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Desenvolvimento e validação experimental de uma metodologia *in house* para amplificação e sequenciamento do genoma completo do Zika vírus

Dissertação apresentada ao Departamento de Microbiologia do Instituto de Ciências Biomédicas da Universidadede São Paulo, para obtenção do Título de Mestre em Ciências.

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Development and validation of an in house method for whole genome amplification and sequencing of Zika virus

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Resumo

ZAKI POUR, SH. Desenvolvimento e validação experimental de uma metodologia in-house para amplificação e sequenciamento do genoma completo do Zika vírus. [Dissertação (Mestrado em Microbiologia)]. São Paulo : Instituto de Ciências Biomédicas, Universidade de São Paulo, 2018.

O zika é um arbovírus emergente. Há evidências para a relação entre o zika e a microcefalia congênita e também com a síndrome de Guillain-Barre. Várias características do vírus são importantes, como a persistência do vírus no sêmen por vários meses, transmissão sexual e evidência de transmissão pré-natal. As mães grávidas infectadas com zika podem dar à luz crianças aparentemente saudáveis que podem apresentar manifestações e complicações tardias. Existe uma clara necessidade de diagnosticar e sequenciar amostras clínicas do ZIKV que circulam na América do Sul, especificamente no Brasil. No entanto, as baixas cargas virais observadas que são observadas comumente em amostras humanas constituem um fator complicador para detecção, amplificação e sequenciamento. Neste projeto, propor projetar um fluxo de trabalho otimizado para o sequenciamento completo do genoma com base no pré-enriquecimento por PCR (reação em cadeia da polimerase) e pools de amplicons.

Palavras-chave: Zika. Reação em cadeia da polimerase. Enriquecimento prévio. Sanger. Sequenciamento de nova geração. Amplificação de genoma viral completo.

Abstract

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Zika is an emerging arbovirus. There is enough evidence for the relation between Zika and congenital microcephaly and also with the Guillain-Barre syndrome. Several characteristics of the virus are important, such as persistence of the virus in semen for several months, sexual transmission and evidence of prenatal transmission. Zika infected pregnant mothers may give birth to apparently healthy children that may show late manifestations and complications. There is a clear necessity of diagnosing and sequencing clinical samples of ZIKV circulating in South America, specifically in Brazil. Nevertheless, the observed low viral loads that are commonly in human samples constitute a complicating factor for detection, amplification and sequencing. In this project, we aim to design an optimized workflow for full genome sequencing based on pre-enrichment by PCR (polymerase chain reaction) and amplicon pools.

Keywords: Zika. Polymerase chain reaction. Enrichment. Sanger. New generation sequencing. Whole genome amplification.

1. Introduction

Zika virus (ZIKV) was first identified in a rhesus monkey in the forest of Ziika in Uganda in 1947 (1). Then the virus was recovered from the mosquito *Aedes africanus*, caught in the same forest in 1948 (2). The first human cases of ZIKV were detected in Uganda and the United Republic of Tanzania in 1952 (3). In 1964 a researcher from Uganda was infected while working with the virus, confirming the Zika virus disease in human (4). Human cases were confirmed, although no hospitalization was reported between the 1960's and 1980's. The disease then moved from Uganda to western Africa and Asia in the first half of the 20th century (5, 6). ZIKV was detected in mosquitoes found in equatorial Asia, including India, Indonesia, Malaysia, and Pakistan from 1969 to 1983 (7). The first ZIKV large outbreak in humans reported in the Pacific Island of Yap in the Federated States of Micronesia with an estimated 73% of residents infected in 2007. Prior to this, only 14 cases of human ZIKV were documented around the world (8). In 2008, a US scientist conducting field-work in Senegal fell ill with ZIKV infection. On his return home to Colorado, he infected his wife and that was the first documented case of sexual transmission of ZIKV (9). In 2013 and 2014, outbreaks occurred in the Pacific: French Polynesia, Easter Island, the Cook Island, and New Caledonia. After infection has been linked to microcephaly in Brazil, thousands of previous suspected infections in French Polynesia were re-investigated in order to establish and confirm a possible association between the ZIKV virus and congenital malformation and to severe neurological and autoimmune complications (10, 11). On March 2014, during the outbreak in French Polynesia, two mothers and their newborns were found infected. The infants possibly acquired the infection by transplacental transmission or during delivery (12). At the same time, during the outbreak in French Polynesia, 1,505 asymptomatic blood donors reported being (polymerase chain reaction) PCR positive for ZIKV alerting the authorities that the virus can be passed on through blood transfusion (13). Brazil notified WHO of an illness with the symptom of rash in north-eastern states on the 29 of March 2015. From February 2015 to 29 April 2015, nearly 7000 mild cases are reported, with no associated deaths. Of 425 blood samples tested, 13% were dengue positive.

