

ZIKA VIRUS: WHAT HAVE WE LEARNT SINCE THE START OF THE RECENT EPIDEMIC?

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ZIKA VIRUS: WHAT HAVE WE LEARNT SINCE THE START OF THE RECENT EPIDEMIC?

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Image: Rubén Bueno Marí.

The considerable number of viral infectious disease threats that have emerged since the beginning of the 21st century have shown the need to dispose global and coordinated responses to fight properly and efficiently against them. Severe acute respiratory syndrome (2003), avian influenza in humans (2005), A(H1N1) pandemic influenza (2009), Middle East respiratory syndrome coronavirus (MERS-CoV) (2012 onward) and Ebola virus disease (2014-2015) are some of the most important examples. The latest emerging and devastating threat was Zika virus, an arbovirus that provoked more than 500,000 suspicious cases in the Americas in 2016 and notable processes of social and medical alarms due to the evidence of a causal link between Zika virus and several congenital injuries, like microcephaly, as well as due to its association with neurological disorders such as Guillain-Barré syndrome in adults (PAHO, 2017). In the framework of this global response and multistrategic approach, the purpose of this Research Topic is to provide updated information and novel researches about control strategies, encompassing virological, entomological and epidemiological data, in order to reach the triad of protagonists of transmission cycles (virus, mosquitoes and humans).

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Editorial: Zika Virus Research

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Editorial on the Research Topic

Zika Virus Research

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The considerable number of viral infectious disease threats that have emerged, since the beginning of the twenty-first century has shown the need to dispose global and coordinated responses to fight properly and efficiently against them. Severe acute respiratory syndrome (2003), avian influenza in humans (2005), A(H1N1) pandemic influenza (2009), Middle East respiratory syndrome coronavirus (MERS-CoV) (2012 onward), and Ebola virus disease (2014–2015) are some of the most important examples (1). The latest emerging and devastating threat was Zika virus, an arbovirus that provoked more than 580,000 autochthonous suspected disease cases in the Americas between 2015 and 2018 (2). Most notably, Zika caused social and medical alarm due to the evidence of a causal link between Zika virus and several congenital injuries, like microcephaly, as well as due to its association with neurological disorders, such as Guillain-Barré syndrome in adults (3). In the framework of this global response and multistrategic approach, the purpose of this research topic was to provide a platform for the publication of updated information and high-quality research papers about control strategies, encompassing virological, entomological, and epidemiological data, in order to reach the triad of protagonists of transmission cycles (virus, mosquitoes, and humans). We received 30 manuscripts, of which 23 were accepted for publication after rigorous peer review processes between March 22, 2016 and October 6, 2017.

Baraka and Kweka and Vythilingam et al. highlight the need to focus the Zika problem not only in the Americas but also to pay attention to what could happen in Sub-Saharan Africa (original region of the virus) and Southeast Asia (where the two most important vectors are well distributed). Recently, some of these concerns have become reality, especially in Southeast Asia where several countries have reported autochthonous cases in the past months (4).

As occurs with the rest of arthropod-borne diseases, detailed knowledge of the biology, behavior, and genetic plasticity of vectors are essential to predict potential outbreaks. Muñoz et al. provided an interesting research regarding the predictability of the conditions conducive to Zika epidemics based on a reproduction number model of the two most important disease vectors, namely *Aedes aegypti* and *Aedes albopictus*, that can be concurrent in disease risk areas. According to this study, “conditions for the occurrence of the Zika epidemic at the beginning of 2015 could have been successfully predicted at least 1 month in advance for several Zika hotspots, and in particular for Northeast Brazil: the heart

of the epidemic.” Similarly, Nejati et al. used several climatic and topographic data to model and forecast which areas may be most prone to the establishment of *Ae. albopictus* in Iran. This interesting investigation is supposed to be the first study in the country to determine the regional probability for the establishment of this invasive mosquito of major concern for public health. Zika virus–vector interactions were explored by Anglero-Rodriguez et al. who found several differences in the tropism of Zika virus in two different *Ae. aegypti* strains and compared how Zika and dengue viruses affect the transcriptome of the vector. Moreover, Hunter also emphasizes the importance of not limiting Zika problems to *Ae. aegypti* presence, since too much information is available in relation to other susceptible mosquitoes, although it is clear that more infectivity studies are needed.

Prevention and control strategies have also been deeply discussed in the topic. One of the most applied examples has been given by Millet et al. that exposed the epidemiological and entomological surveillance program against imported Zika cases in the city of Barcelona (Spain). Rather et al. also reviewed the results of preventive campaigns conducted in different parts of the world, which were based on different approaches like, for instance, avoiding unnecessary travel to infected areas and mosquito bites. They argue that these and other proactive measures could “be employed to effectively combat the epidemic transmission of the Zika virus.”

Definitely, congenital abnormalities have triggered an increasing interest in Zika virus by medical, social, and economic reasons. Rather et al. provided an interesting and deep review about the scientific evidence supporting a causal relationship between ZIKV infection during pregnancy and congenital abnormalities. Coming closer to specific congenital and neurological problems, Yuan et al. commented a very interesting point of view regarding the teratogenic effects of Zika virus and the role of the placenta as natural barrier against the infection (5). Additionally, highlighted findings of Zika virus in cerebrospinal fluid samples from adults with neurological symptoms have been reported by Acevedo et al. in Ecuador.

Different virological aspects have also been well analyzed in the topic. Valadão et al. reviewed the mechanisms by which RNA viruses are recognized by host cells, triggering intracellular stress pathways and inflammatory responses, and how *Flaviviridae* members developed several strategies to counteract these cellular

mechanisms. Furthermore, Giri et al. studied the prevalence of intrinsic disorder in Zika virus proteome (African strain MR 766). Their results revealed abundant intrinsically disordered protein regions (IDPRs) in Zika virus polyprotein, capsid protein, and several non-structural proteins, which could play a role in the pathogenesis of the disease. In an interesting study of the mutational preference direction of Zika virus, Khrustalev et al. contributed with promising and useful data that may have further implications for vaccine development and for the understanding of the evolution of new Zika virus strains.

Short discussions by way of articles commentaries could not be missed in the topic. Craig et al. and Saiz et al. crossed interesting points of view regarding the review article by Saiz et al.

Finally, some of the most relevant sources of knowledge of the Zika Virus research topic reside in several excellent reviews. Munjal et al. gives a great overview of different advances in the development of therapies against Zika virus, while Shukla et al. focus their review on rapid detection strategies of the virus in order to have quick response mechanisms to fight efficiently against the disease and to prevent reaching epidemic levels. Wider approach has been given by Shankar et al., encompassing additional aspects related to virus genome, clinical manifestations, diagnosis, and vaccine development. The four classical steps of epidemic management (transmission, detection, control, and prevention) are also well addressed by Sharma and Lal. Mini reviews about Zika virus emergence at American and worldwide level have been also interestingly written by Fajardo et al. and Rather et al., respectively. With all this information, Saiz et al. elaborated a very pertinent compilation of data and information to arrange all that has been learned at virological, entomological, clinical, and epidemiological level, since the start of Zika epidemic in late 2015.

We want to thank all the authors and reviewers for their valuable contributions to this research topic, and we hope that this collection of reviews, commentaries, and original articles will be helpful for clinicians, researchers, and students seeking for information about Zika virus.

AUTHOR CONTRIBUTIONS

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The Threat of Zika Virus in Sub-Saharan Africa – The Need to Remain Vigilant

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BACKGROUND

News of the recent outbreak of Zika virus (ZIKV) disease in South America, North America, and Europe has generated great interest in the scientific community and general public like (1, 2). The disease is caused by the Zika virus (ZIKV), a mosquito-borne *Flavivirus* transmitted mainly by *Aedes aegypti* and *Aedes albopictus*, mosquitoes that also transmit other viral infections, including dengue virus (DENV), chikungunya virus (CHIKV), and yellow fever virus (YFV) (2). Zika virus was isolated for the first time in Rhesus monkey in 1947 in Zika forest in Uganda and since then, evidence of seroprevalence of ZIKV infection in human has been documented in several African countries (3–5). However, to date, the virus has not been considered a serious threat in the region. Symptoms of Zika virus disease are very similar to those of dengue and chikungunya and include fever, rash, joint pain, or conjunctivitis (6). Furthermore, most of the infections remain asymptomatic; thus, majority of the cases are either misdiagnosed or not detected at all. Worryingly, the recent pandemic in South America has associated ZIKV infection with microcephaly, a condition that results in small heads and underdeveloped brains in infants and neurological complication (Guillain–Barré syndrome); yet, no specific treatment or vaccine for the disease exists (1, 7). The incidences of ZIKV infections are escalating at alarming rates in South, North America, and in Europe and potentially threatening countries in sub-Saharan Africa if migration might play a role in both directions. Increasing urbanization, poor urban planning, changes in climatic factors, and the availability of favorable microecological condition suitable for *Aedes* mosquitoes breeding in sub-Saharan Africa are among factors that escalate mosquito abundance. In the face of such potential threat, there is a need for vigilance and establishment of preparedness measures before a Zika pandemic hits the continent. Such a pandemic would pose overwhelming cost burdens to the health systems and potentially compromise the achievement of the sustainable development goals (SDGs). In this letter, we wish to highlight measures that we believe would be effective in setting up countries' preparedness response and surveillance systems to address the potential Zika virus disease threat in sub-Saharan context.

First, there is need for capacity strengthening with focus on the laboratory facilities and human resources to be able to implement epidemiological surveillance and disease control carry out accurate diagnosis and offer quality case management during outbreaks. The establishment of guidelines for all these aspects of disease management would need to be developed. There is need to support the setting up public health laboratories and strengthening of the existing ones to be able to conduct epidemiological surveillance and sophisticated molecular diagnosis that relies on polymerase chain reaction (PCR) or real-time PCR (RT-PCR) based assays. Currently, there is no ZIKV rapid diagnostic test available at the point of care. Therefore, it is important that health-care professionals

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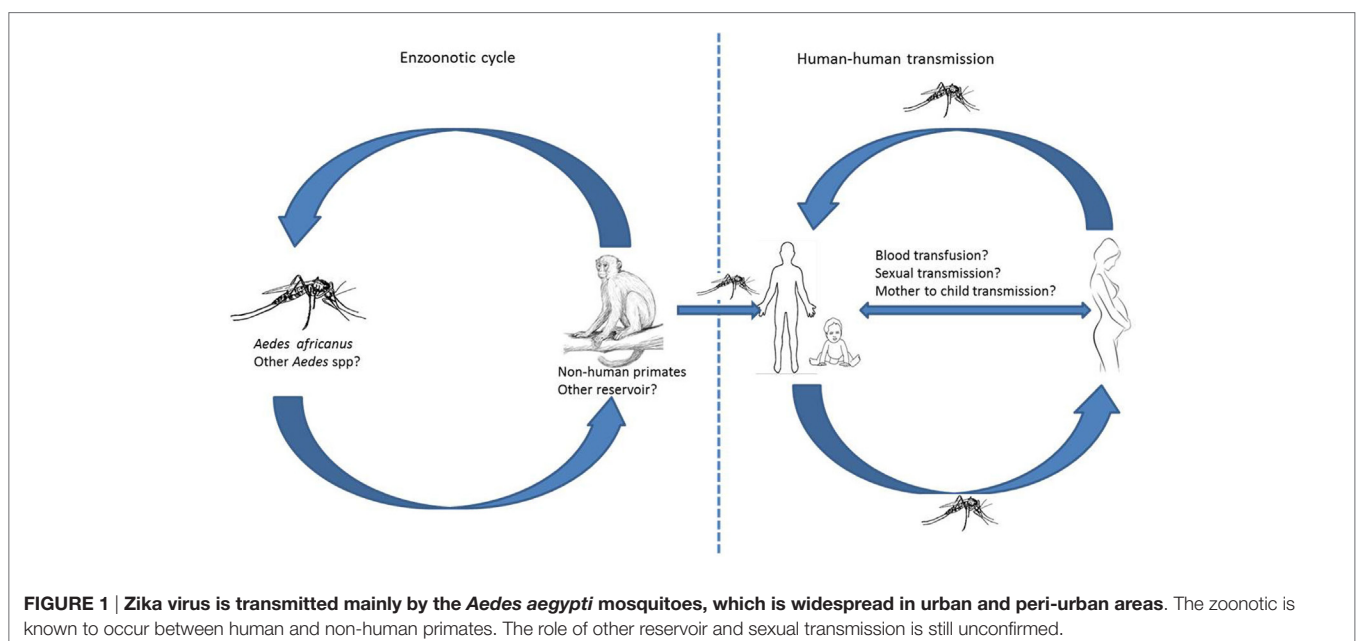
are trained on case diagnosis and management approaches. This has to be in parallel with the improved capacity of laboratories to exclude other severe conditions, such as malaria and bacterial infection. Regional and cross-border networks, such as the East Africa Public Health Laboratory Networking (EAPHLNP), should be strengthened to fill the gaps and the model emulated in other African countries. In this era of increased mobility between countries, the need for regional coordination in sharing of virologic/serotype and vector surveillance data should be underscored. Additionally, there is a shortage of entomologists at regional and district levels to provide technical support in mosquito vector identification and dynamics, which is critical for vector surveillance and control. This shortage of qualified entomologists with technical field expertise is well documented (8). We therefore suggest that serious consideration be given to the training of entomologists so as to fill this gap.

Second, there is a need to raise community awareness and to educate the public on measures that they can put in place to avoid mosquito bites and reduce mosquito breeding habitats. *A. aegypti* are container breeders and integrated approaches that require close community engagement are necessary for their effective control. Community awareness and education will contribute toward the adjustment of risky behavior, such as the failure to cover water storage containers that are in use and improper disposal of old water containers and used car tires. The media should be granted the opportunity by governments to take the lead in supporting community awareness and education efforts on all matters related to epidemiology and control of Zika virus disease more than is currently happening (2).

Third, the role of global travel in the emergence and re-emergence of disease diseases cannot go unnoticed. There is already risk for transmission of Zika virus disease to sub-Saharan African countries in Cape Verde and other regions (3, 9). Expanding

global travel and the shipping industry contribute significantly in the transportation of asymptomatic individuals (10). Strong incentives are needed for surveillance to prevent re-infestation of the Zika virus in sub-Saharan Africa. Attention is particularly needed in the main entry points such as the airports and seaports that are the main gateway from the infected areas. There is need for ministries of health and respective authorities to issue travel alerts and guidance to those visiting to ZIKV-risk countries. For example, pregnant women in any trimester should be advised to highly consider postponing travel and individuals who must visit such countries provided with guidelines and recommendations on the symptoms to look out for and immediately report to the health-care professional on the onset of such symptoms. Also, guidelines and recommendations on personal protection measures to avoid human–mosquito contact, such as the use of repellants and wearing long sleeve clothes, should be emphasized.

Fourth, several key research issues need to be addressed. It will be important to evaluate the role of potential non-human primates in maintaining transmission and/or serving as ZIKV reservoirs. Other proposed routes of transmission, including sexual and maternal (Figure 1), also need further investigation as this will have implications for the epidemiology of the disease. In addition, given that there are no preventative or therapeutic vaccines and point of care diagnostic tests for Zika virus disease, it is important that financial resources to accelerate the discovery and clinical testing of these tools be urgently set aside and made available. With regard to vector control, strategies relevant to the local context are urgently needed to support effective preventive and control measures. The increasing role of climatic factors in relation to *Aedes* mosquito dynamics also needs further exploration as the changes in global temperatures and weather patterns could impact the transmission and spread of the virus. Possible consequences of coinfections between dengue serotypes



(DENV 1–4) and ZIKV virus or coinfection between ZIKV and other prevalent infections in the continent, such as malaria and HIV, also need to be understood. Furthermore, the implication of different ZIKV serotypes in vulnerable groups' particularly pregnant women and children are yet to be understood. With Africa increasingly opening up to the rest of the world due to human migration associated with tourism and business, it is imperative for countries to remain vigilant regarding the threat of the expanding arboviral infections.

The re-emergence and spread of arboviral infections could lead to devastating consequences on the human population, the health-care system and economic progress in the continent. It is

crucial that countries establish harmonized and robust vector control and surveillance systems, which will include the setting up of regional preparedness plans in response to mosquito-borne viruses and investing in capacity building as well as creating community awareness. Investing in research in the development and validation of tools and strategies for the control of the viruses and in understanding of their epidemiology will also be critical.

AUTHOR CONTRIBUTIONS

Both authors, VB and EK contributed equally to the drafting and approved the final manuscript for submission.

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New Paradigms for Virus Detection, Surveillance and Control of Zika Virus Vectors in the Settings of Southeast Asia

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Zika virus (ZIKV) has now become a global public health concern. The vectors for ZIKV are *Aedes aegypti* and *A. albopictus*. Both these mosquitoes are predominant in Southeast Asia and are also responsible for the spread of other arboviral diseases like dengue virus and chikungunya virus. The incidence of dengue has been increasing over the years and this is of concern to public health workers. Simple laboratory tools for the detection of ZIKV is also lacking. In the absence of drugs and vaccine for these arboviral diseases, vector control is the main option for surveillance and control. *Aedes* larval surveys have been the hallmark of dengue control along with larviciding and fogging when cases are reported. However, we need new paradigms and options for control of these vectors. The current situation in Southeast Asia clearly proves that effective strategies for vector control need to be proactive and not reactive. This will be the way forward to control epidemics of these diseases inclusive of ZIKV until a vaccine becomes available.

Keywords: Zika virus, vectors, diagnostic tools, new paradigms, control

INTRODUCTION

Zika virus (ZIKV) which was first discovered from the Rhesus monkey in the Zika forest of Uganda in 1947 (Dick et al., 1952) has now become a global public health concern (Fauci and Morens, 2016; Focosi et al., 2016). ZIKV is a flavivirus and is maintained in a sylvatic cycle which involves non-human primates and the *Aedes* mosquitoes as vectors (*Aedes africanus*, *A. aegypti*) (Haddow et al., 1964; Marchette et al., 1969). ZIKV was only known to cause infection in Africa and Southeast Asia (Haddow et al., 2012). However, in 2007 for the first time ZIKV was reported outside of Africa and Southeast Asia in Yap Island (Hayes, 2009). In Yap Island out of the 185 suspected cases 49 of them were confirmed to be ZIKV and majority of the cases occurred in the older age group (50–55 years) (Duffy et al., 2009). However, during that outbreak there was no death or haemorrhagic complications and the patients only suffered from symptoms like rash, fever, arthritis or arthralgia, conjunctivitis, myalgia, headache, retro-orbital pain, edema, and vomiting (Duffy et al., 2009).

In recent years 2012–2014 there were outbreaks of ZIKV in the Pacific Islands namely Cook Island, Easter Island, French Polynesia, and New Caledonia (Cao-Lormeau and Musso, 2014; Roth et al., 2014). In some of the Pacific Islands especially in 2014 all three viruses ZIKV, chikungunya virus (CHIKV), and dengue virus (DENV) were circulating (Cao-Lormeau and Musso, 2014).

These viruses showed a gradual spread over the years starting in 2007 and becoming more widespread in 2014. It is of great concern to learn that in French Polynesia when 1505 asymptomatic blood donors were screened for ZIKV by RT-PCR, 42 of them were positive (Musso et al., 2014). This seems to implicate how travel by humans can help to spread the viruses to new areas.

From February to April 2015, north eastern states of Brazil reported almost 7000 cases of people having rash and minor illness; of which only a small percentage of them were positive for dengue while tests for other viruses (but not for ZIKV) were all negative (Kindhauser et al., 2016). It was only by early May 2015 it was confirmed that it was ZIKV by RT-PCR and was reported for the first time in the Americas (Kindhauser et al., 2016). By July 12 states in Brazil had confirmed ZIKV cases and by the end of 2015 Colombia, Suriname, El Salvador, Mexico, Guatemala, Paraguay, Venezuela, Honduras, and Panama had reported locally acquired ZIKV (Kindhauser et al., 2016). This implies that the ZIKV will go on spreading to many more countries unless concerted effort is taken on a global scale.

Zika virus which was thought to be just a mild viral disease was later found to cause neurologic symptoms and microcephaly (Oliveira Melo et al., 2016). ZIKV was also found in other body fluids and was also shown to be sexually transmitted (Musso et al., 2015; Mansuy et al., 2016; Venturi et al., 2016). The current situation seems to portray that ZIKV could lead to serious public health concerns on a global scale. In the Americas ZIKV has been circulating along with DENV and CHIKV.

In Southeast Asia it is known that arbovirus diseases like DENV, CHIKV, Japanese encephalitis are serious public health concerns (Dash et al., 2013). In recent years (2014–2015) in Indonesia, a positive case of ZIKV was detected during a dengue outbreak in Jambi province Sumatra (Perkasa et al., 2016). Similarly in Cambodia a confirmed case of ZIKV was reported in 2010 (Heang et al., 2012) and in 2012 in Cebu, Philippines a 15 year old boy was confirmed to be suffering from ZIKV by real time RT-PCR and virus isolation (Alera et al., 2015). Travelers to Thailand were found to be infected with ZIKV on return to their country (Tappe et al., 2014). The Thai Ministry of Health then reviewed cases and found ZIKV infection circulating in Thailand between 2012 and 2014 (Buathong et al., 2015). Due to large outbreaks of dengue and CHIKV in Southeast Asia, which cause similar symptoms, ZIKV may be overlooked in Malaysia (Sam et al., 2016). However, there have been no reports of other neurologic symptoms.

This review will delve into the methods available for the detection of ZIKV, the vectors involved, current tools used for the control of the vectors and finally on the recommendations of new paradigms for surveillance and control of these vectors.

DIAGNOSTIC TOOLS OF ZIKV

Dengue virus and CHIKV share the same mosquito vectors (*A. aegypti* and *A. albopictus*) and potential distribution as ZIKV, and indeed co-circulation is described in the Americas (Rodriguez-Morales et al., 2016). It is difficult to clinically

differentiate between these infections as there is much overlap in symptoms and signs. Laboratory diagnosis takes on added importance as the long-term consequences of these infections are quite different and require specific approaches, for example the follow-up of ZIKV-infected pregnant women. There has been a flurry of new diagnostic assays described recently to complement existing conventional techniques such as cell culture [reviewed by Waggoner and Pinsky (2016)]. To date (July 20, 2016), several PCR and IgM assays for ZIKV have been submitted to the WHO Emergency Use Assessment and Listing Procedure, which assesses and expedites the availability of *in vitro* diagnostics during public health emergencies (World Health Organization, 2016a).

The current gold standard for diagnosis is PCR, which should be carried out on serum samples (within 7 days of illness) or urine (within 14 days) (CDCP, 2016). ZIKV RNA can also be detected in saliva (Bingham, 2016) and semen (Reusken et al., 2016) (the latter for up to 62 days), and there is some evidence that the non-serum specimens urine, saliva, and semen may be more likely to yield positive results than serum (Bingham, 2016; Reusken et al., 2016). Serum samples should also be tested for co-circulating arboviruses such as DENV and CHIKV (Waggoner et al., 2016).

Detection of serum IgM from day five of illness onward is a mainstay for arboviral diagnosis in most diagnostic laboratories in developing countries, as culture and PCR facilities are not widely available. ZIKV IgM can also be detected in the CSF of babies with microcephaly suspected to be due to congenital ZIKV infection (Cordeiro et al., 2016). However, the utility of IgM is much reduced by the extensive cross-reactions seen with past infections of or vaccinations against other flaviviruses, notably DENV, Japanese encephalitis, and yellow fever viruses (Calisher et al., 1989), necessitating the use of the highly specific plaque reduction neutralization test for confirmation (Lindsey et al., 1976; Rabe, 2016). This assay is beyond the scope of most laboratories. The antibodies that cross-react to ZIKV or DENV are mainly targeted to envelope protein domains EDI/II, and can cause antibody-dependent enhancement of infection with either virus (Stettler et al., 2016). In contrast, antibodies to non-structural protein 1 (NS1) are ZIKV-specific and could be used to develop a serological assay that can distinguish DENV from ZIKV infections (Huzly et al., 2016; Stettler et al., 2016). However, negative test results by culture, PCR or serology can never fully rule out ZIKV infection.

The ideal diagnostic test for ZIKV should be affordable, sensitive, specific, user-friendly, rapid and robust, particularly for the developing countries where the vectors exist. One of the WHO's top priorities for ZIKV medical products are multiplex tests for the three arboviruses (ZIKV, DENV, and CHIKV) which share the same mosquito vectors (World Health Organization, 2016b). The ideal test should detect RNA or antigen. The development of NS1 antigen detection assays (including rapid tests) was a major advance for dengue diagnosis. NS1 is secreted by flavivirus-infected cells and is involved in immune evasion and pathogenesis. ZIKV NS1 shares conserved features with DENV and West Nile virus, but has different electrostatic potential at the loop surface, which interacts with host factors and antibodies

(Song H. et al., 2016). Unlike IgM assays, DENV NS1 assays do not seem to demonstrate cross-reactivity with ZIKV (Matheus et al., 2016), apart from a single case report using a particular kit (Gyurech et al., 2016). A ZIKV NS1 assay would theoretically be feasible as an accessible test to reliably differentiate DENV and ZIKV, and several candidate assays are in the pipeline (World Health Organization, 2016b).

Several alternative diagnostic field tools for resource-poor settings have been described (Meagher et al., 2016). These include a synthetic biology approach, whereby isothermal RNA amplification is carried out, and toehold switch RNA sensors induce a color change, with all reagents embedded into a paper-based sensor (Pardee et al., 2016). A point-of-care loop-mediated, isothermal amplification assay with colorimetric detection has also been described (Song J. et al., 2016).

The detection of arboviruses in wild mosquitoes is useful for surveillance or for identifying the vectors of a relatively understudied pathogen (such as ZIKV) (Samuel and Tyagi, 2006). However, there are specific challenges which reduce sensitivity of testing methods. For example, mosquitoes may not be collected from traps for some time, which will lead to drying, rapid loss of viability for culture, and RNA degradation. Pools of triturated mosquitoes may also contain PCR inhibitors and other microorganisms, which may contaminate cultures. The traditional culture techniques for arbovirus diagnosis in mosquitoes, such as inoculation in cells, suckling mice or mosquitoes, and immunofluorescence assay, are in any case too labor-intensive for routine surveillance. Next-generation sequencing is useful for mosquitoes which potentially carry more than one pathogen or during an outbreak with an unknown arbovirus, but it is expensive and requires complex bioinformatics analysis (Bishop-Lilly et al., 2010).

For DENV, the rapid commercial NS1 assays developed for human diagnosis are excellent tools for testing mosquitoes, with the benefits of similar sensitivity to PCR (Tan et al., 2011; Voge et al., 2013), simplicity, and the potential for field use with a hand-held battery-operated homogenizer (Muller et al., 2012). Antigen-capture enzyme immunoassays have been described for detection of other flaviviruses in desiccated mosquitoes kept at ambient temperatures, including DENV (Thenmozhi et al., 2005; Chao et al., 2015) and Japanese encephalitis virus (Tewari et al., 1999). The surveillance of mosquitoes is a potential additional application for future ZIKV antigen assays.

VECTORS OF ZIKV

Aedes aegypti and *A. albopictus* are known to be the vectors of ZIKV (Li et al., 2012; Wong et al., 2013) and these two mosquitoes are also responsible for transmission of DENV and CHIKV. These are container breeding mosquitoes and it is known that the eggs of these mosquitoes can withstand desiccation. Thus *Aedes* mosquitoes are easily dispersed to many areas. It is also known that *A. aegypti* exhibits skip oviposition where it deposit its eggs in many containers (Reiter, 2007).

Zika virus was first isolated from *A. aegypti* from the rural area of Bentong in Pahang, Malaysia in 1965 (Marchette et al., 1969).

Recent studies carried out in Singapore demonstrated that *A. aegypti* was susceptible to ZIKV and by day five almost 60% of the mosquito's salivary glands were positive and on day six 100% were positive (Li et al., 2012). Studies conducted by the same group also demonstrated that *A. albopictus* could transmit ZIKV and by day 10 100% transmission was obtained in mosquito's saliva (Wong et al., 2013).

Zika virus was found naturally infected in *A. aegypti* in 1965 (Marchette et al., 1969) and seropositivity of ZIKV was also reported in 1960s (Dash et al., 2013). Thus, is it possible that the ZIKV has been in Southeast Asia all the time and people have developed immunity to this virus? It has been postulated that ZIKV originated in East Africa and spread to West Africa and Asia thus forming three different genotypes; the Asian genotype further spread to Pacific Islands and the Americas (Lanciotti et al., 2016). Also a case of ZIKV was confirmed in a traveler who visited Sabah, Malaysian Borneo on his return to Germany (Tappe et al., 2015). Thus, there must be other cases that have not been reported, perhaps people would only have suffered mild symptoms and it would not have been detected.

In Gabon there was an outbreak of CHIKV and DENV in 2007 and 2010 (Grard et al., 2014). The predominant vector found was *A. albopictus* and 91 pools of them were screened of which four pools were positive for CHIKV, three pools for DENV and two pools had mixed infection of CHIKV and ZIKV (Grard et al., 2014). When sera samples from humans were screened five were found to be positive for ZIKV (Grard et al., 2014). Here it clearly showed that ZIKV was circulating along with CHIKV and DENV. By screening both human and mosquito pools concrete evidence has been established that ZIKV can be transmitted alongside CHIKV and DENV. This clearly indicates that the trapping of adult mosquitoes and detection of viruses in them is the way forward to prevent epidemics.

It has been estimated that 440,000 to 1,300,000 ZIKV cases have occurred in Brazil (Bogoch et al., 2016), and the virus has finally been isolated from *A. aegypti* in that country (Gretchen, 2016). Now that *A. aegypti* can be easily trapped using the sticky gravid trap, this should be carried out and the vector should be confirmed in all localities. With such a large number of cases one would expect that it would be fairly easy to obtain infected mosquitoes. For example in a dengue prone area in Selangor, Malaysia, we obtained a minimum field infection rate (MIR) of 38.02 per 1000 using the NS1 antigen test kit (Lau et al., 2015). Since it is more difficult to get blood from people living in urban areas and it involves ethical clearance the best way to move forward is to detect the virus in the mosquitoes and to start proper control measures when results are positive.

The same vectors *A. aegypti* and *A. albopictus* are responsible for the spread of DENV and CHIKV and these vectors know no borders. If control measures can be instituted for these vectors the incidence of all these arboviral diseases will also be decreased. It seems like ZIKV is taking the same route as CHIKV (Musso and Gubler, 2015). If that is the case Southeast Asia could be in the forefront for ZIKV outbreak in the very near future. Perhaps the people of Southeast Asia are already immune to the disease, but visitors to the region may get infected and help to spread the disease globally.

VECTOR CONTROL MEASURES

Vector control measures carried out in Southeast Asia for surveillance and control of *A. aegypti* are shown in **Table 1**. It can be seen that vector surveillance and control strategies are mainly targeting the larval breeding sites. This includes the use of chemicals (Lee et al., 1997; Sulaiman et al., 2000, 2002; Chung et al., 2001; Tun-Lin et al., 2009; Huy et al., 2010; Oo et al., 2011; Kittayapong et al., 2012; Saiful et al., 2012; Sommerfeld and Kroeger, 2012), biological agents (Seng et al., 2008a; Lacroix et al., 2012; Hugo et al., 2014; Lazaro et al., 2015; Zuharah et al., 2015), environmental management (Ooi et al., 2006; Tun-Lin et al., 2009; Lee et al., 2013; Lau et al., 2015), and community participation (Chang et al., 2011). We have become over-dependent on chemicals and now the *Aedes* mosquitoes are resistant to most pyrethroids (Ponlawat et al., 2005; Wan-Norafikah et al., 2010; Kooou et al., 2014a,b; Ishak et al., 2015). Studies have also shown that space spraying has not conclusively been effective in reducing dengue transmission (Mount, 1998; Perich et al., 2000; Esu et al., 2010). Thus, when fogging/ULV is carried out impact on the vectors is minimal as shown in some studies (Vythilingam and Panart, 1991; Tanrang and Vythilingam, 2004). This could be one reason why cases of DENV are on the increase in countries in Southeast Asia.

Chang et al. (2011) have suggested that a positive move would be to include all three parameters of dengue transmission – vector density, human cases, and vector infection rate for prediction of early outbreaks. This seems to be the way forward in controlling these arboviral diseases. It has also been demonstrated that the gravid *Aedes* mosquito can be easily captured using sticky traps and the infected mosquito was obtained before human cases were reported (Lau et al., 2015). Studies conducted in different countries have demonstrated that the sticky traps are effective in collecting the *Aedes* mosquitoes when they come to oviposit (Ritchie et al., 2004; Gama et al., 2007; Honório et al., 2009; Chadee and Ritchie, 2010; de Santos et al., 2012; Resende et al., 2013). Thus a proactive approach is needed to test these mosquitoes for the different viruses so that a more positive control approach can be instituted. In Singapore too it has been shown that the sticky trap was able to trap the infected *Aedes* mosquito (Lee et al., 2013). It has also been documented that asymptomatic cases were infectious to *Aedes* mosquitoes and thus silent transmission was ongoing all the time (Duong et al., 2015).

House to house larval surveys have been the hallmark of dengue control program in many countries in Southeast Asia (Cheong, 1967; Ho and Vythilingam, 1980; Cheong et al., 1986; Chang et al., 2011; Mudin, 2015; Hapuarachchi et al., 2016). This method has also been used for the control of CHIKV vectors and can be used for ZIKV vectors. However, current studies have shown that although the *Aedes* house index has been reduced to as low as 0.07–0.14, yet epidemics of dengue have been explosive as reported in Singapore (Hapuarachchi et al., 2016). This has been due to the switch in serotypes as noted in 2007–2008 outbreaks (Lee et al., 2010) and also in 2013–2014 (Hapuarachchi et al., 2016). Singapore being a small and an affluent country can afford to carry out serotyping and sequencing in a timely manner

but still face epidemics of dengue. It is agreed that a multi-pronged approach backed by the epidemiological, virological, and entomological understanding is necessary for the control of vector borne viral diseases. However, entomological activities have always been reactive and thus could be one of the reasons for the current epidemics in many countries.

BIOLOGICAL CONTROL

The biological control approach traditionally reduce vector numbers by means of introducing their natural predators, such as larvivorous fish (Seng et al., 2008a) dragonfly nymphs (Tun-Lin et al., 2009), *Mesocyclops* sp (Lazaro et al., 2015), and *Toxorhynchites splendens* (Zuharah et al., 2015). While these approaches are environmentally friendly, they only affect the immature stages of the mosquito vector. In addition, they are effective only in containers that are constantly filled, such as wells and large containers (World Health Organization, 2012). Now that most breeding sites are cryptic it will be difficult to use biological control.

INSECT GROWTH REGULATOR

Pyriproxyfen an insect growth regulator has been tested under field conditions and it has a unique mode of action where it inhibits the development of the adult mosquito and also when an adult mosquito comes in contact with pyriproxyfen it can help to transfer it to other containers (Invest and Lucas, 2008). Studies carried out in Southeast Asia have shown that low doses were required for the inhibition of adult *Aedes* and the residual activity can be maintained for 11–15 weeks (Vythilingam et al., 2005) in Malaysia, while in Cambodia using a slow release formulation residual activity was effective for 6 months (Seng et al., 2008b). In Philippines pyriproxyfen was successfully used to control the dengue outbreak after typhoon Haiyan (Aumentado et al., 2015).

THE WAY FORWARD

If we learn lessons from malaria control with regards to vectors, it was always targeted toward adult mosquitoes and not so much against the larvae. One reason could be that because it was difficult to find the breeding sites of *Anopheles* mosquitoes and some sites were inaccessible. However, for dengue, the vectors breed in containers and thus control of larvae and source reduction were the initial strategies for dengue control. This has obviously worked since *Aedes* index has been reduced to low levels (Mudin, 2015; Hapuarachchi et al., 2016) yet the cases of DENV infection have increased. Thus, it is timely now to focus on interventions based on the adult population to reduce and prevent epidemics of DENV. If we control the *Aedes* adults, we will automatically reduce outbreaks of ZIKV and CHIKV.

Besides monitoring the adult *Aedes* population it is similarly important to detect the pathogen in the mosquitoes. Currently

TABLE 1 | Current research on *Aedes* vector control in Southeast Asian over the past 25 years.

Countries	Environmental	Chemical	Biological
Cambodia		Temephos (Huy et al., 2010). Pyriproxyfen (Seng et al., 2008b)	Guppy larvivorous fish (Seng et al., 2008a)
Indonesia		Transfluthrin/metofluthrin (Achee et al., 2015)	Mosquitoes with <i>Wolbachia</i> (Rašić et al., 2015)
Laos			<i>Mesocyclops</i> spp. (Lazaro et al., 2015)
Malaysia	Sticky traps (Lau et al., 2015)	<i>Bacillus thuringiensis israelensis</i> (Saiful et al., 2012). Pyriproxyfen (Vythilingam et al., 2005). Cynoff 25ULV (cypermethrin 25 g/l) and Solfac UL015 (cyfluthrin 1.5% w/v) (Sulaiman et al., 2002). Deltamethrin/S-bioallethrin/piperonyl butoxide and cyfluthrin (Sulaiman et al., 2000). ULV-applied bifenthrin (Lee et al., 1997)	Mosquitoes carrying a Dominant Lethal gene, RIDL (Lacroix et al., 2012). <i>Toxorhynchites splendens</i> (Zuharah et al., 2015)
Myanmar		<i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> (Oo et al., 2011). Temephos (Tun-Lin et al., 2009)	Dragonfly nymphs and fish (Tun-Lin et al., 2009)
Philippines		Pyriproxyfen (Aumentado et al., 2015). <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> (Sommerfeld and Kroeger, 2012)	Guppy (Chang et al., 2011)
Singapore	Mosquito traps (Lee et al., 2013)	<i>Bacillus thuringiensis</i> and chemical fogging (Chung et al., 2001)	
Thailand	Screen net covers, mosquito traps, vacuum aspirators (Kittayapong et al., 2012) and treated curtains (Lenhart et al., 2013); Insecticide treated clothing (Tozan et al., 2014). Mosquito trap (Ponlawat et al., 2013)	Temephos (Kittayapong et al., 2012). <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> (Kittayapong et al., 2012).	Copepods (Kittayapong et al., 2012)
Vietnam	Olyset Nets (Tsunoda et al., 2013)	Pyriproxyfen (Tsunoda et al., 2013)	<i>Mesocyclops</i> sp (Lazaro et al., 2015). Mosquito with <i>Wolbachia</i> (Nguyen et al., 2015)

for dengue the NS1 antigen test kit can be used and the procedure is very simple for use by public health workers. Thus what is needed for CHIKV, ZIKV, and other common arthropod borne viruses are very simple tools that can be used by the public health workers. Molecular tools like PCR and real-time PCR are available but these are expensive and need experienced staff and expensive equipment which is not feasible for a control program.

Although it has been stated that in Americas the success of *A. aegypti* eradication was due to perifocal spraying and source reduction (Achee et al., 2015), this will not work in present times because currently it is difficult to persuade people in urban areas to allow indoor residual spraying to be carried out. Besides in Southeast Asia it is also known that the *A. aegypti* like to rest on temporary surfaces like clothes and curtains (Pant and Yasuno, 1973).

Field studies have been carried out on use of insecticide treated curtains and jar covers and these have shown reduction in mosquito population (Kroeger et al., 2006; Lenhart et al., 2013; Tsunoda et al., 2013), however, its efficacy in reducing dengue cases have not been deciphered. Thus although a number of studies were conducted on various methods to monitor adult population (Lee et al., 2013; Ponlawat et al., 2013; Tozan et al.,

2014; Lau et al., 2015) and show promise, the end result of reduction of cases has not been established.

Studies are also on going on genetically modified mosquitoes and one showing promise is the release of insects with dominant lethality (RIDL). In the laboratory these mosquito larvae are bred in water containing tetracycline, however, in the absence of tetracycline these larvae and pupae will not be able to survive. Field studies have been conducted in Cayman Islands, where there was a suppression of 80% of the natural population (Harris et al., 2012) and in Brazil there was a suppression of 85% of the natural population (Achee et al., 2015). Although this method has reduced mosquito populations, it should be noted that only male mosquitoes were released. However, a fool proof method is needed to ensure that females are not released. RIDL females are equally susceptible to dengue virus compared to the wild *A. aegypti*. Besides, evidence is required to show that with the reduction of the *A. aegypti* population it is possible to reduce dengue cases. Each country will also need to obtain approval from regulatory bodies before they can release these mosquitoes. Insectaries will also have to be maintained if these RIDL mosquitoes are to be used. All these come with a cost and countries should be able to afford these expensive methods before they embark on such a program.

Another similar approach is the release of *A. aegypti* with the bacteria of *Wolbachia* sp. *Wolbachia* is naturally found in many arthropods and nematodes but is not found in *A. aegypti* (Werren, 1997). Currently, one of the novel approaches for bio-control is the introduction of *Wolbachia* from naturally infected arthropods into *A. aegypti* to reduce dengue transmission (Moreira et al., 2009; Walker et al., 2011). When an uninfected female *A. aegypti* mates with a *Wolbachia*-infected male, the female will produce eggs but no progeny will develop due to cytoplasmic incompatibility. However, when a *Wolbachia* infected female mates with either infected or uninfected male, all progeny will carry *Wolbachia* (Caragata et al., 2016). Field trials have been conducted in Australia with the release of *A. aegypti* with wMel *Wolbachia* and the frequency has remained at more than 90% for 3 years (Hoffmann et al., 2014). However, field release of *A. aegypti* with wMelPop *Wolbachia* in Vietnam and Australia failed to become successfully established (Nguyen et al., 2015). Studies are also ongoing in Indonesia (Rašić et al., 2015). Thus it will take time for more studies to be conducted before *Wolbachia* infected *A. aegypti* can be used in dengue control program.

Now with ZIKV becoming a huge public health global problem, it is timely that randomized control trials (RCT) need to be carried out in Southeast Asia and prove that some of these paradigms will be able to control and prevent epidemics caused by these *Aedes* mosquitoes. For a start RCT studies should show that the cases of dengue can be reduced (Reiner et al., 2016). If a particular paradigm proves to be successful, it would also work for all the other arboviruses transmitted by *A. aegypti* and *A. albopictus*.

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CONCLUSION

There are several options for ZIKV diagnosis building on existing technologies, which can be used in both humans and mosquitoes. However, most are not available in developing countries, and there remains an urgent need for an accessible RNA/antigen assay, as well as an IgM assay with acceptable specificity against other flaviviruses. While extensive work is ongoing to develop a vaccine, diagnostic kits, and to study the epidemiology of ZIKV, it is equally important to develop new paradigms to control the vectors. We need to learn from the past and thus a more proactive approach is needed to control the vectors and not a reactive one. In the early years besides Africa, ZIKV was known to be circulating Southeast Asia. Thus it is imperative to ensure that Southeast Asia don't become a hub for transmitting the ZIKV to other countries. We need to work together and carry out multi-country RCT for vector control to show that the way forward is to monitor the adult *Aedes* population along with infectious status.

AUTHOR CONTRIBUTIONS

IV, JS, YC, LK, and WW all played a role in the preparation of this manuscript.

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Could the Recent Zika Epidemic Have Been Predicted?

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Given knowledge at the time, the recent 2015–2016 Zika virus (ZIKV) epidemic probably could not have been predicted. Without the prior knowledge of ZIKV being already present in South America, and given the lack of understanding of key epidemiologic processes and long-term records of ZIKV cases in the continent, the best related prediction could be carried out for the potential risk of a generic *Aedes*-borne disease epidemic. Here we use a recently published two-vector basic reproduction number model to assess the predictability of the conditions conducive to epidemics of diseases like Zika, chikungunya, or dengue, transmitted by the independent or concurrent presence of *Aedes aegypti* and *Aedes albopictus*. We compare the potential risk of transmission forcing the model with the observed climate and with state-of-the-art operational forecasts from the North American Multi Model Ensemble (NMME), finding that the predictive skill of this new seasonal forecast system is highest for multiple countries in Latin America and the Caribbean during the December–February and March–May seasons, and slightly lower—but still of potential use to decision-makers—for the rest of the year. In particular, we find that above-normal *suitable conditions* for the occurrence of the Zika epidemic at the beginning of 2015 could have been successfully predicted at least 1 month in advance for several Zika hotspots, and in particular for Northeast Brazil: the heart of the epidemic. Nonetheless, the initiation and spread of an epidemic depends on the effect of multiple factors beyond climate conditions, and thus this type of approach must be considered as a guide and not as a formal predictive tool of vector-borne epidemics.

Keywords: *Aedes*-borne diseases, Zika, dengue, chikungunya, predictability, R_0 model, climate

INTRODUCTION

Zika virus (ZIKV, family *Flaviviridae*, genus *flavivirus*) disease is a viral illness transmitted primarily by the *Aedes aegypti* and *Aedes albopictus* mosquitoes (Abushouk et al., 2016). ZIKV has recently emerged as a major epidemic in Latin America and the Caribbean, with 738,783 suspected and confirmed cases reported to date (PAHO, 2017). Prior studies from Yapp Island suggest that the majority of ZIKV infections are asymptomatic or result in mild disease (Duffy et al., 2009), and initial studies from Latin America suggest that the ZIKV infections are less severe and less febrile than chikungunya (CHIKV) or dengue (DENV) infections (Waggoner et al., 2016). The spread of ZIKV has been accompanied by severe neurological complications, including children born with microcephaly (Calvet et al., 2016; Schuler-Faccini et al., 2016) and people with Guillain-Barré syndrome (Cao-Lormeau et al., 2016; PAHO, 2016b).

In a previous study (Muñoz et al., 2016a), our team analyzed the potential contribution of climate signals acting at different timescales in creating the environmental scenario for the current ZIKV epidemic. We found that suitable climate conditions were present, due to the co-occurrence of anomalously high temperatures and persistent below-normal rainfall in several regions of South America, especially in Brazil, the heart of the epidemic.

These suitable conditions are not only favorable for ZIKV, but in general enhance the probability of both *Aedes* sp. reproduction and viral replication. Due to the fact that ZIKV, DENV, and CHIKV share the same mosquito vectors and seem to have similar temperature dependence for their extrinsic incubation periods (Mordecai et al., 2017), there are advantages in considering the overall eco-epidemiological conditions for the potential risk of transmission of *Aedes*-borne arboviruses rather than focusing on the risk of transmission of only one disease. The effect of rainfall on *Aedes* sp. is more complex than temperature (e.g., Stewart-Ibarra and Lowe, 2013; Stewart Ibarra et al., 2013), because *Aedes* vectors breed in domestic water containers which are more abundant during droughts and water shortages (Chretien et al., 2007). Their presence is also known to increase following unusually high rainfall when peri-domestic breeding sites (discarded containers, flower pots, tires, etc.) are filled with water.

The study of the different environment-virus-vector-human interactions in this field is normally performed using a diversity of mathematical models. Most of them are based on the Ross-McDonald model (Smith et al., 2012) or its generalizations. These models are commonly referred to as compartmental models, normally stratifying the population in susceptible (S), infected (I) and recovered (R) individuals (so-called SIR models). A set

of coupled differential equations is used to describe the evolution of each compartment (Anderson and May, 1991; Murray, 2002). These models vary in complexity, and tend to be classified as homogeneous or heterogeneous models; for further details, see for example (Moreno et al., 2002).

Although these models are most frequently used to diagnose past or present epidemics, they can also be used in predictive mode, even at seasonal scale (see Thomson et al., 2006). Predicting conditions of environmental suitability presents a complex problem, but it is indeed less complex than predicting the occurrence and transmission of the diseases in human populations. The complexity resides in the non-linear interactions between the different components of the coupled disease model system in consideration, in which the effects of population immunity and susceptibility, or different possible immunological interactions between the diseases (e.g., co-infections of DENV and ZIKV) are still not well understood. Nonetheless, some new studies are already considering some of these interactions (for recent ZIKV examples, see Ferguson et al., 2016; Lourenco et al., 2017; Perkins, 2017), underscoring—in addition to the role of climate—the importance of herd immunity and the frequency of viral re-introductions in the modulation of potential future outbreaks.

Here, we develop a new seasonal forecast system to assess suitable climate conditions for the transmission risk of *Ae. aegypti*- and *Ae. albopictus*-borne diseases. We use a two-vector one-host basic reproduction number model driven by state-of-the-art climate forecasts to assess its predictive skill, and we discuss the implications for Latin America and the Caribbean. For brevity, in the following pages we will use “potential risk of transmission” to refer to potential transmission associated with climate conditions suitable for transmission of the aforementioned diseases. Data and general methods are presented in Section Data and Methods, the basic reproduction number model is discussed in Section Two-Vector One-Host Ento-Epidemiological Model, the skill assessment for different seasons of the year is analyzed in Section Skill Assessment and DJF 2014–2015 Forecast, and the concluding remarks are presented in Section Concluding Remarks.

DATA AND METHODS

The domain of study includes Latin America and the Caribbean, and is defined by the boundaries 120–25°W and 60°S–32°N.

The observed monthly temperature and rainfall fields for the period 1950–2015 were obtained from the University of East Anglia Climate Research Unit product version 3.4 (CRUv3.4; Harris et al., 2014), available at a horizontal resolution of 0.5 degrees. These datasets were selected to be consistent with our previous study on a similar topic (Muñoz et al., 2016a). Tests indicated that the results are consistent with other large scale gridded climate datasets, such as the Climate Anomaly Monitoring System (CAMS, Global Historical Climatology Network version 2 Fan and van den Dool, 2008) used in Caminade et al. (2017).

State-of-the-art temperature and rainfall forecasts at monthly timescales were obtained from the North American Multi-Model

Abbreviations: 2AFC, Two-Alternative Forced Choice; CAMS, Climate Anomaly Monitoring System; CHIKV, chikungunya virus; CPT, Climate Predictability Tool; CRU, Climate Research Unit, at East Anglia University; DENV, dengue virus; DJF, December-January-February; JJA, June-July-August; IRI, International Research Institute for Climate and Society, at Columbia University; MAM, March-April-May; NMME, North American Multi-Model Ensemble; SON, September-October-November; PAHO, Pan-American Health Organization; PCR, Principal Component Regression; WHO, World Health Organization; ZIKV, zika virus.

Ensemble project (NMME; Kirtman et al., 2014), at a common horizontal resolution of $1^\circ \times 1^\circ$ degrees. The total of 116 members available was used for the hindcast¹ period of 1982–2010, but only 104 members were used for the December–February 2014–15 forecast due to data availability (no members from the NCAR-CESM1 and NASA-GMAO models). Hindcasts and forecasts correspond to the month prior to the target season; for example, for the December–February season, the hindcast and forecast of November was used.

The vector model used in this work was recently developed by Caminade et al. (2017). For the sake of organization, the basic reproduction number model equations are presented in the next section. The model requires climate information, and thus the observations and NMME forecasts mentioned above were used, the first one for diagnostics and baseline validation, and the second one for the prognostic set up. The model was coded and executed in Matlab at a monthly timescale for a total of 792 months when forcing it with observed data, and 348 months per member when using the NMME hindcasts; each member was run independently before computing the ensemble and seasonal averages. The basic reproduction number model output, forced with both climate observations and hindcasts, is available online at the Latin American Observatory's Datoteca (Muñoz et al., 2010, 2012, 2016b; Chourio, 2016): http://datoteca.ole2.org/maproom/Sala_de_Salud-Clima/ContexHist-Map-1/index.html.es.

When analyzing the model forced with observations, standardization was performed with respect to the 1950–2015 period. Anomalies are defined as the value of the variable being analyzed minus its 1950–2015 average. To analyze inter-annual variability, a 12-month running average was computed. A linear detrending was used.

Skill was assessed using both Kendall's τ and the 2AFC score (Mason and Weigel, 2009), computed using the International Research Institute for Climate and Society (IRI) Climate Predictability Tool, CPT (Mason and Tippet, 2016), version 15.4.7. Kendall's τ is a non-parametric rank correlation coefficient used here to measure the overall association between observations and model output, with positive values indicating that the forecasts are better than using the average expected value (negative values imply that it is better to use the average expected value). The 2AFC score indicates the probability of correctly discriminating an observation in a higher category from one in a lower (e.g., an “above-normal” observation from a “normal” observation) given the forecasts expressed in deterministic form (i.e., the actual model values, and not the probabilities associated with them). The following four seasons were considered: December–February, DJF, March–May, MAM, June–August, JJA, and September–November, SON. A cross-validation window of 5 years was used, for the 1982–2010 period. For each iteration, 5 years were left out and the remainder years were used to build the statistical model, forecasting the middle year of the 5-year window. This window is shifted 1 year into

the future for the next iteration, and so on. The skill reported is the average of the metric computed for each iteration, and it was assessed after magnitude and spatial biases were corrected using a simple Model Output Statistics approach involving a Principal Component Regression (PCR; Mason and Baddour, 2008; Jolliffe and Stephenson, 2012), an option available in the CPT software. For further details see (Mason and Baddour, 2008).

Maps showing the 2AFC score computed using this methodology were produced for each of the seasons considered. Categories for above normal, normal, and below normal were identified in the vector model output using the typical 33.33 and 66.66% thresholds in the corresponding probability density function. Forecast probabilities for each category were computed using the PCR model built with the CPT package.

TWO-VECTOR ONE-HOST ENTO-EPIDEMIOLOGICAL MODEL

Both *Ae. aegypti* and *Ae. albopictus* are considered the most important vectors in Latin America and the Caribbean for the transmission of ZIKV, CHIKV, and DENV (e.g., Lambrechts et al., 2010; Li et al., 2012; Grard et al., 2014; Chouin-Carneiro et al., 2016; Gardner et al., 2016; Muñoz et al., 2016a; Mordecai et al., 2017). These vectors are known to have different susceptibilities to these diseases, as well as different feeding characteristics (Caminade et al., 2017). While *Ae. aegypti* and *Ae. albopictus* are considered to be a domestic and peri-domestic mosquito, respectively, it is in principle possible to find them co-existing in the same place (Li et al., 2014; Kraemer et al., 2015), something that is expected to be even more common in the near future due to global warming (Gardner et al., 2016; Lessler et al., 2016). Hence, we consider that an actionable seasonal forecast system should involve at least these two species for Latin America and the Caribbean. This section presents the model equations used by the prediction system.

As it has been shown by other authors (Turner et al., 2013; Caminade et al., 2017) the equations for the dynamics of a two-vector one-host SIR model, a generalization of the standard Ross-McDonald model (Smith et al., 2012), are

$$\frac{dS_H}{dt} = -\lambda_H S_H \quad (1)$$

$$\frac{dI_H}{dt} = \lambda_H S_H - r I_H \quad (2)$$

$$\frac{dR_H}{dt} = r I_H \quad (3)$$

$$\frac{dS_i}{dt} = \rho_i N_i - \lambda_{Vi} S_i - \mu_i S_i \quad (4)$$

$$\frac{dL_i}{dt} = \lambda_{Vi} S_i - (v_i + \mu_i) L_i \quad (5)$$

$$\frac{dI_i}{dt} = v_i L_i - \mu_i I_i \quad (6)$$

where S_H , I_H , and R_H are the number of susceptible, infectious and recovered hosts, respectively, associated with the *Aedes*-borne disease of interest. S_i , L_i , and I_i are the number of

¹ A hindcast is a retrospective forecast, made using the same methodology of actual forecasts, but for a past period of time. They are usually produced to evaluate forecast skill.

susceptible, latent and infectious vectors of kind $i = 1, 2$ (*Ae. aegypti* and *Ae. albopictus*, respectively). In addition,

$$\lambda_H = \sum_{i=1,2} \frac{1}{N_i} a_i b_i \phi_i m_i I_i \quad (7)$$

$$\lambda_{V_i} = \frac{I_H}{H} a_i \beta_i \phi_i \quad (8)$$

and a_i is the daily biting rate (a function of temperature), b_i is the vector-to-host transmission probability, ϕ_i quantifies the vector's preference for humans, m_i is the vector-to-host ratio (a function of both temperature and rainfall; see Caminade et al., 2017 for details), β_i is the host-to-vector transmission probability, r is the daily recovery rate, and v_i and μ_i are the inverse of the extrinsic incubation period of the virus in days and the mortality rate, respectively, both a function of temperature. As in (Caminade et al., 2017), the vector-to-host-ratio m_i is defined in terms of the probability of occurrence of the vectors (multiplied by 1,000), which was obtained in (Kraemer et al., 2015) using maximum and minimum annual rainfall to account for the presence of water-filled containers, and other environmental variables involving temperature and urbanization; for details see the Materials and Methods section in (Kraemer et al., 2015). H and N_i are the total number of hosts and the total number of the i -th kind of vector, respectively.

This is a 5-compartmental model which includes infectious human host, latent *Ae. aegypti* vectors, latent *Ae. albopictus* vectors, infectious *Ae. aegypti* vectors and infectious *Ae. albopictus* vectors. If Δ and Λ are the new infectious rate appearing in a compartment and the rate at which individuals leave said compartment, respectively, then

$$\Delta = (\lambda_H S_H \quad \lambda_{V1} S_1 \quad \lambda_{V2} S_2 \quad 0 \quad 0)^T \quad (9)$$

$$\Lambda = (r I_H \quad (v_1 + \mu_1) L_1 \quad (v_2 + \mu_2) L_2 \quad -v_1 L_1 + \mu_1 I_1 \quad -v_2 L_2 + \mu_2 I_2)^T \quad (10)$$

The basic reproduction number R_0 is the dominant eigen-value of the next-generation matrix (Caminade et al., 2017)

$$K = \left(\frac{\partial \Delta_m}{\partial x_l} \right)_{x_0} \left(\frac{\partial \Lambda_m}{\partial x_l} \right)_{x_0}^{-1} \quad (11)$$

for $m, l = 1..5$ identifying the different compartments, x , being a vector with the number of individuals in each compartment, and x_0 denoting the disease-free equilibrium state. The only non-zero elements K_{ml} (new infections in compartment m produced by infectious individuals in compartment l) of K are

$$K_{12} = \frac{a_1 b_1 \phi_1 v_1}{(v_1 + \mu_1) \mu_1} \quad (12)$$

$$K_{13} = \frac{a_2 b_2 \phi_2 v_2}{(v_2 + \mu_2) \mu_2} \quad (13)$$

$$K_{14} = \frac{a_1 b_1 \phi_1}{\mu_1} \quad (14)$$

$$K_{15} = \frac{a_2 b_2 \phi_2}{\mu_2} \quad (15)$$

$$K_{21} = \frac{a_1 \beta_1 \phi_1 m_1}{r} \quad (16)$$

$$K_{31} = \frac{a_2 \beta_2 \phi_2 m_2}{r} \quad (17)$$

R_0 is the largest eigenvalue solution of the eigenvalue problem $|K - R_0 I| = 0$:

$$R_0^4 - R_0^2 (K_{21} K_{12} + K_{31} K_{13}) = 0 \quad (18)$$

or

$$R_0 = \sqrt{\frac{a_1^2 \phi_1^2 b_1 \beta_1 m_1 v_1}{(v_1 + \mu_1) \mu_1 r} + \frac{a_2^2 \phi_2^2 b_2 \beta_2 m_2 v_2}{(v_2 + \mu_2) \mu_2 r}} \quad (19)$$

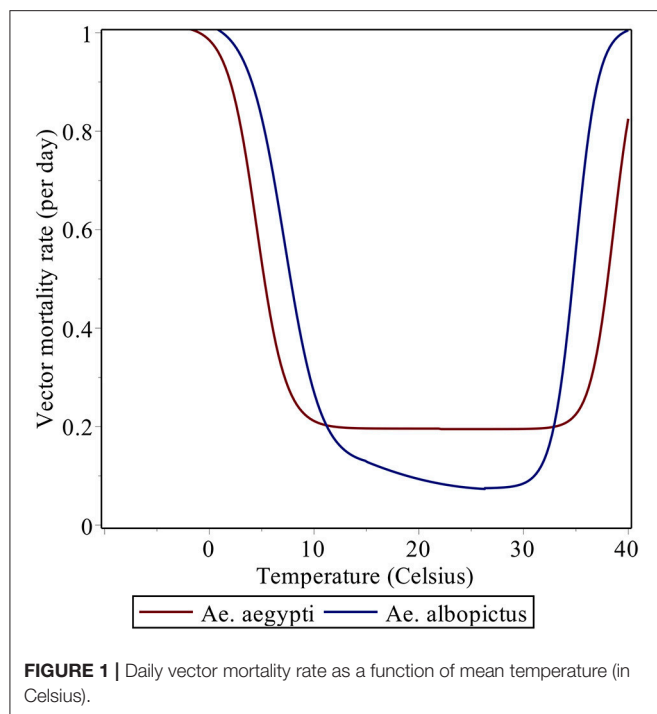
where, as the indices suggest, the first term in the square root corresponds to *Ae. aegypti* and the second one to *Ae. albopictus*. As in (Caminade et al., 2017), we set $R_0 = 0$ in all locations and times for which the total monthly rainfall has not been at least 80 mm during a minimum of 5 months, a condition for stable transmission.

This model has been reported (Caminade et al., 2017) to reproduce well the observed basic reproduction number obtained when using the relatively short record of ZIKV cases available in Latin America. Because of this, we have chosen the same values of the parameters and functional dependence on temperature and rainfall that was used in that study (Caminade et al., 2017).

The basic reproduction number can be understood as the expected number of new cases generated by a single (typical) infection in a completely susceptible population. It is a dimensionless number that can be associated with the potential risk of transmission of the disease, considering only basic environmental, entomological, and epidemiological information. Only values of $R_0 > 1$, which are related to spreading of the epidemic in a fully susceptible population, were considered in this study.

The temperature dependence of certain parameters in the model (for example, the mortality rate μ_i ; see **Figure 1**) strongly controls the spatial and temporal distribution of R_0 . Most of Latin America and the Caribbean typically exhibits high values of R_0 (**Figure 2**). The potential risk of transmission of *Aedes*-borne diseases is higher for the northern half of South America, especially in Brazil, most of Colombia, Venezuela, Guyana, Suriname, and the French Guyana, coastal Ecuador and the Ecuadorian and Peruvian Amazon. Central America and the Caribbean, although to a lesser degree, also exhibit high values of R_0 . Furthermore, with the increasing occurrence of high-temperature records, the frontier is extending farther into southern South America, in countries like Uruguay, which reported the first cases of autochthonous dengue fever in 2016 (WHO, 2016). Nonetheless, places that are too hot decrease the life expectancy of the vectors (roughly speaking, when temperatures exceed 40°C, see **Figure 1**), and thus some regions in the future could start seeing a relative decrease in vector abundance if temperatures keep increasing.

An analysis of the evolution of the suitable conditions for transmission during 2013–2015 (**Figure 3**) complements the study on the associated temperature and rainfall anomalies

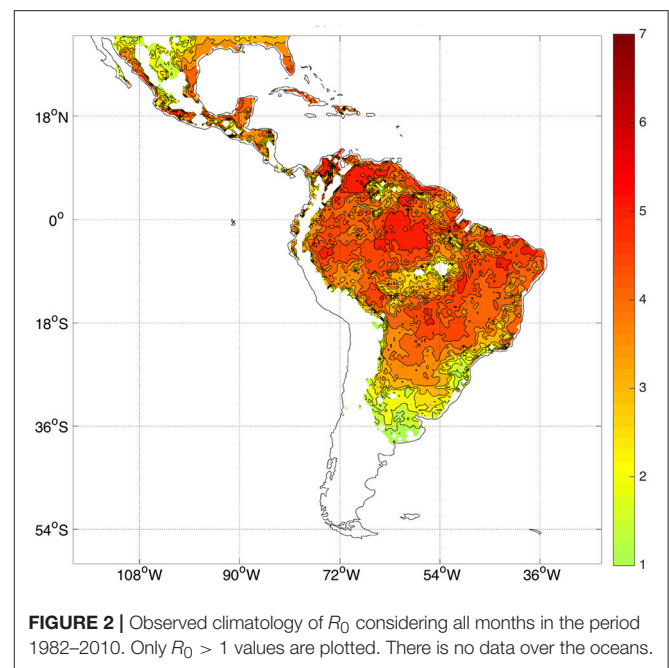


performed previously (Muñoz et al., 2016a). Standardized positive R_0 anomalies present during 2013 in regions of northern South America and northern Brazil became dominant almost everywhere in the northern half of South America, Central America and the Caribbean in 2015; during this year values exceeded one standard deviation in zones of the Brazilian Amazon, the northern Peruvian coast, all of coastal Ecuador, most of northern Colombia and western Venezuela. Standardized anomalies of around two standard deviations occurred in the heart of the Brazilian Amazon.

The neutral standardized anomalies in the Brazilian Nordeste (Northeast), one of the most affected regions in terms of the 2015 ZIKV outbreak, are attributed to the buffering role of the Atlantic Ocean in controlling the local temperatures. Still, neutral standardized anomalies in Nordeste are associated with R_0 ranging between 3.5 and 5.5, indicating a very high potential risk of transmission.

The high values of the 2015 standardized anomalies (Figure 3C) are also consistent with the observed burden of other diseases like dengue; for example, the reported number of dengue cases for Ecuador in 2015 (42,667) was about 3 times larger than the average number of cases for 2011–2014 (14,467.5); for details see (PAHO, 2016a). Nonetheless, unpublished work of our team in Machala (coastal Ecuador) suggests that a high percentage of the 2015 dengue cases reported there are likely to be chikungunya cases. Even if that is the case, the model was able to capture enhanced conditions leading to a larger burden of *Aedes*-borne diseases.

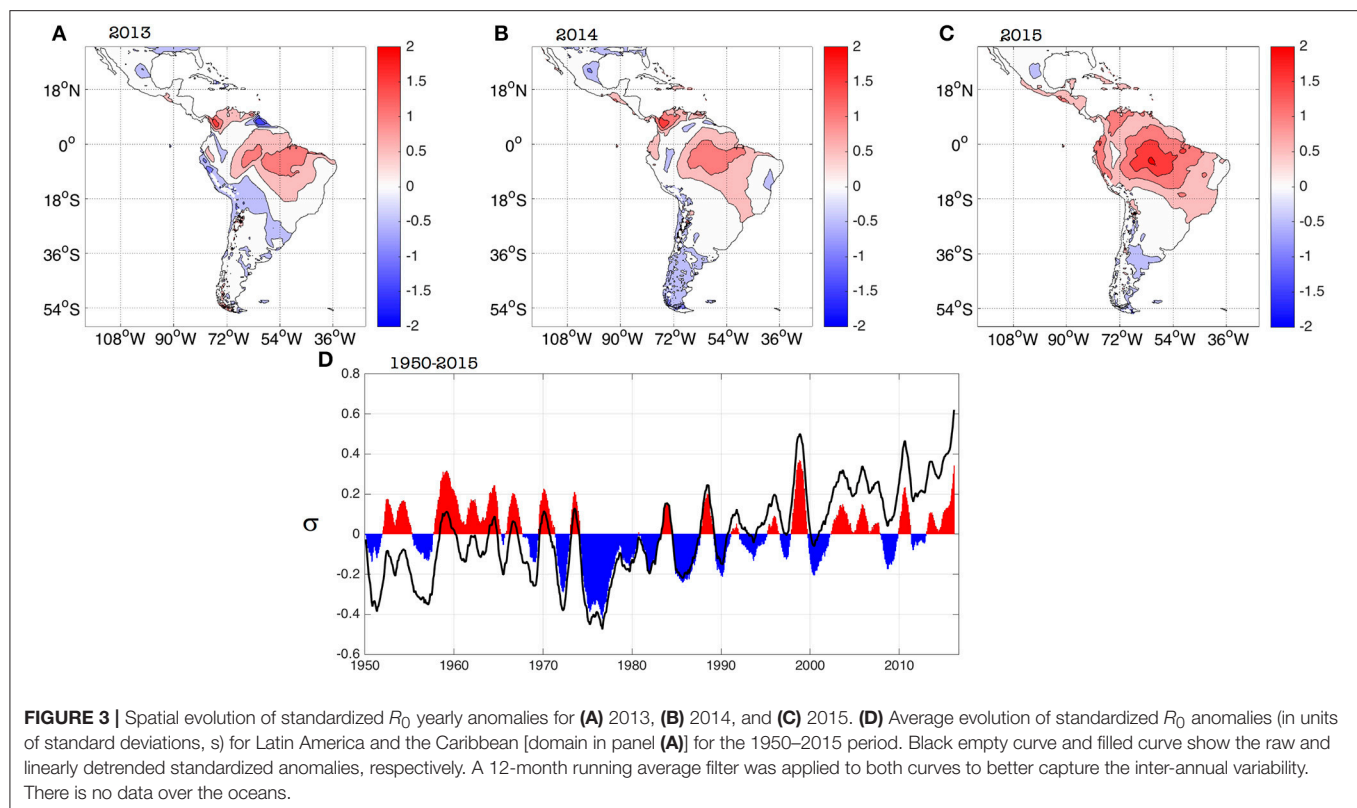
The evolution of the spatially-averaged R_0 standardized anomalies for Latin America and the Caribbean exhibits a clear trend between 1950 and 2015 (black curve in Figure 3D), as



reported by (Caminade et al., 2017), that is consistent with the persistent increase in temperatures observed in the region. Once the longer-term signals are filtered-out, the inter-annual component of the R_0 standardized anomalies (filled curved in Figure 3D), show a peak in 2015 that is the second-highest on record, following the largest one occurred during 1998. This slightly contrasts with the analysis performed by (Caminade et al., 2017); overall Figure 3D is telling the same story as Figure 3 in (Caminade et al., 2017), the main differences due to the use of a different dataset and mostly to the use of a 12-month running average in our case (see Section Data and Methods above). Our interpretation is consistent with our previous study on the 2015 climate conditions (Muñoz et al., 2016a): a superposition of long-term, decadal and inter-annual signals was responsible for the 2015 absolute maximum in the unfiltered time series (black curve in Figure 3D). Although most likely the 2015 El Niño had an important contribution, the maximum cannot be explained only by this inter-annual phenomenon.

SKILL ASSESSMENT AND DJF 2014–2015 FORECAST

A new seasonal forecast system for potential risk of transmission of *Aedes*-borne diseases can be developed by driving the R_0 model discussed in the previous section with a multi-model ensemble of climate predictions at seasonal scale. For this purpose, we have selected the set of coupled global models participating in the North American Multi-Model Ensemble project (Kirtman et al., 2014). Although, our focus is Latin America and the Caribbean, the same system can be used for other regions of the world, and a subset of the NMME models or a completely different



seasonal climate forecast system can be used straightforwardly if that provides higher skill for the particular region of interest.

In brief, the system uses the monthly climate information from each one of the 116 (or 104, if the target period is between 2010 and 2015) realizations of the NMME models to compute the associated value of the basic reproduction number for each grid box in our geographical domain. Although, the forecast horizon is typically 9 months after the initialization month, skill is normally higher for the first few seasons; to illustrate the approach here we focus on the first season starting immediately after the initialization month (e.g., JJA for forecasts initialized in May). After the multi-model ensemble and the seasonal average is computed, the output is corrected using a simple Principal Component Regression, which provided better results than other methods like Canonical Correlation Analysis or the use of the raw model output. For additional details, see Section Data and Methods.

The cross-validated analysis shows that there is relatively high skill ($>60\%$, as measured by the 2AFC metric) for R_0 for all the seasons over the northern half of South America and several regions of Central and North America, and some Caribbean nations (Figure 4). Overall, the skill is higher in DJF and MAM (with Kendall's τ of 0.199 and 0.191, respectively), and minimum in JJA (0.123), SON being in the middle (0.146). These values of Kendall's τ are typical for rainfall predictions in the region, as can be seen in the Validation Maproom of the Latin American Observatory's Datoteca (Muñoz et al., 2010, 2012; Chourio, 2016): http://datoteca.ole2.org/maproom/Sala_de_Validacion/.

Regionally speaking, skill is higher in Mexico in JJA, especially in the south (Figure 4). Central American countries exhibit high skill (above 70% for most of them) for DJF and MAM, with the unskilled values ($<50\%$) occurring in JJA and SON for Panama and Costa Rica. The western Caribbean tends to show higher skill during JJA, while the Central Caribbean and Lesser Antilles during MAM.

The northern part of South America shows relatively high skill ($>70\%$) all year around, with the exception of some regions such as Ecuador, northern Peru, southwestern Colombia, northeastern Venezuela, and northern Guyana which show no skill during JJA and SON (Figure 4). The forecast system has in general low skill or no skill at all for southern South America, with some exceptions, e.g., the Bolivian Amazon in DJF, Paraguay and northern Argentina in SON, and northwestern Uruguay in DJF. Most of Brazil exhibits values of the 2AFC metric that are above 50% in all seasons, although southern Brazil has very low skill in MAM. In general, Chile and central and southern Argentina do not show potential risk of transmission with this model, and thus those regions appear in white in our skill maps (Figure 4).

To illustrate an example of the bias-corrected probabilistic forecasts produced by our system, we now consider the season preceding the first reported case of ZIKV in Brazil (May 2015 Faria et al., 2016, 2017; Kindhauser et al., 2016): DJF 2014–2015. The probabilistic prediction indicates that there were mostly conditions for above-normal risk of transmission in eastern Brazil, which is similar to the observed conditions (Figure 5). Nonetheless, below-normal conditions were in general no

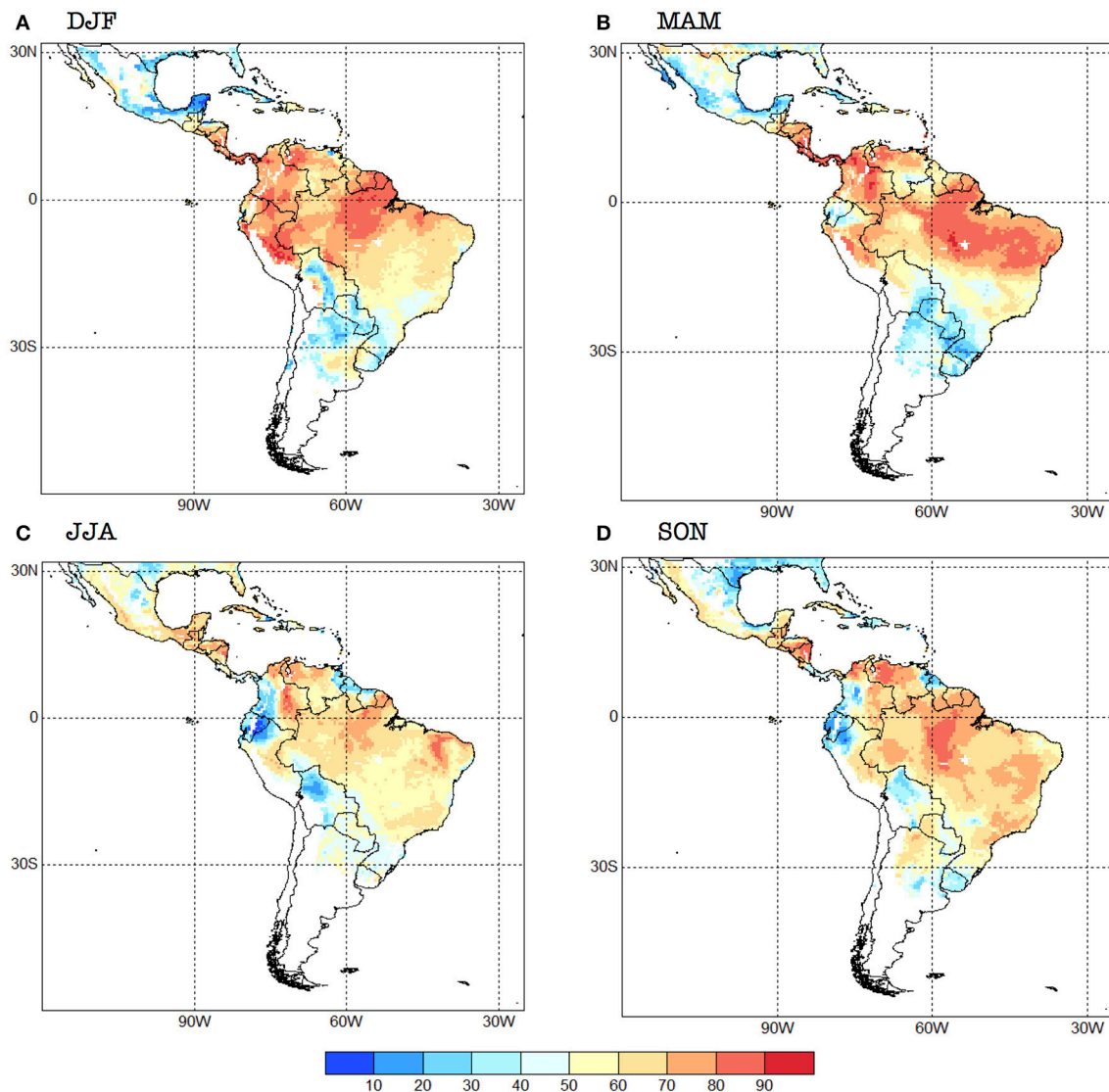


FIGURE 4 | 2AFC skill score for the seasonal forecast system for each one of the four seasons selected: **(A)** DJF, **(B)** MAM, **(C)** JJA and **(D)** SON. Units in %. The 2AFC score is an indication of how often the forecasts are correct; it also measures how well the system can distinguish between the above-normal, normal, and below-normal categories.

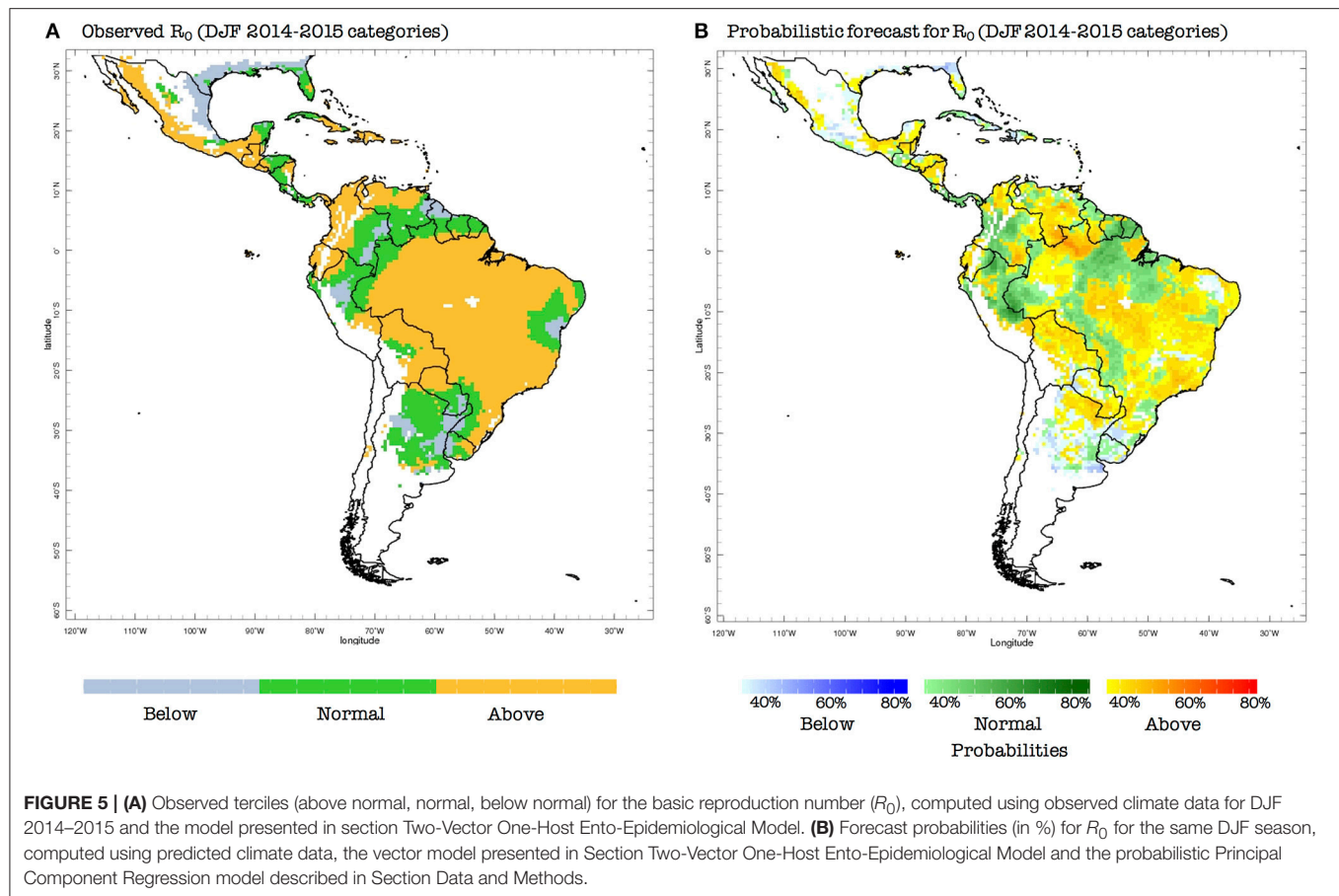
forecast in the ZIKV hotspot places (in Brazil, for example), and as discussed above, the normal category the northern half of South America is already conducive to epidemic conditions. Hence, we claim that this particular forecast, even if not perfect, could have been useful for decision-makers at the time (November 2014), assuming that they already knew that ZIKV was already circulating in the region, which was of course not the case.

The previous example also illustrates why this tool can only be used as a guide for the local and international experts, as these diseases involve complex interactions beyond the presence or not of enhanced environmental (climatic) conditions suitable for the occurrence and transmission of *Aedes*-borne epidemics.

CONCLUDING REMARKS

We have discussed the development and predictive skill of a new probabilistic forecast system to estimate climatic suitable conditions for potential risk of transmission of diseases like ZIKV, DENV, and CHKV. To the best of our knowledge this is the first seasonal forecast system of this type for Latin America and the Caribbean, although it is conceptually similar to a malaria forecast system developed for Africa years ago (Thomson et al., 2006).

Instead of focusing on the different *Aedes*-borne diseases separately (for which some model parameters are still uncertain or are actually unknown, as for example in the case of



zika), our approach addresses suitable conditions for the risk of transmission of these diseases as a whole. This idea is consistent with the information required by international health agencies and general health practitioners. As a matter of fact, although the two-vector model used in this study was developed by Caminade et al. (2017) for zika using some dengue-like parameters, they reported notorious epidemic hotspots for 2015 for Angola and the Democratic Republic of Congo (which reported very active circulation of yellow fever), and for India (which reported high number of dengue cases in the south of the country). Although further verification studies are needed, these results seem to support our argument for a generalized potential risk of transmission of *Aedes*-borne diseases.

From the regional perspective, this forecast system has the potential to help the Pan-American Health Organization (PAHO), the World Health Organization (WHO) and other decision-makers to prepare more detailed epidemiological alerts and guides for zika's surveillance and other arboviruses; to calculate different levels of population at risk and incidence rates for regional assessment, to prepare vector control guidelines for a more integrated management; to plan and support vector control resources an equipment; to organize and program activities and resource mobilization, as well as improve risk communication materials. One of the co-authors (PN) has already started

to explore ways to take advantage of this forecast system at PAHO/WHO.

Our system is a first attempt to provide predictive tools for health practitioners and decision-makers interested in *Aedes*-borne diseases in Latin America and the Caribbean, and can be considered an additional step in the direction followed by other research groups (Kraemer et al., 2015; Carlson et al., 2016; Lessler et al., 2016; Messina et al., 2016; Monaghan et al., 2016; Samy et al., 2016; Caminade et al., 2017).

Indeed, forecasts of health events are designed to change human behavior. Nonetheless, as with the practice of medicine, there are ethical issues to consider. It is possible that there might be negative consequences from an epidemic risk forecast (i.e., incidence, or cases), even if the prediction is skillful. To illustrate this idea, consider that a forecast for ZIKV is provided to the community, indicating that there is above 80% probability of acquiring the disease in Rio de Janeiro during a certain season, but less than 10% probability of infection in Montevideo. People—some of whom could already be infected with ZIKV, or even with a different disease—might decide to travel to Montevideo instead of Rio de Janeiro because of that forecast, thus igniting or being part of a new focus of an epidemic there, that was not predicted and that is partially caused by the original prediction itself. This is an important caveat to be considered by the decision-makers. Another consideration is that

a ZIKV forecast may have negative consequences for tourism, leading to livelihood impacts that may have negative health consequences.

There are a number of important limitations related to our forecast system. As indicated earlier in this paper, by itself this kind of system cannot forecast the occurrence and spread of new epidemics, but only partial conditions for that to happen. The model employed here only considers the effect of climatic conditions, through temperature and rainfall, on disease transmission via the vectors and viruses of interest. Direct human-to-human transmission via sexual intercourse and blood transfusion are outside the scope of this modeling approach. Also, the present version of the model cannot simulate co-infections or mixed states (e.g., a fraction of the population recovered from dengue but still susceptible to zika infections).

One particular way in which the model needs to be improved involves how rainfall is considered. The present version of the model only uses rainfall in a rather simplistic way, without really considering its seasonal characteristics. There are examples in the scientific literature that could be used to improve the representation of rainfall in this type of model (see for example, Magori et al., 2009; Santos et al., 2009; Morin and Comrie, 2010). In addition, it is key to have a good representation in the model of immunity and viral re-introduction (see Ferguson et al., 2016; Lourenco et al., 2017). There is also room to consider a better set of realizations in the ensemble of simulations, varying the ento-epidemiological parameters of the model. These options for further model development will be explored in the near future.

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AUTHOR CONTRIBUTIONS

ÁM and MT established the concept of the study. ÁM obtained the data. All authors undertook the analysis and interpretation of results. ÁM, MT, and AS drafted the manuscript. All authors critically reviewed and revised the manuscript and agreed the final submission.

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Potential Risk Areas of *Aedes albopictus* in South-Eastern Iran: A Vector of Dengue Fever, Zika, and Chikungunya

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The possibility of the rapid and global spread of Zika, chikungunya, yellow fever, and dengue fever by *Aedes albopictus* is well documented and may be facilitated by changes in climate. To avert and manage health risks, climatic and topographic information can be used to model and forecast which areas may be most prone to the establishment of *Ae. albopictus*. We aimed to weigh and prioritize the predictive value of various meteorological and climatic variables on distributions of *Ae. albopictus* in south-eastern Iran using the Analytical Hierarchy Process. Out of eight factors used to predict the presence of *Ae. albopictus*, the highest weighted were land use, followed by temperature, altitude, and precipitation. The inconsistency of this analysis was 0.03 with no missing judgments. The areas predicted to be most at risk of *Ae. albopictus*-borne diseases were mapped using Geographic Information Systems and remote sensing data. Five-year (2011–2015) meteorological data was collected from 11 meteorological stations and other data was acquired from Landsat and Terra satellite images. Southernmost regions were at greatest risk of *Ae. albopictus* colonization as well as more urban sites connected by provincial roads. This is the first study in Iran to determine the regional probability of *Ae. albopictus* establishment. Monitoring and collection of *Ae. albopictus* from the environment confirmed our projections, though on-going field work is necessary to track the spread of this vector of life-threatening disease.

Keywords: *Aedes albopictus*, dengue fever, zika virus, modeling, analytical hierarchy process, geographical information system, remote sensing

INTRODUCTION

The Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894) (*Diptera*: *Culicidae*), is known as a competent vector for at least 22 arboviruses, including Zika, dengue fever (DF), and chikungunya (Gratz, 2004; Wong et al., 2013; Collantes et al., 2015). Several studies have warned about the rapid expansion of *Ae. albopictus* around the globe (Roiz et al., 2011; Rochlin et al., 2013; Kraemer et al., 2015). Despite being incapable of flying a distance greater than 800 m, this species has been able to spread from native tropical and subtropical areas of Southeast Asia to America, Europe and Africa as well as to Indo-Pacific and Australian regions in a matter of decades (Roiz et al., 2011; Bueno-Marí and Jiménez-Peydró, 2015; Kraemer et al., 2015). Specimens of *Ae. albopictus* have been observed in southeast Iran (Doosti et al., 2016), which was not unexpected after detecting dengue seropositivity in residents of the south-eastern province of Sistan and Baluchestan (Chinikar et al., 2013). Iran remains at risk of increasing colonization by *Ae. albopictus* from neighboring Pakistan, where there is a history of DF outbreaks (Mukhtar et al., 2011; Rasheed et al., 2013; Khan et al., 2015; Suleman et al., 2016). Chikungunya and Zika have been identified in mosquitoes and sporadically in humans in Pakistan, with positive serological tests signifying widespread population exposure to this virus (CDC, 2016; Kindhauser et al., 2016).

In the late autumn of 2014, *Ae. albopictus* was first reported in the Sistan and Baluchestan Province of Iran in the southeast of the country bordering Pakistan (Doosti et al., 2016). Prior to that, in 2012, the presence of specific IgG, IgM and viral nucleic acid of dengue virus had been detected in the blood of Iranian residents of the province, and were referred to the arboviruses laboratory of the Pasteur Institute of Iran (Chinikar et al., 2013). Again, in 2014, the dengue virus was detected in blood donors in the Chabahar district of the then province (Aghaie et al., 2014).

Dengue fever and, more recently, the Zika virus are considered serious threats to human health due to their increasing abundance and the adaption of their vectors (Banu et al., 2011; Medlock et al., 2012; Petersen et al., 2016). The transmission risk of these viruses depend on the densities of *Ae. albopictus* which, in turn, depend on climatic parameters that control their habitat. Climatic factors can be used to forecast potential establishment areas of *Ae. albopictus* via modeling, which include: mean annual temperature ($\text{AnnT}^{\text{mean}}$), precipitation, altitude, and relative humidity (RH) (Roiz et al., 2011; Sarfraz et al., 2014; Bueno-Marí and Jiménez-Peydró, 2015; Collantes et al., 2015). One of the most successful techniques in modeling vectors of DF is known as the 'Analytical Hierarchy Process' (AHP) (Aziz et al., 2012). AHP and other climate models can be coupled with Geographic Information Systems (GIS) and remote sensing (RS) to predict areas where disease-vectors may establish, as well as for raising awareness of the status of vector-borne diseases in an effective and timely manner (Roiz et al., 2011; Sarfraz et al., 2014). We utilized these models and methods to forecast the proliferation of *Ae. albopictus* and possible dengue/Zika outbreaks in the southeast of Iran.

Managing outbreaks of dengue or Zika virus would pose significant challenges and could strain a health system already involved in the control of arboviral and parasitic diseases such as Crimean-Congo haemorrhagic fever (Izadi et al., 2004; Mostafavi et al., 2013) and malaria (Vatandoost et al., 2011; Hanafi-Bojd et al., 2012; Nejati et al., 2013). The aim of this study was to identify areas of human health risk posed by the colonization of *Ae. albopictus* in south-eastern Iran and lay the groundwork for future monitoring using GIS and RS.

MATERIALS AND METHODS

Study Area

The study was conducted in the Sistan and Baluchestan province located in southeast Iran, neighboring Pakistan and Afghanistan. It is the largest province in Iran, with an area of 181,785 km², and shares a long border with Pakistan (Figure 1). It has a population of approximately 2,534,000 residents in 19 cities, 37 towns, and 9716 villages with an annual growth rate of 1.05% (SCI, 2011)¹. The climate is generally arid (Amiraslani and Dragovich, 2011) with dust storms and 120-day winds (Alizadeh-Choobari et al., 2014). However, there is substantial climatic diversity due to the dominance of seasonal subtropical high pressures over a large part of the land mass, large internal deserts, the Alpine-Himalayan folded system and the surrounding Jazmurian basin to the west. The influences of the Arabian Sea, to the south, and Hirmand basin, in the north, result in periodic Monsoon systems that can cause a notable ecological phenomenon (SBMO, 2016)². This part of Iran is one of the few with summer rainfall and the main source of humidity stems from the Bengal Gulf streams, coming only in summer (Dinpashoh et al., 2004). Common landscapes in the province are shown in Figure 2.

Identification of Parameters

It was necessary to identify the most important ecological and climatic parameters that affect the survival of *Ae. albopictus* prior to formulating the AHP model. Based on an extensive literature review and consultation with experts on *Ae. albopictus* ecology (Dr. Rubén Bueno-Marí, Dr. Francisco Collantes, and Prof. David Roiz), eight criteria were selected that have the greatest effect on distributions of *Ae. albopictus* populations; these include: precipitation, relative humidity, temperature, land use/anthropization, altitude, wetlands, normalized difference vegetation index (NDVI), soil type, and distance from seaports.

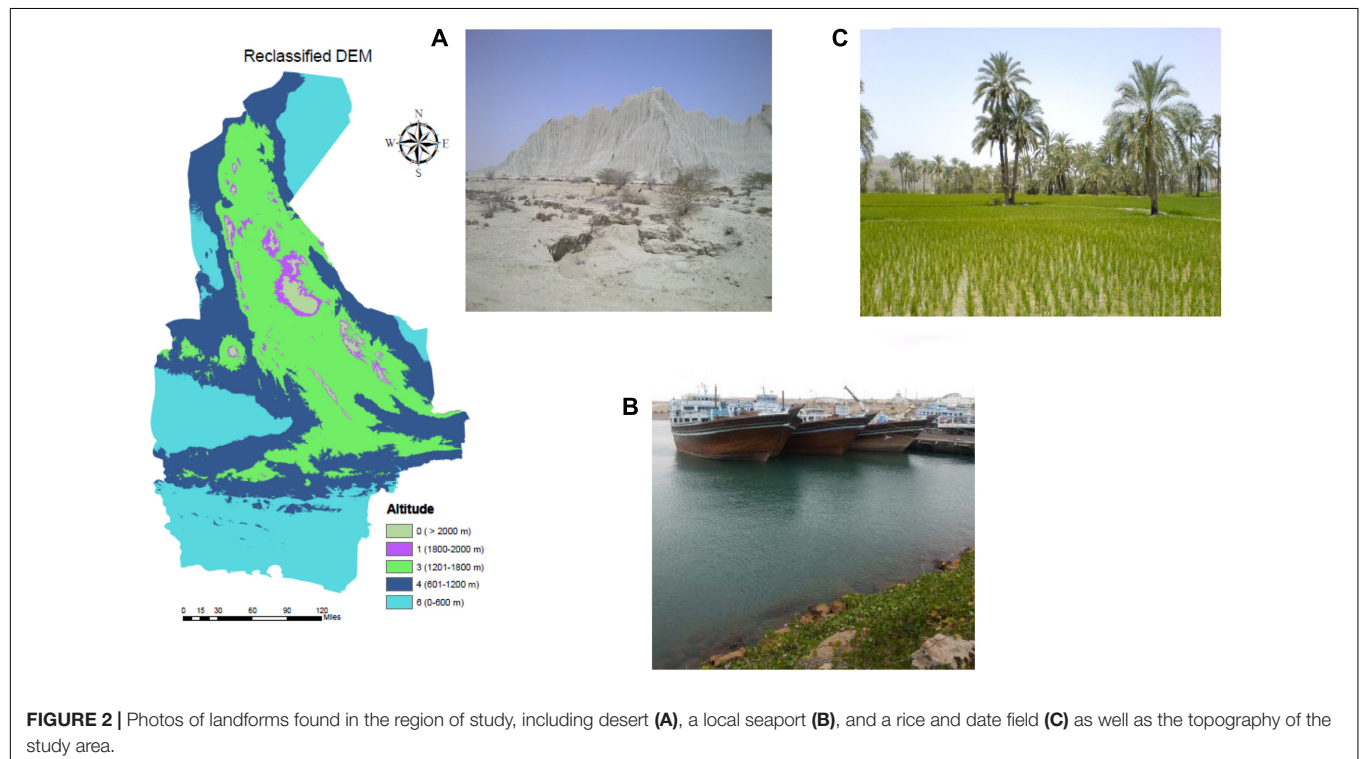
Analytical Hierarchy Process

Formulation of AHP Model

Analytical Hierarchy Process is a structured technique that incorporates both mathematical and psychological components to represent elements of a problem quantitatively in order to facilitate decision-making (Aziz et al., 2012). The priorities and the impact of each element are computed based on the

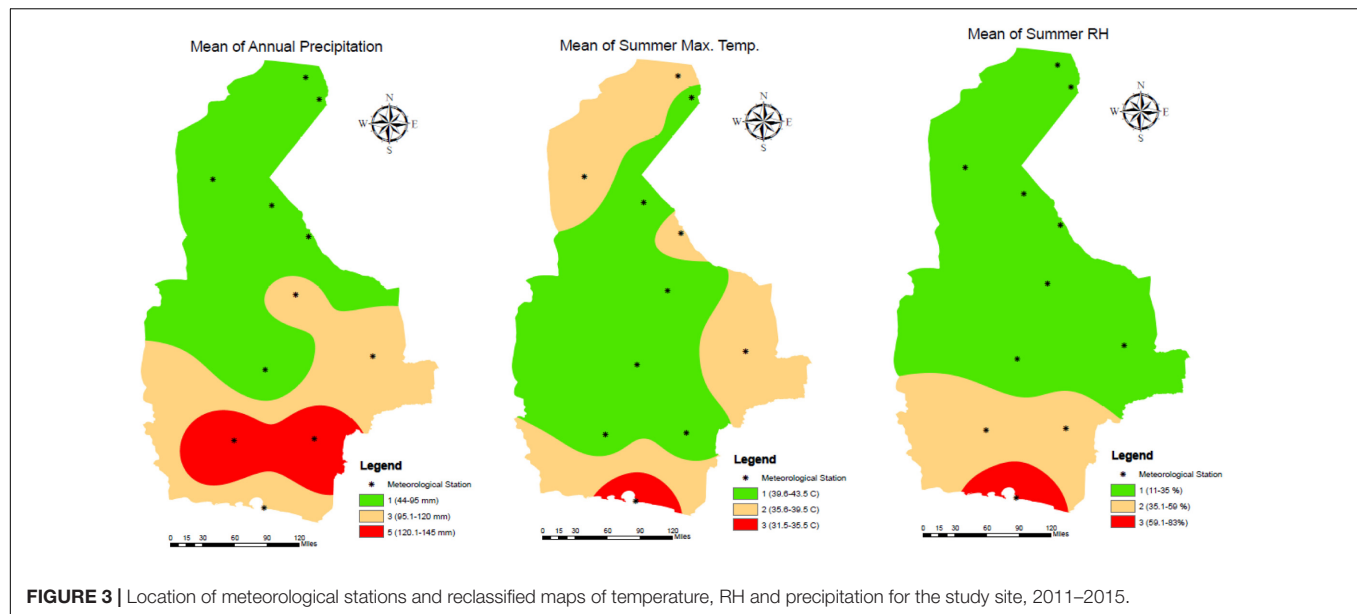
¹<https://www.amar.org.ir/Portals/1/Iran/90.pdf>

²<http://www.sbmet.ir/userfiles/uploads/climatesb.pdf>



score assigned by the judgments (Saaty, 1990). A pairwise comparison matrix that included the eight aforementioned factors was used to survey 11 scholars and experts who have written authoritative articles on *Ae. albopictus* ecology, and are from Belgium, France, Germany, Iran, Italy, Spain, Sri Lanka,

and the United Kingdom. Participants were provided guidelines that asked them to rate the importance of each factor defined by numbers 1–9 or the reciprocal (i.e., 1/2–1/9). The ranking system was as follows: ‘1’ designated equal importance; ‘3’ moderate importance; ‘5’ essential importance; ‘7’ importance;



and ‘9’ absolute importance. Even numbers were designated as intermediate values between the two adjacent judgments. The reciprocal was used to demonstrate the relative importance of variables to each other. For example, ‘1/8’ meant that variable ‘x’ is less importantly than ‘y,’ while the reverse (‘8’) meant that ‘x’ was more important. All responses were averaged based on the geometric mean and the weight and priority of each parameter was obtained by Expert Choice 11 software (Ishizaka and Labib, 2009). This software is a powerful decision-making implementation of AHP.

DATA COLLECTION

Meteorological Parameters

Five-year weather data was collected from 11 meteorological stations across the Sistan and Baluchestan province of Iran. These stations were in the following cities/regions: Zabol: 1, Zahak: 2, Zahedan: 3, Mirjaveh: 4, Nosratabad: 5, Khash: 6, Saravan: 7, Iranshahr: 8, Rask: 9, Nikshahr: 10, and Chabahar: 11 (Figure 3). Meteorological data was collected over 60 months, from January 2011 to December 2015 and is summarized in Table 1. We focused on precipitation, temperature and RH data. Precipitation creates conditions that provide breeding sites and a moist microclimate for adult mosquitoes (Sarfranz et al., 2014). Annual, spring, summer, autumn, and winter precipitation values were not significantly correlated to each other (Correlations test; $P > 0.05$). Pluviometric conditions with annual rainfall between 200 and 500 mm have been reported in established areas (Bueno-Marí and Jiménez-Peydró, 2015). Although studies have shown a correlation between seasonal rainfall and *Ae. albopictus* density, differing views exist in the literature. This species can propagate in some rainfall-independent breeding sites because of its behavior as a container-breeding mosquito (Waldock et al., 2013). Due to the dry climate and low

rainfall of the province, we opted to use annual precipitation (AnnP) based on the average precipitation at each meteorological station.

Aedes albopictus cannot typically survive average temperatures less than -5°C or greater than $+41^{\circ}\text{C}$, though snow cover, in winter, and shade, in summer, can mitigate temperature and provide refugia (Brady et al., 2013). A mean annual temperature ($\text{AnnT}^{\text{mean}}$) of 11°C is also considered as a critical temperature for cessation of *Ae. albopictus* life cycle (Roiz et al., 2011). A variety of temperature calculations were assessed, including the monthly temperature, mean of maximum summer temperature (MxST), mean of minimum winter temperature (MiWT), mean of winter temperature (MWT), and temperature in January. A significant correlation was found between MxST, MiWT, and MWT ($P < 0.05$). Given the arid climate in our study area, we selected MxST as a major constraint in our model.

Conditions of high temperature and low RH have deleterious effects on adult populations (Alto and Juliano, 2001). Humidity also has a direct effect on the durability of breeding sites and larval survival rates, where high RH (68–80%) can increase fecundity (Sarfranz et al., 2014). Among diapause eggs, *Ae. albopictus* can survive the longest at low RH (44%) compared to *Stegomyia* strains (Sota and Mogi, 1992). RH values for annual, spring, summer, autumn, and winter were analyzed and all showed significant pairwise correlation. Without a real difference between each variable, we utilized the mean summer RH (MSR) at each station as a limited factor for modeling.

Remote Sensing Parameters and Image Pre-processing

Images from Landsat and Terra satellites were used to calculate the Digital Elevation Model (DEM), water bodies, urban/rural (residential) areas and NDVI. For all but DEM, calculations were based on 16 images from the Landsat 8 satellite, each covering an area of 185 km^2 .

TABLE 1 | Meteorological data from all weather stations collected from 2011 to 2015.

Station No.	Climate*	Temperature average within 5-year study (°C)		Precipitation average within 5-year study (mm)		RH average within 5-year study		Annual average of days < 0°C*	Average annual of days < -5°C	Average annual of days > 35°C*
		Mean ± STDEV	Confidence interval 95%	Mean ± STDEV	Confidence interval 95%	Mean ± STDEV	Confidence interval 95%			
1	Hot and dry	23.2 ± 1.3	20.6–25.7	44.38 ± 17.4	36.6–52.1	30.8 ± 1.8	27.3–34.5	17	0	155
2	Hot and dry	23.1 ± 1.2	20.7–25.6	57.82 ± 32.1	43.4–72.2	30.1 ± 1.8	26.5–33.7	13	0	165
3	Semi-arid and warm/temperate	19.1 ± 1.0	17.1–21.2	75.42 ± 29.43	62.3–88.6	30.1 ± 1.6	26.9–33.4	43	0	90
4	–	23.9 ± 1.1	21.6–26.1	54.6 ± 37.7	37.8–71.5	22.5 ± 1.5	19.6–25.4	–	0	–
5	–	22.5 ± 1.2	20.0–24.9	87.9 ± 45.5	67.5–108.2	25.2 ± 1.7	21.6–28.7	–	0	–
6	Semi-arid and warm/temperate	20.4 ± 1.0	18.4–22.5	106.0 ± 73.3	73.3–138.8	28.1 ± 1.5	25.0–31.2	20	0	110
7	Semi-arid and warm/temperate	22.3 ± 1.1	20.2–24.3	103.9 ± 56.9	78.3–129.3	28.8 ± 1.1	26.6–31.1	10	0	139
8	Hot and dry	27.2 ± 1.1	25.1–29.4	64.9 ± 42.1	46.1–83.8	26.2 ± 1.1	23.9–28.5	1	0	187
9	Hot	28.5 ± 0.8	26.9–30.1	145.6 ± 89.1	105.7–185.4	38.7 ± 1.4	35.8–41.6	0	0	24
10	Hot	27.9 ± 0.8	26.2–29.6	142.0 ± 39.4	124.4–159.6	38.8 ± 1.2	36.6–40.9	0	0	231
11	Costal	26.5 ± 0.5	25.6–27.5	106.7 ± 50.7	84–129.4	71.9 ± 1.5	68.8–74.9	0	0	13

*Based on the annual report of the provincial meteorological organization (SBMO, 2016)².

A seamless coverage of the study area was prepared by tiling satellite images that had been color balanced and mosaicked using ENVI V.5.0 (EXELIS Visual Information Solutions, Boulder, CO, United States). Residential areas were classified using visual interpretation on virtual color combination ($RGB = \text{bands } 7, 5, \text{ and } 2$). DEM was calculated from 3N and 3B bands taken from the ASTER (Advanced Space-borne Thermal Emission and Reflection Radiometer) sensor on the Terra satellite with a nominal resolution of 25–30 meters for each pixel size. The normalized index of different water (NDWI) was used to calculate the water bodies' parameter. The index was defined as follows: $(B2-B7)/(B2+B7)$, where values greater than 0.2 were considered as bodies of water. NDVI was generated using red and near-infrared bands of Landsat 8 with the following equation: $(B5-B4)/(B5+B4)$.

Classification and Valuation of Factors

Prior to GIS mapping, factors were classified based on literature reviews. To ensure the quality of the classification, we received confirmation from three international scholars (Dr. Rubén Bueno-Marí, Dr. Francisco Collantes, and Prof. David Roiz). Precipitation ranged from 44 to 146 mm and was grouped into three classes: 44–95 mm, 95.1–120 mm, and 120.1–146 mm and attributed three values: 1, 3, and 5. The summer maximum average temperature ranged from 31.5 to 43.5°C and was also grouped into three classes with descending values 3, 2, and 1, with lower values allocated to the high temperatures to signify a deterrent role in adult mosquito survival. The classification maps based on inverse distance weighted (IDW) interpolation of temperature, RH and precipitation are presented in **Figure 2**.

Colonies of *Ae. albopictus* are commonly reported in urban and suburban areas (Devi and Jauhari, 2004; Roiz et al., 2011; Bueno-Marí and Jiménez-Peydró, 2015; Collantes et al., 2015; Dhimal et al., 2015). *Ae. albopictus* is a weak flier with a flight range of less than 1 km (Rochlin et al., 2013). The unaided flight range of this species has been reported to be between 100 and 800 m with the average falling in the range of 100–200 m followed by 200–400 m (Honório et al., 2003; Nihei et al., 2014). Based on these results, we drew concentric circles and hazard bands (buffer) up to 800 m via straight-line distance around any polygons, ploy lines and points of cities, villages, roads, costumes, international airports, railway stations, and sea/ground entrance ports. The land use/anthropization parameter was then grouped into six classes: 0–100 m, 101–200 m, 201–400 m, 401–600 m, 601–800 m, > 800 m and assigned descending values: 6, 5, 3, 2, 1, and 0, respectively.

Altitude was considered as a limitation to the dispersal of *Ae. albopictus* with the threshold set at 2100 m (Dhimal et al., 2015) and greatest expected density of survivorship at 300–600 m (Devi and Jauhari, 2004). Elevation ranged between 0 and 3912 m for the study area and was grouped into five classes and values including 0–600 m (6), 601–1200 m (4), 1201–1800 m (3), 1801–2000 m (1) and > 2000 m (0). The classification map of altitude is shown in **Figure 2**.

Water bodies were attributed a 200 m buffer and classified into two classes: pixels with (2) and without wetlands (1). The vegetation or land cover was used to assign potential breeding

TABLE 2 | The scale, classes, and values of the parameters used in Geographic Information Systems (GIS) mapping.

Criteria	Scale	Min-max in the study area	Classification and value					
			Class 1 (value)	Class 2 (value)	Class 3 (value)	Class 4 (value)	Class 5 (value)	Class 6 (value)
Precipitation	Average of annual precipitation 2011–2015	44–146 mm	44–95 (1)	95.1–120 (3)	120.1–146 (5)	–	–	–
Relative Humidity (RH)	Average of summer RH 2011–2015	11–83%	11–35% (1)	35.1–59% (2)	59.1–83% (3)	–	–	–
Temperature	Average of maximum summer Temperature 2011–2015	31.5–43.5°C	31.5–35.5 (3)	35.5–39.5 (2)	39.5–43.5 (1)	–	–	–
Land use/Anthropization	Villages+Cities	–	0–100 (6)	100–200 (5)	200–400 (3)	400–600 (2)	600–800 (1)	> 800 (0)
Altitudes	Elevation	0–3912 m	0–600 (6)	600.1–1200 (4)	1200.1–1800 (3)	1800.1–2000 (1)	> 2000 (0)	–
Wetlands	Surface water bodies	*200 mbuffer	Wetland (1)	No wetland (0)	–	–	–	–
NDVI	NDVI *100 m buffer	–1–1	–1–0 (1)	0–0.15 (3)	0.15–0.50 (4)	0.50–0.75 (5)	> 0.75 (6)	–
Distance from Border	Sea ports + Entrance points + Customs + Int. airports + Int. rail station	–	0–100 (6)	100–200 (5)	200–400 (3)	400–600 (2)	600–800 (1)	> 800 (0)

habitats (Sarfratz et al., 2014). NDVI ranged from -1 to 1 and was grouped into five classes: -1 to 0 indicated areas without vegetation; 0 to 0.25 indicated low vegetation; 0.25 to 0.50 indicated moderate vegetation; 0.50 to 0.75 indicated high vegetation and 0.75 to 1 designated densely vegetated (or 'very high'), with a value of 6 assigned to areas of very high vegetation and a value of 0 to areas without vegetation.

The 'distance from border' was calculated the same way as land use/anthropization and was grouped into six classes with descending values. The scale, classes and values of each criterion are shown in **Table 2**.

GIS Analysis

Each of the eight factors were added as a layer in ESRI ArcGIS 10.3 software. The IDW was used to interpolate the boundaries between meteorological stations for precipitation, RH and temperature. They were reclassified based on previous classification and valuation. The reclassification was also applied for other layers. Before reclassifying, the straight-line approach was implemented for buffering around cities, villages, seaports, entry points, international airports, and railway station. **Figures 2, 3** show the reclassified map of altitude and meteorological data. Although the distance from a border was conflated with distance from land and sea in certain situations, we considered all seaports, each entry point to the province from Pakistan (ranging from the gravel roads to the paved and asphalted), international airports, costumes, and international railway station for preparation of this layer via punctuation in Google Earth and transferring to the GIS. The final predictive model exhibiting areas with the greatest potential for proliferation of *Ae. albopictus* was developed using Spatial Analyst of ArcMap. The weight of each parameter (layer), previously obtained by AHP, was used for raster calculation. A summary of our methodology is provided in **Figure 4**.

RESULTS

AHP; Criteria Weighting and Priority by EC

After weighing the eight criteria by AHP, the factor with the greatest weight (0.274) and priority was land use/anthropization for assessing the presence of *Ae. albopictus*. The next best criteria were temperature, altitude and precipitation, while proximity to wetland had the lowest weight (0.042) and priority (**Figure 5**). The inconsistency of this analysis was 0.03 with no missing judgments.

Predictive Modeling

Potential Habitable Regions of *Ae. albopictus*

Areas where *Ae. albopictus* has the potential to be present are shown in **Figure 6** based on the representation of the AHP model. The Natural Breaks (Jenks) classification method divided the potential presence of this species into four classes: 0.759 – 1.605 , 1.606 – 2.061 , 2.062 – 2.572 , and 2.573 – 4.1834 . Classes 3 and 4, where values are greater than > 2.06 , can be considered areas

with potential of presence for *Ae. albopictus*. The map shows that southern areas with a coastal climate are most suitable for supporting populations of *Ae. albopictus*.

