Improving the Diagnosis of Zika Virus to Study at Risk Populations

A Thesis Submitted in Accordance with the Requirements and Regulations of the University of Liverpool for

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Declaration

Except for the assistance outlined in the acknowledgements, the work described is my own work and has not been submitted for a degree or other qualification to this or any other university.

Abstract

Background

During 2015-16, Brazil experienced the largest epidemic of Zika virus (ZIKV) ever reported affecting millions of people. ZIKV diagnostics underpin all public health responses to the outbreak; however, accurate detection of Zika antibodies is very challenging because of cross reactivity with the closely related dengue virus (DENV). Chikungunya virus (CHIKV), which also circulates in Brazil causes similar clinical symptoms to both these viruses. Evaluation of the existing serological assays for ZIKV diagnostics had never been carried out in South America. During the outbreak, ZIKV appeared to be associated with a wide range of neurological manifestations meriting further investigation. In addition, understanding the population exposure to ZIKV is crucial to predict future epidemics in Brazil and effective design of vaccination strategies.

Methods

I evaluated the performance of ten Zika diagnostic antibody assays and two dengue antibody assays, individually and in combination using a well-characterised panel of 405 samples from Rio de Janeiro (Brazil). Then, we used a combination of the most accurate diagnostic methods to identify ZIKV among patients with new neurological disease (such as Guillain-Barré syndrome [GBS]) following suspected ZIKV infection I conducted a cross-sectional survey to estimate the prevalence of ZIKV and chikungunya virus (CHIKV) infection following the 2016 arbovirus season (n=1001) and a longitudinal cohort study to investigate the antibody changes over the years 2013, 2014, 2015 and 2016 (n=121). All samples were tested for Zika and chikungunya IgG antibodies. Sociodemographic and socioeconomic indicators as well as geographic heterogeneity were assessed.

Results

My results show that the overall performance of all the commercial ZIKV assays was quite poor. I showed improved accuracy using the in-house Block of Binding (BOB) ELISA and Plaque Reduction Neutralisation Tests (PRNTs). I also demonstrated strong cross-reactivity between Zika and dengue antibody responses, which accounted for the low specificity. Among the patients with neurological conditions recruited (n=35), 22 had evidence of ZIKV, CHIKV or dengue virus (DENV) infection. Zika seroprevalence in Rio de Janeiro was estimated in 2013 (2.0%), 2014 (5.8%), 2015 (19.4%) and at the end of the epidemic in 2016 (33.1%). My sociodemographic analyses have shown a significantly higher exposure to Zika and chikungunya viruses among low socioeconomic status populations.

Conclusions

In this thesis, I present the first systematic evaluation assessing the performance of the currently available serological assays to diagnose ZIKV infection in the Brazilian population. This evaluation highlights the urgent need for improvement and validation of Zika diagnostic assays and surveillance tools in South American populations. I have shown, that Zika virus is associated with a wide range of neurological manifestations, including central nervous system disease. Also, my results measure for the first time the seroprevalence of ZIKV and CHIKV in the metropolitan region of Rio de Janeiro. The results have shown that the majority of the population in Rio de Janeiro is still naïve to Zika and Chikungunya virus and future outbreaks may occur in the coming years.

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"If I have seen further it is by standing on the shoulders of giants"

-Isaac Newton

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List of Abbreviations

ADE	Antibody Dependent Enhancement
ADEM	Acute Disseminated Encephalopathy Mediated
ANOVA	One-way analysis of variance
BOB	Blockade-of-Binding
C	Capsid Protein
CCHF	Crimean-Congo Haemorrhagic Fever
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya Virus
CI	Confidence Interval
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CV	Coefficient of Variation
CZS	Congenital Zika Syndrome
DENV	Dengue Virus
DHF	Dengue Haemorrhagic Fever
DNA	Deoxyribonucleic acid
E	E protein
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FCS	Foetal calf serum
FDA	Food and Drug Administration
FE	Fisher's Exact
Fig	Figure
GADM	Database of Global Administrative Areas
GBS	Guillain-Barré Syndrome
h	hour
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDI	Human Development Index
HDU	Human Development Unit
HI	Hemagglutination inhibition

HRP	Horseradish peroxidase
HSV	Herpes simplex virus
ID	Identification
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIFT	Indirect Immunofluorescence Test
IOC	Institute Oswaldo Cruz
JEV	Japanese Encephalitis Virus
kDa	Kilodalton
km	Kilometres
LAMP	Loop Mediated Isothermal Amplification
LSPR	Localized Surface Plasmon Resonance
Max	Maximum
MEM	Minimum Essential Medium Eagle
min	minutes
Min	Minimum
ml	millilitre
MVEV	Murray Valley Encephalitis Virus
n	Number of Subjects
Na	sample not available / not enough volume
NHP	Non-Human Primates
No.	number
NPC	Neural Progenitor Cells
NS	Non-Structural
OD	Optical Density
OR	Odds Ratio
PAHO	Pan American Health Organization
PBS	phosphate buffered saline
PCI	Per Capita Income
PCR	Polymerase Chain Reaction
PCT	Point of Care testing
PhD	Doctor of Philosophy
PHEIC	Public Health Emergency of International Concern
pNPP	p-NitroPhenylPhosphate

PNS	Peripheral Nervous System
POCT	Point-of-care-test
PPV	Positive Predicted Value
prM	pre-M protein
PRNT	Plaque Reduction Neutralization Test
PU	Panbio Units
R$	Brazilian Reals
RNA	Ribonucleic acid
ROC	receiver operating characteristic
rpm	revolutions per minute
RT	Room Temperature
RT-PCR	Real-Time Polymerase Chain Reaction
RU	Relative Units
SD	standard derivation
sec	seconds
Sens	Sensitivity
SES	Socioeconomic Status
SINAN	Sistema Nacional de Informacao do Ministério da Saude
SLEV	Saint Louis Encephalitis Virus
Spec	Specificity
SPOV	Spondweni Virus
Syst	systemic
TORCH	Toxoplasma gondii, Other, Rubella virus, Cytomegalovirus, Herpes simplex virus
UFBA	Instituto de Ciências da Saúde da Universidade Federal da Bahia
UNDP	United Nations Development Programme
USA	United States of America
UV	Ultra violet
WBC	White blood cell
WHO	World Health Organization
WNV	West Nile Virus
YF	Yellow Fever
YFV	Yellow Fever Virus
ZIKV	Zika Virus

µm	micrometre
µl	microliter
µM	microMolar
µPAD	Microfluidic Paper-based Analytical Devices

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CHAPTER 1.

Introduction

Chapter 1: Introduction

1.1 Overview

Zika Virus (ZIKV) is a vector-borne emergent zoonotic pathogen that belongs to the *Flaviviridae* family. Infection in humans is typically associated with a fever-arthralgia-rash syndrome. ZIKV is transmitted mainly by *Aedes* mosquitoes but also through sexual contact, vertically and through blood transfusion. The majority of ZIKV infections, around 50 to 80%, are thought to be subclinical. However, in late 2015 a massive outbreak in Brazil, during which an association between Zika virus and an increase in severe congenital disease and Guillain-Barré syndrome (GBS) was identified, triggering an international public health response.

Just as I was starting my PhD, the largest epidemic of Zika virus in history was spreading across the whole of Brazil. The increase in neurological complications associated with ZIKV prompted the World Health Organisation (WHO) to declare the Zika virus outbreak a public health emergency of international public health concern in February 2016 [1]. Within a few weeks, in March 2016, I was deployed to Brazil to provide support to the Zika reference laboratory in Rio de Janeiro (Fiocruz) and started the highly needed research challenge of evaluating the assays which local teams were using to diagnose ZIKV. What was initially anticipated to be a few weeks of work in Brazil turned into almost 2 years working in Rio de Janeiro, carrying out multi-disciplinary research in Rio de Janeiro and Recife, two of the regions that had been the most affected by the Zika virus epidemic.

I feel that it is essential to understand the complexity of the impact of ZIKV in Brazil in order to understand the context in which my research and my PhD took part. At the same time as the ZIKV outbreak exploded causing a serious widespread epidemic of near pandemic proportions, Zika virus became a global public health emergency. The drama around Zika virus was highly publicised in every journal and the global news media with an intense focus on Brazil, which was home to the highest number of infected individuals.

During these early stages of the epidemic, there were more unknowns and uncertainties than responses and understanding. This confusion was not only spread among the general population but also among public health specialists, physicians and researchers. Everyone was posing the same questions: What is the exact impact of ZIKV in the population? What is the long-term burden associated with Zika virus infection? What is the best way to know if someone has been infected with Zika virus or not? Is there any treatment? How can we

prevent ZIKV? When and how did ZIKV arrive in Latin America? How many people were going to be exposed to Zika virus? Finally, what should the recommendations be for pregnant woman and vulnerable populations at risk?

When I started my PhD there were too many questions, dilemmas and unknowns to be addressed. It is precisely within this background of change and uncertainty around the largest Zika virus epidemic in history that I have undertaken my PhD.

To address these questions above, identifying, optimising and determining the most appropriate algorithm for Zika virus diagnosis underpins all public health responses to any ZIKV epidemic. This is essential in order to provide the appropriate clinical management and response and to allow for a better understanding of the disease burden in the neonatal population and neurologically affected patients. Finding appropriate diagnostic methods will also help to provide a better understanding regarding the incidence and prevalence of ZIKV in the population. Consequently, this will provide a base to guide future public health measures and research in particular regarding vaccination strategies and potential treatments. The main goal of my PhD was to respond quickly to some of the crucial research questions arising from this emergent epidemic and helping to unravel some of the mystery around Zika virus.

1.2 Zika virus: emergence, clinical features transmission and pathology

1.2.1 Zika virus: an emergency of international public health concerns

ZIKV was firstly identified in 1947 in the Ziika Forest, near Lake Victoria in Uganda by a team of researchers from the Rockefeller Foundation who were studying the sylvatic (jungle) cycle of yellow fever virus. The newly-discovered virus was isolated from the serum of an infected Rhesus monkey that developed fever and viremia [2](Figure 1.1). However, it was not until 1952 when ZIKV was detected for the first time in humans. Dr Dick found ZIKV neutralizing antibodies in the serum of six individuals in Uganda who had not reported symptoms. Interestingly, the author predicted the following in his publication in 1952:

"*The absence of the recognition of a disease in humans caused by Zika virus does not necessarily mean that the disease is either rare or unimportant. (…) The available information suggests that perhaps not only yellow fever but also Zika virus (…) are at the moment well adapted to the human host*" [3].

The author, George Dick, a Scottish virologist from the National Institute for Medical Research in London could have never imagined that this virus that they had just discovered by serendipity, was going to cause a major pandemic 70 years later.

Figure 1.1 The origins of Zika Virus
ZIKV was initially identified in 1947 in Uganda. A) Street sign pointing to the Ziika Forest Research Field Station in the Ziika Forest in Uganda, place that gave a name to the virus. Adapted from: Cohen (2016) [4]; B) Map of Africa showing where Zika Virus was initially identified and isolated. Source: adapted from WHO, The origin of Zika virus. (http://www.who.int/emergencies/zika-virus/timeline/en/)

In 1954, the virus was isolated from a young girl who suffered fever and headache in Nigeria representing the first characterization of disease caused by ZIKV ever reported [5, 6](Figure 1.1 B). ZIKV was detected for the first time outside Africa in 1966 when positive *Aedes aegypti* mosquitoes were found in Malaysia [7].

Before 2006, ZIKV had been isolated from more than 20 mosquito species mainly from the Aedes genus, however confirmed ZIKV cases in humans were rarely reported and appeared only sporadically and in just around 24 countries in Africa and Southeast Asia [7]. Interestingly, seroprevalence data available from various studies performed in Africa and Asia suggest that there have probably been extensive widespread circulation of Zika virus in both continents with the disease spreading unnoticed [8] [9] [10] [11].

In 2007, the first large outbreak of disease associated directly to Zika virus infection was reported in the Island of Yap in the Federated States of Micronesia where 70% of the population was infected [12]. Then, during 2013 and 2014 French Polynesia suffered an outbreak of Zika Virus with an estimated infection rate of around 80% and with more than 30,000 patients presenting to health-care medical facilities in just a few months [13, 14]. During this outbreak, there was an increase in the number of neurological cases among the population including 42 acute cases of Guillain-Barré syndrome (GBS). This represented a 20-fold increase in the incidence of GBS compared with the previous four years. These cases

were reported simultaneously as the outbreak was spreading throughout the islands [15]. During that period, ZIKV outbreaks were also registered in other islands within the Pacific Region [16].

In early 2015, an "*explosive pandemic of Zika virus infection*" started in Brazil coinciding with an outbreak of another emergent virus, chikungunya which had arrived in Brazil only one year earlier [17]. A large number of cases of a fever-rash like illness were reported mainly in the Northeast of Brazil. When physicians ruled-out chikungunya or dengue virus infections scientists from Bahia, in the Northeast of Brazil, identified and confirmed the first locally acquired-Zika case in April 2015 [18]. By the end of 2015, ZIKV had been identified through laboratory testing in most States of Brazil and it was estimated that between 0.5 to 1.5 million individuals had been infected in Brazil [19]. However, what raised the most alarm was the increased incidence of children born with congenital abnormalities, such as microcephaly, following the peak of ZIKV infections. In December 2015, ZIKV RNA was detected in amniotic fluid of two pregnant women whose foetuses had been diagnosed with microcephaly strengthening the link for this association [20]. From the end of 2015, ZIKV spread rapidly from Brazil to other countries and territories in South, Central, North America and the Caribbean.

Following these associations, on the 1st of February of 2016 the World Health Organization (WHO) declared that the link between Zika virus, microcephaly and other neurological disorders constituted a Public Health Emergency of International Concern (PHEIC) lasting until November 2016 [1]. The PHEIC is a formal declaration that designates public health crisis of global reach. Previously, the WHO had only declared it three times for Swine flu (2009), polio (2014) and Ebola (2014).

When the PHEIC for Zika virus was announced multiple countries in South and Central America, as well as the Caribbean, had reported autochthonous transmission of Zika Virus infections including the United States of America. The Pan American Health Organization (PAHO) and the WHO have now declared that they estimate that during 2015 and 2016, over 1 million individuals were affected by ZIKV in the regions of North America and Latin America with 20 fatal cases (excluding deaths associated with Guillain-Barré Syndrome and Congenital malformations [21]) (Figure 1.2). Interestingly, in 2017 ZIKV infection rates declined drastically worldwide. However, the WHO declared that up to March 2018 there were still 71 countries or regions worldwide with autochthonous transmission of ZIKV (See

Figure 1.3). An autochthonous transmission is an infection spread from one individual and acquired in another individual in the same place with local mosquitoes.

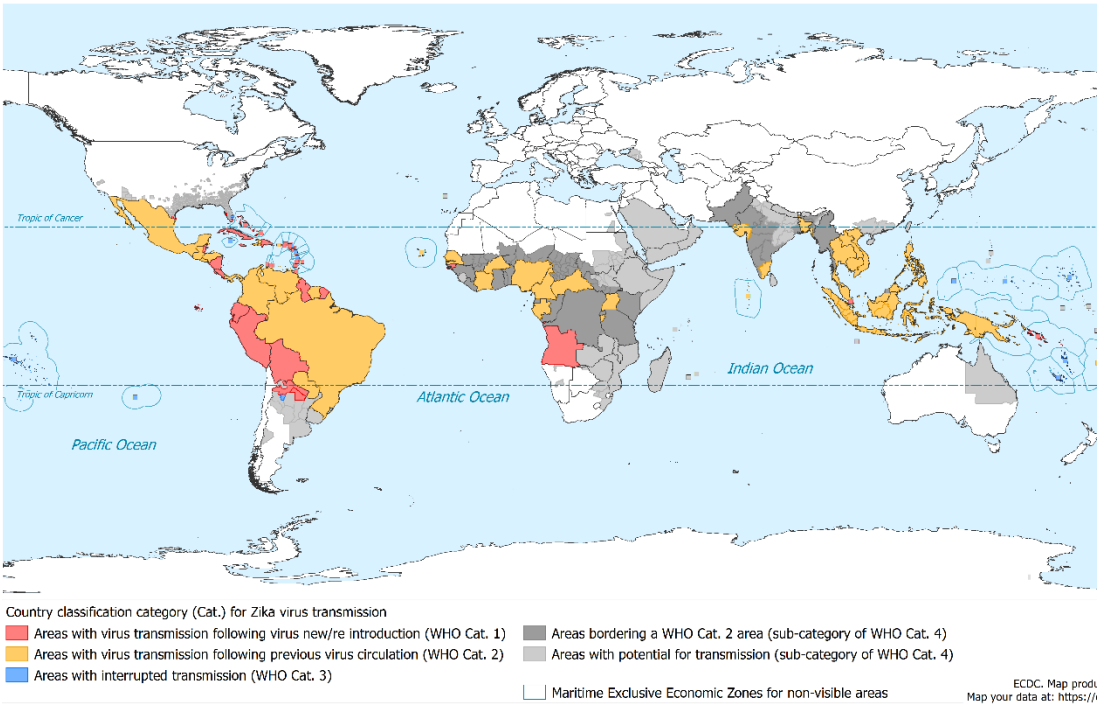


Figure 1.2 Transmission and spread of Zika Virus worldwide
Classification of countries and territories regarding Zika virus transmission following the WHO's Zika virus country classification scheme into categories explained in WHO (2017) [22]. Map adapted from the European Centre for Disease Control (ECDC): ZIKV and updated on the 19th of December 2017.

	WHO Regional Office	Country / territory / subnational area	Total
Category 1: Area with new introduction or re-introduction with ongoing transmission	**AFRO**	Angola; Guinea-Bissau	**2**
	AMRO/PAHO	Anguilla; Antigua and Barbuda; Argentina; Aruba; Barbados; Belize; Bonaire, Sint Eustatius and Saba; British Virgin Islands; Cuba; Curaçao; Dominica; Grenada; Montserrat; Saint Kitts and Nevis; Saint Lucia; Saint Martin; Saint Vincent and the Grenadines; Sint Maarten; Trinidad and Tobago; Turks and Caicos Islands; United States Virgin Islands	**21**
	WPRO	Samoa; Singapore; Solomon Islands; Tonga	**4**
Subtotal			**27**
Category 2: Area either with evidence of virus circulation before 2015 or area with ongoing transmission that is no longer in the new or re-introduction phase, but where there is no evidence of interruption	**AFRO**	Burkina Faso; Burundi; Cabo Verde; Cameroon; Central African Republic; Côte d'Ivoire; Gabon; Nigeria; Senegal; Uganda	**10**
	AMRO/PAHO	Bolivia (Plurinational State of); Brazil; Colombia; Costa Rica; Dominican Republic; Ecuador; El Salvador; French Guiana; Guatemala; Guyana; Haiti; Honduras; Jamaica; Mexico; Nicaragua; Panama; Paraguay; Peru; Puerto Rico; Suriname; Venezuela (Bolivarian Republic of)	**21**
	SEARO	Bangladesh; India; Indonesia; Maldives; Myanmar; Thailand	**6**
	WPRO	Cambodia; Fiji; Lao People's Democratic Republic; Malaysia; Papua New Guinea; Philippines; Viet Nam	**7**
Subtotal			**44**
Category 3: Area with interrupted transmission and with potential for future transmission	**AMRO/PAHO**	Bahamas; Cayman Islands; Guadeloupe; ISLA DE PASCUA – Chile; Martinique; Saint Barthélemy; United States of America	**7**
	WPRO	American Samoa; Cook Islands; French Polynesia; Marshall Islands; Micronesia (Federated States of); New Caledonia; Palau; Vanuatu	**8**
Subtotal			**15**
Category 4: Area with established competent vector but no known documented past or current transmission	**AFRO**	Benin; Botswana; Chad; Comoros; Congo; Democratic Republic of the Congo; Equatorial Guinea; Eritrea; Ethiopia; Gambia; Ghana; Guinea; Kenya; Liberia; Madagascar; Malawi; Mali; Mauritius; Mayotte; Mozambique; Namibia; Niger; Réunion; Rwanda; Sao Tome and Principe; Seychelles; Sierra Leone; South Africa; South Sudan; Togo; United Republic of Tanzania; Zambia; Zimbabwe	**33**
	AMRO/PAHO	Uruguay	**1**
	EMRO	Djibouti; Egypt; Oman; Pakistan; Saudi Arabia; Somalia; Sudan; Yemen	**8**
	EURO	Georgia; Região Autónoma da Madeira – Portugal; Russian Federation; Turkey	**4**
	SEARO	Bhutan; Nepal; Sri Lanka; Timor-Leste	**4**
	WPRO	Australia; Brunei Darussalam; China; Christmas Island; Guam; Kiribati; Nauru; Niue; Northern Mariana Islands (Commonwealth of the); Tokelau; Tuvalu; Wallis and Futuna	**12**
Subtotal			**62**
Total			**148**

Figure 1.3 WHO classification of countries affected by Zika
Data as of the 15th of February 2018 following the Zika Virus country classification scheme into four categories regarding differences in the current transmission levels explained by WHO (2017) [22].

