

REVIEW – Virology

Zika virus: from pathogenesis to disease control

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One sentence summary: A comprehensive review of Zika virus biology, pathogenesis, clinical features, diagnosis and perspectives for vaccine and antiviral drug development.

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ABSTRACT

Zika virus is a mosquito-borne flavivirus discovered in Uganda in 1947. The virus has emerged in recent years and spread in the Pacific Area and the Americas, where it has caused large human outbreaks. The factors involved in the virus's emergence are still unknown, but probably include its introduction in naïve environments characterised by the presence of high densities of competent *Aedes* spp. mosquitoes and susceptible human hosts in urban areas. Unique features of Zika virus infection are sexual and transplacental transmission and associated neurological morbidities, i.e. Guillain-Barré syndrome and fetal microcephaly. Diagnosis relies on the detection of viral nucleic acids in biological samples, while detection of a specific antibody response may be inconclusive because of the broad cross-reactivity of antibodies among flaviviruses. Experimental studies have clarified some mechanisms of Zika virus pathogenesis and have identified potential targets for antiviral drugs. In animal models, the virus can infect and efficiently replicate in the placenta and in the brain, and induce fetal demise or neural damage, recapitulating human diseases. These animal models have been used to evaluate candidate vaccines and promising results have been obtained.

Keywords: Zika virus; pathogenesis; transmission; clinical features; diagnosis; vaccines; antiviral drugs

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne flavivirus that was originally discovered in 1947 (Dick et al. 1952). It was not considered a relevant human pathogen until the large outbreak starting in Brazil in 2015 that revealed an association of ZIKV infection with fetal microcephaly and Guillain-Barré syndrome (Ministério da Saúde (Brasil) 2015; PAHO 2016b). This prompted the World Health Organization (WHO) to declare ZIKV a Public Health Emergency of International Concern on 1 February 2016 (WHO 2016b). In response to this emergency, research on ZIKV was intensified with the aim to provide evidence for the aetiological link between ZIKV infection and neurological sequelae, demonstrate the mechanisms of transmission and pathogenesis, set up accurate diagnostic tests, develop prophylactic vaccines and discover antiviral drugs. Although there are still

knowledge gaps that require investigation, this intense recent research has rapidly produced impressive results, which are summarised and discussed in this review article.

ZIKV BIOLOGY

Zika virus (ZIKV) is a mosquito-borne flavivirus that was first isolated in 1947 from the serum of a febrile sentinel rhesus macaque (*Macaca mulatta*) in the Zika Forest area located near Entebbe in Uganda, and subsequently found also in *Aedes africanus* mosquitoes in the same region (Dick 1952; Dick et al. 1952). The virus belongs to the *Flaviviridae* family, genus *Flavivirus*, which contains several vector-borne viruses relevant for human health, such as dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV),

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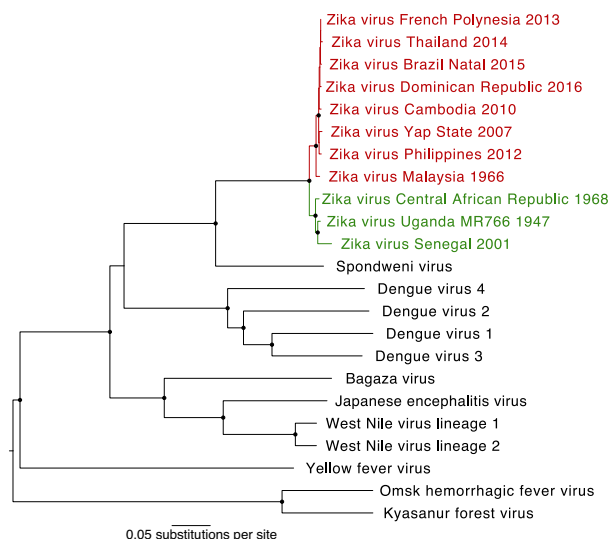


Figure 1. Evolutionary relationships of Zika virus and other flaviviruses based on the whole polyprotein. The multiple alignment of full-length viral polyprotein obtained from different flaviviruses was built with Clustalw2. The phylogenetic tree was inferred using the Neighbor-Joining method with 1000 bootstrap replicates, ignoring all positions containing gaps (3300 amino acids remained in the final datasets). The two Zika virus lineages currently identified are highlighted in red (Asian) and green (African), while black circles indicate nodes confirmed by at least 90% of bootstrap replicates. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The list of NCBI Protein Database accession numbers of sequences included in the phylogenetic tree are as follows: Bagaza virus: YP002790883; Dengue virus 1: P33478; Dengue virus 2: P12823; Dengue virus 3: Q99D35; Dengue virus 4: Q2YHF2; Japanese encephalitis virus: NP_059434; Kyasanur forest virus: ADH95737; Omsk hemorrhagic fever virus: NP_878909; Spondweni virus: YP009222008; West Nile virus lineage 1: YP001527877; West Nile virus lineage 2: AAT02759; Yellow fever virus: AAT12476; Zika virus Brazil Natal 2015: AMB18850; Zika virus Cambodia 2010: AFD30972; Zika virus Central African Republic 1968: AHL43500; Zika virus Dominican Republic 2016: AMN14619; Zika virus French Polynesia 2013: AHZ13508; Zika virus Malaysia 1966: AEN75264; Zika virus Philippines 2012: AMD61711; Zika virus Senegal 2001: AHL43503; Zika virus Thailand 2014: AMD61710; Zika virus Uganda MR766 1947: AMK02027; Zika virus Yap State 2007: ACD75819.

and tick-borne encephalitis virus (TBEV). In addition, a fourth group of viruses found only in insects is included in this genus. Phylogenetic analysis of the *Flavivirus* genus shows a relationship between groups of viruses and their epidemiology and associated diseases and identifies clades of mosquito-borne, tick-borne and no-known-vector viruses, which are further divided into subclades defined by their principal vertebrate host and the vector species involved in transmission (Gaunt et al. 2001). In particular, mosquito-borne flaviviruses include two phylogenetically and epidemiologically distinct groups, namely the neurotropic viruses, such as WNV and JEV, which are transmitted by *Culex* species vectors and have bird reservoirs, and the non-neurotropic viruses, such as DENV and YFV, which are associated with hemorrhagic disease in humans, are transmitted by *Aedes* species mosquitoes and have primate hosts (Gaunt et al. 2001).

In the phylogenetic tree, ZIKV is closely related to DENV and clusters within the Spondweni group (Fig. 1). ZIKV strains are divided into two major lineages, namely the African lineage, which includes strains isolated in Central and Western Africa, and the Asian lineage, which includes strains isolated in Southeast Asia and the Pacific region and the contemporary human ZIKV strains from the Americas (Haddow et al. 2012; Faye et al. 2014; Wang et al. 2016; Fig. 1). The two lineages differ for about

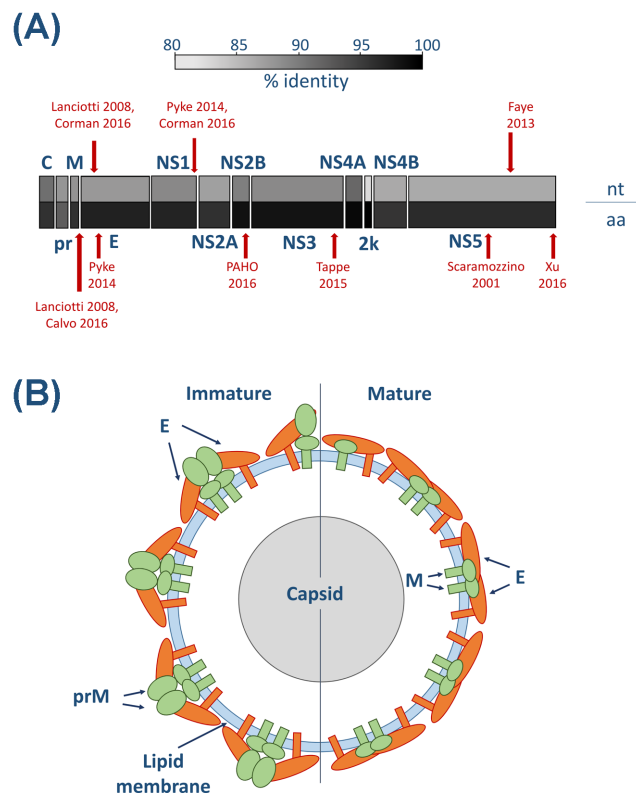


Figure 2. Schematic representation of Zika virus genome and structure. (A) The genomic regions encoding Zika virus mature proteins. Shades of gray represent the nucleotide (nt) and amino acid (aa) identities between African (GenBank: KU720415) and Asian lineages (GenBank: KU527068). Genes on the polyprotein are represented to scale. Coordinates of structural and non-structural genes are based on RefSeq NC_012532. Regions in the Zika virus genome targeted by nucleic acid diagnostic tests are indicated by red arrows. (B) Simplified representation of Zika structure. The 'spiky', immature conformation is shown on the left, while the mature 'smooth' virion generated after cleavage of the pr domain is on the right.