Tests for Chikungunya, Measles, Rubella, Parvoviruses B19, and Enteroviruses were negative. At that time, ZIKV was not suspected and was no tests were carried out for its detection. On May 2015, Brazil National reference laboratory, The Oswaldo Cruz Institute confirmed ZIKV circulating in Brazil and this was the first report of locally acquired ZIKV in the Americas (14). On November 2015, Brazil declared a national public health emergency, as cases of suspected ZIKV associated with microcephaly continued to increase. At the same time, in Brazil it was detected ZIKV genome in blood and tissue samples of a baby with microcephaly (15). Afterward, during January of 2016, it was reported 3,893 suspected of microcephaly and 1,708 cases of Guillain-Barre syndrome in Brazil. On September 2016 WHO concluded that Zika virus infection during pregnancy is the cause of congenital brain abnormalities (14). Finally, on the18 of November 2016, WHO declares the end of the public health emergency of international concern regarding microcephaly, although concerns still prevail in Brazil and more research will be necessary to understand ZIKA evolution, host adaptation, viral virulence and molecular epidemiological investigations (16). Recently Zika was included in the top ten emerging pathogens with the potential of causing a public health emergency and the absence of effective drugs or vaccines by a panel of scientists and public health experts convened by WHO in the January of 2017, also in the Second annual review in the February 2018 (17).

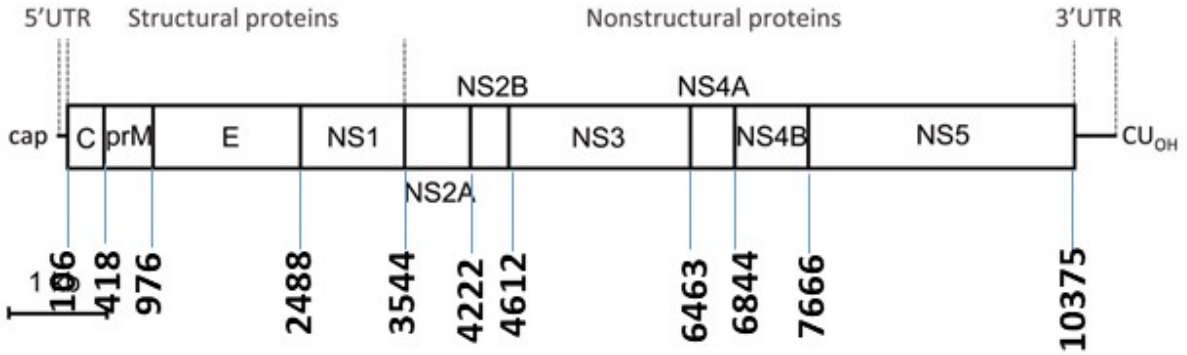
1.1 The Zika virus

Arboviruses (Arthropod-borne viruses) comprise more than 500 viruses transmitted either by insect vectors or spread as a zoonotic agent. Arboviruses are classified according to antigenic and phylogenetic relationships, morphology, and replicative mechanisms. Arboviruses are included in different taxonomic families, including Flaviviridae (genus Flavivirus), Bunyaviridae (genus Nairovirus, Orthobunyavirus, Phlebovirus, and Tospovirus), Togaviridae (genus Alphavirus), Rhabdoviridae (genus Vesiculovirus), Orthomyxoviridae (genus Thogotovirus), and Reoviridae (genus Orbivirus and Coltivirus) according to International committee on taxonomy of viruses (18).

