A REVIEW OF ZIKA VIRUS

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ABSTRACT

One of the most important outbreaks in recent years, the one declared in 2016 by WHO as a public health emergency of international concern, was produced by Zika virus, a member of the Flaviviridae family. The clinical forms of Zika virus infection are, in majority, asymptomatic and self-limited, but can lead to significant long term neurological complications (like microcephaly and Guillain-Barré syndrome).

In this paper we present a synthetic review of the current knowledge on Zika virus with few comments on the first imported Zika virus infection cases diagnosed in Vector-Borne Infections Laboratory in Cantacuzino Institute.

Keywords: Zika virus, microcephaly, diagnosis, prevention

REZUMAT

Una dintre cele mai importante epidemii din ultimii ani, declarată de Organizația Mondială a Sănătății la 1 februarie 2016 ca fiind o urgență de sănătate publică de interes internațional, a fost produsă de virusul Zika, un flavivirus din familia Flaviviridae, care determină la om apariția unor infecții ușoare, majoritar subclinice, dar urmate de posibile complicații neurologice, cum sunt microcefalia și sindromul Guillain-Barré.

În acest articol propus spre publicare, prezentăm o sinteză a cunoștințelor actuale despre virusul Zika, cu accent pe diagnosticul de laborator, împreună cu câteva comentarii legate de diagnosticarea în laboratorul nostru (Infecții Transmise prin Vectori din INCDMM Cantacuzino) a primelor cazuri de infecție cu virus Zika din Romania.

Cuvinte-cheie: Virusul Zika, microcefalie, diagnostic, prevenție

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne flavivirus, a member of the family Flaviviridae and genus Flavivirus, related with dengue, West Nile, yellow fever, Japanese encephalitis and tick-borne encephalitis viruses. From 1947, when it was first time isolated from a rhesus monkey in Zika forest in Uganda, East Africa, it had a large geographical distribution in Central Africa (sub-Saharan), in tropical and subtropical Americas and South-Eastern Asia, causing a widespread epidemic. The virus circulated at enzootic level in Africa and Equatorial Asia as two distinct genetic lineages: African and Asian, although some studies further differentiate the African lineage into West and East African lineages. Phylogenetic studies suggest that ZIKV Asian lineage is responsible for the emergence of infection in the Pacific, Americas, Cape Verde and Southeast Asia [1]. The asymptomatic forms of the disease, the unspecific signs and symptoms and serological cross-reactivity with other related flaviviruses allowed the unnoticed/ silent transmission of the ZIKV infection [2].

The first human infection was reported in 1954 in Nigeria [1] and in 2007 in Yap Island in the Western Pacific [2], the first ZIKV outbreak occurred, followed in 2013-2014 by a larger epidemic in French Polynesia (South Pacific), where the first severe neurological

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syndromes were reported. In 2015, the ZIKV outbreak in Brazil was declared a national public health emergency after local researchers and physicians reported an increase in microcephaly cases in newborns. The virus spread and caused in 2016 outbreaks in other countries in South America, which urged WHO to declare, on the 1st of February 2016, ZIKV a public health emergency of international concern.

In Europe, the first imported case of laboratory-confirmed ZIKV infection was reported in November 2013 by the German public health system in a German traveler returning from Thailand [3]. In Romania, the first three cases of ZIKV infection were reported in 2016 in three travelers returned from French Guiana, Dominican Republic and French Caribbean Island of Martinique.

TRANSMISSION WAYS

ZIKV is a vector-borne flavivirus, transmitted to humans by mosquitoes from genus *Aedes*, especially from species *A. aegypti*, *A. albopictus* being only a competent vector. The transmission of the virus can be also non-vectorial: sexual, maternal-fetal, by blood donations or by body fluids [2, 4, 5]. It was demonstrated that the sexual transmission is possible from both asymptomatic and symptomatic infections [5], the virus being detected in semen for up to 6 months after symptoms onset, and up to 13 days in the female genital tract after symptoms onset [6]. Mouse and human studies revealed that multiple cells and signals like interferon and hormones are implicated in vertical (maternal-fetal) transmission of ZIKV [7].

Maternal-fetal transmission was demonstrated during the epidemics in French Polynesian Islands in 2013 [5], but not all infected women would transmit the virus to the fetus and not all the exposed fetuses would develop a symptomatic infection [8].

PATHOPHYSIOLOGY

ZIKV has a single positive sense RNA genome which encodes a polyprotein with 3 structural (C, PrM, E) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) components [9, 10]. The envelope (E) protein is the major viral protein involved in cell receptor binding and entry and represents a major determinant for viral pathogenesis, but E glycosylation may function differently when the viruses alternate their infections between mammalian and mosquito hosts [11].

