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Zika Virus: a re-emerging pathogen with rapidly evolving public health implications

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INTRODUCTION

History of Zika virus (ZIKV) infection

ZIKV is an enveloped, arthropod-borne (arbovirus) virus characterized by a single positive-stranded RNA genome. ZIKV was firstly isolated in 1948 from a sentinel monkey that was monitored for the infection by Yellow fever virus in the Zika forest of Uganda (Dick *et al.*, 1952). The virus belongs to the family of *Flaviviridae* and it is closely related to other members of the family, mainly Dengue, Yellow fever, West Nile and Japanese Encephalitis Viruses.

As illustrated in *Figure 1*, the first cases of human infection were reported in Nigeria in 1952 (Macnamara, 1954), however, the first recognizable outbreak happened almost sixty years later in 2007 in the Micronesian island of Yap (Duffy *et al.*, 2009). The infection then rapidly moved across the Pacific to Easter Island (Cao-Lormeau *et al.*, 2014) and progressed to South and Central America as well as to the Caribbean in 2015-2016 (Musso, 2015). The explanation of this huge temporal gap occurred between the first human cases and ZIKV recognizable outbreaks are likely consequent to several factors. Firstly, human ZIKV infection is asympomatic in 4 out of 5 individuals (Duffy *et al.*, 2009). Secondly, the symptoms of the infected individuals are usually mild and quite generic, such as elevated blood temperature, cutaneous rash, headache/malaise, non-purulent conjunctivitis, conjunctival hyperemia, arthralgia, myalgia, peripheral edema and gastrointestinal disturbance persisting for a few days and then disappearing (http://www.bmj.com/freezikaresources). These very same symptoms are common to other infections and, in particular, to Dengue that often co-circulates with ZIKV (Lanciotti *et al.*, 2008). Although ZIKV was already circulating in the Yap Islands prior to 2007, the outbreak was only recognized when 3/4 of the island population was acutely and unequivocally infected with this virus (Duffy *et al.*, 2009).

Indeed, the second ZIKV outbreak was recognized in French Polynesia in 2013 as part of the regional surveillance of Dengue infection (Cao-Lormeau *et al.*, 2014). Importantly, an unusual increase of cases affected by the neurological Guillain-Barré syndrome (an acute, immune-mediated peripheral neuropathy that may lead to paralysis typically occurring after viral or bacterial infections including cytomegalovirus, influenza virus and Campylobacter jejuni) was reported in coincidence with the outbreak. Although the majority of individuals with Guillain-Barré syndrome survive, it may take months to years to fully recover (Willison *et al.*, 2016). A case-control study reported that 41 out of 42 patients in French Polynesia infected with ZIKV developed Guillain-Barré syndrome *vs* 55 of 98 patients of a control group without the infection (Cao-Lormeau *et al.*, 2016). The ZIKV symptoms were reported approximately 6 days before the onset of the neurological symptoms supporting the hypothesis of an etiological role of ZIKV infection in Guillain-Barré syndrome. Furthermore, high concentrations of ZIKV were found in the cerebrospinal fluid of a 15 year-old-girl with acute myelitis in Guadalupe, French West Indies (Mecharles *et al.*, 2016) and in a 81 year-old man in France with meningo-encephalitis following a cruise in the Pacific (Carteaux *et al.*, 2016).

Of particular relevance is the capacity of ZIKV to infect the fetus of infected mothers. The first cases were reported at the end of 2015 in the North East regions of Brazil. Epidemiological studies suggested a clear temporal and geographical association between ZIKV infection and the incidence of mothers delivering babies with microcephaly and arthrogryposis of legs and arms (Hazin *et al.*, 2016; Miranda-Filho Dde *et al.*, 2016). In support of this hypothesis, a complete virus genome sequence was obtained from the amniotic fluid of two pregnant Brazilian women whose fetuses were diagnosed with microcephaly (Calvet *et al.*, 2016). Importantly, significant amounts of ZIKV virions were visualized by electron microscopy in the brain of another fetus at autopsy along with grossly abnormal brain tissue (Mlakar *et al.*, 2016). A retrospective study clearly showed an association between ZIKV infection during the first trimester of pregnancy and severe fetal neurodevelopment abnormalities in approximately 1% of the infected women (Cauchemez *et al.*, 2016). An independent Brazilian cohort study reported that 72 out of 88 pregnant women were positive for ZIKV with 29% of ZIKV-positive women showing fetal abnormalities by Doppler ultrasonography (Brasil *et al.*, 2016). Although case-control studies will be necessary to definitively prove a cause-effect relationship (Musso and Baud, 2016), nevertheless these findings are quite compelling in terms of demonstrating a causal relationship between ZIKV infection and severe abnormalities of the brain intra-utero, as recently endorsed by the CDC (http://www.cdc.gov/media/releases/2016/s0413-zika-microcephaly.html).