90% of their nucleotide sequence and 59 amino acids in the polyprotein (Wang et al. 2016; Fig. 2A).

Viral genome and replication

Like other flaviviruses, ZIKV has an icosahedral envelope containing a positive-sense, single-stranded RNA genome of approximately 10.7 kb in length, which is complexed with multiple copies of the capsid protein. The genome encodes a single polyprotein of 3423 amino acids that is processed by viral and cellular proteases into three structural proteins, i.e. the capsid (C), membrane (prM) and envelope (E) proteins that form the virus particle and mediate virus attachment, entry and encapsidation, and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which function in viral genome replication, polyprotein processing and counteraction of host innate antiviral response (Fig. 2A). Similarly to other flaviviruses, the ZIKA genome has two untranslated regions (UTRs) in the 5' and 3' ends of 107 and 429 nucleotides, respectively. The 5' UTR has a type I cap structure, while the 3' UTR lacks a polyadenylate tail and contains conserved structures that probably play different functions during replication in vertebrate and invertebrate hosts (Villordo et al. 2016; Zhu et al. 2016).

Viral replication occurs in the cytoplasm of infected cells (Suthar, Diamond and Gale 2013). Following endocytosis mediated by a still unknown cellular receptor, viral fusion occurs with endosomal membranes leading to uncoating of the viral particle and release of the viral genome, which is immediately translated as a single polyprotein at the endoplasmic reticulum (ER). The polyprotein is processed by viral NS2B–NS3 protease and cellular proteases to generate mature viral proteins. The positive-sense viral RNA genome is then copied to give a negative-sense RNA molecule, which serves as the template for synthesis of the full-length positive-sense genomic RNA. Synthesis of viral genome occurs in association with the ER and is catalysed by a replication complex that includes the NS5 RNA-dependent RNA polymerase and other NS proteins. The new genomes are packaged by the C protein and acquire the envelope while budding from the ER. These immature virions are transported through the cellular secretory pathways, where E glycosylation and cleavage of prM by host furin protease occurs resulting in the formation of mature virions that are released by exocytosis. High-resolution cryoelectron microscopy (Kostyuchenko et al. 2016; Sirohi et al. 2016) showed that the structure of ZIKV is similar to those of DENV and WNV: immature ZIKV virions have a spiky surface composed of 180 copies of each of the E and prM proteins associated as heterodimers and anchored in the bilayer lipid membrane via their transmembrane regions, while mature virions have a smooth surface containing antiparallel E protein homodimers and cleaved M proteins that lie flat on the lipid envelope (Pierson and Diamond 2012; Fig. 2B).

Structure and function of viral proteins

Both structural and NS proteins, mainly E, prM and NS1, contain immunogenic epitopes that are targeted by neutralising antibodies, which can be exploited for the development of prophylactic antibodies and for the design of vaccines against ZIKV, while the NS proteins NS3 and NS5 are the main targets for the rational design of antiviral drugs.

The E protein, whose structure has been determined (Dai et al. 2016; Kostyuchenko et al. 2016; Sirohi et al. 2016), is made of about 500 amino acids with a transmembrane domain and three ectodomains, i.e. DI, DII and DIII, with DI acting as a bridge between DII and DIII. This protein is responsible for recognition of cellular receptors and entry cofactors and represents the main target for neutralising antibodies. Like in other flaviviruses, the tip of domain DII contains the fusion loop, a hydrophobic sequence that inserts into the host cell endosomal membrane during pH-dependent conformational changes that drive fusion; DII is recognised by cross-reactive antibodies among flaviviruses that have low neutralising efficiency. At variance with this, DIII has an immunoglobulin-like structure and contains the receptor-binding site; it is recognised by protective neutralising antibodies.

The structure of ZIKV E protein is similar to the structure of DENV envelope protein, except for differences in glycosylation sites, i.e. a single glycosylation site (Asn154) and an insertion of five amino acid residues in ZIKV E relative to DENV, which has two glycosylation sites (Asn67 and Asn153) in the E protein (Kostyuchenko et al. 2016; Sirohi et al. 2016). Changes in this region might play a role in virus transmission and neurovirulence, as demonstrated for WNV (Beasley et al. 2005). ZIKV E protein has also a unique positively charged patch adjacent to the fusion loop, which might affect attachment to host cells (Dai et al. 2016).

The prM protein consists of three domains, the pr N-terminal domain, a central ectodomain and a C-terminal transmembrane domain. In order to obtain infectious viral particles, the pr domain is first cleaved by a cellular furin protease and then released, resulting in the formation of fusion-competent virions (Pierson and Diamond 2012). Differences in a set of amino acids determine structural changes between the African and Asian ZIKV lineages, which have been hypothesised to be responsible for the increased pathogenicity observed in the recent outbreaks (Wang et al. 2016).

The C protein is required for viral encapsidation. It exists as a symmetric homodimer and is composed of an RNA binding domain and a hydrophobic domain for dimerisation and interaction with the membrane (Ma et al. 2004).

The NS1 protein of flaviviruses is necessary for viral replication and evasion of the immune response. It is glycosylated, associated with lipids and forms homodimers inside the cell, while it forms hexameric lipoprotein particles when it is secreted into the extracellular space (Akey et al. 2014; Brown et al. 2016). In addition, NS1 functions during virus maturation, including interaction with the viral prM and E proteins (Scaturro et al. 2015). ZIKV NS1 has high structural similarities with NS1 of other flaviviruses, but it displays also diverse electrostatic features in the interface involved in interactions with host factors that might affect pathogenesis and immunogenicity (Brown et al. 2016; Song et al. 2016a).

Little is known about NS2A, which is a small and hydrophobic protein that is part of the replication complex and has a role in modulating host antiviral response. In DENV, it has been shown to interact with the ER through five transmembrane segments, while the N- and C-terminal domains are located within the ER lumen and in the cytosol, respectively (Leung et al. 2008; Xie et al. 2013).

NS2B is another small transmembrane protein; through a central hydrophilic domain, it provides a chaperone-like function for NS3, with which it interacts, while the hydrophobic terminal domains serve as anchors for the NS2B–NS3 complex to the ER membrane (Bollati et al. 2010).

The NS3 protein presents two distinct globular domains connected by a short linker. The N-terminal domain is the viral protease, which is responsible for some of the cleavages that occur to the polyprotein and requires the binding of the small NS2B cofactor in order to be functional. Unlike other flaviviruses, ZIKV protease forms a tight homodimer (Lei et al. 2016). The C-terminal domain is a helicase, which is involved in the unwinding of RNA secondary structures during genome replication (Bollati et al. 2010). In ZIKV, the helicase seems to exist as a monomer, as opposed to DENV helicase that functions in dimeric form (Tian et al. 2016).

Both NS4A and NS4B are hydrophobic transmembrane proteins and are essential components of the flavivirus replication complex. Their physical interaction has been demonstrated in DENV (Zou et al. 2015), together with their ability to modulate ER membrane rearrangements that favour the formation of the replication complex (Nemésio, Palomares-Jerez and Villalain 2012). They also seem to interact with NS3, acting as cofactors (Bollati et al. 2010).

Finally, NS5 is the largest and most conserved flavivirus protein, with an N-terminal methyltransferase (MTase) domain and a C-terminal RNA-dependent RNA polymerase (RdRp). While the latter is responsible for the synthesis of viral RNA, the former carries out the 5'-capping of the genome and its methylation. Recently, the crystal structure of DENV NS5 has demonstrated

that the most likely active form of the protein is a homodimer (Klema et al. 2016).

PATHOGENESIS

Virus life cycle and infection dynamics in humans

Little is known about the life cycle of ZIKV and most of the information is extrapolated from the knowledge of the other closely related flaviviruses. ZIKV is transmitted between humans in the urban cycle through the bite of female *Aedes* spp. mosquitoes. The virus is acquired by the mosquito during a blood meal, and upon replication in the body of the arthropod, it reaches the salivary gland and is injected at the subsequent meal into the skin of the new host (Suthar, Diamond and Gale 2013). The human skin at the site of inoculation thus represents the first site of viral replication, and human primary dermal fibroblasts, epidermal keratinocytes and immature dendritic cells have been demonstrated to be permissive to ZIKV infection and replication (Hamel et al. 2015). From the skin the virus spreads to the draining lymph node, where it is amplified, resulting in viraemia and haematogenous dissemination to peripheral tissues and visceral organs. There are few cell types for which ZIKV tropism has been proven so far. Most of the recent experimental *in vitro* studies focused on the cell types involved in transplacental transmission and neural damage, in order to get clues as to ZIKV pathogenesis.

