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Running title: Zika virus cellular preference

Differences in “in vitro” infectivity of Zika virus lineages reveals cellular preference that could implicate in outbreak intensity

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Abstract

Background: Zika virus (ZIKV) is a mosquito-borne pathogen that belongs to the *Flaviviridae* family. Most of ZIKV infections are asymptomatic and, when symptomatic, cause a mild febrile illness characterized by headache, myalgia, fever, rash and non-purulent conjunctivitis. Recently, in the Brazilian ZIKV outbreak more than 200,000 people were notified with the disease and some complications associated with this infection were identified. **Objectives:** Due to the rapid spread of the disease over Central and South America, the aim of this project was to determine differences in infection between an African strain (prototype MR766) and the strain circulating in Brazil (Asian lineage ZikaSPH2015). **Methods:** Analysis of nucleotide and protein sequences of both strains was carried out *in silico*, by comparing their sequences and performing the prediction of the envelope protein structure. Comparative infection with both strains was performed *in vitro* using *Aedes* cell lines and the resulting viral progeny was quantified by RT-qPCR. **Findings:** Although similar effects were observed with both cell lineages, ZikaSPH2015 strain had a better viral fitness in *Aedes aegypti* cells than in *Ae. albopictus*. Analysis of codon usage showed an increased usage of some codons in *Ae. aegypti* and ZikaSPH2015. Immune response evaluation showed fully heterologous protective antibodies, but cytokine gene expression demonstrated that MR766 induces a more intense immune response than ZikaSPH2015. **Main Conclusions:** The results presented here indicate that ZIKV Asian strain replicates more efficiently in *Aedes aegypti* cells, which may explain the higher intensity and distribution of this lineage outbreaks over the African strain ones. In addition, the Asian lineage might have a mechanism that interferes in antiviral response allowing for a prolonged viremia in some individuals leading to the severe clinical implications related to this infection.

Key words: Zika Virus; Phylogeny; Outbreak intensity; Cellular preference

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Introduction

Zika virus (ZIKV) infections have been an increasing concern to public health worldwide due to past years ZIKV epidemics, occurring mostly in the Americas. The complications associated with this disease (Zika), notably the neurological disorders

(Guillain-Barré Syndrome – [GBS] and newborn's microcephaly) have increased worldwide interest in the ZIKV infection and its outcomes.

Overall, data from previous Zika outbreaks have shown that ZIKV infection results predominantly in an asymptomatic infection or a disease similar to a mild form of dengue (Duffy et al. 2009). Unfortunately, due to this similarity, along with the lack of reliable laboratory tests, the diagnosis of Zika disease is still a challenge in countries where several flavivirus circulate.

Similar to other flaviviruses, such as dengue, West Nile, Japanese encephalitis, tick-borne encephalitis and yellow fever viruses, ZIKV is primarily transmitted by arthropods, and belongs to the *Flavivirus* genus within the *Flaviviridae* family, possessing a positive-sense, single-stranded RNA genome with 10,794 nucleotides (Haddow et al. 2012).

ZIKV was originally isolated in 1947 from a monkey used as a sentinel to study the presence of yellow fever virus in the Ziika Forest, Uganda. In the following year, a second isolation was made from a pool of *Aedes africanus* mosquitoes (Dick et al. 1952). Heretofore, two different ZIKV lineages had been described: an Asian and an African. However, there is a study that separates the African lineage in East and West strains, according to nucleotide sequences derived from the NS5 gene (Lanciotti et al. 2008). Some small outbreaks caused by the African lineage had been reported before, but only in 2015, when ZIKV was detected in blood samples from patients in Brazil and was associated with microcephaly, it gained worldwide attention (Campos et al. 2015). It is well known that the important outbreaks that occurred in Yap Island, Micronesia, in 2007, and French Polynesia (2013), were caused by a strain originated in the Southeast Asia (Haddow et al. 2012), as well as the ZIKV isolates in American countries including Brazil, Suriname, Puerto Rico, and Guatemala. Yet, many amino acid variations between Asian and African strains were identified and they can contribute to the heightening of pathogenicity and transmission efficiency of this lineage (Ye et al. 2016).

The Brazilian Ministry of Health estimates that in 2016 there were 215,319 suspected cases of ZIKV infection and, from those, 126,395 were confirmed by a combination of clinical and epidemiological criteria. The Brazilian Midwest region was the geographic region that showed the higher incidence rate in 2016. In opposition to other regions of the world, a total of eight ZIKV confirmed deaths were reported, but up

to the end of March 2017, there was a decrease of 96% on the reported ZIKV cases (Ministry of Health 2017).

The dissemination of ZIKV, as well as the others arboviral infections, is determined by its vector distribution. *Aedes* spp mosquitoes are present throughout the continents and are mainly found in the tropical and subtropical regions. While *Ae. aegypti* prefers urban environments (Ritchie 2014), *Ae. albopictus* occurs mostly in rural habitats (Hawley 1988). There are some reasons why these two mosquito species present a threat to public health as both, infected human host and vectors, can move to arbovirus silent areas, which can lead to the spread of arboviruses into a naïve community (Lounibos & Kramer 2016). Besides, vector and viral genetics, vector competence, environment parameters, such as temperature, rainfall, and human land use, are some intrinsic and extrinsic factors, respectively, that are related to the vector dissemination (Kramer & Ciota 2015).