Map of Risk Areas

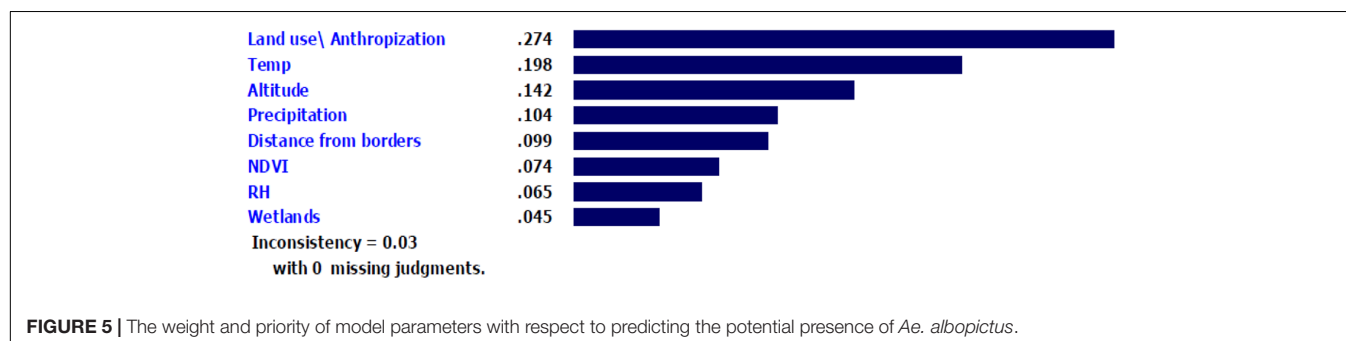
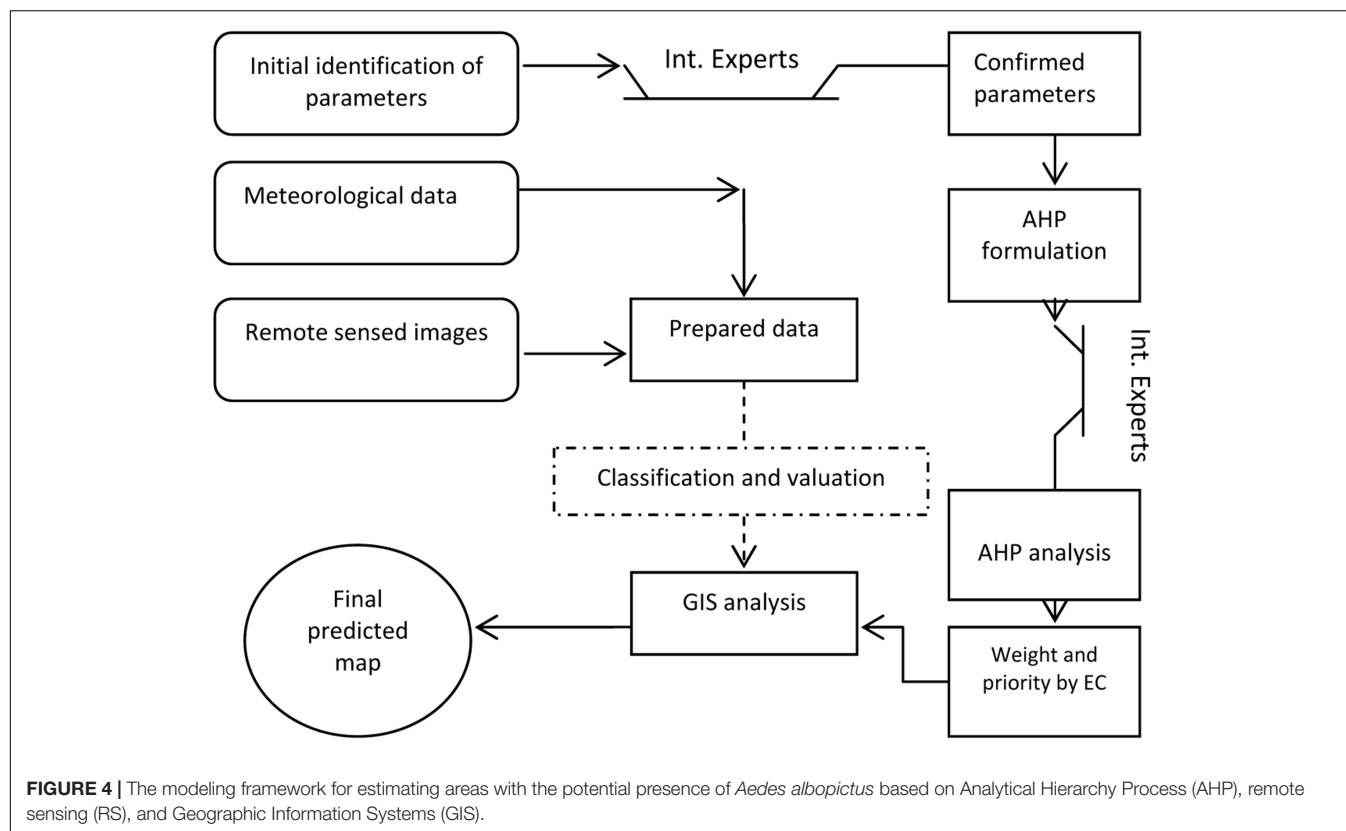
To identify areas with greatest potential risk, we multiplied the estimated presence of *Ae. albopictus* by the main means of dispersal. Therefore, we added the reclassified layer of provincial roads with a maximum 800 m buffer via Euclidian Direction. This map shows the risk areas and most likely habitable regions for *Ae. albopictus*. Again, the potential of presence of this species was classified into four classes with values 0 – 2 based on the Natural Breaks classification method. Classes 3 and 4 with values > 0.49 show the moderate and high-risk areas with potential of presence for *Ae. albopictus* (**Figure 6**). The majority of risk areas are found in the southern part of the province, which include 834 villages. Doosti et al. (2016) previously reported the detection of *Ae. albopictus* in four of these villages (Vashname dorri, Paroomi, Lashar, and Rask; noted in **Figure 6**), confirming the validity of our model and the provided map. Also, some bioclimatic variables and altitudes of the collection points of *Ae. albopictus* are presented in **Table 3**.

DISCUSSION

This is the first study to model areas in Iran at greatest risk for colonization by disease-vectors *Ae. albopictus*. Our model's predictions are in agreement with the most recent field reports, demonstrating the utility of our risk map for forecasting the spread of *Ae. albopictus* and for monitoring and ensuring preparedness for potential DF or Zika outbreaks. To date, only dengue has been detected in Iran (Chinikar et al., 2013), but in neighboring Pakistan, dengue, Chikungunya fever, and sporadic human cases of Zika have been reported along with the detection of infected mosquitoes (Kindhauser et al., 2016). These findings clearly indicate the risk posed by the colonization of south-eastern parts of Iran by *Ae. albopictus*.

Aedes albopictus is not the primary vector of Chikungunya and dengue viruses (Waldock et al., 2013; Collantes et al., 2015; ECDC, 2016) and has a lesser role in the transmission of Zika compared to *Ae. aegypti* (Chouin-Carneiro et al., 2016; Jupille et al., 2016). Yet, the competence of *Ae. albopictus* for Zika transmission has been proven in a laboratory setting (Di Luca et al., 2016; WHO, 2016) and, recently, the African lineage of Zika has been isolated from *Ae. albopictus* in Gabon (Grard et al., 2014). Though there has yet to be a case of *Ae. albopictus* transmission of Zika, the risk should not be ignored as the virus has been isolated in its salivary glands, suggesting a strong likelihood of it acting as a vector for Zika (Ioos et al., 2014).

The selection of valid parameters has an important role in developing a reliable model and can be achieved through knowledge of mosquito ecology (Waldock et al., 2013). We were able to select factors through an extensive literature review and the participation of international scholars. The most heavily weighted parameters identified in our model have been previously used in modeling studies, such as land use, land cover,

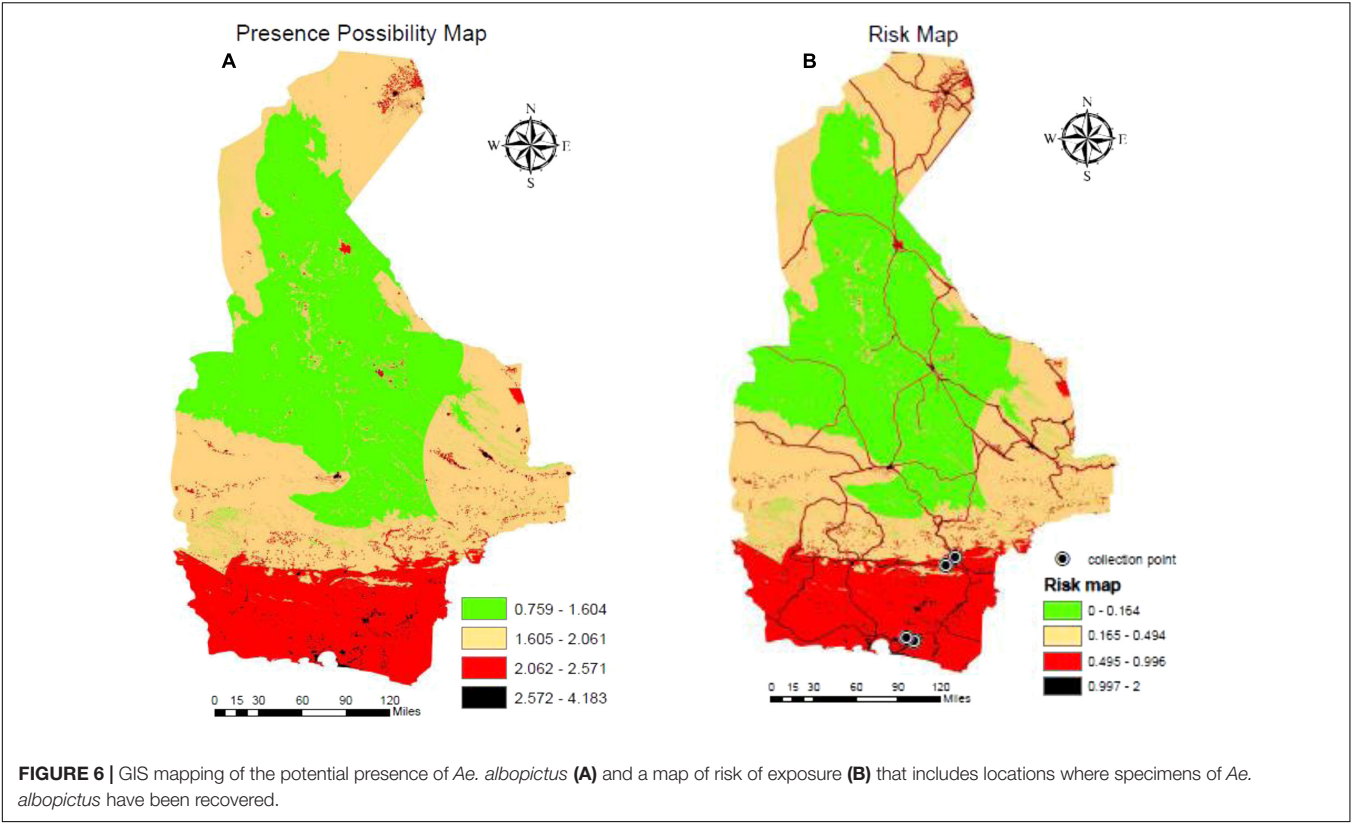


temperature, precipitation, RH, and altitude (Aziz et al., 2012; Rochlin et al., 2013; Sarfraz et al., 2014).

Our finding that land use/anthropization had the highest weight and priority, followed by temperature, altitude, and precipitation, paralleled the findings from a previous study that used AHP to model dengue hotspots in Malaysia (Aziz et al., 2012). Similarly, land use was determined as the second most important variable in a modeling study in the north-eastern United States. Hotspots for *Ae. albopictus* were more urban and suburban due to the affinity of this species to urbanized environments (Rochlin et al., 2013). Villages, too, are likely habitats (Ganushkina et al., 2016) and were where the first collection of this species in Iran occurred. Therefore, we calculated villages similar to cities in our model.

Despite parallels to previous work, our identification and selection of some parameters was different. Most papers have

constrained *Ae. albopictus* fitness using temperatures in January, whereas we utilized the average summer maxima (Takumi et al., 2009; Neteler et al., 2011; Caminade et al., 2012). The temperature in January would be a stronger limiting factor in the Northern hemisphere (Waldock et al., 2013). Due to the dominance of hot and dry climates in our study area, overwintering was easier than escape from summer heat given that minimum temperature for biological activity of *Aedes* mosquitoes is 6–10°C (Dhimal et al., 2015) and temperatures do not drop below 5.5°C in our region of study. On the other hand, the average annual of glacial days (days when the temperature drops below freezing) in each station was rare, while days with a temperature greater than 35°C were common. At temperatures > 40°C, the survival of immature and adult stages of *Ae. albopictus* are negatively impacted, but these conditions are less documented (Waldock et al., 2013). In this region of Iran, it is possible that



summer temperatures are more important for modeling because the most suitable temperature range for reduction of extrinsic Dengue incubation is between 32 and 35°C (Alto and Juliano, 2001).

Relative humidity is considered an effective predictive measure of population dynamics and egg, larval and adult stages of *Ae. albopictus* (Alto and Juliano, 2001; Waldoock et al., 2013). This factor was selected in much the same way as other studies, but, again, with a slight difference. In contrast with other studies that have applied annual RH (Almeida et al., 2005), we used the average RH in summer. In our case, hot and dry regimes with low RH in the summer are likely to be a

stronger limiting factor due to reduction in adult reproduction (Alto and Juliano, 2001). Yet, due to the important role of high RH in increasing abundances (Waldoock et al., 2013; Sarfraz et al., 2014), we allocated greater values to high RH in the classification phase. Similar to low RH, annual rainfall plays an important part in most modeling (Aranda et al., 2006; Dhimal et al., 2015) as a limited factor (Medlock et al., 2006). The importance of this parameter grows under drought conditions when aquatic habitats dry up (Alto and Juliano, 2001). Thus, we weighted our criteria (i.e., decussate values 1, 3, and 5 were chosen for low precipitation to high) since Sistan and Baluchestan province has experienced drought in recent years (Abbaspour and Sabetraftar, 2005; Lashkaripour and Zivdar, 2005).

Apart from meteorological parameters, certain landforms can play an important role in the presence of *Ae. albopictus* (Rochlin et al., 2013). The inclusion of wetlands in our model did not have a significant predictive value. This is consistent with the fact that *Ae. albopictus* is considered a peridomestic mosquito and is known as a container-breeding mosquito that prefers rain-filled containers (Vanwambeke et al., 2011; Dhimal et al., 2015). Wetlands can provide a natural reservoir that can enable it to spread quickly (Dale and Knight, 2008) and small numbers of *Ae. albopictus* have been collected from the wetlands of Valencian autonomous regions (Bernues Baneres et al., 2012). Also in Iran some of larvae of this species were collected from natural breeding sites (Doosti et al., 2016). Thus, we included wetlands in our model, but assigned a low

TABLE 3 | Bioclimatic variables at collection points of *Aedes albopictus*.

Variable	Min	Max	Mean ± SD	Confidence interval 95%
Altitude	8	427	280 ± 235.8	143.9–416.1
Annual precipitation*	106.7	145.6	131.4 ± 21.5	119–143.8
Winter precipitation*	38	62.8	51.6 ± 12.6	44.3–58.9
Annual Temperature*	26.6	28.5	27.6 ± 1.0	27.0–28.2
Winter Temperature*	19.5	21.5	20.5 ± 1.0	20.0–21.1
Summer Temperature*	29.8	33.6	32.5 ± 2.4	31.1–33.9
Annual RH*	38.7	71.8	49.8 ± 19.1	38.7–60.8
Winter RH*	38.1	60.5	59.8 ± 20.9	47.8–71.9
Summer RH*	43.9	83.4	48.8 ± 49.0	47.7–57.6

*Average of the variable during 2011–2015.

value (1) commensurate with the small role of wetlands in its ecology.

The effects of global warming on climate and landforms is commonly argued as the major reason for increased vector-borne diseases. Yet, road construction and urban development have had significant impacts on the spread of these diseases. There is evidence for the occasional transport of *Ae. albopictus* by car and/or trucks in Europe through artificial ovipositor sites such as tires and flowers (Takken and Knols, 2007; Collantes et al., 2015). In our study, roads served as a vector habitat developer layer to obtain the risk map. Roads were reported as important means of dispersal of this species in Vietnam (Higa et al., 2010). This important caused that in a research on the effect of climatic factors driving invasion of *Ae. albopictus* in Northern Italy, some of sampling stations were positioned along the roads as passive transport way of this species (Roiz et al., 2011). We feel that the most innovative models will address the role of roads as contributing factors to the risk of *Ae. albopictus* and believe this research sets an important precedent. In the final risk map, the collection points, reported in a previous research (Doosti et al., 2016), were close to the roads that can confirm this equation.

Among various classification techniques supplied by mapping software, the Natural Breaks method can find cut points for class creation based on the data distribution. There is no specific rule about the number of classes, but its acceptable range is between 4 to 11 classes (Werneck, 2008); in our case it produced 4 classes. Natural Breaks was used for identification of malaria hotspots in India (Srivastava et al., 2009) and was suggested to be the best method for predicting the most severe threat of dengue in a case study in Cambodia (Owen and Slaymaker, 2005). The Equal Intervals as another common classification method might be a good choice in rectangular data distribution (Werneck, 2008). This method has been rather useful for finding areas with high probability of presence of cutaneous Leishmaniasis vectors (Hanafi-Bojd et al., 2015). Our research provides an example of the effective use of Natural Breaks for future modeling work.

Aedes albopictus is considered as a dangerous species that is highly adaptive and present in both tropical and temperate climates (Roiz et al., 2011). In our study, the most suitable areas for colonization, where specimens had previously been collected (Doosti et al., 2016), were in coastal climates with low elevation. The presence of *Ae. albopictus* in lowlands with an altitude of less than 100 m was demonstrated in a region in Nepal with comparable climate (Dhimal et al., 2015). The low rainfall in northern parts of our study area likely limit conditions for establishment of *Ae. albopictus*. In a study in the United Kingdom, annual rainfall of less than 300 mm limited survival. The mean AnnP reported for our region in Iran is well below the 500 mm annual rainfall reported to be sufficient for establishing populations (Medlock et al., 2006). Yet, specimens have been collected from comparable climates in India, where sparse vegetation and water stored in human-made containers (Roiz et al., 2011; Sarfraz et al., 2014) supported populations

similar to what was seen in the south-eastern area of our study site.

For the first time, meteorological, topography, and climatology variables were applied to predict areas in Iran that may support populations of *Ae. albopictus*. We demonstrated the innovative use of AHP and the importance of adding roads during prediction mapping. We recommend further improving RS capabilities as collecting meteorological and climatic data from ground stations is time consuming (Roiz et al., 2011; Sarfraz et al., 2014). We also believe long-term data collection and integration with climate change models is an important next step for refining our model. And, although our model was validated by limited field measurements, further monitoring and refinement will be necessary, as well as expanding to more northern provinces in Iran where the populations of *Ae. albopictus* have been predicted (WHO, 2014; Kraemer et al., 2015). The next advancement will be to model actual fluctuations in the abundances of populations.

ETHICS STATEMENT

This study has been approved by ethical committee, Research Deputy, Tehran University of Medical Sciences with the letter-number: IR.TUMS.SPH.REC.1395.507.

AUTHOR CONTRIBUTIONS

Conception or design of the work: JN, AH-B, HV, and FC. Data collection: JN, AH, MRS, and SM-K. Data analysis and interpretation: ZC, ST, and MMS. Drafting of the article: JN, AH-B, and HV. Critical revision of the article: RB-M and MY-E. All authors read and approved the final version of the manuscript.

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***Aedes aegypti* Molecular Responses to Zika Virus: Modulation of Infection by the Toll and Jak/Stat Immune Pathways and Virus Host Factors**

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Zika (ZIKV) and dengue virus (DENV) are transmitted to humans by *Aedes* mosquitoes. However, the molecular interactions between the vector and ZIKV remain largely unexplored. In this work, we further investigated the tropism of ZIKV in two different *Aedes aegypti* strains and show that the virus infection kinetics, tissue migration, and susceptibility to infection differ between mosquito strains. We also compare the vector transcriptome changes upon ZIKV or DENV infection demonstrating that 40% of the mosquito's midgut infection-responsive transcriptome is virus-specific at 7 days after virus ingestion. Regulated genes included key factors of the mosquito's anti-viral immunity. Comparison of the ZIKV and DENV infection-responsive transcriptome data to those available for yellow fever virus and West Nile virus identified 26 genes likely to play key roles in virus infection of *Aedes* mosquitoes. Through reverse genetic analyses, we show that the Toll and the Jak/Stat innate immune pathways mediate increased resistance to ZIKV infection, and the conserved DENV host factors vATPase and inosine-5'-monophosphate dehydrogenase are also utilized for ZIKV infection.

Keywords: Zika virus, dengue virus, *Aedes aegypti*, innate immunity, vector competence

INTRODUCTION

Aedes mosquitoes are the primary vectors of Zika virus (ZIKV), dengue virus (DENV), and chikungunya virus (CHIKV) which are currently the most devastating arboviral pathogens. DENV causes 400 million infections and 12,500 deaths per year, and ZIKV recently emerged as another arbovirus of major public health concern (Guzman et al., 2010; WHO, 2012; Bhatt et al., 2013; Weaver et al., 2016). Typically, ZIKV infections are mild or asymptomatic; however, in contrast to DENV and CHIKV infections, ZIKV has been associated with Guillain-Barré syndrome in adults, and a heightened risk of microcephaly and other birth defects in prenatally infected infants (Cao-Lormeau et al., 2016; Johansson et al., 2016). At the moment, no preventive or therapeutic drugs exist against these pathogens. Hence, the control of *Aedes* mosquitoes remains the primary approach to limit viral transmission (Morrison et al., 2008).

Currently, the primary DENV and ZIKV control approaches either suppress mosquito populations or render the vector less competent to support viruses infection (Harris et al., 2011; Walker et al., 2011; Carvalho et al., 2015). The most commonly used approach is based on insecticides, but insecticide resistance is becoming increasingly common and controlling spread of arboviruses will require novel approaches. New alternative strategies for DENV and ZIKV control are being developed, and include genetically modified mosquitoes that could block virus transmission, transmission-blocking vaccines and small molecules (Harris et al., 2011; Carvalho et al., 2015; Schmidt et al., 2017). Research in the past few decades on DENV-*Aedes* molecular interactions has been critical to the development of these new vector control approaches. However, the recent public health emergency of the CHIKV and ZIKV epidemics, and the ongoing global threat of yellow fever has emphasized the importance of broadening our understanding to other virus infection systems. A first step toward this goal is to understand how mosquitoes respond to, and manage, infection at the molecular level. Our more extensive knowledge of *A. aegypti* – DENV interactions can greatly leverage and facilitate this effort for other mosquito-arbovirus infection systems.

The mosquito becomes infected with an arbovirus when a female acquires a blood meal on an infected human. The ingested virus first encounters the midgut tissue, where it replicates to produce viral particles. The viral particles then disseminate from the midgut through the hemolymph to other tissues including the salivary glands. There they are further propagated before being transmitted to a new host during a subsequent blood meal. The average extrinsic incubation period (EIP) of both DENV and ZIKV is 7–14 days. The DENV EIP is dependent on numerous factors such as mosquito and virus genotypes, as well as environmental factors such as humidity and temperature (Hardy et al., 1983; Bennett et al., 2002; Black et al., 2002; Salazar et al., 2007; McElroy et al., 2008; Dubrulle et al., 2009; Tchankouo-Nguetcheu et al., 2010; Li et al., 2012; Chouin-Carneiro et al., 2016). Different mosquito populations and strains can vary in permissiveness to virus infection. This variability is driven by the virus' compatibility with host factors and its ability to elicit and evade the action of the mosquito's restriction factors, many of which are components of the insect's innate immune system.

The immune responses of arthropods are, to a significant degree, regulated by the Toll, immune deficiency (Imd), and Janus kinase/signal transducer and activator of transcription (Jak/Stat) signaling pathways. Activation of these pathways leads to the nuclear translocation of transcription factors, resulting in the production of a variety of anti-pathogen effector molecules (Xi et al., 2008; Souza-Neto et al., 2009; Ramirez and Dimopoulos, 2010; Zou et al., 2011; Garver et al., 2013; Clayton et al., 2014). Furthermore, the RNAi pathway, a key antiviral defense system, can degrade viral RNAs. Functional genomics and reverse genetic approaches have demonstrated that the Toll, Jak/Stat, and RNAi pathways exert anti-DENV activity (Sanders et al., 2005; Campbell et al., 2008; Xi et al., 2008; Sánchez-Vargas et al., 2009; Souza-Neto et al., 2009; Ramirez and Dimopoulos, 2010; Sim et al., 2013). However, while the molecular interactions between *A. aegypti* and DENV have been characterized in some detail over

the past decade, the interactions between ZIKV and its mosquito vector have remained largely unexplored.

Here we have initiated the elucidation of *A. aegypti* molecular response to ZIKV infection, and the implication of the mosquito's immune system, and known virus host factors, in modulating vector competence. We demonstrate that different *A. aegypti* strains show different degrees of permissiveness to ZIKV and DENV. We used an RNA sequencing (RNAseq)-based comparative transcriptome analysis of *A. aegypti* responses to ZIKV and DENV infection of the midgut tissue that revealed both conserved and unique responses to infection with the two viruses, involving a variety of functional gene groups, including immunity. We show that the Toll and Jak/Stat pathways are implicated in suppressing ZIKV infection. We also report that key host factors for viral replication are essential for infection with both viruses. Furthermore, our comparative analysis of the ZIKV, DENV, West Nile virus (WNV) and yellow fever virus (YFV) infection-responsive transcriptomes identify potential pan-flavivirus infection-responsive genes that could represent key factors in the general mosquito-virus interactions. This study has significantly furthered our understanding of mosquito-virus interactions with a special emphasis on the largely understudied ZIKV.

RESULTS

Aedes aegypti Strains Show Differential Susceptibility to ZIKV

Genetic variations among *A. aegypti* mosquito populations and strains can influence their susceptibility to different viruses. Here we assessed the possible differences in temporal and spatial (tissue) ZIKV tropism between two *A. aegypti* strains. These strains differ in their permissiveness to DENV at the stage of midgut and salivary gland infection; the Rockefeller (Rock) strain being the more permissive to DENV compared to the Orlando (Orl) strain (Sim et al., 2013). We orally infected mosquitoes by allowing them to feed on blood containing equal titers of ZIKV. Next, we assayed infection intensity and prevalence (Figures 1A–J), by plaque assay, at different days post-infectious blood meal (dpibm) in the midgut, abdomen (at 4, 7, 10, 14 dpibm) and salivary glands (at 10, 14, 21 dpibm). The kinetics of ZIKV midgut infection intensity peak at 7 dpibm, with the Rock being significantly more permissive than the Orl strain at 4–10 dpibm (Figures 1A–C). The kinetics of disseminated infection, as measured by virus titers in the abdomen, were also similar for the two viruses, showing a peak in infection intensity at 14 dpibm (Figures 1D–F); the Rock strain was again more permissive to ZIKV infection at 10 and 14 dpibm. Salivary gland infection intensity and prevalence exhibited a gradual increase from 10 to 21 dpibm and showed a statistically non-significant trend toward being higher in the Rock strain (Figures 1G–I). Our study suggests that the kinetics of ZIKV infection intensity and prevalence differ between the *A. aegypti* Orl and Rock strains. While the Rock strain appeared to be more permissive to ZIKV infection at the stage of midgut and disseminated infection, virus infection intensity and prevalence

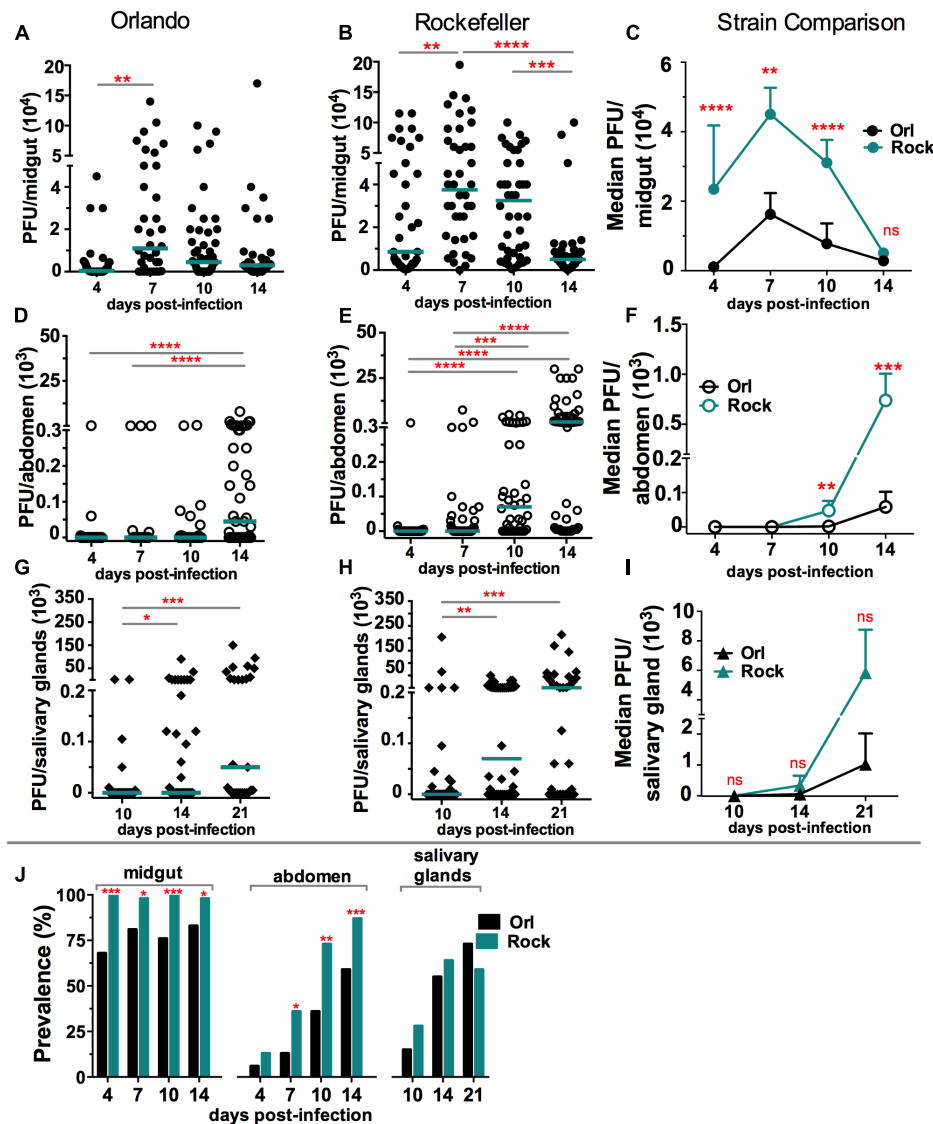


FIGURE 1 | Zika virus (ZIKV) tropism in *A. aegypti*. Orlando (Ori) and Rockefeller (Rock) *A. aegypti* mosquito strains were infected with ZIKV via an infectious-blood meal, and (A–C) midguts, (D–F) abdomens, and (G–I) salivary glands were assessed for infection intensity at different time points. (A) 4 days ($N = 34$), 7 days ($N = 36$), 10 days ($N = 46$), 14 days ($N = 36$). (B) 4 days ($N = 37$), 7 days ($N = 40$), 10 days ($N = 42$), 14 days ($N = 42$). (D) 4 days ($N = 35$), 7 days ($N = 38$), 10 days ($N = 25$), 14 days ($N = 51$). (E) 4 days ($N = 56$), 7 days ($N = 61$), 10 days ($N = 40$), 14 days ($N = 55$). (G) 10 days ($N = 33$), 14 days ($N = 38$), 21 days ($N = 27$). (H) 10 days ($N = 40$), 14 days ($N = 46$), 21 days ($N = 34$). Each dot represents the plaque-forming units (PFUs) per individual tissue from three independent experiments. Bars represent the median. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis test. (C, F, I) show strain comparisons between Ori and Rock; each point represents the median PFU per strain at the given time point per replicate ($N = 3$). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; Mann-Whitney test. (J) The prevalence (percentage) of infected mosquitoes for Ori vs. Rock was compared at each time point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; chi square test.

of ZIKV in the salivary glands were similar between the two mosquito strains.

ZIKV and DENV Infection-Responsive Transcriptomes Show Both Shared and Virus-Specific Responses

An organism's transcriptome provides a detailed snapshot of its physiological state under a given condition. Therefore, transcriptomic infection-responses can be highly informative

for understanding how virus infection affects the mosquito, and how the mosquito responds to counteract infection. While *A. aegypti* transcriptomic responses to DENV infection have been addressed in previous studies, using microarray-based technology, to the mosquito's molecular responses to ZIKV infection were unknown. Here we used a more powerful RNAseq approach to study the mosquitoes' responses, comparatively, to both DENV and ZIKV infection of the midgut tissue at 7 dpim. This tissue and infection stage was chosen because it has proven to be highly informative with regard to characterizing immunity

and other physiological responses to virus infection in previous studies with DENV. We used the more susceptible Rock strain to ensure high infection intensity and prevalence (**Figure 2**). Mosquito cohorts infected with each of the two viruses showed similar viral titers, but a lower prevalence of ZIKV-infected mosquitoes (67%, $P = 0.0422$) compared to those infected with DENV (**Figure 2A**). Infection by either ZIKV or DENV resulted in broad physiological responses entailing both common and unique expression signatures, reflecting possible differences in the interactions between the mosquito and the two viruses.

Infection with either virus resulted in a more prominent enrichment than depletion of transcripts; DENV and ZIKV infection led to an upregulation of 130 and 148 genes, respectively, and downregulation of 82 and 75 genes, respectively (**Figure 2B** and Supplementary Table S1). A comparison of the ZIKV and DENV infection-responsive transcriptomes showed 61% of the genes (135 of 223) to be uniquely regulated by ZIKV infection, whereas 35% (79 of 223) were regulated by both viruses in the same direction, and 4% in opposite direction (**Figure 2C**). A gene ontology functional analysis was then performed on the infection-responsive transcriptomes to determine the representation of specific functional groups and gene families. The defense response (biological processes category) ($P < 0.01$) and two redox activities (monooxygenase and oxidoreductase) in the molecular function category were significantly ($P < 0.05$) enriched in the regulated transcriptome repertoire (**Supplementary Figure S1**). The molecular function category also included numerous cytochrome P450 genes, which play roles in stress response and detoxification.

We then compared both our ZIKV and DENV infection-responsive transcriptomes with the published *A. aegypti* Rock strain YFV, WNV, and DENV2 infection-responsive transcriptomes (Colpitts et al., 2011). This analysis revealed 26 genes that were regulated in the same direction upon infection with all viruses; 13 genes were upregulated, and 13 genes were downregulated (**Figure 2D**). Only 11 of these genes have a predicted function and include factors associated with stress responses, immune responses, and enzymatic activity (**Figure 2E**).

ZIKV Induces Mosquito Immune Responses, with a Bias toward the Toll Pathway

Data mining revealed a significant representation of the immune response ontology category in the ZIKV infection-responsive transcriptome. A closer assessment of immune genes, as classified in Waterhouse et al., 2007, identified 13 infection-responsive genes in ZIKV infected mosquitoes (**Figures 3A,D**). Some of these genes have predicted redundant immune functions, and other have been specifically associated with the mosquito immune pathways: Toll, immune deficiency (Imd), Jak/Stat, and RNAi. Six of the 13 (46%) infection-responsive immune-related genes are putatively linked to the Toll pathway, including Leucine rich repeat (LRRs)-containing proteins, clip-domain serine proteases (CLIPBs), myeloid differentiation 2-related lipid recognition protein (ML) receptors (MD2-like) and cecropin

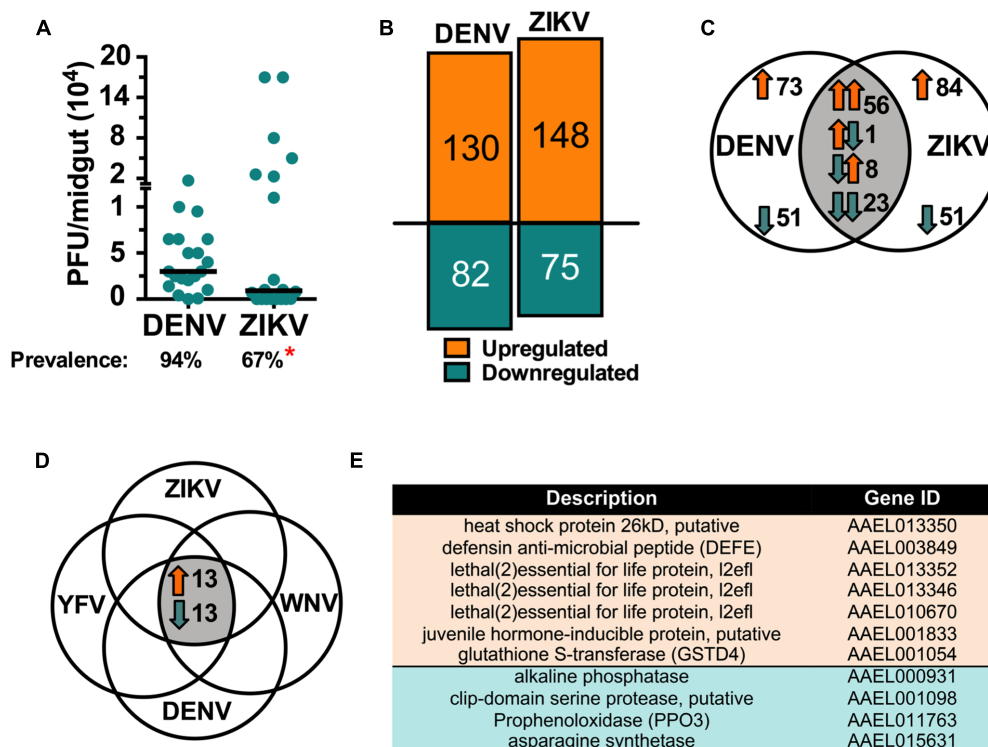
E (CECE) (Xi et al., 2008; Jupatanakul et al., 2014). The long peptidoglycan recognition proteins (PGRP-Ls) are frequently associated with the Imd pathway, but PGRPs have also been linked to the Toll pathway (Dziarski, 2004; Steiner, 2004). However, the specific role of each PGRP is not well understood, and different members may act as activators as well as negative regulators of the immune response (Waterhouse et al., 2007). The infection-responsive pathogen recognition receptor genes, including fibrinogen-related proteins (FREPs) and C-type lectins (CTLs) have not been specifically linked with any of the immune pathways. CECE is the only effector molecule most frequently associated with the Toll pathway; nevertheless, other CECs, defensins (DEF), and lysozymes (LYSC) can be regulated by both the Toll and Imd pathways (Zou et al., 2011). DENV-infected mosquitoes displayed a total of 15 regulated immune genes, with a greater representation of FREP and CTL family genes than in ZIKV-infected mosquitoes (**Figures 3B,D**). Interestingly, infection with either virus elicited upregulation of CECE, DEFA, and DEFE, all of which are common anti-microbial peptides. We also identified immune genes that were differentially regulated upon infection with ZIKV vs. DENV (**Figure 3C**). ZIKV infection specifically induced genes belonging to the ML family, which have been shown to influence DENV infection (Jupatanakul et al., 2014). ZIKV also induced expression of the Dicer-2 (*dcr2*) gene, a key factor of the antiviral RNAi immune pathway.

Activation of the Toll and Jak/Stat Pathways Results in Suppression of ZIKV Infection

Transcriptome data can predict immune pathway activation but does not prove its implication in controlling virus infection. To assess the involvement of the innate immune pathways (Toll, Imd, and Jak/Stat) in regulating *A. aegypti*'s permissiveness to ZIKV infection, we used an RNA-mediated gene silencing approach to activate each of the pathways by depleting pathway-specific negative regulators of the Rock strain before feeding mosquitoes on ZIKV-infected blood. Activation of the Toll and Jak/Stat pathways, through depletion of Cactus and PIAS, respectively, resulted in a significantly lower infection intensity when compared to control mosquitoes treated with GFP dsRNA ($P < 0.001$ and $P < 0.01$, respectively) (**Figure 3E**). A modest decrease in prevalence was observed, but it was not statistically significant ($P = 0.0539$ and $P = 0.8153$). Activation of the Imd pathway through silencing of Caspar did not result in a significant modulation of ZIKV infection ($P = 0.2334$). These results show a similar pattern of immune pathway-specificity to that for DENV infection (Sim et al., 2013), where the Jak/Stat pathway was more potent in suppressing DENV, whereas we observed a greater potency of the Toll pathway in suppressing ZIKV.

The vATPase and IMPDH Genes Act As Aedes Host Factors for Zika Infection

Arboviruses rely on numerous mosquito host factors for replication and infection, and we have previously confirmed that two subunits (VoB and Ac39) of the vacuolar ATPase (vATPase), and inosine-5'-monophosphate dehydrogenase (IMPDH), are



essential DENV host factors (Kang et al., 2014). Others have confirmed the vATPase as a mammalian host factor for ZIKV (Savidis et al., 2016). The vATPase is required for the acidification of endosomes and viral genome release into the cytosol, and IMPDH is required for *de novo* RNA synthesis during viral replication. Silencing of these genes also resulted in reduced ZIKV infection intensity in the mosquito gut and a significant reduction in infection prevalence of IMPDH-depleted mosquitoes. These results show that that vATPase and IMPDH also serve as ZIKV host factors, and that the two viruses use some of these same mosquito factors to complete their replication cycle (Figure 4).

DISCUSSION

The recent ZIKV epidemic highlights the importance of studying the interactions of emerging arboviruses with their mosquito vectors. Despite their similarities in structure and transmission cycle, the outcomes of infection with ZIKV and DENV differ greatly in humans. Here, we performed a comparative study to address similarities and differences between the interactions of ZIKV and DENV with their common mosquito vector. The goal

of this work was to initiate the elucidation of *A. aegypti* responses and immunity to ZIKV, while these features have been studied to some extent for DENV, to support the ongoing development of novel disease control strategies targeting the virus in the vector.

Compatibility between a virus and its mosquito vector is generally determined by virus host and restriction factors. These factors are broadly defined as mosquito proteins that support virus infection (have an agonistic function) or restrict virus infection (have an antagonistic function). Two *A. aegypti* strains, Rock and Orl, have previously been reported to differ in their vector competence for DENV (Sim et al., 2013). Here we show differences in permissiveness for ZIKV, at the midgut and disseminated stages of infection, with the Rock strain being a more permissive vector. Previous comparative transcriptome analyses between the Rock and Orl strains, in conjunction with RNAi-based studies of immune pathways, identified an infection bottleneck for DENV in the Orl strain. The study also showed a greater basal expression level of immune genes in the Orl strain, and implicated the Toll and Jak/Stat pathways in its lesser permissiveness to DENV infection (Sim et al., 2013).

DENV EIP is dependent on the mosquito strain, virus genotype, and environmental factors such as humidity and temperature. A peak of DENV2 infection intensity in the

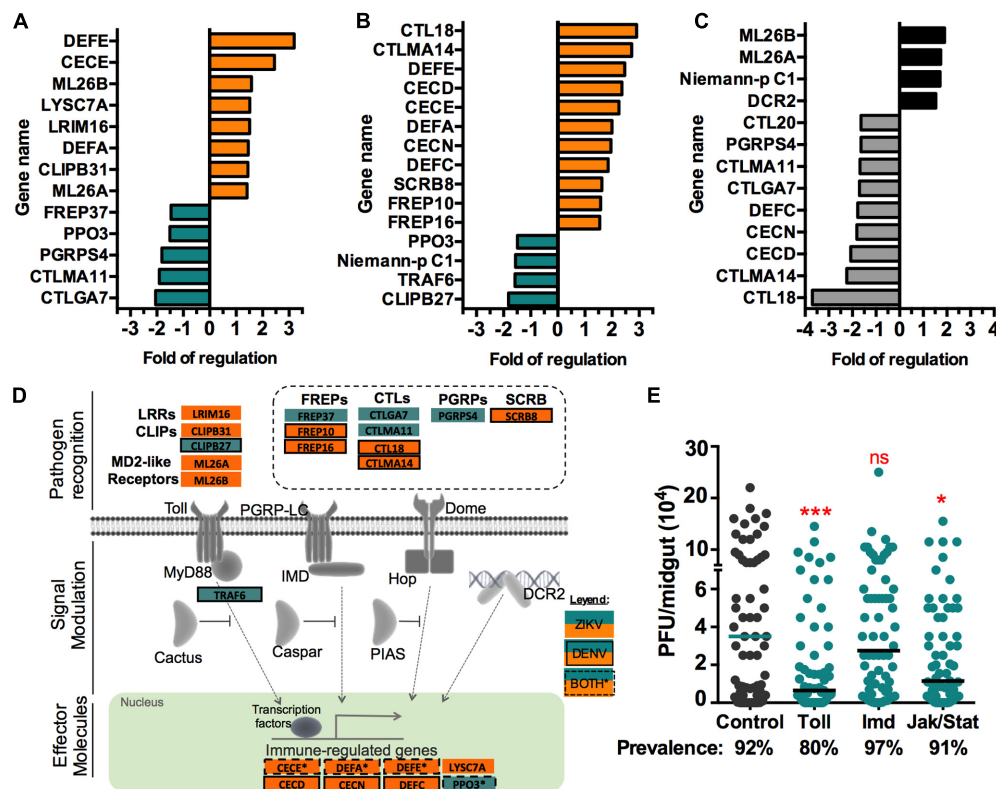


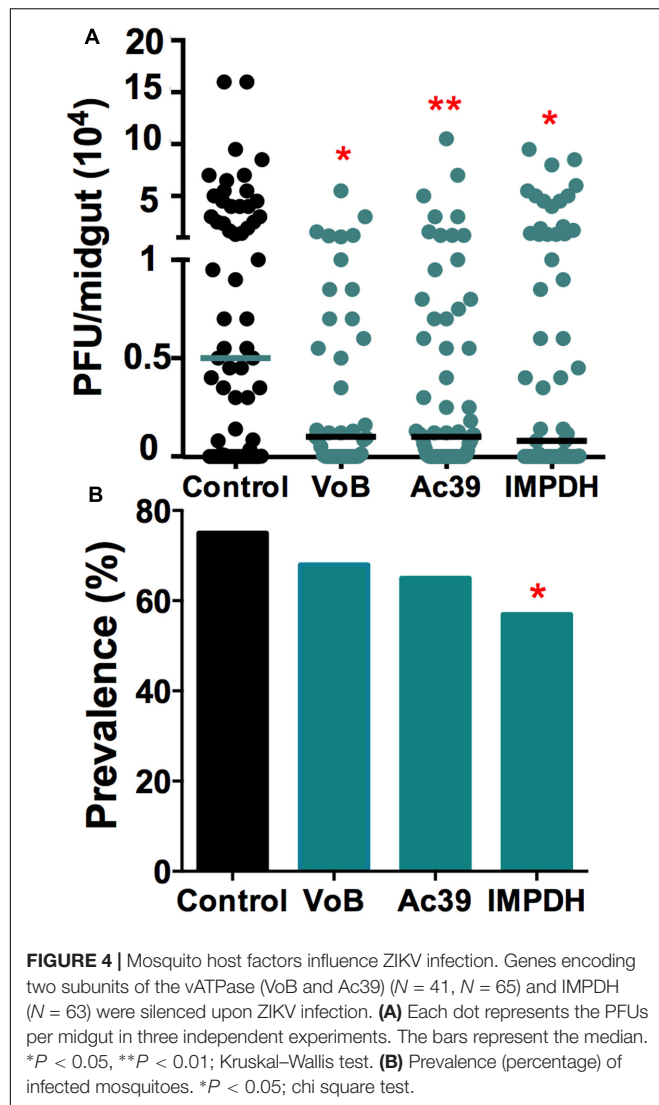
FIGURE 3 | Infection-responsive immune genes. The fold change of putative immune gene transcript abundance upon midgut infection with **(A)** ZIKV or **(B)** DENV, as compared to non-infected blood-fed controls. Orange indicates upregulation, blue downregulation. **(C)** The fold difference in transcript abundance of immune genes between ZIKV- vs. DENV-infected midguts; black and gray indicates genes showing a higher transcript abundance in ZIKV and DENV infected midguts, respectively. **(D)** Schematic representation of the four main immune pathways (Toll, Imd, Jak/Stat, and RNAi). Boxes without lines represent ZIKV infection-responsive genes, boxes with solid lines represent DENV infection-responsive genes, and boxes with dashed lines represent genes regulated upon infection with either virus. **(E)** Immune pathway activation demonstrated through dsRNA-mediated silencing of the negative regulators of the Toll pathway (dsCactus, $N = 65$), Imd pathway (dsCaspar, $N = 66$), and Jak/Stat pathway (dsPIAS, $N = 65$). GFP dsRNA was used as a control ($N = 62$). ZIKV infection was measured as PFU in individual midguts at 7 days post-infection. The bars represent the median. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Mann-Whitney test.

mosquito occurs at 7 dpbm (Salazar et al., 2007), which agrees with the tropism of ZIKV infection in our study. Several studies have described the *Aedes* transcriptome at different time-points during DENV infection. Others have indicated a modest immune gene regulation during the early stages of infection (1–4 dpbm), which is likely due to viral immune evasion and modulation mechanisms (Xi et al., 2008; Sim and Dimopoulos, 2010; Bonizzoni et al., 2012). Transcriptomic studies focusing on later infection time points (7–10 dpbm) documented the activation of the Toll and Jak/Stat pathways by DENV infection (Xi et al., 2008; Souza-Neto et al., 2009).

In the midgut tissue, that first encounters the pathogen, we found differences between the transcriptomic responses to ZIKV and DENV infection in the susceptible Rock strain at the 7 dpbm stage of peak infection. This indicates differences in the molecular interactions between the vector and each of the two viruses. We focused most of our data mining efforts on genes putatively being involved in the mosquito's innate immune system. This immune system plays a crucial role in anti-viral defenses and therefore constitutes an infection

bottleneck in some mosquito-virus combinations (Sanders et al., 2005; Franz et al., 2006; Galiana-Arnoux et al., 2006; Deddouche et al., 2008; Frangkoudis et al., 2008; Xi et al., 2008; Adelman et al., 2013; Sim et al., 2013). Genes belonging to different immune gene families were identified as ZIKV infection-responsive. The Clip-domain serine protease gene, CLIPB31, was regulated upon ZIKV infection. The CLIP gene family comprises over 60 members, some of which have been associated with immune pathway activation as well as melanization and lytic effector mechanisms (Xi et al., 2008; Kanost and Jiang, 2015). Interestingly, at least four CLIP genes (CLIPB13B, CLIPB46, CLIPB5, CLIPB2) have been shown to be induced upon Toll pathway activation (Xi et al., 2008). The transcript abundance of CLIPB31 is also significantly increased in mosquitoes infected with the DENV-blocking endosymbiont *Wolbachia* (Rancès et al., 2012), pointing at a possible link between immune system modulation by the bacterium and virus suppression.

Antimicrobial peptide (AMPs) genes encode short peptides that interact with the pathogen to mediate their elimination



through mechanisms that include lysis and membrane disruption (Kaushal et al., 2016; Zheng et al., 2017). Here we found that AMPs such as LYSCs, DEFs, and CECs, were induced upon ZIKV infection, but their potential role in antiviral defense has not yet been studied (Xi et al., 2008; Zou et al., 2011). We identified members of the ML receptors in the ZIKV infection-responsive transcriptome. The mammalian MD-2 mediates Toll pathway activation through the TLR4 receptor (Stokes et al., 2015). The role of MD-2-like proteins in mosquito immunity is not well understood, but the *Anopheles gambiae* ML1 gene has been linked to both antibacterial and anti-*Plasmodium* defenses, and a *Drosophila* ML gene has also been linked to Imd pathway activation (Dong et al., 2006; Shi et al., 2012; Sandiford et al., 2015). The *A. aegypti* genome contains 26 ML-like gene of which ML26A and ML26B were upregulated upon ZIKV infection. These two genes have been shown to be upregulated in DENV-resistant *A. aegypti* at 18 dpibm (Behura et al., 2011). Another study has shown that ML13 and ML33 are induced by DENV infection in susceptible mosquitoes, and ML33 acts as an agonist

for DENV (Jupatanakul et al., 2014). The fibrinogen domain -containing gene FREP37 was regulated upon ZIKV infection, and this gene has also been shown to be downregulated upon infection with YFV and DENV2 (Behura et al., 2011; Colpitts et al., 2011). DENV-infected mosquitoes showed upregulation of the FREP10 and FREP16 genes that have been previously associated with anti-bacterial responses (Rancès et al., 2012; Ye et al., 2013). FREPs belong to a large gene family, and have been shown to function as putative pathogen recognition receptors for both bacteria and *Plasmodium* (Dong and Dimopoulos, 2009).

Our transcriptomic study revealed a bias toward the Toll pathway upon ZIKV infection, and we further confirmed a role for this pathway in suppressing ZIKV through RNAi-based reverse genetic assays. We also demonstrated that activation of the Jak/Stat pathway contributes to ZIKV suppression. Both the Toll and Jak/Stat pathways have previously been shown to suppress DENV infection in the midgut tissue, thereby corroborating a major role for these immune pathways in suppressing flaviviruses (Sanders et al., 2005; Xi et al., 2008; Fragkoudis et al., 2009; Souza-Neto et al., 2009; Ramirez and Dimopoulos, 2010; Tchankouo-Nguetcheu et al., 2010; Colpitts et al., 2011; Sim et al., 2013). A recent study using a transgenic approach showed that activation of the Jak/Stat pathway in the mosquito's fatbody (contained in the abdomen and thorax) did not affect susceptibility to ZIKV infection while it strongly suppressed DENV infection. This discrepancy with our RNAi-based assays is interesting and suggests possible differences in the immune pathway's spatial (tissue) and temporal antiviral specificity (Jupatanakul et al., 2017).

The RNAi pathway cannot be transiently activated through gene silencing since a negative regulator has yet not been identified, but partial inhibition of the pathway using RNAi to silence dicer-2 (*dcr2*) increases the susceptibility of refractory Orl strain mosquitoes to DENV infection (Sim et al., 2013). We found that *dcr2* expression was significantly higher in ZIKV-infected than in DENV-infected mosquito midguts, suggesting that the RNAi pathway is likely to be involved in suppressing ZIKV infection.

The functional annotation of *A. aegypti* immune genes is based on phylogenomic analysis using the *Drosophila melanogaster* immune gene repertoire, as well as those of other disease-vector mosquitoes (Waterhouse et al., 2007). Many of our ZIKV and DENV infection-responsive genes were of unknown function and are therefore likely to represent additional factors of the mosquito's antiviral defense system.

Of the 88 genes that responded to infection with both viruses (42% of the ZIKV infection-responsive transcriptome), 9 were regulated in opposite directions upon infection with DENV vs. ZIKV. Interestingly, five of these were trypsin genes that were downregulated in DENV- and upregulated in ZIKV-infected midguts. Trypsins have previously been associated with the modulation of DENV infection in *A. aegypti* midguts (Brackney et al., 2010). This suggests that both viruses have the potential to differentially modulate the mosquito's digestive processes.

By comparative analysis of the ZIKV, DENV, YFV, and WNV infection-responsive transcriptomes, derived from our and a previous study, we identified 26 genes that were regulated in

the same direction during infection of *A. aegypti* with the four viruses (Colpitts et al., 2011). Despite possible differences in experimental procedures between the studies, this finding suggests that at least some of these genes are part of the mosquito's general response to virus infection. Three of them represent potential pan-flavivirus infection-responsive immune genes [DEFE, a putative CLIP, and prophenoloxidase 3 (PPO3)]. We also identified three lethal (2) essential for life (l(2)efl) genes, which are relatively unstudied but encode for small heat shock proteins (Kurzik-Dumke and Lohmann, 1995). Heat shock proteins have been linked with apoptosis, a process that is associated with the mosquito's defense against virus infection, and previous studies have suggested their possible interaction with factors of the antiviral Toll pathway (Guo et al., 2010; Gonda et al., 2012). While current research on transmission-blocking approaches are mostly focused on targeting a single pathogen the identification and characterization of mosquito defense systems that can control multiple types of viruses is of particular importance for the development of pan-flavivirus transmission-blocking strategies (Shin et al., 2003; Bian et al., 2005; Adelman et al., 2008; Dong et al., 2011; Zou et al., 2011; Jupatanakul et al., 2017; Simões et al., 2017).

As part of our comparative study of the molecular interactions between the two viruses and the *A. aegypti* vector, we also tested the possible involvement of two DENV host factors in ZIKV infection (Kang et al., 2014). Silencing of two vATPase subunits (VoB and Ac39) and IMPDH revealed that they also play ZIKV host factor functions. Interestingly, a recent study has shown that the mammalian vATPase is a ZIKV host factor (Savidis et al., 2016), indicating conserved components of the cellular machinery involved in sustaining viral replication in both hosts. Virus host factors represent potent transmission-blocking targets through multiple approaches, including transgenic gene deletion, small molecule inhibitors and transmission-blocking vaccines (Bowman et al., 1988; Diamond et al., 2002; Kang et al., 2014; Liu et al., 2014; Dong et al., 2015; Sandiford et al., 2015; Londono-Renteria et al., 2016).

In summary, we report that ZIKV and DENV have a similar tropism and ability to establish infection in low and high infection-permissive *A. aegypti* strains. The study generated new insights regarding the molecular responses of the vector mosquito to infection with ZIKV and DENV, and shows that ZIKV infection elicits quite a different transcriptome response, although many immune genes are similarly regulated by infection with both viruses. The major innate immune pathways, Toll and Jak/Stat, play roles in suppressing both viruses, and vATPase and IMPDH likely represent general flavivirus host factors in both mosquitoes and humans. This study also identifies numerous mosquito genes that are likely to participate in responding to, and controlling, virus infection. Our study has significantly contributed toward the gradually growing knowledge of mosquito-virus interactions, aiding in the further focus on specific factors, defense systems and host factors that can be used to develop novel flavivirus transmission blocking strategies. For example, the spread pathogen-resistance genes in mosquito population has in recent years gained further interest through the development of gene-drive systems

(Franz et al., 2006, 2014; Windbichler et al., 2011; Gantz et al., 2015; Jupatanakul et al., 2017). The development of virus resistant mosquitoes will require knowledge on virus restriction factors and infection-responsive promoters to express the antiviral genes. Furthermore, multiple research efforts are addressing the inhibition of mosquito-encoded virus agonists (host factors) through small molecule inhibitors, transmission-blocking vaccines and gene deletion (Bowman et al., 1988; Diamond et al., 2002; Kang et al., 2014; Liu et al., 2014; Dong et al., 2015; Londono-Renteria et al., 2016). The current lack of protective vaccines and drugs for most arboviruses, and the emergence of mosquito resistance to insecticides, have created an increasing need for additional complementary disease control strategies.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Animal Care and Use Committee of the Johns Hopkins University and the institutional Ethics Committee (permit number: M006H300). Mice were only used for mosquito rearing. Commercial, anonymous human blood was used for virus infection assays in mosquitoes, and informed consent was therefore not applicable.

Cell Culture and Mosquito Rearing

The *A. albopictus* cell line (C6/36) was maintained in MEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% NEA, and 1% penicillin/streptomycin. Baby hamster kidney cells (BHK-21), clone 15, were maintained in DMEM (Gibco) supplemented with 10% FBS, 1% L-glututamine, 1% penicillin/streptomycin, and 5 µg/mL plasmocin (Invivogen). C6/36 cells and BHK-21 cells were incubated with 5% CO₂ at 32°C and 37°C, respectively. *A. aegypti* mosquitoes were maintained on 10% sucrose solution at 27°C and 80% relative humidity with a 14:10 h light:dark cycle.

ZIKV or DENV Infections and Virus Titration

ZIKV strain FSS13025 or DENV NGC were propagated in C6/36 cells, and titers were determined by plaque assay on Vero cells for ZIKV or BHK-21 for DENV. ZIKV or DENV were propagated in C6/36 cells for 6 days; virus was then harvested and mixed with a sterile 1% solution at pH 7.1 (2.18 M sucrose, 38 mM KH₂PO₄, 72 mM K₂HPO₄, 60 mM L-glutamic acid), and stored at –80°C for future experiments. The virus suspension was mixed 1:1 with commercial human blood and supplemented with 10% human serum and 1% 10 mM ATP. Mosquitoes were infected via an artificial membrane feeder at 37°C for 30 min. Midguts, abdomens (without midguts), and salivary glands were dissected, individually collected, and stored at –80°C until used for plaque assays. Virus titration was performed as described in (Sim et al.,

2013), plates were incubated for 4–5 days, fixed and stained with methanol/acetone and 1% crystal violet mixture, washed, and the plaque-forming units counted.

RNA Sequencing and Data Analysis

Sample Preparation

Aedes aegypti Rockefeller strain mosquitoes were fed with a naïve blood meal containing non-infected cell culture or infected with 1×10^7 plaque-forming units (PFU)/mL of DENV2 NGC or ZIKV FSS13025 for 7 days. The midguts were dissected, pools of 20 midguts were collected per group, and three independent biological replicates were assayed. RNA was extracted with the Quick-RNA MiniPrep kitTM (Zymo Research). An Illumina sequencing library was constructed for each of the nine biological samples according to the manufacturer and sequenced on the Illumina NextSeq 500 sequencer. Compressed FASTQ files were extracted, mapped, and aligned to the mosquito transcriptome into the QIAGEN CLC Genomics Server platform¹. The annotated *A. aegypti* (NCBI Taxonomy ID: 1424507) reference transcriptome from the ENSEMBL metazoan database was used for annotation. Across the sequenced samples, between 82 and 85% of the reads aligned to the 17,478 annotated genes were used to assign FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values to those genes. These nine FPKM datasets were then imported into the Partek Genomics Suite v6.6 analytic platform (Partek Inc. St Louis, MO, United States) for further processing and comparison of their experimental classes: ZIKV infection, DENV infection, and naïve blood controls. These raw datasets were quantile normalized, values were converted to log₂ notation, and two-tailed one-way ANOVAs was conducted comparing infected mosquito samples to controls and to themselves. These comparisons yielded each gene's relative expression, as a fold change, and that change's statistical significance, as its *p*-value. These ANOVA results were then imported for further analysis and graphic display into the Spotfire Genomics Suite v9.1.2 platform (TIBCO Spotfire, Boston, MA, United States). The comparisons were filtered to exclude transcripts whose minimum (infected or Control) normalized Log₂(FPKM) value was less than −0.6 to avoid unreliable fold changed caused by stochastic noise in too low signal values. Since the remaining approximately 6K log₂ fold changes showed normal distributions, their standard deviation changes from the mean of no change were established and used to set a 2 SD thresholds for significant differential expression (linear fold changes of 1.40, 1.49, and 1.54 absolute value for ZIKV vs. Cont, DENV vs. Cont, and ZIKV vs. DENV respectively). RNAseq data are available from NCBI GEO with accession number GSE96605.

Pan-Flavivirus Transcriptome Analysis

We used the available transcriptome data of (Colpitts et al., 2011) for DENV, YFV, and WNV, significantly regulated genes with $P < 0.05$, log₂ fold change > 1 or < -1 , regulated in the same direction for at least one-time point per virus. Selected genes were then compared to our RNAseq transcriptome selecting only genes

that were significantly regulated in the same direction for both, ZIKV and DENV.

dsRNA-Mediated Gene Silencing

The *cactus*, *caspar*, and *PIAS* genes were depleted from adult female mosquitoes using established RNAi methodology (Sim et al., 2013). Assays were repeated three times, using GFP dsRNA as a control. Gene silencing was verified at 3 days post-injection of RNA extracted from five whole mosquitoes per biological replicate, and two technical replicates were analyzed by qRT-PCR (Supplementary Figure S2). The ribosomal protein S7 gene was used to standardize and verify gene silencing. The primers used to produce PCR amplicons for dsRNA synthesis and qRT-PCR are given in Supplementary Table S2.

Statistical Analysis

The Graphpad Prism 6 (Graphpad Prism®) software package was used to perform statistical analyses. The particular test used is indicated in the legend of each respective figure. See Supplementary Table S3 for a summary of the statistics.

AUTHOR CONTRIBUTIONS

YA-R, NJ, and GD conceived experiments; YA-R, HM, SK, JC, and NJ performed experiments. YA-R and GD analyzed the data obtained and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.02050/full#supplementary-material>

FIGURE S1 | Gene ontology functional analysis. Biological process, cellular component, molecular function, and immune system terms were analyzed for all significantly regulated genes using ClueGO, a Cytoscape plugin, and the *A. aegypti* genome was used as background. **(A)** Bars show the percent of genes per term, and those with similar functional categories have the same color. **(B)** Pie chart graph shows total genes represented per term, the identified term was

¹ <https://www.qiagenbioinformatics.com>

based on highest enrichment. Asterisk represents significantly enriched terms. * $P < 0.05$, ** $P < 0.01$, Bonferroni test. Only gene terms with more than three genes per category are presented in the graph. (C) List of genes per term.

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FIGURE S2 | Silencing efficiency. qRT-PCR of silenced genes *cactus* (Toll), *caspar* (Imd), and *PIAS* (Jak/Stat). (A) Immune genes. (B) Host factors. Bars represent SEM of three independent experiments.

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Linking Only *Aedes aegypti* with Zika Virus Has World-Wide Public Health Implications

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ZIKV MOSQUITO VECTORS ARE NOT WELL-ESTABLISHED

Zika virus (ZIKV) is an emerging arbovirus in the Americas and as such, we know far less about the mosquito species involved in transmission than many experts, public health authorities, and politicians would have the public believe.

The entomological literature with respect to ZIKV and the supposedly pivotal role that *Aedes aegypti* plays in its transmission is frighteningly scant. Multiple mosquito species have tested positive for the virus in field-collected specimens, including 20 species from genus *Aedes* (in alphabetical order, *A. aegypti*, *Aedes africanus*, *Aedes apicoargenteus*, *Aedes dalzielii*, *Aedes dorsalis*, *Aedes flavicollis*, *Aedes fowleri*, *Aedes furcifer*, *Aedes hirsutus*, *Aedes jamoti*, *Aedes luteocephalus*, *Aedes metallicus*, *Aedes minutus*, *Aedes neoaffricanus*, *Aedes opok*, *Aedes taeniarostris*, *Aedes tarsalis*, *Aedes taylori*, *Aedes unilineatus*, *Aedes vittatus*), as well as six non-*Aedes* species (*Anopheles coustani*, *Anopheles gambiae*, *Mansonia uniformis*, *Eratmapodites inornatus*, *Eratmapodites quinquevittatus*, and *Culex perfuscus*) (McCrae and Kirya, 1982; Haddow et al., 2012; Diallo et al., 2014).

In Malaysia, 58 pools of 1,277 *A. aegypti*, 59 pools of 4,492 *Aedes albopictus*, and 179 pools of 27,636 mosquitoes from 23 other *Aedes* species were surveyed for ZIKV and only a single pool of *A. aegypti* tested positive (Marchette et al., 1969). This was the first isolation of ZIKV outside of Africa (strain P6-740). In Gabon, 137 pools of 2,701 *A. albopictus*, 45 pools of 881 *A. aegypti*, 15 pools of 88 *Aedes simpsoni* complex, 29 pools of 690 *Culex quinquefasciatus*, and 21 pools of another 305 mosquitoes (made up of *An. gambiae*, *Mansonia africana*, *M. uniformis*, *Culex* sp., and *E. quinquevittatus*) were surveyed and two *A. albopictus* pools tested positive for ZIKV (Grard et al., 2014).

From the outset of the ZIKV outbreak in Brazil, the World Health Organization and other authorities have stated that *A. aegypti* is the mosquito that needs to be targeted for control in order to avoid a ZIKV pandemic; sometimes, *A. albopictus* is also included in the warnings. Novel strategies for controlling *A. aegypti* using *Wolbachia*-infected males or Oxitec[®] females (Yakob and Walker, 2016) target only *A. aegypti*. If ZIKV transmission is not being driven by *A. aegypti*, then these strategies will fail to protect people from ZIKV.

In mosquito surveys that followed the ZIKV outbreak on Yap Island in the Federated States of Micronesia, several species of mosquitoes were collected and tested for ZIKV; none was positive. This included a single negative *A. aegypti* specimen as well as 362 *Aedes hensilli*, the most abundant species, and 247 *C. quinquefasciatus*, the second most abundant (Ledermann et al., 2014). Subsequently, *A. hensilli* was experimentally infected with ZIKV MR766 (the original African lineage strain) and, despite relatively low dissemination rates, it was assumed that it served as a vector (Ledermann et al., 2014).

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However, Guerbois et al. (2016) were able to collect mosquitoes during the 2015 ZIKV outbreak in Chiapas State, Mexico. They tested 471 female *A. aegypti* mosquitoes in 55 pools of which 15 were positive for ZIKV by RT-PCR. The second most abundant species was *C. quinquefasciatus* but no pools tested positive for ZIKV. Unfortunately, Guerbois et al. (2016) were only able to isolate virus from 3 of the 15 RT-PCR-positive samples following inoculation onto VERO cells.

Taken together, these data suggest a role for *A. aegypti* in the transmission of ZIKV but the data also leave room for other mosquito species (and other non-vector modes of transmission) to contribute to ZIKV outbreaks.

TRANSMISSION STUDIES INVOLVING *A. AEGYPTI* AND *A. ALBOPICTUS*

In the 1950's *A. aegypti* mosquitoes were fed a blood-meal containing a ZIKV dose of $10^{6.7}$ mouse LD₅₀ per 0.03 mL. There was one successful ZIKV transmission after a group of three infected mosquitoes fed on a rhesus monkey. The authors concluded “until it can be shown that *A. aegypti* can be infected with lower virus doses than those used here, its efficiency as a vector of Zika virus under natural conditions remains uncertain” (Boorman and Porterfield, 1956).

In another study, *A. aegypti* mosquitoes were infected intrathoracically with ZIKV ArD 24280 (an African lineage ZIKV strain isolated from *A. luteocephalus* in 1976) and subsequently the mosquitoes transmitted ZIKV to suckling mice (Cornet et al., 1979). However, for transmission to occur naturally, virus must be ingested during a blood-meal, cross over the midgut epithelium into the hemolymph, disseminate throughout the body, and eventually cross the salivary gland epithelium into the gland's lumen. Then, as a mosquito feeds on a subsequent blood-meal host, she spits saliva (containing virus along with anticoagulants, vasodilators, and salivary peptides) into the host. The experiment with ArD 24280 demonstrated that there was no salivary gland barrier, but it shed no light on the issue of a potential midgut barrier; thus, the study did not provide evidence that *A. aegypti* is a competent vector in the wild. However, the study did compare the ability of *A. aegypti* to transmit YF vs. ZIKV (both via intrathoracic infection) and found that the incubation period for ZIKV was less than for YF. This would suggest that *A. aegypti* is likely an efficient ZIKV vector.

A research group from Singapore infected *A. aegypti* using a blood-meal with ZIKV MR766 at an initial infectious dose of 7.0 Log_{10} tissue culture infectious dose₅₀ (TCID₅₀/mL). They observed both high infection rates and high salivary gland dissemination rates (Li et al., 2012). Unfortunately, saliva was neither collected nor tested so conclusions about transmission could not be made. The same group then conducted very similar experiments with *A. albopictus* but this time they collected saliva and tested it for virus. They found that on day 10 post-infection 100% of mosquitoes ($n = 12$) had ZIKV in their saliva (Wong et al., 2013). These studies provide support for the assumption that *A. albopictus* (and probably *A. aegypti*) are good vectors of ZIKV MR766. Nevertheless, the infection,

dissemination and transmission rates reported in the two studies are exceptionally high compared to what other researchers have found. Although mosquito strain differences and/or viral strain differences might cause these differences, it is possible that mosquito husbandry techniques also may have played a role. During extrinsic incubation, the mosquitoes were fed “10% sugar/vitamin B complex *ad libitum*.” Most (if not all) mosquito transmission studies use only an *ad libitum* sugar meal (although concentrations may vary) for daily maintenance of mosquitoes. It is possible that the addition of vitamin B complex to the sugar meal may be partially responsible for the high infection, dissemination, and transmission rates.

Four *Aedes* spp. from Senegal—*A. aegypti*, *A. unilineatus*, *A. vittatus*, and *A. luteocephalus*—were tested for their potential to transmit African lineage ZIKV isolates (MR766 and HD78788) in the lab. All four species were infected orally. ZIKV-positive saliva was only detected from *A. vittatus* and *A. luteocephalus*. Despite relatively high infection rates in *A. aegypti*, dissemination rates were low (6.3% of 111 and 5.6% of 216 specimens from two different populations) and subsequent transmission rates (i.e., virus in saliva) were zero (Diagne et al., 2015).

Chouin-Carneiro et al. (2016) looked at the susceptibilities of *A. aegypti* and *A. albopictus* to an Asian lineage ZIKV strain (NC-2014-5132), fed at 10^7 TCID₅₀/mL. Similar to the Senegalese study (Diagne et al., 2015), authors found high infection rates but low dissemination and transmission rates for both species. Calculated transmission efficiencies were $3.3 \pm 3.3\%$ for *A. albopictus* and $10 \pm 5.5\%$ for *A. aegypti*. The authors concluded that “*Ae. aegypti* and *Ae. albopictus* were unexpectedly low competent vectors for ZIKV” (Chouin-Carneiro et al., 2016).

Aliota et al. (2016) studied the vector competence of *A. aegypti*, *A. albopictus*, *Aedes triseriatus*, and *Culex pipiens* fed on mice that had been infected with an Asian strain of ZIKV (PRVABC59). After 14 days' incubation, neither *A. triseriatus* nor *C. pipiens* had a disseminated infection or yielded virus in saliva. As positive controls, the researchers used *A. aegypti* and *A. albopictus*. They were able to detect virus in the saliva in 4 of 17 (24%) *A. aegypti* fed 6.83 log_{10} PFU/mL and in 2 of 9 (22%) *A. albopictus* fed 6.02 log_{10} PFU/mL.

In a recent study, Jupille et al. (2016) tested the vector competence of two populations of *A. aegypti* (from the island of Madeira) and two populations of *A. albopictus* (from France) using a ZIKV strain from New Caledonia (NC-2014-5132). They concluded that neither species was very susceptible to ZIKV. Virus was detected in the saliva of 1 of 20 (5%) *A. aegypti* from Funchal and 0 of 20 (0%) *A. aegypti* from Paul do Mar on day 9 post-infection; data were identical for *A. aegypti* on day 14. In contrast, the saliva of *A. albopictus* was negative for ZIKV for all specimens tested on day 9 from both Nice ($n = 24$) and Bar-sur-Loup ($n = 24$) and for *A. albopictus* tested on day 14 from Nice ($n = 24$). The saliva from only 1 of 24 (4.2%) *A. albopictus* from Bar-sur-Loup was positive on day 14.

Richard et al. (2016) tested the vector competence of two species implicated in 2013–2014 ZIKV outbreak in French Polynesia, using ZIKV strain PF13/251013-18. They found that “transmission efficiency was poor in *A. aegypti*” until 14 days post-infection and that *A. polynesiensis* was unable to transmit

ZIKV at all. They concluded that there might be the “possible contribution of another vector for the propagation of ZIKV during the outbreak.”

Ayres (2016) cautioned about placing too much emphasis on *A. aegypti* in the battle against ZIKV, and suggested that *C. quinquefasciatus* might be an important vector in Recife, Brazil. Huang et al. (2016) looked at the vector competence of colonized *C. pipiens* and *C. quinquefasciatus* but were unable to demonstrate infection or dissemination at 7 or 14 days post-infection in mosquitoes held at 28°C. Fernandes et al. (2016) were unable to show transmission in *C. quinquefasciatus*.

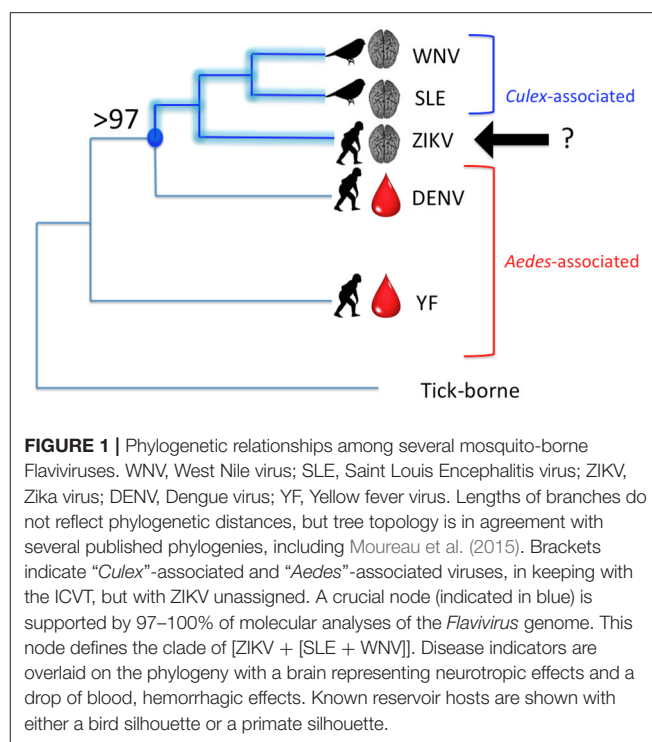
The first published report of the vector competence of *C. quinquefasciatus* comes from Guo et al. (2016). They were able to demonstrate that mosquitoes held at 29°C could transmit ZIKV by bite to suckling mice and furthermore, that the peak time of virus appearance in the salivary glands was day 8 post-infection.

With all of the conflicting reports in the literature, it is prudent to consider other evidence that might shed light on which mosquito species are involved in ZIKV transmission.

EVIDENCE FROM PHYLOGENETICS

There is an additional line of evidence—the evolutionary history of the Flaviviridae—that points to species other than *A. aegypti* as playing key roles in the current ZIKV outbreaks. Numerous authors have reconstructed phylogenies of the Flaviviridae based on amino acid sequences and nucleotide sequences. **Figure 1** is a synthesis of three papers (Kuno et al., 1998; Lanciotti et al., 2008; Moureau et al., 2015). According to the International Commission on Viral Taxonomy (ICVT), West Nile virus (WNV), and Saint Louis Encephalitis virus (SLE) are designated as “*Culex*-associated” viruses whereas Dengue virus (DENV), Yellow Fever virus (YF), and ZIKV are “*Aedes*-associated” viruses (see also Moureau et al., 2015). The “*Culex*-associated” flaviviruses are known for their bird reservoirs and human neurotropic effects (e.g., encephalitis and paralysis) whereas “*Aedes*-associated” flaviviruses such as Dengue virus (DENV) and Yellow Fever virus (YF) are known for primate reservoirs and hemorrhagic diseases.

Phylogenetically, ZIKV clearly belongs to the lineage that contains WNV and SLE, two so-called “*Culex*-associated” viruses. Nodal support for the [ZIKV + [SLE + WNV]] clade ranges from 97 to 100% (Kuno et al., 1998; Lanciotti et al., 2008; Moureau et al., 2015). However, several of these same authors still consider ZIKV to be *Aedes*-associated according to convention. Based on the phylogenetic relationships, the most parsimonious interpretation is to include ZIKV within the *Culex*-associated lineage. This is in line with comments made by Grard et al. (2010) who analyzed the complete coding sequence of *Aedes*-borne flaviviruses [*sensu* ICVT] and concluded that, based on an analysis of amino acid distances in the NS5 gene, ZIKV is “clearly related to *Culex*-borne flaviviruses.” Using this logic, the fact that ZIKV is linked to neurotropic effects—such as fetal brain



abnormalities and Guillain-Barré syndrome—comes as no surprise. Due to the relatively low number of human ZIKV cases prior to 2007, severe neurotropic symptoms were previously unknown, and were unexpected due to the paradigm that ZIKV was a traditional “*Aedes*-borne” virus [*sensu* ICVT] akin to DENV.

Figure 1 also shows the association with disease indicators, namely neurotropic effects (indicated by a brain) or hemorrhagic effects (indicated by a drop of blood). The common vertebrate reservoirs are also indicated with silhouettes of either birds or non-human primates. In terms of disease indicators, ZIKV is more like WNV and SLE, but possibly more like DENV and YF in terms of reservoir hosts. Or is it? A forgotten paper by Okai et al. (1971) reported that 15% of 221 birds collected in Uganda tested positive for ZIKV antibodies by Hemagglutination Inhibition Assays, with the majority of positive birds being Greenbuls (Family Pycnonotidae). The Okai et al. (1971) study would support the addition of a bird silhouette to the ZIKV branch in **Figure 1**.

KEEP AN OPEN MIND

Scientists need to consider the possibility that ZIKV may be more similar to the classic “*Culex*-associated” flaviviruses than it is to the “*Aedes*-associated” viruses by collecting and testing field-collected *Culex* spp., by screening a number of different vertebrates for their potential roles as reservoirs, and by studying the vector competence of *Culex* spp. in the laboratory. Aliota et al. (2016) have reported that colony-reared *C. pipiens* were unable to become infected with ZIKV strain

PRVABC59. Weger-Lucarelli et al. (2016) also showed that colony-reared *C. pipiens*, *C. quinquefasciatus*, and *C. tarsalis* were refractory to ZIKV strain PRVABC59. However, additional studies on the vector competence of *Culex* mosquitoes to other ZIKV strains need to be conducted and published. In certain geographic regions, *C. quinquefasciatus* is a highly peridomestic mosquito and it feeds avidly on humans; furthermore, it has been found in relatively large numbers when mosquito surveillance has been conducted for ZIKV (Grard et al., 2014; Gabon, third most abundant species; Ledermann et al., 2014; Yap, second most abundant species). The World cannot afford to concentrate all of its efforts on monitoring and controlling *A. aegypti* in the fight against ZIKV, especially if there are *Culex* mosquitoes that may also serve as competent vectors.

Consideration ought to be given to the phylogenetic evidence that “*Culex*-borne” flaviviruses have evolved from

ancestral “*Aedes*-borne” flaviviruses (Grard et al., 2010) and that means that an expanded (rather than a restricted) vector range might be expected for ZIKV. Furthermore, based on a data-driven model linking mosquito vector species and vector-virus traits, Evans et al. (2017) have predicted that as many as 35 different mosquito species could be vectors for ZIKV.

Medical entomologists, public health professionals, and politicians are urged to keep an open mind on the issue of which mosquito species need to be targeted for control in the battle against ZIKV.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Imported Zika Virus in a European City: How to Prevent Local Transmission?

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Background: On February 1st 2016 the WHO declared the Zika Virus (ZIKV) infection a worldwide public health emergency because of its rapid expansion and severe complications, such as Guillain-Barré Syndrome or microcephaly in newborn. The huge amount of people traveling to endemic areas and the presence of *Aedes albopictus* in Barcelona increase the risk of autochthonous transmission. The objective of this study was to describe the first ZIKV cases diagnosed in our city and to analyze the surveillance, prevention, and control measures implemented to avoid autochthonous transmission.

Methods: An observational cross-sectional population-based study in Barcelona, Spain was performed. An analysis of the socio-demographic, epidemiological, clinical characteristics, and mosquito control activities of the ZIKV cases detected between January 1st and December 2016 was carried out using a specific ZIKV epidemiological survey of the Barcelona Public Health Agency.

Results: A total of 118 notifications of possible ZIKV infections were received, and 44 corresponded to confirmed cases in Barcelona residents. Amongst these, the median age was 35 years and 57% were women. All cases were imported, 48% were Spanish-born and 52% foreign-born. Dominican Republic was the most visited country amongst foreign-born patients and Nicaragua amongst Spanish-born. The most frequent symptoms were exanthema, fever, and arthralgia. Among the 24 diagnosed women, 6 (25%) were pregnant. There was one case of microcephaly outside Barcelona city. Entomological inspections were done at the homes of 19 cases (43.2% of the total) and in 34 (77.3%) public spaces. Vector activity was found in one case of the 44 confirmed cases, and 134 surveillance and vector control were carried out associated to imported ZIKV cases. In all cases prevention measures were recommended to avoid mosquito bites on infected cases.

Conclusion: Epidemiological and entomological surveillance are essential for the prevention of autochthonous transmission of arbovirosis that may have a great impact on Public Health. The good coordination between epidemiologists, entomologists, microbiologists, and clinicians is a priority in a touristic city with an intense relationship with endemic countries to minimize the risk of local transmission by competent vectors.

Keywords: arbovirus, epidemiology, global health, Guillain-Barré syndrome, microcephaly, public health, mosquito, Zika virus

INTRODUCTION

Arboviruses are a group of viruses transmitted through arthropods and many of them are capable of producing infection in humans. West Nile virus (WNV), Chikungunya (CHIKV), dengue (DENV), or Zika virus (ZIKV) are emerging arboviruses with a high potential of generating epidemic outbreaks (White et al., 2016). ZIKV was discovered in 1947 in Uganda by scientists that were performing a surveillance of the yellow fever virus in a forest called Zika (Hayes, 2009; Bulletin of the World Health Organization, 2016). This virus is transmitted by mosquitoes and *Aedes aegypti* is the main known vector (Ayres, 2016; Chouin-Carneiro et al., 2016). Recent infectivity studies in laboratory conditions revealed that *Aedes albopictus* is also susceptible to ZIKV virus infection, since the virus is replicated, disseminated and can reach to salivary glands. However, the efficiency of this infective process is lower in comparison with *A. aegypti*, which clearly shows the highest ZIKV vector competence among mosquitoes (Chouin-Carneiro et al., 2016; Di Luca et al., 2016; Jupille et al., 2016). Moreover, it is important to note that *A. albopictus* has been found infected also in wild populations in endemic areas as Gabon, in Central Africa (Grard et al., 2014).

The first cases of infection by ZIKV in humans were diagnosed in Uganda and Nigeria in 1952 (Macnamara, 1954). Throughout the second half of 20th century, the ZIKV had expanded to countries in Africa and Asia; India, Egypt, Malaysia, Mozambique, Nigeria, the Philippines, and Vietnam. Until 2007, only 16 cases in humans had been reported. It is at this time, when the ZIKV expands to the Yap Island, in the Pacific (Federal State of Micronesia), where the first great outbreak was reported (Duffy et al., 2009). Other large outbreaks were reported in the French Polynesia (2013–2014) and in Brazil in 2015, where the first ZIKV autochthonous case in Latin America was reported. This outbreak has been the origin of the Public Health crisis initiated in 2016. By the starts of February 2016, local transmission of ZIKV had been reported from more than 20 countries and territories in the Americas (Bulletin of the World Health Organization, 2016).

On February 1st 2016 the World Health Organization (WHO) declared the ZIKV epidemic a worldwide health emergency. This took place after the appearance of three epidemiological alerts

related to outbreaks of congenital microcephaly and Guillain-Barré Syndrome (GBS) cases due to ZIKV in countries such as Brazil, France, USA, and El Salvador (U. S. Department of Health and Human Services, et al., 2016). Although a decline in ZIKV infections has been reported in some countries, or in some regions of countries, surveillance needs to remain high (WHO, 2016a).

Within Europe, Spain is one of the countries with high risk of autochthonous cases of ZIKV infection (WHO, 2016b), due to the presence of *A. albopictus* (Grard et al., 2014; Bueno, 2016) (commonly named “tiger mosquito”) in various regions and due to the great cultural, commercial, touristic, and migratory relationship with Latin America (Díaz-Menéndez et al., 2016). Consequently in April 2016 a National Plan of preparedness and response against DENV, CHIKV, and ZIKV was established. Its objective was to reduce the impact and the risk of establishment of these emerging diseases in Spain (MSSSI, 2016).

In Spain, the first imported case of ZIKV was detected in December 2015–January 2016 (Bachiller-Luque et al., 2016). In Barcelona, both imported ZIKV cases and *A. albopictus* (a competent vector for ZIKV) overlap in space and time. With the objective of controlling the imported ZIKV cases and prevent autochthonous cases, the public health services of the city were reorganized. This rearrangement was included within a more global Surveillance and Control of Arbovirosis Program that was already in place since 2014 that includes other emerging viruses transmitted by *A. albopictus* such as DENV, WNV, and CHIKV (Agència de Salut Pública de Catalunya, 2015; González et al., 2017).

The objectives of this paper were to describe the epidemiological and entomological surveillance for ZIKV cases in Barcelona (Spain) and to describe the improvements in the procedures to control ZIKV and other emerging arbovirosis to reduce the risk of an autochthonous outbreak.

METHODS

Design

A descriptive observational cross sectional population-based study was performed in the city of Barcelona.

Study Period and Population

The notified and confirmed ZIKV cases among residents in Barcelona city from January 1st to December 31st 2016 were studied. All cases were detected and followed by the Epidemiology Service of the Public Health Agency of Barcelona

Abbreviations: CHIKV, Chikungunya; DENV, Dengue; GBS, Guillain-Barré Syndrome; PHAB, Public Health Agency of Barcelona; STD, Sexual Transmitted Disease; SUPCS, Surveillance and Urban Plague Control Service; WHO, World Health Organization; ZIKV, Zika Virus.

(PHAB) through the notifiable disease register. Entomological surveillance period starts on March and finishes on November because the low temperatures prevent vector activity. The cases that did not reside in Barcelona and those that were not confirmed by the laboratory were excluded from the analyses.

ZIKV, CHIKV, and DENV are diseases of mandatory notification in Spain. In Barcelona an active epidemiological surveillance system is carried out based on a close communication among doctors, laboratories and the public health nurses from the Epidemiology Service. The circuit and procedures followed from the notification of a possible case until entomological inspections are listed in **Figure 1**.

Definitions and Case Classification

The case definition used at the PHAB is described in the “National Plan for preparation and response against vector transmitted diseases” and in the “Protocol for surveillance and Control of mosquito transmitted arbovirolos in Catalonia” updated on 14 June 2016. The cases were classified according to clinical, epidemiological and laboratory criteria in four categories

(Díaz-Menéndez et al., 2016; González et al., 2017).

1. *Probable case*: person that complies with the clinical criteria with or without epidemiological criteria and that complies with the laboratory criteria of probable case.
2. *Confirmed case*: person that complies with the clinical criteria with or without epidemiological criteria and that complies with the laboratory criteria of confirmed case.
3. *Imported case*: when the beginning of the symptoms occur until 15 days after abandoning a ZIKV epidemic area.
4. *Autochthonous case*: if there is no record/history of trip to endemic area within the last 15 days previous to the beginning of symptoms (Agència de Salut Pública de Catalunya, 2015).

The days of viremic phase in Barcelona since the arrival date were calculated for each patient. The viremic period was estimated from the natural history of the infection reported in the literature. For the viremic period, we assumed 7 days starting from the day of symptoms onset. However, we also simulate how many people would be viremic in Barcelona with different theoretic values: 8 and 9 days of viremic period.

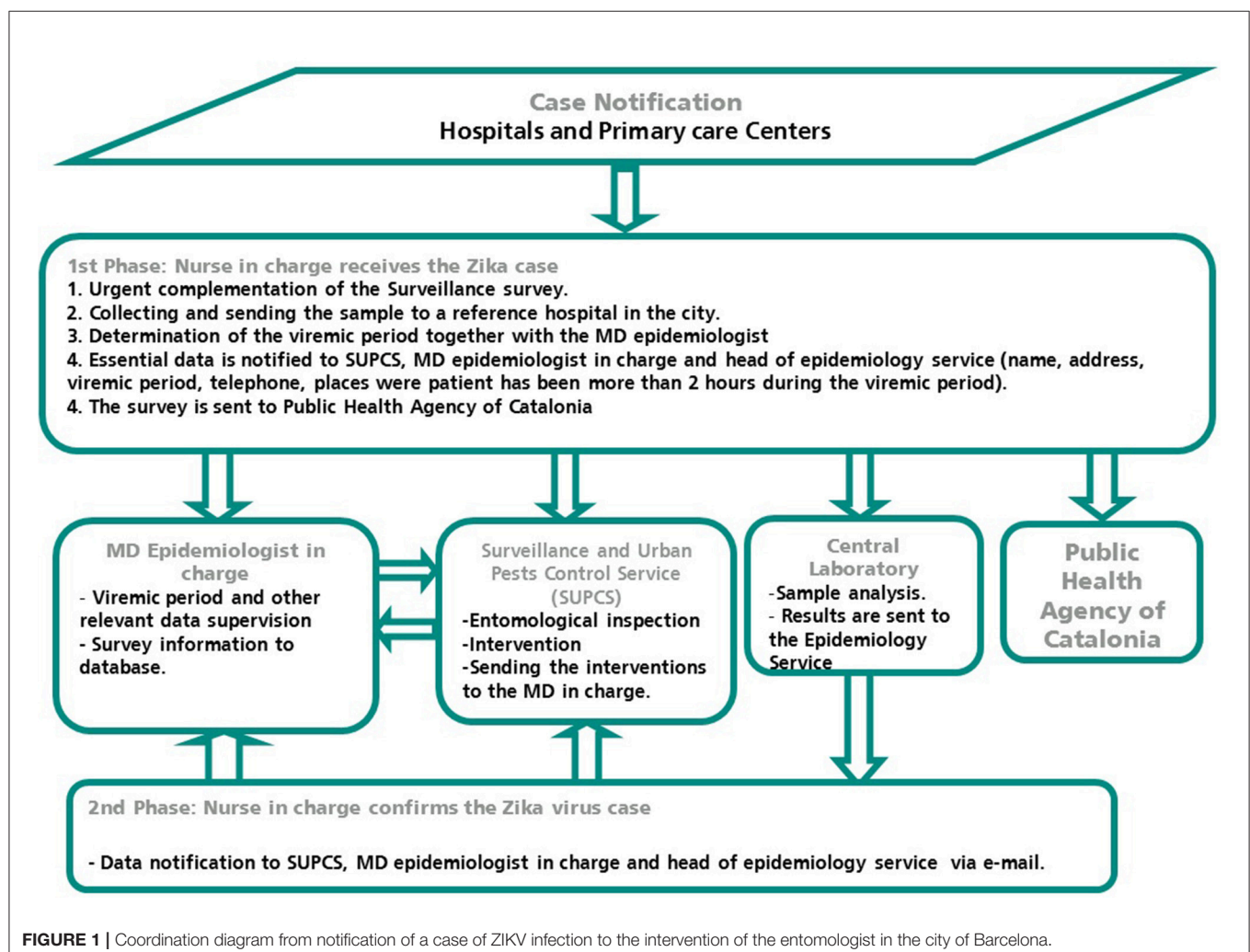


FIGURE 1 | Coordination diagram from notification of a case of ZIKV infection to the intervention of the entomologist in the city of Barcelona.

Variables and Source of Information

The different epidemiological variables were collected from the epidemiological survey of Catalunya specific to ZIKV infection. Socio-demographic variables (age, sex, country of origin, neighborhood/district of residence), clinical variables (date of beginning of symptoms, viremic period, fever, asthenia, arthralgia, arthritis, conjunctival hyperemia, cephalgia, non-purulent conjunctivitis, exanthema, myalgia, complications, hospitalization), diagnostic variables [date of diagnosis, laboratory results (IgG, IgM, PCR)], and epidemiological variables (way of transmission, pregnancy, and gestation week if affirmative, country or countries visited, dates of departure, and arrival to Barcelona, cause of trip, number of bites, place in which bites were received, mobility of the cases and if activities of prevention, confinement, or personal protection were given) were collected. The median time elapsed (in days) between the symptoms onset of patients and the first medical consultation, the notification and the laboratory confirmation, and the implementation of vector control activities were calculated.

The Surveillance and Urban Plague Control Service (SUPCS) at PHAB recorded different information during their entomological inspections related to the cases: places visited during the viremic period, vector activity at case residency, capture of adult vectors with BG traps, detection of mosquito breeding sites, and presence of virus in the vector.

Surveillance Procedures and Vector Control

In order to minimize the risk of ZIKV transmission through local mosquito bites during the viremic period, prevention measures were recommended by the Epidemiology Service. These measures included personal protection measures against mosquito bites (type of clothing, use of mosquito repellent, etc.), and house confinement during the viremic period and recommendations regarding safe sex.

Based on the collected information in the epidemiological survey, the SUPCS carried out an entomological inspection in at least two places: the public space around the case's place of residence and in the patient's own home. All the risk areas for the proliferation of mosquitoes were included in a Geographical Information System (GIS) to speed up the inspections. Gutters, fences, ornamental fountains and small artificial containers, within a buffer of 150 m in relation to the case's place of residence (habitual flying ratio of *A. albopictus* in the urban setting) were included in the GIS.

In our study, informative notes with recommendations to prevent the proliferation of larvae breeding sites in the home were distributed during the inspections of more vulnerable residential areas close to the imported case. The entomological inspections in private homes were analogous to the already described procedures for public areas. The areas were inspected for detecting larvae breeding spot sites and adult mosquitoes. It's worth mentioning that these activities are essential in the reduction of the risk of disease expansion because the contact between vectors and the infected host is likely to occur at home. The need to obtain an authorization from the patient to be able

to carry out the inspection was an important limiting factor in comparison with the interventions in public spaces.

The collected entomological material was examined and identified and females were analyzed in pools for the detection of virus presence by RT-PCR. All the necessary steps for the evaluation of transmission risk were considered in the protocol, including vector activity (inside and around the patient's place of residence), and the efficacy of the control activities. The program monitoring the cases of imported arbovirosis is integrated within a general procedure that monitors and treats monthly the areas with recurrent proliferation of tiger mosquitoes (Bonnefoy et al., 2008; Montalvo et al., 2016).

Statistical Analysis

A descriptive study of qualitative and quantitative variables to characterize the study population was carried out. We computed the frequency distributions of the qualitative variables, and compared proportions using the χ^2 -test, or the two-tailed Fisher test when expected values were <5 . Absolute frequencies were calculated for categorical variables. Social, demographic, epidemiologic and clinical variables were compared according to the country of origin of the infected person (Spanish born or foreign-born). The median and interquartile range (IQR) for continuous variables was calculated and Spanish born and foreign born were compared using the U-Mann Whitney-test. A $p < 0.05$ was considered statistically significant.

Ethical Considerations

This study was approved by the Clinical Research Ethical Committee of Parc Salut Mar (IMAS). In order to guarantee data and registry confidentiality the regulation established by the Organic Law of Personal Data Protection of Spain 15/1999 and Royal Decree 994/1999 about security of computerized files that contain personal data was followed. All ethical principles for investigation in humans defined in the Declaration of Helsinki of 1964 revised and updated by the Worldwide Medical Association (Fortaleza, Brazil 2013) were followed.

RESULTS

Epidemiological Surveillance

During the study period 118 cases were notified, 75 of which (63.6%) were laboratory confirmed. **Figure 2** shows the monthly distribution according to the place of residency (those who lived in the city or outside the city but diagnosed in Barcelona) of these 75 confirmed cases. Forty-four confirmed cases correspond to residents in the city, with an incidence of 2.74 cases per 100,000 inhabitants. The median age was 35 (standard deviation 11.8), 25 (57%) were women, and 19 (43%) men. They were all imported cases, 21 (48%) among Spanish-born population and 23 (52%) among immigrant population. No differences regarding age and sex were observed ($p = 0.2$ and 0.57 , respectively). The number of cases peaked in August, when most of the infections occurred among Spanish-born individuals (**Figure 3**).

When comparing the characteristics of the 44 confirmed cases between Spanish-born and foreign-born, it stands

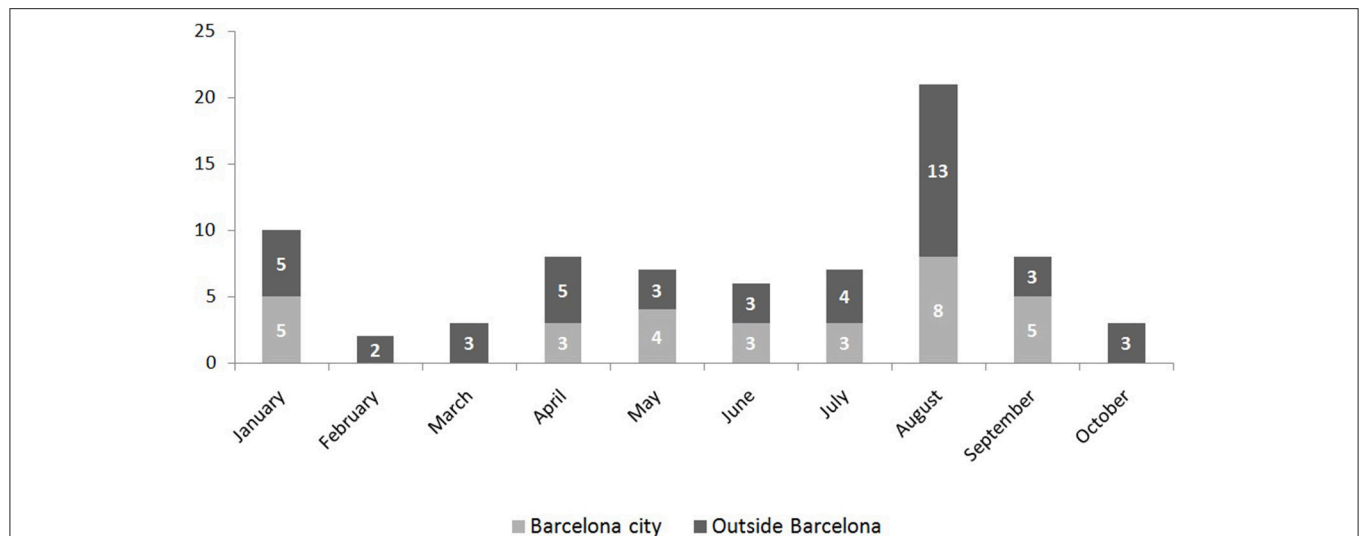


FIGURE 2 | Monthly distribution according to place of residence (living in the city or outside the city of Barcelona) of the first 75 confirmed cases of Zika virus infection in Barcelona. Period: January–December 2016.

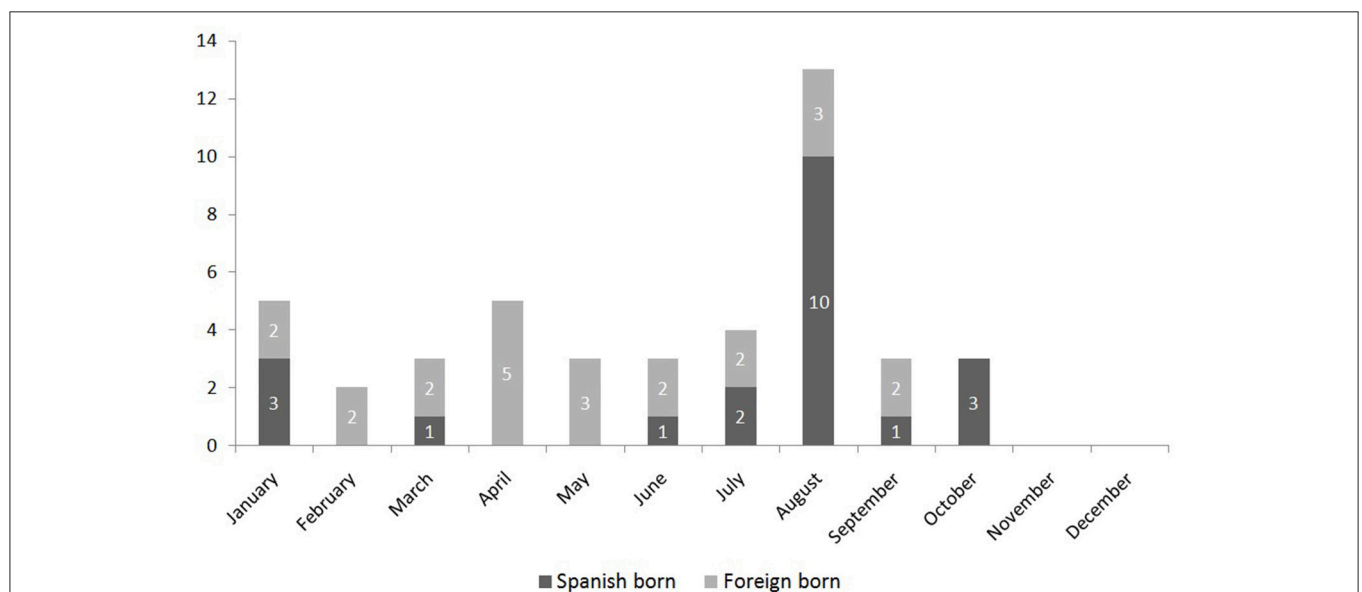


FIGURE 3 | Monthly distribution of the 44 imported cases of Zika virus in Barcelona according to country of birth (Spanish born or foreign born). Period: January–December 2016.

out that the most frequently visited countries where the Dominican Republic and Venezuela amongst the foreign-born and Nicaragua, Colombia, Mexico, and Vietnam amongst the Spanish-born. Fifty-two percent of the cases didn't report being bitten by mosquitoes after arrival to Barcelona. The most frequent clinical symptoms were rash (86%), fever (61%), and arthralgia (59%). No significant differences were found between Spanish-born and foreign-born in relation to the clinical symptoms, visited country or detected bites (Table 1).

A total of 31 cases (70.5%) were arrived in Barcelona during the viremic period and 13 (30%) arrived during the incubation phase and became viremic in the city (Figure 4). The proportion of viremic immigrants for 8–9 days in Barcelona was higher than for the Spanish born (Table 1) (61% vs. 29%), $p = 0.034$.

The median time, for Spanish born, elapsed between the symptoms onset and: (a) the first medical consultation date was 4 days (IQR 2–5); (b) the notification date was 8 days (IQR 7–14); (c) the laboratory confirmation date was 9 days (IQR 6–12), and

TABLE 1 | Comparison of the descriptive characteristics between Spanish born and foreign born for the 44 confirmed cases of Zika virus infection in the city of Barcelona. January–November 2016.

	Spanish-born N (%)		Foreign-born N (%)		Total N (%)	
	21	48%	23	52%	44	
AGE (MEDIAN, SD)	33.5	10.7	36.5	12.7	35	11.8
SEX						
Male	10	48%	9	39%	19	43%
Female	11	52%	14	61%	25	57%
HOSPITALIZATION						
Yes	0	0%	0	0%	0	0%
No	21	100%	23	100%	44	100%
CLINICAL SYMPTOMS						
Fever	14	67%	13	57%	27	61%
Arthralgia	11	52%	15	65%	26	59%
Rash	17	81%	21	91%	38	86%
Myalgia	6	29%	7	30%	13	30%
Cephalaea	9	43%	9	39%	18	41%
Any other symptom	15	71%	12	52%	27	61%
COMPLICATIONS						
	0	0%	0	0%	0	0%
VISITED COUNTRY						
Dominican Republic	2	10%	6	26%	8	18%
Nicaragua	6	29%	2	9%	8	18%
Colombia	3	14%	2	9%	5	11%
Mexico	3	14%	1	4%	4	9%
Venezuela	0	0%	4	17%	4	9%
Vietnam	3	14%	0	0%	3	7%
Honduras	1	5%	1	4%	2	5%
Bolivia	0	0%	2	9%	2	5%
Others	3	14%	5	22%	8	18%
DETECTED BITES						
Yes	10	48%	11	48%	21	48%
No	11	52%	12	52%	23	52%
PREGNANCY						
	2	10%	2	14%	4	16%
DAYS OF RISK IN BARCELONA (VIREMIC PERIOD)						
0 days	10	48%	3	13%	13	30%
1–7 days	5	24%	6	26%	11	25%
From 8 to 9 days	6	29%	14	61%	20	45%
PREVENTIVE RECOMMENDATIONS						
Yes	21	100%	23	100%	44	100%
No	0	0%	0	0%	0	0%

(d) the implementation date of vector control activities was 9 days (IQR 7–14). The median time in days for foreign born was 4 (IQR 2–11), 11 (IQR 8–27), 20 (IQR 13–25), and 13 (IQR 11–28), respectively. No statistically significant differences were observed in these elapsed times between Spanish-born and foreign-born patients except for the lab confirmation days ($p = 0.04$).

Regarding the consequences of the ZIKV infection, no mortality was observed. Regarding morbidity, no GBS was detected. Among the 24 diagnosed women, 6 (25%) were pregnant. There was one case of microcephaly notified to PHAB but the pregnant woman lived outside Barcelona city.

Vector Control

According to the Surveillance protocol of arbovirolos in Catalonia, 34 entomological inspections related to ZIKV were done between 1st April and 15th November. Nine of the 34 cases had high mobility during the viremic period visiting different areas of the city, increasing their exposure to tiger mosquito bites. Only 19 (43.2%) homes could be inspected, since the persons could not be contacted or refused the inspection in the other 15 cases. The activity of *A. albopictus* was low, and was only detected in the public neighborhood of one of the cases (Figure 5).

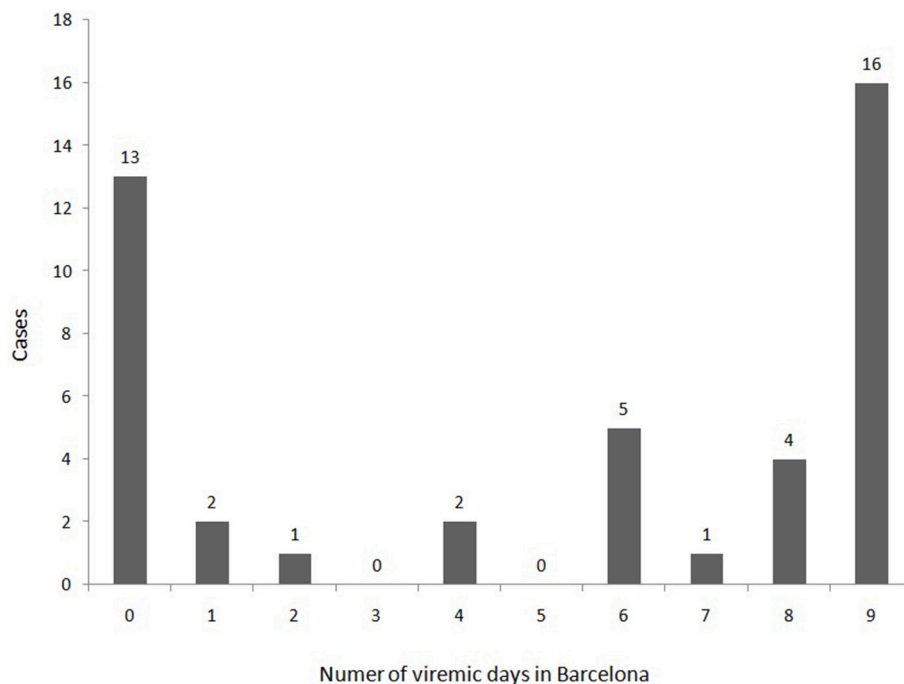


FIGURE 4 | Distribution of the first 44 confirmed cases of Zika virus infection according to the viremic days spent in the city of Barcelona.

There was a seasonal overlap between vector activity, and the arrival of imported ZIKV cases with viremia (**Figure 6**). April, August, and September, are amongst the months of higher overlap and consequently higher risk of local transmission of ZIKV.

To minimize the risk of transmission as much as possible, 122 vector control interventions took place in public spaces and 12 in places of residence of ZIKV cases. Most of the interventions were larvicide treatments in gutters and modifications of elements with risk of hosting mosquito larvae, in most cases in private property. In those cases in which tiger mosquito activity was detected or its presence was suspected, we used BG Sentinel traps for capturing mosquito females. Twelve traps were set, four in public spaces, and eight in private property. Only one pool of mosquitoes was finally analyzed for ZIKV detection and the result of the molecular analysis was negative.

DISCUSSION

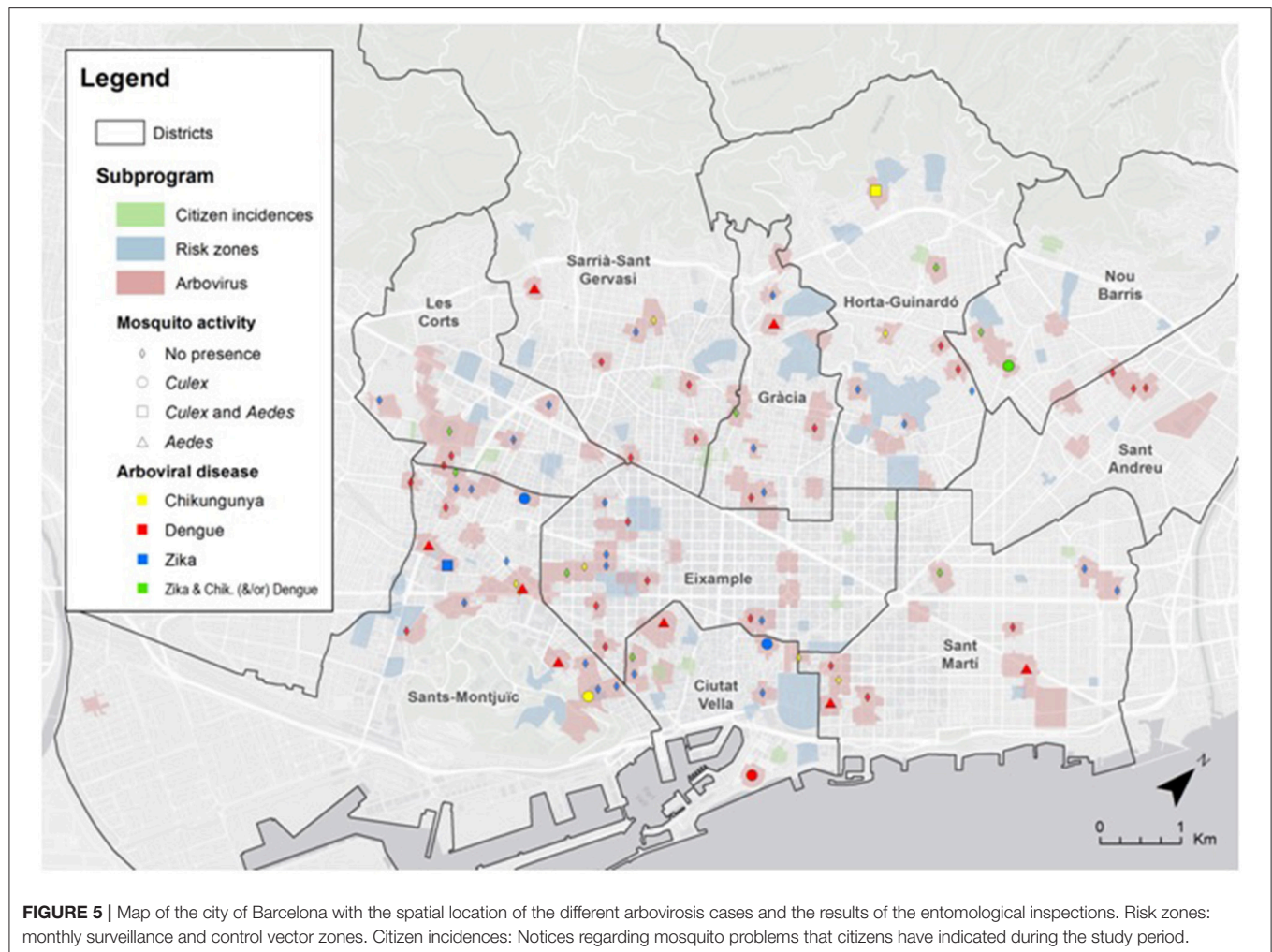
The first year of epidemiological surveillance for ZIKV in the city of Barcelona has allowed us to know very well the profile of ZIKV imported cases. The wide presence of a vector that can transmit the virus (*A. albopictus*) and the population movements increases the risk of introduction of emerging arbovirus like ZIKV. This fact requires surveillance of the imported cases and tasks for vector control. This makes a good coordination between all the different actors, like epidemiologists, clinicians, entomologists, and microbiologists essential in order to avoid ZIKV from becoming a public

health problem that would also affect the tourism in the city.

The imported ZIKV cases profile was that of a traveler that after visiting endemic areas for ZIKV in Latin America, presents clinical symptoms such as fever, rash and arthralgia. No differences in sex, age, clinical symptoms, detected bites, or visited country were observed among Spanish and foreign-born patients. Regarding the visited country, the predominant group was originally from the Dominican Republic, Venezuela, and Nicaragua or Colombia. Other cities in Spain have also reported a high number of cases from Latin American countries. This is due to the mobility of people between Latin America and Spain that takes place for different reasons such as business, cooperation, tourism or visits to friends and relatives. It also responds to the high number of immigrants living in Spain especially since year 2000, and that occasionally travel to their countries of origin (López De Lera, 1995; Díaz-Menéndez et al., 2016). In many of these countries ZIKV is endemic and its expansion is favored by the wide presence and abundance of *A. aegypti*, main vector for ZIKV and other arbovirolosis.