In humans, ZIKV RNA is detectable in blood typically within the first 10 days after infection (i.e. during the first 3–5 days after the onset of symptoms), with viral load peaks occurring at the onset of symptoms (Lanciotti et al. 2008; Campos, Bandeira and Sardi 2015; Gourinat et al. 2015). In the blood, the virus appears to be cell-associated, since viral load is higher in whole blood than in plasma and serum (Lustig et al. 2016). Viraemia is typically low titre (about 1000–10 000 ZIKV RNA copies/mL); however, high level (up to $\sim 10^7$ – 10^9 copies/mL) and/or prolonged viraemia may occur (Lanciotti et al. 2008; Besnard et al. 2014; Driggers et al. 2016; Meaney-Delman et al. 2016b). During the first weeks post-infection, the virus is excreted at relatively high load in urine, saliva and other bodily fluids (Gourinat et al. 2015; Musso et al. 2015c; Prisant et al. 2016; Barzon et al. 2016a,b), consistent with a systemic infection. Viral shedding in saliva and urine has been reported also for other flaviviruses, e.g. DENV (Hirayama et al. 2012; Andries et al. 2015) and WNV (Barzon et al. 2012; Barzon et al. 2013), while a peculiar feature of ZIKV is the tropism for testicular tissue (Lazear et al. 2016) and excretion in semen (Musso et al. 2015c), even for months after clearance from blood (Nicasri et al. 2016; Barzon et al. 2016b). The mechanisms of infection and the cellular reservoir for ZIKV in the testis are unknown.

Following infection, an antibody- and cell-mediated immune response is induced. Specific IgM antibodies against ZIKV have been estimated to appear at 9.1 (95% CI 7.0–11.6) days after infection (Lesser et al. 2016), i.e. approximately 4–7 days after symptom onset, followed by the appearance of IgG antibodies after 2–3 days. IgM antibodies against flaviviruses are usually detectable for 2–3 months, but may also persist for over 1 year, while IgG antibodies usually remain detectable for months or years and probably confer lifelong protection.

Infection of neural cells

Human induced pluripotent stem cell (iPSC)-derived cells represent useful systems to model viral infections in humans (Trevisan et al. 2015). These cells have been exploited to gener-

ate human neural progenitor cells (hNPCs), neurons and cerebral organoids to study ZIKV neuropathogenesis *in vitro* (Garcez et al. 2016; Qian et al. 2016; Tang et al. 2016). In these studies, both the prototype African lineage ZIKV strain MR766 and contemporary Asian lineage strains were used for experimental infections. These strains efficiently infected and replicated in iPSC-derived hNPCs and, less efficiently, in differentiated immature neurons and in iPSCs (Garcez et al. 2016; Tang et al. 2016). Infection induced a marked cytopathic effect (CPE) and cell apoptosis via caspase-3 activation (Tang et al. 2016). At variance with this, persistent viral replication was reported in primary human fetal neural progenitors associated with limited CPE and without induction of cytokine and chemokine production (Hanners et al. 2016). In a model of forebrain organoids, ZIKV infection led to reduced organoid size and thickness and enlarged lumen, reminiscent of the dilated ventricles observed in microcephalic fetuses (Qian et al. 2016). Among different cells in brain organoid structures, ZIKV preferentially infected NPCs rather than neurons and astrocytes and induced cell apoptosis (Dang et al. 2016; Qian et al. 2016). Similar results were obtained by using the most recent ZIKV Brazilian strain (Cugola et al. 2016).

Infection of placental cells

The association between ZIKV infection and fetal microcephaly has been supported by detection of ZIKV RNA and infectious virus in the placenta, amniotic cavity and brain of fetuses with microcephaly (Calvet et al. 2016; Driggers et al. 2016; Mlakar et al. 2016). Transplacental transmission and teratogenic effects have been less frequently observed with other flaviviruses, such as DENV (Paixão et al. 2016) and WNV (Barzon et al. 2015a), thus suggesting that ZIKV may have unique mechanisms to cross the placental barrier and cause fetal damage.

ZIKV infection of placental cells has been demonstrated *in vitro* and *in vivo*. In particular, ZIKV can infect and replicate in human primary cells isolated from mid- and late-term placenta and in villus cytotrophoblasts from first-trimester placenta (Tabata et al. 2016). The highest infection efficiency was observed in amniotic epithelial cells from mid-gestation placenta, characterised by the highest expression of the entry cofactor TIM-1, while late-stage placental cells were less permissive to ZIKV infection (Tabata et al. 2016). Constitutive release of type III interferon (IFN) $\lambda 1$ by trophoblast cells was suggested to play a role in the protection of placental villous syncytiotrophoblasts from ZIKV infection during the late stages of pregnancy (Bayer et al. 2016). ZIKV can also infect and replicate in macrophages isolated from full-term human placenta with higher efficiency than in placental cytotrophoblast (Quicke et al. 2016). Infection led to induction of an antiviral immune response in infected macrophages, but exerted a limited CPE, thus suggesting that these cells might play a role in virus dissemination (Quicke et al. 2016).

Animal models of pathogenesis

Although *in vitro* and *ex vivo* models are useful to understand the molecular mechanism of virus–host interaction, the use of animal models is pivotal to study the pathogenesis of ZIKV infection within pregnant individuals and fetuses and to perform preclinical studies to evaluate the efficacy of vaccines and antiviral molecules.

Infection of neurons and astrocytes leading to CPE was already observed in early studies in mice infected by intracerebral inoculation with the prototype African ZIKV strain, MR766 (Bell,

Field and Narang 1971). The neurotropism of ZIKV was recently confirmed in adult mouse models infected with both contemporary ZIKV isolates and MR766 (Aliota et al. 2016a; Lazear et al. 2016; Rossi et al. 2016).

After subcutaneous or intravenous infection with contemporary ZIKV strains, transgenic mice deficient in the IFN signalling pathway, i.e. mice lacking the IFN receptor and *Irf3*^{-/-} *Irf5*^{-/-} *Irf7*^{-/-} triple knockout mice that produce little IFN α/β , developed neurological disease and exhibited 100% lethality (Aliota et al. 2016a; Lazear et al. 2016; Rossi et al. 2016). At variance with this, no significant morbidity was observed among wild-type mice and single knockout mice lacking different interferon response genes (Lazear et al. 2016), thus suggesting that ZIKV is inefficient in inhibiting the IFN response in mice. Lower lethality was observed with the MR766 ZIKV and in aged mice (Aliota et al. 2016a; Lazear et al. 2016; Rossi et al. 2016).

In infected wild-type mice, viral RNA was detectable mainly in the spleen, while IFN-deficient mice had high viraemia and organ involvement, including spleen, liver, kidney, testis, brain and spinal cord, with the highest viral load in spleen, brain and testis (Aliota et al. 2016a; Lazear et al. 2016; Dowall et al. 2016; Rossi et al. 2016). Interestingly, high and sustained viral load was detected in brain and testis of surviving IFN-deficient mice at 28 days after infection (Lazear et al. 2016).

To model pregnancy and the impact of ZIKV infection on microcephaly, the virus was injected into the cerebroventricular space/lateral ventricle (LV) of the embryo's brain at embryonic day 13.5, equivalent to the late second trimester of pregnancy in humans when cortical neurogenesis occurs. Infected brains had smaller size and enlarged LV compared with controls, with the NPCs being the most damaged cell population (Li et al. 2016; Wu et al. 2016). Vertical transmission of ZIKV and its effect on brain development were demonstrated by injecting intraperitoneally a contemporary ZIKV strain in pregnant mice at embryonic day 13.5. This resulted in infection of radial glial cells of dorsal ventricular zone of the fetuses, leading to a marked reduction of these cells and a decrease in surface area of the cortex (Wu et al. 2016).

