

Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012

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An outbreak of dengue fever in Madeira island was reported in 2012. Clinical and laboratory findings of the first two laboratory-confirmed autochthonous cases are reported. Both cases had fever ($\geq 38^\circ\text{C}$) and petechial rash. Symptoms also included myalgia, asthenia, nausea, vomiting, anorexia, diffuse abdominal pain, and diarrhoea. The two cases were confirmed by serology and one tested positive for a dengue viral sequence. Dengue virus serotype DEN-1 was identified with probable Central or South American origin.

Dengue virus (family *Flaviviridae*, genus *Flavivirus*) is the aetiological agent of dengue fever, a mosquito-borne infection endemic in the tropics and subtropics. The National Institute of Health Dr. Ricardo Jorge (INSA) performs reference laboratory diagnosis of dengue in Portugal. Human cases identified annually in this laboratory are imported from endemic areas. In terms of number of positive samples, these areas include by order of importance, mainly Brazil, but also Timor-Leste, India, Cape Verde, Mexico, Thailand, Angola, Pakistan and Vietnam. In the beginning of October 2012, for the first time, two autochthonous cases of dengue fever from Madeira archipelago, Portugal were diagnosed. These signaled the beginning of an outbreak and the latest published data includes 2,144 reported autochthonous cases of dengue fever in Madeira island [1]. Clinical and laboratory findings of these first two cases are reported.

Case reports

Case 1

On 20 September, 2012 a 16 year-old woman developed febrile illness with temperature up to 38.5°C , nausea, vomiting, anorexia, diffuse abdominal pain and diarrhoea. Five days later she presented at the local hospital, with prostration, and, in the second day after admission developed petechial rash at the upper and lower limbs that spread to the lower abdomen within 24 hours.

The patient, who lives in Caniço in the neighbourhood of the town of Funchal and studies in Funchal, mentioned traveling to Algarve, south of mainland Portugal, six weeks before and had not been vaccinated against flaviviruses such as yellow fever, tick-borne encephalitis and Japanese encephalitis viruses.

The first laboratory findings in the hospital showed thrombocytopenia with minimal platelet count ($65 \times 10^9/\text{L}$, norm: $150\text{--}400 \times 10^9/\text{L}$), leucopenia ($2.4 \times 10^9/\text{L}$, norm: $4.5\text{--}13.5 \times 10^9/\text{L}$) and elevated transaminases (alanine/glutamic pyruvic transaminase (ALT/GPT): 117U/L , norm: $17\text{--}63 \text{U/L}$; aspartate/glutamic-oxaloacetic (AST/GOT): 95U/L , norm: $10\text{--}50 \text{U/L}$). In the Funchal hospital the screening test for dengue was performed by immunochromatography (NADAL dengue fever IgG/IgM) followed by dengue IgM and IgG capture enzyme-linked immunosorbent assay (ELISA) (Panbio). The serum sample taken on day 6 after the onset of febrile illness was positive by immunochromatography, ELISA IgM positive (5.91) and IgG negative (0.07) (cut-off >1.1).

In the National Institute of Health, sera samples were tested by immunofluorescent assay in-house (IgG and IgM) and Euroimmun commercial test (IgM). The serum sample (day 11 post onset) was positive for dengue virus specific IgM with a titre of 256 (cut-off =16) and IgG with a titre of 1,024 (cut-off =32). The serum sample was also tested for immunoglobulins specific to other flaviviruses, such as yellow fever, tick-borne encephalitis, West Nile and Japanese encephalitis viruses by immunofluorescent assays and all assay results were negative. The patient recovered without complications and was discharged from the hospital on the eighth day of hospitalisation.

Case 2

On 27 September 2012, a 44 year-old man developed febrile illness with temperature up to 38.0°C , myalgia

and asthenia. Two days later he visited the local hospital and presented also with petechial rash at the upper and lower limbs.

The patient who lives and works in Santa Luzia, a local administrative unit of the city of Funchal, did not travel abroad and reported no vaccination against yellow fever, tick-borne encephalitis and Japanese encephalitis viruses.

