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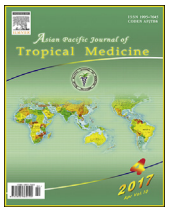

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Advances in research on Zika virus

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ABSTRACT

Zika virus (ZIKV) is rapidly spreading across the America and its devastating outcomes for pregnant women and infants have driven this previously ignored pathogen into the limelight. Clinical manifestations are fever, joint pain or rash and conjunctivitis. Emergence of ZIKV started with a first outbreak in the Pacific area in 2007, a second large outbreak occurred in the Pacific in 2013/2014 and subsequently the virus spread in other Pacific islands. Threat of explosive global pandemic and severe clinical complications linked with the more immediate and recurrent epidemics necessitate the development of an effective vaccine. Several vaccine platforms such as DNA vaccine, recombinant subunit vaccine, ZIKV purified inactivated vaccine, and chimeric vaccines have shown potent efficacy *in vitro* and *in vivo* trials. Moreover, number of drugs such as Sofosbuvir, BCX4450, NITD008 and 7-DMA are ready to enter phase I clinical trial because of proven anti-ZIKV activity. Monoclonal based antibodies offer promise as an intervention effective for use in pregnant women. In this review, we describe the advances in research on ZIKV such as research strategies for the development of antiviral drugs & vaccines, molecular evolution, epidemiology emergence, neurological complications and other teratogenic outcomes as well as pathogenesis.

1. Introduction

ZIKV is a mosquito-borne *flavivirus*, related to West Nile virus (WNV), Dengue Virus (DENV) and Yellow Fever virus (YFV) [1]. The infection has now been an explosive threat with possible outbreaks in Africa, the Pacific Islands, the Americas, and Southeast Asia. ZIKV transmission had been reported in 52 countries from 1 January 2007 to 25 February 2016. Autochthonous, vector-borne transmission of ZIKV has been confirmed in 48 American countries, from January 2015 to December 2016 [2]. ZIKV has been a strong global attraction because of its confirmed causal linkage with microcephaly, a neurological disease that is characterized by a small head and brain leading to developmental issues and death in some cases [1]. ZIKV is vector-borne virus and is transmitted to humans mainly by *Aedes aegypti*, the well-known transmitter of yellow fever, dengue fever and chikungunya virus. Zika viral infection is marked by fever, joint pain or rash and conjunctivitis. The future of ZIKV is unpredictable because it has the potential to follow the path of dengue and chikungunya and the global distribution of both these viruses is closely tied to the trends of urbanization and globalization. Number of arboviral infections such as West Nile virus (WNV), chikungunya fever (CHIKV), Crimean-Congo hemorrhagic (CCHF) fever, dengue, Japanese encephalitis (JE), and Kyasanur forest disease virus (KFDV) spread unprecedentedly in Asia during last few decades.

2. Emergence of ZIKV and epidemic potential

ZIKV first outbreak was documented in year 2007 in the remote island of Yap United States of Micronesia followed by several other outbreaks in Pacific Islands including Easter Island, Cooks Island, Tahiti, French Polynesia, and New Caledonia [3]. Out of 73% of the infected population, 49 cases of Zika human infections were confirmed in Asia and Africa. During 2013 and 2014, the second and major ZIKV outbreak was reported in French Polynesia, the febrile cohort of Zika was estimated at 28.000 (11% of the populace). Therefore,

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ZIKV circulated in the Pacific region including Eastern Island, Fiji, Cook Islands, Solomon, Vanuatu, and New Caledonia in year 2015 [4]. ZIKV emerged in North East Brazil in 2015 and spread at an alarming rate. ZIKV entered in Brazil from the Pacific area because ZIKV strain found in Brazil is similar to French Polynesian ZIKV strain [5]. ZIKV spread in South America followed the pattern similar to co-circulation of arboviruses in the Pacific area. The imported cases of ZIKV from travelers returning from pandemic regions have been documented in Europe, Americas, and Asia. Zika virus epidemic has been declared 'Public Health Emergency of International Concern' by World Health Organization. From Thailand to French Polynesia in 2013, this virus has spread to countries of South and Central America infecting more than one million people. Mostly infection is transient and mild but it is strongly neurotropic and cause teratogenic outcomes in fetuses [6].

Since May 2015, ZIKV has spread to at least 20 countries in the Americas. As of July 2016, ZIKV has been reported in 65 countries whereas microcephaly and other neural defects have been documented in 13 countries. Likewise, increase incidence of Guillain-Barré syndrome have confirmed in 15 countries [7]. Significant increase in microcephaly cases has been observed during the recent outbreak of ZIKV in Latin America. In addition to this, the association of ZIKV with neurological complications as well as its maternal transmission has been firmly established (discussed in Pathogenesis section). ZIKV was reported in Brazil, Caribbean, several Central and South American states by May 2015. About 30 000 cases of ZIKV infection were confirmed in Brazil by 30 January, 2016. ZIKV is still widespread in Brazil with high incidence and transmission notified in 22 of 27 administrative states. A recently published study reported 1 474 cases of the Guillain-Barré syndrome and 164 237 confirmed and suspected cases of ZIKV disease from Bahia, Venezuela, Brazil, the Dominican Republic, El Salvador, Honduras, Colombia, and Suriname during April 1, 2015, to March 31, 2016 [8]. Recent report by Defense Armed Forces Health Surveillance Branch released on 25th January confirmed 198 346 cases of ZIKV and 2 569 cases of microcephaly during January 2015 to January 2017 in Western Hemisphere countries [9].

