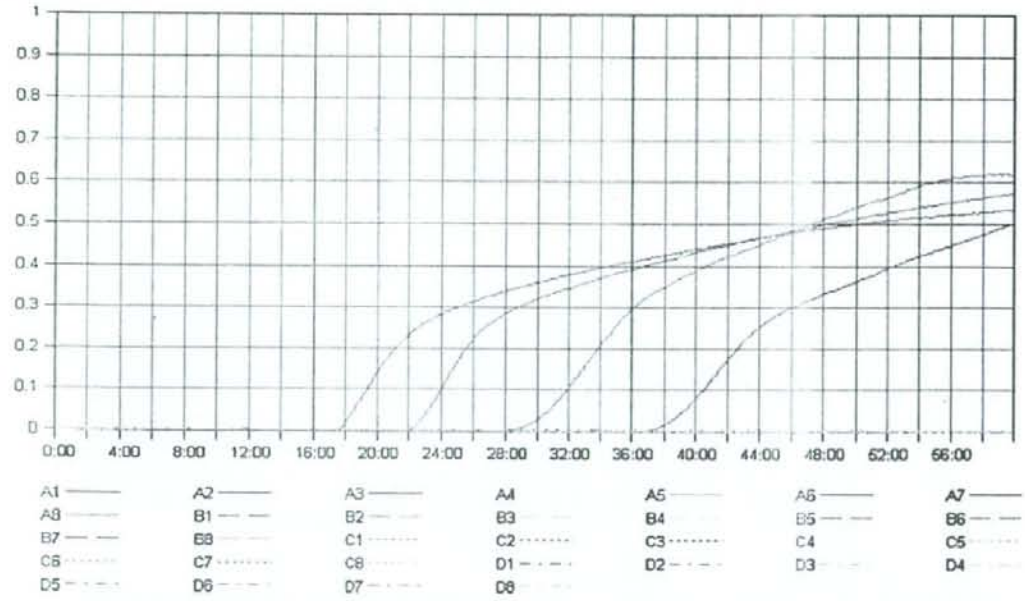

Figure 3. Real-Time monitoring of LAMP amplification showing the amplification curve. X-axis, depicts the time of positivity and Y-axis, shows the turbidity value in terms of O.D. at 400 nm

Rockland, ME, USA) in Tris-borate buffer followed by staining with ethidium bromide and visualisation on a UV transilluminator at 302 nm (Figure 4B).

Naked eye visualisation

In order to facilitate application of LAMP assay in the field, monitoring of amplification can also be carried out with naked eye inspection either in the form of visual turbidity or visual fluorescence.

Visual turbidity

The turbidity of magnesium pyrophosphate can be visually observed. Following amplification, the tubes can be inspected for white turbidity through naked eye after a pulse spin to deposit the precipitate in the bottom of the tube (Figure 4C) [3].

Visual fluorescence

The tube containing the amplified products can also be better visualised in the presence of fluorescent intercalating dye viz; ethidium bromide, SYBR Green I, Calcein, etc. by illuminating with a UV lamp. In practice, usually the visual inspection for amplification is performed through observation of colour change following addition of 1 µl of SYBR Green I (a fluorescent dsDNA intercalating dye) to the tube. In case of positive amplification, the original orange colour of the dye will change into green that can be judged under natural light as well as under UV light (302 nm) with the help of a hand held UV torch lamp. In case there is no amplification, the original orange colour of the dye will be retained. This change of colour is permanent and thus can be kept for record purposes (Figure 4D).

Calcein is another chelating fluorescent detection reagent that can be used in the reaction mixture prior to amplification. Calcein initially combines with manganese ions to achieve a quenching effect. The amplification generates the by-product, pyrophosphate ions, which will bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. From this feature, the presence of fluorescence can indicate the presence of target gene and visual

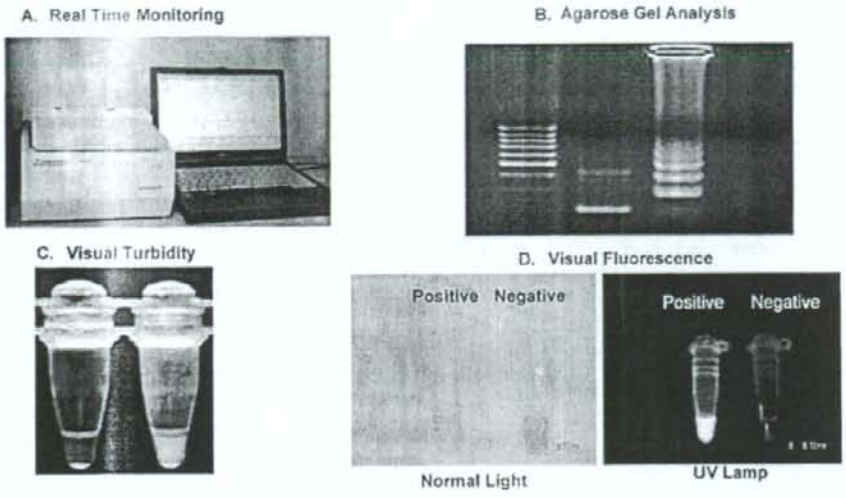

Figure 4. Monitoring of LAMP amplification. (A) The turbidity of magnesium pyrophosphate, a by-product of the reaction, can be detected real-time by a real-time turbidimeter. (B) Agarose gel analysis revealing the typical electrophoresis pattern of LAMP amplified product, which is not a single band but a ladder pattern because the LAMP method forms amplified products of various sizes consisting of alternately inverted repeats of the target sequence on the same strand. (C) Visual turbidity in the form of white precipitate as observed in positive control due to accumulation of magnesium pyrophosphate in proportion to the accumulated amplified products. (D) When the tube containing the amplified products incorporating a fluorescent intercalating dye is illuminated with a UV lamp, the fluorescence intensity increases

detection can be achieved without opening the tube, thus preventing carry-over contamination with post-amplification products.

Sequence specific visual detection of LAMP

In order to detect LAMP products in a sequence-specific manner visually, an extremely simple method was reported by adding a small amount of low-molecular weight polyethylenimine (PEI) to the LAMP reaction solution [7]. A characteristic of this technique is the ability to visually present sequence information of amplicons without using an expensive source of light or a detector. The new detection method described above utilises the unique nature of low-molecular-weight PEI, i.e. it cannot form an insoluble complex with a single-stranded anionic polymer with a low-molecular weight such as an oligo DNA probe, but it can form an insoluble complex with DNA with a high-molecular weight such as LAMP product. Since a large amount of amplification product is created by the LAMP reaction, precipitate of a size that can be easily confirmed with the eyes is generated when PEI is added to the LAMP reaction solution. Moreover, the fact that the amplification is highly efficient means that the amount of labelled probe for detection that can be added is large. As a result of these characteristics, the LAMP reaction followed by addition of PEI yields precipitate with a clear colour and in a size that can be identified visually. If the 5′ end of the internal primer is fluorescently labelled, the LAMP product should be visible, but this approach is not preferred, because the possibility of false positives from self-extension of the labelled primer cannot be excluded. There is no risk of false positives with the oligo DNA probes fluorescently labelled at the 3′ end, so that highly accurate genetic testing can be established. It is necessary to add PEI to the LAMP reaction solution after the LAMP reaction takes place since PEI strongly inhibits the LAMP reaction. However, opening the reaction tube after amplification should generally be avoided to prevent carry-over contamination.

Interpretation of results

Unlike real-time PCR assay, where the positivity is decided on the basis of C_t value, in the case of

LAMP the criterion of positivity is based on the time of positivity (T_p). This varies from virus to virus, based on the designed primer set and nature of the selected template. The cut-off value for positivity by real time RT-LAMP assay for a particular gene can be determined by taking into account the time of positivity (in minutes) at which the turbidity increases above the threshold value fixed at 0.1, which is two times more than average turbidity value of the negative controls of several replicates. In most LAMP amplifications, it is observed after 30–40 min in the amplification cycle.

On agarose gel analysis, the LAMP amplicons reveal a ladder-like pattern in contrast to a single band as observed in PCR. This is due to the cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand.

Sensitivity and specificity of LAMP amplification

In general, the LAMP assay was found to be 10–100 fold more sensitive than PCR with a detection limit of 0.01–10 pfu of virus [8–10]. The specificity of the LAMP amplification is directly attributed to the six sets of primers spanning eight distinct sequences of the target gene that are being used for amplification. Unless all the target genes are available, amplification will not proceed. The authenticity of the LAMP amplified product can be established by digesting with a restriction enzyme cutting a site at one end of the selected target. Further confirmation of the structures of the amplified products can also be accomplished through nucleotide sequencing of the amplified products.

Quantification of gene copy numbers by LAMP assay

The quantification of gene copy number and/or concentration of the viral nucleic acid can be accomplished through generation of a standard curve by plotting known concentration of gene copy number or infectious unit of virus against time of positivity to obtain the amplification signal for that particular concentration. A linear relationship between various concentrations against time of positivity is usually obtained through the real-time monitoring of the amplification (Figure 5). The quantification of gene copies in clinical samples can be extrapolated from a standard curve on the basis of their time of positivity [6].

Advantages of LAMP amplification

The primary characteristics of the LAMP are its ability to amplify nucleic acid under isothermal conditions in the range of 65°C; as a result it allows the use of simple and cost effective reaction equipment. The second characteristic is that LAMP has high specificity and high amplification efficiency. Its specificity is extremely high because it can amplify a specific gene from a human genome specimen discriminating a single nucleotide difference. The high amplification efficiency of LAMP is attributed to no time loss of thermal change because of its isothermal reaction. The reaction can be conducted under optimal temperature of the enzyme and the inhibition reaction at the later stage of amplification is less likely to occur compared with the PCR. It was observed that when nucleic acid is amplified by the LAMP method, the turbidity derived from the precipitate is produced according to the progress of the reaction and thus making it ideal for easy monitoring through naked eye.

