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Profile of nonstructural glycoprotein NS1 as a diagnostic marker in dengue type 2 virus infection

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ABSTRACT

BACKGROUND

Several studies in dengue on cloned or cell culture passaged in wild and attenuated virus have been performed, but each report described different sites of nucleotide and amino acid changes possibly associated with virulence. The aim of this study was to investigate protein expression of dengue type 2 virus infected C6/36 cells through two-dimensional gel electrophoresis followed by N-terminal Edman degradation sequencing to identify target proteins.

METHODS

Two type of samples; dengue type 2 virus-infected C6/36 cells and uninfected C6/36 cells were prepared. Immobilized non-linear pH gradient strips, pH 3-10 were used in isoelectric focusing, and 10% homogeneous gels were used in Sodium dodecyl sulphate - Polyacylamide gel electrophoresis. The target protein spots were subsequently transferred onto polyvinyldifluoride membrane by western blotting and visualized by coomassie brilliant blue for N-terminal sequencing purpose.

RESULTS

This study revealed four target protein spots in dengue type 2 virus-infected C6/36 cells that were not present in uninfected C6/36 cells. The N-terminal sequencing result was D-S-G-C-V-V-S-W-K-N-K which was identical to nonstructural glycoprotein NS1 (Swissprot-database) associated with the replication process of flaviviruses.

CONCLUSION

Nonstructural NS1 might be used as a diagnostic marker and/or as a parameter to evaluate the effect of antiviral agents for dengue type 2 virus infection/replication.

Keywords: *Dengue type 2 virus, non structural NS1 protein, proteomics*

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Profil glikoprotein "non structural" NS1 sebagai marker pada diagnosis infeksi virus dengue tipe 2

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ABSTRAK

LATAR BELAKANG
Pada dengue telah dilakukan beberapa penelitian yang menggunakan cara cloning atau subkultur sel baik yang berasal dari virus yang delemahkan maupun yang tidak dilemahkan, tetapi masing-masing hasil memperlihatkan perubahan asam amino dan nukleotida pada tempat yang berbeda yang berhubungan dengan virulensi. Tujuan penelitian adalah untuk menyelidiki ekspresi protein virus dengue tipe 2 yang di kultur pada sel C6/36 melalui *two-dimensional gel electrophoresis.*

CARA
Sampel yang digunakan terdiri dari dua tipe, yaitu kultur sel C6/36 yang diinfeksi dengan virus dengue tipe 2 dan kultur sel C6/36 yang tidak diinfeksi. Pada teknik *isoelectric focusing* digunakan *immobilized non-linear pH gradient strips* dengan pH 3-10 yang diikuti dengan *Sodium dodecylsulphate-Polycrylamide gel electrophoresis*. Selanjutnya protein target yang diperoleh dipindahkan ke *polyvinyldifluoride membrane* dengan metode *Western blot* dan dilakukan pewarnaan dengan *coomassie brilliant blue* untuk keperluan *N-terminal sequencing*.

HASIL
Hasil yang diperoleh pada ekspresi protein adalah 4 titik ekstra yang ditemukan pada sel C6/36 yang diinfeksi virus dengue tipe 2, tetapi tidak ditemukan pada sel C6/36 yang tidak diinfeksi. Hasil *N-terminal sequencing* menunjukkan urutan sekuens yang identik dengan *nonstructural glycoprotein* sesuai dengan *Swissprot-database* yaitu D-S-G-C-V-V-S-W-K-N-K. Sekuens ini berkaitan dengan proses replikasi *flaviviruses*.

KESIMPULAN
Nonstructural NS1 dapat digunakan sebagai alat bantu diagnosis dan atau parameter untuk mengevaluasi efek antivirus terhadap infeksi atau proses replikasi virus dengue tipe 2.

Kata kunci: Virus dengue tipe 2, protein non structural NS1, proteomik

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INTRODUCTION

Dengue fever (DF), dengue hemorrhagic fever (DHF) and shock syndrome (DSS) are mosquito-borne infectious diseases that have become major international public health concerns. DF and DHF/DSS occur in tropical and subtropical regions around the world, predominantly in urban and semi-urban areas.[1] According to World Health Organization (WHO) estimates, its incidence has increased by a factor of 30 over the last 50 years.[2] It occurs in tropical areas and affect up to 100 million people each year,[3,4] including 500,000

