Molecular characterization of dengue virus 1 from autochthonous dengue fever cases in Croatia

I. C. Kurolt[1], L. Betica-Radić[2], O. Đaković-Rode[1], L. Franco[3], H. Zelena[4], A. Tenorio[3] and A. Markotić[1]

1) *University Hospital for Infectious Diseases 'Dr Fran Mihaljević', Zagreb, Croatia,* 2) *General Hospital, Dubrovnik, Croatia,* 3) *National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain and* 4) *Institute of Public Health Ostrava, Ostrava, Czech Republic*

Abstract

In the summer of 2010, two autochthonous dengue fever cases were detected in Croatia. Here we report the retrospective detection of an additional case of dengue fever, representing the first sustained autochthonous transmission in Europe since 1928. In addition, we present the phylogenetic analyses based on two sequences from the Pelješac peninsula, southern Croatia. The sequences were identified as dengue virus genotype 1 and recovered from two out of the three Pelješac patients in whom infection occurred.

Keywords: dengue virus 1, autochthonous transmission, Croatia, *Aedes albopictus*, arbovirus
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Corresponding author: I. C. Kurolt, University Hospital for Infectious Diseases 'Dr Fran Mihaljević', Research Department, Mirogojska 8, 10000 Zagreb, Croatia
E-mail: ikurolt@bfm.hr

Four viruses, dengue virus (DENV) 1, 2, 3 and 4, are recognized within the DENV group (family *Flaviviridae*, genus *Flavivirus*). Human infection with these viruses may cause: inapparent infection, a mild and self-resolving febrile illness with rash or the more serious dengue haemorrhagic fever/dengue shock syndrome. About two-fifths of the world population reside in DENV-endemic areas, with about 50 million infections annually [1]. The disease is endemic in the Americas, Africa, Asia and Oceania. The last documented epidemic in Europe occurred in 1928 in Greece [2] and other Mediterranean countries [3], where *Aedes aegypti* was the incriminated mosquito vector. However, in Croatia the alternative vector, the invasive *Ae. albopictus*, was first discovered in 2004 [4], and then spread throughout Croatia [5] and ten other Mediterranean countries by 2008 [6]. Furthermore, an epidemic of chikungunya virus infection in northern Italy in 2007 [7], as well as autochthonous circulations of chikungunya in southern France in September 2010 [8], were all associated with *Ae. albopictus*. In addition, two autochthonous DENV infections associated with the same vector emerged almost simultaneously in the south of France and Croatia [9,10].

The first imported Croatian case of DENV occurred in August 2007, in a prominent tourist destination, Dubrovnik. The patient was a woman who had recently returned from India [11]. Since then, five additional imported cases have been treated at the University Hospital for Infectious Diseases, Zagreb, Croatia (A. Markotić, unpublished data). In August 2010, after a DENV infection had been confirmed in a traveller who had returned from his vacation in southern Croatia (Korčula Island and the Pelješac peninsula) to Germany [12], an autochthonous DENV circulation in Croatia was suspected. Two months later, a 54-year-old woman, a resident of Podobuče village on Pelješac, the same village that the German tourist had stayed in, was diagnosed with dengue fever [10,12] as described below.

Retrospectively, we confirmed that her daughter, a 33-year-old female, had contracted dengue fever as well. On 11 August she, too, had been admitted to the Department of Infectious Diseases, Dubrovnik General Hospital. No aetiological diagnosis had been established for the 33-year-old female at the time. However, both mother and daughter had fever (39°C), headache, dry cough, joint and muscle pain, thrombocytopenia, leucopenia, neutropenia and elevated aspartate transaminase.

Until the testing, the acute sera samples of both patients had been stored at the Dubrovnik General Hospital. The presence of NS1 antigen and IgM antibodies to an unspecified DENV was confirmed with two distinct commercial immunochromatographic assays (Focus Diagnostics, Cypress, CA, USA). In addition, IgG antibodies to an unspecified DENV were detected by ELISA in the serum sample from the 33-year-old woman, 2 months after the dengue fever onset.

For the purpose of DENV molecular characterization, phenol-chloroform extraction was performed in order to isolate total RNA from the acute serum samples of both patients, as well as the stored whole blood sample from the first recognized imported Croatian case (August 2007) [11]. In

addition, we decided to include in the analysis the three samples from cases recently imported from India to the Czech Republic and Spain, because only limited data on recent Indian DENV-1 strains were available through GenBank.

The virus was identified using nested RT-PCR, described elsewhere [13]. The 306 bp product was sequenced on an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), using a BigDye termination Kit and the same primers employed to generate the amplicon.

