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Brief communication (Original)

Time course of concurrent infection with dengue virus serotypes 2 and 4 detected in urine

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Background: In hyperendemic areas, concurrent multiserotype dengue virus (DENV) infections commonly occur in both humans and in mosquito vectors. Previous studies have focused on single blood specimens.

Objectives: To illustrate and characterize the time course of mixed infection with DENV serotypes 2 (DENV2) and 4 (DENV4) in a single case.

Materials and methods: Plasma, saliva, and urine were collected from a patient diagnosed with dengue hemorrhagic fever grade II and secondary DENV infection on days 7, 18, and 31 of his illness. DENV RNA detection was performed using 2 DENV-specific reverse transcriptase-polymerase chain reaction protocols. Cloning and sequencing were performed to quantify the major and minor viral populations in dual-serotype-infected specimen(s). Genotypes of both DENV serotypes were characterized.

Results: DENV genome was detected in plasma and saliva only at the first time point (day 7 of illness), but in urine at both the first and second time points (days 7 and 18 of illness). DENV4 serotype was found in all DENV genome-positive specimens. DENV2 serotype was unexpectedly also detected in the first urine specimen. DENV4 as the major and DENV2 as the minor population. The DENV2 serotype was clustered in genotype Asian I and DENV4 serotype in genotype I.

Conclusion: To our knowledge, this is the first report of concurrent multiserotype DENV infection detected solely in urine. Prospective noninvasive investigations may determine the prevalence of this phenomenon. Clinical and public health implications of this finding need to be explored.

Keywords: Concurrent, dengue virus, mixed infection, multi-serotype, saliva, urine

Dengue virus (DENV) infection is a major health problem in tropical and subtropical countries. The virus consists of 4 serotypes: DENV1–DENV4. DENV are positive single-stranded RNA viruses in the family *Flaviviridae*, which contains 3 structural (capsid (C), membranes (M), and envelope (E)) and 7 nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) for a total length of approximately 11 kb. Transmission of this virus occurs via the vectors *Aedes aegypti* and *Aedes albopictus* [1]. A wide range of clinical symptoms is found in infected patients ranging from asymptomatic infection, dengue fever and, dengue hemorrhagic fever (DHF) to dengue shock syndrome [2].

Previous studies by our group and by others have shown that, during acute infection, DENV genome is detectable in blood, saliva, and urine samples [3-6]. In addition, concurrent infections with multiple serotypes can occur in both infected mosquitoes and humans [7-10]. Here, we present a case of dual DENV infections with DENV2 and DENV4 serotypes in the urine at a single time point. The 'minor' serotype disappeared in subsequent specimens.

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Materials and methods

Patient and specimen collection

This study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (COA No. 478/2008 and 877/2009).

Plasma, saliva, and urine were collected from a 28-year-old man hospitalized at King Chulalongkorn Memorial Hospital, Bangkok in late July 2008 after written informed consent was provided by this patient on an approved form. The patient had been febrile for 6 days. Specimens were collected on days 7, 18, and 31 of his illness. He was diagnosed as having DHF grade II according to the World Health Organization criteria [11]. An anti-DENV-specific enzyme-linked immunosorbent assay (ELISA) revealed an IgM:IgG ratio of less than 1.8 **(Table 1)**, suggesting a secondary DENV infection [12]. Stock DENV1 (Hawaii), DENV2 (16681), DENV3 (H87), and DENV4 (814669) were used as positive controls for molecular assays.

Viral RNA extraction and nested reverse transcription-polymerase chain reaction (nested RT-PCR)

Viral RNA was extracted from all collected specimens in a volume of 140 μL each, using QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted viral RNA (5 μL aliquot of the 140 μL sample) was subjected to nested RT-PCR using the primers described by Yenchitsomanus et al. [13]. First round RT-PCR was performed using a Qiagen OneStep RT-PCR Kit with a left and right pair of DENV genome envelope (E) region outer universal primers (DEUL and DEUR) in a total reaction mixture of 50 μL. Reverse transcription was performed at 50°C for 40 min. Activation of HotStarTaq DNA Polymerase (Qiagen) was set at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The first PCR product (1 μL aliquot of the 50 μL reaction mixture) was used for the nested reaction with a left and right pair of DENV genome E region inner or nested universal primers (DENUL and DENUR). The polymerase was activated at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR product was 434 bp for all 4 serotypes. The PCR experiment and the detection step were conducted in separate laboratories. All standard and strict precautions for PCR were carefully followed for all reactions performed.