To achieve a sufficient level of viraemia to seed the placenta, an in utero transmission model of ZIKV infection used pregnant mice deficient for type I IFN signalling, previously mated with wild-type mice in order to produce heterozygous embryos, and subcutaneously infected with a lower dose of virus at embryonic days 6.5 and 7.5, which is equivalent to the first trimester of pregnancy in humans (Miner et al. 2016). This study demonstrated that ZIKV infects the placenta and damages the placental barrier leading to fetal infection, placental insufficiency, intrauterine growth restriction and, in severe cases, fetal demise (Miner et al. 2016). In addition, the virus was identified within trophoblast and fetal endothelial cells in the placenta, consistent with a transplacental route of infection (Miner et al. 2016). Concordant results were obtained by using very high doses of virus inoculated intravenously in immunocompetent pregnant mice (Cugola et al. 2016). Altogether, these studies provided the evidence of a causal link between ZIKV infection and microcephaly (Mysorekar and Diamond 2016).

Although representing a suitable tool to investigate infectious diseases, murine models do not perfectly mimic human infection and fetal development. Compared with these, studies on non-human primate are crucial to recapitulate flavivirus pathogenesis in humans and to investigate the efficacy of antiviral vaccines and drugs (Verstrepen et al. 2014a,b). Studies on ZIKV infection in rhesus macaques, including pregnant monkeys, are ongoing and preliminary data have been published

(Aliota et al. 2016b; Dudley et al. 2016). These studies showed that infection in monkeys recapitulated human infection, with decreased total white blood cells following infection, shedding of viral RNA in urine and oral swab, peak viraemia occurring between days 2 and 6 post-infection and detectable up to day 21, and production of serum neutralising antibodies by day 21 post-infection (Dudley et al. 2016). Interestingly, pregnant animals showed persistent viraemia up to 59 days post-infection (Dudley et al. 2016), in agreement with the data on the persistence of ZIKV in human maternal blood during pregnancy (Driggers et al. 2016; Meaney-Delman et al. 2016b). To investigate the protective effect of immune responses upon first infection, non-pregnant animals were subsequently re-challenged after 10 weeks. Notably, all the animals showed complete protection against re-infection both with homologous Asian-lineage (Dudley et al. 2016) and heterologous African-lineage ZIKV (Aliota et al. 2016b).

Host innate immunity and antiviral restriction factors

Another important focus of ZIKV research was the identification of the host factors involved in the restriction of infection and in the development of antiviral immune responses.

Host factors known to facilitate cellular entry of flaviviruses (Meertens et al. 2012), i.e. DC-SGN and phosphatidylserine receptor proteins (TIM-1, TIM-4, AXL and Tyro3), can also enhance ZIKV infection (Hamel et al. 2015). Among these factors, AXL is highly expressed in the developing human cortex throughout the period of neurogenesis and is particularly overexpressed in radial glia, which represent the neural stem cell population of the human fetal cerebral cortex (Li et al. 2016; Nowakowski et al. 2016), while TIM-1 is the predominant ZIKV entry factor expressed in human placental cells (Tabata et al. 2016).

ZIKV infection triggers inflammatory and innate immune responses in infected cells. In primary human skin fibroblasts, ZIKV induces the expression of pattern recognition receptors (PRRs), such as Toll-like receptor 3 (TLR3), retinoic acid inducible gene-1 (RIG-I), melanoma differentiation associated gene 5 (MDA5), and factors involved in downstream pathways and inflammatory antiviral response, such as IFN regulatory factor 7 (IRF7), IFN α , IFN β and C-C motif chemokine ligand 5 (CCL5) (Hamel et al. 2015). Up-regulation of TLR3 was also demonstrated in ZIKV infected cerebral organoids and neurospheres, and TLR3 activation was responsible for cell apoptosis, organoid shrinkage and dysregulation of neurogenesis induced by ZIKV infection (Dang et al. 2016).

Modulation of the host innate immune response may be exploited to treat viral infection. In this regard, treatment with type I and type II IFNs decreased viral replication (Hamel et al. 2015; Frumence et al. 2016), while inhibition of TLR3 by small interfering RNA (siRNA), but not inhibition of other PRRs, resulted in a strong increase of ZIKV replication in skin fibroblasts, thus suggesting a key role for TLR3 in the induction of an antiviral response against ZIKV (Hamel et al. 2015). Small membrane-associated interferon-induced transmembrane proteins (IFITMs), which are important host restriction factors that inhibit replication of a broad range of viruses, have also been shown to inhibit ZIKV infection (Savidis et al. 2016b) and could represent another therapeutic target for strategies aimed at enhancing host innate antiviral immunity.

Flaviviruses have evolved mechanisms to counteract host innate antiviral pathways, such as masking or sequestering viral RNA from recognition by PRRs, suppressing interferon signalling and evasion of IFN antiviral effects through 2'-O-methylation of viral genome (Suthar, Diamond and Gale 2013). These activities

are carried out by NS viral proteins, among which NS5 exerts a potent inhibition of IFN signalling through its methyltransferase activity and by inactivation of the IFN-regulated transcriptional activator signal transducer and activator of transcription 2 (STAT2). The NS5 protein of ZIKV strongly and specifically binds to human STAT2, prevents its translocation to the nucleus and, like DENV but with a different mechanism, induces its degradation via proteasomes (Grant et al. 2016). Notably, this effect is species specific, since ZIKV NS5 has no effect on mouse STAT2 (Grant et al. 2016), and this can explain, at least in part, the resistance of wild-type mice to ZIKV infection at variance with the high susceptibility of IFN-deficient mice (Aliota et al. 2016a; Lazear et al. 2016; Rossi et al. 2016).

Genome-wide screening studies based on siRNA and CRISPR/cas9 technologies have allowed the discovery of novel human genes required for ZIKV infection that could represent pharmacological targets for antiviral drugs (Marceau et al. 2016; Savidis et al. 2016a; Zhang et al. 2016b). These genes include proteins involved in ER functions, such as signal sequence recognition, protein translocation, degradation and N-linked glycosylation (Marceau et al. 2016; Zhang et al. 2016b). In particular, among these genes, SPCS1, which encodes a component of the signal peptidase complex, was demonstrated to be required for selective processing of flavivirus proteins through a specific signal processing pathway (Zhang et al. 2016b). Other antiviral restriction factors identified by genome-wide screening include the STT3A and STT3B components of the oligosaccharyltransferase complex, which was demonstrated to be required for viral RNA replication (Marceau et al. 2016; Savidis et al. 2016a), the entry factor AXL, and proteins involved in endocytosis (RAB5C and RABGEF), heparin sulfation (NDST1 and EXT1) and the endoplasmic reticulum membrane complex (EMC1, EMC2, EMC4, EMC5), involved in trafficking between the ER and the Golgi (Savidis et al. 2016a).

EPIDEMIOLOGY

After the first isolation of the virus from a sentinel rhesus macaque in Uganda in 1947 (Dick et al. 1952), serological surveys in humans and entomological studies in mosquitoes showed that the virus was endemic in areas in central, western, eastern and northern Africa and in southern and south-eastern Asia (Dick 1952; Haddow et al. 2012).

Only a few sporadic cases or small clusters of ZIKV infection and disease in humans were reported until the recent epidemics (Musso and Gubler 2016), in part because of underestimation due to its unspecific clinical presentation that may be misdiagnosed as other more commonly diagnosed arboviral infections, such as dengue and chikungunya.

The first large outbreak of ZIKV infection occurred in Yap, Federated States of Micronesia, in 2007 (Lanciotti et al. 2008; Duffy et al. 2009). This outbreak, which was identified retrospectively, was estimated to have involved over 70% of Yap residents, corresponding to about 5000 individuals (Duffy et al. 2009). A ZIKV strain belonging to the Asian lineage was responsible for the outbreak (Haddow et al. 2012), while an African lineage ZIKV was involved in another outbreak that occurred in the same year in Gabon (Grard et al. 2014).

A large ZIKV epidemic with more than 30 000 suspected cases was identified in late 2013 in French Polynesia (Cao-Lormeau et al. 2014). During this outbreak, for the first time, an increased incidence of Guillain-Barré syndrome and other severe neurological complications, including fetal microcephaly, was observed (Oehler et al. 2014; Besnard et al. 2016). Then, the virus

spread to other islands in the Pacific region, with most human infections recorded in 2014 in New Caledonia and the Cook Islands (Roth et al. 2014; Dupont-Rouzeyrol et al. 2015; Musso 2015; Musso, Cao-Lormeau and Gubler 2015a). In addition, since 2012 clusters of ZIKV infection were identified in Southeastern Asia and, in October 2015, an outbreak started in Cape Verde, caused by the same ZIKV strain that was circulating in the Americas (ECDC 2016d).