Key words:
Zika Virus, epidemiology, pathology, virus genome and replication.

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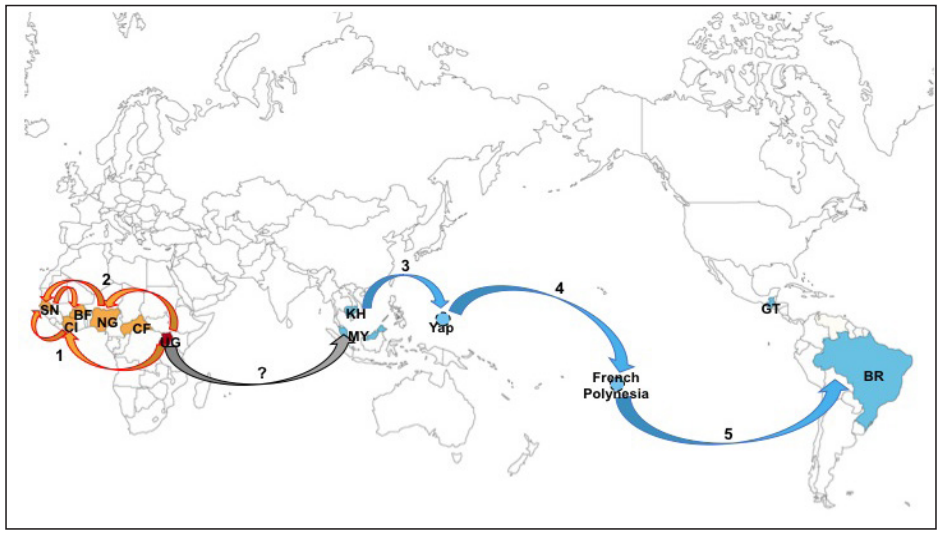

Figure 1 - *Geographical spreading of ZIKV infection. Arrows and numbers from 1 to 5 indicate the sequential spread of ZIKV from Africa to Asia. The different colors of arrows and countries indicates the viral genotype, with the African genotype in orange and the Asian genotype in blue. The grey arrow and the question mark indicate the lacking of data fully supporting the origin of the Asian genotype from Uganda. The country abbreviations is: Brazil (BR), Burkina Faso (BF), Cambodia (KH), Central African Republic (CF), French Polynesia, Guatemala (GT), Ivory Coast (CI), Malaysia (MY), Nigeria (NG), Uganda (UG), Micronesian Yap Island (Yap).*

ZIKV transmission: more than one route

ZIKV transmission mainly occurs through bites of infected blood-feeding *Aedes aegypti* mosquitoes that are present in the tropical and sub-tropical regions sustaining the urban human-mosquito cycle (Musso and Gubler, 2016). The geographical distribution of *Aedes aegypti* remarkably overlaps with evidence of ZIKV infection and the cases reported outside the tropical and sub-tropical regions are related to travellers returning from endemic regions (Hills *et al.*, 2016). However, as millions of people travel to the world areas affected by ZIKV annually, air travel has contributed to spreading of ZIKV infection (Bogoch *et al.*, 2016). Furthermore, although *Aedes aegypti* is the main transmitter of the ZIKV, other types of mosquitoes may be contributing as well. A prime suspect is *Aedes albopictus*, which thrives in more temperate climates; its rapid expansion and vectorial capacity for different arboviruses affect an increasingly larger proportion of the world population (Bonizzoni *et al.*, 2013). As a vaccine preventing ZIKV infection is not yet available, protective measures to avoid mosquito bites and control of mosquito'es dissemination represent nowadays the main tools for preventing ZIKV transmission to humans.

A peculiarity of ZIKV infection (that distinguishes it from other *Flaviviruses*) is its capacity of being transmitted vertically from mothers to their fetuses through the placenta (Adibi *et al.*, 2016). The virus has been detected in the placenta (Martines *et al.*, 2016) and amniotic fluid of mothers carrying fetuses with microcephaly (Calvet *et al.*, 2016), although it is still debated whether the virus crosses the placenta barrier or whether the virus multiplicates in the placental cells.

