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FULL PAPER

# Dengue fever in travellers and risk of local spreading: case reports from Southern Italy and literature update

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SUMMARY

Dengue fever (DF), an arbovirosis caused by Dengue viruses (DV, serotypes 1-4), is responsible for an increasing number of travel-related acute febrile illnesses due to population growth, climate changes, spreading by viremic travellers, and improved laboratory diagnosis. The presence of efficient vectors (mosquito *Aedes albopictus*) has also been described in temperate regions including Italy which is considered the most heavily infected European country. Normally characterized by non-specific signs and symptoms, DF incidence is probably underestimated, especially in non-endemic countries, but the risk of severe forms is substantial. Between August and November 2013, five DF patients (4 males, age 23-38) were observed in the Infectious Disease Clinic (University of Bari, Southern Italy). All had just returned from DF endemic areas (2 French Polynesia, 3 Dominican Republic); 4/5 were hospitalized. Common clinical features included acute febrile syndrome, headache (2 with retro-orbital pain), rash (all patients), two with bleeding manifestations and one with gum bleeding. Laboratory tests demonstrated leukopenia (4 patients), elevated liver enzymes (3 patients), and thrombocytopenia (1 patient). Serum samples for DV antibodies and RNA detection were analyzed by the Regional Arbovirosis Reference Laboratory. Viral RNA was identified in 2/5 patients (DV-4) and seroconversion in the remaining cases. All patients made a complete recovery. Recent literature was reviewed, focusing on epidemiology and vector distribution (especially European and Italian territories), pathogenesis, clinical features, diagnosis, and treatment including vaccine strategies. The occurrence of 5 DF cases during the period of highest vector activity (June-November) in Italy emphasizes the risk of local outbreaks in temperate regions. This paper highlights the importance of clinical alert for dengue also in non-endemic countries.

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## INTRODUCTION

Travel-related illnesses are reported in 20% to 70% of subjects returning from tropical to temperate countries, and albeit associated with low mortality rates, a significant morbidity is observed (Kotlyar *et al.,* 2013). According to international data (Wilson *et al.,* 2007), fever is the most common reason for seeking clinical care in returning travelers. However, the evaluation of this symptom is complex as several factors (geographical areas, incubation period, signs/symptoms) should be considered in order to detect unusual tropical infections which may be unfamiliar to clinicians in non-endemic regions.

Dengue fever (DF), a disease caused by mosquito-borne Dengue viruses (DV, serotypes 1-4), has been recognized as responsible for an increasing number of acute febrile syndromes according to data from the Geosentinel Network over a ten-year period (Leder *et al.,* 2013). This increase may be related to the expansion of DF in previously uninfected nations and urban areas due to several reasons, such as population growth, urbanization, development of peri-urban slums, spreading of virus by infected travelers, and improved diagnostic tools. In 95% of travel-related cases, DF presents non-specific signs and symptoms including fever, headache, myalgia, and cutaneous rash (WHO 2009). Therefore, its incidence is probably underrated because of its similarity with other acute febrile illnesses. However, more severe and potentially fatal forms of Dengue infection (previously named Dengue hemorragic fever - DHF and Dengue shock syndrome - DSS), have been reported in up to 16% of cases (CDC 2010). A recent case report describes the third locally acquired dengue-related death documented in the United States over the past ten years (Sharp *et al.,* 2014), emphasizing the importance of clinicians being aware of this disease even in non-tropical areas.

Another important factor is the expansion of the *Aedes albopictus* mosquito (one of the DV vectors, in addition to the typical *Aedes aegypti*) in several European countries during past decades (Medlock *et al.,* 2012) and its efficiency in dissemination of arboviruses also in temperate regions. Recent reports of autochthonous DF cases in European nations (Croatia in 2010, France 2010 and 2014) and the epidemic of Chikungunya in Italy (2007) suggest a role for this vector in potential arboviral outbreaks in Europe

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(Vega-Rua *et al.,* 2013; Schaffner *et al.,* 2014), especially considering the increasing number of viremic returning travelers from endemic regions. The growing interest in travel-associated illnesses has led to the development of worldwide surveillance networks which collect epidemiological data concerning the return of sick travelers, such as GeoSentinel (a system of the International Society of Travel Medicine and the Centers for Disease Control and Prevention - CDC), TropNet Europe (the European Network on Imported Infectious Disease Surveillance), and EuroTravNet (Gautret *et al.,* 2012).
Herein, we describe five cases of imported DF in a single center in Southern Italy over a short period (4 months), followed by an update of relevant recent literature.