cases of DHF and around 30,000 deaths, mostly among children.[5] It is now endemic in more than 100 countries (the Americas, the eastern Mediterranean, Southeast Asia, and the Western Pacific) and poses a threat to more than 2.5 billion people.[6] The African continent seems less affected by the dengue virus.[5] An epidemic of DHF in the New World took place in Venezuela in 1989; isolates of DEN-1, DEN-2 and DEN-4 were obtained during this epidemic, but the most severe cases were associated with DEN-2 infections.[10] Dengue viruses belong to the *Flavivirus* genus of the *Flaviviridae* family. Flaviviruses are enveloped, single stranded RNA viruses. The dengue genome is approximately 11 kb in size and contains a single open reading frame, which encodes a precursor polyprotein and is flanked by two nontranslated regions (5' and 3' NTR). Co- and posttranslational proteolytic cleavage of the precursor results in the formation of three structural proteins, capsid (C), membrane (M), and envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Among them, the nonstructural protein NS1 related to replication process of flaviviruses. Unusually for a viral glycoprotein, NS1 is produced both membrane-associated and secreted forms[5] both of which are highly immunogenic and can elicit high-titre antibody.[11] NS1 has a predicted molecular mass of 46 to 50 kilodalton[12] due to N-linked carbohydrate chains at position 130 and 207 and 12 invariant cysteine residues.[13] NS1 is not incorporated into the virion and therefore does not elicit virus-enhancing antibodies.[14] The precise function of dengue NS1 protein remains unclear.[15]

Proteomics is widely accepted as a technology in the post genomic era to identify proteins that are either involved in a specific cellular process (cell map proteomics) or exhibit an altered expression profile as the result of some changes in physiological condition (expression proteomics). Since proteins play a central role in the life of an organism, proteomics is instrumental in discovery of biomarkers, such as markers that indicate a particular disease.

The development of two-dimensional electrophoresis (2-DE) was a major step forward of the science in proteomics to identify the proteins of interest. The 2-DE is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissue, or other biological samples.[16] One problem that had to be overcome was the lack of sensitive protein sequencing technology. The first major technology to emerge for the identification of proteins was the sequencing of proteins by Edman degradation.[17] Followed by image analysis and mass spectrometry to quantitify and to characterize proteins.[18] The aim of this study was to investigate protein expression of dengue type 2 virus-infected C6/36 cells through two-dimensional gel electrophoresis followed by N-terminal Edman degradation sequencing to identify target proteins.

MATERIAL AND METHODS

Preparation of uninfected sample

C6/36 cells from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia were maintained in RPMI-1640 medium (Flow Laboratories, U.K.) supplemented with 2% fetal calf serum, and incubated at 28°C. The C6/36 cells were disrupted by repeated fast freezing, three times for 15 minutes, and subsequently pelleted by spinning at 4°C, 2000 rpm for 10 minutes. The pellet was twice washed with 1 ml cold-PBS (Phosphate saline buffer), spun as above, then resuspended with 1 ml lysis buffer (8M urea, 4% Triton-X 100,

2% IPG-phor buffer 3-10), incubated for 1 hour at 4°C and again spun as above. The supernatant was stored at -20°C.

DV2-infected C6/36 cells

Dengue virus-2 (*New-Guinea C* strain) was obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia. Virus stocks were used to infect 80% confluent cell monolayers in RPMI-1640 supplemented with 2% fetal calf serum and incubated at 28°C until cytopathic effect (CPE) was observed (up to day-4), at which stage the supernatant and cell monolayers were harvested. Cell processing used the same method as described for uninfected cells.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DGE) was performed using the Multiphore II Electrophoresis System based on the recommended method by Amersham Biosciences.[19] Sample was diluted in buffer containing 8M urea, 0.5% (v/v), Triton X-100, 0.5% (v/v), IPG-phor buffer 3-10 and 12 mM dithiothreitol (DTT). Pre-cast immobilized dry strips pH 3-10 (Amersham Biosciences) were rehydrated overnight in the same solution. The strips were aligned in a tray that was filled with mineral oil (Amersham Biosciences). Isoelectric focusing was performed in three steps by varying the voltage as suggested by the manufacturer. For the second dimension, the strips were incubated for 15 minutes in a solution consisting of 6M Urea, 1% (w/v) SDS, 30% (v/v) glycerol and 0.3M DTT dissolved in 0.05M Tris-HCl (pH 6.8). This was followed by incubation for another 15 minutes in a similar solution but containing 50mM iodoacetamide instead of DTT. Strips were placed on a Sodium dodecyl sulphate-Polyacylamide gel electrophoresis (SDS-PAGE) 10% homogeneous gel (Amersham Biosciences) and electrophoresed for 100 minutes. Visualization step was done by silver staining. For determination of molecular weight of the target proteins, marker with low range was run together on SDS-PAGE with the molecular weight kit in range of 45 kDa and 66 kDa (Bio-Rad manufacturer).