Viremia

Over 70% of the diagnosed ZIKV cases in our city where viremic (PCR positive on serum) when they sought medical attention and over 36% (16/44) arrived in the city during incubation phase and spend all the viremic phase in our city. Usually tourists spend less time at their destinations than foreign born which also explains that Spanish-born patients more often present viremic phase after arriving. For the foreign-born, the reason for traveling is to visit friends and relatives and therefore normally they stay with their



family and stay for a longer period of time. The cases that started viremic phase after arriving to Spain represented the highest risk for local transmission because of the longest duration of exposure to local vectors while still viremic. Additionally, an important percentage of patients reported mosquito bites after arrival, in many cases overlapping with the viremic period.

The incubation period can vary between 2 and 14 days after infection and the viremic period can be 3–5 days long or longer (Falcao et al., 2016). Nevertheless, the viremic period for ZIKV has not been clearly established, especially in some vulnerable populations such as pregnant women (Suy et al., 2016). The viremic period in our city is estimated from the natural history of the infection, 7 days starting from the day of symptoms onset, not performing a repeated blood test to check the presence of the virus. Recently the CDC stated that the ZIKV viremic period should be extended to 7 days, information that is of great relevance in vector control and autochthonous transmission (Agència de Salut Pública de Catalunya, 2015). According to **Figure 4** it can be deduced that if the viremic phase was considered to be only 5 days, the total days for all cases in viremic period would be 116 days. However, if the viremic

phase is considered to be 9 days, the total days of viremic period are 252 days, almost twice. Therefore, we believe that while the accurate length of the viremic phase is not yet established, the best option to maximize effects of epidemiological surveillance and entomological precautions is to consider a viremic phase of 9 days (Alejo-Cancho et al., 2016).

The duration of the viremic period has also important implications for the establishment of prevention measures. Entomologic inspections and tasks should occur as soon as a suspected case seeks medical advice, in order to reduce the risk of transmission during the viremic phase. Delays in the flow of information between medical and entomological staff may dramatically reduce the effectiveness in control, making them ineffective if the information reaches the entomological staff when the viraemic period has finished.

It is important to highlight that 9 of 34 confirmed cases, in spite of the recommendations given by public health services or the clinic, experienced great mobility during the viremic period. The fact that the clinical symptoms are frequently mild probably had an influence on this. These aspects together with the fact that most of the imported cases overlapped with periods of vector

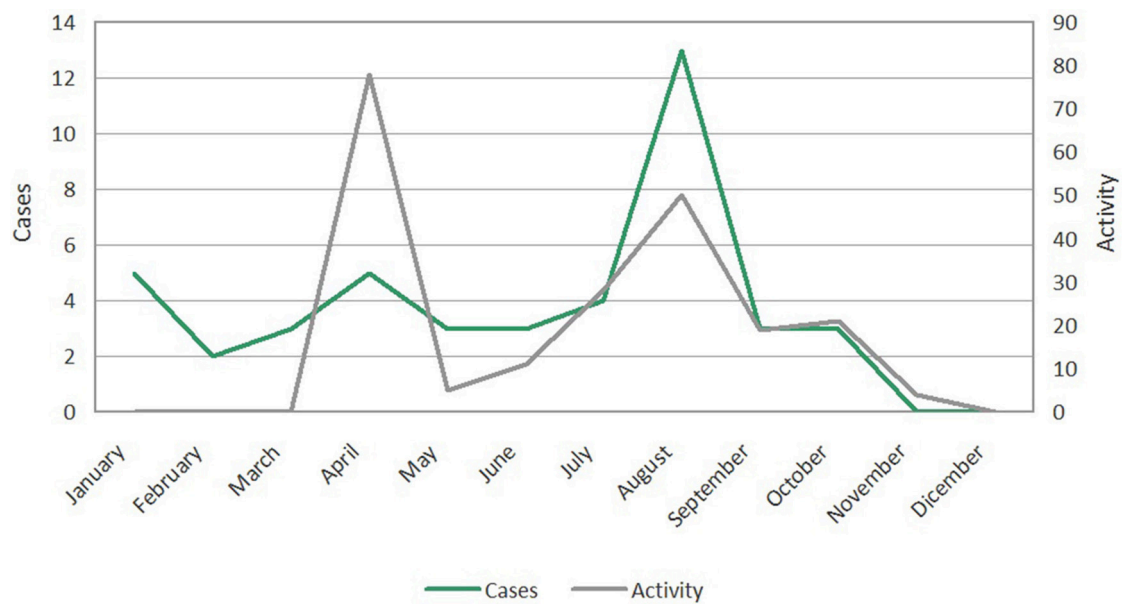


FIGURE 6 | Distribution of the Zika virus and *Aedes albopictus* vector activity (number of larval hatcheries detected on the public road) by month in the city of Barcelona.

activity increased the risk of possible transmission. It is therefore very important to recommend to patients a reduction in their mobility during this period (Marrama-Rakotoarivony and Zeller, 2012).

Zika Virus Consequences

To this date, nor deaths nor cases of SGB related to ZIKV have been reported in Barcelona. However, in a retrospective case-control study that took place after the ZIKV outbreak in French Polynesia (2013–2014), 42 cases of SGB were identified which were also positive for DENV virus and ZIKV. Recently there has been an increase in the SGB incidence in countries like Brazil, Colombia, El Salvador, and Suriname, but the exact cause is still unknown.

Unfortunately, we found one microcephaly case out of 6 babies born from ZIKV infected mothers. We had a high portion of pregnant woman due to the active screening of pregnant woman that is being performed in Catalonia. Not only the doctors but also pregnant woman that travel to a ZIKV endemic area are aware about the risks of microcephaly in newborn and look for medical advice and follow up when they are back home. This ongoing protocol in Catalonia perform not only an screening and follow up of all pregnant woman exposed to the risk of ZIKV but also a long term follow-up of all newborn in order to monitor the development of neurological abnormalities (Bocanegra et al., 2016). A prospective study in Brazil amongst pregnant women infected by ZIKV showed that 29% of the fetus suffered some type of abnormality during pregnancy, including microcephaly and intrauterine restricted development. Fetal abnormalities were detected by Doppler ultrasonography in 12 of the 42 (29%) ZIKV positive women (Brasil et al., 2016; Mysorekar and Diamond, 2016; Reynolds et al., 2017). In light

of this scenario, we need to rapidly evaluate the risk to child development by this emerging disease that is spreading quickly in many territories.

In the context of epidemiological surveillance it is important not to forget that ZIKV could be a sexual transmitted disease (STD). In the latest WHO update, 12 countries have reported STD of ZIKV, not only from man to woman, but also from woman to man, man to man (Deckard et al., 2016) and also could be transmitted by oral sex (World Health Organization, 2016). The exact duration of ZIKV in semen still remains unknown. In a recent study, ZIKV RNA was detected in semen 62 days after the initiation of the symptoms (D'Ortenzo et al., 2016), even though the longest period in which ZIKV has been detected in semen is 188 days (Althaus and Low, 2016; Paz-Bailey et al., 2017). Other routes of transmission include transmission through blood (Barjas-Castro et al., 2016).

Reorganization of the Public Health Services

After the diagnosis of the first imported cases of arbovirolos in our city, it was necessary to face up to the risk the presence of *A. albopictus* represented to public health. For these reason, an epidemiologist and an entomologist were incorporated in the team for surveillance and control of arbovirolos.

As a result, a protocol for the surveillance and control of mosquito-borne arboviruses was established in Catalonia in 2014. It was written by an inter-institutional commission comprised of different experts (clinicians, microbiologists, entomologists, epidemiologists, etc., Agència de Salut Pública de Catalunya, 2015). The importance of surveillance and early detection was key therefore an interdisciplinary group formed by members of the Epidemiology Service and the Vector

Control Service of the PHAB was created. Public health nurses were trained to fully know the arboviruses and to adequately complete the case reporting surveys. The structure (**Figure 1**) was also organized in coordination with the doctors of the hospitals specialized in Travel Medicine. This changes improved communication systems, and accelerated the responses to risk situations that occurred with the arrival of viremic patients. A fundamental aspect was the communication and knowledge of the protocol by all the involved agents. For this reason, PHAB organized training sessions for physicians were held in the main hospitals. The aim was to increase awareness of the importance of each agent involved in the process (Bonney et al., 2008; Bueno, 2016). The information was disseminated through the commission of arbovirology of Catalonia and through TV programs and news reports and at a round table that was organized at the 6th Emerging Diseases Congress in Barcelona (Camprubí, 2016).

In this first year of experience in ZIKV surveillance and control, the delays observed among time elapsed between the symptoms onset and first medical consultation, public health notification, laboratory confirmation, and implementation of vector control activities, were remarkable. The first problem was that the diagnostic delay in the first medical consultation and therefore the notification delay to the public health system. Therefore, the vector control activities were also initiated later, when patient was no longer viremic. So, from public health it is essential to educate patients to consult soon and also that doctors notify all suspected cases of ZIKV quickly.

Vector Control

To this date no autochthonous ZIKV case has been detected in Barcelona. However, they could have been occurred since some cases may go undetected as happened in Croatia for DENV infection (Kurot et al., 2013). There is a clear overlap of the peaks of imported cases and the vector's activity (**Figure 6**). The analysis of the phenology in the vector appearance using a weighting of the risk factors, allows us to identify the months of April, August, and September (in general the months with highest temperatures) as the most favorable for the mosquito species (**Figure 3**). In any case, the phenology of *A. albopictus* matches the predictive models and the observations that have been carried out recently in different Mediterranean cities (Tran et al., 2013; Bueno, 2016).

Local transmission of ZIKV has been observed recently in temperate zones of USA such as Texas and Florida. Among the 1,325 reported cases in 2016, 262 were due to local transmission, and 224 were in pregnant woman (16.9%). After governmental efforts on vector control activities, no new ZIKV cases have been detected by local transmission in Florida. The example of Florida together with Barcelona, underlines the importance of surveillance and vector control activities and close monitoring to prevent ZIKV circulation (Department of Health Daily Zika Update, 2017).

In light of this situation, efforts to intensify traveler's advice, preventive measures and individual protection measures must be maximized. We believe in paying special attention in providing resources to reduce mobility of the cases that are in viremic

period. This would help reduce contact with the vector and decrease possible risk of transmission. A possible solution would be to make home confinement obligatory. We are aware that adherence to this recommendation could be low. In the case of people that are working, an obligatory medical leave could be provided in order to reduce mobility. Another area in which improvement could be made is in the inspection of private properties. These are confined areas where there is a great overlap between vector and viremic patient. In our case only 19 out of 34 homes could be inspected, leaving a significant part of the risk assessment without completion.

In Barcelona, when vector activity was detected, either in larval phase or in adult phase, larvicide or adulticide control actions took place within the framework of the basic criteria of Plague Integrated Control (Bonney et al., 2008). Adulticide spraying can pose several environmental problems especially in urban environments, since they are of large spectrum interventions that can affect non-target organisms. However, although the effect of adulticide treatments is well known to be short-lived, in certain epidemiological contexts a well technified adulticide application is needed to reduce the mosquito population rapidly (Caputo et al., 2016). Consequently, it is a particularly interesting strategy in the framework of imported cases of arbovirus in a concrete place where the vectors are present in high densities. In any case, it is well known that a long temporal lapse between outbreak initiation and the start of control tasks reduces effectiveness of adulticides applications significantly in terms of cost-benefits (Burattini et al., 2008). Therefore, the best approach is to supply reactive adulticides by preventive larvicides as a basis of the control programme, since the treatment of potential breeding sites with larvicides has a proven role in the reduction of adult population at local scale in urban environments (Sochacki et al., 2016).

The activity of vectors detected in the entomological inspections of the ZIKV viremic cases in homes was low. However, additional inspections related to DENV cases detected high activity for five cases during 2016. This fact highlights the need for coordination in the protocols for surveillance and control of any arbovirology in order to reduce the risk of transmission (Lucientes-Curdi et al., 2014). Similar protocols have been implemented in other countries such as France or Italy (Zammarchi et al., 2015; Maria et al., 2016) and recently have also been approved at a national level in Spain (MSSSI, 2016). It is important that these programs for surveillance and control integrate epidemiological issues (with an appropriate identification and follow up of cases and adequate prevention measures) as well as vector issues (vector presence and quantification) (Bonney et al., 2008; Bueno, 2016).

The use of new technology can help improve the systems and knowledge of the territory (mosquito proliferation areas and detection of new competent vectors for transmission of diseases). Applications such as *Mosquito Alert* (<http://www.mosquitoalert.com>) can help improve the early detection system and are of great help in managing this public health problem (Center for Ecological Research and Forestry Applications. Oltra

et al., 2016). *Mosquito Alert* collaborated in locating unidentified breeding sites in private property near confirmed cases of ZIKV.

A. albopictus may not be the only competent vector of ZIKV in Barcelona. Different studies have suggested the possible role of *Culex* sp. as a ZIKV vector due to the occasional detection of the virus in wild populations (Huang et al., 2016). However, viral infection experiments in the laboratory have not confirmed *Culex* as a competent vector (Amraoui et al., 2016; Boccolini et al., 2016; Huang et al., 2016).

Risk of Local Transmission and Preventive Measures

Autochthonous transmission of ZIKV can potentially occur in the Mediterranean countries since imported cases and competent vectors are present. Therefore, it is essential to establish active case surveillance and prevention protocols to avoid local transmission. As previously stated, the most effective prevention measure to avoid local transmission is vector control. Additionally, it is important that healthcare professionals are informed of the potential risk of ZIKV cases, since this will improve early case detection, surveillance procedures and transmission control (Leona, 2016; Red Nacional de Vigilancia Epidemiológica, 2016). Nevertheless, since a low percent of ZIKV are symptomatic, the majority of viremic travelers returning from endemic zones could be unidentified, and therefore possible source of autochthonous outbreak. In this sense, reducing mosquito populations in the city may significantly reduce the risk of autochthonous outbreak because limiting vector control activities to the neighborhood of symptomatic cases, may have limited impact if a significant fraction of ZIKV infections are asymptomatic during the viraemic phase.

In relation to the preventive measures, at an individual level, the imperative recommendation is that all people that travel to endemic areas should take precautions against mosquito bites (use of repellents, mosquito nets, long sleeve clothes) and on arrival should visit a medical center if any symptom develops (Agència de Salut Pública de Catalunya, 2015; Leona, 2016; MSSSI, 2016). At a community level, taking into consideration that in the last few years migratory mobility and the increase of individual's mobility has produced the introduction of emerging diseases such as many arbovirosis, the recommendations would be based on reducing the areas suitable for the reproduction of *Aedes* mosquitoes in around human inhabited areas. These measures would be based on the elimination or protection of small containers that can accumulate water, and the application of measures of control in those water containers that cannot be protected or eliminated. A frequent inspection of these spaces to avoid any mosquito breeding site would be necessary. Individual protection measures should be taken in order to avoid bites, such as the use of long sleeves and long pants, and the use of repellents.

Pregnant women are a priority. The early detection of the cases and effects on fetus and newborns is essential. In pregnant women, the diagnosis has to be confirmed and there

has to be a follow up of the pregnancy and the fetus. Men arriving from areas with local ZIKV transmission should have safe sex during a minimum period of 6 months. Women arrived from endemic areas should have safe sex during 8 weeks (Red Nacional de Vigilancia Epidemiológica, 2016). The pregnant women who have traveled to areas with local ZIKV transmission or that have presented symptoms after the trip should communicate this information during pre-natal visits. The time that a woman has to wait to get pregnant after arriving from an affected area is 8 weeks from the time of arrival or since the time of diagnosis (CDC, 2016; WHO, 2016c).

All these preventive measures together with a good management of imported ZIKV cases and coordination among epidemiologists, clinicians, entomologists, and microbiologists are essential to prevent local transmission and therefore to limit the extension of this emerging disease. The arbovirus surveillance program in Barcelona is an example of the need of a multidisciplinary approach in order to reduce the risk of introduction of the different arbovirosis, amongst them, ZIKV. The coordination between public health and pest control agencies has contributed to the reduction of the risk of autochthonous transmission.

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AUTHOR CONTRIBUTIONS

JM, TM, RB, EC, and JC conceived and designed the work, write the first draft, revised critically and approved the final version. AR, AP analyzed and participated in the interpretation of the data, revised critically and approved the final version. AR, AP, LF, LD, VP, MM, ES, and JF participated in the acquisition and interpretation of the data, revised critically and approved the final version. Zika Working Group in Barcelona participated in the acquisition and interpretation of the data, revised critically and approved the final draft. All authors agree to be accountable for all aspects of the work.

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Prevention and Control Strategies to Counter ZIKA Epidemic

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ZIKA virus (ZIKA) has now become a global phenomenon. Since 2007, evidence of ZIKA transmission has been reported over 72 countries and territories. The transmission of ZIKA has made World Health Organization to categorize the situation under the ambit of a health emergency. This situation is serious because there appears to be a highly tangible link between infection during pregnancy and the occurrence of microcephaly and Guillain-Barré syndrome. In the context of this emergency situation, this review article intends to discuss the prevention and control strategies such as avoiding travel to infected area, being careful from mosquito bites, take precautions to reduce the risk of sexual transmission, and seek medical care for any acute illness with rash or fever. This review is an attempt to analyze the results of those campaigns, keeping in view the variables and constants that affect any such measures. Furthermore, this article will suggest proactive measures that can be employed to effectively combat the epidemic transmission of the ZIKA.

Keywords: ZIKA, diagnoses, prevention, treatment, awareness

INTRODUCTION

ZIKA is a member of family Flaviviridae and the genus *Flavivirus*. The virus is icosahedral, enveloped, non-segmented, single-stranded, 10 kb positive-sense RNA genome that closely relates to the Spondweni virus (Hayes, 2009; Faye et al., 2014; Hafiz et al., 2016). Apart from lipid bilayer and one genome RNA, ZIKA contains three distinct types of structural proteins such as envelope protein (E), M-membrane protein (M/prM) and capsid or core protein (C) and seven non-structural proteins (Lindenbach and Rice, 2003; Dia et al., 2016). ZIKA infection is caused by the bites of daytime-active *Aedes* mosquitoes such as *Aedes aegypti* and *A. albopictus* (Malone et al., 2016). The virus was first isolated from *Aedes africanus* infected rhesus monkey in 1947 and is named after the Ugandan ZIKA Forest (Dick, 1952; Weinbren and Williams, 1958). The parallel research indicated that *A. aegypti* has also the capability of transmitting ZIKA to monkeys and mice (Boorman and Porterfield, 1956). This led to the suspicion that the virus can possibly infect humans. The first major outbreak of ZIKA infection was reported from the Island of Yap in 2007 followed by Brazil in 2015. The most commonly reported symptoms of ZIKA are mild fever, skin rashes, joint pain, myalgia, and conjunctivitis (red eyes). Many people infected with ZIKA do not get sick, people who get sick usually report a number of symptoms, resembling the symptoms of dengue fever (Malone et al., 2016), with a very low mortality rate. This is the primary reason that the prevention and control strategies are rarely implemented in infected population; however, there is a need to prevent further infections. The virus attracted the spotlight from public health officials because of its highly suspected association with maternal-fetal transmission and the microcephaly in the infected

fetus as well as other associated neurological abnormalities in adults with Guillain-Barré syndrome (GBS). Several prevention and control strategies have been discussed and implemented to control the epidemic. The World Health Organization (WHO) currently listed following 72 countries and territories where evidence of ZIKA transmission has been reported since 2007 (WHO, 2016). In 2015 alone, the evidence of ZIKA transmission was reported in 56 countries and territories; in 2016, it was reported in five countries, whereas in 12 countries and territories the evidence of ZIKA transmission could be in or before 2015. Further, since February 2016, 12 countries have reported evidence of person-to-person ZIKA transmission and over 12 countries reported microcephaly and other CNS malfunctions associated with ZIKA. In addition, four countries reported microcephalic babies born from mothers in countries with no endemic ZIKA transmission, but have traveled to ZIKA affected countries in the past. Therefore, to minimize the transmission, and associated effects including fetal microcephaly, the Centers for Disease Control and Prevention (CDC) circulated an advisory, particularly for the expecting women, to avoid or at least postpone their visit to the areas where transmission rate is high (Oliveira et al., 2016), since, the risk of infection could be associated with travelers from Brazil to other countries. From September 2014 to August 2015, nearly 2.8 million people travel to and from Brazil to United States as shown in **Figure 1** (Statista, 2016), suggesting that there is a high risk factor of ZIKA infection.

Epidemiology

Because of the asymptomatic clinical course of ZIKA infection, it is difficult to calculate precise global prevalence. It has not been widely reported owing to its clinical resemblance with other flavivirus infections, and difficulty in differential diagnosis.

The ZIKA has been reported in various hosts including mosquitos, humans, and, monkeys through sporadic case reports, seroprevalence surveys and entomological surveys in 14 different

countries across Africa, Asia, and Oceania (Ioos et al., 2014). The virus may also pass from primary to secondary host through blood transfusion via infected blood cells and sexual contact (**Figure 2**). The ZIKA infection symptoms usually appear in 3–12 days after the vector bite and end within 2–7 days after onset of symptoms (Duffy et al., 2009).

In South America, Brazil reported first outbreak of ZIKA around mid of 2015. Brazil Ministry of Health gave an estimated figure of 440,000–1,300,000 suspected cases of patients infected with ZIKA in December 2015 (ECDC, 2016). In the United States as of May, 2016, a total of 472 cases of ZIKA were reported that are travel-associated. In other US territories, a total of three travel-associated cases and 658 locally acquired cases were reported (CDC, 2016a). Microcephaly and congenital syndrome associated with ZIKA are also prevalent in countries, which have ZIKA outbreak as shown in **Table 1**.

CLINICAL PRESENTATION AND DIAGNOSIS

As previously discussed ZIKA transmits to humans through infected *A. aegypti* and *A. albopictus* mosquitoes. Further, if infects pregnant mother, the virus can pass to the fetus and lead to microcephaly. In Colombia, 12,000 pregnant women were infected with ZIKA and there were no reported microcephaly evidence in their babies as of May 2016 (Rasmussen et al., 2016). However, in April 2016, the CDC declared ZIKA the cause of microcephaly in Brazil (Rasmussen et al., 2016), yet outside of Brazil a similar number of cases have not been reported. In June 15, 2016, the WHO reported seven ZIKA associated microcephaly cases in Colombia and 1500 confirmed cases in Brazil (WHO, 2016). The results of a study in French Polynesia provided the evidence that 1 in 100 pregnancies exposed in the first and second trimester, resulted in microcephaly (Cauchemez et al., 2016). However, for ZIKA, asymptomatic infection is common, and only 20% of infected humans show symptoms like

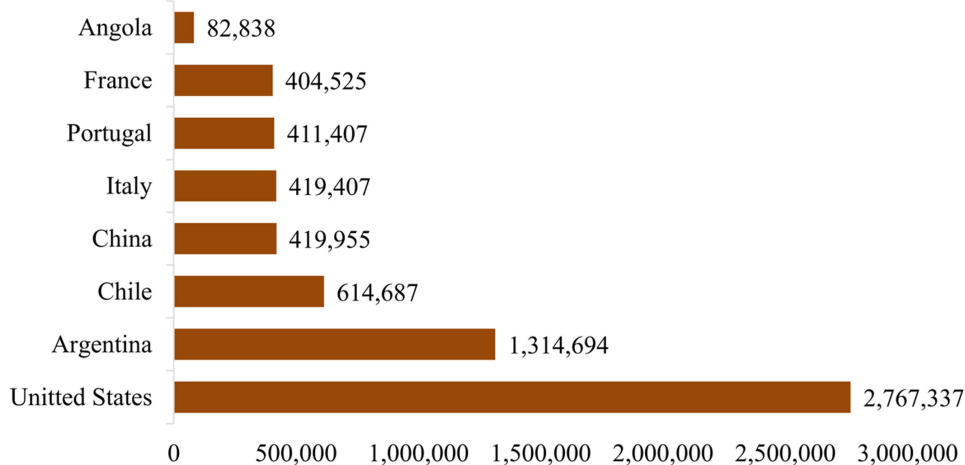


FIGURE 1 | Number of travelers to and from Brazil to other countries (Statista, 2016).

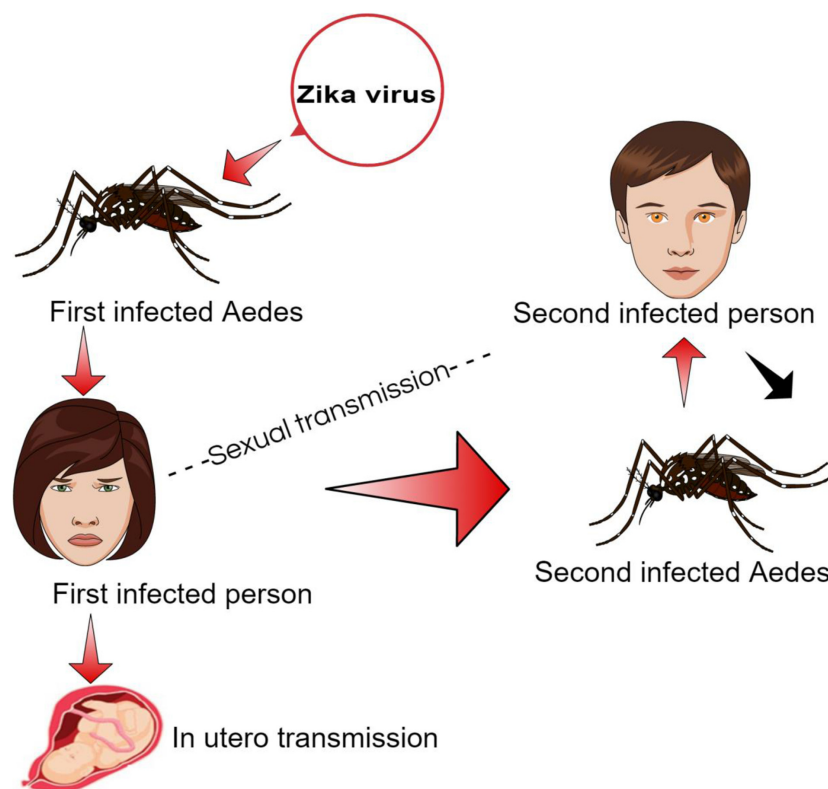


FIGURE 2 | ZIKA transmission cycle in humans.

TABLE 1 | ZIKA microcephaly and congenital syndrome by September 2016 (WHO, 2016).

Location	Number of confirmed cases by September 2016
Brazil	1911
Colombia	40
Martinique	12
USA	23
French Polynesia	8
Panama	5

acute fever, maculopapular rashes, conjunctivitis, and arthralgia. Because of low mortality and mild symptoms, hospitalization ratio is relatively lesser as compared to Ebola like infections (Wojda et al., 2015).

The disease can be diagnosed by patient's clinical signs and symptoms, resembling to other mosquito-borne viral diseases. The complete travel history of the patient to the infected areas can lead the physicians to correct diagnosis. Although the mosquito has been designated as the primary vector for ZIKA transmission, other modes of transmission like blood transfusion and sexual intercourse are also under consideration (Musso et al., 2015).

While the clinical differential diagnosis is not specific, diagnosis can be authenticated by performing a reverse transcriptase-polymerase chain reaction (RT-PCR) according to the CDC-issued guidance. The ZIKA RNA is detectable in serum

after 7th days post-symptom onset. ZIKA RNA has been detected in serum in non-pregnant patients and pregnant patients after 62 days post-symptom onset and 53 days of after last known exposure in an asymptomatic pregnant women (Driggers et al., 2016; Meaney-Delman et al., 2016). ZIKA has been detected from number of body fluids, including urine, blood, saliva, and amniotic fluid (Musso et al., 2014; Barzon et al., 2016; Hills et al., 2016). However, one of the reports suggest that urine samples are more useful for diagnosis of ZIKA infections (Gourinat et al., 2015). The virus specific immunoglobulin M (IgM) as well as neutralizing antibodies can also be detected after 1 week of infection, but these detected antibodies are not specific (Tappe et al., 2014). In addition, other rapid tests such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Lee et al., 2011) and RNA-biosensors (Baeumner et al., 2002) could be used as a first step for virus detection. Therefore, there is a dire need to develop a bedside test for ZIKA, to enable the clinicians for effective diagnosis of the patients infected with the virus.

PUBLIC HEALTH RESPONSE TO EPIDEMIC

Currently, there is no specific vaccine for the preventive treatment of ZIKA infection. However, different groups of scientists are working world over to develop the ZIKA vaccine,

and hopefully will be available in couple of years (Daily Mail, 2016). For the effective disease control and prevention, the surveillance system can be drawn by reviewing the models that have already been used against dengue and chikungunya fever, but it should not be limited to only these two viral diseases (Tami et al., 2016).

The public health approaches that will be implemented for prevention of ZIKA should be capable of acknowledging the urbanization of diseases spreading through animal associated with population explosion, international trade and easy modes of intercontinental transportations. There are five clear strategies delineated by the Weaver (2013) where the interventions by public health authorities can be made. The first strategy is to intercept the enzootic life cycle. In this strategy, it is advised to stop the vector growth in its native environment; however, this strategy would not be feasible for ZIKA as it is difficult to control the vector. Another limitation to the strategy is that there is no available vaccine for ZIKA that could be inoculated in primates. The second strategy is to reduce the exposure of vulnerable subjects to the vector, in case of humans, applying bed nets and mosquito repellents that can decrease the exposure. The third technique that can reduce the disease burden is to limit the vector/source to the urban population. This could be done through control via modulating the vectorial capacity of the *Aedes* mosquito. Limiting the travel to infected areas also minimizes the risk of ZIKA. Fourth strategy could be the most helpful as well as it is an active strategy where the vector reservoirs are eliminated. In the case of ZIKA, proper drainage can reduce the stagnant water reservoirs to inhibit uncontrolled reproduction of mosquitoes. Adequate garbage management could also be used to hinder the vector proliferation. The fifth intervention that can be helpful is to avoid the recurrence of the disease where humans can act as the source of the virus for infection in non-human primates like monkeys. Avoiding mosquito bites to infected humans could be the aiding strategy for the prevention of spill over (Weaver, 2013).

The intervention of public health authorities is important, as the current epidemic is not confined to single geographic location. Despite vector-based transmission, blood transfusion could also be a cause of the spread among the patients having comorbidities. In the recent past, 3% of the blood donors in French Polynesia were screened positive for ZIKA using PCR (Aubry et al., 2015).

It has also been reported that the ZIKA was present in the semen (Musso et al., 2015), therefore leading to a possibility of transmission through the sexual intercourse. If it is a sexually transmitted disease, the approaches used to avoid sexual transmitted diseases can also be employed to avoid the transmission. The contact tracing used in other sexual transmitted disease can be used to identify the other infected patients (LaMotte, 2016).

PREVENTION STRATEGIES

Till date, there is no curative medicine for the ZIKA disease, so to contain the epidemic prophylactic measures are bifurcated

into following approaches: control vector density and personal protection (Daily Mail, 2016).

Control Vector Density

The key measure to interrupt the transmission of viruses is to control the vector density. One of such control measures is Integrated Vector Management (IVM) recommended by WHO. Using the IVM model, overall sustainability, efficacy and cost-effectiveness of the strategy can be improved. The Integrated Management Strategy for the Prevention and Control of Dengue (IMS – Dengue) could be a basic model for ZIKA. Therefore, participation and collaboration of different organizations would play a great role in exploring IVM model. It has been reported that the *A. aegypti* is found in wide range of larval habitats both natural and manmade. There is a critical need of consistent implementation of the three-pronged IVM Model (Gubler and Clark, 1996). Some of the important control measures should include the followings:

- Monitoring of household and common areas to eliminate vector breeding sites, such as water reservoirs and waste drainage pipes.
- Regular cleaning of garbage collection sites.
- Development of physical, biological, and/or chemical methods to control mosquito breeding.
- Use of appropriate insecticides as per WHO recommendations.
- Fumigation of cargoes at ports and borders to prevent transport of larva by various means of transportation.

In addition to the above control measure, one of the key control measures is to avoid contact with vector. Therefore, personal prevention is very important to avoid sickness. Patients suffering with ZIKA infection and their family members must be well-educated about the risk of transmission. Use of mosquito nets, wire-mesh doors and windows, skin protection, and use of repellents are some of the key prevention measures that need to be taken under consideration.

Environmental Management

There are different environmental intervention and managements to control the growth of the *Aedes* species. In this subclass, authorities should be enabled to implement reliable water supply management, proper cleaning and maintenance of water storage systems, adequate solid waste management systems, and desirable alterations in human behaviors and residence systems. This includes proper cleaning of streets, maintenance and modifications of buildings/structures as well as housing units such as use of mosquito screens/nets on windows and mosquito proofing of water storages (Sikka et al., 2016).

Introduction of Bacteria into the Mosquito Population

A bacteria known as *Wolbachia* is present in approximately 60% of insects, commonly known as world's most common reproductive parasite in the world (EDP, 2016). *Wolbachia* reduces the mosquito-to-human transmission events, ultimately reducing the transmission of virus to the humans from mosquito; the introduction of *Wolbachia* to offspring through the female

mosquito's egg will facilitate the beneficial epidemiological effect of securing humans from the bites. When the males with *Wolbachia* will mate with normal female mosquitos, females fail to hatch eggs while on other hand, the infected *Wolbachia* females will hatch eggs and produce offspring that will carry the *Wolbachia* effect (Nguyen et al., 2015). At the start, the technique will have a very limited effect as there will be a very few *Wolbachia* infected mosquitos present, but with the time, number of *Wolbachia* containing mosquitos increase, the significant effect can be observed. This approach has shown significant results to control dengue; it may also contribute significantly to control the ZIKA epidemic by limiting the ZIKA transmission since both ZIKA and dengue share similar clinical manifestation (Lambrechts et al., 2015).

Genetic Tailoring of the Mosquito

Another technique under consideration is to genetically modify the mosquitoes and giving rise to the population of mosquitoes whose offspring are not able to survive. Reducing the population of mosquitoes, ultimately will reduce the mosquito's bites to humans as well as the vulnerable primates. Especially, using *A. aegypti* OX513A that has previously used to control dengue spread and hopefully it will help in controlling ZIKA spread as well (Phuc et al., 2007). *A. aegypti* OX513A is a genetically engineered strain, which is effective due to presence of release of insects carrying a dominant lethal (RIDL) genetic system (Alphey and Alphey, 2014). Similarly, offspring of OX513A, so the wild females will also die; this will keep the threshold of population needed for the disease spread. There are many benefits of the genetically modified vectors, but the most important fact is the dissemination of knowledge regarding capabilities of genetically modified vector to the concerned governmental officials, public health officials, and scientists (Alphey and Alphey, 2014).

Scientists and organizations are working on the genetic modification of the vectors and aware of the fact that transgenic technologies are associated with several environmental and safety concerns that still need to be addressed. There are several

ecological side effects like unintended spread to non-target species; and horizontal transfer of the transgenes that should be properly addressed to avoid any undesired outcome (Gabrieli et al., 2014).

Personal Prevention Measure

In the area of known ZIKA infection outbreak, the patients should avoid further contact with the vector to limit the spread of virus to other healthy people in the community. The community members should go through a proper awareness and education regarding prevention. The community members should be encouraged to act on the steps mentioned in **Table 2**. Insect repellents like *N*, *N*-diethyl-3-methylbenzamide, 3-(*N*-butyl-*N*-acetyl) amino propionic acid ethyl-ester or icaridin can be used. There are no specific recommendations and restrictions regarding use of these mosquito repellents, unless there is any specific warning given on the label of the product (Gibbons, 2002).

In the first week of ZIKA infection, following preventive measures should be followed:

- *Aedes* mosquito bites should be avoided.
- The patients are advised to stay under the bed-nets.
- Another community that is under great risk of getting infected from the patients is the health workers. It is essential to protect the health workers so that other hospitalized patients might not get infected from the workers. In addition, care should be taken during blood donations and organ donations.
- Avoid sexual intercourse when traveling to infected area or when one of the partner is infected with ZIKA.
- Pregnant women are advised not to visit the setting where patients are resided. Similarly, pregnant women are advised not to visit where the epidemic is present.

Vaccine Preparation

Multiple firms are looking to prepare ZIKA vaccine so this virus can be cured and does not ail our next generations. In Japan, Takeda Pharmaceutical, Co. Ltd has created a team to

TABLE 2 | Prevention recommendation (copied from Sikka et al., 2016).

Strategy	Action
Control vector design	<ul style="list-style-type: none"> • Diligent management and control of environmental factors. • Eliminate or reduce vector breeding sites in common areas. • Conduct mass sanitation campaigns to educate the public. • Ensure Mosquitoes are removed within the predetermined radius of critical places like schools, hospitals, transport terminals, using risk stratification paradigms. • In areas with viral activity, use mosquito adulticidal sprays to interrupt ZIKA transmission. • Ensure proper monitoring and follow-up during integrated actions for vector control.
Preventative measure	<p>Individual protection</p> <ul style="list-style-type: none"> • Encourage Individuals to use Bed-nets. • Appropriate clothing to cover exposed skin. • Use repellents. <p>Household/residential protection</p> <ul style="list-style-type: none"> • Encourage Installation and use of wire-mesh screens on doors and windows. • Once per week emptying, cleaning, turning over, and disposal of containers that can hold water inside or outside the houses to reduce any mosquito breeding sites.

investigate the propensity for creating a vaccine (Reuters, 2016). In India, Bharat Biotech is looking in creating vaccine for the virus. It is reported that two possible vaccines are under process in India. In USA, Johnson and Johnson, Pfizer Inc. and Merck & Co. Inc. are evaluating the tendency of previous vaccines to combat ZIKA. Sanofi SA from France also launched a program to create ZIKA vaccine. All in all, pharmaceutical firms around the world are trying to create a vaccine for this virus, suggesting that a ZIKA vaccine is at ground zero. Till then preventive measures is the best way to avoid this virus (Reuters, 2016).

A group of researchers performed a drug repurposing screen of around 6000 compounds and identified some novel compounds that either suppress or inhibit ZIKA infection-induced caspase-3 activity in different neural cells. In addition, 10 different inhibitors of cyclin-dependent kinases inhibited ZIKA replication (Xu et al., 2016). One of these compounds are already existing drug, Niclosaminde and another potentially active against ZIKA is PHA-690509 (Xu et al., 2016). The results

are still preminary and after successfull animal trails might be available to humans.

In another study, ZIKA-117, an antibody derived from the blood of ZIKA infected people potentially protect developing fetuses from the ravages of the ZIKA virus in mice. The antibody treatment inhibit the virus in the mother and protect the fetus (Sapparapu et al., 2016). However, development of ZIKA vaccine would be promising for long-lasting immunity against the virus than short-term antibody treatment.

BRIEF HISTORY AND TRAVELERS RECOMMENDATIONS

In 1947, Alexander Haddon and George Dick first identified ZIKA in monkey while studying yellow fever in the ZIKA forest of Uganda. Subsequently, the same virus was isolated from the *Aedes* species of mosquitoes in the same forest of Uganda. In 1950, antibodies against ZIKA were detected in humans, and in

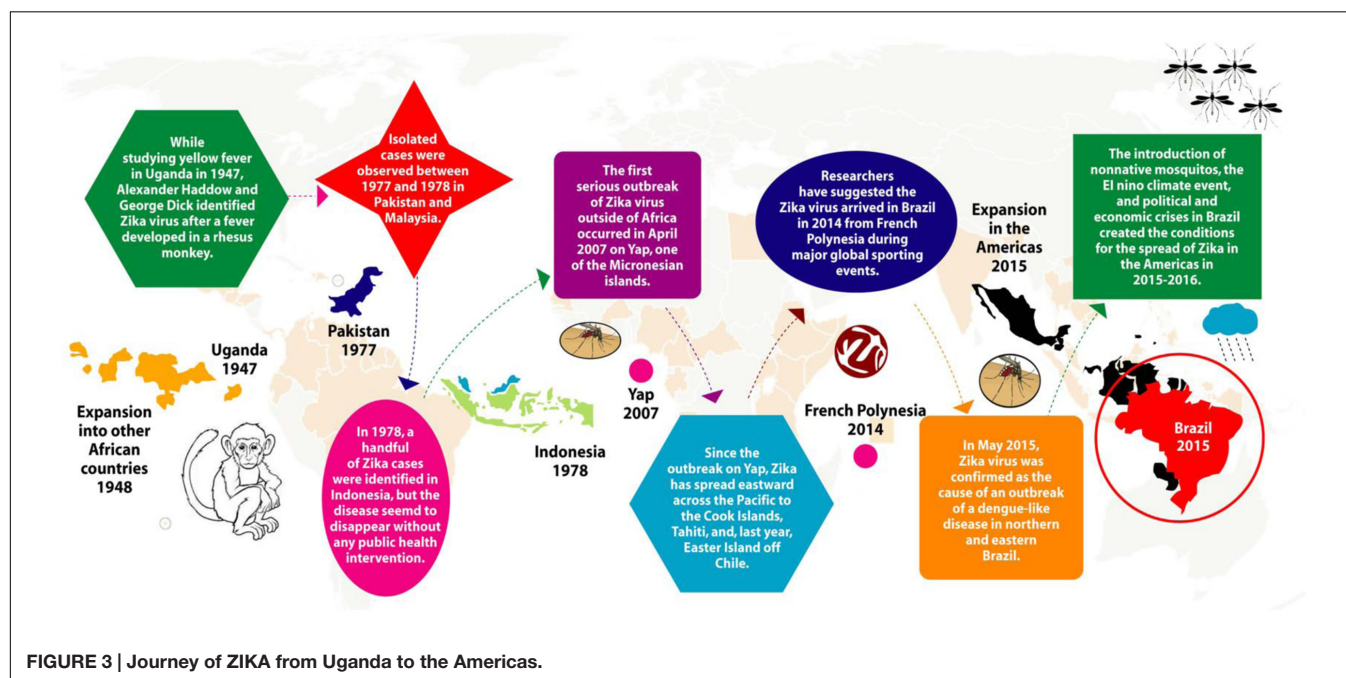


FIGURE 3 | Journey of ZIKA from Uganda to the Americas.

TABLE 3 | Recommendations to travelers (copied from Sikka et al., 2016).

Traveler status	Recommendations
Prior to departure	<ul style="list-style-type: none"> Travelers are advised to protect themselves from mosquito bites during stay. Use Mosquito repellents, wear appropriate clothing to minimize skin exposure. Use insecticides and bed-nets. Educate travelers about signs and symptoms of ZIKA/dengue/chikungunya virus in order to identification and to reduce the time to required medical attention.
During visit	<ul style="list-style-type: none"> Avoidance of mosquito-infested areas. Avoidance of mosquito bites. Proactive and proper use of bed-nets and/or insecticide. Seek professional care in case there are symptoms of ZIKA/dengue/Chikungunya
Upon return	<ul style="list-style-type: none"> Travelers should contact appropriate health care provider in case ZIKA infection is suspected. Due to some symptomatic overlap, this is also applies to dengue and chikungunya viruses.

1968, ZIKA was isolated from humans in Nigeria. With time, the ZIKA spread to other parts of Africa as well as Asia. However, until 2007, no cases of ZIKA infection was found outside Africa and Asia, except an outbreak in Yap Island and French Polynesia in 2013. In 2015, the first case of ZIKA infection was detected in Brazil and has spread to more than 50 countries in the Americas (Figure 3).

Since there is a great risk of ZIKA infection due to travelers traveling from ZIKA infected regions to other non-infected regions, necessary precautions need to be taken. At present, there is no recommendation from the WHO as to travel bans to the affected countries. However, it is the government responsibility to educate the public while traveling to the areas known for epidemic spread (Table 3). Travelers should be advised to carry necessary measures if traveling to ZIKA stricken regions. In addition, appreciate information about the symptoms of ZIKA infection and prevention should be handy at airports, bus terminals, railways stations, and so on. This information could be also printed on travel documents such as air tickets or webpages. It is very important that upon returning from ZIKA prone areas, travelers should contact their healthcare providers before returning home.

CONCLUSION

ZIKA with no doubt took the world as a storm because of *Aedes* mosquitoes in South America. The virus entered and propagated in the country with conditions favorable for ZIKA like high population density, lack of immunity in targets and viral mutations. Global warming is a contributor in widespread of ZIKA as well (Ai et al., 2016). On the face of earth, no country

is safe from ZIKA until preparation of vaccine. Countries having *Aedes* mosquito is on high alert, every day a new case emerges from other part of the world. ZIKA is progressing very fast and next few months are very crucial for stopping the attacks of *Aedes* mosquitoes. High population directly affect the cases of ZIKA. So, if the virus outbreaks in those countries like India, China and USA, it would be impossible to control this virus. The current most workable suggestion to pregnant mothers is to avoid traveling to ZIKA affected areas. This is the best chance we have got till now, more research is necessary on this subject to find the cure for the disease. Cases of ZIKA has to be analyzed in detail to check the relationship of ZIKA and genetic mutation of *Aedes* to help understand why ZIKA is carried out in this type of mosquito (CDC, 2016b).

When ZIKA was identified, it caught us by surprise and no time could be leveraged to do anything in stopping this virus but there are major implications for researchers and doctors in studying ZIKA. It is still unidentified that how many more viruses of this type are present in our atmosphere (Lambrechts et al., 2015). Due to globalization, ZIKA can land anywhere through any channel. Global climate change and urban crowding also give way for ZIKA to grow. Maybe it is time when we need to rethink our public health infrastructure and disease-control strategies.

AUTHOR CONTRIBUTIONS

IR wrote the initial draft of the paper, designed figures and SK updated and proofread the paper. VB and JL did the critical analysis of the data and Y-HP designed, analyzed and approved the paper.

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Zika Virus Infection during Pregnancy and Congenital Abnormalities

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The presence of the Zika virus (ZIKV) infection has gone ahead to be a threat to people based on its adverse impacts. More specifically, the pregnant women have been discouraged from traveling to the areas affected by the ZIKV because of the likelihood of the virus causing congenital abnormalities especially the microcephaly. The pregnant women probably attracted the virus during their first trimester while visiting ZIKV affected territories. Although the ZIKV infected cases have reduced in some parts of countries, the global risk assessment has not been changed. The virus continues to spread geographically to areas where competent vectors are present. At present, there is still no treatment of ZIKV related illness, especially microcephaly.

Keywords: ZIKV, microcephaly, infection diseases, outbreak, mosquito

OVERVIEW

The Zika virus (ZIKV) was discovered during a study conducted in 1947 aimed at establishing the causative agents of yellow fever, dengue, and other West Niles viruses (Dick et al., 1952; Korhonen et al., 2016). The virus was first identified in the blood of a sentinel rhesus monkey in Zika Forest in Entebbe, Uganda (Shors, 2016). The virus was later discovered in a field worker and then spread sporadically to other humans in Africa and Asia and continued to remain insignificant for over six decades from when it was first isolated. It was until its outbreak in Brazil that its spread accelerated rapidly throughout the Americas and other parts of the world raising global health concerns. The increase in the number of women infected with the ZIKV giving birth to children with suspected congenital defects raised fresh concerns by health organizations to research on possible links between the ZIKV infections and brain related defects to the infected mothers during prenatal and postnatal stages of pregnancy. Extensive research was vital to be able to obtain factual proofs rather than mere speculations regarding the seriousness of the infection and on the fetuses and subsequent health status of the newborn babies after the infection. Currently, there are several countries with active ZIKV.

Transmission Areas

These include Aruba, Barbados, Bolivia, Brazil, Colombia, Costa Rica, Cuba, Ecuador, Mexico, Guatemala, Haiti, Panama, Trinidad and Tobago, and American Samoa among others (Centers for Disease Control [CDC], 2017a). Therefore, pregnant women should not travel to areas with active ZIKV transmission. Several studies in the ZIKV affected countries have established adverse effects of the virus on the neonates leading to the bearing of children with various malfunctions associated with the presence of the ZIKV among the pregnant mothers (Centers for Disease Control [CDC], 2016b). The commonly known birth defect covered in this review linked to the presence of ZIKV giving birth to children suffering from microcephaly. Microcephaly is a brain

abnormality characterized by giving birth to children whose heads are abnormally smaller than normal babies irrespective of sex and age (Magauran et al., 2016; Marcondes, 2016). This review is purposely meant to unravel the association between the ZIKV infection during pregnancy, and congenital abnormalities exhibited in children with the virus (McNeil, 2016; Posen et al., 2016). A clear and a deeper insight of the existing relationship can only be achieved through a critical analysis of varied studies about the ZIKV and congenital abnormalities. Therefore, an effort was made to obtain factual information regarding the links between the ZIKV infection and birth-related defects and discard speculative cases of ZIKV as a global menace. Besides, the review assesses the known modes of transmission of the virus from the infected pregnant mothers to the fetuses and how they lead to related brain defects. However, some of the reports have also been cited to discredit ZIKV as the sole cause of all brain damages affecting children before and after birth (Clammer, 2016). Some of these causes of brain damages also include genetic and physical interaction with the toxic chemicals (Jaishankar et al., 2014). The main focus of this review has been put on microcephaly as one of the common congenital birth defects believed to be caused by ZIKV. In most babies, the causes of microcephaly are unknown. State birth defects tracking systems have estimated that microcephaly ranges from two babies per 10,000 live births to about 12 babies per 10,000 live birth in the US (NBDPN, 2013).

The ZIKV is an arthropod-born virus of the Flaviviridae family and is transmitted by the bite of several *Aedes* mosquitoes (Anderson et al., 2016; Focosi et al., 2016; Musso and Gubler, 2016; Petersen et al., 2016). The virus can also be transmitted through the placenta transplant (Besnard et al., 2016), blood transfusion (Venturi et al., 2016), and sexual activities (Hills et al., 2016). The spread of ZIKV remained confined within some specific parts of Sub-Saharan Africa, South East Asia and the America during the 1940s. The confinement of the virus in few countries can be interpreted to mean the high level of reluctance by the World Health Organization (WHO), making countries like the Americas to underestimate its impacts due to insignificant effects on its population before 2015 when it got into Brazil and the Pacific areas. However, the situation changed due to the increasing cases of infants with microcephaly who were born from mothers infected by ZIKV raising the need to carry out research to establish the link between the virus and other birth related defects (Magauran et al., 2016).

THE ASSOCIATIONS BASED ON THE TRENDS OF ZIKV SPREAD

Pan American Health Organization and World Health Organization establish that the first case of ZIKV infection in the human was discovered in 1954 in Nigeria (MacNamara, 1954). Up to this time, the virus was perceived as a preserved issue of African and some parts of Asia. However, this notion changed in 2007 when the pandemic befell Micronesia and Island in the State of Yap with over 5000 infections in a population of 6700 (Duffy et al., 2009). Another outbreak of the infectious virus was reported in French Polynesia in 2013 and 2014 (Hancock

et al., 2014). Other related cases also occurred in 2014 in Pacific Islands like in Cook Islands, Samoa, and American Samoa (Hancock et al., 2014; Tappe et al., 2015; Magauran et al., 2016). The increasing trend in the spreading virus indicated how this dangerous ZIKV infection was able to cause inevitable trouble that the world was likely headed to if the problem was not controlled in time. Consequently, the concomitant increase in the infection of the virus and the cases of microcephaly raised more questions about the association between ZIKV infection and congenital abnormalities among the infected pregnant women (Musso, 2015; Barcellos et al., 2016). The existence of this virus in the Americas was first identified in March of 2015, when an outbreak of an exanthematous illness occurred in Bahia, Brazil (Campos et al., 2015; Zanluca et al., 2015). By October, 2015, ZIKV had spread to at least 14 Brazilian states, and in December 2015, 1.3 million suspected cases were reported by the Brazil Ministry of Health (Hennessey et al., 2016). By September 2015, a tremendous increase in the number of cases of children born with microcephaly was reported, in the areas in which ZIKV was first reported (Schuler-Faccini et al., 2016). The unabated steady increase in the related cases of microcephaly indicates that minimum attempts have been made to curb more effects of the deadly ZIKV. The initial discoveries of the virus were identified from two pregnant Brazilian women whose amniotic fluid tested positive for the ZIKV and were later established in the fetuses which further confirmed the possible relationship between the ZIKV and the causes of microcephaly. More research was imperative to have more samples to choose from and strengthen the previously established links. An extensive research commissioned by the Brazil Ministry of Health served to the purpose of building on the data that had already been carried by other studies of the infection of the virus and congenital defects among the unborn. The research examined the children suffering from microcephaly and how their mothers might have been affected by living or visiting countries with ZIKV during pregnancy. There was a common result obtained from the 35 infant cohorts used during the study (Schuler-Faccini et al., 2016). They all had the lumbar puncture, a situation which made researchers to suspect a link between this defect and the ZIKV. However, clear results were not obtained from the Brazilian Laboratory to conclusively argue that the ZIKV causes microcephaly in children and such brain damages as discovered on the lumber, making it hard to base on this outcome as a clear proof of linkage. The potential relationship that is aimed at in this review is largely based on supported clinical results to ascertain the associations.

There are also statistical data that have been presented to confirm the link between the ZIKV and child birth defects. For example, the epidemiological report for monitoring cases of microcephaly in Brazil established that about 75% cases of microcephaly were reported on February 6th, 2016 which were still under investigation. About 62% of these cases were reported in 2015 and over 37% in 2016. Of all investigations conducted, 15% of the children diagnosed indicated the presence of microcephaly, which was characterized by changes in the central nervous system suggesting an instance of congenital infection in the children. The report also confirmed that out of the total

number of the investigated cases expressing congenital infection, a considerable percentage was identified to have ZIKV through an extensive clinical laboratory test conducted (Clammer, 2016; Naccache et al., 2016). These cases can be used to justify a link between the ZIKV infection and congenital infection, though the actual numbers of children born with microcephaly associated with the ZIKV have not been established.

Effectiveness can be achieved if the association is further broken down into modes of transmitting ZIKV from the environment to the unborn and how the infection can impact on the unborn regarding the brain development. The symptoms of the infection have also been briefly examined to help identify the children with ZIKV related brain infections.

TRANSMISSION OF THE VIRUS

The ZIKV is largely transmitted through a bite by an infected mosquito of the *Aedes* genus majorly found in the tropical areas (Anderson et al., 2016; Focosi et al., 2016; Musso and Gubler, 2016; Petersen et al., 2016). These mosquitoes are similar to those believed to transmit dengue and yellow fever (Sikka et al., 2016). ZIKV transmission is also believed to occur through sexual activities (Hills et al., 2016). The means have been considered a matter of concern due to the perceived adverse impact of the ZIKV on pregnancy and the possible effects on the fetuses (Naccache et al., 2016; Nicastrì et al., 2016). In areas with high transmission rates, infected people can transmit the virus to their partners. It is, therefore, recommended that men and women undergo counseling to know the right time to conceive to avert problems during pregnancy and the deadly outcomes of the ZIKV on the child during pregnancy. Besides, it is imperative that women who are interested in becoming pregnant, but fear due to the risks of ZIKV should have protected sex or use contraceptives (Nicastrì et al., 2016). Safe sex and abstaining should be practiced by mothers during pregnancy for women in areas with active transmission of the virus. In addition, it is recommended that safe sex be practiced or abstinence adopted for men and women who are returning from ZIKV active transmission areas to safe regions to prevent the transmission through sexual intercourse. Transmission through this mode occurs before the symptoms of the illness are realized or during the development of the symptoms. However, these discoveries are limited because they do not establish clear risk-factor duration.

Various evidences have been shown to indicate the possibility of transmitting ZIKV from the mother to the fetuses during pregnancy (Centers for Disease Control [CDC], 2017c). ZIKV RNA was identified in the brain tissue of children with microcephaly who were later reported to have died after their delivery (Branswell, 2016). Studies have shown that the presence of this virus can be detected through ultrasonography by examining the amniotic fluid of the mothers.

There are also suspected possibilities of the virus being transmitted through blood transfusion (Venturi et al., 2016). The assumption here is that other flaviviruses are also transmitted through the same route (Hancock et al., 2014; Nicastrì et al., 2016). There is limited research that has been done to affirm this

truth. The test carried during the French Polynesia established that about 3% of the blood tested had tested positive for the ZIKV.

Monkey bite can also lead to the transmission of the ZIKV. Such a case was identified in Indonesia. It is also suspected that the ZIKV can be transmitted through breastfeeding from the women who have the symptom of the ZIKV during delivery (Bradford, 2016). Such cases are possible if the ZIKV is highly concentrated in the body of the carrier who might, in turn, infect the child. At present, no cases of ZIKV infection associated with breastfeeding have been reported (Centers for Disease Control [CDC], 2017b).

SYMPTOMS IN PREGNANT WOMEN AND POTENTIAL EVIDENCE

According to the WHO, the time between exposure and the symptoms of the ZIKV have not been clearly identified. However, the research assumes that the incubation period is likely to be a few days similar to other related arbovirus infections like dengue. Moreover, over 80% of total women who were infected by ZIKV showed the minimal clinical manifestation of symptoms (Bradford, 2016). The commonly noticed symptoms include fever ($>38^{\circ}\text{C}$), skin rashes, joint pain, malaise, conjunctivitis, headache and fatigue, myalgia, abdominal pain, and vomiting among others (**Figure 1**). Some women had reported rashes that were seen on the faces, palms and soles. However, several studies have also noted that not all these symptoms present in women were infected by the ZIKV. Some of the cases tested showed that some women who were infected had no physical signs (Bradford, 2016; Centers for Disease Control [CDC], 2016c). It can, therefore, be justified through these studies that some of the non-clinical signs are obtained through the principle of generalization raising possible doubts about the universal physical identification of the infected personalities.

MICROCEPHALY

Zika virus has been linked to some of the birth defects experienced by mothers who were infected with the virus during their pregnancy (Centers for Disease Control [CDC], 2017b). The most commonly noted birth outcomes associated with the infection include microcephaly and related brain problems in infants (Campos et al., 2015; Barreto de Araújo et al., 2016). The Center for Disease Control and Prevention defines microcephaly as a birth defect where a child is born with small sized head caused by ZIKV (**Figure 2**). Biologically, the growth and development of the brain happen concurrently with the head. The condition occurs when there is an inadequate development of the brain during the prenatal stage in the head and causing the brain to stop growing after childbirth. The condition can occur in isolation or occur together with other congenital defects prevalent among the fetuses and babies who have been infected by the virus during pregnancy (Jones et al., 2016). Severe microcephaly is characterized by seizure, delay in the development of speech, sitting, standing and walking problems

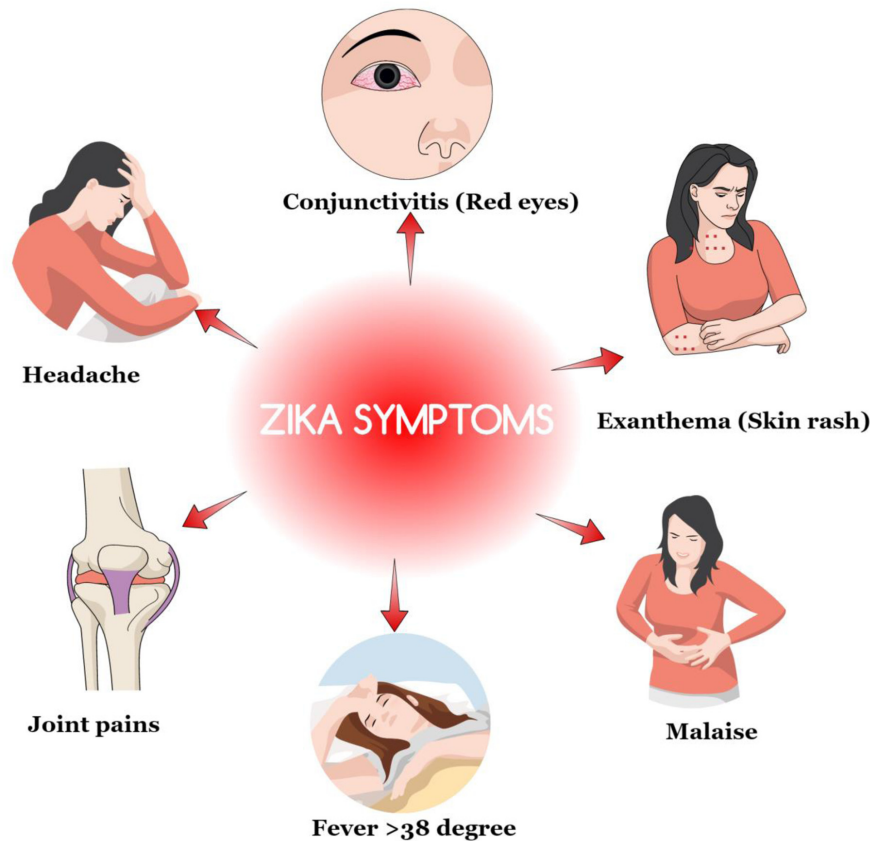


FIGURE 1 | Common noticed symptoms of Zika virus (ZIKV) infection.

(Centers for Disease Control [CDC], 2016a). Children with the condition also experience difficulties in intellectual development which result into decreased learning abilities and influence the normal functioning daily life (Mlaka et al., 2016; Schuler-Faccini et al., 2016). They also experience feeding problems, hearing loss, and vision impairment. A severe condition of this defect is deadly and can only be managed through frequent check-ups and health care monitoring services since they are not treatable. There are two forms of microcephaly like, congenital and acquired (Nicastri et al., 2016). Congenital microcephaly is genetic and is passed from one member of the family whose brains had been infected by the virus causing the defect to the siblings. Acquired microcephaly refers to the case where the brain of the fetus comes into contact with a harmful situation altering the normal growth and development. Fetuses risk acquiring microcephaly in the womb when the pregnant mothers impaired infectious condition specifically with viral infections caused by rubella, chickenpox, and ZIKV. However, other toxic malnutrition and infectious conditions such as hemorrhage state, stroke, and brain injury may also lead to fetus acquired microcephaly. Severe microcephaly is a considered a feature of congenital ZIKV syndrome (Barreto de Araújo et al., 2016). An infant with congenital ZIKV infection was born without showing any signs of microcephaly; however, later experienced slow growth of the head (Van der Linden et al., 2016).

The condition is described as a postnatal microcephaly, which is equally dangerous in the life of the child. These causative agents of microcephaly are important in broadening the understanding of the causes of brain problem. It is, therefore, possible that non-clinical tests can result in a mistaken case of suspected ZIKV on the neonates based on mere observation on physical symptoms.

Furthermore, results from the studies published in the New England Journal Medicine discovered more relationships between the ZIKV infection and the causes of microcephaly and brain anomalies in newborn babies (Michael et al., 2016). The assessment criteria of potential teratogens were used in evaluating the existing data on pregnant mothers. The research coorelated the findings on accumulating evidence to support the existing links of the virus microcephaly during the prenatal development. No research practices were performed making the outcome dependent on the availability of past studies which were accumulated (Centers for Disease Control [CDC], 2016a; Costello et al., 2016). Consistency in the observed defects, was associated with brain anomalies that caused by the presence of ZIKV obtained from the brain tissues of the infants or the fetuses, further supporting the link between ZIKV infection and microcephaly (Kaslow et al., 2014; Rasmussen et al., 2016). This research failed to mention a direct link between the infections rather that accumulating relevant data on the causes of the

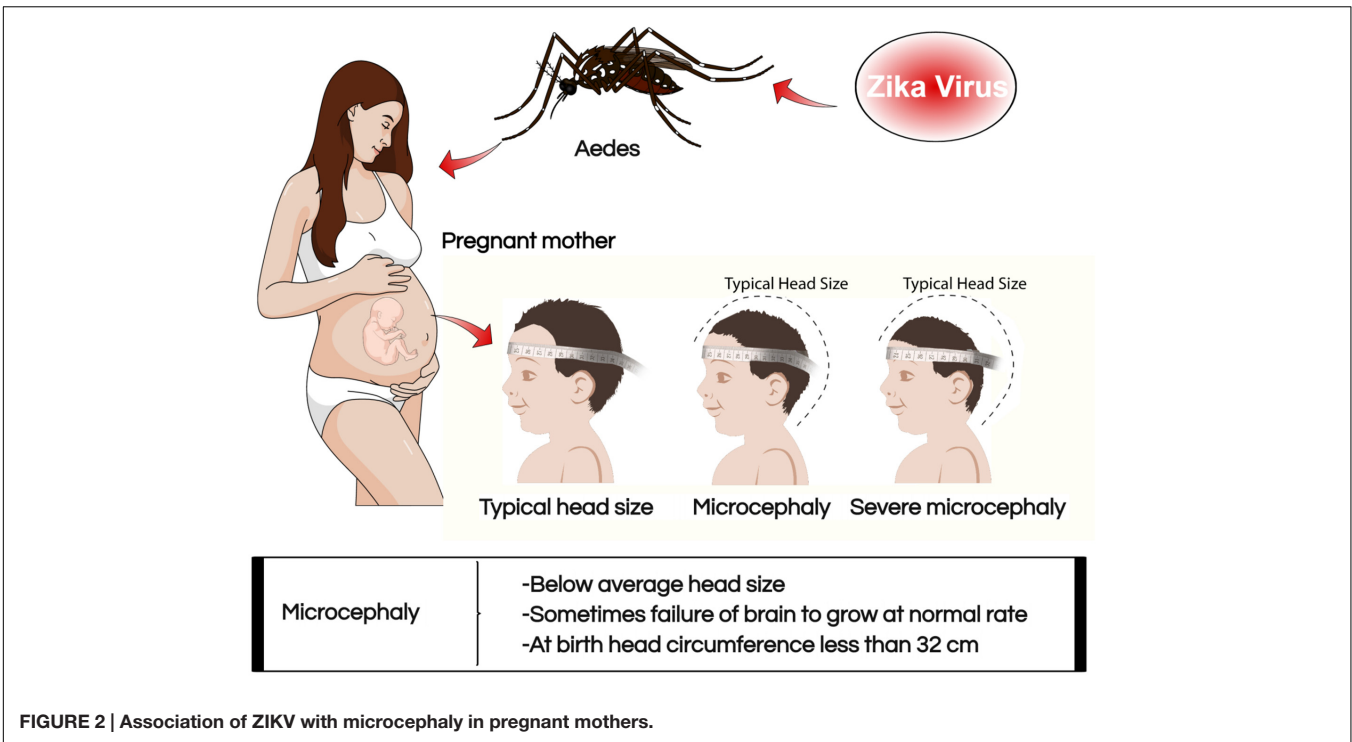


FIGURE 2 | Association of ZIKV with microcephaly in pregnant mothers.

microcephaly among infants. However, the research provides precautionary measures aimed at preventing the possible adverse outcomes of the confirmed cases of ZIKV infection. Nevertheless, no clear risk factors are identified of the virus on the fetuses during pregnancy and the various stages of infection to reduce the burden of ZIKV effects.

Wang and Ling (2016) established causal links between the ZIKV infection and microcephaly. The article by the duo stressed on the previous research outcomes which showed the unusual increase in the number of cases of microcephaly as identified in Pernambuco and their association with ZIKV (Olusanya, 2011; Michael et al., 2016). However, the article adds nothing new to the existing data about the expected cases discovered in Brazil. The article suggests that the infection by the ZIKV can be detected through a standard fetal ultrasound scans on the brain of the fetuses. Fetal abnormalities can be discovered through an ultrasonography among pregnant women who are not showing signs of the infection of the ZIKV. The process can identify factors that deter the normal growth and development of the brain and the subsequent microcephaly which is associated with the Zika virus. The virus is also deadly because it can affect the Central Nervous System, thereby leading to the death of the infant (Nicastri et al., 2016). The article stresses on the effectiveness of using scan in identifying congenital ZIKV syndrome symptoms. A case about two women who were diagnosed of ZIVK can also be used to establish a link between the cause of microcephaly and the ZIKV (Schwartz, 2016a). When the women gave birth, they miscarried, and autopsies were performed on the two fetuses in both cases. Various parts of the head, other organs of the body, such as lungs, heart, skin, spleen, thymus, kidney and the brain tissues were tested

for the presence of ZIKV (McNeil, 2016). It was realized that the fetus whose mother had tested positive for the ZIKV, had traces only in the brain tissue. However, the report is discredited for lack of direct proof of an existing link between the ZIKV and the cause of microcephaly (Shapshak et al., 2015). However, the article is vital in strengthening the link between ZIKV infections and how it leads to congenital birth defects and possible damage to the brain (Zanluca et al., 2015). The reports about this case also indicated that placenta of infected mothers and brain tissues can be used to further understand the link between the means of transmission during pregnancy to the unborn.

The other studies reported also examined the possible link between ZIKV infection and congenital birth defect in the case of microcephaly, which is a common type of brain abnormality believed to be caused by the ZIKV (Microcephaly Epidemic Research Group, 2016; Wang and Ling, 2016). The report noted that the effects of microcephaly depend on its exposure, which can be mild or serious depending on its long-term exposure on the brain, which can be mild or serious in the development of the brain. When the brain is mildly damaged, the child can experience slow development in the brain functions while when it is serious, the child's intellectual, motor can be adversely affected (Simões et al., 2016; Southwell et al., 2016). The study also ascertains other than congenital infections, chromosomal abnormalities like exposure to illicit drugs, craniosynostosis, which result from the premature fusion of bones of the skull and other metabolic disorders (Campos et al., 2015). The research fails to single out ZIKV as the main cause of microcephaly because there are several related brain damages which have varied causative agents making

it hard to get a clear distinction of ZIKV as the sole cause of microcephaly as a congenital infection of the fetuses. Therefore, for areas, which have been considered to have active transmission of Zika virus, attempts should be made to deal with the spread of mosquitoes to eliminate these possibilities of infections. These are considered both personal and national protective measures that can reduce cases of ZIKV infections that results from ZIKV spreading *Aedes* mosquitoes.

CONCLUSION

In this review, an effort was made to unfold a close link between ZIKV infection during pregnancy and congenital abnormalities. The first confirmed evidence of this association is based on the Pan American Health Organization and World Health Organization reports which updated on the status of the ZIKV infection based on the discoveries made in Brazil and French Polynesia in 2015 and 2014, respectively. However, these studies were never based on direct factual proofs, but relied on fetal malformations which were associated with the infection of ZIKV (Hancock et al., 2014; Campos et al., 2015). The related abnormalities which were linked to this infectious virus were the occurrence of several cases of microcephaly and other nervous system defects on the fetus among pregnant mothers who were diagnosed with ZIKV. Moreover, pathologists have also helped to strengthen the link by examining brain tissues of children who contracted the infection from their mothers during pregnancy through autopsy (Schwartz, 2016b). Moreover, the

environment also plays a role in the transmission of the ZIKV. For instance, traveling partners from the areas where the ZIKV infection could transmit through sexual intercourse (Harrower et al., 2016; Plourde and Bloch, 2016). It is, therefore, important to refrain from unprotected intercourse when such cases are suspected within a particular area. However, all the research covered in this review fails to present how one can be completely protected from the risks of the ZIKV infection. Other than the precautionary measures recommended, there are no treatment mechanisms suggested or medical preventive mechanisms like vaccination presented for future safety. Moreover, there is little much direct evidence presented to directly link ZIKV with congenital abnormalities other than microcephaly, which is a single case of brain and head defect. Therefore, there is a need for more research for conclusive outcomes that can be utilized to curb the spread of ZIKV and reduce global health fear about the spread of this infectious virus.

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IR wrote the initial draft, JL and VB designed the manuscript, Y-HP did critical analysis and approved the paper.

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Commentary: Teratogenic effects of the Zika virus and the role of the placenta

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A widespread epidemic of Zika virus (ZIKV) infection was reported recently in South and Central America. The biggest concern to the ZIKV infection is the significant increase of microcephaly in the fetus born to the infected mother (Brasil et al., 2016; Johansson et al., 2016; Mlakar et al., 2016). The placenta acts as a barrier against the infection, due to its multiple unique structural, cellular and immune properties. However, the placenta may also play an important role in the virus transfer. One possibility is that the virus penetrates through the placental barrier. Zika virus could be packaged as a cargo for the placental exosome pathway at the endoplasmic reticulum in trophoblast cells. Then the secretory autophagy-pathway may cause secretion or expulsion of the viral particle rather than its degradation (Zhang et al., 2017). Alternatively, the infection to the placenta may induce several immune responses and lead to brain defects indirectly (Adibi et al., 2016). A recent study indicated that the ZIKV genome could be detected in the amniotic fluid (Calvet et al., 2016), and the complete genome of ZIKV can be also recovered from the fetal brain (Mlakar et al., 2016), all of which confirm that the virus can cross the placental barrier (Mysorekar and Diamond, 2016).

In vitro studies confirmed that ZIKV, but not the closely related dengue virus or West Nile virus, can infect key placental barrier cells efficiently (Richard et al., 2017). Although some reports indicated that Zika virus infection triggers apoptosis and vascular damage in the placenta, which may increase the permeability of the placenta (Aldo et al., 2016; Melo et al., 2016; Miner et al., 2016), two recent studies suggested that most trophoblast cells succeed in blocking ZIKV infection or only permitting a very low viral replication level (Bayer et al., 2016; Quicke et al., 2016). However, no other pathogenic flaviviruses cause congenital defects. Miner and colleagues' work mainly focused on placental and fetal cytological changes. They found that ZIKV infection induces trophoblast apoptosis and vascular damages in the placenta (Miner et al., 2016). El Costa et al. (2016) also demonstrated that ZIKV infects and damages tissue architecture of the fetal placenta, the maternal decidua basalis and umbilical cord. The structure of the placenta is complex, which is comprised of the maternal decidua and the fetal-derived compartments, including the labyrinth and junctional zones. Different types of trophoblast cells reside within all three layers. In the labyrinth-zone, fetal capillaries are lined by fetal blood vessel endothelium, which are segregated from maternal sinusoids by a layer of mono-nuclear trophoblasts and a syncytio-trophoblast bilayer (Adibi et al., 2016; Miner et al., 2016). However, only minimal trophoblast cell death was observed after the infection (Quicke et al., 2016) and thus the placental barrier might remain relatively intact.

TABLE 1 | Time intervals between infections and abnormal findings of fetuses.

Fetus/Case No. (Reference)	Week of gestation at Infection	Week of gestation at the time of first abnormal finding of the fetus or the time of birth	Predicted time length of viral placental transfer (Weeks)
Case 1 (Noronha et al., 2016)	7	Miscarried at 12 weeks (No fetal virus or neurological abnormality was found)	>5
Case 5 (Noronha et al., 2016)	32	Birth at 37 weeks (No fetal virus or neurological abnormality was found)	>5
Case 1 (Soares de Souza et al., 2016)	36	Birth at 38 weeks (subependymal cysts and lenticulostriate vasculopathy at birth, however has a normal neurological development later)	>2
Case 2 (Soares de Souza et al., 2016)	36	Birth at 39 weeks (subependymal cysts at birth, however has a normal neurological development later)	>3
Fetus 24 (Brasil et al., 2016)	12	29	17
Fetus 41 (Brasil et al., 2016)	12	24	12
Fetus 39 (Brasil et al., 2016)	21	30	9
Fetus 17 (Brasil et al., 2016)	22	26	>4
Fetus 12 (Brasil et al., 2016)	22	27	5
Fetus 10 (Brasil et al., 2016)	25	30	5
Fetus 36 (Brasil et al., 2016)	26	35	9
Fetus 38 (Brasil et al., 2016)	27	35	8
Fetus 2 (Brasil et al., 2016)	30	Birth at 34 weeks (Normal at birth)	>4
Fetus 53 (Brasil et al., 2016)	32	Still birth (Fetal death at 38 weeks)	6
Fetus 23 (Brasil et al., 2016)	35	Birth at 40 weeks (Electroencephalogram abnormalities)	5

On the other hand, the placenta continues to produce trophoblast-derived interferons and other trophoblast-specific antiviral factors (Bayer et al., 2016; Quicke et al., 2016). Therefore, most ZIKV was blocked by trophoblast cells and the viral placental transfer might be a time-consuming process.

Placental transfer suggests that ZIKV must be transferred to the embryo at the early brain development stage (e.g., at the first trimester; Mlakar et al., 2016). However, at that time, the embryo has been shielded from the maternal blood, which nevertheless flows into the placenta only after 10 weeks of gestation (Adibi et al., 2016). Consistent with these structure changes, a tissue-level analysis in a case where the mother was infected at 7 weeks and miscarried at 12 weeks confirmed that the trophoblast was not infected by ZIKV at that period (Noronha et al., 2016). Thus, the virus may not reach the embryo before the first trimester.

However, for cases of ZIKV exposure in pregnancy, it is established that the greatest risk of microcephaly is in the pre-conception period and the first trimester (Johansson et al., 2016). In a case of late pregnancy, where infection occurred at 32 weeks, the virus was not detected in the fetal circulation, but it was detected in the placenta (Noronha et al., 2016). Similarly, in two cases of infection at 36 weeks, the virus was not detected in the infant's blood, although the newborns showed subependymal cysts (a kind of cerebral cysts) and lenticulostriate vasculopathy (lenticulostriate artery deformation) and they had normal neurological development for age as of the first postnatal month (Soares de Souza et al., 2016). Therefore, the viral

placental transfer may take some time. Analysis of the clinical data (Brasil et al., 2016; Noronha et al., 2016; Soares de Souza et al., 2016) suggests that the virus may take about 5 weeks to reach the fetus for most cases (Table 1).

As mentioned above, ZIKV infections may also induce acute inflammatory responses with up-regulation of interferons and cytokines and the indirect effects to the fetus (Adibi et al., 2016; Bayer et al., 2016; Mor, 2016; Quicke et al., 2016). Two case reports from Soares de Souza et al. (2016) indicated some congenital brain injuries in the blood ZIKV-negative fetuses (or the virus levels were under the limit of detection). The fetal brain injuries may be caused by the maternal infection indirectly, if the virus did not reach the fetus birth. Besides ZIKV, other flaviviruses also induce the cytokine storm. However, no relevance between any other flavivirus and microcephaly has been reported so far. Thus inflammatory responses may not play a major role in the incidence of lethal microcephaly.

We can postulate that if most ZIKV was cleared before it reaches the fetus, the incidence of microcephaly may be greatly decreased. The interferon therapy might help to clear the virus within 5 weeks. Alternatively, a combination of interferon with vaccines, newly-developed antiviral agents (e.g. 2'-C-methylated nucleosides; Eyer et al., 2016) or exosome-specific inhibitors (Zhang et al., 2017) should be adopted for this therapeutic time window. The infection early in pregnancy may give the virus plenty of time to transfer through the

placental barrier. And if the infection occurs early in pregnancy, the treatment window would be even longer, perhaps over 12 weeks.

AUTHOR CONTRIBUTIONS

SY coordinated the writing and wrote this manuscript together with inputs from all other listed co-authors. SY and QL made equal contributions in finalizing this manuscript.

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Zika Virus, Chikungunya Virus, and Dengue Virus in Cerebrospinal Fluid from Adults with Neurological Manifestations, Guayaquil, Ecuador

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Zika virus (ZIKV), chikungunya virus (CHIKV), and dengue virus (DENV) have been associated with clinical presentations that involve acute neurological complaints. In the current study, we identified ZIKV, CHIKV, and DENV in cerebrospinal fluid (CSF) samples from patients admitted to the Hospital Luis Vernaza (Guayaquil, Ecuador) to the Emergency Room or the Intensive Care Unit, with neurological symptoms and/or concern for acute arboviral infections. Viral RNA from one or more virus was detected in 12/16 patients. Six patients were diagnosed with meningitis or encephalitis, three with Guillain–Barré Syndrome, and one with CNS vasculitis. Two additional patients had a systemic febrile illness including headache that prompted testing of CSF. Two patients, who were diagnosed with encephalitis and meningoencephalitis, died during their hospitalizations. These cases demonstrate the breadth and significance of neurological manifestations associated with ZIKV, CHIKV, and DENV infections.

Keywords: Zika virus, Guillain–Barre syndrome, meningitis, chikungunya virus, dengue virus, cerebrospinal fluid, molecular diagnosis

INTRODUCTION

Zika virus (ZIKV) is an arthropod-borne virus (arbovirus) of the genus *Flavivirus* (family *Flaviviridae*) that is transmitted by the same mosquito vectors as chikungunya virus (CHIKV) and dengue virus (DENV; Waggoner and Pinsky, 2016). Amidst the current outbreak in the Americas, ZIKV has been associated with severe neurological manifestations in adults, including Guillain–Barré syndrome (GBS; Cao-Lormeau et al., 2016; Dirlikov et al., 2016; do Rosario et al., 2016; Dos Santos et al., 2016), acute myelitis (Mecharles et al., 2016), and encephalitis (Carteaux et al., 2016; Soares et al., 2016). GBS has been the best studied neurological presentation in adults, and the association between recent ZIKV infection and GBS has been principally demonstrated in studies that have relied on serologic

testing for ZIKV diagnosis (Cao-Lormeau et al., 2016; Dos Santos et al., 2016). This may be due to difficulties in diagnosing acute ZIKV infections in adults, as detection of ZIKV in CSF has been infrequently reported (Parra et al., 2016; Rozé et al., 2016; Siu et al., 2016; Zambrano et al., 2016) and serologic testing may be difficult to interpret in DENV endemic regions (Waggoner and Pinsky, 2016).

Zika virus, CHIKV, and DENV have co-circulated in many regions of the Americas over the past year. While the neurological complications of ZIKV infection have been the cause of much concern during this outbreak, less attention has been paid to the neurological manifestations associated with acute or recent CHIKV and/or DENV infections (Solomon et al., 2000; Gerardin et al., 2016). Clinical diagnosis cannot reliably distinguish symptomatic infections caused by these viruses (Waggoner et al., 2016b), which complicates the interpretation of results from studies based on reported Zika cases (Dos Santos et al., 2016) or patients with compatible symptoms (Parra et al., 2016). Additionally, co-infections between these viruses may be common in certain settings (Villamil-Gomez et al., 2016; Waggoner et al., 2016b; Zambrano et al., 2016), and the detection of co-infections in cases of CNS disease has not been well described (Zambrano et al., 2016).

The first cases of ZIKV infection in Ecuador were reported in January 2016 in two returned travelers. Shortly thereafter, autochthonous transmission was documented in the country. CHIKV was introduced into Ecuador in 2014, and DENV has been endemic in Ecuador since 1988. Guayaquil, located on the Pacific Ocean, is the largest city in Ecuador, and has a high incidence of arboviral infections. During 2016, ZIKV, CHIKV, and DENV co-circulated in Guayaquil. In the current study, we sought to determine the incidence of ZIKV, CHIKV, and/or DENV detection in cerebrospinal fluid (CSF) from adult patients admitted with neurological symptoms to the Hospital Luis Vernaza, the largest medical center in Guayaquil.

MATERIALS AND METHODS

Clinical Samples

A convenience set of CSF samples from 16 patients was tested for this study. Patients had been admitted to the Hospital Luis Vernaza in Guayaquil, Ecuador and had CSF collected as part of routine care. Patients were admitted from February 1 to August 31, 2016 and were initially evaluated by a neurologist or hospital staff physician. If lumbar puncture was warranted, the procedure was performed and CSF was sent to the Molecular Biology Laboratory and the Hospital Central Laboratory. Following the performance of testing requested by care providers, remaining CSF was stored at -20°C until RNA extraction could be performed. When possible, urine and blood samples collected on the day of CSF collection were also tested.

In addition to molecular testing for ZIKV, CHIKV, and DENV (described below), CSF samples were evaluated by the following methods: Gram stain, bacterial culture (up to 48 h if no growth was observed), Ziehl-Neelsen stain, and India ink stain. CSF PCRs were also performed for herpesviruses [HSV 1, 2, and 6;

cytomegalovirus; Epstein-Barr virus (EBV); and varicella zoster virus, all from DiaPro, Milan, Italy], toxoplasma gondii (DiaPro, Milan, Italy), enterovirus and tuberculosis (Cepheid, Sunnyvale, California) according manufacturer recommendations. Clinical data and the results of additional laboratory tests (e.g., blood cell counts, biochemical, and microbiological results from CSF), electromyography, and radiographic studies were obtained from the medical record. Patients provided written informed consent for diagnostic procedures and laboratory testing recommended by their care providers. For patients with altered cognition, consent was obtained from a surrogate decision maker. The study protocol was reviewed and approved by the Comité de Investigación at the Hospital Luis Vernaza.

Sample Processing and Arbovirus Testing

RNA was extracted from 140 μL CSF for all patients using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and a 60- μL elution volume, according to the manufacturer's instructions. RNA was also extracted from serum ($n = 4$) and urine ($n = 3$) samples, using the same protocol and specimen volumes. RNA was stored at -20°C until testing.

All samples were tested for ZIKV, CHIKV, and DENV using the ZCD assay. This is an internally controlled, multiplex real-time reverse transcription PCR (rRT-PCR), which was performed on a Cobas Z 480 instrument (Roche Diagnostics) using the users defined format, as described previously (Waggoner et al., 2016a; Zambrano et al., 2016). ZIKV, CHIKV, and DENV are detected in separate channels of the Cobas Z, and an assay for RNase P detection serves as a heterologous, intrinsic internal control. A sample was considered positive for a given target (any virus or RNase P) if an exponential curve crossed the instrument-defined threshold in the appropriate channel prior to or at cycle 40. Samples negative for all three viruses and with a positive result for RNase P were considered negative. Each run of the ZCD assay included a no-template control (water), a negative control (positive for RNase P but negative for any pathogen), and positive controls for ZIKV, CHIKV, and DENV.

RESULTS

Cerebrospinal fluid samples from 16 patients were included in this study (Table 1). The mean patient age was 42.1 years (sd 17.4) and 10 patients (62.5%) were male. Results of testing using the ZCD assay are shown in Table 1. Twelve samples (75.0%) tested positive for one or more viruses: ZIKV was detected in nine patients, CHIKV in 11, and DENV in 5. Three patients had evidence of a mono-infection, and nine patients had evidence of a co-infection with two ($n = 5$) or all three viruses ($n = 4$; Table 1). Viral RNA was detected as late as 14 days post-symptom onset. Four individuals tested negative in the ZCD assay but had detectable RNase P, indicating sufficient nucleic acid extraction and the absence of PCR inhibitors.

Clinical information on the 12 positive patients is shown in Table 2. Six patients were diagnosed with meningitis or encephalitis, three patients had GBS, and one patient

TABLE 1 | Demographic information and ZCD assay results from CSF for 16 patients.

Patient	Age (years)	Gender	Days post-symptom onset	ZCD assay results			
				ZIKV	CHIKV	DENV	RNase P
1	18	Male	5	+	+	+	+
2	16	Male	3	–	–	–	+
3	23	Male	1	+	+	+	+
4	54	Female	6	+	+	–	+
5	44	Male	9	+	+	–	+
6	25	Female	14	+	+	+	+
7	47	Male	1	+	+	–	+
8	28	Female	6	+	+	–	+
9	65	Female	1	–	–	–	+
10	48	Male	14	–	+	+	+
11	48	Male	15	–	–	–	+
12	60	Female	5	–	+	–	+
13	53	Female	14	–	+	–	+
14	62	Male	1	+	+	+	+
15	21	Male	8	+	–	–	+
16	62	Male	30	–	–	–	+

TABLE 2 | Clinical information for 12 patients with positive ZCD assay results from CSF.

Patient	Signs and symptoms	Diagnosis	Additional positive results
1	Upper respiratory tract infection, followed by asthenia, leg paresthesias, weakness, dysarthria, decreased muscular strength in legs and left arm (3/5), hyporeflexia, hyporeactive pupils.	Guillain–Barré syndrome. Symmetric motor polyneuropathy	None
3	Encephalopathy, asthenia, oliguria	Encephalitis	None
4	Fever, headache, and lumbar back pain; followed by paresthesias in her hands, feet, and face; left facial paralysis; and dysarthria.	Guillain–Barré syndrome. Motor and sensory axonal neuropathy	None
5	Nosocomial fever of unknown origin, arthralgia	Fever secondary to ZIKV-CHIKV co-infection	None
6	Fever, nausea, vomiting and diarrhea; followed by generalized tonic-clonic seizure, headache, ptosis of the eyelids, neck stiffness.	Meningitis	None
7	Altered wakefulness, generalized tremors and walking difficulties. Fever and tonic-clonic seizure.	Encephalitis	None
8	Fever, generalized weakness, asthenia, anorexia, headache, neck stiffness	Meningitis	HIV/AIDS Toxoplasmosis detected by CSF PCR
10	Fever, vomiting, diarrhea and headache	Meningitis	HIV/AIDS <i>Cryptococcus</i> detected by India Ink and culture
12	Cold symptoms followed by fever, chills, headache, neck stiffness, nausea, vomiting, sensory impairment, photophobia	Meningoencephalitis	EBV detected by CSF PCR
13	Polyarthralgia, fever and headache	Chikungunya fever	Self-reported history of chikungunya
14	Fever, sweating, paraplegia, areflexia, dyspnea, decreased muscular strength in arms	Guillain–Barré syndrome	None
15	Blurred vision in left eye followed by blindness, dysphasia, weakness and decreased muscular strength in legs and arms, lack of sphincter control.	Cerebral vasculitis	None

was diagnosed with CSF vasculitis. In two additional cases, patients had a systemic febrile illness related to a ZIKV-CHIKV co-infection or CHIKV mono-infection. Two patients with meningitis had AIDS and were also diagnosed with toxoplasmosis and cryptococcosis, respectively, during their

admissions. Patients 3 and 12 were both admitted to the intensive care unit with encephalitis and meningoencephalitis, respectively, and died during their hospitalization.

Cerebrospinal fluid findings for eight patients who had ZIKV, CHIKV, and/or DENV detected in CSF without another potential

TABLE 3 | Cytological analysis for patients with ZIKV, CHIKV, and/or DENV detected from CSF without another potential cause for their presentation.

Patient*	Leukocyte count (per μ L)	Neutrophils (%)	Mononuclear cells (%)	Glucose (mg/dl)	Protein (mg/dl)	LDH (U/l)
1	13	38.5	61.5	55	33.3	11
3	5	40	60	79.1	50.9	27
4	3	66.7	33.3	57.1	120.2	16
6	245	18	82	56.1	20.3	25
7	6	83.3	16.7	36.6	76.0	43
13	142	4.9	95.1	31.3	62.0	66
14	2	50	50	68	278	22
15	1	0	100	59.4	54	22

*For patient 5, there was clinical concern for a post-operative infection, though no growth was observed in bacterial cultures.

cause for their symptoms are shown in **Table 3**. Five of eight patients had an elevated white blood cell (WBC) count in the CSF (≥ 5 cells/ μ L), and patients 6 and 13 had marked elevations to 245 and 142 cells/ μ L, respectively. Three patients with CSF leukocytosis had a predominance of mononuclear cells. Patient 7, who presented 1 day post-symptom onset, had a neutrophil predominance. Five patients had elevated CSF protein (> 40 mg/dl), including all three patients with normal CSF WBC counts. Mild elevations in CSF lactate dehydrogenase (LDH) and decreases in glucose were seen, together, in patients 7 and 13. While both patients had CHIKV infections, these changes did not correlate with clinical presentation or disease severity.

In four patients (1, 3, 4, and 12), serum and/or urine samples were available from the same day as the CSF sample. Results from CSF and serum were concordant for 10/12 (83.3%) possible comparisons. In patient 3, DENV was detected in CSF and not serum; in patient 12, ZIKV was detected in serum (and urine) but not in CSF. Patient 4 had concordant results in each specimen type (ZIKV-CHIKV co-infection). Finally in Patient 1, urine tested negative for DENV and CHIKV, though ZIKV, CHIKV, and DENV were all detected in serum and CSF.

Radiographic Findings and Electromyography Testing

Electromyography findings in patients diagnosed with GBS were consistent with the following: motor axonal polyneuropathy (Patient 1); motor and sensory axonal neuropathy and secondary acute demyelination (Patient 4); demyelinating polyneuropathy and secondary axonal damage (Patient 14). Patient 1 had a brain MRI performed, which was normal.

Three additional patients had abnormalities noted on CNS imaging. Patient 3 had a head computed tomography scan showing symmetric alterations in the lenticular nucleus that were confirmed by magnetic resonance angiography as ischemic alterations of vasculitic origin affecting the lenticular nucleus, periventricular thalamic nucleus and the periaqueductal gray matter. Patient 7 had discrete changes detected in the lenticular nucleus by MRI (T2-flair images) as well as electroencephalogram findings consistent with cortical dysfunction. Patient 15 had multiple abnormalities on brain MRI affecting the periventricular regions, the protuberance and the cerebral peduncle. These did not extend to contiguous structures, and findings were most consistent with cerebral vasculitis.

DISCUSSION

In the current paper, we present 16 patients who were admitted to a single center in Guayaquil, Ecuador, with neurological symptoms and/or concern for arboviral illness. We identified twelve individuals with detectable RNA from ZIKV, CHIKV, and/or DENV in CSF. Notably, co-infections were identified in CSF relatively frequently (9/12 positive cases). RNA was detectable early in the course of neurological symptoms, which is consistent with a recent report describing detection of ZIKV RNA in the CSF of a patient with GBS on day 3 (Siu et al., 2016).

Recent reports describing GBS in the setting of ZIKV infections have principally relied on serologic testing and/or clinical symptoms for the detection of Zika cases. In a report by Parra et al. (2016), 66/68 patients with GBS in Colombia had symptoms of a recent ZIKV infection. Of 42 patients tested by RT-PCR, only 17 (25% of the total population) were positive for ZIKV, including 16 urine samples and 3 CSF samples. All patients tested negative for DENV by RT-PCR (Parra et al., 2016). In our experience, clinical symptoms do not accurately differentiate patients with ZIKV, CHIKV, and/or DENV (Waggoner et al., 2016b). Reliance on reported symptoms without diagnostic confirmation may over-emphasize the association between GBS and ZIKV mono-infection, and in our series, the three cases of GBS occurred in the setting of ZIKV co-infections with CHIKV and/or DENV.

It is notable that in the current series, the most severe cases involved patients with encephalitis and meningoencephalitis. Both patients tested positive for CHIKV, including patient 12 who only tested positive for CHIKV, and both patients died in the intensive care unit. A small number of deaths have been reported in the setting of ZIKV infection, though these cases have typically presented with anemia and severe thrombocytopenia, rather than neurological manifestations (Sarmiento-Ospina et al., 2016; Swaminathan et al., 2016).

Increased detection in our case series may have resulted from utilization of a multiplex rRT-PCR with improved sensitivity for ZIKV and DENV detection (Waggoner et al., 2016a) compared to assays that were used in studies referenced here (Cao-Lormeau et al., 2016; Dirlilikov et al., 2016; Parra et al., 2016). This facilitates the testing of all samples for ZIKV, CHIKV, and DENV in a single reaction, where testing with separate assays may not be performed following a single positive result. Arbovirus RNA was detected in CSF as late as 14 days post-symptom onset. As this

specimen type is rarely tested in the setting of acute infections with these pathogens, the duration of RNA detection in CSF is unknown. However, we do not favor that viral detection in these cases was related to past infections, given the paucity of co-infections reported from CSF to date.

Finally, two patients with HIV and AIDS were identified who had ZIKV-CHIKV and CHIKV-DENV co-infections, respectively. Both patients were severely ill, but the contribution of arboviral infection to the clinical picture, in the setting of documented opportunistic infections, is unclear. Only a single case of ZIKV in an HIV-infected patient has been well documented in the literature (Calvet et al., 2016). For DENV, however, AIDS does not appear to be a risk factor for the development of severe disease (Watt et al., 2003), and it is likely that ZIKV, CHIKV, and DENV were incidentally detected in these two cases. Given the small sample size and observational nature of the current study, further conclusions regarding the impact of co-infections on disease manifestations cannot be made, but this warrants further study in endemic regions.

CONCLUSION

Our data demonstrate the breadth of neurological manifestations associated with ZIKV, CHIKV, and/or DENV infections. All three viruses should be considered in the differential diagnosis for patients with new neurological symptoms in endemic areas of the world, and these data further support the use

of a multiplex diagnostic for ZIKV, CHIKV, and DENV testing.

AUTHOR CONTRIBUTIONS

HZ conceived the investigation and supervised the experimental work and data analyses. LR and JL performed the PCR experiments; JW and BP originally described the ZCD assay and provided the primers and probes for the PCR reactions. NA and MR revised and analyzed the medical records of the patients. NA prepared the database with integrated molecular and clinical data. NA, HZ and JW analyzed the data. NA, JW and HZ wrote the manuscript. BP edited the manuscript. All authors revised and approved the final version.

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Interplay between Inflammation and Cellular Stress Triggered by *Flaviviridae* Viruses

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The *Flaviviridae* family comprises several human pathogens, including Dengue, Zika, Yellow Fever, West Nile, Japanese Encephalitis viruses, and Hepatitis C Virus. Those are enveloped, single-stranded positive sense RNA viruses, which replicate mostly in intracellular compartments associated to endoplasmic reticulum (ER) and Golgi complex. Virus replication results in abundant viral RNAs and proteins, which are recognized by cellular mechanisms evolved to prevent virus infection, resulting in inflammation and stress responses. Virus RNA molecules are sensed by Toll-like receptors (TLRs), RIG-I-like receptors (RIG-I and MDA5) and RNA-dependent protein kinases (PKR), inducing the production of inflammatory mediators and interferons. Simultaneously, the synthesis of virus RNA and proteins are distinguished in different compartments such as mitochondria, ER and cytoplasmic granules, triggering intracellular stress pathways, including oxidative stress, unfolded protein response pathway, and stress granules assembly. Here, we review the new findings that connect the inflammatory pathways to cellular stress sensors and the strategies of *Flaviviridae* members to counteract these cellular mechanisms and escape immune response.

Keywords: flavivirus, innate immune response, ER stress, reactive oxygen species, stress granules

FLAVIVIRUS REPLICATION

The *Flaviviridae* family includes several important human pathogens such as Dengue Virus (DENV), Zika Virus (ZIKV), Yellow Fever Virus (YFV), West Nile Virus (WNV), Japanese Encephalitis Virus (JEV), St. Louis Encephalitis Virus (SLE), and Hepatitis C Virus (HCV). In this review, we will describe the inflammation and stress responses triggered by members of flavivirus and hepacivirus genus only. Briefly, the first interaction of Flavivirus with its host cell occurs via several putative receptors (Mukhopadhyay et al., 2005). They play an important role in capturing and concentrating infectious virions, leading to a cascade of events that culminates in the virus and cell membranes fusion. Most Flaviviruses are internalized into the cell by clathrin-mediated endocytosis (Pierson and Kielian, 2013). A low pH-triggered conformational change of envelope (E) proteins in endosomes leads to viral and cellular membranes fusion and virus uncoating. Capsid is released into the cell cytoplasm, where it dissociates and releases RNA viral genome. Flavivirus genome is a single strand positive sense RNA molecule, with a single open reading frame (ORF), which is then translated into one large polyprotein (Iglesias et al., 2009). This polyprotein is targeted to the endoplasmic reticulum (ER), where it is processed by virus and

host's encoded proteases to form the structural proteins (capsid protein C and envelope protein E) and non-structural proteins (NS), which participate in replication, polyprotein processing and virion assembly. Usually, Flavivirus replication takes place at a membranous web associated with the ER (Pierson and Kielian, 2013). Formation of the membranous web is mainly induced by NS viral proteins, and replication is catalyzed by an RNA-dependent RNA polymerase (usually named NS5), via a negative sense RNA intermediate (Mottola et al., 2002; Gillespie et al., 2010). Once capsid proteins coat the genomes copies, immature virions, containing surface E proteins, bud into ER lumen and are transported through the *trans*-Golgi network (TGN). At the TGN, they undergo further glycan modifications and structural cleavage, becoming mature infectious virions, which are transported out of the cell by exocytosis (Mukhopadhyay et al., 2005).

Production of viral progeny interferes with different aspects of cellular metabolism; therefore, viral infection may represent a stress condition to the host cell (Fernandez-Garcia et al., 2009). In order to adapt to the stress, the cells react by transiently inhibiting protein synthesis and restricting the consumption of nutrients and energy, aiming to enhance cell survival and restore homeostasis (Ruggieri et al., 2012; Onomoto et al., 2014). Stress responses triggered by virus infection may occur at multiple levels and include the ER associated stress, mitochondria stress (oxidative stress) and cytoplasmic stress [stress granules (SGs)] (Buchan and Parker, 2009; Montero and Trujillo-Alonso, 2011; Valiente-Echeverría et al., 2012; Zhang and Wang, 2012; Reshi et al., 2014; Gullberg et al., 2015).

All these responses may be triggered by host cell sensing of incoming virus macromolecules, including genome RNA, double-stranded RNA intermediates, and proteins. Virus sensing is achieved by pattern recognition receptors (PRRs), which recruit adaptor molecules and stimulate transcription factors, leading to the expression of type I interferons (IFN) and proinflammatory cytokines (Chang et al., 2006; Kato et al., 2006; Boo and Yang, 2010; Muñoz-Jordán and Fredericksen, 2010). Those, in turn, may regulate the expression and activation of PRRs themselves and of other mediators, which are involved in the control of translation, mitochondrial function, and cell death or survival, contributing to inflammation and stress. Viruses are, then, confronted with the consequences of cell stress and emerging evidences suggest that they not only interfere with the interferon system, but also manipulate the programs of cell-induced stress to promote viral replication (Garaigorta and Chisari, 2009; Aguirre et al., 2012; Green et al., 2014).

Notably, prolonged stress may result in cell death. Indeed, activation of PRR and IFN receptors (IFNR) are usually associated to stimulation of different cell death pathways (Li et al., 2004; Fulda et al., 2010). In turn, intracellular mediators activated by stress responses or released after tissue damage may also stimulate PRRs and adaptor molecules, amplifying the inflammatory response. The overall inflammation and stress are important for limiting virus replication and dissemination and for tissue repair, but may be harmful to the host if an exacerbated, uncontrolled response is triggered (Inohara and Nuñez, 2003).

Recent findings have been demonstrating an intimate interaction between RNA immune sensors and cell stress pathways, which may be central for the success of virus replication controlling (Smit et al., 2011; Perera-Lecoin et al., 2013). This review will focus on the cellular stress responses triggered by flavivirus and HCV replication, addressing the connection between those responses with virus sensing and inflammation, and discussing the virus strategies to counteract these pathways.

FLAVIVIRUS SENSING COMPLEXES

Virus RNA may be sensed by RIG-I-like receptors (RLR) or toll-like receptors members (TLR), depending on RNA structure, cellular location, and infected cell type. Monocytes, macrophages and non-immune cells, such as endothelial cells, epithelial cells and hepatocytes usually sense RNA virus through RIG-I and/or TLR3; whereas TLR7 is highly expressed and is the major RNA sensor in plasmacytoid dendritic cells (pDC; Sun P. et al., 2009; Tsai et al., 2009; Nasirudeen et al., 2011; Qin et al., 2011; da Conceição et al., 2013). Activation of either RLRs or TLRs may promote the secretion of interferons and proinflammatory cytokines. The former induces autocrine and paracrine stress responses, such as inhibition of protein synthesis, RNA editing and, potentially, cell death, contributing to control viral replication and dissemination. Proinflammatory cytokines and chemokines, such as IL-6, IL-8, TNF- α and Rantes, recruit and activate other cell types to the infected tissue, amplifying inflammation. These mediators may also contribute to tissue lesion due to activation of cell death pathways, and induction of oxidative stress, among other mechanisms, which will be discussed later (He et al., 2011; O'Leary et al., 2012; Lucas and Maes, 2013). All these effects may contribute to the control of virus replication, but also to enhancement of inflammatory response and disease severity.

Virus components and cellular metabolites generated upon virus replication may also stimulate inflammasome complexes, leading to the secretion of inflammatory IL-1 β and IL-18 and, eventually, to cell death (Poeck et al., 2010; Negash et al., 2013; Chen et al., 2014).

Cytoplasmic RNA Sensing

Virus RNA present in the cytoplasm may be sensed by RLR, including RIG-I and MDA5. These are cytoplasmic helicases, composed by a RNA-binding domain at the C-terminal region (CTD), associated to a central DExD/H helicase domain with an ATP-binding motif, and a caspase recruitment domain (CARD), located at the N terminus (Kowalinski et al., 2011) (Figure 1). RLRs recognize, by their RNA-binding domain, signatures present in several RNA virus. dsRNA bearing an uncapped 5'triphosphate end (5'ppp) with a minimum of 20 nt length was showed to be essential for optimal RIG-I sensing, whereas long dsRNA, lacking triphosphate end are preferentially recognized by MDA5 (Kato et al., 2006). Once the receptor is activated, CARD domain associates to the adaptor molecule MAVS (or IPS, VISA, or Cardif), which is located at the outer

membrane of mitochondria (Gack et al., 2007) (**Figure 1**). In steady state conditions, CARD domain is masked by CTD, avoiding intrinsic activation of the receptor. The binding of viral RNA signatures leads to an ATP-dependent conformational change of CTD, allowing exposition of CARD (Kowalinski et al., 2011) (**Figure 1**). CARD domain is then targeted by the ubiquitin ligases tripartite motif protein 25 (TRIM25), which promotes the polyubiquitination of lysine-63 (K63), RIG-I oligomerization, and MAVS recruitment, being essential to RIG-I-induced antiviral responses (Gack et al., 2007; Jiang et al., 2012) (**Figure 1**). In addition, ubiquitination of CTD by the ring finger proteins 135 (RNF135 or Riplet), which are upregulated upon virus infection, also facilitates RIG-I-mediated viral recognition (Oshiumi et al., 2009, 2010).

RIG-I-MAVS interaction may then form complexes with other adaptor proteins including: STING (stimulator of interferon genes); ERIS (ER interferon stimulator); TRADD (TNFR-associated death domain); FADD (Fas-associated death domain protein); RIP1 (receptor-interacting protein 1); TRAF2, 3, 6 (TNFR-associated factors 2, 3, and 6) and caspases (Takahashi et al., 2006; Ishikawa and Barber, 2008; Yoshida et al., 2008; Zhong et al., 2008; Sun W. et al., 2009; Rajput et al., 2011). After full activation of the receptor, MAVS recruitment, together with the formed complexes activates TBK and IKK, triggering the stimulation of IRF3 and NF- κ B. Activated IRF and NF- κ B transcription factors are translocated to the nucleus, where they induce the expression of interferons and proinflammatory cytokines, respectively (**Figure 1**).

Activation of RIG-I, MDA5, MAVS, STING, IRF3, and NF- κ B had been reported to mediate sensing of dsRNA intermediates during Flavivirus replication. Those pathways were, then, associated to regulation of IFN production and IFN-mediated responses, as well as to cellular stress or cell death/survival. Depending on the virus and the infected cell type, RNA virus can be distinguished by RIG-I or MDA5, or both receptors may be synergistically stimulated (Kato et al., 2006; Nasirudeen et al., 2011).

Both RIG-I and MDA5 were shown to be up regulated and involved in IFN- β induction upon DENV and WNV infection in hepatocytes and endothelial cells (Loo et al., 2008; Nasirudeen et al., 2011; da Conceição et al., 2013). RIG-I, MDA5 and MAVS were, then, associated to increased secretion of inflammatory mediators, which have been also observed in patients' plasma. Unexpectedly, RLR silencing did not affect viral replication, indicating that those molecules might be more associated to inflammation than to viral replication.

Zika Virus infection of human fibroblasts also resulted in upregulation of RIG-I and MDA5, what might be associated to the observed production of IFN- α and IFN- β , increased expression of IRF7 and upregulation of IFN stimulated genes (ISG), such as OAS and ISG15 (Hamel et al., 2015).

On the other hand, JEV and HCV are recognized only by RIG-I (Sumpter R. et al., 2005; Kato et al., 2006). The polyuridine motif of HCV 3' untranslated region (UTR) genome region and its replication intermediates are the PAMP substrates of RIG-I, that activates IRF3, thereby inducing the expression of IFN- α/β and antiviral/interferon-stimulated genes that limit

infection (Sumpter R. et al., 2005; Saito et al., 2008). During HCV infection, RIG-I was also associated to the induction of cellular apoptosis through the TRAIL pathway and the death receptors DR4 and DR5 (Eksioglu et al., 2011) (**Figure 1**). Activation of RIG-I-MAVS/STING pathways during JEV infection in neurons was associated to increased production of interferon, proinflammatory cytokines and reduction of intracellular levels of virus (Nazmi et al., 2011, 2012). On the other hand, it was demonstrated that JEV infection induced the expression of microRNAs, such as miRNA15b, which negatively regulates RIG-I signaling, contributing to virus escape from innate immune response (Zhu et al., 2015).

Vesicular RNA Sensing

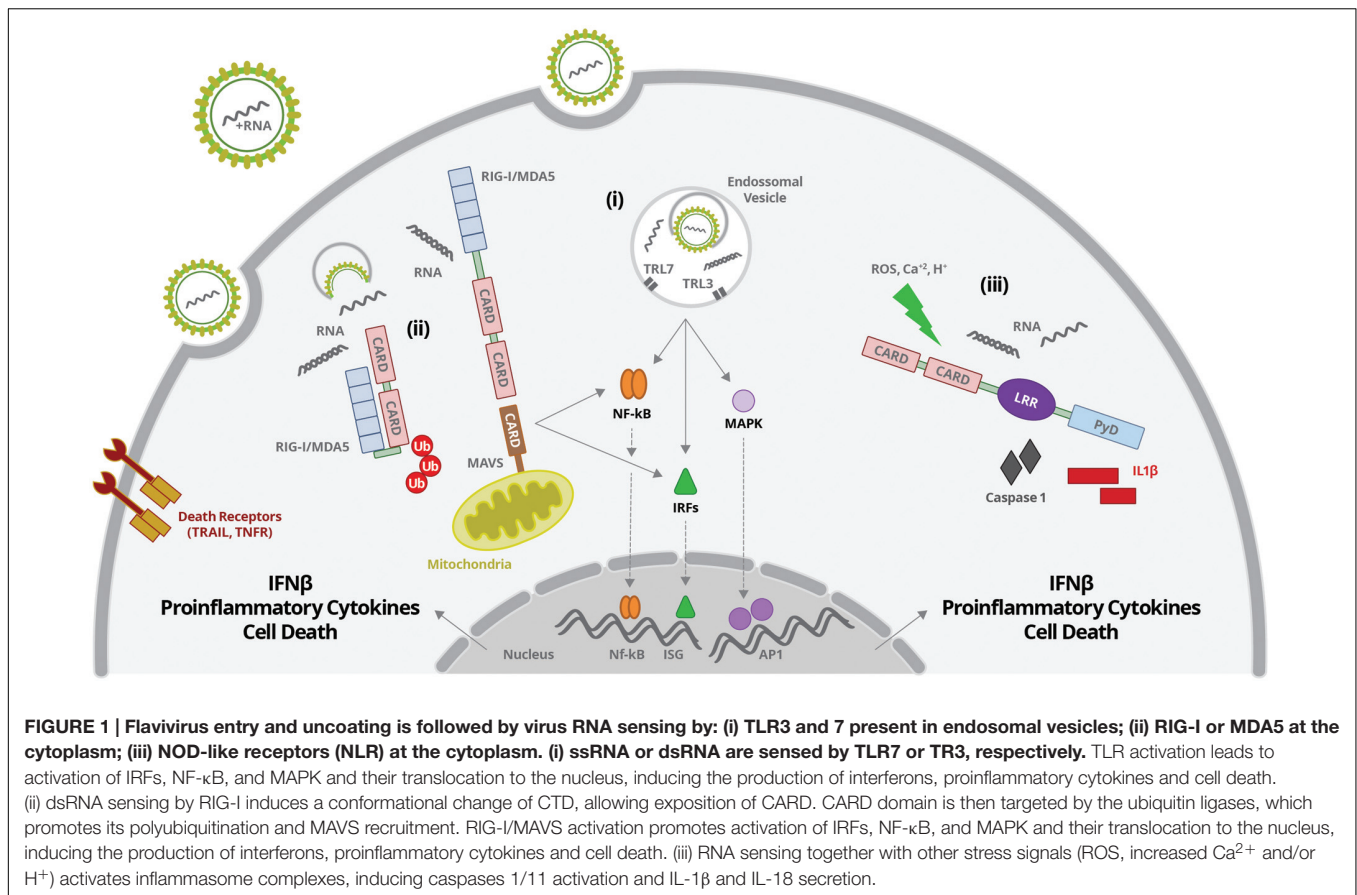
Flavivirus genome may also be sensed by TLR, located at endosomal vesicles (Nazmi et al., 2014) (**Figure 1**). TLRs are composed by leucine-rich repeats (LRRs) luminal domain, which is responsible for PAMP recognition; a transmembrane domain; and the Toll/IL-1 receptor domain (TIR), which faces the cytoplasm and associates to downstream signaling molecules (Kawai and Akira, 2010). TLRs may signal through two different pathways: one dependent on the recruitment of the adaptor molecule MyD88, and other involving the adaptor TRIF, which is independent on MyD88. Immune response against Flaviviruses and HCV involves TLR3 and TLR7 activation, which recognize dsRNA and ssRNA containing uridine rich motifs, respectively (**Figure 1**).

After TLR7 engagement, MyD88 is recruited and forms a complex with proteins IRAK1 and IRAK4 (interleukin-1 receptor-associated kinases 1 and 4), TRAF3, and TRAF6 (TNF receptor-associated factors 3 and 6). These complexes stimulate MAPK, IKK and TBK, which will activate and promote the nuclear translocation of AP1, NF- κ B, IRFs 3 and 7 transcription factors, inducing the expression of type I interferons, pro-inflammatory cytokines and chemokines (Hemmi et al., 2002; Lin et al., 2010; von Bernuth et al., 2012; Zhou et al., 2013) (**Figure 1**).

Toll-like receptor3 recruits the adaptor molecule TRIF, which also interacts with TRAF3 and 6, activates TBK1 and IKK, stimulating NF- κ B and IRF3, and cytokine secretion. TRAF6 also interacts with RIP, which stimulates TAK-1 complex, promoting the activation of NF- κ B and MAPK (Hemmi et al., 2002; Kawasaki and Kawai, 2014). Activation of TLR3 or TLR7, therefore, promotes the secretion of interferons and proinflammatory cytokines. Also, endosomal TLR may sense self-RNAs released by damaged cells, indicating another cross talk pathway between stress, lesion and innate immune response (Takemura et al., 2014).

Dengue Virus RNA is recognized by TLR3 in monocytes (Tsai et al., 2009; Nasirudeen et al., 2011). Also, TLR3 may synergize with RIG-I and MDA5 for the induction of interferons after DENV infection of hepatocytes (Nasirudeen et al., 2011). Interestingly, DENV infected cells may be sensed by pDCs, in a pathway involving cell-to-cell contact and RNA-dependent TLR7 activation, promoting the production of IFN α , inducing, therefore, an antiviral state (Décembre et al., 2014).

