นิพนธ์ต้นฉบับ

Reactivities of antibodies of dengue virus infected patients by Western blotting

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Objectives : *To study the reactivities of antibodies from dengue virus infected patients compared with non infected cases by Western blotting under reducing conditions. To determine the relative immunogenicities and antigenic relationships of DEN-1-4 proteins.*

Research design : *A cross - sectional study*

Methods : *One hundred and six serum samples were collected from dengue virus infected patients (36 cases: acute and convalescent sera) and non infected cases (34 cases). The antibodies were investigated by biotin - streptavidin Western blotting under reducing conditions of sodium dodecyl sulfate - polyacrylamide gel electrophoresis.*

Results : *Acute sera of dengue virus infected patients recognized the positive protein bands of DEN-1 (MW 56 and 70 kd), DEN-2 (16 and 56 kd) and DEN - 4 (56 kd) are different from non - dengue infected patients with statistical significance, p - value < 0.05. In convalescent sera, the difference were observed in DEN - 1 (49, 56, 70 and 94 kd), DEN -2 (56 and 60 kd), DEN - 3 (56 kd) and DEN - 4 (60 kd), p - value < 0.05. The antibodies from convalescent sera increased in the intensity of the protein bands at MW of 29 kd (NS4a) significantly when compared with acute sera, p - value < 0.05. The specific proteins of any dengue serotype*

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recognized by the sera from dengue virus infected patients were observed at 45 kd (NS1) and 56 kd (E) in acute and convalescent phases, respectively (p value < 0.05).

Conclusions : *The biotin-streptavidin Western blotting test can be used to study the reactivities of antibodies and characterize dengue proteins under reducing conditions thus the purified proteins might be useful for diagnosis, study of antibody response and vaccine development for dengue hemorrhagic fever.*

Key words : *Dengue virus infection, Antibody reactivities, Biotin - streptavidin, Western blotting.*

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วัตถุประสงค์ : *เพื่อศึกษาปฏิกริยาของแอนติบอดีในซีรัมของผู้ป่วยติดเชื้อไวรัสเด็งกิว เปรียบเทียบกับผู้ที่ไม่ติดเชื้อไวรัสเด็งกิวด้วย วิธีเวสเทอร์น บล็อตติ้งในสภาวะรีดิวซ์ และเพื่อตรวจหาอิมมิวโนเจนิกซิตีและความสัมพันธ์ของแอนติเจนไวรัสเด็งกิว ทั้ง 4 ซีโรทัยป์*

รูปแบบการวิจัย : *การศึกษาภาคตัดขวาง*

วิธีการทำวิจัย : *เก็บซีรัมจำนวน 106 ตัวอย่าง จากซีรัมเริ่มป่วยและซีรัมระยะพักฟื้น ในผู้ป่วยติดเชื้อไวรัสเด็งกิวจำนวน 36 ราย และผู้ที่ไม่ติดเชื้อไวรัสเด็งกิวจำนวน 34 ราย แยกแอนติเจนด้วยกระแสไฟฟ้าในวุ้นชนิดโพลีอะครีลาไมด์ในสภาวะรีดิวซ์ และทำปฏิกริยากับแอนติบอดีในซีรัม ใช้วิธีไบโอติน-สเตรปตาวิดิน เวสเทอร์น บล็อตติ้ง*