Serotype-specific seminested RT-PCR

Serotype-specific RT-PCR was performed on the specimens determined to be DENV-positive by the nested RT-PCR above. The protocol was described by Lanciotti et al. [14]. The reverse transcription was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, a 5 μL aliquot of the 140 μL sample of viral RNA was incubated with the DV2 primer in a total volume of 10 μL at 65°C for 5 min, and chilled on ice for 2 min. The cDNA synthesis was performed at in a total volume of 20 μL 50°C for 50 min, and then 85°C for 5 minutes. Subsequently, RNase H (1 μL) was added and incubated at 37°C for 20 min. A first-round PCR was performed using a 2.5 μL aliquot of each cDNA reaction mixture with DENV serotype-specific primers DV1 and DV2 and HotStarTaq DNA Polymerase in a total reaction mixture of 50 μL). The polymerase was activated at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The first-round PCR product (1 μL aliquot of the 50 μL reaction mixture) was used as the template for the seminested reaction. The primers were D1 and a specific primer for each of the 4 serotypes (DV1 and type-specific (TS)1, DV1-TS2, DV1-TS3, and DV1-TS4). Twenty-five cycles of the same PCR conditions as used for the first round were performed. The PCR products were 482 bp for DENV1, 119 bp for DENV2, 290 bp for DENV3, and 392 bp for DENV4.

Cloning and sequencing

Each 434 bp of nested RT-PCR product was ligated into a pCR8/GW/TOPO TA Cloning Kit (Invitrogen) and transformed into StrataClone SoloPack Competent Cells (Stratagene, Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. Ten to 15 colonies from each specimen were sequenced using both universal forward and reverse primers (M13 forward and T7 promoter) by 1st Base (Selangor Darul Ehsan, Malaysia). Each chromatogram was checked using Chromas Lite (version 2.1.1) and corrected manually. The DENV serotype identification in each sequence was investigated using the BLAST algorithm in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The DENV genotype was analyzed using DENV genotype determination tool in Viral Bioinformatics Research Center [10]. A number of clones representing each serotype in each specimen were investigated.

Results

Patient characteristics

The 28-year-old man hospitalized with 4 days of high undifferentiated fever (39.2°C) with chills and headache denied prior clinical DENV infection. The patient was hemoconcentrated (with peak hematocrit of 47.2%), had leukopenia (with nadir leukocyte count of 2,020 per mm^3), and thrombocytopenic (with nadir platelet count of 8,000 per mm^3). Urinalysis revealed 1+ protein, but otherwise was unremarkable. Aspartate transaminase was elevated at 181 IU/L and alanine transaminase was elevated at 165 IU/L. The patient was febrile for 2 more days in hospital and then made an uneventful recovery upon supportive management. Clinical diagnosis was DHF with significant fluid leakage.

DENV genome detection in plasma, saliva, and urine

The patient was concurrently infected with DENV2 and DENV4. DENV RNA was detected in all specimen types at the first time point (day 7 of illness), but at the second time point (day 18 of illness) the virus was found in urine only. DENV4 was detected in all DENV-positive specimens, while DENV2 was found in the first urine specimen only. No DENV genome was detected in any specimens collected at the third time point (day 31 of illness) (**Table 1 and Figure 1**).

Table 1. ELISA and RT-PCR results for each specimen type on different days

Day of illness	ELISA results (plasma)		RT-PCR results		
	IgM (units)[a]	IgG (units)[b]	Plasma	Saliva	Urine
7	26	93	DENV4	DENV4	DENV4 + DENV2
18	49	113	–	–	DENV4
31	55	106	–	–	–

[a]The cut-off value of IgM was 40 units. [b]The cut-off value of IgG was 100 units.