Upregulation of TLR3, but not TLR7 was also observed in human fibroblasts infected by ZIKV (Hamel et al., 2015). In



fact, both cytoplasmic (RIG-I and MDA5) and vesicular (TLR3) PRRs were upregulated and might synergistically account for the enhanced expression of antiviral response stimulated after ZIKV infection, however, the specific role of each receptor was not investigated yet (Hamel et al., 2015).

Toll-like receptor-mediated protection was also observed during WNV infection in neurons, although this effect had not been so evident upon infection of other cell types (Daffis et al., 2008). In addition, in an *in vivo* experimental model, TLR3 showed to be essential for WNV penetration in the brain and for the induced inflammatory response (Wang et al., 2004). On the other hand, MyD88-deficient mice presented increased susceptibility to WNV infection, indicating the participation of other TLR in WNV protection and pathogenesis (Szretter et al., 2010). Regarding HCV, TLR3 was strongly involved in the induction of IFNs, which showed to be important to keep low levels of viral replication (Eksioglu et al., 2011).

TYPE I INTERFERONS AND INDUCTION OF ANTIVIRAL STATE

A major virus-induced response upon virus sensing in a range of different cell types is the production of type I IFN. During flavivirus infection, IFN production is stimulated by RIG-I, MDA5, TLR3, and TLR7 triggering a variety of cell responses

affecting cellular physiology and virus replication (Muñoz-Jordán and Fredericksen, 2010). Increased IFN production induced by activation of PRRs also contributes to the amplification of the response, by increasing the expression of PRRs themselves and subsequent signal transduction.

The essential role of type I IFNs for the control of Flaviviruses replication is clearly evidenced in experimental mouse models, which do not express IFN receptors or IFN-related signaling molecules, such as STAT. Although adult wild type mice are not susceptible or do not develop classical disease when inoculated with most flaviviruses, infection of A129 mice, lacking type I IFN receptor, result in systemic and fatal infection induced by ZIKV and YFV (Meier et al., 2009; Rossi et al., 2016). Similarly, DENV infection of AG129 mice, which lacks both type I and type II IFN receptors, or infection of STAT2-deficient mice, lead to fatal infection, associated to viral replication in multiple organs, and vascular alterations, including hemorrhage (Williams et al., 2009; Ashour et al., 2010). These data indicate that both innate and adaptive responses are crucial for dengue protection.

Type I IFN binding to IFN receptors (IFNAR) expressed at the surface of the infected or bystander cells promote the expression of a number of ISGs, including PKR, OAS/RNase L, IFITs, and others, which then modulates protein synthesis, RNA degradation, autophagy and apoptosis (Li et al., 2004; Chakrabarti et al., 2012).

One of the first described antiviral element up-regulated by interferon was the double-stranded RNA-dependent protein kinase (PKR; Xu and Williams, 1998). When activated, it phosphorylates the guanine nucleotide exchange factor eIF2B from recycling eIF2 to its active GTP-bound form, the α -subunit of translation initiation factor eIF2- α (eIF2- α ; Aguirre et al., 2012). This leads to the shutoff of protein synthesis and, thereby, inhibition of viral replication. PKR is activated by binding to dsRNA, and could be, therefore, classified as a RNA sensor (Onomoto et al., 2012). However, PKR is more accurately characterized as a critical sensor of cell stress and virus infection, which activates stress responses and inflammation through dsRNA recognition domain (Wu and Kaufman, 1997; Balachandran et al., 1998). The function of PKR in flavivirus-mediated stress responses will be further detailed in the cytoplasmic stress section.

Other ISGs also indirectly modulate the immune response, such as RNase L, that activates RIG-I/MAVS pathways, leading to increased IFN- β production triggered by HCV RNA (Malathi et al., 2010). However, the effect of RNase L in IFN production may vary depending on the virus and the cell type, given that different cell types express different isoforms and levels of OAS genes (Banerjee et al., 2014). Therefore, increased IFN production is one of the key elements for an antiviral response, by amplifying the inflammatory response, by regulating cellular metabolism and consequently affecting virus production. The function of several others ISGs are beyond the scope of this review and we will discuss here how viral RNA and protein sensing and IFN signaling can be associated to cellular stress.

CELLULAR STRESS PATHWAYS

ER Stress Response [Unfolded Protein Response (UPR) Pathway] and Inflammation

Endoplasmic reticulum is the major site where secreted and transmembrane proteins are synthesized and folded in eukaryotic cells. Likewise, a large amount of viral proteins, including envelope proteins, are synthesized at the ER, which is, therefore, an essential organelle for viral replication and maturation (He, 2006). Indeed, most Flavivirus replicates at a membrane web associated to ER and promote membrane-remodeling events that are driven by hydrophobic transmembrane non-structural viral proteins (Blázquez et al., 2014). Depending on the physiological state and environmental conditions, the protein flux into the ER may vary substantially. In virus-infected cells, the cellular translation machinery is orchestrated by the infecting virus to produce large amounts of viral proteins, which ultimately disturbs ER homeostasis and causes ER stress (Liu et al., 2009; Peña and Harris, 2012; Zhang and Wang, 2012).

Endoplasmic reticulum stress comprises multiple stress response pathways including oxygen and nutrient deprivation, calcium dysregulation, misfolded protein recognition and N-linked glycosylation inhibition (Zhang and Wang, 2012; Sen et al., 2014). Nevertheless, they all converge on the

unfolded protein response (UPR). UPR restores the cellular normal function by attenuating protein translation and activating the signaling pathways associated to increased production of molecular chaperones required for protein folding (Zhang and Wang, 2012).

The UPR consists of three branches of signaling pathways named after the transmembrane ER stress sensors: PKR-like ER protein kinase (PERK), activating transcriptional factor-6 (ATF6), and inositol-requiring protein-1 (IRE1; Garg et al., 2012) (**Figure 2**). BiP is the ER molecule that coordinates all the UPR pathways to restore the cell homeostasis. BiP is a member of heat shock proteins that binds to properly folded and misfolded proteins (Bertolotti et al., 2000). In normal cells, BiP associates with the luminal domains of PERK, ATF6 and IRE1 blocking the activation of UPR pathways (Sou et al., 2012). Under stress conditions, such as Flavivirus infection, BiP transiently associates with viral glycoproteins folding intermediates, releasing PERK, ATF6, and IRE1 to activate UPR pathways (Benali-Furet et al., 2005; Pincus et al., 2010) (**Figure 2**). BiP releasing from PERK or IRE1 allows the homodimerization of each protein through their luminal domain, which induces their autophosphorylation and subsequent activation. Activation of PERK inhibits protein synthesis through phosphorylation of the eIF2- α (Liu et al., 2009; Garg et al., 2012). IRE1 activation leads to the transcription of a subset of genes encoding protein-degradation enzymes (Bertolotti et al., 2000; Pincus et al., 2010). In parallel, the release of BiP from ATF6 promotes the translocation of ATF6 from ER to the Golgi apparatus, where it is cleaved and activated. Activation of ATF6 stimulates the transcription of genes encoding chaperones that refold misfolded proteins (Shen et al., 2005). The three branches of UPR do not operate independently, and the tight temporal control and crosstalk among them constitute an intricate signaling network. Apoptosis is induced when cells are unable to recover from ER stress (Menu et al., 2012).

Endoplasmic reticulum stress-inducing agents synergistically activate type I IFN response (He, 2006; Smith, 2014). In addition, there are increasing evidences that UPR pathway directly enhances cytokine production due to activation of pro-inflammatory transcription factors. Upon ER stress, XBP1 is spliced by IRE1, thereby generating functional spliced XBP1 (XBP1s; Savic et al., 2014) (**Figure 2**). The XBP1 is not only an important component of the UPR pathway, but also an important transcription factor. Spliced XBP1 can then be translocated to the nucleus and interact with a conserved site downstream of *ifnb1* gene, enhancing IFN- β production (Zeng et al., 2010). Virus-triggered UPR pathway also activate IRF3, in the absence of additional stimuli, and this transcription factor binds to the *ifnb1* enhancer sequence, independently of XBP1 (Sato et al., 2000; Sakaguchi et al., 2003; Liu et al., 2012). Little is known about how IRF3 is activated, but the signaling pathway seems to be dependent on calcium release induced by ER stress. Activation of both XBP1 and IRF3 seems to prime the type I IFN response when ER stress is present (Perera-Lecoin et al., 2013) (**Figure 2**).

NF- κ B activation is also induced by UPR pathway, leading to the production of proinflammatory cytokines, such as TNF- α and IL-6. Different members of UPR pathway were associated

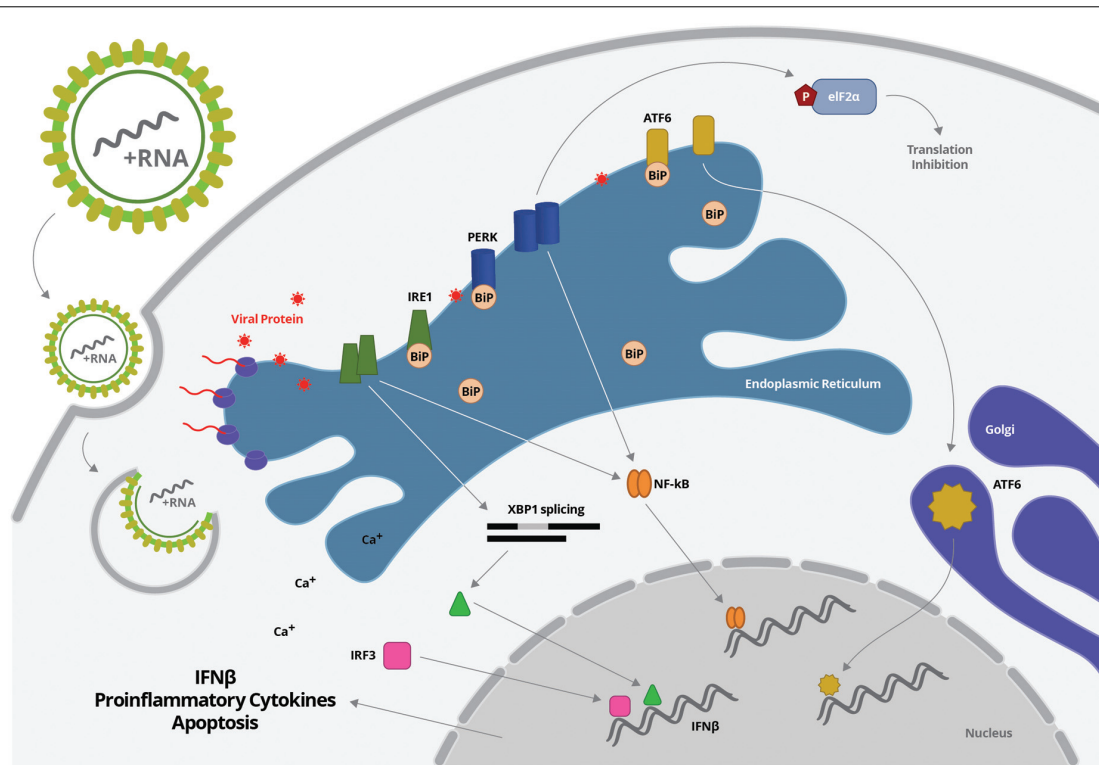


FIGURE 2 | Flavivirus entry and uncoating is followed by virus RNA translation, which takes place mostly in association to ER membrane. Increased protein synthesis may disturb ER homeostasis, inducing unfolded protein response, characterized by BiP transiently dissociation and release of PERK, ATF6, and IRE1. BiP releasing from PERK or IRE1 allows the homodimerization of each protein through their luminal domain, which induces their autophosphorylation and subsequent activation. PERK or IRE1 can activate NF-κB. IRE1 also mediates XBP1 splicing, generating functional spliced XBP1 (XBP1s), which is translocated to the nucleus and interact with a conserved site downstream of *ifnb1* gene, enhancing IFN-β production. Activation of PERK inhibits protein synthesis through phosphorylation of the eIF2-α. In parallel, the release of BiP from ATF6 promotes the translocation of ATF6 from ER to the Golgi apparatus, where it is cleaved and activated. Activation of ATF6 stimulates the transcription of genes encoding chaperones that refold misfolded proteins.

to NF-κB activation, including PERK and IRE-1 (Deng et al., 2004; Tam et al., 2012) (Figure 2). In addition, a crosstalk pathway between ER and mitochondrial stress, leading to reactive oxygen species (ROS) accumulation and ER calcium release, was also reported to induce NF-κB activation (Pahl and Baeuerle, 1997). In a positive feedback loop, the resulting inflammatory cytokines can trigger ER stress through induction of further ROS production (oxidative stress) and increased release of calcium from the ER, interfering with chaperone function (Zhang and Kaufman, 2008). Therefore, UPR, or specific pathways within the UPR, can promote inflammatory cytokine production serving as an internal “danger” signal, complementing cellular viral sensors in alerting a cell to invasion and boosting subsequent antiviral immune response (Smith, 2014).

In a sterile inflammation model of ER stress, it was demonstrated that UPR contributes to inflammasome stimulation, promoting IL-1β secretion and cell death (Kim et al., 2013). In this model, activation of IRE1α induced thioredoxin-interacting protein (TXNIP), which activated NLRP3 inflammasome, causing procaspase-1 cleavage and IL-1β secretion (Lerner et al., 2012). In fact, inflammasome-mediated IL-1β secretion after ER stress had been demonstrated in different cell types and it was associated to NF-κB-mediated pro-IL-1β

expression, increased ROS production and activation of TXNIP, promoting NLRP3 activation (Kim et al., 2015). Whether UPR pathway, oxidative stress and inflammasome activation crosstalk during flavivirus infection needs further investigation.

It is not surprising that viruses have also evolved mechanisms to counteract UPR pathways to promote their infection. This generally involves regulation of stress response proteins and several molecular chaperones to modulate UPR and increase ER folding capacity that will be addressed below for each specific flavivirus.

Flavivirus Infection and ER Stress

Several members of the *Flaviviridae* family including WNV, JEV, DENV, and HCV activate the UPR pathway in a variety of mammalian cells (Su et al., 2002; Tardif et al., 2002; Yu et al., 2006; Medigeschi et al., 2007; Umareddy et al., 2007; Sun W. et al., 2009; Klomporn et al., 2011; Peña and Harris, 2012; Thepparit et al., 2013). The hallmarks of UPR pathway activation observed for Flavivirus was IRE1 mediated splicing of XBP-1, and BiP overexpression in infected cells. By triggering the XBP1 signaling pathway, flaviviruses take advantage of this cellular response, as it is beneficial for viral production and alleviate virus-induced cytotoxicity (Yu et al., 2006).

Regarding DENV infection, it was demonstrated that nonstructural protein NS2B and NS3 are potent inducers of *xbp1* splicing (Yu et al., 2006). Also, in liver cells infected with DENV2, it was observed that PERK and ATF6 were not associated with BiP, resulting in increased eIF-2 α phosphorylation (Thepparit et al., 2013).

Japanese Encephalitis Virus infection triggers the UPR pathway in neuronal cells, resulting in apoptotic cell death by robust expression of CHOP/GADD153, a death-related transcription factor that down-regulates Bcl-2 and raises the production of ROS (Liao et al., 2002). ER-mediated UPR induced by JEV also involves stress-inducible p38 mitogen-activated protein kinase (p38 MAPK) activation that could contribute to stimulate CHOP induction at the post-translational level (Su et al., 2002).

West Nile Virus infection also disturbs ER homeostasis leading to activation of all three branches of UPR pathway. Early *xbp1* splicing was detected by 24 h post WNV infection and splicing kinetics corresponded to WNV titers, suggesting that the activation of IRE1 pathway is due to increasing viral load in the ER (Medigeschi et al., 2007). ATF6 cleavage was also detected upon WNV infection, but no change in *atf6* mRNA levels was observed, indicating that this regulation occurs at a posttranscriptional level. WNV infection also leads to eIF-2 α phosphorylation by PERK activation and CHOP expression, mediating apoptosis that limited WNV replication (Medigeschi et al., 2007).

The processing and folding of HCV core occurs at the ER and it is strictly dependent on interaction with the ER membrane (Santolini et al., 1994). HCV core expression has been reported to modulate calcium oscillations in T lymphocytes (Bergqvist et al., 2003). In addition, liver cells expressing HCV core showed increased calcium release from ER, which was taken by mitochondria, resulting in high levels of ROS production and decreased antioxidant levels. These events resulted in transitional mitochondria permeability, triggering oxidative stress and apoptosis, which will be better characterized afterward (Choi et al., 2004; Benali-Furet et al., 2005).

Little is known about the role of virus-induced PRR activation on UPR pathway and vice-versa. However, the regulation of RLR, TLR and NLRP3 downstream signaling transduction by ER stress responses had been largely reported in other disease models, such as autoimmune and inflammatory diseases and bacterial infections (Menu et al., 2012; Jiang et al., 2013; Kim et al., 2013; Eckard et al., 2014; Savic et al., 2014). In fact, ER responses were associated to activation of NF- κ B, ROS formation and activation of transcription factors that could potentially regulate innate immune responses, as previously described.

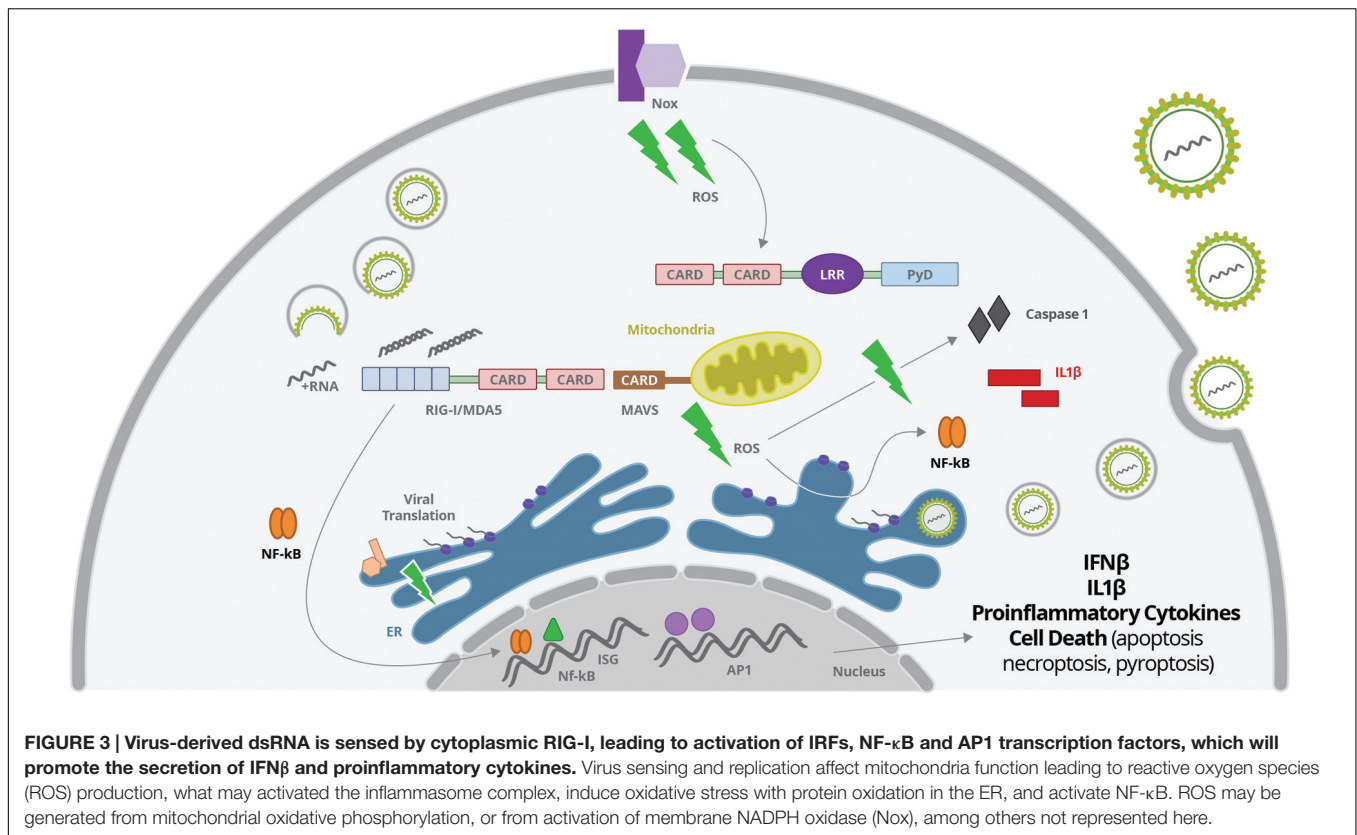
Endoplasmic reticulum stress and inflammation might also function in a paracrine way, as observed in other disease models (Garg et al., 2012). It was reported that macrophages cultured with conditioned medium from ER-stressed cells became activated and underwent ER-stress themselves (Mahadevan et al., 2011). Although this mechanism had not been demonstrated for virus infection, this sort of “transmissible stress” could also amplify the inflammatory response and control virus dissemination.

Mitochondria Stress, ROS Production, and Virus Sensing

Oxidative stress is an event of enhanced formation of so-called ROS in the cell. ROS is a general term indicating a set of molecules and radicals including hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radical (HO; Malhotra and Kaufman, 2007; Reshi et al., 2014). ROS are usually produced during the processes of aerobic metabolism, and ongoing stress, such as exposure to UV light or X-rays, and virus infection (Mittler et al., 2011; Reshi et al., 2014). ROS induces cellular stress through direct interaction with the biological molecules such as proteins, lipids and nucleic acids. One particular ROS species is superoxide (O₂⁻), which is generated by incomplete electron transfers in the electron transport chain in mitochondria (Paracha et al., 2013). Upon production, O₂⁻ molecules are rapidly metabolized into hydrogen peroxide (H₂O₂). Intermediate concentrations of H₂O₂ (and other ROS) result in activation of NF- κ B, and activating protein-1 (AP-1), that up-regulate several antioxidant and inflammatory pathways, including ISGs (Schreck et al., 1991, 1992). Many different enzymes at the mitochondria, ER, peroxisomes and other cell compartments are involved in the synthesis of ROS (Figure 3). ROS may be generated from mitochondrial oxidative phosphorylation, or from activation of NADPH oxidase, xanthine oxidase, cyclooxygenase, and lipoxygenases, whereas ROS source may differ depending on the initial stimuli (Harjith et al., 2014).

Intracellular scavengers confine ROS production and create a gradient surrounding its source. Therefore, ROS signaling is compartmentalized allowing signal specificity. Disturbing of this compartmentalization by virus replication usually results in increased ROS production, accumulation or efflux, promoting oxidative stress (Reshi et al., 2014). Flavivirus replication in association to ER membranes may result in protein oxidation in ER and production of ROS that results in oxidative stress (Paracha et al., 2013) (Figure 3). Other antiviral cellular response, such as autophagy, is also associated with increase of ROS production by accumulation of dysfunctional mitochondria (Nakahira et al., 2011; Scherz-Shouval and Elazar, 2011).

Indeed, viruses frequently modify mitochondrial function through direct interaction of viral proteins with mitochondrial components. Some Flaviviruses changes the steady-state levels of the mitochondrial chaperone prohibitin that disturb the mitochondrial respiratory chain leading to overproduction of ROS (Dang et al., 2011). Also, viral core proteins can bind to the outer mitochondrial membrane, or can be imported into the matrix or to the intermembrane space (Okuda et al., 2002; Ivanov et al., 2013). Viral proteins can also bind to membrane sites closely associated with the mitochondria, such as the mitochondria associated membrane (MAM) fraction of the endoplasmic reticulum (Chan and Gack, 2015; Vassallo and Gastaminza, 2015). Importantly, Flavivirus dsRNA sensing involving RIG-I activation is directly related to MAVS signal transduction, which requires recruitment of this adaptor to the mitochondrial outer membrane protein (Soucy-Faulkner et al., 2010; Chan and Gack, 2015) (Figure 3). Therefore,



mitochondrial alteration may directly affect MAVS-stimulated interferon production and inflammatory response.

Nox-derived ROS was reported to be one of the factors related to activation of NF-κB and IRF 3, and subsequent production of IFN-β and IFIT upon RIG-I activation (Soucy-Faulkner et al., 2010; Asdonk et al., 2012; Olganier et al., 2014b; Kim et al., 2015). Protein sensing by NOD-like receptors (NLRs) was also associated to ROS production induced by Nox2 activation (Lipinski et al., 2009) (Figure 3).

The role of oxidative stress in TLR-mediated inflammation was demonstrated in association to TLR4 activation in macrophages and neutrophils, but much less is known regarding virus-mediated activation of TLR3 and TLR7 (Asehnoune et al., 2004; Park et al., 2004). However, it was recently reported that TLR7 activation by RNA virus may enhance Nox activation and oxidative stress, and this may also be true for Flavivirus infection (Lin et al., 2000; Ke and Chen, 2012; Ivanov et al., 2013; Paracha et al., 2013; Olganier et al., 2014a).

Flavivirus Infection and Oxidative Stress

Reactive oxygen species are induced by a number of different Flaviviruses, including HCV, JEV, and Dengue. In many cases, ROS induced by viral infection have been linked with innate antiviral signaling pathways.

Changes in redox status have been associated with increased dengue severity. Adult patients experiencing dengue fever showed altered levels of circulating antioxidants, high levels of biochemical markers of lipid peroxidation (peroxidation

potential), and protein oxidation (Malondialdehyde MDA and 4 hydroxyalkenals 4-HDA), as well as increased antioxidant enzymatic activity of superoxide dismutase (SOD; Gil et al., 2004; Klassen et al., 2004). Significant difference was found in protein carbonylation (PCOs) and protein-bound sulphhydryl (PBSh) groups in patients with severe dengue. These results suggest a possible role for oxidative stress in plasma proteins and lipid oxidation during DENV-induced pathogenesis (Soundravally et al., 2008; Seet et al., 2009).

Oxidative stress promoted by HCV has been shown to manipulate antioxidant systems, leading to chronic disease (Ke and Chen, 2012; Ivanov et al., 2013; Paracha et al., 2013). Markers of oxidative stress were observed *in vivo* in chronic hepatitis C patients and transgenic mice, as well as in cell lines infected with HCV (Yamaguchi et al., 2005; Ivanov et al., 2013). Almost all HCV proteins, including E1, E2, NS3, NS4B, and NS5A, trigger oxidative stress in HCV infected cells, but HCV core protein is the most potent regulator (Okuda et al., 2002; Thorén et al., 2004; García-Mediavilla et al., 2005; Pal et al., 2010; Ivanov et al., 2011; Ming-Ju et al., 2011). A recent work with human hepatocarcinoma cells (Huh7) showed that oxidative stress caused by HCV core occurs by several independent pathways, such as induction of TGFβ1-dependent expression of NADPH oxidase, upregulation of cytochrome P450 2E1 transcription and expression of ER oxidoreductin 1α, according to the region of HCV core protein (Ivanov et al., 2015). Moreover, HCV core-induced oxidative stress was also shown to induce RNA damage, leading to enhanced HCV genome heterogeneity and allowing the virus

to escape from immune system, as well as from antiviral drugs (Serono et al., 2011). IL-1 β secretion induced by HCV was also dependent on ROS production, in association to activation of NLRP3, ASC and caspase 1, but not on RIG-I sensing, demonstrating that oxidative stress participates in inflammation induced by *Flaviviridae* viruses (Chen et al., 2014).

Cytoplasmic Stress or Stress Granules

RNA molecules in the cytoplasm can be either directed to active sites of translation represented by polysomes, or may be packed into RNA cytoplasmic granules, called P bodies and SGs, which prevent RNA translation (Anderson and Kedersha, 2006). In fact, when cells undergo environmental stress such as heat shock, UV irradiation, nutrient restriction, hypoxia, ER stress, or viral infection, mRNAs molecules are released from polysomes and the general translation is arrested (Anderson and Kedersha, 2006; Valiente-Echeverría et al., 2012; Reineke and Lloyd, 2013). Different mRNAs in this scenario can be either storage or degraded into SGs and P bodies, which are cytoplasmic granules consisting of RNA-proteins aggregates. P bodies are more dedicated to RNA degradation mechanisms, including RNA association with proteins involved in the RNA induced silencing complex (RISC; Beckham and Parker, 2008; Balagopal and Parker, 2009; Lavut and Raveh, 2012). Those mechanisms will not be addressed in this review, since it is also observed under normal physiological conditions. On the other hand, SG were recently described as being part of the cellular response to stress generated by viral infection, especially RNA viruses, such as Flavivirus (Valiente-Echeverría et al., 2012; White and Lloyd, 2012; Onomoto et al., 2014).

The critical event to promote SG formation is the phosphorylation of eIF2, preventing the assembly of the active ternary preinitiation complex eIF2-GTP-tRNA^{Met}, which results in polysome disassembly and blockade of translation initiation (Kedersha et al., 2002; Weber et al., 2008; Valiente-Echeverría et al., 2014) (**Figure 4**). As mentioned earlier, eIF2 phosphorylation can be mediated by a family of serine/threonine kinases including HRI, PKR, PERK/PEK, and GCN2 (Kedersha et al., 2005; Mazroui et al., 2006). The intracellular localization of each kinase is associated to the different pathways and stress sensors that culminate in the final event of eIF2 phosphorylation and translation inhibition. The HRI kinase (eIF2K1) is activated in heme deprivation and oxidative stress with ROS release; PKR is the cytoplasmic kinase (eIF2K2) activated by viral infection and foreign dsRNA; PERK/PEK (eIF2K3) is associated to ER and related to UPR pathway, as described before; and GCN2 (eIF2K4) is activated by amino acid starvation and UV irradiation (Bertolotti et al., 2000; Daito et al., 2014; Jain et al., 2014). Those kinases cause the phosphorylation of the eIF2- α at Ser51, which impairs its binding to eIF2B, inhibiting GDP-GTP exchange, which is necessary for ^{Met}tRNA position on the 43S ribosomal subunit and initiation of translation (Kedersha et al., 2002). Other mechanisms independent of the phosphorylation of eIF2 have also been described (Mazroui et al., 2006; White and Lloyd, 2012).

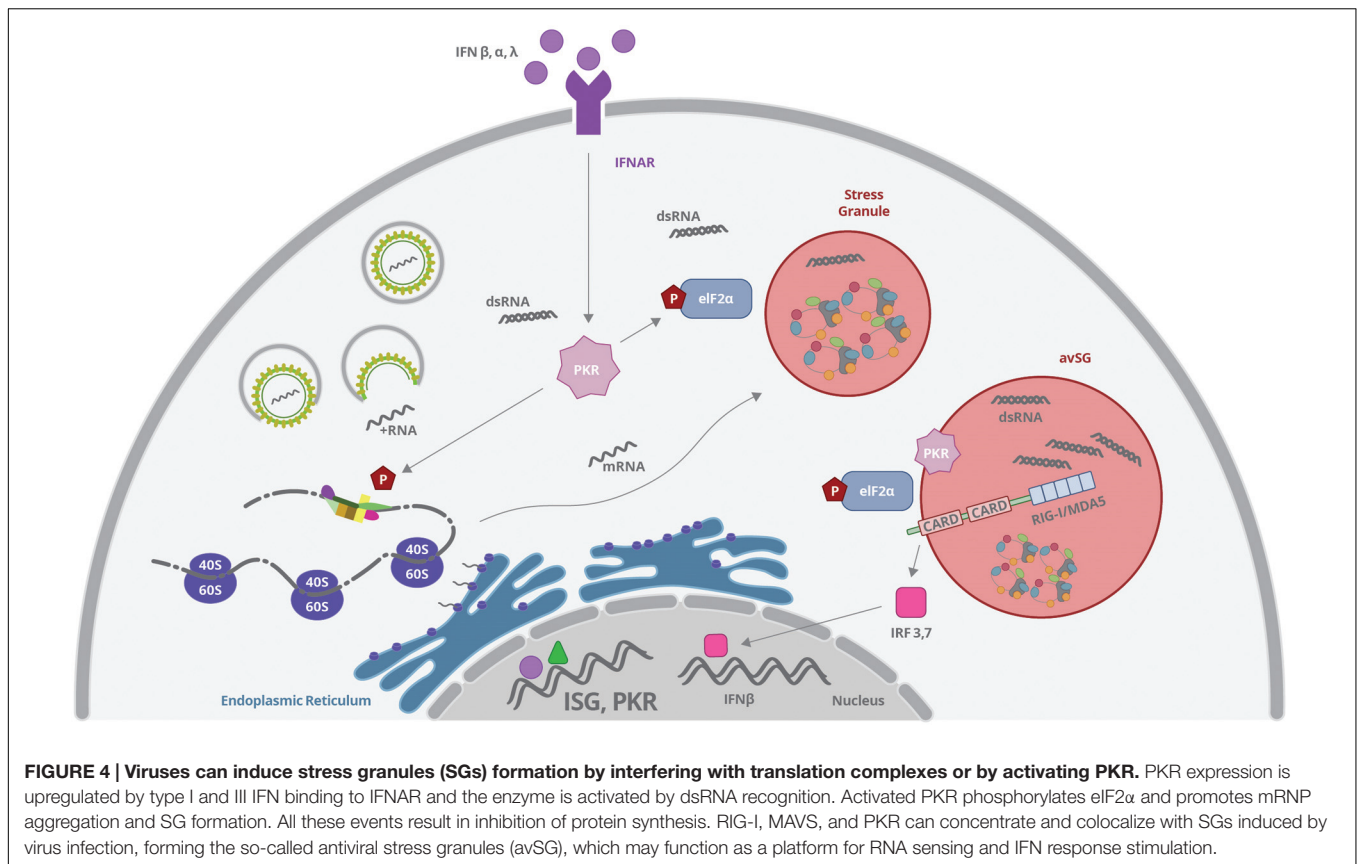
Stress granules are very dynamic sites and are formed from condensation of stalled translation initiation complexes including

43S and 48S ribosomal preinitiation complexes (Kedersha et al., 2005). However, these translation complexes can be rapidly released to resume protein synthesis when stress conditions are ceased. The molecular mechanism by which SG condense is still in debate and involves the self-oligomerization of key constituent RNA-binding proteins, such as Ras-Gap SH3-binding protein (G3BP1), T-cell restricted intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR; Kedersha et al., 1999; Piotrowska et al., 2010; Valiente-Echeverría et al., 2014). Moreover, hundreds of RNA-interacting proteins and an siRNA were described to be located into SG including: translation initiation factors (eIF4E, eIF4G, eIF4A, eIF4B, eIF3, and eIF2), poly(A)-binding protein (PABP1) and others RNA binding proteins that regulate mRNA structure and function (Kedersha et al., 2005; Anderson and Kedersha, 2009). These RNA binding proteins include: human antigen R (HuR); dsRNA-binding protein (Staufen1); polysomal ribonuclease 1 (PMR-1); the posttranscriptional regulator Smaug (SAMD4A); tristetraprolin (TTP); T-cell restricted Fragile \times Mental Retardation Protein (FXMR/FXR1); RNA helicase (RCK/p54/DDX6); cell cycle associated protein 1 (caprin1); HuR; Y-box binding protein 1 (YB-1), that activates the 5' UTR of G3BP1 mRNAs and cytoplasmic polyadenylation binding protein (CPEB; Anderson and Kedersha, 2006).

The composition of SGs can vary depending on the type of cell stress, however, stalled translation complexes are common components of these RNA granules. SGs induced by virus infection are enriched in G3BP1 and RNA binding protein Sam68, which is part of signaling transduction pathways during alternative splicing, RNA-3'/UTR formation and cell cycle regulation (Piotrowska et al., 2010; Finnen et al., 2012; Valiente-Echeverría et al., 2014).

SG Formation and RNA Virus Sensing

Viruses can induce SGs formation by interfering with translation complexes such as eIF4G or eIF4A (White and Lloyd, 2012). However, induction of SG upon virus infection is more commonly associated to activation of PKR by recognition of viral dsRNA (**Figure 4**). Activated PKR phosphorylates eIF2 α , as described before, and promotes mRNA aggregation induced by G3BP protein (Tsai and Lloyd, 2014). Interestingly, it has been recently demonstrated that cytoplasmic virus sensor and interferon responsive genes such as RIG-I, MDA5, MAVS, and PKR can concentrate and colocalize with SGs induced by virus infection, forming the so-called antiviral stress granules (avSG; Onomoto et al., 2012; Langereis et al., 2013) (**Figure 4**). Some authors propose that avSG may function as a platform for RNA sensing and IFN response stimulation (Onomoto et al., 2012; Yoo et al., 2014). On the other hand, inhibition of protein synthesis induced after SG formation may diminish the translation of IFN-induced genes as a viral strategy to prevent immune response (Onomoto et al., 2014). Different groups have been investigating these events and, although some of them have been using surrogate dsRNA agents (like poli I:C), it is plausible to assume that transient avSG formation may be a common phenomenon upon virus infection, including Flaviviruses.



RIG-I translocation to SG containing PKR, OAS, and RNase L was demonstrated upon viral infection or viral RNA transfection (Chakrabarti et al., 2012). In this model, PKR activation was essential for the formation of RIG-I containing SG, and was necessary for IRF3 dimerization and viral-mediated IFN response (Figure 4). PKR activation was completely dependent on the presence of virus RNA (Gilfoy and Mason, 2007). The complete signaling pathway involved in the formation and response of avSG is not fully understood, although it was recently reported that dsRNA-activated MAVS can directly interact with PKR, through CARD domain, promoting PKR activation, and subsequently eIF2- α phosphorylation followed by SG assembly (Zhang et al., 2014). Silencing of MAVS or PKR limited SG formation induced by dsRNA, and it was proposed that association of MAVS with PKR might directly affect the dsRNA-dependent dimerization of the latter. However, the role of RIG-I and MDA5 was not as important as MAVS in the system, suggesting the involvement of additional undefined pathways.

Although PKR had been demonstrated to be a key element for the generation of avSG as a platform that allows interaction between antiviral proteins and non-self RNA ligands, it might have different roles, depending on the stimulated cell type (Onomoto et al., 2012; Yoo et al., 2014). PKR silencing resulted in decreased IFN- β production in hepatoma cells infected with DENV, but not in fibroblast nor macrophages (Li Y. et al., 2013). In addition, decreased protein synthesis induced by PKR activation might, in fact, inhibit the synthesis of ISG proteins, in

spite of increased mRNA expression triggered by the RNA sensor (Garaigorta and Chisari, 2009).

Modulation of SG Assembly by Flavivirus

Since viral propagation completely depends on the host translational machinery, induction of SG by virus infection is usually transitory and most viruses suppress SG assembly at some point of their replicative cycle. In general, flaviviruses such as WNV, DENV, and JEV block arsenite-induced stress granule formation by hijacking multiple SG components, including TIA1, TIAR, G3BP1, and Caprin-1 (Onomoto et al., 2014).

Li et al. (2002) first demonstrated that SG proteins directly interact with WNV RNA and proteins (Li et al., 2002). They observed that TIA-1 and, mostly, TIAR interacts with minus strand 3' terminal stem loop RNA (3' SL RNA) of WNV, which is the initiation site of genomic RNA synthesis (Li et al., 2002). Interaction of virus RNA with TIA-1/TIAR seemed to facilitate WNV replication and, TIAR knockout cells exhibited decreased WNV replication when compared with control cells (Li et al., 2002). DENV RNA also binds specifically to TIA-1, TIAR, and G3BP (Emara and Brinton, 2007; Bidet et al., 2014). In addition, a recent study showed that caprin-1 interacts with the DENV 3'UTR suggesting that this genomic region is a site for assembly of SG proteins (Ward et al., 2011). Both flavivirus, DENV and WNV, inhibited SG formation and eIF2- α phosphorylation, preventing the shutoff of host proteins translation, and favoring virus RNA translation (Emara and Brinton, 2007). It is likely,

therefore, that recruiting SG proteins to different compartments may allow virus translation or RNA replication and prevents innate immune response as a consequence of SG assembly.

Japanese Encephalitis Virus core protein also recruited several SG-associated proteins, including G3BP and USP10, in a way dependent on caprin-1 binding (Katoh et al., 2012). These interactions were associated to the suppression of SG assembly, resulting in increasing viral replication. SGs enriched with G3BP1/eIF3/eIF4B were also detected upon TBEV infection. Depletion of TIA-1 or TIAR resulted in increased production of new infectious virus, indicating that SGs harboring TIAR and TIA-1 inhibit TBEV replication (Albornoz et al., 2014).

Hepatitis C Virus also exploits SG machinery by recruiting PKR-eIF2- α phosphorylation pathway as a strategy for viral escape (Garaigorta and Chisari, 2009; Garaigorta et al., 2012). It has been reported that IFN treatment of HCV-infected cells induced phosphorylation of PKR and eIF2- α , thereby inhibiting *de novo* cellular protein synthesis, including translation of antiviral interferon-stimulated genes. Indeed, IFN-stimulated proteins like MxA and USP18 levels were inversely correlated with the amount of SGs in HCV infected Huh7 cells, suggesting that interferon-stimulated gene translation was inhibited in SG-containing infected cells (Garaigorta et al., 2012). Activation of PKR, however, did not inhibit translation of HCV proteins, probably due to IRES-dependent synthesis (Garaigorta and Chisari, 2009).

Early and late stages of HCV replication seems to be dependent on SG components. Short hairpin RNA (shRNA) knockdown experiments suggested that TIA-1, TIAR, and G3BP1 were required for efficient HCV RNA and protein accumulation at early time points after HCV infection (Garaigorta et al., 2012). In addition, G3BP1, ATX2, PABP1, and USP10 colocalized with HCV core protein at lipid droplets, suggesting that they might facilitate RNA packaging and virus assembly (Ariumi et al., 2011; Pager et al., 2013). G3BP1 was showed to interact with the 5' end of the HCV minus-strand RNA and with NS5B protein suggesting that G3BP1 is required for HCV replication. Indeed, RNAi mediated depletion of SG components decreased the expression of HCV core and NS5A proteins, and inhibited assembly and release of HCV virions (Pager et al., 2013).

In this sense, the subversion of another SG-associated pathway has been recently unveiled and involves the ubiquitous ATP-dependent RNA helicase DDX3X, which is a pivotal cell factor for HCV replication and SG assembly (Li Q. et al., 2013; Pène et al., 2015). Interaction of DDX3X with HCV 3' UTR had been previously shown to induce IKK- α activation and cellular lipogenesis, which benefited virus assembly (Li Q. et al., 2013). It was then demonstrated that DDX3X recognition by HCV 3'UTR and IKK- α activation, promoted its redistribution to G3BP-containing SG and posterior association with HCV core protein at lipid droplets (Pène et al., 2015). Knockdown of DDX3X and multiple SG components inhibited HCV infection, suggesting that dynamic associations of DDX3X, HCV RNA and host proteins at lipid droplets surfaces might play a crucial role in HCV replication (Pène et al., 2015).

Ruggieri et al. (2012) showed that HCV exhibited a stress response oscillation as a mechanism to prevent long-lasting translation repression (Ruggieri et al., 2012). Changes in active and repressed phases of RNA translation were observed during HCV infection of IFN- α treated Huh7 liver cells, inducing highly dynamic assembly/disassembly of cytoplasmic SGs. In this case, eIF2- α phosphorylation was counteracted by GADD34 upregulation, a regulatory subunit of protein phosphatase 1 that reverted the phosphorylation of eIF2- α , reactivating translation (Ruggieri et al., 2012).

Inflammasome Activation upon Virus-Induced Stress

Virus sensing and the resulting metabolites available upon cell stress may be intimately associated to the activation of inflammasome complexes, which are a signaling platforms, containing a PAMP/DAMP sensor from NLR or PIHYN families, an adaptor protein containing CARD domain (ASC) and caspase 1 (Henao-Mejia et al., 2012). Stimulation of the inflammasome ultimately leads to activation of caspase 1/11, leading to IL-1 β and IL-18 secretion and, sometimes pyroptosis cell death (Poeck et al., 2010). Inflammasome activation may be triggered by different signals, including infection, altered concentration of secondary metabolites, or ion influx (Figures 1 and 3). A first signal, usually triggered by other innate immune receptors such as TLR or RIG-I, induces the production of immature pro-IL-1 β and pro-IL-18. A second signal, then, leads to caspase 1/11 activation, which cleaves pro-IL-1 β and pro-IL-18, generating mature cytokines, which may then be secreted (Lupfer et al., 2015; Vanaja et al., 2015).

NLRP3 is the best-known NLR associated to inflammasome activation. Although the whole mechanism associated to NLRP3 activation is still not fully understood, it is well known that, once activated, it associates to ASC and procaspase 1, promoting its activation (Motani et al., 2011; Zhou et al., 2011). NLRP3 is activated by several stimuli, such as ATP, uric acid and cholesterol crystal, among others. Several NLRP3 activators are associated with ROS production, which, in turn may be either important for NLRP3 activation or expression (Bauernfeind et al., 2011; Rahman and McFadden, 2011; Zhou et al., 2011). In fact, NLRP3 inflammasome is very sensitive to ROS and most signals leading to activation of NLRP3 inflammasome, including virus infection, are ROS-dependent (Bauernfeind et al., 2011) (Figures 1 and 3). In addition, cell death and release of cellular content, such as ATP may also promote inflammasome activation in bystander cells.

NLRP3 activation may take place at the mitochondria and MAVS had been reported as a possible adaptor protein associated to NLRP3 recruitment to this organelle (Zhou et al., 2011; Subramanian et al., 2013). In this case, MAVS participation might not depend on RIG-I activation. In fact, MAVS association to RIG-I or NLRP3 were reported to be mutually exclusive in some systems (Subramanian et al., 2013). MAVS interaction with either RIG-I or NLRP3 could be dictated by the expression levels of each sensor, or by the available PAMP, and both elements might be determinant to the subsequent

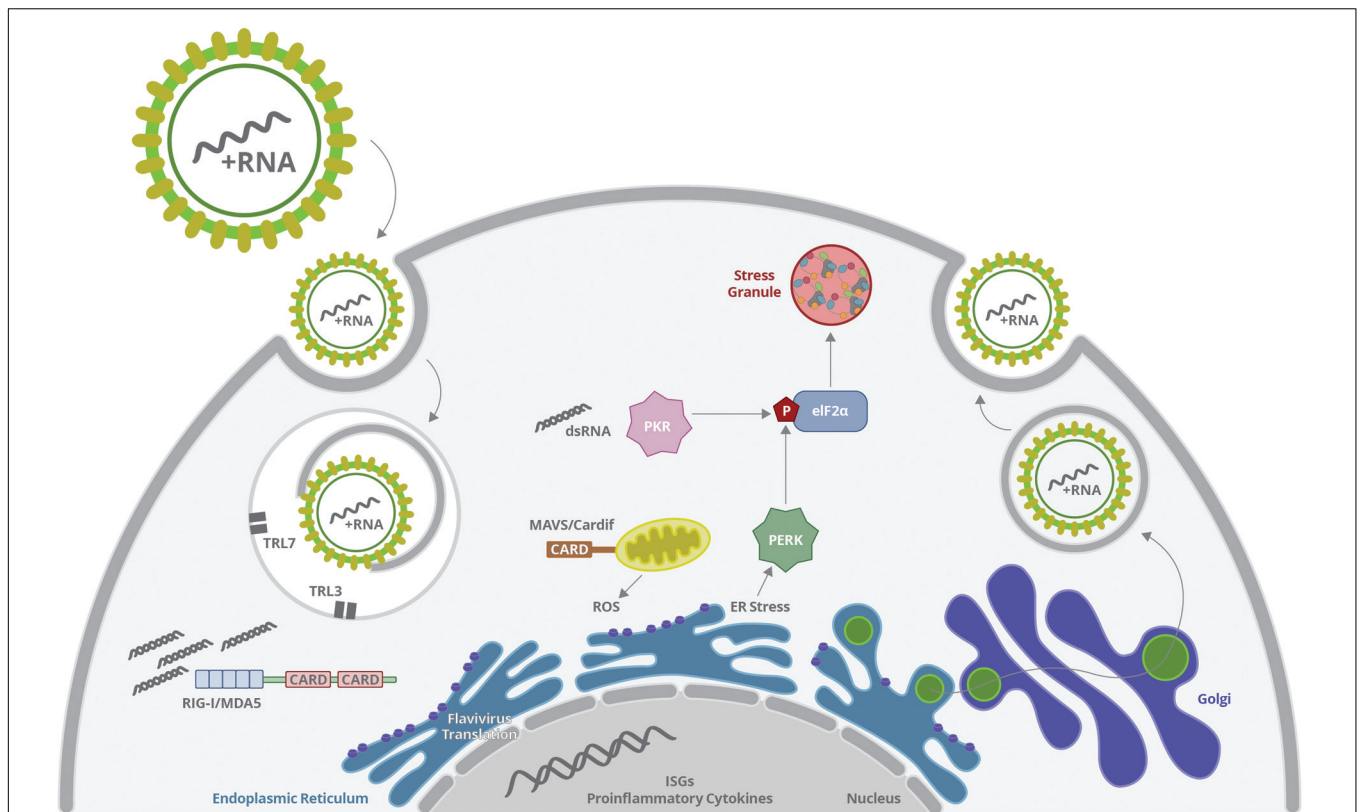


FIGURE 5 | Flavivirus infection is initiated by virus endocytosis, envelope fusion and genome uncoating, releasing virus RNA. Genomic RNA or dsRNA generated during replication may be sensed by vesicular TLR, RIG-I, MDA5, or PKR. Activation of TLR and RIG-I promotes ISG and inflammatory genes expression; whereas PKR stimulation promotes eIF-2 α phosphorylation and stress granules formation. In parallel, virus protein translation takes part mostly at the ER, altering ER homeostasis and promoting UPR. Virus sensing and replication affect mitochondrial function, inducing increased ROS production and oxidative stress, what may activate other inflammatory signal and affect cell survival. Virus scape mechanisms are triggered at different levels in different cell types, allowing some level of progeny release.

triggered signaling pathway, resulting in either IFN- β or IL-1 β production. RIG-I might also directly activate inflammasomes, in a MAVS-independent way (Poeck et al., 2010). Alternatively, RLR and inflammasome might be sequentially stimulated. In epithelial infection with influenza virus, it was demonstrated that RIG-I was the primary viral RNA sensor, inducing IFN- β secretion (Loo et al., 2008; Pothlichet et al., 2013). RIG-I-induced IFN then up-regulated TLR3 and NLRP3 and all these mediators (RIG-I, TLR3, NLRP3, and IFN) were necessary for caspase 1 activation and IL-1 β release (Pothlichet et al., 2013).

Caspase 1 has more than a hundred substrates, indicating that inflammasome may function far beyond the inflammatory response (Henao-Mejia et al., 2012). Cell death is one possibility downstream inflammasome activation, and this event may be a complex process involving cross modulation of adaptor proteins associated to different death mechanisms. In most cases, inflammasome-dependent caspase 1 activation leads to pyroptosis, which may contribute to the amplification of the inflammatory response by promoting the release of intracellular content (Bergsbaken et al., 2009; Labbé and Saleh, 2011; Miao et al., 2011).

Hepatitis C Virus may activate different virus sensing and inflammation pathways, depending on the cell type. TLR3 showed to be essential for type I IFN production in hepatocytes upon HCV infection, whereas TLR7 was essential for this response in pDC (Chattergoon et al., 2014). On the other hand, in monocytes, activation of TLR7 by HCV virions was associated to activation of the inflammasome pathway, promoting the secretion of IL-1 β and IL-18 in a NLRP3-dependent and infection independent pathway (Chattergoon et al., 2014). Other authors could not observe activation of inflammasome by HCV virions, but also found that HCV RNA was able to directly stimulate these platforms dependent on NLRP3, ASC, caspase 1 and ROS (Chen et al., 2014).

Activation of NLRP3 inflammasomes was also observed upon dengue infection of macrophages, promoting the secretion of IL-1 β and pyroptosis (Wu et al., 2013). Interestingly, it was recently demonstrated that inflammasome activation might also take place in platelets, and was induced by dengue infection (Hottz et al., 2013). Activation of NLRP3 and increased ROS production promoted IL-1 β release from platelets, which were then able to increase vascular permeability. Taken together, these data suggested that inflammasome activation by dengue might

be a key mechanism for disease pathogenesis, and might be associated to altered platelet and vascular function, cell death of immune cells, and fever (Hottz et al., 2013).

Increased IL-1 β and activation of NLRP3 inflammasomes were also detected during infection of flaviviruses associated to CNS, including JEV and WNV (Kaushik et al., 2012; Kumar et al., 2013). In a mouse experimental model of WNV infection, IL-1 β secretion was associated to inhibition of virus replication in neurons, increased production of IFN- β , modulation of pro-inflammatory cytokines, and protection from neuronal cell death and tissue damage in CNS (Ramos et al., 2012; Kumar et al., 2013).

Virus Infection Inducing Stress and Death

Prolonged stress induced by any of the mechanism described above may result in cell death. For example, ablation of RIG-I or MAVS in a number of virus infection models resulted in increased cell survival, indicating a direct role of dsRNA sensing for the induction of cell death (Chattopadhyay et al., 2010; Yu et al., 2010). In fact, RIG-I activation stimulates several apoptosis-related genes, either by a direct effect of IRF3 activation or through secondary IFN-mediated responses, which can also amplify the response induced by IRF. RIG-I activation by HCV, for example, promoted TRAIL-mediated apoptosis, although it was not clear whether this was a direct effect of RIG signaling or a secondary, IFN-mediated effect (Yang et al., 2011). Other studies reported that IRF-3 translocation to the nucleus directly stimulated the expression of ISG, such as IFIT1 and IFIT2 (ISG54 and ISG56), which activation was previously associated to caspase3-dependent, mitochondrial associated apoptosis (Stawowczyk et al., 2011).

It was also proposed that IRF3-induced apoptosis might happen independently of its transcription factor activity. In this sense, IRF3 activation by cytoplasmic dsRNA-RIG-I complex induced its association to proapoptotic Bax, leading to the translocation of both proteins to the mitochondria and induction of Bax-mediated, caspase 9-dependent apoptosis (Chattopadhyay et al., 2010). According to this model, activated IRF3 may either translocate to the nucleus or to the mitochondria, but the mechanisms regulating both pathways are not completely understood.

Interestingly, it had been showed that caspase activation induced by virus infection resulted in MAVS cleavage and consequent inhibition of interferon production, but did not result in increased virus titers (Yu et al., 2010). Therefore, caspase activation upon RIG-I stimulation may help to control the inflammatory response, suggesting a close relation between virus sensing, stress signals and inflammation.

Activation of RLR/MAVS pathway may also result in necroptotic cell death. Stimulation of pDC with poli:C, a dsRNA surrogate, induced the release of cathepsin D from the lysosomes to the cytoplasm, where it associated to MAVS, and was recruited to a complex formed by MAVS-caspase 8-RIPK1 in the mitochondria. Cathepsin D cleaved caspase 8, which probably promoted its release from mitochondria, allowing activation of RIPK and

initiation of necroptosis (Zou et al., 2013). The consequence of necrosis is the release of several cellular constituents, such as nucleic acids and ATP, which may then stimulate other receptors expressed in bystander cells, like inflammasome-related sensors.

Finally, other cell mediators produced after virus sensing may also modulate survival of infected and bystander cells. Engagement of TNFR by TNF- α can result in cell survival or promote apoptosis or necrosis, which will depend on NF- κ B activation, or on the balance between RIPK1 and caspase 8 activation. Therefore, the other signals triggered by virus infection in association to TNFR-driven signaling will then dictate the cell fate.

Importantly, apoptotic or necrotic death is not always the cellular fate upon virus infection, due to rescue mechanisms, such as inhibition of protein synthesis and autophagy. For instance, virus-induced ROS accumulation stimulates RLR- and NLRP-mediated responses. However, these events may also stimulate the autophagy machinery, which will then limit ROS and mitochondria signals and restrain PRR downstream responses. A crosstalk between cellular rescue mechanisms and PRR-induced downstream signaling is, then, important to control virus replication while maintaining tissue homeostasis.

CONCLUSION

The RNA genome and proteins of *Flaviviridae* members can be sensed by several cellular mechanisms, which will induce immune responses and activate cellular stress pathways, including include ER stress, oxidative stress and stress granules formation, restricting virus production (Figure 5). However, viruses develop several mechanisms to subvert the innate antiviral responses by sequestering cellular partners related to inflammation and stress in order to delay protein translation inhibition, inhibit interferon responses and cell death. Virus scape mechanisms may be triggered at different levels in different cell types, allowing some level of progeny release.

The interplay between innate immune response and cellular stress pathways is just beginning to be elucidated and several key questions remains unclear. *Flaviviridae* members infect different cell types, and the virus sensing molecules and the persistence of cellular stress will depend directly on virus intracellular trafficking through the cell compartments. Thus, it is not only important to follow multiple components of inflammation and cellular stress during infection, but also to examine the functional consequences of these cellular responses to the disease pathogenesis. Some Flavivirus described here are related to aggressive pathologies such as Hemorrhagic Fever and neurological disorders. In addition, the biology and pathogenesis of other Flavivirus, such as ZIKV is largely unknown. The balance between stress, inflammation and antiviral responses will determine a successful control of virus dissemination or the development of severe disease. Since stress responses and innate immunity likely crosstalk at multiple levels, these pathways may be exploited as a broad spectrum antiviral strategy.

AUTHOR CONTRIBUTIONS

AV, RA, and LA wrote the review. RA and LA critically revised the review. LA was responsible for conception and design of the review.

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Intrinsically Disordered Side of the Zika Virus Proteome

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Over the last few decades, concepts of protein intrinsic disorder have been implicated in different biological processes. Recent studies have suggested that intrinsically disordered proteins (IDPs) provide structural plasticity and functional diversity to viral proteins that are involved in rapid replication and immune evasion in host cells. In case of Zika virus, the roles of protein intrinsic disorder in mechanisms of pathogenesis are not completely understood. In this study, we have analyzed the prevalence of intrinsic disorder in Zika virus proteome (strain MR 766). Our analyses revealed that Zika virus polyprotein is enriched with intrinsically disordered protein regions (IDPRs) and this finding is consistent with previous reports on the involvement of IDPs in shell formation and virulence of the *Flaviviridae* family. We found abundant IDPRs in Capsid, NS2B, NS3, NS4A, and NS5 proteins that are involved in mature particle formation and replication. In our view, the intrinsic disorder-focused analysis of ZIKV proteins could be important for the development of disorder-based drugs.

Keywords: Zika virus, intrinsically disordered proteins (IDPs), intrinsically disordered protein regions (IDPRs), molecular machinery, viral proteome, cellular proteome

INTRODUCTION

In 1947, Zika virus (ZIKV) was first identified in Uganda through a monitoring network of sylvatic yellow fever in rhesus monkeys (Dick et al., 1952). Outbreaks of ZIKV-related disease have been recorded throughout southern Africa with a high number of birth defects and abnormalities including microcephaly, intracranial calcification, and fetal death (Petersen et al., 2016). As many other members the *Flaviviridae* family, ZIKV is an arbovirus transmitted through the infected arthropods (by the bites of the infected mosquitoes from the *Aedes* genus, *Ae. aegypti* and *Ae. albopictus*). Therefore, distribution of Zika infection is mainly associated with the distribution of *Aedes* mosquito vectors that can be found in different parts of the world (Wikan and Smith, 2016). World Health Organization (WHO) has declared Zika related problems as public health emergency of international concern. Therefore, it is of utmost urgency to find out mechanism of pathogenesis of this virus and to develop therapeutics. A recent study on immunocompetent mouse model has strengthened the previous observations that ZIKV infection might cause neurological defects in fetuses (Lazear et al., 2016). This virus is transmitted through mosquitoes, as well as via blood transfusion and also from mother to fetus during pregnancy (Wikan and Smith, 2016). Reports also suggest the possibility of sexual transmission (Grischott et al., 2016).

ZIKV belongs to the *Flaviviridae* family, genus *Flavivirus*, which includes several important human pathogens, such as West Nile virus (WNV), Dengue virus (DENV), Yellow fever virus

(YFV), and Japanese encephalitis virus (JEV). Recently, ZIKV structure has been solved by cryo-electron microscopy (Sirohi et al., 2016). Genome of this virus includes a single-stranded RNA consisting 10794 bases along with two non-coding regions known as the 5' NCR and the 3' NCR. The open reading frame (ORF) of the ZIKV, concerning the protein expression order is as follows: 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. It codes for a single polyprotein that is posttranslationally cleaved into three structural proteins (Capsid (C), Precursor membrane (prM) protein, and Envelope (E) protein), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (see **Figure 1**).

The major focus of this article is on intrinsic disorder-centered analysis of ZIKV proteome. In general, intrinsically disordered proteins (IDPs) are recently recognized class of proteins that lack stable three-dimensional structure in their native state but still functional. Structurally, these proteins are highly heterogeneous and include random coils, pre-molten globules, molten globules, proteins with large flexible linkers, and hybrid proteins containing ordered and disordered regions (Wright and Dyson, 2015). Lack of structure in IDPs and intrinsically disordered protein regions (IDPRs) allow the interaction of proteins with several partners, thereby regulating multiple signaling pathways. These multiple interactions are attributed to fast binding kinetics of IDPs/IDPRs that is regulated through coupled folding and binding mechanisms (Gianni et al., 2012). Disordered regions provide greater capture radii that increase the probability to interact with partners. This mechanism is known as the fly casting mechanism (Shoemaker et al., 2000). Commonly, viruses have highly compact genome and contain disordered protein regions. This could be one of the reasons for high mutagenic capacity of viruses (Xue et al., 2014). Recent studies have reported that core proteins of the viruses from the *Flaviviridae* family contain significant amount of IDPRs (Xue et al., 2014; Goh et al., 2016). A functional correlation between intrinsic disorder and protein function has been established in proteomes of other *Flaviviridae* family members, such as Dengue Virus (Meng et al., 2015) and Hepatitis C Virus (HCV) (Fan et al., 2014).

In our view, the intrinsic disorder-focused analysis of ZIKV proteins could be important for the development of new

disorder-based drug strategies (Cheng et al., 2006; Uversky, 2010, 2012). Current drug development strategies have shown to utilize combination of conventional drug design and computational approaches to target dynamic ensembles of IDPs (Ambadipudi and Zweckstetter, 2016). In the current work, we have analyzed the penetrance of intrinsic disorder in the ZIKV proteome. Further, we have correlated the abundance of structural disorder with functionality of ZIKV proteins. This study provides a novel direction for elucidating the mechanism of virus-host interaction. Some proteins have been already used as drug targets in other flaviviruses, such as NS3 helicase, envelope glycoproteins, NS2B-NS3 (serine protease) and NS5 (RNA-directed RNA Polymerase) (Li et al., 2008; Poh et al., 2009; Mayhoub et al., 2011). Therefore, results of our current work should be considered before inhibitor designing.

MATERIALS AND METHODS

Reviewed and experimentally validated polyprotein sequence (UniProt ID: Q32ZE1) of the Zika virus strain Mr766 was used for the disorder analysis. There are several protein intrinsic disorder predictors developed, such as multiple members of the PONDR[®] family [e.g., PONDR[®] FIT (Xue et al., 2010), PONDR[®] VLXT (Romero et al., 2001), and PONDR[®] VSL2 (Obradovic et al., 2005), IUPred (Dosztanyi et al., 2005a), GlobPlot (Linding et al., 2003b), DisoPred (Ward et al., 2004), SPRITZ (Vullo et al., 2006), DisEMBL (Linding et al., 2003a)], etc. Many of these predictors have been assessed for accuracy within the frames of the Critical Assessment of Protein Structure Prediction (CASP) (Deng et al., 2012). Since many of these predictors are considering phenomenon of intrinsic from different angles, it is advisable to use several computational tools while looking for the abundance of intrinsic disorder in query proteins. Therefore, PONDR[®] FIT (Xue et al., 2010), PONDR[®] VLXT (Romero et al., 2001), PONDR[®] VSL2 (Obradovic et al., 2005), and IUPred (Dosztanyi et al., 2005a) were used in our study for disorder prediction in polyprotein of ZIKV. We also extended our analysis over each individual protein derived from the ZIKV polyprotein. Here, scores above 0.5 are considered to correspond to the disordered residues/regions. PONDR[®] VSL2B is one of the more accurate stand-alone disorder predictors

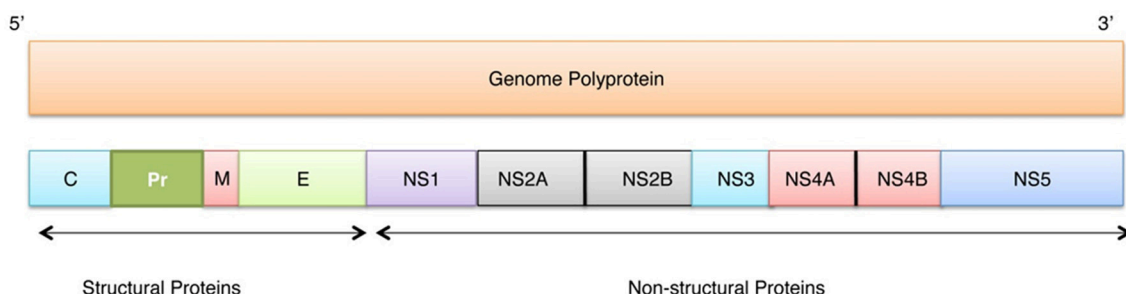


FIGURE 1 | Localization of individual proteins within the genome polyprotein of Zika virus (Q32ZE1). Top bar shows ZIKV RNA (10974 bases) that translates into polyprotein of 3418 residues (bottom bar) that at maturation is cleaved into three structural proteins (Capsid (C), Precursor membrane (prM), and Envelope protein (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

(Peng et al., 2005; Peng and Kurgan, 2012; Fan and Kurgan, 2014), PONDR® VLXT is known to have high sensitivity to local sequence peculiarities and can be used for identifying disorder-based interaction sites (Dunker et al., 2001), whereas a metapredictor PONDR-FIT is moderately more accurate than each of the component predictors (Xue et al., 2010), PONDR® VLXT (Dunker et al., 2001), PONDR® VSL2 (Peng et al., 2005), PONDR® VL3 (Peng et al., 2006), FoldIndex (Prilusky et al., 2005), IUPred (Dosztanyi et al., 2005a), TopIDP (Campen et al., 2008). IUPred was designed to recognize IDPRs from the amino acid sequence alone based on the estimated pairwise energy content, where it was hypothesized that globular proteins are composed of amino acids which have the potential to form a large number of favorable interactions, whereas IDPs/IDPRs do not have unique 3D structure because their amino acid composition does not allow sufficient favorable interactions to form (Dosztanyi et al., 2005a,b).

Often, IDPs/IDPRs are involved in protein-protein interactions and molecular recognitions (Dunker et al., 2002a,b, 2008; Tompa, 2002; Oldfield et al., 2005; Dunker and Uversky, 2008; Uversky and Dunker, 2010; Uversky, 2013b). There are numerous reports emphasizing that IDPs/IDPRs are able to undergo at least partial disorder-to-order transitions upon binding, which is crucial for recognition, regulation, and signaling. Among these potential functional sites are short order-prone motifs within long disordered regions that are able to undergo disorder-to-order transition during the binding to a specific partner. These motifs are known as molecular recognition feature (MoRF), and they can be identified computationally (Oldfield et al., 2005; Cheng et al., 2007). We used ANCHOR algorithm to identify potential disorder-based binding sites (Dosztanyi et al., 2009; Meszaros et al., 2009). This approach relies on the pairwise energy estimation approach developed for the general disorder prediction method IUPred (Dosztanyi et al., 2005a,b), being based on the hypothesis that long regions of disorder contain localized potential binding sites that cannot form enough favorable intrachain interactions to fold on their own, but are likely to gain stabilizing energy by

interacting with a globular protein partner (Dosztanyi et al., 2009; Meszaros et al., 2009).

RESULTS AND DISCUSSION

Intrinsic Disorder in ZIKV Polyprotein

In crystallography-based protein structure characterization it is assumed that the disordered regions cannot crystalize, being present in the form of regions with missing electron density, and only ordered regions have the propensity for crystal formation (Uversky, 2013a). In addition to this, crystallization conditions frequently contain various additives (presence of PEG, high salt concentrations, etc.), which make these conditions to be different from the natural environment. Therefore, a computational analysis of disorder based on the amino acid sequence alone using various programs may provide a great advantage to analyze the disorder in proteins (Uversky, 2013a).

Despite being a big threat, the holistic understanding of ZIKV proteins in both ordered and disordered perspective has not been established as of yet. Despite obvious interest to Zika virus, crystallographic data are currently available only for four ZIKV proteins, NS1, NS2B-NS3 protease (residues 49 to 95 of NS2B covalently linked via Gly₄-Ser-Gly₄ to the N-terminal protease domain (residues 1 to 170) of NS3), NS3 (only Helicase domain), M and E proteins (see below).

In this study we have computationally evaluated the predisposition of ZIKV polyprotein for intrinsic disorder (see **Figure 2**) and also studied intrinsic disorder propensity of all individual proteins derived from this polyprotein: Capsid protein C (residues 2-122, which includes protein C (residues 2-104) and the ER anchor for the protein C (residues 105-122), which is removed in mature form by serine protease NS3), precursor membrane protein prM (residues 123-290, which is further divided to peptide pr (residues 123-215) and small envelope protein M (residues 216-290)), an envelope protein E (residues 291-790), and seven non-structural proteins: NS1 (residues 791-1142), NS2A (residues 1143-1368), serine protease subunit NS2B (residues 1369-1498), serine protease NS3 (residues 1499-2115),

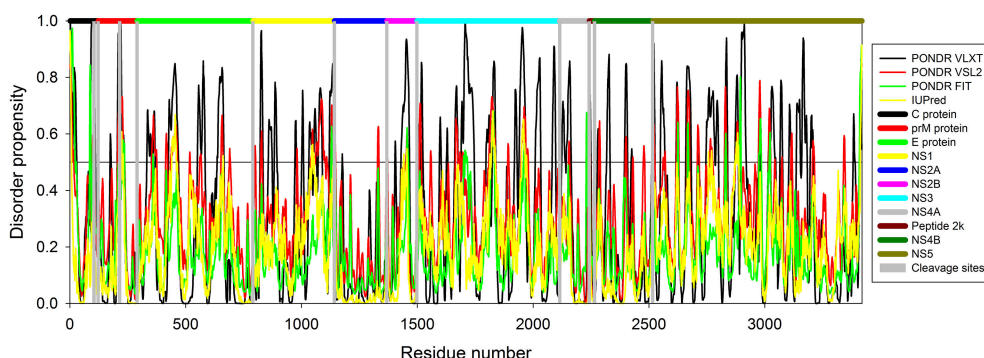


FIGURE 2 | Intrinsic disorder predisposition of Zika virus polyprotein (Q32ZE1). Disorder propensity is evaluated by PONDR® VLXT (black line), PONDR® VSL2 (red line), PONDR® FIT (green line) and IUPred (yellow line). Colored bars at the top of plot shows localization of the individual proteins. Corresponding cleavage sites leading for the generation of mature individual proteins are shown by gray bars. Disorder scores above the threshold 0.5 characterize residues/regions predicted to be disordered.

NS4A (residues 2116-2242), peptide 2k (residues 2243-2265), NS4B (2266-2516), and RNA-directed RNA polymerase NS5 (residues 2517-3419). It is recognized that disordered regions provide structural flexibility leading to binding promiscuity often involved in cellular regulations (Xue et al., 2014; Wright and Dyson, 2015). Often, disordered or flexible regions contain sites of proteolytic cleavage, since proteolytic digestion is known to occur much faster in unstructured than in structured protein regions (Fontana et al., 1986, 2004; Novotny and Brucoleri, 1987; Iakouchcheva et al., 2001). This suggests that the sites of preferential cleavage should be preferentially located within the regions that lack stable structure or possess high structural flexibility. Earlier, this specific use of intrinsic disorder/flexibility for generation of mature viral proteins from the polyprotein was reported for two other representatives of the *Flaviviridae* family, HCV (Fan et al., 2014) and Dengue Virus (Meng et al., 2015). In agreement with these considerations, **Figure 2** shows that, within the polyprotein, cleavage sites leading to the generation of mature ZIKV proteins are preferentially located within the disordered or flexible regions or at least in close proximity to the regions with increased flexibility. **Figure 3** further zooms into this phenomenon and shows that in the vicinity of the vertical gray lines that correspond to the cleavage sites, the per-residue disorder propensity scores evaluated by at least one of the disorder predictors used in this study typically spike to relatively high values.

Analysis of the IDPRs in ZIKV Structural Proteins

C Protein

Structural proteins in ZIKV consist of Capsid, prM and Envelope proteins. Capsid protein contains 102 residues and forms icosahedral capsid (30 nm in diameter) of the virus (Kuno and Chang, 2007), where the genomic RNA of ZIKV is encapsulated. Disordered regions of capsid protein include its N- and C-termini, with an overall predicted percent of intrinsic disorder (PPID) of 33.3% calculated from outputs of four predictors used in our study (**Table 1** and **Figure 4A**).

These observations are consistent with the results of previous studies on the disorder predisposition of proteins in flaviviruses (Goh et al., 2016). *In vitro* studies have implicated the role of intrinsic disorder for chaperone-like activities, such as viral genome packaging (Ivanyi-Nagy et al., 2008). In the case of Dengue virus, detailed functional study of capsid protein revealed that its N-terminal disordered region is responsible for carrying out multiple interactions necessary for mature virus particle formation (Martins et al., 2012). Furthermore, disordered N-terminal region interacts with phospholipids of lipid droplets (Martins et al., 2012). Similar studies have correlated the role of capsid disorder with the diverse functions in other flaviviruses, such as YFV and WNV (Ivanyi-Nagy and Darlix, 2010). In other flaviviruses, it was shown that high virulence is correlated with the disorder levels of capsid protein (Goh et al., 2016). We assume that high abundance of disorder in capsid of ZIKV may provide an insight to uncover the mechanism of pathogenesis of this virus.

prM Protein

Next to capsid is the protein known as prM that acts as a chaperone for envelope protein. prM protein shows a central role in transition of immature virus particle to mature form, which is infectious, virulent, fusogenic, and can adhere to host cell membrane (Zhang et al., 2003). Immature viral particles of flaviviruses are noninfectious and are characterized by their “spiky” shape, possessing 60 trimeric E-prM heterodimer spikes (Zhang et al., 2003). The mature viral particle is smooth and contains 90 dimeric E:M heterodimers (Kuhn et al., 2002; Zhang et al., 2013). The low-pH environment of the trans-Golgi network is crucial for the maturation of viral particles, since it leads to the conformational changes of the surface glycoproteins needed for the cleavage of prM by the host protease furin to generate the pr peptide and mature protein M (Kuno and Chang, 2007). Subsequent removal of the pr peptide leads to the exposure of the ~12-amino acid-long fusion loop on the E protein, which, in the immature virus, is protected by the pr peptide (Yu et al., 2008). With exposed fusion loop, the virus is prepared for the low pH-mediated endosomal fusion (Yu et al., 2008). Therefore,

TABLE 1 | Some physicochemical and intrinsic disorder properties of Zika virus proteins.

Protein name	Length (M.W., kDa)	pI	PPID _{VLXT}	PPID _{VSL2}	PPID _{FIT}	PPID _{IUPred}	PPID _{mean}
C	103 (11.73)	12.0	40.8	34.0	36.9	12.6	33.3
prM	168 (19.01)	8.55	23.8	22.6	16.7	10.1	19.0
Pr	93 (10.51)	6.05	20.4	17.2	14.0	6.6	16.1
M	75 (8.51)	10.11	28.0	29.3	20.0	14.7	24.0
E	500 (54.09)	6.48	27.4	18.0	3.8	4.6	7.6
NS1	352 (40.08)	6.17	33.0	26.7	7.1	6.0	10.8
NS2A	226 (23.97)	10.34	7.5	7.5	9.7	3.1	5.7
NS2B	130 (13.77)	4.44	37.7	14.6	17.7	7.7	16.2
NS3	617 (68.41)	8.22	36.6	18.2	9.4	9.2	12.8
NS4A	127 (13.70)	5.82	40.2	12.6	23.6	6.3	16.5
NS4B	251 (26.94)	9.10	19.1	12.4	10.4	4.0	6.4
NS5	903 (103.02)	8.67	33.6	16.1	7.0	3.8	8.6

both proteins eventually generated from the prM are functionally important, where Pr peptide protects the fusion loop of the E protein, and M protein acts as a transmembrane protein in the mature viral particle (Yu et al., 2008).

ZIKV prM contains 168 amino acids. In addition to the disordered N- and C-termini there is a long disordered central region in prM located between residues 80 and 120. This region contains the furin cleavage site (see **Figure 4B** and **Figure 3**). A multitool computational disorder analysis shows that the overall disorder of prM is 19.0% (see **Table 1**). Since the proteolytic cleavage of prM generates two functionally important proteins, the pr peptide and M protein (Yu et al., 2008). The analysis of both proteins separately revealed the PPID of 16.1% and 24% for pr and M proteins respectively (see **Table 1**). Despite high level of predicted disorder, M protein is able to form stable structure, being complexed with the E protein (see **Figure 4D**). Structurally, ZIKV M protein is characterized by N-terminal soluble loop (M loop) which contain two short α -helices (residues 6–10 and 21–39) and two transmembrane α -helices (residues 40–52 and 56–71) connected by very short loop that forms the stem and the transmembrane part of this protein embedded in the lipid bilayer. (Liu et al., 2004; Sirohi et al., 2016). Curiously, although the

soluble M loop is predicted to be mostly disordered and contains little regular secondary structure in the E-M complex, it is crucial for stabilization of the E-M dimer, being intercalated into the E protein structure. Therefore, it is likely that this region of the M protein undergoes functional disorder-to-order transition.

E Protein

At the next step, we analyzed the envelope protein E (500 residues) that participates in the membrane fusion between host late endosomes and virion. Protein E heterodimerizes with M, and this complex stabilizes E protein likely due to the chaperone-like activity of M (Hamel et al., 2015). The averaged PPID of 7.6% is observed in E protein (**Table 1**), which is rather low. However, several IDPRs of different length are predicted in the ZIKV E protein (**Figure 4C**). Viral capsid consists of 180 copies each of the E glycoprotein and the M protein anchored in a lipid membrane (Sirohi et al., 2016). Structurally, E protein consists of four domains; stem transmembrane domain and three ectodomains t external surface of the viral capsid and are mostly β -structural (see **Figure 4D**).

Previous studies on other flaviviruses (e.g., Dengue virus and West Nile virus) demonstrated that glycosylation of the E protein

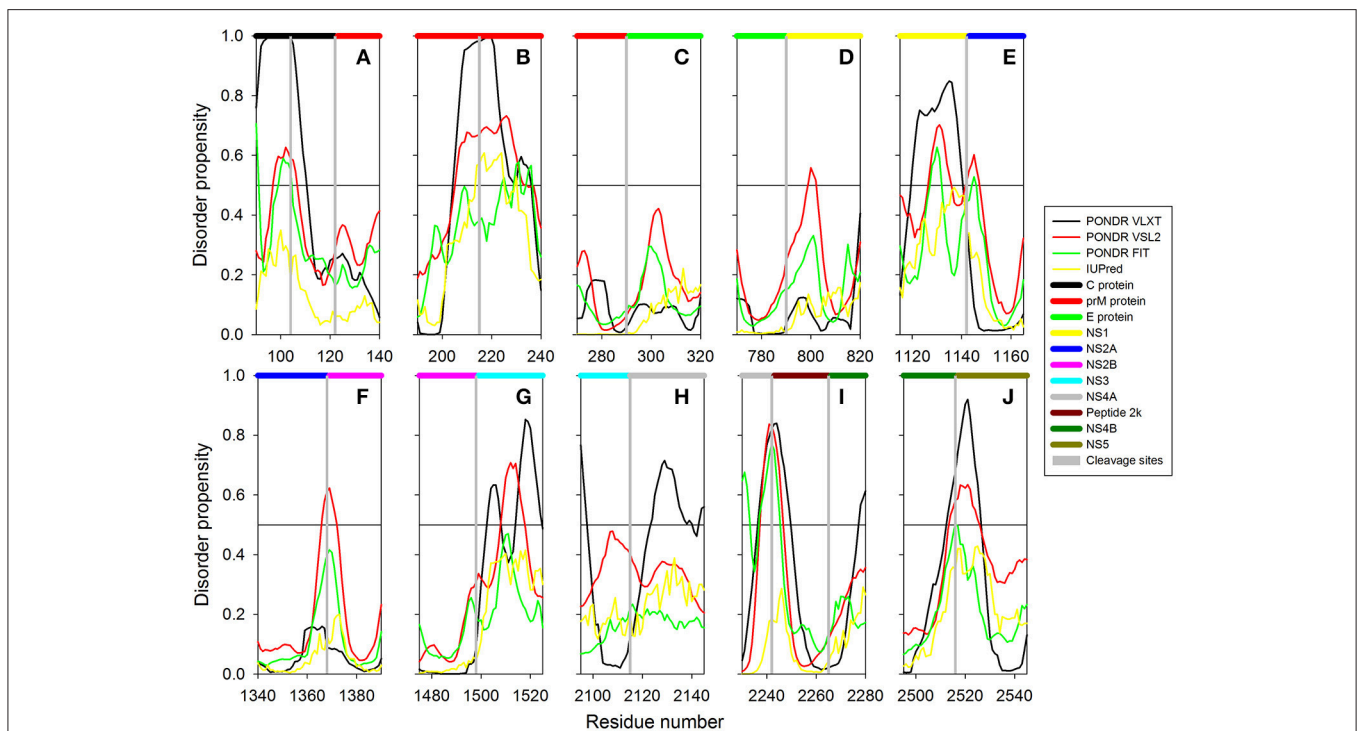


FIGURE 3 | The role of intrinsic disorder in maturation of individual proteins of Zika virus. Plots shows position of cleavage sites (gray vertical bars) in relation to disorder profiles at the junction between the individual proteins within the polyprotein. **(A)** A cleavage site between proteins C (black horizontal bar) and prM (red horizontal bar). A cleavage site at the position 104 within the pro-protein C leading to the removal of propeptide (residues 105–122) is also shown. **(B)** A cleavage site within the prM protein leading to generation of proteins Pr and M. **(C)** A cleavage site between the proteins prM (red horizontal bar) and E (green horizontal bar). **(D)** A cleavage site between the proteins E (green horizontal bar) and NS1 (yellow horizontal bar). **(E)** A cleavage site between the proteins NS1 (yellow horizontal bar) and NS2A (blue horizontal bar). **(F)** A cleavage site between the proteins NS2A (blue horizontal bar) and NS2B (pink horizontal bar). **(G)** A cleavage site between the proteins NS2B (pink horizontal bar) and NS3 (cyan horizontal bar). **(H)** A cleavage site between the proteins NS3 (pink horizontal bar) and NS4A (gray horizontal bar). **(I)** Cleavage sites between the protein NS4A (gray horizontal bar) and the peptide 2k (dark red horizontal bar) and the peptide 2k (dark red horizontal bar) and protein NS4B (dark green horizontal bar). **(J)** A cleavage site between the proteins NS4B (dark green horizontal bar) and NS5 (dark yellow horizontal bar).

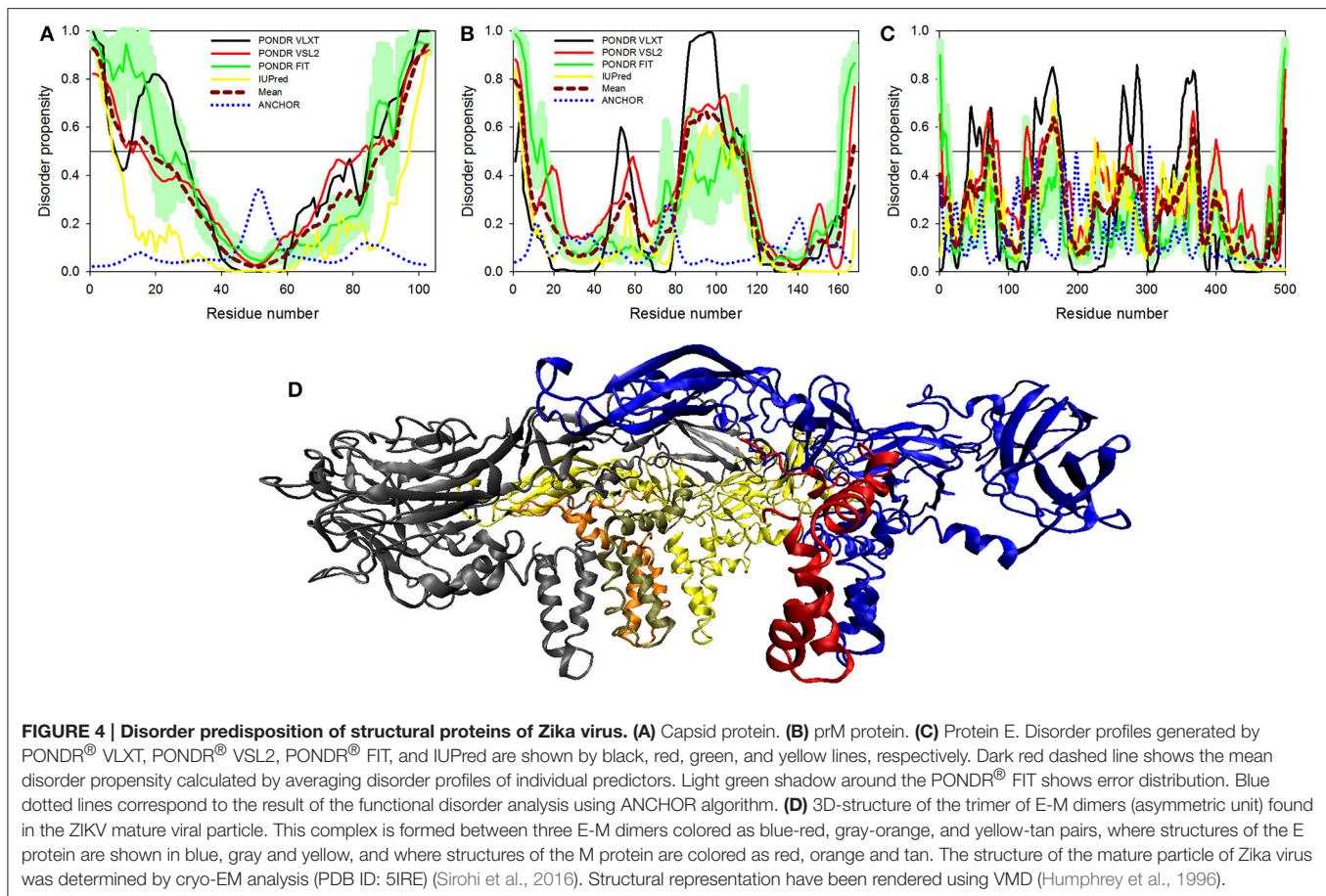


FIGURE 4 | Disorder predisposition of structural proteins of Zika virus. (A) Capsid protein. **(B)** prM protein. **(C)** Protein E. Disorder profiles generated by PONDRL[®] VLXT, PONDRL[®] VSL2, PONDRL[®] FIT, and IUPred are shown by black, red, green, and yellow lines, respectively. Dark red dashed line shows the mean disorder propensity calculated by averaging disorder profiles of individual predictors. Light green shadow around the PONDRL[®] FIT shows error distribution. Blue dotted lines correspond to the result of the functional disorder analysis using ANCHOR algorithm. **(D)** 3D-structure of the trimer of E-M dimers (asymmetric unit) found in the ZIKV mature viral particle. This complex is formed between three E-M dimers colored as blue-red, gray-orange, and yellow-tan pairs, where structures of the E protein are shown in blue, gray and yellow, and where structures of the M protein are colored as red, orange and tan. The structure of the mature particle of Zika virus was determined by cryo-EM analysis (PDB ID: 5IRE) (Sirohi et al., 2016). Structural representation have been rendered using VMD (Humphrey et al., 1996).

at specific sites provides the ability to attach to different cell types (Beasley et al., 2005; Pokidysheva et al., 2006; Miller et al., 2008). Cryo-EM structure of mature ZIKV viral particle revealed that the glycosylation site is located at the loop region nearby the fusion peptide (Sirohi et al., 2016). Besides being needed for pH-mediated endosomal fusion, the ZIKV fusion loop (residues 98–110) can act as an epitope and react with different antibodies. **Figure 4C** shows that although this region is predicted to be mostly ordered, it has some weak tendency for the presence of disorder-based binding sites as evidenced by the output of the ANCHOR algorithm. In fact, the ANCHOR-based analysis of protein E revealed the absence of disordered binding regions that function via undergoing a disorder-to-order transition upon binding to a globular protein partner. However, the fusion loop is located in close proximity to the fifth highest spike found within the ANCHOR profile of protein E (see **Figure 4C**). Furthermore, our disorder analysis revealed that the longest disorder region found in ZIKV E protein (located within the 120–180 region) contains the glycosylation site (Asn154) (Sirohi et al., 2016). High disorder propensity of the loop encompassing the glycosylation site is in line with the reported sequence variability of this region among ZIKV strains (Faye et al., 2014) and in other flaviviruses (Sirohi et al., 2016), suggesting that local structural dynamics and sequence variability could be of functional importance for this protein. In fact, sites of various enzymatically-catalyzed

posttranslational modifications in proteins are commonly located within their IDPRs (Iakoucheva et al., 2004; Pejaver et al., 2014). Therefore, finding intrinsic disorder in functionally important regions of the ZIKV E protein signifies the potential therapeutic importance of its IDPRs.

Disorder Analysis of the ZIKV Non-structural Proteins

There are seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) in ZIKV. Six of the NS proteins (NS2A to NS5) in ZIKV are known to be engaged in the formation of a replication complex on the cytoplasmic side of the endoplasmic reticulum membrane (Song et al., 2016). In addition to the viral non-structural proteins this replication complex also contains several host cofactors (Salonen et al., 2005).

NS1 Protein

The glycoprotein NS1 is considered as a key molecule in replication, immune evasion and pathogenesis of flaviviruses. NS1 is also secreted out into extracellular space as hexameric lipoprotein particles that are involved in multiple interactions with various components of immune system and host cell molecules (Suthar et al., 2013). In fact, it is believed that NS1 can be responsible for the diverse clinical consequences of infection caused by flaviviruses (Kuno et al., 1998; Cheng et al.,

2009). Recently, the crystal structure of a C-terminal fragment of ZIKV NS1 (PDB ID: 5IY3) has been solved (Song et al., 2016). The sequence of this crystallized C-terminal domain (residues 176–351) along with the sequence of the full-length NS1 protein was used in our disorder analysis. This analysis revealed that the full-length NS1 is characterized by the PPID of 10.8% (see **Figure 5A** and **Table 1**), whereas the crystallizable C-terminal fragment (PDB ID: 5IY3) shows the PID of 14.1%. Structurally, NS1 is arranged in a rod-like head to head dimers. Each dimer has 20 β -strand showing ladder-like arrangement on one surface and a complex arrangement of several loops on an opposite surface (see **Figure 5B**) (Song et al., 2016). Typically, loops between the β -strands are short, with the exception to the spaghetti loop connecting strands $\beta 4$ and $\beta 5$ (residues 218–272), which lacks regular ordered structure and is predicted to contain significant amount of disorder (see **Figure 5A**). Furthermore, a potential glycosylation site is located within the short loop between the $\beta 3$ and $\beta 4$ strands (residues 207–209), which is also predicted to have high level of disorder. Therefore, the high abundance of disorder in NS1 is correlated with the complex arrangement of loops in 3D crystal structure (Song et al., 2016) (see **Figure 5B**) and has functional significance. A phylogenetic analysis based on the amino acid sequences of NS1 proteins from 10 flaviviruses revealed some unique sequence characteristics of ZIKV NS1 that positioned it in the individual phylogenetic group (Song et al., 2016). It was emphasized that there is a very large variability in positively and negatively charged surfaces in central loop regions of the NS1 proteins from DENV, WNV, and ZIKV (Song et al., 2016). This variability in loop regions of flaviviruses may be implicated in the diversity of their pathogenicity. In Dengue and WNV, these loops of NS1 have been implicated in immune pathogenesis, whereas in ZIKV, this mechanism needs to be explored (Suthar et al., 2013).

Proteins NS2A and NS2B

Non-structural proteins from NS2 to NS5 are involved in the formation of replication complex which is located on the endoplasmic reticulum membrane. NS2A possesses several important functions, such as involvement in viral RNA synthesis, virus-induced membrane formation, and inhibition of interferon α/β response (Xie et al., 2015). **Table 1** shows that, being the most ordered of the ZIKV proteins, NS2A has the PPID of 5.4%. However, NS2B is on the other side of spectrum, being characterized by a high abundance of disordered residues with PPID of 16.2 % (**Figures 5C,D**). **Figure 5D** shows that NS2B contains a central long disordered region of 37 residues (residues 62–98). This region is responsible for interaction with the NS3 protease (Murray et al., 2008). In the case of DENV it was shown that a central hydrophilic region of NS2B binds to NS3 and is needed for the formation of an active NS2B-NS3 protease complex. NS2B also stabilizes NS3 by acting as a chaperone. In previous studies on other members of the *Flaviviridae* family (Dengue virus and WNV), the structure of NS2B in a complex with NS3 was determined (Erbel et al., 2006). Curiously, recent crystallographic analysis of the hybrid protein constituting a central of the ZIKV NS2B (residues 49–95) covalently linked via the Gly₄-Ser-Gly₄ artificial linker to the N-terminal protease

domain of NS3 (residues 1–170) revealed that the NS3-binding region of NS2B wraps around the globular NS2B domain (see **Figure 5E**; PDB ID: 5LC0) (Lei et al., 2016). This structure clearly indicates that this NS2 region is disordered in its unbound form and folds upon binding to the globular protease domain of ZIKV NS3 protein. In the perspective of folding upon binding found for many IDPs, this seems to be a similar mechanism where protein acquires active conformation and function only by binding disordered partner as also evidenced in case of the model IDP systems such as KIX and cMyb (Gianni et al., 2012).

NS3 Protein

NS3 is a bifunctional enzyme that consists of two domains, such as the N-terminal protease domain (residues 1–167) and the C-terminal helicase domain (residues 168–617), which are essential for the polyprotein processing and the viral replication, respectively (Luo et al., 2015). NS3 consists of 617 residues, 12.6% of which are predicted to promote disorder. The longest disordered region found in this protein consists of 57 residues (residues 191–247) and is located within the helicase domain (**Figure 6A**). According to a recent report, Dengue virus NS3 contains the N-terminal proline-rich disordered region that plays a critical role in replication and virus particle formation (Gebhard et al., 2016). As it was already mentioned, the ZIKV NS3 protease domain (residues 1–170) was recently crystallized as a part of the hybrid protein containing the NS3-binding region of the ZIKV NS2B covalently linked by the Gly₄-Ser-Gly₄ peptide to N-terminus of the NS3 protein. No structural information was obtained for the 31 residue-long region connecting NS2B and NS3 and containing C-terminal tail of the NS2B, the Gly₄-Ser-Gly₄ linker, and the N-terminal 14 residues of the ZIKV NS3 containing the aforementioned proline-rich region (see **Figure 5E**; PDB ID: 5LC0) (Lei et al., 2016). Remaining part of the NS3 protease domain (except to its last 3 residues) was well-resolved and represents two β -barrels with strand orders AI-BI-CI- α I-DI-EIa-EIb-FI and AII-BIIa-BIIb-CII-DII-EIIa-EIIb-FII (Lei et al., 2016). Recently, a crystal structure of the NS3 helicase domain of ZIKV has been solved (PDB ID: 5JMT) (Tian et al., 2016) (see **Figure 6B**). In contrast to the DENV NS3 helicase, ZIKV has a monomeric helicase molecule. NS3 helicase is characterized by mostly ordered tertiary structure, with loops serving as connectors between the three domains of this protein (Tian et al., 2016). It was pointed out that the cleft between Domain I and II contains the NTPase active site and includes Walker A (or motif I or P-loop, residues 193–204) and B motifs (or motif II, residues 285–292) that play an important role in recognizing NTP and Mn²⁺ or Mg²⁺ cations (Caruthers and McKay, 2002; Tian et al., 2016). **Figure 6A** shows that both of these motifs are located within IDPRs. Although these loops are characterized by the considerable intrinsic flexibility, it was pointed out that they are highly conserved among flaviviruses and play an important role in binding and catalysis of NTP (Tian et al., 2016). Curiously, NS3 is one of the two ZIKV proteins that are predicted to have some disorder-based binding sites identified by ANCHOR. In fact, **Figure 6A** shows that there are 6 such sites in ZIKV NS3 (residues 1–4, 79–81, 147–150, 261–264, 311–314, and 374–376)

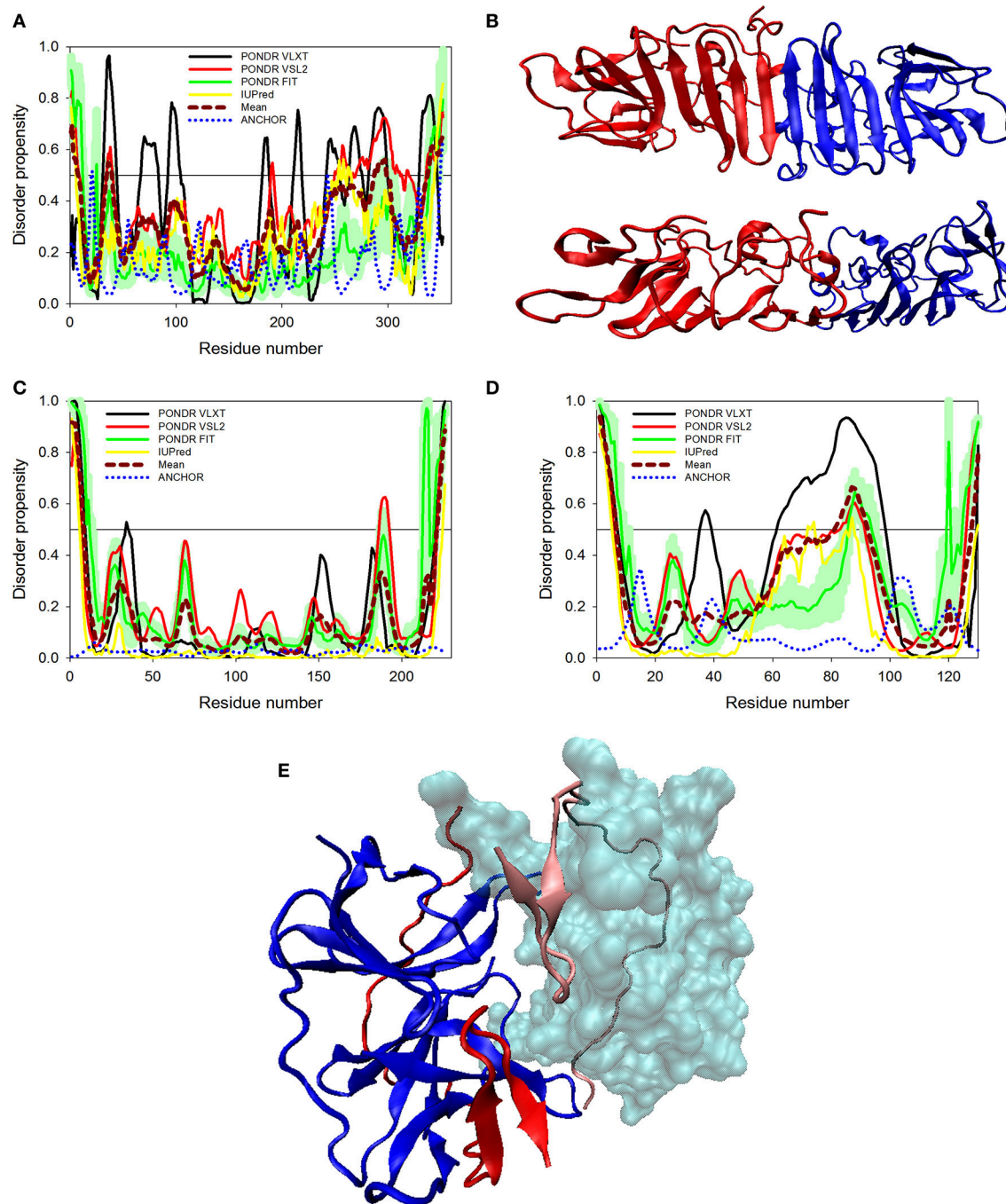


FIGURE 5 | Disorder predispositions of non-structural proteins NS1 (A), NS2A (C) and NS2B (D). Disorder profiles generated by PONDRL[®] VLXT, PONDRL[®] VSL2, PONDRL[®] FIT, and IUPred are shown by black, red, green, and yellow lines, respectively. Dark red dashed line shows the mean disorder propensity calculated by averaging disorder profiles of individual predictors. Light green shadow around the PONDRL[®] FIT shows error distribution. Blue dotted lines correspond to the result of the functional disorder analysis using ANCHOR algorithm. (B) 3D-structure of the head-to-head dimer of the NS1 protein, where the β -ladder side (top) and loop arrangement (bottom) are shown. Structure is based on the PDB ID: 5IY3 (Song et al., 2016). Structures of monomers within a dimer are shown as blue and red cartoons. (E) 3D-structure of a hybrid protein containing NS3-binding region of the NS2B protein covalently linked to the protease domain of ZIKV NS3 protein (PDB ID: 5LC0) (Lei et al., 2016). This hybrid protein is crystallized as a tight dimer. Structures of the NS3-binding region of the NS2B protein are shown as red or pink ribbons in chains (A,B) of this dimer, respectively. Structures of the protease domain of NS3 protein are shown as blue ribbon or cyan surface in chains (A,B), respectively. For chain (B), this domain is shown as a transparent surface to simplify visualization of the NS2 chain wrapped around it. Structural representation have been rendered using VMD (Humphrey et al., 1996).

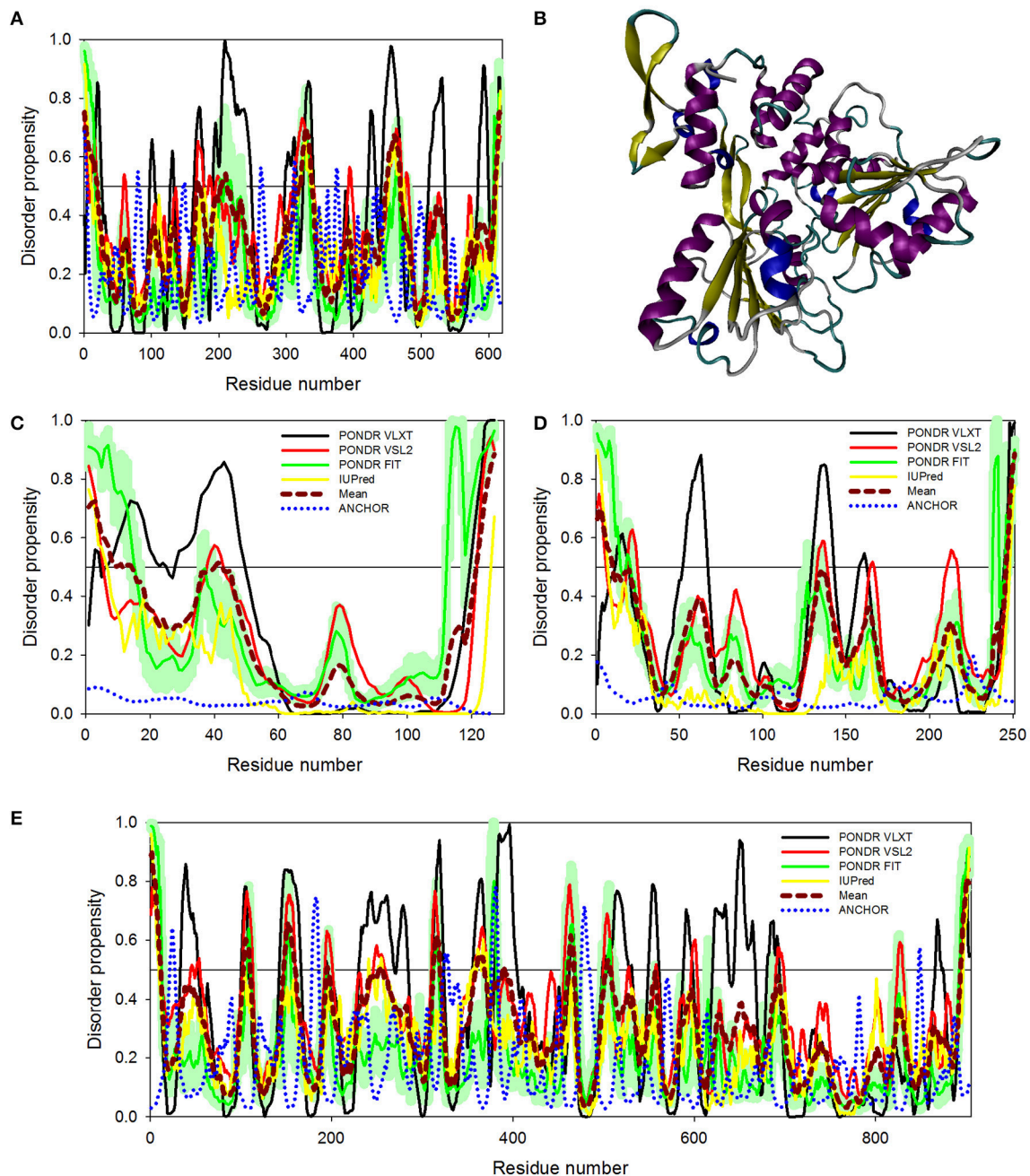


FIGURE 6 | Disorder predispositions of non-structural proteins NS3 (A), NS4A (C), NS4B (D), and NS5 (E). Disorder profiles generated by PONDRL® VLXT, PONDRL® VSL2, PONDRL® FIT, and IUPred are shown by black, red, green, and yellow lines, respectively. Dark red dashed line shows the mean disorder propensity calculated by averaging disorder profiles of individual predictors. Light green shadow around the PONDRL® FIT shows error distribution. Blue dotted lines correspond to the result of the functional disorder analysis using ANCHOR algorithm. (B) 3D-structure of the helicase domain of the ZIKV NS3 protein (PDB ID: 5JMT) (Tian et al., 2016). Structure is colored according to the secondary structure content. Structural representation have been rendered using VMD (Humphrey et al., 1996).

which although were predicted by ANCHOR but then were filtered out by the algorithm because of their small size (they are ranging in length between 3 and 4 residues and ANCHOR uses the length threshold of 6 residues). Nevertheless, our analysis shows that ZIKV NS3 has several functionally important IDPRs.

NS4A and NS4B Proteins

Although non-structural proteins NS4A and NS4B from flaviviruses have not been crystallized as of yet, their biological roles in Dengue virus have been investigated experimentally. (Zou et al., 2015) NS4A functions by introducing rearrangements in membrane of host endoplasmic reticulum. These changes lead to

the formation of virus-induced membranous vesicles. NS4A also control the ATPase activity of the NS3 helicase, whereas NS4B inhibits the interferon-induced host STAT1 phosphorylation and nuclear translocation (Kuno and Chang, 2007). **Table 1** and **Figures 6C,D** show that although NS4A is predicted to be rather disordered, having the PPID of 16.5%, the level of intrinsic disorder in the NS4B protein is noticeably lower (its PPID is of 6.4%).

NS5 Protein

The NS5 protein is the largest ZIKV protein (903 residues) and has high degree of sequence similarity with the Dengue virus NS5 protein. In DENV, crystal structure of the full-length NS5 has been solved recently (PDB ID: 4V0Q) (Zhao et al., 2015), and structures of the methyltransferase domain (PDB ID: 1L9K) (Egloff et al., 2002), and the RNA-dependent RNA polymerase (RdRp) domain (PDB ID: 2J7W) of the NS5 (Yap et al., 2007) are also available. The NS5 is the most conserved protein amongst flaviviruses, exhibiting enzymatic activities that play vital roles in virus replication. In fact, it has the N-terminal domain (residues 1–262 in DENV3) that belongs to the S-adenosyl-L-methionine (SAM)-dependent methyltransferase (MTase) superfamily (Egloff et al., 2002) and the C-terminal domain (residues 273–900) which serves as the RNA-dependent RNA polymerase (RdRp) that synthesizes the anti-genome and progeny genome (Yap et al., 2007). Since NS5 contains two functional domains with several key enzymatic activities crucial for the viral RNA replication in the host cell, this protein represents one of the major targets for the design of antiviral inhibitors. Our analysis revealed that despite being the multifunctional enzyme, ZIKV NS5 contains numerous IDPRs (in a range of 20) and is characterized by the PPID of 8.6%. Furthermore, ANCHOR analysis revealed the presence of 4 disorder-based binding sites (residues 22–27, 179–186, 324–329, and 377–383), with two more disorder-based binding sites that were filtered out by the algorithm (residues 476–484 and 846–849). Our analysis also revealed that most of the NS5 disordered regions are located in the central part of the protein, with the longest disordered

region containing 40 amino acid residues (see **Figure 6E**). These findings of NS5 are also correlating well with the presence of long disordered region in DENV and JEV NS5 proteins.

CONCLUDING REMARKS

In conclusion, our analysis revealed that all ZIKV proteins contain disordered regions. These proteins are involved in diverse mechanisms of virus survival and immune evasion in other flaviviruses. Till now, the mechanisms of the ZIKV pathogenesis have not been deciphered in detail. Therefore, our study that considers ZIKV proteins from the protein intrinsic disorder perspective may provide novel insights that can help elucidating the molecular mechanisms of virus host interaction. In general, the highly dynamic and flexible nature of the disordered proteins or proteins containing IDPRs has shown to play major roles in disease development and progression (Uversky et al., 2008; Babu et al., 2011). As disordered regions are attractive and challenging drug targets, new drug development strategies should be developed to find small molecule inhibitors that could target IDPRs and could serve as effective antivirals. Therefore, detailed biophysical analysis of ZIKV disordered proteins is required to develop effective new antiviral therapeutics.

AUTHOR CONTRIBUTIONS

RG and VU: Conception and design, analysis and interpretation of data, writing and review of the manuscript and study supervision; DK and NS: acquisition of data, analysis and interpretation of data, writing of the manuscript.

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Mutational Pressure in Zika Virus: Local ADAR-Editing Areas Associated with Pauses in Translation and Replication

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Zika virus (ZIKV) spread led to the recent medical health emergency of international concern. Understanding the variations in virus system is of utmost need. Using available complete sequences of ZIKV we estimated directions of mutational pressure along the length of consensus sequences of three lineages of the virus. Results showed that guanine usage is growing in ZIKV RNA plus strand due to adenine to guanine transitions, while adenine usage is growing due to cytosine to adenine transversions. Especially high levels of guanine have been found in two-fold degenerated sites of certain areas of RNA plus strand with high amount of secondary structure. The usage of cytosine in two-fold degenerated sites shows direct dependence on the amount of secondary structure in 52% (consensus sequence of East African ZIKV lineage)—32% (consensus sequence of epidemic strains) of the length of RNA minus strand. These facts are the evidences of ADAR-editing of both strands of ZIKV genome during pauses in replication. RNA plus strand can also be edited by ADAR during pauses in translation caused by the appearance of groups of rare codons. According to our results, RNA minus strand of epidemic ZIKV strain has lower number of points in which polymerase can be stalled (allowing ADAR-editing) compared to other strains. The data on preferable directions of mutational pressure in epidemic ZIKV strain is useful for future vaccine development and understanding the evolution of new strains.

Keywords: Zika virus, mutational pressure, pauses in replication, ADAR-editing, ribosome stalling

INTRODUCTION

Zika virus (ZIKV) is a pathogenic mosquito borne virus that became a Health emergency in February, 2016 (WHO, 2016) (<http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/>). ZIKV is a member of flaviviridae family and transmitted by Aedes vector to cause severe neurological disorders such as fetal microcephaly and Guillain-Barré syndrome (Cao-Lormeau et al., 2016; Heymann et al., 2016). ZIKV infection has been known since 1940s, when the studies on yellow fever virus (YFV) yielded the isolation of Zika strain in Uganda (Wikan and Smith, 2016). But the expansion of geographic range of ZIKV has been observed after the significant outbreak infecting over 65% of population in Yap Island, Micronesia in 2007 (Duffy et al., 2009). The rapid spread of ZIKV during the outbreak

in French Polynesia (2013), as well as the Brazilian outbreak in 2015 has increased the risk of infection worldwide. A surge in the number of fetal microcephaly cases in Brazil has gained the attention toward ZIKV infection (Butler, 2016). The evidences of ZIKV vertical transmission during pregnancy have raised alarms and posed a situation of global threat due to high epidemic and less effective control measures of infection (Coyne and Lazear, 2016). This results into the drastic increase in clinical and research framework to find effective strategies to encounter ZIKV infection (Giri et al., 2016).

ZIKV is a positive sense single stranded RNA virus with genomic size of ~10.7 kb. The genome of ZIKV encodes a single polyprotein that is cleaved into three structural and seven non-structural proteins. There are three major lineages of ZIKV: from West Africa, East Africa, and Asia. The Asian lineage has been evolved to give rise to modified strains detected in 2007 that are responsible for neurological complications (Haddow et al., 2012). The epidemiological evidences had revealed the transmission of ZIKV originated from Yap in 2007 to other Pacific islands, South, and Central America (Angeletti et al., 2016). The reference strain of ZIKV from GenBank belongs to the East African lineage (“type 1” in this study). West African lineage is referred to as “type 2” in this work, while Asian lineage (together with strains from the recent outbreak) goes under the name “epidemic strain,” or “type 3”.

Viral genomes are likely to show highest mutational frequencies and evolutionary evidences in living world. The fast mutation rate of RNA virus genomes creates different populations of viruses from a single culture termed as viral quasi-species. This kind of variation is thought to be reversible: the frequencies of different quasi-species vary during the infection process in the response to immune pressure and other factors. The pattern of mutations in viral genome is never random. Some types of nucleotide mutations always occur more frequently than other types. This situation is known under the name directional mutational pressure (Sueoka, 1993). Mutational pressure introduces irreversible changes in the nucleotide content of the whole viral population. For example, increased rates of A–G mutations will slowly make the usage of G higher in all the possible quasi-species. The causes of such unequal rates of mutations in RNA viruses may be different: error-prone polymerase, RNA editing, oxidative damage (Gros et al., 2002). One of the enzymes involved in viral RNA-editing is known as ADAR (double-stranded RNA-specific adenosine deaminase; Tomaselli et al., 2015). Expression of this enzyme is stimulated by the increase of the level of alien RNA in a cell (Tomaselli et al., 2015). However, some viruses provoke ADAR expression (including those from Flaviviridae family: Hepatitis C virus from hepacivirus genus and Bovine viral diarrhea virus from pestivirus genus), while others do not. Interestingly, just certain strains of Dengue virus promote ADAR expression (Umareddy et al., 2008). The direct consequence of RNA editing by ADAR is the increase of A–G transition rates (Tomaselli et al., 2015). So, one can suspect that genome of a given virus is edited by ADAR if the level of G in its RNA is high (Cuevas et al., 2016). High level of G in viral RNA increases the percentage of nucleotides forming doublestranded fragments. That is how ADAR-editing leads to

rearrangements of secondary structure of RNA and the inclusion of previously unavailable adenine residues into doublestranded fragments.

