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Case Report

First detection of Zika virus infection in a Croatian traveler returning from Brazil, 2016

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Abstract

In the last few years, several imported cases of Zika virus (ZIKV) infection were reported in European countries. We report the first imported ZIKV infection case in a Croatian traveler returning from Brazil. The patient presented with a low-grade fever, pruritic rash, general weakness, myalgia, arthralgia and edema of the legs and recovered completely within a week. ZIKV infection was confirmed by detection of IgM/IgG antibodies using enzyme-linked immunosorbent assay (ELISA) and confirmed by plaque-reduction neutralization test (PRNT). ZIKV IgM antibodies cross-reacted with dengue virus (DENV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) in ELISA. In indirect immunofluorescence assay (IFA), IgM cross-reactivity was found only with DENV-3. ZIKV IgG antibodies cross-reacted with DENV in both ELISA and IFA. PRNT for DENV was negative. Control serology performed on days 64 and 98 after disease onset showed a decline in cross-reactive heterologous DENV IgG antibodies compared to persistently high titer of homologous ZIKV IgG antibodies.

Key words: Zika virus; imported; Croatia.

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Introduction

Zika virus (ZIKV) is an arthropod-borne virus of the family *Flaviviridae*, genus *Flavivirus*, Spondweni serocomplex [1]. After the first isolation from a febrile *rhesus* monkey in 1947 (Uganda), virus was confined to Africa and only sporadic human cases were reported [2]. In 2007, the outbreak involving 185 cases occurred on the Yap Island, Federated States of Micronesia [3]. In the following years, ZIKV spread across the Pacific causing outbreaks in French Polynesia (2013-2014) [4], Easter Island (2014) [5], the Cook Islands (2014) [6] and New Caledonia (2014) [7]. In 2015, ZIKV was confirmed in Vanuatu, Solomon Islands, Samoa, and Fiji [8]. Since early 2015, autochthonous transmission was documented in Brazil, and the first outbreak was documented in May 2015, in Bahia [9]. Since then, ZIKV infections have been detected in several countries in South and Central America and the Caribbean [10].

In urban environments, virus is transmitted between humans by the bite of *Aedes* mosquitoes. Both *Ae. aegypti* and *Ae. albopictus* mosquitoes are competent vectors for the ZIKV [11]. Transmission through blood/blood products, sexual transmission and transplacental/perinatal transmission have also been reported [12-14].

In Europe, the first imported case of Zika fever was reported in 2013 in a German traveler returning from Thailand [15], and thereafter imported cases were continuously noted [16-19].

The majority of ZIKV infections (~80%) are asymptomatic. Most commonly reported clinical symptoms include fever, rash, conjunctivitis, arthritis and/or arthralgia, myalgia and fatigue. Retro orbital pain, edema of the extremities and lymphadenopathy may occur [10]. In areas with epidemic ZIKV circulation, spontaneous abortions and fetal microcephaly following maternal infection during pregnancy were reported [20]. Neurological diseases associated with ZIKV infection include meningoencephalitis, myelitis and Guillian-Barré syndrome [10,21,22].

We report clinical and serology findings of a Croatian traveler with ZIKV infection imported from Brazil.

Case Report

Epidemiological data, clinical presentation and laboratory parameters

A previously healthy, Croatian traveler in her late 20s returned from Brazil at the beginning of March. After a four-week trip in Brazil (16 January-12 February 2016), the patient visited Portugal where she spent the next two weeks. On 14 February, the patient developed low-grade fever (37.5°C), pruritic rash on face and trunk, general weakness, myalgia, arthralgia and edema of the legs. Symptomatic treatment with paracetamol and antihistamines for pruritic rash was recommended. The patient fully recovered within a week. She had noted numerous mosquito bites despite using repellents. Previous flavivirus vaccination (tick-borne encephalitis, yellow fever) was not reported. Upon return to Croatia, the patient was asymptomatic. Physical examination was normal as well as laboratory parameters: erythrocytes $5.05x10^{12}$/L (3.86-$5.08x10^{12}$/L), hemoglobin 134 g/L (119-157 g/L), leukocytes 6.46×10^{9}/L (3.40-9.70×10^{9}/L), C-reactive protein 7.56 mg/L (0.00-5.00 mg/L), bilirubin 4.3 µmol/L (3.0-20.0 µmol/L), aspartate aminotransferase 23 U/L (8-30 U/L), alanine aminotranspherase 13 U/L (10-36 U/L), gamma glutamyl transferase 11 U/L (9-35 U/L), urea 3.88 mmol/L (2.80-8.30mmol/L), creatinine 70 µmol/L (49-90 µmol/L).

