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ORIGINAL ARTICLE

Phenotypic and genotypic characterization of dengue virus isolates differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome

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Abstract Dengue viruses (DENV) cause 50-100 million cases of acute febrile disease every year, including 500,000 reported cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Viral factors have been proposed to influence the severity of the disease, but markers of virulence have never been identified on DENV. Three DENV serotype-1 isolates from the 2007 epidemic in Cambodia that are derived from patients experiencing the various clinical forms of dengue were characterized both phenotypically and genetically. Phenotypic characteristics *in vitro*, based on replication kinetics in different cell lines and apoptosis response, grouped isolates from DF and DHF patients together, whereas the virus isolate from a DSS patient showed unique features: a lower level of replication in mammalian cells and extensive apoptosis in mosquito cells. Genomic comparison of viruses revealed six unique amino acid residues in the membrane, envelope, and in non-structural genes in the virus isolated from the DSS patient.

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Introduction

Dengue viruses (DENV) (serotypes 1, 2, 3 and 4) belong to the genus *Flavivirus*, in the family *Flaviviridae*. They are enveloped viruses with a positive-sense single-stranded RNA genome of approximately 10 700 bases. Upon infection, the genome is translated in the cell cytoplasm into a polyprotein, which is secondarily cleaved into three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [1].

Dengue is the most frequent arthropod-borne viral infection in the world affecting humans, with one hundred million estimated cases per year. It is endemic in more than one hundred countries and more than 500 000 severe forms of the disease, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), are reported annually, most of them affecting children under 15 years of age [2]. DENV has adapted completely to humans and is maintained in large urban areas in the tropics in human-mosquito-human transmission cycles that no longer depend on animal reservoirs. Such reservoirs, however, can still be found in the jungles of Africa and Southeast Asia in mosquito-monkey-mosquito transmission cycles [3].

Most of dengue infections remain asymptomatic. Apparent disease due to dengue virus varies from a

relatively self-limited though debilitating febrile illness, dengue fever (DF), to the more severe and potentially life-threatening diseases DHF and DSS. The molecular mechanisms underlying dengue illness and the exact factors contributing to disease progress, however, are far from well understood. Host factors, such as age, gender, nutritional status, and genetics are known disease parameters. Furthermore, previous DENV infections and maternal antibodies seem to interfere and affect the outcome of an ongoing DENV infection [4–6].

Epidemiological data indicate, however, that viral factors should also influence the outcome of the disease and that the virulence differs among DENV strains, including strains within the same serotype [7–9].

Although dengue virus strains are easily differentiated on a genotypic basis, no phenotypic profile of "virulent" or "avirulent" strains has been established, except for a few strains that have been attenuated for vaccine purposes.

DENV isolates from children representing the three clinically distinct forms of dengue disease were characterized genotypically by genomic sequencing and phenotypically by analyzing replication kinetics, apoptosis, and adaptation to cell culture. Regarding phenotypic characteristics (replication kinetics and apoptosis patterns), the strain isolated from a patient experiencing DSS, the most severe form of dengue disease, differed significantly from the DENV isolates obtained from patients with DF and DHF. This strain also showed a remarkable ability to rapidly adapt to *in vitro* culture. Interestingly, at the genomic level, this strain exhibited six unique amino acid substitutions.

These observations group DENV obtained from DF and DHF cases together, whereas the DSS virus isolate stands alone when comparing virus isolate characteristics. Together, clinical observations and *in vitro* data suggest that the virus isolated from a DSS case can be distinguished from those obtained from DF and DHF patients, which appear similar.

Materials and methods

DENVs

Three dengue serotype 1 viruses isolated during the 2007 outbreak from patients living in the Kampong Cham province, Eastern Cambodia, were used during this study (Table 1). The isolates were obtained from patients experiencing the three distinct clinical forms of dengue disease: DF, DHF, and DSS, according to the WHO classification [10]. Blood samples were drawn between days 2 and 6 after onset of disease. These samples were collected during the DENFRAME study (a project supported by European Union) and immediately anonymized as stated in the research protocol. The DENFRAME project was approved by the National Cambodian Ethics Committee, and a patient's enrolment was subject to obtaining a written consent signed by the patient or, for those under 16 years old, their legal representatives. The patients specifically agreed that their blood samples may be stored and that further tests on these samples may be undertaken in the future to further understand dengue disease.

