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Laboratory findings in Zika infection: The experience of a reference centre in North-West Italy

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ABSTRACT

Background: Zika virus (ZIKV) remains a public health concern due to its association with fetal malformation and neurologic disease.
Objective: To report a reference centre experience on ZIKA virus (ZIKV) infection in travelers from epidemic countries from January 1 to September, 30, 2016 in Italy North-West (a geographic area covering 4.424 million inhabitants, corresponding to almost 73% of Italy North-West area).
Study design: One hundred and twelve febrile travelers were studied to rule out a tropical fever [e.g. malaria, dengue (DENV), chikungunya (CHIKV), West Nile (WNV) and ZIKV]. Molecular tests for detecting ZIKV RNA were applied on serum or urine as well as IgG and IgM specific serology.
Results: ZIKV was the most frequent "tropical infection (11.6%) with 12 infected travelers and one sexual partner of an infected traveler. At the time of the diagnosis, ZIKV RNA was detected in the blood from 9 patients (69%) within 7 days from symptom onset; afterwards, the virus was detected only in urine (5 patients) and ZIKV IgM was reactive in 9 patients (69%). Travelers with ZIKV infection tested negative for DENV, CHIKV, WNV and malaria and completely recovered. Other infections identified in travelers were DENV (5 patients, 4.5%), CHIKV (1, 0.9%), malaria (*Plasmodium vivax*, 1, 0.9%), measles (1, 0.9%) and tuberculosis (1, 0.9%).
Conclusions: The etiologic diagnosis of a febrile illness in travelers where ZIKV is endemic is highly desirable as they are sentinel of a challenging epidemiology including the risk of autochthonous transmission in non endemic countries where the competent or carrier vector is present.

1. Background

Zika virus (ZIKV), a mosquito-borne member of the *Flaviviridae* family, is the focus of a great public health concern due to its association with fetal malformation and neurologic disease. While ZIKV was previously limited to sporadic cases in Africa and Asia, but in the year 2015 it rapidly spread to Brazil and throughout the Americas and Caribbean causing 0.5–1.5 million human infections [1–5]. Moreover, in 2016, due to the Olympic Games in Brazil, an increasing number of travelers from the Americas have been observed during the period of vector activity (May–November). Neither an effective treatment nor a vaccine is available; therefore, prevention, surveillance and accurate diagnosis are the primary public health response. Since 2007, 31 of the 70 countries and territories reporting evidence of ZIKV transmission (67 from the year 2015 onwards) reported also the association of the disease with microcephaly and other CNS malformations [6]. Although a decline of Zika infection has been recently reported by the last WHO estimates, vigilance needs to remain high [7] and increasing reports of imported ZIKV cases have been observed throughout Europe, United States and Asia [6,8–11].

From a public health perspective the etiologic diagnosis of febrile travelers from countries where ZIKV is epidemic, is highly desirable and

Abbreviations: ZIKV, Zika virus; PCR, Polymerase Chain Reaction; RT-PCR, Reverse Transcription PCR; rtRT-PCR, real time RT-PCR; PRNT, Plaque Reduction Neutralization Tests; $PNRT_{80}$, PRNT ≥ 80% of plaque reduction; GEN, Genesig Advanced Kit; ALT, Altona Diagnostics RealStar Zika virus RT-PCR Kit 1.0; RU, relative units; ELISA, Enzyme-Linked Immunosorbent Assay; CV, coefficient of variation; Ct, cycle threshold

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efforts in this direction are highly recommended, as travelers can be a sentinel of rapidly challenging epidemiology including the risk of autochthonous transmission in non epidemic countries where the competent or carrier vector is present.

Currently, the diagnosis of ZIKV infection relies on the detection of the virus by Reverse Transcription Polymerase Chain Reaction (RT-PCR)-based tests especially useful during the acute phase of the disease (that is within 7 days from symptom onset) and the identification of specific antibodies. Accurate detection of ZIKV in biological samples can be highly demanding due to the low viral load and the short viraemic period, implying the risk of false-negative results in the acute phase. To increase the diagnostic sensitivity in this stage of the disease, testing other specimens than plasma/serum, such as whole blood [12], urine and more recently saliva [13] is now highly recommended as viral shedding is longer in these compartments [8,14]. As observed by Gourinat [15] and Bingham [16] urine might be the preferred specimen to identify acute/recent ZIKV disease because viral RNA can be detected at higher levels for longer periods of time than in serum; moreover, urine has the advantage of a much easier sample to collect than blood. ZIKV serology should be considered in those patients with a high suspicion of ZIKV infection in whom ZIKV RNA is negative by RT-PCR and whose symptoms last for more than one week. Serology has substantial limitations [17], mainly due to the high cross-reactivity among flavivirus antibodies, but recently, ELISA tests based on ZIKV NS1-antigen have been developed for a more specific identification of ZIKV antibodies [18]. Final evaluation of serologic results needs to be confirmed by the detection of neutralizing antibody that remains the gold standard, although this test is restricted to reference laboratories due to its high complexity [19].