1.2.2 Zika virus transmission, pathogenesis and clinical disease

Zika virus is an enveloped single-stranded RNA virus that belongs to the Flavivirus genus. ZIKV is an arbovirus (arthropod-borne virus) since it is mainly transmitted via infected mosquitoes from the Aedes genus [23]. These mosquitoes are also the primary vectors for dengue and chikungunya viruses, with *Aedes aegypti* considered the most competent species [24]. During this outbreak, it has been demonstrated that, albeit at a lower efficiency and frequency, ZIKV can also be transmitted through vector-independent routes. Mother-to-child transmission during Zika infection, known as vertical transmission, is now well

documented. Additionally, during the last few months there has been extensive evidence to show transmission of ZIKV via sexual contact. This is noteworthy as it is a characteristic unique to ZIKV amongst the flaviviruses [25, 26] [27]. This may have played a bigger role than originally thought in the ZIKV outbreak in Latin America. Furthermore, transmission has also been reported through blood transfusion, laboratory-acquired incidents and via secondary non-sexual contact. Interestingly, recent studies have demonstrated that ZIKV can be shed in a variety of body fluids including sexual fluids, CSF and tears [28].

Two geographically distinct lineages of ZIKV have been described: the African lineage and the Asian lineage which can be further divided into three genotypes (West African, East African and Asian) [29]. Although it was estimated that around 40 - 80% of the infections of ZIKV could be asymptomatic, most individuals experiencing symptoms have a very mild disease with fever, non-purulent conjunctivitis, pruritic and maculopapular rash and in some cases a weak arthralgia [30]. This clinical presentation in children and adults is generally a self-limiting illness, which is clinically similar to dengue fever, chikungunya infection and many other tropical fevers. Moreover, many of these pathogens are transmitted through the same vector and share similar geographical distribution [23]. Due to these clinical similarities, diagnosis without further laboratory testing can be significantly challenging leading to potentially high rates of misdiagnosed and underreporting especially in areas endemic for various arboviruses.

1.2.2.1 Zika virus pathogenesis

The pathogenesis of ZIKV remains to be elucidated however it has been documented that ZIKV is an intensely neurotropic virus which has been shown to induce both central and peripheral neuronal cell death. Zika targets mainly Neural Progenitor Cells (NPCs) but also other neuronal cells in all stages of maturity as well as neural retinal cells resulting in increased inflammation, reduced cellular proliferation and apoptosis [31]. These cell types are involved in brain processes such as proliferation and migration of glial progenitor cells as well as the development of neural pathways and myelination. These processes are still relevant during the course of childhood and adolescence, however, they are most important during the development of the brain of the foetus.

Several in vitro and animal models have been developed to better understand the pathogenesis of ZIKV during pregnancy (in the foetuses) and in developing children and during neurologic disease. Experiments performed in Non-Human Primate (NHP) models demonstrated substantial pathology in the CNS and ZIKV spread to different parts of the NHP

such as the placenta, foetal brain and foetal liver and also the maternal brain, eyes, spleen, and liver [32]. Interestingly, NHPs had a specific T-cell response following ZIKV infection and they were protected from re-infection [33].

The neurotropic properties of Zika, its damage to developing neural cells, and its impact on the development of the human brain raise serious concerns about the neurological sequelae of postnatal ZIKV infection.

1.2.2.2 Treatment for Zika Virus Infection

There are no specific antiviral drugs available yet for Zika virus and the current most used treatment is supportive care for the fever, the itching and pain. Following accurate diagnostic testing and once dengue has been ruled out as potential agent causing the infection, aspirin and other non-steroidal anti-inflammatory drugs can be prescribed. Recent *in vitro* studies suggest that interferon treatment may be used against Zika infection and further studies are being concluded to understand this [34]. A few recent publications have highlighted the capacity of Sofosbuvir to inhibit ZIKV replication and infection [35, 36].

1.2.2.3 Congenital complications associated with Zika Virus infection

In 2015, the association between ZIKV and congenital malformations had not yet been suggested and the full extent of complications that ZIKV could trigger or cause in newborns had not even been hypothesised. The initial inquiries about the relationship between ZIKV and congenital malformations were first remarked upon by the obstetrician Dr Adriana Melo from Campina Grande (Paraíba, Brazil) who in August 2015 started to raise the alarm and share her concerns with other Brazilian specialists and public health experts [37].

Figure 1.4 Map showing Paraiba and Rio de Janeiro (Brazil)
The association between ZIKV and congenital microcephaly was established for the first time in Paraiba. First arrow shows Paraiba, Second arrow shows Rio de Janeiro. Map adapted from: www.viagemdeferias.com/Brasil.

Her discovery came after a few months in which a large number of people in the region suffered from exanthematous disease with a very specific rash that did not fit with the traditional dengue or chikungunya clinical presentation. Within less than two weeks she admitted into her consultation (in Paraiba, one of the poorest regions of the country) two pregnant woman. When she realised that they both were carrying foetuses suffering with very severe anomalies that she had previously not observed, Dr Melo recognised that there was something previously undocumented with significant clinical implications beginning in Brazil. The ultrasounds of these two patients revealed that the foetuses were severely compromised with a smaller-than-average head and severe brain malformations [37]. Laboratory testing of amniotic fluid from the two pregnant women showed that they were both positive for ZIKV RNA. This emphasised the very likely causal relationship between ZIKV and congenital anomalies [20].

As of the 4th of January 2018, a total of 27 countries and regions within the Americas have reported confirmed cases of congenital disease associated with Zika [38] . There have been over 3,700 confirmed congenital cases that follow the PAHO/WHO definition: *"a live newborn who meets the criteria for a suspected case of congenital syndrome associated with*

Zika virus and Zika virus infection was detected in specimens of the newborn, regardless of detection of other pathogens" [39]. The case definition for congenital syndrome associated with Zika virus infection is available here: PAHO [40]. Moreover, it is probable that this number may be an underestimation due to difficulties in the detection of ZIKV RNA or antibodies in the specimens of the newborns and due to lack of access to public health services in some regions of Latin America.

Following the isolation of ZIKV from brain tissue and cerebrospinal fluid (CSF) of neonates born with severe neurological sequelae and after identifying ZIKV in placental tissue of mothers who suffered from Zika infection during their pregnancy, the association between flavivirus infection and congenital birth defects was considered strong [41]. A review of the existing evidence using established scientific criteria and conducted by experts from the Centre for Disease Control and Prevention (CDC) concluded in April 2016 that Zika virus is a cause of microcephaly and other severe brain defects [42]. The authors highlighted that microcephaly was just "the tip of the iceberg of what we could see in damaging effects on the brain and other developmental problems" associated with ZIKV.

Research has demonstrated that, during pregnancy, ZIKV is able to cross the placenta which can lead to severe pregnancy outcomes including placental insufficiency, foetal growth restriction, foetal demise and central nervous system (CNS) complications [43]. It has been estimated that around 30% of the infants infected with ZIKV during pregnancy will develop congenital abnormalities that can lead to serious sequelae such as loss of brain parenchyma, ventriculomegaly, microcephaly or blindness [44]. The full spectrum of sequelae associated with Zika during pregnancy is still being investigated, but includes manifestations such as craniofacial disproportion, spasticity, seizures, irritability and brainstem dysfunction such as feeding difficulties, ocular disruptions or calcifications [45]. This full clinical presentation is now defined as the Congenital Zika Syndrome (CZS).

Reviewing the available evidence, it has recently been proposed that Zika virus should be considered an emerging TORCH agent that can seriously threaten public health. The TORCH refers to infection of a developing foetus or newborn by any of the following group of infectious agents: **T**oxoplasmosis, **O**ther agents, **R**ubella virus, **C**ytomegalovirus and **H**erpes simplex virus (HSV). All of these listed pathogenic microorganisms may cause a mild illness in the infected mother then, infect the foetus through vertical transmission triggering the development of several anomalies. As ZIKV infection has demonstrated the above-

mentioned characteristics, similar guidelines as to the ones used for TORCH cases should be followed [46].

However, the extent of the impact of Zika virus infection during pregnancy is still being unravelled and several multi-centric cohorts monitoring the progress of children suspected to suffer CZS are currently being undertaken in order to try understanding the incidence, burden and range of abnormalities associated with ZIKV during gestation [47]. Accurate ZIKV diagnostics, promptly performed, in ZIKV-suspected pregnant women are key to guiding appropriate clinical management of cases and providing informed advice regarding the potential legal termination of pregnancy.

1.2.2.4 ZIKV causes much more than congenital disease: Neurologic complications

Neurological complications triggered by infections represent an enormous challenge to physicians due to the potential high morbidity and mortality as well as the difficulties involved in their treatment. Infections of the central nervous system (CNS) and Peripheral Nervous System (PNS) are rare for most pathogens. The mechanical barriers of the host (such as the blood-brain barrier) as well as the immune response are in most cases effective in preventing infection of the nervous system [48]. Nevertheless, a small number of arboviruses can have CNS involvement such as CHIKV, WNV, DENV and, the recently demonstrated Zika Virus [49].

Zika virus infection was associated with neurological complications for the first time during the outbreak in French Polynesia when it was observed a marked 20-fold increase in the number of Guillain-Barré syndrome (GBS) cases [50]. Further evidence followed in 2016 with reports from Colombia where ZIKV RNA was detected in 17 GBS patients and several studies have found an increased incidence of GBS during the ZIKV outbreak across Latin America [51, 52].

In addition to the marked increase in GBS cases other rare presentations in adults such as acute myelitis, meningoencephalitis and facial paralysis increased during the outbreaks [53]. Furthermore, several case reports have associated ZIKV with myelitis, encephalitis, meningoencephalitis, acute disseminated encephalomyelitis, Miller-Fisher syndrome and myasthenia gravis. All of this evidence suggests that the full spectrum of neurological complications found in children and adult (non-congenital related) may be much wider than what was initially hypothesised.

To the best of our knowledge, only a few ZIKV cases with neurological involvement have been described among children or adolescents so far. One suspected case of neuropsychiatric and cognitive symptoms in a 16-year-old who travelled to South America and one case of hemiparesis and myelitis [54, 55].

It is still unknown what the long-term effect of ZIKV-associated neurological infections will be. However, it has now been demonstrated that long-term neurological complications are also frequent with other acquired arboviral infections, with sequelae manifesting in the survivors during the years following primary infection [56].

Recently, Lebov and Brown *(*2018) concluded that the high level of neurotropism of ZIKV to CNS and PNS cells demonstrates the possibility that undetected damage may occur in neural cells following ZIKV infection and potentially causes subtle neurological damage [47]. The authors hypothesised that this may occur in a percentage of individuals following ZIKV infection and it may be unnoticed by physicians and public health experts. This would mean that it may take years to understand the neurological burden of ZIKV infections. Further investigations are needed to understand the implications of pre-existing dengue infections, together with recent Zika infections, in the pathogenesis of GBS. GBS is an acute monophasic paralyzing illness which is usually provoked by a preceding infection.

Accurate diagnosis among individuals suffering from acute neurologic disease is crucial as it has an effect on the clinical management of the patient and can direct which treatment is the most appropriate.

During the ZIKV epidemic, multiple arboviruses were co-circulating and a specific viral diagnosis was key to anticipating, preventing and managing subsequent complications. For instance, dengue positive patients require aspirin whilst those with CHIKV infection need to be monitored for the treatment of acute arthralgia and long-term arthritis [17], thus highlighting the breadth of possible responses and the need for accurate diagnosis. When multiple arboviruses are co-circulating and when the specific aetiological agent is not identified it can lead to longer hospital stays as well as unnecessary use of certain treatments such as antibiotics.

In chapter 4, I will give further details regarding the challenges of ZIKV diagnosis for neurological patients as well as the wide range of neurological complications that are associated with Zika virus.

1.3 Emergent zoonotic arboviruses

Over the last decade, awareness regarding the need to increase the preparedness to fight emergent zoonosis has improved. The WHO defined emergent zoonosis as "*a zoonosis that is newly recognized or newly evolved or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range*" such as Ebola, chikungunya, dengue or ZIKV [57].

Emergent zoonosis generally occurs due to a combination of factors including environmental changes, modifications in human or animal demography and changes in the pathogens themselves. This subsequently can contribute to the spread of pathogens or vectors that were not found in that region before. Moreover, there are roles for local traditions regarding food and religious customs play in the emergence of these pathogens which need to be considered [58].

Over the last few years, the emergence and spread of zoonotic viruses to new regions have become much more rapid due to several factors. Gould *et al.* (2017) exemplified this as the quick adaptation of arthropod vectors to live in urbanised habitats, the increase in international travel and failing to contain the spread and density of vectors [59].

Flaviviruses are arboviruses (arthropod-borne viruses) and over the last few decades, they have become a concerning global health threat. Arboviruses are transmitted mainly via mosquitoes but flies, midges and ticks can also transmit some arboviruses [60].

In addition to the flaviviruses, the most clinically relevant emergent arboviruses are the Alphavirus such as chikungunya or Mayaro virus [61], the Bunyaviridae such as La Crosse Virus or California Encephalitis Virus and the Reoviridae such as Colorado tick fever [62]. Some of these viruses cause predominantly acute CNS disease whereas some others just cause a fever-like disease.

Among those, chikungunya virus (CHIKV) has played a relevant role during the ZIKV outbreak. This is because Brazil and many countries in South America were experiencing simultaneous outbreaks of ZIKV and CHIKV during 2015 and 2016. Chikungunya (named from the Swahili word – which roughly translates as "disease that bends up the joints") was identified for the first time in Tanzania in 1952 and in the recent years it has spread to cause large epidemics around the tropics [63]. In 2013, it was reported for the first time in Latin America quickly spreading to cause large epidemics across the continent and affected Brazil in particular. Chikungunya virus, like ZIKV, is also transmitted by the Aedes mosquitoes and has a very

similar clinical presentation with severe joint pains that can persist for months. Moreover, it occasionally causes neurological complications in children and adults such as myelitis, encephalitis and GBS [49]. Despite CHIKV co-circulating in many Zika-affected areas (including Colombia and Brazil) and both viruses being shown to cause neurologic disease, their respective roles in potential co-infections or concomitant infections has not yet been understood or systematically assessed.

During my PhD, I have addressed this challenge which will be further described in Chapter 4. Moreover, I have also worked on understanding the relationship between the incidence of Zika and chikungunya viruses in the population of Rio de Janeiro in Chapter 5.

1.3.1 Importance of emergent flaviviruses

Flaviviruses belong to the *Flaviviridae* family which have a total of 70 different antigenically related species. They are enveloped virus with icosahedral and spherical geometries, a diameter of around 50 µm and are positive-sense, single-stranded RNA viruses. The RNA genome of flaviviruses is approximately 11 kb. It encodes a single large polyprotein which is proteolytically processed to yield three structural proteins: the envelope protein (E), capsid protein (C) and membrane precursor protein (prM). It also yields seven non-structural components (NS) proteins which are called: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. NS1 is a highly conserved region as all flaviviruses' NS1 genes share a high degree of homology. NS1 genes are typically 1056 nucleotides in length, encoding 352-amino-acid polypeptides [64] [65].

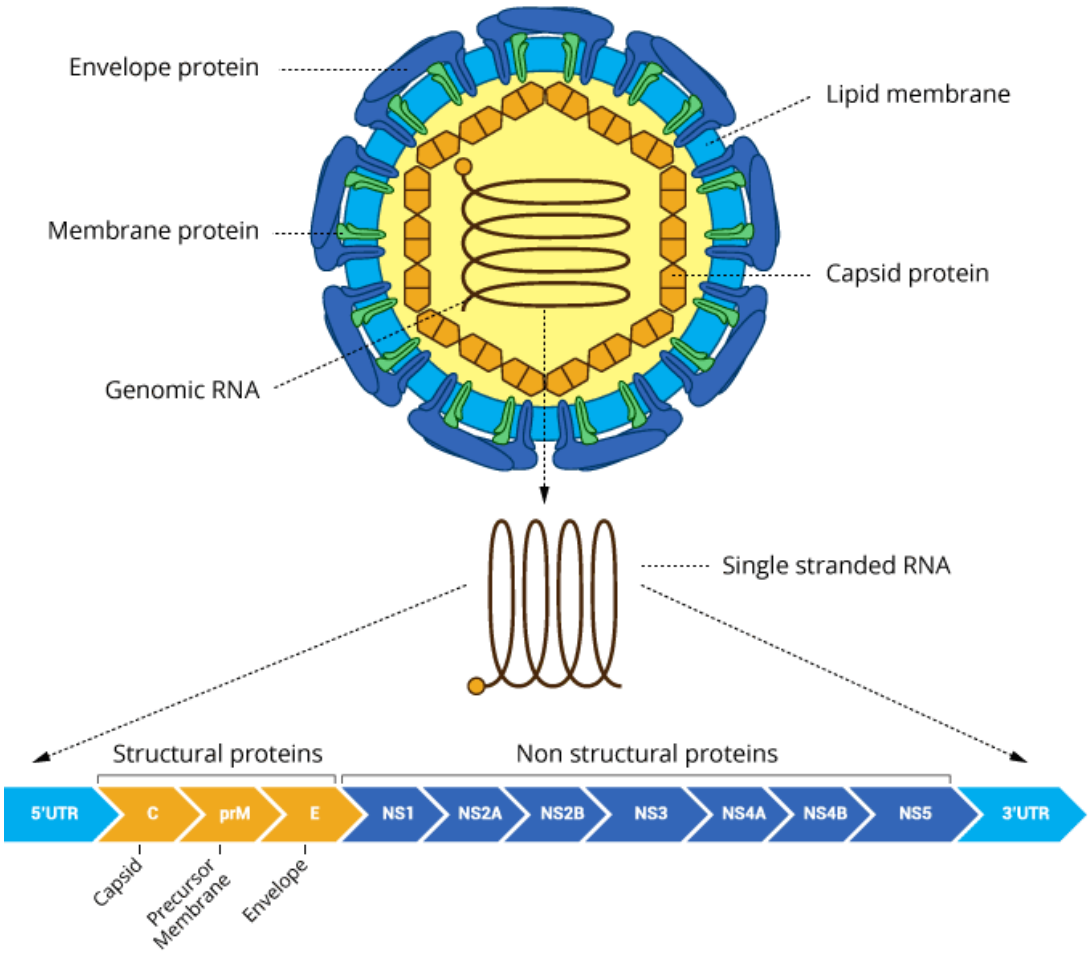


Figure 1.5 Schematic diagram of the structure of Flavivirus species.
Flaviviruses are around 50 µm, contain a single-stranded genomic RNA which encodes three structural proteins (E, PrM and C) and 7 Non-structural. (Adapted from https://neuronewsnight.com/flavivirus1)

Most of the flaviviruses are transmitted via mosquitoes or ticks and are classified as arboviruses. During the last few decades, a number of flaviviruses have emerged in specific regions around the globe. In particular these include: Japanese encephalitis virus (JEV) in South East Asia and Pacific islands; St Louis encephalitis virus (SLEV) in the United States and occasionally in Canada and Mexico; Murray Valley encephalitis virus (MVEV) which is endemic in northern Australia and Papua New Guinea; Usutu virus (UV) in Africa and Spondweni virus (SPOV). SPOV is a flavivirus which is genetically closely related to ZIKV and therefore there exists a high risk of misdiagnosis, though it has only been detected in Sub-Saharan Africa [59, 66].

The most clinically relevant flaviviruses that have emerged in multiple regions around the world and both in the Northern and Southern hemispheres during the last few decades include West Nile virus (WNV), yellow fever virus (YF) and dengue virus (DENV). These three flaviviruses are of relevance in Brazil due to their emergence over the last few years and their potential cross-reaction antibody responses with ZIKV. WNV has been detected in horses in various regions of Brazil and recently reports of fatal human cases have raised the concerns of international public health institutions [67-69].

1.3.2 Importance of dengue in Brazil

Dengue Virus (DENV) is a zoonotic infection that like ZIKV and CHIKV is mainly transmitted via Aedes mosquitoes. DENV is the most prevalent emerging arbovirus with a growing incidence estimated in around 390 million infections per year of which around 96 million have clinical manifestations. Nevertheless, the exact number of cases worldwide is probably underreported [70]. Dengue has spread rapidly across most regions of the world except for continental Europe.

The first DENV outbreak in the American continent was recorded in 1635 in the French Antilles and it is believed that the virus has an African origin. DENV does not appear to have established a sylvatic existence in the forests as dengue seem to circulate predominantly amongst humans and Aedes species of mosquitoes [71]. Dengue and Zika viruses have similar epidemiology and transmission cycle in urban environments.

DENV has five different serotypes (DENV1-5), although DENV1-4 have spread further worldwide and have circulated across Brazil for the last three decades. The four serotypes differ by approximately 30 to 35% in their amino acid sequence and envelope protein, while the difference between DENV and ZIKV is around 41 to 46% [72]. Approximately, around 50% of dengue infections are symptomatic and the clinical presentation can be similar to ZIKV with a rash-fever-arthralgia syndrome. Dengue can also cause both peripheral and CNS disease [73]. In addition, a proportion of patients suffer from severe haemorrhagic DENV fever which can lead to death. Secondary DENV infections are associated with a more severe clinical form of the disease [74]. Whether previous DENV infection may play a role in severity of ZIKV complications has not been fully elucidated yet and I will further analyse this in Chapter 3 and 4. Also, the question of whether previous dengue exposure leads to antibody cross-reactivity when conducting Zika testing had not been addressed prior to the beginning of my PhD and this will be discussed in Chapters 3 and 4.