It is important to state that the direction of mutational pressure may not be constant throughout the whole length of the viral genome (Khrustalev et al., 2015b). The same situation (local mutational pressures) has been found in certain bacterial and eukaryotic genes (Khrustalev et al., 2014, 2015a). Finding the cause of the deviation from the general mutational pattern may be even more useful procedure than estimating the general direction of mutational pressure. Autonomic transcription is thought to be the cause of local mutational pressure in bacteria and eukaryotic organisms (Khrustalev et al., 2014, 2015a). However, there is no autonomic transcription of small coding regions in Zika virus. In this article we described a hypothesis of translation-associated mutational pressure occurring in regions of RNA-plus strand situated after the sequences on which ribosome is stalled. We also hypothesize that RNA-dependent-RNA-polymerase stalling may open up the door to RNA-editing enzymes acting on fragments of RNA situated after such “stop-signals” as G-quadruplexes.

The knowledge on the main directions of mutational pressure should be used in vaccine design studies. One of the applications of this knowledge is in the choice of a best vector for DNA vaccine. The closer the pattern of mutations in the virus to the pattern of mutations in the vector, the higher the chance that vaccine vector will cover the spectrum of variants which occur during mutagenesis of wild type virus (Khrustalev et al., 2015b). Another application is in the determination of the less mutable fragments of coding regions. Such fragments should have the lowest amount of highly mutable nucleotides, especially, in first and second codon positions (Khrustalev et al., 2015b). Our findings have emphasized the importance of local mutational pressures analysis in understanding epidemics and disease tracking of ZIKV infection.

MATERIALS AND METHODS

We used 79 complete sequences of Zika virus available in GenBank. All sequences have been aligned with MEGA 7.0 program using MUSCLE algorithm (Kumar et al., 2016). After that we deleted short sequences from 5′ and 3′-ends to let them start and end at the same position. So, actual first nucleotide in our sequences has number 218 in the reference Zika sequence (NC_012532.1), while the last nucleotide has number 10088 in that sequence. We analyzed only the coding region of the virus.

The phylogenetic analysis has shown three main clusters of Zika sequences (Tamura-Kumar method for evolutionary distances calculation, and minimum evolution method for the phylogenetic tree construction; Kumar et al., 2016). The first cluster (type) contains 13 sequences together with the reference one (East Africa lineage). The second cluster (type) contains nine sequences (West Africa lineage). The third cluster (Asian lineage) includes 55 sequences mostly from the recent outbreaks of the infection (2014–2016). There are also two sequences that occupy uncertain positions in the dendrogram (between two types of the

virus), which we did not include in the following steps of the study. All sequences are available in the Supplementary Material File “Data Sheet 1.xlsx”

For each of the three types of virus we built a consensus sequence that has been analyzed with the help of “VVTAK Sliding Window,” QGRS Mapper, and RNAFold algorithms. Consensus sequences are available as Supplementary Material File “Data Sheet 3.xlsx” Nucleotide usages in four- and two-fold degenerated sites from third codon positions have been calculated by the “VVTAK Sliding Window” algorithm. We used the length of a sliding window equal to 150 codons (450 nucleotides) and the step of a sliding window equal to 1 codon. Positions of sequences able to form G-quadruplexes have been found by the QGRS Mapper (Kikin et al., 2006). Secondary structure of a single RNA strand (stems and loops) has been predicted using the RNAFold algorithm (we used centroid prediction; Hofacker and Stadler, 2006). The length of a sliding window for secondary structure prediction was equal to 400 nucleotides; the step was equal to 200 nucleotides. Reverse complement sequences have been created with the help of MEGA 7.0 program (Kumar et al., 2016). We executed QGRS and RNAFold predictions on reverse complement sequences as well.

Codon usage has been calculated in three consensus sequences with the help of MS Excel. Average codon usage in coding regions of *Homo sapiens*, *Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, and *Aedes aegypti* has been taken from the Codon Usage Database (Nakamura, 2000). There are just 10 codons with extremely low (<1%) usages in primates: CGU; CGA; UCG; UUA; CUA; AUA; GUA; CCG; ACG; GCG. We identified positions of sequences three codons in length in which at least two rare codons are situated throughout the length of each consensus sequence. In *Aedes aegypti* the number of rare codons is higher than in primates (there are 15 rare codons: UUA; CUU; CUA; AUA; GUA; UCU; UCA; CCU; ACA; UGU; CGA; CGG; AGA; AGG; GGG). Because of this the frequency of groups of rare codons usage is 5.375 times higher in the coding region of East Africa Zika lineage if we consider *Aedes aegypti* codon usage instead of *Homo sapiens* one. For West Africa Zika lineage this ratio is 6.021; for Asian Zika lineage it is equal to 5.680. So, rare codons for the *Aedes aegypti* are distributed relatively equally through the whole coding region of the viral genome, while rare codons for primates are distributed none equally: there are some long regions free from the groups of rare codons. So, we focused on the description of the distribution of rare codons for primates along the length of Zika coding region in the current study.

To confirm the existence of correlation between guanine usage in two-fold degenerated sites (G2f3p) on the plus strand of RNA and the percent of nucleotides in “stems” of the same strand we calculated the coefficient of correlation between G2f3p and the percent of stems. We calculated G2f3p usage in windows 150 codons in length and the percent of nucleotides in stems in windows 400 nucleotides in length. Five consequent pairs of these two windows (with centers at the same nucleotides) are referred to as a “dot.” So, the coefficient of correlation between G2f3p and the percent of stems has been calculated in each dot along the length of a nucleotide sequence. To find the area with a correlation between the percent of “stems” and the usage

of guanine in two-fold degenerated sites we took into account the sequence of “dots” in which the correlation has been found interrupted by maximum three dots without such correlation. The same kind of analysis has been applied for guanine usage in four-fold degenerated sites (G4f) and the general guanine usage (G) with the secondary structure of the RNA plus strand; for cytosine usage in two-fold degenerated sites (C2f3p), in four-fold degenerated sites (C4f) and the general cytosine usage (C) with the secondary structure of the RNA minus strand.

The difference between nucleotide usage in two-fold degenerated sites in windows of 150 codons in length between consensus sequences of epidemic and reference Zika strands has been calculated with the help of MS Excel.

Directions of mutational pressure have been estimated using the “VVTAK VarInvar” algorithm in windows 400 codons in length with a step equal to 200 codons. This operation has been performed for the complete set of sequences from each of the three lineages. If the usage of a nucleotide is higher in invariable sites than in all stable sites (which stay two-fold degenerated or four-fold degenerated in all the sequences from the alignment), then the usage of such nucleotide is increasing (Sueoka, 1993). If the usage of a nucleotide is lower in invariable sites than in all stable sites, then the algorithm postulates that the usage of such nucleotide is decreasing (Sueoka, 1993). The algorithm takes into consideration just four-fold degenerated sites and two-fold degenerated sites from third codon positions. Results of such calculations are available as Supplementary Material File “Data Sheet 2.xlsx”

Variable two-fold degenerated sites have been found with the help of the “VVTAK VarInvar” algorithm in each of the three alignments of Zika sequences. Stable two-fold degenerated sites have been found with the same algorithm.

To check whether adenine in nucleotide sequences specific for ADAR2 editing mutates more frequently than in all positions, we calculated the usage of UAG, UAU, AAG, and AAU trinucleotides in three consensus sequences of Zika lineages and their reversed complements, and counted the number of mutated adenines in centers of these trinucleotides. The percent of mutated adenines in these ADAR2-specific motifs has been compared with the overall number of mutated adenines using two-tailed *t*-test.

RESULTS

Nucleotide Usage Biases along the Length of the Consensus Open Reading Frame of the East African Zika Strains

Nucleotide usage in four-fold degenerated sites represents the direction of the most frequent types of nucleotide mutations. Since all the mutations in such sites are not leading to substitutions in amino acid sequence of a corresponding protein, they are considered to be neutral for the evolution of a protein. Of course, mutations in four-fold degenerated sites may influence the fate of the protein (by the way of the influence on RNA structure, on RNA interference, on the usage of more or less frequent codons, etc.), but not its primary structure (Cristina et al., 2016). So, the influence of natural selection on four-fold

degenerated sites is lower than that on all other sites of a coding region.

In the consensus sequence of the reference Zika virus strain adenine usage prevails in four-fold degenerated sites (see **Figure 1**). However, its actual usage (A4f) is not something constant through the whole length of the sequence. In some sliding window (150 codons in length) A4f is higher than 40%, in others it is lower than 25%. In general, mutations of three other nucleotides to adenine occur at higher rates than mutations of adenine to three other nucleotides. Because of this, adenine usage is high in four-fold degenerated sites. In some fragments of ZIKV RNA, this type of mutation (from other nucleotides to adenine) is either occurring faster or fixing more easily producing the “waves” of A4f in the graph (**Figure 1**).

The usage of nucleotides in two-fold degenerated sites from third codon positions represents the preferable direction of transitions. Indeed, just a transition (and not transversions) is neutral in two-fold degenerated sites from third codon positions. Transversions occurring in such sites lead to amino acid replacements. In the reference Zika strain guanine usage in two-fold degenerated sites (G2f3p) demonstrates several high peaks through the length of the coding region (**Figure 2**). Interestingly, the usage of adenine in two-fold degenerated sites is rather low. Only in two fragments closer to the 3′-end of the RNA plus strand the usage of A2f3p suddenly increases (**Figure 2**). The usage of cytosine in two-fold degenerated sites is also high in several fragments of the coding region.

According to the distribution of adenine usage in four- and two-fold degenerated sites, transversions leading to the growth of adenine usage are responsible of the high A4f levels. In contrast, transitions from G to A are less frequent than transitions from A to G in the most of the fragments of viral RNA plus strand.

The most probable mechanism of A–G transitions is the deamination of adenine leading to the formation of inosine that usually forms hydrogen bonds with cytosine (George et al., 2014). The process may be spontaneous or enzymatic. In the latter case enzymes from adenosine-RNA-deaminase (ADAR) family bind double-stranded (and not single-stranded) fragments of RNA and perform RNA-editing. So, Zika RNA plus strand may collect A to G transitions in both two- and four-fold degenerated sites, but then the level of G in four-fold degenerated sites decreases due to transversions (since G4f is lower than A4f).

The level of adenine should grow mostly due to transversions. The probable mechanism of its growth is a frequent oxidation of guanine on the RNA minus strand resulting in C to A transversions on the RNA plus strand. Such process can occur on the RNA plus strand as well. However, RNA minus strand may serve as a collector of mutations: multiple RNA plus strands are synthesized from the same RNA minus strand. Therefore, each next RNA plus strand should have more C to A transversions. If adenine deamination occurs on the RNA minus strand, then it should increase the usage of C2f3p on the RNA plus strand.

In general, we can observe that mutational pressure has two opposite directions in the same virus. Guanine usage is growing due to A to G transitions leading to the increase of G2f3p, and not G4f. Adenine usage is growing due to C to A transversions leading to the increase of A4f, and not A2f3p. Moreover, there

are some areas in which G2f3p is much higher than in other areas.

Secondary Structure of RNA Strands from East African Zika Strains

Secondary structure of a single RNA strand forms because nucleotides make hydrogen bonds with each other. Perfect or imperfect inverted repeats usually make hairpins. Some of such hairpins play important roles in the viral lifecycle. For example, hairpins from 3′ or 5′-end of viral genome usually participate in replication (Thurner et al., 2004). Hairpins are also involved in the regulation of transcription and translation (Hofacker and Stadler, 2006). However, the most of the hairpins formed by RNA may not play any significant functional role. Obviously, they occur not just in untranslated, but in translated parts of RNA. Theoretically, “stems” of hairpins from Zika RNA plus strand should be prone to ADAR editing more than “loops” (single-stranded fragments). If a relatively long fragment of RNA has more “stems” than “loops,” then the usage of G2f3p should grow inside it. Therefore, we calculated the amount of nucleotides in “stems” (according to the RNAfold predictions) in fragments 400 nucleotides in length along the length of Zika RNA plus strand. Surprisingly, the correlation between the percent of nucleotides in “stems” and the usage of G2f3p has been found only in one long area of the RNA plus strand (from codon 600 until codon 1,730), as it is shown in **Figure 3**.

If we consider the possibility of ADAR editing of the RNA plus strand, then we should consider the possibility of ADAR editing of the RNA minus strand as well. In such case, the regions of RNA minus strand with high level of nucleotides forming “stems” should be also enriched by guanine. In other words, there should be correlation between the content of “stems” on the RNA minus strand and the usage of C2f3p on the RNA plus strand. As one can see in **Figure 4**, this correlation can be observed in two long areas: from codon 460 until codon 1,130; from codon 1,730 until codon 3,200.

According to our results, for some fragments of RNA their secondary structure (namely, the percent of nucleotides forming hydrogen bonds with each other) influences the nucleotide content in two-fold degenerated sites from third codon positions, but in other fragments there is no such dependence.

The Usage of Rare Codons along the Consensus Open Reading Frame of East African Zika Strains

Codon usage usually demonstrates intriguing patterns of non-randomness. Some biases in codon usage can be directly explained by the mutational pressure theory. For example, in Zika virus one can expect high usage of codons ending with adenine, if those codons contain four-fold degenerated sites in their third positions. However, certain codons may demonstrate extremely low usage in a giving organism or a group of closely related organisms. It is thought that the level of tRNAs for such codons is low because of the low copy number of corresponding genes, their repression or problems with corresponding tRNA-aminoacyl-synthetases (Wolin and Walter, 1988; Letzring et al.,

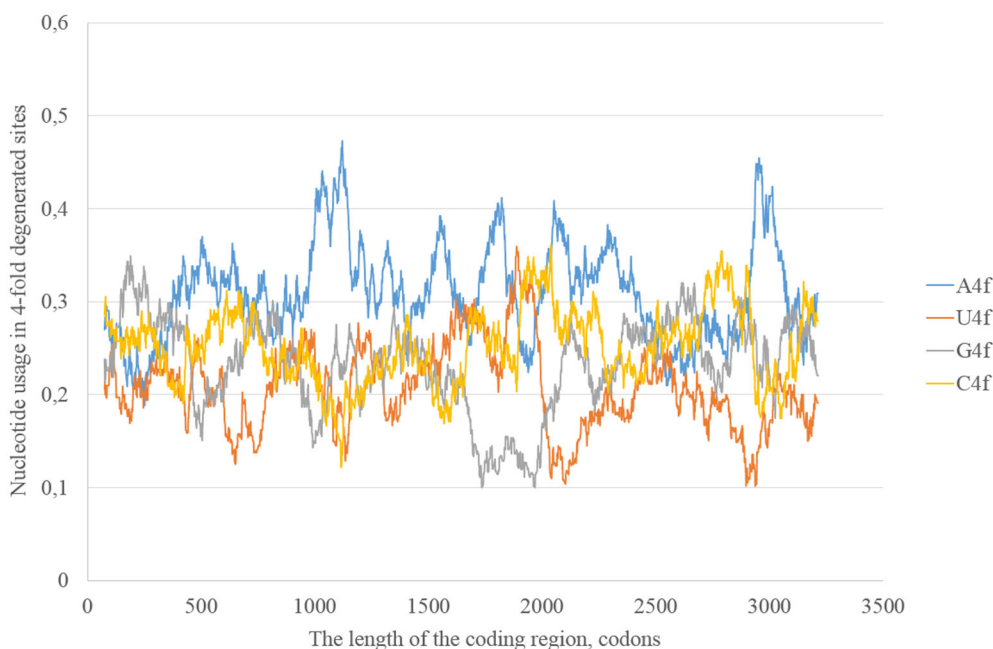


FIGURE 1 | Nucleotide usage in four-fold degenerated sites along the length of the consensus sequence for East African (type 1) ZIKV strains.

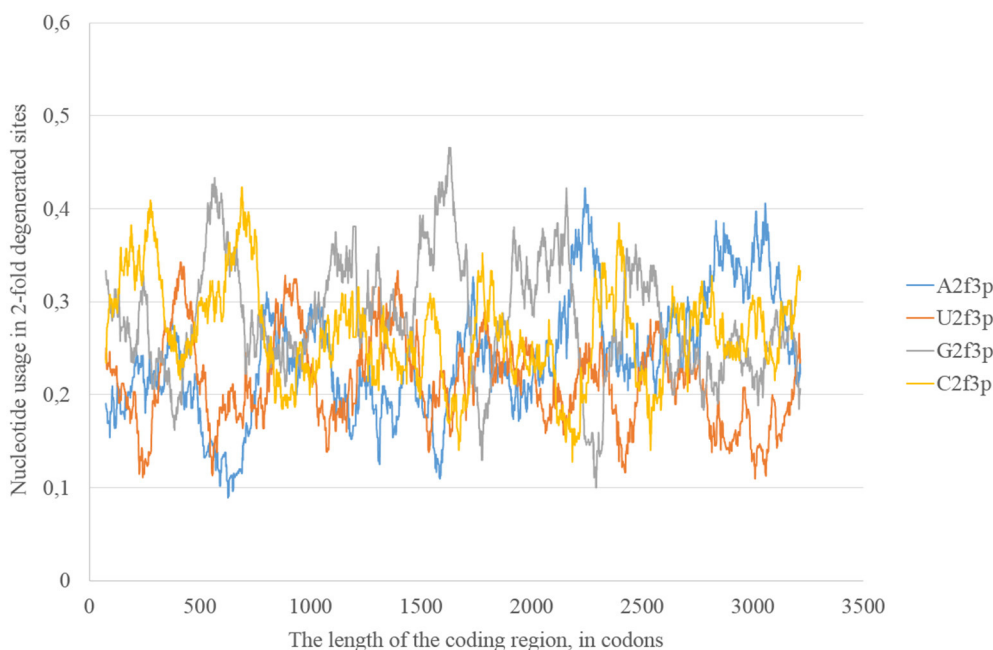


FIGURE 2 | Nucleotide usage in two-fold degenerated sites from third codon positions along the length of the consensus sequence for East African (type 1) ZIKV strains.

2010). Alternative hypothesis stays that there may be repression of the usage of CpG and ApU dinucleotides causing the decrease of usages of codons containing such combinations (Tulloch et al., 2014). Anyway, the usage of certain codons decreases. After that, the system dealing with such rare codons begins to work

slower (since there is no more strong negative selection keeping its velocity on a high level). Finally, rare codons become able to cause translation pausing (Rosenblum et al., 2013; Dana and Tuller, 2014). Ribosome stalls for a longer period of time when there are several rarely used codons situated near each other

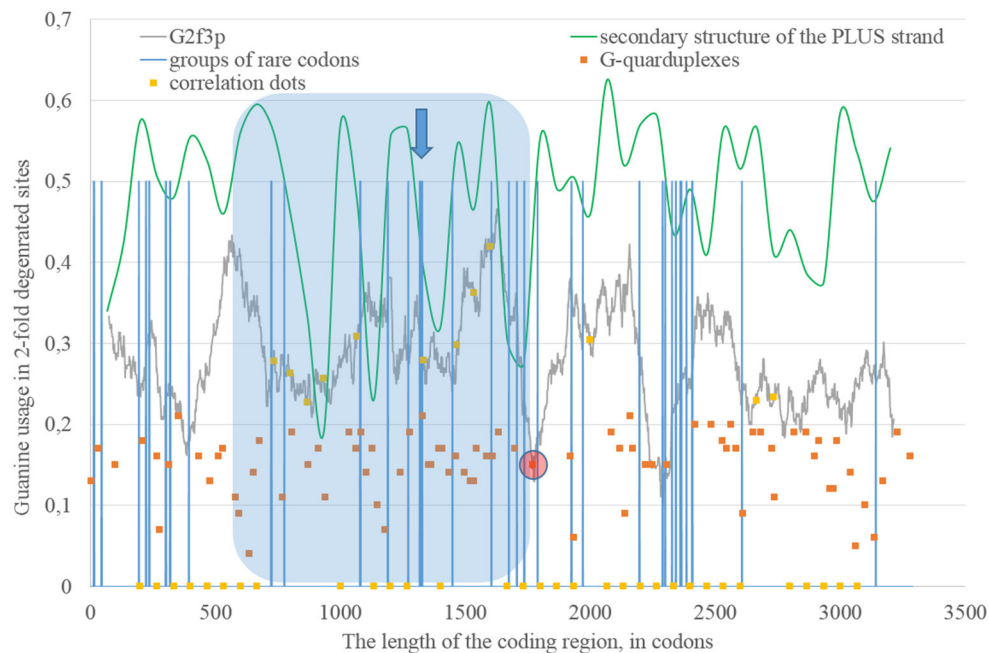


FIGURE 3 | The area of correlation (blue area) between the amount of nucleotides forming secondary structure of the RNA plus strand from ZIKV type 1 and the usage of guanine in two-fold degenerated sites from third codon positions (G2f3p). Positions of groups of rare codons and predicted G-quadruplexes are shown. If correlation dots are situated on the G2f3p line, then the coefficient of correlation between G2f3p and the percent of stems in five windows (150 codons and 400 nucleotides in length, respectively) is higher than 0.3.

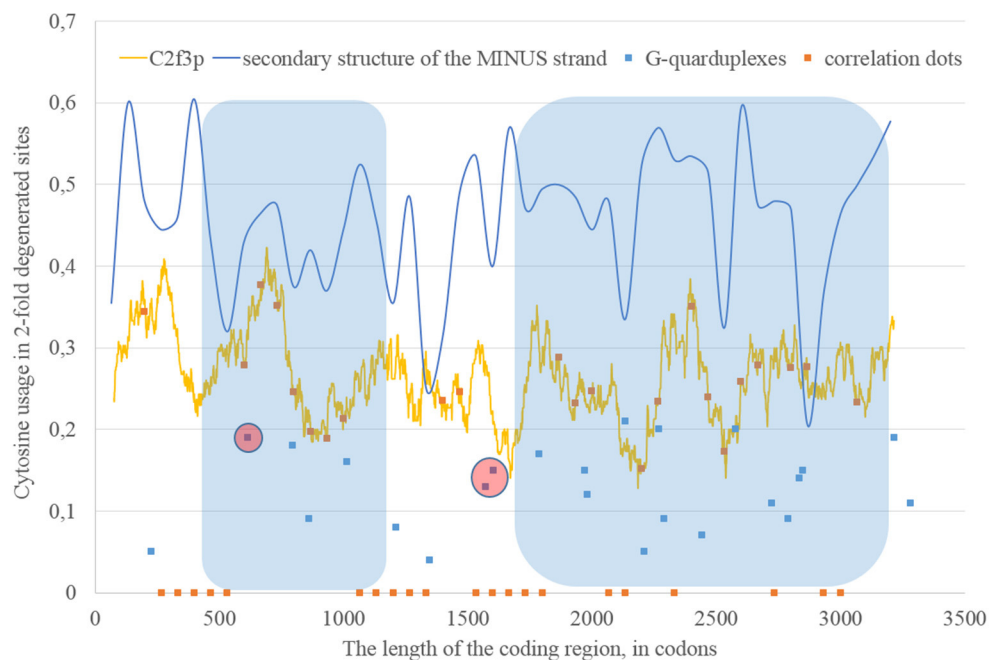


FIGURE 4 | The areas of correlation (blue areas) between the amount of nucleotides forming secondary structure of the RNA minus strand from ZIKV type 1 and the usage of cytosine in two-fold degenerated sites from third codon positions (C2f3p). Positions of predicted G-quadruplexes are shown. If correlation dots are situated on the C2f3p line, then the coefficient of correlation between C2f3p and the percent of stems in five windows (150 codons and 400 nucleotides in length, respectively) is higher than 0.3.

(Wolin and Walter, 1988). If such translational pause happens, the fragment of RNA in the 3'-direction from stalled ribosome becomes exposed to oxidative damage and RNA editing. Indeed, viral RNA plus strand should first be cleaned from proteins (Byk et al., 2016), then its secondary structure should be unwound by an enzyme with RNA-helicase activity (Jaramillo et al., 1991; Marintchev, 2013). If ribosome stalls somewhere in the middle of RNA, then the fragment in the 3'-direction will have enough time to form the secondary structure again and to be bound by ADAR. If it is so, then there should be a group of rarely used codons before the beginning of the area with the correlation between the percent of nucleotides in "stems" and G2f3p. However, in **Figure 3** one can see that there are no such groups of rarely used codons in the beginning of the area, but the strongest "stop signal" for ribosome can be found in the middle of that area.

We showed the positions of groups of rarely used codons (in human and primates) in **Figure 3**. Indeed, in the middle of the abovementioned area there is a sequence represented below: GUA/GUA/GAC/CCU/AUU/GUG/GUA/GGA/CUA/CUG/UUA. There are five rarely used codons among 12. Therefore, there is a high probability that ribosome will be stalled in this sequence during translation of Zika RNA plus strand in the beginning of the infection (after the entrance of viral genome into the cytoplasm).

Therefore, there may be a kind of a local translation-associated mutational pressure in Zika virus. Probably, the area of correlation from the **Figure 3** may be divided into two parts. The second part of this area really starts from the ribosome "stop signal." There should be another mechanism responsible of the existence of the first part of the area of correlation.

G-Quarduplexes along the Length of RNA Strands for East African Zika Strains

The fragment of viral RNA may become exposed to oxidative damage and RNA-editing enzymes in case of a pause in replication. One of the causes of RNA-dependent-RNA-polymerase stalling is the formation of G-quarduplexes by a single-stranded RNA (Cea et al., 2015). G-quarduplexes are formed by guanine-rich fragments of RNA. Positions and scores of G-quarduplexes have been predicted for Zika RNA plus strand (**Figure 3**) and RNA minus strand (**Figure 4**) with the help of the QGRS Mapper. The number of suspected regions is rather high for RNA plus strand, relatively to RNA minus strand. Interestingly, there are two regions that may form G-quarduplex near the beginning of the area with the correlation between the percent of nucleotides in "stems" on RNA plus strand and G2f3p (**Figure 3**). There are also such regions that may form G-quarduplexes before or soon after the start of both areas with the correlation between the percent of nucleotides in "stems" on RNA minus strand and C2f3p (**Figure 4**). Therefore, we cannot except the possibility of RNA-polymerase stalling before abovementioned areas. It is known that after the pause in replication viral RNA-dependent-RNA-polymerase continues the replication at significantly lower rate (Vilfan et al., 2008). Then the rate of replication may become normal again (Vilfan et al., 2008). It is also confirmed that one of the DNA-editing

enzymes (Activation induced cytosine deaminase—AID) binds single-stranded fragments of DNA from immune cells (and introduces mutations in them) only in case if the rate of transcription is low enough to let it make its job (Canugovi et al., 2009). So, a "big wave" of G2f3p from **Figure 3** might grow so high because that area is edited by ADAR during the translation (when the 3' area after the sequence of rare codons is cleaved from capsid proteins) and during replication (when the 5' area after the G-quarduplex is unwound). Several "low waves" of G2f3p from **Figure 3** are not so high, probably, because they are affected by RNA-editing enzymes only during the polymerase stalling.

We can hypothesize about local replication-associated mutational pressure in Zika virus due to ADAR-editing of its plus and minus RNA strands during the pauses in replication. The hypotheses described above has been confirmed on consensus sequences of two other Zika lineages.

The Study of the Consensus Sequence of Zika Type 2 (West African Lineage)

In the consensus sequence of Zika type 2 cluster nucleotide usage biases are similar (in terms of overall nucleotide usages) to those from Zika type 1 consensus sequence, but rather different in positions and heights of some peaks (**Figure 5**). The distribution of "stems" is also different for two types of the same virus.

Long areas with the correlation between the percentage of nucleotides forming "stems" on the RNA plus strand and the level of G2f3p are located between codons 80 and 460, 1,730, and 2,530 (**Figure 6**). There are numerous G-quarduplex sequences at the 5'-end of the first area and the group of rare codons inside it. There is the predicted G-quarduplex with the highest score situated near the 5'-end of the second area and there are two rare "CUA" codons going one after another before the beginning of that area (near its 3'-end). About 220 nucleotides downstream of the abovementioned G-quarduplex with the highest score there is another G-quarduplex sequence that was shown to be conserved in Flaviviruses (Fleming et al., 2016). It demonstrates the highest thermal stability *in vitro* among other sequences, which are able to form G-quarduplexes tested in that study (Fleming et al., 2016).

Long areas with the correlation between the percentage of nucleotides forming "stems" on the RNA minus strand and the level of C2f3p are located between codons 330 and 1,670, as well as between codons 2,670 and 3,200. There are predicted G-quarduplexes soon after the beginning of both abovementioned areas (**Figure 7**).

The Study of the Consensus Sequence of Epidemic Zika Type (Asian Lineage)

The epidemic Zika type has some distinctive properties; however, the general direction of mutations in four-fold degenerated sites is the same as in other types of Zika: A4f demonstrates the highest peaks (**Figure 8**). There are two areas with the correlation of "stems" content and G2f3p on the RNA plus strand (**Figure 9**) from codon 730 until codon 1,730, from codon 2,000 until codon 2,670. There are no groups of rare

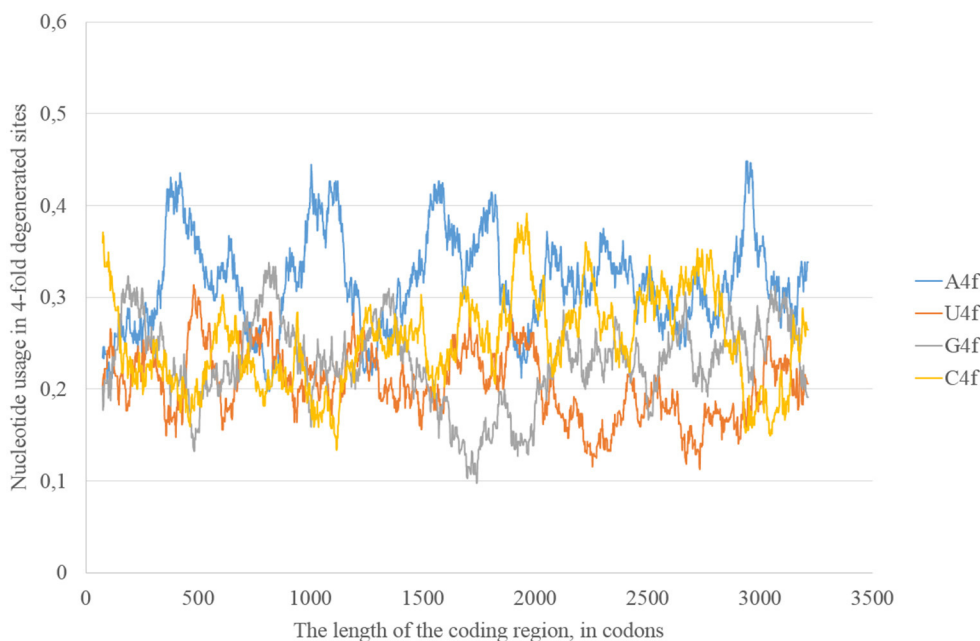


FIGURE 5 | Nucleotide usage in four-fold degenerated sites along the length of the consensus sequence for West African (type 2) ZIKV strains.

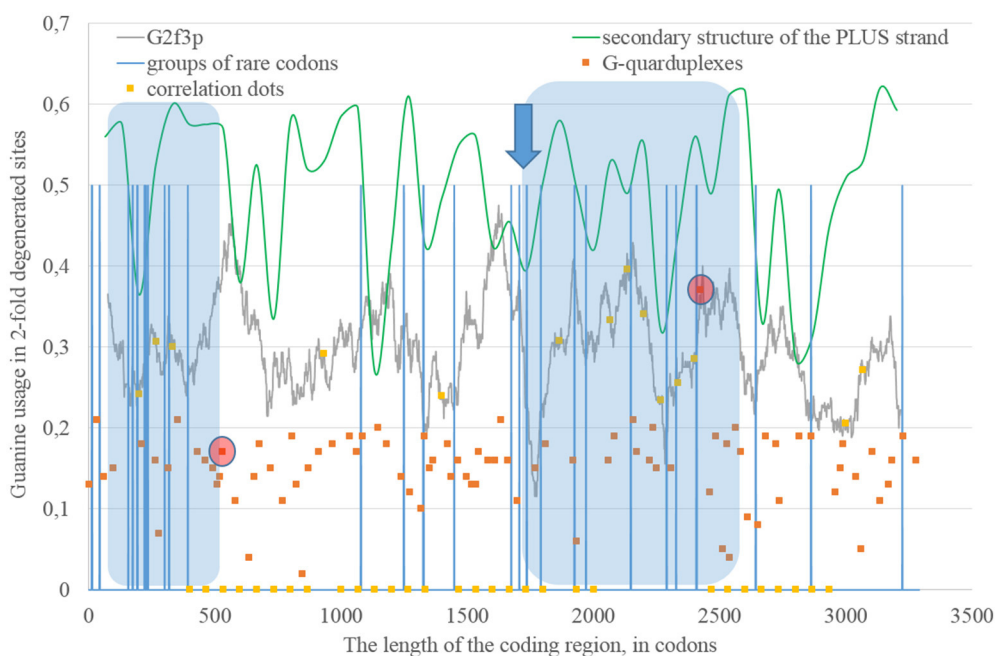


FIGURE 6 | The areas of correlation (blue areas) between the amount of nucleotides forming secondary structure of the RNA plus strand from ZIKV type 2 and the usage of guanine in two-fold degenerated sites from third codon positions (G2f3p). Positions of groups of rare codons and predicted G-quadruplexes are shown. If correlation dots are situated on the G2f3p line, then the coefficient of correlation between G2f3p and the percent of stems in five windows (150 codons and 400 nucleotides in length, respectively) is higher than 0.3.

codons before the first area, but there is a predicted G-quadruplex in its 3'-end (Figure 9). It means that the polymerase of epidemic Zika virus may be stalled on the RNA plus strand near the codon 1,730 and not the ribosome near the codon 730.

There is also the second area with the correlation between the percent of “stems” on the RNA plus strand and G2f3p (from codon 2,000 until codon 2,730) situated after the set of rare codons given below: CGU/GUC/AUA/GAU/UCC/AGG/AGA/UGC/CUA/AAG/CCG/GUC/AUA.

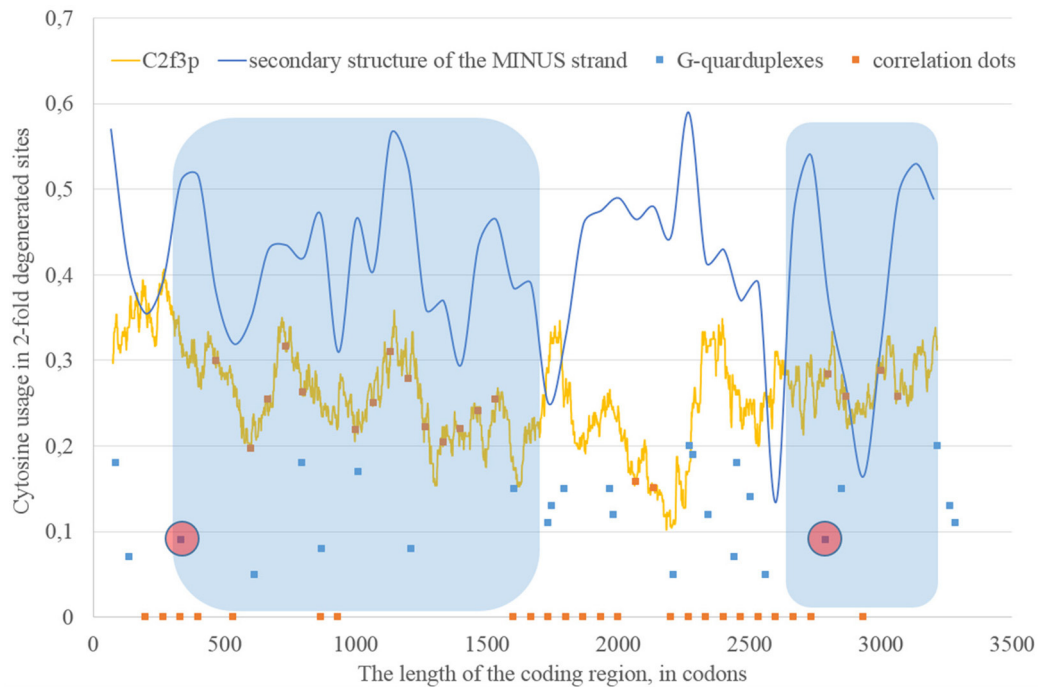


FIGURE 7 | The areas of correlation (blue areas) between the amount of nucleotides forming secondary structure of the RNA minus strand from ZIKV type 2 and the usage of cytosine in two-fold degenerated sites from third codon positions (C2f3p). Positions of predicted G-quadruplexes are shown. If correlation dots are situated on the C2f3p line, then the coefficient of correlation between C2f3p and the percent of stems in five windows (150 codons and 400 nucleotides in length, respectively) is higher than 0.3.

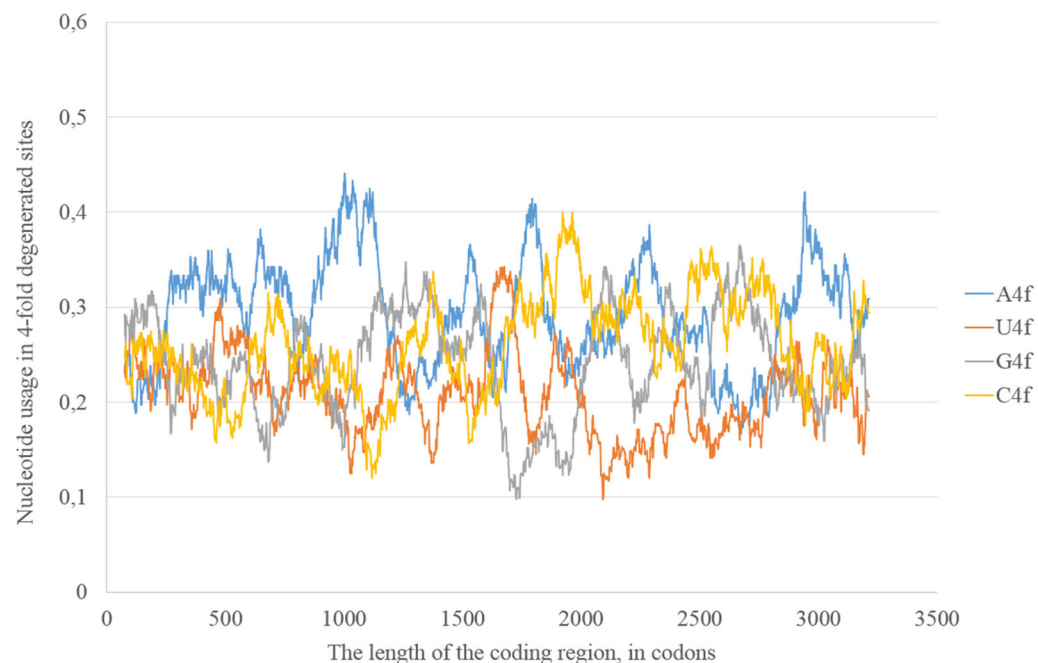


FIGURE 8 | Nucleotide usage in four-fold degenerated sites along the length of the consensus sequence for epidemic (Asian; type 3) ZIKV strains.

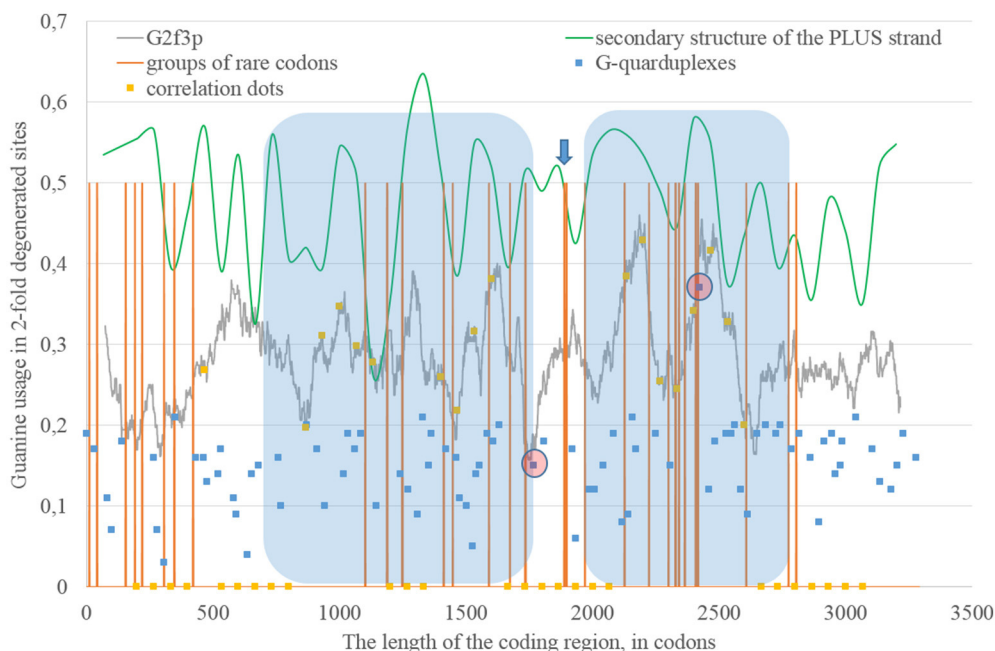


FIGURE 9 | The areas of correlation (blue areas) between the amount of nucleotides forming secondary structure of the RNA plus strand from ZIKV type 3 (epidemic) and the usage of guanine in two-fold degenerated sites from third codon positions (G2f3p). Positions of groups of rare codons and predicted G-quadruplexes are shown. If correlation dots are situated on the G2f3p line, then the coefficient of correlation between G2f3p and the percent of stems in five windows (150 codons and 400 nucleotides in length, respectively) is higher than 0.3.

The most probable G-quadruplex can be found inside the second area from **Figure 9**, as well as the nearby G-quadruplex region that has already been studied *in vitro* (Fleming et al., 2016). Interestingly, the first area from the **Figure 9** (putatively replication-associated one) demonstrates lower “waves” of G2f3p, while the second area (putatively both replication and translation-associated one) has higher “waves.” Similar situation has been described for Zika type 1 and type 2.

The number of “dots” with the correlation between the percent of “stems” on the RNA minus strand and the usage of C2f3p on the RNA plus strand is relatively low for the epidemic Zika virus. We can distinguish two areas of correlation: from codon 600 until codon 1,270 and from codon 1,460 until codon 2,530. The first area starts from the predicted G-quadruplex. There is no any G-quadruplex sequence in the beginning of the second area. Probably, there is just a residual correlation between C2f3p and the percent of nucleotides in “stems” of RNA minus strand in the first half of the first area, while the “stop signal” for polymerase is situated downstream.

The changes in positions of rare codons, in secondary structure and in positions of G-quadruplexes (together with other factors) cause mosaic distribution of changes in nucleotide usage if we compare consensus sequence of epidemic lineage with the one for East African lineage (**Figure 11**). In some areas the usage of adenine in two-fold degenerated sites is higher in the epidemic sequence relative to the reference one, in others the usage of guanine is higher (**Figure 11A**). The same situation can be seen with uracil and cytosine usages (**Figure 11B**). However, the overall tendency for all the types of Zika virus genomes is as

follows: adenine is growing in four-fold degenerated sites, while guanine is growing in two-fold degenerated sites.

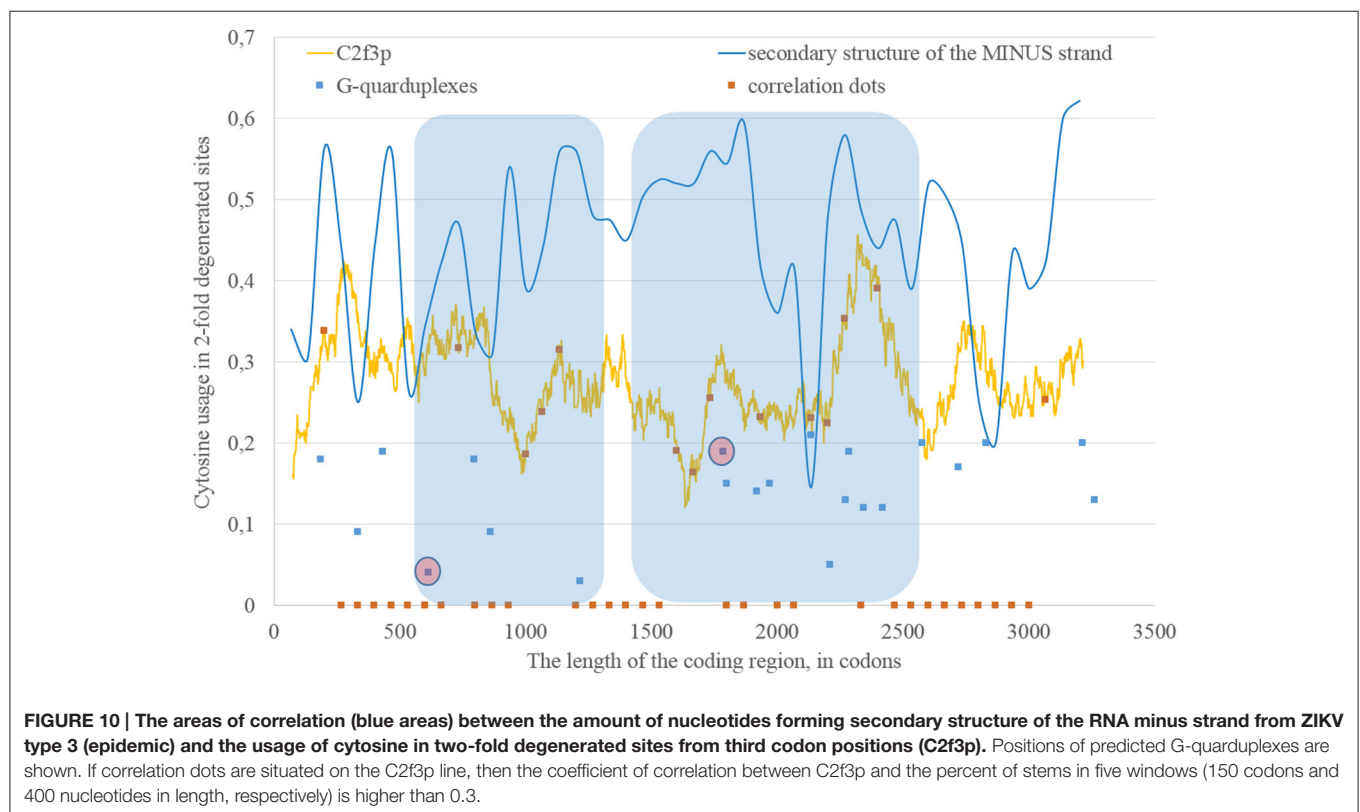
Directions of Mutational Pressure along the Length of Zika Sequences

Nucleotide usage biases are reflections of the mutational pressure, while that kind of reflection is similar to the starlight: we still see them after (sometimes) millions of years since the cause of those biases had disappeared. That is why it is necessary to estimate the direction of mutational pressure using the method taking into account nucleotide mutations in the whole alignment of sequences. The “VVTAK VarInvar” algorithm finds what types of nucleotides are less mutable than others by the way of comparison of their usages in invariable and in all stable sites. That method confirmed (**Table 1**) that throughout the most of the length of all three Zika types adenine and guanine usages are not decreasing in two-fold degenerated sites, while uracil and cytosine in two-fold degenerated sites are quite mutable. There are several deviations from the common tendency. The usage of uracil is growing only in one area from the epidemic Zika genome (from codon 200 until codon 800). Cytosine usage is growing in the region from codon 2,000 until codon 2,600 for the epidemic Zika type. Indeed, there is a peak of C2f3p usage in that region which is associated with the peak of the secondary structure content of the RNA minus strand (**Figure 10**). Interestingly, the usage of adenine is decreasing in the epidemic Zika virus in the nearby area (from codon 2,200 until codon 2,800), that can be observed in **Figure 11A**.

TABLE 1 | Directions of mutational pressure in two-fold degenerated sites from third codon positions along the length of type 1 (including reference sequence), type 2, and type 3 (including epidemic one) Zika genomes.

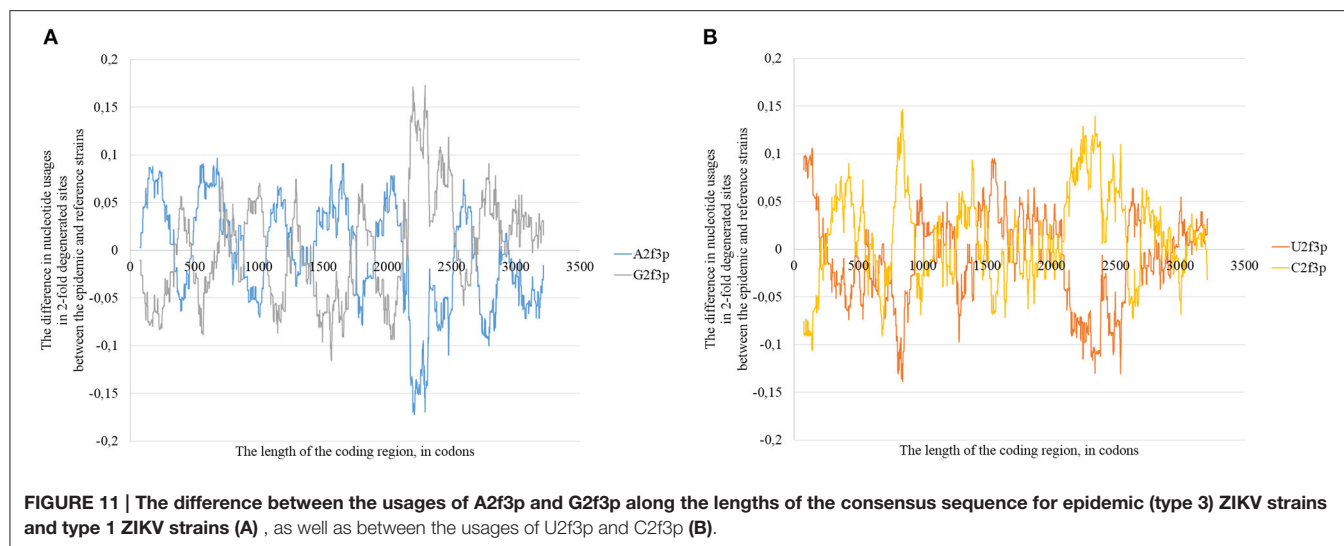
Zika type codons	A2f3p			U2f3p			G2f3p			C2f3p		
	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)
1–400	+	=	+	–	–	–	+	+	+	–	–	–
200–600	–	=	–	–	–	+	+	+	+	=	–	–
400–800	–	=	+	–	–	+	+	+	+	–	–	–
600–1000	+	=	+	–	=	=	+	=	+	–	–	–
800–1200	+	–	+	–	=	–	+	+	+	–	=	–
1000–1400	+	+	+	–	–	=	+	+	+	–	–	–
1200–1600	=	=	+	=	–	–	+	+	+	=	–	–
1400–1800	+	=	+	–	–	–	+	+	+	–	–	–
1600–2000	+	+	+	–	–	–	+	+	+	–	–	–
1800–2200	+	+	+	–	–	–	+	+	+	–	–	–
2000–2400	+	=	=	–	=	–	+	=	+	–	=	+
2200–2600	+	+	–	–	–	–	+	+	+	–	–	+
2400–2800	+	=	–	–	=	–	+	+	+	–	=	–
2600–3000	+	=	+	=	=	–	+	=	+	–	=	–
2800–3200	+	+	+	=	=	–	+	=	+	–	–	–

The increase of the usage of a given nucleotide is shown by “+” symbol, the decrease is shown by “–,” the equilibrium is shown by “=.”



The same kind of analysis for four-fold degenerated sites showed that adenine and guanine usages are growing due to the decrease of uracil and cytosine usages in all the three types of Zika (Table 2). However, there are some local deviations from this tendency. The largest deviation can be found in

epidemic Zika virus (from codon 1,200 until codon 2,200), where cytosine usage is growing. This area (there are actually two peaks of C4f in that area in Figure 8) makes an overlap with the area of cytosine usage growth in two-fold degenerated sites.



Nucleotide Content and Secondary Structure of RNA: What Is the Cause and What Is the Consequence?

It is known that the higher the usage of guanine, the higher the amount of secondary structure in the fragment of RNA. Guanine is able to form the most stable wobble base pair “G:U.” Because of this fact, computer algorithms consider “G:U” pairs when they predict the secondary structure of nucleic acids (Hofacker and Stadler, 2006). Taking this fact in consideration, one can suggest that the elevated usage of guanine is the cause, while the high percent of nucleotides in “stems” is the consequence. In this study we state that the situation is opposite: the percent of secondary structure is the cause, while the guanine usage is the consequence. To find the answer to this question for Zika virus we found the “dots” of correlation between the guanine usage and the percent of “stems” in RNA plus strand, as well as between the cytosine usage and the percent of “stems” in RNA minus strand, for three locations of guanine and cytosine residues. If the correlation is better in two-fold degenerated sites, than in four-fold degenerated sites and in all the sites occupied by guanine, then it is more likely that the secondary structure is the cause of mutational pressure. If the situation is opposite, then it is more likely that nucleotide usage is the cause of changes in secondary structure.

In our case (Table 3) for the RNA plus strand and the usage of guanine the answer is unclear. In Zika type 1 the percent of “dots” with the correlation is approximately the same if we calculate it for G2f3p, G4f and G. For Zika type 2 and type 3 the highest number of “dots” with correlation belongs to the total guanine usage. However, in Zika type 3 the difference between the percent of “dots” for G and G2f3p is lower. So, one may suggest that variations in total guanine usage should influence the amount of secondary structure on the RNA plus strand. This suggestion may be disproved by the data from Figure 12A where it is clearly seen that the variations in total usage of

G are much narrower than variations in G4f and, especially, in G2f3p.

For the secondary structure of RNA minus strand and the usage of cytosine (in RNA plus strand) the tendency is clear. The highest percent of “dots” with correlation has been found for C2f3p and not for C4f or the total usage of C. It means that the percent of “stems” in RNA minus strand is the factor determining cytosine usage mostly in two-fold degenerated sites. Interestingly, the number of “dots” with such correlation for epidemic Zika (31.82%) is lower than those for two other types of Zika (52.27 and 45.45%). There is no even a correlation between the usages of C2f3p and C4f ($R = -0.013$) along the length of consensus sequence for epidemic Zika virus (Figure 12B).

In all three types of Zika virus the rates of C to T and T to C transitions are higher than the rates of G to A and A to G transitions. This fact is approved by the comparison of the ratio between variable sites containing T or C and A or G in two-fold degenerated sites with the ratio of all two-fold degenerated sites containing T or C and A or G in third positions. As one can see in Table 4, the real ratio between variable sites is always higher than the expected ratio. This fact is the evidence that RNA minus strand of Zika virus becomes a target for ADAR-editing more frequently than RNA plus strand.

Mutations in ADAR2-Specific Trinucleotide Motifs

It is known that ADAR2 enzyme prefers to deaminate adenines situated in four trinucleotide motifs: UAG; UAU; AAG; and AAU (Lehmann and Bass, 2000). The overall percentage of adenine residues that mutated at least in a single sequence from the East Africa lineage of Zika (relative to the consensus sequence) is equal to 6.32%. The percentage of mutated adenines in UAU, AAG, and AAU trinucleotides is approximately the same as an overall percentage (the differences are insignificant). In contrast, the percentage of mutated adenines in UAG motif (29.03%) is significantly higher than the overall percentage ($P < 0.05$). In

TABLE 2 | Directions of mutational pressure in four-fold degenerated sites along the length of Zika type 1 (including reference sequence), type 2, and type 3 (including epidemic one) genomes.

Zika type codons	A4f			U4f			G4f			C4f		
	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)	1 (ref)	2	3 (epid)
1–400	+	+	+	–	–	–	+	+	+	–	–	–
200–600	+	+	+	–	=	–	+	+	+	–	–	–
400–800	+	+	+	–	=	–	+	=	+	–	–	–
600–1000	+	+	+	–	–	–	+	–	+	–	=	–
800–1200	+	+	+	–	–	–	–	+	+	–	=	–
1000–1400	+	+	=	–	–	–	=	+	+	–	=	–
1200–1600	+	+	–	–	–	–	=	+	+	–	–	+
1400–1800	+	+	+	–	–	–	+	+	–	–	–	+
1600–2000	+	+	+	–	–	–	+	+	+	–	–	+
1800–2200	+	+	=	=	–	–	=	+	+	–	–	+
2000–2400	+	+	+	–	–	–	–	=	+	–	=	–
2200–2600	+	+	+	=	–	–	=	=	+	–	=	–
2400–2800	+	+	+	–	–	–	=	+	+	–	=	–
2600–3000	+	=	+	–	=	–	=	=	+	=	=	–
2800–3200	+	+	+	–	=	–	+	=	+	–	=	+

The increase of the usage of a given nucleotide is shown by “+” symbol, the decrease is shown by “–,” the equilibrium is shown by “=.”

the RNA minus strand of the East Africa Zika lineage adenine residues in UAG motif mutated in 21.34% of cases, adenine residues in UAU motifs mutated in 23.08% of cases, while the overall percentage of mutated adenine residues is equal to 11.10%. These data give additional confirmation of the ADAR editing of Zika virus genome.

The same test has been applied to West Africa lineage of Zika. On the RNA plus strand the percentage of UAG motifs with mutated adenine is equal to 16.30%. This percentage is significantly higher than the overall percentage of mutated adenines (4.14%). However, the percentage of AAU motifs with mutated adenine residues (1.67%) is significantly lower than the overall percentage of mutated adenines. On the RNA minus strand percentages of UAG (12.82%) and UAU (13.68%) motifs with mutated adenine residues are higher than the overall percentage of mutated adenines (7.30%), but the difference is not significant.

In the RNA plus strand of epidemic Asian Zika lineage the percentage of UAG motifs with mutated adenine is significantly higher than the overall percentage of mutated adenine (18.18 vs. 6.28%, $P < 0.05$). However, the percentage of UAU motifs with mutated adenines (2.60%) is significantly lower than the overall percentage. In the RNA minus strand of epidemic Zika lineage the percentage of UAG motifs with mutated adenine residues is significantly higher than the overall percentage of mutated adenines (24.14 vs. 10.76%, $P < 0.05$).

In general, the rate of adenine mutations is elevated in one of the four ADAR2 specific trinucleotide motifs (UAG). In some cases described above the rate of adenine mutations may be lower in such motifs as UAU and AAU. In our opinion, such motifs are completely GC-poor, and so the probability that they will form secondary structure is low. First of all, to be edited by ADAR each motif must be included in the double stranded region.

TABLE 3 | The percent of “dots” with the correlation between the amount of “stems” and the nucleotide usage for consensus sequences of three types of Zika.

Zika type	Type 1 (ref)	Type 2	Type 3 (epid)
Plus strand/G	29.55	50.00	52.27
Plus strand/G4f	29.55	22.72	40.91
Plus strand/G2f3p	27.27	31.82	40.91
Minus strand/C	34.09	25.00	29.55
Minus strand/C4f	27.27	34.09	20.45
Minus strand/C2f3p	52.27	45.45	31.82

If overall GC-content of a sequence is high, trinucleotides like UAU and AAU have a higher chance to be included in a double stranded region, and so to be edited by ADAR. If a sequence has average or low GC-content, then UAG trinucleotide has the highest probability to form secondary structure and to let ADAR2 to edit adenines in its preferable motif.

DISCUSSION

The implications of Codon usage bias (CUB) can be explained by mutational pressure and translational selection (Zhao et al., 2015). CUB can be influenced by definite factors and show marked consequences in different organisms (Guo et al., 2012). Synonymous codon usage can reveal the evidences of evolution for individual genes. Compositional constraints and translational selection are thought to play a major role in accounting for nucleotide usage variations in different organisms, but for some bacterial species it has already been approved that the CUB is primarily affected by strand-specific mutational pressure (Guo

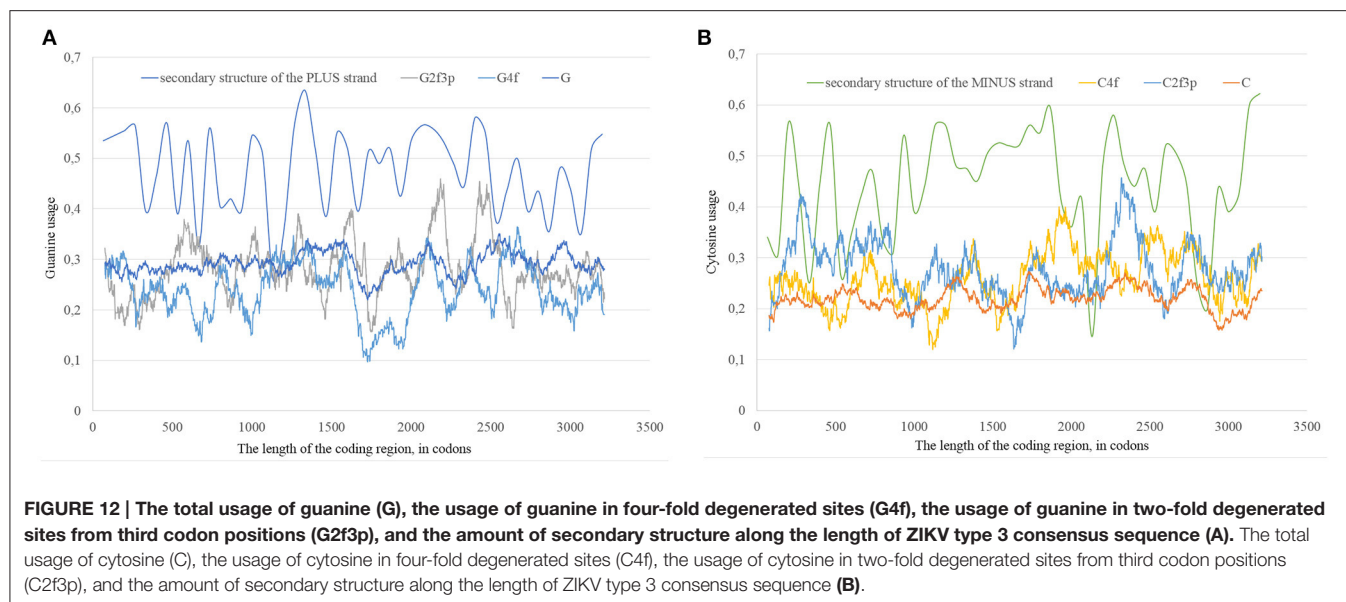


TABLE 4 | Comparison between the real ratio of TC and AG variable sites in two-fold degenerated sites and their expected ratio along the length of Zika type 1 (including reference sequence), type 2, and type 3 (including epidemic one) genomes.

Zika type	Type 1 (Ref)		Type 2		Type 3 (Epid)	
	TC/AG Real	TC/AG Expected	TC/AG Real	TC/AG Expected	TC/AG Real	TC/AG Expected
1–400	2.0	1.0	1.4	1.0	2.6	1.0
200–600	1.5	1.1	1.6	1.0	1.8	1.1
400–800	1.5	1.2	2.1	1.1	1.3	1.0
600–1000	2.6	1.1	1.3	1.1	1.5	1.0
800–1200	2.1	0.9	0.9	0.8	2.0	0.9
1000–1400	2.0	0.9	2.0	0.8	1.6	0.9
1200–1600	1.7	1.0	3.0	1.0	2.7	1.0
1400–1800	2.0	1.0	2.8	0.9	3.1	1.0
1600–2000	2.1	0.9	3.5	0.9	2.9	0.9
1800–2200	1.3	0.8	3.0	0.8	2.6	0.8
2000–2400	1.1	0.9	1.0	0.8	0.9	0.9
2200–2600	2.0	0.8	1.8	0.8	0.9	0.8
2400–2800	1.7	0.9	1.2	0.9	1.1	0.9
2600–3000	1.2	0.8	0.9	0.8	1.8	0.8
2800–3200	1.4	0.8	1.1	0.8	1.7	0.7

and Yuan, 2009). On one hand, CUB may be adapted to the levels of tRNAs in the given organism, on the other hand, levels of tRNAs may also be adapted to the existing CUB. These two processes should slowly work together in case if the accuracy and velocity of translation plays a significant role in the survival of a given species. One may expect that at a constant mutational pressure CUB of genes (especially highly expressed ones) will finally become adapted to the requirements of translation machinery (and vice versa). However, a sudden change in the mutational pressure direction may cause a disadaptation of CUB and the levels of corresponding tRNAs. Especially interesting situation exists when there are different CUBs along the length of the same coding region. According to our data, the number of

groups of rare codons for epidemic ZIKV strain is less than that for type 1 ZIKV (50 vs. 56), but it is higher than that for the type 2 ZIKV (47). So, we cannot state that the epidemic ZIKV has the most adopted CUB to the translation system of primates.

Probably, there is also a kind of positive natural selection fixing those variants of viruses which have the less number of G-quadruplex regions. Indeed, the lower the number of G-quadruplexes, the higher the velocity and accuracy of replication (Stanton et al., 2016). Currently it is thought that eukaryotic organisms developed special mechanisms to prevent formation of G-quadruplexes both in DNA (Lehmann and Bass, 2000) and RNAs (Stanton et al., 2016), while prokaryotic organisms seem to avoid the usage of DNA fragments prone to form

G-quadruplexes (Lehmann and Bass, 2000). It is hard to suggest that the eukaryotic machinery for G-quadruplexes unfolding can work well with viral RNA in the period of acute infection. So, probably, viruses follow a strategy of prokaryotic organisms and avoid the usage of sequences that cause pauses in replication. According to our results, RNA minus strand of epidemic ZIKV really has just 22 possible G-quadruplex regions, while type 1 ZIKV has 24 such regions, and type 2 ZIKV has 27. The decrease of the number of G-quadruplexes in the progenitor of epidemic ZIKV strains might result in the faster replication of daughter RNA plus strands and to the decrease of the length of regions prone to ADAR-editing.

Usually the strand of RNA that has a longer period of life in cells collects more nucleotide mutations and inherits them to the progeny (Khrustalev and Barkovsky, 2011). Many viruses are known to have more purine nucleotides (adenine and guanine) in their mRNAs than pyrimidine nucleotides (uracil and cytosine; Cristillo et al., 2001). Zika virus is not an exception from this rule (van Hemert and Berkhout, 2016). In ZIKV, RNA minus strand should play the role of the collector of mutations. The usage of uracil and cytosine is decreasing in ZIKV genome. However, there are certain areas of genome in which cytosine usage is growing. In comparison to reference ZIKV strain, the epidemic strain has remarkable difference in the distribution of cytosine usage in two-fold degenerated sites along the RNA plus strand. This corresponds to the distribution of guanine usage in minus strand of RNA. There are regions in which the amount of secondary structure dictates the usage of guanine in sites synonymous for A–G transitions. Existence of these regions can be explained for ADAR-editing of RNA minus strands during pauses in replication caused by polymerase stalling on G-quadruplex regions.

Even though there is a significant bias in nucleotide usages, the sum of guanine and cytosine usages (G+C) is close to 50% in Zika and other Flaviviruses (Jenkins et al., 2001). In general, adenine and guanine usages are growing in Zika genome. Adenine is more stable in four-fold degenerated sites, while guanine is more stable in two-fold degenerated sites from third codon positions. Certain local peaks of guanine usage in two-fold degenerated sites are linked to the areas with high amount of secondary structure on the RNA plus strand. Possible reasons behind are the ADAR-editing of RNA plus strand during pauses in translation caused by ribosome stalling on groups of rare codons and pauses in replication caused by polymerase stalling on G-quadruplex regions.

The number of regions of RNA minus strand demonstrating traces of ADAR-editing is lower for epidemic Zika sequences than for other types of the same virus. Probably, the number of points at which polymerase makes pauses on the RNA minus strand (including G-quadruplexes) is lower for epidemic type of Zika.

This feature can be the cause of more accurate and fast replication of the viral strain in recent outbreaks of the decade.

CONCLUSIONS

In this article, the mutational pressure direction of reference and epidemic ZIKV lineages has been estimated using nucleotide usage biases comparison and the analysis of nucleotide content in invariant and variable sites. The difference in nucleotide usage of three lineages has shown that genomic variations may be linked with the increased virulence of epidemic lineage by the mechanism of the decrease of number of points in which RNA-polymerase can be stalled during replication. The results have shown that guanine usage is growing in ZIKV RNA plus strand due to adenine to guanine transitions, while the nucleotide usage of adenine in four-fold degenerated sites prevails in ZIKV genome due to cytosine to adenine transversions. In certain areas of RNA plus strand of both reference strain and epidemic strain the usage of cytosine in two-fold degenerated sites shows direct dependence on the amount of secondary structure in minus strand RNA. There are also certain areas with the correlation between guanine usage in two-fold degenerated sites and the amount of secondary structure in plus strand. The presence of high amount of secondary structure and conserved G-quadruplexes in genomic RNA has resulted in increased ADAR-editing of RNA plus and RNA minus strands of ZIKV. These variations arisen due to areas associated with ADAR-editing and may resulted into the origin of epidemic strains. The lower amount of areas associated with ADAR-editing in the RNA minus strand of Asian ZIKV lineage could be the major cause behind the rise in the number of outbreaks in past decade.

AUTHOR CONTRIBUTIONS

RG and VVK: conception and design, review of the manuscript and study supervision. VVK, TAK: acquisition of data, analysis and interpretation of data. VVK, TAK, NS, and RG: writing of the manuscript.

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SUPPLEMENTARY MATERIAL

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Commentary: Zika Virus: the Latest Newcomer

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Keywords: epidemiology, surveillance, disease surveillance, Zika virus (ZIKV), outbreaks, communicable diseases, emerging, communicable disease epidemiology

A commentary on

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We commend Saiz et al. (April 19) (Saiz et al., 2016) for their contribution to the growing body of evidence linking Zika virus infection and Guillain-Barré syndrome (GBS). With 13 countries or territories now reporting increased incidence of GBS and/or laboratory confirmation of Zika virus infection in GBS cases (World Health Organization, 2016b) the authors' contribution is timely and builds the case for heightened vigilance for the detection and response to Zika virus outbreaks using syndromes associated with infection or its complications.

Saiz et al. note the surge in recently reported GBS cases, temporally and geographically linked with Zika virus infection, highlighting a near 20-fold increase in case numbers following the French Polynesia outbreak (Oehler et al., 2014). In their paper the authors neglect to discuss the potential effect enhanced surveillance, currently being implemented by many Zika-affected and at-risk countries, may have on data collected, data comparability and the situational "picture" presented.

An example of the effect of enhanced surveillance following a high profile outbreak comes directly from the Global Polio Eradication Initiative (Global Polio Eradication Initiative, 2016). In 2015 countries that were either wild polio virus endemic (Afghanistan and Pakistan) or most recently endemic (Nigeria and India), where there is clearly intensive surveillance, rates of Acute Flaccid Paralysis (AFP)—the surveillance marker indicator for polio surveillance and the most common caused by GBS (Olivé et al., 1997; World Health Organization, 2014)—detection was up to 18 times higher than expected when compared with the average performance of countries in the same World Health Organization (WHO) Regions. (Craig et al., 2016; World Health Organization, 2016a).

While it is difficult to quantify what proportions of the noted increases in GBS/AFP cases are (i) due to actual excess case occurrence associated with recognized Zika virus outbreaks or (ii) the result of frenzied case finding where previously surveillance slumbered, caution should be exercised in assuming that these increases mirror the scale of Zika virus epidemics. The greater utility may be in considering trends before surveillance is enhanced once local occurrence of illness is confirmed. Further research to quantify the effect of enhanced surveillance in relation to the ongoing Zika situation, and during other public health emergencies, is necessary.

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AC, BP, and DD conceived the ideas presented in the article. AC led the drafting of the article with significant input from BP and DD. AC led the submission process.

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Response: Commentary: Zika Virus: the Latest Newcomer

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We really appreciate Craig et al. (2016) for their commended to our contribution to the current knowledge about Zika Virus (ZIKV), and their comments to the possible relationship between Guillain-Barre syndrome (GBS) and ZIKV infection (Saiz et al., 2016). Even more, we absolutely agree with their interpretations and recommendations pointing to the necessity of further research to accurately quantify the effect of enhanced surveillance related to ZIKV circulation and associated risks. In fact, we clearly stated in the abstract of our review (Saiz et al., 2016) and throughout the text, including our final remarks, that clarifying whether there is a causal link between ZIKV infection and GBS is a currently unavoidable goal.

In this regard, recently, we have specifically addressed what it is currently known about ZIKV infections in relation with neurological manifestations (Blázquez and Saiz, in press), and stated that, beside the reported data pointing to a relationship between ZIKV infection and GBS, so far, a causal association has not been yet solidly established.

GBS is a clinical syndrome of multiple autoimmune etiologies and the most common and severe acute paralytic neuropathy (van den Berg et al., 2014; Willison et al., 2016). In many cases GBS appears to be associated with antecedent infectious diseases (Winner, 2001), and sporadic arboviral infections with dengue virus, DENV (Garg et al., 2015; Simon et al., 2016), West Nile virus, WNV (Sejvar, 2004), or chikungunya virus, CHIKV (Wielanek et al., 2007) have already been associated to GBS. In fact, Malone et al. (2016) have suggested that the concomitant regional increase in DENV and CHIKV infections may have contributed to the recently registered increase in GBS incidence.

In any case, and beyond the considerable efforts carried out by the scientific community and the national and international health authorities focused on deciphering ZIKV infection and its consequences, sufficient resources should be allocated to provide the necessary tools to evaluate the potential mechanisms of ZIKV association to GBS.

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All authors conceived the ideas presented in the article. J-CS led the drafting of the article with inputs from all other contributors. AV-C led the submission process.

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Advances in Developing Therapies to Combat Zika Virus: Current Knowledge and Future Perspectives

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Zika virus (ZIKV) remained largely quiescent for nearly six decades after its first appearance in 1947. ZIKV reappeared after 2007, resulting in a declaration of an international “public health emergency” in 2016 by the World Health Organization (WHO). Until this time, ZIKV was considered to induce only mild illness, but it has now been established as the cause of severe clinical manifestations, including fetal anomalies, neurological problems, and autoimmune disorders. Infection during pregnancy can cause congenital brain abnormalities, including microcephaly and neurological degeneration, and in other cases, Guillain-Barré syndrome, making infections with ZIKV a substantial public health concern. Genomic and molecular investigations are underway to investigate ZIKV pathology and its recent enhanced pathogenicity, as well as to design safe and potent vaccines, drugs, and therapeutics. This review describes progress in the design and development of various anti-ZIKV therapeutics, including drugs targeting virus entry into cells and the helicase protein, nucleosides, inhibitors of NS3 protein, small molecules, methyltransferase inhibitors, interferons, repurposed drugs, drugs designed with the aid of computers, neutralizing antibodies, convalescent serum, antibodies that limit antibody-dependent enhancement, and herbal medicines. Additionally, covalent inhibitors of viral protein expression and anti-Toll-like receptor molecules are discussed. To counter ZIKV-associated disease, we need to make rapid progress in developing novel therapies that work effectually to inhibit ZIKV.

Keywords: Zika virus, drugs, therapies, microcephaly, Guillain-Barré Syndrome

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne virus belonging to the Spondweni serocomplex in the genus *Flavivirus* of the family *Flaviviridae* that has become a new threat following the Ebola virus epidemic (Singh et al., 2016). The expanding ZIKV epidemic was declared an emergency by the World Health Organization on February 1, 2016 (Fajardo et al., 2016; WHO, 2016).

ZIKV is a single-stranded RNA virus that encodes a single polyprotein that is cleaved to form mature proteins, i.e., the capsid, envelope (E), and precursor of membrane and non-structural proteins. Other flaviviruses such as dengue virus (DENV), yellow fever virus (YFV), and West Nile virus (WNV) are closely related to ZIKV. In the last six decades since its discovery, ZIKV has been considered a mild human pathogen, but recently it has emerged as threat to global health, showing increased virulence, rapid spread, and an association with microcephaly and grave neurological complications like Guillain-Barré syndrome (GBS) (Cao-Lormeau et al., 2016; Carteaux et al., 2016; Mlakar et al., 2016; Sarno et al., 2016).

Zika virus has a wide tissue tropism in an experimental rhesus macaque model, infecting the hemolymphatic system, lymph nodes, spleen, cardiopulmonary, gastrointestinal, integument, and genitourinary tissues, along with the adrenal gland, spinal cord, and cerebrospinal fluid (Coffey et al., 2017). Additionally, it has been reported in muscles, kidneys, bladders, and in excreted urine (Gourinat et al., 2015). In males, ZIKV can infect testes (Govero et al., 2016), prostate and seminal vesicles, explaining the long-term persistence of viremia in semen, even after virus is no longer detectable in blood. In the female reproductive system, virus can be maintained in the vagina, uterus (Hirsch et al., 2017), vaginal epithelium (mice), and in uterine fibroblasts (Miner and Diamond, 2017). Miner and Diamond (2017) demonstrated the wide tissue tropism of the virus in Hofbauer cells, trophoblasts, and endothelial cells from the placenta. In addition, ZIKV was found to infect the cornea, neurosensory retina, optic nerve, aqueous humor, and tears. ZIKV infection in eyes results in uveitis (Furtado et al., 2016), and the persistence of the virus in cerebrospinal fluid and lymph nodes appears to enhance activity of rapamycin (mTOR), proinflammatory, and anti-apoptotic signaling pathways and reduce extracellular matrix signaling (Aid et al., 2017).

Zika virus adapts to human hosts by altering NS1 codon usage to facilitate viral replication and to increase viral titers (de Melo Freire et al., 2015). Furthermore, ZIKV placental transfer and its ability to infect neuronal tissue of growing fetuses is evident (Martines et al., 2016; Mlakar et al., 2016).

The complications of ZIKV infection are intensified by the unavailability of effective prophylactics, vaccines, or therapeutics. The spread of ZIKV, which, earlier, was limited to small geographical areas, has been facilitated by globalization, unplanned urbanization, poor sanitation, inadequate health services, and the emergence of insecticide resistance in mosquito vectors. Mosquitoes, mainly *Aedes aegypti* and *Ae. albopictus*, play a primary role in ZIKV transmission (Musso and Gubler, 2016). In addition, sexual transmission; male-to-female, female-to-male, and male-to-male transmissions have been reported by Hamer et al. (2017). A mathematical modeling study conducted by Gao et al. (2016) indicated that sexual activity contributed to 3.044% of transmission. During the typical incubation period of 2–7 days, despite the relatively low viral loads in people, infected human patients serve as a source of ZIKV (Foy et al., 2011). After viremia declines, convalescence begins, during which time a person is no longer infectious to a mosquito; however, they remain infective to other human hosts, with a low infection rate.

The convalescent stage ends with establishment of long lasting immunity (Gao et al., 2016).

Zika virus vaccines in development include inactivated virus, nucleic acid-based vaccines (DNA or RNA), live vector vaccines, subunit vaccines, virus-like particles, and recombinant ZIKV. Because of its devastating effects, effective therapeutic agents and a vaccine are urgently needed. Presently, there are several drugs reported to be useful in treating ZIKV, a few of which are repurposed drugs. Efforts to develop effective drugs have increased worldwide, and a few compounds are in phase I trials (Alam et al., 2017; Ali et al., 2017). The present review discusses recent advances in and prospects for the design and development of various anti-viral drugs and therapeutics for ZIKV infection, including the identification of novel drug targets. The updated information compiled here will contribute to the design and development of additional effective drugs and pharmaceuticals to curtail the ill effects of ZIKV.

ADVANCES IN THE DESIGN AND DEVELOPMENT OF ANTI-ZIKV DRUGS AND THERAPIES

Specific anti-viral drugs are not yet available to combat ZIKV. Acetaminophen is used to control fever and pain, anti-histamines are used for pruritic rashes, and fluids are administered to prevent dehydration in ZIKV-infected patients. However, certain drugs such as acetylsalicylic acid (aspirin) and non-steroidal anti-inflammatory drugs (NSAIDs) are contraindicated because they increase the risk of internal bleeding, and other flaviviral infections, including DENV and chikungunya virus, can cause hemorrhage (Mukherjee and Era, 2016; Musso and Gubler, 2016). ZIKV actively replicates in and causes death of neurons. Research for developing anti-viral drugs for Zika is going on fast and compounds like Sofosbuvir, 7-DMA, BCX4450, and NITD008 are currently entering a phase I trial (Ali et al., 2017).

Recently, several drugs and therapeutic candidates have been explored to determine the most effective treatment regimens to combat ZIKV and its severe clinical complications, which are being described in the following sections.

Interferons as Anti-virals

Activation of the innate immune system by viruses leads to the release of interferons (IFNs), which are responsible for the elimination of viruses and for immune regulation. In an *in vitro* cell culture system developed for ZIKV cultivation, IFN- α , IFN- β , and IFN- γ have been shown to inhibit viral replication (Contreras and Arumugaswami, 2016). Type I interferons have shown dose-dependent inhibition of ZIKV replication in a cell culture study that used quantitative RT-PCR (Goebel et al., 2016). The inverse has been documented by Bowen et al. (2017); they demonstrated ZIKV's ability to evade in the presence of type I interferon responses by degrading STAT2 signaling molecules. Trophoblastic cells secrete IFN- λ 1, which exhibits anti-viral activities against single-stranded RNA viruses. In an *in vitro* model, conditioned medium obtained from PHT cells

has been found to inhibit ZIKV growth in trophoblastic and non-trophoblastic cells by stimulating the secretion of IFN- λ 1 (Bayer et al., 2016).

Inhibition of Virus Entry into Cells

Inhibition of viral entry into a cell can serve as the first line of defense against ZIKV infection. ZIKV first binds to cell receptors, including AXL (Nowakowski et al., 2016), DC-SIGN, Tyro3, TIM, and TAM (Hamel et al., 2015), and then enters cells by clathrin-dependent endocytosis. ZIKV entry is severely hampered in human microglial cell line (CHME3) by silencing the clathrin heavy chain (a component essential for clathrin-coated vesicle formation) and dynamin-2 (a GTPase, required to pinch off endocytic vesicle from the plasma membrane). TIM receptors mediate viral entry after binding with viral phosphatidylserine and phosphatidylethanolamine (Jemielity et al., 2013). TIM1-mediated entry of DENV-2, WNV, and EBOV is inhibited by duramycin-biotin, which has less profound hemolytic effects and does not exhibit cellular cytotoxicity (Richard et al., 2015). The same TIM1 receptors are also involved in ZIKV entry; therefore, these drugs can be evaluated for the prevention of ZIKV entry. Peptide (GQASNGVFIHWGKFDSFGIAV) derived from the Japanese encephalitis virus (JEV) E protein stem is able to prevent ZIKV infection with IC₅₀ even at the nanomolar scale (3.93 nM). It also decreases the viral load and prevents histopathological damages in brain and testes in AG6 mouse, and attenuates the inflammatory response (Chen et al., 2017).

Modes of entry of ZIKV and various drugs inhibiting viral entry and replication have been depicted in **Figure 1**.

Blocking of Receptor Binding

After screening, more than 2000 molecules for their ability to inhibit ZIKV replication, nanchangmycin, a polyether obtained from *Streptomyces nanchangensis* that possesses insecticidal and anti-bacterial activity has been shown to block ZIKV replication in U2OS cells in *in vitro*. It is considered to act by targeting AXL receptors and blocking clathrin-mediated endocytosis. However, the exact mechanism of action of nanchangmycin is unknown (Rausch et al., 2017). Two small drug-like molecules, ZINC33683341 and ZINC49605556, both identified through homology modeling *in silico*, have been reported to inhibit ZIKV E protein by binding the viral receptors. Antiviral activities of ZINC33683341 have been confirmed in *in vitro* test. Thus, such viral inhibitors may be candidate molecules for ZIKV drugs after further research and clinical validation (Fernando et al., 2016).

Inhibition of Endosomal Fusion

Fusion of the endosome to lysosome is a critical step in releasing virus from endosomes. Obatoclax is a potential anti-neoplastic and pro-apoptotic synthetic small molecule Bcl-2 inhibitor. Its mesylate salt is reported to reduce the acidity of endolysosomal vesicles in *in vitro* model. Bcl-2 antagonists are effective only against viruses that require a low pH for fusion and entry, such as ZIKV, WNV, YFV, and others. Despite this limitation, Obatoclax works as a broad-spectrum anti-viral agent (Varghese et al., 2016). However, in clinical phase I and II trials while

treating hematological and myeloid malignancies, Obatoclax did not produce satisfactory results, possibly due to inadequate inhibition of Bcl-2 family proteins. Chloroquine, which is an anti-malarial drug, raises endolysosomal pH and inhibits ZIKV infection in human brain microvascular endothelial cells, human neural stem cells, and mouse neurospheres (Delvecchio et al., 2016). Similarly, SaliPhe, a molecule under pre-clinical study and vATPase inhibitor, was tested as an inhibitor of endocytosis to obstruct ZIKV infection (Adcock et al., 2017). Griffithsin, a lectin isolated from the red alga *Griffithsia* sp., is a potent flaviviral entry inhibitor. It can cross-link high-mannose oligosaccharides present on the viral E glycoproteins and has shown wide anti-viral activity against HIV (Alexandre et al., 2011), HPV (Levendosky et al., 2015), HSV (Nixon et al., 2013), HCV (Takebe et al., 2013), and SARS (O'Keefe et al., 2010). Squalamine, a FDA approved cationic chemical, which act by disturbing electrostatic interactions between the virus and host membranes during fusion and budding (Zasloff et al., 2011), has been found well tolerated as component of eye drop in clinical studies conducted on human participants. Therefore, such potent drugs can be used as an anti-viral agent against ZIKV too.

Inhibition of Virus Replication

The single-stranded RNA genome encodes a polyprotein, which is proteolytically cleaved into three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The NS5 protein, an RNA-dependent RNA polymerase, plays an important role in viral RNA synthesis and inhibits IFN signaling by binding to STAT2 (Grant et al., 2016). ZIKV NS3 protein exhibits helicase activity that is essential for viral replication. The helicase domain of NS3 is activated by GTP γ S (triphosphate), which facilitates the unwinding and translocation of RNA at the time of replication. The ZIKV helicase, along with NS5, is an attractive target for ZIKV drug development. Small membrane-associated interferon-inducible transmembrane proteins (IFITMs) are intrinsic immune system defenses that are able to inhibit replication of several pathogenic viruses. Both IFITM1 and IFITM3 have been reported to inhibit early stages of infection and replication of ZIKV in HeLa cells with the predominant role played by IFITM3 (Savidis et al., 2016). Cavinafungin, an alanine-containing lipopeptide of fungal origin, has recently been found to inhibit ZIKV polyprotein processing and cleavage of host protein signal peptides through inhibition of host endoplasmic reticulum signal peptidase in *in vitro* model (Estoppey et al., 2017). Synthetic 25-hydroxycholesterol has been shown to inhibit ZIKV entry into the host in an *in vivo* assay using mouse and rhesus macaque models (Li et al., 2017). An *in vitro* study conducted in Vero cells using compounds such as ribavirin, CMX001, T-705, and T-1105 showed that T-705 (favipiravir) and T-1105 were able to reduce cell death caused by ZIKV (Cai et al., 2017). Thus, these compounds that inhibit ZIKV replication in cell culture need to be explored further so that they can be used safely against ZIKV.

Numerous drugs involved in inhibition of virus replication have been portrayed in **Figure 2**.

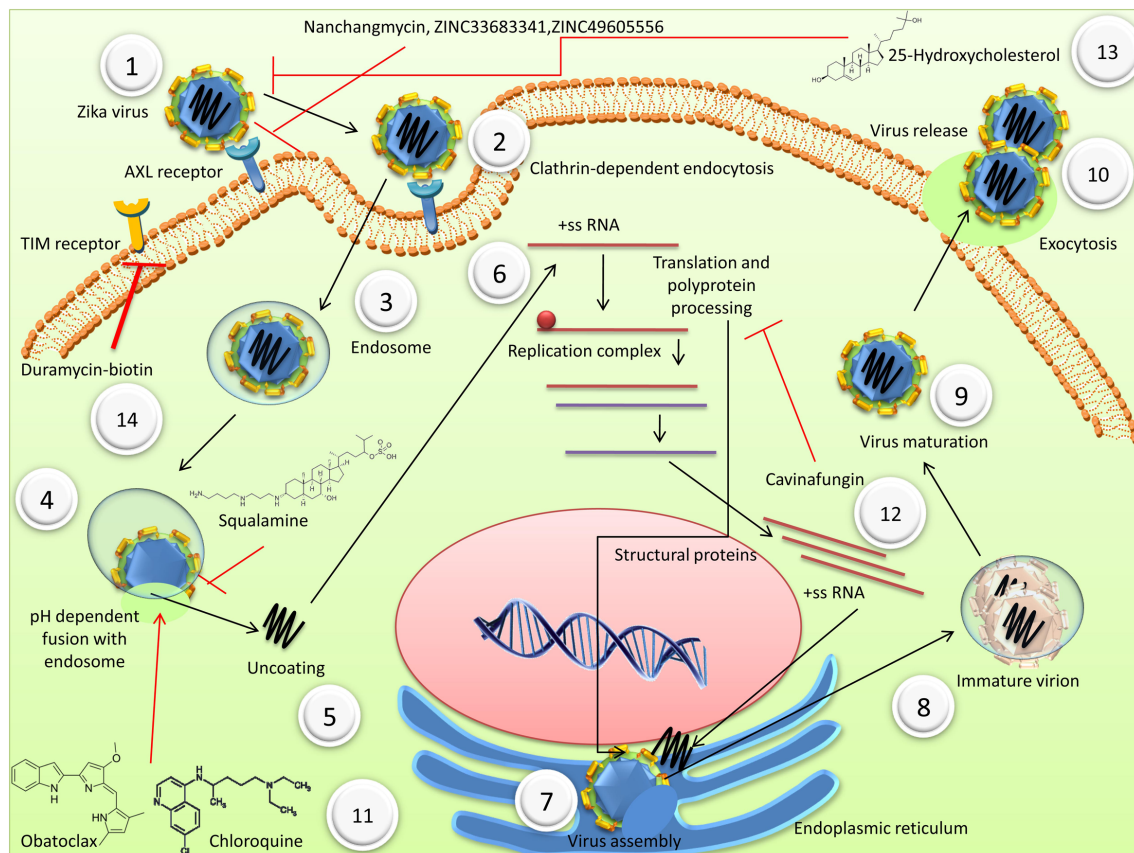


FIGURE 1 | Mode of entry of Zika virus (ZIKV) and various drugs inhibiting viral entry and replication (1) ZIKV binds to cell receptors including AXL, DC-SIGN, Tyro3, TIM, and TAM. (2) Clathrin-dependent endocytosis. (3) Endosome mediated transport of ZIKV. (4) Fusion of virus membrane with host endosomal membrane, which depends on the pH. (5) Uncoating (6) The positive-sense genomic ssRNA is translated into a polyprotein, which is cleaved into all structural and non-structural proteins. Replication occurs at the surface of endoplasmic reticulum in cytoplasmic viral factories. A dsRNA genome is synthesized from the genomic ssRNA(+) (7) Virus assembly takes place at the endoplasmic reticulum. (8) At the endoplasmic reticulum, virions bud and are transported to the golgi apparatus. (9) In the golgi, prM protein is cleaved and maturation of the virion takes places. (10) Virions are released by exocytosis. (11) Obatoclax and chloroquine inhibit the acidic environment of endolysosomal vesicles. Squalamine, a cationic chemical, disturbs the electrostatic interaction between virus and host membranes during fusion and budding. (12) Cavinafungin, an alanine-containing lipopeptide of fungal origin, inhibits ZIKV polyprotein processing and also the cleavage of signal peptide of host proteins. (13) Nanchangmycin, a polyether obtained from *Streptomyces nanchangensis*; small drug-like molecules, ZINC33683341 and ZINC49605556 block the receptor thus inhibiting the ZIKV entry. (14) TIM1 mediated entry is inhibited by Duramycin-biotin.

Inhibition of NS5

The flaviviral NS5 protein has two major catalytic domains. The first domain is the RNA-dependent RNA polymerase (RdRp), and the second one is a methyltransferase domain (Egloff et al., 2002). Conserved regions in ZIKV NS5 MTase and RdRp structures have been identified, so that current anti-virals targeting these regions in flaviviruses like DENV, WNV, and YFV may be used against ZIKV (Duan et al., 2017). NS5 is also involved in IFN antagonism. Mutant ZIKV, which is unable to prevent IFN-stimulated signaling can grow in IFN-deficient cell lines and has vaccine potential. Structural analysis of NS5 will provide information to design small molecule inhibitors that may prevent NS5-mediated interferon inhibition (Dar et al., 2017).

Inhibition of the RdRp domain

Nucleoside analogs may be incorporated into the viral nascent RNA chain during RdRp activity, causing premature termination

of RNA synthesis (Kok, 2016). After screening a library of nucleoside analogs, 2'-C-methylated nucleosides were found to be most efficient at terminating synthesis because they were selective for ZIKV, and there was no cytotoxicity or adverse effects on cellular proliferation observed (Eyer et al., 2016). These nucleosides do not target the active RdRp site; rather, they terminate elongation of the nascent viral RNA chain. Another nucleoside analog, 7-deaza-2'-C-methyladenosine, developed initially as an HCV replication inhibitor, was also evaluated for inhibition of ZIKV replication in Vero cells and a mouse model. Using a mouse model deficient in IFN- α/β and the IFN- γ receptor (AG129 strain), intraperitoneal inoculation of ZIKV with approximately 2 plaque-forming units (pfu)/animal resulted in disease. Inoculated animals were treated with 7-deaza-2'-C-methyladenosine at a dose of 50 mg/kg/day. The drug delayed the onset of viremia and virus-induced morbidity and mortality in infected mice (Zmurko et al., 2016). Despite demonstrating

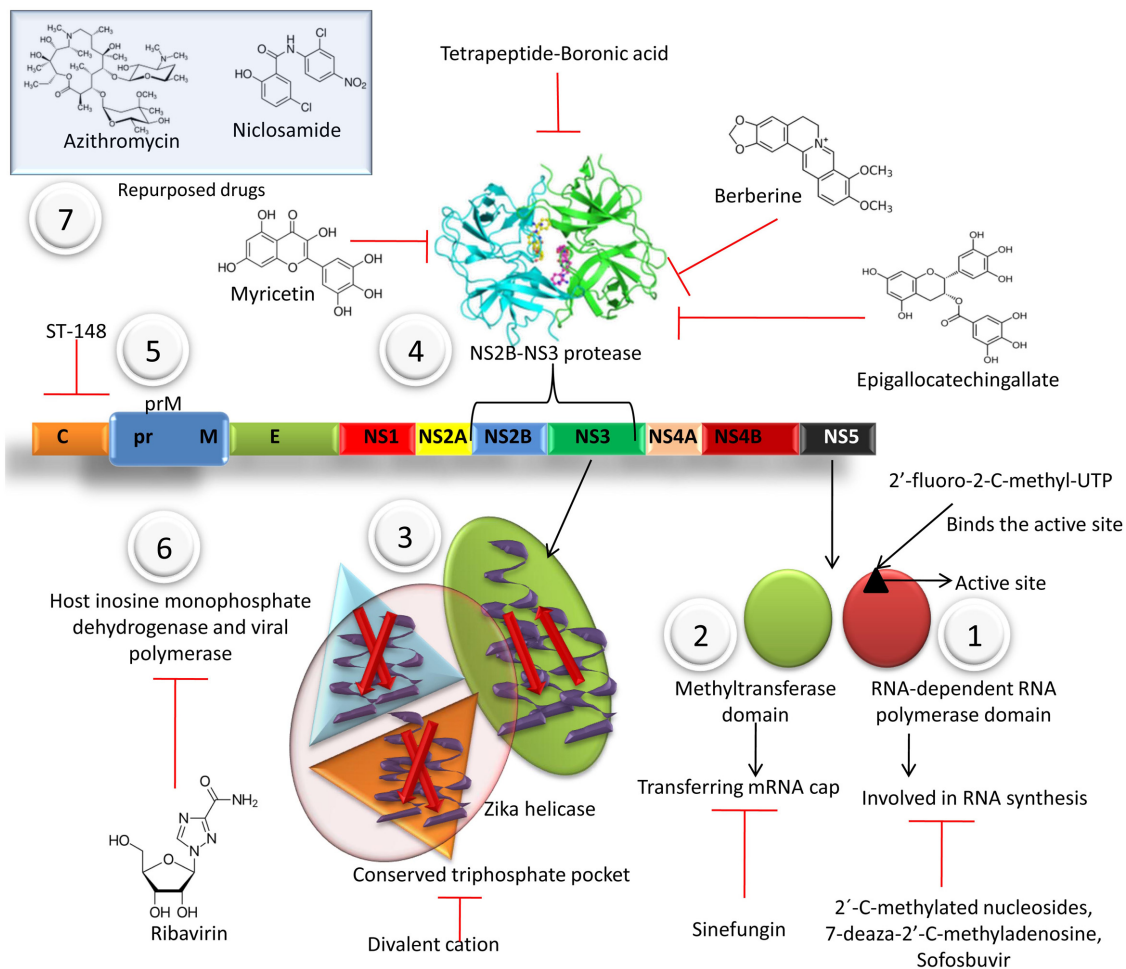


FIGURE 2 | Various drugs involved in inhibition of virus replication at different stages. (1) Flaviviral NS5 has two major catalytic domains: RNA-dependent RNA polymerase (RdRp) and methyltransferase domain. Nucleoside analogs like 2'-C-methylated nucleosides, 7-deaza-2'-C-methyladenosine, Sofosbuvir may incorporate during the polymerase activity of RdRp in the viral nascent RNA chain and cause premature termination of RNA synthesis. The 2'-fluoro-2-C-methyl-UTP binds to the active site on NS5. (2) Methyltransferase domain is responsible for transferring mRNA cap. Sinefungin, an adenosine derivative, isolated from *Streptomyces griseoleus*, inhibit S-adenosyl-1-methionine (SAM), the natural substrate for methyltransferases and inhibit the methyltransferase activity. (3) Helicase crystal structure reveals a conserved triphosphate pocket and a positively charged tunnel for the accommodation of RNA. The helicase-activation is inhibited in the presence of divalent cation, due to extended conformation adopted by GTPyS in such conditions. (4) Tetrapeptide-Boronic acid is a potent inhibitor of NS2B-NS3 protease. Berberine, Myricetin, Epigallocatechingallate binds with affinity to NS3 protease and also inhibit the ZIKV replication. (5) Small-molecule inhibitor ST-148 inhibits capsid. (6) Ribavirin inhibits host inosine monophosphate dehydrogenase and viral polymerase. (7) Repurposed drugs like Chloroquine, azithromycin, niclosamide are used to treat ZIKV infection.

efficacy in animal models, the drug remained unsuccessful in the phase I clinical trial conducted. Adenosine analog NITD008 was found to be effective against flaviviruses including ZIKV in both *in vitro* and *in vivo* studies and exhibited reduced viremia in mice (Deng Y.Q. et al., 2016). Unfortunately, in pre-clinical animal testing, it was found to be too toxic to be suitable for human trials.

Sofosbuvir (Sovaldi) is a nucleotide analog inhibitor that is commercially available for the treatment of chronic HCV infection. Its active metabolite is 2'-fluoro-2-C-methyl-UTP, which binds to the active site of NS5 (Reznik and Ashby, 2017). It has also shown the ability to inhibit ZIKV infection in human tumor cell lines and human fetal-derived neuronal stem cells (Bullard-Feibelman et al., 2017; Reznik and Ashby, 2017). In

clinical phase I and II studies, the regimen containing sofosbuvir was found to be clinically safe and efficacious (Mangia and Piazzolla, 2014). It is a class B drug and can be used in men and non-pregnant women to prevent tissue damage. The 2'-C-ethynyl and 2'-C-methyl analog of 5'-triphosphates were found to be incorporated by the RdRp of ZIKV and therefore to efficiently terminate the elongating RNA chain (Lu et al., 2016); hence, they may be candidate for the design of better anti-virals against ZIKV.

Methyltransferase domain inhibition

The NS5 protein contains methyltransferase, which is responsible for transferring the mRNA cap. An NS5 methyltransferase null mutant was found to be lethal for the virus. Targeting the NS5 methyltransferase structural domains may prevent ZIKV

propagation (Zhang C. et al., 2016). Structural analysis of ZIKV-NS5 aided in the identification of methyltransferase inhibitors based on *in silico* analysis, which could lead to the identification of hotspots for drug designing and development of anti-ZIKV drugs (Coutard et al., 2016; Stephen et al., 2016). Furthermore, sinefungin, an adenosine derivative originally isolated from *Streptomyces griseoleus*, is a potential anti-fungal and anti-parasitic compound that competitively inhibits S-adenosyl-1-methionine (SAM), the natural substrate for methyltransferases, as well as methyltransferase activity. On the basis of structural computational analysis, sinefungin was able to attach to GTP and GDP analogs and might be useful in enhancing their affinity toward the enzyme for greater selectivity and inhibition of ZIKV replication (Hercik et al., 2017). Severe toxicity was observed with this drug in animal studies conducted in dogs and goats while its usage as an anti-parasitic agent (Robert-Gero et al., 1989), which has hampered its clinical use. Therefore, less toxic and better tolerated derivatives must be obtained and tested against ZIKV.

Inhibition of NS3 (Helicase)

An NS3 inhibitor can be used to impede ZIKV infection. The helicase action of NS3 is inhibited by divalent cations that cause GTPyS to adopt an extended conformation (Cao et al., 2016). An understanding of the interactions between NS3 and GTPyS has led to the identification of small molecules that inhibit ZIKV. The ZIKV helicase crystal structure has revealed a conserved triphosphate pocket and a positively charged tunnel that accommodates the RNA. This critical substrate-binding pocket may be a good target for anti-virals. Tian et al. (2016) reported that the helicase of ZIKV is closely related to those of other members of the family *Flaviviridae*. Therefore, drugs that target the helicase of these viruses could also be explored for the control of ZIKV.

Inhibition of the NS2B-NS3 Protease

The crystallographic structure of the NS2B-NS3 protease of ZIKV indicated that tetrapeptide-boronic acid would be a potent inhibitor of the NS2B-NS3 protease (Lei et al., 2016). Based on studies of surface plasmon resonance and the kinetics of protease inhibition, Lee et al. (2016) identified several small molecular inhibitors of the protease. In addition, the group identified a “pre-open conformation” for the ZIKV NS2B-NS3 protease by X-ray crystallographic analysis. A molecular docking analysis revealed that berberine, an FDA-approved quaternary ammonium salt used against DENV, has a high binding affinity to the NS3 protease of ZIKV (Sahoo et al., 2016). Further, a ‘Hybrid Combinatorial Substrate Library’ approach has also been used to determine the substrate specificity of the NS2B-NS3 protease for the design of phosphonate-containing protease inhibitors (Rut et al., 2016). Lim et al. (2017) tested 22 plant polyphenolic compounds for their ability to inhibit *Escherichia coli*-expressed ZIKV NS2B-NS3 protease. Inhibition of protease activity was evaluated by a fluorescence resonance energy transfer-based assay. Among all compounds tested, myricetin showed the strongest inhibition of the NS2B-NS3 protease, followed by luteolin, epicatechin

gallate, gallic acid, gallic acid, and epigallocatechin gallate. CN-716, a capped peptidomimetic boronic-acid compound, has been found to form ZIKV NS2B-NS3 protease inhibitor complex, which might have biological importance in inhibiting ZIKV replication (Lei et al., 2016). Structural and functional insights gained through crystallographic techniques will accelerate the discovery of structure-based anti-ZIKV compounds.

Nucleoside Biosynthesis Inhibitors

Nucleoside biosynthesis inhibitors have broad anti-viral activities, but limited numbers of such compounds are available for clinical use. Inhibition of host inosine monophosphate dehydrogenase (IMPDH) and viral polymerase is the key to the anti-viral activity of ribavirin against flaviviruses (Crance et al., 2003). Mycophenolic acid (MPA) is an IMPDH inhibitor and exhibited potent dose-dependent anti ZIKV activity (EC_{50} of $<0.32 \mu M$) in cell culture experiments that were confirmed using qRT-PCR (Goebel et al., 2016). Contrary to the above finding, Adcock et al. (2017) found MPA to be not very effective ($EC_{50} > 50 \mu M$) with observation of significant cytotoxicity and cytopathogenic effects in a high-throughput assay. Dihydroorotate dehydrogenase (DHODH), a host enzyme which is responsible for pyrimidine biosynthesis, is a possible enzyme target for antiviral research. Brequinar, an inhibitor of this enzyme, exhibited anti-ZIKV activity with an EC_{50} (half maximal effective concentration, which is a common measure of a drug's potency; the lower the EC_{50} , the more effective the drug is) at submicromolar levels, but a low therapeutic index for Brequinar has restricted its clinical use.

Capsid inhibition

In comparison with targeting E protein, the capsid has gained less research attention. It is a dimeric protein with a positively charged surface and hydrophobic core pocket. A single small-molecule inhibitor (ST-148) has been identified, which, despite poor oral bioavailability, was shown to decrease the viremia and viral loads of DENV-1–4, Modoc virus, YFV, and HCV. ST-148 was screened in a panel of 20,000 chemically diverse molecules using a high-throughput assay. It is non-mutagenic and selectively inhibits flaviviruses (Byrd et al., 2013). ST-148 mediates the self-interaction of capsid proteins and imposes structural rigidity, disturbing the assembly and disassembly of DENV particles (Scaturro et al., 2014). The concept of capsid protein stabilization may also be applicable to ZIKV.

Computer-Aided Drug Design

Before clinical studies, there are three essential phases of research: high-throughput computer or *in silico* drug design, medium-throughput *in vitro* drug testing, and low-throughput *in vivo* drug testing (Basak and Nandy, 2016). Understanding the ZIKV structure would aid in designing anti-viral therapies to curtail ZIKV infections (Cox et al., 2016). ZINC64717952 and ZINC39563464 have been found to block MTase and RdRp, respectively, based on a computational docking analysis (Ramharack and Soliman, 2017). The NS5 polymerase was inhibited by an andrographolide from *Andrographis*, whereas bisabolol and levomenol from *Matricaria recutita* and *Myoporum*

crassifolium, respectively, blocked NS3 protease in virtual screening (Feranchuk et al., 2016). *In silico* techniques to identify enzyme blockers allow simultaneous assessments of various compounds with limits financial or experimental resource costs. A virtual screen of 36 million compounds from the MCULE database led to the selection of two molecules, MCULE-8830369631-0-1 and MCULE-9236850811-0-1, with inhibition constant (K_i) values of 0.08 and 0.30 μ M, respectively (Onawole et al., 2017).

OpenZika, an IBM world community grid project, was used to identify drug molecule docking for various ZIKV structures. This platform allows data to be shared with researchers worldwide to facilitate the speedy discovery of anti-ZIKV drugs (Ekins et al., 2016). To elucidate therapeutically essential components like siRNAs, miRNAs, and sgRNAs (CRISPR/Cas9 targets) for ZIKV, an integrative multi-omics platform, ZikaVR¹, is available. This platform offers other functions, including whole-genome alignment, codon information and bias assessments, phylogenetic deduction, and information regarding glycosylation sites and primer design (Gupta et al., 2016). The therapeutics based on enzyme, nucleoside, and capsid inhibitors are currently in their infancy and much more work needs to be carried out to bring these to clinical grounds. Computational analysis allows high throughput screening of potentially active molecules, however, *in vivo* validation is a prior requisite to move them from bench to bedside.

Drug Repurposing

Drugs take decades to develop and test for efficacy and safety. Since there is presently no approved vaccine or drug available for ZIKV, the major focus of researchers, therefore, is on attempting drug repurposing. Scientists are evaluating repurposing of several FDA approved drugs against ZIKV infections. In this direction, a few promising drug candidates have been shortlisted by adapting various screening methodologies. For example, chloroquine, a 4-aminoquinoline, readily increases the pH of acidic vesicles (Akpovwa, 2016) and inhibits a conformational change essential for fusion between the virus envelope and endosomal membrane (Smit et al., 2011). *In vitro* studies revealed that chloroquine decreases the number of ZIKV-infected neural cells in different cell models and protects cellular death (Delvecchio et al., 2016). Other anti-malarial drugs such as quinacrine, mefloquine, and GSK369796 also demonstrate anti-ZIKV activity by inhibiting autophagy (Balasubramanian et al., 2016). During the screening of a library of FDA-approved drugs, both established anti-virals like bortezomib and mycophenolic acid and compounds with no previously reported anti-viral activity (e.g., daptomycin) were found to inhibit ZIKV replication in human cervical, placental, neural stem, and primary human amniotic cells (Barrows et al., 2016). Xu M. et al. (2016) screened a panel of compounds containing FDA-approved drugs, drugs in clinical trials, and pharmacologically active compounds to suppress infection-induced caspase activity. Human neural progenitor cells and glial SNB-19 cells infected with ZIKV were used as models to quantify ZIKV-induced caspase-3 activity. Of these

compounds, a pro-caspase inhibitor, emricasan, successfully protected both neural cell monolayers and three-dimensional organoid cultures of neural cells by decreasing ZIKV-induced caspase-3. Similarly, screening of 725 FDA-approved chemically diverse compounds in ZIKV-infected Huh7 cells at a 20- μ M concentration led to the selection of lovastatin, a drug used to reduce cholesterol; 5-fluorouracil used as a cancer treatment; 6-azauridine, a broad-spectrum antimetabolite; palonosetron, which is used to treat chemotherapy-induced nausea and vomiting; and kitasamycin, a macrolide antibiotic. The selection criteria included a selectivity index, maximum activity, and the EC₅₀ of compounds (Pascoalino et al., 2016).

Niclosamide, clinically given to treat helminths infection, can protect ZIKV-infected cells and inhibit virus replication (Xu M. et al., 2016). Bortezomib and sorafenib are anti-cancer drugs possessing anti-viral activity and have been well tolerated in phase I clinical trials (Cheng et al., 2016). Azithromycin, a commercially available antibiotic, was also found to inhibit ZIKV proliferation in cultured brain cells, suggesting a possibly drug to prevent GBS and microcephaly (Retallack et al., 2016). Recently, bromocriptine, a drug indicated for the treatment of pituitary tumors, Parkinson's disease, and type 2 diabetes, was shown to inhibit ZIKV replication *in vitro*, possibly by occupying the active site of the ZIKV-NS2B-NS3 protein. A fluorescence-based enzymatic assay also revealed that bromocriptine inhibits the activity of the ZIKV-NS2B-NS3 protease, possibly by occupying the active site pocket. In addition, bromocriptine, along with type I interferon, exhibits synergistic anti-ZIKV activity (Chan et al., 2017). Suramin is an approved drug used to treat trypanosomal human sleeping sickness and is available for prophylactic and therapeutic uses in children. It inhibits the early steps of ZIKV binding/entry and decreases the number of infectious ZIKV progeny virions (Albulescu et al., 2017; Tan et al., 2017). The safe pediatric anti-protozoan and anti-viral drug nitazoxanide was found to affect post-attachment steps of ZIKV infection at or below a 10 μ M dose (Cao et al., 2017). The anti-ZIKV activity of nitazoxanide is not less than that of niclosamide (Xu M. et al., 2016), but poor absorption of niclosamide might reduce its utility.

Hyperactivation of the N-methyl-D-aspartate receptor (NMDAR), mediated by enhanced glutamate release, may result in the accumulation of high levels of Ca²⁺ in neurons, and this may further lead to apoptosis or necrosis of neural cells. Neurodegeneration in ZIKV disease possibly occurs due to the excitotoxicity of glutamate. FDA approved NMDAR antagonistic drugs to treat Alzheimer's disease (namely memantine, MK-801, agmatine, and ifenprodil) were found to prevent neuronal cell death caused by ZIKV under *in vitro* conditions without reducing viral titers (Costa et al., 2017). Memantine was found to bind non-competitively with NMDAR, while blocking only the pathologically active NMDAR and leaving its physiological activity unaffected (Sirohi and Kuhn, 2017). Memantine is also listed in pregnancy category B drugs by the FDA, hence it could be used safely to reduce neurological complications associated with ZIKV infection (Sirohi and Kuhn, 2017). Practical usage of repurposing earlier approved drugs could help in formulating fascinating approaches to counter ZIKV and its associated complications for which purpose

¹<http://bioinfo.imtech.res.in/manojk/zikavr/>

more research work is needed before moving into clinical trials.

Development of Pregnancy-Safe Drugs

The ability of ZIKV to infect fetuses and cause severe disease requires the development of drugs that function during pregnancy and that are safe for both the pregnant mother and fetus. The drugs must be able to cross the placental barrier to reach the fetus and to cross the blood-brain barrier to reach neural cells, the main targets of ZIKV. Khandia et al. (2017) summarized FDA-approved category B drugs (adequate animal study data shows no risk to fetuses, but controlled studies on pregnant women are unavailable) and category C drugs (animal studies revealed few teratogenic effects on fetuses, but control studies on pregnant women are unavailable; however, the potential benefits of using the drug may outweigh the risks). The list contains several drugs including the FDA category B drugs sofosbuvir (Sacramento et al., 2017), azithromycin (Retallack et al., 2016), niclosamide (Xu M. et al., 2016), palonosetron (Pascoalino et al., 2016), mefloquine (Balasubramanian et al., 2016), and daptomycin B (Barrows et al., 2016), category C drugs chloroquine (Delvecchio et al., 2016), amodiaquine, quinacrine hydrochloride (Balasubramanian et al., 2016), auranofin, clofazimine, deferasirox, methoxsalen, micafungin, sertraline-HCl, fingolimod, ivermectin, digoxin (Barrows et al., 2016), and seliciclib (Xu M. et al., 2016), which could be repurposed for treating ZIKV infection.