In addition, both amplification and detection of gene can be completed in a single step, by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. It provides high amplification efficiency, with DNA being amplified 10^9–10^{10} times in 15–60 min. Because of its high specificity, the presence of the target gene sequence can easily be detected just by judging presence of amplified products. There is no need for a step to denature double stranded DNA into a single stranded form. LAMP assay has the great advantage of monitoring amplification by SYBR Green I dye mediated naked eye visualisation and by real-time monitoring by using an inexpensive turbid meter according to the situation. The particular importance is the substantial reduction in time required for the confirmation of results by RT-LAMP assay in 30 min as compared to 3–4 h in case of RT-PCR (Table 1).

Applications of LAMP assay

LAMP is a gene amplification method with a variety of characteristics and applications in a wide

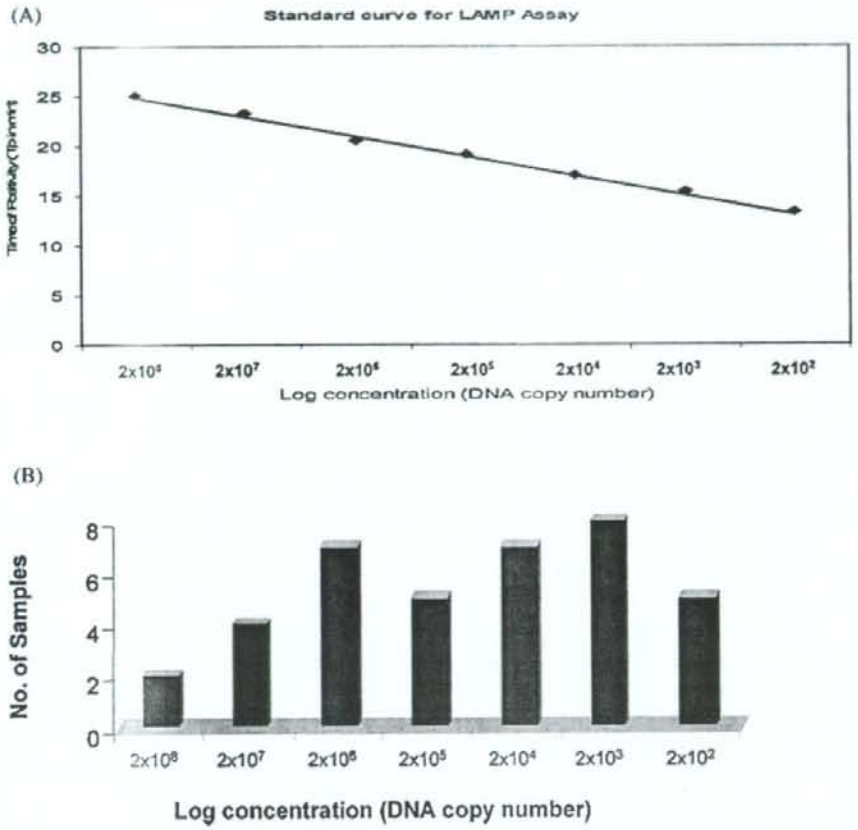

Figure 5. (A) Standard curve for LAMP assay generated from the amplification plots between 10 fold serially diluted plasmid construct and time of positivity (Tp). (B) Quantitative determination of virus concentration in clinical samples employing standard curve

range of fields, including clinical diagnosis, single nucleotide polymorphism (SNP) typing and quantification of template DNA. In particular, LAMP is considered to be effective as a gene amplification method for use in gene point-of-care testing (g-POCT) devices.

LAMP in clinical diagnosis

Although the inception of LAMP refers back to 1998, it became popular only after 2003 following the emergence of West Nile and SARS viruses. Since then LAMP assay has increasingly been adapted by researchers mostly from Japan for the clinical diagnosis of emerging diseases (Table 2).

LAMP has been successfully applied for rapid and real-time detection of both DNA and RNA viruses. However, most of the published research has been directed towards RNA viruses, maybe due to the increased incidence of RNA viruses in recent past in the form of major epidemics having significant public health importance. A one-step single tube real-time accelerated RT-LAMP assay for rapid detection of each of several recently emerged human viral pathogens viz; Dengue, Japanese Encephalitis, Chikungunya, West Nile, SARS, highly pathogenic avian influenza (HPAI) H5N1, Norwalk viruses have been developed and evaluated [8–15]. In comparison to conventional RT-PCR, RT-LAMP assay demonstrated

Table 1. Comparative advantages and disadvantages of different molecular diagnostic techniques

	Conventional PCR	Real-time PCR	LAMP
Advantage	• Alternate gold standard for isolation in absence of live agent • Early confirmatory diagnosis • Widely used molecular diagnostic format	• Simultaneous amplification and detection during exponential amplification • Real-time monitoring of amplification as it happens • Quantitative, thus useful for monitoring the viral load • Lower carry over contamination due to closed tube operation • Increased sensitivity due to fluorescent chemistry • High throughput analysis due to software driven operation	• Isothermal field-based gene amplification without requiring thermal cycler • Amplification can be accomplished with waterbath/heating block • Real-time as well as quantitative • Higher amplification efficiency and sensitivity • Naked eye visual monitoring either through turbidity or colour change by fluorescent intercalating dye (SYBR Green I)
Disadvantage	• Qualitative (Yes or No format) • End-point detection in plateau phase with non spurious amplification • Post-PCR handling leading to carry over contaminations • Less sensitive, thereby missing borderline cases with low gene copy numbers • Time consuming (3–4 h) • Requirement for thermal cycler and gel documentation system	• Expensive detection equipments and consumables • Requirement for fluorescent probe • Restricted to referral laboratory with good financial support	• Complicated primer design (requirement for six primers) • Two long primers of HPLC grade purity • Restricted availability of reagents and equipment in some countries • Laboratory based

10–100 fold more sensitivity with a detection limit of 0.01–10 pfu of virus in all these cases.

The usefulness of LAMP for amplification of DNA viruses was also reported for HPV (Human papillomavirus) type—6, 11, 16 and 18, HSV, VZV, CMV, Adenovirus and BK virus (Table 2) and found to be superior in terms of sensitivity, specificity, rapidity and simplicity, and can potentially be a valuable tool for the detection of HPV DNA compared to PCR and real-time PCR [16–27].

Among the animal viruses, a one-step, RT-LAMP assay was reported for detection of Foot and Mouth Disease virus (FMDV) in less than 1 h in a single tube without thermal cycling [28]. A fragment of the 3D RNA polymerase gene of the virus is amplified at 65°C in the presence of a

Table 2. Applications of LAMP to the rapid detection of viral diseases of humans and animals

Host	Virus	References
Human		
DNA	Adenoviral keratoconjunctivitis	[26]
	Human papillomavirus type 6, 11, 16 and 18	[16]
	Varicella-zoster virus	[24]
	Herpes simplex virus and varicella-zoster virus	[17]
	Cytomegalovirus	[25]
	Herpes simplex virus	[18,19]
	Human herpesvirus 6	[20,21]
	Human herpesvirus 7	[22]
	Human herpesvirus 8	[23]
	BK virus	[27]
RNA	Severe acute respiratory syndrome (SARS) coronavirus	[10]
	West Nile virus	[9]
	Japanese encephalitis virus	[11,12]
	Norovirus	[15]
	H5 avian influenza virus	[14]
	Chikungunya virus	[13]
	Dengue viruses (1,2,3 & 4)	[8]
Animal		
DNA	Plum pox virus	[33]
	Newcastle disease virus	[29]
RNA	Canine distemper virus	[30]
	Canine parvovirus	[31]
	Foot-and-mouth disease virus	[28]
	Viral haemorrhagic septicaemia virus (VHS)	[32]

primer mixture and both reverse transcriptase and *Bst* DNA polymerase. Compared with real-time PCR, RT-LAMP was consistently faster, and ten copies of FMDV transcript were detected in 22 min. LAMP has also been utilised for diagnosis of Newcastle disease, Canine distemper, Canine parvo, Viral haemorrhagic septicemia virus and Plum pox viruses [29–33].

LAMP in SNP typing

LAMP-based SNP typing is an accurate, rapid and simple method that may be useful especially for point-of-care testing. Because of the high specificity of the LAMP method, only the target gene will be amplified from gene samples containing homologous nucleotide sequences when using LAMP-based SNP typing [34,35]. Furthermore, because of the characteristics of its amplification reaction, the LAMP method discriminates a single nucleotide difference at each cycling step of the DNA replication, through both 'sense and antisense strand' reactions, and the type of SNP can easily be detected just by amplifying the DNA containing SNP in a single step. Due to the simplicity and rapidity of the LAMP method, simple detection of SNP typing can be achieved within 30 min. The products of the LAMP reaction, which was performed in the presence of an intercalating dye, were detected within 30 min without any post-reaction sample manipulation. With the use of four primers designed to recognise six distinct regions, only the target gene is strictly and specifically amplified even in coexistence with its homologous gene. The reaction is so specific as to strictly discriminate single nucleotide difference.

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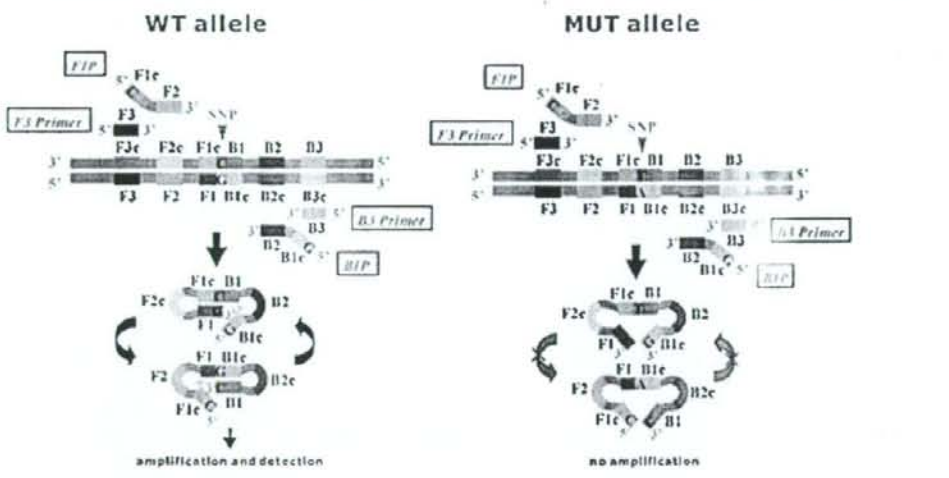

Figure 6. The basic principles of mutant detection using Wild Type (WT) primers. The FIP and BIP are designed to contain a SNP nucleotide (in this case of Wild Type allele) at 5′ end, respectively. Using the WT primers, when the target gene is the WT allele, DNA synthesis from dumbbell-like starting structure proceeds and the LAMP amplification cycling continues. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan.