Table 1 Epidemiological and clinical characteristics associated with dengue virus serotype 1 isolates used in the study

ID	Epidemic year	Sex	Age	Type of antibody response	Clinical outcome
R0627319	2007	M	7	Secondary	DF
R0627321	2007	F	5	Indeterminable	DHF
R0808258	2007	F	9	Secondary	DSS

Each isolate had been isolated at the Institut Pasteur in Cambodia (IPC) in the standard cell line C6/36, derived from *Aedes albopictus* (CRL 1660, ATCC), and thereafter had undergone only one *in vitro* passage (P1) in order to avoid mutations that might be associated with tissue culture adaptation. Cell culture supernatant was stored at −80°C prior to shipment on dry ice to the French Army Biomedical Research Institute (IRBA, Marseille). The isolates were serotyped at IPC by nested reverse transcriptase polymerase chain reaction according to the Lanciotti procedure modified by Reynes et al. [11].

Cells

Mammalian Vero cells (CCL-81, ATCC) were grown at 37°C, 5% CO_2, in cell culture medium 199 (Gibco) with 5% fetal calf serum (FCS). The *A. albopictus* cell line C6/36 was grown at 28°C, in cell culture medium Leibovitz-15 with 5% FCS and 2% tryptose phosphate (TP).

Immunofluorescence with HB112 and J2 for detection of DENV infection and DENV titration by fluorescent focus assay (FFA)

5×10^5 C6/36 cells per well were seeded in 96-well plates (Corning) and incubated for 24 h prior to infection. Viruses, duplicates of 10-fold dilutions, were prepared with cell culture medium (FCS-free), and 50 µL of diluted virus was inoculated in each well and incubated for 2 h. To immobilize the virus, an overlay medium (0.7% high-viscosity carboxymethyl cellulose (CMC), Sigma-Aldrich, 1X MEM-medium, 2% TP, 5% FCS) was added on top of the cells, followed by a 72-h incubation. The overlay medium was discarded, and the cell monolayer was gently washed once with cell culture medium before fixation with paraformaldehyde (3.7%, pH 7.4) and incubated for 15 min at

room temperature. The cell monolayer was then washed once with PBS (1X) followed by a 5-min incubation with Triton-X 100 (0.5 %) in order to permeabilize the cells. Two washes with PBS (1X) followed before adding a blocking solution (2.5% FCS, 1% BSA in PBS (1X)), and the cells were incubated at 37°C for 30 min. A primary panflavi anti-envelope monoclonal antibody (MAb) (HB112^{TM}, ATCC) or a MAb for staining double-stranded RNA (J2, English and Scientific Consulting, Hungary) was added to the fixed cells and incubated for 30 min at 37°C, followed by two washes with PBS (1X) before applying a goat anti-mouse IgG A488-conjugate (1:250 dilution, Gibco) and incubating at room temperature for 45 min. In order to stain the eukaryotic nuclei, Dapi (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) was added at a 1:5000 dilution, and the cells were incubated for 30 min at 37°C. The monolayer was then washed three times with PBS (1X), and plaque-forming units (PFU) were counted under a microscope.

Viral RNA extraction and real-time one-step quantitative RT-PCR

Viral RNA was extracted from lysed cells and cell supernatant using a QIAmp Viral RNA Mini Kit. RT-PCR reactions were set up according to the manufacturer's instructions (SuperScript III Platinum One-Step Quantitative RT-PCR System, Invitrogen) as already described [12].

Kinetics of DENV replication in various cell lines

Replication kinetics was investigated under three different conditions: on mosquito C6/36 cells at 28°C, on mammalian Vero cells grown at 37°C (5% CO_2) and on Vero cells at 40°C (5% CO_2). The C6/36 cells were seeded to confluence at 170,000 cells per well (3.8 cm^2) in a 12-well plate, and the Vero cells were seeded to confluence at 110,000 cells per well. The cell medium was discarded, and the monolayer of C6/36 cells was washed once with L-15 medium (with 2% tryptose phosphate broth). The monolayer of Vero cells was washed once with PBS (1X). An inoculum of 500 µL per well was used for infection at a multiplicity of infection (MOI) of one. The virus was diluted in cell medium (FCS-free), and virus free-cell culture medium was used for mock infection. The cells were then incubated for 2 h at the appropriate temperature. The inoculum was removed, and the cells were gently washed with PBS (1X). Two mL per well of the appropriate cell medium (supplemented with 5% FCS) was added, followed by incubation at the corresponding temperature. Samples of both supernatant and cells were harvested daily throughout the week and used for viral RNA extraction and virus titration. C6/36 cells were harvested by scraping them off and dissolving them in 2 mL of cell culture medium. Vero cells were harvested by adding 500 µL trypsin 2-3 minutes before adding 1.5 mL of cell culture medium to obtain a homogenous cell suspension.