The NS1 protein, a factor of pathogenicity present in all flaviviruses, necessary for virus replication and for immune evasion, is implicated in the generation of antibodies, being highly immunogenic, and is involved in direct and antibody-mediated attack onto target cells. The extrapolation of these functions of NS1 from flaviviruses, especially from dengue, to Zika virus must be done with caution [10]. Intimate pathogenic mechanisms of ZIKV infection are still unknown but there is clinical and laboratory evidence that proves the implication of ZIKV in the pathogenesis of neurologic syndromes affecting humans (adults and children). Endothelial cells (which are part of placental and blood brain barrier, the two main barriers to be crossed by ZIKV to cause severe disease) seem to play an important role in further understanding the mechanisms of virus dissemination and congenital disease, including CNS alterations [11].

The high rates of microcephaly among children born to mothers with proven acute ZIKV infection during pregnancy and detection of viral RNA in the amniotic fluid, placenta, brain tissue of fetuses and infants with microcephaly, provided strong evidence for considering ZIKV as an etiologic agent of CNS anomalies [5, 12].

The pathogenesis of ZIKV associated Guillain-Barré syndrome is still unknown: there are multiple neuropathogenic and immune hypotheses.

IMMUNITY

The specificity of the antibody response in primary and secondary flaviviruses infection is different: following the primary infection there is often a very specific response, allowing

serological identification of the infecting virus; during the secondary flavivirus infection (dengue, yellow fever, West Nile), which frequently occurs in endemic ZIKV areas, or in persons previously vaccinated against a flavivirus (tick-borne encephalitis-TBE, yellow fever or Japanese encephalitis) and exposed to a related flavivirus, the antibody response becomes less specific, cross-reacting with multiple flaviviruses antigens [12]. A previous exposure to a flavivirus might produce a higher neutralizing antibody titer than the titer against the virus with which the person was most recently infected [13].

During the development process of an adaptive immune response the virus is rapidly cleared from the plasma [14].

It is presumed that ZIKV, like other flaviviruses, provide humoral lifelong protective antibody response against reinfection, although this assumption should be further confirmed. Studies in rhesus macaques suggest that immunity against one ZIKV lineage confers immunity against both ZIKV lineages [15]. Despite the existence of two genetic lineages (African and Asian) and three genotypes (West African, East African, and Asian), there is one ZIKV serotype [16].

CLINICS

In most cases, ZIKV infection causes a mild, self-limited illness (50 to 80% are asymptomatic virus infections) [17]. Among flaviviruses, Zika and dengue virus share similar symptoms of infection, transmission cycles, and geographic distribution. After an incubation period of 3-12 days unspecific symptoms develop, which include rash (predominant), fever, arthralgia (involving the small joints of the hands and feet), retroocular headache, and conjunctivitis. Symptoms of the acute phase last 2 until 7 days [5]. The convalescence period is short and, commonly, patients recover fully. Despite the low percentage of symptomatic cases and relatively mild forms of illness there are some redoubtable complications of ZIKV infection in infants and adults: microcephalia and, respectively, Guillain-Barré syndrome [5]. In October, 2015, in Brazil, an increase in microcephaly cases was observed by neurologists and a potential link between maternal ZIKV infection and a congenital syndrome was identified. In February, 2016, the incidence of microcephaly increased twenty-fold only in 6 months after ZIKV outbreak in Brazil and, consequently, WHO declared this epidemic a public health emergency of international concern [18].

Besides microcephaly, other major cardiac, cerebral, ocular anomalies were observed in fetuses and neonates that might lead to severe mental retardation and substantial motor disabilities, and visual and auditory impairments [5]. Some studies reported infants born with a normal head circumference, who developed microcephaly after birth (growth deceleration) [19]. Even asymptomatic women may transmit the infection to the fetus [20], which underscores the importance of timely diagnosis of ZIKV infection in pregnant women. Prospective follow-up of exposed and infected infants is also crucial.

Guillain-Barré syndrome was the first reported severe complication of ZIKV infection in adults. The link between ZIKV and Guillain-Barré syndrome was confirmed by a case-control study conducted in French Polynesia [21].