The first indication that ZIKV could be transmitted by sexual intercourse came from a case report of an American man who contracted ZIKV infection in Senegal in 2008 and transmitted the infection to its wife after returning home (Foy *et al.*, 2011). Additional cases (a total of 9 to date) of sexual ZIKV transmission were related to symptomatic males of three different countries not associated to vector-mediated infection (USA, France and Italy) infecting their partner (Moreira *et al.*, 2016; Venturi *et al.*, 2016). Importantly, ZIKV isolation from semen was successfully achieved and the presence of viral sequences was detected by polymerase chain reaction (PCR) in semen up to 60 days since the onset of the symptoms, suggesting that the virus can persist much longer in the male genital compartment than in peripheral blood (Musso *et al.*, 2014c).

To prevent the possibility that ZIKV transmission could occur by blood transfusion, an extensive screening was implemented in French Polynesia since 2013. Up to 3% of blood donors, asymptomatic at the time of the blood donation, were indeed found to be positive for ZIKV by PCR (Musso *et al.*, 2014a). In the USA, these results have raised concern on the safety of blood from donors considered at risk of infection because of their travelling profiles, living or having had sexual contact in the endemic areas in the previous 4 weeks. In order to increase blood safety, treatment of blood and blood products with Amotosalen and UV light, a system that inactivates the RNA genome of several pathogens, was demonstrated to be effective against ZIKV (Aubry *et al.*, 2016) as it was earlier demonstrated in the case of SARS Corona and Dengue viruses, respectively (Pinna *et al.*, 2005; Musso *et al.*, 2014c). This inactivation process is of particular interest to prevent plasma transfusion-transmitted ZIKV infections in areas such as French Polynesia, where several arboviruses are co-circulating.

Virus genotype and variability

To date, phylogenetic analyses strongly support the existence of two distinct lineages of ZIKV (i.e. African and Asian). The prototypic African MR766 strain has been identified as the common ancestor of two independent ZIKV spreads from Uganda to Western Africa (Faye *et al.*, 2014). The first introduction of the virus has been traced to Senegal and Ivory Coast whereas the second spread involved firstly Central African Republic and Nigeria, then Senegal, Ivory Coast and Burkina Faso (*Figure 1*). Thus, co-circulation of two ZIKV lineages has been suggested to occur at least for Senegal and Ivory Coast (Faye *et al.*, 2014).

Sequence analysis of NS5 and envelope (E) genes (later discussed) stimulated the hypothesis that the Asian genotype originated from viruses exiting out of Africa (Faye *et al.*, 2014). Noteworthy, previous *Flavivirus* studies suggested that their spreading patterns are informative only

if they are based on whole, or near-complete, genome sequences (Nunes *et al.*, 2012; Pybus *et al.*, 2012). Therefore, other hypotheses should be taken into account, such as a parallel evolution of both genotypes from a common ancestor. Nevertheless, ZIKV spread in South-East Asia from Malaysia to Cambodia and Micronesia (Haddow *et al.*, 2012) and, lately, to the Pacific area (Cao-Lormeau *et al.*, 2014; Musso *et al.*, 2014b; Pyke *et al.*, 2014; Tognarelli *et al.*, 2016) has been linked to a genetically diverse Asian genotype (*Figure 1*).

A recent sequence analysis of the ZIKV Brazilian isolates with a "molecular clock" approach supports the hypothesis of a common ancestor of the Asian genotype closely related to the Polynesian lineage (Faria *et al.*, 2016). Thus far, evidences indicate that ZIKV spreading within Americas arose from Brazil where the introduction of the virus likely occurred in 2014 (Musso, 2015; Zanluca *et al.*, 2015) or even earlier in 2013 (Faria *et al.*, 2016). Indeed, Brazilian isolates do not cluster together, but result interspersed among all American isolates with a high degree of diversification, suggesting the existence of the common ancestor of an American lineage of the virus in Brazil.

A potential source of ZIKV diversification is the possibility of recombination between different viral strains (Faye *et al.*, 2014). Breakpoints as markers of recombination events have been identified in both NS5 and E genomic regions of African strains. Since recombination has not been documented in other *Flaviviruses*, these findings need to be confirmed by more extensive analyses. However, in support of this hypothesis, the mutation rate of ZIKV has been estimated ranging from 0.98 to 1.06×10^{6} nucleotide substitutions/site/year (Faria *et al.*, 2016), which is higher than the evolutionary rate of other *Flaviviruses* (Pybus *et al.*, 2012).