The basic principles of LAMP-SNP typing rely on the use of wt primers. The FIP and BIP are designed to contain a SNP nucleotide (wt allele) at 5′ end. Using the wt primers, when the target gene is the wt allele, DNA synthesis from dumbbell-like starting structure proceeds and the LAMP amplification cycling continues (Figure 6). In contrast, when the target gene is the mutant (MUT) allele, no DNA synthesis proceeds from the dumbbell-like structure and the LAMP amplification cycling does not occur. Even if DNA synthesis proceeds for one step due to miscopy, the amplification reaction is either halted in other steps or is delayed since repetition of this reaction continually checks at each cycling step of the DNA replication. By simply incubating genomic DNA and reagents, including fluorescent detection reagent, at a constant temperature (60°C) for a fixed period of time, SNP typing can be achieved by determining whether amplification has taken place.

FUTURE DIRECTIONS

LAMP has all the characteristics required of real-time assays (high sensitivity, quantitative) along with simple operation for easy adaptability to field conditions. The combination of the LAMP technology and the new detection method described here can overcome several factors that have been preventing true practical application of simple g-POCT. The integration of isothermal amplification and electrophoresis onto microchips could lead to LAMP on Chips for quick and accurate identification of disease producing genes at the patient's bed side. Thus LAMP is considered to be effective as a gene amplification method for g-POCT devices, which can be used for simple genetic testing whenever and wherever necessary. If these characteristics of the LAMP method are used effectively, it should be possible to develop simple genetic testing devices that have not been realised yet despite a strong awareness of their necessity, in a wide range of fields, including infectious disease testing, food inspection and environmental testing.

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Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga

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ABSTRACT

A sulfated polysaccharide, named fucoidan, from the marine alga *Cladosiphon okamuranus* is comprised of carbohydrate units containing glucuronic acid and sulfated fucose residues. Here we found this compound potently inhibits dengue virus type 2 (DEN2) infection. Viral infection was inhibited when DEN2, but not other serotypes, was pretreated with fucoidan. A carboxy-reduced fucoidan derivative in which glucuronic acid was converted to glucose did not inhibit viral infection. Elimination of the sulfated function group from fucoidan significantly attenuated the inhibitory activity on DEN2 infection with <1% fucoidan. DEN2 particles bound exclusively to fucoidan, indicating that fucoidan interacts directly with envelope glycoprotein (EGP) on DEN2. Structure-based analysis suggested that Arg323 of DEN2 EGP, which is conformationally proximal to one of the putative heparin binding residues, Lys310, is critical for the interaction with fucoidan. In conclusion, both the sulfated group and glucuronic acid of fucoidan account for the inhibition of DEN2 infection.

Dengue virus is an envelope virus that causes human diseases, such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. There are four serotypes of dengue virus, type 1 (DEN1) to type 4 (DEN4), which have similar clinical manifestations and epidemiology in tropical and subtropical regions of the world where more than two billion people are at risk of infection [1–3]. The viruses are circulated and amplified by transmission to humans through *Aedes* mosquitoes [4]. The adsorption of viruses to the host cell surface is the initial and critical step for viral infection. Envelope glycoprotein (EGP) is a viral membrane protein involved in the early events of dengue virus infection, such as binding of the virus to the host cell surface and fusion between viral and host cell membranes [5,6]. EGP consists of three functional domains (domains I, II and III). Domain III is critical for virus adsorption to host cell receptors [7]. Recent X-ray crystallography and NMR studies demonstrated the three-dimensional structures of EGP or domain III of flaviviruses, dengue virus type 2, 3 and 4 [8–10]. Putative receptor molecules for dengue viruses have been reported, such as sulfated proteoglycans. Sulfated and non-sulfated carbohydrate molecules on the surface of host cells seem to be involved in the interaction with flaviviruses [11,12]. The receptor molecules used for binding and entry of dengue viruses are apparently distinct between cell types and virus serotypes [13,14]. However, the molecular features of cellular receptors and the molecular mechanisms of virus entry have yet to be fully elucidated.

Dengue virus belongs to the family Flaviviridae the same family as Japanese encephalitis and yellow fever viruses, which are controlled by specific vaccinations. However, no licensed dengue vaccines or anti-dengue agents are clinically available.

Fucoidans are sulfated polysaccharides extracted from marine brown seaweeds that possess some biological activities similar to those of heparin [15,16]. Previous studies have shown that fucoidan mediates significant biological effects on mammalian cells. Particularly, fucoidans from brown seaweeds show anti-inflammatory and anti-coagulant activities [16]. These fucoidans show the anti-viral effects against infectious diseases, such as human immunodeficiency virus (HIV), herpes simplex virus and cytomegalovirus [17]. Recently, it was reported that sulfated polysaccharides from the red seaweeds and sulfated galactomannans from seeds of *Mimosa scabrella* inhibit *in vitro* and *in vivo* infection of flaviviruses, such as dengue and yellow fever viruses [18,19]. As these polysaccharides show no toxicity or irritation in humans, fucoidans may useful as anti-viral agents as well as anti-coagulant and anti-inflammatory agents. However, there have been very few studies of relationships between the biological activities of fucoidans and molecular structures.

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The chemical structure of the *Cladosiphon* fucoidan has recently been described in detail [20,21]. The *Cladosiphon* fucoidan has one sulfate group for every two molecules of fucose. Furthermore, this fucoidan has one glucuronic acid residue for every six molecules of fucose as a branched chain.

In the present study, we examined the anti-dengue virus activity of fucoidan from the marine alga, *Cladosiphon okamuranus*. We also investigated molecular mechanisms of susceptibility of four serotypes of dengue virus to the fucoidan by structure-based analyses.

Materials and methods

Materials. Fucoidan and its derivatives were provided by Yakult Central Institute for Microbiological Research, Tokyo, Japan. These compounds were prepared as described previously [20]. All other chemicals were of the highest quality commercially available.

Cell culture and viruses. BHK-21 was cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 5% FBS. Dengue virus type 1 (DEN1), D1/Lao/03 strain, type 2 (DEN2), ThNH-7/93 strain (GenBank Accession No. U31949), type 3 (DEN3), D3/BDH02-01 strain (Accession No. AY496871), and type 4 (DEN4), ThD4-17/97 (Accession No. AY618989) were propagated in C6/36 cells as described previously [22].

Inhibition of virus infection by fucoidan and its derivatives. Virus infection was determined by focus-forming assay using BHK-21 cells as described previously [12]. BHK-21 cells were seeded onto 96-well plates and cultured for 24 h at 37 °C in DMEM supplemented with 2% FBS. After removal of medium, the virus–fucoidan premixtures were then inoculated for 2 h at 37 °C onto the cells. After washing with serum-free DMEM, overlay medium was added and plates were incubated at 37 °C for 43 h. Infectious foci were detected with human anti-dengue antisera, followed by HRP-conjugated goat anti-human immunoglobulin. Virus infectivity was determined as focus-forming units (FFU). The optimal titer of inoculated virus was predetermined such that more than 50 foci appeared per well.

Solid-phase virus-binding assay. The binding activities of DEN2 for fucoidan and its derivatives in solid-phase virus-binding assay were evaluated as described previously [23]. Briefly, fucoidan or its derivatives in phosphate-buffered saline were immobilized on wells of plastic plates (Universal BIND 1 × 8 Stripwell; Corning Inc., Corning, NY) by UV-crosslinking. After blocking with PBS containing 1% BSA, the plates were incubated for 1 h at 28 °C with virus solution. After washing, the plates were incubated for 1 h at 28 °C with human anti-dengue antiserum, followed by HRP-conjugated goat anti-human immunoglobulin. The complexes were detected by incubation with *o*-phenylenediamine. The absorbance was measured at 492 nm.

Preparation and cellular binding of DEN2 labeled with a fluorescence dye, DiO. A lipophilic dye, DiO (3,3′-dilinoleyloxacarbocyanine perchlorate, *FAST* DiO™; Molecular Probes, Eugene, OR) was used to label virus particles as described previously [24]. All procedures for labeling of the virus were carried out without light. The virus (7.0×10^6 FFU/ml) was incubated at room temperature for 10 min in VP-SFM (Invitrogen, Carlsbad, CA) containing 6.4 μM *FAST* DiO. The labeling solution contained 8% PEG 6000 and 2.2% NaCl at the final concentration. The solution was then kept at 4 °C overnight. The labeled virus was sedimented (8700g) at 4 °C for 1 h and resuspended in PBS. The labeled virus was stored at −80 °C before use.

Direct binding activity of labeled dengue viruses to cultured cells was performed as follows. BHK-21 cells were seeded onto 96-well plates and cultured at 37 °C in DMEM supplemented with 10% FBS. After blocking with DMEM containing 2% BSA, the plates were incubated at 4 °C for 2 h in DMEM containing dengue virus (10^6 FFU/ml). The bound virus was lysed at room temperature for 10 min with 1% Triton X-100 solution. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission). The virus-binding activity was determined from the quantity of DiO associated with the cell surface.

Sequencing of dengue virus cRNA. A fragment of domain III of the DEN1 EGP gene was reverse transcribed and amplified from RNA extracted from the purified virus using *Taq* DNA polymerase, and the PCR products were directly sequenced.