3. ZIKV genome

ZIKV has single-stranded positive-sense RNA genome about 10.7 kb in length enclosed in a capsid and surrounded by a membrane. Single polypeptide that is encoded by the genome of Zika virus experience post translational chopping done by viral and host proteases leading to formation of seven non-structural proteins that are NS1, NS2A, NS2B, NS3, NS4A NS4B, NS5 along with three structural proteins such as envelope (E), pre-membrane (prM), and capsid (C). Cellular entrance, connection and fusion is induced by envelope proteins, premature fusion is averted by pre-membrane (prM), and capsid protein combines Zika nucleic acid to structure nucleocapsid. Regulation of viral replication and transcription along with stimulation of host antiviral responses is regulated by non-structural proteins [6]. A previous study based on nucleotide sequences of NS5 gene indicated three lineages of ZIKV: East African (one strain examined), Asian (one strain examined), and West African (three strains examined). While based on phylogenetic analysis of entire genomes, two genetic lineages exist for ZIKV that corresponds to Asian and African geographical areas. African strains are further categorized into two groups: Nigerian cluster MR766 prototype cluster (isolated in Uganda) whereas, Asian clade is also classified into two groups Malaysian and Micronesian strains [10]. Initially the Asian lineage caused an outbreak of feverish disease in Yap Island, Federated States of Micronesia, in 2007. It emerged again in 2013 and 2014 and caused a large pandemic in French Polynesia [11], transmitting to Oceania and arriving in America at Easter Island by 2014 [4]. Recently, in early 2015, ZIKV was documented in several Brazilian provinces, mainly in the Northeastern region. Up to date preliminary results from molecular analysis of 17 genomes in the public domain stressed a probable alteration in the fitness of the Asian lineage via an adaptation of the NS1 codon usage [12].

4. Molecular evolution

ZIKV have experienced several recombination events unlike other *flaviviruses* [13]. About 13 recombination events have been reported in the primary analysis using sequencing tool RDP (Recombination Detection Project). Genomic breakpoints have been detected by Rec-HMM at several different alignment positions such as 9 580 to 9 631, 9 007 to 9 132, 5 181 to 5 238, and 1 044 to 1 095. Genetic breakpoints were observed in only E and NS5 regions of genome. Only one event in E sequences has been observed near 414th and 1065th site region of E gene producing 9 viral strains ArA982, ArA27106, ArA27407, ArA27443, ArA27096, ArA27290, ArA27101, HD78788, and ArA986. Likewise, in NS5 gene of strains ArD157995, ArD158084, and ArB1362 one recombination event was detected near 1581 and 2152 (Table 1) [10].

The recurrent gain and loss of N-linked glycosylation site in the E protein could be associated with mosquito-cell infectivity [14]. Researchers have also identified 5 amino acid sequences that contain probable epitopes. These 5 amino acid sequences contained 10 amino acid positions where entropy was >0.0. These entropic positions may cause mutational escape of the ZIKA virus from the immune response of infected individual, this will form basis of anti-ZIKV vaccine design [15].

In Ceara, Brazil, eight new amino acid changes have been observed in ZIKV genome obtained from microcephaly infected fetus and none of these mutations have previously been reported in any microcephaly cases. Two lineages have been proposed based on eleven amino acid changes identified in phylogenetic character mapping [3].

Crystal structure of ZIKV NS1 gene that acts as host-interaction molecule has been found to play an important role in *flavivirus* replication, immune evasion and pathogenesis. NS1 have also been identified as an elongated hydrophobic surface for membrane association and a polar surface that varies to considerable degree among *flaviviruses* [13]. The comparison of genome and polyprotein sequences of pre-epidemic and epidemic ZIKV shows that all coding regions of phylogenetic tree were same except non-structural 2B (NS2B) coding region. Further research is still needed to understand the biological importance of genomic alterations. Likewise, another study has highlighted the challenges in vaccine design due to sequential differences that occur at precursor membrane, envelope, NS2A, NS3, NS4B, and NS5 between different ZIKV strains responsible for differential pathogenesis. NS3 and NS5 inhibitors from other *flaviviruses*, may form the basis of ZIKV intervention strategies [16]. A study showed that in a viral genome of 10 272 bases mutation rate vary between 12 and 25 bases a year [17].

Table 1
Recombination events detected in ZIKA virus genomes [10].

Begin	End	Recombinants	RDP	GENECONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
3119	4261	ArD142623	5.66E-38	1.34E-37	6.35E-42	2.79E-17	1.69E-16	5.54E-14	NS
93	805	ArD128000	1.76E-33	5.25E-34	1.42E-33	5.69E-05	3.79E-13	1.37E-14	4.83E-11
1352	1835	ArD157995	2.10E-33	1.07E-28	3.98E-18	1.10E-09	9.02E-10	2.43E-10	3.84E-09
5161	5222	ArD157995	2.39E-28	1.19E-22	NS[#]	1.33E-09	2.39E-09	NS[#]	NS
8271	9002	ArD142623	3.05E-18	5.69E-28	3.25E-21	2.91E-13	4.27E-11	2.22E-21	NS
5096	5555	ArD128000	3.83E-25	3.76E-23	3.13E-25	6.35E-10	2.82E-10	4.62E-11	2.01E-03
5096	5555	ArD7117	3.83E-25	3.76E-23	3.13E-25	6.35E-10	2.82E-10	4.62E-11	2.01E-03
3003	3530	ArD128000	1.12E-23	1.32E-21	9.45E-24	1.91E-08	9.25E-09	4.18E-09	2.89E-05
847	1272	ArD158084	1.34E-15	5.94E-13	3.99E-16	1.85E-08	8.28E-07	7.55E-04	NS
847	1272	ArD157995	1.34E-15	5.94E-13	3.99E-16	1.85E-08	8.28E-07	7.55E-04	NS
5631	6498	ArD142623	5.58E-23	2.31E-22	1.12E-24	1.21E-13	3.17E-12	1.99E-19	NS
1	634	ArD142623	5.71E-24	1.35E-21	1.33E-14	7.65E-05	9.08E-05	2.04E-15	NS
9560	9802	ArD142623	1.77E-12	1.25E-11	4.63E-13	8.56E-05	2.75E-06	NS	NS
9003	9176	ArD157995	1.77E-11	2.15E-06	2.78E-06	6.08E-04	NS	NS	NS
9003	9176	ArD158084	1.77E-11	2.15E-06	2.78E-06	6.08E-04	NS	NS	NS
7172	7406	ArD142623	2.87E-06	1.68E-09	9.18E-08	1.12E-06	3.02E-06	9.75E-07	NS