Mosquitoes are the main vectors for most arboviruses, although other biting flies, midges, and ticks may also transmit these diseases. Humans are understood as important hosts because of the huge outbreaks observed in the Americas. Humans transmit disease to female mosquitos during blood feeding. The virus replicates in midgut cells and moves to the hemocoel (the primary body cavity of most invertebrates, containing circulatory fluid) infecting subsequently the salivary glands, from where is transmitted back to humans.

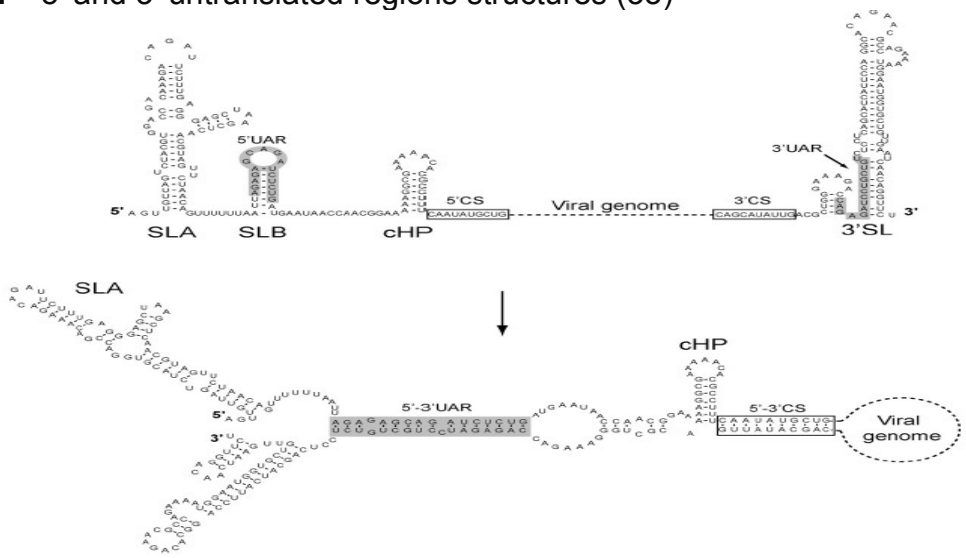
ZIKV belongs to the genus Flavivirus in the family Flaviviridae. The genus Flavivirus includes 53 other viral species, as well as the Dengue, Yellow Fever, Saint Louis encephalitis and West Nile viruses (19). ZIKV belongs to the Spondweni serogroup which shares serological cross-reactivity and similar clinical presentations. It has a small virion of approximately (50 - 60) nm in size (20), single-stranded RNA of positive sense, around 11 kb in length (21), with a single ORF (open reading frame) flanked by 5' (106nt) and 3' (428nt) UTRs (*untranslated region*) at both ends. It has 5' cap structure at 5' end and not polyadenylated at the 3' end, but makes a secondary loop structure that leads to the formation of a subgenomic flavivirus RNA (sfRNA) that is abundant non coding subgenomic RNA in infected cells through genomic RNA degradation by the host XRN1 exonuclease. The sfRNA is an extension of the 3' UTRs and it is essential for pathogenicity (22, 23). The single ORF then encodes a polyprotein precursor, which is cleaved by both host and virus enzymes, resulting the tree structural viral proteins C (capsid), preM (pre-membrane), E (envelope) and seven non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (24). NS1 is essential for virus replication and inhibition of complement-mediated immune response (25) and makes multimers with different functions during the infection cycle, including dimers involved in the replication complex in vesicles and hexamers, complexed with lipids that are secreted to the extra cellular environment. NS3 combines helicase/NTPase, serine protease, and RNA triphosphatase activity (26 - 28). NS2B is a cofactor for the protease activity of NS3. NS5 contains a methyltransferase and RNA-dependent RNA polymerase