## MATERIALS AND METHODS

In the period between August and November 2013, five cases of Dengue infections in travelers coming from endemic areas were registered in the Clinic of Infectious Diseases, University Hospital - Policlinico, Bari, Italy. All five patients were enrolled in our observational study. After written informed consent, data regarding medical history, clinical course of infection, laboratory and microbiological investigations were collected.
All laboratory diagnostic tests were performed at the Regional Reference Laboratory for Arbovirosis (U.O.C. Igiene, Azienda Ospedaliero-Universitaria Policlinico Bari). Based on the patient's journey to endemic areas for DF and on the clinical manifestations, serum samples were collected for DV antibodies and RNA detection. Moreover, both *real time* PCR and serology for Chikungunya virus (CHIKV) were also performed due to the overlapping clinical features and geographic areas of interest.
Viral RNA was extracted from serum samples using High Pure Nucleic Acid Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. *Real-time* PCR (Light Cycler 2.0 system, Roche Diagnostics) was performed to detect viral genomic RNA in human samples, targeting the E1 gene for CHIKV and the conserved 3-UTR region of the genome of DV (LightMix Dengue Virus and LightMix Chikungunya Virus, Roche TIBMOLBIOL, Germany).
Serological analysis for specific anti-DV and anti-CHIKV antibodies was performed using the ELISA test, with commercially available kits (Anti-Dengue virus Elisa -IgM and Anti-Dengue virus Elisa-IgG, Euroimmun, Germany; NovaLisa Chikungunya Virus IgM μ-capture, NovaLisa Chikungunya Virus IgG, NovaTec Immunodiagnostic, Germany), thus permitting the differentiation between acute- and convalescent-phase serum samples.
Serotyping of DV was performed using conventional reverse transcription-nested polymerase chain reaction (RT/nested PCR) according to the protocol previously described (Lanciotti *et al.,* 1992). In the case of confirmed DF diagnosis, serial serum samples were also collected to document seroconversion.

## RESULTS

All five cases were young, aged 23-38-years, born in Southern Italy; only one patient had been living abroad (South America) for six months and was visiting his family in Italy, while the remainder were tourists. Four of these patients were hospitalized, while the fifth was an out-patient whose diagnosis had been previously made at another clinical center.

**Case 1**. A 35-year-old man residing in Santo Domingo for the last six months came to the Emergency Unit of our hospital after one week of fever (peak value 38°C), asthenia, gum bleeding, and a recent urinary tract infection, previously treated with phosphomycin in Santo Domingo. The clinical examination demonstrated only dehydration and blood tests revealed a normal white cell count (WBC, 5.17 x$10^6$/μL) with lymphopenia (11%); haemoglobin (Hb) and platelets (PLT) were within the normal range. After two days, body temperature reached 39.8°C with shivering, retro-orbital headache and mucosal bleeding; blood count showed a WBC reduction to 3.72 x$10^6$/μL. An empiric antibiotic treatment with ceftriaxone for six days was initiated together with hydration; paracetamol was administered for fever above 39°C. Serological and microbiological investigations were performed and, on the third day, RT-PCR resulted positive for DV. On the fourth day, fever was reduced by crisis; the next day, a 24-hour rash was observed over the entire body, followed by a slight increase in hepatic enzymes three times above the upper normal limit (ULN). The patient was discharged after 14 days of hospitalization. At a two-week follow-up, laboratory findings had returned to normal. Lastly, the patient was informed about the risk of a secondary infection in case of returning to the Dominican Republic.

**Case 2**. A 32-year-old man, returning from a two-week holiday in French Polynesia, arrived at the Emergency Unit in the second half of August after two days of fever with shivers (peak value 39.5°C) and lipothymia; he was then hospitalized. The initial physical examination was normal, while blood tests showed a mild lymphomonocytosis (lymphocytes and monocytes 50.4% and 10.9%, respectively), Hb 13.2 g/dl and increased inflammatory markers including C-reactive protein (CRP), erythrocyte sedimentation rate, ferritin, and $\beta_2$ microglobulin. After two days, an itchy rash appeared over the entire body (including his palms and soles), which lasted for a couple of days and resolved from head to toe with desquamation. An empiric antibiotic therapy (ceftriaxone) was initiated for this patient after collecting samples for microbiological investigations, which provided a diagnosis of DF. He was discharged after 11 days of hospitalization with a complete resolution of symptoms.