ผลการศึกษา : *แอนติบอดีในซีรัมระยะเริ่มป่วยของผู้ติดเชื้อไวรัสเด็งกิว จับกับโปรตีนของไวรัสเด็งกิว ซีโรทัยป์ 1 ที่น้ำหนักโมเลกุล 56 และ70 กิโลดาลตัน ซีโรทัยป์ 2 ที่น้ำหนักโมเลกุล 16 และ56 กิโลดาลตัน และ ซีโรทัยป์ 4 ที่น้ำหนักโมเลกุล 56 กิโลดาลตัน แตกต่างจากซีรัมของผู้ไม่ติดเชื้อไวรัสเด็งกิว อย่างมีนัยสำคัญทางสถิติ ค่าพี น้อยกว่า 0.05 ในซีรัมระยะพักฟื้น พบความแตกต่าง ที่โปรตีนของไวรัสเด็งกิว ซีโรทัยป์ 1 น้ำหนักโมเลกุล 49, 56, 70 และ 94 กิโลดาลตัน ซีโรทัยป์ 2 ที่น้ำหนักโมเลกุล 56 และ 60 กิโลดาลตัน ซีโรทัยป์ 3 ที่น้ำหนักโมเลกุล 56 กิโลดาลตัน และ ซีโรทัยป์ 4 ที่น้ำหนักโมเลกุล 60 กิโลดาลตัน ค่าพี น้อยกว่า 0.05 แอนติบอดีในซีรัมระยะพักฟื้น จับกับโปรตีนของไวรัสเด็งกิวซีโรทัยป์ 2 ที่ 29 กิโลดาลตัน ให้แถบสีเข้มขึ้น มากกว่าในซีรัมระยะเริ่มป่วย อย่างมีนัยสำคัญทางสถิติ โปรตีนของไวรัสเด็งกิว ซีโรทัยป์ใดซีโรทัยป์หนึ่ง จะทำปฏิกริยาจำเพาะกับแอนติบอดีในซีรัมระยะเริ่มป่วย ที่ 45 กิโลดาลตัน และในซีรัมระยะพักฟื้นที่ 56 กิโลดาลตัน ค่าพี น้อยกว่า 0.05*

สรุป : *วิธีไบโอติน-สเตรปตาวิดิน เวสเทอร์น บล็อตติ้ง สามารถใช้ศึกษาปฏิกริยาของแอนติบอดีต่อโปรตีนของไวรัสเด็งกิว ในสภาวะรีดิวซ์ เพื่อเป็นหนทางนำไปสู่การทำให้โปรตีนบริสุทธิ์ สำหรับใช้ในการวินิจฉัยโรค การศึกษาปฏิกริยาจำเพาะของแอนติบอดี และการพัฒนาวัคซีนในโรคไข้เลือดออก*

คำสำคัญ : *การติดเชื้อไวรัสเด็งกิว, ปฏิกริยาของแอนติบอดี, วิธีไบโอติน-สเตรปตาวิดิน เวสเทอร์น บล็อตติ้ง*

Dengue fever (DF), dengue hemorrhagic fever (DHF) and the most severe dengue shock syndrome (DSS) in young children are caused by dengue viruses (DEN - 1, DEN - 2, DEN - 3 and DEN - 4) which are members of the genus Flavivirus. The mature virion of flaviviruses contains three structural proteins including envelope (E) glycoprotein (MW 51-60 kd), core (C) protein (13 - 16 kd) and membrane (M) - associated proteins (8 - 8.5 kd). Nonstructural (NS) proteins consist of NS1 (44 - 49 kd), NS2a (16-21 kd), NS2b (12-15 kd), NS3 (67 - 76 kd), NS4a (24 - 32 kd), NS4b (10 - 11 kd) and NS5 (91 - 98 kd).[1] The flavivirus genome structure and the expression and possible functions of the viral proteins have been described.[2] Studies of antibody reaction to dengue viral proteins have been reported using sera from humans[3-6] and rabbits.[7,8] The antibodies from dengue hemorrhagic fever patients which reacted to dengue polypeptides were demonstrated by viral - antigens - strips/enzyme immunoassays under non - reducing conditions since the structural proteins were quite sensitive to 2 - mercaptoethanol (2 - ME) and the heat treatment conditions in sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE).[3,4] In our previous study, a highly sensitive and specific method for detection of dengue IgM antibodies was achieved by a use of biotin - streptavidin system adapted in an enzyme - linked immunosorbent assay.[9] Thus, the biotin - streptavidin was applied to the Western blotting test and the reactions of antibodies directed against dengue viral proteins in the presence of 2 - ME and heat treatment were observed. We describe here the reactivities of antibodies from dengue virus infected patients compared with non - infected cases to dengue proteins under reducing conditions of SDS - PAGE and detection by biotin - streptavidin immunoblotting. In addition, the relative immunogenicities and antigenic relationships of DEN - 1 - 4 proteins were also determined.