Homology modeling. The Swiss-Model automated comparative protein modeling server (http://swissmodel.expasy.org//SWISS-MODEL.html) [25] was used for comparative structural analysis to model three structures of DEN1 (D1/Lao/03 strain), DEN2 (ThNH-7/93 strain) and DEN4 (ThD4-17/97) domain III onto those of DEN3 (PDB ID: 1UZG) [9], DEN2 (PDB ID: 1OKE) [6] and DEN4 (PDB ID: 2H0P) [10] proteins, respectively. DEN3 (D3/BDH02-01 strain) domain III is shown on the basis of the structure of PDB accession number 1UZG. VMD 1.8.6, OpenGL [26] tools running on UNIX were used to visualize all figures.

Results and discussion

Anti-dengue virus effects of fucoidan and its derivatives

Although previous studies indicated that sulfated polysaccharides from other natural sources showed anti-dengue virus activity, the molecular mechanisms of the inhibitory effects of these compounds have not been elucidated [18,19]. Fucoidan from the marine alga *C. okamuranus* was used for viral infection assay in the present study. This fucoidan was chosen as an anti-viral agent for the following reasons. Recently the carbohydrate structure has been defined well. The *Cladosiphon* fucoidan is comprised of a repeating unit of sulfated fucose and glucuronic acid residues [20]. In addition, its derivatives have been generated by chemical modifications [20], such as elimination of the sulfated group or reduction of carboxylic acid resulting in a desulfated derivative termed FD and a carboxy-reduced derivative termed FC, respectively (Fig. 1A). Fucan, a fucose polymer, was used for control experiments. Similar to other fucoidans, the *Cladosiphon* fucoidan mediates a variety of biological effects on mammalian cells. This fucoidan was more effective for healing of gastric ulcers than that from *Fucus vesiculosus* [21]. The *Cladosiphon* fucoidan also showed an inhibitory effect on the adhesion of *Helicobacter pylori* to carbohydrate ligands [27]. In dengue virus infection, the *Cladosiphon* fucoidan significantly inhibited DEN2 infection to BHK-21 cells in a dose-dependent manner (Fig. 1B). Treatment of the virus with 10 μg/ml fucoidan reduced the infectivity by 20% compared with that in untreated cells. The inhibitory activity of fucoidan is equivalent with that of heparin, which is a competitive entry inhibitor, as described previously [11,28]. Three types of fucoidan derivative were simultaneously examined for effects on infection of BHK-21 cells with DEN2. Sulfation is required for anti-dengue virus activity of glycan [28]. As expected, desulfation from fucoidan (FD or fucan) showed marked suppression of inhibitory activity (Fig. 1B). Interestingly, carboxy-reduction knocked out the effect of fucoidan against DEN2 infection. These findings strongly suggest that the glucuronic acid residue as well as sulfated fucose are essential for the inhibitory activity of fucoidan. Four dengue virus serotypes were premixed with fucoidan at various concentrations, and the premixtures were inoculated onto BHK-21 cells. The results are summarized in Table 1. The effects of fucoidan on DEN2 infection were much greater than those on the other dengue serotypes examined. Particularly, fucoidan did not inhibit DEN1 infection of BHK-21 cells under the experimental conditions sued here. This observation strongly suggested that the

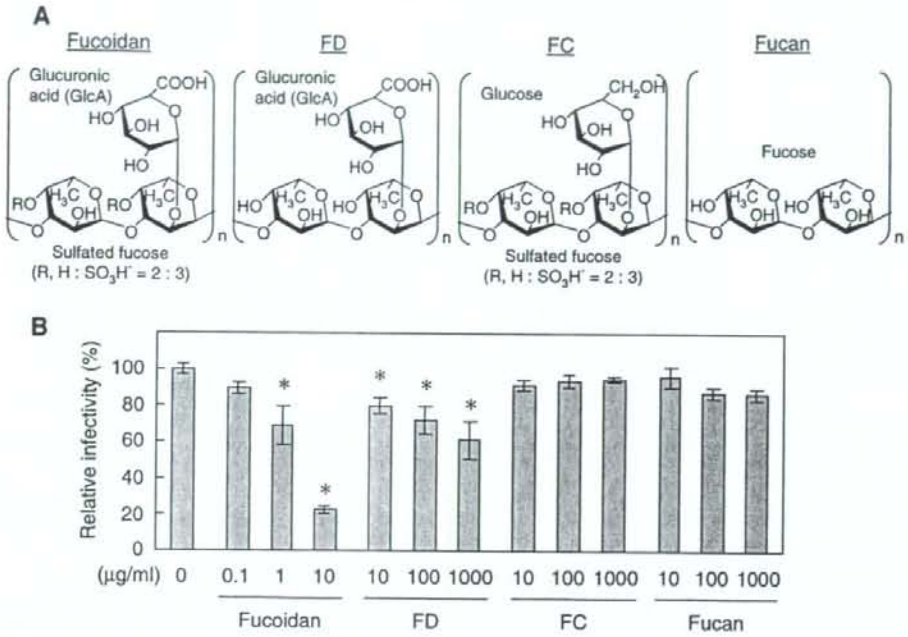


Fig. 1. Structures of fucoidan from *Cladosiphon okamuranus* and its derivatives used in this study. (A) Structures showing the repeating unit of polysaccharides. FC, a derivative prepared by reduction of the glucuronic acid residue in fucoidan; FD, a derivative obtained by elimination of the sulfated group in fucoidan; Fucan, fucose polymer. (B) Effects of fucoidan and its derivatives on DEN2 infection. Values indicate the averages of infectivity ratios of DEN2 with polysaccharides at the indicated concentrations relative to viral infection alone. Bars show standard deviation of triplicate measurements. Statistical significance was determined by *t*-test ($^{*}P < 0.01$). The results shown are representative data from three independent experiments.

Table 1
Effect of fucoidan on viral infection

Virus (serotype)	IC_{50} (μg/ml)
DEN1	>1000
DEN1	4.7
DEN1	500
DEN1	365

IC_{50}, concentration of 50% of inhibition. Infection experiments were performed as described in Materials and methods.

inhibitory action of fucoidan on viral infection depends on distinction of EGP structures based on amino acid residues that may influence interaction of the virus with fucoidan.

Binding activity of DEN2 to fucoidan and its derivatives

To clarify the structural determinant responsible for the interaction with DEN2, we examined DEN2 binding to fucoidan and its derivatives by solid-phase virus-binding assay. Previously, we established a direct binding assay with carbohydrate molecules for influenza viruses [23]. In the present study, we applied the assay for determination of the direct binding dynamics of fucoidan to DEN2. Fig. 2A shows the results of solid-phase virus-binding assay. The virus particles showed significant binding activity to native fucoidan immobilized on plastic plates in a dose-dependent manner, but did not bind to other derivatives. This observation clearly indicated that both glucuronic acid and sulfated fucose residues were involved in the interaction with DEN2.

In addition, we examined the effects of fucoidan and its derivatives on the direct binding of DEN2 to BHK-21 cells. In accordance with a previous report by van der Schaar et al. [24], the virus particles were labeled with the fluorescent probe, DiO. The labeled DEN2 was used for determination of cellular binding activity. Fig. 2B shows binding of the labeled virus to the cells in the presence or absence of compounds. Although fucoidan marginally inhibited the cellular binding of DEN2, the inhibition was dose-dependent. Heparin as a positive control also showed inhibition of virus binding. In comparison to infection experiments, the inhibitory activity of the virus binding was apparently lower than expected (see Fig. 1B). As fucoidan is thought to inhibit virus binding to the cells in a competitive manner, lower activity may be observed when fucoidan was used at the same concentration as in the infection experiments. On the other hand, fucoidan derivatives such as FD or FC did not inhibit binding of DEN2 to BHK-21 cells. This result strongly suggested that both glucuronic acid and sulfated fucose residues accounted for inhibition of the virus binding to BHK-21 cells. Taken together with the findings of infection experiments, both glucuronic acid and sulfated fucose residues of the *Cladosiphon* fucoidan appear to critically affect the interaction of DEN2 with cellular receptors.

Sequencing and modeling analyses of dengue virus types 1–4

Of the dengue virus serotypes, the DEN2 strain ThNH-7/93 was highly susceptible to the *Cladosiphon* fucoidan. The inhibitory effect of fucoidan on ThNH-7/93 infection was almost 100-fold greater than that on infection by DEN3 or DEN4 strains (Table 1). Another strain, DEN1 (D1/Lao/03), showed no susceptibility to fucoidan under our experimental conditions. To elucidate the molecular basis of susceptibility of the *Cladosiphon* fucoidan, the