Recent published report in Nature showed dependence of ZIKV spread on NS1 translational selection and demonstrated that present Asian lineage epidemic is related to NS1 codon usage adaptation to human housekeeping genes that could promote viral titer and viral replication. Furthermore, several epitopes of NS1 protein are common between ZIKV and dengue virus [12]. Occurrence of mutation threonine residue to alanine C terminal of NS5 sequences has been observed in Panama, Colombia, Martinique, and Mexico. Comparison of Asian and African lineages led to H383Q, P391S, R29N, and N273S. The study also revealed that NS5 amino acid mutation is associated with neurotropsim of ZIKV [18].

Guangyu Sun analyzed 158 currently available full-length ZIKV genomes (as of Nov 2016) and found no complete recombination events however, same recombination event was identified for 3 genomes KF383117 (in PrM), KF383113 (in PrM), and KF383118 (in Env) [19]. A recent study revealed that ZIKV infection increased total P53 level, and nuclear accumulation of P53 Ser15 phosphorylation correlates with apoptosis induction and genotoxic stress. Moreover, small group of P53 effector proteins played their role as critical mediators of many genetic microcephaly syndromes including ZIKV-induced microcephaly [20]. In contrast to this, recent analysis of all ZIKV polyprotein mutations exhibited that microcephaly is a feature of ZIKV itself it is not a consequence of any particular amino acid substitution.

5. Symptoms

The symptoms of ZIKV infection are similar to other *flaviviruses* such as chikungunya or dengue infection which can be easily misguided for one of those infections (Table 2). Incubation duration is usually between (3–12) days. Symptoms of Zika viral infection may include headache, conjunctivitis, fever, rash, myalgia, and arthralgia. Other less common symptoms include: diarrhea, anorexia, abdominal pain, constipation, and dizziness. Most Zika infected individuals experience mild or no symptoms. Approximately 25% of infected patients develop symptoms (2–10) days after illness, including fever, joint pain, rash, red eyes, and headache [21]. The infected person is usually recovered completely and fatalities are rare.

According to Pan America Health Organization (PAHO), commonly reported clinical symptoms in ZIKV infected individuals of America are exanthema (skin rash) and mild fever often accompanied by joint or muscle pain as well as conjunctivitis whereas, autoimmune and neurological complications are infrequent in contrast to epidemics in Brazil and Polynesia. As of 29th December 2016, 48 American countries have confirmed autochthonous, vector-borne transmission of ZIKV since the start of 2015 [2]. Most of symptoms of ZIKV are similar to *flaviviruses* but unlike other *flaviviruses*, the ZIKV efficiently use the cell-surface receptor AXL to enter the fetal bloodstream to reach fetal tissues and lead to microcephaly in neonates.

Table 2
Comparison of Dengue, Zika and Chikungunya symptoms.

Clinical Features	DENV	ZIKV	CHIKV
Onset post infection	(4–7) d	1 and 5 become ill	(3–7) d
Fever	>38 °C	No or mild fever	High fever >38 °C
Headache	More common	Common	Common
Rash	Common	Very common	More common
Itch	Common	Common	Common
Joint pain (arthralgias)	Yes	Common	Very common
Muscles pain (myalgias)	More common	Common	Common
Red eyes (conjunctivitis)	None	Very common	Yes/none
Thrombocytopenia	Very common	Less common	None
Low level of blood cells and platelets	Very common	None	Common
Bleeding disorders	Very common	None	Yes/none
Shock	Yes/none	None	None
Recovery	(6–7) d (only DF)	(4–7) d	<1 week

6. Neurological complications

Two conditions linked with ZIKV have made the outbreak potentially more alarming. The first condition observed in French Polynesia during Zika virus outbreak of 2013–2014 is development of microcephaly, Guillain-Barré syndrome (GBS) and neurological complications associated with Zika virus such as post-infectivity autoimmune neuropathy which may cause fatality, paralysis or muscle weakness.

About 4000 cases of microcephaly linked with ZIKV have been reported in Brazil and this incidence is 20-fold more than 2010 to 2014. Microcephaly has been considered as an irreversible and most serious neurological complications caused by ZIKA virus. Evidence of the virus has been found in the placenta and amniotic fluid of mothers and in the brains of newborns. The first domestic case of microcephaly in fetus was observed in Hawaii on January 15, 2016, whose mother had lived in Brazil. Later on, many other cases of microcephaly in new borns were reported in Texas, Illinois, and Florida in women who were back from international tour [22].

A case control study performed during an outbreak have revealed that individuals infected with GBS were previously exposed to Zika virus infection in contrast to controls that exhibited 0.24 cases of GBS per 1000 Zika virus infections. A study demonstrated that level of anti-ganglioside antibodies decreased in patients infected with GBS associated with Zika virus as compared to those patients who acquire GBS because of other reasons thus signifying that there were other mechanisms that might affect GBS caused as a result of ZIKV [23]. According to a study investigated in Brazil, Venezuela, Colombia and El Salvador, GBS associated with Zika virus have been linked temporally with diffuse demyelinating disorder [24]. Microcephaly is the most serious neurological complication linked with ZIKV. An alarming increase in microcephaly cases have been observed in infants born to pregnant women infected with Zika virus. Intrauterine Zika virus infections have been known to cause cerebral calcifications in different areas of brain or fetuses. In utero-fetus recovered during 32nd week of gestation exhibited different abnormalities such as calcifications in the cortical and sub-cortical parts of the parietal, occipital and frontal lobes of the cerebral cortex [25]. Other brain anomalies such as hydrops fetalis and hydranencephaly observed in microcephalic fetuses led to foetal termination. Virological and pathological analysis of fetal imaging demonstrated the vertical transmission of ZIKV from mother to child during the end of first trimester of pregnancy [26]. Optical abnormalities such as optic nerve and retinal disorders have also been observed in 30% of infants with assumed Zika virus linked microcephaly [27].