(RdRp) domains and is necessary for genome replication also capping of nascent RNA (29).**Figure 1 –** ZIKV genome organization based on the MR-766 isolate



Untranslated regions play a fundamental role by RNA cyclization during the Flaviviruses replications (Figure 2) (30). This complementary requirement between 5' and 3' untranslated regions not only showed by RNA secondary structure prediction, but also by using infectious clones and replicon systems of DENV and WNV (31, 32). These RNA elements within the UTRs include 5' stem loops A and B (5' SLA and 5'SLB respectively), 5' and 3' upstream AUG, 3' cyclization sequence, 3' short hairpin structure (sHP), the highly-conserved 3'SL and the 5' cyclization sequence, and the capsid-coding region hairpin element (cHP) that lies within the ORF The translation initiator AUG at the 5' UTR found to be complementary to the region present at 3' SL (stem loops) which is called cyclization sequence 5'-3' UAR (Figure 2) (33).

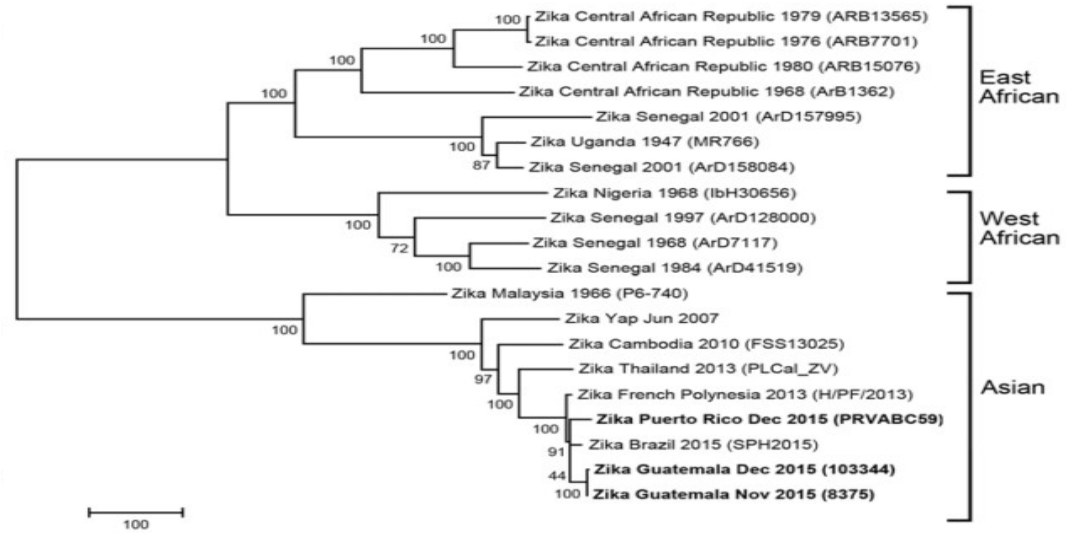
Figure 2 – 5’ and 3’ untranslated regions structures (33)



The transmission to humans occurs through the bite of infected mosquitoes. After the entrance of the virus, it interacts with host cell receptors like DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-Integrin) known as CD209, and also TAM family of the receptor, tyrosine kinase (Tyro-3, Axl, and Mer). With the attachment of viral envelope protein (E) and mediation of host cell receptor internalization occurs by endocytosis and the virus fuses with the endosome. Low pH causes the release of genomic RNA into the host cell cytoplasm. The positive ssRNA in replication vesicles is translated into a large polyprotein that is subsequently cleaved into mature structural and non-structural proteins. Negative ssRNA then is synthesized from the positive ssRNA serving as a template strand for viral genome replication by the viral-encoded RdRp and finally assembling. Polyprotein will be made. Then the polyprotein is cleaved into separate, mature proteins. Replication takes place at the surface of Endoplasmic Reticulum (ER). Virus assembly occurs in the endoplasmic reticulum and the virion buds at the ER and is translocate to the Golgi apparatus. The prM is cleaved into the Golgi and then the mature virion is released by exocytosis (34 - 40).