A recent study has shown that *Aedes* mosquitoes are not very competent vectors regarding ZIKV transmission, and population susceptibility may be the main factor for the rapid spread of this infection (Chouin-Carneiro et al. 2016). Since most of the large ZIKV outbreaks were characterized by Asian rather than African lineage infections, the aim of this study was to perform a comparison between both lineages, at a molecular and cellular level, and investigate possible factors associated with the Asian lineage that could have some epidemiological implications on the recent dissemination of Zika to many countries of the world.

Material and Methods

Virus stock

African strain MR766 (ATCC[®] VR-84) and Asian strain ZikaSPH2015 (Cunha et al. 2016) stocks were made in VERO cells (ATCC[®] CCL-81). After infection, cells were monitored for cytopathic effect (5-7 days) and the supernatants were collected and titrated by plaque assay in VERO cells. Virus titer was determined by plaque forming units per mL of viral stock. Replication curve was obtained for both strains on cells infected with a M.O.I. (multiplicity of infection) of 0.1 (10^5 cells and 10^4 virus), and supernatant was collected at 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, and 216 hours after infection.

***In vitro* viral interference investigation**

Experiments of viral interference and co-infection were performed in VERO, *Aedes albopictus* (C6/36 – ATCC[®] CRL-1660) and *Aedes aegypti* (ATCC[®] CCL-125) cells. VERO cells were seeded at a concentration of 10^5 cells per well (24 wells plate) using DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic, and incubated at 37°C with 5% of CO₂. C6/36 and *Ae. Aegypti* cells were grown in L15 (Leibowitz) medium supplemented with 10% FBS, 10% Tryptose and 1% antibiotic/antimycotic, and were incubated at 28°C. All three cell lineages were seeded 24 hours prior to infection. Four experimental groups were designed where cells were infected in two different times, initially with the so-called “first infection” (No Infection; either African or Asian lineages; or both lineages). Cells were incubated for one hour to ensure virus adsorption at room temperature on a rocking platform, and a “second infection” was performed after a PBS washing (3 times) (no virus; homologous infection; heterologous infection; or both infections) as described for the “first infection”. After the “second infection”, cells were washed 3 times with PBS and incubated with maintenance medium (1% FBS). The supernatants were collected 48 hours after the “second infection”, centrifuged to avoid cells debris contamination and kept frozen at -80°C until samples were processed.

RNA Extraction and RT-qPCR

RNAs from supernatant were extracted with the Nucleospin RNA Virus kit (Macherey-Nagel[®]) according to the manufacturer's protocol. A set of NS2A region-based primers and lineage-specific probes were designed to amplify viral RNA in a multiplex assay, using a one-step amplification protocol (Taqman[®] Fast Virus 1-Step Master Mix). The forward primer (5'-TTTGCTTTGGCCTGGTTGGCAAT-3') and reverse primer (5'-TCCCAGGGCCATGACAAATGG-3') was used to amplify both strains, and each virus infection was detected by two different probes, a VIC fluorescent probe (6-VIC/TAACATCACCTTGGCAATCC/TAMRA) and a FAM probe (6-FAM/CAACATCGCTCTACCAATCT/TAMRA), specific to Asian and African lineages, respectively.

Cytokine expression profile

To evaluate the cytokine expression profile, PMBC (Peripheral Mononuclear Blood Cells) from 3 different healthy individuals, naïve to ZIKV infections, were pooled and

infected with a M.O.I. of 1 (10^4 cells and 10^4 virus), incubated in RPMI-1640 medium, and collected by centrifugation (800G) 48 hours after infection. Experimental groups were: negative control (no infection), infected either with African or Asian strains. Total mRNA was extracted, integrity verified by denaturing agarose gel (using Guanidine Thiocyanate) and cDNA was synthesized from a total of 200ng of RNA using random primers. The expression of Th1, Th2, Th17 and Treg cytokines (IL-6, TNF- β , IFN- γ , IFN- α , IFN- β , IL-13, IL-5, IL-4, STAT3, RORA, RORC, TBX21, FoxP3, IL-10, TGF- β , IL-23, IL-17A, IL-12, GATA3) was analyzed by Real-Time PCR, normalized to GAPDH and β -actin gene expression using a PCR array custom kit (RT² Profiler PCR arrays [CAPI10120C]; SABiosciences, Frederick, USA) in a 7500 Fast Thermocycler (Applied Biosystems[®]). Data were evaluated by relative quantification, according to Livak & Schmittgen (Livak & Schmittgen 2001).

Cross-protection against ZIKV strains

Antibody protection was evaluated using serum of BALB/C mice (provided by the Animal Facility from University of Sao Paulo – School of Medicine) immunized by intraperitoneal inoculation of 10^4 plaque-forming units (PFU) of each strain. Twenty-one days after inoculation, whole blood was collected, clarified by centrifugation, and neutralization assays were performed by plaque reduction neutralization test (PRNT) using serum from homologous and heterologous strains. PRNT was performed on VERO cells (cell monolayers grown on 24-well plates) and dilutions of mouse sera were made with DMEM media, no FBS, using a two-fold serial dilution, from 1/20 to 1/10,240. Viruses (approximately 35 PFU of ZIKV per dilution) and serum dilutions were then incubated overnight at 4°C. Shortly, Asian lineage was incubated either with a mouse serum that was previously inoculated with Asian or African lineages, and the same procedure was performed with African lineage. The mixture serum/virus was added to the confluent monolayer and plates were incubated one hour at 37°C to allow virus adsorption to cells; washed twice with PBS, and then, a semi-solid carboxymethyl cellulose (CMC) medium (1.5% in DMEM) was added to the cells. Cells were incubated 5 days at 37°C, 5% CO₂ when the overlay was removed, cells fixed with 10% formalin in PBS and then, stained for 15 minutes in 1% crystal violet.

