Molecular epidemiology of dengue fever cases imported into Romania between 2008 and 2013

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Summary  Background: Dengue fever is the commonest arthropod-borne infection worldwide. In recent years, rapid growth in global air travel has resulted in a considerable increase in the incidence of imported cases. In Romania it is now the second most frequent cause for hospitalization (after malaria) in patients arriving from tropical regions.

Methods: Serological and molecular diagnostics were applied to samples obtained between 2008 and 2013 from travelers with suspected dengue. Molecular typing was performed by RT-PCR followed by sequencing of the E-NS1 junction.

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1. Introduction

Dengue fever is the most widely distributed arthropod-borne infection worldwide. According to World Health Organization 2.5 billion humans living in tropical and subtropical regions are at risk of acquiring dengue infection [1], with an estimated 390 million cases per year [2]. The dengue viruses (DENVs) comprise a distinct serocomplex of the family Flaviviridae in the genus Flavivirus [3,4]. There are four divergent serotypes (DENV-1 to DENV-4), based on genetic and antigenic properties. These display 63–68% amino acid sequence homology [5,6]. In addition, a new viral serotype (DENV-5) has been identified in a human outbreak in Malaysia in 2007; there is evidence that this circulates among macaques [7]. Serotypes 1–4 are further classified into genotypes according to nucleotide divergence of up to 6% in the envelope glycoprotein coding region. DENV-1 comprises five genotypes (I–V) with a broad distribution: Southeast Asia, China, East Africa (genotype I); Thailand (genotype II); Malaysia (genotype III); West Pacific islands and Australia (genotype IV); America, West Africa and Asia (genotype V) [8]. Six genotypes have been proposed for DENV-2: Asian I (Southeast Asia and East Asia); Asian II (Southeast Asia, East Asia, Oceanica, Caribbean region and the Americas); Asian/American (Southeast Asia, Caribbean region and Americas); a cosmopolitan genotype (India, South-Central Asia, East Asia, East Africa, Middle East, Southeast Asia and Oceanica); an American genotype (India, Caribbean region, South America, Central America, Oceanica and Trinidad) and sylvatic genotype (West Africa and Malaysia) [9]. DENV-3 isolates are assigned to four genotypes: genotype I (Indonesia, Malaysia, Philippines and South Pacific islands), genotype II (Thailand, Vietnam and Bangladesh), genotype III (Sri Lanka, India, Africa and Samoa), and genotype IV (Puerto Rico, Latin and Central America and Tahiti) [10]. Phylogenetic analysis indicates the existence of four genotypes within the DENV-4 serotype: genotype I (Thailand, the Philippines, Sri Lanka and Japan), genotype II (Indonesia, Malaysia, Tahiti, the Caribbean and the Americas), genotype III (Thailand) and genotype IV (sylvatic strains from Malaysia) [10].

The virus is transmitted by mosquitoes of the genus Aedes, primarily Aedes aegypti and Aedes albopictus, and is maintained in urban cycles in humans and sylvatic cycles in non-human primates [1,11]. Spillover from the sylvatic cycle to the urban cycle has been documented [12,13]. The infection can be asymptomatic or range from an undifferentiated acute febrile illness to “classic” dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [14]. At present there are no specific treatments or commercially available vaccines [15].

One of the largest epidemics of dengue ever recorded occurred world-wide was in refugee camps in Greece (1927–1928), with at least 650,000 cases and 1,000 deaths. The vector was Ae. aegypti [16]. In recent years there has been a sharp rise in the number of imported DF in Europe, attributable to a rapid rise in international air travel and high rates of transmission in endemic countries [17]. The introduction of Ae. albopictus followed by rapid colonisation has given rise to autochthonous cases in mainland France (2010, 2013) and Croatia (2010) [18–20]. In addition, more than 2,000 cases were recorded in Madeira (2012), an autonomous region of Portugal and therefore technically a part of Europe. In this case the vector was Ae. aegypti, a recent arrival in the archipelago [21,23]. The rapid dissemination and establishment of the Ae. albopictus in Southern Europe, and the intense increase in traveling fuel the introduction of dengue in non-endemic areas [24]. This species was detected in 2012 in Bucharest, Romania, and has remained present in subsequent years (Prioteasa et al., personal communication). Dengue is now included in the list of notifiable communicable diseases; laboratory diagnosis by the Romanian National Reference Centre for Vector-Borne Infections was implemented starting 2008. In the present study we present the molecular epidemiology of imported DF cases in Romania between 2008 and 2013.