Use of Convalescent Serum

Recently, neutralizing activity of human convalescent serum against ZIKV has been demonstrated in a standard plaque reduction neutralization test (Li et al., 2016). Further, a decreased number of ZIKV-infected brain cells in ICR albino fetal mice were observed after treating pregnant mice intraperitoneally with convalescent serum. Furthermore, ZIKV-mediated caspase activity was reduced, indicating the utility of convalescent serum in limiting ZIKV infection and cell death. Convalescent serum also reversed thinning of the cortical plate (CP) and ventricular zone (VZ)/subventricular zone (SVZ) observed in the brains of ZIKV-infected fetal mice. Therefore, the use of convalescent serum for the treatment of ZIKV-infected pregnant women and whether it can protect against brain abnormalities in fetuses should be assessed. A study by Wang S. et al. (2016) demonstrated the suppression of ZIKV infection in pregnant mice with a reduction in caspase-3-activated cells using convalescent serum with high amounts of neutralizing antibodies. Convalescent serum also inhibited progenitor cell death in infected fetal brain tissue, thereby preventing microcephaly. ZIKV-confirmed convalescent human serum was able to neutralize multiple strains of infectious ZIKV or ZIKV RVPs, indicating that ZIKV is circulating as a single serotype (Wang S. et al., 2016). Additionally, antibodies present in convalescent serum can cross the placental, as well as the blood-brain barrier, of fetuses; thus, it is a good candidate for the treatment of infected pregnant women.

The convalescent serum should be free from ZIKV and other blood-borne pathogens prior to transfer, therefore heat

treatment at $58.0 \pm 1.0^\circ\text{C}$ for 590 ± 10 min or solvent/detergent (S/D) treatment is commonly employed. S/D treatment with 1% (wt/wt) tri-n-butyl phosphate (TBP) and 1% (wt/wt) octoxynol-9 at $30.0 \pm 1.0^\circ\text{C}$ and pH 6.9–7.4 for 60 min completely inactivated ZIKV (Kühnel et al., 2017). A photochemical, amotosalen, quickly intercalates into DNA and RNA strands and forms covalent adducts with pyrimidine, thereby inhibiting replication and transcription of the virus. Plasma samples treated with amotosalen and UVA-light are safe for use in patients, as they are free from viable ZIKV particles, even in the presence of a detectable amount of ZIKV viral RNA (Aubry et al., 2016).

Use of Neutralizing Antibodies

A human monoclonal (mAb) antibody against DENV named C10 has been found to neutralize ZIKV E protein. Using an electron microscope, C10-ZIKV interactions were studied in extracellular (pH 8), early (pH 6.5), and late endosomal (pH 5.0) stages. At all of the tested pHs, C10 bound ZIKV E protein at different positions. At pH 8.0, it bound at the intradimer interface; at pH 6.5, it bound the virus surface; and at pH 5.0, it blocks raft structure. Of note, different structural rearrangements of the virus were blocked by C10 antibodies as depicted in visualization under electron microscope (Zhang S. et al., 2016), suggesting its broad applicability at different stage of infection. Out of the panel of human mAbs derived from patients previously infected with ZIKV, ZIKV-117 mAb was found to broadly neutralize the African and Asian-American lineages of ZIKV. The mAb recognized the unique quaternary epitope on the E protein dimer-dimer interface and effectively reduced ZIKV infection, maternal-to-fetal transfer, and tissue pathology and mortality (Sapparapu et al., 2016).

Three-dimensional cryo-electron microscopy showed that ZIKV-117 Fabs cross-link with monomers in surface E glycoprotein dimers and between neighboring dimers, thereby preventing the structural reorganization of E protein monomers and requiring the formation of fusogenic E protein trimers (Hasan et al., 2017). Neutralizing antibody 2A10G6, which targets the highly conserved fusion loop region of flavivirus E proteins, binds with high affinity and can neutralize ZIKV in a mouse model (Dai et al., 2016). Moreover, of 13 human mAbs from a single ZIKV patient, two mAbs (Z23 and Z3L1) potently bound and neutralized ZIKV, but it did not cross react with any DENV strains (Wang Q. et al., 2016).

Strategies to Limit Antibody-Dependent Enhancement (ADE)

Studies regarding the phenomenon of antibody-dependent enhancement (ADE), in which viremia is increased in the presence of pre-existing cross-reactive, poorly neutralizing antibodies against a heterologous flavivirus strain, are controversial. In few cases, pre-existing cross-reactive antibodies have shown to increase ADE, while in some experiments such antibodies exhibited therapeutic potential. For instance, preincubation of human myeloid cells (U937), which are poorly permissive for ZIKV, with convalescent serum obtained from subjects who resolved DENV infections, resulted in increased

ZIKV infectivity (Dejnirattisai et al., 2016). These results were confirmed by Charles and Christofferson (2016), who used a DENV serotype 2-derived mAb (4G2) to demonstrate ADE in ZIKV infections. A contradictory finding is reported by Pantoja et al. (2017) who reported reduction in viremia in DENV-exposed rhesus macaques, when compared with naïve animals. Antibodies to WNV have also shown cross-reactivity with ZIKV E protein, resulting in viremias that were at least 35-fold higher than those of the controls, and studies to determine the role of anti-WNV antibodies in enhancing ZIKV revealed a pattern like that observed with anti-DENV antibodies. WNV may enhance ZIKV *in vitro* as well as *in vivo*; however, the amplitude of enhancement is less than that of DENV (Bardina et al., 2017). Because 4G2 is widely used as an anti-flavivirus mAb, the possibility of using this mAb for other flaviviruses such as JEV and YFV or to limit WNV mediated ADE of ZIKV should be considered.

The two EDI/II cross-reactive mAbs developed against ZIKV (ZKA78) and DENV (DV82) were tested for their capacity for ADE of DENV and ZIKV infection in animal models. Further, in an AG129 mouse model, wild-type mAbs ZKA78 and DV82 (without the LALA mutation), when administered prior to DENV-2 infection, resulted in severe disease and death of mice on the 5th day post-infection, suggesting that the DENV infections were affected by the presence of pre-existing ZIKV antibodies (Stettler et al., 2016). However, antibodies against the envelope dimer epitope 1 (EDE1) region were shown to neutralize ZIKV, in addition to all four DENV serotypes, indicating their potential immune-therapeutic potential in ZIKV infections (Swanstrom et al., 2016). The mouse mAb 2A10G6 reported to bind the conserved 98DRXW101 motif of the FL loop is a broadly neutralizing antibody. It not only neutralizes DENV1-4, but also protected A129 mice against ZIKV infection (Dai et al., 2016). Hence, it seems that the antigenic epitopes against which the antibody is generated is the deciding factor in developing/not developing ADE. The epitopes resulting in poor neutralization lead to ADE, while the strongly neutralizing antibodies have therapeutic potential. Information regarding common epitopes may be useful in determining strategies to limit ADE (Xu X. et al., 2016).

To develop therapeutic mAb candidates, LALA mutations in the Fc region of antibodies have been examined (Figure 3). The LALA mutations are a leucine (L)-to-alanine (A) substitution at positions 234 and 235 (LALA) in the Fc region of IgG antibody. The binding of the Fc region of an antibody with gamma receptors (FcγRs), expressed on various immune cells, triggers their effector function. In the case of ZIKV or DENV, the virus-antibody immune complex is internalized by FcγRs and can result in ADE. Introduction of the LALA mutations into the Fc region abolishes its ability to bind to FcγRs (Arduin et al., 2015). Such engineered antibodies are incapable of interaction with FcγRs and hence eliminate ADE from prior DENV infections *in vitro* and *in vivo* (Williams et al., 2013).

Anti-ZIKV mAbs with the LALA mutation have been engineered for therapeutic and prophylactic purposes. The EDIII-specific neutralizing mAb ZKA64, which has the LALA mutations in the Fc region, blocked ADE in ZIKV infections

in K652 cells in the presence of convalescent serum and completely protected A129 mice from lethal ZIKV challenge when administered one day prior to or post challenge (Stettler et al., 2016). Given the high potency and *in vitro* and *in vivo* efficacy of LALA mutant neutralizing antibodies, creation of such mutants appears to be a safe and promising approach to inhibit ADE in patients living in areas which co-circulating flaviviruses. The LALA mutations and substitution at amino acid position 297 (N297A) in the Fc region of an antibody reduces binding with FcγRs and C1q complement. Thus, such engineered antibodies may play an important role in therapeutics and prophylaxis (Arduin et al., 2015). DENV human mAb SIgN-3C, which strongly neutralizes ZIKV when the LALA mutations are introduced, did not induce ADE, showed a reduced viral load in fetal organs, and prevented virus-induced fetal growth retardation. This indicates the prophylactic potential of the antibody (Kam et al., 2017). In addition, mAbs such as Z23 and Z3L1 (mentioned in above section), which specifically neutralize ZIKV only and neither bind to nor neutralize any of the four DENV serotypes, are also of great importance in limiting ZIKV-associated ADE. In fact, a patent has been granted to Baehner et al. (2015), who modified the Fc region of human antibody, reducing its affinity for FcγRs by 1.15 to 100 folds and resulting in the inhibition of signaling cascades that lead to downstream immune response such as ADE in the case of DENV and ZIKV infection.

Use of Herbal Drugs

Herbal drugs are of increasing interest because of the development of anti-microbial resistance in microbes and owing to their cost effectiveness. Curcumin, a common food additive, is able to reduce ZIKV infectivity by hindering the virus binding to host cell in a dose- and time-dependent manner, without having adverse effects on cellular viability (Mounce et al., 2017). Recently, a Chinese semi-synthetic formulation from *Andrographis paniculata* named xianping, in combination with other antiviral and symptomatic treatments was administered to treat Zika fever in a patient admitted in Ganxian People's Hospital. The patient recovered within 7 days after starting treatment that included this medication (Deng Y. et al., 2016). Quercetin, a flavonoid present in fruits, vegetables, leaves, and grains, has been found to inhibit Zika NS2B-NS3pro enzymatic activity in a dose-dependent manner. Commercially available quercetin has been reported to inhibit the ZIKV protease with an IC₅₀ of 26.0 ± 0.1 μM (Roy et al., 2016).

In vitro studies with a polyphenol, (-)-epigallocatechingallate (EGCG), found in green tea have shown inhibition of ZIKV (Carneiro et al., 2016). The structures of ZIKV NS2B-NS3 protease, NS3 helicase, NS5 methyltransferase, and NS5-RdRp were generated by homology modeling using the BLOSUM80 scoring matrix by Byler et al. (2016) and molecular docking with virtual library of phytochemicals was carried out. Out of 2263 plant-derived secondary metabolites tested, 43 compounds docked with at least one of the ZIKV enzymatic proteins. Some of these include balsacone B from *Populus balsamifera*, kanzonol V from *Glycyrrhiza glabra*, cinnamoylchinoxanthol from *Echinacea*, cimiphenol from *Actaea racemosa*, and rosmarinic

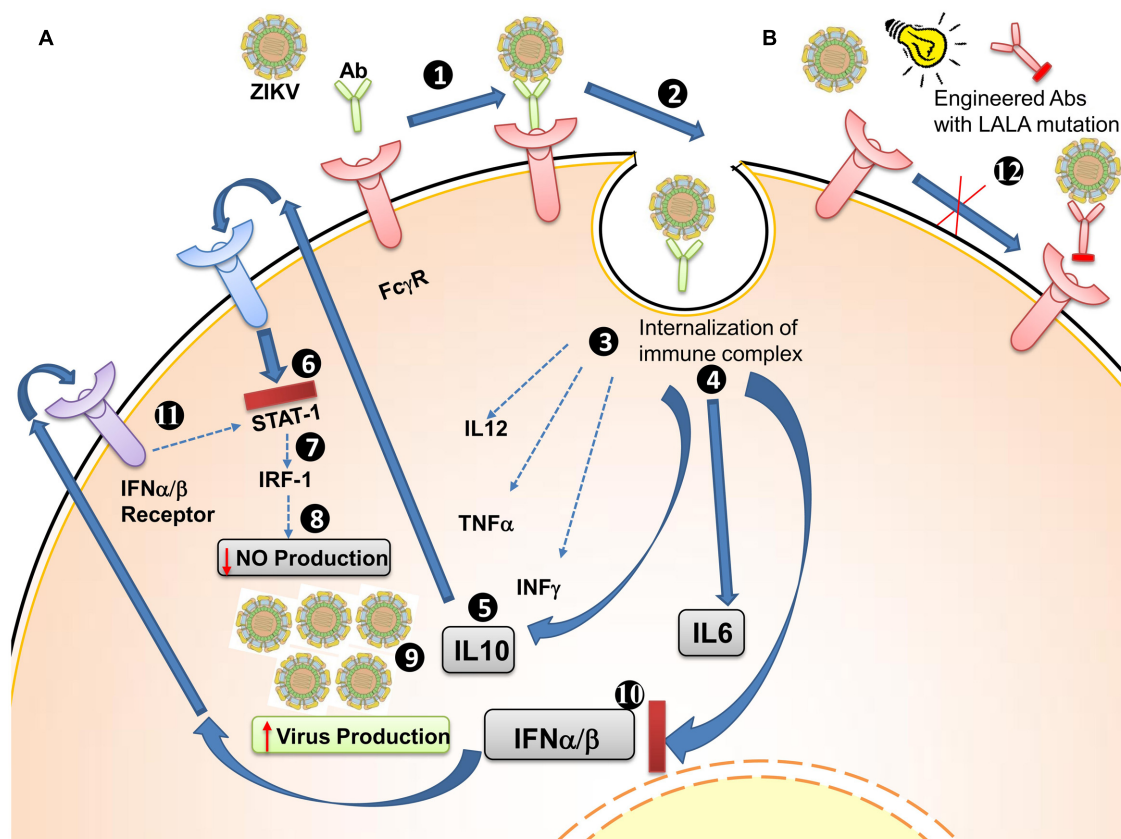


FIGURE 3 | Antibody-dependent enhancement (ADE) of Zika virus (ZIKV) and strategies to limit it. **(A)** General ADE mechanism. (1) FcγRs are receptors present on the surface of various immune cells. Binding of antibody along with ZIKV results in (2) internalization of immune complexes into cells, (3) a reduction of IL12, TNFα, and IFNγ expression levels, and (4) increased levels of IL6 and IL12 (5). IL10 acts in an autocrine manner and binds to its own receptor (6), inhibiting the JAK-STAT pathway (7), which leads to reduced IRF1 production (8) and reduced IFN-stimulated response element (IRF1) production, resulting in decreased nitric oxide production. Nitric oxide, a diffusible radical antimicrobial and anti-viral, is reduced (9), which results in an increase in the number of infectious virus particles (10). IFNα/β are inhibited (11), diffuse out from the cell (12), and bind to their own receptors to reduce JAK-STAT signaling. **(B)** Antibodies engineered to prevent ADE (12). Engineered antibodies having LALA mutations in their Fc region are able to neutralize virus but fail to bind to FcγRs, thus preventing ADE.

TABLE 1 | Patents of novel innovations useful for the treatment of various flaviviral diseases [readily adaptable for Zika virus (ZIKV) treatment].

S. No.	Patent title	Patent number	Date of publication/Application	Legal Status	Inventors' Reference
1.	Carba-nucleoside analogs for antiviral treatment	WO2009132123A1	29.10.2009	Application	Cho et al., 2009
2.	Carba-nucleoside analogs for antiviral treatment	US8012942B2	06-09-2011	Granted	Butler et al., 2001
3.	Inhibitors of Flaviviridae viruses	WO2011088345A1	21.07.2011	Application	Canales et al., 2011
4.	Compositions and methods for treatment of viral diseases	WO2008033466A2	20.03.2008	Application	Johansen et al., 2008
5.	Antiviral agents for treatment of Flaviviridae infections	US20040266723A1	30.12.2004	Application	Otto et al., 2004
6.	Methods and compositions for treating Flaviviruses and Pestiviruses	US6812219B2	02.11.2004	Granted	LaColla and Sommadossi, 2004
7.	Compounds and pharmaceutical compositions for the treatment of viral infections	US7951789B2	31.05.2011	Granted	Sommadosi et al., 2011
8.	Antibody Fc variants	US 8969526 B2	03.03.2015	Granted	Baehner et al., 2015

TABLE 2 | Therapies available/ possible for ZIKV treatment.

S. No.	Strategy to combat ZIKV	Assessed molecules	Modus Operandi	FDA pregnancy category	Reference
1.	Substrate binding pocket of helicase protein	Conserved triphosphate pocket Positively charged tunnel for the accommodation of RNA	Inhibit ZIKV replication	–	Tian et al., 2016
2.	Inhibitors of NS3 protein	Protease activity essential for its replication Presence of divalent cation	Inhibit ZIKV replication Inactivation of helicase domain by extended conformation of GTPyS	–	Sahoo et al., 2016 Cao et al., 2016
3.	Inhibition of NS2B-NS3 protease	Phosphonate inhibitor	Inhibit ZIKV replication	–	Rut et al., 2016
4.	Inhibition of viral entry	Obatoclastax ZINC33683341 and ZINC49605556	Inhibit acidic environment in endolysosomal vesicles Bind with viral receptor and inhibit entry	– –	Varghese et al., 2016 Fernando et al., 2016
5.	Nucleoside inhibitors	2'-C-methylated nucleosides 7-deaza-2'-C-methyladenosine Sofosbuvir 2'-C-ethynyl analog of 5'-triphosphates Active metabolite 2'-fluoro-2-C-methyl-UTP NITD008 adenosine analog	Cause premature termination of RNA synthesis Bind to active site present on NS5 Cause premature termination of RNA synthesis	– B –	Kok, 2016 Zmurko et al., 2016 Govero et al., 2016 Lu et al., 2016
6.	Interferon	Type I interferons- IFN- α and IFN- β Human trophoblast (PHT) cell culture conditioned media secreting type III IFN and IFN- λ 1 IFN- α , β , γ	Antiviral defense system inhibits ZIKV replication Inhibit ZIKV replication Inhibit ZIKV replication	– – –	Deng Y.Q. et al., 2016 Schneider et al., 2014 Bayer et al., 2016
7.	Neutralizing antibodies	mAbto E proteindimer-dimer interface mAb 2A10G6 to FLE region mAb ZIKV-117 to E region mAb Z23 and Z3L1 mAb C10- against E region	Reduce ZIKV infection, maternal to fetal transfer and tissue pathology Protect from ZIKV infection <i>in vivo</i> Broadly neutralize several ZIKV lineages. Inhibit ZIKV replication Inhibit ZIKV replication	– – – –	Contreras and Arumugaswami, 2016 Sapparapu et al., 2016 Dai et al., 2016
8.	Convalescent serum	Polyclonal neutralizing high titer serum	Reduction in caspase activity Prevent thinning of the cortical plate (CP) and ventricular zone (VZ)/ subventricular zone (SVZ) Prevention of neural progenitor cell death in infected fetal brain tissue and prevention of microcephaly	– – –	Zhang S. et al., 2016 Li et al., 2016 Wang Q. et al., 2016
9.	Drug repurposing	Niclosamide PHA-690509 Emricasan Seliciclib Bortezomib Mycophenolic acid Auranofin Vermectin Daptomycin	Inhibit ZIKV replication Cyclin-dependent kinase (CDK) inhibitor studied for use in treating cancer; Known to interfere with gene expression Protect brain cells of developing fetuses against viral damage by inhibiting apoptosis Cyclin-dependent kinase (CDK) inhibitor Replication inhibition in human cervical, placental, neural stem and primary human amniotic cells unknown Antiparasitic; inhibits viral protein functioning Cause bacterial membrane depolarization and a potassium ion efflux.	B D – D D D C C B	Xu M. et al., 2016 Barreyro et al., 2015; Xu M. et al., 2016 Xu M. et al., 2016 Xu M. et al., 2016 Barrows et al., 2016 Mastrangelo et al., 2012; Barrows et al., 2016 Silverman et al., 2003; Barrows et al., 2016

(Continued)

TABLE 2 | Continued

S. No.	Strategy to combat ZIKV	Assessed molecules	Modus Operandi	FDA pregnancy category	Reference
		Sertraline	Inhibit phospholipase A1 and phospholipase D	C	Rainey et al., 2010; Barrows et al., 2016
		Pyrimethamine	Dihydrofolate Reductase Inhibitor and block purine and pyrimidine synthesis	C	Barrows et al., 2016
		Cyclosporine A	Immunosuppression by selectively inhibiting cytokine-induced DNA binding of activator protein-1 and NF- κ B.	C	Doller et al., 2007; Barrows et al., 2016
		Azathioprine	Inhibits purine synthesis	D	Lennard, 1992; Barrows et al., 2016
		Vinblastine	Anticancer; Microtubule inhibitor	D	Kouznetsova et al., 2014
		Vinorelbine/Navelbine	Anticancer Microtubule inhibitor	D	
		Vincristine	Anticancer Microtubule inhibitor	D	
		Nocodazole	Anticancer Microtubule inhibitor	–	
		Sunitinib	Anticancer Kinase inhibitor	D	
		Toremifene	Anticancer Estrogen receptor modulator	D	
		Daunomycin	Anticancer Topoisomerase Inhibitor	D	
		Clemastine	Antiallergic, hay fever, rhinitis histamine antagonist	B	
		Digoxin	Antiarrhythmic Na ⁺ -K ⁺ pump inhibitor	C	
		Colchicine	Primary for gout, microtubule inhibitor	C	
		Propafenone	Antiarrhythmic sodium channel blocker	C	
		Dronedarone	Antiarrhythmic multichannel blocker	X (not for use in pregnancy)	
		Maprotiline	Antidepressant adrenergic uptake inhibitors and histamine antagonist	B	
		Thiothixene	Antipsychotic dopamine antagonist	–	
		Clomipramine	Antidepressant serotonin uptake inhibitors and histamine antagonist	C	
		Trifluoperazine	Antipsychotic, antiemetic dopamine antagonist	–	
		Benztrapine	Anticholinergic, antihistamine histamine antagonist and cholinergic antagonist	B2	
		Azithromycin	Antimicrobial protein synthesis inhibitor	B	
		Clarithromycin	Antimicrobial protein synthesis inhibitor	C	
		Mebendazole	Anthelmintic microtubule inhibitor	C	
		Albendazole	Anthelmintic microtubule inhibitor	C	
		Azithromycin	Antibacterial	B	Retallack et al., 2016
		Quinacrine, Mefloquine, and GSK369796	Antimalarial drug inhibiting autophagy	C	Balasubramanian et al., 2016
		Sinefungin	Antifungal antibiotic	Not approved	Hercik et al., 2017
		Suramin	African trypanosomiasis	Not approved	Albulescu et al., 2017
		Nitazoxanide	Antiprotozoan drug	B	Cao et al., 2017
		Memantine	For treating Alzheimer's Diseases	B	Costa et al., 2017
		Kitasamycin	Broad spectrum antimicrobial;	–	Saiz and Martín-Acebes, 2017
		Lovastatin	Inhibits cholesterol 247 biosynthesis.	X	
		Nordihydroguaiaretic acid	Anticancer drug	Not approved	
		PF-429242	Impairs the onset of HCV infection.	–	
		Fatostatin	Fat synthesis blocker	–	
		6-azauridine	Inhibits <i>de novo</i> pyrimidine synthesis	D	Adcock et al., 2017
		Finasteride	For treatment of benign prostatic hyperplasia	X	
		Mycophenolic acid	Immunosuppressant	D	Goebel et al., 2016

(Continued)

TABLE 2 | Continued

S. No.	Strategy to combat ZIKV	Assessed molecules	Modus Operandi	FDA pregnancy category	Reference
10.	Herbal therapies	Preparation of <i>Alternanthera philoxeroides</i> , <i>Andrographispaniculata</i> , <i>Azidarachta indica</i> , <i>Euphorbia hirta</i> , <i>Eupatorium perfoliatum</i> , <i>Tinosporacordifolia</i> , and <i>Psidiumguajava</i>	Broad spectrum antiviral activity	–	Parida et al., 2002; Jiang et al., 2005; Apostol et al., 2012; Sriwilajaroen et al., 2012; Tang et al., 2012; Ching et al., 2016; Saxena et al., 2016
		Anti-inflammatory drugs + <i>Andrographispaniculata</i>	Inhibit ZIKV replication	–	Deng Y.Q. et al., 2016
		Andrographolide	Inhibition of NS5 polymerase pocket	–	Feranchuk et al., 2016
		Bisabolol and levomenol from <i>Matricariarecutita</i> and <i>Myoporumcrassifolium</i>	Inhibition of NS3 protease	–	
		Epigallocatechin gallate (EGCG) found in green tea	Inhibitory effect (Inhibition through yet unknown mechanisms)	–	Carneiro et al., 2016
		Quercitin and myricetin (flavonoid)	Allosteric inhibition of NS2B-NS3 protease	–	Lim et al., 2016, 2017; Roy et al., 2016
		Balsacone B, kanzonol V, cinnamoylchinaxanthol, cimiphenol and rosemarinic acid	Inhibitory effect (Inhibition through yet unknown mechanisms)	–	Byler et al., 2016
11.	Other strategies	Methyltransferase inhibitors	Inhibition of NS5	–	Stephen et al., 2016
		Methyltransferase inhibitor-QL-XII-47 and QL-XII-54	Inhibition of viral protein expression	–	de Wispelaere et al., 2016
		Bithionol	Inhibition of caspases	C	Leonardi et al., 2016
		Amotosalen combined with UVA light	Viral inactivation	–	Aubry et al., 2016
		Metadichol	Binds to Vitamin D receptor and displays virus	–	Raghavan, 2016

acid from *Rosmarinus officinalis*. Such common medicinal plants may serve as a source of herbal anti-virals. Such studies encourage the findings of structure-based drug (Byler et al., 2016). Several bioactive components, including alkaloids, flavonoids, saponins, tannins, terpenoids, essential oils, and herbs, such as *Azidarachta indica* and *Tinospora cordifolia*, have shown anti-flaviviral activities against DENV, JEV, and YFV infections. Such antiviral agents can also be explored for their efficacy against ZIKV and be used as complementary alternative medicine (Parida et al., 2002; Kiat et al., 2006; Meneses et al., 2009; Tang et al., 2012; Roy et al., 2015; Ching et al., 2016; Gómez-Calderón et al., 2017).

Compounds used for the treatment of various flaviviral diseases that are readily adaptable to ZIKV are presented in Table 1.

Other Strategies

Certain compounds such as QL-XII-47 and QL-XII-54 are quinolines (covalent inhibitors of DENV) that act by inhibiting viral E and NS5 protein expression without significantly affecting the host housekeeping protein GAPDH. Due to structural similarities between flaviviral E and NS5 proteins, similar compounds can be employed to combat ZIKV infection (de Wispelaere et al., 2016). Host caspases have been found to mediate the lethality of multiple pathogenic agents, based on the HapMap Project of B lymphoblastoid cells from a cohort of persons of African, European, and Asian ancestry. Bithionol

(an FDA-approved drug) inhibits caspases and has been found to be effective in reducing the negative effects of ZIKV and bacterial and plant toxins. Thus, elucidation of such host proteins that further disease can be used to design drugs for ZIKV (Leonardi et al., 2016). Nordihydroguaiaretic acid (NDGA) alters the lipid metabolism of a host by intervening in the sterol regulatory element binding protein (SREBP) pathway. More recently, inhibitors of the SREBP pathway, including NDGA and its methylated derivative tetra-O-methyl nordihydroguaiaretic (M₄N), PF-429242, and fatostatin, have also been found to reduce WNV and ZIKV replication. These drug candidates may serve as effective anti-viral agents against ZIKV (Merino-Ramos et al., 2017).

After ZIKV binds to nerve cells, Toll-like receptor (TLR)-3 is activated, leading to the dysregulation of genes participating in neurogenesis, axon guidance, and differentiation. TLR3 agonist poly (I:C) and thiophenecarboxamidopropionate compounds act as high-affinity competitive inhibitors of TLR3, and prevented a reduction in the size of ZIKV-treated neurospheres (Dang et al., 2016). Metadichol, a nanoemulsion of policosanols, binds to the Vit D receptor and stimulates the immune system. It can displace viruses bound to the Vit D receptor, thereby blocking viral entry into host cells. Metadichol has shown activity against ZIKV, EBOV, SARS coronavirus, JEV, WNV, and YFV. It is sold as a nutritional supplement in some Asian countries and is well tolerated; hence, it can be used as a safe and broad-spectrum

anti-viral agent (Raghavan, 2016). Similarly, testing of other broad-spectrum anti-viral agents for anti-ZIKV activity will aid in rapid drug discovery to combat this virus.

An overview of recent advances in the design of drugs and therapies for ZIKV is presented in **Table 2**.

A few recent therapies include cytokines, TLRs, siRNA, RNA interference, probiotics, immunomodulatory interventions, and nanodrug delivery. These approaches have gained momentum and are being examined for optimum benefit and safety in humans and their companion animals. Prospective aspects of these valuable therapies could be given a due focus for designing and developing effective drugs, medicines, therapeutics and immunomodulatory pharmaceuticals for the treatment of ZIKV infections. Molecular and genetic analyses for a more in-depth understanding of ZIKV pathogenesis would facilitate the identification of novel targets and development of safer and more effective drugs to counter ZIKV effectively.

CONCLUSION AND FUTURE PERSPECTIVES

Zika virus, an arbovirus, shares several characteristic features with other members of the *Flavivirus* family. Recent evidences of autoimmune complications (GBS) and maternal-to-fetal transmission of virus leading to microcephaly has accumulated. Using state-of-the-art methods to formulate effective diagnostics, anti-viral drugs, therapeutics, vaccines, and prevention and control strategies would aid in addressing this emergent virus. Some recent therapies have shown promise in inhibiting ZIKV infections and associated disease. These therapies include limiting viral entry into cells, targeting the ZIKV helicase protein, use of nucleoside analogs like 2'-C-methylated nucleosides and 7-deaza-2'-C-methyladenosine to terminate nascent RNA strand formation, and use of antibodies that bind to ZIKV but do not neutralize it, reducing the risk of ADE. ADE is of major concern in the application of ZIKV therapies in geographical regions where other flaviviruses are endemic. Thus, to limit ADE, antibodies are being engineered to contain a modified Fc region. Modification of the Fc region of antibodies not only hampers their attachment to FcγRs to inhibit internalization of the immune complex, but also reduces complement binding, preventing ADE. In the future, several such mutations may be identified, and humanized mAbs can be genetically engineered to prevent ADE. Combination use of such engineered antibodies might be evaluated for synergistic effects in other therapeutic and prophylactic regimens.

Encouraging results with repurposed drugs, as shown by the use of chloroquine, a malaria drug, has led to the screening of several other FDA-approved drugs, including niclosamide, emricasan, and daptomycin, palonosetron, kisasamycin, and many more, for ZIKV treatment. Another valuable strategy for the discovery of ZIKV preventives and anti-virals is the use of computational analysis.

More insights into genetic and molecular mechanisms associated with the recent increase in virulence of ZIKV could aid in the design and development of safer and more potent drugs and therapeutics against ZIKV. Along with identifying novel drug targets, therapeutics, and vaccines, strengthening of appropriate prevention and control measures, including mosquito control, could help in limiting ZIKV infections, its associated complications, and its potential for further spread. It is time for researchers, pharmaceutical companies, policy makers, regulators, and funding agencies to identify and implement strategies to counter ZIKV globally.

What We Are Still Lacking

Since the declaration of the Zika epidemic as an international public health emergency by WHO in 2016, research on ZIKV has increased many fold. However, there are areas that still need to be addressed.

(1) The percent contribution of each route of ZIKV infection is not precisely understood. Presently, based on mathematical modeling study, sexual transmission has been estimated to account for upto 3% of transmission, but contributions by other routes of infection are yet to be studied. This knowledge may be helpful in designing precisely targeted inhibitory molecules to block infection at site of entry.

(2) Many FDA approved drugs have been tested for efficacy against ZIKV; which can be repurposed for treating ZIKV infection in human. However, to date, no FDA category A drug has been identified clinically safe for use in mothers and fetuses.

(3) For engineered mAb, only two mutations that prevent internalization of immune complexes, i.e., LALA and N297A substitutions, have been identified. More such mutations must be identified for optimal efficacy and synergism.

AUTHOR CONTRIBUTIONS

All the authors substantially contributed to the conception, design, analysis and interpretation of data, checking and approving final version of manuscript, and agree to be accountable for its contents. AM and RK initiated this review compilation; KD reviewed, analyzed, and edited; RK designed tables; AM, RK, and KK designed the figures; SS and RT covered critical aspects on drug and vaccine development; YM and RS reviewed virological aspects and analyzed data; DK reviewed biotechnological and bioinformatics advances; HI and SK overviewed immunotherapeutic aspects and drug development.

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Rapid Detection Strategies for the Global Threat of Zika Virus: Current State, New Hypotheses, and Limitations

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The current scenario regarding the widespread Zika virus (ZIKV) has resulted in numerous diagnostic studies, specifically in South America and in locations where there is frequent entry of travelers returning from ZIKV-affected areas, including pregnant women with or without clinical symptoms of ZIKV infection. The World Health Organization, WHO, announced that millions of cases of ZIKV are likely to occur in the USA in the near future. This situation has created an alarming public health emergency of international concern requiring the detection of this life-threatening viral candidate due to increased cases of newborn microcephaly associated with ZIKV infection. Hence, this review reports possible methods and strategies for the fast and reliable detection of ZIKV with particular emphasis on current updates, knowledge, and new hypotheses that might be helpful for medical professionals in poor and developing countries that urgently need to address this problem. In particular, we emphasize liposome-based biosensors. Although these biosensors are currently among the less popular tools for human disease detection, they have become useful tools for the screening and detection of pathogenic bacteria, fungi, and viruses because of their versatile advantageous features compared to other sensing devices. This review summarizes the currently available methods employed for the rapid detection of ZIKV and suggests an innovative approach involving the application of a liposome-based hypothesis for the development of new strategies for ZIKV detection and their use as effective biomedical tools.

Keywords: detection, diagnosis, immunoassay, liposome, PCR, Zika, virus

OVERVIEW: WHAT IS ZIKA AND HOW DID IT BECOME EPIDEMIC?

Zika virus (ZIKV), the causative agent of the infectious disease Zika fever, is a positive-sense RNA virus that belongs to the family *Flaviviridae*, genus *Flavivirus*, and is similar to Dengue virus (DENV), yellow fever virus, Japanese encephalitis virus, and West Nile virus (Sikka et al., 2016). ZIKV was first isolated from *Rhesus macaques* in Uganda in 1947. Previously, only sporadic cases of negligible concern associated with human ZIKV infection were reported (Hayes, 2009). Now, ZIKV infections have become epidemic throughout the world (Charrel et al., 2016).

In the north-eastern states of Brazil, the public health authorities recently confirmed autochthonous transmission of ZIKV with the first known reported case of ZIKV infection in mainland South America (Campos et al., 2015; Zanluca et al., 2015), followed by 26 countries, including countries in the European Union and the outermost regions of the Americas, such as Barbados, Bolivia, Brazil, Colombia, Costa Rica, Curacao, Dominican Republic, Ecuador, El Salvador, French Guiana, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Nicaragua, Panama, Paraguay, Puerto Rico, Saint Martin, Suriname, the US Virgin Island, and Venezuela (Pan American Health Organization [PAHO], 2016; World Health Organization [WHO], 2016). An increased frequency of ZIKV infection among world travelers has been reported in European countries, including Austria, Denmark, Finland, France, Germany, Ireland, Italy, Portugal, the Netherlands, Spain, Sweden, Switzerland, and the UK (European Centre for Disease Prevention [ECDC], 2016).

The virion of ZIKV consists of an approximately 11 kb positive-sense RNA with a single capsid and two membrane-associated envelope proteins (M and E) (Leyssen et al., 2000; Daep et al., 2014; Charrel et al., 2016). Recent outbreaks of ZIKV infections have become fatal on a daily basis in the Americas, where this obscure viral candidate has been placed at the forefront of global healthcare. The reported occurrences of ZIKV infections are thought to be transmitted mainly by the mosquito species *Aedes aegypti* and *Aedes albopictus*. Infections have now dramatically increased in highly populated areas of South, Central, and North America due to the increased frequency of the international travel from Zika-infected areas (Bogoch et al., 2016). Considering the calamity of ZIKV infection, there is an urgent need to develop rapid detection methods for ZIKV along with DENV, which shares common clinical symptoms with ZIKV. The purpose of this review is to provide a complete update of the various analytical methods for virus detection, such as molecular, immunological, sensor-based and other detection assays, along with the advantages and limitations of these strategies. Furthermore, we suggest innovative hypothetical approaches for the development of liposome-based rapid detection assays for ZIKV detection, which will provide new insight to medical professionals for controlling this widespread epidemic virus candidate.

DIAGNOSTIC FEATURES AND CHALLENGES

ZIKV, an emerging flavivirus, shares common clinical symptoms with DENV and chikungunya virus (CHIKV). The outbreaks caused by these viruses present a large number of diagnostic challenges. The clinical manifestations of ZIKV involve similar clinical symptoms to DENV and CHIKV, which include fever, exanthema, conjunctivitis, retro-orbital headache, and arthralgia (Cardoso et al., 2015). The diagnosis of viral infection has specific management implications for medical personnel. The identification of DENV requires a routine follow-up to examine thrombocytes along with hematocrit, whereas for CHIKV,

chronic arthralgia should be assessed due to its high prevalence. In the case of ZIKV, a detailed diagnosis of sexual and maternal-fetal transmission should be performed to confirm the risk of congenital microcephaly in newborn babies (Fauci and Morens, 2016). A variety of arboviral infections (arthropod-borne; DENV is the most common arboviral infection) may have similar clinical presentations; therefore, their circulation may be under-reported if specific diagnostic tools have not been implemented. However, there are several drawbacks in ZIKV diagnosis due to the lack of availability of diagnostic tools and the frequent cross-reactivity of antibodies between flaviviruses, which have resulted in several limitations in the use of serology (Musso et al., 2015). Commonly, no routine testing of virus cultures is performed, and an antigenic detection test is lacking at present (Musso et al., 2015; Saiz et al., 2016).

The symptoms of ZIKV infection usually tend to be mild, and the initial symptoms can escape notice, reducing the opportunity to collect a sample. Although the viremic period has not been completely defined, viral RNA has been detected in serum after the onset of symptoms up to day 10. In addition, RNA particles of ZIKV have been detected in urine over an extended period in the acute phase, leading to the possibility of considering an alternative sample type. Evidence suggests that serum samples should be taken during the first 5 days after the onset of symptoms supported in some more detailed studies (Musso et al., 2015). Symptoms of microcephaly associated with ZIKV during the development of newborns in the uterus have been reported (Oduyebo et al., 2016). For the diagnosis of infant microcephaly, a complete analysis of head circumference is requested (Kallen, 2014), as the diagnostic parameters for severe microcephaly include a head circumference more than 3 standard deviations below the mean (Von der Hagen et al., 2014). Testing should be performed in pregnant women with positive or inconclusive results from ZIKV testing. If diagnostic parameters confirm possibility of congenital ZIKV infection in an infant, further clinical evaluation should be performed in follow-up. Fever is a common presenting symptom in patients testing positive for arboviruses due to their association with multiple illnesses; hence, it is suggested to eliminate differential diagnoses (Kelsner, 2016). Patients with DENV and ZIKV present with temperatures $>40^{\circ}\text{C}$ and $<38.5^{\circ}\text{C}$, respectively. ZIKV is usually self-limiting, with symptoms lasting 2 to 7 days. Jaundice is a distinguishing clinical presentation of yellow fever virus and can aid in identifying patients with ZIKV virus. The presence of nausea, vomiting, and bleeding may be helpful in identifying DENV. Any of the above symptoms in an individual who has been exposed to ZIKV indicates the possibility of ZIKV infection, and immediate serum testing should therefore be performed (Centers for Disease Control and Prevention [CDC], 2015a,b).

AVAILABLE DETECTION METHODS FOR ZIKV

Diagnosis of ZIKV in the laboratory is usually based on the serum analysis employing viral RNA or antibody-based detection assays (Al-Qahtani et al., 2016; Haug et al., 2016). However,

molecular technique-based assays are considered more reliable due to the cross-reactivity of IgM antibodies among the candidate flaviviruses during serological analysis (Haug et al., 2016). Consistent efforts are being made regarding the development of various detection strategies for virus detection (Figures 1 and 2). However, there is still a need to search for and improve upon more sensitive and reliable detection methods for the challenging virus candidate ZIKV for daily diagnoses. Developed and applied methods for the detection of ZIKV are listed in Table 1.

Molecular Methods

Molecular detection of viruses is a two-step process that includes virus detection in propagated cell culture and viral genome-based detection through molecular amplification using polymerase chain reaction (PCR)- or real-time PCR-based detection methods. In propagated cells, the detection of a virus is confirmed by its cytopathic effect in cell culture, and positive results are confirmed using tissue culture with an infectious dose 50 (TCID₅₀) in plaque assays (Hamza et al., 2011). During the last several years, there has been

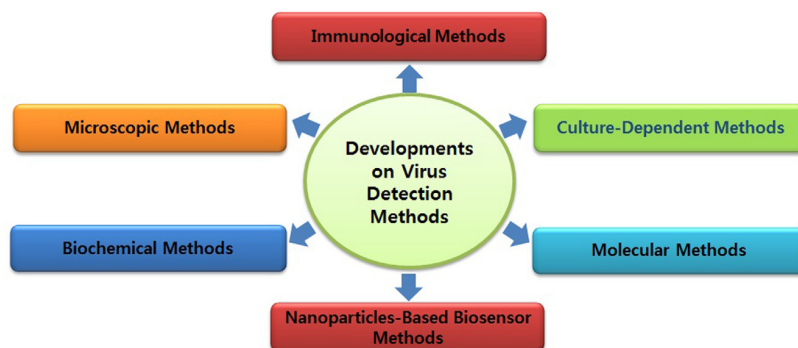


FIGURE 1 | Developments in various pathogen detection strategies.

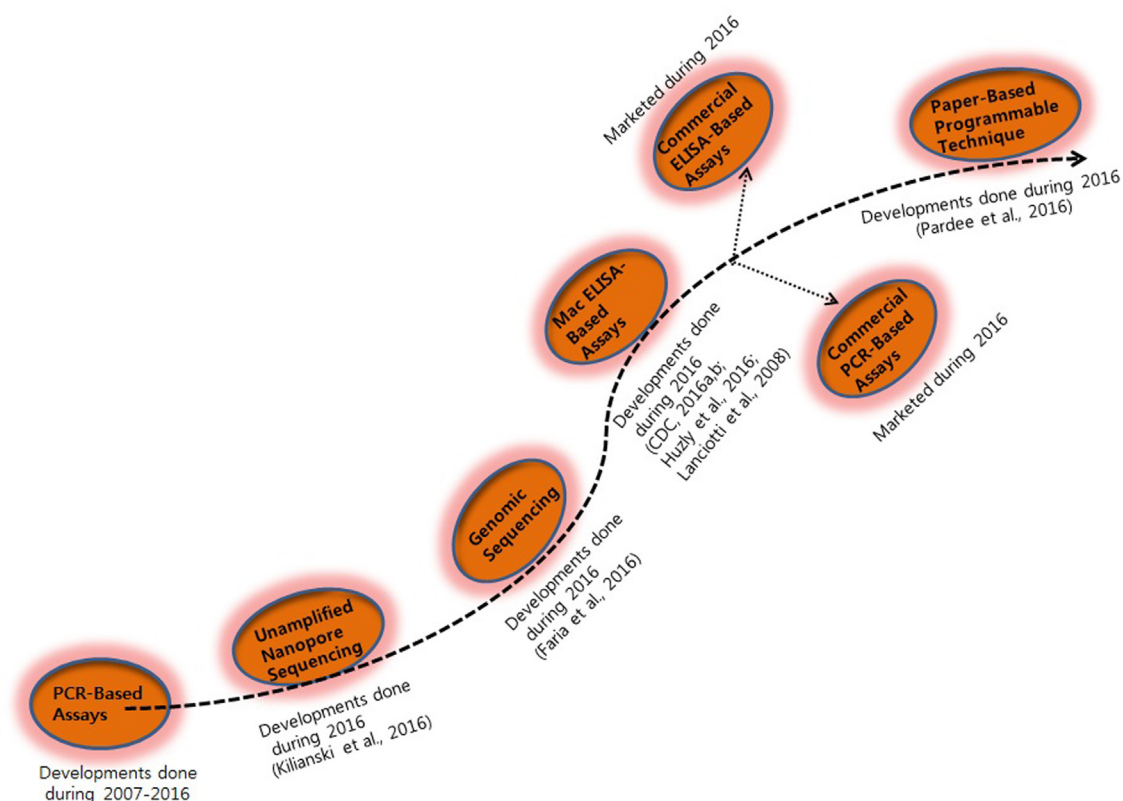


FIGURE 2 | Consistent major year-wise efforts in the development of detection assays for Zika virus.

TABLE 1 | Methods available and applied for Zika virus detection.

Method	Sample analyzed	Virus strain	Detection limit	Reference
Real-time reverse transcription-PCR	Serum specimens	Flavivirus/Zika virus (ZIKV)	Tested for positive/negative samples	Lanciotti et al., 2008
Real-time reverse transcription-PCR	Serum specimens	Flavivirus/ZIKV	Tested for positive/negative samples	Lanciotti and Tesh, 2011
One step real-time reverse transcription-PCR	Human serum	ZIKV	0.05 plaque forming unit (pfu) in less than 3 h	Faye et al., 2013
Viral metagenomic next-generation sequencing	Amniotic fluid samples	ZIKV	Tested for positive/negative samples	Calvet et al., 2016
Complementary DNA synthesis followed by PCR	Human serum	ZIKV	33.7 pfu/mL	Faye et al., 2008
Recombinant polymerase amplification assay	Virus culture	Dengue virus (DENV)	Analytical sensitivity between 10^5 and 10^3 RNA molecules	Wahed et al., 2015
Real-time-PCR	Virus cell culture/Human serum	Flavivirus/ZIKV	140 copies viral RNA/reaction	Balm et al., 2012
Multiplex real-time reverse transcription-PCR	Virus cell culture/Human serum	Flavivirus/ZIKV/DENV/Chikungunya virus	10^8 to 10 copies/ μ L for ZIKV and from 10^8 to 10^2 copies/ μ L for Dengue-1	Waggoner et al., 2016
Real-time-PCR	Saliva/Blood	ZIKV	Tested for positive/negative samples	Musso et al., 2015
Genomic sequencing	Serum/Blood/Amniotic fluid/New born babies	ZIKV	Tested for positive/negative samples	Faria et al., 2016
Paper-based synthetic gene networks	Virus cell culture (ZIKV RNA genome)/Plasma samples	ZIKV	1.7×10^6 copies/mL	Pardee et al., 2016
Zika-specific reverse transcriptase-PCR	Human serum	ZIKV	No information available	Charrel et al., 2016
Zika MAC-ELISA	Virus cell culture/Human serum	ZIKV	No information available	Centers for Disease Control and Prevention [CDC], 2016a,b; Lanciotti et al., 2008
Indirect immunofluorescent assay	Human serum	ZIKV	Tested for positive/negative samples	Tappe et al., 2014
Antigen detection and immunoglobulin M capture ELISA	Virus cell culture/Human serum	Mosquitoes transmitted virus/Yellow fever virus	1.0×10^3 pfu/100 mL	Adungo et al., 2016
Real time-PCR	Human urine	ZIKV RNA	Tested for positive/negative test	Gourinat et al., 2015
Instrument-free point-of-care molecular detection (reverse-transcription loop-mediated, isothermal amplification assay)	Virus cell culture	ZIKV	5 pfu	Song et al., 2016
Streptavidin-magnetic nanoparticles coupled oligonucleotide detection based on loop-mediated isothermal amplification	Virus cell culture/Human serum	ZIKV	1 aM synthetic ZIKV oligonucleotide	Tian et al., 2016
Liposome-based detection assay	Virus cell culture	DENV	10 pfu/mL	Baeumner et al., 2002
Multi-analyte single-membrane biosensor	Virus cell culture/Human serum	DENV	50 RNA molecules for serotype 2, 500 RNA molecules for serotypes 3 and 4, and 50,000 RNA molecules for serotype 1	Zaytseva et al., 2004

a revolution in virus detection methods using real-time PCR based assays, which can be performed rapidly and produce specific, sensitive, and reproducible results for virus detection at a very low concentration (Sanchez et al., 2007). All of these parameters are interconnected and depend mostly on the target sequences of primers and probes and provide absolute specificity and a balance between high sensitivity,

broad reactivity, and reliability of quantification. (Seifi et al., 2012).

Polymerase chain reaction-based methods have numerous advantages compared with conventional assay methods, such as rapidity, quantitative measurement, low contamination rates, and easy standardization (Sanchez et al., 2007; Seifi et al., 2012). Although a real-time reverse transcription PCR assay is available

for the detection of Micronesian ZIKV strains in Africa and Asia, it does not cover the genetic diversity and geographic distribution of ZIKV (Hayes, 2009). Current diagnostic techniques for ZIKV infection are time-consuming and are mainly based on specific detection of antibodies or isolation of viruses from animals or mosquitoes (Digoutte et al., 1992; Hayes, 2009; Faye et al., 2013). However, standard PCR-based techniques such as real-time PCR and quantitative real-time PCR provide a rapid, specific, and sensitive method for early detection of ZIKV (Foy et al., 2014). Faye et al. (2013) developed a quantitative real-time PCR assay for the detection of ZIKV in mosquitoes in the field with high analytical sensitivity. It was found that the assay was able to detect 37 ZIKV isolates. These findings confirmed that the use of real-time reverse transcription PCR assays could be a useful tool for the detection of ZIKV in virus pandemic areas where viruses including DENV and CHIKV also occur (Faye et al., 2013).

Calvet et al. (2016) developed a real-time quantitative PCR assay for the detection of ZIKV in pregnant women using viral metagenomics and gene sequencing analysis. The real-time quantitative PCR analysis confirmed that the developed assay was able to detect ZIKV infection in amniotic fluid; however, the same assay showed negative results for the detection of ZIKV infection in urine and serum samples. Escadafal et al. (2014) tested the efficacy of a recombinant polymerase amplification assay for the detection of yellow fever virus using a nucleic acid detection method, which confirmed positive results. Detection of DENV was also confirmed using a recombinase polymerase amplification assay, supporting the suggestion that this assay could be a useful tool for the detection of ZIKV (Wahed et al., 2015). Due to the rapid global increase in ZIKV and concern about the rapid increase of microcephaly cases in newborns, the development of specific and sensitive point-of-care-testing methods has become a major public health priority (Sikka et al., 2016). Because ZIKV shares a common platform of clinical manifestation with DENV and CHIKV, a specific and sensitive one-step real-time PCR assay was tested for the presence of ZIKV in 88 Dengue and Chikungunya-negative sera samples collected from the patients representing with DENV-like illness in Singapore (Balm et al., 2012). The assay displayed specific detection of ZIKV with a low detection limit (140 copies of viral RNA), suggesting that this assay could be useful for viral detection in a variety of environmental samples (Balm et al., 2012). Recently, Waggoner et al. (2016) developed and evaluated a multiplex real-time reverse transcription PCR assay for simultaneous detection of ZIKV. The developed assay improved the detection limit of ZIKV relative to the corresponding real-time reverse transcription PCR assay, and 31 samples were found to be positive for ZIKV (Waggoner et al., 2016). The findings of this study suggested that improved sensitivity for ZIKV detection is needed, given the low viremia detected in clinical samples and the current lack of accurate alternative diagnostics, such as serology (Lanciotti et al., 2008; Musso et al., 2015). Additionally, the developed assay identified 17 co-infections in ZIKV-positive patients, suggesting its potential for application as a multiplex diagnostic test for viral detection.

Furthermore, Kilianski et al. (2016) developed an unamplified RNA/complementary DNA (cDNA)-hybrid

nanopore sequencing assay for the detection of RNA viruses, including Middle East respiratory syndrome, Venezuelan equine encephalitis virus, Ebola, and ZIKV. The developed method was able to detect Venezuelan equine encephalitis virus within 3 hours of acquisition and achieved differentiation from other viral genomes, facilitating strain-level identification. Nanopore sequencing is a novel genomics technology, which has potential applications for routine biosurveillance, clinical diagnosis, and outbreak investigation of virus infections by rapid sequencing of unamplified RNA/cDNA hybrids. Kilianski et al. (2016) sequenced unamplified poly(A)-tailed viral RNA using a rapid cDNA library preparation coupled with real-time data analysis to determine its potential application for pathogen genomic characterization. This approach for pathogen identification and characterization differs from the previously used methods on the MinION platform (Hoenen et al., 2016). Biased techniques, such as amplicon sequencing, have proven to be effective in complex sample backgrounds in which titers of the target pathogen might be low, but such approaches limit characterization to known pathogens and require additional viral genome amplification (Wang et al., 2015; Quick et al., 2016). The great potential of the use of a RNA/cDNA hybrid approach in field studies has been confirmed in western Africa (Hoenen et al., 2016; Quick et al., 2016). Generally, high virus titers in clinical samples are necessary for virus detection before genome sequencing, hence utilizing an RNA/cDNA hybrid approach for genomic ZIKV characterization could be a feasible strategy, especially for genomic library preparation and for reducing the time required for strain-level identification (Faria et al., 2016).

Although PCR-based molecular techniques have aided in the easy detection of a variety of viruses, there are still several limitations to these methods. It has been noted that these methods are prone to miss detections, favoring false negative results and requiring proper quality control measures. However, precautions have been taken to overcome the problem of inhibition, including sample dilution analysis, the use of a small sample size, and adaptation of the PCR assay employing chemical reagents such as Tween (Al-Soud and Radstrom, 2001; Rutjes et al., 2005; Butot et al., 2007). Another limitation of PCR-based methods is their inability to differentiate between infectious and non-infectious viruses. Although several approaches have been used to overcome this limitation, the utilization of integrated systems based on the molecular detection of viruses after cell culture infection is considered one of the most efficient methodologies (Reynolds et al., 2001; Rodriguez et al., 2009). Additionally, integrated cell culture-PCR assays allow rapid virus detection with selective enumeration of infectious viruses, though the long incubation period required to reveal a cytopathic effect is a major limitation.

Paper-Based Synthetic Gene Networks for ZIKV Detection

The aim of synthetic biology focuses on re-engineering of the molecular components of cells to exploit the power of biology for which molecular biologists have developed whole-cell

biosensors, synthetic probiotic candidates, new drug sources, green technology, and chemical resources (Zhang et al., 2012; Torella et al., 2013; Fossati et al., 2014; Kotula et al., 2014). As recently reported, paper disk diagnostic tools show significant efficacy as rapid and low-cost screening tools for the screening of blood, urine, and saliva samples for specific identification of virus strains. The assay methodology is based on the color change of a paper disk to purple, indicating the presence of virus, or yellow, in the absence of virus. Recently, Pardee et al. (2016) reported some biotechnological parameters that dramatically lower costs and technical barriers in the development of synthetic biology-based diagnostics. In this regard, programmable RNA sensors referred to as “toehold switches” could be the first technology to be rationally applied to bind virtually any sense RNA sequence (Green et al., 2014). Second, a freeze-dried, paper-based, cell-free protein expression platform allows the application of these toehold switch sensors outside of laboratories by providing an abiotic, sterile method for the distribution and storage of genetic circuits at room temperature (Pardee et al., 2014). Concerning the above findings, combination of these techniques could provide a rapid and inexpensive platform for the development of easy-to-use virus diagnostic sensors. In the context of the ZIKV outbreak, paper-based sensors offer a solution to the critical challenges involved in diagnosis of the virus. Standard serological methodologies involving antibody detection present limitations due to their cross-reactivity with other flaviviruses detected in previously infected patients; thus, nucleic acid-based and isothermal nucleic acid amplification detection methods are recommended for accurate detection of viruses (Lanciotti et al., 2008; Tappe et al., 2014; Zammarchi et al., 2015). However, such techniques are relatively expensive, require technical expertise to run and interpret, and involve equipment that is incompatible with use in remote and low-resource locations, where surveillance and containment are critically needed.

Immunoassays

The current ZIKV outbreak represents a great threat specifically for pregnant women. Because the antibody response during the pregnancy stage may differ from that in non-pregnant individuals, more precautions should be taken when determining the ZIKV immune responses in pregnant women. In flavivirus infections, immunoglobulin M (IgM) antibodies typically develop within a few days after the onset of illness and can generally be detected up to three months (Charrel et al., 2016). In addition, immunoglobulin G (IgG) antibodies are developed within a few days after development of IgM antibodies and can be detected for several months.

Recently (February 26, 2016), the Centers for Disease Control and Prevention (CDC) requested that a letter be issued authorizing the emergency use of the Zika IgM antibody capture enzyme-linked immunosorbent assay (Zika MAC-ELISA) for the presumptive detection of ZIKV-specific IgM in human sera or cerebrospinal fluid (Centers for Disease Control and Prevention [CDC], 2016b). The Zika MAC-ELISA is employed for *in vitro* qualitative detection of ZIKV-specific IgM antibodies in human sera. In addition, testing of ZIKV has been applied based on CDC

clinical and epidemiological criteria for ZIKV which include clinical signs and symptoms associated with ZIKV infection and/or history of residence in or travel to a geographic region with active ZIKV transmission at the time of travel or other epidemiologic criteria that may indicate ZIKV testing (Centers for Disease Control and Prevention [CDC], 2016a). As reported previously, immune responses under ZIKV infection have only been described in a small number of patients ($n = 11$), during the ZIKV virus outbreak in Yap (Lanciotti et al., 2008). When MAC-ELISA for IgM and capture ELISA for IgG with the whole viral antigen (inactivated virus) and monoclonal antibodies (MAbs) were applied, IgM was found to appear as soon as 3 days after the onset of symptoms, while IgG appeared after 10 days in a patient with no history of previous flavivirus infections (Johnson et al., 2000; Martin et al., 2000). Subsequently, it became possible to detect neutralizing antibodies against ZIKV as early as 5 days after the onset of fever. Euroimmun (2016) has developed the first complete test package for the serological detection of ZIKV infections. The ELISAs and indirect immunofluorescence assays allow the determination of specific antibodies (IgM, IgG) against a variety of viruses in the blood of infected patients.

Huzly et al. (2016) reported that the use of putative cross reacting sera in ELISA tests from patients with Euroimmun anti-ZIKV IgG and IgM antibodies against ZIKV showed high specificity, confirming the applicability of Euroimmun ELISA for specific detection of virus in patients exposed previously to flavivirus or vaccine. The results suggest that this ELISA method could be an effective diagnostic tool for the screening and counseling of patients with a possibility of ZIKV virus infection, especially pregnant women and travelers commuting from ZIKV-endemic regions (Huzly et al., 2016). To confirm the ability of the ZIKV ELISA to detect ZIKV antibodies, Tappe et al. (2014) analyzed serum samples collected from 10 patients in Brazil suffering from acute ZIKV infection. Laboratory results confirmed that the indirect immunofluorescent assay was able to define immunofluorescent assay titers for anti-ZIKV IgM (1: 1,280 to 1: > 20,480) and anti-ZIKV IgG (1: 320 to 1: > 20,480) (Tappe et al., 2014). These samples were previously confirmed to be negative for IgM and IgG against DENV and for the DENV nonstructural protein-1 (DENV NS1) antigen.

Along with ZIKV and DENV, yellow fever is another acute viral infection transmitted through mosquito bites. It is strongly believed that the current emergence and re-emergence of yellow fever and other arboviruses is partly attributed to the increased migration of people from disease-endemic regions and the expanding establishment of the vector. The use of flavivirus antigens in the development of MAbs and diagnostic tests has not been well documented (Vazquez et al., 2009; Nunes et al., 2011; Okamoto et al., 2012; Kwallah et al., 2013; Escadafal et al., 2014). The application of MAbs in immunoassays offers the advantage of high specificity, due to their ability to bind to specific epitopes of antigens. This specific binding is useful in reducing the incidence of cross-reactivity, which is reported to negatively impact the use of many serological kits (Modis et al., 2003, 2005). Adungo et al. (2016) also reported the development, characterization, and evaluation of MAbs to yellow fever virus. Diagnostic application of these MAbs was tested in antigen detection and

IgM capture ELISA. The results suggested that MAb-based antigen detection ELISA enabled the detection of virus in 40 culture supernatants containing titers of approximately 1,000 plaque forming units (pfu). Concerning the above findings, the developed approach can be applied for the development of improved detection kits for ZIKV. One of the limitations of this test is the possibility of false positive results in patients with a history of infection with other flaviviruses. Additional testing of equivocal and positive specimens and/or other patient-matched specimens, as specified in the CDC-issued algorithm, is therefore required to confirm ZIKV infection (Centers for Disease Control and Prevention [CDC], 2016a).

Magnetic Nanoparticles-Based Assays

Zika fever is a zoonotic infection caused by ZIKV, which shares the limelight with other well-known members of the flavivirus family, such as DENV, yellow fever virus, West Nile virus, and Japanese encephalitis virus (Tilak et al., 2016). Consequently, there is an urgent need to develop effective methods for the surveillance of ZIKV. As ZIKV and DENV share sequential homology, there is a high possibility that there may be detection principles and vaccine development strategies that are applicable to both viruses with some modifications.

One approach that could be useful for the detection of DENV involves concentrating virus particles using ultracentrifugation and polyethylene glycol-mediated precipitation. Although these methods have been used successfully for variety of viruses, ultracentrifugation shows numerous practical limitations, such as the great amount of time required, which may increase the false-positive rate when applied for PCR analysis (Roth et al., 1999). Regarding polyethylene glycol-mediated precipitation, although it is simple and easy to perform, it interferes with subsequent PCR analysis (Novotny et al., 1992). A possible alternative to these methods could be application of magnetic beads coated with molecules that efficiently bind to the virus. These beads allow viral particles to be captured and assist in the concentration of viral particles through the application of magnetic field.

A number of magnetic nanoparticles, such as iron, nickel, or cobalt, have been successfully used for biomedical and environmental applications due to their highly/specific surface area and the ease of the magnetic collection of target materials adsorbed by the magnetic nanoparticles (Safarikova and Safarik, 2001; Pankhurst et al., 2003). However, these beads generally exhibit inherent chemical instability, limiting their application in the fields of biological and environmental science (Saraswati et al., 2012). To overcome this limitation, magnetic nanoparticles have typically been encapsulated with a protective shell of graphite, silica, or polymer for chemical stability (Saraswati et al., 2012). Similarly, Sakudo et al. (2016) developed a novel technology for the sensitive detection of DENV using graphite-encapsulated magnetic nanoparticles conjugated with anti-DENV antibody. Their assay method demonstrated that antibody-integrated magnetic beads were useful for capturing DENVs. The capturing of DENV1-4 using antibody-integrated magnetic beads was confirmed by the results of the real-time PCR analysis, showing that the captured fraction contained

DENV genomic RNA (Sakudo et al., 2016). Therefore, this method may be used in combination with real-time PCR for the detection of DENV and may increase the sensitivity of viral detection for the diagnosis of DENV. Based on these findings, it can be hypothesized that this methodology could be extended for the accurate detection of ZIKV through the immobilization of an anti-ZIKV antibody on the functionalized surface of graphite magnetic nanoparticles. The modified graphite magnetic nanoparticles can then be assessed for their ability to capture ZIKV, and the concentrated virus can subsequently be detected through the application of PCR-based amplification procedure.

In the case of hydrophobic graphite-encapsulated magnetic nanoparticles, which present limitations in various biomedical applications, the particles can be modified appropriately to achieve improved binding properties, allowing them to efficiently recognize and bind to molecular targets, including antibodies, antigens, and receptors (Poplawska et al., 2014). Improvement of amino group functionalization can increase the reactivity and hydrophobic nature of graphite particles with the desirable functionality of graphite (Saraswati et al., 2012). A promising method for the amino functionalization of magnetic graphite nanoparticles could be to use inductively coupled radiofrequency plasma, an environmentally friendly method that requires a short duration for the reaction to reach completion, thus effectively introducing amino groups (Saraswati et al., 2011). Furthermore, alteration of plasma discharge conditions can help to optimize the degree of surface derivatization with the amino groups of graphite magnetic nanoparticles. With these modifications of graphite magnetic nanoparticles, efficient surface immobilization of antibodies against various pathogens, including anti-influenza virus and anti-*Salmonella* antibodies, could be achieved (Sakudo et al., 2015).

Recently, Tian et al. (2016) developed an attomolar ZIKV oligonucleotide detection method based on loop-mediated isothermal amplification and susceptometry. It was demonstrated that hydrodynamic volumes of streptavidin-magnetic nanoparticles were dramatically increased after a successful loop-mediated isothermal amplification reaction. The hydrodynamic volumes are probed as Brownian relaxation frequency shifts, which can be employed to quantify the ZIKV oligonucleotide. The proposed detection system can recognize 1 aM synthetic ZIKV oligonucleotide in 20% serum with a total assay time of 27 min. The results suggested that this could be a promising strategy that may give rise to new possibilities for diagnosing and controlling ZIKV infection.

With the increasing incidence of Dengue infection in developing countries where DENV is endemic, the rapid applicability of diagnostic tests is required for disease control. Several immunological approaches have been applied for laboratory diagnosis of DENV infection. These methods include detection of the virus (by cell culture and immunofluorescence-based), detection of virus antigen (by ELISA), detection of anti-DENV antibody (by hemagglutination inhibition, complement fixation test, and neutralization tests), and detection of virus nucleic acid (by real-time reverse transcription-PCR). The

hemagglutination inhibition is the most widely accepted serological technique in developing countries; however, ELISA has become the technique that is most often applied for the serological diagnosis of DENV infection in DENV epidemic regions due to its stability, simple handling procedure, and no need of any complicated equipments.

Developments in the Liposome-Based Detection of ZIKV/DENV

The characterization and detection of viruses is a labor-intensive and time-consuming process. Upon the emergence of new viral pathogens with a high frequency and catastrophic outbreaks, there is always a need for improved detection and identification methods to control the proliferation of these pandemic viral candidates. Currently, at laboratory scale, only PCR-based molecular techniques provide accurate detection of a variety of viruses based on trial-and-error methods using a cell culture-based procedure. PCR-based methods require thermal cycling instruments and transformation of DENV/ZIKV genomic RNA to DNA (Kow et al., 2001; Zaytseva et al., 2004). Zaytseva et al. (2004) developed a multi-analyte single-membrane biosensor for the serotype-specific detection of DENV, which showed 92% reliability in DENV serotype determination. The assay exhibited a detection limit of 50 RNA molecules for serotype 2; 500 RNA molecules for serotypes 3 and 4; and 50,000 RNA molecules for serotype 1, following isothermal amplification of the target sequences. The developed biosensor can be considered as an applicable portable, inexpensive, and easy to use tool, representing an alternative to current detection methods based on more expensive and time-consuming methods, such as ELISA or tissue culture.

Although these techniques are readily available, they are expensive and present engineering challenges regarding miniaturization and field utilization. In addition, PCR products, which consist of double-stranded DNA, must be denatured before being subjected to a probe hybridization-based detection method (Baeumner et al., 2002). In contrast, biosensors based on liposome technology have been successfully used for the development of rapid, inexpensive, and field-utilizable detection systems with potential for the detection and quantification of RNA molecules (Esch et al., 2001). We provide an update on the development of liposome-based detection methods for DENV or ZIKV, as the two viruses share majority of their genetic information. Liposome-based immunoassays show applicability in various areas of food chemistry, food microbiology, nanobiotechnology and diagnostic or clinical microbiology, for the detection of foodborne pathogens, toxins, and hazardous components in a variety of environmental and human samples. Liposome immunoassays involve a liposome-encapsulating marker, prepared with a phospholipid composition and coupled to either an analyte or antibody through a suitable procedure (Shukla et al., 2012). There have been various recent advances in methods related to liposome immunoassays, including immunoliposome (liposome coupled to antibody)-based ELISA, immunoliposome-based magnetic separation, and liposome-based immunochromatographic strips used in various pathogen detection applications. Baeumner et al. (2002) developed a

liposome biosensor-based method for the detection of DENV that was sensitive, rapid, and serotype specific. The biosensor assay was based on utilizing a membrane-based DNA/RNA hybridization system with liposome amplification. The generic DNA probe was coupled to dye-encapsulating liposomes, and conserved or DENV serotype-specific probes were immobilized on a polyether sulfone membrane strip. Liposomes were mixed with the amplified target sequence and then applied to the membrane. The mixture was allowed to migrate along the test strip, and the liposome target sequence complexes were immobilized in the capture zone via hybridization of the capture probe with the target sequence. The amount of liposomes present in the immobilized complex is directly proportional to the amount of target sequence present in the sample and can be quantified using a portable reflectometer (Baeumner et al., 2002). Connelly et al. (2012) also developed a method involving microfluidic pre-concentration coupled to liposome-based signal amplification to efficiently detect viruses in environmental water and clinical samples. Subsequently, an electrochemical liposome-based assay was developed to facilitate direct detection of virus-liposome-bead complexes using a magnet (Connelly et al., 2012). The integrated current signals from the lysis of captured liposomes resulted in detection of the viral titer in an environmental sample. Although a number of established liposome-based methods are available for the detection of different viruses in various samples, no specific and sensitive liposome-based detection method has been developed for ZIKV.

Liposome-Based Detection Assays in Medical/Clinical Use

A number of liposome-based assays are also being used in biomedical and therapeutic applications as clinical diagnostic assays. For pathogen detection, liposome-based lateral flow assays coupled with nucleic acid sequence-based amplification have been proved to represent an effective alternative for the rapid and sensitive detection of viable bacterial pathogens (Hartley and Baeumner, 2003; Baeumner et al., 2004). Edwards et al. (2010) developed a fluorescent dye-encapsulated liposome tagged with DNA aptamers and used it for the detection of alpha-thrombin via an aptamer-based assay in human plasma. The advantages of DNA aptamers over antibodies include stability; production via inexpensive, consistent chemical synthesis; ease of modification in terms of both labeling and selective changes in the sequence to achieve affinity selectivity and enhanced stability; potential for regeneration; smaller size; and production against toxic or poorly immunogenic targets (Tombelli et al., 2005; Balamurugan et al., 2008). In these assays, dye-encapsulating liposomes are used for signal enhancement, providing extremely low detection limits in the assays. In addition to fluorescent markers and enzymes, liposomes can be used to encapsulate a variety of electrochemical markers. Feng et al. (2010) and others (Sofou and Sgouros, 2008; Sajja et al., 2009) developed and demonstrated the utility of a bifunctional immunoliposome system targeting glioma cells both *in vitro* and *in vivo*, providing the potential for drug delivery and imaging in tumors. Lehtinen et al. (2012) also reported an immuno-targeting system involving liposomal drug carriers to

treat ovarian carcinoma. Additionally, immunoliposomes have been used to deliver contrast agents and radionuclides for diagnostic imaging and therapy (Torchilin, 2000; Erdogan et al., 2008). Semiconductor quantum dots conjugated to liposomes are used for imaging and the immunoliposomes have successfully been observed *in vitro* and *in vivo* (Weng et al., 2008).

Under this subtitle, we intended to elucidate the variety of ways in which liposomes have been used as analytical reagents to date. These methods relying on a tagged liposome could provide numerous ways for signaling molecules to be released to provide a signal. Therefore, it can be stated that these liposomes/immunoliposomes show potential for application in various industrial and medical sectors, including for virus detection through liposome immunoassays.

COMMERCIALLY AVAILABLE TEST KITS FOR ZIKV DETECTION

As a future consideration, commercial serology tests will become increasingly available for the detection of a variety of viruses. To our knowledge, there are few commercial tests that are expected to hit the commercial market in very near future. ZIKV IgG or IgM ELISA kits based on utilizing a double-antigen sandwich ELISA will be introduced by MyBiosource in USA. However, no information on the type of antigens involved or the specifics of validation for determining cross-reactivity is available. Biocon Diagnostics in Canada offers a rapid finger-prick assay based on a mixture of ZIKV NS1 and envelope protein that can detect IgM and IgG viral antibodies. The company indicates a specificity of 99%, but no specifications regarding the validation procedure are given. Euroimmun in Germany offers an IgM/IgG immunofluorescence assay and an IgM/IgG ELISA based on a NS1 protein. The ZIKV immunofluorescence assay is offered in a mosaic slide, together with assays for DENV1-4 and CHIKV. The provided information indicates cross-reactivity with antibodies directed against tick-borne encephalitis virus, West Nile virus, and DENV for both the IgG and IgM assays (Charrel et al., 2016). Furthermore, validation data indicate a wide range of specificities and sensitivities for the IgM and IgG ZIKV immunofluorescence assays depending on the validation cohort. However, the provided values are difficult to interpret as

the description of the cohorts is too brief. In addition, these data should be interpreted with great caution as positivity was only rated in the cut-off dilution. This could mean that the specificity can be different than given (higher) as the results were not scored as end-titers. The use of end-titers would provide a window for differentiating the cross-reactivity measured. The Euroimmun ZIKV ELISA is based on recombinant NS1 protein which leads to a reduction of cross-reactivity with other flavivirus antibodies to maximal values of 18.8% (IgG) and 8.3% (IgM). Euroimmun appears to be the only manufacturer that is actually providing detailed validation data, and its data clearly address and illustrate the above-mentioned difficulties with cross-reactivity in flavivirus sero-diagnostics (Euroimmun, 2016).

Musso et al. (2015) performed a molecular detection of ZIKV in saliva samples after RNA extraction using NucliSENS® easyMAG® System (BioMérieux) according to manufacturer's recommendations. Extracted RNA was used for further real-time PCR detection system using two real-time primers/probe amplification sets specific for ZIKV (Musso et al., 2015). The analysis confirmed 210 positive results from 748 blood samples (28.1%) and 182 positive results from 319 saliva samples (57.1%) as the overall number of real-time PCR-positive ZIKV samples, suggesting that ZIKV RNA was more frequently detected in saliva samples than blood samples (Musso et al., 2015). Updated information on newly developed commercial detection kits for ZIKV detection is given in Table 2.

RECENT RESEARCH BASED ON A SPECIFIC HYPOTHESIS

The limitations of current diagnostic tests for ZIKV have been described previously. These considerations highlight technical challenges in test development and the limits of our scientific understanding of the virus at this stage of the ZIKV response. In our previous work, we reported polyclonal antibody production, purification, and applicability in various liposome-based assays (Shukla et al., 2012, 2016). There are currently no available studies on liposome-based assays for the detection of ZIKV, and only few such studies on DENV are available, which were based on liposomes. Considering the various assays developed for DENV and other viruses, we propose a strategy for developing

TABLE 2 | Update on commercially available detection kits for Zika virus.

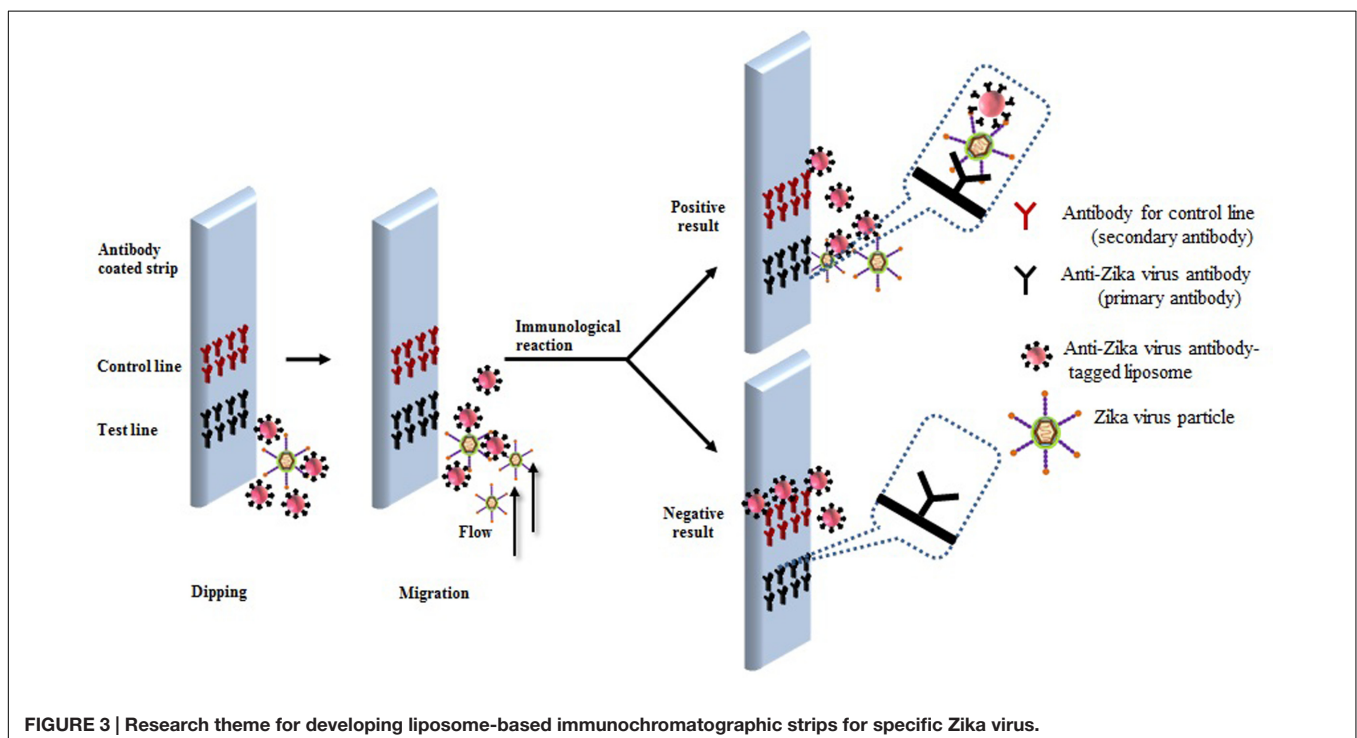
Commercially available detection kits	Company
Immunoglobulin G (IgG) and immunoglobulin M (IgM) ELISA kit for Zika virus (ZIKV)	MyBiosource (USA)
Rapid finger prick assay kit (based on ZIKV nonstructural protein-1 and envelope protein)	Biocon Diagnostics (Canada)
IgM and IgG immunofluorescence kit for ZIKV	Euroimmun Diagnostics (Germany)
ZIKV IgM ELISA, Mac ELISA kit for ZIKV	InBIOS Diagnostics (USA)
Real star ZIKV real-time polymerase chain reaction kit	Altona Diagnostics (Germany)
Immunochromatographic test strips for ZIKV	Tanaka Diagnostics (Japan)
Fast-Track diagnostics ZIKV test kit based on-multiplex real-time polymerase chain reaction by TaqMan technique	Fast-Track Diagnostics (Malta)
ZIKV/DENV test kit based on RNA, qualitative real-time Reverse Transcriptase Polymerase Chain Reaction and ZIKV antibody (IgM), MAC-ELISA	Quest Diagnostics (USA)

a liposome-based assay for the detection of ZIKV. Thus, a specific liposome-based assay can be applied with our developed liposome particles to develop a liposome-based detection system for analyzing ZIKV in clinical samples using anti-ZIKV antibody.

In several reports, we have described the principle of various liposome-based assays for detecting pathogens (Shin and Kim, 2008; Shukla et al., 2012, 2016). Liposome immunoassays involve a liposome-encapsulating marker (sulforhodamine B dye) and are prepared with a phospholipid composition, coupled to either an analyte or antibody through a suitable procedure (Shukla et al., 2012, 2016), after which the assay is carried out in a routine manner. The detectable signal of these assays is produced upon the lysis of the liposome and the release of the encapsulated markers. Shin and Kim (2008) reported that as the size of the liposomes increased, a higher fluorescence signal was obtained due to a greater number of sulforhodamine B molecules encapsulated in the liposome. Various advanced methods related to immunoliposome assays have recently been developed, including immunoliposome-coupled ELISA, immunoliposome-coupled magnetic separation, and immunoliposome-based immunochromatographic strips used in various applications.

A predicted model of liposome-based immunochromatographic strips for viral detection is summarized in **Figure 3**. Briefly, the assay will involve an antigen capture zone (test line) on a nitrocellulose membrane strip, which is exposed to the target antigen, consisting of a virus particle in a solution containing immunoliposome. Liposomes are encapsulated in sulforhodamine B and tagged with anti-ZIKV antibody specific for the binding of the specific ZIKV particle. In the presence of the ZIKV particle, the immunoliposome

and ZIKV particle complex migrates along the test strip via capillary action and subsequently binds to the capture antibody zone of the membrane, producing a purple band. However, some of the immunoliposomes do not bind the ZIKV particles at the test line and continue to migrate and bind to the control line of membrane, resulting in the development of a purple band, showing a positive result for the presence of ZIKV particles (**Figure 3**). In the absence of ZIKV particles in test samples, the immunoliposomes are not able to bind to the specific antigen (ZIKV) and they therefore migrate and bind at control line of the test membrane, leading to the development of a purple line in the control line only, confirming a negative result for ZIKV particle detection (**Figure 3**). In addition, there is another simple liposome-based detection strategy utilizing a liposome immunoassay, in which detection is measured via the lysis of liposome particles (Shukla et al., 2016). Sulforhodamine B is a self-quenching molecule when encapsulated at a high concentration within lipid and phospholipid vesicles to form sulforhodamine B-encapsulated liposomes. Therefore, the integrity of the sulforhodamine B-encapsulated antibody-tagged liposomes with a target virus particle can be determined by measuring the increase in fluorescence after lysis of the immunoliposome particles (**Figure 4**). Furthermore, there might be another strategy for developing a combined assay involving immunoliposomes and immunomagnetic particles for the sensitive detection of ZIKV particles as shown in **Figure 5**. The assay procedure can be divided into the following three steps: immunomagnetic concentration and separation of target ZIKV; reaction of the immunoliposome particles with virus particles attached to immunomagnetic nanoparticles;



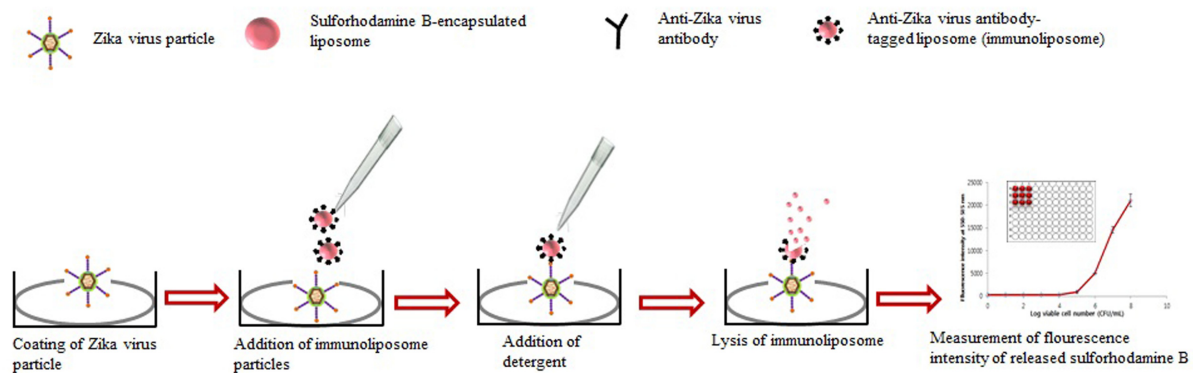


FIGURE 4 | Strategy for developing liposome immunoassay-based fluorescence immunoassay for the detection of Zika virus.

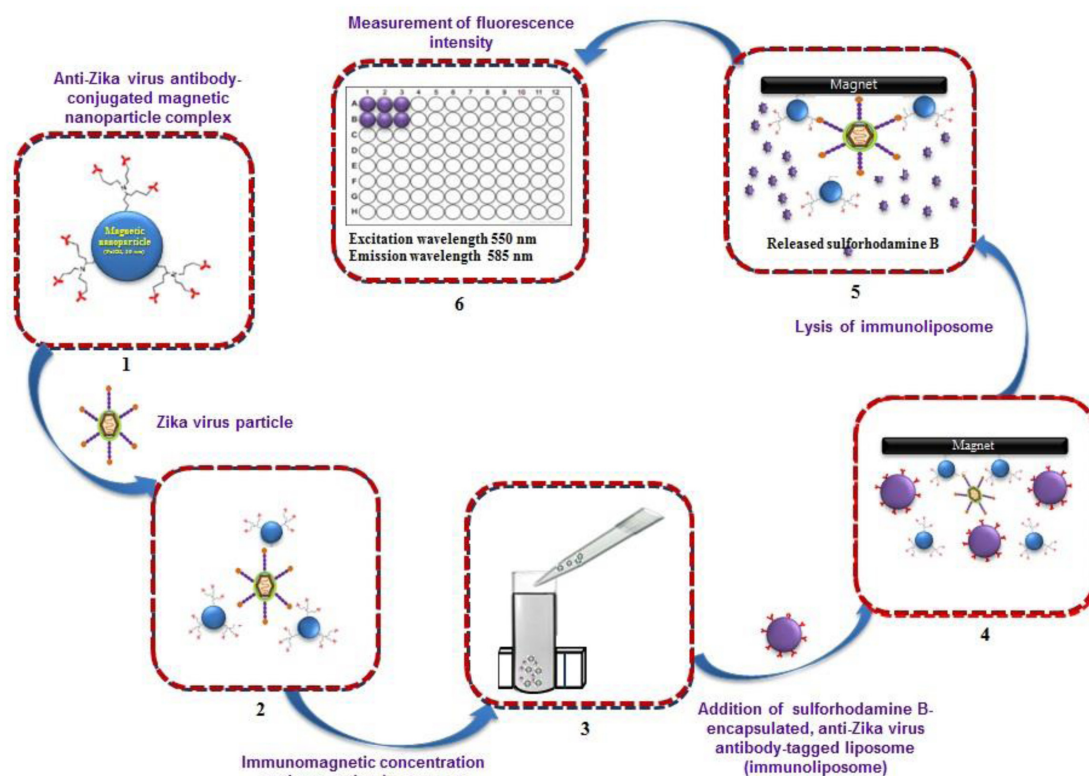


FIGURE 5 | Strategy for developing immunoliposome and immunomagnetic particle-based sensitive detection of Zika virus. (Retrieved from Shukla et al., 2016).

and fluorescence signal generation, as the principle of the developed assay discussed in our earlier research (Shukla et al., 2016).

Another important possibility is the development of liposome-based multiplexing that would allow simultaneous detection of several similar gene sequences of viral pathogens in a single sample, particularly for ZIKV, DENV, and CHIKV. In addition to ZIKV, DENV, and CHIKV, the choice of pathogens for a multiplexed diagnostic tool should consider clinically consistent

presentations. Not all pathogens will be relevant to all settings; nonetheless, the need to differentiate CHIKV, DENV, and ZIKV for both clinical and epidemiologic purposes is of immediate importance and is likely to remain relevant in the future. In multiplexed assays, and particularly those that measure RNA from CHIKV, DENV, and ZIKV, it is important that the analytical sensitivity for ZIKV not be compromised. Possibilities for the interpretation of such multiplexing liposome-based kits, such as those involving immunochromatographic strips, are illustrated

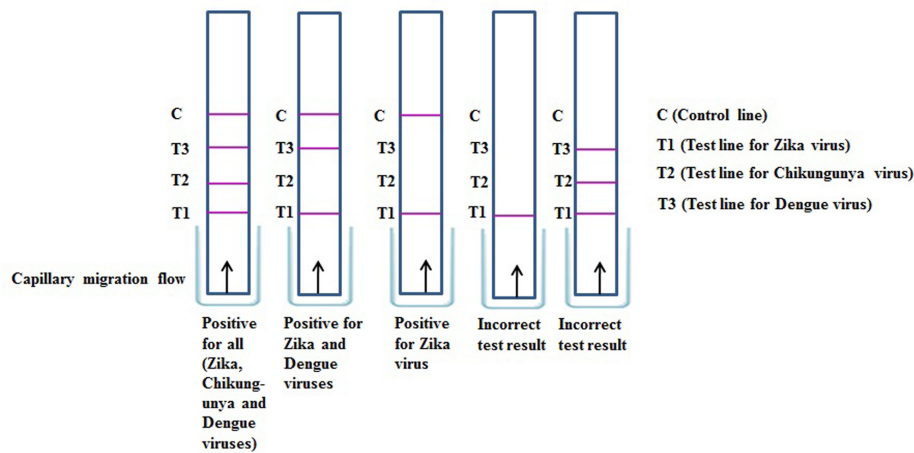


FIGURE 6 | Result interpretation of immunochromatographic strip assay for simultaneous detection of Zika, Chikungunya, and Dengue viruses.

in **Figure 6**. To obtain accurate results in multiplexing assays that may be based on liposome-based immunochromatographic strips, the development of a common antibody against ZIKV, DENV, and CHIKV might provide useful results. Multiplex tests should be able to detect the ZIKV antibodies in cases of concurrent infection with both pathogens, and the analytic limit of detection for ZIKV antibodies should be the same for singleplex and multiplex tests. Multiplexing using dual analytes in a single test (e.g., nucleic acid and immunoassay testing) (Chen and Zhu, 2016) may also greatly improve the diagnosis of acute ZIKV infection. For example, a test that could simultaneously detect both ZIKV RNA and anti-ZIKV IgM would cover the entire time period of acute ZIKV infection and might be particularly useful given the limited reliability of patient self-reporting of the onset of fever and other symptoms.

Although few antibody-based ZIKV detection kits are available, we have presented a potentially easy method for the development of pathogen detection kits based on laboratory-produced antibodies and liposomes, which could provide a cheaper alternative for pathogen detection compared with expensive commercial pathogen detection kits. In the near future, studies should attempt to confirm the practical applications of liposome-based virus detection assays in various food samples contaminated with foodborne viruses, including ZIKV.

SAFETY PRECAUTIONS FOR LABORATORIES WORKING ON ZIKV

Regarding laboratory safety, ZIKV is classified as a biological safety level 2 pathogenic agent. These pathogens must be handled according to the biosafety guidelines of microbiological and biomedical laboratories, and risk assessment should be performed for each laboratory for any specific procedure used in the laboratory. Because of the major concern regarding congenital microcephaly and ZIKV infection, precautions

should be taken by pregnant women while working in the laboratory, and regular ZIKV testing should be maintained in the laboratory. The CDC recommends that each laboratory should perform risk assessment when implementing any new viral methodology in the laboratory as a safety precaution (Centers for Disease Control and Prevention [CDC], 2016a).

CONCLUSION

The widespread ZIKV epidemic in the Americas and Asia has led to the urgent need to develop rapid, sensitive, and specific assays for virus detection with regular monitoring of viral infection. Rapid detection of the virus in field-collected specimens can accelerate the application of appropriate mosquito control measures that could prevent transmission and disease among human populations. Early diagnosis of ZIKV infection, supportive care, symptomatic treatment, and referral of children with microcephaly to specialized care are all necessary measures to improve the neuro-development of affected children. The hypothesis and strategies that we have presented herein provide a relevant platform, emphasizing the importance of liposome-based detection methods in ZIKV diagnosis for improving global health. In overall view, the best advantage of these hypothesized assays is easy handling, no requirement of any sophisticated instruments, rapid detection efficacy, and cost effectiveness than other molecular approaches. On the other side, major limiting factor of these hypothesized assays especially for the detection of viral infections including ZIKV, DENV, and others is their antigen-antibody-based detection ability. Identification of major antigenic and protective epitopes of the target virus particles importantly needed to understand the antibody response while developing these detection strategies. In brief, virus particles can easily go for mutation, and antigenic variations by which an infectious virus alters its surface proteins called antigenic structure eventually result in the differentiation of virus strains.

These antigenic variations make difficulty in the process of specific antibody production, thus, may affect in the developmental process of these diagnostic assays. In conclusion, the present review summarizes and updates the methods available for the detection of ZIKV in human clinical samples. New insights and interpretations should be obtained by studying the mechanisms of several similar detection strategies that are being developed. Innovative approaches based on liposome particles should be of interest for the detection of ZIKV, particularly in relation to previously developed liposome-based strategies, which may provide new insights for future research and strategies for the development of fast and sensitive assays for ZIKV infections.

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AUTHOR CONTRIBUTIONS

MK and SS conceived and designed the review theme. S-YH and SHC helped in writing and reviewing the paper.

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Recent Perspectives on Genome, Transmission, Clinical Manifestation, Diagnosis, Therapeutic Strategies, Vaccine Developments, and Challenges of Zika Virus Research

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One of the potential threats to public health microbiology in 21st century is the increased mortality rate caused by Zika virus (ZIKV), a mosquito-borne flavivirus. The severity of ZIKV infection urged World Health Organization (WHO) to declare this virus as a global concern. The limited knowledge on the structure, virulent factors, and replication mechanism of the virus posed as hindrance for vaccine development. Several vector and non-vector-borne mode of transmission are observed for spreading the disease. The similarities of the virus with other flaviviruses such as dengue and West Nile virus are worrisome; hence, there is high scope to undertake ZIKV research that probably provide insight for novel therapeutic intervention. Thus, this review focuses on the recent aspect of ZIKV research which includes the outbreak, genome structure, multiplication and propagation of the virus, current animal models, clinical manifestations, available treatment options (probable vaccines and therapeutics), and the recent advancements in computational drug discovery pipelines, challenges and limitation to undertake ZIKV research. The review suggests that the infection due to ZIKV became one of the universal concerns and an interdisciplinary environment of *in vitro* cellular assays, genomics, proteomics, and computational biology approaches probably contribute insights for screening of novel molecular targets for drug design. The review tried to provide cutting edge knowledge in ZIKV research with future insights required for the development of novel therapeutic remedies to curtail ZIKV infection.

Keywords: mosquito-borne flavivirus, Zika virus (ZIKV), current animal models, vaccine development, computational drug discovery, novel molecular targets, ZIKV research

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne flavivirus (family Flaviviridae) pertinent to West Nile virus, dengue virus (DENV), and yellow fever virus. ZIKV carry a positive RNA which is single stranded (Abushouk et al., 2016). ZIKV has been isolated on many instances from *Aedes africanus* although there was no indication to cause human disease (Petersen et al., 2016). The ZIKV was mostly restricted to African continent and later it was reported in South East Asia (1980) (Saiz et al., 2016). An outbreak of ZIKV divulged in Yap Island, Federated States of Micronesia in 2007. Prior to 2007, 14 cases of human infections were reported (Avsic Zupanc and Petrovec, 2016). An outburst occluded in French Polynesia in 2013 along with dengue pandemic

and during this outburst, primitive malformations such as Guillain–Barre syndrome (GBS) and microcephaly were noticed in patients (Musso and Gubler, 2016). The areas infected by ZIKV as per the current knowledge is shown in **Table 1**.

The ZIKV infection accreted severe global health issues due to the rise in number of GBS correlated reports and exuberant microcephaly cases in infants and fetus in Brazil by the end of 2015 (Saiz et al., 2016). The serosurveys determined wider geographic distribution which included East Africa, Malaysia, India, Nigeria, Thailand, Egypt, Philippines, and Vietnam (Petersen et al., 2016).

More than 5600 microcephaly cases in neonates have been well-documented which delineate greater than 20-fold with respect to historical average of past 5 years by 2016. The mortality rate due to microcephaly associated with ZIKV was registered to be approximately 120 by Brazilian health authorities (Noronha et al., 2016).

OUTBREAKS

According to the recent report, there were active circulations of ZIKV nearly all Caribbean and Latin American countries (World Health Organization [WHO], 2017b). Even though the transmission of virus was reported 60 years ago in Africa, the awareness of ZIKV to cause potential threats was revealed post-Brazilian and French Polynesian outbreaks (Baud et al., 2017). The circulation of the virus was recently reported in Southeast Asia and Africa, however; the incidence in these areas was undefined (World Health Organization [WHO], 2017b). The detection of infection in travelers was the basis for ZIKV circulation in Maldives or Vanuatu (Musso et al., 2016). Further, ZIKV has been caused minor outbreaks in Florida and Texas, United States. Recent advances in the diagnostic practices and surveillance system provides examination and reporting of autochthonous cases (Baud et al., 2017). Recent reports revealed that three cases of ZIKV infections in Ahmedabad, Gujarat, India by Ministry of Health and Family Welfare, Government of India (World Health Organization [WHO], 2017a). The cases of ZIKV in India was confirmed through reverse transcription polymerase chain reaction (RT-PCR) by routine laboratory surveillance (World Health Organization [WHO], 2017b). It is estimated that most of the tropical and subtropical areas in the world are at risk for ZIKV, yellow fever virus, chikungunya, and DENV. Studies suggested that the transmissions of ZIKV are evident in 84 countries across the world since April 2007 to March 2017 (Baud et al., 2017).

GENOME

ZIKV virion nucleocapsid exhibits icosahedral symmetry which is approximately 50–60 nm in size as shown in **Figure 1A** (Dasti, 2016). The genome is 10,794 kb in length which consists of single-stranded, positive sense RNA, flanked by two non-coding regions (5' and 3' NCR) and single open reading frame (ORF) coding for

a polyprotein 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. It is further cleaved into three structural proteins such as capsid (C), envelope (E), membrane precursor (prM) and seven non-structural (NS) proteins which are essential for the replication and assembly of the virus (Faye et al., 2014; Hamel et al., 2016; Rather et al., 2017). The major structural and NS proteins present in ZIKV genome is shown in **Figure 1B**. The three-dimensional structures of gene products of major structural and NS proteins are available till date are illustrated in **Figures 1C–H**. The virus initially infected in wild primates causing erratic “spillover” infections in human when the full-length of ZIKV genome was announced in 2007. It is assumed that after the loss of NS1 codon from the genome, the virus recently adapted to humans (Abushouk et al., 2016). In a study conducted in 2014 with 37 isolates showed that the virus has attained molecular changes to become accustomed to *Aedes dalzielii* by losing a glycosylation site (N154) in their protein envelope (Faye et al., 2014).

The ORF codes a polyprotein of approximately 3400 amino acids which is predicted to be cleaved into mature viral proteins (Saiz et al., 2016). The 5' UTR which is 106 nucleotides in size displayed conserved folding pattern in mosquito-borne flavivirus (Ye et al., 2016). The genome lacks poly-A tail at the 3' end and stops in conserved 5'-CU-3'. The G + C content ranges from 50.94 to 51.26%. The sHP-3' SL structure at 3'-UTR and the Y-shaped stem loop (SLA) structure at 5'-UTR in the flavivirus genome are among the most conserved secondary RNA structures, which is also expected to present in ZIKV. The conserved short sequences at 3' terminal consists of 5'-ACAG-3' and 5'-CU-3' in the top loop of the smallest sHP-3' SL structure (Zhu et al., 2016).

The lipid bilayer (surrounded by 180 units of glycoprotein E and M) involved in the binding of cell receptors form the envelope (Silva and Souza, 2016). E protein (approximately 53 kDa) is the chief surface protein which functions in diverse aspects of binding, membrane fusion and replication cycle (Faye et al., 2014). In flavivirus genome, *cis* acting RNA elements plays crucial role in viral replication, translation, and pathogenesis (Ye et al., 2016).

The two known ZIKV lineages belong to African lineage (divided into two clades signifying two different introductions) and Asian lineage are differentiated by complete genetic analysis of RNA sequences (Chang et al., 2016). A strong conservation has been observed at nucleotide level among all ZIKV strains with less than 12% divergence. The existing strain belongs to Asian subtype consisting of more than 99.7% of nucleotides and 99.9% of sequence identity with French Polynesian strain of 2013 outbreak as per the phylogenetic analysis (Enfissi et al., 2016). The conservations present in ZIKV strains are essential for diagnostic assays that depend on specific sequence and epitopes (Petersen et al., 2016).

The region of glycoprotein E which is contiguous to Asn 154 glycan depicts the largest structural deviation from other flavivirus. Novel anti-ZIKV vaccines and drugs can be developed based on the knowledge of the function of glycans and proteins (Boeuf et al., 2016).

TABLE 1 | Table depicting ZIKV cases reported as of August 2017 and category classification by WHO (European Centre for Disease Prevention and Control [ECDC], 2017).

Country	Region	Classification category of country for ZIKV transmission as per WHO	Number of cases reported
American Samoa	American Samoa	Category 3 (interrupted transmission areas)	51
Angola	Angola	Category 1 (virus transmission areas followed by new/re introduction of virus)	2
Argentina	Chaco, Formosa, Salta, Tucuman	Category 1 (virus transmission areas followed by new/re introduction of virus)	4
Bahamas	Bahamas	Category 1 (virus transmission areas followed by new/re introduction of virus)	25
Bangladesh	Bangladesh	Category 2 (virus transmission areas following previous circulation of virus)	1
Brazil	Acre, Alagoas, Amapa Amazonas, Ceara, Distrito Federal, Espirito Santo, Goias, Mato Grosso, Mato Grosso Do Sul, Minas Gerais, Para, Paraiba, Parana, Pernambuco, Piaui, Rio Grande Do Norte, Rio Grande Do Sul, Rondonia, Roraima, Santa Catarina, Sao Paulo, Sergipe, Tocantin Bahia, Maranhao Rio de Janeiro	Category 1 (virus transmission areas followed by new/re introduction of virus) Category 2 (newly documented intense transmission areas) Category 2 (virus transmission areas following previous circulation of virus)	27 46
Colombia	Colombia	Category 1 (virus transmission areas followed by new/re introduction of virus)	23
Costa Rica	Costa Rica	Category 1 (virus transmission areas followed by new/re introduction of virus)	30
Dominican Republic	Dominican Republic	Category 1 (virus transmission areas followed by new/re introduction of virus)	2
India	Gujarat, Tamil Nadu	Category 2 (virus transmission areas following previous circulation of virus)	31
Indonesia	Bali, Bangka Belitung, Banten, Bengkulu, Daerah Istimewa Yogyakarta, Dki Jakarta, Gorontalo, Jambi, Jawa Barat, Jawa Tengah, Jawa Timur, Kalimantan Barat, Kalimantan Selatan, Kalimantan Tengah, Kalimantan Timur, Kepulauan-Riau, Lampung, Maluku, Maluku Utara, Nanggroe Aceh Darussalam, Nusa Tenggara Barat, Nusa Tenggara Timur, Papua, Papua Barat, Riau, Sulawesi Barat, Sulawesi Selatan, Sulawesi Tengah, Sulawesi Tenggara, Sulawesi Utara, Sumatera Barat, Sumatera Selatan, Sumatera Utara	Category 2 (virus transmission areas following previous circulation of virus)	8
Malaysia	Malaysia	Category 2 (virus transmission areas following previous circulation of virus)	25
Mexico	Aguascalientes, Baja California, Baja California Sur, Campeche, Chiapas, Coahuila, Colima, Guerrero, Hidalgo, Jalisco, Michoacan, Morelos, Nayarit, Nuevo Leon, Oaxaca, Puebla, Quintana Roo, San Luis Potosi, Sinaloa, Sonora, Tabasco, Tamaulipas, Veracruz, Yucatan, Zacatecas	Category 1 (virus transmission areas followed by new/re introduction of virus)	57
Philippines	Philippines	Category 2 (virus transmission areas following previous circulation of virus)	33
Thailand	Thailand	Category 2 (newly documented intense transmission areas)	202
United States of America	Cameron Broward, Miami-Dade, Palm Beach, Pinellas	Category 1 (virus transmission areas followed by new/re introduction of virus) Category 3 (interrupted transmission areas)	232
Vietnam	Vietnam	Category 2 (newly documented intense transmission areas)	

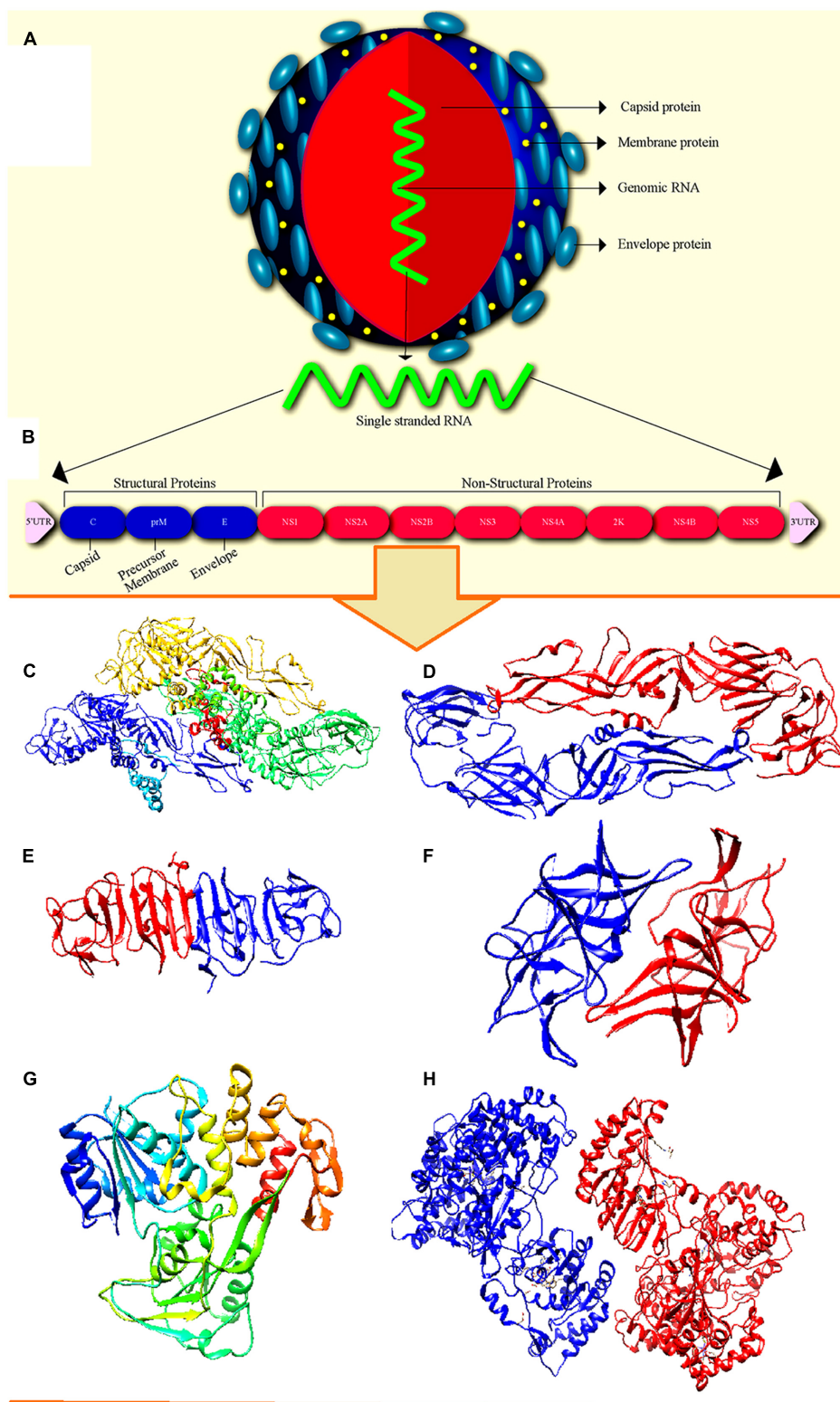


FIGURE 1 | Structure of Zika virus (ZIKV). **(A)** Ultra structure of ZIKV with major surface proteins. **(B)** Genomic RNA of ZIKV exhibiting major structural and non-structural proteins. The crystal structure of available structural and non-structural protein of ZIKV. **(C)** The cryo-EM structure of ZIKV (PDB: 5IRE). **(D)** Structure of envelop protein (PDB: 5JHM). **(E)** Structure of NS1 protein (PDB: 5IY3). **(F)** Structure of the NS2b-NS3 complex (PDB: 5GXJ). **(G)** Structure of NS3 helicase (PDB: 5JPS). **(H)** Structure of NS5 protein (PDB: 5U0B).

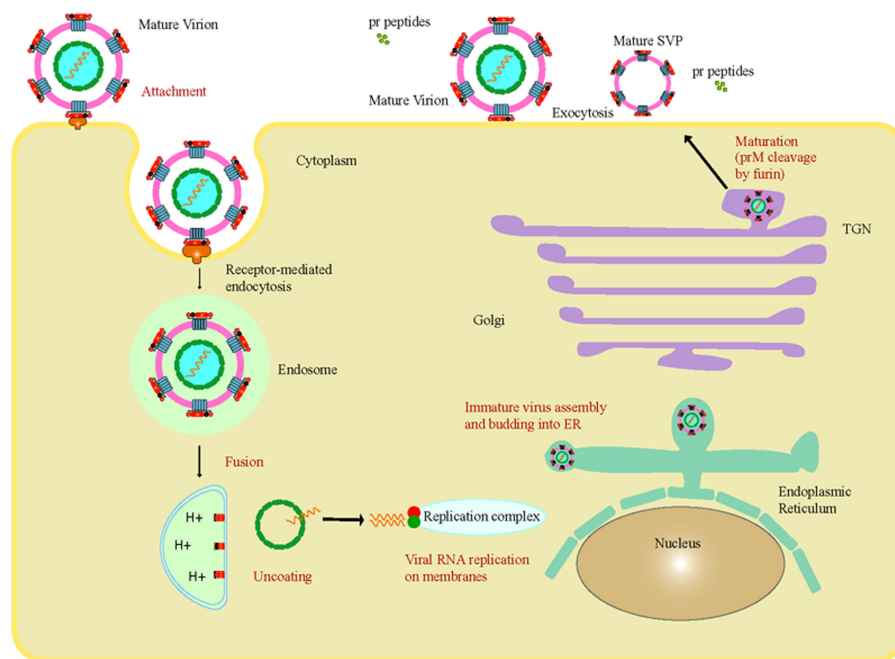


FIGURE 2 | The major events occurred in the replication and maturation of ZIKV inside the host cell followed by their release into the environment resulted in spread of the disease.

VIRAL MULTIPLICATION AND PROPAGATION

The combination of viral envelope with endosome membrane from the host cell set off the mechanism of penetration of flavivirus genome into the cytoplasm by a method triggered by acidic pH inside the cellular endosomes (Saiz et al., 2016). The host cell membrane of flavivirus is known to produce suitable environment for viral replication in endoplasmic reticulum (ER). The major events occurred in the replication and maturation of ZIKV inside the host cell followed by their release into the environment causing spread of the infection is illustrated in **Figure 2**. Most of the arboviruses are known to replicate within the skin dendrites at the primary inoculation site, later, it spreads to the regional lymph nodes followed by the blood stream (Abushouk et al., 2016).

The replication cycle is completed in four stages, i.e., translation of RNA into viral proteins, replication of viral RNA (vRNA), assembly of viral particle in ER and release of the virions (Hamel et al., 2016). The replicates of ZIKV were found in the salivary gland and mid-gut of *Aedes* mosquitoes and *in vitro* cultured mosquito cells C6/36 when infected by blood meal experimentally (Li et al., 2012). The viral infectivity and replication is enhanced by salivary gland products of the mosquito (Malone et al., 2016).

There are various relations with cellular organelles may exist to assist the viral replication, evasion, and propagation in the cellular cytoplasm, even though the exact process needed to be studied (Hamel et al., 2016). It has been suggested in infected mice that the virus replicates mainly in the brain cells, nervous

and astroglial cells, whereas, it can replicate *in vitro* in cultured monkey cell lines such as LLC-MK2 or Vero (Saiz et al., 2016).

Defective neurogenesis and anomalous activation of autophagy was caused by the inhibition of Akt-mTOR pathway due to ZIKV infection of human fetal neural stem cells (fNSCs). Autophagy developed as vital ancient immune response during evolution of lysosome-mediated catabolic process (Liang et al., 2016). Some viruses such as flaviviruses took over the cellular autophagy pathways to benefit their life cycle even though the host have progressed autophagy to maintain cellular homeostasis and circumvent viral infection (Heaton and Randall, 2011). Autophagy is induced in multiple cell types including fNSCs due to ZIKV infection (Liang et al., 2016). The various stages present in ZIKV replication are obscure, nevertheless, they are assumed to be similar to other members of flavivirus (Hamel et al., 2016).

TRANSMISSION

The transmission of the diseases through mosquito bite is a common scenario in ZIKV infection. The existence of ZIKV in the pharynx and saliva of infected patients could be persistent with probable transmission. Following the primary isolation of *Aedes aegypti* (other than *A. africanus*) from a group of mosquitoes garnered in Malaysia (1966), it furnished the indication of the first transmission cycle of ZIKV in urban areas (Saiz et al., 2016).

Mosquito bites are the major mode of transmission; however, few cases of non-vector-borne infections have been reported, known as perinatal transmission (Hamel et al., 2016; Rather

et al., 2017). The probable modes of perinatal transmission are transplacental, during delivery and at the time of breastfeeding (Besnard et al., 2014).

The occurrence of viable ZIKV particles in blood bags might have severe ramification in pregnant women (Silva and Souza, 2016). Various non-vector modes of ZIKV transmission are congenital and sexual. Separation of the virus or anti-ZIKV antibodies was indicated in various animal reservoirs from domestic and wild animals and numerous non-human primates (Plourde and Bloch, 2016).

The existence of ZIKV nucleic acids were authenticated among 2.8% of asymptomatic blood donors and transmitted infection by transfusion was observed in French Polynesia. Viable ZIKV particles have been isolated from the saliva and urine collected from two acute infected patients, hence; they were recognized as vectors for the transmission (Silva and Souza, 2016). The identification of ZIKV RNA and proteins in conserved microcephalic fetus of Brazilian women at the eighth week of gestation provided the first acceptable proof of vertical transmission (Shastry et al., 2016). The other vectors for ZIKV are *Aedes furcifer*, *A. vittatus*, *A. dalzieli*, *A. metallicus*, *A. hirsutus*, *A. unilineatus*, *A. africanus*, *A. taylori*, *A. hensillican*, and *A. luteocephalus* (Dasti, 2016). Various species of mosquito such as *A. albopictus* and *A. aegypti* causes difficulties in control and transmission of ZIKV (Saiz et al., 2016). These mosquitoes are also broadening the multiplication of dengue and chikungunya virus (Centers for Disease Control and Prevention [CDC], 2016).

STUDIES IN ANIMAL MODELS

Subcutaneous Route

The crucial requirement to understand the features of ZIKV infection paves path for enhanced research on animal models. In a study conducted by Kumar et al. (2017), immunocompetent guinea pigs sensitive to PRVABC59 (American) strains of ZIKV was utilized. A total of 10^6 plaque-forming units of the virus were inoculated into the guinea pigs (Dunkin-Hartley) by virtue of the subcutaneous route leading to the recognition of clinical features. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to determine the viral load in the tissues, protein levels of cytokine, anti-ZIKV neutralizing antibody, and viremia followed by plaque reduction neutralization test (PRNT) and multiplex immunoassay. The symptoms observed were lethargy, hunched back, fever, reduced mobility, and ruffled fur. PRNT presented the anti-ZIKV neutralizing antibody in the infected guinea pigs and the infection led to rise in multiple cytokines level and increased level of growth factors in the serum. The virus was observed to be replicated in the brain and spleen (Kumar et al., 2017).