1.3.3 Importance of Yellow fever in Brazil

Yellow fever virus (YFV) causes generally a short febrile illness, although, in around 15% of infected cases, it can cause a haemorrhagic disease that can lead to liver failure, jaundice, bleeding and loss of life. Yellow fever (YF) is endemic in tropical regions of Africa and South America [75]. Even though large urban outbreaks of yellow fever have occurred several times throughout history, currently, most yellow fever cases result from human exposure to jungles or forests [75].

The first epidemic of Yellow fever in Brazil was reported in 1685 in Recife. YFV transmission is also via mosquitoes. In Brazil, it is predominantly maintained in tropical forests in a sylvatic cycle and generally involves non-human primates [76]. During 2017 -2018, Brazil experienced the largest outbreak of yellow fever in decades. Almost 500 cases were reported with over 154 related deaths. As a response, massive vaccination campaigns were taken forward. The current vaccine is considered the single most important measure for preventing yellow fever because it offers lifelong protection with a single dose [77]. Unfortunately, fatal side effects have been reported to be associated with the YF vaccine in a small proportion of individuals [78]. The recent outbreaks have shown that there is sustained virus circulation in the country which is now expanding to areas that previously were not considered at risk such as Rio de Janeiro and Sao Paolo, the largest metropolis in the country with a population of over 30 million people between both cities [79]. The role that this vaccination programme which resulted in a highly flavivirus immunised population plays in the pathogenesis of ZIKV and cross-reactivity of diagnostic assays is not yet fully understood.

1.4 Laboratory diagnosis of flavivirus infections

As clinical diagnosis of the different flavivirus is not specific due to a very broad clinical picture in most patients, laboratory diagnosis of flaviviral infections is essential in order to reliably confirm the aetiology of the illness. Moreover, laboratory diagnosis of flavivirus is crucial as it has a direct influence towards the clinical management of the patients [80].

In the last few years, significant international advances have sought to develop and improve diagnosis of flavivirus infections. Antibody testing, molecular technologies such as PCR and RT-PCR and virus isolation are some of the techniques used for the diagnosis of flavivirus infections. However, other diagnostic tests are often employed such as antigen-capture procedures, or immune fluorescent techniques. In order to understand the reason behind

why different diagnostic tests are used depending on the moment the patient presents to the health centre, it is necessary to understand the kinetics of flavivirus infections.

1.4.1 Kinetics of flavivirus replication and host response

Humoral antibodies are generated early on following flavivirus infection and constitute the main basis of antibody-mediated immunity. This immunity is considered life-long. For instance, it has never been recorded that an individual has suffered yellow fever twice [81]. Infection with any of the four serotypes of dengue virus induces life-long protective immunity but only to that serotype, and therefore, it does not confer long-term protection against potential further infections by the other dengue serotypes [82]. Flaviviral infections are generally characterised by having a short-viremia following acute presentation. In most cases, viremia associated with flavivirus infection peaks and disappears within the first week.

The immune response to dengue infection is the most understood immune response among the flavivirus group. Therefore, I will summarise it here, as a general representation of flaviviral immune responses. As shown for dengue virus infection in the diagram in Figure 1.6 when a dengue-infected individual begins experiencing symptoms there are elevated levels of virus in the bloodstream. The virus can be found in serum, plasma, circulating blood cells and in certain tissues. Viremia may last between 2 to 7 days (or even more in CNS disease patients) roughly corresponding with the period of fever [83]. The acquired immune response consists of the production of Immunoglobulin M (IgM) and Immunoglobulin G (IgG) antibodies which are predominantly directed against the virus envelope proteins [84]. Anti-dengue antibodies can be detected up to several days following the onset of acute illness. IgM antibodies may remain elevated for 2 to 3 months while IgG antibodies generally persist for decades. The soluble non-structural protein 1 (NS1) is released into the bloodstream by the virus and can be detected from the onset of disease during the first few days. Interestingly, detecting high NS1 levels during the first 72h from symptom onset is a strong prognostic biomarker of the severity of dengue disease [85].

Understanding the dynamics of the humoral response is fundamental. Sometimes two samples (at acute-phase and convalescent-phase) are needed in order to diagnose flaviviral infections. For DENV, among specimens tested at day 6 from symptom onset, both viral RNA and anti-viral IgM antibodies may be undetectable [86]. In dengue, there are two patterns of immune response clearly distinguishable depending on whether the individual has a primary (first dengue or other flavivirus infection) or a secondary (anamnestic) dengue infection. The diagram in Figure 1.6 shows the classical primary immune response to DENV.

Secondary dengue infections are characterised by higher levels of IgG antibodies with lower levels of IgM dengue antibodies. Secondary dengue infections account for most cases of Dengue Haemorrhagic Fever (DHF) [83]. DHF is a severe disease characterised by acute fever, haemorrhagic manifestations and a tendency to develop shock syndrome [87]. Following a DENV infection, most dengue antibodies are weakly neutralizing and able to bind to multiple Dengue virus serotypes (not only the specific serotype that caused the infection). It has been suggested that the increased risk of developing DHF following a secondary dengue infection is due to a phenomenon called antibody dependent enhancement (ADE). This phenomenon postulates that weakly neutralizing antibodies from the first dengue virus infection bind to the secondary dengue virus and enhance infection leading to severe disease in a proportion of cases [88].

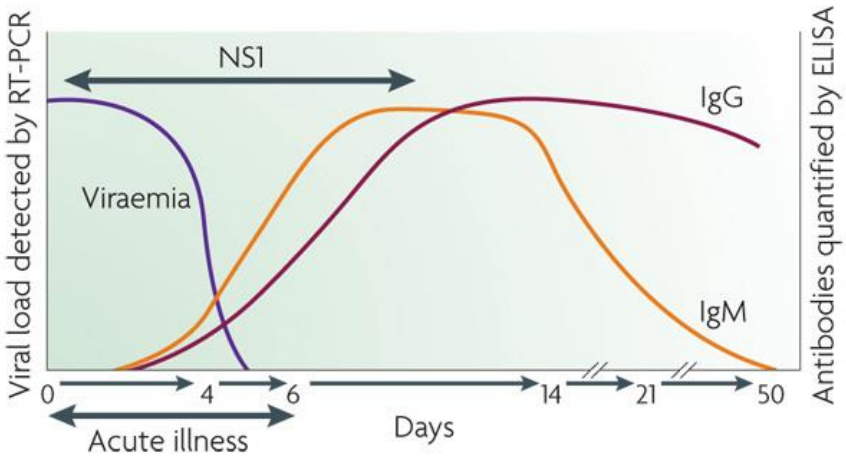


Figure 1.6 Diagram showing the dengue virus, antigen and a primary immune response to dengue virus infection

Understanding pathogen-specific antibody responses are crucial for choosing the appropriate diagnostic technique to be used. Acute symptoms of dengue are experienced when high levels of virus in the bloodstream are reached. The virus triggers a rapid response that is generally developed between 3 to 7 days post symptom onset. The subject's B cells start generating IgM and IgG antibodies which are being released into the bloodstream and lymph fluid. IgM antibodies generally decrease a few weeks following symptom onset. NS1: Non-structural protein 1.

Most studied flavivirus follow a similar antibody pattern as the described above. Nevertheless, some exceptions include West Nile Virus (WNV) kinetics which has a period of viremia that declines prior to the onset of disease symptoms (that is generally CNS disease). Therefore, molecular detection methods are not the most successful techniques for the diagnosis of this flavivirus [89]. Determining the exact aetiology of a flaviviral infection is extremely relevant clinically and epidemiologically. Nevertheless, this is generally very challenging due to the broad serological cross-reactivity between viruses and the

phenomenon known as the original antigenic sin, which are anamnestic responses of the antibodies to previous infections with related viruses.

1.4.2 Frequent methods for laboratory diagnosis of flavivirus

Specific distinction of the flavivirus agent causing an infection can be challenging due to cross-reactivity responses. Over the last few years, diagnosis of flavivirus infection has advanced and improved drastically both in the field of nucleic acid-based diagnosis and in the development of serological assays [90]. Moreover, the most widely used algorithms are based on conducting a battery of assays to detect related viruses for differentiation (WHO Laboratory). The most relevant flaviviral methods can be classified as serological, molecular or virus isolation.

1.4.2.1 Virus culture assays for diagnosis of flavivirus

Virus isolation has been the traditional diagnostic technique for detecting many flaviviruses such as DENV. For virus isolation, a specimen is collected from a suspected patient and is then cultured with a cell line of either mosquito or mammalian origin. The virus can also be isolated in live mosquitoes. Even though virus isolation is widely used and a positive isolation is considered a definitive positive, this method is not practical for diagnostic purposes as it may take between days to weeks to yield a result [91]. Moreover, identifying the human specimen or tissue for performing the viral isolation can be quite challenging for many flaviviruses including Japanese encephalitis or Tick-borne encephalitis [88] [92].

1.4.2.2 Molecular assays for diagnosis of flavivirus

Specific aetiological diagnosis of arboviral infections has increased greatly since the advent of molecular methods such as the Polymerase Chain Reaction (PCR) and Real-Time Polymerase Chain Reaction. These methods have indeed become routine laboratory diagnostic procedures for acute patients suspected of flaviviral infection as they are quicker than traditional viral cultures [93]. Molecular techniques are highly sensitive and results may be ready in just a few hours. PCR is widely used for diagnosis of other disease pathogens and is widely available in most diagnostic laboratories worldwide.

While PCR is considered a highly specific technique, it is dependent on ensuring that the primer sequences that are being used are specific and not detecting and amplifying other related viruses. Moreover, PCR needs to be performed in a well-prepared area thus preventing assay contamination. This requires specially designed areas for carrying out these procedures [80]. Moreover, sensitivity could be reduced due to inhibitor factors in the patients' sample including high levels of proteins or leukocytes. The exact time of collection

of the samples used for the PCR testing is also crucial. PCR testing not performed in the first 1 to 7 days in the course of the disease (for example, a traveller returning from flavivirus exposed area) may generate false negative results. With most flaviviruses, samples need to be tested with molecular techniques in the very first days following acute onset of symptoms, otherwise the amount of virus present may be too low or may be "cleared" from the body fluids tested resulting in false negative results [94].

Additionally, the applicability of molecular techniques also varies depending on the flavivirus and the long-term viremia. DENV is characterised for high viremias that last a few days, while other flaviviruses produce barely detectable viremias in humans [95, 96].

Multiplex PCRs are techniques that amplify multiple targets in a single PCR assay by using multiple primer pairs in the same reaction mixture. Recently, this technique has become very common for flavivirus diagnosis with detection of up to the 4 serotypes of DENV, or up to 8 medically relevant flaviviruses [97] [98].

1.4.2.3 Serological assays for diagnosis of flavivirus

There are several different types of serological assays available for diagnosis of flavivirus including: IgM and IgG antibody –capture enzyme-linked immunoabsorbent assay (ELISA), hemagglutination inhibition (HI) assays, indirect immunofluorescent antibody tests (IIA), complement fixation assays, dot-blot assays, Western blotting or plaque reduction neutralization tests (PRNTs). These assays are based on antibody detection and regarding flavivirus diagnosis they generally present a window of detection that starts after day 4 post symptoms onset, as the relevant antibody response is elicited generally a few days after onset of symptoms [99].

Detection of flavivirus infections by serology is very challenging in areas of the world where more than one flavivirus are circulating (such as yellow fever, dengue, Japanese encephalitis and now Zika virus) due to the shared cross-reactive epitopes of the flavivirus and the hence, cross-reactivity of the antibody response. These cross-reactive responses may lead to false positive results. Further details on this will be discussed in Chapter 3.

Traditional approaches, which measure antibodies on the surface of the virus, generally target the premembrane (prM) and/or envelope (E) proteins. Moreover, recently methods targeting the non-structural protein 1 (NS1) have also been developed. These methods include plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI) tests, indirect immunofluorescence assays (IFAs), and IgM and IgG antibody-capture enzyme-

linked immunosorbent assays ELISAs [100]. High-throughput IgM and IgG capture ELISAs have become routine in most flavivirus laboratories and with particular those that follow assay automation.

1.5 Laboratory diagnosis of Zika Virus

The ZIKV Diagnostic panorama has shifted dramatically through the last two years of my PhD studies. This has been a rapidly developing area in which the emergency of this epidemic prompted major tackling of important public health research needs. Throughout my PhD, I tried to address some of these needs with a particular focus on improving the diagnosis of Zika Virus in Brazil.

Laboratory diagnosis and surveillance of Zika virus in Brazil and South America has been very challenging due to several reasons. Below, I summarize the ones that I believe are the most relevant:

- No commercial diagnostic assay for ZIKV diagnostics was available at the beginning of the outbreak
- In-house assays for ZIKV diagnostics had been not optimised or evaluated in South American populations before this outbreak
- Due to the emergency of this epidemic, many assays were approved for use or commercialization and they were implemented across the Americas without prior appropriate systematic evaluation
- Some Zika virus assays were evaluated but they were tested on travellers, who had very different seroexposure compared to the local population
- In Brazil, there potentially exists extensive antibody cross-reactivity with endemic flaviviral diseases such as dengue.

Before 2016 and the emergence of Zika as a public health threat, there were no commercially available diagnostic assays for detection of ZIKV RNA or ZIKV antibodies. Similarly in South America, before this outbreak, Zika virus testing was not available for routine diagnosis and reagents to perform *in-house* molecular testing of ZIKV were only available in a few laboratories across the continent. In April 2015, an increased number of patients with a rash-fever clinical picture was seen in ambulatory clinics and healthcare centres in various regions of the Northeast of Brazil. It was then when Dr Antonio Carlos Bandeira from Santa Helena hospital in Bahia (Northeast of Brazil) got in touch with the virologists Dr Gubio Soares Santos

and Dr Silvia Inês Sardi in the Instituto de Ciências da Saúde da Universidade Federal da Bahia (UFBA) [37]. At the end of April 2015, after conducting PCR testing in tens of serum samples, they reported that the virus that was causing a rash fever outbreak across the State of Bahia was a virus that "had only been detected a few times in history": the Zika Virus [18]. This was the first laboratory-confirmed ZIKV case in Brazil and Dr Silva and Dr Gubio would have never imagined the impact of their discovery. The virus which they had found after running just "one simple PCR assay", was going to change the life of thousands of people from the whole continent [37].

After their announcement and following the rapid spread of the virus firstly across Brazil and lately across several countries in South America, ZIKV testing became gradually available in most diagnostic laboratories all over the globe. Following the declaration from the WHO of the Zika virus epidemic as a PHEIC in 2016, assay manufacturers and research centres across the world started to work intensively on ZIKV. In particular, there have been important efforts trying to develop, evaluate and distribute novel assays that could detect ZIKV RNA antibodies or antigens.

1.5.1 Types of diagnostic assays for ZIKV:

Typically, there are three types of diagnostic techniques employed for ZIKV detection: 1) detection of the viral particle or its components via PCR or RT-PCR; 2) immunoassays and 3) virus isolation. These techniques detect either ZIKV RNA, viral proteins (such as NS1) or antibodies or the live virus respectively. These methods are used both with clinical specimens and for detection of ZIKV in mosquitoes. The key advantages and disadvantages of each of those methods are summarised in Table 1.1.

Table 1.1 Characteristics of some frequently used diagnostic assays for Zika Virus

Diagnostic Method	Detected ZIKV Agent	Application	Advantages	Disadvantages
Serological Methods				
IgM antibody capture enzyme-labelled immunoabsorbent assay (ELISA)	IgM	Identification of acute or recent infection	Quick and easy to perform	Brief window of detection Different test results between primary and secondary infections
IgG antibody capture enzyme-labelled immunoabsorbent assay (ELISA)	IgG	Identification of a long-term acquired ZIKV infection or a recent infection	Quick and easy to perform	Low specificity in flavivirus exposed populations
Indirect fluorescent antibody Assays	IgM and IgG	Detection of virus IgM and IgG antibodies	Easy to perform	Low accuracy
Neutralization Assays (PRNT, VNT)	IgM and IgG	Differentiating between flaviviral infections	Highly specific	Time-consuming, labour intensive. Requires live virus cultures
Molecular methods				
Real-Time – Polymerase Chain Reaction (RT-PCR)	RNA	Detection of ZIKV viremia	Quick, sensitive	Short window of detection in ZIKV patients
Triplex RT-PCR (DENV, CHIKV, ZIKV)	RNA	Detection of RNA of ZIKV, CHIKV and DENV	Quick, multiple diagnosis in one	Short window of detection in ZIKV patients
Viral Culture				
ZIKV viral culture	Zika Virus (Live particle)	Detection of infective viral particles	Highly specific	Challenging if samples have low viremia or following freeze, thaw cycles

In March 2016, the WHO published some interim guidelines for laboratory diagnostic testing of ZIKV [101] recommending the following strategy:

- **Molecular** diagnosis of ZIKV in patients presenting with less than seven days from onset of symptoms
- **Serological** diagnosis and/or molecular detection for patients presenting seven or more days following symptom onset.

The WHO recommended that a negative result via Nucleic Acid Testing (NAT) assay (molecular testing) on samples collected seven or more days after symptom onset should be interpreted with caution as viremia drops after 7 days and it may not be detectable. The recommended NAT method to detect the presence of ZIKV RNA was RT-PCR and the suggested primers sequences are described by Charrel *et al. 2016* [102]. The serological testing recommended by the WHO included enzyme immunoassays (EIAs) and immunofluorescence assays (IFA) detecting Anti-ZIKV antibodies as well as neutralization assays such as plaque-reduction neutralization tests (PRNTs). However, the WHO highlighted the challenges regarding interpretation of ZIKV diagnostics when other flaviviruses may also be present.

The particular context of this ZIKV epidemic needs to be highlighted. It affected mainly areas with an extensive circulation of dengue and chikungunya viruses. Consequently, correctly identifying the infecting agent became essential to provide the appropriate clinical management to the suspected-ZIKV patients. Another challenge was to determine the window of detection of ZIKV RNA and ZIKV antibodies.

1.5.1.1 Virus culture and molecular techniques for ZIKV diagnosis

As mentioned above, during the early stages of infection, ZIKV replicates within infected cells and viremia develops. Current RNA detection of ZIKV is performed via RT-PCR (real-time – Polymerase Chain Reaction). There are different targets used and one of the most popular is the NS5 protein region. Overall RT-PCR (both quantitative and semi-quantitative) are specific, sensitive and rapid methods for diagnosis of acute infections during the viremic phase, which is generally the first few days after symptom onset.
The window of positivity for ZIKV PCR as well as the best sample type to use for ZIKV diagnosis has been discussed in the literature [103]. One of the challenges of the use of PCR for ZIKV diagnosis is the low viremic levels in body fluids. Other difficulties are the high degradability of ZIKV RNA as well as the challenges of post PCR amplification and sensitivity to freeze-throw cycles.

Viral isolation is considered a highly reliable diagnosis with very high specificity but very labour-intense and time-consuming, and therefore, tedious and inefficient. Viral isolations are performed by inoculation of the patient's sample to some cell culture and allowing them to grow [104].

1.5.1.2 Serological diagnosis of ZIKV

Serological assays for ZIKV detection are based on techniques that measure antibodies in serum or body fluids. They can also identify antigens in blood, tissue or secretions using immunochemical techniques. The principle of these assays relies on the detection of anti-Zika IgM and IgG antibodies [105]. The main serological techniques developed for ZIKV diagnostics include Enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays (IFAs) and neutralizing assays.

1.5.1.2.1 Enzyme-linked immunosorbent assays (ELISAs)

Enzyme-linked immunosorbent assays (ELISAs) are one example of serological assays. An ELISA assay is a plate-based assay methodology that is designed for detecting and quantifying (through a colorimetric reaction produced via the conjugate enzyme reacting with the substrate) antibodies and proteins such as the Anti-ZIKV antibodies. They are often called enzyme immunoassays (EIA). With this technique, it is crucial that there is a highly specific antibody-antigen interaction [106]. The main types of ELISA for ZIKV diagnosis are direct and indirect ELISAs as shown in Figure 1.7.

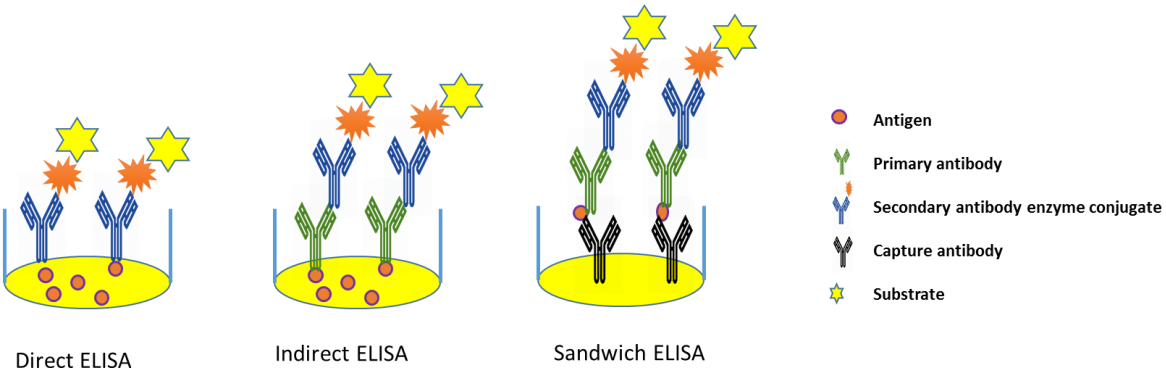


Figure 1.7 Diagram of common ZIKV ELISA formats: indirect, direct and sandwich ELISA assays

During the ELISA technique, the antibody detection is generally accomplished by assessing the magnitude of the response of the conjugated enzyme activity reacting with the substrate and producing a colorimetric measurable product. As the reactants of the ELISA are immobilized to the microplate wells' surface, it is possible to separate the specifically-bound

and not bound materials during the assay. Several washing steps are conducted during ELISA assays in order to wash away the non-specifically bound antibodies and thus allowing only for specific detections. The colorimetric reaction only occurs if the specific antibody binding to the target pathogen has taken place [107].