2. Material and methods

2.1. Patients and laboratory diagnosis

Sera from travelers with clinical suspicion of dengue fever after return from dengue-endemic regions were received by the National Reference Centre for Vector-Borne Infections (“Cantacuzino” N.I.R.D.M.I.) from Public Health Departments and from Clinical Hospital of Infectious and Tropical Diseases “Dr. Victor Babeș” in Bucharest. The samples were tested for DENVs antibodies with IgM and IgG indirect ELISA (Euroimmun, Lübeck, Germany). Paired sera were requested but convalescent serum was not available.
for all patients. Sera taken in the acute phase of the disease (up to eight days from fever onset) were subjected to molecular analysis. A case was classified as probable when IgM was detected in serum obtained during the acute phase. Confirmed cases were defined by IgM and IgG, seroconversion for IgG in paired sera, and/or detection of DENVs nucleic acid in sera by RT-PCR and DNA sequencing.

2.2. Molecular typing

Viral RNA was extracted from 140 µL of serum using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Reverse transcription and PCR were carried out using primers described earlier [25] that amplify a fragment located within the E and NS1 viral genomic regions junction. The amplicons were gel purified (Wizard® SV PCR and Gel Clean-Up System; Promega, Madison, WI) and sequenced with BigDye Terminator v3.1 on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The DNA sequences were visually inspected and aligned with BioEdit version 7.0.5.3 [26]. Phylogenetic analysis was conducted using Mega 6 software [27], Neighbor-joining algorithm with 1,000 bootstrap replicates. Sequences obtained in this study were submitted to GenBank.

3. Results and discussion

From 2008 to 2013, serum samples from 37 suspected patients returning from endemic countries were tested for the presence of IgM and IgG dengue-specific antibodies (Table 1). The patients declared a history of recent travel in Asia, Africa and Europe (Madeira). One patient, a cruise ship worker, took a voyage in Southeast Asia and islands in the Indian Ocean. For six DF suspected patients we did not receive any information regarding their traveling history.

Twelve DF cases were confirmed in patients with recent travel to India, Indonesia, Vietnam-Thailand, Angola, Kenya and Portugal (Madeira). Three more cases, returning from Vietnam-Thailand, India and Sri Lanka, were classified as probable (Table 1).

Nine DNA sequences were obtained from patients returning from India, Indonesia, Vietnam-Thailand and Portugal (Madeira). The isolates were of DENV-1 (genotypes I and V), DENV-2 (cosmopolitan genotype) and DENV-3 (genotypes I and III) (Table 2). In half of the confirmed and probable cases, the infected persons arrived during the Ae. albopictus season in Romania, months May to October.

3.1. DENV-1 phylogenetic analysis

Five out of the nine analyzed sequences cluster into DENV-1. The first sequence (GenBank acc. no. HE565699), belonging to genotype I, was obtained from the serum of a patient working on a cruise ship in 2011 who declared a history of travel in Southeast Asia (Vietnam and Thailand) and islands of Indian Ocean and was repatriated to Romania from Maldives after the illness onset. The phylogenetic analysis indicates 100% identity between our sequence and sequences obtained in recent years in Southeast Asia (Cambodia, Malaysia, Thailand, Vietnam) where genotype I is circulating [8]. A similar strain was involved in a major outbreak in Colombo, Sri Lanka (2012) after its introduction in 2009 [28,29]. Phylogeographic analysis performed by Ocwieja et al. indicates that the origins of this strain might be the virus causing severe dengue in Thailand (2001) and which later spread to neighboring Cambodia and Vietnam [29]. Two other sequences clustering into genotype I of DENV-1 were obtained from patients who traveled to Indonesia in 2013 (GenBank acc. no. HG918038-9). These were remarkably similar to sequences obtained in 2004, 2011 and 2013 in China, as well as Singapore (2003) and Malaysia (2005). Two sequences fell into genotype V of DENV-1. The first was obtained from a patient returning from India in 2008 (GenBank acc. no. FR874940) and was identical to others obtained from viremic subjects in that country in 2010 and 2011. Similar sequences had been obtained from India (1982 and 2009) and Sri Lanka (1997 and 2004). It is noteworthy that the first autochthonous cases of DF in Croatia were due to DENV-1 in the same cluster of