Materials and Methods

Dengue antigens

Dengue antigens produced by sucrose acetone extraction from the brains of suckling mice were obtained from Virus Research Institute, Department of Medical Science, Ministry of Public Health, Nonthaburi. They included DEN -1 (Hawaii), HA 5,120, DEN - 2 (Tr 1751), HA 2,560, DEN - 3 (H 87), HA 5,120, and DEN - 4 (H 241), HA 10,240. The lyophilized antigens were resuspended in distilled water before use. Chemicals were of reagent grade obtained from Sigma chemical Co. (St. Louis, MO, USA) except as indicated.

Human sera

Serum samples were collected from children aged under 15 years who had been admitted to Ratchaburi Hospital and Suan Phung Hospital between June and July, 1995. Thirty six patients in the study group included dengue fever (7 cases), dengue hemorrhagic fever (28 cases) and dengue shock syndrome (1 case) who were diagnosed by clinical and laboratory findings and classified according to WHO criteria[10] (1997). Acute sera were collected from the dengue virus infected patients within a day of hospital admission, and during the 3 - 5th day after onset of illness. Convalescent sera were collected before discharge from the hospital, and during the 6-10th day after onset of the disease. Thirty four sera were also collected from non - dengue virus infected

patients as the control group. All sera were confirmed by dot blot enzyme immunoassay (Diagnostic Biotechnology, Singapore). All dengue virus infected patients had secondary infections. The total serum samples (106 samples; 36 acute and 36 convalescent sera from dengue virus infected patients and 34 sera from non - infected cases) were assayed by Western blot tests.

Western blotting

Western blotting was performed essentially according to the procedure described by Towbin et al [11] and Burnette.[12] In brief, the dengue antigens (DEN - 1, DEN - 2, DEN - 3 or DEN - 4) in the 45 μg amount of protein determined by the Lowry method [13] was mixed with reduced sample buffer containing 5% 2 - ME and boiled at 100°C for 3 minutes. The protein antigen was loaded in the well with 10% separating gel with 3% stacking gel and resolved by SDS - PAGE according to Laemmli.[14] Protein molecular weight (MW) standards were run on each gel. Protein was transferred to 0.45 – μm - pore - size nitrocellulose paper (Schleicher & Schuell, Dassell, Germany) by use of a mini vertical gel electrophoresis (BRL, Life technologies, Gaithersburg, MD). The nitrocellulose membrane was directly visualized by staining with 0.1% Amido black. For immunological detection of protein bands, the membrane was blocked by 5% skim milk in Tris - saline, pH 7.4 and incubated at room temperature for 1 hr on a rocking platform. Next, the serum sample at a dilution of 1:25 in Tris - saline, pH 7.4 containing 2% BSA was added to the membrane and incubated at room temperature for 2 hours. The membrame was washed with Tris - saline, pH 7.4 for 15 minutes followed by Tris - saline, pH 7.4 containing 0.05% nonidet P - 40 twice for 30 minutes each and then washed with Tris - saline, pH 7.4 for 15 minutes. Biotinylated - goat anti - human immunoglobulins diluted 1:500 was added to the membrane and incubated at room temperature for 2 hours. After the washing step, horseradish peroxidase-streptavidin (Vector Laboratories, Burlingame, CA) at a dilution of 1:1,000 was added. It was then incubated at room temperature for 2 hours and washed again. Antibody bands were visualized with 4 - chloro - 1 - naphthol in Tris - saline, pH 7.4. The reaction was stopped after 10 minutes incubation with distilled water. Finally, the membrane was dried on a couple of filter papers and stored away from light.