Microcephaly as well as other brain and ocular abnormalities has been observed in new borns in 11 studies (7 case series/reports & 4 observational studies). Intrauterine growth retardation and microcephaly was observed in four studies [26].

Ocular abnormalities linked with ZIKV include chorioretinal macular atrophy, focal pigment mottling, optic nerve abnormalities, intra-ocular calcifications, cataracts, microphthalmia, optic disc cupping, conjunctival injections, lens subluxation, foveal reflex loss, bilateral iris coloboma, scarring, and muscular hypoplasia [25]. Evidence of ZIKV linked acute myelitis [28] and meningoencephalitis [29] has been published in The Lancet and NEJM respectively. Findings of study conducted by Tricarico PM *et al.* showed the ability of ZIKV to infect and replicate U87-MG glial cells [30]. Likewise, number of other evidences has proved the causal relationship of ZIKV with neurological complications.

7. ZIKV pathogenesis

There is insufficient literature available on the pathogenesis of ZIKA virus. Immature dendritic cells, epidermal keratinocytes, and human dermal fibroblasts have been found to be permissive to ZIKV infection. The AXL, DC-SIGN, TIM-1 and Tyro entry/adhesion factors allow the entry of ZIKA virus. Replication of ZIKV triggers an antiviral immune response and produce type I interferon in infected cells [31]. Likewise, during the acute phase of ZIKA infection, T cells such as (Th1, Th2, Th9, and Th17) are activated [32].

Animal models are very much necessary to understand the pathogenesis of ZIKV infection. Lazear *et al.* have introduced mouse model to study Zika virus vaccine and anti-viral testing. High viral loads are observed in testes, brain, and spinal cord of mice that lack IFN a/b signaling. This finding was consistent with ZIKV infection in humans.

Radial glial cells are the primary neural progenitor cells for development of cortex in fetus. A study showed signs of infection in radial glial cells of dorsal ventricular zone in response to intraperitonial injection of ZIKV strain in pregnant mice. Therefore, significant reduction of cortex founder cells in neonates was observed. The findings of study supported the concept of vertical transmission of ZIKV and development of brain anomalies in fetus [33]. Brazilian ZIKV ($ZIKV^{BR}$) strain has been shown to cause birth defects such as intrauterine growth restriction and microcephaly in mice. $ZIKV^{BR}$ cross the placenta, target cortical progenitor cells, induce cell death and impair neurodevelopment [34]. ZIKV promotes cells death by inhibiting cell-cycle progression and cause death of human cortical progenitor cells (hCNPCs) [35].

Dang *et al.* developed human embryonic stem cell derived organoid to recapitulate first trimester fetal brain growth. ZIKV was found to infect neural progenitor cells in neurosphere models. ZIKV activated TLR-3 (Toll like receptor-3) in cerebral organoids that led to attenuation of neurogenesis [36].

Nowakowski *et al.* elucidated the underlying cellular and molecular mechanisms that associated ZIKV infection to neurological defects. The expression of receptors involved in entry of several enveloped viruses including ZIKV across diverse cell types in the developing brain was examined. High level of mRNA expression of candidate viral entry receptor AXL was observed in human radial glial cells, endothelial cells, astrocytes, and microglia in developing human cortex therefore, AXL has been proved as a candidate ZIKV entry receptor in neural stem cells and its expression is conserved in human cerebral organoid system [37].

Accumulating evidence suggests that there is causal link between ZIKV and microcephaly specifically during the first trimester of pregnancy; however, the mechanism behind this link needs more in-depth research. In addition to this, the association of ZIKV with microcephaly has been extensively studied using animal models. Cui *et al.* found the efficient replication of ZIKV in the embryonic mouse brain leading to apoptosis, cell-cycle arrest, and inhibition of neural precursor cell differentiation that resulted in cortical thinning and microcephaly [38]. Another study shows ZIKV impairs proper mitotic apparatus function that exerts teratogenic effects [39]. Likewise, another study exhibited the role of gangliosides in ZIKV associated neurological complications [36]. Apart from direct neurotrophic effects of ZIKV, Garcez

et al. demonstrated the ZIKV-linked neurological alterations through 3D culture models that exhibited cell death in human neural stem cells that ultimately affected the growth of organoids as well as formation of neurospheres [40]. The strong neurotropism of ZIKV provides the sound evidence of its association with microcephaly as well as other neurological complications such as GB syndrome, meningoencephalitis, and myelitis. The study of murine models proves placenta as a portal permissive for replication as well as infection. KM Aagaard *et al.*, demonstrated that ZIKV-FLR strain can replicate in human placental trophoblasts without host cell destruction, thereby serving as a likely permissive reservoir and portal of fetal transmission with risk of latent microcephaly and malformations [41].

8. ZIKV transmission

Several different routes of ZIKV transmission are described below.

8.1. Vector transmission

ZIKV has been isolated from Aedes apicoargenteus, Aedes aegypti (Ae. aegypti), Aedes africanus (Ae. africanus), Aedes luteocephalus (Ae. luteocephalus), Aedes furcifer and Aedes vitattus mosquitoes (Table 3). Aedes hensilii was the most commonly found mosquito species during the ZIKV outbreak of Yap Island that occurred in 2007 [42].