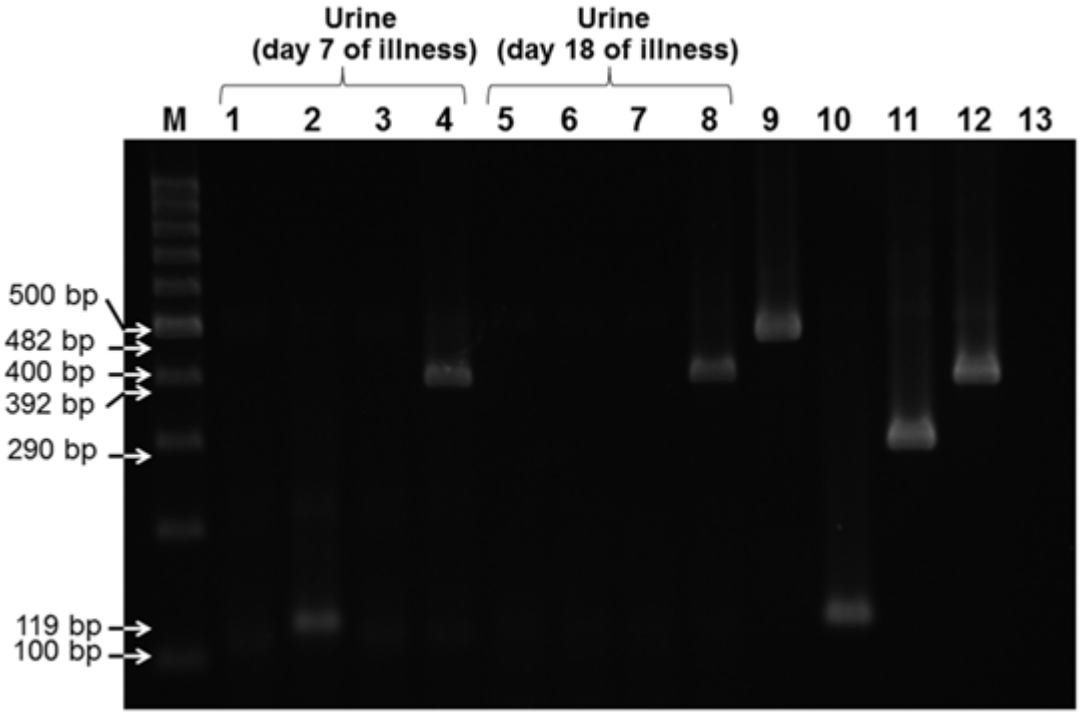

Figure 1. The 2% agarose gel electrophoresis of serotype-nested RT-PCR products of positive specimens collected at different times. Lanes 1–4 are from urine collected on day 7 of illness. Lanes 5–8 are from urine collected on day 18 of illness (lanes 1 and 5 using dengue virus serotype-specific primer DV1 and type-specific primer (TS1); lanes 2 and 6 using primers DV1 and TS2; lanes 3 and 7 using primers DV1 and TS3; lanes 4 and 8 using primers DV1 and TS4). Lanes 9–12 are positive controls

Major and minor serotype populations in dual-serotype-infected urine

In the 15 analyzed clones from the first urine with biserotype infection by DENV2 and DENV4, 11 clones were of DENV4, and the 4 others of DENV2. Clones from the single-serotype specimens were all of DENV4 (**Table 2**).

Genotype analysis in each DENV population

All DENV4 clones were clustered in genotype I, whereas all DENV2 clones were clustered in genotype Asian I (**Table 2**).

None of the above cloned sequences were similar to DENV sequences and strains used or analyzed in our laboratory in the previous few months.

Discussion

Concurrent multiserotype infections by DENV have been described in several dengue-prevalent countries [7, 8, 10, 15-17]. The proportion of such patients could be as high as 5%–15% [16, 17], and the number of serotypes as many as 3 [7, 8, 15]. These concurrent infections were confirmed by both serotype-specific RT-PCR [7, 8, 10, 15-17] and viral isolation [7, 16]. Clinical features and severity in monoserotype and multiserotype infections do not appear to be different [8].

DENV has been shown to be excreted in urine and saliva [3-5]. To our knowledge, our group was the first to demonstrate presence of the virus in urine [4], and to show that the urinary virus is still alive and culturable by mosquito inoculation [18]. The kidneys are consistently recognized among target organs for the virus. Findings include inflammatory changes of and presence of DENV-specific antigen or genome in renal tissues of fatal cases [19-23].

The present report notably combines knowledge and challenges to complex dengue pathogenesis. Clearly, without the DENV molecular assays performed in urine specimens, the patient would have been diagnosed as singly infected with DENV4 alone, based on DENV4 as the only serotype consistently detected in plasma and saliva. While its clinical importance is yet to be elucidated, the phenomenon of concurrent multiserotype DENV infection is probably more common than has been recognized.

Conclusion

Persistent and mixed DENV serotype infections in plasma, saliva, and urine specimens from different sources at different time points can occur in the hyperendemic regions of DENV infection. Our findings may contribute to a new understanding about the pathogenesis of DENV infection.

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The authors have no conflicts of interest to declare.

Table 2. The serotype and genotype analysis of each clone in each specimen type

Specimen	Serotype[a]	No. of selected clones	DENV4 population		DENV2 population	
			No. of clones	Genotype	No. of clones	Genotype
Plasma (7)	4	14	14	I	0	ND
Saliva (7)	4	12	12	I	0	ND
Urine (7)	2 + 4	15	11	I	4	Asian I
Urine (18)	4	13	13	I	0	ND

The number in parentheses represents the day of illness. [a]Serotype of dengue virus (DENV) in each specimen was based on the serotypic RT-PCR results. ND = not determined.

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