Genetic variations of MR766 strain sequences and glycosylation sites have been reported (Haddow *et al.*, 2012). However, this observation could be more the result of serial passages in culture of the viral isolates, as previously observed for West Nile virus strains (Chambers *et al.*, 1998), rather than representing true virus variability *in vivo*. A recent sequence analysis has investigated whether amino acid changes of Brazilian ZIKV could be linked to the increased occurrence of microcephaly (Faria *et al.*, 2016). However, no association between mutations and clinical manifestation were identified as none of the analyzed amino acidic changes predicted a significant variation in the physico-chemical properties of the viral proteins. Therefore, according to the current knowledge, genetic divergence of the Asian genotype does not seem to account for its pathogenetic profile. Thus, it is conceivable that any drug or vaccine product developed against ZIKV should be protective against all strains. At diagnostic level, the close relatedness between ZIKV and other *Flaviviruses* generates challenges in developing *ad hoc* algorithms to distinguish the different viruses in order to obtain reliable data in terms of their spreading and, in perspective, to apply specific therapeutic or preventative measures.

ZIKV genome and replication

Besides the ZIKV full-length RNA genome sequences published in Genbank (http://www.ncbi.nlm.nih.gov), very few data are available on the ZIKV life cycle, the regulation of its viral transcription and protein synthesis. Therefore, the information reported below refers to general features of *Flaviviruses*.

As illustrated in *Figure 2*, the ZIKV genome is a positive stranded RNA of ~11 kb that encodes a single open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (UTR), as reported (Blitvich and Firth, 2015). These 5'UTR and 3'UTR regions contain the regulatory elements that fold in stem-loop structures and regulate the synthesis of the viral RNA (Villordo *et al.*, 2016). The genome is translated into a single polyprotein that is subsequently cleaved by both the viral NS3 serine protease and by the cellular protease Furin into 3 structural proteins: membrane precursor (prM), capsid (C) and (E) protein. In addition, the synthesis of 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) is required for virus replication and assembly (Blitvich and Firth, 2015) (*Figure 2*). The E protein is involved in the recognition of a yet unidentified receptor at the surface of the host cell and then mediates the fusion process between the viral envelope and the intracellular membranes. The implications of the discovery of such receptor(s) in terms of drug and vaccine development do not require further comments.

Although not yet well defined, the different stages of ZIKV replication are considered to be similar to those of other members of the *Flavivirus* genus. Typically, the virus has to complete 4 major stages to finalize its replicative cycle:

1) translation of genomic RNA into viral proteins;

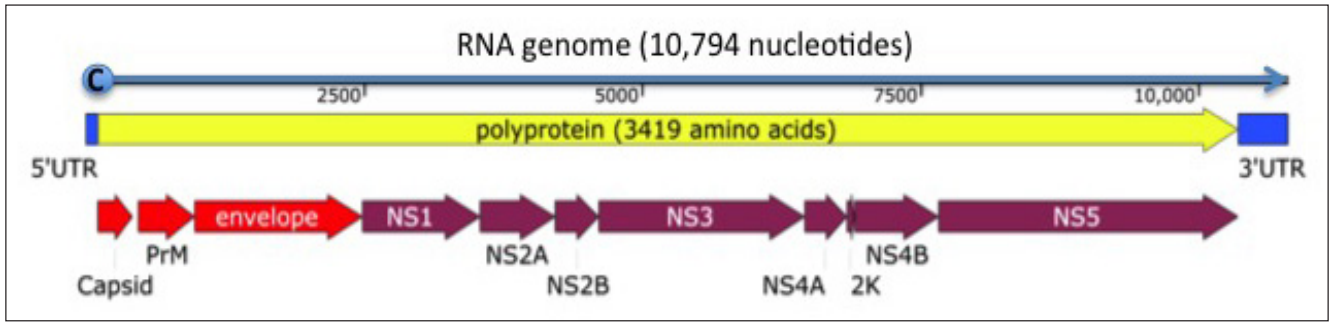

Figure 2 - *Genomic organization of ZIKV. The ZIKV strain MR766 genome and proteins (GenBank accession: AY632535) is reported. The image was generated with the SnapGene software (http://www.snapgene.com/). The 5' and 3' end of the genome is characterized by 2 UTR of 106 and 428 nucleotides, respectively. The genome 5' end has a methylated nucleotide cap (C) for canonical cellular translation. The 3' terminus is not polyadenylated but it forms loop structures. Almost the whole genome is translated in a single polyprotein of 3,419 amino acids that is processed co- and post-translationally by host and viral proteases. Structural proteins are in red whereas regulatory proteins are in violet.*

2) transcription of viral RNAs and their translation into viral proteins;
3) assembly of virus particles in the endoplasmic reticulum (ER);
4) virion release from the infected cell.