DIAGNOSIS

The diagnosis of ZIKV infection is difficult due to its non-specific signs and symptoms and the difficulty of laboratory diagnosis, especially serological one. Antigenically closely related to other flaviviruses, ZIKV produce an antibody response very difficult to interpret due to the high cross-reactivity with dengue, West Nile, yellow fever virus antibodies. Serologic results must be interpreted in the context of the immunologic status of the individual (vaccination for yellow fever or TBE, immune deficiency), previous possible flaviviral infections, as well as flavivirus endemicity in the region of exposure.

Serology for ZIKV diagnosis relies on the detection of specific antibodies: IgM antibodies

in a single serum sample collected after 6-7 days (some studies indicate 4-5 days) and up to more than 12 weeks after clinical onset [5, 22] for a probable case, and sero-conversion or four-fold increase of specific ZIKV antibodies titers in a pair of serum samples at 2 to 3 weeks interval for a confirmed case [23]. In a suspicion of ZIKV infection the confirmation is mandatory, especially in a person resident in or returned from a geographic area with endemic transmission of other flaviviruses (dengue, West Nile, yellow fever). Specific IgG antibodies appear later, usually from day 8 to 10 and remain detectable for months.

All positive or inconclusive IgM ELISA results should be confirmed by a plaque reduction neutralization test (PRNT), a complex technique only available in few laboratories. However, recent evidence suggests that a 4-fold higher titer by PRNT might not discriminate between anti-Zika virus antibodies and cross-reacting antibodies in all persons who have had a previous history of flavivirus infection (dengue and West Nile) or vaccination against another flavivirus (yellow fever) [12, 24]. Thus, a more conservative approach to interpreting PRNT results is now recommended to reduce the possibility of missing the diagnosis of either Zika or dengue virus infection [25]. Performing additional/complementary tests in order to discriminate between different flavivirus infections is mandatory because the results will guide clinical management which is very important mainly in dengue infections in order to reduce the risk for hemorrhage and shock [12]. Therefore laboratories should receive clinical and epidemiological information for establishing their investigation strategy and conducting ZIKV diagnosis, including date of illness onset, travel history (date and locations), past flaviviral immunization records and pregnancy status.

The molecular diagnosis of ZIKV infection is more accurate and consists in reverse transcription polymerase chain reaction (RT-PCR) targeting the envelope gene or NS5 region of viral genome from different clinical samples (blood and urine being the samples of choice; the persistence of virus genome in urine is even longer than in whole blood [5]: up to 39 days after exposure [5]). The optimum period of time for blood and urine detection of virus genome is the acute phase, during the first 5-6 days after symptoms onset [5], but a negative PCR test in samples collected 5–7 days after symptoms onset does not exclude flavivirus infection and therefore serological testing (IgM detection) should be considered.

Comparative with the detection of ZIKV RNA in blood, the detection in saliva has the advantage of being non-invasive, but it does not extend the period during which an acute infection can be diagnosed with PCR [26].

There are several commercial assays available for ZIKV genome detection. As viral load in patient samples is usually low, the use of a pan-flavivirus by PCR assay and subsequent sequencing may not be successful.

In order to facilitate a more rapid and accurate diagnosis of ZIKV infections in a context of ZIKV emergence in the last two years, ECDC, CDC and PAHO-WHO elaborated multiple diagnostic algorithms for cases in residents to or travelers from endemic areas, for pregnant or preparing for pregnancy women [27], for children. All these algorithms rely on the following laboratory diagnostic criteria for confirming the cases of ZIKV infection: detection of ZIKV nucleic acid or detection of ZIKV antigen or isolation of ZIKV from a clinical specimen, detection of ZIKV specific IgM antibodies in serum sample(s) and confirmation by neutralization test, seroconversion or four-fold increase in the titer of ZIKV specific antibodies in paired serum samples.

An overview of laboratory tests for Zika virus infection diagnostic is synthesized in Table 1. [23].

Frequently, the diagnosis of ZIKV infections requires an association of molecular and serological methods. For persons with suspected Zika virus disease, a positive RT-PCR result confirms Zika virus infection, and no antibody testing is indicated [12], but a negative RT-PCR result does not exclude Zika

Table 1. Laboratory tests for Zika virus infection diagnostic

Acute phase									Convalescent phase													
	Onset of symptoms↓↓																					
Days after onset		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	…	1 month	2 months	3 months	6 months
Laboratory assays																						
Sample type																						
Molecular assays	RT-PCR[1]																					
Serum/plasma																						
Saliva																						
Urine																						
Semen																						
Viral isolation	CC[2]																					
Serum/plasma																						
Urine																						
Semen																		21				
Serology	ELISA[3]/IFA[4]																					
Serum	IgM																					
	IgG																					
Serum	NT[5]																					

Notes:

Optimal period of use per current knowledge

Sub-optimal period for detection per current knowledge

(1) RT-PCR - reverse transcription polymerase chain reaction; (2) CC - cell culture (mammalian, mosquito cells)
(3) ELISA - enzyme-linked immunosorbent assay; (4) IFA – immunofluorescent assay; (5) NT – neutralisation test

virus infection (due to a possible inaccuracy in reporting the onset of illness or decline in viremia level) and a testing of serum IgM antibody for Zika and dengue virus infections should be performed.