Susceptibility to infection with either lineage was investigated by observing the survival rates of interferon α/β receptor-deficient mouse (A129 – 32045-JAX | IFN- $\alpha\beta$ R-). Mice were inoculated intraperitoneally with 10^5 PFU of each lineage to evaluate the development of disease and followed until their death.

***In silico* molecular analysis of ZIKV strains**

Phylogenetic tree of ZIKV lineages was generated using MEGA 7.0 software, using whole genome sequences deposited into NCBI (National Center for Biotechnology Information) website. Alignment of nucleotide sequences and amino acids were performed to determinate homology between MR766 and ZikaSPH2015 strains. Analysis of codon usage bias was performed for *Ae. aegypti*, *Ae. albopictus*, VERO cells and human cells according to the *Codon Usage Database* (<http://www.kazusa.or.jp/codon/>).

Prediction of the three-dimensional envelope protein structure of ZIKV lineages was performed using the *RaptorX* software, in which it is possible to predict secondary and tertiary structures. The amino acid sequences of the envelope protein of each strain (MR766 and ZikaSPH2015) were inserted into the software and the domains of each protein were highlighted following standard colors (Domain I – Red; Domain II – Yellow; and Domain III – Blue) with the *PyMol Molecular Graphics System* software.

Ethics

Ethical approval was granted by the Ethical Committee on Research of the Hospital das Clinicas of Ribeirao Preto - School of Medicine of University of Sao Paulo (CEP – Comitê de Ética em Pesquisa - protocol # 1.428.859), in order to work with PBMCs from a healthy group of patients on a zika virus surveillance study. All samples were anonymized and tested for previous or current infections (dengue and zika). An informed written consent was required in order to participate in this study, signed individually before blood collection. All procedures with animals were performed in accordance with the Ethics Committee on Animal Use (CEUA – Comitê de Ética no Uso de Animais). Animal research protocol was approved by a local committee (CEUA – FMRP from University of Sao Paulo – protocol #214/2014).

Results

Virus titer and cytopathic effect

The ZIKV titers, measured by plaque assay, were 2.5×10^6 PFU/mL for African strain MR766 and 2×10^6 PFU/mL for Asian strain ZikaSPH2015, respectively. To avoid cycles of freeze/thaw on viral stocks, several aliquots of 50 μ L were prepared and kept at -80°C freezer. Both strains were able to induce cytopathic effect in VERO cells after 4-6 days of infection, monitored by light microscope, and no difference was observed in the size of plaques produced. Additionally, the replication curve for both strains was similar, with a slight difference at the eclipse phase (the period when the virus enter the cells) and the time to maintain the replication plateau (after 48 hours) (Figure 1). It is clear from this experiment that ZikaSPH2015 strain enters the cells more readily but does not replicate as well as MR766 after reaching the replication plateau. In addition to the fact that both viruses presented the same type of plaques, there is no difference in replication pattern between these two viruses.

Viral interference *in vitro*

Since both strains used produced similar plaques after VERO cell infection, viral load from both strains, expressed as viral RNA copies, were quantified by RT-qPCR after sequential infections using same and/or different strains, MR766 (A) and ZikaSPH2015 (B). Results show that the Asian strain ZikaSPH2015 replicates at a higher rate in *Ae. aegypti* and VERO cells regardless of whether the strain was used in primary infection, secondary infection or in concomitance with the African strain MR766 (Figure 2A and 2B). Although ZikaSPH2015 strain kept its ability to infect C6/36 cells, there was a significant increase of MR766 viral load in *Ae. albopictus* cell line, in comparison to the Asian strain, showing that the African strain replicates better than Asian in C6/36 cells (Figure 2C). Furthermore, when VERO and *Ae. aegypti* cells are co-infected with both strains, the Asian strain suppresses viral production of the African strain (Figure 2A and 2B) and the same result is not observed in *Ae. albopictus* cells.

Cytokine expression profile

Cellular response to each strain infection was analyzed comparing the cytokine gene expression in PBMCs. Fold change values were obtained after normalization with non-

infected cells. mRNA levels were normalized using geometric average of housekeeping genes GAPDH and β -actin. For the majority of analyzed cytokines, there was a higher expression level after infection with African than Asian strain indicating that a stronger immune response against the MR766 strain, especially by Th1 effector cells. The transcriptional factor RORC and the cytokine IL17A were more positively modulated after infection with the Asian than African strain, and both are immunological mediators from Th17 pattern (Figure 3), a pattern known to be involved in the immune response against extracellular bacteria and fungi. Statistics were calculated using a One-Way ANOVA test, and significant difference (*) was given with $p < 0.05$.

Cross-protective response against ZIKV strains

Antibody protection after ZIKV inoculation in mice was evaluated through neutralization assays in VERO cells by incubating each virus strain and its homologous or heterologous hyperimmune serum from mice. Antibodies produced against both strains were able to neutralize ZIKV infection from both, homologous and heterologous incubation, with higher titers observed, as expected, with homologous strain (Figure 4). Outcomes resulting from inoculation of ZIKV strains into A129 mice did not differ for both viruses since both groups of mice succumbed to the infection and died 8 days after inoculation.

***In silico* molecular analysis of ZIKV strains**

Similar to other phylogenetic studies, results obtained in this study shows that alignment of all available full genomic ZIKV sequences deposited into NCBI website splits the sequences into two distinct clades, grouping strains derived from the Asian or African lineages (Figure 5). Differences in nucleotide or amino acid composition fluctuated when all protein segments were analyzed, with an average of 88.88% and 96.60% of nucleotide and amino acid homology, respectively (Table 1).