**Case 3**. A 27-year-old woman, who was the partner of Case 2, also returning from French Polynesia, arrived at our clinic one day after him. She presented fever and nausea, hypotension (95/50 mmHg), leukopenia (2.76 x$10^6$/μL) with monocytosis (16.2%), mild thrombocytopenia (136 x$10^3$/μL), an increase in inflammatory markers (CRP 32.4 mg/L and D-dimers 1.11 mg/L) and a slight alteration of coagulation parameters. Clinical examination demonstrated lymphoadenopathy and hepatosplenomegaly and two small petechiae on the right ankle. Clinical conditions worsened the following day, with fever rising to 39.4°C, deep asthenia, headache, reduction of WBC to 1.00 x$10^6$/μL and PLT to 71x$10^3$/μL. In addition to parenteral hydration, the administration of GM-CSF and antibiotic therapy was deemed necessary. RT-PCR was positive for Dengue virus. On the third day of hospitalization, she became afebrile and an itchy rash appeared on her legs (starting from

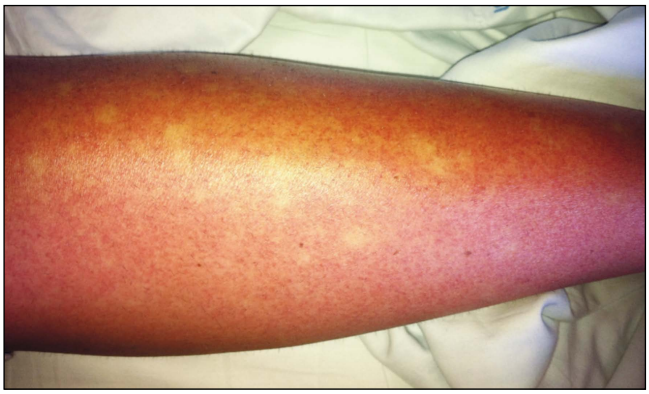

**Figure 1 -** *Dengue fever associated cutaneous rash.*

the back of feet) (*Figure 1*) which solved after a few days with desquamation. As in Case 1, an increase in hepatic enzymes (AST 7 X ULN and ALT 3 X ULN) was observed. The patient was discharged after 14 days of hospitalization.

**Case 4**. A 23-year old man arrived in our Emergency Unit in mid-November after five days of fever (39°C) with chills, asthenia, nausea, vomiting and sore throat which did not improve despite antibiotic therapy with amoxicillin-clavulanate. The day before admission, a diffuse rash had appeared, thus the antibiotic therapy had been shifted to a macrolid by the patient's general practitioner who suspected allergy. At examination, he presented no rash, but a hyperaemic pharynx, hypertrophic tonsils, and lymphoadenopathy. A blood count showed an increase in anti-streptolysin O (499 UI/ml), leukopenia ($3.38x10^6$/µL) and hepatic enzymes three times above the ULN. His medical history revealed that he had returned two weeks previously from a trip to the Dominican Republic. Moreover, a retro-orbital headache was noted a few days before hospitalization. Therefore serum samples for DV infection were collected and serology confirmed the diagnosis. He left the hospital after seven days.

**Case 5**. Less detailed information is available for the fifth patient, who was not admitted to hospital. He was a 38-year old man presenting fever, headache, myalgia and a skin petechial rash on his legs. He had returned from the Dominican Republic a few days before, where he had already been diagnosed with DF. He refused hospital admission and was then advised to rest, stay properly hydrated and avoid NSAIDs. At the following visit after two weeks he was asymptomatic.
*Table 1* lists the non-specific signs and symptoms (Kalayanarooj 2011) observed in our five patients with acute febrile illness. Except for the gum bleeding in case 1, no other warning signs (WHO 2009) (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, lethargy, restlessness, liver enlargement >2 cm, increase in haematocrit concurrent with rapid decrease in platelet count) were found.

### *Virological and serological follow-up*

*Table 2* summarizes the results of laboratory tests for DV. Tests for CHIKV resulted negative in all five cases. Cases 1 and 3 were viremic, since DV RNA was confirmed by *real time* PCR; in these two cases, serotyping revealed DV-4.