Statistical analysis

The reactivities of antibodies directed against dengue virus antigens were determined visually and scored as 1+, 2+, 3+ or 4+. The samples giving $\geq$ 2+ were considered positive. The difference between dengue and non - dengue cases and the magnitude of relative immunogenicities from acute to convalescent sera of dengue cases were analysed by Chi-square or Fisher exact test. Antigenic relationships among DEN -1 - 4 were determined for specific proteins by the Cochran Q test. A p - value of < 0.05 was considered statistically significant.

Results

Comparison between serum samples from dengue virus infected patients and non – infected cases

The separated DEN - 1 - 4 viral proteins were electrotransferred onto nitrocellulose paper and reacted with sera obtained from dengue and non - dengue virus infected patients by Western blotting. The protein

bands with estimated MW between 16 - 260 kd were recognized. According to a previous study, the peptides of dengue viruses were in the range of 16 - 97.4 kd.[1] Using acute serum samples, the intensified protein bands of dengue virus infected patients which differentiated from non - infected cases were observed in DEN -1 (56 and 70 kd), DEN - 2 (16 and 56 kd) and DEN - 4 (56 kd) with statistical significance ($p < 0.05$), as shown in Table 1. In convalescent sera, the differences of protein bands were found in DEN -1 (49, 56, 70 and 94 kd), DEN - 2 (56 and 60 kd), DEN - 3 (56 kd) and DEN - 4 (60 kd) with statistical significance ($p < 0.05$), as shown in Table 2.

Relative immunogenicities of dengue proteins reacted to antibodies

The immunogenicities of dengue viral antigens were analysed by determination of the frequency of positive sera which showed increases in intensity. The reactive protein bands were present at MW of 21, 23, 29, 43 - 97.4 kd of DEN -1 - 4 in acute sera and were more intense in convalescent sera. After analysis of difference in all 36 dengue virus infected cases, however, only DEN - 2 with MW of 29 kd reacted to antibodies from convalescent sera (9 cases) and increased in the intensity significantly when compared with acute sera (3 cases), p - value < 0.05.

Table 1. Comparison of the reactivities of antibodies from acute sera of dengue virus infected patients and non cases to dengue proteins by Western blotting.

Dengue virus protein, kd	Dengue virus infected patients N = 36, No. (%)	Non cases N = 34, No. (%)	X^2-test	p- value*
DEN-1, 56 (E)				
positive	18 (50.0)	4 (11.7)		
negative	18 (50.0)	30 (88.3)	11.86	0.0005
DEN-1, 70 (NS3)				
positive	17 (47.2)	7 (20.6)		
negative	19 (52.8)	27 (79.4)	5.51	0.01
DEN-2, 16 (C)				
positive	12 (33.3)	3 (8.8)		
negative	24 (66.7)	31(91.2)	6.24	0.01
DEN-2, 56 (E)				
positive	17 (47.2)	5 (14.7)		
negative	19 (52.8)	29 (85.3)	8.58	0.003
DEN-4, 56 (E)				
positive	13 (36.1)	4 (11.7)		
negative	23 (63.9)	30 (88.3)	5.64	0.01

* statistical significance, p - value < 0.05

Table 2. Comparison of the reactivities of antibodies from convalescent sera of dengue virus infected patients and non cases to dengue proteins by Western blotting.