8.2. Transmission through blood

Blood-borne transmission of *flaviviruses* of West Nile virus and Dengue virus has been reported. About 2%–8% blood donors were tested positive for ZIKV during French Polynesisa outbreak. Two more cases of transfusion transmitted ZIKV were reported in Brazil. AABB (American Association of Blood Banks) has declared ZIKV as high risk agent and introduced precautionary measures such as NAT (nucleic acid test) or pathogen inactivation of blood products to control transfusion-transmitted ZIKV. Blood safety authorities have been alerted regarding post-transfusion Zika fever based on study that revealed 3% (42 out of 1505) of blood donors positive for ZIKV by PCR [43].

8.3. Transmission through saliva

In January 2016, ZIKV has been isolated in cell-culture from the salivary sample of person with febrile illness who had returned from Dominican Republic to Italy. About 29 days after the onset of symptoms, viral load was found to be higher in saliva and urine than in blood and the genome sequencing of ZIKV was related to Latin American strains [44]. During the largest ZIKV outbreak of French Polynesia that occurred from October 2013 to March 2014, patients were tested negative in blood for ZIKV by RT-PCR therefore, salivary samples were used as an alternative. About 1 067 samples (only blood, only saliva or both samples together) were collected from 855 patients and were tested for ZIKV using RT-PCR. Results showed that ZIKV was more frequently confirmed in saliva samples than blood samples. For 182 individuals with both samples collected, tests were positive for 16 (8.8%) in blood while negative in saliva whereas tests were positive for 35 (19.2%) in saliva while negative in blood. Saliva sample is of particular interest because it has raised the chances of molecular detection of ZIKV at acute phase of ZIKV infection and more appropriate solution when the blood is difficult to collect especially in children and neonates.

8.4. Transmission through urine

There are many evidences that show transmission of ZIKV through urine. In Rio de Janeiro, five saliva samples and nine urine samples collected from nine patients with acute phase symptoms of ZIKV infection were inoculated with vero cell culture and tested by RT-PCR, RT-qPCR, and NAT-Zika. ZIKV

Table 3
Mosquito ZIKA isolates [43].

Year	Country	Species (no. of strains)
1948	Uganda	*Ae. africanus*
1956	Uganda	*Ae. africanus*
1969, 1970	Uganda	*Ae. africanus*, *Ae. apicoargenteus*
1962, 1963	Uganda	*Ae. africanus*
1964	Uganda	*Ae. africanus*
1968	Senegal	*Ae. luteocephalus*
1969	Senegal	*Ae. luteocephalus*, *Ae. furcifer-taylori*, *Ae. gambiae*
1969	Nigeria	*Ae. luteocephalus*
1969	Malaysia	*Ae. aegypti*
1972–1977	Senegal	*Ae. furcifer-taylori*, *Ae. luteocephalus*, *Ae. dalzieli*, *Ae. vitatus*, *Mansonia uniformis*
1975	Malaysia	*Ae. aegypti*
1976–1980	Central African Republic	*Ae. africanus*, *Ae. opok*
1988–1991	Senegal	*Ae. furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. aegypti*, *Ae. neoafricanus*, *Ae. dalzieli*, *Ae. fowleri*, *Ae. minutes*, *Ae. vittatus*
1999	Ivory Cost	*Ae. aegypti*, *Ae. vittatus*, *Ae. furcifer*
2007	Gabon	*Ae. albopictus*
2011	Senegal	*Ae. furcifer*, *Ae. luteocephalus*, *Ae. africanus*, *Ae. vittatus*, *Ae. taylori*, *Ae. dalzieli*, *Ae. hirsutus*, *Ae. metallicus*, *Ae. aegypti*, *Ae. unileaetus*, *M. uniformis*, *Culex perfuscus*, *Ae. coustani*
2011	Southern Senegal	*Aedes*, *Mansonia*
Not available	Not available	*Ae. flavicollis*, *Ae. grahamii*, *Ae. jamoti*, *Ae. taeniarosistris*, *Ae. tarsalis*, *Eretmapodites inornatus*,*Eretmapodites quiquevittatus*

was isolated one from saliva and one from urine sample. Viral load was found to be higher in urine samples than saliva samples. The study also revealed that out of two isolated strains only one was isolated from urine suggesting other factors such as acidic nature of fluid also affects the infectivity of virion [45]. Detection of ZIKA virus in urine of patients more than 10 days after onset of disease have proved urine sample useful for diagnosis of ZIKV infections.

8.5. Transmission through breast-milk

ZIKV has been detected in breast milk of three lactating mothers and two of three newborns showed symptoms of ZIKV. More evidence is still needed to support this conclusion of transmission of ZIKV through breast milk and other perinatal transmission routes [46].

8.6. Sexual transmission

There are several evidences that show sexual transmission of ZIKV. In Tahiti, French Polynesia, high ZIKV RNA load was confirmed in semen of patient who sought treatment of hepatospermia during outbreak that occurred in December 2013 [47].

Female to male transmission of ZIKV was reported in New York City (NYC) Department of Health and Mental Hygiene (DOHMH) as a result of condomless vaginal intercourse because woman partner had just recently returned to New York from ZIKV infected area and rRT-PCR of woman showed that she was viremic at the time of sexual intercourse. Virus was transmitted to male partner due to exposure with vaginal fluids or menstrual blood [48].

Another evidence of sexual transmission of ZIKV has recently been published in NEJM. In February 2016, RT-PCR of 24 year old woman who had not travelled to any Zika infected areas and had not received any blood transfusion, was positive for Zika virus because she had seven episodes of sexual contact with 46 year old man who was back from Rio de Janeiro and showed symptoms of ZIKV infection [49]. Sexual transmission of ZIKV has been confirmed in two cases on the basis of serological tests (plaque reduction neutralisation) performed on stored serum samples that were registered in May 2014 in Florence, Italy ex-Thailand [50]. Likewise, another case of sexual transmission of ZIKV has also been documented in Texas, USA [51].