Serology results

Three serum samples were collected on days 32, 64 and 98 after disease onset. Serologic testing was performed at the National Reference Laboratory for Arboviruses, Croatian National Institute of Public Health. IgM/IgG antibodies to ZIKV were detected using a commercial ELISA based on recombinant ZIKV NS-1 antigen (Euroimmun, Lübeck, Germany). Serology for dengue virus (DENV) and chikungunya virus (CHIKV) was performed using a commercial ELISA (DENV; Euroimmun, Lübeck, Germany) and IFA test (Arbovirus mosaic: ZIKV, DENV 1-4, CHIKV). In addition, samples were tested for potential cross-reactivity with other flaviviruses using ELISA (West Nile virus; WNV, Usutu virus; USUV, tick-borne encephalitis virus; TBEV, Euroimmun, Lübeck, Germany) and/or IFA (Flavivirus mosaic; yellow fever virus; YFV, Japanese encephalitis virus; JEV, WNV, TBEV, Euroimmun, Lübeck, Germany).

ELISA test was performed in microtiter strips. In the first reaction step, diluted serum samples were incubated with the antigen in the wells. To detect the bound antibodies, in the second reaction step, enzyme-conjugate (peroxidase-labeled anti-human IgM and IgG antibodies) was added. After adding the chromogen/substrate solution (TMB/H_2O_2), photometric measurement of the color intensity was made at a wavelength of 450/620 nm.

In IFA test, slides coated with antigen were incubated with the diluted serum samples. In the second step, the attached antibodies were stained with fluorescein-labeled anti-human IgM and IgG antibodies. The fluorescence was read with UV microscope.

Before the determination of IgM antibodies in both ELISA and IFA, IgG antibodies were removed by immunoabsorbtion using IgG/RF-absorbent (anti-human IgG antibodies) to prevent rheumatoid factor of IgM class from reacting with specifically bound IgG (false positive IgM result), as well as to prevent specific IgG displacing IgM from the antigen (false negative IgM result).

In order to confirm ELISA and IFA tests results, a plaque-reduction neutralization test (PRNT) was also performed at the Istituto Superiore di Sanità, Roma, Italy, for ZIKV and for DENV. PRNT was carried out in six-well tissue culture plates with subconfluent VERO cell monolayers (approximately 70% confluence). The following viruses were used: serotype 2 DENV (NGB strain), and 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) [23]. Infectivity titration of each viral strain was performed by plaque assay using VERO cells. Patients sera were diluted 1:10 in serum-free maintenance medium and heat-inactivated. Equal volumes (100 µl) of DENV/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. After 1 h incubation at 37°C and 5% CO_2, the inocula were aspirated and the wells were overlayed with a mixture of one part 2% Gum Tragacanth and one part of supplemented medium (2× MEM, 2.5% inactivated FCS and 2% 1M HEPES). The plates were incubated at 37 °C and 5% CO_2 for 7 (DENV) - 4 (ZIKV) days, and then were stained with 1.5% crystal violet. A titration of DENV/ZIKV with three dilutions in duplicate (the working dilution, 1:2 and 1:8 dilutions) was performed in each assay and used as a control for the assay. Neutralizing antibody titers was calculated as the reciprocal of the serum dilution that gives an 80% reduction of the number of plaques as compared to the virus control. PRNT80 ≥10 are considered positive.

Serology results are presented in Table 1. At initial testing (day 32), ZIKV IgM (ratio 1.67) and IgG antibodies (>200 RU/mL) were detected using ELISA. IgM cross reactivity was detected with DENV IgM and IgG antibodies using ELISA and IFA (Figure 1). Moreover, ZIKV IgM antibodies cross-reacted with WNV and TBEV in ELISA test. Serological tests for CHIKV were negative. Control serology performed on day 64 showed a decline of ZIKV IgM antibodies (ratio 1.11) and high IgG antibodies (>200 RU/mL). In the third sample taken on day 98, the result of ZIKV IgM was equivocal (ratio 1.03) while titer of IgG antibodies was still high (160 RU/mL).

ZIKV neutralizing antibodies were confirmed using a PRNT. PRNT for DENV was negative. According to the ECDC proposed case definition for surveillance of ZIKV infection, detection of ZIKV IgM and IgG antibodies with PRNT80 titer ≥ 1:10 confirmed ZIKV infection [24].