**Table 1 -** *Non-specific patient signs and symptoms associated with fever.*

| *Signs and symptoms* | *Patient* | | | | |
|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* |
| Headache | yes | yes | yes | yes | yes |
| Retro-orbital pain | yes | no | no | yes | no |
| Myalgia | yes | no | no | no | yes |
| Arthralgia/ bone pain | yes | no | no | no | no |
| Rash | yes | yes | yes | yes | yes |
| Bleeding manifestations* | yes | no | yes | no | no |
| Leukopenia (WBC ≤5,000 cells/mm³) | yes | yes | yes | yes | NA |
| Platelet count ≤50,000 cell/mm³ | no | no | yes | no | NA |
| Hematocrit (Hct) rising 5-10% | no | no | no | no | NA |

*petechiae, epistaxis, gum bleeding, hematemesis, melena, or positive tourniquet test
NA: not available.

**Table 2 -** *Laboratory diagnostics.*

| *Patient* | *Sample* | *Day* | *Laboratory* | | |
|---|---|---|---|---|---|
| | | | *RT-PCR* | *IgM* | *IgG* |
| Case 1 | 1. | 2nd | POS | NEG | NEG |
| | 2. | 4th | POS | NEG | NEG |
| | 3. | 7th | NEG | POS | NEG |
| | 4. | 9th | NEG | POS | DOUBT |
| | 5. | 11th | NEG | POS | POS |
| | 6. | 23rd | NEG | POS | POS |
| Case 2 | 1. | 4th | NEG | POS | NEG |
| | 2. | 7th | NEG | POS | POS |
| Case 3 | 1. | 3rd | POS | NEG | NEG |
| | 2. | 6th | NEG | POS | NEG |
| | 3. | 16th | NEG | POS | POS |
| Case 4 | 1. | 3rd | NEG | POS | POS |
| Case 5 | 1. | 1st | NEG | POS | POS |

For case 2, the diagnosis was confirmed by specific IgM antibodies and subsequent detection of IgG in a second sample. In cases 4 and 5, the diagnosis was confirmed by the concomitant presence of specific IgM and IgG in the same sample. Follow-up samples were available for three patients (case 1, case 2 and case 3) and seroconversion was detected in all of them.

## DISCUSSION

### *Epidemiology and vector distribution*

In 2012, Dengue was defined by the WHO as the most important mosquito-borne viral disease worldwide (Murray *et al.,* 2013) and it is endemic in over 125 nations (Ratnam *et al.,* 2013). Global estimates suggest that up to 3.6 billion people live in tropical and subtropical areas with potential transmission of DV and that approximately 50 to 200 million dengue infections, 500,000 episodes of severe dengue (including ex DHF/DSS forms), and over 20,000 dengue-related deaths occur annually (Murray *et al.,* 2013). A