Evolution of DENV in persistently infected Vero cells

Four T-25 flasks were seeded with 433,000 Vero cells each and infected with one dengue virus isolate each at an MOI of one. The cell medium was discarded and the cell monolayer washed once with PBS (1X) prior to infection and incubated for 2 h at 37°C, 5% CO_2. The inoculum was then discarded and the cells washed with PBS (1X). Ten mL of medium 199 (5% FCS, Invitrogen) was added to each flask, and the cells were incubated for one week at 37°C, 5% CO_2. Seven days post-infection, 2 mL of each supernatant was transferred to a new T-25 flask containing 433,000 cells and infected as described above. This supernatant was then used to infect new cells seven days later, and so on for ten weeks.

The cells from the old flasks were split by first washing with PBS (1X) and then trypsinizing (Gibco), and 1/7 of the cells in a total volume of 10 mL were transferred to a new T-25 flask and incubated for a week. Those cells were subsequently split each week into a new T-25 flask, and the infection was thereby maintained until week 10, when the experiment was terminated.

Samples were harvested each week, and Lab-Tek slides (Nunc) were prepared in parallel to the cell culture flasks to perform immunofluorescence.

Apoptosis TUNEL assay

Vero and C6/36 cells were grown on Lab-Tek chamber slides (0.8 cm^2 per well). Vero cells were seeded with 20,800 and 41,600 cells per well to 50%, and 100% cell confluence, respectively, at the start of infection. C6/36 cells were seeded with 5×10^5 and 16×10^5 cells per well to approximately 50%, and 100% cell confluence, respectively. The cells were infected at an MOI of one and incubated for 24, 48, or 72 h or six days. The cells were then stained for the presence of dengue virus antigen using the HB112 and J2 MAbs, as well as for apoptotic cells using TUNEL staining (terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling). Apoptosis was detected using an In Situ Cell Death Detection Kit Red (Roche) according to the manufacturer's instructions, and cells were stained for 1 h at 37°C. Staurosporine (Sigma) was used as a positive control for apoptosis [13]. One micromolar staurosporine was added to Vero and C6/36 cell cultures, followed by 24 and 48 h of incubation at 37°C and 28°C, respectively.

Sequencing

Viral genomic RNA was isolated from infectious supernatant from the first passage propagated in C6/36 cells, which is believed to confer the lowest possible level of selective pressure, using a QIAamp Viral RNA Mini Kit (QIAGEN). RT was performed using the SuperScript First Strand Synthesis System for RT-PCR (Life Technologies) with gene-specific primers or by self-priming. Phusion Hot-Start High Fidelity DNA Polymerase (Finnzymes) was used to generate overlapping PCR fragments of approximately 1000 nucleotides, which were purified by gel electrophoresis followed by gel extraction using a MinElute Gel Extraction Kit (QIAGEN) and eluted in 10 µL water. DENV1-virus-specific primers were used for two-directional sequencing. Primers were designed for sequencing both strands (Sanger sequencing using ABI3739XL technology and the PhredPhrep program) of the PCR product, from which consensus sequences were assembled.

Results

FFA titration on C6/36 cells

The most common method to titrate DENV for a long time has been based on cytopathic effect (CPE) in monkey Vero cells or rodent BHK-21 cells. This method is widely used for high-passage laboratory DENV strains. Unfortunately, most of primary (or low-passage) DENV isolates from patient sera do not give any CPE on these cells. To overcome this problem, a FFA using C6/36 cells was developed for DENV titration. C6/36 cells are commonly used for DENV isolation and propagation, which explains why these cells were the natural choice for the development of the FFA for virus titration. As seen in Fig. 1, distinct foci (clearly separated from each other) appeared as the virus was more diluted, resulting in a manageable number of foci to be counted for titre estimation.

Depending on the DENV strain, a decrease in the titre was observed after a freezing step at −80°C. For all of our experiments with a specified MOI, it was important to ensure that the initial titre of each strain was stable at each step of freezing and thawing. We have tested different freezing solutions containing sucrose and/or HEPES. The titre was stable for two years at −80°C for viruses frozen in 0.5 M sucrose and 50 mM HEPES and also after few freezing/thawing cycles (data not shown).