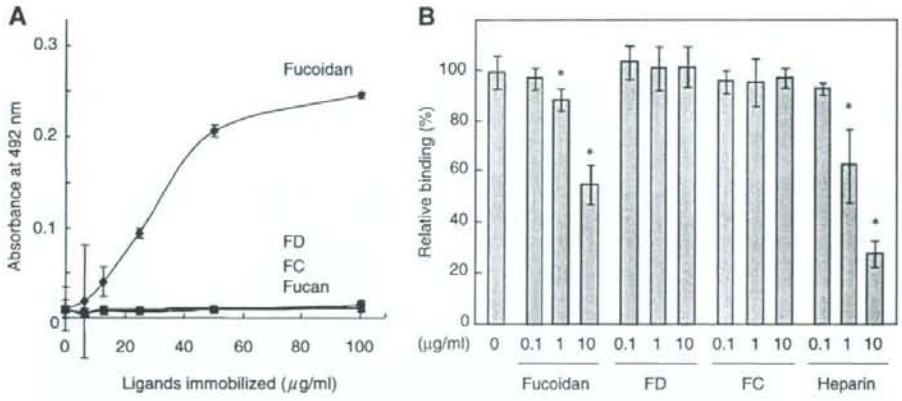
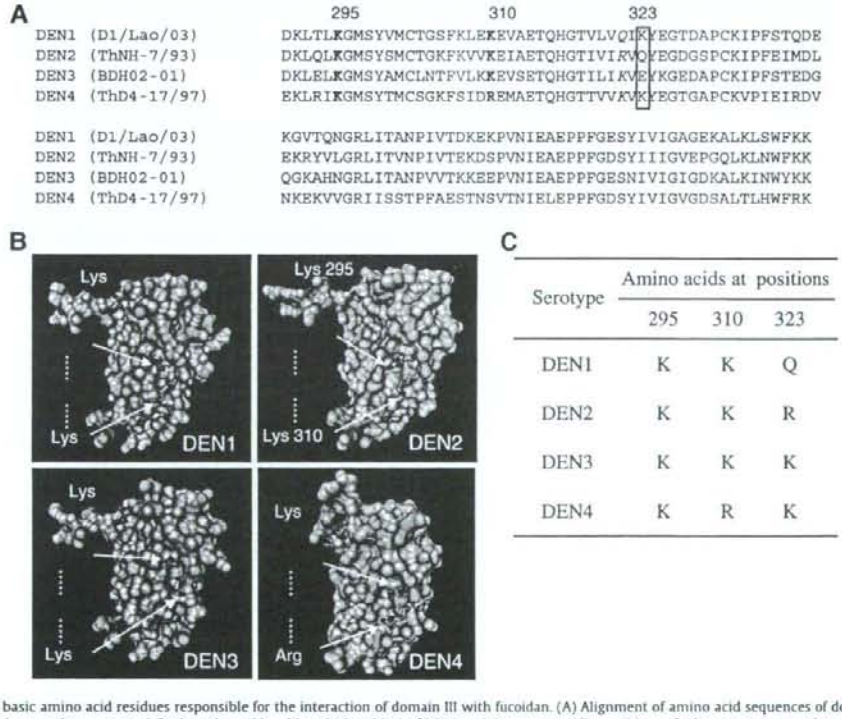


Fig. 2. Functional interaction of fucoidan with DEN2 particles. (A) DEN2 binding to fucoidan and its derivatives immobilized on plastic plates. Wells were coated with fucoidan (diamonds), FD (squares), FC (triangles), and fucan (circles) at the indicated concentrations. Values indicate the averages of viruses bound to each sample. Bars show standard deviation of triplicate measurements. The results shown are representative data from three independent experiments. (B) Inhibitory activity of fucoidan on DEN2 binding to BHK-21 cells. Values indicate the averages of cellular binding ratios of DEN2 with polysaccharides at the indicated concentrations relative to virus alone. Bars show standard deviation of triplicate measurements. FC, a derivative prepared by reduction of the glucuronic acid residue in fucoidan; FD, a derivative obtained by elimination of the sulfated group in fucoidan. Heparin was used as a positive control. Statistical significance was determined by *t*-test (*$P < 0.01$). The results shown are representative data from three independent experiments.

nucleotide sequence of DEN1 EGP was examined and multiple-alignment analysis of the amino acid sequences of the proteins of the four-serotype strains was performed (Fig. 3A). Basic amino acid residues at positions 295 and 310 are critically involved in the interaction with sulfated glycosaminoglycans such as heparin and highly sulfated heparan sulfate, as described previously [29].



Serotype	Amino acids at positions		
	295	310	323
DEN1	K	K	Q
DEN2	K	K	R
DEN3	K	K	K
DEN4	K	R	K

Fig. 3. Prediction of basic amino acid residues responsible for the interaction of domain III with fucoidan. (A) Alignment of amino acid sequences of domain III of envelope glycoprotein among dengue virus types 1–4. Basic amino acid residues 295 and 310 of DEN2 and the corresponding residues of other serotypes are shown in bold. Amino acid residue 323 (boxed) may be critical for the interaction with fucoidan. (B) The structures of domain III are shown on the basis of the three-dimensional structures. Distances between nitrogen atoms of side chains of Lys310 (Arg310 in DEN4) and Arg323 in DEN2 (Gln323 in DEN1, and Lys323 in DEN3 and DEN4) are shown as red dotted lines. (C) Summary of consensus amino acids at positions 295, 310 and 323 of domain III of the four serotypes. Search of protein database was carried out using protein query of each serotype domain III by blastp algorithms.

Both basic amino acid residues (Lys295 and Lys or Arg310) were conserved in all strains examined. The results of our infection and binding experiments clearly demonstrated distinct susceptibilities to fucoidan among dengue serotypes. Fucoidan strongly inhibited ThNH-7/93 infection and binding to BHK-21 cells. DEN 3 and 4 strains were moderately susceptible to fucoidan. On the other hand, fucoidan did not affect D1/Lao/03 infection. In addition, our structure-based experiment demonstrated that glucuronic acid residues are one of the critical determinants for fucoidan function. These findings strongly suggest that some basic amino acid residues on ThNH-7/93, but not DEN1 strain, may account for susceptibility to fucoidan. One putative candidate amino acid was identified in the EGP domain III region between the four-serotype strains. As the positive charge of arginine is greater than that of lysine, the basic amino acid residue at position 323 may contribute to the interaction of the virus with the glucuronic acid residue of fucoidan. Substitution of arginine (or lysine) to glutamine at position at 323 in the EGP domain III may diminish the interaction of D1/Lao/03 with glucuronic acid (carboxylic acid) residues. The locations of three amino acid residues at positions 295, 310, and 323 in domain III were estimated in homology-modeled structures of four-serotype strains generated based on the domain III structures reported previously [8–10]. In Fig. 3B, the overall structures of the domains are similar. The amino acid sequence of domain III of D3/BDH02-01 is identical to that of DEN3 protein (PDB accession number 1UZG). Therefore, other domain III proteins were modeled on the basis of the three-dimensional structures, as reported previously. In all structures, the amino acid residue at position 323 is located between residues at 295 and 310. The distance between Arg323 and Lys310 in DEN2 domain III is closer to those between Lys323 and Lys310 or Arg310 in DEN3 or DEN4, respectively. This prediction also suggested that the packed positive charges on domain III enhance the interaction with negative charges of fucoidan. Protein database search by blastp algorithms demonstrated that the three amino acid residues at positions 295, 310 and 323 of domain III were conserved among all strains of each serotype registered (Fig. 3C), meaning that the amino acid residue at position 323 accounts for susceptibility of dengue virus serotypes to fucoidan. Taken together, these observations strongly suggest that not only substitution of Arg (or Lys) to Gln at position 323 but also the distance from position 310 may cooperatively contribute to the susceptibility of dengue virus to fucoidan.

In the present study, we determined unique carbohydrate determinants involved in anti-dengue virus activity of fucoidan from the marine alga *C. okamuranus*. Using structure-based analyses, we also elucidated the molecular mechanisms of the susceptibilities of four dengue virus serotypes to the fucoidan. Information on these carbohydrate residues may facilitate the development of effective anti-dengue virus agents.

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PLoS NEGLECTED TROPICAL DISEASES

Protective and Enhancing HLA Alleles, HLA-DRB1*0901 and HLA-A*24, for Severe Forms of Dengue Virus Infection, Dengue Hemorrhagic Fever and Dengue Shock Syndrome

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Abstract

Background: Dengue virus (DV) infection is one of the most important mosquito-borne diseases in the tropics. Recently, the severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), have become the leading cause of death among children in Southern Vietnam. Protective and/or pathogenic T cell immunity is supposed to be important in the pathogenesis of DHF and DSS.

Methodology/Principal Findings: To identify HLA alleles controlling T cell immunity against dengue virus (DV), we performed a hospital-based case control study at Children's Hospital No.2, Ho Chi Minh City (HCMC), and Vinh Long Province Hospital (VL) in Southern Vietnam from 2002 to 2005. A total of 211 and 418 patients with DHF and DSS, respectively, diagnosed according to the World Health Organization (WHO) criteria, were analyzed for their characteristic HLA-A, -B and -DRB1 alleles. Four hundred fifty healthy children (250 from HCMC and 200 from VL) of the same Kinh ethnicity were also analyzed as population background. In HLA class I, frequency of the HLA-A*24 showed increased tendency in both DHF and DSS patients, which reproduced a previous study. The frequency of A*24 with histidine at codon 70 (A*2402/03/10), based on main anchor binding site specificity analysis in DSS and DHF patients, was significantly higher than that in the population background groups (HCMC 02-03 DSS: OR = 1.89, P = 0.008, DHF: OR = 1.75, P = 0.033; VL 02-03 DSS: OR = 1.70, P = 0.03, DHF: OR = 1.46, P = 0.38; VL 04-05 DSS: OR = 2.09, P = 0.0075, DHF: OR = 2.02, P = 0.038). In HLA class II, the HLA-DRB1*0901 frequency was significantly decreased in secondary infection of DSS in VL 04-05 (OR = 0.35, P = 0.0025, Pc = 0.03). Moreover, the frequency of HLA-DRB1*0901 in particular was significantly decreased in DSS when compared with DHF in DEN-2 infection (P = 0.02).

Conclusion: This study improves our understanding of the risk of HLA-class I for severe outcome of DV infection in the light of peptide anchor binding site and provides novel evidence that HLA-class II may control disease severity (DHF to DSS) in DV infection.

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Introduction

Dengue virus (DV) infection has become one of the most important mosquito-borne diseases in the tropics [1]. An estimated 50 million people worldwide are infected with DV each year [2]. There are two principal severe forms of DV infection, namely dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Other severe forms such as encephalitis and spastic tetraparesis occur less frequently [3]. Children younger than 15 years of age account for 90% of the severe cases in Southeast Asian countries [4] where the DHF/DSS incidence has increased about 5 fold more rapidly since 1980 than in the previous 30 years [5]. These severe forms of DV infection have become the leading cause of death among children in Southern Vietnam [6].