The first laboratory findings in the hospital showed thrombocytopenia with minimal platelet count ($74 \times 10^9/L$, norm: $150-400 \times 10^9/L$), leucopenia ($2.7 \times 10^9/L$, norm: $4.5-13.5 \times 10^9/L$), very high creatine kinase (CK) (1,146 ng/ml, norm: 20–200 ng/ml), and slightly elevated AST/GOT (58 U/L, norm: 10–50 U/L), high lactate dehydrogenase (LDH) (395 U/L, norm: <246 U/L). A sample collected on day 2 post onset was positive by immunochromatography, ELISA IgM positive (5.32), unusually at day 2, and IgG negative (0.18). The IgM antibodies in cases of dengue infection are usually detected after the fifth day following the onset of symptoms suggesting that maybe, in this case, the date of the onset was not properly determined.

In the National Institute of Health the serum sample (day 4 post symptom onset) tested by immunofluorescent assay was positive for dengue virus specific IgM with a titre of 1,024 (cut-off =16) and IgG with a titre of 512 (cut-off =32). The serum sample was also subjected to immunofluorescent assays for immunoglobulins specific to other flaviviruses, such as yellow fever, tick-borne encephalitis, West Nile and Japanese encephalitis viruses and all assay results were negative.

The patient recovered without complications and was discharged from the hospital on the fourth day of

hospitalisation. Twelve days after the onset of symptoms he returned for further evaluation, and the blood count had returned to normal (leukocytes $7.2 \times 10^9/L$, norm: $4.5-13.5 \times 10^9/L$) and platelets ($326 \times 10^9/L$, norm: $150-400 \times 10^9/L$).

Molecular laboratory analysis

In the National Institute of Health laboratory, nucleic acids were extracted from ethylenediaminetetraacetic acid (EDTA) blood samples using NucliSens easyMAG platform (bioMérieux). The presence of dengue virus RNA was checked by one-step conventional reverse transcription-polymerase chain reaction (RT-PCR) using generic flavivirus primers targeting the non-structural protein 5 (NS5) gene [2,3] followed by dengue specific assays including multiplex RT-PCR targeting the core-pre-membrane (CprM) region [4,5] and real time RT-PCR [6].

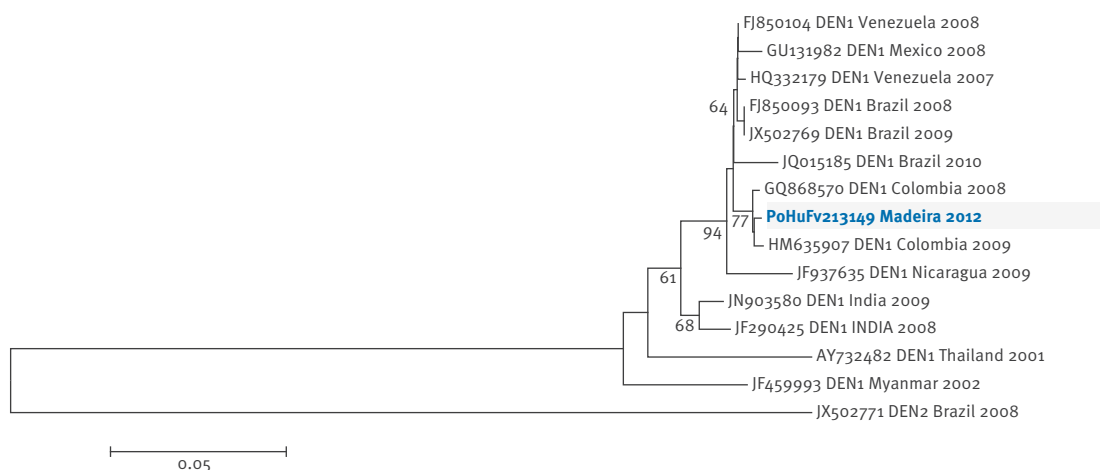
Fragments obtained by nested-PCR (NS5, 163 bp and CprM region, 454 bp) were sequenced bi-directionally. The 454 bp CprM partial polyprotein gene sequence was deposited on the GenBank database under accession number KC248375. Similarity searches were made within the GenBank data set using the basic local alignment search tool (BLAST) BLASTN algorithm [7].

For case 1, RT-PCR for flaviviruses was negative as well as the results from both dengue specific assays (conventional multiplex and real time RT-PCR) on day 11 post onset of disease. For case 2, RT-PCR for flaviviruses was positive as well as dengue specific real time RT-PCR and conventional multiplex RT-PCR on day 5 post onset.

