CLONING AND APPLICATION OF RECOMBINANT DENGUE VIRUS prM-M PROTEIN FOR SERODIAGNOSIS OF DENGUE VIRUS INFECTION

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Abstract. We studied the use of the precursor to the M structural protein (prM) found only on the surface of mature dengue virus as a target protein to detect dengue virus infection. Recombinant D2-16681 prM-M protein was constructed and tested for immunogenicity with dengue and Japanese encephalitis patient sera by Western blot analysis and indirect ELISA. The sensitivity and specificity of indirect ELISA were 48.1 and 85.5%, respectively, and Western blot assay were 23.1 and 98.7%, respectively, for detection of dengue virus. Although the sensitivity of the indirect ELISA is low, the indirect ELISA using recombinant D2-16681 prM-M proteins as antigen may be used for early detection of dengue virus infection.

Keywords: dengue virus, prM-M, recombinant protein, ELISA, Western blot

INTRODUCTION

Dengue virus infection causes a spectrum of illness: acute febrile illness with dengue fever, dengue hemorrhagic fever and dengue shock syndrome (Jacobs *et al,* 2000; Gould *et al,* 2001). Dengue virus is a single positive-stranded RNA virus, which encodes for three structural proteins, nucleocapsid (C), membrane (M) and envelope (E) and seven non-structural (NS) proteins (Rice *et al,* 1985). The prM is a precursor of the M protein and not found on the surface of other flaviviruses making the prM a potentially useful target protein for differentiating it from other flaviviruses (Cardosa *et al,* 2002).

Laboratory diagnosis of dengue virus infection relies on the isolation of infectious virus, detection of virus-specific RNA or antigens or identification of specific antibodies (serological diagnosis) (Deubel and Murgue, 2006). Most serodiagnostic methods for detecting dengue virus infection use whole virus as an antigen, which results in false positives due to cross-reactivity with other flaviviruses. This type of detection is costly and poses a biohazard risk.

We used an indirect enzyme-linked

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immunosorbent assay (ELISA) and Western blot analysis using dengue virus prM-M recombinant protein, expressed by *E. coli* and purified by electroelution, as an antigen, to determine anti-dengue virus prM-M antibodies in dengue cases.

MATERIALS AND METHODS

Sample collection

A total of 195 serum samples, consisting of 60 paired sera from dengue suspected cases, among whom 52 pairs were later confirmed to be dengue cases by capture ELISA, and 75 sera from a comparative group, were used in this study. The 60 paired dengue serum samples were collected from patients admitted to hospitals in Nong Khai and Nakhon Phanom, Thailand, from June to September 2002. The enrolled cases were aged 5-15 years. Acute and convalescent sera were collected from each patient. The acute sera were collected within a day of hospital admission and convalescent sera were collected within a day of hospital discharge. Thirty serum samples, with antibodies to Japanese encephalitis virus, were obtained from the Department of Virology, AFRIMS. Forty-five single serum samples from normal healthy schoolchildren were used as negative controls. According to WHO criteria (WHO, 1997), 14 (26.92%), 34 (65.38%) and 4 (7.69%) patients were clinically diagnosed as having DF, DHF and DSS, respectively. This study was approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok; approval number: MUTM 2006-050.

Construction and cloning of recombinant D2-16681prM-M DNA

Dengue virus type 2, strain 16681 (D2-16681) was obtained from the Department of Virology, AFRIMS. The prM-M gene was amplified by reverse transcriptase-polymerase chain reaction, D1 forward: 5'-CGT AAG GAT CCA CCA CAC ATG ATC GTC A- 3' and D2 reverse: 5'-AGG AGT GAA TTC TGT CAG TAA GAT GAA A- 3'. The PCR product was digested with *Bam*HI and *Eco*RI prior to cloning into *pRSET-B* (Invitrogen, Carlsbad, CA). The recombinant DNA was transferred to *E.coli* strain BL21(DE3)pLysS by electroporation. The transfered cells were grown in selective media, LB-agar supplemented with 100 g/ml of amplicillin and 40 g/ml of chloramphenicol. The clones were confirmed with DNA sequencing.

Extraction and purification of recombinant D2-16681prM-M protein

A positive clone of the recombinant prM-M protein was produced at 37°C while shaking overnight in selective media. The cell pellet was extracted by being lysed in 3 ml of BugBuster®Master Mix (Novagen, Madison, WI), washed with LEW buffer followed by LEW containing 8 M urea at pH 8.0. Separation of soluble and insoluble fractions was carried out by centrifugation of the sonication product. The extracted recombinant prM-M protein was purified with an Electro-Eluter machine, Model 422 (Bio-Rad, Hercules, CA) according to the company's instructions.