Dengue virus protein, kd	Dengue virus infected patients N = 36, No. (%)	Non cases N = 34, No. (%)	X^2- test	p - value*
DEN-1, 49 (NS1)				
positive	19 (52.8)	10 (29.4)		
negative	17 (47.2)	24 (70.6)	3.93	0.04
DEN-1, 56 (E)				
positive	14 (38.9)	4 (11.7)		
negative	22 (61.1)	30 (88.3)	6.73	0.009
DEN-1, 70 (NS3)				
positive	18 (50.0)	7 (20.6)		
negative	18 (50.0)	27 (79.4)	6.59	0.01
DEN-1, 94 (NS5)				
positive	8 (22.2)	0		
negative	28 (77.8)	34 (100)	-	0.005#
DEN-2, 56 (E)				
positive	20 (55.5)	5 (14.7)		
negative	16 (44.5)	29 (85.3)	12.71	0.0003
DEN-2, 60 (E)				
positive	9 (25.0)	2 (5.9)		
negative	27 (75.0)	32 (94.1)	4.83	0.02
DEN-3, 56 (E)				
positive	16 (44.4)	5 (14.7)		
negative	20 (55.6)	29 (85.3)	7.36	0.006
DEN-4, 60 (E)				
positive	15 (41.7)	1 (2.9)		
negative	21 (58.3)	33 (97.1)	14.87	0.0001

* statistical significance, p - value < 0.05

Fisher exact test

Antigenic relationships of dengue viral proteins

Most dengue virus infected patients had antibodies against all DEN 1 - 4. Nevertheless, the antibodies reacted specifically to proteins of any dengue serotype at MW of 45 kd in acute sera (Table 3) and 56 kd in convalescent sera (Table 4) with statistical significance, p - value < 0.05.

Table 3. Frequency of dengue virus infected patients whose antibodies in acute sera reacted to dengue viral proteins, total of 36 cases.

Molecular weight, kd (viral protein)	DEN - 1 No. (%)	DEN - 2 No. (%)	DEN - 3 No. (%)	DEN - 4 No. (%)
16 (C)	12 (33.3)	12 (33.3)	12 (33.3)	13 (36.1)
18 (prM)	5 (13.9)	5 (13.9)	4 (11.1)	3 (8.3)
21	16 (44.4)	15 (41.7)	9 (25.0)	14 (38.9)
23	4 (11.1)	6 (16.7)	4 (11.1)	4 (11.1)
27 (NS4a)	7 (19.4)	4 (11.1)	5 (13.9)	6 (16.7)
29	7 (19.4)	3 (8.3)	3 (8.3)	8 (22.2)
31	5 (13.9)	9 (25.0)	4 (11.1)	5 (13.9)
43 (NS1)	9 (25.0)	7 (19.4)	9 (25.0)	11 (30.6)
45*	20 (55.5)	15 (41.7)	12 (33.3)	10 (27.8)
49	15 (41.7)	15 (41.7)	8 (22.2)	11 (30.6)
51 (E)	13 (36.1)	13 (36.1)	13 (36.1)	11 (30.6)
56	18 (50.0)	17 (47.2)	11 (30.6)	13 (36.1)
58	1 (2.8)	1 (2.8)	2 (5.6)	-
60	6 (16.7)	7 (19.4)	8 (22.2)	7 (19.4)
70 (NS3)	17 (47.2)	12 (33.3)	11(30.6)	12 (33.3)
91 (NS5)	12 (33.3)	7 (19.4)	9 (25.0)	7 (19.4)
94	3 (8.3)	8 (22.2)	3 (8.3)	4 (11.1)
97.4	9 (25.0)	5 (13.9)	3 (8.3)	4 (11.1)

* statistical significance by Cochran Q test

Table 4. Frequency of dengue virus infected patients whose antibodies in convalescent sera reacted to dengue viral proteins, total of 36 cases.