Immundblotting and immund detection

Western blotting was performed according to the standard method.[20] Nova Blot Kit of Multiphore II Electrophoresis System (Pharmacia, Uppasala, Sweden) was used to transfer the protein to nitrocellulose membrane (Protran-Schlicher and Schuell-0.45 µM). The unit was run at 0.8mA/cm^2 for 2 hours. After transfer was completed, immunodetection procedure was performed using hyperimmune anti rabbit sera against DV2 and amplified alkaline phosphatase goat anti-rabbit immunoblot assay kit (Bio-Rad). The reaction was stopped by washing the membrane with distilled water.

Preparation of sample for Edman sequencing

Western blotting was performed as per standard method.[20] Nova Blot Kit of Multiphore II Electrophoresis System (Pharmacia, Uppasala, Sweden) was used to transfer the protein to polyvinyldifluoride (PVDF) membrane (0.45 µm). To vasilitate the N-terminal sequencing process, the infected C6/36 cells were run together on SDS-PAGE with the molecular weight kit. After wards, the protein spots from SDS-PAGE were transferred to PVDF membrane and visualized with coomassie brilliant blue staining (Figure 2), then sent for N-terminal Edman degradation sequencing (Midwild Analytical, USA).

RESULTS

This investigation of dengue protein expression revealed four target protein spots on

DV2-infected C6/36 (Figure 1a), but not on uninfected C6/36 (Figure 1b). The result of immunoblotting and immunodetection of infected C6/36 cells showed the target protein spots (Figure 2a) were picked by antibody against DV-2 and were visualized as a purple color (Figure 2b). The molecular weight of the protein spots in infected C6/36 cells was 50 kDa in (Figure 3a) and immunoblotting of infected C6/36 cells onto PVDF membrane with coomassie blue staining for N-terminal sequencing purpose was showed Figure 3b. N-terminal sequencing determined the protein spots to be D-S-G-C-V-V-S-W-K-N-K (Midwild-Analytical,USA), which was identical to nonstructural glycoprotein NS1 (Swissprot-database) associated with the replication process of flaviviruses.

DISCUSSION

In this study, the aim of this study was to observe the protein expression of uninfected and infected C6/36 cells by DV2 through 2-DGE. This study found protein spots in DV2-infected C6/36 cells which were absent in uninfected C6/36 cells (Figure 1). Immunoblotting was done to prove the target protein spots originally came from DV2 infection. Based on Swiss-prot database, the 50 kDa protein spots turned out to have the following sequence: D-S-G-C-V-V-S-W-K-N-K, which was determined to be homologous to non structural NS1. Glycoprotein NS1, present in all flaviviruses, appears to be essential for virus viability. NS1 is produced both membrane-associated an secreted forms[5] both of which are highly immunogenic and can elicit high-titre antibody.[11] This is presumably because NS1 is present on the surface of infected cells and is efficiently secreted.[21] In the post genomic era, proteins are coming back into focus because it has been realized again than whole genome sequence information alone is not sufficient to explain and predict cellular phenomena, as it is largely the proteins that execute and control the majority of cellular activities. There are several reasons for focusing on the analysis of proteins, such as the level of mRNA expression frequently does not represent the amount of active protein in a cell[22] or the genome sequence does not describe post translational modification, which may be essential for protein function and activity. In this study, the protein spots did not migrate to the same level, probably due to the different glycosylation patterns.[13] NS1 protein expression through proteomics technique might be of use as a diagnostic marker and/or as a parameter for evaluation of the efficacy of antiviral agents in DV2 infection. The value of using NS1 as a surrogate marker of infection is supported by Young *et al* who assessed the potential of capture ELISA as a diagnostic assay[23] and Huang *et al* who showed high level expression of recombinant dengue viral NS1 protein used as a diagnostic antigen.[24] There are no known reports from other researchers on this protein using identical or similar techniques.

CONCLUSION

The application of NS1 separated by 2-DGE may provide an alternative approach for monitoring or as a parameter for evaluation the efficacy of anti-viral agents against DV2 infection/replication.