Four distinct dengue virus serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) are recognised [8]. Sequence

FIGURE

Phylogenetic analysis of a viral sequence derived from an autochthonous case of dengue fever in Madeira, Portugal, October 2012



The phylogeny was inferred based on the core-pre-membrane (CprM) nucleotide sequences (454 bp) by neighbour-joining method using molecular evolutionary genetics analysis (MEGA) version 5 software. Distance matrices were calculated using Kimura two-parameter model. Bootstrap values obtained from 1,000 replicate trees are shown for key nodes. The sequence derived from the autochthonous case of dengue fever in Madeira is highlighted in blue.

analysis of NS5 and CprM partial sequences derived from case 2 by BLASTN and a preliminary phylogenetic analysis based on CprM nucleotide sequences ascertain DEN serotype 1 identified as related to viruses circulating in Latin America namely Colombia, Venezuela, and the Roraima region in northern Brazil [7] (Figure). The virus isolated in Madeira is also associated with DEN-1 strains recognised as belonging to genotype V (e.g. in the Figure, GenBank accession numbers: JX502769, JQ015185), a genotype that represents most of the strains collected in the Americas, West Africa and a limited number of strains collected from Asia [9].

For the case 2, the viral isolation in Vero E6 cells was achieved and the full genome sequence is in progress.

Discussion and conclusions

Infection with dengue virus causes a wide spectrum of human disease, from asymptomatic infections, to classic dengue fever and to haemorrhagic disease. More severe disease is usually associated with secondary infections with heterologous serotypes [10,11]. Between early October 2012 and the beginning of 2013, 2,144 autochthonous dengue virus infections were reported in the island of Madeira. There were no reports of severe disease and a decrease of new cases in the last weeks of 2012 was observed [1].

Dengue virus is transmitted by *Aedes* mosquitoes, namely *Aedes (Ae.) aegypti* and *Ae. albopictus*. *Ae. aegypti* was introduced in Madeira probably from a Caribbean country (personal communication, M Melim, 2009) and its presence was detected for the first time in 2005, in the city of Funchal [12]. Although vector control measures to eradicate or to reduce the spread of this invasive mosquito species were taken, it became the most abundant mosquito species namely in the sites selected for the national vector surveillance programme in 2010 and 2011 [13].

The first cases of the dengue fever outbreak in Madeira, as the majority of all cases, happened in Funchal, the urban environment where the mosquito *Ae. aegypti* was first found [12]. In 2012, the combination of the high vector density with multiple breeding sites in the city [13] and lack of immunity of the population are likely to explain the dimension of the outbreak.

After the initial cases presented here and as of the end of November 2012, the National Institute of Health laboratory tested an additional 43 samples by RT-PCR, 28 of which were found positive. In all of these cases DEN-1 serotype was identified.

The circulation of the four serotypes of dengue in the Caribbean region and South America is described [8]. Hence as it has not yet been identified how the mosquito and the virus were introduced in Madeira, we can assume that there is a risk of new introductions, and spread of new virus serotypes in the next years. As it is well known, the absence of long-term cross-immunity

among the different dengue serotypes allows for multiple sequential infections with heterologous serotypes and the occurrence of cases with more severe disease.

Vector control and surveillance in the territory to avoid the mosquitoes' introduction to other regions of Madeira archipelago and to the mainland is necessary and has already been implemented by the local health authorities.

After 2012, the clinical cases should also be evaluated and serotyped due to the risk of new virus introductions.

In cases of dengue outbreaks, the laboratory has an essential role for early detection and reporting of the first cases. This contributes to the decision to implement vector control measures. Laboratory tests are important for precise identification of the aetiological agent and to retrace its origin. The reference laboratory also provides a quality control for other laboratories and helps with the continuous monitoring of suspected cases

Conflict of interest

None

Authors' contributions

All authors collaborated in the work and in the manuscript presented here. MJA: laboratory coordination at INSA and manuscript preparation; PLF, GA: clinical data and laboratory techniques in Madeira; MJA, FA, HO, LZZ: molecular diagnosis at INSA; MJA, TL, PP: serological diagnosis at INSA; LZZ, HZ: data analysis and suggestions to the manuscript.

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