One of the biggest challenges in the use of ELISAs for diagnosis of ZIKV is the fact that the duration of antibody persistence in body fluids has not been fully established neither the number of days following symptom onset for the antibodies to be produced. This is something that we will further explore in chapter 3. Moreover, these methods suffer several limitations including the intrinsic variations obtained when using different sample types, cross-reactivity and difficulty of interpretation when antibodies against more than one flavivirus are present in the same specimen

1.5.1.3 ZIKV neutralization assays

Neutralization assays are currently considered the 'gold standard' for the serological differentiation of flavivirus infections. Plaque Reduction Neutralization Tests (PRNT) are based on serial dilutions of the suspected patients' sera and incubation with known concentrations of the virus (ZIKV or another flavivirus) in cell culture medium and followed by an overlay that will allow the cells to survive [108]. After incubation with a variable amount of time (days to weeks), the cell culture is checked for plaque formation which reveals the amount of virus still present. In the presence of specific neutralizing antibodies from the serum, plaques do not develop. This can be quantified and compared to the number of plaques in the cells that have virus only which are one of the controls of the experiment [105]. Then the dilution at which a 50 or 90% of the plaques have been reduced in the patient sample experiments calculated. The WHO suggests comparing the PRNT titres between ZIKV and DENV to determine the specificity of the antibodies. ZIKV Plaque Reduction Neutralization Tests are the standard detection reference of neutralizing antibodies (which are a class of IgG antibodies that neutralize) for most flaviviruses. This assay is certainly quite challenging for routine diagnostics for technical reasons: the assay requires days to weeks to obtain the result, it needs live virus cultures, high amount of technical time and generally offers low reproducibility.

1.5.2 Evaluating laboratory assay performance for ZIKV diagnosis

When selecting a diagnostic assay, the WHO recommends following the ASSURED criteria. Assays should be "Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to end-users" [109]. These criteria were applied to try

identify the most appropriate serological assay for ZIKV detection in a resource-limited setting such as Brazil.

Evaluation of diagnostic assays is fundamental before its use in routine diagnostics and in research in order to ensure that it is appropriate to use that assay. The laboratory where these evaluations are performed needs to maintain high proficiency in their testing procedures. The WHO recommends that for the evaluation of assays, the test's optimal diagnostic accuracy needs to be investigated and determined in practice under real-life conditions with sample specimens from the local population. Then quality controls need to be carried out as well as proficiency testing. Moreover, evaluations should be performed following the STARD guidelines [110]. STARD stand for Standards for Reporting of Diagnostic Accuracy Studies. These were developed with the objective "to improve the completeness and transparency of reports of diagnostic accuracy studies".

1.6 Seroprevalence studies for Zika Virus

In 2016 when I began my research in Brazil, the seroprevalence of Zika Virus in the South American population was unknown. Given the high proportion of asymptomatic cases, population serology is the only way to understand the scale of exposure to this arbovirus in the population. Seroprevalence studies are important because they facilitate estimates of the proportion of the population who are immunologically naïve and thus at risk of future infection [111]. This has enormous implications for guiding public health measures, including vaccination strategies. Moreover, the estimates of the proportion of people affected give a perspective on the rates of complications of these arboviruses. Even though arboviruses are a major constituent of emerging infectious diseases in Brazil, limited data is available on the prevalence, distribution and risk factors for transmission within this geographical region.

In order to estimate the burden of ZIKV, some studies have used a range of empirical or extrapolative methods as well as disease-modelling approaches. However, the most accurate and reliable data for this empirical assessment of the population exposure to a virus such as ZIKV comes always from seroprevalence studies [112, 113].

1.6.1 Types of seroprevalence studies

There are different types of study designs that can be applied to conduct seroprevalence research. Studies can be designed involving a general population or a specific population subgroup. The WHO in their guide to design serosurveys for dengue virus suggests that the

most fundamental distinction between seroprevalence studies is either the determination of viral incidence or investigating viral prevalence [114]. Incidence measures the number of new cases of ZIKV in the population over a specific period of time. Prevalence measures the number of cases of Zika virus at a particular point in time. In addition to incidence and prevalence, the different epidemiological study designs differ primarily in the manner in which information is obtained from the population and period. Longitudinal studies (cohort studies) involve repeated observation of study participants over time. Finally, cross-sectional studies measure the presence or absence of antibodies to the arbovirus at a particular point in time.

As part of my thesis, I carried out two seroprevalence linked studies: a cross-sectional and longitudinal observational study which will be further described in Chapter 5.

1.7 The scope of this thesis

Since late 2015, large and geographically widespread epidemics of ZIKV have occurred around the world with Brazil the most affected country. Accurate diagnostic assays underpin the public health responses to this outbreak. However, a lack of specific assays, cross-reactive reactions and challenges in identifying sensitive assays has complicated diagnoses of Zika virus. Moreover, without appropriate diagnostic assays the extent of the burden of Zika virus infections in the general population, as well as in pregnant female and neurologic patients cannot be determined with reliability.

Therefore, this thesis focuses on the improvement of serological assays for Zika virus diagnosis. In particular, I aimed to tackle the following challenges:

- Are the commercial ZIKV serological assays accurate?
- Can we modify the cut-off of these assays to improve their performance in the Brazilian population?
- Can we develop or optimize novel methods for ZIKV antibody detection?

Having answered those questions, I then look into addressing the following:

- Can we use these diagnostic assays effectively to study at-risk populations?
- Is Zika virus associated with neurological disease?
- What is the prevalence of Zika Virus in the population of Rio de Janeiro?
- What has been the prevalence of Zika Virus following the recent epidemic waves?

CHAPTER 2.
Materials and Methods

Chapter 2: Materials and Methods

2.1 Ethics and governance

The procedures described in Chapter 3, and 4 were carried out in accordance with the ethical standards of the Instituto Nacional de Infectologia Evandro Chagas (Brazil). The study protocol was approved by its Comitê de Ética em Pesquisa (reference CAAE 0026.0.009.000–07). The procedures in Chapter 5 were approved by the Ethics Committee of Fundacao Oswaldo Cruz, Brazil (Fiocruz/IOC, number 2.476.733). All specimens were anonymised prior to testing. No identifiable data was shared, published or distributed.

2.2 Serological diagnostic assays for ZIKV diagnoses

Several different methods for serological detection of ZIKV antibodies were conducted including ELISA assays, immunofluorescence assays and a rapid Point of Care Test (POCT). The commercial assays used in this investigation claimed to have highly reproducible results during independent evaluations and were approved for use in Brazil by the Brazilian Ministry of Health.

2.2.1 Key general laboratory practice when conducting ELISA assays

I want to highlight that when conducting the evaluations of the ELISA assays good laboratory practice was ensured at all times. These key aspects taken into account:

- **Samples:** All the serum samples were allowed to thaw at 4 ^{o}C or at room temperature (RT) between 22 and 26 ^{o}C. Repeated cycles of freeze-thaw were avoided because they can affect the quality and integrity of the samples. I recognise that a key learning point from this PhD was that adequate organisation of samples; box and freezer maps were extremely useful both for ensuring reduction of biohazard risks but also for a more efficient practice of serological diagnostics. Another learning outcome of my months carrying out serologic assays was the importance of clear and unique sample labelling that can resist the freeze-thaw cycles.

- **Equipment**: The equipment underwent regular quality control checks. Only equipment that had successfully passed the recent quality control investigations was used.
- **Safety:** Moreover, adequate approved protocols, risk assessments, COSSH forms and use of adequate personal protective equipment were maintained throughout the whole of the laboratory investigations. Working in a safe environment when handling human samples was always ensured and potential biohazard risks were always minimised.
- **Quality of the assays:** Appropriate temperature conditions were ensured for all reagents. Moreover, potential risks of cross-contamination of samples were avoided. All the assays included quality control checks such as minimum and maximum absorbance levels of the positive, negative and calibrators provided. Assay plates were not considered valid if controls were out of the expected range. If the absorbance results from a plate assay did not meet the quality control criteria, the plate was repeated. All assays tested in this chapter met the absorbance criteria ranges.

2.2.2 The IgM and IgG ZIKV NS1 ELISA assays

The IgM and IgG Non-Structural Protein 1 (NS1) anti-Zika Virus (ZIKV) ELISAs from Euroimmun (Lubeck, Germany), use recombinant Zika NS1 as the ZIKV antigen. This is an indirect, colourimetric ELISA that detects IgM human antibodies generated against ZIKV infection. We performed all of the procedures following the manufacturer's instructions. Briefly: the manufacturer provided a 96 well plate which was pre-coated with recombinant ZIKV NS1. The pre-coated plate was incubated for 1 h at 37 ^{o}C in an incubator with 100 µl of diluted patient samples, calibrator, positive and negative control into each individual microplate well. Sera were diluted (10 µl of serum + 1000 µl buffer diluent to make a 1:101 dilution factor). Then, plate was washed 3 times with wash buffer (1x) using an automatic plate washer. Following this, 100 µl of enzyme conjugate (goat peroxidase-labelled anti-human IgM) was added and incubated for 30 min at room temperature (RT). Then, wells were washed again 3 times and a chromogen/substrate solution was added. Plates were incubated in the dark for 15 min at RT. This was followed by the addition of 100 µl/well of sulphuric acid stop solution. The OD of each well was then measured using a spectrophotometer at 450 nm with a 620 nm reference wavelength. The colour intensity is then related to the absence or presence of anti-ZIKV IgM or IgG antibodies in the patient sample. Prior to measuring, the microplate was shaken slightly as recommended in order to ensure

homogeneous distribution of the solution in the wells and the absorbance was read within 30 min. The procedures for the IgM and IgG NS1 ELISAs were identical, however, reagents were specific for each of the assay kits and the IgG assay used an enzyme conjugate which was peroxidase-labelled anti-human IgG. A diagram of the assay procedure is shown in Figure 2.1.

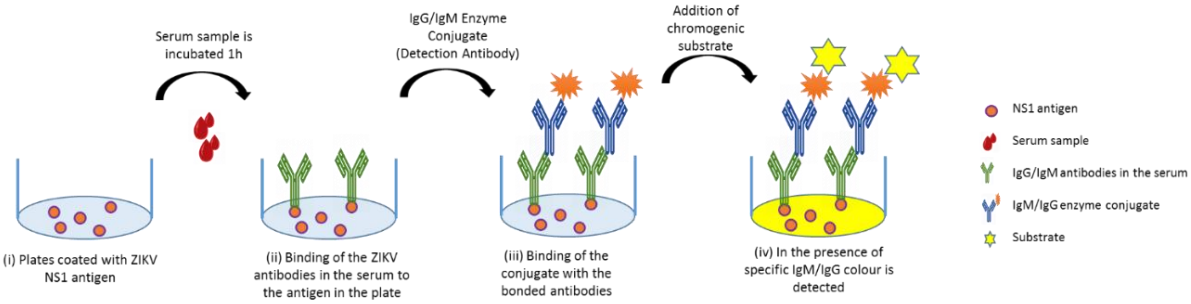


Figure 2.1 Principles and protocol for the IgM and IgG Anti-ZIKV NS1 ELISAs
Diagram showing the key steps in the commercial IgM and IgG ZIKV Euroimmun ELISAs

Both the IgM and IgG results are calculated semi-quantitatively and results are expressed as recommended by the manufacturer, by calculating the ratio of the Optical Density (OD) of the patient sample or control over the OD of the calibrator (as shown below).

$$\text{IgM and IgG NS1 ELISA Ratio } = \frac{OD\ of\ the\ patient}{OD\ of\ the\ calibrator}$$

A specimen was considered IgM positive if ratio ≥1.1; borderline ≥0.8 and <1.1; negative ≤0.8. Cut-offs for both assays were determined by the manufacturer.

2.2.3 IgM ZIKV µ-capture ELISA.

The Novagnost® Zika Virus IgM µ-capture ELISA (NovaTec Immunodiagnostica GmbH, Germany) which is based on the ELISA µ-capture technique. It uses ZIKV NS1 as antigen. Assays were conducted according to the manufacturer's protocol.

Briefly: samples were diluted with sample diluent buffer with 10 µl of serum + 1000 µl buffer diluent to make a 1:101 dilution factor. Then 100 µl of controls (1x negative, 2x cut-off, 1x positive) and 100 µl of diluted samples were pipetted to each of the wells of a pre-coated microplate and incubated for 1 h at 37 ^{o}C in an incubator. Then, the plate was washed 3 times with wash buffer (1x) using an automatic plate washer. Following this, 100 µl of conjugate was added and incubated for 30 min at RT. Then, wells were washed again 3 times and 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added. The plates were incubated in the dark for 15 min at RT. This was followed by the addition of 100 µl/well

of stop solution. As recommended, I ensured constant timing between dispensing substrate and adding stop solution. The OD of each well was then measured using a spectrophotometer at 450 nm with a 650 nm reference wavelength. Absorbance was measured within 30 min. An illustration of the assay procedure shown in Figure 2.2.

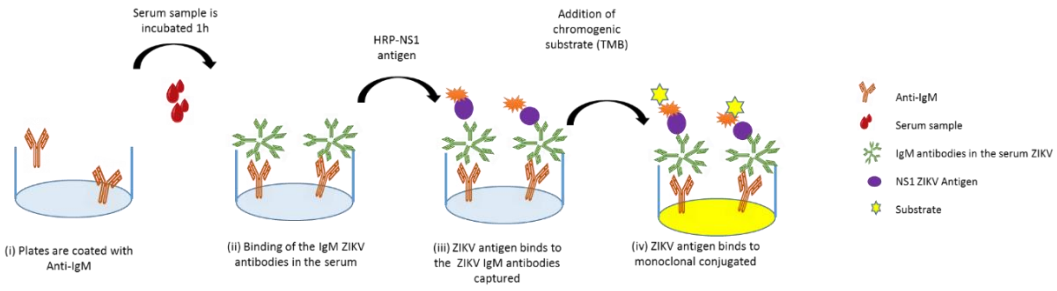


Figure 2.2 Principles and protocol for the IgM µ-capture ZIKV ELISA
Diagram showing the key steps in the commercial IgM ZIKV assay from Novagnost®(NovaTec).

Results were calculated as recommended by the manufacturers in Novagnost® Units by calculating the ratio of the Optical Density (OD) of the patient sample or control over the OD of the average value of the two cut-off controls multiplied by 10 as shown below:

$$\textbf{IgM } \mu - \textbf{capture } (\textbf{Novagnost® Units}) = \frac{OD\ of\ the\ patient}{OD\ of\ the\ average\ value\ of\ the\ two\ cutoff\ controls}\ x\ 10$$

A specimen was considered IgM positive if ratio ≥11.5; borderline ≥8.5 and <11.5; negative ≤8.5.

For the purpose of visually comparing the results of the IgM ZIKV µ-capture ELISA and the other assays, most of the graphs that I show in this chapter express the antibody titres for the IgM µ-capture assay in Units of Ratio instead of Novagnost® Units. Ratio Units do not multiply by 10 the ratio between OD of patient and OD of the cut-off as shown below:

$$\textbf{IgM } \mu - \textbf{capture } (Ratio) = \frac{OD\ of\ the\ patient}{OD\ of\ the\ average\ value\ of\ the\ two\ cutoff\ controls}$$

The ratio results I obtained had titres comparable to other assays. This calculation does not alter the classification between positive/equivocal or negative, nor the proportion of antibodies produced and allows better comparisons of ratios among different assays. With this new calculation, a sample was considered positive if Ratio ≥1.15; equivocal if ratio is between ≥ 0.85 and <1.15; and negative if ratio <0.85. For clarity, every figure presented in this thesis specifies if the results are represented as Ratio Units or as Novagnost® Units.

2.2.4 IgM ZIKV CDC MAC ELISA

The FDA CDC-designed IgM antibody capture ELISA also known as the ZIKV MAC ELISA was performed as previously described by using the US Centers for Disease Control and Prevention (CDC) emergency use authorisation protocol (CDC Fort Collins, CO, USA)[115]. Prior to implementation, the assay was tested and evaluated by the experienced team in the flavivirus reference laboratory to ensure that all the concentrations and site-specific parameters were adjusted accordingly.

Briefly, 96-well microtiter plastic plates were coated with goat anti-human IgM antibody and incubated at 4 °C overnight (day 1 of the assay). On day 2, plates are blocked with 200 µl of blocking buffer per well during 30 min at RT. During this incubation, negative control (negative sera), positive control (known positive ZIKV with known antibody titre) and patient sera were used at 1:400 dilution. Then, plates were washed 5x with wash buffer using an automatic plate washer. Following this, controls and patient serum were added to 6 wells each and incubated for 1 h at 37 ^{o}C. Another 5x washes are conducted just after the incubation. Then, Vero cell ZIKV antigen (1:500 dilution) was added in triplicate for each patient / control. Diluted normal antigen was added to the other 3 wells and the plate was incubated overnight at 4 °C. On day 3, plates were washed again 5x and 50 µl/well of diluted conjugated monoclonal flavivirus antibody (6B6C-1), a chimeric monoclonal antibody specific for flavivirus conjugated to horseradish peroxidase, diluted at 1:1,500) was added to each well followed by a 1 h incubation at 37 ^{o}C. Following this, the plate was washed 5x and 75 µl of TMB substrate were added to each well. The plate was incubated at RT for 10 min and 50 µl of stop solution was added to stop the reaction. Finally, the absorbance was measured with a spectrophotometer at a wavelength of 450 nm within 10 minutes after addition of the stop solution. A diagram of the assay procedure is shown in Figure 2.3.

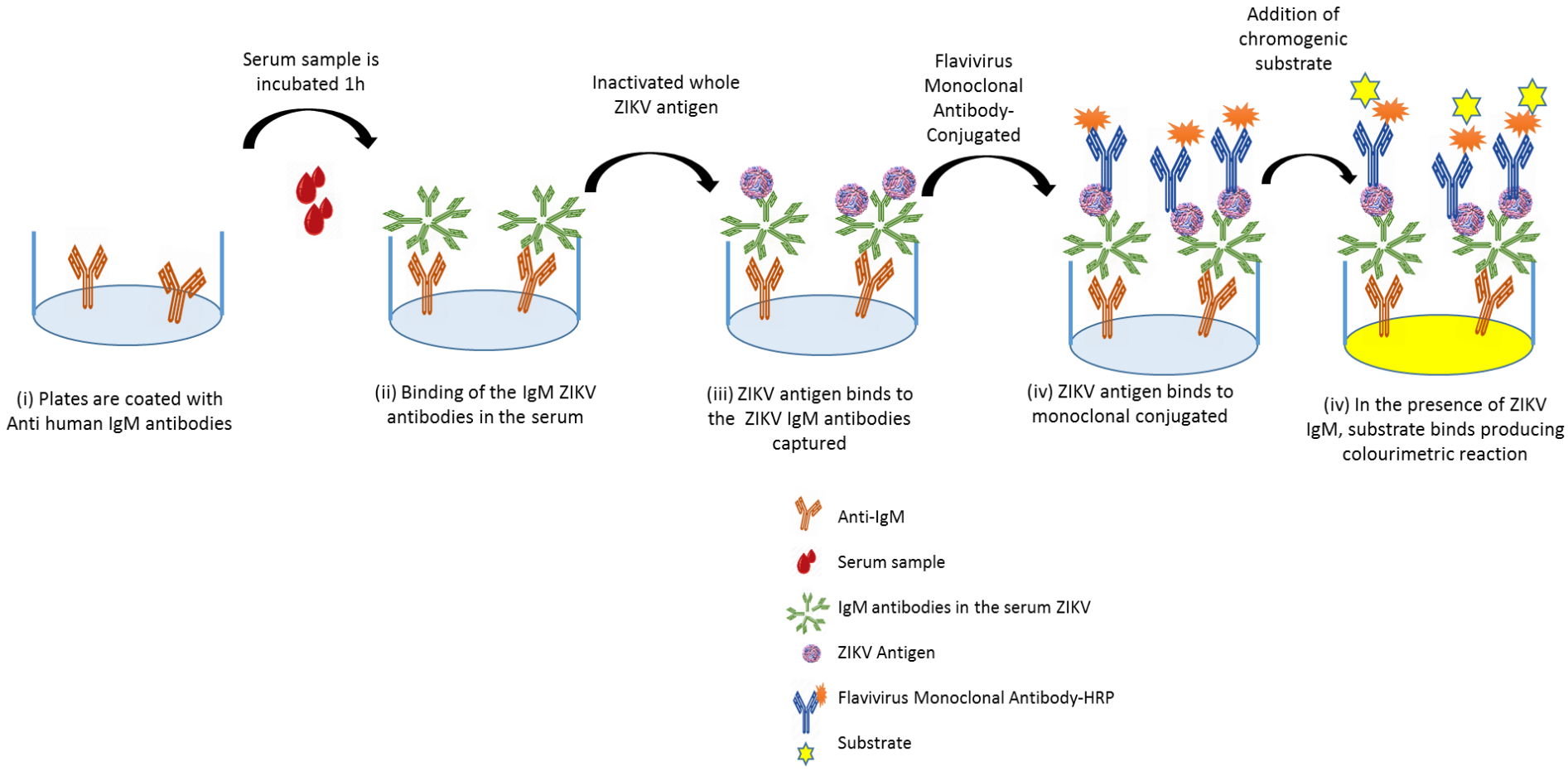


Figure 2.3 Diagram representing the principles and protocol for the CDC MAC ELISA

Results were reported as recommended by the CDC, based on the Positive to Negative ratio $\frac{P}{N}$. P is obtained as the mean OD of the test serum reacted on ZIKV antigen and N is the mean OD of the normal human serum/OD negative-control serum reacted with the ZIKV antigen. The simplified calculation is shown here below:

$$\textbf{IgM MAC ELISA}(\frac{\textbf{P}}{\textbf{N}}) = \frac{P\ (OD\ patient\ serum\ with\ the\ ZIKV\ antigen)}{N\ (OD\ patient\ serum\ with\ the\ Normal\ antigen)}$$

Results were reported as recommended by the CDC protocol as positive if $\frac{P}{N}$≥3; equivocal if f $\frac{P}{N}$≥2 but $\frac{P}{N}$<3 and negative if f $\frac{P}{N}$<2.