Analyzation of recombinant D2-16681prM-M protein by Western blot

SDS-PAGE was mixed into 4% (w/v) acrylamide in the stacking gel and 15% (w/v) acrylamide in the separating gel and processed in a vertical direction with a constant voltage of 200 V for 1 hour. The proteins were then electrotransferred to a nitrocellulose membrane. An immunoblot was performed using a 1:500 mouse anti-histidine tag followed by goat anti-mouse conjugated with horseradish peroxidase (HRP). The blot was developed using

substrate 3,3'-di-aminobenzidine (DAB).

Protein dialysis

All fractions of purified protein were pooled and placed in a dialysis bag. The protein was dialyzed against 0.01 M PBS (pH 7.4) at 4°C for 2 days; the buffer was changed twice a day. The protein solution was transferred to a collecting tube. The purified protein was detected using a Quick Start™ Bradford protein assay (BioRad, Hercules, CA).

Capture enzyme-linked immunosorbent assay (Capture ELISA)

The ELISA protocol has been described previously by the Department of Virology, AFRIMS (Innis *et al*, 1989). Dengue and Japanese encephalitis virus infections were determined based on IgM. When the ratio of dengue IgM to Japanese encephalitis IgM was greater than 1.0, it was defined as a dengue virus infection. When the ratio was less than 1.0, it was defined as Japanese encephalitis infection.

Western blot analysis

The immunoblot was performed using 1:50 diluted sera in 1% skim milk with 0.05% TBS-T followed by goat anti-mouse serum conjugated with HRP. The blot was developed using the substrate 3, 3'-di-aminobenzidine (DAB).

Indirect enzyme link-immunosorbent assay (Indirect ELISA)

In order to optimize the concentration of recombinant D2-16681 prM-M protein, checkerboard titration was performed; the appropriate concentration was used to determine antibodies against recombinant prM-M protein in all serum samples.

Briefly, a 96-well microtiter plate was coated with 100 l of 5-2,000 ng/ml recombinant prM-M protein followed by 50 l of 1:100 to 1:4,000 control serum diluted in PBS. Then, 50 l of 1:500 to

1:2,000 dilution of goat anti-human IgG was conjugated with HRP and visualized with 50 l of substrate buffer. The results were measured at 492 nm with an ELISA reader (TECAN, Melbourne, Austria).

Data analysis

The McNemar test was used to determine differences in proportions among the results of the capture ELISA, the indirect ELISA assay and the Western blot. The relationship between the clinical data and the results of the indirect ELISA assay and Western blot analysis were determined with the Spearman rank correlation test. Statistical analyses were performed with SPSS version 17.0 (SPSS, Chicago, IL).

Galen's method (Galen, 1979) was used to determine the sensitivity, specificity and predictive values of the Western blot assay that used the recombinant protein as an antigen. The capture ELISA was used as a reference assay.

RESULTS

In this study, we amplified prM-M gene of DENV-2 strain 16681 by PCR. After the 0.5 kbp PCR product was purified by a PCR DNA extraction kit, it was cloned into the pRSET-B expression vector. The positive clone of the recombinant D2-16681 prM-M DNA was screened by colony PCR, which gave a 0.5 kbp PCR product, as shown in Fig 1A. DNA sequencing confirmed the collected sequence and orientation of the D2-16681 prM-M gene was successfully cloned in the pRSET-B vector (Fig 1B).