large study based on global population data estimates that in 2010 there were 96 million apparent Dengue infections, borne by Asian countries (70%), the Americas (14%), Africa (16%) and Oceania (0,2%), respectively (Bhatt *et al.,* 2013). According to the same study, an additional 294 million unapparent infections occurred worldwide in 2010. These cases represent a potential reservoir of infection, with important implications for DV population dynamics or, for example, development of future vaccine programs.
All our patients had visited endemic countries for DF. In particular, three acquired DF in the Dominican Republic, the other two in French Polynesia. This is concordant with data for the ten-year period 2000-2010 from the Geosentinel Network (Leder *et al.,* 2013). Regarding the Dominican Republic, several local outbreaks and cases of imported disease are reported (CDC 2010) and a recent study includes this country for development of a plan to optimize disease surveillance and outbreak response (Badurdeen *et al.,* 2013). French Polynesia is reported by CDC as the most affected Pacific area with 35,689 cases between 2001 and 2008 (WHO 2009).
Although, as previously shown, Dengue is generally considered a tropical disease, several factors may enhance the risk of introduction or re-introduction of one or more DV vectors (Rogers *et al.,* 2014). In particular, increasing international travel and trade, migration, population growth, rapid urbanization and climate change might play a role in the rise of DF transmission and importation. Moreover, the distribution and/or survival of vectors also in non-endemic areas, and changes in the vectorial capacity of mosquitos may be provoked as well (Jaenisch *et al.,* 2013). DF epidemics occurred in southern Europe in the past (Greece, 1927-1928) when the vector *Aedes aegypti* was locally present. After disappearing for several decades, this disease and its vectors (mostly travel-related, as stated previously) have recently increased again in Europe (Van den Berg *et al.,* 2013; Schmidt-Chanasit *et al.,* 2012; Baaten *et al.,* 2012), where autochthonous cases have been reported (Gjenero-Margan *et al.,* 2011; La Ruche *et al.,* 2010; Schmidt-Chanasit *et al.,* 2010; Marchand *et al.,* 2013; Frank *et al.,* 2013; Schaffner *et al.,* 2014). According to these considerations, it is not surprising that the European Commission has recently launched a large research programme focusing on a comprehensive control of DF (Jaenisch *et al.,* 2013) with the aim of improving diagnosis, surveillance, treatment and prevention (including vaccine strategies) of this disease. With the same purpose, a regional partnership between WHO, the European Mosquito Control Association (EMCA) and the European Centre for Disease Prevention and Control (ECDC), also involving the VBORNET (a network of medical entomologists and public health experts), has been established to increase awareness and further comprehension of arboviral disease and to assist European countries in the early DF detection and prompt response to potential outbreaks (Van den Berg *et al.,* 2013).
Regarding Italy, several reports of imported DF cases observed in Northern regions are available (Gobbi *et al.,* 2012; Pierro *et al.,* 2011; Burdino *et al.,* 2011). In particular, a recent, large case study based on data from ten regions over a three-year period documented 109 cases of DV infection reported to the National Institute of Health (ISS) and the Ministry of Health (Napoli *et al.,* 2012). To our knowledge, no specific data for Southern Italy are available thus far. In particular, our region is close and easily connected by ferries to the Balkan countries, where autochthonous DF cases have been detected (Gjenero-Margan *et al.,* 2011). Furthermore, our study identifies a high number of DF cases over a relatively short time period and in a single centre, when compared to previous Italian reports. In fact, four out of five cases were detected within three weeks, while throughout the 2010-2012 period, only five cases were notified to the Italian National Institute of Health (Italian Board of Health 2013) from our region.
Italy has been shown to be the country in Europe most heavily infested by the Dengue vector *A. albopictus* (Medlock *et al.,* 2012), which is highly active between June and November (Napoli *et al.,* 2012). It is remarkable that two of our patients were viremic when they returned. Therefore, the combination of these factors (viremic travelers reaching a geographic area which is highly infested by a competent vector) may lead to a substantial risk of DF transmission and potential local outbreaks. Moreover, several factors such as climate change could alter the distribution and/or survival of vectors also in non-endemic areas, changing the vectorial capacity of mosquitos (Jaenisch *et al.,* 2013). A recent study drew up DF risk maps according to climate changes, showing Mediterranean and Adriatic coasts as well as Northern Italy as areas of highly increased risk (Bouzid *et al.,* 2014). According to these considerations, the possibility of hospital isolation for viremic subjects with DF should be evaluated to avoid a potential spread of the disease.

### *Pathogenesis*

Dengue virus is a small single-stranded RNA virus belonging to the genus *Flavivirus*, family Flaviviridae. There are four distinct serotypes (DEN-1 to DEN-4); different genotypes within each serotype, as well as intrahost variability (viral quasispecies) have been described (WHO 2009). Diverse serotypes are classified based on the serological reactivity to the envelope proteins involved in viral binding and entry into host cells. On the other hand, viral non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are thought to play a role in immune-escape mechanisms (Spiropoulou *et al.,* 2013). The transmission of DVs to human hosts is mediated by the *Aedes* female mosquitos. In particular, *Aedes aegypti* is the most important and efficient vector, mainly distributed in tropical and subtropical regions, followed by *Aedes albopictus* and, less frequently, by *Aedes polynesiensis* (Tang *et al.,* 2012). After attacking a viremic human host, the vector becomes infectious within 8-12 days, defined as the extrinsic incubation period (EIP). The duration of this period is influenced by climatic factors, such as environmental temperature: at higher temperature, DVs replicate faster, shortening the EIP. Conversely, the intrinsic incubation period (IIP) is defined as the time between human DV infection and the onset of clinical symptoms (Chan *et al.,* 2012), usually 4-10 days (according to WHO definitions).
In the human host, DVs inoculate different cell types, such as keratinocytes, Langerhans cells, and dendritic cells. Infected cells, together with activated T lymphocytes, generate a cytokine cascade which might have both a pro-inflammatory and a protective role. However, the exact contribution of these mediators to the severity of DF has not been clearly defined thus far. Another crucial point is the endothelial activation mediated by vasoactive fragments of the complement system and by different cytokines such as IL-6, IL-8, TNF-α, MCP -1, and VEGF, resulting in