2.2.5 Zika Virus Block-of-binding Assay

The Block-of-binding assay is an NS1-based competition ELISA using a Zika specific monoclonal antibody (BOB) was developed by the Swiss company Humabs® and it has been previously described [116]. This assay is based on a competitive principle. If Anti-Zika NS1 antibodies are present in the serum sample tested, they will compete with the monoclonal anti-Zika antibodies for the ZIKV NS1 antigen coated in the wells. Thus, when no anti-ZIKV NS1 antibodies are present in the sample, the substrate will bind and there will be a chromogenic reaction. For a graphic illustration of the assay protocol, see Figure 2.4.

Briefly, on Day 1: 1 µg/mL of ZIKV NS1 (produced with the ZIKV MR766 strain from the Native Antigen Company, Oxford, UK) were prepared in coating buffer (PBS). Then, 96 well polystyrene plates (Nunc MaxiSorp) were coated with 50 µl of the diluted ZIKV NS1. Plates were incubated overnight at 4 °C in a protected locked box.

Also on Day 1, samples were inactivated with lysis buffer. The lysis buffer was prepared in PBS with 2% Triton X-100 (Sigma-Aldrich, UK), 0.1% ProClin 950 (Sigma-Aldrich, UK), 1% Bovine Serum Albumin (Sigma-Aldrich, UK) and 2% Tri(n-butyl)phosphate (Sigma-Aldrich, UK). Samples were incubated overnight at 4 ^{o}C in a solution 1:1 with lysis buffer (25 µl of sample and 25 µl of lysis buffer).

On Day 2, the coating mixture is aspirated with an automatic plate washer. Then, plates were blocked for 1 hour at room temperature with 200 µl 1% Bovine Serum Albumins (BSA) in filtered blocking buffer (PBS). Then plates were washed with PBS + 0.05% Tween20 (Sigma-Aldrich, UK). Following this, 50 µl of control and serum specimens diluted 1:5 (in PBS + 1% BSA) were added to the ZIKV NS1-coated 96-well ELISA plates. The plates were incubated for 1 h at RT. Then, 50 µl of biotinylated antibody (cold anti-NS1 ZKA35) at 20 ng/mL was added. The plates were incubated at RT for 15 min. Following this, plates were washed with wash

buffer (PBS + 0.05% Tween20) four times using the automatic plate washer. After this, 50 µl of IgG alkaline-phosphatase-conjugated streptavidin (secondary antibody) was added to all wells. The plates were incubated for 1 h at RT.

Finally, the substrate p-NitroPhenylPhosphate (from Sigma-Aldrich, UK) was added and plates were incubated for 30 min protected from the light. The reaction was stopped with 2M of Sulphuric Acid. Plates were read in an ELISA reader at 405 nm. When low or no colour develops it suggest that anti-ZIKV specific NS1 antibodies are present in the serum sample tested. If colour is developed, the sample is considered negative for ZIKV antibodies. The percentage of inhibition was calculated as follows:

$$Percentage\ of\ inhibition\ (\%) = 1 - \frac{OD\ sample - OD\ negative\ Control}{OD\ Positive\ Control - OD\ negative\ Control} x100$$

As described in [116], the percentage of inhibition of 50% was determined to be the best cut-off for this assay.

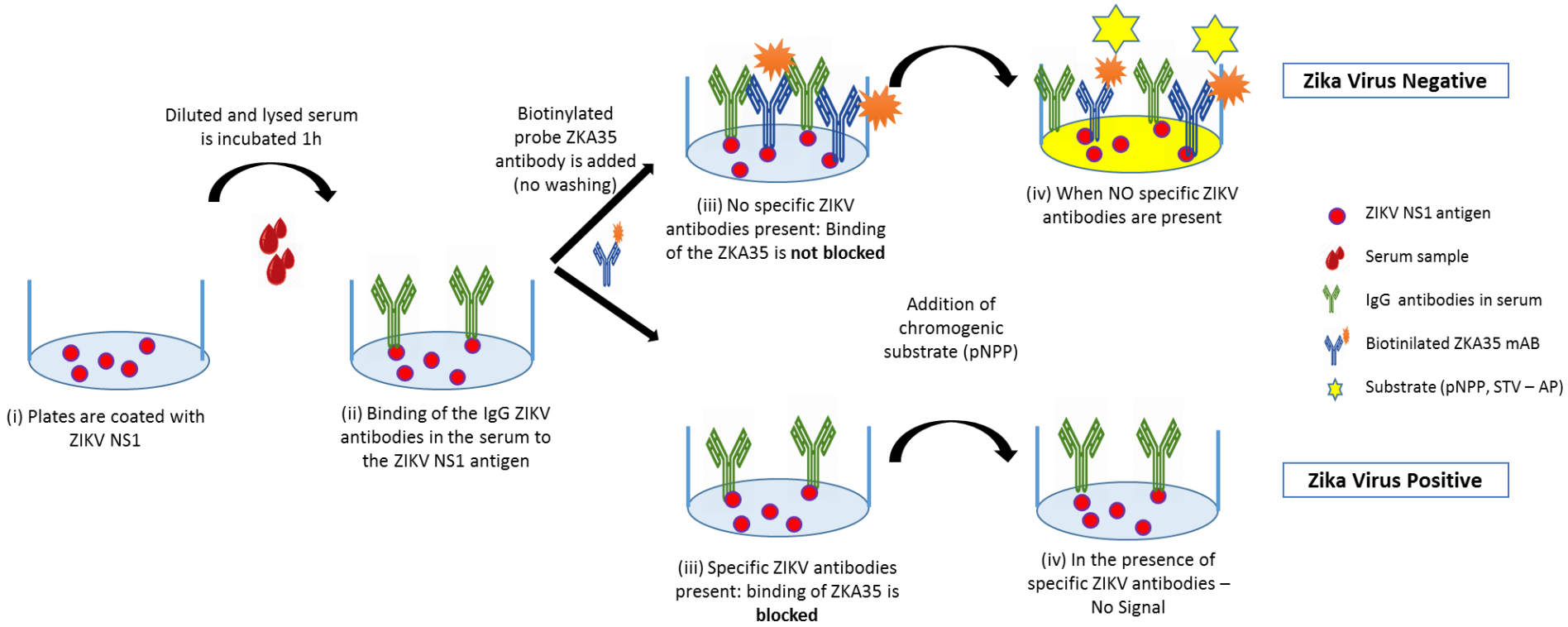


Figure 2.4 Principles and protocol for the Block-of-Binding (BOB) assay

2.2.6 Zika Virus Green BOB Assay

The Green BOB assay is a Zika competitive enzyme-linked immunoabsorbent assay that detects antibodies specifically to the NS1 region of the ZIKV. It has been developed by collaborators in Public Health England as a modification of the Block-of-binding (BOB) assay. The Green BOB assay is based on an ELISA format and it also uses the same ZIKA35 Mab as described above.

The Green BOB is based on a one-step incubation competitive protocol. If the Anti-ZIKV antibodies are present in the sample, they will compete with the monoclonal anti-ZIKV antibodies that are conjugated to HRP for the fixed amount of recombinant ZIKV NS1-antigen that is already pre-coated in the plate wells. For a graphical illustration of the assay, see Figure 2.5.

Briefly, 25 µl of serum specimen, positive and negative controls were added to the wells of a ZIKV NS1 pre-coated 96-wells ELISA plate. Conjugate was prepared at working strength of anti-ZKA35 coupled with horseradish peroxidase (HRP) in stabiliser solution and diluted with the conjugate diluent. Then, 75 µl of prepared conjugate were added. The mixture is incubated for 2 h at 37 ^{o}C. Then, plates were washed 5 times with wash buffer. Then, 100 µl of TMB substrate is added to each well and plates are incubated for 30 min at 37 ^{o}C. Following this, 50 µl of stop solution (2M sulphuric acid) was added to each well to stop the reaction. The absorbance is read in a plate reader at 450 nm (reference wavelength at 630 nm). The results are calculated as follows:

$$\text{Patient Green BOB Ratio} = \frac{Patient\ OD}{\left(\frac{Mean\ OD\ \ 4x\ Negative\ Control + Mean\ OD\ 2x\ \ Positive\ Control}{2}\right)}$$

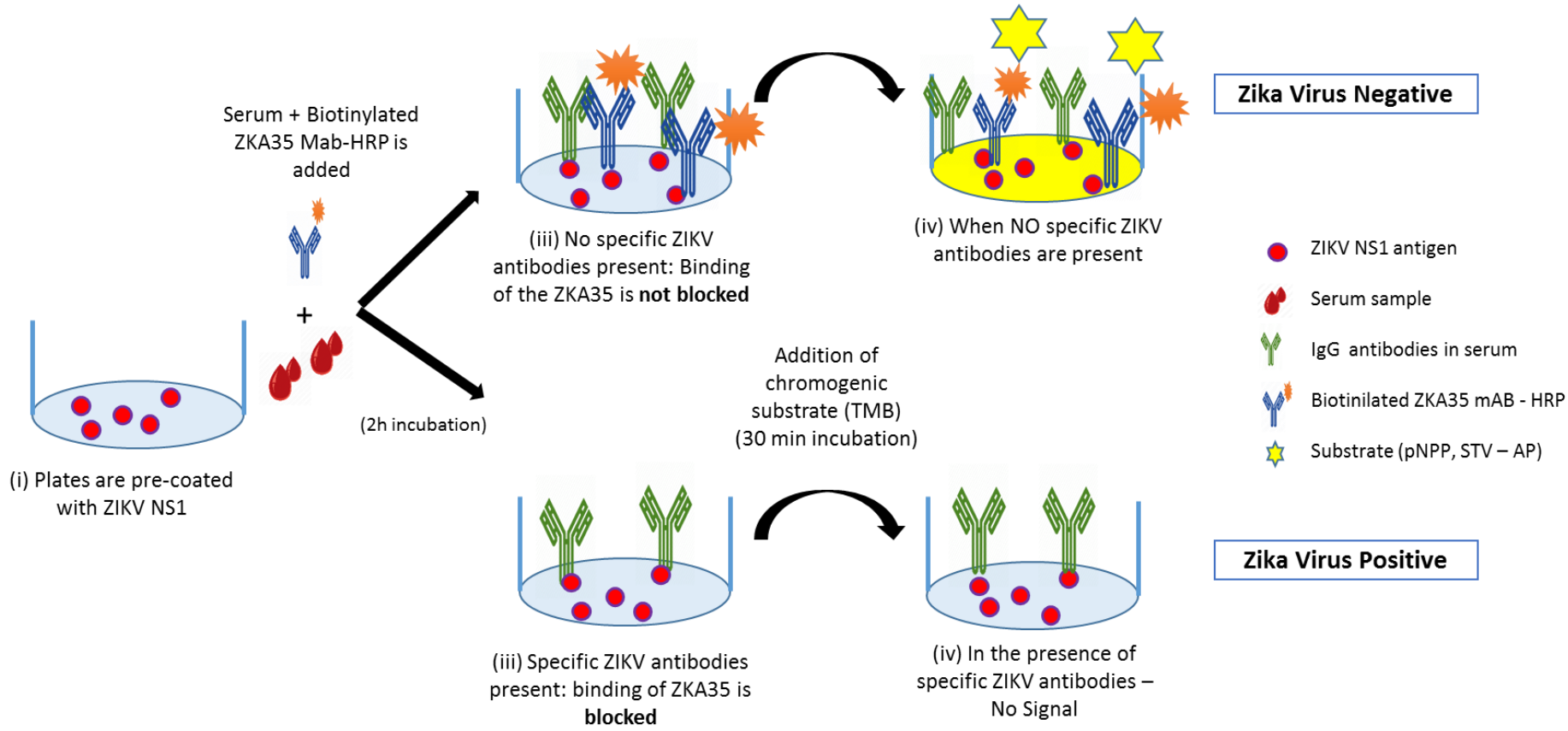


Figure 2.5 Principles and protocol for the Green Block-of-Binding (BOB) assay

2.2.7 Plaque Reduction Neutralization Tests (PRNTs)

ZIKV assays were conducted in Liverpool using the Brazilian ZIKV Strain PE-243, already described by Donald *et al*. (2016) [117]. ZIKV was expanded by three passages in C6/36 cells. The PRNTs tested in Liverpool were performed adapted from the protocol developed by Russell *et al.* (1967) for DENV neutralization assays [118]. The PRNT assays performed in Brazil, used the same virus, but had minor modifications in the method.

Briefly, fifty percent PRNT titres ($PRNT_{50}$) were measured using Vero cells for all study samples. Cells were seeded at a density of 300 000 cells/ml and grown in Minimum Essential Medium Eagle (MEM) with 10% FBS and 1% penicillin/streptomycin solution for 24h prior the assay. The same batch of cells and ZIKV stock was used to minimize variation in the assay (both in Liverpool and in Brazil). For PRNT50, sera were heat inactivated for 30 min at 56 ^{o}C prior dilution in MEM. Then, 80 plaque-forming-units (PFU) of ZIKV virus was added to the wells with the serum dilutions. Then, plates were incubated at 37 ^{o}C in a 5 % CO_2 incubator for 1 h with gentle rocking every 15 minutes. Then, 50 ml of each dilution of the mixture serum/virus was inoculated in duplicate to 24 well plates. Plates were then incubated at 37 ^{o}C in 5% CO2 for 1 h. Then, plate-wells were covered with 500 ml of semi-solid medium [MEM 10% FBS, 10% carboxymethylcellulose (3%), 1% penicillin/streptomycin]. Following incubation for 5–6 days at 37 ^{o}C in 5% CO2, the medium was discarded and the cell monolayer was fixed with formalin solution (3. 5 M) for 1 h and stained with Crystal Violet (0. 5 ml/well) or Neutral Red (0.5/well). Plates were counted. The PRNT positivity was defined based on a >50% reduction in plaque counts ($PRNT_{50}$) at the serum dilution used (1:100) based on previous optimizations conducted in Brazil [119]. Further details will be shown in Chapter 3. Positive and negative assays were included. As part of the Liverpool evaluations, two staining techniques were employed: crystal violet (Sigma, UK) and neutral red (Sigma, UK).

2.2.8 Single-use point-of-care tests (POCT) for ZIKV diagnosis

The DPP® Zika IgM/IgG is a Rapid Diagnostic Test (RDT) for ZIKV diagnosis for use with an automatic reader. The TR DPP® is a rapid diagnostic assay for the detection of ZIKV antibodies in whole blood, serum or plasma. It is based on an anti-ZIKV Single-use point-of-care test (POCT) format. It has been developed by Bio-Manguinhos (Fiocruz) in collaboration with Chembio Diagnostics (USA) and the test is based in the immunochromatographic technology

and uses a dual platform. This assay has been approved "for use under Emergency Use Authorization (EUA)" in the USA.

A photo of the kit is shown in Figure 2.6. The assay uses a combination of ZIKV NS1 specific antigens are attached to a solid phase. The kit also includes buffer solutions to allow the reaction to move through the solid phase, which separates both the IgM and IgG antibodies. The assay can be used with: serum, plasma, finger stick whole blood and venepuncture whole blood.

The principle of the assay is based on a conjugate that has colloidal gold attached which is added to the reaction after migration of the sample + buffer mixture through the solid phase. Then, the conjugate binds to the complex of antigen and ZIKV specific antibody generating a pink/red line in the case ZIKV antibodies are present. If no ZIKV antibodies are present, there should be no pink/red line. The kit includes a control that should always generate a response. The kit uses a small electronic reader (the DPP® Micro Reader) to allow quantification of the ZIKV antibody response which is shown in Figure 2.6 A.

The assay was performed following the manufacturer's instructions. Briefly, 10 µl of serum were mixed with 150 µl of dilution buffer. A hundred µl of the mixture were transferred to the Point 1 in the POCT assay. The assay was incubated for 5 min at RT. Then 9 drops of conjugate (Kit solution number 2) were added to the assay. The test was incubated for 10 min at RT and the results were read with the DPP® Micro Reader.

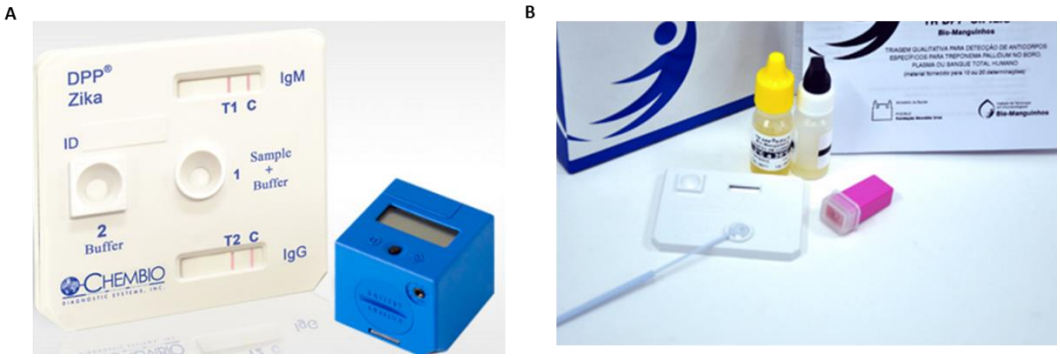


Figure 2.6 Rapid Diagnostic Test (RDT) for ZIKV diagnosis: the DPP® Zika IgM/IgG
Test works with just one droplet of serum (10 µl)/ patient. Assay Kit shown A) Adapted from: http://chembio.com/dpp-zika-igmigg-system-ce-marked/. Image shows the single-Point-of-Care (POC) assay and electronic reader. B) Image adapted from www.bio.fiocruz.br/index.php/noticias/1296-testes-rapidos.

2.2.9 Indirect immunofluorescence test for Zika Virus detection

The concept of the IFA assay technique is based on the following: the test is washed to remove any unbound antibody then a suitable anti-species antibody conjugated to a

fluorescent marker is added. This secondary antibody will bind to any antibodies present in the sample that had bound to the antigen. The tests are incubated, then washed again, before being read by microscopy. The fluorescent marker glows with a bright apple green fluorescence under the UV light indicating the presence of specific antibodies bound to the antigen. Finally, the pattern of fluorescence observed in the sample is able to confirm if there has been a specific reaction.

We evaluated two IFA assays produced by Euroimmun (Lubeck, Germany). The manufacturer's evaluations reported excellent specificity and sensitivity (>90%). The two assays evaluated are:

1- IIFT Arbovirus Fever Mosaic 2 assay IgG
2- IIFT Arbovirus Profile 3 IgG

An image of both assays is shown in Figure 2.7. The kits are designed for the qualitative or semi-quantitative in vitro determination of IgG antibodies for a range of arboviruses in patient samples. They are based on cells infected with different arboviruses that are incubated with patient samples. If a positive reaction occurs, the specific IgGs in the sample attach to the antigens. Then, the attached antibodies are stained with fluorescein-labelled anti-human antibodies which are made visible with a fluorescence microscope. Each slide contains Biochips with 6 and 10 positions each (respectively for the IIFT Mosaic 2 and Profile 3). Positive and negative controls are provided with the assay. Each assay targets the following pathogens:

1- **IIFT Arbovirus Fever Mosaic 2.** Designed for specific detection of 6 pathogens including ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV.
2- **IIFT Arbovirus Profile Mosaic 3.** Designed for specific detection of 10 pathogens including the 6 above mentioned in the IIFT Mosaic 2 [ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV] but also Tick-Borne Encephalitis Virus (TBEV), Yellow Fever Virus (YFV), West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV).