A prediction of the tertiary structure of envelope protein was evaluated to verify differences between the strains. No difference was found in the predicted structures and, both strains had the best match with a cryo-EM structure of the Envelope protein (5gzrA (Wang et al. 2016)) (Figure 6).

Analysis of the tRNA codon usage for both strains demonstrated that, for the 16 amino acids that had a discrepancy between the strains, 12 (75%) had a difference in

percentage of codon usage for both genomes, in the sense that ZikaSPH2015 strain and *Ae. aegypti* cells had an increased usage of codons that were unusual for MR766, which in counterpart had more similarities with *Ae. albopictus* cells (Table S1). This result is in agreement with the observation that the Asian lineage used on this study replicated better in *Ae. aegypti* cells than in *Ae. albopictus* cells.

Discussion

ZIKV was first isolated in 1947, but it took almost a decade to confirm its ability to cause illness in humans. Until the early 2000s, ZIKV remained quiescent with only a few isolated reports of human disease confined to some African countries. A series of large outbreaks were reported more recently, beginning in 2007 with the first outbreak outside Africa occurring in Yap Island, Micronesia. In 2013-2014, French Polynesia was affected by a large-scale outbreak, with nearly 11% of the population infected by ZIKV. From there, the virus spread to the South Pacific Ocean reaching New Caledonia in 2014, and then caused the largest outbreak in the Americas, especially, but not only, in Brazil where confirmed cases of ZIKV infection could be traced back to 2015 (Song et al. 2017).

Interestingly, all ZIKV recent outbreaks reported so far, as well as Zika-related complications, such as Guillain-Barré Syndrome and microcephaly, have been associated with the Asian lineage of the virus. Some factors could explain this association, such as lack of reported cases in the early years after its isolation due to inadequate surveillance (Hamel et al. 2017) and could have been underreported due to the mild nature of ZIKV infections, or even a more pathogenic attribute, evolutionarily acquired, by the Asian strain. Other explanations to these disease complications might be related to race, time of infection and size of the outbreaks but the evolutionary ability to cause severe disease must not be overlooked.

Some few attempts to explain the comparative differences in infectivity between both strains have been reported. Some authors (Hamel et al. 2017) showed a more intense antiviral response in the first hours of infection with an Asian strain, but this evidence was changed over the hours. Chouin-Carneiro *et al.* (Chouin-Carneiro et al. 2016) demonstrated that *Aedes* mosquitoes infected in the laboratory with both strains, although susceptible to infection, these vectors were not good vectors for ZIKV, and the fast spread of the disease in the Americas could have occurred by a large naïve population.

In the work presented here, a comparative study of both ZIKV lineages was performed in order to contribute to the knowledge about the replicative behavior of these strains, and to try to shed some light on the possible reasons for the spread of the Asian lineage in the last years. Both strains had similar *in vitro* infectivity pattern, with no difference found in replication curves, cytopathic effect and morbidity/mortality in a immunocompromised mouse (A129) used as a model for this arbovirus infection (Dowall et al. 2016; Orozco et al. 2012; Partidos et al. 2011). Additionally, cross-protective antibody neutralization was found to be efficient for both viruses and infection with any of the strains conferred long-lasting immunity against the homologous strain.

Although some infectious features were observed to be similar between both strains, expression of immunological molecules was differentially modulated 48 hours after infection of human PBMC of healthy individuals. It is known that a rapid and efficient detection of invading microorganisms and consequent triggering of proinflammatory response are of paramount importance for the initial containment and control of infection. Through analysis of fold change of cytokine mRNA expression, it is clear that cytokines were significantly more expressed after infection with African rather than Asian strain, especially Th1 and Th2 patterns. It is clear that MR766 strain induced a more potent activation of cells expressing INF- β , TNF- α , IL-12 and IL-6 and these cytokines may act in containing the infection by the African lineage more efficiently than the Asian lineage. Also, IL-12 induces Th1 effector cells that might contain the African strain rapidly while the efficient control of Asian strain infection may take longer, allowing for the deleterious effect observed with the Asian strain. These findings would vary from individual to individual as microcephaly is not seen with every pregnant women infected with ZIKV and could explain the reports of prolonged ZIKV viremia in some individuals. Furthermore, IL-10 is an inflammatory cytokine that can regulate the amount of tissue damage upon a viral infection and repression of this cytokine could lead to a more severe outcome of the disease, especially for its ability to downregulate Th1 effector cells. This repression was observed after Asian infection and this finding could explain the more severe complications observed with this strain infection. Additionally, IL-17 is associated to tissue damage and have also the ability to downregulate Th1 response (Rouse& Sehrawat 2010). IL-17 was the most

expressed cytokine after Asian strain infection, might have downregulated the Th1 response, and interfered with the optimal antiviral response.

Finally, most of the analyzed cytokines in this work were relatively suppressed after infection with the Asian compared to the African strain, which could implicate in an evolutionary mechanism acquired by this ZIKV strain in order to evade the immune system. Expression of immunological mediators is needed to stimulate adaptive immunity and promote cessation of a pathogen infection, and its down-regulation could affect the intensity of the disease or even contribute to secondary infections (Mehta et al. 2015). Despite the fact that there is no comparative clinical studies regarding the expression of cytokines in patients infected with these strains, the *in vitro* results presented here could implicate that the Asian strain generates a less effective initial immune response, which could lead to viral persistence (Paz-Bailey et al. 2017) or have an association with adverse outcomes of the disease.