Molecular weight, kd (viral protein)	DEN - 1 No. (%)	DEN - 2 No. (%)	DEN - 3 No. (%)	DEN - 4 No. (%)
16 (C)	10 (27.8)	9 (25.0)	9 (25.0)	8 (22.2)
18 (prM)	5 (13.9)	4 (11.1)	1 (2.8)	4 (11.1)
21	19 (52.7)	17 (47.2)	16 (44.4)	14 (38.9)
23	8 (22.2)	8 (22.2)	9 (25.0)	9 (25.0)
27 (NS4a)	5 (13.9)	6 (16.7)	5 (13.9)	6 (16.7)
29	4 (11.1)	9 (25.0)	5 (13.9)	8 (22.2)
31	6 (16.7)	11 (30.6)	5 (13.9)	10 (27.8)
43 (NS1)	11 (30.6)	12 (33.3)	10 (27.8)	8 (22.2)
45	18 (50.0)	17 (47.2)	10 (27.8)	15 (41.6)
49	19 (52.8)	20 (55.6)	14 (38.9)	18 (50.0)
51 (E)	16 (44.4)	13 (36.1)	11 (30.6)	17 (47.3)
56*	14 (38.9)	20 (55.5)	16 (44.4)	10 (27.8)
58	-	-	1 (2.8)	-
60	12 (33.3)	9 (25.0)	10 (27.8)	15 (41.7)
70 (NS3)	18 (50.0)	9 (25.0)	11(30.6)	13 (36.1)
91 (NS5)	13 (36.1)	12 (33.3)	10 (27.8)	15 (41.7)
94	8 (22.2)	8 (22.2)	3 (8.3)	4 (11.1)

*statistical significance by Cochran Q test

Discussion

A combination of SDS - PAGE under reducing conditions and biotin - streptavidin Western blotting was carried out to study the antibody's reactivities in dengue virus infected patients. Although 5% 2-ME was used as a reducing agent in preparation of the virus samples followed by heat treatment at 100° C for 3 minutes, all protein bands of the dengue viruses could be detected in the range of 16-97.4 kd. Churdboonchart et al,[3] using SDS - PAGE, stated the presence of DEN - 2 protein bands reacting to antibodies in the range of 19-130 kd but disappearance of bands at 19 - 21 kd, 48 - 50 kd, 54 - 60 kd and 68 - 71 kd after treatment with 2 - ME. However, three peptides of 20, 46 and 57 kd were recognized by the rabbit sera in denaturing methods of heating the dengue virus samples at 100°C for 5 minutes.[8] The presence of biotin - streptavidin in the immunological detection of dengue antibodies displayed more intense color of the positive protein bands than without it (data not shown). It is likely that the biotin - streptavidin increases the sensitivity of detection by Western blotting.

The antibodies of dengue virus infected patients reacted to specific viral proteins significantly different from non - infected cases indicating that the combination of proteins such as C (13-16 kd), E (51-60 kd), NS3 (67-76 kd) and NS5 (91-98 kd), may be the represented proteins for dengue diagnosis. The 92-98 kd reactive band of NS5 specific for only dengue patients' sera was also reported.[5] NS3 and NS5 were defined previously for diagnosis of dengue infection.[4] The majority of strong, intense protein bands were observed in DEN -1 - 4 with high molecular weights (43-97.4 kd) because of higher immunogenicities rather than low molecular weight proteins. Among those polypeptides recognized, E protein of DEN-2 with 56 Kd appeared as a broad intense band. The E proteins of flaviviruses are known to contribute to several important biological functions and to be the most immunogenic in giving rise to neutralizing antibodies and protective immunity in mice.[15, 16] The intense color of the protein band at 29 kd (NS4a) of DEN-2 increased significantly from acute to convalescent sera. The antibody response in the convalescent phase was higher than in the acute phase and was demonstrated as sero-conversion to in all types of dengue virus.[3]

Regarding DEN 1 - 4 viral proteins, the antibodies reacted specifically to any dengue serotype at 45 kd (NS1) in acute sera and at 56 kd (E) in convalescent sera of dengue virus infected patients. Anti-dengue NS1 antibody occurred at a significantly higher frequency in acute phase serum samples from Indonesian patients with secondary infections.[6] NS1 was purified and developed for the study of protection from dengue infection.[15, 17, 18] The recognition of synthetic oligopeptides from NS1 and NS3 by sera from dengue virus-infected children was demonstrated.[19]

The developed biotin-streptavidin Western blotting under reducing conditions is useful to characterize protein antigens reacing specifically to antibodies from sera of dengue virus infected patients. The purification of such dengue proteins will be of value for diagnostic purposes, study of antibody response and vaccine development for prevention and control of DHF.

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