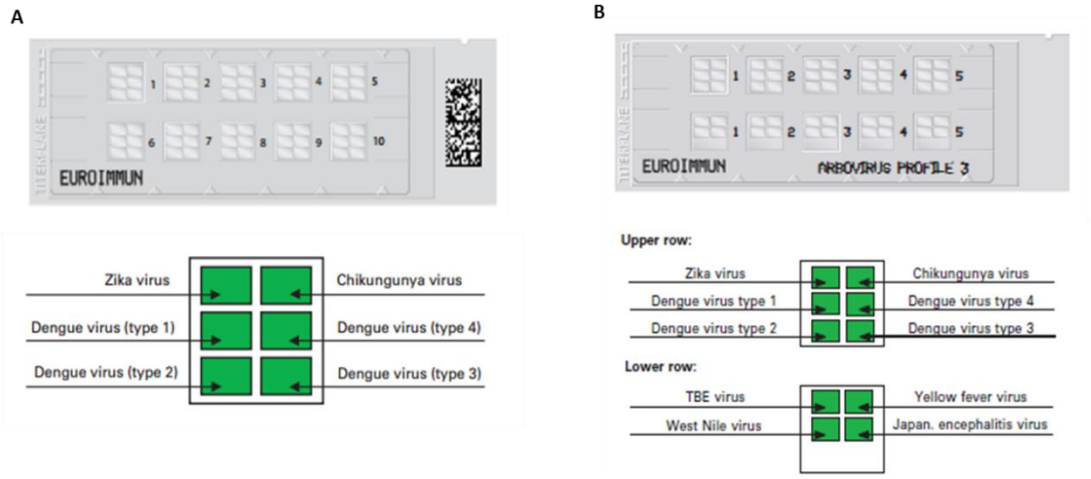


Figure 2.7 Immunofluorescence assays evaluated from Euroimmun.
Showing the test slide and the biochip composition for both assays evaluated. A) IIFT Arbovirus Fever Mosaic 2. Assay designed for specific detection of ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV and B) IIFT Arbovirus Profile 3 allows specific detection of 10 pathogens [ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV] but also Tick-Borne Encephalitis Virus (TBEV), Yellow Fever Virus (YFV), West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV). Image adapted from www.euroimmun.com.

I followed the manufacturer's instructions. Briefly, 30 µl of serum (diluted 1:10) is added to each slot in the slide (10 slots / slide) avoiding bubbles. Then, the reaction is incubated for 30 min at RT. Following this, the Biochip slides are then washed with PBS-Tween. After this, the slides were immersed in PBS-Tween for 5 min. Then, 25 µl of fluorescein labelled anti-human globulin was added to each reaction field in the slides. Slides were incubated for 30 min at RT protected from light. Following this, the slides were flushed again with PBS-Tween and incubated in a cuvette filled with PBS Tween for 5 min protected from the light. Then, embedding medium was added to a cover glass (less than 10 µl/ slide) and placed over the side of the slide with BIOCHIPs just after drying it. Fluorescence was read with a light microscope using an objective of 20X and an excitation filter of 450:490nm, with colour separator at 510 nm and blocking filter at 515 nm. Assay interpretation: a fluorescence pattern was considered a positive reaction. Positive and negative controls were provided with the kit. A diagram is shown in Figure 2.8.

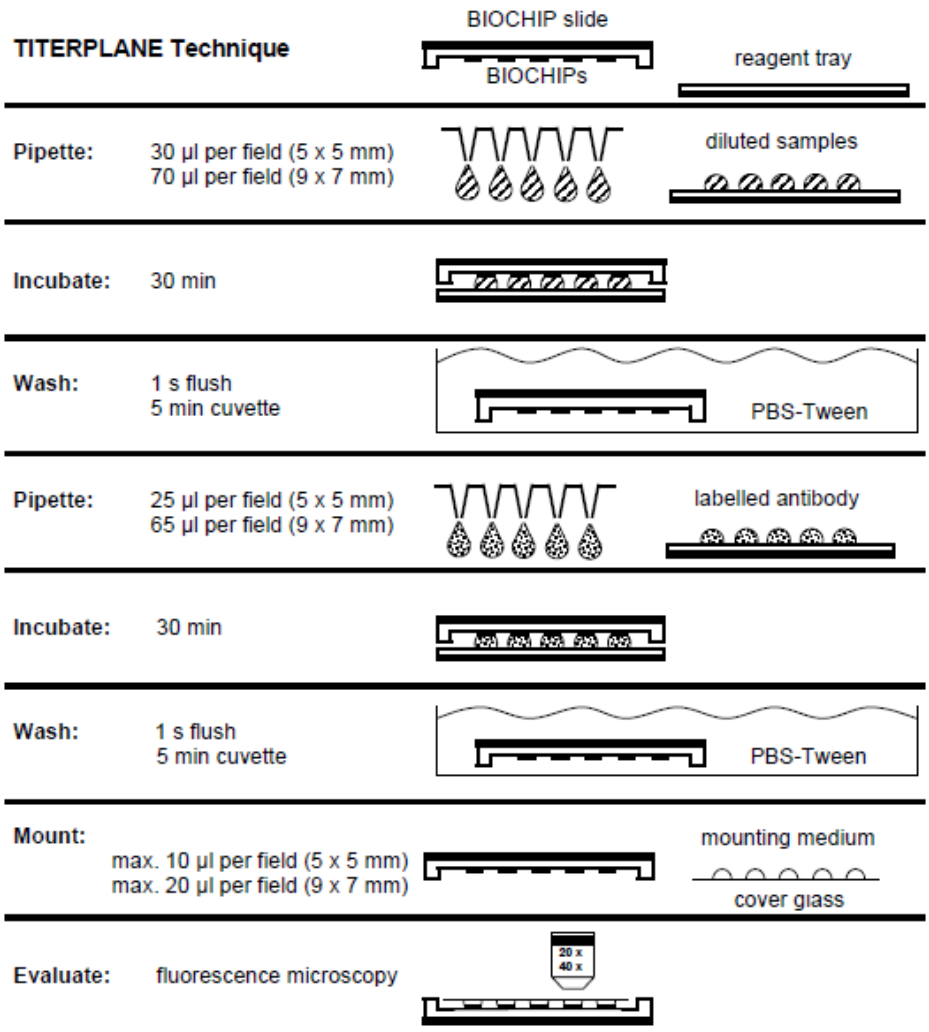


Figure 2.8 Summary procedure for performance of the IIFT assays
Protocol summary with the procedure for indirect immunofluorescence assay both for the IIFT Arbovirus Mosaic 2 and the IIFT Arbovirus Profile 3. Image adapted from www.Euroimmun.ch.

2.3 Serological Diagnostic Assays for DENV and CHIKV diagnosis

2.3.1 IgM DENV capture ELISA

For the qualitative detection of DENV IgM antibodies to dengue antigen in serum, Panbio dengue IgM capture ELISA (Cat. No. E-DEN01M, Alere, UK) was performed following the manufacturer's manual.

Briefly, for the IgM DENV ELISA, 100 µl of diluted patient serum, 1x positive, 1x negative control and 3x calibrators (1:100) were added to the anti-human IgM-coated microwells and incubated for 1 h at 37°C. The lyophilised dengue antigen (lyophilised vial with stabilised dengue viral antigens: DENV 1 to DENV 4) was reconstituted in buffer (as described by the manufacturer) and mixed well with an equal volume of HRP-conjugated monoclonal

antibody (mAb) tracer to make the mix Antigen-mAb. After the incubation, the assay plate was washed 6 times with washing buffer (at a concentration of 1X) with an automatic plate washer to remove unbound serum. Then, the captured dengue-specific antibodies are detected by adding 100 µl of the antigen-mAb mix to each well and incubated for 1 h at 37 °C. Then, the assay plate was washed 6 times and 100 µl/well of TMB substrate were added. Plates were incubated for 15 min at RT in the dark. After the incubation, stop solution was added and absorbance was read at 450 nm in a spectrophotometer reader with a reference filter (620 nm). The colour intensity is then related to the absence or presence of anti-dengue IgM antibodies in the patient sample. A diagram is shown below in Figure 2.9

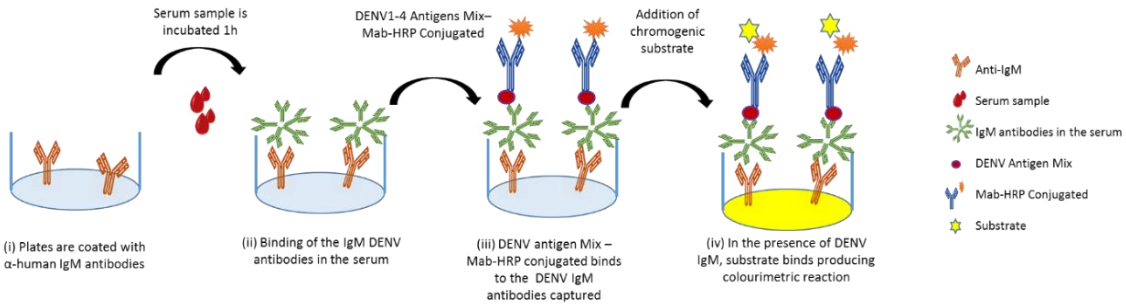


Figure 2.9 Diagram representing the principles and protocol for the IgM DENV Capture ELISA

The OD results from the commercial IgM Capture ELISA were interpreted as recommended by the manufacturer and samples were considered positive for recent dengue infection according to the ratio calculations suggested by the manufacturer:

$$\boldsymbol{IgM\ DENV\ ELISA\ Index\ Value} = \frac{OD\ patient\ serum}{Average\ OD\ of\ the\ calibrators\ x\ Calibration\ Factor}$$

The calibration factor was specific for each assay lot number. The interpretation of the results was performed as recommended by the manufacturers with patients being classified as "presumptive positive" if Index value>1.1; borderline if Index >0.9 and <1.1; negative if Index<0.9. The manufacturers indicate that for interpretation purposes a negative result means that there is no detectable IgM antibody and they inform that "*the result does not rule out dengue infection, an additional sample should be tested in 7-14 days if early infection is suspected*".

The manufacturers report that the antibody titre can also be measured in Panbio Units instead of Index values as follows:

$$IgM\ DENV\ ELISA\ Panbio\ Units = \frac{OD\ patient\ serum}{Average\ OD\ of\ the\ calibrators\ x\ Calibration\ Factor} x10$$

The interpretation of the results in IgM Panbio Units is therefore proportional as with Index Values but multiplied 10 times. It was performed as recommended by the manufacturers with patients being classified as positive if Panbio Units>11; borderline if Panbio Units between >9 and <11; and negative if Panbio Units<9.

2.3.2 IgG Indirect DENV ELISA

For detection of DENV IgG antibodies, the IgG DENV capture ELISA (Cat. No. E-DEN02G) (Panbio, Alere, United Kingdom) was performed. The manufacturers indicate that the assay is able to determine levels of IgG antibodies against the four dengue serotypes (DENV-1 to 4). The assay was performed according to the manufacturer's instructions. Briefly, for the IgG indirect DENV ELISA, 100 µl of diluted patient serum, positive, negative control and calibrator (1:100) were added to the anti-human IgG-coated microwells added and incubated for 1 h at 37 °C. After the incubation, the assay plate was washed 6 times with washing buffer (1X) with an automatic plate washer to remove unbound serum. Then, the captured dengue-specific antibodies are detected by adding 100 µl of the conjugate and incubated for 1 h at 37 °C. Afterwards, the assay plate was washed 6 times and 100 µl/well of TMB substrate were added and incubated for 15 min at RT in the dark. After the incubation, stop solution was added and absorbance was read at 450 nm in a spectrophotometer reader with a reference filter (620 nm). The colour intensity is then related to the absence or presence of anti-dengue IgM antibodies in the patient sample. A more detail representation of this IgG Indirect DENV ELISA is shown in Figure 2.10.

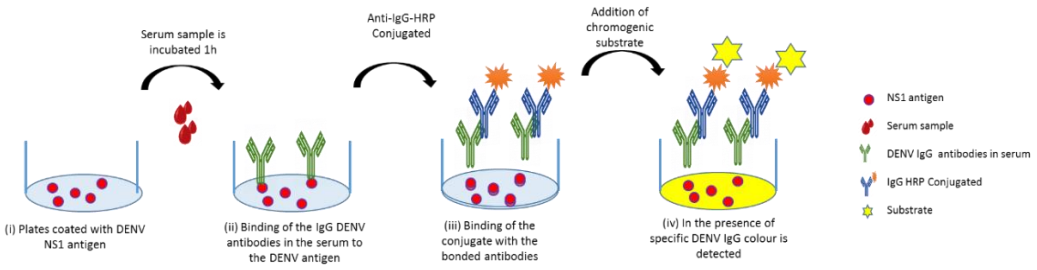


Figure 2.10 Diagram representing the principles and protocol for the IgG DENV Indirect ELISA

Interpretation of the absorbance results obtained from the commercial IgG DENV capture ELISA was performed as recommended by the manufacturers and samples were considered positive for previous or recent dengue infection according to the ratio calculations suggested by the manufacturer:

$$\boldsymbol{IgG\ DENV\ ELISA\ Index\ Value} = \frac{OD\ patient\ serum}{Average\ OD\ of\ the\ calibrators\ x\ Calibration\ Factor}$$

The calibration factor was specific for each assay lot number. The interpretation of the results was performed as recommended by the manufacturers with patients being classified as positive if Index >1.1; borderline if Index between >0.9 and <1.1; and negative if Index <0.9. A negative result can be interpreted as no evidence of a past dengue infection.

The manufacturers report that the antibody titre can also be measured in Panbio Units instead of Index values as follows:

$$\boldsymbol{IgG\ DENV\ ELISA\ Panbio\ Units} = \frac{OD\ patient\ serum}{Average\ OD\ of\ the\ calibrators\ x\ Calibration\ Factor}\ x10$$

The interpretation of the results in Panbio Units is therefore proportional as with Index Values but multiplied 10 times. It was performed as recommended by the manufacturers with patients being classified as positive if Panbio Units >11; borderline if Panbio Units between >9 and <11; and negative if Panbio Units <9.

2.3.3 IgM CHIKV ELISA

The IgM anti-CHIKV enzyme-linked immunosorbent assay (ELISA) from Euroimmun (Lubeck, Germany) were performed following strictly the manufacturer's instructions. This ELISA kit provides an in vitro assay for human IgG antibodies against CHIKV for the diagnosis of chikungunya fever and differential diagnosis of haemorrhagic fever. The kit contains plates with pre-coated wells with recombinant chikungunya virus antigens. The protocol can be summarised as follows: The pre-coated plates were incubated for 1 h at 37 ^{o}C in an incubator with 100 µl of calibrator, CHIKV positive, negative controls or diluted patient samples into each individual microplate wells. Serum samples were diluted (10 µl of serum + 1000 µl buffer diluent to make a 1:101 dilution factor). Then, the plate was washed 3 times with wash buffer (1x) using an automatic plate washer. Following this, 100 µl of enzyme conjugate (goat peroxidase-labelled anti-human IgG) was added and incubated for 30 min at room temperature (RT). Then, the wells were washed again 3 times and a chromogen/substrate solution was added. Plates were incubated in the dark for 15 min at RT. This was followed by the addition of 100 µl/well of sulphuric acid stop solution. The OD of each well was then measured using a spectrophotometer at 450 nm with a 620 nm reference wavelength. The colour intensity is then related to the absence or presence of anti-CHIKV IgG antibodies in the patient sample. Prior to measuring, the microplate was slightly shaken as recommended in order to ensure homogeneous distribution of the solution in the wells and the absorbance was measured within 30 min. For a graphical visualization of the assay, see Figure 2.11.

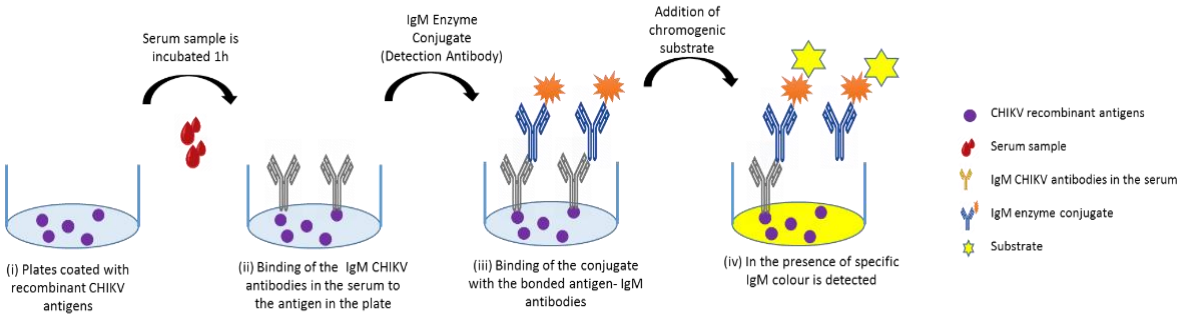


Figure 2.11 Diagram representing the principles and protocol for the IgM CHIKV ELISA (Euroimmun)

2.3.4 IgG CHIKV ELISA

The IgG anti-CHIKV enzyme-linked immunosorbent assay (ELISA) from Euroimmun (Lubeck, Germany) were performed following strictly the manufacturer's instructions. The assay uses a mix of recombinant CHIKV antigens. This is an indirect, colourimetric ELISA that detects IgG human antibodies generated against CHIKV infection. Briefly: the manufacturer provided 96 well plates which were pre-coated with a proprietary mixture of recombinant CHIKV antigens. The pre-coated plates were incubated for 1 h at 37 ^{o}C in an incubator with 100 µl of calibrator, CHIKV positive, negative controls or diluted patient samples into each individual microplate wells. Serum samples were diluted (10 µl of serum + 1000 µl buffer diluent to make a 1:101 dilution factor). Then, the plate was washed 3 times with wash buffer (1x) using an automatic plate washer. Following this, 100 µl of enzyme conjugate (goat peroxidase-labelled anti-human IgG) was added and incubated for 30 min at room temperature (RT). Then, the wells were washed again 3 times and a chromogen/substrate solution was added. Plates were incubated in the dark for 15 min at RT. This was followed by the addition of 100 µl/well of sulphuric acid stop solution. The OD of each well was then measured using a spectrophotometer at 450 nm with a 620 nm reference wavelength. The colour intensity is then related to the absence or presence of anti-CHIKV IgG antibodies in the patient sample. Prior to measuring, the microplate was slightly shaken as recommended in order to ensure homogeneous distribution of the solution in the wells and the absorbance was measured within 30 min. For a more graphical visualization of the assay, see Figure 2.12. CHIKV IgG results are calculated semi-quantitatively and results are expressed as recommended by the manufacturer, by calculating the ratio of the Optical Density (OD) of the patient sample or control over the OD of the calibrator (as shown below).

$$\textbf{IgG CHIKV ELISA Ratio} = \frac{\text{OD of the patient}}{\text{OD of the calibrator}}$$

A specimen was considered IgG positive if ratio ≥1.1; borderline ≥0.8 and <1.1; negative ≤0.8. Cut-offs for the assay was determined by the manufacturer. An individual with previous exposure to CHIKV was defined as a subject with positive IgG CHIKV antibodies detected in the serum.

The IgG CHIKV ELISA have shown in previous multi-centric independent investigations high sensitivity between 88% to 100% and specificity of 95% [120, 121].

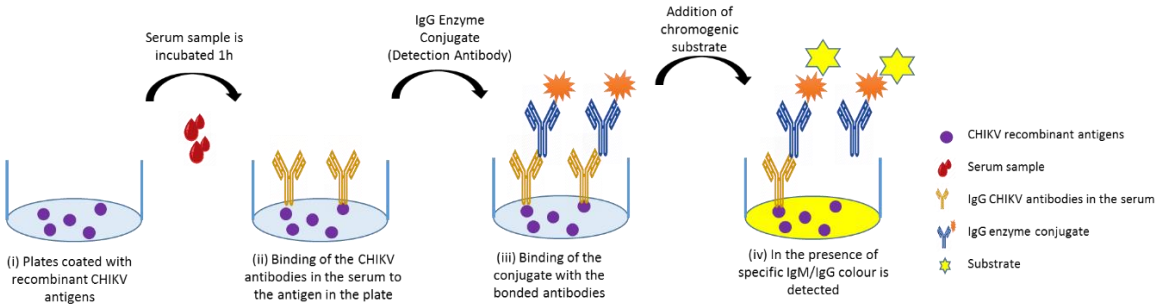


Figure 2.12 Diagram representing the principles and protocol for the IgG CHIKV ELISA (Euroimmun)

2.4 Molecular Diagnostic Assays

Molecular detection of pathogen RNA was performed for ZIKV and DENV though RT-PCR.

2.4.1 RNA extraction

The individuals in our ZIKV panel with clinical presentations indicative of recent ZIKV infection (during 2015 and 2016) were tested for detection of ZIKV nucleic acid material. RNA was extracted using the QIAamp Mini Elute (Qiagen, Brazil) following the manufacturer's instructions. Briefly, 140 µl of well-mixed serum at RT were added with 280 µl of AVL buffer and 2.8 µl of carrier RNA (1 µg µl−1 in AVE buffer). The mix was vortexed for 15 s and incubated at RT for 10 min. After, 280 µl of ethanol (96–100%) was added to the mixture which was then transferred to the QIAamp spin column and centrifuged at 6000 x g for 1 min. Following this, the QIAamp spin column was transferred to a new tube and 500 µl of AW1 were added. The QIAamp spin column is centrifuged at 6000 x g for 1 min again and transferred to a new collection tube discarding the filtrate. We added 500 µl of AW2 and columns were centrifuged at 20,000 x g for 3 min. Then, the QIAamp spin column was transferred to a new empty collection tube and centrifuged at 20,000 x g for 1 min. Then, the QIAamp spin column was transferred to a new empty collection tube and 60 µl of elution buffer was added to each column. Finally, the RNA was eluted by centrifuging at 6,000 x g

for 1 min. As RNA is easily degradable – especially in hot temperatures-such as the ones in Rio de Janeiro where the assays were performed, the eluted RNA was immediately stored at 4 ^{o}C (if the PCR was going to be performed on the day) or at -20 ^{o}C if the PCR was going to be performed at a later time point. A negative extraction control made up of 140 µl of water was included in every batch.