In Brazil, the first case of ZIKV infection was notified in May 2015 in an HIV-infected patient from Rio de Janeiro, Brazil (Calvet et al. 2015). However, subsequent reports indicated that the virus was already circulating in the country at least since January–March 2015 (Campos, Bandeira and Sardi 2015; Cardoso et al. 2015; Zammarchi et al. 2015; Zanluca et al. 2015; Brasil et al. 2016a), while phylogenetic and molecular clock analysis of viral genome sequences predicted that the virus was introduced from French Polynesia to Brazil between May and December 2013 (Faria et al. 2016).

Subsequently, ZIKV rapidly spread from Brazil, where about 500 000 to 1 500 000 cases of ZIKV disease were estimated to have occurred from the beginning of the outbreak to early February 2016 (WHO 2016d), to other countries of South America, Central America and the Caribbean, where thousands of cases were also reported, most of which were from Colombia and Venezuela (PAHO 2016a; WHO 2016c). In July 2016, four cases of ZIKV infections probably transmitted by local mosquitoes were reported in the state of Florida, USA (PAHO 2016c).

Several cases of imported ZIKV infection have been recorded in Europe, North America, Asia and Australia, including infections in pregnant women (ECDC 2016b; CDC 2016c). In addition, autochthonous ZIKV infections due to possible sexual transmission have been recorded in the USA and Europe (CDC 2016a,b,c).

TRANSMISSION

Vector-borne transmission

ZIKV is transmitted between humans by *Aedes* species mosquito vectors (mainly the highly anthropophilic *Ae. aegypti* mosquito), with humans representing the amplifying host. A sylvatic cycle of ZIKV transmission between non-human primates and arboreal zoophilic *Aedes* spp. mosquitoes also exists in African and Asian forests, in which humans may be incidentally infected (Hayes 2009). In Africa, the virus has been isolated or detected by PCR in *Ae. africanus*, *Ae. furcifer*, *Ae. vittatus*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. albopictus* and other *Aedes* species mosquitoes (Dick et al. 1952; Diallo et al. 2014; Grard et al. 2014), while in Asia it has been detected in *Ae. aegypti*, which is considered the main ZIKV epidemic vector outside Africa (Marchette et al. 1969). *Aedes hensilli*, the most abundant mosquito species in Yap Island and the competent one for ZIKV transmission (Ledermann et al. 2014), was conceivably the main vector for ZIKV transmission during the 2007 outbreak, even though the virus could not be detected in any mosquito samples (Duffy et al. 2009). *Aedes aegypti* and *Ae. polynesiensis*, the most common mosquito species in French Polynesia, were probably involved in local ZIKV transmission (Cao-Lormeau et al. 2014; Roth et al. 2014), while *Ae. aegypti* and possibly also *Ae. albopictus* are considered the vectors responsible for transmission in Brazil and in other countries in South and Central America, although reports on ZIKV isolation from field mosquitoes are lacking (Gardner, Chen and Sarkar 2016; Guérbois et al. 2016; Wang et al. 2016). Both *Ae. aegypti* and *Ae. albopictus* are competent vectors for ZIKV (Li et al. 2012; Wong et al. 2013; Grard et al. 2014; Chouin-Carneiro et al. 2016), but transmission

from *Ae. albopictus* is less efficient than from *Ae. aegypti* due to a longer extrinsic incubation period (Di Luca et al. 2016).

Non-vector-borne transmission

While the vast majority of ZIKV infections in humans are transmitted through mosquito vectors, other modes of transmission have been demonstrated, including sexual transmission, transplacental and perinatal transmission, blood transfusion, and laboratory acquired infections.

Several cases of sexual transmission of ZIKV infection have been described so far (Foy et al. 2011; D'Ortenzio et al. 2016; Davidson et al. 2016; Deckard et al. 2016) and linked to viral shedding in semen (D'Ortenzio et al. 2016) and vaginal fluids (Prisant et al. 2016). Among vector-borne flaviviruses, sexual transmission is a unique feature of ZIKV. Notably, infectious ZIKV has been recovered in semen up to 24 days after symptom onset (D'Ortenzio et al. 2016), viral RNA has been detected for over 6 months after onset (Nicastri et al. 2016; Barzon et al. 2016b), and cases of sexual transmission occurring weeks after the index case have been described (Turmel et al. 2016).

Perinatal transmission of ZIKV was reported during the outbreak in French Polynesia in 2013 from two mothers who acquired the infection a few days before delivery (Besnard et al. 2014). ZIKV RNA was detected in their breast milk, while no infectious virus was isolated in cell culture (Besnard et al. 2014). At variance with this, infectious ZIKV was isolated from breast milk of another woman with symptoms at the time of delivery (Dupont-Rouzeyrol et al. 2016). Although not demonstrated by these reports, it cannot be excluded that ZIKV may be transmitted through breastfeeding. Although rare, transmission through breast milk has been reported for other vector-borne flaviviruses, i.e. DENV (Barthel et al. 2013), WNV (CDC 2002) and YFV (Kuhn et al. 2011). However, because of the benefits of breastfeeding, the Centers for Disease Control and Prevention (CDC) encourages mothers to breastfeed even in areas of endemic activity of these viruses.

Transplacental transmission of ZIKV in humans has been extensively documented in cases of fetal microcephaly by detection of the virus in the amniotic fluid (Calvet et al. 2016; Oliveira Melo et al. 2016; Schuler-Faccini et al. 2016), in fetal and placental tissues (van der Eijk et al. 2016) and in the brain of microcephalic fetuses (Mlakar et al. 2016; Martinez et al. 2016a,b; WHO 2016d).

Since most ZIKV infections are asymptomatic, there is the risk of transmission through blood and organ donations (Musso et al. 2014; Barjas-Castro et al. 2016; CIDRAP 2016; CDC 2016b). During the outbreak in French Polynesia, 42 of 1505 (3%) asymptomatic blood donors were found to be positive for ZIKV RNA (Musso et al. 2014), while 68 of 12 777 (0.53%) blood donations were reactive for ZIKV RNA in Puerto Rico in April–June 2016 (Kuehnert et al. 2016).

Human-to-human transmission of ZIKV via other substances of human origin has not been demonstrated so far. Infectious ZIKV has been isolated from urine and saliva (Bonaldo et al. 2016; Barzon et al. 2016a; Zhang et al. 2016a), where the virus may be excreted at high titre and for a longer time than it is detectable in blood. A human case of ZIKV infection following a monkey bite was reported, but mosquito-borne transmission could not be excluded (Leung et al. 2015). Shedding in urine and saliva has been demonstrated for other flaviviruses, e.g. DENV (Korhonen et al. 2014; Andries et al. 2015) and WNV (Barzon et al. 2012, 2013), but, so far, urine and saliva have never been implicated in the spread of these viruses.

CLINICAL PRESENTATION AND OUTCOME

In about 80% of cases, ZIKV infection is asymptomatic (Duffy et al. 2009). In patients with symptoms, the disease presents as a febrile illness that may be misdiagnosed as dengue or chikungunya. Symptoms occur after an incubation period of 3–12 days, are usually mild and last for 4–7 days without severe complications (Duffy et al. 2009; Lesser et al. 2016). However, during the recent outbreaks, severe symptoms and sequelae were reported, including Guillain-Barré syndrome and other neurological disorders, haemorrhagic complications and even death.

Zika disease

Among symptomatic patients, the most common symptoms include macular or papular rash (90%), fever, typically low grade (70%), arthralgia (60–70%), fatigue (70%), non-purulent conjunctivitis or conjunctival hyperaemia (55%), myalgia (45%) and headache (45%), while other symptoms, e.g. retro-orbital pain, oedema, vomiting, sore throat, uveitis and lymphadenopathy, are less frequent (Duffy et al. 2009; Campos, Bandeira and Sardi 2015; Cardoso et al. 2015; Furtado et al. 2016; Meltzer et al. 2016; Thomas et al. 2016; Tognarelli et al. 2016; Weitzel and Cortes 2016; Brasil et al. 2016a). A typical feature of ZIKV infection is the maculopapular rash that is often pruriginous and starts on the face and/or trunk and then spreads throughout the body, but may be also focal and fugacious (Brasil et al. 2016b).

In areas of co-circulation of ZIKV, DENV and chikungunya virus (CHIKV), like Brazil, where multiple infections are probably not uncommon (Cardoso et al. 2015; Villamil-Gómez et al. 2016), no increase in the incidence of severe disease has been reported, thus suggesting the absence of synergistic effects (Dupont-Rouzeyrol et al. 2015; Sardi et al. 2016; Zambrano et al. 2016).