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of suspected cases tested</th>
<th>Number of confirmed cases</th>
<th>Number of probable cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>2</td>
<td>1 (India)</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>7</td>
<td>4 (Indonesia, Thailand, Angola, Kenya)</td>
<td>0</td>
</tr>
<tr>
<td>2012</td>
<td>11</td>
<td>3 (Vietnam-Thailand, Madeira Island, India)</td>
<td>1 (Vietnam-Thailand)</td>
</tr>
<tr>
<td>2013</td>
<td>16</td>
<td>4 (Indonesia-3, Vietnam-Thailand)</td>
<td>2 (Sri Lanka, India)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>12</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Genotype</th>
<th>Year</th>
<th>History of traveling</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
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<td>DENV-1</td>
<td>I</td>
<td>2011</td>
<td>Vietnam-Thailand</td>
<td>HE565699</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>2012</td>
<td>Madeira Island</td>
<td>HG918038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>Indonesia</td>
<td>HG918039</td>
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<td></td>
<td></td>
<td>2013</td>
<td>Indonesia</td>
<td>FR874940</td>
</tr>
<tr>
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<td>Cosmopolitan</td>
<td>2012</td>
<td>Madeira Island</td>
<td>HF955512</td>
</tr>
<tr>
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<td>I</td>
<td>2011</td>
<td>Indonesia</td>
<td>HF955511</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>2012</td>
<td>Vietnam-Thailand</td>
<td>FR822184</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
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<tr>
<td></td>
<td></td>
<td>2013</td>
<td>Vietnam-Thailand</td>
<td>HE800554</td>
</tr>
</tbody>
</table>
isolates [30]. The second sequence (GenBank acc. no. HF955512) falling into genotype V of DEN-1 was amplified from the serum of a patient traveling to Madeira in 2012 at the time of the outbreak mentioned above [22]. Our sequence is 99.94% identical with a sequence derived from an isolate obtained in 2008 in Colombia, South America. As shown earlier, the isolate from Madeira probably originated in Brazil, Columbia or Venezuela [31,32].

3.2. DENV-2 phylogenetic analysis

The only DENV-2 sequence obtained in this study (GenBank acc. no. HF955511) was from a patient arriving from India in 2012. A similar sequence was found in 2011 in an Irish patient with an unknown travel history. Phylogenetic analysis indicates that this isolate belonged to the cosmopolitan genotype of DENV-2. Similar sequences were previously obtained from patients in India as early as 1996 or in the neighboring country Bangladesh or in Sri Lanka.

3.3. DENV-3 phylogenetic analysis

DENV-3 sequences belong to genotypes I and III. One sequence (GenBank acc. no. FR822184) from a patient arriving from Indonesia (2011) clusters with genotype I sequences retrieved from East Timor (2000), Australia (2008), as well as endemic viruses from Indonesia (2004). The Indonesian sequences belong to the Sumatran-Javan clade, a viral lineage that appears to have a superior level of evolutionary fitness and epidemic potential [33]. The sequences belonging to genotype III (GenBank acc. no. HE800554 and HG918040) were obtained from two patients arriving from Vietnam-Thailand in 2012 and 2013, respectively. The two sequences are highly similar to each other and share a high degree of similarity with sequences obtained from Cambodia (2011) and Pakistan (2005–2009). As previously shown, the Pakistanis strains emerged from isolates already circulating in Indian subcontinent before 2005–2006 [34].

4. Conclusions

This is the first report on the molecular epidemiology of DF imported into Romania. Given the small number of cases, the widespread geographic origins of these cases is remarkable, a graphic demonstration of the global mobility of this virus. DF is a potentially serious infection. For this reason it should be included in differential diagnosis in patients with fever and recent travel in endemic regions. Molecular investigation of samples from viremic patients is of interest for typing the viruses, characterizing their genome and detecting their origin and evolution in endemic regions. Autochthonous cases are likely wherever competent vectors are present, which now includes continental Europe.

Contributors

Sorin Dinu, Ioana R. Pânculescu-Gâţeţ and Gabriela Oprisan performed molecular analysis. Daniela Bădescu and Cornelia S. Ceianu performed the serological diagnosis. Simin A. Florescu and Corneliu P. Popescu performed clinical diagnosis. Anca Sirbu managed dengue fever surveillance. Sorin Dinu and Ioana R. Pânculescu-Gâţeţ wrote the paper. Leticia Franco advised regarding the molecular approach and provided a critical review of the manuscript. Cornelia S. Ceianu coordinated the study.

Conflict of interest

None.

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