Replication kinetics of wild-type DENV isolates obtained from patients experiencing various degrees of dengue illness severity

We investigated the replication kinetics of various clinical DENV isolates in order to characterize them phenotypically in mammalian and mosquito cell lines. Viruses from supernatants and cells were titrated by FFA and by quantification of total viral RNA using a quantitative RT-PCR. Similar kinetics were obtained using these two methods.

All of the isolates replicated with similar efficiency in the mosquito cell line C6/36, with close to 100% of cells infected (Figs. 2a, 4).

In mammalian Vero cells at 37°C, strains isolated from patients experiencing DF and DHF showed identical growth kinetics, reaching viral titres of 10^6 RNA copies/ml (around day 5-6 post-infection). However, the isolate obtained from the patient with the shock syndrome showed restricted replication in Vero cells, with a loss of more than one log in titre (Fig. 2). Immunofluorescence with a pan-flavi anti-envelope monoclonal antibody and with a MAb detecting active virus replication (J2, targeting double-stranded RNA) showed a constantly low level of cells infected with the DSS virus (around 10%) compared to DF and DHF isolates, which infected 100% of the cells (Fig. 3).

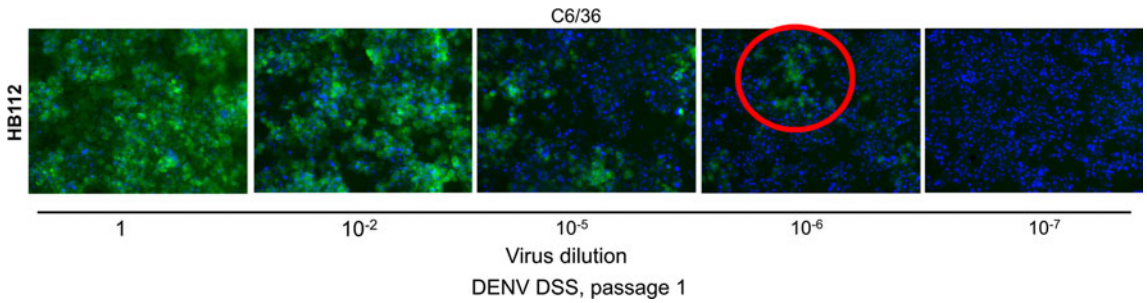

Fig. 1 Titration of a DENV serotype 1 DSS isolate on C6/36 cells, passage 1, stained with HB112 antibody (green) and Dapi (blue). The red circle indicates a fluorescent focus that is well separated from its surroundings

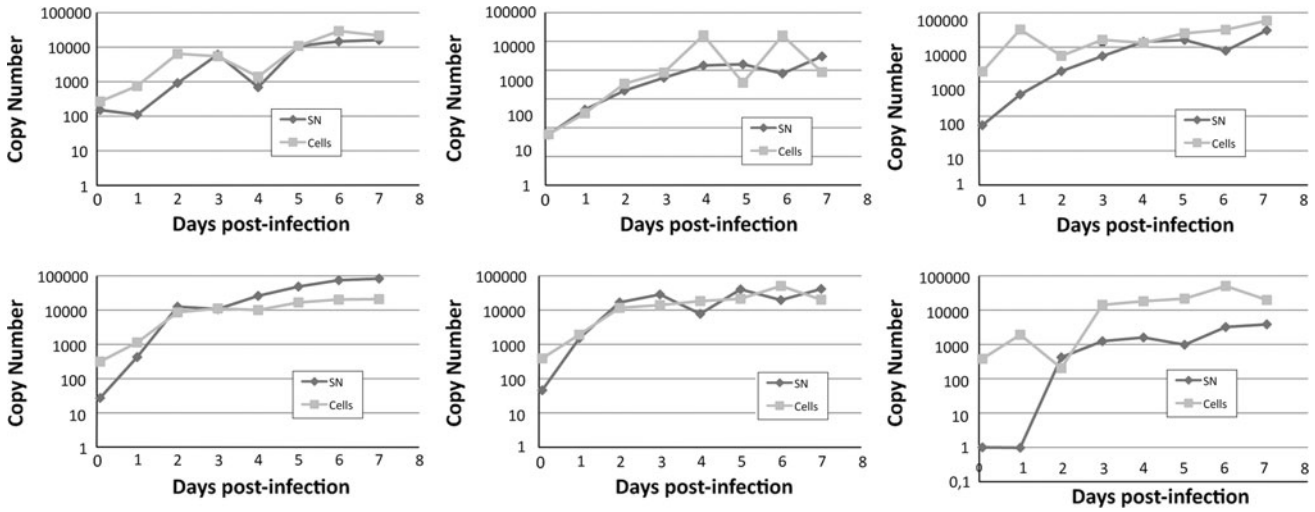

Fig. 2 Replication kinetics of DENV isolates in mosquito C6/36 cells and in mammalian Vero cells at 37°C determined by measuring the copy number by quantitative RT-PCR in supernatants and in cells. These data are the average of three independent experiments. The standard deviation error bars are too small to be seen in this figure on a log scale (see supplementary data)