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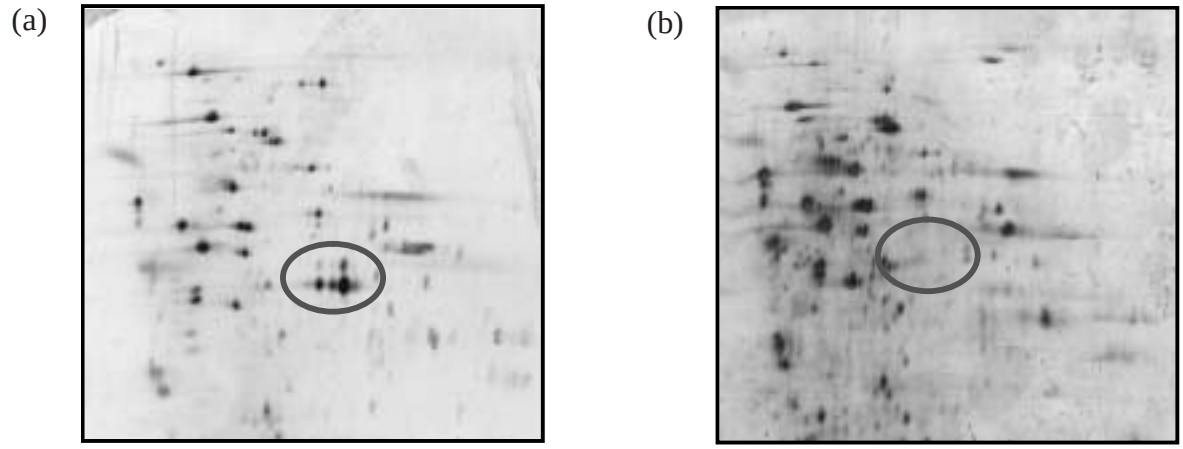

Figure 1. Protein expression of (a) Infected C6/36 cells with DV2 and (b) Uninfected C6/36 cells

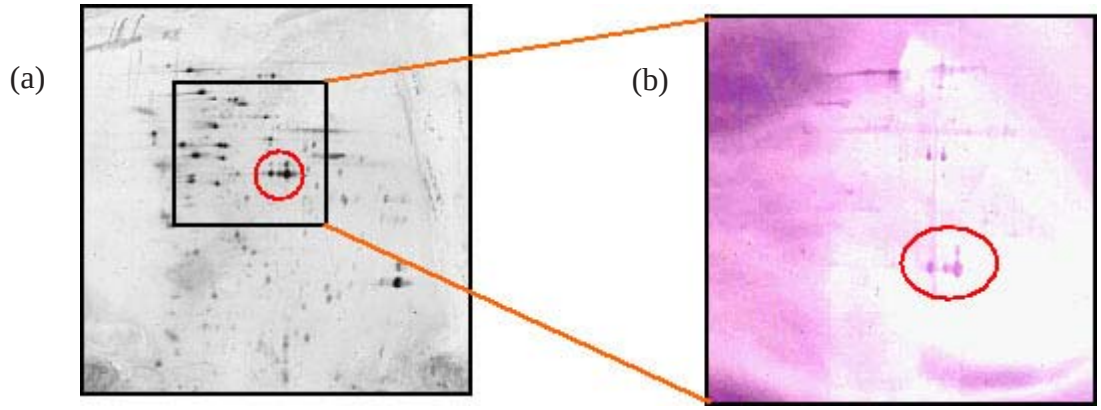

Figure 2. Protein expression of (a) Infected C6/36 cells with DV2 and (b) Immunoblotting and immunodetection of infected C6/36 cells with specific antibody against DV2 onto nitrocellulose membrane

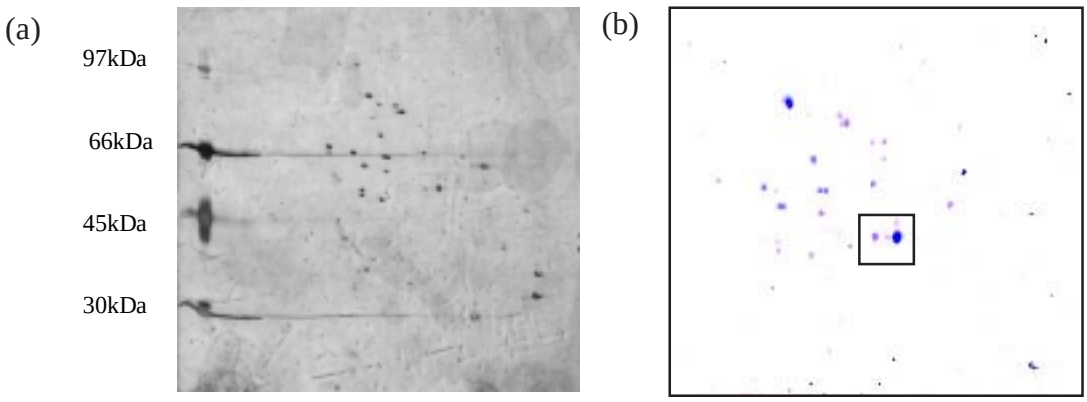

Figure 3. Protein expression of (a) Infected C6/36 cells with the molecular weight of target protein spots and (b) immunoblotting of infected C6/36 cells onto PVDF membrane with coomassie blue staining

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