2. Objective

To report ZIKV infection in febrile travelers returning from epidemic areas (Venezuela, Dominican Republic, Puerto Rico, Mexico, Costa Rica), who referred at the regional Centre for Infectious Diseases, Amedeo di Savoia Hospital, Turin, Italy (a geographic area characterized by the widespread presence of the ZIKV vector *Aedes albopictus* and serving 4.424 million inhabitants), between January 1 and September 30, 2016 [19,20]. Ours represents a significant cohort of ZIKV imported infection in North-West Italy within the national surveillance system that since January 2014 has been enhanced for ZIKV (Ministero della Salute. Sorveglianza dei casi umani di chikungunya, dengue, West Nile Disease ed altre arbovirosi e valutazione del rischio di trasmissione in Italia 2015. 0020115-16/06/2015-DGPRE-COD_UO-P. 2014:1–135) and accounts for approximately 60 notified ZIKA cases in the year 2016. (http://www.iss.it/binary/arbo/cont/Casi_confermati_Chikungunya_Den_Zika.pdf; data not-published).

3. Study design

3.1. Patients and samples

Febrile travelers (N = 112) returning home from ZIKV epidemic areas reporting symptoms and signs such as fever, maculopapular and itchy rash and/or arthralgia/myalgia who referred to the outpatient Clinic of Travel Medicine at the regional reference Centre for Infectious Diseases, Amedeo di Savoia Hospital, Turin, from January, 1, to September, 30, 2016 were studied. A suspicion of ZIKV infection was posed. Patients underwent hematological, biochemical, microbiologic, serologic and virological examinations to rule out a tropical fever [e.g. malaria, dengue (DENV), chikungunya (CHIKV), West Nile (WNV) and ZIKV fever] and the Italian Ministry of Health and WHO case definition for ZIKV, CHIKV, DENV and WNV disease were followed [21].

3.2. Diagnostic tests for ZIKV infection

One hundred eighty-five specimens were processed (130 serum and 55 urine) for ZIKV molecular testing and/or ZIKV serology.

For the detection of ZIKV RNA in serum and urine, a CE-marked real time (rt) RT-PCR assays (RealStar Zika virus RT-PC Kit 1.0, Altona Diagnostics, Germany) was used, while quantification of ZIKV RNA in positive samples was performed with the Genesig Advanced Kit (Primerdesign Ltd, United Kingdom) using a plasmid as standard curve from 2×10^5 to 2 copies/μl after ZIKV RNA extraction from 600 ml of serum and urine using the semi-automated system NucliSENS easyMAG (Biomérieux, France), according to manufacturer's protocols. Performances of the two molecular tests was done with the Asian reference strain ZIKV H/PF/2013 kindly provided by the Istituto Superiore di Sanità (ISS), Rome.

All patients underwent ZIKV IgG and IgM antibody detection with a commercial ELISA assay (Euroimmun AG, Germany) against ZIKV NS1 protein [18]; confirmation with Plaque Reduction Neutralization Tests (PRNT) was performed at the ISS, as previously described [22], in those patients in whom serology was positive but ZIKV RNA was not detectable in serum/urine (therefore classified as probable cases according to the Italian Ministry of Health and WHO case definition for ZIKV virus disease [21]). Briefly, the assay was prepared in six-well tissue culture plates with sub confluent VERO cell monolayers and the ZIKV H/PF/2013 strain of the Asian genotype (kindly provided by Dr. Isabelle Leparc-Goffart of the French National Reference Center on Arboviruses in Marseille). Patient sera were diluted 1:10 in serum-free maintenance medium. Equal volumes (100 μl) of ZIKV dilution containing approximately 80 Plaque Forming Units (PFU) and serum dilutions, were mixed and incubated overnight at 4 °C. Subsequently, VERO cells plates were infected with 200 μl/well of virus-serum mixtures in duplicate, incubated at 37 °C (5% CO_2) for 4 days, and stained with 1.5% crystal violet. A titration of ZIKV with three dilutions in duplicate was performed in each assay as control and neutralizing antibody titers were calculated as the reciprocal of the serum dilution that gave an 80% reduction of the number of plaques ($PRNT_{80}$), compared to the virus control. $PRNT_{80} \geq 10$ were considered positive.

Diagnosis of DENV, CHIKV and WNV infection was ruled out with rapid tests against to NS1 DENV antigen, DENV IgM and IgG (Panbio ALERE, I) and indirect immunofluorescence for both CHIKV and WNV IgG and IgM (Euroimmun AG, Lubek, Germany). A multiplex rtRT-PCR was applied to detect DENV and CHIKV RNA in serum (Dengue/Chik, FastTrack Diagnostics assay) and WNV RNA was identified with the West Nile Virus ELITe MGB® Kit (ELITechGroup, Italy).

4. Results

According to WHO case definition, ZIKV infection was identified in 13 out of 112 febrile (11.6%) patients (Table 1): 12 were travelers (ID#1-8 and ID#10-13), one was the sexual partner of an imported case (ID#9). Twelve patients (92%) reported maculopapular rash (with itching in half of them); 9 (69%) arthralgia/myalgia, 4 (30.7%) headache and/or retrorbital pain, 3 presented with conjunctivitis (23%), malaise and fatigue (23%) and 1 with diarrhea (7.7%). Leukopenia (< 4000 WBC/μl) was present in 4 patients, while impaired liver function in two. Paracetamol and steroids for uncontrollable itching were administered and all patients completely recovered within 1 week without hospitalization.

A case most likely of sexual transmission of ZIKV was suspected in the female partner (ID#9) of a male traveler (ID#5) returning home from the Dominican Republic with ZIKV infection. The partner (patients ID#9) had no history of travelling abroad and of mosquito bites, but reported a sexual intercourse with travel ID#5 after his returning home and falling ill. The female partner had fever, a maculopapular itchy rash and ZIKV RNA was detected in urine, but not in serum. Other body fluids from the two partners were not available for testing, therefore, a