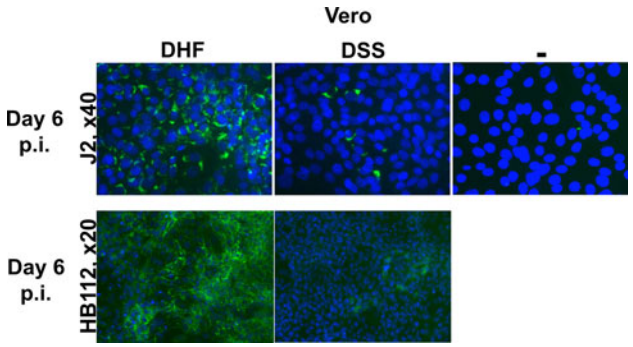

Fig. 3 Replication of DHF and DSS isolates at day 6 p.i. in Vero cells stained with J2 antibody (magnification X40) and DAPI (nucleus) or stained with HB112 antibody and DAPI (magnification X20)

Finally, there was no difference in the kinetic for the three DENVs when infecting Vero cells at 40°C (data not shown).

Apoptosis due to DENV infection

We studied apoptosis in C6/36 and Vero cells to investigate whether the DSS isolate differed from the DF and DHF isolates, as was observed in the replication kinetics. The cells were stained at four time-points (+24, +48, +72 h and +6 days p.i.) in order to follow the development of apoptosis along the course of infection. The experiment was repeated at two different levels of cell confluence, since this parameter influences the degree of apoptosis.

None of the DENV isolates induced a significant degree of apoptosis on Vero cells, regardless of whether the cells were fully confluent or only 50% confluent (data not shown).

The DF and DHF virus isolates did not induce a significant degree of apoptosis on C6/36 cells, regardless of cell confluence (Fig. 4). With the DSS isolate, however, at least 30% of the C6/36 cells were already undergoing apoptosis during the first 24 h of infection, and the number of apoptotic cells continued to increase to 50% during the following days (Fig. 4). This did not depend on the initial cell confluence at the time of infection (apoptosis in 50% and 100% confluent cells was compared). The apoptotic signal one day after infection was found in surrounding, not yet infected cells, rather than in infected cells (Fig. 4). Virus titration and viral RNA quantification using real-time quantitative RT-PCR confirmed that all the three DENV isolates had similar replication kinetics in C6/36 cells (data not shown). Thus, the DSS isolate caused a much higher degree of apoptosis in C6/36 cells compared to the DENV DF and DHF isolates, regardless of replication kinetics.

Adaptation of DENV isolates in persistently infected mammalian Vero cells

After having observed the strong replicative restriction of the DSS strain in Vero cells, we investigated the ability of the virus to establish a persistent infection compare to other isolates. Infected Vero cells were propagated for ten weeks by splitting them weekly, as well as by transferring the infectious supernatant to new uninfected Vero cells. All three DENV isolates had the characteristic of establishing a persistent infection in VERO cells for at least ten weeks (Fig. 5).

For the DSS isolate, the two first weeks of infection showed the same low level of replication with 10% of cells infected, as observed during the kinetics' experiment. However, after 3 weeks, there was an increase in replication,