Infection by any of the 4 serotypes of DV, DEN-1, -2, -3, and -4, may result in no symptoms, dengue fever (DF, without any serious complications), DHF or DSS. DF is characterized by high fever; severe headache; retro-orbital, muscle, bone or joint pains;

Author Summary

Dengue has become one of the most common viral diseases transmitted by infected mosquitoes (with any of the four dengue virus serotypes: DEN-1, -2, -3, or -4). It may present as asymptomatic or illness, ranging from mild to severe disease. Recently, the severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), have become the leading cause of death among children in Southern Vietnam. The pathogenesis of DHF/DSS, however, is not yet completely understood. The immune response, virus virulence, and host genetic background are considered to be risk factors contributing to disease severity. Human leucocyte antigens (HLA) expressed on the cell surface function as antigen presenting molecules and those polymorphism can change individuals' immune response. We investigated the HLA-A, -B (class I), and -DRB1 (class II) polymorphism in Vietnamese children with different severity (DHF/DSS) by a hospital-based case-control study. The study showed persons carrying HLA-A*2402/03/10 are about 2 times more likely to have severe dengue infection than others. On the other hand, HLA-DRB1*0901 persons are less likely to develop DSS with DEN-2 virus infection. These results clearly demonstrated that HLA controlled the susceptibility to severe forms of DV infection.

and rash. DHF is characterized by the development of plasma leakage near the time of defervescence, thrombocytopenia, coagulation abnormalities, and hemorrhage. Hypotension with massive hemorrhage and plasma leakage characterize the most serious form of DHF called DSS [3]. DHF in this study corresponds to a milder form of DHF that WHO criteria define as grade I and II, and DSS to a grade III and IV DHF. The four criteria in the WHO case definition for DHF (fever, hemorrhage, thrombocytopenia and plasma leakage) were claimed not so practical because serious cases sometimes lack one or more of them, particularly hemorrhage and thrombocytopenia. Instead the Integrated Management of Childhood Illness (IMCI) has recently proposed the use of the terms "dengue" and "severe dengue" for the symptomatic patients with no emphasis on those two signs [7]

The pathogenesis of DHF/DSS is not yet completely understood. Host immunity has been extensively analyzed, including studies of antibody-dependent enhancement (ADE) [8], complement activation [8], anti-platelet antibodies [8], suppressed Th1/predominant Th2 functions [4] and the production of cytokines [9,10] and cytotoxic factors [4]. Virus factor has also been studied. DEN-2 viral strains were associated with DHF/DSS in Thailand and Vietnam; DEN-3 strains have been predominant in the DHF/DSS in Sri Lanka, Indonesia and India; while in Latin America, DEN-2,-3,-4 have all been commonly associated with severe disease [8,11]. In addition to those, still unidentified variations in immune response, virus virulence, and host genetic background are considered to be risk factors contributing to disease development [1].

Several genetic factors potentially influencing the severity of dengue infection, such as vitamin D receptor (VDR), Fcγ receptor II (FcγRII), interleukin-4 (IL-4), interleukin-1 receptor antagonist (IL-1RA), and mannose-binding lectin (MBL) were investigated in Vietnamese [12]. Among them, the T allele at position 352 of the VDR gene was associated with resistance to DSS as well as homozygotes for the arginine variant at position 131 of the FcγRII gene were shown to be protective against DSS. Additionally, the G allele at position -336 of DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) was reported to be protective against DF among the Thai population [13].

Dengue virus, like other members of the *Flaviviridae* family, increases the expression of HLA class I and II molecules on infected cells [14]. HLA-controlled immune response may be responsible for the immunopathology of DV infection [14]. The host HLA allele profile influenced the reactivity of DV-specific T cells [15,16] however, there have been only a few informative studies on HLA association with the severity of DV infection [17,18]. In the present study, we investigated the HLA-A, HLA-B, and HLA-DRB1 polymorphisms in the Vietnamese population and the association of HLA alleles with two different clinical forms of severe DV infection during the years 2002–2005.

Materials and Methods

Study subjects

The study was performed at two hospitals, the Children's Hospital No. 2 in Ho Chi Minh City (HCMC) and the Center for Preventive Medicine in the Vinh Long Province (VL) of the Mekong Delta. The enrolment was consecutive sequence of children hospitalized at each hospital. The inclusion criteria at the entry point in the hospital were age 6 months to 15 years old, Kinh race, and unrelatedness. The subjects enrolled initially were 325, 356 and 403 in HCMC 2002–2003 (HCMC 02-03), VL 2002-2003 (VL 02-03) and VL 2004–2005 (VL 04-05), respectively. DV infection of the patients was diagnosed by clinical symptoms and history at the admission time. After hospitalization, the patients were diagnosed by the well established serology as described below.

WHO classification criteria [3] was applied after a review of case report form (CRF) of each patient. The DHF classification required fever or a history of acute fever, bleeding manifestation, and signs of plasma leakage which include hemoconcentration, ascites, or pleural effusion with evidence of thrombocytopenia. Hemoconcentration (more than 20% increase in Hct) was evaluated by estimating the percent increase in the Hct by comparing two values recorded at 2 different timings: the maximum value in defervescence day and the value before discharge. The ascites, or pleural effusion were detected by echography or X-ray. The DSS classification required DHF manifestation plus evidence of clinical hypovolemic shock (tachycardia and narrow pulse pressure (<20 mmHg))

It was previously reported that the sensitivity of WHO criteria for DSS in Vietnam was 82%, mainly due to the lack of evidence for thrombocytopenia [19]. Therefore, we basically followed the WHO criteria but included patients lacking the significant reduction of platelet count, which accounted for no more than 11% of all DHF/DSS cases. Our classification met the requirement of the simplified classification system of IMCI, which is based on plasma leakage as a hallmark of severe dengue (DHF/DSS) [7].

Healthy unrelated school children living in HCMC and VL (250 and 200 subjects respectively) who had no symptoms of dengue virus infection were collected as a background population control group for the genetic study. In these control groups, 13 cases (5.2%) in HCMC and 16 cases (8.0%) in VL were seropositive for IgM ELISA to DV, indicating recent asymptomatic DV infection.

This study was approved by the institutional ethical review committees of the Institute of Tropical Medicine, Nagasaki University, and the Pasteur Institute in Ho Chi Minh City. Informed consent was obtained from the parents or legal guardians of the subjects upon enrollment.

Sample collection and serological diagnosis

Blood samples were collected from patients with suspected dengue infection at the time of enrolment to the study and in the

convalescent phase prior to discharge from the hospital. Plasma samples were used for titration of anti-DV IgM and IgG antibodies, virus isolation, and RT-PCR for determination of viral serotype; buffy coat samples were used to extract genomic DNA by using the QIAamp DNA blood kit (Qiagen, Hilden, Germany). DV infection was defined by previously established serologic criteria for IgM/IgG ELISAs to DV (DEN 1–4) and Japanese encephalitis virus (kit of Pasteur Institute HCMC) in paired sera, collected at least three days interval [20]. IgM and IgG ELISAs were considered positive if the ratio of optical density (OD) of test sera to OD of negative control sera was ≥2.3 [3]. The cases were diagnosed as secondary infection when DV IgM-to-IgG ratio was <1.8 [20].

Dengue virus serotyping

Acute plasma samples were inoculated into C6/36 (*Aedes albopictus*) cells, the virus was then obtained and the dengue virus serotype was identified using the direct or indirect fluorescent antibody technique monoclonal antibodies supplied by the Centers for Disease Control and Prevention (Fort Collins, CO, USA) [21]. Viral RNA was also extracted from the same acute plasma samples with the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) for detection of DV and confirmation of its serotype as described [21]. Briefly, cDNA from the DV genome RNA was amplified with the Ready-to-go RT-PCR test kit (Amersham, MA, USA) using consensus primer set (D1 and D2) [22]. Then, the serotype was determined by semi-nested PCR to amplify serotype-specific fragments from the regions encoding the capsid and membrane proteins of DV using the specific primer sets (TS1, TS2, TS3, and TS4) [22].

HLA typing

HLA-A, -B and -DRB1 were typed by using LABType SSO class I A and B and class II DRB1 locus kits (One Lambda, CA, USA) and the Luminex LX 100 IS system (Hitachi, Japan). IS 2.3 typing software (Luminex Corporation, TX, USA) was used to determine the genotype. The presence of alleles of interest were confirmed by PCR with sequence-specific primers (PCR-SSP) [23] (HLA-A*24) and sequencing-based typing (HLA-DRB1*0901) [according to the standard protocol 10-B of the 13th International Histoconpatibility Workshop (http://www.ihwg.org/protocols/sbt/sbtprot.htm)]. HLA-A, HLA-B, and HLA-DRB1 genotype distributions were checked with Hardy-Weinberg equilibrium.

Statistical analysis

The frequencies of individuals carrying particular alleles (phenotype frequencies) were compared between categories of the case group (DHF or DSS) and the population background group. The difference in frequency was evaluated by the odds ratio (OR) with Fisher exact 95% confidence intervals (95% CI), and Exact Fisher two sided P using StatsDirect statistical software, version 2.6.4. The locus-wise correction of the P value (Pc) for multiple tests was made by multiplying the P value by the number of the major alleles whose phenotype frequencies were more than 5% in either the patient or population background group. The correction factors (CF) were 8 for HLA-A, 13 for HLA-B and 12 for HLA-DRB1. Difference yielding Pc values less than 0.05 was considered statistically significant.

Results/Discussion

Clinical features of the patients

Demographic and clinical features of the study subjects are shown in Table 1. An important clinical findings in this study was hepatomegaly which was clearly more prevalent in DSS than in DHF patients (in HCMC 02-03: 90.8% vs. 47.9%, in VL 02-03: 98.8% vs. 88.6%, and in VL 04-05: 93.8% vs. 78.0%, ORs above 4, footnote b of Table 1). There was substantial evidence of hepatomegaly in Asian patients [24,25] as well as in South American patients [26] with DHF/DSS. Our results therefore agree with the previous study by Pham *et al.* which propose hepatomegaly as one of the clinical predictors of DSS in children [27].

