Concurrent Isolation of Chikungunya Virus and Dengue Virus from Co-infected Case Imported from Singapore

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Abstract

We report a cluster of two imported cases returned from Singapore to Taiwan, one was co-infected with chikungunya virus and dengue virus type 2, the other was infected with the same dengue virus. Both viruses were successfully isolated from the co-infected case using antibody neutralization and plaque purification technique.
Dengue fever, caused by a flavivirus in the family *Flaviviridae* is the most prevalent arboviral disease in tropical and subtropical regions of Asia, Pacific and Caribbean islands, and Central and South America (1). Chikungunya, caused by an alphavirus in the family *Togaviridae*, is endemic to Africa and Asia (2). Both diseases are transmitted to humans by day biting *Aedes aegypti* and *Ae. Albopictus* mosquitoes, and both diseases have similar clinical symptoms, including fever, rash and joint pains as well as headache, fatigue, nausea, vomiting and muscle pain; a laboratory test is required to distinguish between the two diseases. Thus, many risk factors for chikungunya virus (CHIKV) and dengue virus (DENV) infections are the same or similar. The urban mosquito *Aedes aegypti*, is the primary vector of both viruses throughout most of their geographic range, although *Ae. Albopictus* was recently identified as the main vector of the recently emerged CHIKV E1-226V variant of the African genotype (3).

The explosive epidemics of chikungunya in Indian Ocean islands and India since 2004 and the worldwide increase in travel have facilitated the expansion of different strains of CHIKV of African genotype into overlap areas where dengue is endemic (4). As a result, co-circulation of CHIKV and DENV were reported in various geographic areas including India, Sri Lanka, Gabon, Cameroon, Madagascar, Malaysia, Indonesia, Singapore and Thailand. Consequently, a few studies showing patients co-infected
with CHIKV and DENV were reported in India, Sri Lanka, Malaysia, and Gabon (5-9). Although molecular and serologic evidence demonstrated or suggested co-infections in the above reports, neither CHIKV nor DENV were isolated from these patients. Successful isolation of both viruses will be needed to conduct basic and applied research on CHIKV and DENV biology, immunology, and pathogenesis, as well as the development of laboratory diagnosis, antiviral drugs, and vaccines.

The first and only concurrent isolation of CHIK and DENV-2 viruses was reported by Myers and Carey from a single blood specimen taken from a patient in the acute phase of a dengue-like illness in South India in 1964 (10). In their study, the dominance of CHIKV in the co-infected patient’s serum along with growth competition, prevented the initial isolation of DENV-2; Isolation was finally accomplished through the pretreatment of the acute serum sample with CHIKV-specific mouse antibody followed by inoculation into infant mice for in vivo growth. Here we report only the second case confirmed by actual isolation of CHIKV and DENV-2 from a patient returned from Singapore, using an in vitro cell culture technique.

The two imported cases, reported by our hospital surveillance system were part of a group tour to Singapore during 17-20 April, 2009. One patient (Case 1) was
co-infected with CHIKV and DENV-2 and the other, a sibling of Case 1, (Case 2) was infected with the same DENV-2 strain. Table 1 shows the summary data of Case 1 reported as a suspected dengue case on 23 April, 2009. He had symptoms of fever, headache, vomiting, arthralgia, rash and skin itch. Molecular screening for flavivirus and alphavirus infections using multiplex 1-step SYBR Green I-based real-time reverse transcription PCR (RT-PCR) (11-12), showed positive reactions to both alphavirus and DENV infections, suggesting the possibility of co-infection. Confirmation using specific primers showed positive reactions to CHIKV and DENV-2, respectively. The co-infection results were later confirmed by positive seroconversion of both CHIKV-specific and DENV-specific IgM and IgG antibodies in day 24 convalescent phase serum samples. Case 2 had symptoms of fever, headache, muscle pain and abdominal pain. The DENV-2 strain was successfully isolated from a day 4 acute phase serum sample of Case 2 by in vitro cell culture using the C6/36 cell line.