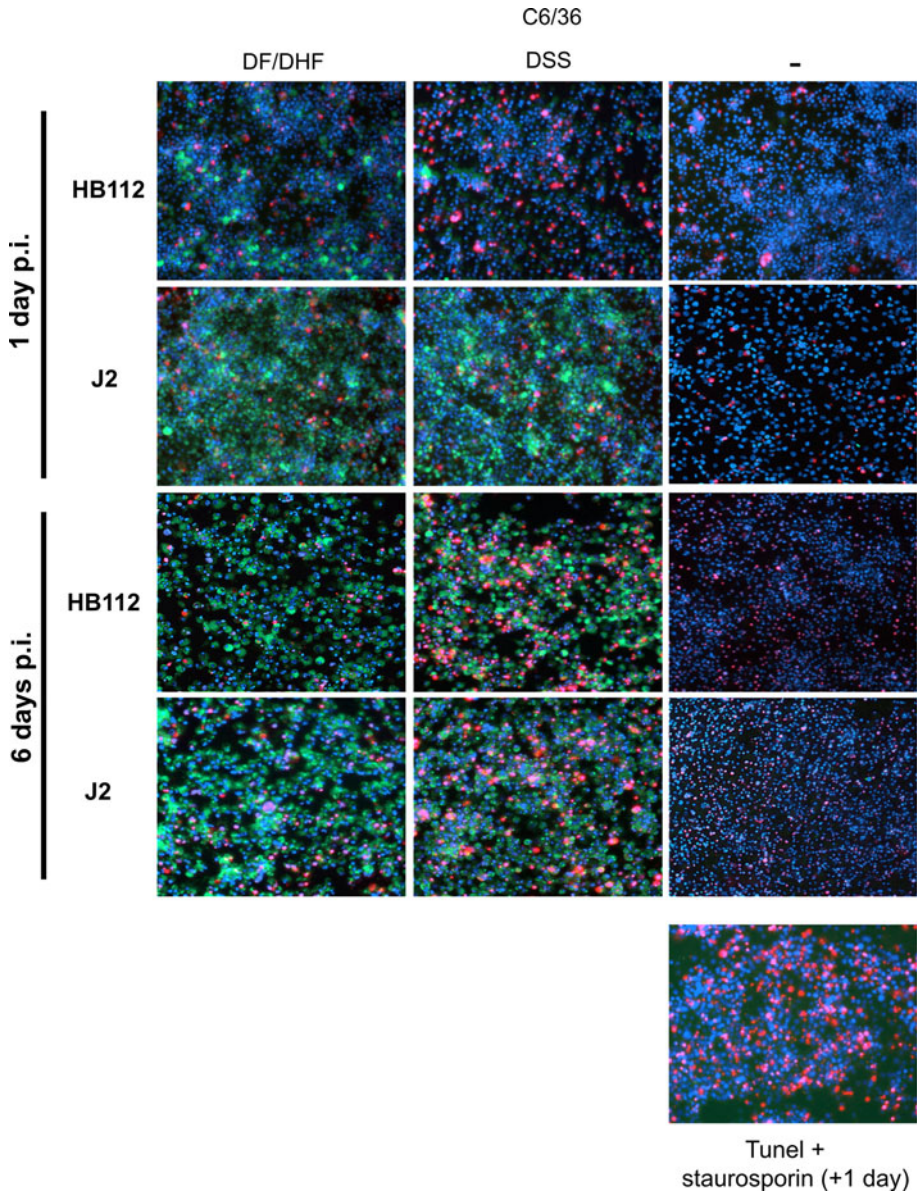

Fig. 4 Apoptosis on confluent C6/36 cells at 24 h postinfection and 6 days postinfection. TUNEL staining (red) and DAPI staining (nucleus, blue) combined with either HB112 or J2 antibodies (green)

with 50% of the cells infected. At week four, there were no longer significant differences in replication activity between the three isolates with 100% of cells infected, leading to viral titres as high as 10^6 to 10^8 FFU/ml (Fig. 5). The passaged viruses showed the same characteristics both for the passaged infected cells and the passaged infectious supernatant. Thus, the DSS isolate could achieve adaptation to Vero cells in only 3-4 *in vitro* passages despite the low level of replication during the first two passages.

Genomic analysis of the three DENV isolates

Complete sequences of viral genomes for the DF (accession number: HQ624983), DHF (accession number: HQ624984) and DSS (accession number: FJ639694) isolates were obtained. These three isolates belong to genotype 1. There are numerous sequence variations at the amino acid level between these three DENV-1 strains, which were isolated during the same outbreak (Table 2). The sequence of the DSS isolate differed from the other two by six amino acids, with mutations located in the membrane gene, the envelope gene, and the non-structural (NS) 1, NS3 and NS5 genes.

The adapted DSS isolate after five passages of Vero cells was also sequenced, and four amino acid changes had appeared in the envelope, 2K, NS4b and NS5 genes (Table 2).

Discussion

Epidemiological, phylogenetic, clinical and experimental data indicate that viral factors could influence the severity of dengue infection [7–9, 14, 15].