an increased vascular permeability which is responsible for the complications of severe DF forms (Spiropoulou *et al.,* 2013). The pathogenesis of the transient thrombocytopenia which frequently complicates DF is poorly understood, even if many mechanisms have been suggested. In the early phase of the infection, DV can directly infect bone marrow, resulting in a transient depressive effect on megakaryocytes (Arya *et al.,* 2013). A second mechanism, demonstrated both in vitro and in vivo, is that DV might have a toxic effect on platelets: in the course of secondary DV infection, viral replicating RNA has been detected in platelets which express DV antigens on their surface (Noisakran *et al.,* 2010). Lastly, immune-mediated mechanisms have been suggested, based on a complement-mediated IgM-induced PLT lysis and an ADP-induced inhibition of PLT aggregation. Moreover, platelets are also destroyed by cross-reactive antiplatelet autoantibodies directed toward DV proteins, especially NS1 (Noisakran *et al.,* 2001).
Infection by a specific DV serotype results in lifelong immunity against it, but not against the remaining three serotypes, even if a temporary cross-protection towards different serotypes within 2-3 months of the primary infection has been described. Secondary infection has been associated with a higher risk of developing severe DF forms (WHO 2009). This association has been explained by the antibody-dependent enhancement (ADE) model in which cross-reactive, non-neutralizing antibodies produced during primary infection bind to epitopes on the surface of a heterologous infecting virus, thus promoting its entry into Fc-receptor-bearing cells (monocytes, dendritic cells) (Tang *et al.,* 2012). This leads to an intense host immune response with a massive release of inflammatory cytokines and markers, some of which (soluble CD4 and CD8, TNF-α, IL-10, IL-2 receptor) may contribute to causing plasma leakage (Halstead 2007). The presence of previously mentioned inflammatory markers, as well as the activation (and subsequent apoptosis) of cross-reactive memory T-cells might be correlated to the severity of DV disease. The same ADE model has been hypothesized to explain the common severity of primary DV infection in infants born to dengue-immune mothers, who passively acquire cross-reactive antibodies at birth (WHO 2009).

### *Clinical symptoms and signs*

Dengue infection is a systemic disease with a wide variety of clinical aspects including both severe and non-severe clinical manifestations after an average incubation of 7 days (range 4-10). Generally, primary dengue infection may develop as an undifferentiated fever, similar to other viral infections, while DF is an acute febrile illness with severe frontal headache, retro-orbital pain, myalgia, arthralgia and a maculopapular to petechial rash (Wright *et al.,* 2012). In particular, skin rash is described as an "island of white in a sea of red" (Vijayalakshmi *et al.,* 2013) and it may persist during the recovery phase (*Figure 1*). Leucopenia and thrombocytopenia may occur. DF is characterized by a sudden onset followed by three phases: febrile, critical and recovery. High-grade fever lasts 2-7 days and usually ends with a crisis (Henchal *et al.,* 1990), whereas a longer duration is improbable and may represent an exclusion criterion for DF diagnosis (Simmons *et al.,* 2012). The fever often presents a biphasic trend (Morrison *et al.,* 2010) with rash and lymphadenopathy generally observed after the first febrile peak, and a second peak lasting 2-3 days ending with the peeling of the rash. A profound weakness follows. Mild hemorrhagic manifestations, like petechiae and mucosal bleeding, may be observed. Around the time of defervescence, the critical phase begins because of plasma leakage lasting 24-48 hours, and usually preceded by progressive leukopenia followed by a rapid drop in platelet count. This critical phase can be mild, with some patients rapidly improving soon after defervescence, or in other cases it can be severe, especially if the capillary permeability is not compensated by fluid therapy, and shock occurs with the risk of multiorgan failure. If the patient survives the 24-48 hour critical phase, reabsorption of the extravascular compartment fluid takes place and general conditions improve. On the contrary, severe bleeding, pleural effusion or ascites, persistent vomiting, altered level of consciousness, and organ failure may worsen leading to death (WHO 2009).
The clinical features of our patients were similar to the majority of imported DF cases described in the literature, that is, acute non-specific febrile illness, often self-limiting. It is likely, therefore, that several patients with mild forms may not seek clinical care and that the true incidence of imported DF cases is underestimated. For this reason, awareness of the disease and clinical knowledge of DF symptoms by European clinicians should be improved to allow early detection and better management of DF cases.
Several complications characterize severe dengue. Three pathogenic mechanisms have been hypothesized for neurological findings (Verma *et al.,* 2011): firstly, viral neurotropism leading to encephalitis, meningitis, and myelitis; secondly, encephalopathy due to a secondary central nervous system (CNS) involvement during systemic complications, such as plasma leakage and/or electrolytic disequilibrium (e.g. stroke and hypokalemic paralysis); lastly, postinfectious immune-based mechanisms, such as acute disseminated encephalomyelitis, Guillain Barré syndrome and optic neuritis. Among them, encephalitis and encephalopathy are the most common: while encephalitis is caused by the direct CNS infection, encephalopathy has a multifactorial pathogenesis into severe dengue including cerebral edema, cerebral hemorrhage, hyponatriemia, hepatic failure, renal failure and cerebral hypoxia (Murthy, 2010). Ophthalmic complications have also been described, including macular edema and hemorrhage because of thrombocytopenia (Chan *et al.,* 2006). Moreover, in severe dengue, electrolyte and acid-base imbalances may occur. Both hyperglycemia and hypoglycemia have been observed, even in the absence of diabetes mellitus, as well as fluid overload with large pleural effusions and ascites, with the risk of acute respiratory distress (WHO 2009).
The current classification of DF is based on the 2009 WHO guidelines for the diagnosis and management of dengue infection, which follows the previous guidelines published in 1997. While dengue infection was formerly divided into three different categories (DF, DHF and DSS), the new classification considers it a continuous spectrum of disease, in which the main feature to be considered is the presence or absence of warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increase of hematocrit with decreasing platelets). In fact, several studies demonstrated that this criterion is more sensitive for the identification of severe disease than the 1997 classification. However, a more accurate definition of warning signs is