Haemorrhagic complications

Laboratory tests are generally in the normal range, including blood cell and platelet counts and liver and kidney function tests. Leucopenia, mild thrombocytopenia and increased transaminases have, however, been described and in some cases associated with severe bleeding disorders requiring hospitalisation (Karimi et al. 2016; Sarmiento-Ospina et al. 2016; Thomas et al. 2016). Mild haemorrhagic symptoms (petechiae, minor mucosal bleeding), but not severe bleeding, were reported in 21% of 119 ZIKV infections that occurred in Rio de Janeiro, Brazil, January–June 2015 (Brasil et al. 2016a). Haematospermia was reported in men with ZIKV infection (Foy et al. 2011; Musso et al. 2015c).

Guillain-Barré syndrome and other neurological complications

The epidemics of ZIKV infection in French Polynesia and South and Central America have been linked with Guillain-Barré syndrome (GBS), a serious autoimmune disease causing acute or subacute flaccid paralysis that is often triggered by infection. The aetiological link between ZIKV infection and GBS was initially suggested by the temporal coincidence between peaks in incidence of the two clinical conditions (Broutet et al. 2016; Cao-Lormeau et al. 2016), while the first documented case of GBS associated with ZIKV infection was reported from French Polynesia in 2013 (Oehler et al. 2014). A case-control study on 42 GBS patients diagnosed between November 2013 and February

2014 during the outbreak in French Polynesia (Cao-Lormeau et al. 2016) demonstrated that all GBS patients had neutralising antibodies against ZIKV compared with 56% in the control group of patients with febrile illness, and most had IgM antibodies against ZIKV. Most patients with GBS had experienced a transient illness in a median of 6 days before the onset of neurological symptoms, suggesting a recent ZIKV infection. Based on a 66% attack rate of ZIKV infection in the general population, the risk of GBS was estimated to be 0.24 per 1000 ZIKV infections (Cao-Lormeau et al. 2016). A typical feature of the GBS was the rapid progression from onset of neurological symptoms to the nadir and the short plateau phase. None of the patients died, but about 50% were still unable to walk without assistance at 3 months after discharge. No association was observed between the occurrence of GBS and a history of DENV infection, thus suggesting that a previous DENV infection probably did not provide cross-protection nor pose a risk of immune enhancement of ZIKV infection (Cao-Lormeau et al. 2016).

Other neurological conditions have been associated with ZIKV infection, as shown in cases of acute meningoencephalitis (Carteaux et al. 2016) and myelitis (Mécharles et al. 2016), in which ZIKV RNA was detected and/or isolated in cell culture from cerebrospinal fluid (CSF).

Microcephaly and other congenital birth defects

The recent outbreaks have also shown an association between ZIKV infection and congenital malformations including microcephaly in newborns (Rasmussen et al. 2016). Also for this condition, the aetiological link was suggested by the temporal coincidence of 20-fold increased incidence of cases of microcephaly in newborns in the Northeast Region of Brazil (Broutet et al. 2016; França et al. 2016; Kleber de Oliveira et al. 2016; Microcephaly Epidemic Research Group 2016; Schuler-Faccini et al. 2016) and in French Polynesia (Besnard et al. 2016; Cauchemez et al. 2016), and the first trimester of pregnancy was identified as the period of major risk for microcephaly (Cauchemez et al. 2016). In addition, the association between ZIKV infection and microcephaly was supported by several case reports and case series of laboratory-confirmed or presumed ZIKV infection during pregnancy (Mlakar et al. 2016; Pacheco et al. 2016; Brasil et al. 2016c).

The risk of transplacental transmission of ZIKV and fetal damage in humans is still unknown: preliminary data from an ongoing prospective study in Rio de Janeiro, Brazil, reported the presence of fetal abnormalities in 12 of 42 (29%) ZIKV-positive pregnant women (Brasil et al. 2016c). In this study, fetal abnormalities were observed in the fetuses of women who were infected at any week of gestation (Brasil et al. 2016c). At variance with this, preliminary data from a longitudinal study carried out in Colombia did not identify any apparent anomaly in 616 infants born from women who were infected during the third trimester (Pacheco et al. 2016). On the other hand, several cases of microcephaly have been diagnosed from woman with asymptomatic ZIKV infection, in whom the gestation period of infection could not be defined (França et al. 2016; Pacheco et al. 2016; Sarno et al. 2016).

The outcome of ZIKV infection during pregnancy ranges from miscarriage to the delivery of apparently healthy babies (França et al. 2016; Meaney-Delman et al. 2016a; Brasil et al. 2016c). A surveillance study in Brazil reported a higher rate of first-week morality, and a smaller head circumference in a series of 602 confirmed or probable cases of congenital ZIKV syndrome than in a control group of newborns, in whom the diagnosis of ZIKV

infection was excluded (França et al. 2016). Malformations reported in fetuses and newborns with congenital ZIKV infection include intrauterine growth restriction, with or without accompanying microcephaly, cerebral and placental calcifications, oligohydramnios or anhydramnios, agenesis of corpus callosum, agyria, hydrocephalus, ventriculomegaly, brain atrophy, hydrops fetalis, arthrogryposis and macular chorioretinitis (Driggers et al. 2016; Mlakar et al. 2016; Oliveira Melo et al. 2016; Sarno et al. 2016; Martines et al. 2016a; Meaney-Delman et al. 2016a; Brasil et al. 2016c). Approximately 30% of children with suspected ZIKV infection in *utero* have evidence of significant retinal and optic nerve abnormalities (de Paula Freitas et al. 2016; Ventura et al. 2016).

In agreement with clinical findings, laboratory tests have demonstrated the presence of ZIKV antigens and nucleic acids in fetal tissues, placenta and amniotic fluid samples of cases of early pregnancy loss or elective termination of pregnancy, and in the brain and placenta of fetuses with microcephaly (Calvet et al. 2016; Driggers et al. 2016; Mlakar et al. 2016; Sarno et al. 2016; van der Eijk et al. 2016; Martines et al. 2016a,b; Meaney-Delman et al. 2016a).

The long-term sequelae of congenital ZIKV infection are unknown and the reported microcephaly cases might represent only the severe end of the spectrum, while mild cognitive or functional disorders might occur in newborns with less severe infection (Leal et al. 2016; Oliveira Melo et al. 2016). In addition, the role of cofactors, e.g. other infections, genetic defects and alcohol use, in ZIKV-associated birth defects is unknown and warrants further research (Rasmussen et al. 2016).

LABORATORY DIAGNOSIS

The diagnosis of ZIKV infection is quite difficult because of the overlap of symptoms with other arboviral diseases and because of the broad cross-reactivity among flaviviruses of the antibodies induced by infection. Thus, laboratory diagnosis of acute ZIKV infection relies on the use of molecular tests for the direct detection of viral nucleic acids in blood and other biological specimens.

Due to the high costs of molecular methods, if capacity for testing is limited, some categories of exposed subjects should receive priority, as recommended by the European Centre for Disease Prevention and Control (ECDC) (ECDC 2016c). These categories include pregnant women with suspicion of congenital malformation of the fetus and those with a history of Zika-like infection during pregnancy. Exposed individuals with Guillain-Barré syndrome or other neurological symptoms are also candidates for testing. For surveillance and vector control purposes, testing should be done in travellers returning from endemic countries to areas where competent *Ae. albopictus* or *Ae. aegypti* mosquito vectors are present, due to the risk of introduction of the virus, as well as in clusters of autochthonous cases presenting with Zika-like symptoms in non-endemic areas but with competent mosquito vectors (Septfons et al. 2016).

Nucleic acid test (NAT) screening of donors is performed in some countries to prevent ZIKV transmission through blood and organ donations (Kuehnert et al. 2016). ZIKV testing has been also recommended as pre-conception screening in men and women exposed to the risk of ZIKV infection, in exposed semen donors and in exposed partners of pregnant women (Oduyebo et al. 2016; Petersen et al. 2016; WHO 2016a).

Case definition

Routine laboratory techniques for the diagnosis of acute ZIKV infection include molecular methods for the detection of ZIKV RNA in blood and urine and enzyme-linked immunosorbent assays (ELISAs) or immunofluorescence assays (IFAs) for the detection of ZIKV IgM and IgG antibodies in serum. Virus neutralisation assays, required to confirm the specificity of antibodies detected by ELISA or IFA, and virus isolation in cell cultures are performed only in specialised reference laboratories.

According to the ECDC, a case of ZIKV infection is defined as confirmed in the presence of at least one of the following laboratory criteria: detection of ZIKV RNA or antigens in a clinical specimen; viral isolation from a clinical specimen; detection of ZIKV-specific IgM antibodies in serum samples and confirmation by neutralisation test; seroconversion or four-fold increase in the titre of ZIKV-specific antibodies in paired serum samples. A case is defined as probable if ZIKV-specific IgM antibodies are detected in serum but further confirmatory tests are not available (ECDC 2016a).