For patients with serum specimens collected <7 days after onset of symptoms, a negative RT-PCR result on both serum and urine suggests that there was no recent infection, but further serological testing on paired serum samples is necessary [12]. However, a negative IgM antibody test, in the absence of RT-PCR testing, might reflect specimen collection before development of detectable antibodies and does not rule out infection with the viruses for which testing was performed. For specimens collected 7 days to 12 weeks after onset of symptoms, a negative IgM antibody result to both Zika and dengue viruses rules out recent infection with either virus [12].

In cases with positive, equivocal, or inconclusive results of IgM antibody testing against either Zika or dengue virus, PRNT against Zika and dengue viruses (or other flaviviruses endemic to the region where exposure occurred) should be performed.

A discriminatory (4-fold higher) neutralizing antibodies titer for ZIKV, as

compared to the other flaviviruses tested, is confirmatory for infection with Zika virus [12]. A positive PRNT (a titer ≥10, which is the starting serum dilution usually used to establish the presence of virus-specific neutralizing antibodies) for Zika and one or more flaviviruses provides evidence of a recent infection with a flavivirus but excludes identification of the specific virus of currently infection [12].

The interpretation of a negative PRNT against ZIKV depends on the moment of specimen collection: for a specimen collected 7 days before onset of symptoms the result might reflect an early collection, before development of detectable neutralizing antibodies, and an RT-PCR testing is necessary; for a specimen collected 7 days after onset of symptoms, the result excludes ZIKV infection [12].

Laboratory confirmation of fetal infection during pregnancy is also challenging; the results of molecular testing for ZIKV in blood, urine and amniotic fluid can be negative, despite existing fetal infection [5].

Positive result of ZIKV PCR in an infant sample (serum, urine) confirms the diagnosis of congenital ZIKV infection, and negative result associated with negative ZIKV IgM antibodies sustain the absence of congenital ZIKV infection. Positive IgM antibodies and negative PCR support a probable case of congenital ZIKV infection. In this case PRNT for Zika and dengue viruses should be performed.

A negative result of ZIKV PRNT suggests that the infant's ZIKV IgM test is a false positive, but a positive PRNT opens a discussion regarding the results, due to the persistence of mother antibodies until 18 months in the infant serum. PRNT cannot distinguish between mother and infant antibodies but PRNT might help confirm or rule out infection in a sample collected from an infant aged 18 months whose initial sample collected at birth was IgM positive or equivocal: if PRNT results at 18 months are negative, the infant is considered not to have congenital ZIKV infection; if PRNT results are positive, congenital ZIKV infection is presumed, but postnatal infection cannot be excluded, especially among infants living in an area with ZIKV active transmission [28].

In response to the WHO alert, in Romania, the National Center for Surveillance and Control of Communicable Diseases (NCSCCD) published on the website of NIPH the methodology for ZIKV surveillance, following the criteria of ECDC. In 2016, the first three cases of imported ZIKV infection were registered in Romania, two cases hospitalized in Victor Babes Hospital for Tropical Diseases in Bucharest and one case in the University Hospital of Infectious Diseases, Cluj-Napoca. All three cases were diagnosed în the National Reference Laboratory for Vector Borne Infections in Cantacuzino Institute, Bucharest. The diagnosis was both serologic and molecular, and very challenging, due to inconclusive results of serology for the first serum samples in two of cases [29, 30].

Commercial enzyme-linked immunosorbent assay (ELISA) detecting IgM and IgG antibodies against ZIKV NS1 protein, considered to have a higher specificity (but a lower sensitivity), was used for serological ZIKV diagnosis. In-house real-time PCR targeting NS5 segment of genome was the test chosen for molecular diagnosis because it can be performed rapidly and is highly specific when performed on urine collected less than 14 days after symptoms onset [5].

The protocol was validated using RNA positive control provided by Robert-Koch Institute as well as RNAs of other flaviviruses (dengue and West Nile virus) to check for cross-reactivity. The primers and probes were chosen from literature and produced by our molecular biology reagents provider [31].