8.7. Vertical transmission

There are lot of evidences that provide basis of vertical transmission of ZIKV and its association with fetal brain development. During French Polnesian epidemic, two cases of ZIKV have been documented. ZIKV RNA was observed in serum sample of both mothers and newborns.

Likewise, in Brazil pregnant women were found to deliver babies with congenital teratogenic disorders specifically microcephaly [52].

Another case-study belonging to Spain showed that ZIKV was detected in the amniotic fluid during 17th week of pregnancy. Fetal malformations such as brain microcalcifications and arthrogryposis were also observed in ultrasound scan [53].

World Health Organization (WHO) has declared ZIKV as Public Health Emergency of International Concern (PHEIC) because of increasing risk of vertical transmission.

ZIKV was also confirmed in Brazilian woman during 8th week of pregnancy. Viral proteins were observed in macrophages present in chronic villi and these immune cells were playing their role as Trojan horses triggering vertical transmission. ZIKV proteins have also been observed in chronic villi in one case of miscarriage. These evidences support the conclusion that ZIKV reach placental tissue in pregnant women [54].

9. ZIKV diagnosis

There are two types of diagnosis for ZIKV. The first type involves the detection of the antibodies produced as a result of ZIKV infection. The second type is based on detection of viral components or/and entire virus particle. Virus isolation, immunoassay, and RT-PCR have been developed to detect live virus, viral proteins and ZIKV RNA, respectively. ZIKV RNA can be detected in serum, urine and saliva samples obtained at the acute phase of the infection using RT-PCR with more specificity, and have low sensitivity outcomes [55].

Several studies explained the detection of anti-ZIKV antibodies (IgM) as early as 3 days of infection whereas, identified by day 8 in most of individuals. Neutralizing antibodies developed within the early 5 days of start of infection [55]. According to documented studies with associated *flaviviruses*, the development of serological detection assays using Zika envelope or NS1 proteins and sub-viral particles encoding mutations in the extremely cross-reactive fusion loop in domain II might have increase specificity of serological assays considerably [56]. RT-PCR that is performed in urine up to 10 to 14th day from disease onset or in blood up to the 15th day confirms diagnosis [57]. ZIKV RNA can be diagnosed using amniotic fluid, tissue and blood samples from microcephalic newborns. The detection of ZIKV RNA and complete viral particles in amniotic fluid provides an irrefutable proof vertical transmission of ZIKV. Viral antigens have been detected in mononuclear cells in brain tissue of neonates. Brain imaging reveals brain anomalies and ophthalmologic examination reveals visual disabilities. This proves that ZIKV possess enough potential to pass the placental barrier, infect fetal neural cells, multiply, and cause neurological damage to fetus [58].

Recent development of SYBR Green based one-step real-time RT-PCR assay has proved that real-time assay allows highly specific and sensitive detection of ZIKV in cell samples. ZIKV titer as low as 1 PFU/mL can easily be detected with this assay [59].

ZIKV infected cell lines were analyzed using transmission electron microscopy. ZIKV isolated from patient's blood was isolated in C6/36 mosquito cell line followed by inoculation of viral suspension in vero cells. Like other *flaviviruses* especially dengue virus, ZIKV also confirmed the susceptibility of the Vero cell line to ZIKV replication [60].

Analyses of tissue samples from a microcephalic aborted fetus provided detailed information at the microscopic, histological, and immunocytochemical levels about neurological damage induced by a congenital infection with Zika virus [61].

10. Animal models

Number of animal models have been established to study the efficacy of novel therapeutics as well as mechanisms behind pathogenesis, dissemination, host immune responses to ZIKV in

developing fetuses, pregnant mothers, and adults [62]. ZIKV strain (MR 766, Uganda 1947) has been passaged serially in brains of mice for 64 more than 100 times [63].

New mouse models with contemporary ZIKV isolates are being developed since last two years. Several other researchers have analyzed potential of mice deficient in innate immunity to favor the replication and infection of ZIKV. Mice lacking the Ifnar1 gene, including A129 mice and Ifnar1−/− C57BL/6 mice, or mice deficient in Irf3, Irf5, and Irf7 (Irf3-86/-Irf5−/−Irf7−/− 87 TKO) transcription factors developed severe disease similar to WT C57BL/6 mice inoculated with ZIKV Dakar 1984 followed by treatment with a blocking anti-IFNAR1 monoclonal antibody (mAb) before and after the inoculation of virus [64].

ZIKV-infected A129 Ifnar1−/− 93 mice developed ZIKV infection for all tested ages. Mice deficient in type I and type II IFN receptors (AG129) was more prone to ZIKV disease [65]. The pathogenesis of ZIKV associated eye diseases can be studied in Ifnar1−/− 115 mice. Recently, ZIKV associated teratogenic outcomes in fetuses as well as reproductive disorders in males have been studied in mouse models [66]. Infection of pregnant Ifnar1−/− 184 C57BL/6 mice with ZIKV strain H/PF/2013 caused fetal brain injury, neuronal cell death and placental infection followed by demise [67]. Mouse models have also been utilized to investigate the sexual transmission of ZIKV [68]. In addition to this, mice deficient in type I IFN signaling by treatment with anti-IFNAR1 mAb have been used to evaluate the therapeutic efficacy of mouse and human anti-ZIKV monoclonal antibodies. Immunocompromised mice models are being used to test the anti-viral efficacy of small molecules [69]. Non-human primates (NHPs) also are being used to evaluate aspects of ZIKV biology, pathogenesis, and therapeutic efficacy of vaccines against it [70].

11. ZIKV vaccine strategies

Currently, there are licensed vaccines for four *flaviviruses*: YFV (live attenuated), TBEV (inactivated), JEV (both inactivated and live attenuated), and DENV (recombinant chimeric live attenuated). To date, there is no antiviral drug or vaccine available against ZIKV however, several vaccine platforms have entered in clinical trials [71].