The recombinant proteins were all purified from cell-free supernatants by an electro-elution method using electro-eluter Model 422 (Bio-Rad, Hercules, CA). The electro-eluted protein fractions were pooled and determined by SDS-PAGE followed by Coomassie brilliant
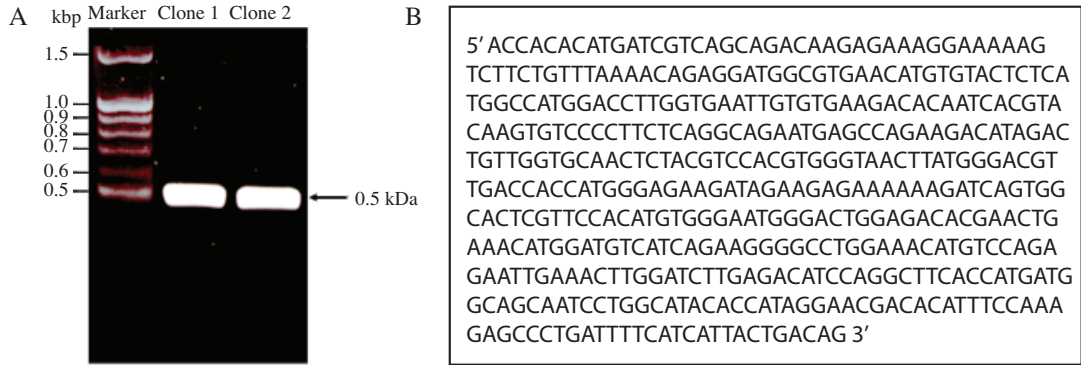
Fig 1–The prM-M gene was cloned in the pRSET-B vector and then the recombinant DNA was transfered into *E. coli* BL21(DE3)pLysS. A. The PCR product of the inserted prM-M. B. The DNA sequence and orientation of the D2-16681 prM-M gene.
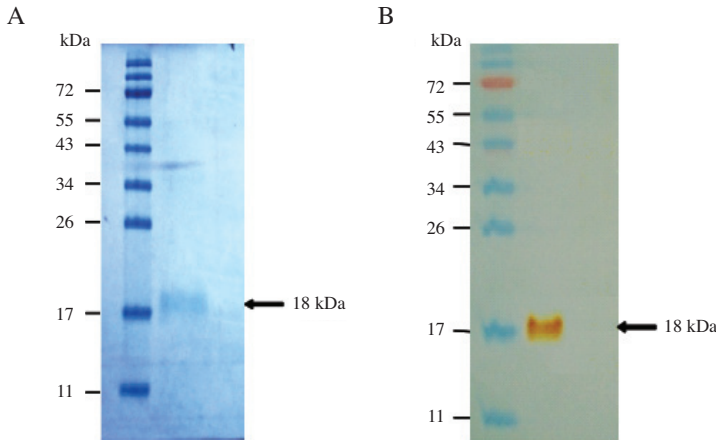

Fig 2–SDS-PAGE and Western blot analysis of the purified recombinant D2-16681 prM-M protein. The urea extracted recombinant D2-16681 prM-M protein was purified by electro-elution. The 18 kDa purified recombinant D2-16681 prM-M protein is indicated by the arrow on the right side. A. The purified protein loaded onto 15% polyacrylamide gel followed by Coomassie blue staining. B. The purified protein immunoblotted onto the nitrocellulose membrane and reacted with a mouse anti-histidine tag.

blue staining and confirmed by Western blot analysis, which revealed the 18 kDa protein that reacted with the mouse anti-histidine tag (Fig 2).

To test the reactivity of the purified recombinant D2-16681 prM-M protein, a total of 179 serum samples were used. All the samples were examined for anti-dengue virus IgM and IgG antibodies by capture ELISA and all were positive. None of the normal healthy controls or Japanese encephalitis virus group were positive for dengue IgM or IgG by capture ELISA.

Western blot analysis was conducted on all serum samples (Table 1). A positive reaction was finding an 18 kDa recombinant D2-16681 prM-M protein. The results are shown in Fig 3.

To optimize the indirect ELISA using recombinant D2-16681 prM-M protein as antigen, various concentrations of recombinant D2-16681 prM-M protein, serum samples and conjugated mouse anti-human IgG-HRP were used. The
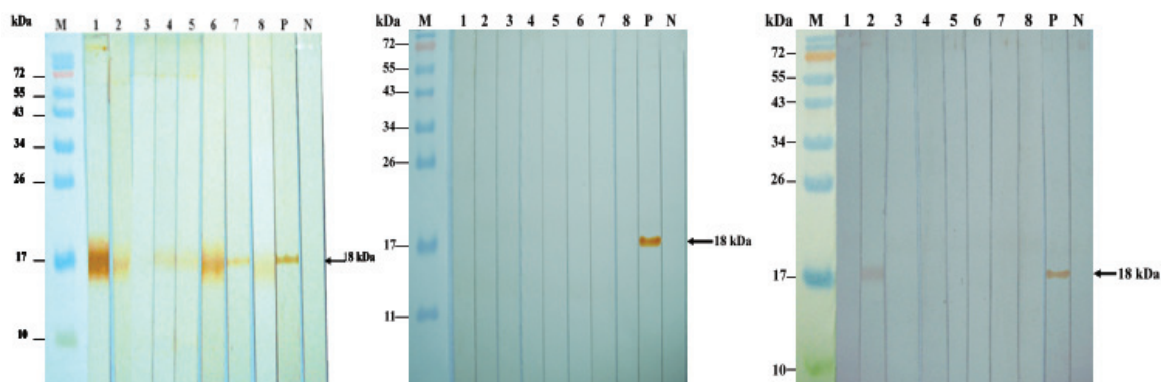
Fig 3–Western blot analysis using recombinant D2-16681 prM-M protein as antigen among dengue cases, normal controls and Japanese encephalitis infected cases. The recombinant protein was immunoblotted onto a nitrocellulose membrane and reacted with 1:100 diluted sample serum, followed by mouse anti-human IgG-HRP as a secondary antibody. The position of the recombinant D2-16681 prM-M protein is indicated by an arrow on the right side. Lane M; standard protein molecular weight; lanes 1-8, serum samples; lane P, positive control; lane N, negative control.