The virion has an icosahedral capsid enclosed by a lipid envelope with a diameter of 40-70 nm (Sirohi *et al.*, 2016). ZIKV replication occurs in the cellular cytoplasm and, like all other *Flaviviruses*, multiple relationships with cellular organelles might occur to facilitate virus replication, evasion and propagation, although the exact details remain to be determined.
The identification of such host factors and the characterization of their interactions with viral RNA and proteins are of crucial importance to understand ZIKV replication and identify potential "druggable" targets.

Pathogenesis of ZIKV infection

Since the virus discovery, ZIKV was described as a neurotropic virus (Dick, 1952; Bell *et al.*, 1971). At that time, the virus was serially passaged in mice by inoculation either in the brain or by the intra-peritoneal route. In order to obtain consistent morbidity and mortality results, the virus was adapted by almost 90 passages. Of note, the resulting virus was almost exclusively capable of replicating in the brain of mice inoculated intra-cerebrally whereas viremia, if present, was transient and likely a spillover from the brain. Importantly, when mice were infected via the intra-peritoneal route, minimal signs of brain infection were present in mice older than 2 weeks, whereas mice of 7 days of age showed unequivocal infection of the central nervous system (Dick, 1952; Bell *et al.*, 1971). In support of these old findings, very recently, *in vitro* studies have demonstrated that human neural progenitor cells derived from induced pluripotent stem cells are indeed permissive to ZIKV productive infection (Tang *et al.*, 2016). ZIKV replication in neural progenitor cells was shown to induce an alteration of gene expression and in particular of those genes that regulate cell-cycle progression and cell death, as shown by an increased levels of Caspase-3 expression in infected cells (Tang *et al.*, 2016). These results have been confirmed by an independent study whereby human neural stem cells were grown as neurospheres (i.e. clusters of neuronal stem cells) and cerebral organoids, i.e. bundles of human tissue that have some features of the early human brain in the first trimester (Garcez *et al.*, 2016). Indeed, viral particles were clearly detected in ZIKV-infected neurospheres with evidence of swollen mitochondria and nuclear alterations. The infection caused both changes of neurosphere morphology and their dramatic reduction in infected cultures 6 days after infection (Garcez *et al.*, 2016). The same effects were observed in brain organoids in which a significant reduction of their size was observed 11 days post-infection (Garcez *et al.*, 2016). Although these *in vitro* experiments were conducted only with the historical ZIKV MR766 strain and not with recent viral isolates, they nevertheless raise critical questions about direct pathological effects on neurons and other neural cell types that may also involve an additional potential damage exerted by local inflammation. It is still unclear whether ZIKV infects neural stem cells of adult humans (Bond *et al.*, 2015).
ZIKV virus is transmitted to humans by mosquito bites, by injecting the virus into the skin and submucosa of the mammalian host.
Thus, the skin and submucosa play a critical role in either preventing or promoting the infection. Human skin cells, including isolated keratinocytes and fibroblasts, are permissive to ZIKV infection and replication. In addition, ZIKV replication was detected in human skin explants and the associated-keratinocytes showed the appearance of cytoplasmic vacuolation with picnotic nuclei indicative of cells undergoing apoptosis (Hamel *et al.*, 2015).
The epithelial cell line A549 is also permissive to ZIKV infection and virus replication induces the production of soluble type I interferons (IFNs) and pro-inflammatory cytokines along with cell death (Frumence *et al.*, 2016). Research into this area might uncover common mechanisms for receptor-mediated infection, viral pattern recognition by innate immunity receptors leading to IFN production aiming at halting spreading infection.
By analogy with other *Flavivirus* infections, antigen presenting cells including dendritic cells (DC) are infectable by ZIKV (Hamel *et al.*, 2015).
The role of attachment receptors such as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) as well as of other proteins such as AXL, Tyro3 and, to lesser extent, TIM-1 on their cell surface remains to be clearly defined (Hamel *et al.*, 2015; Nowakowski *et al.*, 2016).

Closing Remarks

The underlying reasons for the emergence of Zika virus in the past decade are unknown. Recent global increases in the incidence and spread of Dengue, Chikungunya, and now Zika virus - all hosted by *Aedes aegypti* as primary vector - suggest common underlying mechanisms for their emergence, such as globalization and urbanization. Other possible explanations include viral mutations affecting transmission and/or virulence together with the introduction of the infection in previously unexposed populations leading to epidemic spread. Further research will be required to determine whether the recently observed associations and likely cause-effect relationship with adverse birth outcomes and Guillain-Barré syndrome are simply explained by an increased incidence of infection combined with an increased diagnostic attention or whether they reflect a change in the virulence and host cell tropism of ZIKV.

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