Discussion

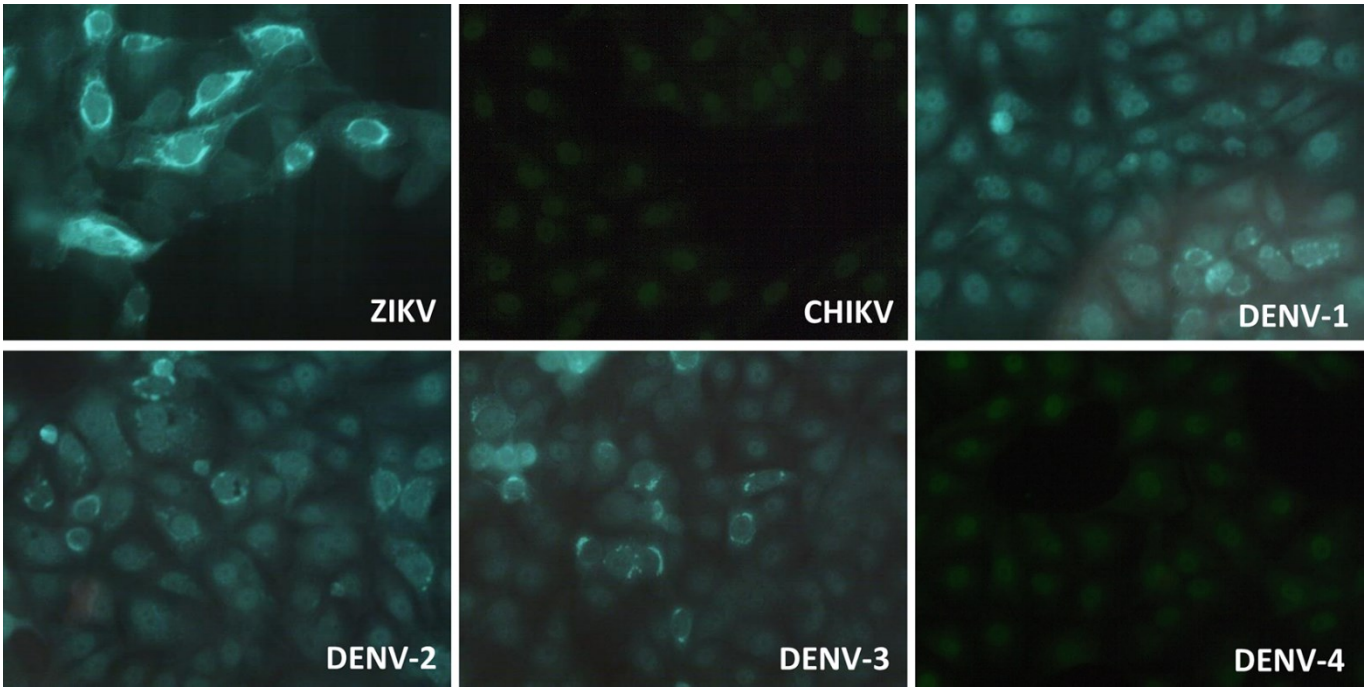
Many ZIKV imported cases in travelers returning to Europe were reported after 2013 [15-19,25]. Due to similar clinical symptoms and geographical distribution