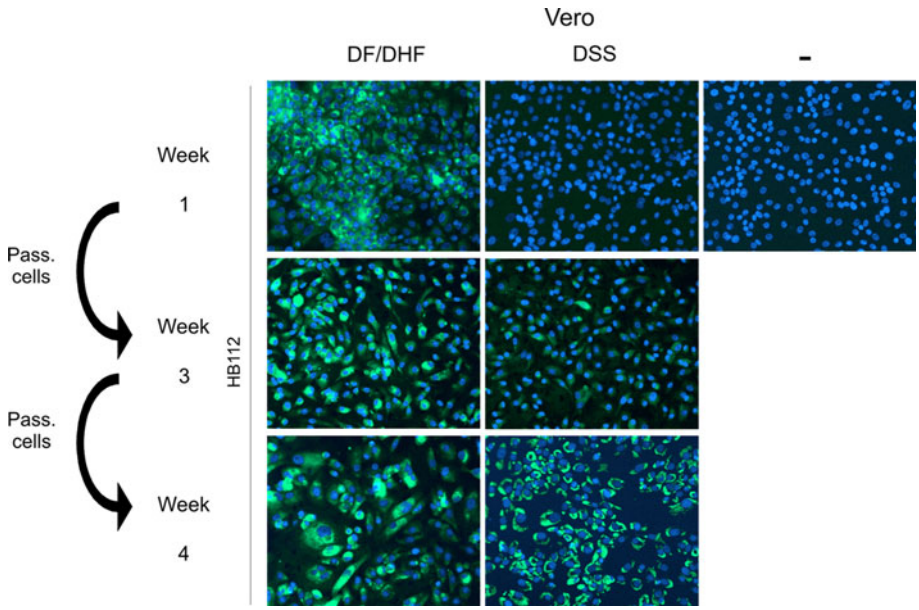

Fig. 5 Adaptation of DENV isolates to VERO cells. Immunofluorescence with HB112 antibody (green) and DAPI staining (blue, nucleus) 7 days postinfection on weeks 1 to 4 of passage of infected VERO cells

This study was aimed at investigating phenotypic as well as genotypic differences between clinical DENV serotype-1 isolates from Cambodian patients experiencing various degrees of dengue disease severity (DF, DHF and DSS). These virus isolates, which were from the same outbreak, represented a good model to investigate if phenotypic properties associated with a specific genetic background could characterize them. It should be noted at this point that this study is preliminary and is limited by the number of isolates used. However, the data obtained showed that both at the genotypic and phenotypic level, differences could be found associated with different clinical outcome.

Previous laboratory experiments have shown that DENV could adapt to cells *in vitro* very quickly, associated with genetic and phenotypic changes [16, 17]. In our study, we also showed that the characteristic of the DSS isolate replicating less efficiently in mammalian cells was lost after three *in vitro* passages and was associated with changes in the viral genome. These results emphasize the importance of using primary viral isolates, or viruses rescued from infectious clones, when studying DENV characteristics, i.e., virulence. Laboratory-adapted strains have passage-acquired mutations that may involve loss of wild-type virus features that are of significant importance for understanding DENV virulence.

Some *in vitro* experiments on human peripheral blood leukocytes have shown differences in DENV replication correlating with the severity of the disease [18]. We observed a similar phenomenon in mammalian Vero cells, with differences in DENV replication depending on the DENV isolate. The clinical DENV isolates derived from patients exhibiting the non-complicated form DF and the more severe form DHF replicated efficiently in Vero cells compared to the DSS virus isolate, which showed a very low level of replication, corresponding to a lower percentage of infected cells. This restricted replication of the DSS isolate in Vero cells could be due to the establishment of a persistent infection. Ten passages of Vero cells infected by DF, DHF or DSS isolates showed that the three isolates are able to establish a persistent infection in mammalian cells. This characteristic has already been described for a laboratory-adapted strain of DENV2 in human mononuclear cell lines [19]. In mosquito cells, none of the three DENV serotype 1 isolates studied showed any differences at the level of viral replication. However, the DSS isolate induce a high level of apoptosis in C6/36 cells as early as 24 h postinfection. Whether apoptosis is the result of infected cells releasing pro-apoptotic mediators rather than being a direct effect of viral infection is difficult to distinguish because most of the C6/36 cells were infected. However, Kanthong et al. recently observed 40% apoptosis in C6/36 cells persistently infected with DENV and treated with 5-kDa filtrate from a supernatant of C6/36 cells acutely infected with DENV [20]. Therefore, it could be hypothesized that the replication of the DSS isolate in C6/36 cells induces the production of apoptosis-inducing cytokines.