Nucleic acid testing

During the acute phase of infection, diagnosis relies on the detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) in blood, urine and saliva specimens. In this phase, molecular methods are preferred with respect to the detection of antibodies because of their high specificity and sensitivity (Waggoner and Pinsky 2016). Several *in house* real-time RT-PCR assays have been developed that target conserved regions in ZIKV genome (Fig. 2A; Faye et al. 2008, 2013; Lanciotti et al. 2008; Pyke et al. 2014; Tappe et al. 2014; Corman et al. 2016), including a multiplex test for the simultaneous detection of ZIKV, DENV and CHIKV RNA (Waggoner et al. 2016), with good sensitivity and specificity for contemporary ZIKV strains of the Asian lineage (Lanciotti et al. 2008; Corman et al. 2016; Waggoner and Pinsky 2016). Commercial real-time RT-PCR assays, suitable for full automation and high-throughput platforms, have been recently released onto the market, but data on their analytical and clinical performance are not available yet in the literature. Detection of ZIKV RNA can be performed also by *in house* pan-flavivirus RT-PCR methods, which broadly amplify genomic sequences of flaviviruses, followed by sequencing for virus identification (Scaramozzino et al. 2001). This analysis can be used as a confirmatory test for possible ZIKV infection, but also as a screening test for flavivirus infection (Barzon et al. 2015b).

The sensitivity of molecular tests is highest during the first week after symptom onset, when patients are still viraemic (Bingham et al. 2016), even though viral RNA can be detected for longer times in blood and especially in saliva and urine (Musso et al. 2015b; Bingham et al. 2016; Lustig et al. 2016; Barzon et al. 2016a, 2016b). Testing whole blood has a higher sensitivity than testing plasma and serum, and positive ZIKV RNA results have been reported in whole blood even after 2 months from the onset of symptoms (Lustig et al. 2016).

Molecular methods can be applied to detect ZIKV RNA in a variety of biological specimens, such as amniotic fluid, CSF, pharyngeal swab, semen and placenta, or in biopsies of tissues collected *post mortem*. However, these analyses should be carried on in specialised laboratories.

Next generation sequencing has been also used for the detection and full genome sequencing of ZIKV in clinical samples, such as blood, amniotic fluid, fetal brain tissue and placenta (Calvet et al. 2016; Faria et al. 2016; Mlakar et al. 2016; Sardi et al.

2016; van der Eijk et al. 2016). While still performed by experienced reference laboratories mainly for research purposes, with the technical advances and reduction of costs brought by the new sequencing platforms, next generation sequencing is expected to become in the future a routine technology in clinical laboratories for the detection and discovery of infectious agents (Lavezzo et al. 2016).

Sequencing of the full or partial viral genome is useful not only for pathogen identification and characterisation, but also for epidemiological studies and for the design of improved molecular tests based on updated information on viral diversity. Since the ZIKV genome is relatively small, sequencing can be easily performed also by Sanger sequencing of overlapping amplicons.

Serological testing

In house and commercial ELISA, IFA and immunoblot assays have been developed for detection of ZIKV IgM and IgG antibodies in serum (Rabe et al. 2016). Detection of ZIKV IgM antibodies can be performed also in CSF for the diagnosis of neuroinvasive disease and microcephaly. In this regard, ZIKV IgM antibodies were present in the CSF of 30/31 neonates with microcephaly tested within 40 days after birth (Cordeiro et al. 2016).

A problem with ZIKV immunoassays is the extensive cross-reactivity of IgM and IgG antibodies with heterologous flaviviruses, especially DENV, induced by a previous infection or vaccination (Lanciotti et al. 2008; Rabe et al. 2016). Immunoassays based on ZIKV NS1 antigen for the detection of ZIKV-specific IgM and IgG antibodies have shown less cross-reactivity than tests based on the E protein or the whole virus, probably because of the structural diversity of ZIKV NS1 from the protein of other flaviviruses (Huzly et al. 2016). Anyway, a positive result from ELISA and IFA should be confirmed by a neutralisation assay, which is the most specific test for flavivirus serology (Rabe et al. 2016).

Another problem with flavivirus serology is the so-called 'original antigenic sin' phenomenon, which is typically observed during secondary flavivirus infection after a previous infection or vaccination with a heterologous flavivirus, such as DENV, YFV and JEV (Lanciotti et al. 2008). In the early phases of a secondary flavivirus infection, IgM and IgG antibodies and neutralising antibodies against the original flavivirus are mostly induced, while IgM antibodies against the infecting flavivirus may remain undetectable (Lanciotti et al. 2008; Rabe et al. 2016). For example, a patient with acute ZIKV infection and a previous dengue may have high levels of serum DENV IgM and/or IgG antibodies during the first days after symptom onset, before the appearance of ZIKV-specific antibodies (Lanciotti et al. 2008). In this case, a neutralisation assay may be useful to demonstrate an increase of specific antibodies in paired serum samples taken during the acute and convalescent phase (Lanciotti et al. 2008). However, in patients with secondary flavivirus infection, even a neutralisation assay may not be conclusive because broad neutralising antibodies against multiple flaviviruses are also induced (Lanciotti et al. 2008; Rabe et al. 2016).

Neutralisation tests are performed with infectious virus in reference laboratories with experience in flavivirus diagnosis. Neutralisation can be carried out as a standard plaque reduction neutralisation test (PRNT) or as a microneutralisation assay. Because partial cross-neutralisation may occur among flaviviruses, neutralisation tests should include DENV and other related flaviviruses, besides ZIKV (Rabe et al. 2016). A neutralising antibody titre ≥ 10 PRNT₉₀ (i.e. the reciprocal of the serum dilution reducing the number of plaques >90%) against ZIKV in a serum

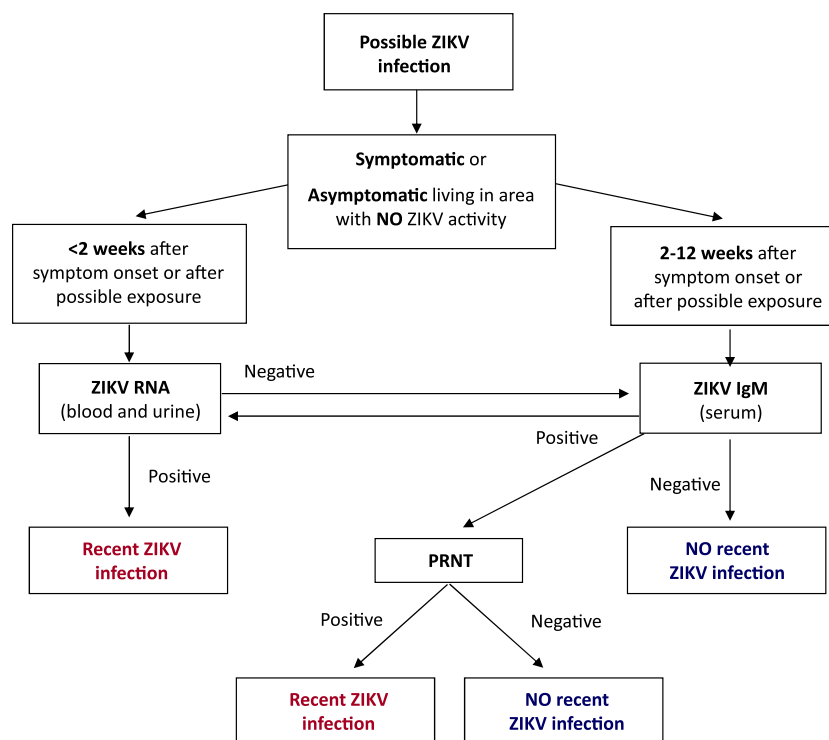


Figure 3. Algorithm for the laboratory diagnosis of Zika virus infection.

sample collected >7 days after illness onset or exposure, together with negative PRNT90 (i.e. <10) against other flaviviruses, is considered specific for ZIKV infection (Rabe et al. 2016).

Based on these considerations and on current guidelines (Oduyebo et al. 2016), an algorithm for the laboratory diagnosis of ZIKV infection is proposed in Fig. 3.