Table 1
Western blot analysis of recombinant D2-16681 prM-M protein among dengue cases, normal healthy controls and Japanese encephalitis virus infected cases.

Western blot analysis	DEN cases		Normal control		JEV infection	
	No.	%	No.	%	No.	%
Positive	12	30	0	0	1	3.33
Negative	40	70	45	100	29	96.67
Total	52	100	45	100	30	100

DEN, dengue virus; JEV, Japanese encephalitis virus

greatest difference in OD value between a positive and negative reaction for each concentration were selected as the optimal condition, resulting in a 25 ng/ml concentration of recombinant D2-16681 prM-M protein; a 1:2,000 diluted serum concentration and a 1:1,000 concentration of conjugated mouse anti-human IgG-HRP was used for this assay. The cut-off value for this test was determined using 45 serum samples from normal health controls. The

mean and the standard deviation (SD) for the OD were 0.678 and 0.34, respectively. When the mean + 2SD value was equal to 1.02, none of the normal controls were positive. Therefore, an OD of 1.02 was used as the cut-off value.

The reactivity of the purified recombinant D2-16681 prM-M protein was tested against all serum samples by indirect ELISA assay. In this study, IgG against D2-16681 prM-M protein was determined

Table 2
Indirect ELISA for recombinant D2-16681 prM-M protein among
dengue cases, normal healthy controls and Japanese encephalitis infected cases.

Indirect ELISA assay	DEN cases		Normal control		JEV infection	
	No.	%	No.	%	No.	%
Positive	25	48.08	0	0	12	40
Negative	27	51.92	45	100	18	60
Total	52	100	45	100	30	100

DEN, dengue virus; JEV, Japanese encephalitis virus

Table 3
Comparison between Western blot and indirect ELISA for the detection of dengue
infection compared to capture ELISA.

Factor	Western blot analysis	Indirect ELISA
Sensitivity	23.08%	48.08%
Specificity	98.67%	85.54%
Efficacy	67.72%	69.29%
p-value	p<0.05	p<0.05

in 52 paired serum samples from dengue cases and 30 serum samples from Japanese encephalitis cases. The 45 samples from normal healthy controls were used to set the cut-off point for this assay. Twenty-five dengue cases (48.08%) were positive for recombinant D2-16681 prM-M protein and 27 cases (51.92%) were negative. The recombinant D2-16681 prM-M protein was found in 12 Japanese encephalitis virus infection cases (40%). None of the normal healthy control serum samples were positive (Table 2).

To determine the sensitivity, specificity and efficacy using Western blot analysis and indirect ELISA, Galen's method and a McNemar test were used. The sensitivity, specificity and efficacy of the Western blot analysis using recombinant D2-16681 prM-M protein as antigen were 23.08, 98.67

and 67.72%, respectively (Table 3). The observed agreement between the Western blot analysis and the capture ELISA was 33.33%. The McNemar test showed a significant difference (p<0.05).

The sensitivity and specificity of the indirect ELISA using recombinant D2-16681 prM-M protein as antigen were 48.08 and 85.54%. The efficacy of the indirect ELISA tested was 69.29% (Table 3). The correlation between the indirect ELISA and capture ELISA was 55.0%. There were 12 false positive results (40%) when testing the samples from Japanese encephalitis infection cases, which is high compared to the Western blot. The McNemar test was significantly different (p<0.05).