2.4.2 RT-PCR

RT PCR reactions were conducted for detection of specific RNAs from ZIKV, DENV1, DENV2, DENV3 and DENV4.

2.4.2.1 General RT-PCR good Laboratory Practice

With the purpose of ensuring good laboratory practice and reducing the possible risk of cross-contamination when performing RT-PCR, several precautions were taken:

- **Avoiding contamination:** RNase-free was used on surfaces where the experiments took place. Also, ethanol (70%) was sprayed across surfaces prior to working. Preparation of the master mix solutions and the PCR set up was performed in different separate rooms.
- **Avoiding sample degradation**: all reagents were kept cold or on ice until they were used to avoid degradation of the solutions.
- **Adequate use of PCR Controls:** Each run included at least two negative controls: 1) negative extraction control (water sample that had been extracted together with the same batch of samples) and 2) negative water control (PCR reaction mix with 5 µl of water instead of patient RNA. One positive control was added for the ZIKV PCR (ZIKV RNA extracted from known virus stock controls that had been isolated from local patients) and for the DENV PCR controls, one positive control was added for each pathogen: RNA from DENV1, DENV2, DENV3 and DENV4. Results on the controls were always checked and high quality control levels were ensured for each plate.
- **General practice:** PCR master mix and PCR reactions were mixed by vortex, plate spinner or by pipetting up and down where appropriate. Samples were run in duplicates.

The primers and probes used for all the RT-PCRs are described in Table 2.2 and the RT-PCR thermocycler protocols are described in Table 2.1.

2.4.2.2 Zika virus RT-PCR

ZIKV RNA was amplified by RT PCR in single-plex reactions set up to 25 µl of final volume each following the previously described protocol by Lanciotti *et al., 2008* [97, 122]. ZIKV PCR primers and thermocycler conditions are shown in Table 2.1 and Table 2.2. All PCR reactions were performed using SuperScript III without Rox (Invitrogen). The following conditions were used for each ZIKV PCR reaction: 0.25 µl of enzyme mix (Taq Polymerase and reverse transcriptase), 10 µl of 2X RT-PCR buffer, 1.25 µl of forward and reverse primers (at a concentration of 10 µM), 0.5 µl of the ZIKV specific probe (at a concentration of 10 µM) and 6. 8µl of nucleic-acid-free water. Finally, in a separate room, 5 µl of positive control, negative controls and extracted patient samples are added to the mixture. Data was analysed using the RT-PCR software from Applied Biosystems. CT values<38 were classified as positive for ZIKV RNA. CT values >38 and <41 were repeated in duplicate. CT>41 were considered negative

2.4.2.3 Chikungunya Virus RT-PCR

CHIKV RNA was amplified by RT PCR in single-plex reactions set up to 25 µl of final volume each following the previously described protocol by Lanciotti *et al., 2007.* CHIKV PCR primers and thermocycler conditions are shown in Table 2.1 and Table 2.2. All PCR reactions were performed using SuperScript III without Rox (Invitrogen). The following conditions were used for each CHIKV PCR reaction: 0.25 µl of enzyme mix (Taq Polymerase and reverse transcriptase), 10 µl of 2X RT-PCR buffer, 1.25 µl of forward and reverse primers (at a concentration of 10 µM), 0.5 µl of the CHIKV specific probe (at a concentration of 10 µM) and 6.8 µl of nucleic-acid-free water. Finally, in a separate room, 5 µl of positive control, negative controls and extracted patient samples are added to the mixture. Data was analyzed using the RT-PCR software from Applied Biosystems. Ct values < 38 were classified as positive for CHIKV RNA. Ct values >38 and <41 were repeated in duplicate. Ct > 41 were considered negative

2.4.2.4 Dengue Virus RT-PCR

Multiplex dengue PCR was performed as previously described by Santiago *et al., 2013* [97] for detection of DENV-1, DENV-2, DENV-3 and DENV-4. Briefly, four-plex reactions were set up to 25 µl each. All PCR reactions were performed using SuperScript III without Rox (Invitrogen). Each multiplex RT-PCR reaction included 5 µl of RNA that were mixed with the following reagents: 2.2 µl of nuclease-free water, 12.5 µl of 2 X mix, 0.5 µl of forward and reverse primers for DENV-1 and DENV-3 (at a final concentration of 1 µM) and 0.24 µl, of forward and reverse for DENV-2 and DENV-3 (at a final concentration of 0.5 µM. Then 0.45

µl of TaqMan probe (final concentration of 180 nM) and 0.5 µl of SuperScript III RT Taq mix were added to a final volume of 25 µl. Single-plex PCR reactions for each one of the DENV serotypes with only their respective primers and controls were prepared. Reactions were placed in a thermocycler from Applied Biosystems. The fluorescence capture was set to capture emissions through the following channels: FAM, HEX, Texas Red and Cy5 in each well. Data was analyzed using the RT-PCR specific software from Applied Biosystems. Ct values < 38 were classified as positive. Ct values >38 and <41 were repeated in duplicate. Ct >41 were considered negative.

Table 2.1 Thermocycler protocols for PCR reactions.

*Denaturing, annealing and extension steps are repeated the number times as indicated in column "number of cycles". ** Extended number of cycles compared to the original protocol to allow for better visualization.

Protocol	Start temperature (°C)	Duration (min)	Inactivation (°C)	Duration (min)	Denaturing temperature (°C) *	Duration (sec)	Annealing temperature (°C)*	Duration (min)	Extension temperature(°C)*	Duration (sec)	Number of cycles**	Final hold temperature (°C)	Duration (min)
Zika RT-PCR	50	1	95	15	94	30	60	3	68	30	45	4	∞
CHIKV RT-PCR	50	1	95	15	94	30	60	3	68	30	45	4	∞
Dengue (1-4) Multiplex RT-PCR	50	1	95	15	94	30	55	1	68	120	45	4	∞

Table 2.2 Sequences and position of primers and probes used in PCR reactions

Target Pathogen	Forward Primer	Genome position	Reverse Primer	Genome position	Probe	Genome position	Fluorophore
Zika Virus	CCGCTGCCCAACACAAG	1086–1102	CCACTAACGTTCTTTTGCAGACAT	1162–1139	AGCCTACCTTGACAAGCAGTCAGACACTCAA	1107–1137	FAM
CHIKV RT-PCR	TCACTCCCTGTTGGACTTGATAGA	6856-6879	TGACGAACAGAGTTAGGAACATACC	6981-6956	AGGTACGCGCTTCAAGTTCGGCG	6919-6941	FAM
Dengue Virus 1	CAAAAGGAAGTCGTGCAATA	8936–8955	CTGAGTGAATTCTCTCTACTGAACC	9023–9047	CATGTGGTTGGGAGCACGC	8961–8979	FAM/BHQ-1
Dengue Virus 2	CAGGTTATGGCACTGTCACGAT	1426–1447	CCATCTGCAGCAACACCATCTC	1482–1504	CTCTCCGAGAACAGGCCTCGACTTCAA	1454–1480	HEX/BHQ-1
Dengue Virus 3	GGACTGGACACACGCACTCA	701–720	CATGTCTCTACCTTCTCGACTTGTCT	749–775	ACCTGGATGTCGGCTGAAGGAGCTTG	722–747	TR/BHQ-2
Dengue Virus 4	TTGTCCTAATGATGCTGGTCG	884–904	TCCACCTGAGACTCCTTCCA	953–973	TTCCTACTCCTACGCATCGCATTCCG	939–965	Cy5/BHQ-3

2.5 Statistics and data Analysis

2.5.1 Calculation of ROC Curves, sensitivity and specificity

Diagnostic performance of the ELISAs was assessed by calculating the probability of a true positive (sensitivity), a true negative result (specificity) and by generating receiver operating characteristic (ROC) curves as follows [123].

Before defining sensitivity, specificity and other statistical measures of assay performance, I believe that it is relevant to clarify a few terms used in these definitions. Regarding the terms true/false positive/negative result, the true/false part refers to the assigned classification given by the diagnostic test which can be correct or incorrect. Meanwhile, the positive/negative part actually refers to the real state of the sample from each patient (positive or negative) for the tested infection.

The sensitivity, also known as the probability of detection, it is defined as the probability of a positive result in the test for a patient who has the infection:

$$Sensitivity\ (\%) = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives} x100$$

The specificity is defined as the probability of a negative result in the test for a patient that has NOT got the infection.

$$Specificity\ (\%) = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives} x100$$

The precision or Positive Predicted Value (PPV) is the percentage of patients with a positive test who actually have the infection:

$$Precision\ or\ PPV\ (\%) = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ positives} x100$$

The Negative Predicted Value (NPV) is the probability that an infection is absent given a negative test result and can be defined as:

$$NPV\ (\%) = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ negatives} x100$$

The accuracy is defined as the overall probability that a diagnostic assay will correctly classify a patient as positive or negative for a particular infection. (no. = number). It can be defined as follows:

$$Accuracy\ (\%) = \frac{number\ of\ true\ positives + number\ of\ true\ negatives}{no.of\ true\ positives + no.of\ true\ negatives + no.of\ false\ negatives + no.of\ false\ positives} x100$$

Here, optimal cut-off values for the ELISAs were investigated to maximize the combination of sensitivity and specificity.

Cut-off values for each diagnostic assay are specific values above/below the one in which a result becomes positive or negative for each assay. One of the most commonly used methods to investigate the effectiveness of a diagnostic test is performing receiver operating characteristic (ROC) curve analysis [124]. ROC curves provide graphical visualization of the sensitivity and specificity at all the cut-off values possible for a particular test. The larger the area under the curve, the better the performance of the assay.

The sensitivity, specificity and accuracy of the assays was determined using MedCalc Statistical Software, version 16.2.0 [MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2016]). ROC curves were generated using PRISM GraphPad v7 (GraphPad Software, La Jolla, CA.

Samples with borderline antibody results were re-tested when enough reagents and sample volume were available. If samples were still borderline, they were considered negative for calculation of sensitivity, specificity and ROC curves.

2.5.2 Calculation of Kappa

Through this thesis, I will also be using the kappa statistic also known as Cohen's Kappa. Kappa is a tool that controls for random agreement factor. When no gold standard diagnostic assay exists, the Kappa coefficient is generally used [125]. It is a form of correlation for measuring agreement of two or more clinical/diagnostic categories using two or more methods. Kappa is better than % agreement because it provides the proportion of agreement after eliminating the agreement of just by chance. If Kappa equals to 0, it means the correlation is no better than chance.

Coefficient of variation was calculated as:

$$\text{Coefficient of Variation (CV)} = \frac{Standard\ Deviation\ (SD)}{Mean\ of\ the\ 5\ Green\ Bob\ Ratio\ results}\ x\ 100$$

2.5.3 Socioeconomic and sociodemographic data

In chapter 5, I compare the exposure to ZIKV and CHIKV infections among population from different socioeconomic status. The following indicators of SES, income, health, education

and income were investigated and their definition is as follows. Definitions translated and adapted from the Atlas Database Dictionary [126].

- **HDI: Human Development Index**. Geometric mean of the Indexes of Income, education and longevity with equal weights. Patients in our study were classified into 5 groups depending on their HDI between very low HDI to very high HDI as follows:
    - HDI Very high if index is between 0.85 and 1 (maximum)
    - HDI High if index is between 0.75-0.85
    - HDI Medium if Index is between 0.650-0.75
    - HDI Low if Index is between 0.550-0.650
    - HDI Very Low if index is less than 0.550
- **HDI Income or Income index**: obtained from the index of income per capita:

  $$\text{Income index} = \frac{\text{Observed value or indicator} - \text{Income (minimum value)}}{\text{Income (maximum value)} - \text{Income (minimum value)}}$$

  In which the minimum and maximum income values adapted in August 2010 were: R$ 8.00 e R$ 4033.00 (Brazilian Reals)

- **HDI Longevity or longevity index**: Geometric mean of life expectancy at birth index following this calculation:

  $$\text{Longevity index} = \frac{\text{Observed value or indicator} - \text{minimum value}}{\text{maximum value} - \text{minimum value}}$$

  Minimum and maximum value are calculated for 25 and 85 years.

- **HDI Education or Education Index:** Composite of education within the HDI calculation. Geometric mean of schooling frequency (2/3) and sub-index of school levels (1/3).
- **Index of school attendance**: Frequency of young population (<18years) attending school. It is calculated with the arithmetic mean of % children 5-6 years old at school, % children 11-13 years school with basic school completed, % young 15-17 years old with basic school completed and % 18-20 years old with basic school completed.
- **Schooling Index:** Index of adult population (>18years) with basic education completed.
- **Child Mortality:** Number of children who are not expected to survive the first year of life (calculated per 1000 children born alive).
- **Life expectancy at birth:** Mean of years that someone is expected to live the year when the census was done.

- **Unemployment rate** (%): Proportion of the economically active population (EAP) over 18 years old with no job (at least up to a week before the Census was done in 2010) and that were looking for a job during the previous month before the Census was conducted.
- **Percentage of extremely poor** (%): Proportion of individuals with a per capita household income under R$70 per month (calculated in August 2010). This is calculated among individuals with a permanent residence.
- **Gini Index** of inequality: Measure of the degree of inequality in the distribution of individuals in terms of the household income per capita. The Gini index equals 0 when there are no inequalities (household income is the same among all individuals in a particular area). The highest inequalities will tend to 1. It only measures individuals with particular permanent households.
- **The Per Capita Income (PCI)** or average income measures the average income earned per person in a given area (city, region, country, etc.) in a specific year. It was measured in Brazilian Reals in August 2010.

2.5.4 Statistical methods and graphical representations

Statistics were performed using PRISM GraphPad version 6.0 and Microsoft Excel. A one way ANOVA was used to assess the differences among groups. Non-parametric Mann–Whitney U tests were used to determine differences among two groups. A value was considered statistically significant if P-value<0.05. This was represented as: * = p-value<0.05; **=p-value<0.005 and ***=p-value<0.0005.

Figures and graphics were generated using PRISM GraphPad. Diagrams were generated using Microsoft Power Point and Inscape version 0.92.3.

CHAPTER 3.

Evaluating and optimizing diagnostic assays to improve antibody detection of Zika Virus and understanding immune responses to Zika virus

Chapter 3: Evaluating and optimizing diagnostic assays to improve antibody detection of Zika Virus and understanding immune responses to Zika virus

3.1 Overview Chapter 3

When I first arrived Brazil in March 2016 and held our first few meetings with the team of experts in the Zika Reference Laboratory in Rio de Janeiro (IOC, Fiocruz, Brazil), I found that one of the most urgent needs was to evaluate and improve the serological diagnostics for ZIKV. Alarm had spread across the population following the sensationalist messages expressed in the local newspapers and TV. As a result of this, a large number of individuals, including pregnant woman, were presenting to their physician and health centres wanting to know if they had suffered Zika. Due to the mild nature of this illness many were presenting a few days following symptom onset. Moreover, in many neurological cases, with a suspected association with ZIKV, symptoms began over a week following the presentation of the acute rash-fever. I realised serological diagnostic assays were key to the public health response in this outbreak and this became my priority. At that point, there was no published evidence on the evaluation of these methods and only a few assays were commercially available. Therefore, I set as my first objective, to evaluate the accuracy of the serological methods used to diagnose ZIKV infections following acute phase of infection.

Then, as a second objective, I sought to further investigate if newer methods could help to resolve the ongoing challenge of providing accurate diagnostics for ZIKV infections.

Thus, I aimed to optimize and evaluate another two different ELISA assays used to detect ZIKV antibodies and a neutralization assay, the plaque reduction neutralization tests (PRNT). Additionally, I also investigated the use of assays with different formats including a lateral flow assay for use as a Rapid Diagnostic Test (RDT) and two immunofluorescence assays.

3.2 Introduction

Zika virus (ZIKV) diagnostics underpin all public health responses to the outbreak and accurate results are essential to guide appropriate clinical management of suspected patients, particularly of vulnerable groups such as patients affected by neurological disorders and gestating women. Both false negative and false positive diagnosis may trigger

catastrophic consequences, especially amongst pregnant women. As a demonstration of this, during the explosion of the ZIKV epidemic, it has been suggested that the rate of illegal abortions increased, with enormous health risks that this entails for pregnant woman [127]. During the ZIKV outbreak, governments from countries such as Colombia, Jamaica and Ecuador advised women to avoid becoming pregnant for a period of months to over a year. This is impractical and neglectful advice because around 40% of pregnancies worldwide are unplanned [128]. Abortion is also illegal or highly restricted in many countries affected by Zika virus, leaving vulnerable women with few options. This led to an increase in illegal abortions in Latin America, with severe risks for maternal health [127].

In 2016, both the WHO and the American Centres for Disease Control and Prevention (CDC) issued laboratory diagnostic algorithms which recommend serological testing for patients presenting a few days after symptom onset when molecular detection of ZIKV was reported to become less sensitive [129, 130]. However, accurate detection of ZIKV antibodies can be very challenging. We still have limited understanding of the antibody responses to ZIKV in different populations. This is a particular concern in Latin America where extensive co-circulation of other flaviviruses has occurred over the last 30 years [131]. Recent studies have shown that over 50% of pregnant Brazilian women have been reported as anti-DENV Immunoglobulin G (IgG) positive [132]. In my research, I have shown that this percentage of IgG positivity among the population may be higher than 80% (See *Chapter 5*). Similarly, yellow fever vaccination has been reported to have good coverage in most regions of Brazil, especially after the recent yellow fever outbreaks [133, 134].

In 2016, the WHO emphasised the need for field validation of available serological tests [135]. This prompted prioritisation of the research described in this chapter including search, selection and collection of adequate panels and reagents during my first months in Brazil.

In April 2016, the first validation study evaluating ZIKV commercial assays was published in Eurosurveillance [106, 136, 137]. They reported “high specificity and sensitivity” of the commercial IgM and IgG NS1 ZIKV ELISAs (Euroimmun, Germany) using mainly samples from returned travellers for their evaluation. However, this was a relatively small study that lacked samples collected systematically from flavivirus exposed populations.

In early 2016, the Food and Drug Administration (FDA or USFDA) from the United States Department of Health and Human Services issued an Emergency-Use-Authorization (EUA) protocol for the MAC ELISA assay developed by the CDC. Their emergency use authorization

protocols are generally intended to "*make available diagnostic and therapeutic medical devices to diagnose and respond to public health emergencies*" [138].

The CDC declared that the ZIKV MAC –ELISA "*is intended for the qualitative detection of Zika virus IgM antibodies in human sera or cerebrospinal fluid (CSF) that is submitted alongside a patient-matched serum specimen, collected from individuals meeting CDC Zika virus clinical criteria (e.g., clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika virus transmission at the time of travel, or other epidemiologic criteria for which Zika virus testing may be indicated).*" Since then, most routine Zika Reference Laboratories in Brazil began to implement this assay.

3.2.1 Cross–reactivity among flaviviruses

One of the main challenges of flavivirus antibody detection is the cross-reactive antibody responses generated following individual flavivirus infection.

It is well established that the immune responses generated following flavivirus infection contain both type specific and cross reactive antibodies. Published reports from the 1970s demonstrated flavivirus cross-reactive antibody responses using macrophage assays in animal models [139]. Several studies have investigated the nature of these cross-reactive responses, with the objective of improving the specificity of serological diagnosis of flaviviruses, with a particular focus on DENV [140]. Additionally, cross-reactive responses have also been investigated for other flaviviruses such as yellow fever (YF), Tick-Borne-Encephalitis (TBE) and West Nile Virus (WNV) [141]. To date, diagnosis and surveillance for most of these pathogens are still based in assays detecting IgM or IgG antibodies.

To better understand the ZIKV specific antibody responses in flavivirus exposed populations, it is important to consider if ZIKV elicits a phenomenon called Antibody-Dependent Enhancement (ADE) responses. ADE occurs as a result of a secondary flavivirus infection and it has been extensively described for sequential dengue infections (Figure 3.1). This phenomenon is mediated through disease-enhancing activity of cross-reactive antibodies [142].

It occurs when antibodies present in the body from an earlier infection (for example a primary dengue virus infection), bind to the dengue virus particle when a second infection with a different DENV serotype occurs [143]. As the pre-existing antibodies from the primary infection cannot neutralize the new Dengue virus, the newly formed complex, which is

composed of the antibody + virus particles attaches to circulating monocytes receptors (Fcy receptors). Thus, antibodies from the primary DENV infection help the second DENV to infect monocytes in a much more efficient way; this contributes to an increase in the overall replication of the virus and to a higher risk of suffering severe complications of dengue such as dengue haemorrhagic fever (DHF). This has been shown in several studies which demonstrate that levels of dengue antibodies in children with previous DENV exposure set them to a higher risk of developing a severe dengue disease [144]. It has been hypothesised and investigated that ADE may play a role in ZIKV antibody responses. Recent in vitro studies have suggested that ZIKV elicits an ADE response in DENV exposed models [145].