still necessary to permit a better identification of patients who require hospitalization and those who can be treated as outpatients (Hadinegoro, 2012).

*Diagnosis*

The diagnosis of DF is based on laboratory tests which should be chosen by the physician based on the phase of disease. According to the DF natural history, viral RNA and NS1 antigen from peripheral blood can be detected in primary DV infection throughout the febrile phase. In patients with secondary infection, viral RNA may be detectable for a shorter time (usually 2-3 days), while NS1 antigen persists in blood some days longer (Hynes 2012). Serological tests for IgG and IgM antibodies, on the other hand, usually lead to positive results only after defervescence, during the early post-febrile period lasting a few weeks (Halstead 2007). Therefore, the combined use of these tests during the course of the disease would increase the likelihood of obtaining a definite diagnosis.

Viral isolation by inoculation of mosquitos, mouse brain or cell lines with blood, serum or plasma from viremic patients is highly specific, but sensitivity is rather low and, because of its costs and the need for complex facilities and well-trained operators, this technique is not routinely used in clinical practice (Tang *et al.,* 2012).

Viral RNA detection by Reverse Transcriptase PCR (RT-PCR) is a rapid, highly sensitive and specific method, which also allows identification of the viral serotype and acquisition of a quantitative measure of viral load (Ratnam *et al.,* 2013). The reliability of this technique is related to the time of its execution, which should be in unison with the febrile period.

Identification of NS1 antigen from blood or serum with ELISA assay represents another method for early DF diagnosis providing high specificity but variable sensitivity (Kosasih *et al.,* 2013); moreover, NS1 levels have been related to disease severity (Tang *et al.,* 2012). The period of highest sensitivity of NS1 detection, according to a recent study (Ratnam *et al.,* 2013), varies in travelers and coincides with the 6-7$^{th}$ day from clinical onset compared to endemic populations whose highest sensitivity is reported on 2$^{nd}$-3$^{rd}$ day.

Detection of anti-DV antibodies (IgG and IgM) is the most common diagnostic tool for DF, used to complement or, if not available, to replace virological laboratory diagnosis. It is based on immunoenzymatic (ELISA) or immunochromatographic assays (Marli Tenório Cordeiro, 2012). A rapid test suitable for bedside use is also available, even if it is less sensitive than ELISA (Tang *et al.,* 2012). IgM levels increase during the first two weeks of illness, then they might persist for 139 to 179 days depending on whether the infection is secondary or primary, respectively. An increasing IgM titer observed on subsequent serum samples confirms a definitive DF diagnosis (Tang *et al.,* 2012). False positive results have sometimes been obtained in patients with malaria, leptospirosis or with previous DF episodes. IgG can be detected after 10 days from illness onset during primary infection, then lasting lifelong. A method to distinguish between primary and secondary infection is a DV specific IgM/IgG ratio. However, this test requires reaching a better standardization (WHO 2009). Serotype identification is not possible with serological methods.