Regarding the phenotypic properties of the three different isolates, the DSS isolate behaved differently than the other two isolates. This differentiation was also found in another recent study using a genome-wide microarray to look at the transcriptional profiles of samples from patients presenting with the different clinical forms of DENV infection [21, 22]. The lower level of replication of the DSS isolate in mammalian Vero cells fits the observations

Table 2 Amino acid differences between DF, DHF and DSS isolates in the open reading frame

Amino acid position in polyprotein	Amino acid position in protein	Gene or region	Virus and amino acid			
			DF	DHF	DSS	DSS adapted
75	75	Capsid	Asn	Ser	Ser	Ser
109	109		Val	Met	Met	Met
158	43	Membrane	Phe	Leu	Leu	Leu
171	56		Ser	Thr	Thr	Thr
229	114		Gly	Ser	Gly	Gly
277	162		Pro	Pro	Ser	Pro
335	56	Envelope	Val	Ile	Ile	Ile
450	171		Ser	Thr	Thr	Thr
484	205		Lys	Lys	Lys	Arg
755	476		Leu	Leu	Phe	Phe
891	115	NS1	Lys	Lys	Arg	Arg
1099	323		Lys	Arg	Arg	Arg
1168	40	NS2a	Phe	Leu	Leu	Leu
1199	71		Met	Thr	Thr	Thr
1345	217		Lys	Arg	Arg	Arg
1594	118	NS3	Ser	Ser	Pro	Pro
1807	331		Val	Leu	Val	Val
2225	3	2K	Asp	Asp	Asp	Asn
2265	20	NS4b	Glu	Gly	Gly	Gly
2404	159		Val	Val	Val	Leu
2496	2		Gly	Gly	Gly	Arg
2512	18	NS5	His	Gln	Gln	Gln
2527	33		Met	Thr	Met	Met
2543	49		Thr	Thr	Ile	Ile
2628	134		Val	Ile	Val	Val
3018	524		Ser	Pro	Pro	Pro
3044	550		Lys	Arg	Arg	Arg
3058	564		Asn	Lys	Lys	Lys
3280	786		Val	Ile	Ile	Ile
3298	804		Ala	Thr	Thr	Thr
3324	830		Ser	Ser	Asn	Asn
3326	832		Glu	Gly	Gly	Gly

made by Duong *et al.*, whose data indicate lower levels of virus in patients suffering from DSS compared to patients with DF [23]. This differs from earlier reports that lay the foundation for the current dogma: the higher viremia, the worse the outcome [24].

A significant number of sequence differences were detected by the complete sequencing of the three DENV serotype 1, genotype 1 isolates, showing that different viruses are circulating during an outbreak. By looking at the complete sequences published in GenBank of fifteen DENV-1 genotype 1 isolates from the 2007 epidemic in Cambodia, 69 positions with amino acid variations were found. But even if some variations are unique to one isolate, it can be seen that all of the isolates analyzed are similar to DF sequence or to DHF/DSS sequences. Two sequenced isolates from GenBank are identical to our DSS isolate. The DSS virus had six unique amino acid changes compared to the two other viruses isolated from patients with DF or DHF. Two of these six amino acid substitutions (P277S and L476F) are located in the transmembrane regions of the membrane and envelope proteins. The transmembrane region of the envelope is involved in virion assembly and is required for translocation of the NS1 protein [25, 26]. The role of this domain in virion production could explain a part of the restricted replication observed with the DSS isolate in mammalian cells. The

other mutations were in non-structural genes (NS1-K115R, NS3-S118F, NS5-T49I, NS5-S830N). NS1, and more specifically, the secreted form of NS1, associated with complement has a potential role in the vascular leakage responsible for the shock syndrome [27]. Finally, NS3 and NS5 are proteins with enzymatic activities that are important for viral replication. All of these mutations could be implicated in the *in vitro* and *in vivo* phenotypic characteristics of the DSS isolate. None of the amino acid differences that were found affect the known glycosylation sites or disulfide bonds. It should again be emphasized that our study and our results are preliminary. Further studies are required in order to understand and identify the virulence markers of DENV. More strains of the different DENV serotypes and for each clinical form should be studied for association between phenotypic and genotypic characteristics. Finally, reverse genetic and *in vivo* studies will be required to further identify virulence markers for DENV.

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