From the co-infected patient, CHIKV was readily isolated from day 2 acute phase serum sample using the C6/36 cell line. However, initial isolation of DENV-2 was not successful, likely due to inferior growth competition with the dominant CHIKV. To eliminate the CHIKV, neutralization was attempted by pre-treatment of the acute phase serum with a day 17 convalescent serum from a CHIK patient (12).
This serum had high titer CHIKV-specific antibodies, but no DENV-specific antibodies. Briefly, the acute phase serum from Case 1 was mixed with CHIKV convalescent serum at the ratio of 1:2 for 1 hour at 37°C, then seeded in BHK-21 cells in a 6-wells plate overlaid with methylcellulose prepared in MEM-5% FBS. The culture was incubated at 37°C for 5 days and single plaques were picked for expansion in Vero cells. All 24 clones were DENV-2 isolates confirmed by immunofluorescence test and RT-PCR (1).

The nucleotide sequences of the envelope gene of CHIKV and DENV-2 strains were sequenced as previously described and submitted to GenBank (accession nos. HM067743, HM0677746 and HM0677747) (11-13). Figure 1 showed the phylogenetic tree of CHIKV constructed on the basis of complete envelope gene nucleotide sequence (4). The strain (CHIK/Singapore/0904aTw) was grouped into the Central/East/South African genotype with an E1-226V mutation, a different lineage from our previous Singapore isolate having an E1-226A, and is closely related to strains isolated from Malaysia. Figure 2 showed the phylogenetic tree of DENV-2 constructed on the basis of complete envelope gene nucleotide sequences (14). These two isolates, D2/Singapore/0904aTw and D2/Singapore/0904bTw, derived from Case 1 and Case 2, respectively, had 100% nucleotide identity in envelope gene and belonged to the Cosmopolitan genotype. Our data are in agreement with recent reports.
that E1-226V mutant CHIKV strains grouped in Central/East/South African genotype and DENV-2 strains grouped in Cosmopolitan genotype were predominant epidemic strains circulated in Singapore in 2009 (15-16). As various genotypic strains may differ in epidemic potential and virulence, molecular epidemiological surveillance can provide valuable information in decision-making regarding patient care, outbreak investigation and control measures (17-18).

Successful isolation of both viruses from the co-infected case remains a challenge since the dominant virus will usually overgrow the minor virus. This problem can be overcome by combination of virus neutralization using convalescent human serum with high titer antibodies followed by in vitro plaque purification. Our results showed that this is a very efficient way to concurrently isolate both CHIKV and DENV-2. Similar approach has been used to isolate two DENV serotypes, a DENV-1 and a DENV-4 strain from a co-infected patient (19). With the expectation that co-infection cases with DENV and CHIKV will be increased in the future due to increased transmission of both viruses in various areas of India, Southeast Asia, and Africa, enhanced surveillance to clinically and diagnostically differentiate CHIKV and DENV infections is needed for early recognition of virus invasion and local transmission, better patient care and timely control measures.
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1 References


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Figure legend:

Figure 1. Phylogenetic analysis of the complete envelope 1 (E1) gene (1317 nt) of chikungunya virus (CHIKV) isolate from an imported case returned from Singapore co-infected with CHIKV and dengue virus. Sequence obtained in this study is designated by boldface type. CHIKV strains with the E1-A226V mutation are indicated. Viruses were identified by using the nomenclature of virus/country/strain/year of isolation/GenBank accession number. The analysis was performed using MEGA 4 software, by using the neighbor-joining (maximum composite likelihood) methods. Bootstrap support values > 60 are shown (1,000 replicates). The scale bar on the left indicates number of nucleotide substitutions per site.