The response of T-cells and innate immunity to ZIKV has been studied in mouse models. The production of α , β (type I), γ (type II), and λ (type III), interferon (IFN) and copious IFN-stimulated genes were stimulated by ZIKV that prevented the infection (Bayer et al., 2016; Hamel et al., 2016; Quicke et al., 2016). The mechanism of immune evasion was species specific which contributed to the lack of ability of ZIKV to replicate vigorously

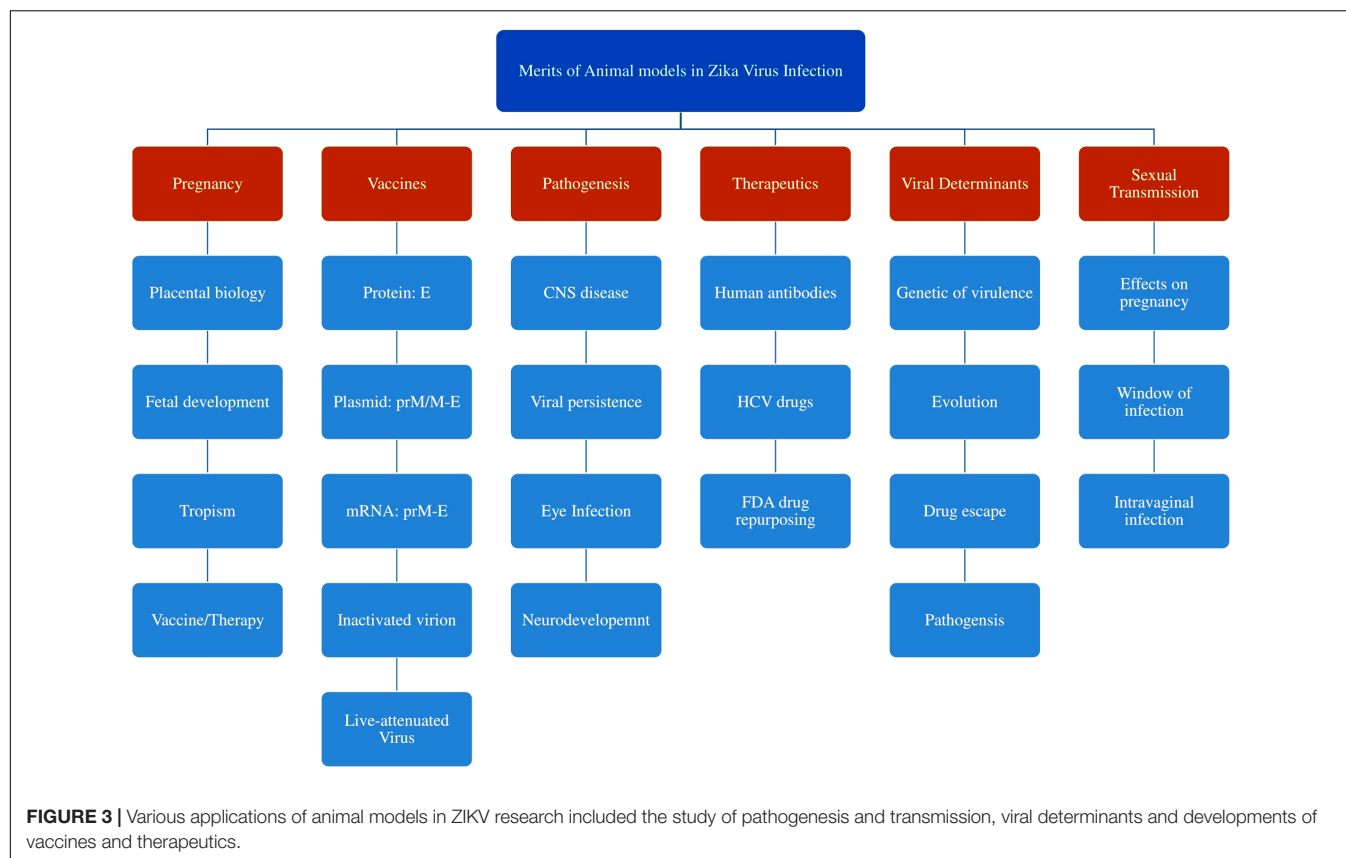
which is the root cause of disease in immunocompetent mice (Mysorekar and Diamond, 2016). A defensive role for CD8⁺ T cells was defined in the studies of mouse models of numerous flavivirus. It was observed that animals lacking MHC class I or CD8 possess condensed capacity for viral clearance and adoptively transferred cells might be protective. Subsequent to flavivirus infection, CD8⁺ T cell responses were easily detectable and both virus type specific and cross reactive determinants were targeted (Pierson and Graham, 2016).

The infection caused by Asian-lineage of ZIKV is affiliated with fetal abnormalities. However, poor knowledge of the mechanism has led to the study of rhesus macaque animal model. The study exhibited the injection of vRNA in the plasma approximately 10 days. The infection evoked an immune response such as neutralizing Ab that enable protection in the case of further infection and T-cell response specific for the virus. Nonetheless, the vRNA was detected in saliva and urine after being cleared from blood and further, the presence in CSF indicated that probable existence of ZIKV in some tissues at low levels (Dudley et al., 2016).

A number of clinical studies have examined malformations in the eye and pathology of newborns to mothers infected with ZIKV during pregnancy. Eye diseases in neonates with ZIKV included optic neuritis, colobomas, bilateral iris, chorioretinal atrophy, intraretinal hemorrhages, blindness, and lens subluxation. Uveitis is a viral infection due to the inflammation of uveal tissues (choroid, iris, retina, and ciliary body) which can result in the permanent loss of vision, if untreated (Miner et al., 2016). The anterior chamber of the eye enclosed vRNA from the sample fluid signifying the replication of ZIKV within the eye at a certain phase of their clinical syndrome (Furtado et al., 2016). Even weeks after the resolution of viremia and clinical symptoms, ZIKV infection to the eye and testis in the human and animal models were provided information that immune privileged organs might influence the replication (Lazear et al., 2016). Direct targeting of the cells in fetal eyes might cause congenital ocular infection by ZIKV. On the other hand, neurodevelopmental defect which is observed in microcephaly cases is due to non-existence of an infectious cause (Miner et al., 2016).

Intra-vaginal Route

Recent study revealed that the ability of ZIKV vectors to transmit via sexual routes. The study demonstrated by the introduction of the virus into AG129 mice and LysMCre⁺ IFNAR^{fl/fl} C57BL/6 via vaginal route post-hormonal treatment. AG129 mice failed to persist infection in di-estrus-like phase while, infection during estrus-like phase demonstrated resistance. However, LysMCre⁺ IFNAR^{fl/fl} mice in di-estrus-like phase showed short-term infection. The viral replication occurred in brain and spleen along with viremia in vaginal washes 10 days of post-infection. Thus, the study indicated that the occurrence of transgenerational transmission accompanied by variation in hormones of female reproductive tract with prolonged prevalence of viral replication (Tang et al., 2016). The applications of studies in various animal models in Zika viral research is shown in **Figure 3**.



Clinical Manifestations

Signs and Symptoms

ZIKV infections in most cases are alleged to be asymptomatic or faintly symptomatic. Most of the generally described symptoms include fever, arthralgia, rash, fatigue, myalgia, conjunctivitis, and headache. The growth stage from bite of mosquito to outbreak of the symptom is approximately 3–12 days in humans (Plourde and Bloch, 2016). After which a mild fever with wide range of symptoms are appeared for 2–7 days. The outburst of maculopapular rashes shown by more than 90% of patients is the major clinical symptom that describes ZIKV infection (Hamel et al., 2016). Typically, whole blood count is normal even if the count changes inconsistently non-specific (mild neutropenia, mild lymphopenia, and mild to moderate thrombocytopenia) (Campos et al., 2015). An inherent incubation period of 4–5 days within the human host later, infecting another vector during blood feeding where it spend an extrinsic incubation period of 8–12 days and propagates via saliva of the vector to another host (Abushouk et al., 2016). Only approximately 18% cases of ZIKV infections were recorded to be symptomatic, where it causes self-limiting, mild disease with up to 10 days of incubation period, normally flawed with other arboviral infections such as chikungunya or dengue (Saiz et al., 2016).

GBS includes the damage of peripheral nervous system with loss of myelin insulation in facial palsy, muscle dysfunction and myalgia. Fetal head circumference below average for gestational age with majority resulting in disabilities such as physical

disability and intellectual retardation is defined as congenital microcephaly (Hamel et al., 2016). The prevalence of GBS or microcephaly presented to be analogous between symptomatic and asymptomatic cases (Koenig et al., 2016). The current outbreak in Brazil provided prodigious attention to microcephaly cases mostly due to the significant increase in infant microcephaly cases (Dasti, 2016).

The noteworthy cellular death of ZIKV infected neural stem cells provided by the latest studies open the role of inhibition on fetal brain development by ZIKV (Tian et al., 2016). A study revealing the viral neutropism in mice that were infected intraperitoneally and development of the disease in mouse brains that were directly infected provide credible neuropathological relation between ZIKV and CNS anomalies. In contradiction to dengue, fatality from acute ZIKV infection is limited; however, it has been reported in children with sickle cell disease in Colombia. In ZIKV infected patients, hemorrhagic signs have not been reported (Koenig et al., 2016).

There is limited knowledge on the presence of ZIKV in congenitally infected newborns but the virus is observed to be linked with microcephaly. There was a failure in the detection of ZIKV during the initial physical examination when the infected mother (26 weeks of pregnancy) experienced symptoms and gave birth to a microcephaly infected male child in 2016 in Sao Paulo, Brazil. The weight, length, and head circumference of the infant was observed to be 3095 g, 48 cm, and 32.5 cm, respectively. A reduced brain parenchyma, preferentially in the frontal and

parietal lobes, foci of calcification in the sub-cortical area and compensatory dilatation of the infratentorial supraventricular system was observed by magnetic resonance imaging technique. The saliva and urine were tested for the virus on the 54th day by qRT-PCR. The studies depicted that all the three assays were positive for ZIKV RNA with 1.4×10^5 , 4.1×10^4 , and 5.4×10^3 copies per milliliter in the serum, saliva, and urine, respectively. A high degree of similarity was observed in the infant with bootstrap value of 98.5% when RNA sequencing of urine samples were performed along with positive results for IgM and IgG. The detection of ZIKV RNA in the serum continued on day 67 with 2.8×10^4 copies per milliliter by qRT-PCR. On day 216, there was failure to detect ZIKV RNA in the serum and ZIKV-specific IgG titer was elevated (>320) in comparison with the first and second samples (average titer, <99). However, the newborn showed delay in neuropsychomotor development, with spastic hemiplegia and global hypertonia, with severe damage to the right dominant side by the age of 6 months (Oliveira et al., 2016).

Recent Diagnostic Strategies

The symptoms of ZIKV infection are non-specific and can be confound with symptoms of other diseases such as chikungunya and dengue caused by arbovirus, the differential laboratory diagnosis plays vital role in the regions where the virus co-circulates and hence, ZIKV infection can be misdiagnosed (Zanluca and Dos Santos, 2016). Asymptomatic individuals might be important reservoirs for virus transmission and have obscure diagnostics. All the currently available diagnostics have been used to test symptomatic individual (Boeuf et al., 2016). Date of onset of the illness for ZIKV infection is intricate to ascertain because of sporadic and frequent mild fever (Gourinat et al., 2015).

Laboratory methods analyze ZIKV infection by the detection of viral nucleic acid and viral antibody or antigen (Zanluca and Dos Santos, 2016). During symptomatic period of infection, various laboratory parameters and symptoms provide information on ZIKV infection such as leucopenia, thrombocytopenia, serum lactate dehydrogenase, γ -glutamyl transferase, and elevated protein markers. The commercial assays and PCR-based assays accepted by the Communauté Européenne and serological assays permitted by US Food and Administration (FDA) can be used in emergency situations (Plourde and Bloch, 2016).

In the earlier stages, mainly during primary acute infections, techniques such as solid phase immunosorbent assay and hemagglutination were used to diagnose flaviviral infection (Chang et al., 2016). The detection of viral nucleic acid was performed by RT-PCR and IgM capture enzyme-linked immunosorbent assay (MAC-ELISA). Even though the exact onset timing and duration of IgM antibody response to the virus can be detected by MAC-ELISA has not been established, clear understanding which implies that IgM become visible as viremia waves within the first week after the onset of symptom and persevere for several months. The Arboviral Diagnostics and Reference Laboratory at CDC developed IgM ELISA to detect the samples obtained from Yap Island outburst (Saiz et al., 2016).

RT-PCR analysis of serum samples attained inside the first week of clinical illness and MAC-ELISA testing of samples that were not tested or found negative by RT-PCR was expected to have the highest diagnostic yield. The uses of RT-PCR were found in detecting ZIKV in amniotic fluid, breast milk, semen, saliva, and blood products. The utility of urine samples for diagnosis of ZIKV infection demonstrated that ZIKV RNA detectable at an elevated load longer period than in serum (Gourinat et al., 2015). The positive results should be confirmed by neutralization assay such as PRNT which offers greater specificity, whereas, some patients with the probability of being infected by another flavivirus exhibit fourfold or higher rise in neutralizing antibody titers against other flavivirus (Zanluca and Dos Santos, 2016). Neutralizing properties of ZIKV antibodies have been subjected to PRNT and played a major role in the diagnosis and case classification (confirmed, probable, suspected, and no ZIKV infection) of several patients during 2007 outbreak on Yap Island (Marano et al., 2016).

In accordance with the recent CDC guidelines for testing pregnant women likely to be exposed to ZIKV, there was a strong explanation for testing asymptomatic pregnant women as long as sufficient laboratory facilities are available (Boeuf et al., 2016). The most specific diagnostic approach is the molecular amplification of serum samples and which is the ideal testing method for ZIKV for the period of acute phase of illness. However, serological testing is not recommended as ZIKV IgM might be undetectable during acute phase (Plourde and Bloch, 2016).

Limitations

The following are the major demerits to be considered during the diagnosis of ZIKV infections (Charrel et al., 2016; Dasti, 2016; Hamel et al., 2016):

- As a result of identical clinical features with other infections of arbovirus such as chikungunya and dengue, the diagnosis of ZIKV is challenging.
- Due to the failure in differential diagnosis, most often, ZIKV is diagnosed as DENV at the incipient stage.
- The scarcity of sensitive and specific laboratory test makes it complicated to detect the virus in the samples collected from the patients.
- Antibodies of ZIKV infection are often cross-reactive with other flavivirus such as yellow fever, dengue, West Nile Virus, Murray valley encephalitis, Kunjin virus and that which frequently co-circulate with ZIKV, confines the use of serology and hinders the regular performance of the viral culture. The lacks of antigenic detection contribute one of the major challenges in the diagnosis.
- Even if PCR-based testing of saliva, blood, and urine is highly recommended, accessibility of the preferred amount of nucleic acids in various samples possess significant encumbrance.
- The short viremic duration demonstrated an impediment in the detection of significant antibodies.

- To run molecular tests in an outburst situation, the laboratories require acquaintance and experience of quality control and validation, ability to access rapid reagents and other necessities from supply chains, and to augment the high throughput testing approaches.

Prevention and Control

The presence of *Aedes* mosquito enables ZIKV to invade newer areas and impose worldwide risk as there are no preventive approaches or vaccines for ZIKV. The control measures are depending on the eradication of mosquito vector breeding foci (Wahid et al., 2016; Zanoluca and Dos Santos, 2016). Methods that sustain the environment and preserve the effectiveness should be employed for efficient control of ZIKV (Chang et al., 2016). With the advent of high level research, effective and safe ZIKV vaccine probably developed in the near future. An initiative for the development of ZIKV vaccine was started by National Institutes of Health (NIH) in 2015 (Wahid et al., 2016).

Mosquito Control Measures

Entomological surveillance is the fundamental aspect for any vector control program. Countries at greater risk of *Aedes* introduction require vigilant monitoring at the entry point (World Health Organization [WHO], 2016). The control of *A. aegypti* relies on an integrated approach which included the exclusion of breeding sites of *A. aegypti* mosquito, the application of insecticides and larvicides to eliminate fully developed mosquitoes. In order to battle ZIKV infection, mosquito breeding grounds should be primary measured which is carried out by targeting mosquito breeding through eradication of probable egg laying sites with the aids of insecticide treatment or by drying the wet environments (Plourde and Bloch, 2016). Research is being carried out for strategies to accomplish mosquito elimination. For example, in order to curb the breeding or proliferation of the vector species, a survey is being conducted by WHO on the release of sterile irradiated mosquitoes (Shastri et al., 2016). Prevention and control of ZIKV infections are primarily directed toward evading the mosquito bites accountable for transmission of the disease (Saiz et al., 2016).

Control Measures for Public

New recommendations were provided by FDA for blood and organ donation as donors are asymptomatic at the time of blood donation. An additional proficient tactic to thwart blood-borne transmission of infectious agent is pathogen reduction technology mainly for unknown pathogens (Marano et al., 2016). As of 2016, for donors who are at risk to ZIKV infection in areas without active ZIKV transmission, the FDA advises that donation should be delayed for 4 weeks. FDA suggested that blood or blood products must be obtained from areas in US without active transmission of ZIKV for those residing in the areas of active ZIKV transmission (Food and Drug Administration [FDA], 2016). Those women who are pregnant and ones trying to conceive should not travel to virus affected areas as suggested by ECDC (European Centre for Disease Prevention and Control) and CDC. It is also advised to use

precautionary measures such as condoms, while traveling or residing in areas of active virus circulation (Centers for Disease Control and Prevention [CDC], 2016). A travel alert of level 2 (practice enhanced precautions) has been issued by CDC for the people traveling to regions such as El Salvador, Guatemala, Haiti, Brazil, Mexico, Panama, French Guiana, Paraguay, Suriname, Honduras, Commonwealth of Puerto Rico, and Venezuela which have been affected by active transmission of ZIKV (Shastri et al., 2016).

Environmental Control Measures

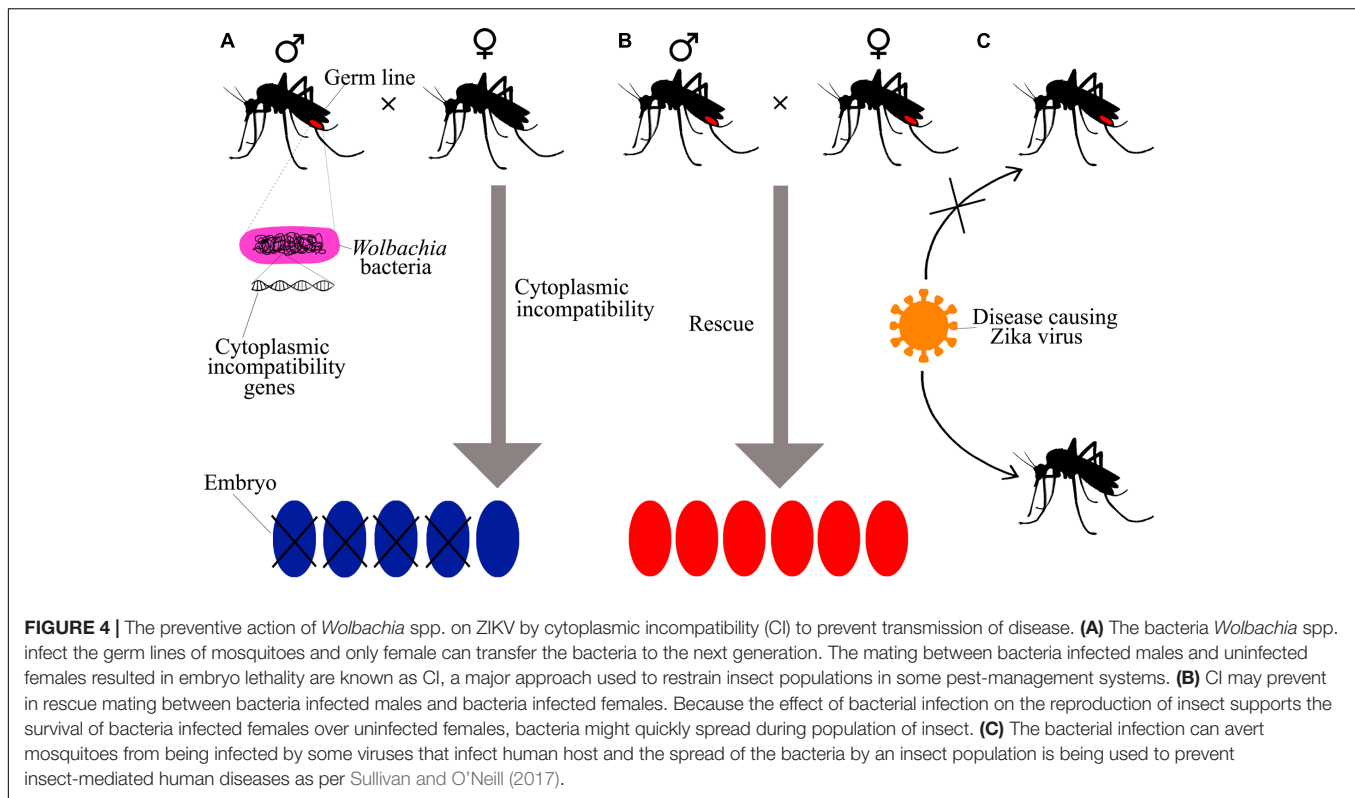
The use of insecticides and exclusion of tiny pools of sluggish water are the major vector control strategies (Basarab et al., 2016). The main aspect considered to combat ZIKV is the knowledge about the breeding grounds of mosquitoes such as stagnant water ponds, unattended furniture, watery polythene bags, old automobile tires, risk factors associated with gardening, and plants containing water. However, for medium-large containers that hold water for domestic uses should be enclosed with tight fitting covers to protect from the introduction of eggs by female mosquitoes (World Health Organization [WHO], 2016). The main recommendations for epidemic areas of travel are as follows:

- Prefer complete covering by cloths.
- Accepted mosquito repellents to be used.
- Reside in air conditioned or screened rooms.
- Bed nets to be used if screened or air conditioned rooms are not available or while sleeping in the open.
- Secure eating habits.

Bacteria Prevents Transmission

The intracellular bacterium *Wolbachia pipientis* reported to be capable candidate for arbovirus control and prevention. *Wolbachia* is found in various species of insect which included butterflies, ants, mosquitoes, bees, and beetles wherein they show endosymbiotic influence on the host replication (Aliota et al., 2016). Cytoplasmic incompatibility (CI) is the usual type of manipulation that permits rapid population dissemination. CI maintains high frequency by patterns of crossing sterile individuals that can provide reproductive benefit to the females carrying bacteria (Molloy et al., 2016). In *A. aegypti*, *W. pipientis* strain wMel applied in field testing that attained at high population frequency (Hoffmann et al., 2014). Since *A. aegypti* has no native *Wolbachia* symbionts, the wMel strains of the bacteria were incorporated into *A. aegypti* which condenses the lifespan of female mosquitoes (Aliota et al., 2016). The proposed mechanism of prevention of ZIKV by *W. pipientis* is shown in **Figure 4**.

The upregulation of innate immune genes has been observed to be added to the phenotype; however, it is not necessary for blockage of viral transmission. Further, the mechanism behind the pathogen inhibition remains uncertain. Since the infected mosquito exhibit decreased fitness in small-scale field releases, *W. pipientis* is not regarded as a biocontrol agent (Nguyen et al., 2015).



RECENT ADVANCEMENTS

Vaccines

Various vaccines are being developed recently such as recombinant protein subunit and DNA-based vaccine, an inactivated ZIKV vaccine. Subunit vaccines are required in multiple doses to stimulate an immune response although they are safe and can be developed in shorter timeline (Shan et al., 2016). In India, Bharat Biotech, a premier Biotech industry, is dynamically developing two vaccine candidates, i.e., recombinant vaccine and inactivated vaccine which destruct the ability to replicate, whereas the immune system can still detect it. These two candidates are undergoing pre-clinical trials. Recent studies revealed that ZIKV was found to be sensitive to the antiviral effects of type 1 and type 2 IFNs (Fellner, 2016). Anti-viral nucleoside analogs GS-5734 and BCX4430 are currently in the first and second phases of clinical trials (Mumtaz et al., 2016). Further, recent studies showed that ZIKV infection could act in response to IFN therapy (Hamel et al., 2015).

Inactivated Zika vaccine in the purified form was recently developed by Walter Reed Army Institute of Research (WRAIR) where four phase 1 trials have been initiated. A joint collaboration between National Institute of Allergy and Infectious Diseases (NIAID) and University of Pennsylvania, Moderna/Valera and GlaxoSmithKline (GSK) investigated several mRNA vaccines that are identical to DNA vaccines. The candidate developed by Moderna/Valera has been assessed in phase 1 clinical trial (National Institute of Allergy and Infectious Diseases [NIAID], 2017).

AGS-v is a vaccine considered to be acted against several mosquito-borne infections. It is found to set off an immune response to the proteins present in mosquito saliva instead of specific virus. Four synthetic proteins found in mosquito salivary glands are utilized to formulate the test vaccine. The proteins are intended to trigger modified allergic responses that lead to the prevention of infection on being bitten by the mosquito. This vaccine currently evaluated for phase 1 clinical trial at NIH Clinical Center in Bethesda, MD, United States (National Institute of Allergy and Infectious Diseases [NIAID], 2017).

Therapy and Treatment

An accurate understanding of ZIKV biology is essential for the development of anti-ZIKV therapeutics, vaccines, and improved diagnostics (Boeuf et al., 2016). The treatment for ZIKV infection is completely supportive as no explicit antiviral therapy is established yet (Koenig et al., 2016). Currently, no drugs are permitted for the treatment but numerous nucleoside analogs have some antiviral activity in cell culture such as 2'-C methylated nucleosides like 7-deaza-2'-C methyladenosine (7DMA) and 2'-C methylcytidine (2CMC) and Ribavirin, Favipiravir and T-1105. Initially, 7DMA and 2CMC were developed for the treatment of hepatitis C which is distantly linked to ZIKV. Studies have shown that these compounds have antiviral activities against other flavivirus (Mumtaz et al., 2016). Brazil's Butantan Institute, Sao Paulo was the first group to announce the development of ZIKV vaccine (Fellner, 2016). To lower the risk of hemorrhage, aspirin and non-steroidal anti-inflammatory drugs should be evaded (Zanluca and Dos Santos, 2016). The administration of fluids

TABLE 2 | The potential drug candidates suggested being ideal toward ZIKV and these probably provide insights in future vaccine developments.

Compounds	Compound source
ChEMBL/PubChem:29	FDA approved antiviral drugs
Quinacrine, pyronaridine	FDA drugs that are not antivirals but
Chloroquine and amodiaquine	have shown antiviral activity
Kinase inhibitors	
Chlorcyclizine	
NTCP inhibitors vs HepB	
Quinacrine, berberine	FDA approved drugs active <i>in vitro</i> or
Amodiaquine	<i>in vivo</i> vs dengue virus
Prochlorperazine	
H-89, MPP, BIBU 1361	Other compounds from HTS screen vs
Diverse molecules	dengue virus, yellow fever, etc.
ChEMBL:90–95	Compounds from ChEMBL datasets
PubChem:96–98	Compounds from PubChem

and Acetaminophen (Paracetamol) or Dipyrone is suggested to manage fever and pain. The incidence of Reye's syndrome can be prevented in children below 10 years by eschew of aspirin, while acetaminophen can be used as an alternative.

An integrated advancement in traditional Chinese medicine and western medicine were used to cure ZIKV infection. Xiyanping was used for the treatment of latest case of ZIKV due to the antiviral properties of this drug (Logan, 2016). Intravenous injection of 250 mg Xiyanping was prescribed daily as an antiviral agent in the infectious secluded wards. Chloramphenicol eye drops were prescribed to relieve conjunctival congestion along with Ibuprofen. Further techniques have been suggested by WHO advisory group to manage ZIKV infections (Fellner, 2016). Some of the future medicine toward ZIKV is shown in **Table 2**.

Epigallocatechin gallate (EGCG), a polyphenol (catechin) found in *Camellia sinensis* (green tea) provides intense antiviral activities against the viruses such as herpes simplex virus (HSV), influenza virus (FLU), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) (Carneiro et al., 2016; Elfiky, 2016). Through direct interaction with lipid envelope, the admission of virus into host cell is inhibited by EGCG causing a consequent obliteration of virus particle. Previous studies were showed that EGCG has low membrane permeability, chemically unstable, and is quickly metabolized by the organism. Recent studies have shown that EGCG is noxious to various cell lines at elevated concentration (Carneiro et al., 2016). Over the short period of time, neutralizing antibody preparations, development and testing of antiviral and medicines designed to block Fc receptor interaction are some of the strategies suggested by medical counter measure to manage ZIKV infections.

Advances in Computational Biology for Drug Development

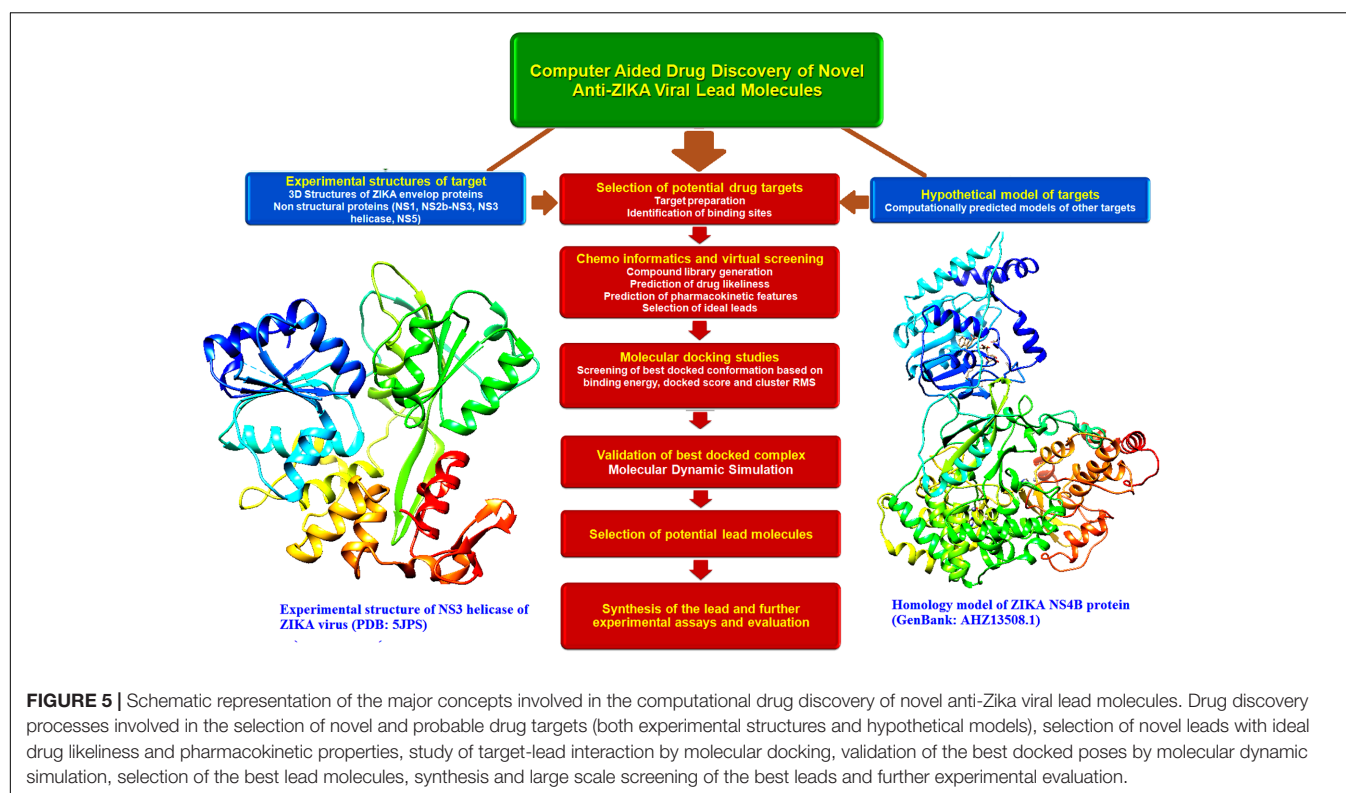
A quick development of therapeutic requires the swift analysis of mechanism underlying the pathogenesis of the ZIKV (Basharat et al., 2016). The prM, Env, and NS proteins can be considered as potential drug targets. In the NS3 and NS5 drug-binding pockets, ZIKV shares sequential homology and presumable structural similarities with other flavivirus (Cox et al., 2015). As an early

stage of computational protocol, the accessible crystal structures from various parts of the virus and novel homology models were used. However, to process large databases of compound libraries, more sophisticated docking techniques such as Blind Docking simulations could be consequently applied. Furthermore, ligand-based virtual screening (LBVS) methods could be useful for the screening of novel lead candidates. Recent studies have shown that the application of LBVS in discovering potential inhibitors against viruses. The electronic description of biological processes such as molecular dynamics and quantum mechanical calculations, could be applied to further filter the structures that lead the prediction of more accurate binding energies between the receptor and ligand (Cerón-Carrasco et al., 2016). The recent advancement and major concepts (Ramharacka and Soliman, 2016; Munjal et al., 2017) involved in the screening of novel anti-ZIKV lead molecules by computer aided discovery approaches are shown in **Figure 5**.

Recently, small number of FDA-approved drugs such as IFN (34.3 IU/ml), Ribavirin (143 µg/ml), 6-Azauridine (1.5 µg/ml), and Glycyrrhizin (384 µg/ml) were tested against ZIKV (Ekins et al., 2016a,b). The phosphorylation in viral protein assists in replication and interrupts the normal host-cell functions that are responsible for pathogenesis. Additionally, phosphorylation and dephosphorylation of protein residues might be involved in cell signaling. Computational biology tool such as ViralPhos exclusively tailored for phosphorylation analysis of viral proteins and probes the virus substrate motif which aids in the identification of potential phosphorylation sites in the viral protein (Basharat et al., 2016).

To control more than 60% of human protein-coding genes, small non-coding RNAs (sncRNAs) which are termed as MicroRNAs (miRNA) that regulate post-transcriptional gene expression by translational repression were examined (Friedman et al., 2009). The influences of ZIKV/human-host interaction by miRNAs are presented in two hypotheses. Firstly, miRNAs which provide advantages linked with viral and cellular gene expression could be transcribed by the virus (Klase et al., 2013). Secondly, the direct interaction between viral genomes and cellular miRNAs enhanced the potential of viral replication. The human genome miRNAs are found to be possessed few complementary sequences with ZIKV genomes, predicted miRNAs can be targeted to develop anti-ZIKV lead molecules. These miRNAs can be retrieved from ZIKV-CDB database (Pylro et al., 2016). The database is an open-source platform which aids in sharing and identification of potential targets. A valuable knowledge base is provided by the ZIKV-CDB to support researchers to targets the predicted miRNAs. The association between neurobiological development in infants and ZIKV infection can also be steered by experimental investigation. The Genomic and Computational Biology Group continuously curate and maintains the database (Pylro et al., 2016).

Furthermore, there is high scope using alternative approaches other than molecular modeling-based methods. For example, techniques such as advanced machine learning methods (i.e., deep learning) are appropriate to capture complex statistical patterns between thousands of descriptors extracted from



drug compounds. Better predictions can be made using Deep Neural Networks (DNN) in comparison with standard machine learning tools based on Kaggle competition data sets. A deep machine learning network was developed to identify the site for epoxidation and to differentiate epoxidized and non-epoxidized molecules (Skalsky and Cullen, 2010; Cerón-Carrasco et al., 2016).

Challenges for ZIKV Research

The most challenging aspect of ZIKV research is the demonstration of ZIKV-induced *in vivo* congenital alterations. The *in utero* infection on pathology has not been reported in mice, and it is difficult to model this pathology in wild-type pregnant mice. The peripherally inoculation of ZIKV to AG129 pregnant mice causes fetus infection and congenital variations, because flaviviral infection leads to the development of viremia in mice when compared to the ones infected in humans (Rossi et al., 2016).

The nucleoside analogs such as 7DMA and T-705 Favipiravir are proficient for the treatment of several viral infections. However, the application of nucleoside analogs for the treatment of ZIKV can be unsafe in pregnant women. Currently, there are protocols established to assess the safety of Ribavirin treatment during pregnancy. The gestational use of nucleoside analogs is suggested when benefits exceed in comparison to the probable fetal harm. The safety of most nucleoside analogs should be studied for ZIKV-induced congenital malformations to examine whether these drugs exhibited competence in infected adults (Ribeiro et al., 2016).

The preservation of an intact immune response in mice models is essential; however, there are issues with inoculation of the virus to brain. The murine models that exist with sustained viremia are supreme to the compounds such as neutralizing antibodies and antiviral agents, which target ZIKV for preclinical testing. To elucidate the scientific challenges, various mice models of infection are required in ZIKV research (Ribeiro et al., 2016).

An alternative vector strategy to abate the transmission of ZIKV by mosquitoes was demonstrated by the introduction of the bacterium *Wolbachia* into the *A. aegypti* population. Thereby, the replication of ZIKV curtails within the mosquito and delays the distribution of virus to the salivary glands of mosquitoes. The existing limitation for the application of vector control strategies in ZIKV affected areas are the probability of environmental damage and public distrust of genetically modified organisms (Rajah et al., 2016).

CONCLUSION

ZIKV infections are spreading across the world and possessed great threat to the public health. The infection often associated with neurological disorders and severe birth defects. Several vector and non-vector-borne forms of transmission are reported which aid in spreading the disease. The similarity of ZIKV with other flavivirus such as DENV and West Nile virus is worrisome. Further, more severe complications such as pathogenesis and cellular mechanism are unknown. Hence, there is a high demand to undertake ZIKV research as thrust areas and develop strategies

to ascertain therapeutic remedies to curtail the infection. There are several researchers and industries are working toward the development of effective anti ZIKV vaccines. The development of vaccine requires clear understanding of the pathogenesis and genome structure of the virus. An interdisciplinary environment of *in vitro* cellular assays and computational biology approaches probably contribute insights for the screening of molecular targets in drug design. An improvement in the existing strategies of vector control, awareness programs among people and

opening novel outlook in ZIKV research to study the current challenges and limitations are the vital component in the disease management.

AUTHOR CONTRIBUTIONS

AS and AP collected the data and prepared the complete manuscript, SS reviewed, edited, and revised the manuscript.

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Zika Virus: Transmission, Detection, Control, and Prevention

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Zika virus (ZIKV) is a mosquito-borne *Flavivirus* discovered in Uganda in the 1940s. To date, three major ZIKV outbreaks have been reported. ZIKV infections have known to be primarily asymptomatic while causing mild illness in a few cases. However, the recent emergence and spread of ZIKV in the Americas has resulted in the declaration of “Public Health Emergency of International Concern” due to the potential association between the infection and prenatal microcephaly or other brain anomalies. In Brazil, a 20-fold increase in prenatal microcephaly cases and 19% increase in Guillain-Barré Syndrome (GBS) cases were reported in 2015, as compared to the preceding year. The probable deleterious effects of ZIKV infection prompt the urgent development of diagnostics and therapeutics. To this end, the existing evidences supporting the increasingly common prenatal microcephaly and GBS association and the current known ZIKV transmission dynamics, modes of detection (molecular and serology-based), and current control strategies are summarized in this review. This review also emphasizes the importance of understanding ZIKV transmission in order to design a sensitive yet cost and time-efficient detection technique. Development of an efficient detection technique would subsequently allow for better surveillance and control of ZIKV infection. Currently, limited literature is available on the pathogenesis of ZIKV, hence, focusing on the modes of ZIKV transmission could potentially contribute to the understanding of the disease spectrum and formulation of targeted treatment and control.

Keywords: *Flavivirus* infection, arbovirus, sylvatic cycle, microcephaly, Guillain-Barré syndrome, Zika diagnosis

INTRODUCTION TO ZIKA VIRUS

Zika virus (ZIKV), a mosquito-borne *Flavivirus* belonging to the *Flaviviridae* family, is an emerging pathogen that is spreading rapidly across the Americas, raising concerns in the forefront of global healthcare (Ayres, 2016). The virus is closely related to other members of the *Flavivirus* genus (positive-sense, single-stranded RNA viruses), including the dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Japanese encephalitis virus (JEV) (Lazear and Diamond, 2016). Although, ZIKV infection is reported to be subclinical in approximately 80% of the cases, the virus has recently raised a “Public Health Emergency of International Concern” due to the dramatic increase in the cases of prenatal microcephaly and Guillain-Barré Syndrome (GBS) in ZIKV endemic regions (Basarab et al., 2016). Microcephaly is characterized by at least two standard deviation reduction in brain volume intellectual and motor disabilities, and behavioral issues (Petersen L. R. et al., 2016). Multiple development factors, such as genetic, environmental, and infectious exposure, during pregnancy are known to contribute to the onset of prenatal microcephaly. Therefore, further efforts are required toward eliminating any

potentially associated confounding factor (Weaver et al., 2016). GBS, on the other hand, is a rare autoimmune disorder of the peripheral nervous system which could result in muscle weakness, paralysis, or even death (Lazear and Diamond, 2016). The plausible association between ZIKV infection and prenatal microcephaly/GBS is yet a matter of debate among researchers. It was suggested that the rise in the number of prenatal microcephaly and GBS cases could potentially be attributed by increased awareness and/or misdiagnosis (Mlakar et al., 2016).

To date, the exact ZIKV transmission dynamics have not been established. ZIKV has been isolated from humans, non-human primates, and multiple species of mosquitoes, suggesting a complex transmission network (Lazear and Diamond, 2016). Investigation of other potential inter-human modes of ZIKV transmission, such as sexual, blood-related, or maternal transmission, has allowed refinement of precautionary measures for ZIKV prevention and novel modes of ZIKV detection. Greater understanding of ZIKV transmission dynamics could also further aid in the development of a precise, rapid and simple tests for ZIKV detection in humans and mosquitoes. The robust detection method could in-turn improve the control of ZIKV, further preventing it from spreading around the globe (Pardee et al., 2016). With the severity of ZIKV associated diseases and the urgent need to develop methods to control its spread, in this review we aim to provide consolidated up-to-date available information on ZIKV associated prenatal microcephaly and GBS, ZIKV transmission dynamics, current molecular and serology-based modes of ZIKV detection, and the latest ZIKV control strategies in place.

Epidemiology

ZIKV was first isolated from the blood of a febrile sentinel rhesus monkey in the Zika Forest of Uganda in 1947 (Dick et al., 1952; Lanciotti et al., 2008; Haddow et al., 2012). In the following years, ZIKV was isolated from various species of *Aedes* mosquitoes (Mlakar et al., 2016; Slavov et al., 2016). The first case of ZIKV infection in human was reported in Nigeria in 1954 (MacNamara, 1954). Since then, a number of significant outbreaks have been reported, prominently in the African and Southeast Asian region (Pan American Health Organization, 2016c).

The first major ZIKV outbreak occurred in 2007 on the Yap Island, Federated States of Micronesia, with approximately 75% of the population being affected within a period of 4 months (Saiz et al., 2016). Subsequently, in 2013 and 2014, ZIKV epidemic was reported in the French Polynesia, Cook Islands, Ester Islands, and New Caledonia. In 2015, ZIKV outbreak was reported in Brazil and henceforth has spread across

the Latin America, Caribbean, and other parts of the world causing a pandemic (Lazear and Diamond, 2016). As of March 2016, ZIKV had spread to 33 countries in the Americas, with approximately over 1.5 million cases reported (Ayres, 2016; Petersen L. R. et al., 2016). By July 7th 2016, autochthonous cases of ZIKV had been reported in 40 countries within the Americas (Pan American Health Organization, 2016b). The exact global prevalence of ZIKV infection has not been reported due to the absence of a standardized protocol for differential diagnosis and clinical resemblance to other *Flavivirus* infections. In addition, ZIKV is known to be self-limiting (asymptomatic in approximately 80% of the cases), hence, it is likely that the infection is underdiagnosed/underreported in a disease-endemic setting (Gourinat et al., 2015).

It is anticipated that ZIKV would further spread around the globe, particularly via viremic travelers or the movement of infected mosquitoes (Petersen L. R. et al., 2016). More recently, autochthonous ZIKV transmission as well as cases of ZIKV transmission via sexual activity were reported in the United States (Centers for Disease Control and Prevention, 2016b; Hills et al., 2016). Currently, autochthonous vectorial ZIKV transmission has not been reported in Europe, although imported and locally sexually transmitted cases have been reported (Tappe et al., 2014; Saiz et al., 2016; Venturi et al., 2016).

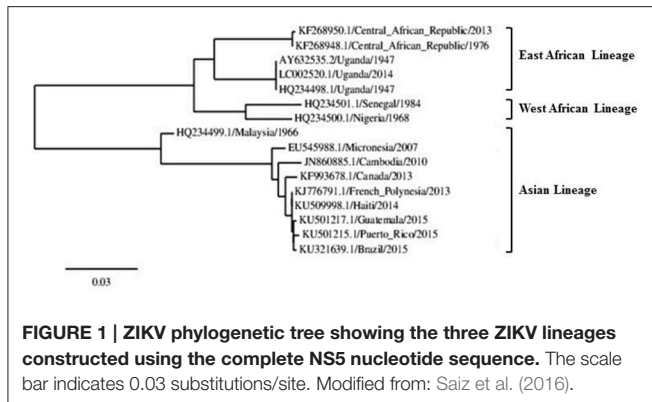
Continuous global surveillance is advised as situations may change following the start of warmer weathers, allowing ZIKV mosquito vectors to become active (Imperato, 2016; Saiz et al., 2016).

Molecular Classification

Although, limited molecular data of the ZIKV genome sequence from human isolates are currently available, sufficient data exists to determine the evolutionary patterns of the virus (Petersen L. R. et al., 2016). Through ZIKV genome sequencing and phylogenetic analysis of several human isolates, three geographically distinct lineages of ZIKV have been reported, including the East African, West African, and the Asian strains (see **Figure 1**). It is postulated that ZIKV originated in the Eastern region of Africa and then spread toward the West (Lanciotti et al., 2016). In the late 1960s, the first Asian ZIKV was isolated in Malaysia and then subsequently, the virus spread across southeast Asia (Marchette et al., 1969). It is postulated that the Asian ZIKV strain emerged as a result of accumulation of mutations in the ZIKV genome, hence, introducing new molecular interacting partners with the host cell factors and changes in disease pathogenicity, vector competence, and epidemic potential (Lazear and Diamond, 2016; Wang et al., 2016; Weaver et al., 2016). At this point, it must be noted that when compared to other RNA viruses, fixation of a mutation in the genome of an arbovirus faces greater constraints due to the presence of important genes required for replication in mammals and invertebrates (Holmes, 2003; Hamel et al., 2015).

The current ZIKV outbreaks in the Americas, and previously in the French Polynesia and Yap Islands, are reported to be most closely related to the Asian strain (see **Figure 1**; Haddow et al., 2012; Imperato, 2016). Through genome analysis and phylogenetic studies, it was found that the ZIKV currently

Abbreviations: CHIKV, Chikungunya virus; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; DENV, Dengue virus; ELISA, Enzyme-Linked Immunosorbent Assay; ER, Endoplasmic reticulum; GBS, Guillain-Barré Syndrome; HPV, Human papillomavirus; HSV, Herpes simplex virus; Ig, Immunoglobulin; JEV, Japanese encephalitis virus; NASBA, Nucleic acid sequence-based amplification; NS5, Non-structural viral protein 5; PM, Prenatal microcephaly; PRNT, Plaque reduction neutralization test; qRT-PCR, Real time quantitative RT-PCR; RT-PCR, Reverse transcription polymerase chain reaction; RT-PCR, Reverse transcription polymerase chain reaction; TBEV, Tick-borne encephalitis virus; UTR, Untranslated region; WHO, World Health Organization; WNV, West Nile virus; YFV, Yellow Fever virus; ZIKV, Zika virus.



circulating in the Americas share >99% identity with ZIKV isolates from the French Polynesia outbreak and is 89% identical to the African strain (Lanciotti et al., 2016; Lazear and Diamond, 2016). The high genomic similarity of the ZIKV strains circulating in the Americas allow for targeted drug and therapeutic development (Petersen L. R. et al., 2016). Recent studies focusing on the phylogenetic relationship between ZIKV and other *Flaviviruses* have also contributed to the understanding of the nature of ZIKV. Upon constructing phylogenetic trees based on the non-structural viral protein 5 (NS5) and the structural viral protein E, it was found that although the ZIKV has the potential to affect the central nervous system, particularly in neonates, it however is not particularly related to other encephalitic viruses (Wong et al., 2016).

Virology and Pathogenesis

ZIKV, a biosafety level-2 pathogen, has an enveloped positive-sense, single-stranded RNA genome with a size of approximately 10,676 bp and is known to be closely related to the Spondweni virus (Charrel et al., 2016; Wong et al., 2016). ZIKV virions are approximately 60 nm in size and spherical in shape (Charrel et al., 2016). The ZIKV genome encodes for a single polyprotein (approximately 3400 amino acids) that is subsequently processed by host and viral proteases into ten different proteins, consisting of three structural and seven nonstructural proteins (see **Figure 2**; Saiz et al., 2016). **Table 1** summarizes the function of each ZIKV protein based on the general information collectively available for *Flaviviruses* (Wong et al., 2016).

Currently, little information is available regarding ZIKV pathogenesis, as compared to other members of the *Flavivirus* genus. Generally, arboviruses (mosquito-borne viruses) are known to replicate in dendritic cells and subsequently disseminate to the lymph nodes and bloodstream (Diamond et al., 2004). Within the cells, replication of *Flaviviruses* is known to occur in the cytoplasmic region however, ZIKV antigens have been observed within the cell nuclei. Hence, it is suggested that ZIKV replication may differ from that of other *Flaviviruses* (Buckley and Gould, 1988). In addition, according to a study by Priyamvada et al. (2016), the potential association between ZIKV and prenatal microcephaly/GBS may be attributed by the introduction of ZIKV into a population with high flaviviral background (e.g., prior exposure to DENV). The immunity

established against other *Flaviviruses* may play a role in the modulation of ZIKV pathogenesis (Fajardo et al., 2016).

To study ZIKV pathogenesis, efforts have been put toward the investigation of murine models with different manifestations of ZIKV infection (Shah and Kumar, 2016). A study by Dick (1952) showed motor weakness and paralysis in mice intracerebrally infected with ZIKV strain M766 isolated from the brain of young infected mice. Previous studies have also suggested that glycosylation of viral E protein is associated with the ability of *Flaviviruses* to cause a pandemic (Shirato et al., 2004). Recently, E protein analysis of multiple pathogenic ZIKV strains circulating in the Americas indicated positive glycosylation patterns. In contrast, majority of the other ZIKV strains were found to lack E protein glycosylation (Baronti et al., 2014; Berthet et al., 2014). These findings suggest that E protein glycosylation may be indicative of ZIKV pathogenicity (Saiz et al., 2016).

Host Cell-virus Interaction

According to a study by Hamel et al. (2015), ZIKV host cell entry and endocytosis occurs via interactions of viral E proteins with host cell adhesion factors, such as DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) and multiple members of the phosphatidylserine receptor family. The acidic environment within the endosome promotes viral envelope and endosome membrane fusion (low pH promotes E glycoprotein rearrangement) hence, allowing the release of ZIKV RNA genome into the cytoplasm for the initiation of translation (Saiz et al., 2016). Subsequently, translated viral proteins aid in viral genome replication at the surface of the endoplasmic reticulum (ER) (Hamel et al., 2015). Within the ER, positive strand viral RNA is packaged to form immature virions. The virus matures in the trans-Golgi network, upon the cleavage of prM into M protein. ZIKV is then released into the surroundings via exocytosis (Roby et al., 2015). Scarce information is available regarding host cell response to viral genome replication. According to Hamel et al. (2015), ZIKV replication induces innate viral response and transcription of interferon stimulated genes.

Symptoms

Up to 80% of human ZIKV infections appear to be asymptomatic, with a small subset of cases presenting with mild clinical symptoms similar to other flaviviral and influenza infections (Marano et al., 2016; Shah and Kumar, 2016). Clinical manifestation in symptomatic cases tend to appear after an incubation period of 3 to 12 days and are reported to be characterized by fever, rashes, myalgia, arthralgia, conjunctivitis, gastrointestinal disturbance, and headaches (Buathong et al., 2015; Basarab et al., 2016). However, major concern is associated with the steep increase in reported cases of prenatal microcephaly and GBS in the Americas after the recent ZIKV outbreak (Mlakar et al., 2016). To this regard, it must be pointed out that prenatal microcephaly and GBS have been implicated in other flaviviral infections, such as by DENV and WNV (Weaver et al., 2016). Teratogenic effects of flaviviral infections have been reported to target the eyes and brain. Recent studies have suggested ZIKV infections to be highly neurotrophic, with a few cases reporting

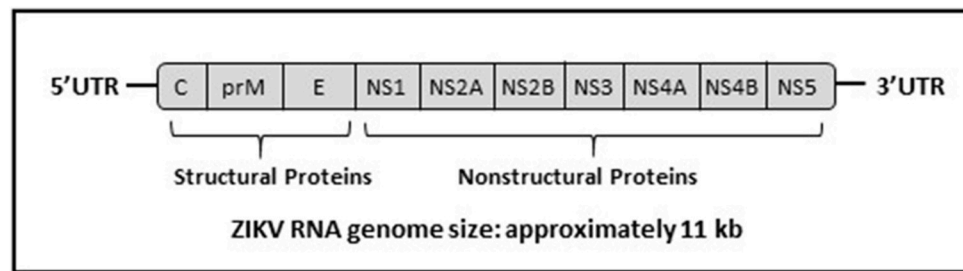


FIGURE 2 | Genomic structure of ZIKV flanked by the 3' and 5' untranslated region (UTR). Redrawn from: Marano et al. (2016).

association with bilateral macular and perimacular lesions (Mlakar et al., 2016; Ventura et al., 2016). Such complications following ZIKV infection were undocumented in the 1950s. Hence, it is evident that ZIKV genetic evolution (emergence of Asian lineage) has resulted in increased virus pathogenicity (Oehler et al., 2014; Rasmussen et al., 2016).

In 2015, amid the ZIKV epidemic, an astounding 20-fold increase in prenatal microcephaly cases were reported in Brazil, as compared to 2014 (Fauci and Morens, 2016). Since October 2015 up until now, approximately 4000 ZIKV infection-related prenatal microcephaly cases have been reported in Brazil, causing over 40 infant deaths (Higgs, 2016). During the outbreak, Paraíba (north-east Brazil), a ZIKV endemic state, was reported to have significantly increased cases of prenatal microcephaly, from 5.7 per 100,000 live births in 2010 to 99.7 per 100,000 live births (Basarab et al., 2016). In addition, 1708 cases of GBS were reported in 2015 in Brazil, a 19% increase as compared to the preceding year (Basarab et al., 2016). Besides that, a case-control study based on the French Polynesia ZIKV outbreak indicated that patients with GBS were more likely to have a history of ZIKV as compared to the control group (Cao-Lormeau et al., 2016). Another study reported 20-fold increase in GBS cases following the French Polynesia outbreak (Oehler et al., 2014).

A recent study on mouse models reported teratogenic effects, such as neuronal cell death and microcephaly, in pups born to SJL mice infected with ZIKV during pregnancy (Cugola et al., 2016). The precise mechanism by which ZIKV causes prenatal microcephaly or GBS is yet unknown. Increased incidences of microcephaly and GBS in regions positive for ZIKV circulation and evidence from clinical and epidemiological studies have increasingly been pointing toward a plausible causal association (Rasmussen et al., 2016). **Table 2** summarizes the studies supporting the plausible association between ZIKV infection and prenatal microcephaly and GBS.

Life Cycle

To date, ZIKV has been isolated from a vast range of organisms, including humans, non-human primates (apes, monkeys, and orangutans), and mosquitoes. Antibodies against ZIKV have also been detected in vertebrates (rodents, birds, sheep, goats, cattle, reptiles), hence, suggesting their potential role in the circulation of ZIKV (Johnson et al., 1977). The African ZIKV

lineage is thought to be maintained via the sylvatic/enzootic transmission cycle primarily between non-human primates (apes and monkeys) and mosquitoes, with humans as incidental hosts. However, humans have most likely become the prominent host for the Asian ZIKV lineage (Althouse et al., 2016; Basarab et al., 2016). Through evolution, ZIKV has gained the ability to sustain transmission in a human-endemic cycle (suburban-urban transmission cycle) thus, allowing humans to serve as the carrier, multiplier, and source of ZIKV for uninfected mosquitoes (see **Figure 3**; Saiz et al., 2016). The suburban-urban transmission cycle is thought to cause and sustain epidemics (Lazear and Diamond, 2016). Current research on ZIKV life cycle focuses on determining the possibility and impact of ZIKV sylvatic cycle establishment within the Americas. Such studies have highlighted the importance of targeted surveillance of the susceptible animal population for enzootic ZIKV (Fauci and Morens, 2016).

ZIKV TRANSMISSION DYNAMICS

Mosquito-Borne Transmission

ZIKV transmission to humans occur primarily through bites of an infected, day-dwelling female *Aedes aegypti* or *Aedes albopictus* mosquito, similar to the transmission of chikungunya virus (CHIKV) and DENV. *A. aegypti* mosquitoes are confined to the tropical and sub-tropical regions, hence, limiting ZIKV transmission potential (Petersen L. R. et al., 2016). However, *A. albopictus* mosquitoes are known to be geographically distributed throughout the tropical, subtropical, and temperate regions, hence, allowing for greater transmission potential (Thomas et al., 2012). The exact incubation period for ZIKV before the mosquito becomes capable of transmitting the virus is yet unknown. However, according to Hayes (2009), the extrinsic incubation period of ZIKV in mosquitoes is suggested to be approximately 10 days. Currently, it is presumed that uninfected mosquitoes are capable of acquiring ZIKV by feeding on an infected human (approximately during the time of clinical manifestation in humans). To this regard, further studies must be conducted to confirm that the viral titer level in the serum of infected individuals is sufficient to infect a naïve mosquito (Grard et al., 2014).

TABLE 1 | Function and cellular localization of ZIKV proteins (Kostyuchenko et al., 2016; Lazear and Diamond, 2016; Wong et al., 2016).

Viral proteins	Protein localization	Function
E	Structural surface protein	Host receptor binding, host cell fusion, and viral entry
prM, M	Structural surface protein	E protein stabilization and host cell fusion
C	Structural core protein	Binds to viral RNA for nucleocapsid formation
NS1		Viral replication
NS2a		Viral transcription and assembly
NS2b		NS3 cofactor for appropriate serine protease function
NS3	Not a part of the virus particle. Encoded by the viral RNA genome and translated using host cellular machinery	Serine protease, RNA helicase, and triphosphatase activity
NS4a		Viral replication
NS4b		Viral replication
NS5		Viral replication, RNA-dependent RNA polymerase, RNA capping, methyltransferase activity

To date, ZIKV has been isolated from 17 different *Aedes* mosquito species as well as *Culex perfuscus*, *Mansonia uniformis*, *Anopheles coustani*, and *Anopheles gambiae* mosquitoes (Ayres, 2016; Saiz et al., 2016; Slavov et al., 2016). It is postulated that the enzootic maintenance of ZIKV occurs through these mosquito species however, ZIKV transmission to humans is contributed only by a subset of these species (Lazear and Diamond, 2016). To date, vector competence and maintenance of the suburban-urban ZIKV transmission has been reported in *A. aegypti*, *A. albopictus*, *A. hensilli* (responsible for Yap Island outbreak), and *A. polynesiensis* (responsible for French Polynesia outbreak) (Imperato, 2016; Lazear and Diamond, 2016). The principle vector currently responsible for spreading ZIKV within the Americas include the *A. aegypti* and *A. albopictus* species (Petersen L. R. et al., 2016). The involvement of a diverse range of mosquito species in the maintenance of ZIKV suggests that the transmission dynamics of ZIKV is complex (Althouse et al., 2016).

Sexual Transmission

Multiple cases of male-to-female ZIKV transmission have been reported thus, raising the concern of a novel mode of ZIKV transmission in the human semen (Imperato, 2016). In 2011, a case study reported ZIKV transmission from an infected male to his female partner via sexual intercourse after the patient returned from Senegal to the United States (Foy et al., 2011). Serologic testing detected ZIKV RNA in both male and female partners. A similar study indicated the development of ZIKV infection in a female (confirmed via RT-PCR on serum sample) 13–14 days after having sexual intercourse with an infected male who had recently returned from the Caribbean (Hills et al., 2016). Three other similar cases have also reported coherent findings (Foy et al., 2011; Hills et al., 2016). In all the cases reported, the female partners had not traveled out of the United States and local mosquito-borne transmission was not considered due to vector absence within the geographical location. Until recently, ZIKV has been thought to be transmitted only from males to their sexual partner. On 15 July 2016, the first female-to-male sexual

transmission of ZIKV was reported, further raising concerns that ZIKV could spread more widely (Centers for Disease Control and Prevention Newsroom, 2016; Davidson et al., 2016; Santora, 2016).

Recently, a study reported detection of ZIKV RNA via qRT-PCR in the semen of infected males up to 188 days after the onset of symptoms, even after viremia had cleared (serum negative for ZIKV RNA) (Nicastri et al., 2016). The detection of high infectious viral load and ZIKV RNA in semen suggest prolonged potential for sexual transmission (Atkinson et al., 2016; Mansuy et al., 2016; Nicastri et al., 2016). However, the mechanism underlying the sexual transmission of ZIKV from a male to the female partner is yet unknown. ZIKV is by far the first arbovirus to be detected in human semen (Musso et al., 2015b).

Recent studies have reported the detection of ZIKV RNA and infectious viral load in the saliva and urine of infected individuals (Gourinat et al., 2015; Barzon et al., 2016). Distinguishing between sexual and salivary/urinary transmission of ZIKV becomes difficult due to the correlated nature of behavior associated with sexual activity (Foy et al., 2011; Musso et al., 2015a). Verification of ZIKV transmission via sexual interaction could significantly change the epidemiology of ZIKV as ZIKV RNA was found to be detectable in semen over a longer period of time, as compared to blood serum (Foy et al., 2011; Atkinson et al., 2016).

Blood Transfusion-related Transmission

During the French Polynesia outbreak, ZIKV RNA was detected in approximately 3% of asymptomatic blood donors (acute phase of infection) thus, making blood transfusion a novel potential mode of ZIKV transmission (Musso et al., 2014; Basarab et al., 2016). ZIKV transmission via blood transfusion is plausible as ZIKV infections are primarily asymptomatic and blood transfusion-related transmission of other *Flaviviruses* have been reported (Marano et al., 2016; Shah and Kumar, 2016). The first confirmed case of blood transfusion-related ZIKV transmission has been recently reported in Brazil (Centers for Infectious Disease Research and Policy, 2016). To address this issue, on 19 February 2016, the WHO issued strict guidelines for

TABLE 2 | Summary of reported association between ZIKV infection and prenatal microcephaly (PM) / GBS.

Country	Year	No. of patients	Symptom	Sample tested	ZIKV detection method	References
Slovenia	2015	1	PM	Fetal brain tissue	qRT-PCR	Mlakar et al., 2016
Hawaii	2015	1	PM	Not Reported	Laboratory confirmation (method not described)	Hawaii Department of Health, 2016
Brazil	2015	2	PM	Fetal brain tissue	RT-PCR & Anti-ZIKV ELISA	Martines et al., 2016
Brazil	2015	2	PM	Amniotic fluid	qRT-PCR & ZIKV sequencing	Calvet et al., 2016
Brazil	2015	1	PM	Fetal blood & tissue	Genome detection (method not described)	Pan American Health Organization, 2016a
Brazil	2015	2	Miscarriage	Placental tissue	RT-PCR & Anti-ZIKV ELISA	Martines et al., 2016
Brazil	2015	1	PM	Fetal cerebral cortex, medulla oblongata, cerebrospinal, & amniotic fluid	qPCR	Sarno et al., 2016
Brazil	2015	12	PM	Fetal cerebrospinal fluid	Anti-ZIKV IgM ELISA	Lazear and Diamond, 2016
Brazil	2015–2016	12	Fetal Abnormalities	Maternal urine and/or blood	qRT-PCR	Brasil et al., 2016
Brazil	2016	1	PM	Fetal brain tissue, membranes, placenta, & umbilical cord	qRT-PCR	Driggers et al., 2016
French Polynesia	2013	1	GBS	Serum	Anti-ZIKV IgG ELISA & PRNT	Oehler et al., 2014
French Polynesia	2013–2014	42	GBS	Serum	Seroneutralization assay & Anti-ZIKV IgM/IgG ELISA	Cao-Lormeau et al., 2016
Martinique	2015	2	GBS	Urine	RT-PCR	World Health Organization, 2016a
Brazil	2015	7	GBS	Not Reported	Lab Confirmed	World Health Organization, 2016c
Brazil	2015	4	GBS	Serum or cerebrospinal fluid	RT-PCR and/or Anti-ZIKV IgM ELISA	Araujo et al., 2016
Suriname	2015	2	GBS	Not reported	RT-PCR	World Health Organization, 2016c
Puerto Rico	2015–2016	5	GBS	Serum	RT-PCR and/or Anti-ZIKV IgM ELISA	Dirilikov et al., 2016
Venezuela	2016	6	GBS	Not Reported	RT-PCR	World Health Organization, 2016b
Venezuela	2016	1	GBS	Serum & cerebrospinal fluid	qRT-PCR	Mécharles et al., 2016

PM, Prenatal microcephaly; GBS, Guillain-Barré syndrome; RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, real time quantitative RT-PCR; Ig, Immunoglobulin; ELISA, Enzyme-Linked Immunosorbent Assay.

blood transfusion/donation in regions where ZIKV was endemic (Imperato, 2016). In multiple countries, such as in Europe, United States, and Canada, donated blood is screened via nucleic acid testing to detect WNV RNA (O'Brien et al., 2010; Centers for Disease Control and Prevention, 2013; Pupella et al., 2013). Adopting the same approach, continuous efforts are in place to formulate a simple yet precise test to detect ZIKV in donated blood. An alternative option is to avoid blood donation from individuals within ZIKV endemic regions or with recent history of travel to those regions (Lazear and Diamond, 2016). To further improve transfusion safety, pathogen reduction technologies are also being utilized to render pathogens inactive (Marano et al., 2016). Nevertheless, further studies need to be conducted to detect ZIKV and its transmission in donated blood (Saiz et al., 2016).

Maternal Transmission

Prenatal Transmission

ZIKV has reportedly been detected in microcephalic neonates born to mothers with a history of ZIKV infection during pregnancy (Besnard et al., 2014; Centers for Disease Control and Prevention, 2016a). It is postulated that ZIKV has the ability to cross the placenta and subsequently, infect fetal nervous tissues. The suggested mechanism is supported by the evident detection of ZIKV RNA and antigens in the amniotic fluid, placenta, and fetal brain tissue as well as visualization of ZIKV particles in fetal brain via electron microscopy (Calvet et al., 2016; Lazear and Diamond, 2016; Petersen L. R. et al., 2016). It is a known fact that the placenta acts as an effective immunological barrier between the mother and the fetus, protecting the fetus from microorganisms in the mother's circulation. The

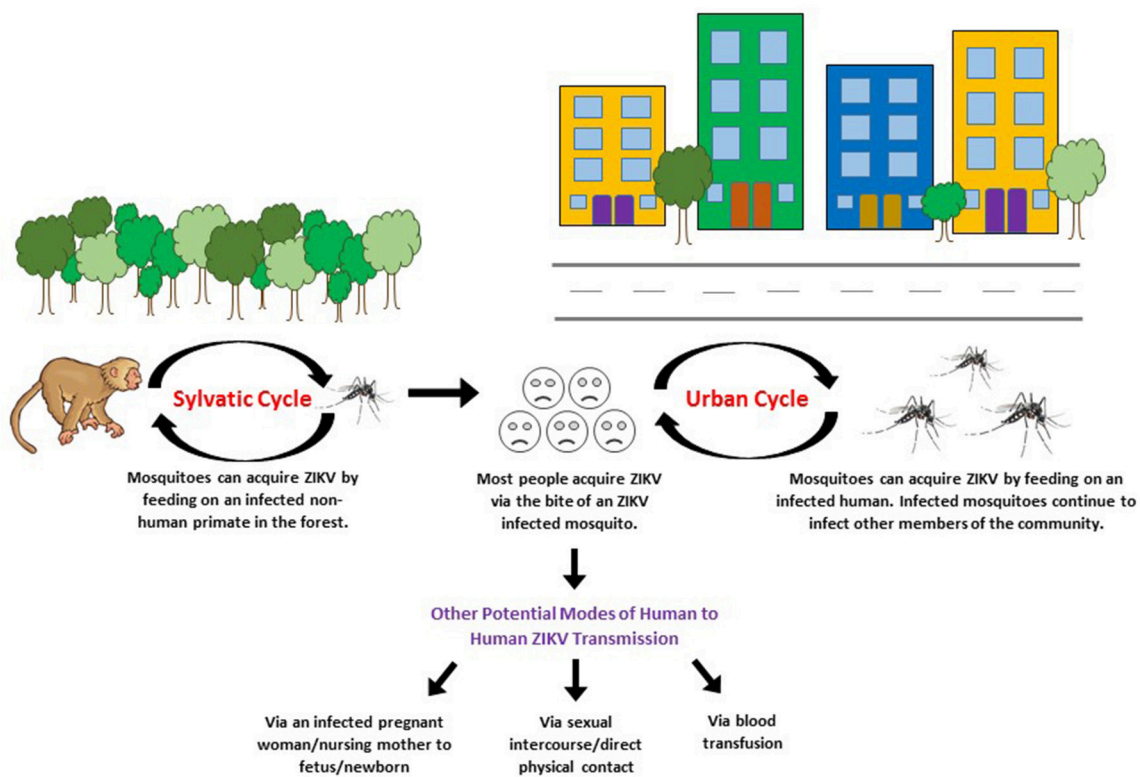


FIGURE 3 | Sylvatic/enzootic and urban transmission cycle of ZIKV. Redrawn based on the information obtained from: Besnard et al. (2014), Musso et al. (2014), Musso et al. (2015b), Swaminathan et al. (2016), Weaver et al. (2016).

mechanism used by ZIKV to circumvent the placental barrier is yet to be discovered (Bayer et al., 2016). A recent study on mouse models discovered that ZIKV infection during pregnancy resulted in placental damage and fetal death, further supporting the trans-placental route of transmission (Miner et al., 2016). The potential of ZIKV to undergo *utero* transmission has raised global concerns as regions positive for ZIKV circulation, such as Brazil, have recently reported a tremendous increase in the cases of prenatal microcephaly. Accumulating evidences, as shown in **Table 1**, suggest the potential ability of ZIKV to be transmitted to the fetus and the potential role of ZIKV in the development of prenatal microcephaly (Lazear and Diamond, 2016).

Nursing Mothers

ZIKV RNA and infectious viral particles have been detected in high loads in the breast milk of infected mothers (Dupont-Rouzeyrol et al., 2016). This introduces a novel transmission mechanism in which ZIKV transmission occurs from the mother to the nursing child. According to a mother-infant pair study, ZIKV RNA was detected in the breast milk and serum of two mothers and in the serum of their respective infants (Besnard et al., 2014). Particularly, serum sample from one of the infants tested positive via RT-PCR after breastfeeding. However, ZIKV replication was not detected upon inoculation of the breast milk on Vero cells hence, making transmission via breast milk

uncertain yet plausible. Other potential confounding mother-to-child ZIKV transmission routes should be further investigated. *Flavivirus* transmission, such as DENV and WNV, via breast milk have been previously reported (Ognjan et al., 2002; Barthel et al., 2013).

Transmission by Physical Contact

In September 2016, the first case of ZIKV transmission via direct physical contact was reported in the United States, further suggesting a sophisticated and complex ZIKV transmission mechanism (Swaminathan et al., 2016). The study reported the transmission of ZIKV from an infected patient (Patient 1: 73-year-old) to his healthy son (Patient 2: 38-year-old). Patient 1 had returned to the United States from the southwest coast of Mexico, where ZIKV transmission had been recorded, 8 days prior to hospitalization in Salt Lake City. Patient 1's serum assay for ZIKV via real-time PCR was positive, with an estimation of a very high viral load. In addition, high-throughput RNA sequencing of the ZIKV isolated from Patient 1 revealed 99.8% similarity to the genome sequence of a ZIKV strain circulating in mosquitoes in Chiapas, Mexico, in 2016. Patient 1 died 4 days after hospitalization. Subsequently, 5 days after Patient 1's death, Patient 2 developed ZIKV symptoms. On day seven post-symptom onset, Patient 2's urinalysis via PCR assay and serum IgM antibody test were positive for ZIKV, although blood serum analysis for ZIKV via PCR was negative. Patient 2 had visited

Patient 1 during hospitalization and reported to have wiped Patient 1's watering eyes without gloves and assisted a nurse in repositioning Patient 1. None of the health care workers who had contact with the patients reported having symptomatic illness. Since the *Aedes* mosquito species known to transmit ZIKV are absent in the Salt Lake City area and Patient 2 had not recently traveled to a ZIKV endemic region and had not had sex with a partner with recent travel history to such areas, it is most likely that Patient 2 acquired the ZIKV infection from Patient 1, whose sweat or tears may have contained infectious ZIKV (Swaminathan et al., 2016).

MODES OF DETECTION

The recent ZIKV outbreak in the Americas and its continuous spread, along with increased likelihood of causal association with prenatal microcephaly and GBS has prompted a search for a low-cost and rapid ZIKV detection method (Pardee et al., 2016; Vorou, 2016). The present ZIKV outbreaks have been reported to be of the Asian lineage, hence, current research focuses on developing Asian strain-specific detection assays (Charrel et al., 2016). As mentioned earlier, scarce information is available regarding the pathogenesis of ZIKV hence, understanding of the ZIKV transmission dynamics could potentially aid in the development of a robust detection technique. To date, standardized tests for ZIKV detection have not yet been developed (Fauci and Morens, 2016). In addition, clinical presentations of ZIKV infection appear to be highly similar to other arboviral infections, such as DENV and CHIKV infection, hence, potentially confounding diagnosis (Basarab et al., 2016; Fauci and Morens, 2016). In 2015, 224 dengue patients were screened for ZIKV infection, with seven out of 10 individuals testing positive for ZIKV infection (Agencia Fiocruz de Noticias, 2016). However, it must be noted that ZIKV diagnosis and confirmation is challenging due to cross-reactivity and low viremia (Gourinat et al., 2015). During ZIKV testing, cross-reactivity to other *Flaviviruses* often occurs due to close-relatedness and co-circulation of other *Flaviviruses* in ZIKV endemic regions (ZIKV infection is secondary) (Basarab et al., 2016; Charrel et al., 2016). Detection of ZIKV is best during the acute-phase, however, it is difficult to determine the period for onset of symptoms as majority of the cases are asymptomatic (Shah and Kumar, 2016). **Figure 4** summarizes multiple techniques for ZIKV infection diagnosis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)-Based Detection

Real-time and conventional RT-PCR are the most common approaches utilized in diagnostic labs owing to their specificity and ability to differentiate ZIKV from other flaviviral infections (Wong et al., 2016). RT-PCR allows for rapid, specific, and reliable ZIKV RNA detection during the acute-phase, as compared to other modes of detection (Marano et al., 2016). The development of ZIKV specific primers for nested RT-PCR has been reported to increase specificity (Grard et al., 2014). Specific RT-PCR molecular assays have been developed for the

detection of Asian and African ZIKV strains. Often, the ZIKV envelope genes (prM/E protein coding regions) are targeted for amplification due to their unique characteristics which allow for differentiation from other *Flaviviruses* (Musso et al., 2015b; Marano et al., 2016). Two specific sets of primers for the Asian ZIKV strain have been tested and established (Lanciotti et al., 2008). To further increase specificity, the use of TaqMan probe is recommended (Charrel et al., 2016). Commercial kits (for research purposes) for ZIKV RNA detection via RT-PCR have recently entered the market (Charrel et al., 2016).

Peripheral blood samples are predominantly used for PCR-based assays (Wong et al., 2016). However, RT-PCR on blood and serum samples is associated with reduced sensitivity due to low viremia in humans (Vorou, 2016). More recently, detection of higher viral RNA load over a longer duration was reported in urine and semen samples (Musso et al., 2015a,b). Related studies have coherently reported detection of higher DENV and WNV RNA load over a longer duration in urine samples, as compared to blood serum (Musso et al., 2015b). ZIKV RNA has also been reported to be detected in the saliva of infected individuals, often more readily compared to blood samples (Musso et al., 2015a). The choice and combination of samples chosen for testing is highly dependent on the stage of infection (see **Table 3**). It is recommended to perform RT-PCR on both blood and saliva/urine samples in order to increase test sensitivity, particularly during the late stage of infection (Gourinat et al., 2015; Musso et al., 2015a). In addition, alternative sampling of urine or saliva reduces invasiveness and hence, is advantageous for diagnosis in neonates and infants (Charrel et al., 2016). For prenatal testing, amniotic fluid is predominantly collected for molecular analysis. A positive RT-PCR for ZIKV RNA is suggestive of intrauterine infection and plausible reduction in fetus fitness (Petersen E. E. et al., 2016). Ultimately, products of ZIKV RNA specific RT-PCR amplification, regardless of sample source, can also be sequenced and aligned against established ZIKV genome sequences for confirmation (Musso et al., 2015b).

Antibody-Based Detection Immunoglobulin (Ig) G/M Enzyme-Linked Immunosorbent Assay (ELISA)

IgM/IgG ELISA involves the detection of ZIKV-specific antibodies in the serum (Huzly et al., 2016). IgM antibodies are known to develop within a few days post onset of symptoms and can last up to 3 months. IgG antibodies on the other hand, develop after IgM and can last from a few months to years. IgM specific to ZIKV have been developed at the Centers for Disease Control and Prevention, Atlanta (Hayes, 2009). However, studies have reported complications during diagnosis due to sera cross-reactivity of ZIKV IgM to antibodies against other *Flaviviruses*, often in patients with a history of flaviviral infection or vaccination (Charrel et al., 2016). According to Hayes (2009), cross-reactivity was predominantly noted with DENV, as compared to other *Flaviviruses*. IgM against ZIKV was detected in the serum as early as 3 days post onset of symptoms. However, in certain cases, IgM was detected after the 8th day post-symptom onset, thus introducing uncertainties in diagnosis. Commercial kits (for research purposes) for rapid

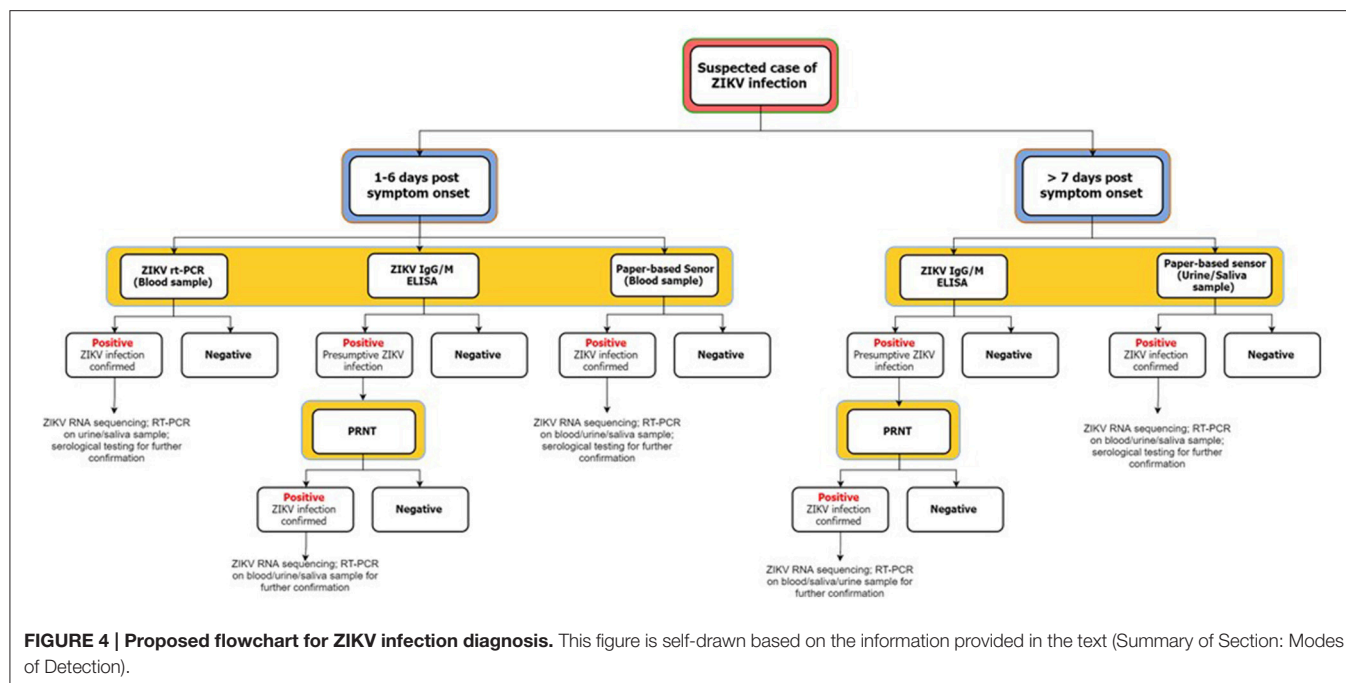


TABLE 3 | Molecular detection of ZIKV RNA from different human sample types.

Sample type	Primary detection technique	Duration for detection	References
Blood	qRT-PCR	Within approximately 5–7 days after onset of symptoms	Vorou, 2016
Serum	qRT-PCR & Antibody-based detection	Within approximately 5–6 days after onset of symptoms	Pan American Health Organization, 2016a
Semen	qRT-PCR	ZIKV RNA detected up to 62 days after onset of symptoms	Atkinson et al., 2016
Urine	qRT-PCR	Within approximately 15 days after onset of symptoms	Gourinat et al., 2015
Saliva	qRT-PCR	ZIKV RNA detected up to 20 days after onset of symptoms	Musso et al., 2015a

ZIKV IgM/IgG ELISA detection are readily available in the market (Charrel et al., 2016).

Plaque Reduction Neutralization Test (PRNT)

PRNT is used for virus-specific neutralizing antibody titer quantification (Rabe et al., 2016). The test is reported to have improved specificity compared to ELISA hence, it is often used in addition to ELISA to rule-out false positive antibody response (Hayes, 2009; Oehler et al., 2014; Charrel et al., 2016). The PRNT was used in addition to ELISA for diagnosis and confirmation of ZIKV in 185 patients during the French Polynesia outbreak (Duffy et al., 2009). To perform the PRNT, firstly, serum sample from the patient was diluted and mixed with a suspension of ZIKV. Subsequently, the mixture was poured over a monolayer of cells, often Vero or LLC-MK2 cell lines (Lednicky et al., 2016). The cells were subsequently covered with a thin layer of agar to avoid viral movement. PRNT against other *Flaviviruses* are concurrently performed as a control (Rabe et al., 2016). At least a 4-fold increase in ZIKV-specific neutralizing antibody titer is recommended for confirmation of ZIKV infection (Pan American Health Organization, 2016a). However, interpretation of results could be complicated if high *Flavivirus* background

is observed in the patient, often due to history of vaccination against *Flaviviruses* (Lanciotti et al., 2008; Rabe et al., 2016).

Toehold Switch Sensor and CRISPR/cas9-Based Detection

A novel, rapid, and low-cost method for ZIKV RNA detection has recently been suggested by Pardee et al. (2016). The study introduces a pipeline for ease of ZIKV RNA detection and ZIKV strain differentiation using a cell-free approach. Firstly, an RNA sensor, also known as the toehold switch sensor, programmable to bind and detect essentially any specific RNA sequence was developed. For ZIKV detection, the sensors were designed to bind specifically to ZIKV RNA and become activated at concentrations as low as 30 nM. Subsequently, the sensors were embedded into paper and freeze-dried. This increased sensor stability and ease of distribution to ZIKV endemic regions. The simple design of the paper-based sensor allows for rapid mass production at a cost as low as US\$1 per sensor (Dockrill, 2016).

For detection using the paper-based sensor, total RNA must first be isolated from blood, serum, saliva, or urine and then subjected to ZIKV genome region specific amplification using the nucleic acid sequence-based amplification (NASBA) technique in order to boost ZIKV RNA signaling (Pardee et al.,

2016). Subsequently, the amplified product is to be applied to the ZIKV RNA-specific sensor. Samples positive for ZIKV RNA are visually distinguishable upon the change of sensor color (from yellow to purple). The viral load in the sample could also be determined via semi-quantitative analysis (as described in Pardee et al., 2016). Recently, a CRISPR/Cas9 based module has also been coupled to the NASBA system hence, allowing different ZIKV strains to be distinguished with single-base resolution (Pardee et al., 2016). Due to the high specificity of the sensor to the targeted ZIKV genomic region, cross-reactivity to other closely related *Flaviviruses* has been eliminated. Serum, urine, and saliva samples from infected patients have been tested using the novel innovation. ZIKV RNA was successfully documented, with higher loads reported in urine samples as compared to serum and saliva (Pardee et al., 2016).

CONTROL MEASURES

Vaccine Development

Vaccines have been successfully developed for protection against multiple *Flaviviruses*, such as YFV, TBEV, JEV, and DENV (Lazear and Diamond, 2016; Weaver et al., 2016). To date, no vaccine against ZIKV has entered the clinical stage, therefore, suggesting that ZIKV vaccine establishment is yet multiple years away (Lazear and Diamond, 2016). Recently, an Indian biotech company claimed that it has two ZIKV vaccine candidates awaiting pre-clinical trials (Macdonald, 2016). SynCon Pharmaceuticals (USA) has also developed a DNA-based vaccine against ZIKV, which is expected to enter clinical trials by the end of this year (Saiz et al., 2016). Current ZIKV vaccine development strategies have been targeted toward adaptation of existing *Flavivirus* vaccine platforms (e.g., inactivated or live-attenuated virus, *Flavivirus* chimera, glycoprotein subunit technology). The growing threat of an explosive global spread of ZIKV has drawn an alarming interest among the scientific community to develop a suitable murine model for vaccine development (Shah and Kumar, 2016). Since low genetic variation is observed in different ZIKV strains, it is likely that a single vaccine may be effective against all circulating ZIKV strains. However, the effect of pre-existing immunity against other *Flaviviruses* on the immunity against ZIKV must be further investigated (Lazear and Diamond, 2016). In addition, it must be noted that *Flavivirus* vaccine development is limited by the nature of outbreaks, being sporadic and unpredictable. Therefore, rapid vaccine production to counter the quick spread of ZIKV may pose difficulties. Besides that, preemptive vaccination in anticipation of an outbreak may appear to be prohibitively expensive (Fauci and Morens, 2016).

Antiviral Therapeutics

Since ZIKV vaccine clinical trials are yet underway, more efforts are being put toward developing antiviral therapeutics against ZIKV for immediate control (Lazear and Diamond, 2016). Hitherto, there is no established antiviral treatment available for flaviviral infections (Weaver et al., 2016). Current

ZIKV infection treatment is symptomatic, often through the use of analgesics and antipyretics (Petersen E. E. et al., 2016). Human neural progenitor cells derived from induced pluripotent stem cells have been developed for use as an *in vitro* platform for therapeutic compound screening (Tang et al., 2016). Often, drugs with inhibitory activity against a specific step in the viral life cycle are targeted. Over the past decade, significant research has been conducted toward the development of drugs against DENV. A few drugs that have shown *in vitro* inhibition of DENV replication include mefenamic acid, tetracyclines, amodiaquine, and chloroquine (Wong et al., 2016). Due to the similarity between ZIKV and DENV, much of the knowledge-base for DENV drug discovery can potentially be applied for the development of anti-ZIKV therapeutics (Weaver et al., 2016). To date, no ZIKV drug screening studies have been published. Nevertheless, the most challenging obstacle to overcome in the field of drug development would be the search of therapeutics for infected pregnant women (Lazear and Diamond, 2016).

Preventative Strategies

Collective responsibility and engagement for integrated vector management, particularly through the removal of stagnant water and use of insecticides (diethyltoluamide/ethyl butylacetylaminopropionate)/larvicides, is greatly emphasized due to the lack of vaccines and antiviral therapeutics against ZIKV (Lazear and Diamond, 2016; Pan American Health Organization, 2016a). Although, *A. aegypti* and *A. albopictus* are primarily responsible for the current ZIKV outbreak, vector control strategies and vector-pathogen interaction of all possible mosquito species are advised to be considered owing to ZIKV's ability to evolve (Ayres, 2016). It is also recommended to wear long-sleeved shirts and long pants, even potentially insecticide-impregnated clothing, in order to minimize vector contact (Basarab et al., 2016; Weaver et al., 2016). In addition, men who have a recent travel history to ZIKV endemic regions are advised to refrain from having unprotected sexual intercourse with their pregnant partner (Oster et al., 2016). For asymptomatic travelers returning from ZIKV endemic regions, barrier contraception for 28 days is recommended (Wong et al., 2016).

The unexpected potential link between ZIKV infection and microcephaly has resulted in increased prenatal surveillance in ZIKV endemic regions (Lazear and Diamond, 2016). Currently, it is recommended that public health authorities in ZIKV endemic regions provide access to contraceptives, prenatal care, and safe abortion services. Efforts toward educating the population, particularly in ZIKV endemic regions and travelers, regarding the potential routes of ZIKV transmission and preventative measures should be greatly emphasized (Lazear and Diamond, 2016). Increased vigilance toward imported cases of ZIKV infection and increased surveillance of individuals returning from ZIKV endemic regions would most certainly reduce autochthonous ZIKV transmission and global spread (Marano et al., 2016). In addition to the current existing surveillance systems, more emphasis should also be put into appropriate ZIKV diagnosis and monitoring of the potentially associated teratogenic and

neurological complications (Pan American Health Organization, 2016a). Standard healthcare precautions should also be taken to eliminate mosquitoes from healthcare facilities in order to prevent autochthonous ZIKV transmission (Wong et al., 2016).

Entomological Surveillance

Entomological surveillance allows for early detection of a potential virus outbreak, vector distribution and density, and evaluation of vector control strategies (Basarab et al., 2016). Faye et al. (2014) have designed a specific, rapid, and sensitive one-step qRT-PCR assay (primers targeting the ZIKV NS5 gene) for the fast detection of mosquito-originated ZIKV isolates from Africa and Asia. Several new technologies have shown to be promising for vector control in ZIKV endemic regions or upon detecting ZIKV positive mosquitoes in a new region (Yakob and Walker, 2016). The most simplified and time and cost-efficient strategy for reducing the mosquito population is through the introduction of lethal mosquito traps. According to a study by Barrera et al. (2014), implementation of lethal traps in two urban areas in Puerto Rico resulted in approximately 50–70% reduction of *A. aegypti* mosquitoes. A more technical approach would be through the introduction of genetically modified male mosquitoes carrying a dominant lethal gene expressed at the larval stage which causes death in offspring upon mating with wild female mosquitoes (Wise de Valdez et al., 2011). Although, this approach has the potential to significantly reduce the mosquito population, scaling up may be technically and financially challenging (Weaver et al., 2016). Another potential strategy, which has shown positive potential for DENV control, is through the use of the endosymbiotic relationship between *Aedes* mosquitoes and the *Wolbachia* bacteria. The endosymbiotic relationship interferes with virus replication in the mosquitoes (inhibitory effect). However, the potential of the virus to evolve and overcome the inhibitory effect of the endosymbiotic relationship must be taken in to consideration (Ritchie et al., 2015).

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CONCLUSIONS

The recent ZIKV outbreaks in the Americas have raised alarming concerns regarding the possible association of ZIKV infection with unexpected clinical manifestations, such as prenatal microcephaly and GBS. The risks and severity of ZIKV infection have been difficult to evaluate due to the poor understanding of ZIKV transmission dynamics and the absence of standardized ZIKV detection technique. To this end, evidence from published reports suggesting the potential association of ZIKV infection with prenatal microcephaly and GBS have been summarized in this review. In addition, this review discussed the current advances in ZIKV transmission and detection and emphasized the importance of understanding transmission dynamics for the subsequent development of a rapid cost-effective and time-efficient ZIKV detection assay and control strategy. Lastly, strong emphasis on the implementation of stringent surveillance systems (for humans and mosquitoes) as a preventive strategy is advised, particularly in tropical regions where the potential for ZIKV outbreak is more likely.

Further studies could investigate the association between ZIKV infection and microcephaly/GBS through case-controls studies in order to rule out potential etiological confounding factors. Future studies could also explore other potential ZIKV reservoirs and further investigate the ZIKV pathogenesis pathways and host cellular response to aid the development of a robust detection assay, ZIKV vaccine, and antiviral therapeutics.

AUTHOR CONTRIBUTIONS

AS collected data, compiled data and wrote the manuscript, SL edited and reviewed the manuscript.

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Emergence and Spreading Potential of Zika Virus

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Zika virus (ZIKV) is an arthropod-borne *Flavivirus* (family *Flaviviridae*) closely related to dengue, yellow fever and West Nile viruses. ZIKV remained neglected, confined to enzootic transmission cycles in Africa and Asia, until the first significant outbreak was reported in Micronesia in 2007. Subsequent epidemics of growing incidence occurred in French Polynesia and other South Pacific Islands, and recently, in the Americas. The latter and currently ongoing outbreak of unprecedented incidence revealed the association of ZIKV infection with the occurrence of severe congenital malformations and neurological diseases, leading to a widespread concern about its potential to pose a global public health threat. Serological and molecular data suggest that the genetic and geographic diversification of ZIKV may be greatly underestimated. Here we discuss several ecological and epidemiological aspects, together with the evolutionary processes that may have driven the emergence and abrupt spread of ZIKV in the Americas.

Keywords: ZIKV, emerging infectious diseases, genetic variability ZIKV, molecular evolution, spreading potential ZIKV

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne virus that belongs to the Spondweni serocomplex in the *Flavivirus* genus of the *Flaviviridae* family. ZIKV is closely related to dengue virus (DENV), yellow fever virus (YFV), and West Nile virus (WNV) (Kuno et al., 1998). Although ZIKV enzootic activity was repeatedly observed in different regions of Africa and Asia, significant human outbreaks have only recently occurred, in particular during current American epidemics (Haddow et al., 2013). Its high variability, great adaptability to vectors and hosts, as well as its association with neurological diseases and fetus malformations, has converted ZIKV in one of the biggest challenges for global health as regards to prevention, detection and prospect for control.

EPIDEMIOLOGY

Zika virus was first detected in 1947 in rhesus monkeys, during a yellow fever routine surveillance in the Zika Forest in Uganda, where one year later was also isolated from a pool of *Aedes africanus* mosquitoes (Dick et al., 1952). The first human disease related with ZIKV was reported in 1954 in Nigeria (Macnamara, 1954). ZIKV enzootic activity was repeatedly observed in different regions of Africa and Asia, but only 14 human cases were reported until 2007 (Faye et al., 2014), when the first significant ZIKV outbreak occurred in Yap Island (Micronesia), leading to infection of 73% of the residents older than 3 years (Duffy et al., 2009). Later on, in 2013, a huge epidemic event occurred

in French Polynesia with around 30,000 symptomatic cases that included low fever, maculopapular rash, arthralgia and conjunctivitis (Musso et al., 2014). ZIKV outbreaks were subsequently reported in different Pacific islands including New Caledonia, Solomon Islands, Cook Islands, Fiji, Samoa, Vanuatu, and Easter Island (Cao-Lormeau et al., 2014; Dupont-Rouzeyrol et al., 2015; Musso and Gubler, 2016). In March 2015, ZIKV was reported in Salvador, Brazil (Campos et al., 2015; Zanluca et al., 2015), and rapidly spread throughout the Americas, leading to autochthonous cases in 40 countries of this continent by July 7th, 2016 (PAHO, 2016).

Zika virus ZIKV infections in humans are mainly asymptomatic, causing in some patients a mild, self-limited febrile illness that can be accompanied by other clinical symptoms like rash, arthralgia or conjunctivitis (Simpson, 1964). This was the usual clinical picture until the French Polynesian outbreak in 2013, when severe neurological complications were observed. Epidemiological data from this outbreak documented a 20-fold increase from expected in the incidence of Guillain-Barré syndrome (GBS) (Oehler et al., 2014), as well as meningoencephalitis (Carteaux et al., 2016) and acute myelitis (Mécharles et al., 2016) cases. GBS was also associated with ZIKV infections during current epidemics in the Americas in 12 countries (Dirlikov et al., 2016; dos Santos et al., 2016; PAHO, 2016; Rozé et al., 2016). In addition, the latter outbreak coincided both in time and geographic location with an increase in the number of infants born with microcephaly and other central nervous system malformations (Schuler-Faccini et al., 2016; Ventura et al., 2016). By July 2016, 1,638 cases of congenital syndrome associated with ZIKV infection were confirmed in Brazil, with reported cases of this disorder in Colombia, El Salvador, French Guiana, Martinique, Panama, Puerto Rico, and United States (PAHO, 2016). Subsequent retrospective studies also revealed congenital cerebral malformations in newborns during French Polynesian outbreak of 2013 (Besnard et al., 2016; Cauchemez et al., 2016). Accumulating evidence supports the association between ZIKV infection and birth defects, including the detection of ZIKV RNA, viral particles, and/or viral antigens in placenta, amniotic fluid and fetal tissues, being the latter studies performed on microcephalic fetuses after miscarriage or neonatal death (Calvet et al., 2016; Ladhani et al., 2016; Martinez et al., 2016; Meaney-Delman et al., 2016; Mlakar et al., 2016; Oliveira Melo et al., 2016; Rasmussen et al., 2016; Sarno et al., 2016). Moreover, a recent study reports the detection of anti-ZIKV IgM antibodies in cerebrospinal fluids of 30 out of 31 neonates with microcephaly, strongly suggesting a congenital infection with ZIKV (Cordeiro et al., 2016). In addition, experimental data indicated that ZIKV infects human neural progenitor cells attenuating their growth (Hughes et al., 2016; Li et al., 2016; Nguyen et al., 2016; Tang et al., 2016).

As previously discussed, until 2007 our knowledge of this ZIKV was restricted to limited confirmed cases in Africa and Asia. However, on the basis of entomological, epidemiological and seroprevalence studies, it can be deduced that the incidence, prevalence and dispersion of ZIKV have been significantly underestimated (Musso and Gubler, 2016). Human serosurveys suggest that ZIKV might be endemic in most part of Africa

and South-East Asia (Dick et al., 1952; Smithburn, 1952, 1954; Hammon et al., 1958; Pond, 1963; Fagbami, 1979; Petersen et al., 2016), although it is important to note that the specificity of the serological studies used is uncertain due to the significant cross-reaction between different flaviviruses (Lazear and Diamond, 2016). This silent circulation may be explained by the fact that most ZIKV infections are asymptomatic, and clinical manifestations are generally mild and can be mistaken with other arboviral infections, leading to significant misdiagnosis and underreporting (Haddow et al., 2012). For instance, patients of Micronesian outbreak of 2007 were initially diagnosed with dengue fever (Lanciotti et al., 2008; Duffy et al., 2009). The same happens in most tropical and subtropical regions where other non-specific diseases like dengue and/or chikungunya are endemic (Nhan and Musso, 2015). Moreover, as ZIKV is not commonly tested in routine diagnostic assays, a considerable number of cases are expected to remain undetected. For example, recently retrospective analyses revealed a widespread distribution of ZIKV in Thailand (Buathong et al., 2015), as was previously suggested by the confirmation of imported cases from travelers returning from that country (Fonseca et al., 2014; Tappe et al., 2014). Several other reports of imported cases from tourists that have visited different Asian and South Pacific Islands reveal that circulation of ZIKV remain silent in different countries (Deng et al., 2016; Korhonen et al., 2016; Zhang et al., 2016).

TRANSMISSION

Zika virus circulation has been mainly reported in sylvatic enzootic transmission cycles, involving arboreal mosquitos and non-human primates (Darwish et al., 1983; Hayes, 2009; Faye et al., 2014). Several mosquitoes species have been related with African and Asian jungle cycles, especially *A. africanus* (Dick et al., 1952; Haddow et al., 1964; Berthet et al., 2014; Diallo et al., 2014), as well as other *Aedes spp.* mosquitoes (Marchette et al., 1969; Cornet et al., 1979b; Fagbami, 1979; McCrae and Kirya, 1982; Akoua-Koffi et al., 2001; Berthet et al., 2014; Diallo et al., 2014). In enzootic transmission cycles, humans and other mammals may act as occasional dead-end hosts (Kenney and Brault, 2014). ZIKV antibodies have been detected in several vertebrates (Darwish et al., 1983; Haddow et al., 2012), suggesting that other animals may be involved in natural transmission cycles.

Humans accidentally infected may potentially act as hosts leading to urban cycles, if they exhibit high and sustainable viremia (Kuno and Chang, 2005; Duffy et al., 2009). The role of a bridge vector between both ecologically distinct transmission cycles is essential for these events to occur. Several mosquito species have been studied in terms of their host choice to evaluate their potential as bridge vectors of different arboviruses (Kaddumukasa et al., 2015). *A. vittatus* have been suggested to link both ZIKV cycles in Africa (Diallo et al., 2014). *A. albopictus* was found to be implicated in DENV cycle switching in Asia (Vasilakis et al., 2011; Hanley et al., 2013), and therefore, it is likely to play the same role with ZIKV. Moreover, experimental data have shown *A. albopictus* adaptability to transmit ZIKV

(Wong et al., 2013; Chouin-Carneiro et al., 2016; Di Luca et al., 2016), as has been observed at least in Gabon in 2007 (Grard et al., 2014). However, *A. aegypti* seems to have the highest vectorial capacity in urban cycles (Lord et al., 2015), and has been related with most human outbreaks, including current American epidemics (Boorman and Porterfield, 1956; Marchette et al., 1969; Cornet et al., 1979a; Olson et al., 1981; Li et al., 2012; Weaver et al., 2016). Both *A. aegypti* and *A. albopictus* are anthropophilic mosquitoes that are widely distributed throughout tropical and subtropical regions and are also competent vectors for other arboviruses like DENV, YFV and chikungunya virus (CHIKV) (Musso and Gubler, 2016). Other mosquito species, like *A. hensilli* and *A. polynesiensis*, have been, respectively, responsible for ZIKV outbreaks in Yap Island in 2007 (Ledermann et al., 2014) and French Polynesia in 2013–2014 (Musso et al., 2014), suggesting that the potential role of other *Aedes* spp. as additional vectors should not be ruled out.

Although mosquito-borne transmission is the most common route for ZIKV infection, current South Pacific/American outbreaks revealed other modes of biological transmission. In particular, the elevated number of newborns with microcephaly raised concern about maternal-fetal transmission during pregnancy. As previously discussed, growing evidence support trans-placental transmission, and perinatal transmission of ZIKV has also been reported in French Polynesia (Besnard et al., 2014). Furthermore, recent studies have demonstrated that ZIKV RNA can be found in semen 62 and 93 days after onset of symptoms, although virus infectivity was not tested as cell culture assays were not performed (Atkinson et al., 2016; Mansuy et al., 2016). This finding is in line with the amount of sexually transmitted cases that have been lately reported (Musso et al., 2015; Deckard et al., 2016; D'Ortenzio et al., 2016; Frank et al., 2016; Hills et al., 2016; Turmel et al., 2016; Venturi et al., 2016). Moreover, recently published data suggest the first case of female to male sexual transmission (Davidson et al., 2016). Despite the potential of sexual transmission to promote an epidemic has been predicted to be unlikely (Yakob et al., 2016), these findings indicate that this route of transmission may contribute to dispersal of ZIKV greater than initially thought.

GENETIC VARIABILITY OF ZIKV

Zika virus is a single-stranded, positive sense, RNA virus with a genome of approximately 10,794 nt in length. Its genome carries a single open reading frame (ORF) that encodes three structural (capsid (C), precursor of membrane (prM) and envelope (E)) and seven non-structural (NS) proteins, with 2 flanking non-coding regions (5' and 3' NCR) (Kuno and Chang, 2007). Several phylogenetic analyses have been performed in order to investigate the genetic diversity of ZIKV, using both partial and complete genome regions (Lanciotti et al., 2008; Haddow et al., 2012; Faye et al., 2014; Enfissi et al., 2016; Fajardo et al., 2016; Faria et al., 2016; Gong et al., 2016; Shen et al., 2016; Wang et al., 2016). Full-length ORF sequences analyses reveal that the high error rate of RNA-dependent RNA polymerase has driven ZIKV to evolve into

two different genetic groups, denominated African and Asian-American lineages (see **Figure 1**) (Haddow et al., 2012; Enfissi et al., 2016; Faria et al., 2016).

African lineage comprises strains isolated in Burkina Faso, Central African Republic, Cote d'Ivoire, Gabon, Nigeria, Senegal, and Uganda, and can be further divided in two sub-lineages, as was indicated by phylogenetic approaches restricted to RNA-polymerase (NS5) (Lanciotti et al., 2008) or E (Faye et al., 2014) coding regions. On the basis of phylodynamic studies, Faye et al. (2014) suggested that the most common recent ancestor of all reported ZIKV strains circulated around 1920 in Uganda, from where it spread to West Africa and afterward to Malaysia, giving rise to Asian (currently Asian-American) lineage (Faye et al., 2014). This genetic group has been better characterized, especially after current epidemics when several full-length sequences were obtained. This lineage clusters together ancestral Malaysian strains (1966) with variants from Micronesia (2007), Cambodia (2010), Philippines (2012), Thailand (2014), and all ZIKV strains reported in the ongoing American outbreak (**Figure 1**, in blue), which are closely related to French Polynesian variants of ZIKV epidemics of 2013. Recent studies indicated that currently ZIKV strains circulating in the Americas emerged from a single introduction of an ancestor that existed in French Polynesia between August 2013 and June 2014 (Fajardo et al., 2016; Faria et al., 2016). This hypothesis is in line with observed phylogenetic patterns, as French Polynesian variant roots the American cluster (**Figure 1**). However, seven variants recently obtained from Chinese travelers returning from Fiji and Samoa (**Figure 1**, in red), share a different evolutionary history, revealing that two different sub-lineages are responsible of current ZIKV epidemics in the Americas and in South Pacific Islands (Deng et al., 2016; Zhang et al., 2016). Moreover, a recent study indicated that these imported Chinese strains share an ancestor with American variants that circulated around May 2013 (Fajardo et al., 2016), coinciding with the time when the ancestor of all American isolates was circulating (Faria et al., 2016). This finding indicates that two different evolutionary routes were followed by ancestral strains that emerged in French Polynesia, giving rise to different contemporaneous sub-lineages circulating in the Americas and in South Pacific Islands, revealing that ZIKV diversification may be greatly underestimated (Fajardo et al., 2016).

Unfortunately, there are no other full-length sequences available from the Pacific Islands outbreak, which limits our interpretation of the evolutionary patterns, diversity and pathogenicity of currently circulating ZIKV strains. In fact, the lack of complete genome sequences distributed over time and space has been one of the major historical limitations to explore in detail the phylogenetic relationships of ZIKV variants and their spatio-temporal distribution. For instance, recent reports have suggested that another genetic group, African lineage II, is revealed when phylogenetic relationships are deduced through the analysis of E and NS5 coding regions (Gong et al., 2016; Shen et al., 2016). This lineage group together one variant of Cote d'Ivoire of 1980 and six strains isolated in Senegal between 1998 and 2001 (Faye et al., 2014). Furthermore, both African and Asian-American clusters seem to be rooted by this lineage, which also suggests a greater ZIKV genetic and geographic



FIGURE 1 | Maximum-likelihood analysis of all complete ORF sequences of Zika virus (ZIKV) available. The phylogenetic tree was performed using PhyML software (Guindon et al., 2005) under a GTR+G+I substitution model, and edited with FigTree program v1.4.2 (available at: <http://tree.bio.ed.ac.uk>). Strains are indicated by their accession number, year and country of isolation. Sequences derived from variants isolated during current American outbreak and from imported strains from Fiji and Samoa (Deng et al., 2016; Zhang et al., 2016), are indicated in blue and red, respectively. Bootstrap values >90% support major nodes but are not shown for visualization purpose. The bar at the bottom of the tree denotes nucleotide distance.

diversification than expected (Shen et al., 2016). This hypothesis was also supported by Gong et al., who calculated a genetic distance of 0.212 substitutions/site between the E gene of two contemporary isolates of Senegal, belonging to African lineages I

and II, respectively. Considering evolutionary rates of the order of 10^{-3} substitutions/site/year, as recently estimated for American ZIKV strains (Fajardo et al., 2016; Faria et al., 2016), ZIKV may have been circulating in Africa for more than 100 years

(Gong et al., 2016). Therefore, the genetic characterization of variants of this lineage would provide essential information to investigate the origin, geographic dispersion and genetic diversity of ZIKV.

ZIKV SPREADING POTENTIAL

The emergence and dispersal of ZIKV in the Americas reminds us of the route that was previously followed by other arboviruses, like DENV and CHIKV, which were first detected in Africa, and spread subsequently to Asia and the Americas. This migration pattern is associated with ZIKV capacity to adapt to urban vectors, like *A. aegypti*, which allows its expansion into human environments. A general characteristic that is shared between different arboviruses is their high mutation rates, which provide them with the possibility to explore different phenotypical changes in their continually evolving process to adapt to different vectors and hosts (Kuno and Chang, 2005). For instance, a single amino acid change in its surface glycoprotein allows CHIKV to switch its competent vector to *A. albopictus* (Tsetsarkin et al., 2007). Therefore, genetic changes play a major role in adaptation of arboviruses to different hosts and vectors, as has been also reported in DENV (Messer et al., 2003; Bennett et al., 2010) and WNV (Malkinson et al., 2002; Brault et al., 2007; Moudy et al., 2007).

The recent epidemics in South Pacific and the Americas with its unprecedented association with microcephaly and GBS cases, led to the proposal of different hypothesis to explain this emergence. It is likely that as a consequence of different genetic changes, ancestral Asian lineage gave rise to epidemic strains that become better adapted to humans, leading to infections with higher viremia levels, enhancing trans-placental transmission and modulating changes in cell tropism (Musso and Gubler, 2016; Weaver et al., 2016). Moreover, recent analyses have suggested a bias in the codon usage of ZIKV to increase its fitness in humans (Freire et al., 2015; Cristina et al., 2016; Russell, 2016). This hypothesis is supported by the short-term diversification observed among recent isolates (Figure 1), revealing the expansion of ZIKV into a large naïve population (Weaver et al., 2016). It has also been hypothesized that the rare congenital malformations and immunological disorders may be in fact low frequency events in ZIKV infections, that are now exposed due to the extent of recent outbreaks (Musso and Gubler, 2016). Furthermore, recent studies have suggested that pre-existing DENV antibodies may enhance

ZIKV infection through an antibody-dependent enhancement mechanism, similar to what happens in secondary infections of DENV (Dejnirattisai et al., 2016; Paul et al., 2016). If this is confirmed it would have huge implications for disease pathogenesis, considering that current epidemics are occurring in regions where most of the population has already been exposed to DENV. Additionally, this mechanism of infection enhancement, together with ZIKV adaptability to vectors/hosts, the widely distribution of competent mosquitoes and the unusual non-vectorial human-to-human transmission routes observed, can help to interpret the reasons for the suddenly explosive emergence of ZIKV in the Americas and its potential to spread into other geographic regions.

CONCLUSIONS

Current ZIKV epidemics occurring throughout the American continent represent the most recent example of a mosquito-borne virus introduction into previously unaffected areas with immunologically naïve population. This unexpected emergence follows recent arrival and spreading of DENV, WNV, and CHIKV, which clearly responds to human activities that promotes optimal ecological environments for vectorial activity. Although ZIKV has remained almost ignored for half a century, entomological, epidemiological and molecular studies have strongly indicated that its incidence, geographic dispersion and genetic diversity have been significantly underestimated. Its adaptability to different mosquito species allowed ZIKV to spread into efficient urban transmission cycles helped by the widespread distribution of competent vectors. Furthermore, unprecedented routes of transmission for flaviviruses, including maternal-fetal and sexual intercourse, may contribute to increase its spreading potential.

AUTHOR CONTRIBUTIONS

AF, JC, and PM contributed to the elaboration of this minireview. All authors read and approved the final manuscript.

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Zika Virus: An Emerging Worldwide Threat

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ZIKA virus (ZIKV) poses a severe threat to the world. Recent outbreaks of ZIKV after 2007 along with its quick transmission have made this virus a matter of international concern. The virus shows symptoms that are similar to those caused in the wake of dengue virus (DENV) and other flaviviruses, which makes it difficult to discern the viral infection. Diagnosis is further complicated as the virus cross-reacts with antibodies of other viruses. Currently, molecular diagnosis of the virus is being performed by RT-PCR and IgM-captured enzyme-linked immunosorbent assay (MAC-ELISA). The real brunt of the virus is, however, borne by children and adults alike. Case studies of the ZIKV outbreaks in the French Polynesia and other places have suggested that there is a close link between the ZIKV and Guillain-Barre syndrome (GBS). The GBS has closely followed in areas facing ZIKV outbreaks. Although solid evidence is yet to emerge, clinical data integration has revealed a large number of ZIKV patients having GBS. Moreover, the amniotic fluids, blood cord, and miscarriage tissues of mothers have been detected with ZIKV, which indicates that the virus either gets transferred from mother to fetus or seeks direct entry in the fetus, causing microcephaly and other brain anomalies in the newborn babies. Studies on mice have confirmed the link between the ZIKV infection during pregnancy and microcephaly in babies. Reports have highlighted the sexual transmission of the ZIKV, as it has been detected in the semen and saliva of affected persons. The intensity with which the ZIKA is spreading can collapse the health sector of several countries, which are poor. A comprehensive strategy is a need of an hour to combat this virus so as to prevent its transmission and avert the looming threat. At the same time, more research on the cure of the ZIKV is imperative.

Keywords: ZIKV, disease, infection, vaccines, diagnosis

INTRODUCTION

ZIKA virus (ZIKV) belongs to the family of flaviviruses (Weaver, 2017a) that entails other viruses, such as yellow fever, dengue, Japanese encephalitis, and West Nile. The virus was first isolated from a rhesus monkey that was being used as one of the sentinel animals in a research program, which covered yellow fever. Soon after, the virus followed to infect humans in Nigeria in 1954

(Schuler-Faccini et al., 2017). It is transmitted through the arthropod vectors having serological overlapping with viruses like dengue virus (DENV) and West Nile Virus (Korhonen et al., 2016). Following the infection in 1954, there were about fourteen documented cases of the ZIKV prior to 2007 (Weaver, 2017b). The virus was only present in Asia and Africa and did not cause any major outbreaks. However, 2007 and 2013 saw two major outbreaks of ZIKV, reported from the Pacific Island of Yap and French Polynesia, respectively (Hall, 2017). The virus quickly spread to the American continent and 33 countries of America were hit by March 2016 (Hennessey et al., 2016). The symptoms were characterized by mild fever, arthralgia, conjunctivitis, and rash. The initial reports were of the opinion that ZIKV can cause mild febrile illness. The Brazilian cases of ZIKV, however, were reportedly linked to fetal microcephaly of ZIKV-infected mothers (Mlakar et al., 2016). Simultaneously, several patients of the ZIKV in French Polynesia showed neurological symptoms such as Guillain-Barre syndrome (GBS) (Ioos et al., 2014). These reports along with the speedy spread of the virus were indications of the latent disorders associated with the ZIKV and its potential of becoming a global threat.