Based on the genome sequencing and phylogenetic trees, three distinct genotypes were identified, West African (Nigerian cluster), East African (MR766 prototype cluster), and Asian (41- 43), (Figure 3).

Figure 3 – Maximum likelihood phylogenetic tree of ZIKV



6. Discussion

Along the whole process from sample to the end there are some factors that can play a fundamental role and therefore change the final results. We will now discuss some of them in details.

6.1 Choice of clinical samples.

It is critical to do an early sample collection because usually, the symptoms appear 4-7 days after the onset with the higher titer of a viral copy in body fluid. However, following the appearance of symptoms viral load decreases. ZIKV is detectable in saliva and urine more than in the blood. In urine, viral load is higher than blood with a peak at around 5 to 7 days but is not detectable for more than 20 days after the clinical onset (72). ZIKV RNA was detectable in nasopharyngeal swabs while negative in serum (73, 74).

6.2 PCR inhibitors.

The effect of inhibitors in PCR reactions could be considered, especially in fresh urine, EDTA containing Vacutainers and in amplifications involving a low amount of viral copy or degraded genome. PCR inhibitors can be divided into two groups: organic and inorganic. Organic compounds, including bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulfate (SDS), humic acids, tannic acid, melanin, different proteins, such as collagen, myoglobin, hemoglobin, lactoferrin, immunoglobulin G (IgG), Heparin, also proteinases (75, 76). Sometimes sample dilution can be a solution to reduce the effect of PCR inhibitors, but not in our case with a low amount of viral RNA (77, 78). Inorganics (*e.g.*, salts and metals) are less problematic in virus-containing samples subjected to nucleic acids extraction protocols we used. However, most of the known inhibitors are organic compounds.6.3 Enhancers for better amplifications.

There are some enhancers and additives that can be added to cDNA and PCR reaction mixes, which increase efficiency and sensibility, improving the enzymatic activity of both, reverse transcriptase (RT) and DNA polymerase. In the case of an extremely low amount of viral genome amplification, these can be helpful. For instance, using 3.5 to 0.1 M of Betaine reduces Tm (melting temperature) facilitating GC-rich region amplification. BSA (bovine serum albumin) in a concentration of 0.01 µg/µL to 0.1 µg/µL is useful when attempting to amplify templates that contain PCR inhibitors, such as melanin. 2-10% of DMSO (dimethyl sulfoxide) reduces secondary structure and is particularly beneficial for better processing of GC-rich templates. But when used in concentrations above 10%, it will reduce polymerase activity. Formamide reduces also secondary structure and GC-rich templates. Non-ionic detergents such as Tween 20 also stabilize Taq polymerase and may also suppress the formation of the secondary structure of the concentration of 0.1-1%.

Moreover, choosing which cDNA synthesis kit should be used is also important. According to our own observation, we got better results when we make cDNA with the

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) rather than using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem).

6.4 cDNA synthesize

Sometimes just a gene or specific region is subject to be amplified and not the whole genome. In this case, synthesizing the cDNA with specific reverse primer can be a solution for low input RNA can be convenient (79). Nested or Semi-nested PCR also can be favorable for better detection and amplification of low input genomic material.

Finally, sequencing DNA rather than RNA needs less effort, is less time consuming and reduces the cost noticeably. Moreover, by the implementation of these techniques we are allowed to increase the amount of genomic material that can be sequenced by Sanger sequencing, which is still routinely used especially in the case of specific genes, genotyping, detection of SNV (single nucleotide variation), biological amplification by culture and cloning. Furthermore, it can be useful for the confirmation of PCR positive material, determine the source of an outbreak rapidly, understanding the molecular evolution of emerging viruses.

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