Figure 2. Phylogenetic analysis of the complete envelope gene (1485 nt) of dengue virus type 2 isolates from two imported cases (Case 1 and Case 2) returned from Singapore. Sequences obtained in this study are designated by boldface type. Viruses were identified by using the nomenclature of virus/country/strain/year of isolation/GenBank accession number. The analysis was performed using MEGA 4 software, by using the neighbor-joining (maximum composite likelihood) methods.
Bootstrap support values > 75 are shown (1,000 replicates). The scale bar on the left
indicates number of nucleotide substitutions per site.
Table 1. Summary data of imported case (Case 1) co-infected with CHIKV and DENV from Singapore

<table>
<thead>
<tr>
<th>Patient</th>
<th>Case 1 (brother)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infections</td>
<td>CHIKV and DENV-2</td>
</tr>
<tr>
<td>Age</td>
<td>12</td>
</tr>
<tr>
<td>Gender</td>
<td>male</td>
</tr>
<tr>
<td>Travel period in</td>
<td>17-20 April 2009</td>
</tr>
<tr>
<td>Singapore</td>
<td></td>
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<tr>
<td>Onset of disease</td>
<td>22 April 2009</td>
</tr>
<tr>
<td>Clinical symptoms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fever</td>
</tr>
<tr>
<td></td>
<td>Headache</td>
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<tr>
<td></td>
<td>Vomiting</td>
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<td></td>
<td>Arthralgia</td>
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<td></td>
<td>Rash</td>
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<td></td>
<td>Skin itch</td>
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<td>Laboratory findings</td>
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<tr>
<td>Real-time RT-PCR</td>
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<tr>
<td>Day2</td>
<td></td>
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<tr>
<td>CHIKV+</td>
<td></td>
</tr>
<tr>
<td>$10^{5.6}$ PFU/mL</td>
<td></td>
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<tr>
<td>DENV-2+</td>
<td></td>
</tr>
<tr>
<td>$10^{1.3}$ PFU/mL</td>
<td></td>
</tr>
<tr>
<td>Virus isolation</td>
<td>CHIKV and DENV-2</td>
</tr>
<tr>
<td>DENV IgM/IgG</td>
<td></td>
</tr>
<tr>
<td>(ELISA OD value)</td>
<td>0.13/0.106 (day 2)</td>
</tr>
<tr>
<td></td>
<td>1.383/0.702 (day 24)</td>
</tr>
<tr>
<td>CHIKV IgM/IgG</td>
<td></td>
</tr>
<tr>
<td>(ELISA OD value)</td>
<td>0.087/0.098 (day 2)</td>
</tr>
<tr>
<td></td>
<td>2.074/1.611 (day 24)</td>
</tr>
</tbody>
</table>
Figure 1. A phylogenetic tree showing the evolutionary relationships of different strains of *Chikungunya virus*.

Key:
- Central/East/South African
- Asian
- West African

Strain codes and references:
- CHIK/Malaysia/0901aTw/2008/FJ807895
- CHIK/Malaysia/0812aTw/2008/FJ807892
- CHIK/Singapore/122508/2008/FJ445502
- CHIK/Malaysia/Mal2008/2008/GQ168719
- CHIK/Singapore/0904aTw/2009/HM067743 (Case 1)
- CHIK/India/91170/2006/EF451148
- CHIK/India/0812cTw/2008/FJ807894
- CHIK/Bangladesh/0810aTw/2008/FJ807898
- CHIK/India/IND-06 RU/1/2006/EF027137
- CHIK/Singapore/0611aTw/2006/FJ807896
- CHIK/Mauritius/1225/2006/EU564334
- CHIK/India/IND-00-MH4/2000/EF027139
- CHIK/Malaysia/MY002MR/2006/EU703759
- CHIK/India/IND-63-WB1/1963/EF027140
- CHIK/Indonesia/0811aTw/2008/FJ807891
- CHIK/Senegal/37997/1983/AY726732
- CHIK/Tanzania/S27/1953/NC004162
Figure 2.