Virus isolation in cell culture

ZIKV is a Biosafety Level 2 pathogen in the EU (with the exception of the UK where the virus is a risk group 3 pathogen), USA and Canada (ECDC 2016c). Isolation of ZIKV in cell culture is not performed for routine diagnosis because it is a poorly sensitive and time-consuming test. Thus, viral isolation is done mainly for research purposes in reference laboratories. The virus can be isolated and grown in several cell lines, including African green monkey Vero cells, Rhesus monkey LLC-MK2 kidney cells, BHK-21 cells and *Ae. albopictus* C6/36 cells. A cytopathic effect, characterised by foci of cells rounding and detaching from the monolayer, can be observed generally 4–5 days after sample inoculation. So far, ZIKV has been successfully isolated from human serum, urine (Bonaldo et al. 2016; Zhang et al. 2016a), saliva (Bonaldo et al. 2016; Barzon et al. 2016a), semen (Musso et al. 2015c; D'Ortenzio et al. 2016), CSF (Carteaux et al. 2016), amniotic fluid (van der Eijk et al. 2016), and breast milk (Dupont-Rouzeyrol et al. 2016). Isolation of the virus in cell culture indicates potential infectivity of biological samples.

Rapid tests

Development of instrument-free rapid point-of-care tests is crucial for diagnosis and surveillance of arbovirus diseases, since these infections typically occur in resource-poor settings where infrastructures, expensive equipment and trained personnel are

not available. Rapid tests have been recently developed and implemented as prototype point-of-care tests. In particular, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay allowed detection of ZIKV RNA in saliva samples with high sensitivity in less than 40 min (Song et al. 2016b). Another rapid test exploited synthetic biology technologies to generate a paper-based switch RNA biosensor coupled with a CRISPR/Cas9 module to detect and discriminate ZIKV genotypes with single-base resolution (Pardee et al. 2016).

VACCINES AND ANTIVIRAL MOLECULES

Vaccines

Currently, there are no approved vaccines against ZIKV. However, several academic institutions and industries are intensively working on the development of vaccines, and a phase I trial in humans has already started (Cohen 2016; Martins, Dye and Bavari 2016; Morrison 2016). Platforms exploited for ZIKV vaccine development include inactivated viral particles, virus-like particle nucleic acid-based vaccines (DNA and RNA), live vectored vaccines (e.g. vectors based on recombinant adenovirus, alphavirus, vaccinia virus, measles), subunit protein vaccines (e.g. E ecto-domain; NS1) and recombinant live vaccines (e.g. YF17D chimeric virus) (Morrison 2016; Tripp and Ross 2016). Immunogenicity and efficacy data have been reported from mouse and monkey studies that evaluated three different vaccine types: a DNA vaccine based on full-length ZIKV prM-E, a purified inactivated ZIKV vaccine, and a vaccine based on a recombinant rhesus adenovirus serotype 52 vector expressing ZIKV prM-E (Abbink et al. 2016; Larocca et al. 2016). All these vaccines induced neutralising antibodies and conferred sterilising immunity and complete protection against a challenge with different strains of contemporary ZIKV. Moreover, in vaccinated animals, the titre of neutralising antibodies

correlated with the degree of protection; passive transfer of IgG purified from vaccinated animals conferred protection to recipient mice; and protection was maintained after depletion of CD4⁺ and CD8⁺ T lymphocytes, thus indicating the T cell-mediated immunity was not required for vaccine efficacy (Abbink et al. 2016; Larocca et al. 2016). Finally, the monkey study showed that among the three vaccines, a single shot of the adenovirus vector-based vaccine induced the best immune response (Abbink et al. 2016).

However, before generalising from these promising results to humans, important problems of flavivirus immunity should be addressed. The first problem is represented by the enhancing effect that cross-reactive antibodies elicited by previous infection or vaccination with a DENV serotype may have on subsequent infection by a different DENV serotype, through the mechanism of antibody-dependent enhancement (ADE; Guzman, Alvarez and Halstead 2013). This phenomenon is mediated by cross-reactive, low-level and/or poorly neutralising antibodies that bind the virus and target the immune complex to the Fc-receptor expressing myeloid cells, thus facilitating infection (Guzman, Alvarez and Halstead 2013).

The risk of ADE is not remote for ZIKV, even if a single serotype exists for this virus (Dowd et al. 2016). In fact, plasma immune to DENV and human monoclonal antibodies specific for DENV envelope, especially those binding to epitopes in the fusion loop, have been shown to cross-react with ZIKV and to drive ADE of ZIKV infection *in vitro* (Dejnirattisai et al. 2016; Priyamvada et al. 2016). On the other hand, poorly neutralising antibodies against domains I and II of ZIKV E protein can enhance DENV infection by ADE (Stettler et al. 2016; Zhao et al. 2016). This is explained by the antigenic similarity among these viruses: the different DENV serotypes differ by 30–35% from each other in amino acid sequence of the E protein, while the DENV serocomplex differs from ZIKV by 41–46%.

A second problem with the development of ZIKV vaccines is linked to the ‘original antigenic sin’ phenomenon, which has been observed in patients exposed to multiple DENV serotypes, but also in patients with ZIKV infection secondary to DENV or other flavivirus infections or vaccinations, who develop higher neutralising antibody responses to the initial infecting virus/vaccine than to the subsequent infecting virus (Lanciotti et al. 2008). Therefore, theoretically, a pre-existing immunity for another flavivirus might interfere with subsequent ZIKV vaccination.

Monoclonal antibodies

Monoclonal antibodies that can neutralise ZIKV could be used to prevent or treat ZIKV infection. Recent studies have characterised neutralising epitopes in ZIKV and have isolated monoclonal antibodies that potentially neutralise ZIKV and protect mouse models from lethal ZIKV infection (Barba-Spaeth et al. 2016; Stettler et al. 2016; Swanstrom et al. 2016; Zhao et al. 2016). These neutralising antibodies target epitopes mapped in the DIII domain of ZIKV E and quaternary epitopes displayed on infectious virions (Stettler et al. 2016; Zhao et al. 2016). Although sera from DENV patients generally have poor neutralising activity against ZIKV (Swanstrom et al. 2016), a category of monoclonal antibodies isolated from DENV patients that target quaternary epitopes at the interface between two subunits of the E protein dimer could potentially cross-neutralise ZIKV (Barba-Spaeth et al. 2016; Swanstrom et al. 2016) and be protective in lethal murine models. These monoclonal antibodies could be used to prevent or treat ZIKV infection in at-risk categories, such as

exposed pregnant women, immunocompromised patients, or patients with severe Zika disease.

Antiviral drugs

Specific antiviral drugs are not available for use in humans to treat ZIKV infection nor for other members of the *Flavivirus* genus, and therapy for patients with ZIKV infection is only supportive. Different strategies have been pursued to discover broad spectrum antiviral drugs by targeting flavivirus proteins, host proteins required for viral replication, or host factors involved in antiviral innate immune response, and some candidates have been identified (Makhluf, Kim and Shresta 2016). In the development of antiviral agents against ZIKV, a key issue to be taken into consideration is safety and lack of teratogenicity, since the target patients for treatment are mainly pregnant women.

A recent study identified inhibitors of ZIKV infection by screening among 774 drugs already approved by the Food and Drug Administration (Barrows et al. 2016). The identified drugs included compounds known to be active against flaviviruses (e.g. bortezomid, ivermectin, cyclosporin A and mycophenolic acid) and other molecules, like daptomycin, sertraline-HCl and pyrimethamine, with unexpected antiviral activity (Barrows et al. 2016). These compounds were demonstrated to reduce ZIKV infection in different cell lines *in vitro* (Barrows et al. 2016). Other studies evaluated the anti-ZIKV activity of nucleoside analogues as inhibitors of viral polymerase and demonstrated that 2'-C-methylated nucleosides reduced ZIKV titre in infected Vero cells, without significant cytotoxicity (Eyer et al. 2016; Zmurko et al. 2016). In particular, 7-deaza-2'-C-methyladenosine was shown to reduce viraemia and to delay ZIKV-induced morbidity and mortality in an experimental mouse model (Zmurko et al. 2016).

SUMMARY AND CONCLUSIONS

After the declaration of the WHO that ZIKV is a Public Health Emergency of International Concern, scientific research has rapidly produced in a few months a huge amount of data on this virus that was almost unknown to the scientific community before. Although relevant knowledge gaps remain and warrant further research, new information has been obtained on the biology, pathogenesis, epidemiology, transmission modalities and clinical features of ZIKV infection. By exploiting previous experience and research achievements with other flaviviruses, such as DENV and WNV, this knowledge has led to the design and development of promising candidate vaccines and to the search for antiviral molecules. In addition, programmes for the surveillance, diagnosis and control of ZIKV infection in humans and the management of patients have been implemented and continuously updated as new data and information were obtained.

Conflict of interest. None declared.

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