Recent study was designed for prediction of T cell and B cell epitopes that form the basis of vaccine design against ZIKV. Several B and T cell epitopes that are conserved among ZIKV strains were collected from 12 different countries. Highly antigenic peptides among T cell epitopes are QTLTPVGRL and IRCIGVSNRDFV in case of major histocompatibility complex (MHC) class I and MHC class II respectively. Interactions of B cell epitopes with HLA-B7 are studied using molecular docking. However, these predicted epitopes could play important role in development of vaccine against ZIKV [72].

A recent study published in Nature revealed that researchers developed DNA vaccine that express series of deletion mutants as well as full-length ZIKV pre-membrane and envelope (prM-Env). The latter provided protection against ZIKV indicated by the absence of viremia that was observed after inoculating mice with vaccine. Protective efficacy was correlated with Env-specific antibody titers. Passive protection was easily conferred by adoptive transfer of purified IgG from vaccinated mice. However, depletion of CD4 and CD8 T lymphocyte in vaccinated mice did not abrogate protective efficacy. These results exhibited that ZIKV can be easily controlled by inactivated virus vaccine or single-shot subunit vaccine in mice. This study suggests that ZIKV vaccine for humans will be developed in near future [73]. This DNA vaccine has shown promising results in nonhuman primate model as well as mice. Another study revealed that inoculation of 1 μg of Zika Virus strain PRVABC59 based inactivated vaccine in Balb/c mice developed higher antibody after four weeks of vaccination. The same vaccine have also shown sufficient protection in rhesus monkeys [74].

Pardi *et al.* suggested the use of RNA vaccine named modified mRNA (mRNA-LNP) as a safe and efficient therapeutic agent against ZIKV because of its good efficacy in mice and non-human primates even with a single dose of 50 μg [75]. The recent development of effective two adenovirus based vaccines named Ad5.ZIKV-Efl (NCT02887482) and MNA-ZIKV-rEfl (NCT028404870) is also a major breakthrough in the area of ZIKV therapeutics [76].

Vaccine Research Center (VRC), National Institute of Allergy and Infectious Disease (NIAID) is currently working on development of ZIKV vaccine based on vaccine platform for other *flaviviruses* such as live-attenuated investigational ZIKV vaccine is based on a similar strategy that is being used for DENV vaccine, DNA plasmid based vaccine similar to WNV vaccine that has been proved safe, effective, and immunogenic in a phase 1 clinical trial, and a ZIKV vaccine that uses a genetically engineered version of vesicular stomatitis virus that has been studied for Ebola vaccine by NIAID.

These vaccine approaches are at initial stages with plans to test the ZIKV vaccine candidates in animal models and tissue culture. It is possible to develop as single vaccine that is effective against all circulating strains of ZIKV because of low strain diversity of ZIKV [77]. Given sufficient resources, it is reasonable to estimate that candidate ZIKV vaccines could be available for clinical evaluation in 2017 [78].

12. ZIKV treatment strategies

Development of medical countermeasures such as therapeutics and vaccines need thorough animal based research with diverse clinical manifestation of ZIKV infection. Insufficient literature is available regarding ZIKV in non-human primates whereas, rhesus monkey is an exception because of isolation of ZIKV from its serum sample and a currently initiated study to evaluate Zika viral infection dynamics in three rhesus macaques [70].

A systematic analysis of ZIKV infectivity and illness through different routes such as intravenous, intradermal, and subcutaneous in various strains of mice at various ages is required. Such studies include genetically diverse mice panels, for instance Collaborative Cross mice, to recognize genetic susceptibility loci that are linked with human infection to develop infectivity models for vaccine and therapeutic testing [79].

NIAID utilized its existing anti-viral drug screening program for other *flaviviruses* including YFV, DENV WNV and Japanese encephalitis to generate a trial that could test drug compounds for probable anti-viral activity against ZIKV infection [80]. The inhibitory drugs against *flaviviruses* must be brought to clinical trials to target ZIKV. Antiviral drug screens have been developed for the identification of inhibitors against fusogenic activity of helicase, protease activity of NS3 and E protein, methyltransferase activities of NS5, and RNA-dependent RNA polymerase, however, an advance pre-clinical development is

still in progress [81]. An study has suggested that important component of viral RNA replication *i.e*, ZIKV helicase is an attractive target for therapy [82].

Other countermeasures include repurposing drug screens of FDA-approved 'orphan' drugs that targets ZIKV infection are under research [83] or viral proteins that need oligomerization has appeared as a potent strategy. Anti-viral drugs directed against *flavirus* infection or cell-intrinsic defense might also be monitored. Antibody-based therapy or inactive transfer of antibodies especially neutralizing mononclonal antibodies that target Zika virus may also emerge as immunetherapy similar to reports with other *flaviviruses* [84]. Zika virus therapeutics is still in infancy because victims are pregnant women and designing and testing of novel anti-viral drugs in pregnant females is challenging because of hindrances that may pose threats to unborn live and health of mother.

The structural stability of ZIKV enables its survival in harsh conditions of urine, saliva, and semen. Therefore, drugs or antibodies that destabilize the structure may control the spread of virus [85]. Antibodies should be selected carefully to minimize risk of diseases enhancement. Two strategies that can be used for ZIKV antiviral development includes repurposing existing clinical compounds and the development of bona fide inhibitors of ZIKV replication and infection [78].

Researchers have recently reported that human monoclonal antibodies and immune sera have the ability to neutralize ZIKV in cell culture and found to be protective in a lethal murine model. The development of class of antibodies [envelope dimer epitope 1(EDE1)] control life-threatening ZIKV infection and increases the possibility of repurposing DENV vaccines to trigger cross-protective immunity in ZIKV infected individuals [86]. This data form the basis of antibody therapy to treat pregnant women infected with other viruses responsible for microcephlay such as rubella virus and cytomegalovirus. In addition to this, the identification of a functionally conserved epitope between DENV and ZIKV increases the chance that vaccine may produce neutralizing antibodies against both viruses.