Moreover, the differences in virulence and infectivity could be a result of genomic mutations that could induce conformational protein changes and improve the fitness of the strain. It has been shown that some mutations in the envelope protein of chikungunya virus have implications in infectivity of the virus and adaptation to *Aedes albopictus* mosquitoes (Tsetsarkin et al. 2009). However, comparative analysis of the percentage of nucleotide homology between African and Asian strains was found to be lower than amino acid homology (Table 1), and *in silico* analysis of the conformational folding of the envelope protein (Figure 6) from both strains did not show striking differences between them, which make it harder to find a specific mutation that could explain this possible increase in Asian strain infectivity.

On the other hand, it was observed a difference in codon usage in *Aedes* species, with a predominant use of certain codons by *Ae. aegypti* and the Asian strain (Table S1). Although this finding cannot explain increased pathogenicity, it could suggest a mechanism by which this virus lineage had such fast spread to several parts of the world and caused outbreaks in areas where this mosquito is prevalent. Kraemer et al. documented the infestation levels of *Ae. albopictus* and *Ae. aegypti* worldwide, and this last species was widely disseminated, especially in those countries where Zika virus outbreaks were reported, such as Brazil (Kraemer et al. 2015). In agreement with these findings, viral replication, a correlate of infectivity, was higher with the Asian strain in

in vitro experiments using cells of *Aedes* mosquitos (Figure 2), albeit this observation is somehow difficult to observe in nature.

In conclusion, based on the results obtained in this work, it could be inferred that ZIKV Asian strain, somehow, acquired the ability to replicate more efficiently in *Aedes aegypti* mosquitoes resulting in a more intense outbreaks where there was an association of high prevalence of *Ae. aegypti* and a susceptible population. In addition, the Asian lineage might have developed a mechanism that leads to a weakening of the initial immunological antiviral response and consequently, to an increase of comorbidities associated with the infection. In spite of the data shown here allow us to raise these hypotheses, more studies are needed to confirm these findings and contribute to a better understanding of the mechanisms involved in the severe outcomes of ZIKV infections.

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Table 1. Percentage of nucleotide and amino acid homology between MR766 (African) and ZikaSPH2015 (Asian) strains.

Protein	% nucleotide homology	% amino acid homology
Capsid	91,74	95,20
Pr-M	88,45	92,31
Membrane	88	96
Envelope	87,72	96,83
Ns1	89,60	97,23
Ns2a	87,30	95,88
Ns2b	88,80	98,61
Ns3	89,10	98,18
Ns4a	90,25	99,32
Ns4b	88,58	97,21
Ns5	88,14	95,86

Figure 1. Replication curves for MR766 and ZikaSPH2015 strains in VERO cells, demonstrating a similar pattern of replication between them. All collection points are represented as average and standard deviation of triplicates.

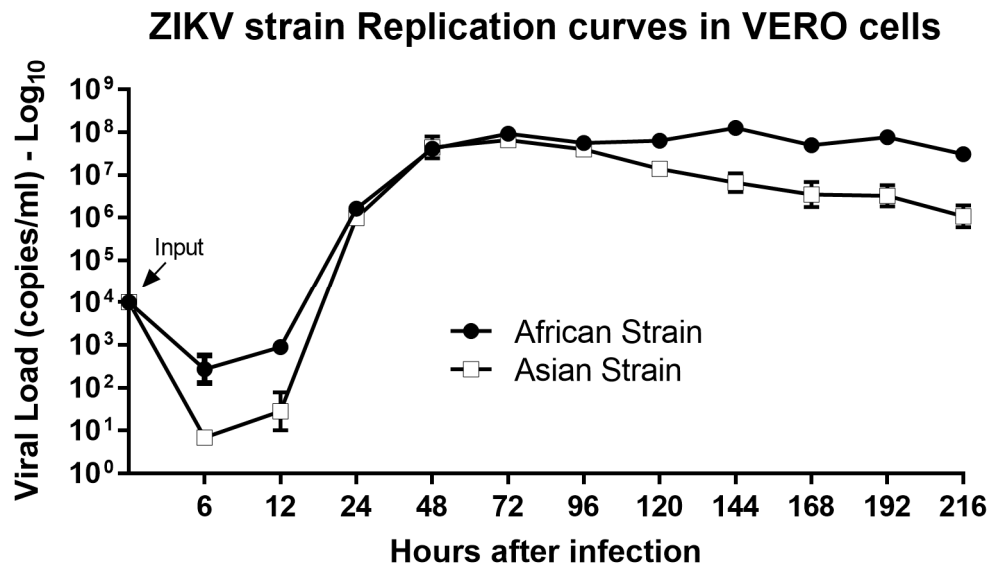


Figure 2. Viral load measurements after sequential infections in different *Aedes* sp. and Vero cell lines. Y-axis shows quantification in a log scale and X-axis shows sequential infections made by A (MR766), B (ZikaSPH2015) or X (no infection). Sequence of infection is shown separated by a slash. All points are represented as average and standard deviation of triplicates.