Another point of note was concerning the issue of significance of secondary infection of DV in the pathogenesis of severe cases. Although secondary infection has been reported to confer most of the risk of disease severity, it is also known that patients without evidence of secondary infection substantially contribute to severe cases in Taiwan [28] and Thailand [29]. Our study is similar to that study in Thailand 2003 [29], where the patients with high IgM/IgG ratio consist of nearly 60% of DSS and 40% of DHF. The data still support the notion of disease risk associated with secondary infection (footnote c of Table 1).

It is worth to mention that four percents of our patients were under 1 year of age and all had primary DV infection. The presence of non neutralizing or low level of neutralizing maternal antibodies may play a role in the development of severe disease. Halstead *et al.* pointed out that infants are a high risk group for

Table 1. Study subjects

	Ho Chi Minh City (HCMC)			Vinh Long province (VL)				
	HCMC 02-03			VL02-03		VL04-05		
	DSS	DHF	Controls	DSS	DHF	DSS	DHF	Control
	n = 152	n = 117	n = 250	n = 170	n = 35	n = 96	n = 59	n = 200
Mean age (years) ± SD	9.1 ± 3.2	10.6 ± 3.2	7.8 ± 4.4	9.9 ± 3.0	8.6 ± 4.2	8.6 ± 3.4	8.8 ± 4.2	10.9 ± 2.2
Male (%)[a]	80 (52.6)	70 (59.8)	143 (57.0)	78 (45.9)	16 (45.7)	37 (38.5)	36 (61.0)	110 (55.0)
Hepatomegaly (%) [b]	138 (90.8)	56 (47.9)	nd	168 (98.8)	31 (88.6)	90 (93.8)	46 (78.0)	nd
Percent increase in Hct (%) ± SD	36.6 ± 16.5	27.4 ± 9.8	nd	34.5 ± 20.9	31.2 ± 17.2	26 ± 19.9	23.3 ± 7.7	nd
Secondary infection (%)[c]	81 (53.3)	50 (42.7)	nd	95 (55.9)	13 (37.1)	80 (83.3)	46 (78.0)	nd

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome, SD: standard deviation, nd: not done, P: Fisher's exact test, Hct: hematocrit.
[a]Male among DSS patients in VL04-05 vs. VL controls: P = 0.008
[b]Hepatomegaly among DSS patients vs. DHF patients, in HCMC 02-03: OR = 10.74, P<0.0001, in VL 02-03: OR = 10.84, P = 0.008, in VL 04-05:OR = 4.24, P = 0.005
[c]Secondary infection in DSS VL04-05 vs. HCMC 02-03: P<0.0001, VL04-05 vs. VL02-03: P<0.0001 and in DHF VL04-05 vs. HCMC 02-03: P<0.0001, VL04-05 vs. VL02-03: P = 0.0001
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Table 2. Dengue virus serotypes and disease severity (2002–05)

	Number of cases (%)				DSS vs. DHF	
	DSS (n = 43)		DHF (n = 44)		OR (95% CI)	P
DEN-1	10	(23.3)	6	(13.6)	1.90 (0.56–7.11)	0.3
DEN-2	30	(70.0)	20	(45.5)	2.77 (1.05–7.37)	0.03
DEN-3	3	(7.0)	10	(22.7)	0.26 (0.04–1.11)	0.07
DEN-4	0	(0.0)	8	(18.2)	0.05 (0.003–0.88)	0.006

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome
OR: Odds ratio, 95% CI: Fisher exact 95% confidence intervals, P: Fisher's exact test
doi:10.1371/journal.pntd.0000304.t002

Table 3. Major alleles of HLA-A, -B, -DRB1 in Vietnamese

HLA	Allele	Total
HLA-A	*01, *02, *11, *24, *26, *29, *30, *33	8
HLA-B	*07, *13, *15, *27, *35, *38, *40, *44, *46, *51, *55, *57, *58	13
HLA-DRB1	*0301, *0403, *0405, *0701, *0803, *0901, *1001, *1202, *1401, *1501, *1502 *1602	12

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DHF/DSS, and need to be studied to further understand primary dengue infection [30].

DV serotypes and disease severity

Virus serotype was tested on 388 patients whose blood samples collected within five days after the onset of symptoms, mostly on days 4 and 5. As shown in Table 2, DV was identified in 87 DHF/DSS patients, which gave a detection rate of 22.4%. This was far lower than many other studies but consistent with a previous observation that the sensitivity to detect DV is dulled in the present of neutralizing antibody [20]. Similar low detection rate was reported in Bangladesh study in 2002 [31], where virus serotype could only be identified in 8% of samples.

DEN-2 constituted the majority of the total isolates, 50 (57.5%) followed by 16 DEN-1 (18.4%), 13 DEN-3 (14.9%) and 8 DEN-4 (9.2%). The correlation between DEN-2 infection and the severe clinical forms (Table 2) provided additional proof for higher virulence of this serotype because previous study reported that patients with DEN-2 had a larger pleural effusion index than those infected by other virus serotypes [32].

Phenotype frequencies of HLA-A, HLA-B and HLA-DRB1

We identified 16 HLA-A alleles, 47 HLA-B alleles and 36 HLA-DRB1 alleles in the study subjects. Major alleles (phenotype frequencies having more than 5% in either the patient or healthy background groups) are shown in Table 3 and Table S1, accounted for about 80–90% of the total phenotypes. We analyzed only these major alleles for the evaluation of the risk of disease severity because rare alleles would have little impact on population risk. There were no significant difference in phenotype frequencies of the major alleles in the 2 population background groups (Table S1), and these data were compatible with data from a previous study [17] on the same Kinh ethnic in Southern Vietnam.

HLA-A*24 was positively associated with DSS or DHF

As shown in Table 4 and 5, HLA-A*24 increased in frequency among DHF and DSS patients when compared with the control in all 3 sample groups, HCMC 02-03 (DSS: OR = 1.62, P = 0.04, Pc = 0.3; DHF: OR = 1.35, P = 0.26), VL 02-03 (DSS: OR = 1.51, P = 0.08; DHF: OR = 1.34, P = 0.4), and VL 04-05 (DSS: OR = 1.80, P = 0.029, Pc = 0.2; DHF: OR = 2.71, P = 0.0019, Pc = 0.02).

Certain alleles of the HLA-class I genes were previously found to be associated with DHF or DSS [1]. In a study of Thai patients, Stephens *et al.* found that HLA- A*0207 and HLA-B*51 were susceptible to both DHF and DSS [18]. It was also reported that HLA-A*29 and A*33 were protective against DHF and DSS in Cuban [33] and in Vietnamese patients, respectively [17]. The

Table 4. Phenotype frequencies of HLA-A*24 and HLA-DRB1*0901 in primary and secondary infection and population background groups

Phenotype		HCMC						VL									
		HCMC 02-03						VL 02-03				VL 04-05					
		DSS		DHF		Control		DSS		DHF		DSS		DHF		Control	
		No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
1. HLA-A*24																	
1.1	A*24	53	(34.9)	36	(30.8)	62	(24.8)	57	(33.5)	11	(31.4)	36	(37.5)	28	(47.5)	50	(25.0)
1.2	A*24 Primary infection	28	(39.4)	25	(37.3)	62	(24.8)	28	(37.3)	8	(36.4)	3	(18.8)	7	(58.3)	50	(25.0)
1.3	A*24 Secondary infection	25	(30.9)	11	(22.0)	62	(24.8)	29	(30.5)	3	(23.0)	33	(41.3)	21	(45.7)	50	(25.0)
2. HLA- DRB1*0901																	
2.1	DRB1*0901	33	(21.7)	31	(26.5)	61	(24.4)	35	(20.6)	6	(17.1)	14	(14.6)	15	(25.4)	63	(31.5)
2.2	DRB1*0901 Primary infection	15	(21.1)	18	(26.9)	61	(24.4)	17	(22.7)	2	(9.1)	3	(18.8)	4	(33.3)	63	(31.5)
2.3	DRB1*0901 Secondary infection	18	(22.2)	13	(26.0)	61	(24.4)	18	(18.9)	4	(30.8)	11	(13.8)	11	(23.9)	63	(31.5)

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome
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Table 5. Association of HLA-A*24, -DRB1*0901 allele with severe forms

	HCMC			VL					
	HCMC 02-03			VL 02-03			VL 04-05		
	OR (95% CI)	P	Pc	OR (95% CI)	P	Pc	OR (95% CI)	P	Pc
1. HLA- A*24									
1.1 DSS vs. control	1.62 (1.02–2.58)	0.04	0.3	1.51 (0.94–2.44)	0.084	-	1.80 (1.03–3.13)	0.029	0.2
1.2 DSS with primary infection vs. controls	1.97 (1.08–3.56)	0.024	0.2	2.38 (1.30–4.32)	0.0032	0.03	0.69 (0.12–2.67)	0.77	-
1.3 DSS with secondary infection vs. controls	1.35 (0.74–2.42)	0.3	-	1.76 (0.99–3.09)	0.045	0.36	2.11 (1.17–3.77)	0.009	0.07
1.4 DHF vs. control	1.35 (0.80–2.25)	0.26	-	1.34 (0.57–3.16)	0.4	-	2.71 (1.41–5.16)	0.0019	0.02
1.5 DHF with primary infection vs. controls	1.13 (0.63–2.00)	0.67	-	2.29 (0.78–6.20)	0.1	-	4.2 (1.08–17.43)	0.018	0.1
1.6 DHF with secondary infection vs. controls	0.86 (0.37–1.83)	0.7	-	1.20 (0.20–4.89)	0.73	-	2.52 (1.22–5.13)	0.007	0.056
1.7 DSS vs. DHF	1.20 (0.70–2.09)	0.51	-	1.1 (0.48–2.67)	1	-	0.66 (0.33–1.35)	0.24	-
2. HLA- DRB1*0901									
2.1 DSS vs. control	0.86 (0.51–1.42)	0.63	-	0.56 (0.34–0.93)	0.018	0.2	0.37 (0.18–0.72)	0.0018	0.02
2.2 DSS with primary infection vs. controls	0.83 (0.41–1.62)	0.64	-	0.64 (0.32–1.22)	0.18	-	0.50 (0.09–1.92)	0.4	-
2.3 DSS with secondary infection vs. controls	0.89 (0.46–1.66)	0.77	-	0.51 (0.26–0.95)	0.026	0.3	0.35 (0.16–0.72)	0.0025	0.03
2.4 DHF vs. control	1.12 (0.65–1.90)	0.69	-	0.45 (0.15–1.18)	0.10	-	0.74 (0.36–1.48)	0.42	-
2.5 DHF with primary infection vs. controls	1.14 (0.58–2.17)	0.75	-	0.22 (0.02–0.95)	0.027	0.3	1.09 (0.23–4.24)	1	-
2.6 DHF with secondary infection vs. controls	1.09 (0.50–2.26)	0.86	-	0.97 (0.21–3.63)	1	-	0.68 (0.29–1.49)	0.37	-
2.7 DSS vs. DHF	0.77 (0.42–1.41)	0.39	-	1.25 (0.46–3.98)	0.82	-	0.50 (0.20–1.23)	0.14	-

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome, OR: Odds ratio, 95% CI: Fisher exact 95% confidence intervals, P: Fisher's exact test, Pc: corrected P.
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inconsistency of these HLA associations may be the result of the differences in ethnicity, geographic location or the diversity of the predominant virus during the study periods [2,34].