Table 1. Serology results of Zika virus positive patient

Virus	Sample	ELISA IgM (ratio)[a]	ELISA IgG (RU/mL)[b]	IFA IgM (titer)[c]	IFA IgG (titer)	PRNT (titer)
Zika virus	I	Positive (1.67)	Positive (>200)	ND[d]	ND	80
	II	Positive (1.11)	Positive (>200)	Positive (10)	Positive (3200)	ND
	III	Equivocal (1.03)	Positive (160)	ND	ND	ND
Dengue virus	I	Positive (3.92)	Positive (29)	ND	ND	Negative
	II	Positive (4.40)	Positive (30)	DENV1 Negative DENV-2 Negative DENV-3 Positive (10) DENV-4 Negative	DENV-1 Positive (10) DENV-2 Positive (10) DENV-3 Positive (100) DENV-4 Negative	ND
	III	Positive (4.40)	Equivocal (20)	ND	ND	ND
Usutu virus	I	ND	Negative (5)	ND	ND	ND
	II	ND	Negative (14)	ND	ND	ND
	III	ND	Negative (10)	ND	ND	ND
Chikungunya virus	I	ND	ND	Negative	Negative	ND
	II	ND	ND	Negative	Negative	ND
Yellow fever virus	I	ND	ND	Negative	Negative	ND
	II	ND	ND	Negative	Negative	ND
	III	ND	ND	Negative	Negative	ND
West Nile virus	I	Positive (1.70)	Negative (8)	Negative	Negative	ND
	II	Positive (2.12)	Equivocal (20)	Negative	Negative	ND
	III	Positive (1.81)	Negative (12)	Negative	Negative	ND
Tick-borne encephalitis virus	I	Positive (1.21)	Negative (12)	Negative	Negative	ND
	II	Positive (1.34)	Negative (14)	Negative	Negative	ND
	III	Positive (1.26)	Negative (14)	Negative	Negative	ND
Japanese encephalitis virus	I	ND	ND	Negative	Negative	ND
	II	ND	ND	Negative	Negative	ND
	III	ND	ND	Negative	Negative	ND

I sample (day 32), II sample (day 64), III sample (day 98); [a]ratio (extinction of sample/extinction of calibrator) < 0.8 negative, 0.8-1.1 equivocal, > 1.1 positive; [b]RU/mL < 16 negative, 16-22 equivocal, > 22 positive; [c]titer = the highest serum dilution for which fluorescence is visible; [d]ND = not determined.

Figure 1. Arbovirus mosaic; indirect immunofluorescence assay (IgG antibodies) of patient with ZIKV infection (fluorescence intensity: ZIKV positive, DENV 1-3 weak positive, DENV 4 negative, CHIKV negative), 400 x magnification.



as well as possible co-infections with dengue and chikungunya, DENV and CHIKV should be considered in the differential diagnosis in febrile travelers [7,26,27]. In a recently published Nicaraguan study, only the presence of rash and fever differed between patients with ZIKV, DENV and CHIKV infection. Rash was significantly more commonly detected in ZIKV-positive patients (91.4%) than among DENV- and CHIKV-positive patients (50.0% and 56.3%, respectively). In addition, patients with ZIKV infection were significantly less likely to be febrile $\geq$ 38°C (7.4%) compared to patients with DENV (28.6%) and CHIKV infection (33.7%) [28]. Clinical symptoms reported in our patient (low grade fever, pruritic rash, edema of the legs) were suggestive of ZIKV, rather than DENV and CHIKV infection.

Serology is the most commonly used routine diagnostic method for arboviral infections. At the end of the acute phase of infection, serology is the method of choice for diagnosis [29]. However, due to cross-reactive properties of flaviviruses, serology is challenging. Our patient showed cross-reactive antibodies, too. IgM antibodies were broadly reactive in ELISA (ZIKV, DENV, WNV and TBEV). This finding is somewhat unusual, since cross-reactivity is usually more common for IgG antibodies. However, a serological study after the Yap outbreak showed that ZIKV-infected patients could be positive for IgM antibodies to heterologous flaviviruses [30]. IFA showed higher specificity in IgM detection compared to ELISA in the patient presented in this study (IgM positivity was found to ZIKV and DENV-3). Similar results are reported by other authors [31,32]. IgG reactivity to ZIKV and DENV in both ELISA and IFA was also found in our patient. These findings may suggest possible ZIKV and DENV co-infection as well as residual antibodies from previous DENV infection. However, demonstration of ZIKV neutralizing antibodies, together with negative DENV PRNT confirmed ZIKV infection in this case.

Our results showed the importance of repetitive sampling. Control serology performed two and three months after disease onset showed a decline in cross-reactive heterologous DENV IgG antibodies (equivocal in the third sample) in contrast to persistently high titer of homologous ZIKV IgG antibodies.

Due to intensive travelling, ZIKV represents an important public health concern. Although Croatia is not endemic area for ZIKV, due to the establishment of *Ae. albopictus* in several Croatian regions, importation of virus by returning viremic travelers or tourists could result in a local disease transmission. Since circulation of several flavivirus infections which may present with rash such as WNV and USUV disease were documented in Croatia [33], these viruses should be also included in the differential diagnosis of febrile

diseases with rash during the arbovirus transmission season. Timely diagnosis is important to prevent spreading of emerging arboviruses in new regions where competent vectors are present. Permanent vector control measures should be regularly performed, particularly in areas with established *Ae. albopictus* population.

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