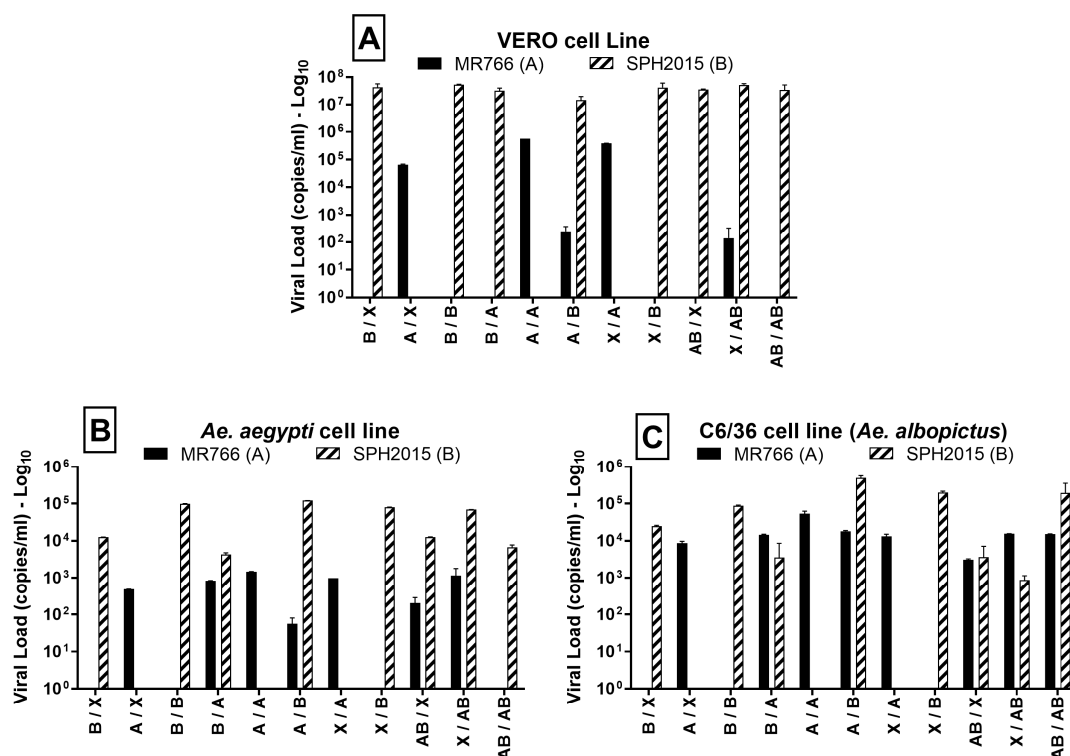


Figure 3. Cytokine expression profile of human PBMC after infection with Zika virus strains. All experiments were made in triplicates, and Fold Change calculated using non-infected cells. * Statistical difference ($p < 0.05$).

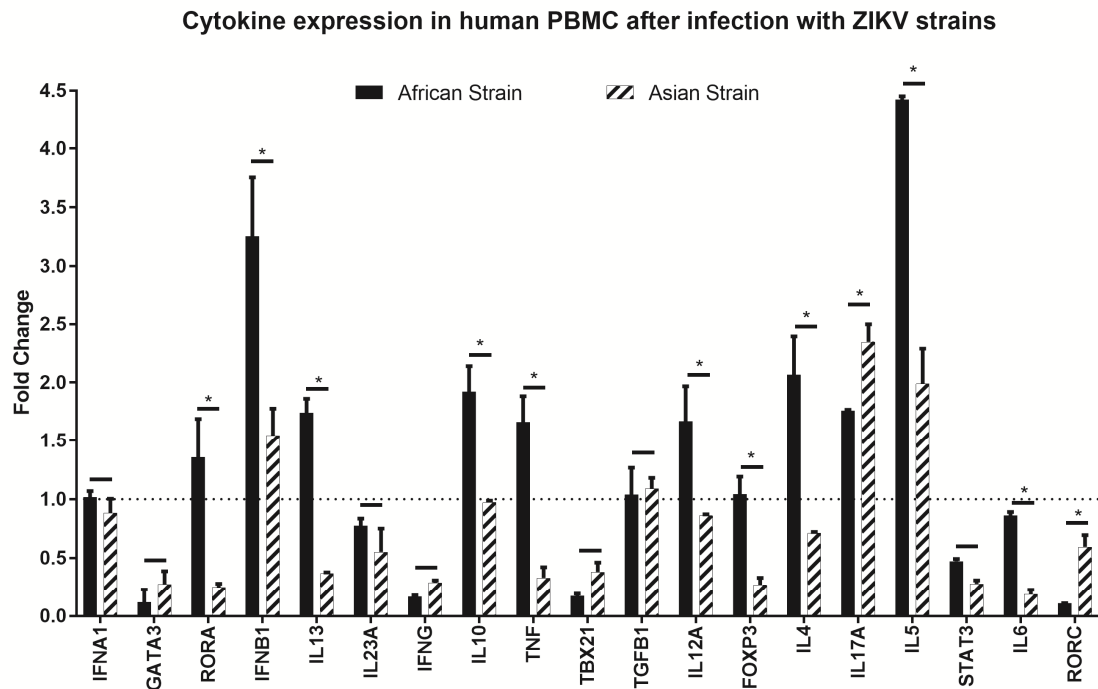


Figure 4. Neutralization assays using homologous or heterologous virus strain/serum showing that infection with either virus is protective against the other.