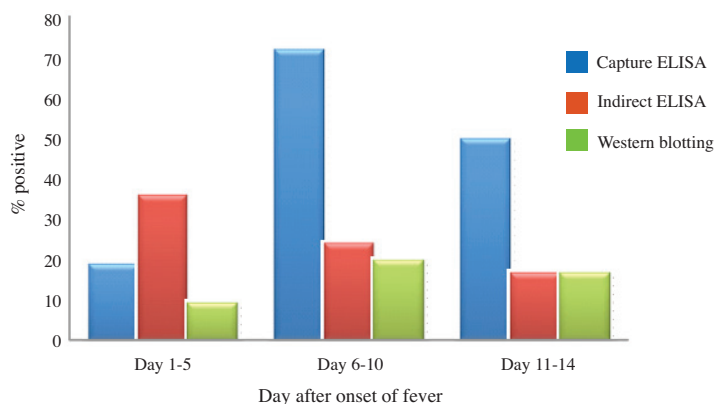Fig 4 shows the anti-dengue antibody results by days of fever. The indirect

Fig 4–Comparison of dengue infection using capture ELISA, indirect ELISA and Western blot.

ELISA using recombinant D2-16681 prM-M protein as antigen was positive for anti-dengue antibodies from days 1 to 5 of fever with a peak of 58.33% on day 5. The capture ELISA using whole virus as antigen had positive results during the convalescent phase, 6-14 days after the onset of fever, with the peak (100%) on day 9. The Western blot using recombinant D2-16681 prM-M protein as antigen gave a relatively low percentage of positive results compared to the capture ELISA and the indirect ELISA.

The indirect ELISA using recombinant D2-16681 prM-M protein as antigen gave its best results 1-5 days after the onset of fever.

DISCUSSION

The prM protein is found only on the surface of mature dengue viruses and anti-prM antibodies do not react with Japanese encephalitis viruses (Cardosa et al, 2002). However, anti-prM antibodies may react with epithelial cells, endothelial cells, T cells and mediated antibody-dependent enhancement (ADE) leading to DHF and DSS (Huang et al, 2008). Thus, we used prM protein as an antigen to develop a method to detect dengue infection.

The Western blot had 23.08% sensitivity and 98.67% specificity with one false positive with Japanese encephalitis infection. The sensitivity was lower than the capture ELISA. The preparation of the test may have caused a change in the antigen caused by denaturing which happens in preparation; however, the ELISA assay uses the natural form of the protein (Goldsby et al, 2003). Cardosa et al (2002) found anti-prM antibodies from Japanese encephalitis viruses do not react with prM from dengue viruses using Western blot analysis. The false positive case in our study may have been caused by a mixed or previous detection.

The indirect ELISA assay gave a 48.08% sensitivity and 85.54% specificity compared to the capture ELISA. The relatively low sensitivity may be due to a difference in the tested antigen. The capture ELISA used the whole virus while the indirect ELISA assay used only the prM-M protein. The low sensitivity may also have been due to the high cut-off value. The relatively high value cut-off may have been due to the patient serum used to prepare the test. A milder host immune response may have resulted in less severe dengue disease (Endy et al, 2002). The indirect ELISA assay had a 40% false positive rate with Japanese encephalitis infection cases. This may have been due to the difference between the liner and the conformation epitope of the protein antigen.

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REFERENCES

Cardosa MJ, Wang SM, Sum MS, Tio PH. Antibodies against prM protein distinguish between previous infection with dengue and Japanese encephalitis viruses. *BMC Microbiol* 2002; 2: 9-14.

Deubel V, Murgue B. Dengue. In: Service MW, ed. The encyclopedia of arthropod-transmitted infections. Wallingford (UK): CAB International, 2001: 133-43.

Endy TP, Nisalak A, Chunsuttiwat S, *et al*. Spatial and temporal circulation of dengue virus serotypes: A prospective study of primary school children in Kamphaeng Phet, Thailand. *Am J Epidemiol* 2002; 156: 52-9.

Galen RS. The predictive value of laboratory testing. *Orthop Clin North Am* 1979; 10: 287-97.

Goldsby R, Kindt TJ, Osborne BA, Kuby J. Immunology: enzyme-linked immunosorbent assay. 5th ed. New York: WH Freeman and Company, 2003: 57-75.

Gould EA, De Lamballe X, Zanotto PMD, Holmes EC. Evolution, epidemiology, and dispersal of flaviviruses revealed by molecular phylogenies. *Adv Virus Res* 2001; 57: 71-103.

Huang KJ, Cheng YT, Lin YS, Huang JH, Liu HS. Anti-PrM antibody as an autoantibody in dengue virus infection. *Am J Infect Dis* 2008; 4: 59-67.

Innis BL, Nisalak A, Nimmannitya S, *et al*. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 1989; 40: 418-27.

Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM, Young PR. Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction. *FASEB J* 2000; 14: 1603-10.

Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* 1985; 229: 726-33.

World Health Organization (WHO). Dengue haemorrhagic fever diagnosis, treatment, prevention and control. Geneva: WHO, 1997: 84 pp.