The results of HLA class I association from the present study reproduced a previous Vietnamese study (309 cases and 251 controls) by Loke *et al.* in which HLA-A*24 association with DHF and DSS was found, with OR 1.54 and P value 0.021 [17]. Considering such reproducibility in different study periods, within the same ethnic group and in the same region, give us the confidence to assign an association of HLA-A*24 and DHF, DSS even though our P value just reached the significant level before Bonferoni correction.

HLA-A*24 with Histidine at codon 70 was positively associated with DSS and DHF

It is known that HLA-A*24 alleles fall into at least two subtypes by the difference at codon 70 of alpha 1 domain, which together with codons 9, 45, 63, 66 and 67 composes peptide binding pocket B for P2 anchor position [35]. In the present study, there were 4 different A*24 alleles, A*2402, A*2403, A*2407 and A*2410 (Table 6). A*2407 is different from the others at codon 70 where histidine (CAC) is changed to glutamine (CAG). The frequencies of A*24 with histidine at codon 70 in DSS and DHF patients were significantly higher than that in the population background groups (HCMC 02-03 DSS: OR = 1.89, P = 0.008, DHF: OR = 1.75,

Table 6. Phenotype frequencies of HLA-A*24 subgroups

A*24	HCMC						VL									
	HCMC 02-03						VL 02-03				VL 04-05					
	DSS		DHF		Control		DSS		DHF		DSS		DHF		Control	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
1. A*24 with histidine at codon 70																
A*2402	46	(30.3)	34	(29.1)	43	(17.2)	53	(31.2)	8	(22.9)	34	(35.4)	18	(30.5)	38	(19.0)
A*2403	0	(0.0)	1	(0.9)	2	(0.8)	1	(0.6)	1	(2.9)	1	(1.0)	2	(3.4)	1	(0.5)
A*2410	2	(0.0)	0	(0.0)	4	(0.8)	0	(0.0)	1	(2.9)	0	(0.0)	1	(1.7)	4	(2.0)
Total	48	(31.6)	35	(29.9)	49	(19.6)	54	(31.8)	10	(28.6)	35	(35.5)	21	(35.6)	43	(21.5)
2. A*24 with glutamine at codon 70																
A*2407	5	(3.3)	2	(1.7)	13	(5.2)	3	(1.8)	1	(2.9)	1	(1.0)	7	(11.9)	9	(4.5)

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome
doi:10.1371/journal.pntd.0000304.t006

Table 7. Association of HLA-A*24 with histidine at codon 70 and severe forms of DV infection

	HCMC		VL			
	HCMC 02-03		VL 02-03		VL 04-05	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
DSS vs. control	1.89 (1.16–3.09)	0.008	1.70 (1.04–2.79)	0.03	2.09 (1.18–3.70)	0.0075
DHF vs. control	1.75 (1.02–2.98)	0.033	1.46 (0.58–3.44)	0.38	2.02 (1.01–3.95)	0.038

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome, OR: Odds ratio, 95% CI: Fisher exact 95% confidence intervals, P: Fisher's exact test. This analysis was perform after the confirmation of A*24 association, so that the correction of P value did not apply.
doi:10.1371/journal.pntd.0000304.t007

P = 0.03; VL 02-03 DSS: OR = 1.70, P = 0.03, DHF OR = 1.46, P = 0.38; VL 04-05 DSS: OR = 2.09, P = 0.0075, DHF OR = 2.02, P = 0.038, Table 7). On the other hand, A*24 with glutamine at codon 70, A*2407 in our samples, did not show any significant difference in frequency (Table 6). Similar behavior of this allele was documented in Indian HIV study [36].

The association of HLA-A*24 in the VL 04-05 samples was stronger than in VL 02-03 and HCMC 02-03, possibly due to higher secondary infection rate in the VL 04-05 samples. Memory T cells might be important in the A*24 associated pathogenesis of DHF/DSS. HLA-A*24 is reported to be one of the potential restricting alleles for NS3 dengue viral protein and CTLs recognizing NS3 peptides coupled with HLA-A*24 were cross-reactive between serotypes because NS3 is highly conserved [37]. Two previous Vietnamese studies reported an identification of the DEN-2 NS3 556–564 peptide that was restricted by HLA-A*24 [17,38], but further study is needed to clarify the role of A*24-restricted CD8 T-cells in the pathogenesis of DHF/DSS.

HLA-DRB1*0901 was negatively associated with DSS

As shown in Table 4 and 5, the HLA-DRB1*0901 allele significantly decreased in frequency in DSS patients in VL 04-05 (OR = 0.37, P = 0.0018, Pc = 0.02) when compared to the control, mainly in secondary infection (OR = 0.35, P = 0.0025, Pc = 0.03), not in primary infection. This trend was also observed in the other 2 groups of samples but did not reach statistical significance (VL 02-03: OR 0.51, P = 0.026, Pc = 0.3; HCMC 02-03: OR = 0.89, P = 0.77).

Certain HLA class II alleles have been reported to confer a protective effect in infections of human immunodefiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) [39]. In dengue virus infection, protective DRB1*07 and DRB1*04 alleles were recently reported. DRB1*07 was protective against DF, DHF and DSS and DRB1*04 was protective against secondary DF in Cuban population [34], DHF or DSS in Mexican population [40]. Possibly there is a common primary associated gene closely located to DRB1 locus, such as HLA-DQB1 and HLA-DRB4, for those alleles (DRB1*07 and DRB1*04) and Vietnamese DRB1*0901. One of the most frequent haplotypes among Oriental populations (including Kinh ethnic) is DRB1*0901-DQB1*030302. DQB1*030302 is also reported to be in disequilibrium with DRB1*0701 [41], however there was no reduced frequency of DRB1*0701 in DSS patients compared to controls in our data. Furthermore, haplotypes found in South America are DRB1*0701-DQB1*0201 and HLA-DRB1*0403-DQB1*0302, not DQB1*030302, which suggests that the association of these protective alleles could not be elucidated by a common DQB1 allele. The possibility of primary disease-protective association with DRB4*01 (encoding DR53 sero-specificity) remains to be examined because of DRB4*01 haplotypic association with DRB1*07, *04 and *0901 was found in all populations which have been studied [42], although neither DRB1*07 or DRB1*04 exhibited difference in frequency in our samples.

Protective effect of HLA-DRB1*0901 against DSS development from DHF was evident when having DEN-2 infection

To see the effect of virus serotype on the HLA susceptibility, we analyzed the HLA alleles in the DEN-2-positive patients and those that were positive for DEN-1, DEN-3, or DEN-4 (DEN-2-negative). As shown in Table 8, HLA-DRB1*0901 frequency was significantly decreased in DSS compared with DHF among DEN-2- positive patients (OR = 0.13, P = 0.02). The effect of DRB1*0901 appeared to be DEN-2 specific because DEN-2-negative population did not show significant difference, although this may be due to the small sample size as Breslow-Day statistic for the uniformity of odds ratio did not reach significant level (P = 0.14).

The HLA-DRB1*0901 allele has also been reported at higher frequency in noncirrhotic HCV patients than in patients with cirrhosis [43,44], suggesting protective effect of this allele against tissue destruction and disease progression. Here, when we focus on DEN-2 infection, a higher frequency of HLA-DRB1*0901 in DHF than in DSS patients was found. The protective effect of this allele against DSS development from DHF could be considered as a new finding in dengue infection. It is generally believed that DSS is preventable by intense clinical management, in fact a certain number of patients, however fall to shock in spite of the early efforts of physicians. Although the pathogenesis of DHF/DSS is still unclear, clinical studies have suggested that the host immune response shifted to Th2 dominant in most DSS cases [45]. Because

Table 8. Phenotype frequency of HLA- DRB1*0901 allele in DHF or DSS patients with DEN-2 or other serotypes

Serotype	DSS	DHF	DSS vs. DHF	P
	No. (%)	No. (%)	OR (95% CI)	
DEN-2 (+)	n = 30	n = 20		
DRB1*0901 (+)	2 (6.7)	7 (35.0)	0.13 (0.01–0.86)	0.02
DRB1*0901 (−)	28 (93.3)	13 (65.0)		
DEN-2 (−)	n = 13	n = 24		
DRB1*0901 (+)	3 (23.1)	7 (29.2)	0.73 (0.10–4.22)	1
DRB1*0901 (−)	10 (76.9)	17 (70.8)		

DHF: dengue hemorrhagic fever, DSS: dengue shock syndrome,
DEN-2 (−): consisted of DEN-1, DEN-3, and DEN-4 as shown in Table 2.
OR: Odds ratio, 95%CI: Fisher exact 95% confidence intervals, P: Fisher's exact test.
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