Using the implemented ClustalW algorithm in MEGA5 [14], a 222 bp sequence (nucleotides 2198–2419), from the study sequences covering the E-NS1 junction, was aligned with additional sequences obtained from GenBank. As it was not possible to obtain a longer sequence, the phylogenetic analysis was limited to this 222 bp sequence, allowing the usage of more entries available from GenBank.

The sequences obtained from the two Croatian patients in 2010 were 100% identical to each other and 98% identical to the sequence obtained from the first case imported into Croatia (2007).

All samples belong to DENV-1, genotype V, American/African genotype, within the Indian II lineage (Fig. 1). The Croatian autochthonous cases and the cases imported to the Czech Republic and Spain cluster together within the Indian II lineage. Sequences isolated during previous years, including the 2007 case imported from India to Croatia [11], as well as the sequences from India, Sri Lanka and the Comoros, form another cluster within the same lineage.

Following the detection of an autochthonous circulation in Podobuče village, Pelješac peninsula, Croatia, where in August 2010 a 33-year-old woman had contracted DENV-1, and after more than 80 years of DENV absence in Europe, the first autochthonous dengue infection was molecularly characterized in Croatia. Both the woman's mother and the aforesaid German tourist, who had stayed in the same village [10,12], later developed dengue fever.

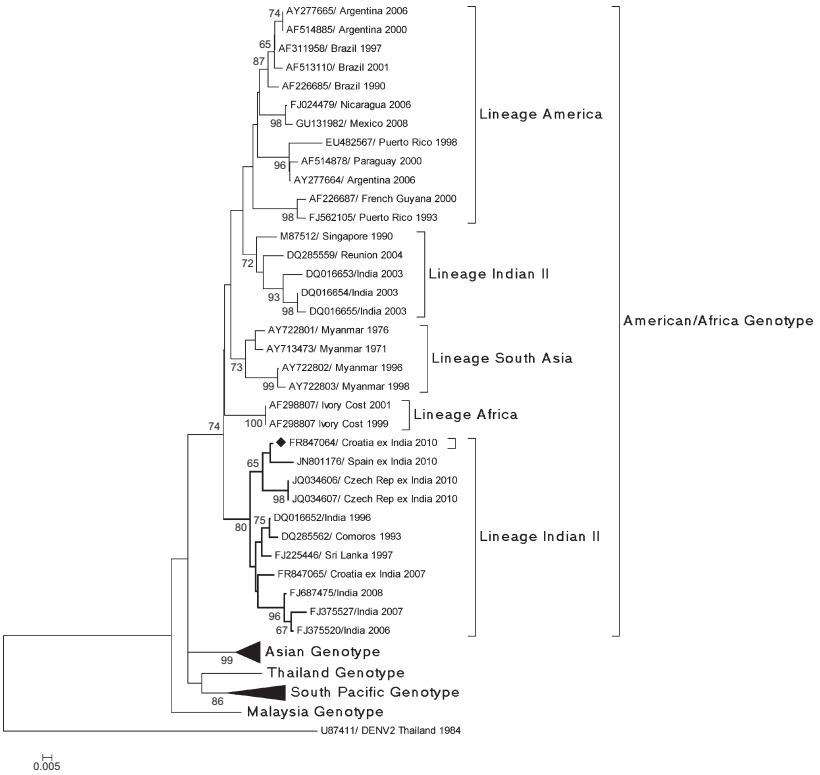

FIG. 1. Phylogenetic tree from DENV serotype 1 strains and isolates based on a 222 bp fragment from the E-NS1 junction, corresponding to the nucleotides 2198–2419 of the DENV1 reference strain. This tree was inferred using the Neighbour-Joining method, employing the p-distance Substitution model including Transitions and Transversions, was constructed by bootstrap analysis of 1000 replicates using MEGA5 software and rooted by the DENV serotype 2 strain 16681 (Accession No U87411). The numbers at the nodes indicate percentage bootstrap replicates (of 100); values below 65% are not shown. Scale bar indicates nucleotide substitutions per site. The sequence obtained from the two autochthonous cases is marked with a rhombus.

This finding suggests that the recent small cluster of autochthonous cases probably originated from a DENV-1-infected person arriving in Croatia from the Indian subcontinent at an earlier time in 2010, and that the virus established itself in the local *Ae. albopictus* population.

In conclusion, we would like to emphasize that this is the first molecular and phylogenetic characterization of autochthonous DENV, which has reappeared in Europe after 80 years. Although the first Croatian autochthonous DENV-1 cases were detected in an isolated peninsula village, with no evidence that this virus has spread elsewhere, infection with this virus represents a potential threat to Croatia and other European countries where *Ae. albopictus* is now widespread.

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Transparency Declaration

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