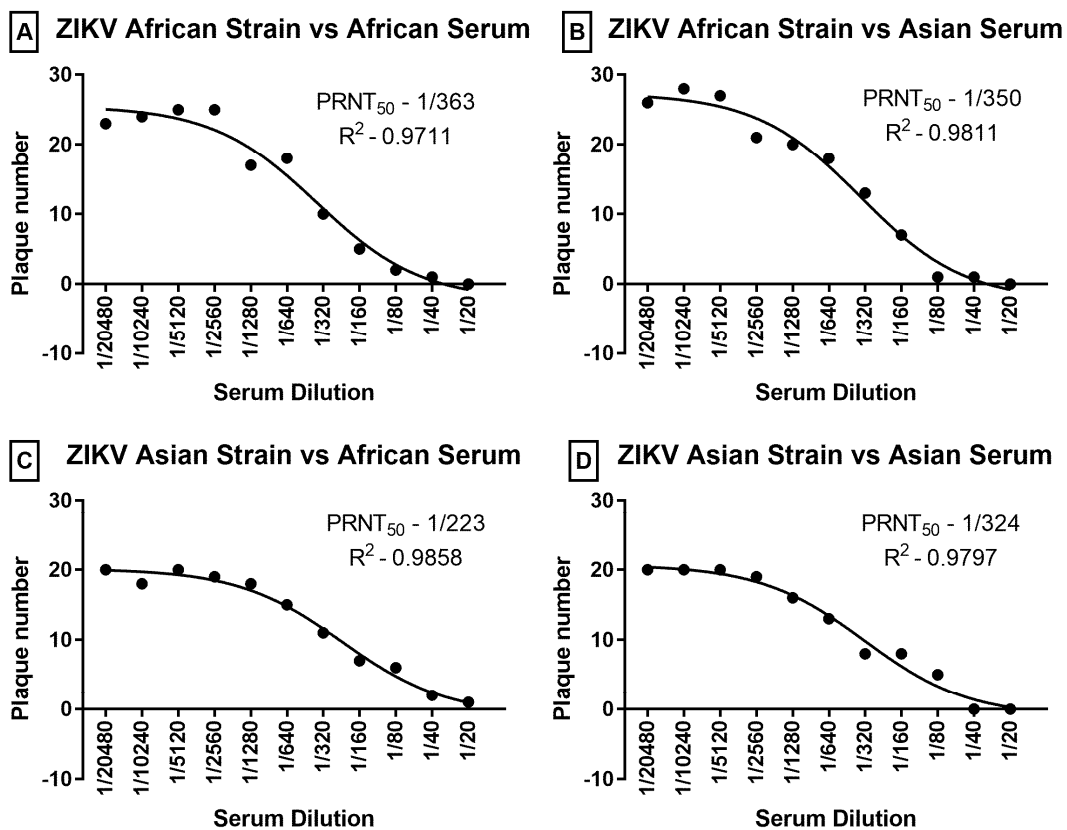


Figure 5. Phylogenetic analysis of Zika virus complete sequences by Maximum Likelihood method. The numbers above/below the branches indicate the distance between sequences, ranging from 0 (no difference) to 1. Phylogenetic analyses were conducted in MEGA7 software.

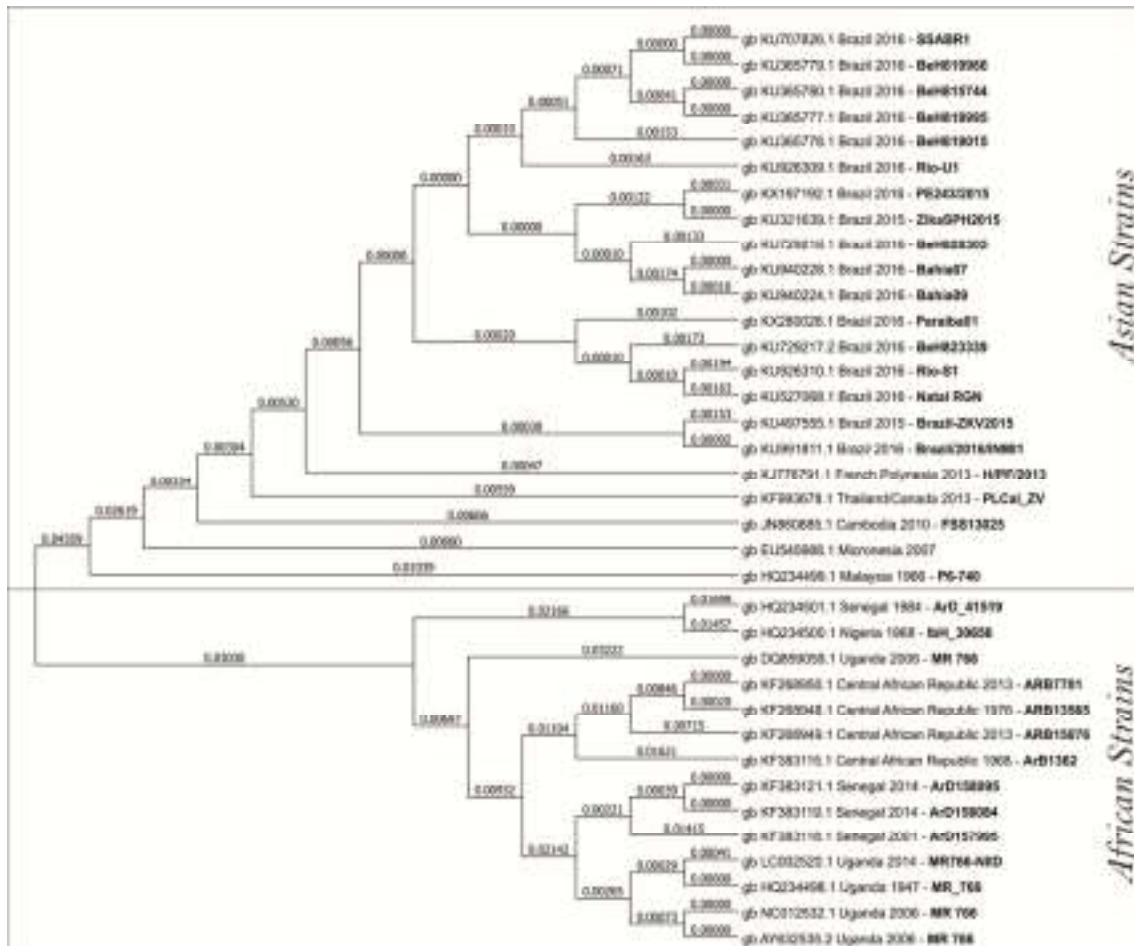


Figure 6. Predicted structure of envelope protein using *RaptorX* software. Protein domains were highlighted using *PyMol Molecular Graphics System* software following standard colors (Domain I – Red; Domain II – Yellow; and Domain III – Blue).