ZIKV disease confirmation in the patient from Cluj hospital, was both serological and molecular, but serological obvious positive results were obtained late in time, only on day 18, by IgM seroconversion of the third serum sample (moderate positive values) and IgG borderline results.

On the contrary, molecular tests were positive on day 5 from onset of the disease in a urine sample. A second urine sample on day

8 was also positive. NS1 dengue antigen was negative [30].

The first patient from Bucharest hospital, returned from Martinique, tested negative for dengue virus antibodies, but positive for ZIKV RNA in a serum sample three days post onset and in a urine sample 9 days post onset. Confirmation of diagnosis was also serologic: 9 days after clinical onset the seroconversion of IgM and IgG was documented.

The most challenging case was the second case from Bucharest, a child resident in French Guiana, recently vaccinated for yellow fever, with nonspecific clinical signs of flaviviral infection, diagnosed by PCR in a urine sample 3 days post illness onset. A serum sample collected on the same day tested negative for viral RNA presence. The acute serum sample was negative for specific ZIKV IgM and IgG antibodies against nonstructural protein (NS1) and for WNV IgM and dengue virus IgM antibodies, but positive for yellow fever IgG antibodies. On day 19 the second serum sample was inconclusive for ZIKV IgM, negative for ZIKV IgG (also against NS1), positive for yellow fever IgG (as expected), and surprisingly positive for dengue IgG and West Nile IgG [29]. A possible explanation for these results is the history of yellow fever vaccination in this child which triggered, during the ZIKV infection as a second infection with a flavivirus, a highly cross-reactive antibody response. In fact, lack of detectable antibody response in some ZIKV infected patients has been reported [32]. The NS1 antigen based ZIKV ELISA may show low sensitivity as well [33].

All three cases were more rapidly and accurately diagnosed with the molecular tools, urine samples being the most reliable samples for PCR testing.

RECOMMENDATIONS FOR ZIKA VIRUS TESTING

During the last years of ZIKV epidemics, CDC and WHO elaborated guidelines for ZIKV testing in different situations and for different individual immune status. The most important recommendations from the point of view of social impact are those regarding the preparing for conception of both men and women, the pregnant women and the newborn children and infants [34, 35]:

- m**en** and women with ZIKV disease should wait at least 6 months and, respectively, 8 weeks after symptoms onset before attempting conception;
- asymptomatic men and women should wait at least 8 weeks after the last date of possible exposure before attempting conception;
- all pregnant women should be tested if they have had possible exposure to ZIKV, including sexual exposure, by both RT-PCR, on serum and whole blood, and serology at any time [36, 37], (Table 2).
- for newborn child with possible congenital ZIKV infection, RT-PCR should be performed within the first 2 days of birth on both serum and urine, and IgM ELISA should be performed on serum.

The vector-borne diseases are difficult to prevent and control. The number and the spreading area of human vector-borne cases are increasing, and new vector-borne diseases have been reported in the last 10-12 years [38]. Therefore, sustained efforts are focused for a rapid development of vaccines and therapeutics. However, until a vaccine anti ZIKV will be available, the recommendations for preventing mosquito bites for all residents of and visitors of areas where Zika virus is spreading, are safer: wearing long sleeved shirts and long pants, using insect repellents, staying and sleeping in screened-in or air-conditioned rooms, etc.

These recommendations for preventing ZKV infection may be considered exaggerated, but we must think at the dramatic complications which could appear and at the fact that even long term complications are possible.

The current experience with ZKV epidemics is preparing us for another possible emerging public health event in the future.

Conflicts of interest: We have no conflicts of interest to disclose.

Table 2. Algorithm for investigating cases suspected of Zika virus infection

Case under investigation	Days after onset of symtoms	Days/weeks after last possible exposure*	Type of sample	Recommended tests
A. If symptomatic	1-5/6		Serum + urine	RT-PCR Zika
	≥6		Serum +/ urine	PCR Zika in urine until 10 days after onset; IgM Zika in serum and IgG Zika in a second serum after 2-3 weeks
B. **If pregnant and symptomatic**, with possible exposure* and no residence in areas with Zika virus transmission		1-14 days	idem A.	idem A.
C. If pregnant and asymptomatic, with possible exposure* and no residence in areas with Zika virus transmission		2-12 weeks	serum	IgM and IgG Zika

*Possible exposure: travel in areas with Zika virus transmission; or sexual contact with a male who had been or has residence in an area with Zika virus transmission (https://www.cdc.gov/zika/geo/index.html)

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