Number of NIH compounds and FDA approved drugs such as have shown potent anti-ZIKV activity *in vivo* as well as *in vitro* SaliPhe, CID 91632869, NITD, pyrimidine synthesis inhibitors e.g, brequinar, 6-azauridine and finasteride, saliphenylhalamide, gemcitabine, obatoclax, kitasamycinm, lovastatin, 6-azauridine, palonosetron, 5-fluorouracil, emricasan, niclosamide, ten cyclin-dependent kinases specifically PHA-690509, PHA-690509, sofosbuvir, Type I Interferons, mycophenolic acid (MPA), chloroquine, 2′-C-methylated nucleosides, azithromycin, T-1105, 2′-C-methylcytidine (2CMC), CX4430 and GS-5734 [87–95]. Accumulating evidence suggest the potent anti-ZIKV activity of number of monoclonal antibodies. ZIKV-117 was found to be the most inhibitory antibody that prevented the replication of ZIKV and fetal disease in mice [96]. Moreover, another study demonstrate that administration of convalescent serum from a subject recovered from ZIKV in pregnant mice resulted in suppression of ZIKV likewise, adoptive transfer of purified IgG from vaccinated mice triggered passive protection [73].

13. Prevention and control of ZIKV

Social behavior like window screens, air conditioning, indoor lifestyles, proper clothing, and vector control actions can prevent extensive ZIKV outbreaks. Imported cases from endemic areas where competent vectors are present can commence ZIKV infection in ZIKV free regions because of tourists and travelers. There are no antiviral measures against ZIKV currently in practice therefore, there is a dire need to develop vector controlling strategies to control arbovirus related infections.

The rapid spread of ZIKV infection can only be controlled by mosquito control. Public health officials must introduce integrated vector management to practice mosquito surveillance strategies such as wearing clothes that cover much of the body, determine the abundance of mosquito in a geographical area, take precautionary measures to reduce the exposure to vector by applying mosquito repellants and nets, insecticides, larvicides, pesticides, and proper clothing to avoid mosquito bites, vector control efforts must be linked with communication efforts like running effective public campaigns to raise awareness for personal protection measures, evaluate the effectiveness of vector control practices sleeping under a bed net, making sure that breeding sites for mosquitoes (standing water in pots and used tires) are eliminated. Reducing mosquito populations with insecticides may also help to reduce the risk of infection and finally clinicians must report suspected or confirmed cases of ZIKV to public or state health departments because timely identification prevents the disastrous outbreaks by practicing proper control measures. Due to astonishing rise in microcephaly associated ZIKV and the unavailability of precautionary practices and information to control ZIKV infection in pregnant women have raised public health authorities to issue some guidelines related to pregnancy including delayed conception. In fact, CDC is emphasizing regular prenatal examination and ultrasound screening of pregnant females after returning from countries which are experiencing ZIKV outbreak. In order to reduce the risk of ZIKV, public health facilities must facilitate the community with prenatal care, safe abortion, and contraceptives.

Recurrent thoughtful calls against emerging pathogens have been made globally and further strict action is needed to control their uncontrolled transmission. Biopharmaceutical can play an eminent role in this response, but it cannot resolve this problem independently. Progress can possibly be accomplished only if government, regulatory, educational and commerce partners work together to solve the scientific, safety, manufacturing, clinical trial and public health challenges in the way of vaccine achievement.

14. Future strategies

The strategies that have previously been used for development of clinically efficacious vaccines against *flaviviruses* are being adopted for design of live attenuated and subunit ZIKV vaccine. More than 500 million doses of YFV vaccine have been administered since its development in 1937. Complementary approaches such as development of antiviral drugs and monoclonal antibodies are also being investigated [1]. Recently, chemoinformatic study of database of Zika-related genes ($N = 69$) identified 35 druggable targets such as growth factors, enzymes, receptors, cytokines and adhesion molecules. Eight of these protiens (IL-6, LTA, PPIB, PLAT, VEGFA, TNF, CCR5, HLA-DRB1) are currently drug targets. Active ($IC_{50} < 100$ nM) Rule of Five (RO5) compliant and toxicophore negative CHEMBL compounds were identified for nine of ZIKV lead targets (CCR5, CCL2, CASP1, AXL, CXCL8, NPM1, EIF2AK2, CTSS, and TYRO3). The RO5 compliant and toxicophore-negative lead compounds identified in the study offer

a rationale for rapid verification in ZIKV cell culture models for drug discovery [97]. Broad-spectrum antiviral drug that can target diverse *flaviviruses* including ZIKV is being developed under the research project funded by National Institute of Health [77].

15. Conclusions

ZIKV was firstly documented in 1964, and since then it has been reported in numerous countries. First major outbreak occurred in 2007 in Yap Island in the Federated States of Micronesia followed by another outbreak in French Polynesia in 2013. In 2015, number of cases of ZIKV was reported in Brazil, several Caribbean Islands and all Americas. ZIKV epidemic has continued to evolve in 2016, and is spreading at an alarming pace. ZIKV is transmitted by vector *Aedes* mosquito, Sexual and vertical transmission have also been reported. ZIKV has been found to cross placenta and impair neuro-developement in animal models and there are lots of evidences that have strengthened the causal link of ZIKV with neurological complications in neonates. Usually ZIKV infection is diagnosed by RT-PCR of saliva, urine, and blood samples. Development of antiviral drugs, vaccinations, immune sera and monoclonal antibodies have been found to be protective in animal models but the investigation in humans is still to be performed.

Conflict of interest statement

The authors declared that they have no competing interests.

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