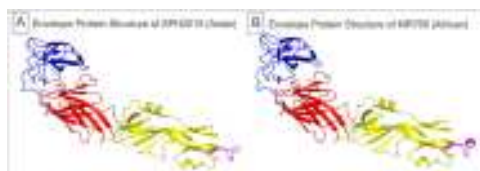


Table S1. Codon usage table of Zika virus strains (percentage) and mosquitoes species (per thousand)

Amino acid	Codon	Percentage on Genome		Codon Usage Per Thousand	
		MR766	ZikaSPH2015	<i>Ae. albopictus</i>	<i>Ae. aegypti</i>
Phenylalanine (F)	UUU	48.91%	51.04%	9.96‰	11.81‰
	UUC	51.09%	48.96%	31.21‰	30.52‰
Leucine (L)	UUA	6.37%	5.11%	3.51‰	4.95‰
	UUG	21.97%	22.04%	16.69‰	18.88‰
	CUU	14.65%	12.78%	7.29‰	9.52‰
	CUC	17.20%	16.61%	12.97‰	11.46‰
	CUA	9.87%	11.19%	8.49‰	7.66‰
	CUG	29.94%	32.27%	25.67‰	32.24‰
Isoleucine (I)	AUU	26.75%	30.81%	12.90‰	17.53‰
	AUC	43.60%	37.21%	32.40‰	27.89‰
	AUA	29.65%	31.98%	6.94‰	7.09‰
Valine (V)	GUU	19.78%	20.67%	15.01‰	17.19‰
	GUC	26.01%	28.95%	22.16‰	17.83‰
	GUA	11.35%	9.40%	8.7‰	9.88‰
	GUG	42.86%	40.98%	22.3‰	20.67‰
Serine (S)	UCU	14.92%	14.76%	6.24‰	8.80‰
	UCC	14.43%	16.19%	16.06‰	15.67‰
	UCA	24.88%	25.71%	5.54‰	8.84‰
	UCG	8.96%	6.19%	19.50‰	18.49‰
	AGU	17.41%	16.19%	9.05‰	12.19‰
	AGC	19.40%	20.96%	12.34‰	14.45‰
Proline (P)	CCU	19.72%	16.31%	4.42‰	8.60‰
	CCC	26.06%	28.37%	14.17‰	10.69‰
	CCA	46.48%	44.68%	13.46‰	15.42‰
	CCG	7.74%	10.64%	18.09‰	16.78‰
Threonine (T)	ACU	23.35%	25.11%	8.56‰	10.92‰
	ACC	27.31%	28.63%	23.91‰	20.20‰
	ACA	40.53%	34.80%	7.78‰	9.57‰
	ACG	8.81%	11.46%	13.25‰	13.72‰
Alanine (A)	GCU	27.46%	27.82%	19.07‰	19.15‰
	GCC	33.45%	32.39%	34.99‰	26.06‰
	GCA	28.52%	27.47%	11.43‰	13.22‰
	GCG	10.57%	12.32%	11.92‰	12.15‰
Tyrosine (Y)	UAU	43.82%	37.93%	8.70‰	11.20‰
	UAC	56.18%	62.07%	22.79‰	23.66‰
Histidine (H)	CAU	45.20%	38.96%	7.01‰	11.03‰
	CAC	54.80%	61.04%	11.57‰	15.32‰
Glutamine (Q)	CAA	50.00%	38.96%	11.01‰	17.22‰
	CAG	50.00%	61.04%	25.67‰	25.29‰
Asparagine (N)	AAU	36.19%	32.04%	14.17‰	19.93‰
	AAC	63.81%	67.96%	30.29‰	30.43‰
Lysine (K)	AAA	40.31%	43.62%	20.76‰	23.22‰
	AAG	59.69%	56.38%	50.84‰	35.23‰
Aspartic Acid (D)	GAU	40.26%	46.80%	23.00‰	31.73‰
	GAC	59.74%	53.20%	24.89‰	25.07‰
Glutamic Acid (E)	GAA	47.95%	46.54%	34.43‰	33.95‰
	GAG	52.05%	53.46%	27.84‰	24.87‰
Arginine (R)	CGU	6.45%	7.80%	14.66‰	10.96‰
	CGC	11.06%	9.63%	12.90‰	10.07‰
	CGA	5.07%	3.21%	9.47‰	9.46‰
	CGG	9.21%	10.09%	11.85‰	8.46‰
	AGA	42.86%	39.91%	5.61‰	5.13‰
	AGG	25.35%	29.36%	4.00‰	4.29‰
Glycine (G)	GGU	12.95%	13.36%	21.25‰	17.85‰
	GGC	16.50%	16.94%	18.3‰	16.80‰
	GGA	49.19%	42.99%	20.76‰	24.08‰
	GGG	21.36%	26.71%	8.06‰	6.00‰