Lastly, a study performed in Brazil in 2013 suggested a predictive model for early DF diagnosis based on clinical and laboratory criteria found up to the 3$^{rd}$ day from the onset (cutaneous rash, conjunctival hyperemia, leucopenia) which were identified as the most frequently associated with confirmed DF, compared to other acute febrile illnesses. These clinical criteria might be useful in limited resource settings endemic for DF, in which the importance of early diagnosis is greater to reduce mortality (Daumas *et al.,* 2013).

In both our viremic patients, RT-nested PCR led to the detection of DV serotype 4 which, to our knowledge, is rather unusual in the geographic areas from which the patients were returning. In particular, in the Dominican Republic DV-1 and DV-2 are more common, according to WHO data (WHO 2009), and several recent outbreaks of DV-3 infection have also been described (Brathwaite Dick *et al.,* 2012). In French Polynesia, on the other hand, serotypes 2 and 3 caused DF outbreaks in 1997 and 1989, respectively (Murgue *et al.,* 1999) while DV-4 was responsible for a 1979 epidemic (Deparis *et al.,* 1998). However, it is interesting to note that the same serotype 4 caused an outbreak in the nearby Federated States of Micronesia during the period 2012-2013 (CDC 2013).

Since 2010, our Regional Reference Laboratory for Arbovirosis has received clinical samples for serological and molecular diagnosis in cases of suspected arboviral infections throughout the region. Moreover, an active surveillance of Dengue and Chikungunya diseases was set up in Italy in June 2011 (Italian Board of Health 2011) by the National Institute of Health, and officially in Puglia in July 2011 (Public Health and Occupational Safety Office of Apulian Region 2011), thus improving the possibility for better case identification and control.

*Treatment*

DF treatment is only symptomatic with analgesics, such as paracetamol, avoiding NSAIDs and salicylic drugs because of the risk of hemorrhage. Rehydration by oral or intravenous fluid therapy with adequate solutions should be considered depending on the haematocrit level, plasma leakage and patient's liquid loss. Hospital admission is not always necessary. In severe cases, transfusion of red blood cells may be required for anemia and, if necessary, assisted ventilation and dialysis (Adehossi 2012). No specific antiviral treatment is available as yet. A recent randomized, double-blind placebo controlled trial with balapiravir, a polymerase inhibitor initially developed for treatment of chronic HCV hepatitis (this trial was discontinued because of toxicity during long-term treatment), did not demonstrate significant effects on clinical, virological and laboratory parameters, despite its documented *in vitro* activity against DVs (Nguyen *et al.,* 2012).

*Vaccine*

At present, there is no available vaccine for prevention of DV infection. Reasons which hinder vaccine development include: incomplete knowledge of the protective immunity against the virus, the scarce reproducibility of animal models (since humans are the only hosts of this virus), and the need to obtain a quadrivalent vaccine active against four serotypes to avoid the risk of ADE. Four types of Dengue vaccines are currently in development, based on live attenuated viruses, chimeric live attenuated viruses, inactivated viruses or sub-units, and nucleic acid (WHO 2009). A live attenuated tetravalent dengue vaccine developed by Sanofi Pasteur (CYD-TDV) is at the most advanced step, reaching phase IIb and III of evaluation (WHO 2014). It

contains four recombinant viruses based on the use of prM and E genes from each dengue serotype, and capsid proteins of the attenuated yellow fever vaccine virus (Ratnam *et al.,* 2013). Phase two studies showed that the vaccination with three-dose CYD-TDV was well-tolerated and elicited a neutralizing antibody response against all four serotypes both in seropositive and seronegative participants (Dayan *et al.,* 2013). In particular, with a three-dose vaccine administered at 0, 4, and 12-15 months, it was shown that the first TDV dose induced a neutralizing humoral response mainly against DENV-4 and DENV-2, while the second and the third dose increased the seroconversion rate for all four serotypes (Morrison *et al.,* 2010). These results paved the way to a possible preventive vaccine, but its efficacy must be demonstrated in large scale and multicenter studies in a variety of epidemiological backgrounds and among all age groups (WHO 2012). Before introduction of a dengue vaccine into the national immunization program in endemic areas, any possible interaction with other vaccinations also needs to be verified. Moreover, studies are ongoing for optimizing the vaccine time schedule for international travelers.

## CONCLUSIONS

In conclusion, our experience emphasizes the importance for physicians to maintain a high level of alert regarding dengue fever, even in non-endemic areas, considering its potential severity in single cases and, above all, the concrete risk of local outbreaks.

### Conflicts of interest

All the authors declare they have no competing interest.

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