ZIKA VIRUS TRANSMISSION

The transmission of ZIKV shows a high degree of variance. In Africa, the virus has adopted the sylvatic transmission cycle mode, which involves various species of *Aedes* mosquitoes and non-human primates, such as rhesus monkeys (Figure 1). Whereas, in the Asia, the sylvatic transmission cycle of ZIKV is yet to be reported (Diallo et al., 2014), where the ZIKV has adopted the transmission passages from the human-mosquito, and human-human transmission cycle. The most widely common vectors of ZIKA are mosquitoes from *Stegomyia* and *Diceromyia* sub-genera of *Aedes*, *Aedes africanus* and *A. furcifer*. *A. aegypti* and *A. albopictus* have been the primary vectors for majority of the ZIKV outbreaks (Ciota et al., 2017). However, in case of Yap, and Polynesia outbreaks, the *A. hensilli* and *A. polynesiensis* were the vectors of ZIKV, respectively (Musso et al., 2014). *A. aegypti* and *A. albopictus* are being considered as vectors with low vectorial competence (Santos and Meneses, 2017); however, with high vectorial capability, where low vectorial competence reduces the ability of the mosquito to acquire and transmit the ZIKV to other susceptible hosts. High vectorial capability, however, increases the efficiency of arthropods in transmitting the virus and is based on the number of bites, its longevity, and the population density of the mosquitoes among other factors. The high vectorial capability of *A. aegypti* and *A. albopictus* is attributed to many factors, such as close imperceptible bite and close association with humans (Chouin-Carneiro et al., 2016). Distribution of *A. aegypti* and *A. albopictus* is also a significant factor in the transmission of ZIKV. Moreover, there are other mosquito species, which could serve as a mode of transmission, fortunately, however, their vectorial capacity is remarkably low, and thus prevents further exacerbation of ZIKV problem (Diallo et al., 2014).

NON-MOSQUITO TRANSMISSION

There are adequate reports that ZIKV has the capability to be transmitted from a mother to her fetus during the pregnancy. Virus particles and RNA were detected in the amniotic fluid of fetus (Calvet et al., 2016). Additionally, the ZIKV viral antigens also marked the placenta and miscarriage tissues of infected mothers (Meaney-Delman et al., 2016). Recent study by Pagani et al. (2017) reported that primary human endometrial stromal cells are greatly permissive to ZIKV infection and supports its *in vitro* replication. Perinatal transmission of ZIKV was also reported in French Polynesia outbreak. A study also suggested that routes of perinatal transmission are mainly transplacental, breastfeeding, close contact between mother and baby during delivery (Colt et al., 2017). ZIKV sequences have been detected in the semen 62 days after the onset of symptoms. The data available hint the possible transmission of the virus through vaginal and oral sex (Hills et al., 2016; Russell et al., 2016). Nonetheless, transmission role of other biological fluid, such as pre-ejaculation semen, and saliva transmission cannot be ruled out (Cowper's gland). Another non-mosquito transmission could be the blood transfusion (Bierlaire et al., 2017). During French Polynesia 3 of donated blood samples were tested positive for ZIKV.

The use of an animal model to study ZIKA infection is crucial for fundamental studies and development of effective interventions. In recent years, significant efforts have been made to develop mouse and non-human primate models to study ZIKA infection (Dudley et al., 2016). Subcutaneous inoculation of ZIKA in non-human primate resulted in the development of fetal brain lesions (Waldorf et al., 2016) and neonatal pigs were found highly susceptible to ZIKA infection (Darbellay et al., 2017). Also, a parallel study reported that subcutaneous administration of Asian-lineage ZIKA in pregnant rhesus macaques resulted in highly efficient maternal-fetal ZIKA transmission (Nguyen et al., 2017). The results propose that maternal-fetal ZIKA transmission could be frequent in human pregnancies. Another study by Haddow et al. (2017) reported high infection rates among adult macaques after intra-vaginal or intra-rectal inoculation. It proposes that ZIKA infection by sexual intercourse could increase the chances of spread of ZIKA in regions where the virus has not been reported.

VIROLOGY

The ZIKV belongs to the family, Flaviviridae, genus Flavivirus. The virus is an arthropod-borne virus or arbovirus. The infectious particle of the virus known as virion is surrounded by a lipid membrane embedded by the viral membrane protein (Protein M), and envelope protein (Protein E). The virions are icosahedral, enveloped and contain a single-stranded, non-segmented RNA genome of the positive strand (Cortese et al., 2017) that encodes seven non-structural and three structural proteins. These are expressed as a singular polypeptide, which undergoes cleavage (White et al., 2016). The virion seeks the entry into the host cell by clathrin-mediated endocytosis. Removal

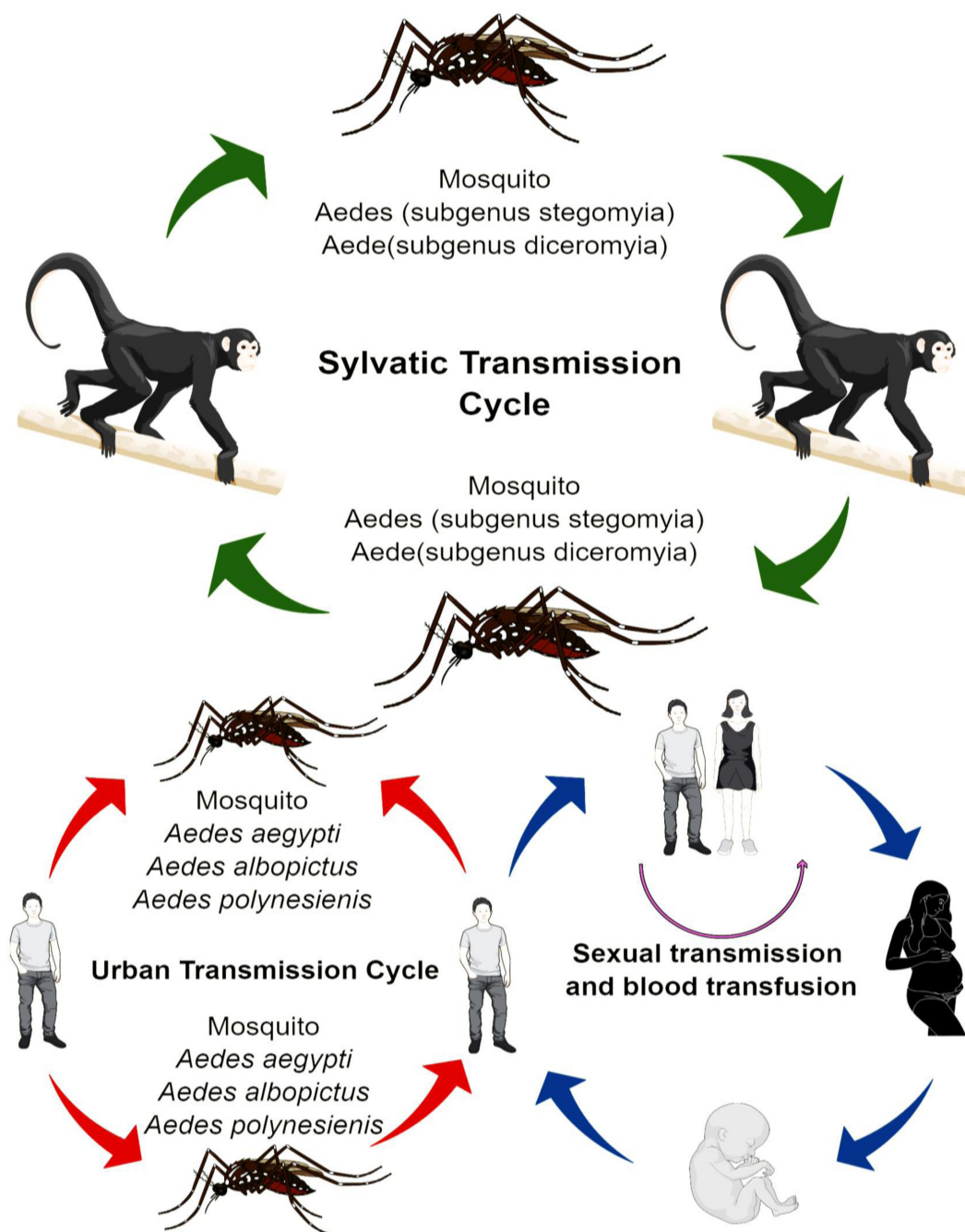


FIGURE 1 | Transmission of ZIKA.

of the envelope is followed by the disruption of nucleocapsid, and the genome is released into the cytoplasm. The genomic RNA of ZIKV then replicates in the cytoplasm of the infected host cells. The genome of the virus is translated by translational apparatus of the host cell, and results into the formation of single polypeptide that is proteolytically cleaved into the individual viral proteins, PreM, C, and non-structural proteins NS1 to NS5 (Figure 2).

ACUTE FEBRILE ILLNESS AND NEUROLOGICAL COMPLICATIONS

The diagnosis of undifferentiated febrile illness is challenging as its relevant symptoms overlap with other common infections (Maria et al., 2013). The adults are more vulnerable to acute febrile illness that is marked by popular rashes, arthritis, non-purulent conjunctivitis, headache, vomiting, and myalgia

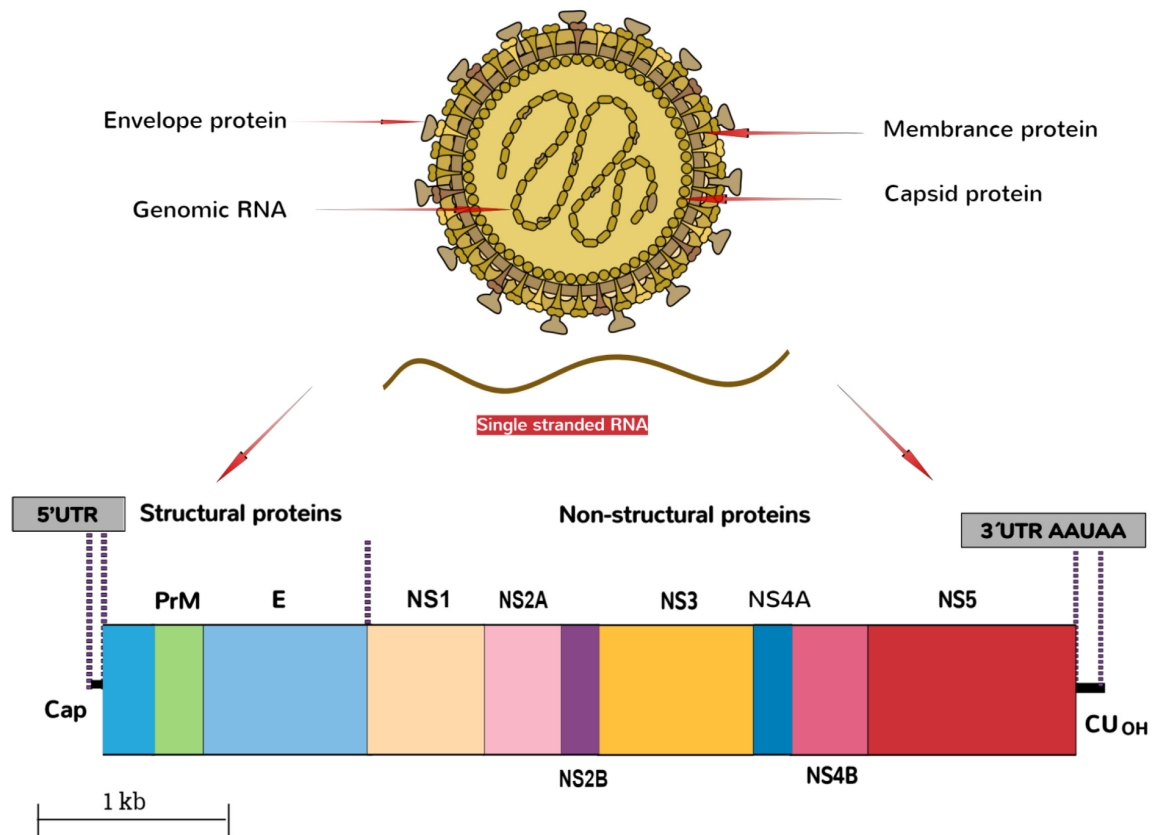


FIGURE 2 | Schematic representation of ZIKA particle and its genome organization.

(Zammarchi et al., 2015). Numerous case studies in the French Polynesia have determined the relationship between GBS and ZIKV (De Oliveira et al., 2017), where GBS is a disorder of the immune system that is characterized by sensory abnormalities, autonomic dysfunction, and weakness due to nerve root or peripheral damage (Dirlikov et al., 2017). During the ZIKV outbreaks, record numbers of patients were found to have GBS (Uncini et al., 2016). Subcutaneous bleeding and hematospermia were other symptoms associated with GBS followed by ZIKV syndrome (Lyle et al., 2016). According to European Center for Disease Prevention and Control [ECDC] (2016), the data of French Polynesia outbreak hints a possible link between GBS and ZIKV, which was later validated by French Ministry of Health in Martinique. In neonates, the neurological complications are on the rise unabated. Microcephaly in Brazil has dramatically increased in the last 3 years. This alarming situation has compelled the government to declare it as a national health emergency, which was further reciprocated by WHO (2016) (Gulland, 2016). Rasmussen et al. (2016) have reviewed and evaluated the available data on the ZIKV infection during pregnancy and concluded that there was a causal relation between ZIKV infection and microcephaly along with other serious brain anomalies. Other studies have also shown evidences from the available reports of clinical data that proves the causal relation between ZIKV and microcephaly due to the viral infection

throughout the length of pregnancy leading to consistent cases of birth defects (Mlakar et al., 2016). Vermillion et al. (2017) also conducted a study on the pregnant mice by inoculating the uterine wall of the immunocompetent mice to understand the transplacental transmission. Results showed that the ZIKV infection during pregnancy caused several fetal abnormalities, which included microcephaly, intrauterine growth restriction, and enlarged ventricle. These recent studies have established a direct link of microcephaly with the ZIKV infection in a pregnant mother.

DIAGNOSIS

The diagnosis of ZIKA is primarily being done either by RT-PCR-based tests or neutralization assay where immune globulin IgM is detected by IgM-captured enzyme-linked immunosorbent assay (MAC-ELISA) (Tappe et al., 2014). Viral nucleic acid can be traced in the serum within 1 week after the onset of clinical illness. However, there is no solid evidence about the response and persistence of IgM antibody-mediated neutralization. The data of other mosquito-borne flaviviruses suggest that formation of IgM antibody takes places within 10 days after onset of clinical illness and can persist for more than 2 months (Kalra et al., 2014). The greatest challenge in the diagnosis of ZIKV is

the cross-reactivity of the flaviviruses. Sometimes ZIKV infected patients evoke positive MAC-ELISA for DENV, and other related viruses (Tauro et al., 2016). The differentiation of closely related antibodies of closely related viruses could overcome by plaque reduction neutralization test (PRNT); however, high cost and labor-intensive reasons do not make it an ideal choice for clinicians (Roehrig et al., 2008). Moreover, the problem of cross-reactivity gets further compounded by the Hoskins effect (Original antigenic sin) (Lanciott et al., 2016). Due to the vaccination or natural infection of closely related flaviviruses, the memory response is more robust to the former than a freshly entered virus. Even this problem could not be solved by high accuracy of PRNT (Mohammed et al., 2012). Furthermore, the saliva and serum of the patients were subjected to RT-PCR and were found sensitive in detection (Rozé et al., 2016). There is still no reliable test for diagnosis of prenatal ZIKV infection, although RT-PCR can be performed on amniotic fluid, and other tissues like blood chod, and so on.

PREVENTION AND CONTROL

At present, there is no vaccine available in the market against ZIKV, thus it is imperative that coordinated, multidimensional, and comprehensive strategies are made to deal with any eventuality. Like other flavivirus infections, the treatment for ZIKV is entirely based on the symptoms. The primary strategy to adopt and to deal with ZIKA outbreak is restricting the vectorial capacity of *A. aegypti* viruses by eliminating their breeding sites, application of larvicides, use of mosquito repellents, bed nets, avoiding sleep in the day, and to maintain the green and clean environment (Banks et al., 2014). The sexual transmission of ZIKV could be prevented by avoiding the unprotected sex, and sexual contact with the persons who are vulnerable of getting infected or traveling from virus prone areas (Petersen et al., 2016). Interference at the genetic level of bacteria like *Wolbachia* could be very beneficial in restricting the transmission of ZIKV (Weaver, 2013). The entry of *Wolbachia* bacteria in a cycling pool of vector will have a cascading effect as the population of *Wolbachia* carrying mosquitoes will expand with each cycle of reproduction (Nguyen et al., 2015). Moreover, the use of genetically modified mosquito strains has been found effective in DENV, and thus are likely to be used against ZIKV as well. *A. aegypti* OX513A is a genetically modified (GM) strain, leading to a reduction of the local population of *A. aegypti*. The GM male mosquitoes can mate with wild type females and thus can eliminate them (Alphey and Alphey, 2014). In order to prevent the mother-fetus transmission, pregnant women should avoid unnecessary travels to the ZIKV affected areas. In addition, the government must alert the people who are traveling to affected areas.

FUTURE OUTLOOK

The cross-reactivity of serological assays and mild symptoms of ZIKV make it difficult for scientists to gauge it at the

preliminary stage. The risk of ZIKV multiplies in the areas where DENV is an epidemic as both belong to the family of flavivirus. The emergence of ZIKV is still an enigma for the scientific community; however, a general trend has been observed across the globe in which transmission of DENV, and chikungunya infections are followed by ZIKV infection (Musso et al., 2015). The simultaneous outbreaks of DENV, and other flaviviruses give a kind of refuge to ZIKV as it is being either misdiagnosed or getting undiagnosed given the reason of lack of standard molecular diagnostic test. Another important aspect of ZIKV endemic transmission could be the change of virulence by *A. aegypti*. The expansion of urbanization and transmission of flavivirus have been very much proportional. The greatest challenge before the scientific community is to save the incoming generation (newborn babies) from the onslaught of ZIKV infection. The relationship between the GBS in French Polynesia is very alarming and could further exacerbate child healthcare in the world. The rise in microcephaly and GBS in the aftermath of ZIKV outbreaks is a serious matter of concern and requires proper treatment so that the disease does not nip out tulips (children) in the bud. In order to develop an effective strategy against ZIKV, and other flaviviruses, there is a need to systematically identify, and address the loopholes in virus research. Diagnostic assays for the ZIKV are garnering intense interest and there is hope that in the near future promising strategies for the improved diagnostics of ZIKV will translate into therapeutic and preventive tools. Development of animal models for fetus development would further deepen the understanding of the transmission of ZIKV from mother to fetus. The present clinical data must be integrated, and new reliable, affordable molecular diagnostic tests must be developed to cope up with ZIKA endemic. Climate change is also a contributive factor in the transmission of many flaviviruses. New reports have outlined that rising temperature of earth suits the breeding patterns of mosquitoes. Globalization of trade is another route for the transmission of the virus specifically due to the rubber tires of transportation vehicles serving as breeding sites for mosquitoes. For the time being, an accurate vaccine for the treatment of the ZIKV is unavailable, therefore, there is little that can be done to reverse the adverse effects that the virus is having on the babies in the form of microcephaly and GBS. New and efficient ways of vector controlling mechanism must be introduced, and influence of environmental factors on ZIKV emergence should be understood in an elaborate manner. Although herd immunity can slow the transmission rate to some extent, it cannot be a replacement for the appropriate vaccination. Hence, it is imperative that efforts to develop therapeutic tools like a vaccine against ZIKV must continue unabated to develop a cure as soon as possible.

CONCLUSION

Zika virus is a threat of international concern that requires immediate attention. Success against this threat can only be achieved by continuing the extensive research regarding this virus to be able to find an appropriate vaccine to tackle with

the menace. The relationship, mode of action, and transmission among DENV, chikungunya and ZIKV infections should also be verified. A breakthrough in either of the mentioned viruses could be a milestone in the history of medicine as all of these viruses have a high degree of genetic similarity because they belong to the family of flavivirus. Until then, a comprehensive multidimensional strategy must be employed to strengthen public awareness in this regard and control the spread of the ZIKV by curbing its transmission through sexual intercourse, travel to affected areas and global trade.

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IR and JBL wrote the paper. VB design the paper. WP and JL did the critical analysis and approved the paper.

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Corrigendum: Zika Virus: An Emerging Worldwide Threat

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Zika Virus: What Have We Learnt Since the Start of the Recent Epidemic?

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Zika is a viral disease transmitted mainly by mosquitoes of the genus *Aedes*. In recent years, it has expanded geographically, changing from an endemic mosquito-borne disease across equatorial Asia and Africa, to an epidemic disease causing large outbreaks in several areas of the world. With the recent Zika virus (ZIKV) outbreaks in the Americas, the disease has become a focus of attention of public health agencies and of the international research community, especially due to an association with neurological disorders in adults and to the severe neurological and ophthalmological abnormalities found in fetuses and newborns of mothers exposed to ZIKV during pregnancy. A large number of studies have been published in the last 3 years, revealing the structure of the virus, how it is transmitted and how it affects human cells. Many different animal models have been developed, which recapitulate several features of ZIKV disease and its neurological consequences. Moreover, several vaccine candidates are now in active preclinical development, and three of them have already entered phase I clinical trials. Likewise, many different compounds targeting viral and cellular components are being tested in *in vitro* and in experimental animal models. This review aims to discuss the current state of this rapidly growing literature from a multidisciplinary perspective, as well as to present an overview of the public health response to Zika and of the perspectives for the prevention and treatment of this disease.

Keywords: flavivirus, epidemiology, microcephaly, Guillain-Barré syndrome, antivirals

INTRODUCTION

Since the beginning of the 21st century, a number of infectious disease threats have emerged that are deemed to be such a risk as to demand a global response. Most have been respiratory viral diseases – severe acute respiratory syndrome (2003), avian influenza in humans (2005), A(H1N1) pandemic influenza (2009), and Middle East respiratory syndrome coronavirus (MERS-CoV)

(2012 onward). Ebola virus disease, transmitted to others by direct contact, bucked that trend when a large outbreak in several West African countries claimed over 11,000 lives from 2014 to 2015. Very few would have predicted the most recent infectious disease emergency would involve a vector-borne virus, Zika virus (ZIKV), causing congenital malformations and other neurological disorders.

ZIKV is a flavivirus (*Flaviviridae* family) transmitted by mosquitoes. The virus has been isolated from many mosquito species, although it seems that the vectors of the natural transmission cycle are mosquitoes of the genus *Aedes* (Diagne et al., 2015). As any other flavivirus, the viral genome is composed of a single-stranded RNA molecule of positive polarity about 10 kb in length that encodes a single open reading frame (ORF) flanked by two untranslated regions at both ends (Kuno and Chang, 2007).

ZIKV was first isolated in 1947 from the serum of a febrile sentinel monkey in the Zika Forest, hence its name, and 1 year later from *Aedes africanus* mosquitoes caught in the same forest (Dick et al., 1952). Since then, it was confined to Africa until its first detection in Asia in the 1980s. Subsequently, human outbreaks were reported in 2007 in the Micronesia and in 2013 in the French Polynesia (Saiz et al., 2016). However, ZIKV was an almost neglected pathogen until its recent introduction into the Americas.

It is not the direct effect that ZIKV has on those infected that is the main concern, as the vast majority will be either asymptomatic or else experience a relatively mild illness and an uneventful recovery. Rather, it is the sequelae of infection – Guillain-Barré syndrome (GBS) and microcephaly and other congenital malformations – that cause the morbidity and mortality associated with the infection. As a result, the World Health Organization (WHO) declared a public health emergency of international concern (PHEIC) (WHO, 2016e), elements of which were later integrated into risk assessments by the European Centre for Disease Prevention and Control (ECDC, 2016).

This review discusses several aspects of the biology, epidemiology, transmission and health consequences of ZIKV infection, including findings from *in vitro* and *in vivo* models. Disease control measures, such as vaccine development and the public health response to ZIKV outbreaks, are also reviewed.

EPIDEMIOLOGY

The emergence of new pathogens has been the reality and a prominent feature of the 21st century. It constitutes a global challenge to public health, especially in developing countries. Arboviruses such as Dengue virus (DENV), Chikungunya and ZIKV are paradigmatic examples of such a statement.

ZIKV virus is a flavivirus first discovered in 1947 in the Zika forest of Uganda, in a captive sentinel rhesus monkey during a yellow fever (YF) surveillance disease activity (Dick, 1953). In 1952, the presence of human cases was demonstrated by a mouse protection test in the sera of indigenous residents of Uganda and Tanganyika (Smithburn, 1952). During 1958, the isolation of two strains of the virus were made in mosquitoes (*Aedes africanus*)

in Lungu forest (Weinbren and Williams, 1958). The virus was also detected during the decades of 1960–1980 in sentinel Rhesus monkeys and on mosquitoes (mainly the genus *Aedes*) in other countries across equatorial Africa. Sporadic human cases of a mild disease were reported (Saluzzo et al., 1981).

A number of serological studies in the 1950s provided some evidence that ZIKV was widespread throughout Asia (Wikan and Smith, 2016). The presence of ZIKV in Asia was confirmed in 1969 when the virus was isolated from an *Aedes aegypti* mosquito in Malaysia (Marchette et al., 1969).

In April 2007, ZIKV spread its usual geographic range and was detected outside Africa and Asia for the first time when an outbreak occurred on Yap Island in the South Western Pacific Ocean, as an emerging pathogen (Hayes, 2009). Sera from acutely ill patients were sent to the Centers for Disease Control and Prevention (CDC) Arbovirus Diagnostic and Reference Laboratory in Fort Collins, Colorado, where 10 of 71 samples (14%) were found positive for the virus, as they contained ZIKV RNA according to reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay (Hayes, 2009). It has been found that the attack rates of ZIKV infection were higher among females than males and among older persons than younger persons. In contrast, the prevalence of IgM antibody against ZIKV was higher in male participants (possibly due to their greater exposure to mosquitoes) and was relatively equally distributed across age groups (Hayes, 2009).

Although wind-blown mosquitoes can travel distances of several hundred kilometers over the open ocean, introduction of the virus by travel or trade involving an infected person or an infected mosquito is considered the most likely source of the Yap Island outbreak, especially because no monkeys were present on the island during the outbreak (Hayes, 2009; Kindhauser et al., 2016).

The fact that ZIKV caused such a widespread outbreak on Yap Island, numbering more than 100 confirmed and probable cases, is striking. The absence of any evidence that viral mutation could explain changes in the epidemiological behavior of the virus has led to several other explanations being postulated, including a lack of population immunity; that means that regular exposure to infection by populations in Africa and Asia may have prevented large outbreaks in those regions, such as those seen on the Pacific Islands and in the Americas (Kindhauser et al., 2016).

Another possible reason for this change may lay on the probable under-reporting, which could explain missing reports of previous outbreaks, especially due to the clinical similarities of mild illnesses associated with ZIKV, DENV, and Chikungunya infections, and the frequent co-circulation of all three viruses (Paixao et al., 2016).

Since 2008, the availability of information about the virus has increased, including data on epidemiology, causal associations, and possible sexual transmission. Clinical and serologic evidence suggested that a US-American scientist contracted ZIKV infections while working in Senegal in 2008, and then transmitted this arbovirus to his wife after his return home. Direct contact was implicated as the transmission route, most likely as a sexually transmitted infection (Foy et al., 2011). Additional evidence was found during a ZIKV outbreak in French Polynesia (2013): the

virus was isolated from the semen of a patient in Tahiti that sought treatment for hematospermia (Musso et al., 2015).

Retrospective surveys identified an unusual and heterogeneous cluster of congenital brain malformations and brainstem dysfunction in fetuses and newborns over a limited period following a ZIKV epidemic in French Polynesia (Besnard et al., 2016), prompting the conclusion of causal association (Araujo et al., 2016).

In 2016, ZIKV spread rapidly throughout the Americas after its initial appearance in Brazil in May 2015. In 2016, 48 countries and territories in the Americas had reported more than 532,000 suspected cases of Zika, including 175,063 confirmed cases. In addition, 22 countries and territories reported 2,439 cases of a congenital syndrome associated with Zika. Five countries had reported sexually transmitted Zika cases (The Pan American Health Organization [PAHO]/World Health Organization [WHO], 2015; Ikejezie et al., 2017).

In Brazil, the most heavily affected country, a very rapid dispersion of ZIKV was identified, mainly in the Northeast region. This area has the lowest vaccine coverage for YF and notified 65.7% of all cases (De Goes Cavalcanti et al., 2016). Only from March to September 2016, which is considered the highest transmission period in Brazil, as evidenced by a large number of dengue cases observed over years, there was an incidence rate of ZIKV infections of 69.22 cases/100,000 newborns, a mortality coefficient of 5.37 deaths/100,000 newborns and a case fatality rate of 7,750 deaths/100,000 cases. The highest incidence and mortality were found in the Northeast region with 201 cases/100,000 newborns and 4 deaths/100,000 newborns, respectively (SVS/MS, 2017).

The rapid spread of ZIKV disease in the Americas was aided by the lack of effective control of vectors of other arboviruses like DENV and Chikungunya, in several countries of Latin America (Rodriguez-Morales, 2015). For decades, vector control programs have failed, due to the adaptation capacity and biological efficiency of the vectors (Al-Abdely, 2016). Additional challenges for population-based surveillance of ZIKV disease include remote locations, porous borders with neighboring countries, and underreporting by health care providers, all of which can limit the ability to ascertain all cases of ZIKV disease. Underreporting of cases would be particularly marked if they occur in areas that are not considered to be at risk for ZIKV disease, such as those at elevations of more than 2,000 m above sea level (Pacheco et al., 2016).

To summarize, the global emergence (Figure 1) of certain arboviruses, such as ZIKV, that in the past were considered to be restricted to specific geographical areas and to cause a mild disease of sporadic behavior, indicates the importance of such diseases to epidemiology and public health, from the perspective of adaptation potential, certain and probable vectors, surveillance and control, and also communication, interdisciplinary investigation and intersectoral cooperation. In other words, “new threats from infectious diseases may emerge from unexpected places, and we need strategies in place that we can roll out to rapidly gain an understanding of the transmission, pathogenesis, and control of previously little-known pathogens to protect global public health” (Lessler et al., 2016).

Maybe it is time to reconsider research horizons, and to pay attention to the integral circle of host, agent, environment and vector. Health promotion approaches are still valid, and can be an alternative for the global reality of health inequities and weakening of public services (Caprara and Ridde, 2016).

BIOLOGY OF THE ZIKV

Molecular Classification and Phylogeny

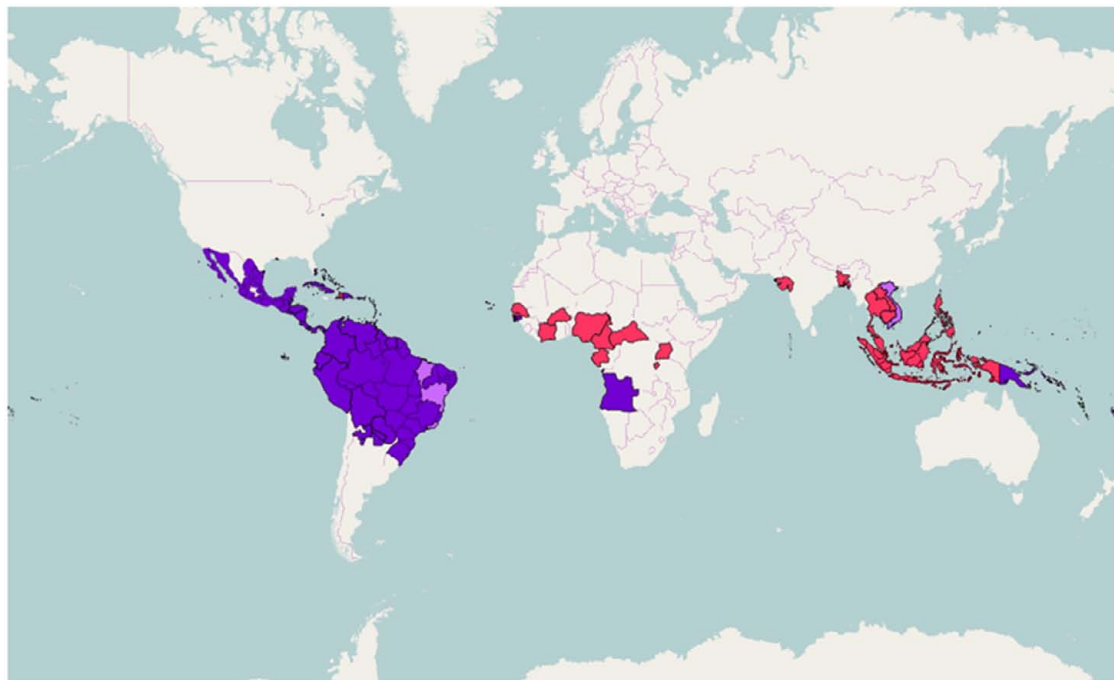
Historically, ZIKV has been classified into the Spondweni serogroup, genus *Flavivirus* (*Flaviviridae*), which includes two species: ZIKV and Spondweni virus (Casals, 1957). Further molecular classifications confirmed these relationships between both species (Kuno et al., 1998; Haddow et al., 2012). Nowadays, ZIKV isolates can be grouped into two or three major lineages (Figure 2). These lineages correspond to the African lineage, the Asian lineage (that includes the American strains) and a neglected lineage circulating in Africa (designated African II) that would constitute a sister group to both African (which should be renamed to African I) and Asian lineages previously identified (Faye et al., 2014; Gong et al., 2016; Wang L. et al., 2016; Li Y. et al., 2017). All these phylogenetic analyses indicate that ZIKV originated from Africa and then spread to Asia, Pacific islands, and throughout the Americas. The introduction of ZIKV into the Americas most probably occurred by a single introduction of an Asian strain of ZIKV between May and December 2013 (Faria et al., 2016). Remarkably, despite the genetic differences between ZIKV strains, the antigenic relationships between strains support the existence of a single viral serotype which may be of crucial importance for the design of ZIKV vaccines (Dowd et al., 2016a).

Genome

The viral genome is composed of a single-stranded RNA molecule of positive polarity about 10 kb in length (Kuno and Chang, 2007). In a similar manner to cellular mRNAs, ZIKV genome includes a cap structure at its 5' end, but in contrast to cellular mRNAs, ZIKV genome lacks a 3' poly(A) tract and ends with CUOH (Figure 3A). The genome contains a single ORF flanked by two untranslated regions located at the 5' and 3' ends of the genome (Kuno and Chang, 2007). ZIKV genome also contains three conserved sequences (CS 1 to CS3) that may mediate genome cyclization between 5' and 3' terminal regions of the genome. Notably, the organization of the CS in the 3' end of ZIKV is different from that of other mosquito-borne flaviviruses (Kuno and Chang, 2007). Besides genomic RNA, it has been described that due to the presence of a multi-pseudoknot structure in the genomic RNA that confounds a cellular exonuclease, ZIKV-infection also produces subgenomic flaviviral RNAs (sfrRNAs) within infected cells that play relevant roles in innate immunity evasion and viral pathogenesis (Akiyama et al., 2016; Donald et al., 2016).

Proteins

The polyprotein encoded by the single ORF in ZIKV is cleaved by cellular and viral proteases into 10 mature proteins (three structural and seven non-structural proteins) (Figure 3A).



Country classification category (Cat.) for Zika virus transmission

- Areas with virus transmission following virus new/re introduction (WHO cat. 1)
- Areas with virus transmission following previous virus circulation (WHO cat. 2)
- Areas with new documented intense transmission (WHO cat.2)
- Areas with interrupted transmission (WHO cat. 3)

FIGURE 1 | Classification of countries and territories regarding vector-borne Zika virus transmission. Map of the current Zika virus transmission based on the European Center for Disease Prevention and Control (ECDC) adaptation of the World Health Organization (WHO)'s Zika virus country classification scheme (<https://ecdc.europa.eu/en/publications-data/current-zika-virus-transmission-list-countries-ecdc-adaptation-whos-zika-virus> - accessed 16 July 2017). The map was generated using GADM database of Global Administrative Areas shapefiles (<http://www.gadm.org/>) and Openlayers plugin within QGIS 2.18.9 (Development Team, 2017, available at <http://www.qgis.org/en/site>).

The sequence of the cleavage sites of ZIKV follows the patterns established for other mosquito-borne viruses (Kuno and Chang, 2007). The three structural proteins [the capsid (C), premembrane/membrane (prM/M), and envelope (E) proteins] participate in the assembly of the virions. The C protein conform the core of the virions together with the RNA. The E protein should mediate the binding to the cellular receptor of the virus, promotes the fusion of the virions with the target membranes, and constitutes the main target for the induction of antibodies (Dai et al., 2016; Stettler et al., 2016; Wang Q. et al., 2016; Zhang C. et al., 2016). The E protein is *N*-glycosylated at Asn 154 in most ZIKV strains. This glycosylation is important for particle protein expression and secretion, viral packaging, and infectivity (Mossenta et al., 2017). The cleavage of prM into M protein promotes the maturation of the virions from “spiky” immature particles to “smooth” mature particles (Prasad et al., 2017). In a similar way to that described for other flaviviruses (Martin-Acebes and Saiz, 2012), the seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) should account for a variety of functions that go from replication of viral

RNA, morphogenesis of viral particles, induction of membrane rearrangements, and viral factory development to modulation of host immune response. The NS1 protein, whose structure has been resolved (Song et al., 2016; Xu X. et al., 2016), participates in flaviviral replication and exhibits immunomodulatory activities. Since NS1 protein is secreted from infected cells, it largely induces antibodies within infected hosts that can be suitable for diagnostics purposes (Dai et al., 2016; Steinhagen et al., 2016). Regarding NS2A, to our knowledge, there are no specific studies addressing the function of this protein in ZIKV. On the other hand, NS3 is a trypsin-like serine protease involved in polyprotein processing (Gruba et al., 2016; Lei et al., 2016) that also exhibits helicase activity, which plays a pivotal role in viral RNA replication enabling RNA unwinding (Tian et al., 2016). Apart from its involvement in viral polyprotein processing, NS3 can cleave cellular factors such as FAM134B, hence modulating the autophagic response within ZIKV-infected cells (Lennemann and Coyne, 2017). NS2B acts as a cofactor necessary for the activity of this protein, and the crystal structure of NS2B-NS3 complex has been resolved under different circumstances

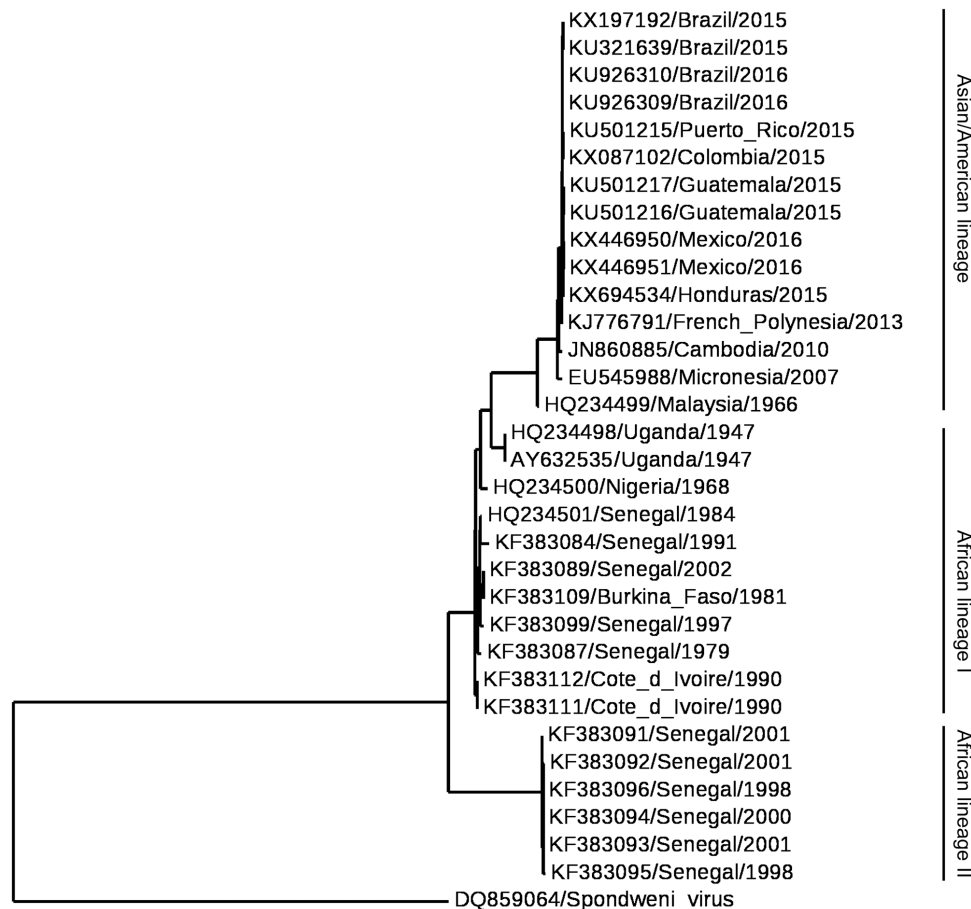


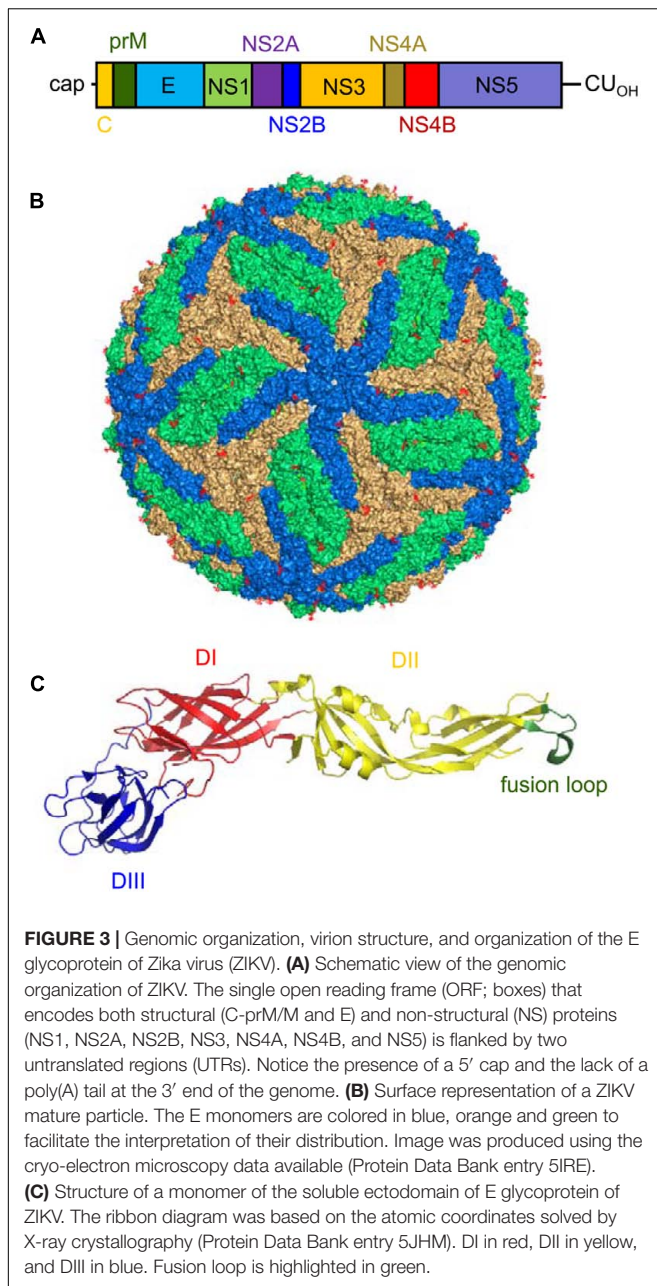
FIGURE 2 | Phylogram of Zika virus (ZIKV). The phylogenetic tree was based on the sequence of NS5. Multiple alignment was performed using MUSCLE (Edgar, 2004) and the tree was constructed by the Maximum Likelihood method using PhyML (Guindon et al., 2010) and Phylogeny.fr (Dereeper et al., 2008). Spondweni virus was included as the outgroup control.

(Lei et al., 2016; Phoo et al., 2016; Zhang C. et al., 2016). Due to the relevance of NS2B-NS3 function in the ZIKV life cycle, the search for inhibitors of the enzymatic activity of this complex is at the front line of antiviral discovery against ZIKV (Cao et al., 2016; Sahoo et al., 2016; Lee et al., 2017; Rut et al., 2017). NS4A and NS4B deregulate Akt-mTOR signaling to inhibit neurogenesis and induce autophagy (Liang et al., 2016). In addition, the expression of NS4A has been also related to activation of the cellular stress pathway involving Tor1 and type 2A phosphatase activator Tip41 (Li G. et al., 2017). NS5 is the viral RNA-dependent RNA polymerase that is in charge of genome replication constituting a major target for antiviral design (Lu et al., 2017; Xu et al., 2017). Furthermore, the analysis of the structure of the methyltransferase domain of NS5, which is responsible for capping the 5' end of the viral genomic RNA, also provides new opportunities for the design of antiviral compounds (Coloma et al., 2016; Stephen et al., 2016; Zhang C. et al., 2016; Coutard et al., 2017; Zhou et al., 2017). Besides its function in genome replication and capping, NS5 from ZIKV also contributes to viral multiplication by inhibiting interferon signaling (Grant et al., 2016). Although a great effort has been

performed to decipher the structure and function of some of the ZIKV NS proteins, many issues still remain to be analyzed, as the more detailed knowledge of their function would probably provide valuable information about the pathogenesis of ZIKV, and would also greatly contribute to the development of antiviral strategies against this pathogen.

Virion

Early filtration studies suggested that the size of ZIKV particles was about 30 to 45 nm in diameter (Dick, 1952). Further transmission electron microscopy showed that the virions were spherical particles with an overall diameter of 40 to 43 nm displaying a central electron dense core being 28 to 30 nm in diameter (Hamel et al., 2015). Nowadays, cryo-electron microscopy reconstructions of mature virions have provided a detailed view of the structure and organization of mature ZIKV particles (Kostyuchenko et al., 2016; Sirohi et al., 2016). The internal core of the particle is composed of the genomic RNA molecule complexed with multiple copies of the capsid (C) protein, enclosed within a lipid membrane derived from the endoplasmic reticulum of the host cell. The E and M proteins



(180 copies of each protein) are anchored to this lipid membrane via their transmembrane regions. Since the M protein is a small protein hidden under the E protein layer, the outer layer of the viral particles consists of an icosahedral protein shell basically composed of the E protein (**Figure 3B**). This outer shell exhibits the characteristic herringbone structure in the virion similar to that of other flaviviruses. The E proteins are arranged as 90 anti-parallel homodimers, with three dimers lying parallel to each other forming a raft. The ectodomain of the E protein that protrudes from the lipid bilayer is organized in three different domains: DI, DII, and DIII (**Figure 3C**). DI acts as a bridge between DII and DIII. The tip of DII contains the hydrophobic fusion loop that interacts with cellular membranes for viral

fusion (Kostyuchenko et al., 2016; Sirohi et al., 2016). DIII is the target for most neutralizing antibodies, whereas antibodies against DI/DII are poorly neutralizing (Stettler et al., 2016; Zhao et al., 2016). As mentioned above, the E protein is glycosylated at Asn 154 in most ZIKV strains, and this glycan protrudes from the surface of the particle. Remarkably, the region surrounding the glycosylation site structurally greatly differs from other related flaviviruses (Kostyuchenko et al., 2016; Sirohi et al., 2016). Also, in contrast to that described for DENV, ZIKV particles are structurally stable even when incubated at 40°C (Kostyuchenko et al., 2016). Although it has been proposed that this thermal stability may have implications for virus survival in body fluids such as saliva or semen (Kostyuchenko et al., 2016), other studies discard that the unique pathobiology of ZIKV may be only the cause of its thermal stability (Goo et al., 2016).

MODES OF TRANSMISSION

Vectorial Transmission

ZIKV was isolated for the first time from a sentinel allocthonous monkey in Uganda in 1948; just 1 year later the sylvatic *Aedes africanus* was found infected in the same site of the Zika Forest, and again in 1958, 1964, and 1969, caught both from the upper canopy and from ground level. Since then, during the period when the records are still mainly restricted to Africa many *Aedes* species reported harboring ZIKV: *Ae. vitatus*, *Ae. hirsutus*, *Ae. unilineatus*, *Ae. metallicus*, *Ae. apicoergenteus*, *Ae. opok*, *Ae. dalzieli*, *Ae. luteocephalus*, *Ae. tayliri* (Haddow et al., 1964; Hayes, 2009; Faye et al., 2013; Diallo et al., 2014; Vorou, 2016). Out of Africa, *Ae. hensillii* was incriminated in the Yap Island as the prevalent species during the outbreak and it was demonstrated experimentally capable of transmission, while in French Polynesia *Ae. polynesium* probably contributes to the transmission (Duffy et al., 2009; Ledermann et al., 2014; Richard et al., 2016).

The scenario of potential worldwide spread of ZIKV changed radically when *Ae. aegypti* and *Ae. albopictus* were identified as vectors during the outbreak in Brazil (Ferreira-de-Brito et al., 2016). Actually, *Ae. aegypti* had already been demonstrated competent for ZIKV in 1956 and isolated from Malaysia in 1969. In addition, it was incriminated in the French Polynesia and Indonesia outbreaks and retrospectively in Africa (Marchette et al., 1969; Richard et al., 2016). *Ae. albopictus* was associated with the outbreak in Gabon in 2007 as this Asian mosquito successfully replace the native *Ae. aegypti* in many areas of Africa (Grard et al., 2014), and it was also shown to be a potential vector in Singapore (Wong et al., 2013). These ubiquitous two mosquito species, with their dynamic adaptation to urban environments, capacity to breed in cryptic containers, to survive to adverse seasons or to be dispersed passively by humans (adults, desiccated eggs-*Ae. aegypti*), to tolerate temperate climates and even keep sylvatic niches (*Ae. albopictus*), together with the increase in the last decades of social trends as those of urbanization, traveling (speed and number of people involved), migration and climate extraordinary events, generates the current epidemic risk momentum.

Further, species that belong to genera other than *Aedes* as *Culex perfuscus*, *Anopheles coustani*, *An. gambiae s.l.*, *Mansonia uniformis* were found infected with ZIKV in Africa (Vorou, 2016), but the isolations only prove that these mosquitoes recently fed on a viremic vertebrate. On the other hand, the vector competence is the innate capability to acquire and sustain the pathogen, and transmit it to a host, so many experiments on competence were performed in alternative vectors, mainly with *Cx pipiens* and *Cx quinquefasciatus*. The results showed arguments that support (Franca et al., 2016; Guedes et al., 2016; Guo et al., 2016) or reject (Aliota et al., 2016b; Amraoui et al., 2016; Boccolini et al., 2016; Fernandes et al., 2016; Hall-Mendelin et al., 2016; Huang Y.J. et al., 2016; Weger-Lucarelli et al., 2016; Hart et al., 2017) *Culex* vector competence. To understand so many disparate results, the protocols should be analyzed and compared taking into account several issues that include the virus-vector origin (geographical coherence), and virus and vector past history in the laboratory (number of passages/generations) that made them genetically quite different to the complex wild circulating ones (Berthet et al., 2014; Bennett et al., 2016; Chouin-Carneiro et al., 2016; Wang L. et al., 2016).

However, even if the vector competence is assessed, it is rather different from vector capacity. The last concept attempts to explain the likelihood of effective human-vector contact and transmission, and depends not only on innate vector characteristics but also on local density, host range, and blood feeding behavior, biting rate, survival rate and colonization success (interspecific competence), as well as transovarial (Thangamani et al., 2016) and horizontal transmission. Therefore, even the presence of *Ae. aegypti* does not confirm this species as the primary vector (Diagne et al., 2015; Di Luca et al., 2016; Weger-Lucarelli et al., 2016), and an undescribed sylvatic cycle in Asia should not be discarded (Althouse et al., 2016).

Focused in the main known urban vectors, *Ae. aegypti* and *Ae. albopictus*, databases and maps of current or forecasted distribution were developed as a proxy of risk maps for ZIKV or *Aedes*-borne arbovirus (Kraemer et al., 2015a,b; Messina et al., 2016). The modeling of simulated risk is usually driven by temperature, precipitation, elevation, land cover and modulated by variables as seasonal or year-round abundance and density (population dynamics), vector biting and mortality rates, and extrinsic incubation period (Carlson et al., 2016; Escobar et al., 2016; Messina et al., 2016; Samy et al., 2016; Attaway et al., 2017). However, besides the accuracy and the assumptions of the model itself, some methodological matters require usually in-depth considerations from the mapping to assess the actual risk, as the space and time scale consistency between data and conclusions, accuracy and representativeness of field-collected data, and the particularities at smaller time or spatial scales (Jian et al., 2016; Misslin et al., 2016; Fischer et al., 2017; Li X. et al., 2017). Hence, the Latin America outbreak was explained by 2015–2016 ‘El Niño-Oscillation South’ 2015–2015 at continental level, but also at sub-regional level in Brazil it was explained by year to year variability (drought 2013–2015) and decadal variability followed by long-term trends as climate

change (warm 2014–2015) (Munoz et al., 2016; Caminade et al., 2017). Furthermore, biological topics, as vector competence of local vectors (Gardner et al., 2016), vector competence between species (Camara et al., 2016), and the timing and location of vector or virus introduction (Robert et al., 2016; Walther et al., 2017) can change the probability and magnitude of transmission. Nevertheless, the anthropogenic factors usually are the main ones that trigger actual epidemics, even through climatic extreme events (Ahmed and Memish, 2017), and so some modeling in border areas includes also travel between borders and socioeconomic factors (Monaghan et al., 2016), while drivers of non-vectorial transmission still need better epidemiological elucidation (Guzzetta et al., 2016).

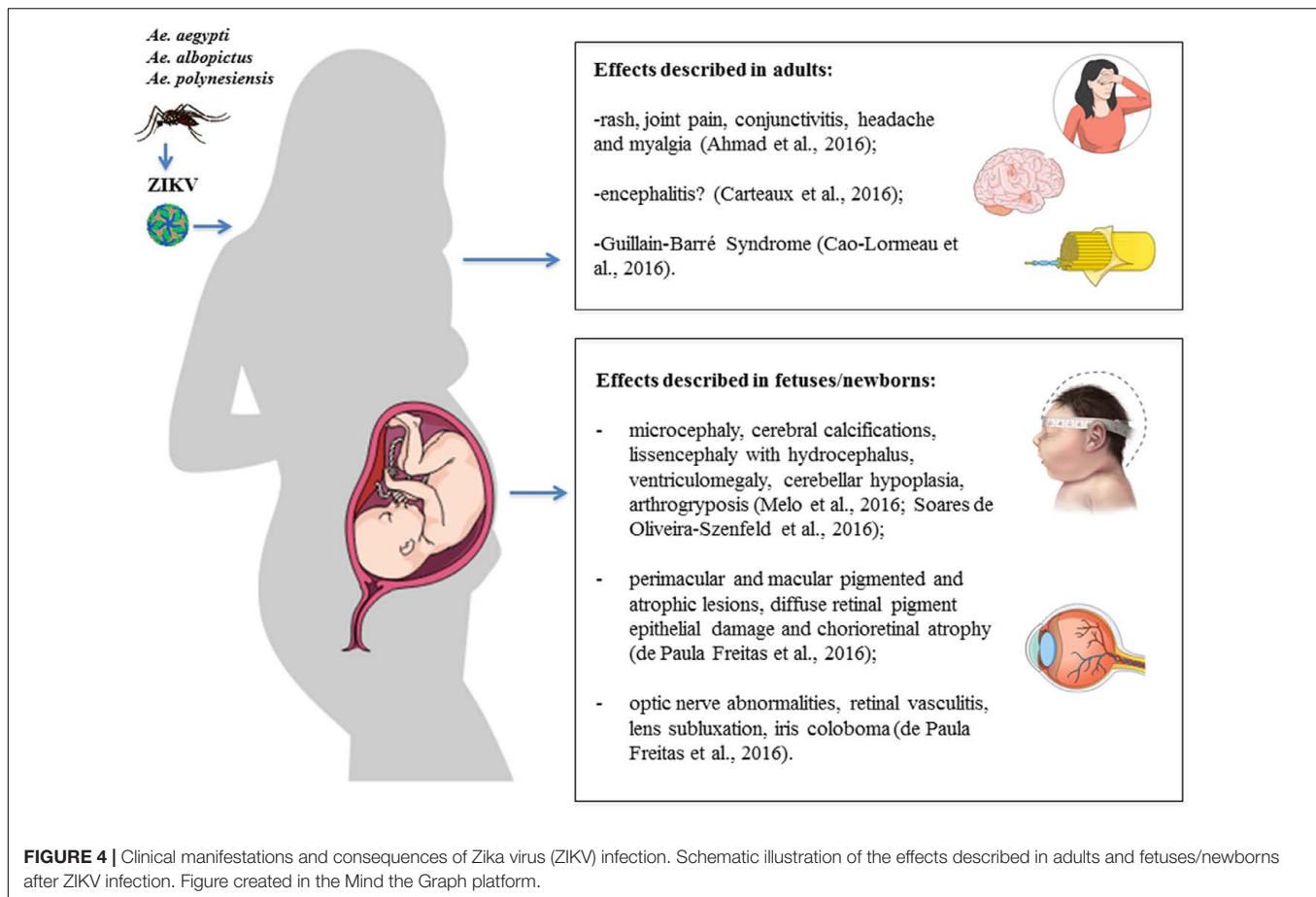
Non-vectorial Transmission

Since the first report of probable sexual transmission of ZIKV by Foy et al. (2011), many studies were published showing evidence of male-to-female, male-to-male and female-to-male sexual transmission by unprotected vaginal, oral or anal intercourse (Moreira et al., 2017). This hypothesis has been strengthened by numerous reports showing the long-term detection of ZIKV RNA and the isolation of infectious ZIKV from semen (Musso et al., 2015; Matheron et al., 2016; Nicastrì et al., 2016; Moreira et al., 2017). ZIKV RNA has also been detected in female genital tract samples beyond viremia, albeit more transiently than in semen: it became undetectable around 3 weeks after symptom onset (Prisant et al., 2016, 2017; Murray et al., 2017). Moreover, Penot et al. (2017) have isolated infectious ZIKV from vaginal samples collected 3 days after the onset of symptoms from a woman with controlled HIV infection.

Remarkably, the vertical transmission of ZIKV (Besnard et al., 2014; Brasil et al., 2016; Calvet et al., 2016; Driggers et al., 2016; Oliveira Melo et al., 2016) has become a major public health challenge, as will be discussed below. It still needs to be demonstrated, however, whether ZIKV can be transmitted through breastfeeding. Infectious ZIKV particles have been isolated from the breast milk of 1 mother and ZIKV RNA was detected in the breast milk of 3 symptomatic mothers (Besnard et al., 2014; Dupont-Rouzeyrol et al., 2016). Other reported forms of non-vectorial transmission include non-sexual person-to-person contact (Swaminathan et al., 2016), and transmission by blood transfusion (Motta et al., 2016).

CLINICAL MANIFESTATIONS

It has been estimated that the ZIKV infection may be symptomatic in 18–57% of cases, in which it causes a mild, self-limiting disease with an incubation period of up to 10 days (Duffy et al., 2009; Aubry et al., 2017). Symptomatic patients may develop fever and influenza-like symptoms relatively common in arboviral infections, such as rash, joint pain, conjunctivitis, headache and myalgia (Ahmad et al., 2016). These relatively mild symptoms last a few days and uncommonly result in hospitalization (Duffy et al., 2009). More recently, however, ZIKV infection has been associated with neurological and



ophthalmological complications, including GBS in adults and microcephaly in fetus and newborns (Figure 4).

Neurological Complications in Adults

Two cases of encephalopathy syndromes, with seizures or electroencephalographic changes, were seen in Martinique as part of ZIKV infection, probably due to encephalitis (Roze et al., 2016). Carteaux et al. (2016) reported a case of an 81-year old man from France who was admitted to an Intensive Care Unit 10 days after a cruise to Pacific islands, with fever and decreased level of consciousness, being diagnosed with meningoencephalitis and had a positive RT-PCR for ZIKV in his cerebrospinal fluid. The patient later recovered and was discharged after 17 days, with cognitive function fully recovered after 38 days and residual weakness in his left arm. In Brazil, a 47-year old pregnant patient was admitted to an Intensive Care Unit with confusion, dysarthria and lower limb weakness, 4 days after presenting a rash and arthralgia (Soares et al., 2016). The patient was diagnosed with encephalitis and had a positive PCR for ZIKV in her urine and IgM ZIKV antibody in her cerebrospinal fluid and serum, and passed away after 11 days (Soares et al., 2016). Since these observations have only recently been described, very little is known about the frequency of a direct central nervous system (CNS) infection by ZIKV. A possible confounder is

the simultaneous occurrence of various arboviruses, such as Chikungunya and DENV, since these arboviruses can cause a direct CNS invasion with myelitis, encephalitis, and meningitis (Moulin et al., 2016).

Throughout the ZIKV epidemic in French Polynesia, an increase in the number of patients presenting with GBS was seen (Paploski et al., 2016). Other arboviral infections such as dengue, chikungunya, Japanese encephalitis, and West Nile fever (WNV) have been associated with GBS (Ravi et al., 1994; Lebrun et al., 2009; Leis and Stokic, 2012; Verma et al., 2014). Cao-Lormeau et al. (2016) published a case-control study reporting the occurrence of 42 cases of GBS after a Zika outbreak between October 2013 and April 2014, in comparison to 5–10 cases in the same period in previous years. All 42 patients had neutralizing antibodies against ZIKV, whereas only 56% of neutralizing antibodies were found in the serum from a control group of 98 patients without GBS. The majority of GBS patients (93%) had detectable ZIKV IgM and 88% had a systemic febrile disease with symptoms that corresponded to ZIKV infection before the development of the neurological symptoms. This cohort of ZIKV associated GBS was classified electrophysiologically as acute motor axonal neuropathy (AMAN) with a rapid onset of disease (4 days to reach the plateau). Approximately one-third of patients required intensive medical therapy with mechanical ventilation

(Cao-Lormeau et al., 2016). Anti-glycolipid antibodies were found in 31% of patients. However, anti-ganglioside antibodies typical of AMAN were rarely present (Cao-Lormeau et al., 2016). Dos Santos et al. (2016) performed an analysis of ministry of health websites and International Health Regulations channels to compare the occurrence of GBS before and after the Zika outbreak. They found an increase of 877% in Venezuela, 400% in Suriname, 211% in Colombia, 172% in the Brazilian state of Bahia, 150% in the Dominican Republic, 144% in Honduras and 100% in El Salvador.

Neurological Complications in Newborns

Zika infection has been confirmed in newborns with microcephaly, and an up to 20-fold increase in the number of microcephaly cases in the French Polynesia, Brazil and other Latin American countries (Brasil et al., 2016; Jouannic et al., 2016; Schuler-Faccini et al., 2016; Ventura et al., 2016a). After the French Polynesia epidemic between October 2013 and April 2014, 14 cases of fetuses and newborns with brain abnormalities (six of them without microcephaly) and five cases with brainstem dysfunction were observed, much higher numbers than those expected outside the epidemic period. Symptomatic cases were related to infections acquired during the first trimester, which is consistent with other congenital infections, such as rubella, cytomegalovirus, and toxoplasmosis (Besnard et al., 2016). However, some severe forms have been described in infections acquired after 20 weeks of gestation (Brasil et al., 2016). There is not always evidence of infection of mothers or fetuses by ZIKV but its presence in the amniotic fluid has been documented (Brasil et al., 2016). The rate of fetal transmission and the actual incidence of serious congenital infections remain unclear. The virus has been detected in fetal cerebral tissues by RT-PCR and electron microscopy (Mlakar et al., 2016).

Brasil et al. (2016) included 345 pregnant women with a history of rash within 5 previous days in a cohort study from September 2015 to May 2016. A total of 182 (53%) tested positive for ZIKV in the blood, urine, or both, and outcomes were available for 125 of these patients. There were nine fetal deaths, and the remaining 116 ZIKV-positive women gave birth to 117 live infants, 42% of which had grossly altered clinical and/or neuroimaging findings, four of them with microcephaly. Melo et al. (2016) followed 11 infants with congenital ZIKV syndrome from gestation to 6 months in Brazil, identifying neurological injuries that included lissencephaly with hydrocephalus, ventriculomegaly, microcephaly, reduction in cerebral volume, cerebellar hypoplasia and arthrogryposis. Soares de Oliveira-Szejnfeld et al. (2016) performed radiological investigation in 17 patients with confirmed ZIKV infection and 18 with presumed ZIKV. The authors reported that neuroimaging abnormalities were similar between both groups, with the more frequent being ventriculomegaly, corpus callosum, infections, intracranial calcifications in the junction of the gray-white matter or in the thalamus and/or basal ganglia.

Ophthalmological Complications

Ventura et al. (2016a) first described ocular lesions in three infants of ZIKV-infected mothers in Brazil, who presented

with microcephaly and cerebral calcifications. Mothers and their children underwent ophthalmological evaluation by biomicroscopy and fundus examination. None of the infected mothers presented eye lesions; however, their infants had gross pigment mottle in the macula and no foveal reflex. ZIKV infection was not tested by RT-PCR, but this was the first warning of its deleterious effects on the developing visual system. The same group also reported on a 57-day infant without microcephaly that had chorioretinal scar on the macula of the left eye (Ventura et al., 2016b). Later, the same group published a study with 40 infants with microcephaly, and reported that eye lesions were more frequent in infants whose mothers had symptoms during the first trimester and infants who had smaller cephalic diameters (Ventura et al., 2016c).

A report by de Paula Freitas et al. (2016) evaluated 23 out of 27 women with suspected infection by ZIKV who had presented clinical manifestations during gestation. Eighteen of them presented them during the first trimester; four occurred in the second trimester and one in the third trimester of gestation. The newborns were studied by ophthalmic examination and, of 29 children (58 eyes), 10 (35.5%) ocular abnormalities were identified in 17 eyes (29.3%). The main fundus results were perimacular and macular pigmented and atrophic lesions, diffuse retinal pigment epithelial damage and chorioretinal atrophy, which in some cases presented very severe forms affecting the macula. They also reported the occurrence of optic nerve abnormalities, mainly hypoplasia, and other findings such as retinal vasculitis, lens subluxation and iris coloboma. In the study, the authors described bilateral findings in 2/3 of the children examined. The authors determined that congenital ZIKV infection was associated with potentially blinding eye disease, including bilateral irreversible macular and perimacular lesions and involvement of the optic nerve (de Paula Freitas et al., 2016).

MODELS OF ZIKV-INDUCED NEURAL DAMAGE

Well before the first suspected cases of ZIKV-associated neurological disorders in humans, there were reports showing the marked neurotropism of ZIKV strains isolated in Uganda (Dick, 1952; Bell et al., 1971). These studies briefly described the neuropathological changes induced by the intracerebral inoculation of ZIKV in newborn and adult mice, such as the severe neuronal degeneration and reactive astrocytosis in the hippocampus of newborn Webster Swiss mice inoculated with ZIKV (Bell et al., 1971). Only recently, however, after the recent outbreak of ZIKV in South and Central America, the study of the vulnerability of neural cells to ZIKV infection has become a focus of intense research.

In Vitro and *Ex Vivo* Models of ZIKV Infection in Neural Cells and Tissues

In vitro studies using human induced pluripotent stem cells (iPSC) have allowed the investigation of the consequences of ZIKV infection in different types of human neural stem cells

(NSC), neural progenitor cells (NPC) and their progeny, as well as in cerebral organoids (Cugola et al., 2016; Garcez et al., 2016; Souza et al., 2016; Tang et al., 2016). Human organotypic fetal brain slices (Onorati et al., 2016; Retallack et al., 2016), human NPC derived from the fetal brain (Hanners et al., 2016; Liang et al., 2016; Onorati et al., 2016) and immortalized cells lines also have been employed to study the neurovirulence of ZIKV.

Tang et al. (2016) were the first to show that the MR766 strain of ZIKV (from Uganda) infected iPSC-derived forebrain-specific human NPC, leading to cell-cycle dysregulation and apoptosis, whereas human iPSC and immature neurons exhibited lower levels of infection. In line with these evidence, Garcez et al. (2016) showed that, while both ZIKV (MR766 strain) and DENV 2 (16681 strain) were capable of infecting human NSC, only ZIKV induced apoptosis in NSC, impaired the formation of neurospheres and decreased the growth rate of human brain organoids. Further studies revealed that brain organoids represent an interesting platform for the study of ZIKV-associated microcephaly (Cugola et al., 2016; Dang et al., 2016; Qian et al., 2016; Wells et al., 2016; Gabriel et al., 2017). For instance, Qian et al. (2016) exposed brain organoids at different stages of cortical neurogenesis to ZIKV (MR766) for 24 h. They showed that most of the infected cells were NPC, although the virus could be detected to a lesser extent in immature neurons, intermediate progenitor cells, and astrocytes. Infection of early stage organoids decreased the number of proliferating cells and induced apoptosis in infected and non-infected cells. As a consequence, the ventricular zone and the neuronal layer were thinner and the ventricles were enlarged in infected organoids, resembling some of the characteristics of microcephaly.

ZIKV Induces Apoptosis, Autophagy and Mitotic Abnormalities in NSC/NPC

Studies using an Asian strain of ZIKV (FSS13025, isolated in Cambodia) (Zhang F. et al., 2016) or ZIKV isolated from recent outbreaks in Brazil (Cugola et al., 2016; Souza et al., 2016; Garcez et al., 2017; Sacramento et al., 2017), Puerto Rico (Hanners et al., 2016; Wells et al., 2016) and French Polynesia (Ghouzzi et al., 2016) have shown the ability of the virus to infect and induce apoptosis of human NPC.

Another common feature of the different strains of ZIKV is the capacity to inhibit the proliferation of NPC. In this regard, Liang et al. (2016) screened the effects of ten ZIKV-encoding potential proteins and found that the ectopic expression of two proteins (NS4A and NS4B, alone or in combination) inhibited neurosphere formation, decreased the proliferation rates of NSC derived from human fetuses, and reduced their capacity to differentiate into neurons and astrocytes. They showed that the co-expression of NS4A and NS4B induced autophagy, by impairing Akt/mTOR signaling, and suggested that the efficient replication of ZIKV requires autophagy (Liang et al., 2016). Infection with either the Brazilian ZIKV or the Cambodian ZIKV strain FSS 13025 caused mitotic abnormalities and increased the number of neural stem/progenitor cells with supernumerary centrosomes (Onorati et al., 2016; Souza et al., 2016; Garcez et al., 2017). Supernumerary foci of centriolar proteins have also been found after ZIKV infection (Polynesia

strain PF-25013-18) in untransformed human retinal epithelia RPE-1 cells and human CHME3 microglial cells, but not in the NPC line ReN (Wolf et al., 2017). Onorati et al. (2016) have proposed a model in which ZIKV infection activates RIG-I-like receptors, cytoplasmic sensors of different forms of dsRNA (which are present during the replication of ssRNA virus) (Thompson and Locarnini, 2007). This would, in turn, cause the relocation of phosphorylated TANK-binding kinase 1 (pTBK1) from centrosomes to mitochondria - where pTBK1 could take part in an innate antiviral immune response - resulting in mitotic impairments in neocortical neuroepithelial stem cells and radial glial cells (Onorati et al., 2016). Interestingly, the relocation of pTBK1 to the mitochondria was also induced by human cytomegalovirus (HCMV), another TORCH syndrome pathogen, but not by DENV 2 (16681 strain), despite the fact that both viruses induced apoptosis in human neocortical neuroepithelial stem cells derived from prenatal specimens (Onorati et al., 2016). The importance of ZIKV-induced centrosomal abnormalities was reinforced by recent findings from Gabriel et al. (Gabriel et al., 2017), who suggested a link between centrosomal structural defects in ZIKV-infected human NPC and the premature differentiation of NPC, which would result in the depletion of the NPC pool.

Mechanisms of ZIKV Entry in Human Cells and the Antiviral Response

Although the knowledge of the cell biology of ZIKV is still scarce, recent advances have provided insights on the life cycle of this pathogen. ZIKV can bind to target cells using adhesion factors such as DC-SIGN and phosphatidylserine binding receptors (Hamel et al., 2017) from which Axl appeared as the main receptor for the entry in human skin fibroblasts (Hamel et al., 2015), microglia (Meertens et al., 2017), astrocytes (Retallack et al., 2016; Meertens et al., 2017) and blood-brain barrier endothelial cells (Liu et al., 2016). Surprisingly, Axl does not seem to be necessary for the entry of ZIKV in NPC and cerebral organoids (Wells et al., 2016; Meertens et al., 2017), despite the high expression of this receptor in NSC and NPC (Cugola et al., 2016; Liu et al., 2016; Nowakowski et al., 2016; Onorati et al., 2016; Meertens et al., 2017). Replication and assembly of progeny virions of ZIKV take place onto modified membranes derived from the endoplasmic reticulum. Thus, ZIKV-infected cells exhibit the characteristic ultrastructural alterations of flavivirus-infected cells (Dick, 1952; Offerdahl et al., 2017). In addition, ZIKV infection provokes changes in the pattern of cellular and viral RNA methylation (Lichinchi et al., 2016) and induces a major impact on the transcriptome of the host cell (Rolfe et al., 2016).

It has been shown that ZIKV (MR766) infection increases the expression of Toll-like receptor 3 (TLR3) (Dang et al., 2016), another innate immune receptor that recognizes dsRNA (Thompson and Locarnini, 2007), in iPS-derived human cerebral organoids and neurospheres. Treatment with the TLR3 agonist poly(I:C) decreased the size of neurospheres in a similar fashion to the infection with ZIKV. Nevertheless, treatment with a TLR3 competitive inhibitor only provided a modest protection against the deleterious effects of ZIKV in neurospheres and cerebral

organoids (Dang et al., 2016). In another study, it was shown that while poly(I:C) induced the secretion of inflammatory mediators by human NPC, infection with a ZIKV strain isolated in Puerto Rico (ZIKV-PRVABC59) was not capable of inducing such a response – despite inducing apoptosis. In addition, ZIKV did not induce a type I Interferon response in NPC (did not induce IFN- α secretion) and did not stimulate cytokine secretion in THP-1 human monocytic cells (Hanners et al., 2016). ZIKV infection (MR766) was also shown to downregulate several immune response genes in a human microglial cell line (Tiwari et al., 2017). Suppression of the innate immune response in human CHME3 microglial cells infected with ZIKV was shown to depend on the kinase activity of Axl (Meertens et al., 2017).

Other studies, however, have found that ZIKV is capable of inducing an immune response in human fetal brain microglia (Lum et al., 2017) and other cell types. Human embryonic stem cells-derived cranial neural crest cells (CNCC) were induced to secrete high concentrations of several cytokines and growth factors, such as IL-6, PAI-1, LIF, and VEGF, after the infection with ZIKV (MR766 or H/PF/2013 strains). CNCC supported ZIKV replication and only a small fraction of the cells died after infection (Bayless et al., 2016). Moreover, ZIKV (PF-25013-18) induced the upregulation of the transcription factor IRF7 and several interferon-stimulated genes in human skin fibroblasts and lung epithelial A549 cells (Hamel et al., 2015; Frumence et al., 2016). Interestingly, the pattern of antiviral response induction in human astrocytes can differ between two ZIKV strains (H/PF/2013 and African HD 78788 strains) (Hamel et al., 2017).

Are There Any Differences in the Neurovirulence of Different ZIKV Strains?

Taken together, current evidence indicates that ZIKV preferentially infects NPC, inducing apoptosis, autophagy and interfering with mitosis (**Figure 5**). ZIKV replicates in NPC and the surviving cells produce the virus for several weeks (Hanners et al., 2016). All strains of ZIKV currently tested have been shown to induce nearly the same cytopathological effects in NPC and few studies have addressed whether there are mechanistic differences among the strains. Cugola et al. (2016) showed that, while the African strain reduced the number of neurons in non-human primate cerebral organoids, the Brazilian strain failed to replicate and did not change the number of neurons, suggesting that the Brazilian virus strain underwent adaptive changes in human cells. Gabriel et al. (2017) have also found some differences in the cellular outcome by comparing the effects of two strains of ZIKV isolated during the recent outbreaks and the African strain MR766 in iPSC-derived human NPC and cerebral organoids. Furthermore, Zhang F. et al. (2016) compared the effects of infecting iPSC-derived human NPC with an Asian ZIKV isolate (FSS13025) or the African strain (MR766). Both strains induced the same alterations in NPC (cell death and decreased proliferation) and caused similar gene expression changes: downregulation of genes involved in cell cycle, DNA repair, and DNA replication, coupled to the upregulation of genes involved in cell death and unfolded protein responses. Comparing the effect of both strains, they

found that TP53 (coding for the tumor suppressor protein p53) was significantly upregulated following the infection with the Asian strain, but only marginally upregulated after the infection with the African strain. Accordingly, p53 inhibitors reduced the activation of caspase-3 more efficiently in NPC infected with the Asian strain (Zhang F. et al., 2016). Activation of p53 has also been reported after the infection of iPSC-derived human NPC with a ZIKV strain isolated from French Polynesia (PF13). The activation of p53 was not restricted to NPC expressing high levels of viral antigens (Ghouzzi et al., 2016), suggesting that this might be an early event or an indirect effect of ZIKV infection in neighboring cells. Other cell types, such as microglia, astrocytes, and endothelial cells, can also be infected (Delvecchio et al., 2016; Lum et al., 2017; Meertens et al., 2017), but it is unknown how the interplay among different cell types contribute to the outcome of microcephaly.

Animal Models of ZIKV Infection

Immunodeficient Mouse Models

Several studies have used mice deficient in type I (A129 mice) or type I/II interferon receptors (AG129) to establish models of robust ZIKV infection in juvenile and adult animals. Aliota et al. (2016a) showed that the subcutaneous injection of a French Polynesian strain of ZIKV produced a lethal infection in AG129 mice. Animals exhibited early serum viremia and high viral loads in several organs, which was accompanied by signs of illness (including weight loss and lethargy) and brain degeneration. One of the most striking observations was the extensive infiltration of neutrophils in the brain. Animals had to be euthanized 7–8 days post-infection due to the rapid progression of the disease. Zmurko et al. (2016) infected AG129 mice with the African strain of ZIKV (MR766) and reported that the first signs of disease appeared at 10 days after the intraperitoneal inoculation. Mice developed acute neutrophilic encephalopathy and had to be euthanized, on average, at 14 days post-inoculation. Acute multifocal neutrophilic encephalitis, inflammatory lesions in the cerebellum and multifocal neutrophilic myelitis were also observed after the subcutaneous inoculation of AG129 mice with the Malaysian strain of ZIKV (P 6-740). In this model, all mice died within 21 days after the infection (Julander et al., 2017). Similar findings were reported after the infection of A129 mice with an African strain (MP1751) (Dowall et al., 2016), a Cambodian strain (FSS13025) (Rossi et al., 2016), or a French Polynesian strain of ZIKV (H/PF/2013) (Lazear et al., 2016). Rossi et al. (2016) compared the effects of ZIKV infection in both mouse strains and found that AG129 mice had more severe neurological symptoms than A129 mice, although there were no differences in other parameters, such as time to death and weight change, suggesting that type II interferon signaling might also influence certain aspects of the disease. Lazear et al. (2016) established a lethal infection in triple knockout mice deficient for IRF3, IRF5 and IRF7, which virtually do not produce interferon- α/β , and showed that these mice are more vulnerable to the intravenous injection of ZIKV than A129 mice, although there were no differences when the subcutaneous route was used. They also showed

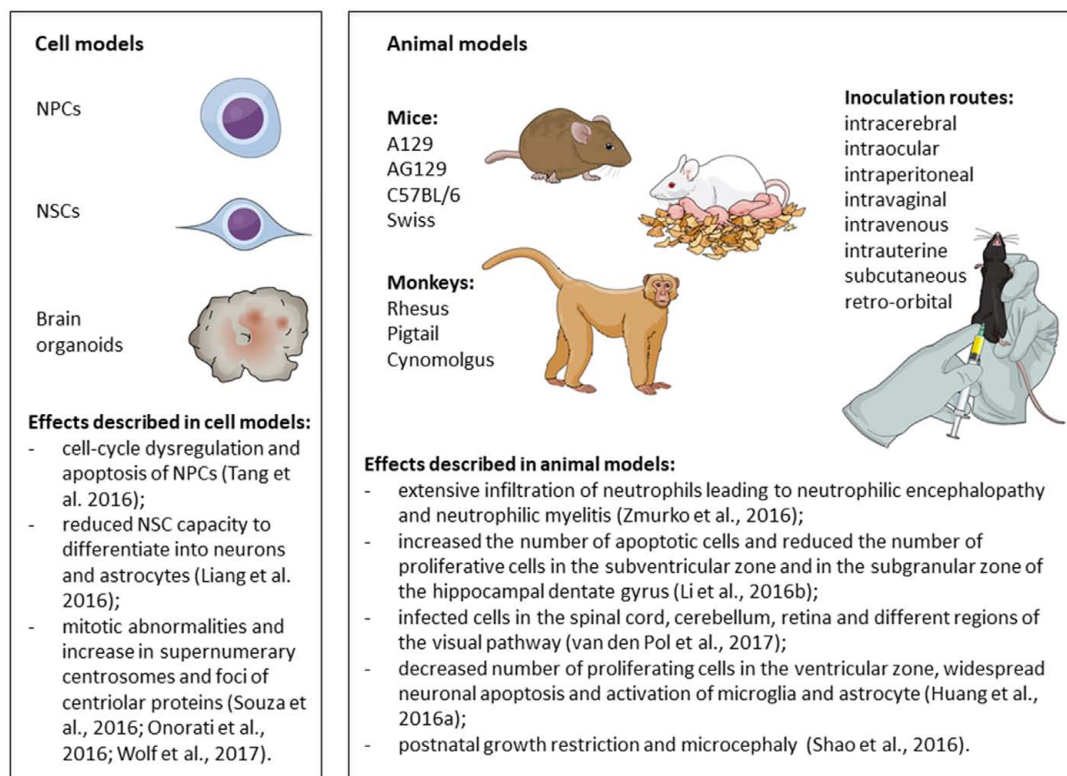


FIGURE 5 | Mechanisms of Zika virus (ZIKV)-induced neural damage. Schematic illustration of the main findings from *in vitro* and *in vivo* models of Zika virus infection. Figure created in the Mind the Graph platform. NPCs: neural progenitor cells; NSCs: neural stem cells.

that the African strain MR766 was less pathogenic to A129 mice than the French Polynesian ZIKV strain (H/PF/2013). Another study using triple knockout mice (IRF3, IRF5, and IRF7 knockout) observed that ZIKV also has a tropism for NPC and immature neurons in the adult brain (Li H. et al., 2016). In this study, mice infected with a Cambodian strain of ZIKV (FSS13025) through a retro-orbital injection exhibited signs of viral illness and hindlimb weakness. The viral envelope protein was detected mainly in the subventricular zone and in the subgranular zone of the hippocampal dentate gyrus, two neurogenic regions in adults. Immunohistochemical analysis revealed that the infection increased the number of apoptotic cells and reduced the number of proliferative cells in these two neurogenic niches.

Immunocompetent Mouse Models

Several studies have demonstrated that immunocompetent mouse strains (CD1, C57BL/6 and 129Sv/Ev mice) are resistant to the subcutaneous or intraperitoneal inoculation of ZIKV and do not develop signs of illness (Dowall et al., 2016; Lazear et al., 2016; Rossi et al., 2016).

Immunocompetent mice only developed the disease when infected during the neonatal period (Lazear et al., 2016; Fernandes et al., 2017). For instance, van den Pol et al. (2017) inoculated C57BL/6 mice on the day of birth with an Asian strain of ZIKV (FSS13025) via the intraperitoneal route. They

found that astrocytes were the first targets of the virus in the brain, which was followed by the infection of neurons. Infected cells were also found in the spinal cord, cerebellum, retina and different regions of the visual pathway. Infection was often lethal and caused growth restriction and motor dysfunction.

Fernandes et al. (2017) inoculated newborn Swiss mice with ZIKV (Brazilian strain SPH 2015), through the intracerebral or the subcutaneous routes. Animals from both groups showed neurological symptoms and severe illness, but the disease progressed faster in the intracerebral group. Histopathological findings were similar to what had been described in A129 and AG129 juvenile and adult animals. An interesting observation was that animals infected through the subcutaneous route additionally exhibited myelopathy, although the brain injury was less severe. Huang W. C. et al. (2016) infected C57BL/6 mice with ZIKV (MR766) at two different time points (either at postnatal day 7 or 21) through the intracerebral route. Infection of postnatal day 7 mice decreased the number of proliferating cells in the ventricular zone and resulted in widespread neuronal apoptosis and in the activation of microglia and astrocytes throughout the brain at 4 days post-infection, when the animals already exhibited neurological symptoms. At this time point, 21-day-old infected mice had severe paralysis, but the regional pattern of neuronal apoptosis was different and less prominent.

Three studies have also reported the effects of injecting ZIKV into the lateral ventricles of C57BL/6 and 129S1/SvImJ

mouse embryos. Shao et al. (2016) observed postnatal growth restriction and microcephaly in newborn pups infected with a Mexican strain of ZIKV (MEX1-44) at embryonic day 14.5. Microscopic analysis of their brains revealed cortical thinning, massive neuronal death, glial activation and abnormal vascular density and permeability. Li C. et al. (2016) injected an Asian strain of ZIKV (SZ01) in the lateral ventricles of embryonic day 13.5 mice. They confirmed the tropism of ZIKV for NPC, although at a later time-point (5 days post-infection) almost all cells in the brain were positive for ZIKV. They also found that ZIKV induced the death of immature and mature neurons and reduced NPC proliferation and differentiation, which resulted in a thinner cortical layer. In both studies, there was no evidence of disruption of cortical lamination and the global transcriptome analyses of infected brains showed the upregulation of genes involved in immune-response-related and apoptosis pathways (Li C. et al., 2016; Shao et al., 2016). Wu et al. (2016), however, found the virus mainly in the ventricular zone and striatum of postnatal day 1 mice that were infected at embryonic day 13.5.

Thus far, animal models corroborate the evidence from *in vitro* and *ex vivo* studies, indicating that NPC are highly susceptible to ZIKV infection (Figure 5). Postmitotic neurons were also shown to be targets of infection in the developing brain and spinal cord of immunocompetent animals and in the brain of young and adult transgenic mice that do not mount an antiviral interferon response. Hindlimb paralysis and neurological symptoms were found in almost all models. Moreover, these models allowed the investigation of important questions raised by clinical and epidemiological observations, such as the link between ZIKV infection during pregnancy and the occurrence of microcephaly, and the possibility of sexual transmission of ZIKV.

Models of Sexual and Vertical Transmissions

The vertical transmission of ZIKV requires the ability of the virus to cross the placental barrier, as well as the developing or developed blood-brain barrier (BBB). Cugola et al. (2016) infected C57BL/6 or SJL pregnant mice on day 10–13 of gestation with a Brazilian strain of ZIKV. Newborns from the SJL ZIKV-infected mice had a high viral load in the brain, intra-uterine growth restriction (IUGR), cortical malformations and ocular defects. These alterations, however, were not observed in pups from C57BL/6 ZIKV-infected mice, indicating that the virus was not able to cross the placenta in this mouse strain, a finding that was supported by a recent study by van den Pol et al. (2017). This is in contrast with the findings of Wu et al. (2016), who injected ZIKV isolated from a patient contaminated in Samoa in the peritoneal cavity of pregnant C57BL/6 mice at embryonic day 13.5. They detected viral RNA in 5 out of 9 placentas and found the virus in radial glial cells localized in the ventricular zone of the fetal brains, 3 days after infection. ZIKV infection reduced the number of proliferating cells in the ventricular zone and intermediate zone and decreased the outer perimeter of the cortex of mice fetuses, although there was no difference in relative thickness of cortical layers. Recently, Xavier-Neto et al. (2017) showed a window of susceptibility between 5.5

and 9.5 days *post coitum* for ZIKV-induced teratogenesis in FVB/NJ and C57BL/6J WT mice. Their model of ZIKV (HS-2015-BA-01, isolated from a Brazilian patient) injection through the jugular vein of pregnant mice caused gross and generalized malformations, IUGR and neural tube defects in the embryos/fetuses.

Another strategy was employed by Miner et al. (2016) who crossed A129 female mice with wild-type (WT) males. Pregnant mice were infected with ZIKV (H/PF/2013) through the subcutaneous route on embryonic days 6.5 or 7.5. This model resulted in high levels of ZIKV RNA in the placenta (1000-fold greater than in the blood), placental abnormalities (vascular injury and apoptosis of trophoblasts) and fetal demise and resorption. IUGR and a large number of apoptotic cells in the brain were found in the remaining fetuses. Alternatively, they treated pregnant mice with a blocking anti-interferon alpha/beta receptor subunit 1 (MAR1-5A3) 24 or 48 h before the inoculation of ZIKV at embryonic days 6.5 or 7.5. This protocol did not cause fetal demise but caused mild IUGR and placental and fetal infection.

Another interesting observation from animal studies is that the brain and testes are the main sites of ZIKV replication in juvenile A129 and AG129 mice (Rossi et al., 2016) and that the virus can persist in the brain and testes for up to 28 days after infection in adult A129 mice (Lazear et al., 2016). Importantly, ZIKV infection (African strain Dakar 41519, and Asian strain H/PF/2013), although at lower levels, caused testis damage, oligospermia and decreased sex hormone production in WT mice treated with anti-Ifnar1 blocking antibodies (Govero et al., 2016). Testicular damage was also observed after the intraperitoneal inoculation of ZIKV (Asian strain SMGC-1) in A129 mice and after the intratesticular injection of ZIKV in WT mice (Ma et al., 2016). Future studies are warranted to determine whether the testicles are affected in ZIKV-infected men.

The possibility of ZIKV transmission through sexual contact was investigated by Yockey et al. (2016), who demonstrated that ZIKV (Cambodian strain FSS13025) replicates in the vaginal tract of WT mice after the intravaginal inoculation. However, while WT mice did not develop disease, A129 mice developed hindlimb paralysis and died within 9 days. However, it was shown that this model of intravaginal infection in WT mice can lead to IUGR if the maternal infection occurs during early pregnancy, even in the absence of viremia. The presence of ZIKV in neurons and glial cells in the fetal brain was also demonstrated. On the other hand, vaginal ZIKV exposure of pregnant A129 mice resulted in viremia, placental infection and fetal demise (early infection at embryonic day 4.5) or severe IUGR (infection at embryonic day 8.5). In conclusion, it has been shown that ZIKV can infect the fetal brain and cause developmental abnormalities (IUGR, for instance) in immunocompetent fetuses if there is a high viral load in the maternal vagina and/or blood, or if the virus is directly inoculated in the brain. Finally, Vermillion et al. (2017) developed a model of transplacental transmission in immunocompetent CD1 mice, in which ZIKV was directly inoculated into the uterine wall at embryonic day 10. At postnatal day 0, they found evidence of cortical thinning and microglial activation in the brain of pups from ZIKV-infected dams.

Non-human Primate Models and Alternative Models

Although the models that use animals that do not mount an interferon response might not be useful for the screening of compounds that target the components of this anti-viral pathway, these models might be useful for testing other therapeutic strategies. Indeed, there is evidence that the ZIKV NS5 protein inhibits type I interferon response in human cells in a species-specific fashion, which might explain why WT mice are more resistant to the infection (Grant et al., 2016). Studies using animal models, therefore, represent an important step for the development of new therapies, especially when in conjunction with *in vitro* and *ex vivo* models (Delvecchio et al., 2016; Retallack et al., 2016; Zmurko et al., 2016; Julander et al., 2017; Sacramento et al., 2017). This includes the utilization of non-human primate models in preclinical translational studies. It has been shown that the infection of Indian-origin rhesus macaques with French Polynesian, South American, Puerto Rican or Thai ZIKV strains, via the subcutaneous route, results in transient viremia and uremia, prolonged presence of ZIKV RNA in cerebrospinal fluid, lymph nodes and colorectal tissue, as well as in the persistent presence of infectious ZIKV in the semen (Dudley et al., 2016; Li X.F. et al., 2016; Osuna et al., 2016; Aid et al., 2017). ZIKV infection induced T-cell responses and protected non-human primates from ZIKV re-infection or from heterologous ZIKV infection (Dudley et al., 2016; Osuna et al., 2016). Pregnant macaques infected at mid-first semester, however, exhibited persistent viremia, although the amniotic fluid was negative for ZIKV (Dudley et al., 2016). Moreover, Adams Waldorf et al. (2016) showed the vertical transmission of ZIKV (strain FSS13025, from Cambodia) after the subcutaneous inoculation of a pregnant pigtail macaque at 119 days of gestation. This model caused fetal brain lesions, including white matter injury and gliosis, brain growth arrest and an increase in the number of apoptotic cells in the subependymal zone, a neurogenic niche. ZIKV RNA was detected in both maternal and fetal brains and in the placenta at 6 weeks after inoculation, which might explain the persistent maternal viremia observed in other studies. Models of ZIKV infection in *Cynomolgus* monkeys have also been developed (Koide et al., 2016; Osuna et al., 2016), showing that these animals are susceptible to infection to ZIKV isolates from Cambodia and Puerto Rico, but not to an African ZIKV strain (IBH30656). In search of alternative and less expensive models, Goodfellow et al. (2016) have demonstrated that ZIKV induced a microcephaly like phenotype in chicken embryos. Finally, a model of intraocular ZIKV inoculation in mice (van den Pol et al., 2017) has already been developed and will permit the investigation of ZIKV-induced dysfunction of the retina and visual pathway.

ZIKV as a Causative Agent of Neurological Pathologies

Taken together, the large amount of data generated over the last years indicate that ZIKV can cause neural damage in an age-dependent manner. The causal relationship between ZIKV and birth defects has been inferred by using the Shepard's criteria for proof of human teratogenicity and the Bradford Hill's criteria for

evidence of causation (Rasmussen et al., 2016). The publication of several experimental studies in animals showing the maternal-fetal transmission of ZIKV and the consequences of congenital infection have reinforced this conclusion. By analyzing data from epidemiologic and experimental studies, Krauer et al. (2017) concluded that ZIKV is a trigger of GBS and found evidence of causality between ZIKV and congenital abnormalities. However, the full spectrum of neurological complications and the long-term sequelae of ZIKV infection, even in the absence of microcephaly, remain to be determined. The mechanisms of neural damage at different developmental stages and the contribution of potential cofactors (nutritional status, viral load, previous infection with DENV, etc.) to the development of adverse outcomes also deserve further investigation.

PUBLIC HEALTH RESPONSE AND DISEASE CONTROL MEASURES

Declaration of a PHEIC

Since the beginning of the 21st century, a number of infectious disease threats have emerged that were deemed to be such a risk that they demanded a global response. Although the transmission dynamics of the various pathogens causing these global emergencies differed, the overarching principles of the public health response were the same – development of policies and procedures, risk communication, effective surveillance, and use of disease control measures to mitigate the risk of infection in the population. These principles formed the bulk of the advice by the Emergency Committee on ZIKV to the Director-General of the WHO when the association between ZIKV and microcephaly and other neurological disorders (e.g., GBS) were declared to be a PHEIC on 1 February 2016 (WHO, 2016e). In addition to the public health measures, other key elements of that advice were that Zika-affected countries should prepare health services; research and development efforts should be increased; national authorities should ensure the rapid and timely reporting and sharing of information of public health importance relevant to this PHEIC; and there should be no restrictions on travel and trade as a result of the outbreak.

Policies and Protocols

Because of the novel and unanticipated nature of the public health emergency relating to ZIKV, there were no specific pre-existing policies and protocols to guide the public health response. In December 2015, the European Centre for Disease Prevention and Control published a rapid risk assessment on the unfolding epidemic (ECDC, 2015) and, soon after, disease control agencies around the world developed public health and clinical guidelines. These guidelines were largely focused on women of reproductive age, their partners, and their clinicians, providing advice on how infection could be prevented, and on testing and management in the event of possible exposure (Petersen et al., 2016; WHO, 2016b). Microbiological testing for ZIKV is not straightforward and was not widely available at the outset of the epidemic, so the development of laboratory guidelines was another early focus.

Guidelines development accelerated following the WHO announcement that the world was experiencing a PHEIC. And as the scientific understanding of the risk and clinical manifestations of ZIKV infection on unborn children was rapidly evolving, guidelines required frequent updating. Over time, the guidelines for various agencies became more consistent with one another.

Surveillance

Surveillance activities for ZIKV in areas where transmission is occurring focus on recording case numbers, complications of the infection, and vectors. The objectives of the surveillance for human cases are to monitor the geographical distribution and temporal trend of infection; characterize disease presentation; identify complications related to the infection; identify non-vector borne routes of transmission; and monitor the effectiveness of containment measures (WHO, 2016d). In countries which are receptive to the virus but without local transmission, the key surveillance objectives are to ascertain imported cases and undertake vector monitoring. In countries where there is no chance of local transmission by vectors, the focus is on identifying imported cases.

The transmission dynamics of ZIKV mean that when it is introduced to an immunologically naive population, an epidemic ensues until a level of population immunity is reached that sees the end of the outbreak. Modeling shows that this period will last approximately 3 years, with a further decade or more before large epidemics are once again possible (Ferguson et al., 2016). It is important for a country to know where on the epidemic curve it is at any point in time, so that the risk to the population is understood and communicated to them, control measures can be instituted, and planning health services undertaken. Although the mild nature of ZIKV disease means that few will come to the attention of health authorities, an understanding of trends can be observed if a robust surveillance system is in place that allows counting of the relatively small proportion of cases that do seek medical help. The more robust systems are those where notification of ZIKV infection to public health authorities is included in the list of reportable infectious diseases mandated by law. Population-based serological studies can be undertaken in Zika-affected countries to better understand the level of population immunity at a point in time.

Estimating the risk of complications from ZIKV is difficult as baseline data for benchmarking are often unavailable and neither accurate denominator data (number of mothers infected), nor numerator data (number of congenital malformations), is available in most of those countries directly affected by ZIKV. Robust surveillance systems for measuring rates of congenital anomalies do exist but tend to occur in countries where ZIKV is not endemic. One such system is the US Zika Pregnancy Registry (USZPR), which provides perhaps the most accurate estimate of risk – of 442 women with completed pregnancies and laboratory evidence of recent Zika infection, 6% of fetuses or infants overall had one or more brain abnormalities and 4% had a finding of microcephaly (Honein et al., 2017). The rate was the same for symptomatic as for asymptomatic women and by far the greatest risk was for women who were infected in their first trimester.

The complication of GBS is rare, estimated to be 2.4 cases per 10,000 people infected during the 2013–2014 outbreak in French Polynesia (Cao-Lormeau et al., 2016). Estimates of the incidence of GBS from a number of Central and South American countries affected by ZIKV range from 2.0 to 9.8-fold higher than pre-epidemic baselines, which pose a significant burden to communities and health systems (Dos Santos et al., 2016).

The aims of vector surveillance are to determine when and where competent vectors are active in a country, where vector control efforts should be focused, and the effectiveness of vector control programs. As a consequence of dengue fever epidemics, most affected countries can rely on existing vector control programs. However, such programs are resource-intensive and need to be well-planned to ensure an efficient use of resources. Trapping sites should be placed to ensure representativeness of the geographical area, and fixed in place to allow determination of changes in mosquito densities over time (WHO, 2016f). Sampling should involve counting of both larval and adult forms of known mosquito vectors to help inform the use of larvicides, adulticides or both. Ideally, synthesis of all the information should be combined with geographic information systems (GIS) data to allow prediction of disease transmission scenarios and focus risk communication to the public. Another important component is the periodic determination of insecticide sensitivity to help selection of authorized biocides used in control efforts.

Communication to High-Risk Groups

Raising awareness of the risk of ZIKV infection to those groups at risk is of critical importance. The population groups to be targeted will vary according to whether they are in a region or country where there is ZIKV activity. In those areas where ZIKV transmission is occurring, the entire population needs to be aware of the disease and vigilant of the risk in order for them to be motivated to undertake risk mitigation measures. Apart from the general public, specific stakeholder groups in the risk communication strategy should include pregnant women, women of reproductive age and their partners, community organizations, schools, health care workers, the media, local and international organizations involved in reproductive health, and local policymakers (WHO, 2016c). Key messages should include prevention of unintended pregnancies by the use of reversible contraception methods; using insect repellents, mosquito nets and other mosquito avoidance measures; and assisting in local vector control activities, such as reducing the mosquito breeding sites on private property. This last issue is essential, because the majority of breeding sites of *Ae. albopictus* and *Ae. aegypti* in urbanized areas are usually found on private property, where the simple preventive measure of water source elimination by owners can significantly reduce the risk.

The advice of health ministries of some Zika-countries for women to defer pregnancy for considerable periods to lessen the risk to their newborns is unprecedented and controversial. Apart from the attendant population planning risks of a distorted population profile resulting from a diminished birth cohort, some of these countries have high rates of unplanned pregnancies, strict abortion laws, a lack of sexuality education programs in

schools, and poor access to contraception, leading to difficulties in implementation of this policy (Ahmed, 2016).

Because of the rarity of the complication of GBS, raising awareness among clinicians is likely to be the more effective risk communication strategy. However, the fact that cases may have mild, or no, symptoms of ZIKV infection preceding it means that clinicians won't necessarily be aided by a clinical prompt, hence delays in diagnosis are likely.

In countries where ZIKV is not active and not receptive to the virus, the focus of awareness-raising is on those citizens planning to travel to Zika-affected areas, in particular, to individuals or couples who are pregnant or planning to become pregnant. Avoiding or deferring travel is advised, and if the person does decide to travel, the messages focus on mosquito avoidance and contraception advice, and symptoms to be aware of on return. Again, the high rate of asymptomatic infection means that the advice needs to relate to anyone traveling to endemic areas, not just those who develop symptoms. Based on available evidence of risk of sexual transmission of ZIKV, the WHO recommends men and women returning from areas where transmission of ZIKV is known to occur to abstain from unprotected sex for at least 6 months upon return to prevent ZIKV infection through sexual transmission and that women who are planning a pregnancy wait at least 6 months before trying to conceive to ensure that possible ZIKV infection has cleared (WHO, 2016b). Disease control agencies from individual countries advise a lesser risk period for women to defer pregnancy following their (or their partner's) return from Zika-affected areas (CDC, 2017).

A third category includes those countries where ZIKV infection is not endemic but are receptive to the virus as they harbor competent vectors. The key population health messages in these countries relate to raising awareness of the symptoms of ZIKV infection so that cases are ascertained early and disease control measures rapidly instigated. Travelers from these countries to endemic areas need to be especially aware of risk mitigation measures whilst traveling to endemic areas, as well as symptoms of the infection, as they are at risk of triggering an outbreak on their return.

Disease Control Measures

Disease control methods available for the fight against ZIKV are limited. There is neither an effective vaccine against the virus, nor anti-viral drugs to reduce the viremic period during which a competent vector with hematophagous behavior can amplify the disease. Vector control measures are, therefore, crucial and reduction of contact between hosts and vectors form the basis of disease control strategies worldwide.

Two broad types of complementary vector control strategies can be applied. First, individuals must take personal responsibility for avoiding mosquito bites and, second, government and non-government organizations must implement vector surveillance and control programs at global, national and local levels.

Recently, innovative methods to reduce mosquito populations have been shown great promise in laboratory and field conditions. Examples include the employment of insect sterile techniques (Alphey et al., 2010), introduction of *Wolbachia* strains (Jeffries

and Walker, 2016) and genetically modified organisms (Beisel and Boëte, 2013), and these may be introduced in future years as adjuncts to conventional mosquito control programs.

In countries that do not have Zika activity but harbor competent vectors of the disease, disinsection of arriving airplanes is an important consideration. The WHO recommends that these countries undertake a risk assessment, and if it concludes that a disinsection program is indicated, that it should be conducted according to standard WHO recommendations (WHO, 2016a).

Risk of Transmission from Blood Transfusion

A study of blood donors during the outbreak in French Polynesia, which found that 3% were positive for ZIKV by PCR while asymptomatic (Musso et al., 2014), has led to concerns about the risk of transmission of ZIKV infection during the transfusion of blood products. The risk is small, however, with only one report of infection caused by transfusion of platelets (Motta et al., 2016).

Some countries require blood donors who have traveled to Zika-affected areas defer their donation for a period after their return or after any sexual contact with a known case (CDNA, 2016). Tests to screen blood donations for ZIKV are available and, despite them not being licensed by the United States Food and Drug Administration (US FDA), it recommends screening all donations in the US and the removal of any positive samples from the blood supply (FDA, 2016).

PERSPECTIVES

Vaccines

Vaccines for several flaviviruses have been produced during the past decades, some of them being already in the market, such as those for YF, tick-borne encephalitis (TBE), or WNF. These vaccines have been produced using different strategies: inactivated or live-attenuated viruses, recombinant proteins and recombinant subviral particles expressed in different heterologous systems, chimeric backbone viruses, or naked cDNA, among others (Martin-Acebes and Saiz, 2012). Thus, after the explosive spread of ZIKV in the Americas that quickly raised social and health worldwide concern because of its possible association with severe neurological pathologies (Blazquez and Saiz, 2016), it was reasonable to think that similar strategies can be applied to ZIKV (Saiz et al., 2016).

In this way, an alum-adjuvanted whole inactivated ZIKV (ZPIV) vaccine candidate recently showed complete protection against detectable viremia in challenged mice (Larocca et al., 2016) and later on in rhesus macaques (Abbink et al., 2016). On the other hand, although live attenuated vaccines are in widespread use for several viral infections, they are usually contraindicated for pregnant women, and in some instances for children, and therefore, they are not a primary target for ZIKV, even though WHO has reported no evidence of increased adverse pregnancy outcomes when licensed vaccines of this kind have been used (WHO, 2014). Similar issues apply for recombinant heterologous viral vectored vaccines and, thus, none of them have been licensed to date, even though a vaccine candidate

that incorporates ZIKV pre-membrane and envelope (prM-E) proteins into a rhesus adenovirus serotype 52 viral vector has recently shown complete protection in challenged monkeys 4 weeks after vaccination with a single dose (Abbink et al., 2016).

On the other hand, until now, and though DNA vaccine technology has been available for many years, no such a human vaccine has been licensed; however, a DNA-ZIKV expressing the full-length ZIKV prM-E proteins induced complete protection against viremia both in mice (Larocca et al., 2016) and rhesus macaques (Abbink et al., 2016). Likewise, a recombinant vaccine ZIKV/JEV prM-E DNA constructs, in which the ZIKV prM signal sequence was replaced with the analogous JEV sequence to improve expression, has shown high levels of protection against viremia in challenged rhesus macaques (Dowd et al., 2016b). Even more, adoptive transfer of purified IgG from mice vaccinated with a ZIKV plasmid DNA vaccine conferred passive protection (Larocca et al., 2016), as did those from mice and rhesus macaques inoculated with an inactivated ZIKV vaccine (Abbink et al., 2016). Similarly, mice treated with a monoclonal antibody against the Domain III of ZIKV-E protein were protected from lethal ZIKV infection (Stettler et al., 2016).

As today, more than 30 vaccine candidates are in active preclinical development, and three have been already approved by the FDA to enter phase I clinical trials (WHO, 2017). NCT02809443 (GLS-5700, Inovio Pharmaceuticals and GeneOne Life Sciences) is a synthetic DNA plasmid vaccine which encodes for the pM-E regions of ZIKV that is being tested in healthy volunteers. This candidate is also in a phase I clinical trial in endemic areas in DENV seropositive adults (NCT02963909). NCT02840487 (VRC-ZKADNA085-00-VP, Vaccine Research Center, NIAID) is also composed of a single closed-circular DNA plasmid that encodes the prM-E proteins from ZIKV. NCT02963909 (NIAID) is an alum adjuvanted ZIKV purified inactivated vaccine (ZPIV) that has entered clinical trial in healthy flavivirus-naïve and flavivirus-primed subjects.

However, due to the characteristics of ZIKV infection, several specific concerns should be kept in mind when developing vaccine candidates against the virus. Thereby, given the possible association of the viral infection with congenital abnormalities (Blazquez and Saiz, 2016), the primary target of the vaccine would be pregnant women and women of childbearing age, although men may also be a target, as ZIKV has been detected in semen pointing to a sexual transmission route. In addition, there are also concerns over the possible interaction of preexisting flavivirus immunity with neutralization and/or enhancement, since several flavivirus cocirculate in the ZIKV endemic areas, including DENV for which an antibody dependent enhancement (ADE) effect has been described. Indeed, the relationships between the immune response to ZIKV and previous DENV infection has been recently demonstrated (Dejnirattisai et al., 2016; Stettler et al., 2016). However, no such ADE effect has been observed in ZIKV infected animal models challenge with WNV (Vázquez-Calvo et al., 2017a). Based on an ecological study, a possible protective effect of YZ fever vaccination has been discussed (De Goes Cavalcanti et al., 2016).

Furthermore, as there is no guarantee that experimental promising results will be reflected in the clinical evaluation of vaccine candidates, and based on the previous experience from the Ebola epidemic, where substantial delays occurred before the stakeholders established the necessary agreements, WHO has launched a target product profile (TPP) describing the preferred and minimal product characteristics for a vaccine targeted to the proposed priority populations (Vannice et al., 2016), which certainly will have to be updated in the coming months once new data are available. In fact, there are tests that take months to be evaluated (stability, neurovirulence, toxicity, etc.), but that should be addressed even under emergency circumstances. Moreover, as noted by Dittmer (2016), besides the technical and ethical aspects, several questions arise regarding ZIKV vaccine campaign implementation that should be considered: Do we need a sophisticated expensive vaccine? Is it economically worthy to develop a vaccine and large-scale clinical trials? Moreover, although vaccines potentially provide powerful tools for the control of viral pathogens, as mentioned before, the development of possible adverse effects derived from ADE of infection of related flavivirus (i.e., DENV) should also be extensively considered before starting large-scale vaccination campaigns.

Antivirals

As mentioned before, nowadays there are no approved specific antiviral agents against any flavivirus (Menendez-Arias and Richman, 2014), and treatment is generally directed to symptom relief with analgesics and antipyretic. However, in the past months, several drugs have been tested *in vitro* and *in vivo* as antiviral candidates (Saiz and Martin-Acebes, 2017), including the screening of different compounds libraries, such those already approved by the FDA, and the repurposing of drugs already used in clinic for other diseases, many of which are broad spectrum molecules.

For instances, different nucleoside analogs/derivatives that target viral polymerases, such as 2'-C-methylated nucleosides, have shown to inhibit ZIKV multiplication in cell culture (Eyer et al., 2016; Zmurko et al., 2016; Hercik et al., 2017), as did Sofosbuvir and BCX4430 (Bullard-Feibelman et al., 2017; Julander et al., 2017; Sacramento et al., 2017), which also induced greater survival rates in treated experimental immunodeficient mice (Bullard-Feibelman et al., 2017; Julander et al., 2017). Likewise, the pyrimidine synthesis inhibitors NITD008, CID 91632869, finasteride, brequinar, 6-azauridine, gemcitabine, and 5-fluorouracil reduced viral multiplication to different levels (Pascoalino et al., 2016; Adcock et al., 2017; Kuivanen et al., 2017). Even more, NITD008 improved survival rates in treated mice infected with ZIKV (Deng et al., 2016). In addition, by means of a high-throughput screening of over 40,000 compounds, it has been shown that non-peptidic small molecules targeting other viral proteins, such as the N2B-NS3 trypsin-like serine-protease, were capable of inhibiting ZIKV multiplication in cell culture (Lee et al., 2017).

On the other hand, it has been recently reported that passive transfer of human neutralizing antibodies to pregnant mice suppressed ZIKV replication and prevent microcephaly

(Sapparapu et al., 2016; Wang et al., 2017), as did a monoclonal antibody against the Domain III of ZIKV-E protein protected against lethal ZIKV infection in a murine model (Stettler et al., 2016). Even more, compounds present in many natural products that also target the viral particle, such as the polyphenols epigallocatechin gallate and delphinidin chloride, also exhibit anti-ZIKV activity, probably through a virucidal effect (Carneiro et al., 2016; Vázquez-Calvo et al., 2017b).

Besides drugs targeting viral components, those directed against cellular factors implicated in the viral life cycle have also been assayed, as they are less prone to induce the emergence of resistant virus. In this line, by screening different libraries and bioactive molecules and by drug repurposing, different inhibitors of ZIKV infection were uncovered, including the fusion inhibitors SaliPhe, monesin, and niclosamide (Xu M. et al., 2016; Adcock et al., 2017; Kuivanen et al., 2017), as well as others molecules that affect different cellular pathways, like bortezomib, sertraline (Barrows et al., 2016), palonosetron (Pascoalino et al., 2016), tenovir-1 (Kuivanen et al., 2017), obatoclax (Rausch et al., 2017), PHA-690509, and emirascan (Xu M. et al., 2016). The immunosuppressants cyclosporine A, mycophenolic acid, and azathioprine have also been tested with promising results (Barrows et al., 2016). Likewise, hypolipidemic drugs like lovastatin, PF-429242, fatostatin, nordihydroguaiaretic acid, and tetra-O methyl nordihydroguaiaretic acid have demonstrated inhibitory activity against ZIKV in cell culture (Pascoalino et al., 2016; Merino-Ramos et al., 2017).

Finally, antiparasitics and antimalarials, such as ivermectin, chloroquine, quinacrine, mefloquine, GSK-36796, and pyrimethamine (Barrows et al., 2016; Delvecchio et al., 2016;

Balasubramanian et al., 2017), as well as antibiotics like nanchangmycin, daptomycin and kitasmycin (Barrows et al., 2016; Pascoalino et al., 2016; Rausch et al., 2017) have also been shown to reduce ZIKV multiplication. Nevertheless, and despite the great effort made by the scientific community, it will take time until any drug against ZIKV will be commercially available.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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