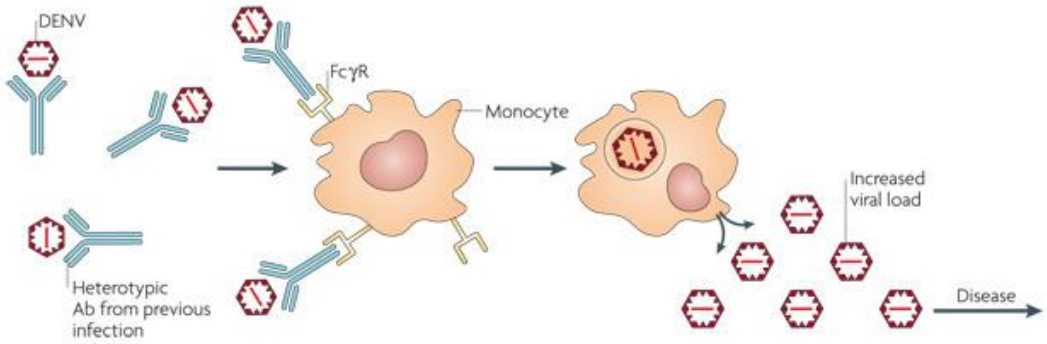


Figure 3.1 Model of antibody-dependent enhancement (ADE) of dengue infection
The host antibodies bind to the surface proteins of the flavivirus but do not inactivate the virus. The immune response attracts numerous macrophages, which the virus is able to then infect because it has not been inactivated. Image adapted from Whitehead, S. S. *et al*. (2007) [146]

3.2.2 Targets for ZIKV serological diagnosis

Understanding the types and degree of cross-reactive responses is key to design appropriate diagnostic assays. The Non Structural protein 1 (NS1 protein) has been used extensively as a target for DENV and WNV assays and it has been the main target in many of the diagnostic assays that I have investigated (IgM μ-capture NovaTec ELISA and IgM and IgG NS1 Euroimmun). The NS1 is a dimer protein with the molecular weight ranging between 46 to 55 kDa that plays a key role in diagnosis, viral replication, pathogenesis and protection. Interestingly, in DENV infections, the NS1 is found both as a secreted lipoparticle and associated with the cell membrane [147]. In recent studies, it has been shown that NS1 has some features that are conserved among flaviviruses, as well as key differences such as its electrostatic characteristics [148]. In contrast, other assays, such as the CDC-MAC ELISA, contain the whole virus and in particular the envelope (E) glycoprotein. The flavivirus E

protein (which is an external structural protein) mediates fusion processes and is one of the main targets for neutralizing antibodies [149]. Recently, a high level of structural similarity between the E proteins of ZIKV and other flaviviruses such as DENV, yellow fever and others has been demonstrated [150, 151].

3.2.3 The context of the development of the ZIKV blockade –of-binging (BOB) assay

The development of the BOB assay started with a key study aiming to better understand the ZIKV antibody responses. Sttetler *et al.* (2016), managed to isolate 119 monoclonal antibodies from 4 ZIKV infected patients, (two of them DENV naïve, and two DENV immune) [152]. Then, they selected the antibodies that bound to both the ZIKV NS1 and/or the ZIKV E proteins. They selected the antibodies that had the capacity to neutralize ZIKV infection. Then, these antibodies were compared with a panel of Monoclonal antibodies previously isolated from DENV infected donors. Of the ZIVK reactive antibodies isolated – 41 bound to NS1. ZIKV and DENV NS1 share over 50% amino acid identity [152]. It is noteworthy that the antibodies isolated from DENV naïve individuals differed from those from DENV immune individuals. Interestingly, they also noted that these antibodies isolated from individuals exposed to ZIKV infection and DENV naïve, were to a large extent ZIKV-specific and did not cross-react to DENV NS1. Due to their interest to develop diagnostic assays that could discriminate DENV type specific antibodies, they identified antigenic sites on the NS1 region that were targeted by ZIKV but not by cross-reactive monoclonal antibodies (Figure 3.2). Then, the monoclonal antibodies to two sites of ZIKV NS1 including monoclonal antibody ZKA35 were used as a probe in a blockade-of-binding (BOB) assay which detected ZIKV antibodies specifically but not DENV-specific serum antibodies to NS1. This showed the potential to develop this antibody into a diagnostic assay, which subsequently was the basis of the ZIKV BOB assay. [116]

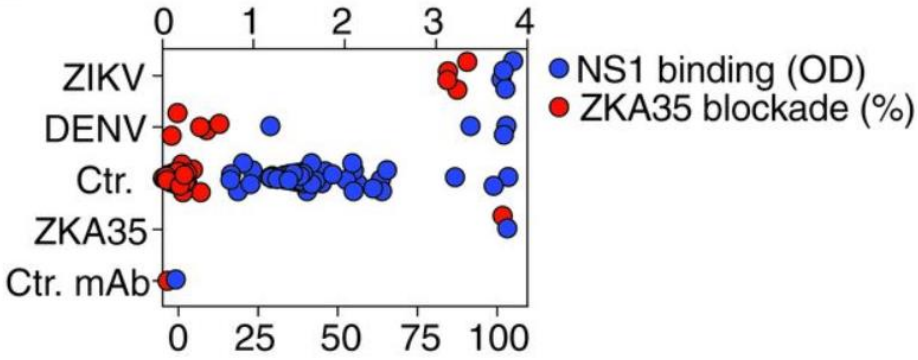


Figure 3.2 Principle on which the Blockade-of-binding (BOB) ZIKV assay is based
Plasma from ZIKV infected patients tested for their capacity to bind NS1 and to inhibit the binding of the ZKA35 to NS1. Red dots show plasma inhibiting the binding of the biotinylated mAb ZKA35 to NS1. Blue dots show the capacity to bind to NS1. Subjects included: 4 ZIKV infected, 5 DENV infected and 45 control donors. Image adapted from Stettler *et al*. Science (2016*)*

Then a modified version of the ZIKV BOB assay was developed: The Green BOB assay. The Green BOB assay is a Zika competitive enzyme-linked immunoabsorbent assay that detects antibodies specifically to the NS1 region of the ZIKV developed by PHE in collaboration with Humabs.

3.2.4 Plaque reduction neutralization tests (PRNTs)

The plaque reduction neutralization test (PRNT) is a neutralization assay that can be used to quantify virus-specific neutralizing antibodies. The PRNT assay was first described in the 1950s, and was later adapted for dengue virus infections by Russell *et al.,* 1967 [118]. PRNTs are currently considered the "gold standard" method for detection and quantification of the circulating levels of anti-ZIKV neutralizing antibodies. However, PRNT specificity may be affected by the production of flavivirus' cross-reactive neutralizing antibodies, especially after multiple DENV infections. This phenomena is common in a country such as Brazil where the four serotypes of Dengue have been circulating for over three decades [153]. Many variations of the PRNT assay are in current use and neither the assay nor its performance conditions have been standardised or harmonised yet. Guidelines on the conduct of flavivirus PRNT have been published by the World Health Organization (WHO) [154].

3.2.5 IFA assays

Immunofluorescence assays (IFA) have been used for flavivirus diagnosis for decades. They can be used to differentiate the IgM and IgG responses to flaviviral infections [155]. The assay generally involves using test plates or slides with immobilised cells infected with virus attached by chemical fixation. The patient serum samples are incubated on a slide. Then, if

a patient's virus-specific antibodies against the antigen are present in the sample they will be detected with a fluorophore-conjugated anti-IgM or IgG immunoglobulin.

There are two main classes of IFA techniques: primary or direct and secondary or indirect fluorescence (Figure 3.3). Indirect assays are widely used for flavivirus diagnosis. They use two antibodies: a primary antibody which specifically binds to the flavivirus antibodies, and the secondary antibody, which carries the fluorophore, recognises the primary antibody and binds to it. Some of the advantages of this type of assays is that they are easy-to-perform and diagnostic results can be obtained within a few hours.

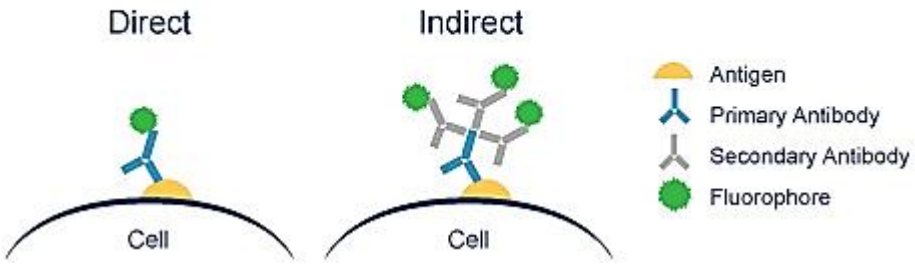


Figure 3.3 Diagram showing Immunofluorescence assays (IFA)
There are two classes of IFA assays: primary (direct) and secondary (indirect). Image adapted from www.Abcam.co.uk/

3.2.6 Point-of-care testing (POCT)

Point-of-care testing (POCT), also known as bedside testing are diagnostic devices that can be performed near the place where the patient is receiving care and take 20-30 mins to complete, which enables testing to be conducted in primary health centres. Enormous international efforts have been conducted to try developing POCT for diagnoses of emergent viruses including ZIKV. A POCT for accurate detection of both ZIKV IgM and IgG antibodies is needed to perform a rapid diagnosis at the bedside. This would facilitate a rapid diagnosis and contribute to patient management.

3.3 Aims of this chapter

Here, I conducted an evaluation using well-characterised panels of ZIKV and non-ZIKV clinical samples from subjects from Brazil to determine the sensitivity and specificity of different assays for ZIKV diagnosis. My main goals in this chapter are:

1) To report assay accuracy for four ZIKV serological assays among the Brazilian population
2) To examine the time window of detection of ZIKV IgM antibodies
3) To explore the biological variability of antibody responses
4) To better understand the effects of the cross-reactivity between Zika and dengue viruses

Then, I further tried to improve the diagnosis of ZIKV by aiming the following:

5) Optimising and evaluating a novel ZIKV blockade-of-binding (BOB) assay for serological diagnosis of ZIKV and a modified version called the Green BOB assay
6) Optimizing and evaluating PRNT assays in the Brazilian population and other non-ELISA methods for ZIKV diagnosis including two Immunofluorescence assays (IFAs) and a POCT for ZIKV IgM and IgG detection.

3.4 Materials and Methods

3.4.1 Ethics statement

The procedures that I employed in this chapter were carried out in accordance with the ethical standards of the Instituto Nacional de Infectologia Evandro Chagas (Brazil). The study protocol was approved by its Comitê de Ética em Pesquisa (reference CAAE 0026.0.009.000–07).

3.4.2 Study population and sample selection - Cohort tested in Brazil

The evaluation of the Zika and dengue assays was conducted at the reference Flavivirus Laboratory at the Institute Oswaldo Cruz (IOC) in Rio de Janeiro (Brazil). In order to fully understand the specificity and sensitivity of the assays, it was crucial to design a comprehensive panel with confirmed ZIKV positive samples and known non-ZIKV control samples. Specimens were submitted for routine diagnostics to one of the following reference laboratories within IOC: the Regional Flavivirus Reference Laboratory, National Influenza and Measles Reference Laboratory and the Regional Hepatitis Reference Laboratory.

In total, 405 sera specimens from 307 participants from the State of Rio de Janeiro that had their samples submitted to the reference laboratories at the Institute Oswaldo Cruz (Brazil) were analysed in three different cohorts. Further details can be found in Table 3.1.

Sensitivity was assessed using 169 sera specimens from individuals reporting rash-fever symptoms and with positive detection of ZIKV RNA by RT-PCR (which was considered the

gold standard method). Specimens were collected during 2015 and 2016 coinciding with the peak of the ZIKV epidemic in Rio de Janeiro (cohort 1, n=71). For most of the patients, two or more sequential serum samples were collected (Table 3.2). Zika virus positive cases and non-ZIKV cases were both distributed between male and female with 55.4% and 44.6% respectively in the study population. Age distribution was unimodal and the median age of the individuals was 24.5. Among the ZIKV confirmed group, the median day of sample collection was day 7 (range 1 - 276). Eighty percent of the samples from ZIKV positive individuals were collected between 1 and 20 days after symptom onset.

Assay sensitivity was also investigated for the cohort divided into three groups depending on the number of days post-symptom onset when the sample was collected: 1 to 5 days (Acute-phase), 6-13 days (Early convalescent-phase) and >14 days (late convalescence-phase).

I assessed the specificity of the assays using a non-ZIKV control panel that includes sera from individuals collected in/or before 2013. As the ZIKV epidemic was reported to have started in Rio de Janeiro in 2015 (Cohort 2 and 3) [156]. The non-ZIKV control panel included Cohort 2 and Cohort 3. Cohort 2 included a total of 184 subjects with confirmed (PCR and IgM positive) exposure to dengue (serotypes 1-4 [n=90]), yellow fever vaccination (n=19), measles or Rubella (n=40) or other infections / control population (n=35). DENV confirmed cases with both clinical manifestations of symptoms and laboratory confirmation with both positive IgM DENV antibodies and RT-PCR positivity for the serotypes DENV1 (21), DENV2 (17), DENV3 (21) and DENV4 (31). Cohort 3 was composed of 52 subjects that represent a control population attending a hepatitis clinic in Fiocruz in 2013.

Serum specimens that had been stored at a minimum temperature of -20 ^{o}C and had no history of repeated freeze-thaw cycles were used. Due to limitations on reagents availability and volume restrictions, not all the samples were tested in all the assays.

Chapter 3

Table 3.1 Characteristics of the study population

Main characteristics of the study population used in this study. Total number of samples, proportion of male/female and proportion of samples included for each group. Abbreviations: No., number, Year, Year of sample collection, *Sex was not documented for 18 patients. The population data for the subgroups denoted by roman superscript letters [a] through [e] add up to 100%: a. represents subjects in Cohort 1, 2 and 3. b. Subjects in cohort 1. c. Dengue Positive subjects. d. Flavivirus positive subjects. e. Non flavivirus infections subjects.

		No. of subjects	No. of samples	% of subjects	Year
Total		**307**	**405**	**-**	**2002-2016**
Sex	Female	164	**-**	55.4%	-
ZIVK Positive (Cohort 1)					
	Total ZIKV	**71**	**169**	**23.1 (71/307)[a]**	**2015-2016**
	1 Sample	5	5	7.0 (5/71)[b]	2015-2016
	2 Sample	55	110	77.5(55/71)[b]	2015-2016
	3 or more samples	11	54	15.5(11/71)[b]	2015-2016
Controls – ZIKV Negative (Cohort 2)					
	Total ZIKV Negative	**184**	**184**	**59.9(184/307) [a]**	**2002-2013**
Flavivirus Positive (DENV& yellow fever)		109	109	59.2(109/184)[e]	2002-2013
DENV positive		90	90	82.6 (90/109)[d]	2002-2013
	DENV 1	21	21	23.3(21/90)[c]	2010-2011
	DENV 2	17	17	18.9(17/90)[c]	2008,2010,2011
	DENV 3	21	21	23.3(21/90)[c]	2002,2007,2008
	DENV 4	31	31	34.4(31/90)[c]	2012,2013
Yellow fever		19	19	17.4(19/109)[d]	2003-2007
Measles or Rubella		40	40	21.7(40/184)[e]	2011-2012
Other non-flavivirus infections / Population controls		35	35	19.0(35/184)[e]	2011-2012
Control population (Cohort 3)	**Total Cohort 3**	**52**	**52**	**16.9(52/307) [a]**	**2013**

Table 3.2 Breakdown of serial collections of the ZIKV study population.
Number of samples collected for each subject in the ZIKV Panel (Cohort 1). At least the first serum collected for each subject was ZIKV PCR positive. Collection: number of sequential serum specimens

Zika Panel (Cohort 1)	ZIKV Positive Subjects	Total Samples	Year of Collection
1 Collection	5	5	2015-2016
2 Collections	55	110	2015-2016
3 Collections	7	18	2015-2016
>4 Collections	4	33	2015-2016
TOTAL	71	169	2015-2016

3.4.3 Study population – Cohort tested in Liverpool

The PRNT methods were optimized in Liverpool with a panel of 16 samples from well-characterised subjects (n=15). Patients had different levels of exposure to Dengue and Zika virus. The set included 1 ZIKV PCR confirmed specimen (52 years old, British male, returned from Mexico). This sample had the following antibody levels: Zika IgG Positive (Index value 2.278)/ Zika IgM Positive (Index value 4.485). The cohort also included 1 subject who was also positive for two different DENV serotypes (OX08) (male, British-Indian) and 2 sera from a female volunteer (25 years) taken before and after travelling to a ZIKV exposed area (Brazil) (Sample LIV14). The other 12 subjects were non-ZIKV exposed healthy volunteers.

3.4.4 Serological Diagnostic Assays

3.4.4.1 The IgM and IgG ZIKV NS1 ELISA assays

The IgM and IgG NS1 anti-ZIKV ELISAs from Euroimmun (Lubeck, Germany) use recombinant Zika non-structural protein 1 (NS1) as the ZIKV antigen. This is an indirect, colourimetric ELISA that detects IgM human antibodies generated against ZIKV infection. I performed the procedures following the manufacturer's instructions as described in Chapter 2.

3.4.4.2 IgM ZIKV μ-capture ELISA.

The Novagnost® Zika Virus IgM μ-capture ELISA (NovaTec Immunodiagnostica GmbH, Germany) is based on the ELISA μ-capture technique. It uses ZIKV NS1 as antigen. Protocol described in further detail in Chapter 2.

3.4.4.3 IgM CDC MAC ELISA

The FDA CDC-designed IgM antibody capture ELISA also known as the ZIKV MAC ELISA was performed as previously described by using the US Centers for Disease Control and

Prevention (CDC) emergency use authorization protocol (CDC Fort Collins, CO, USA)[115]. Results were reported as recommended by the CDC protocol as positive if $\frac{P}{N}$ ≥3; equivocal if f $\frac{P}{N}$ ≥2 but $\frac{P}{N}$ <3 and negative if f $\frac{P}{N}$ <2.

3.4.4.4 DENV IgM capture ELISA

For the qualitative detection of DENV IgM antibodies Panbio dengue IgM capture ELISA (Cat. No. E-DEN01M, Alere, UK) was performed following the manufacturer's manual. Further details are shown in Chapter 2..

3.4.4.5 IgG Indirect DENV ELISA

For detection of DENV IgG antibodies, the IgG DENV indirect ELISA (Cat. No. E-DEN02G) (Panbio, Alere, United Kingdom) was performed. The manufacturers indicate that the assay is able to determine levels of IgG antibodies against the four dengue serotypes (DENV-1 to 4). The assay was performed according to the manufacturer's instructions and a further detailed protocol is shown in Chapter 2.

3.4.4.6 BOB Assay

The Blockade-of-binding (BOB) assay is an NS1-based competition ELISA using a Zika specific monoclonal antibody developed as described in [116]. This assay is based on a competitive principle. Thus, when no anti-ZIKV NS1 antibodies are present in the sample, the enzyme labelled Mab will bind and there will be a chromogenic reaction. A percentage of inhibition of 50% was determined to be the best threshold for this assay. Patients exhibiting higher percentages of inhibition than 50% are considered positive for IgG ZIKV antibodies. More details are described in Chapter 2.

3.4.4.7 Green BOB Assay

The Green BOB assay is a Zika competitive enzyme-linked immunoabsorbent assay that detects antibodies specifically to the NS1 region of the ZIKV. It has been developed by collaborators in Public Health England as a modification of the Blockade-of-binding (BOB) assay. The Green BOB assay is based on an ELISA format and it also uses the same ZIKA35 mAb as described above. The main differences between BOB and Green BOB assays are the following:

1- Plates are pre-coated with NS1 ZIKV Antigen.
2- Serum concentration is modified to reduce the amount of serum required.
3- The concentrations of the reagents had been adjusted

4- No previous serum lysis step or serum dilution step is needed, aiming to reduce the time of the assay.
5- No incubation step of the serum in the plate before adding the Anti-ZKA35 mAb.
6- The assay can be performed in only one day and it can be completed in less than 4 hours.
7- The Anti-ZKA35 Monoclonal antibody is conjugated to HorseRadish Peroxidase (HRP).
8- The substrate used is 3,3',5,5'-Tetramethylbenzidine (TMB) instead of p-Nitrophenyl Phosphate (pNPP).

The Green BOB is based on a one-step incubation competitive protocol. If the Anti-ZIKV antibodies are present in the sample, they will compete with the monoclonal anti-ZIKV antibodies that are conjugated to HRP for the fixed amount of recombinant ZIKV NS1-antigen that is already pre-coated in the plate wells. For a graphical visualization of the assay, and further details on the protocol, see Chapter 2.

3.4.4.8 Plaque Reduction Neutralization Tests (PRNTs)

The protocol I used was adapted from protocol used for DENV and JEV PRNT in the Liverpool group, based on the previously described protocol [118]. Testing was conducted both in Liverpool and in Brazil. Further details on the protocol can be found in Chapter 2.

3.4.4.9 Single-use point-of-care (POC) assays for ZIKV diagnosis (DPP® Zika IgM/IgG)

A Rapid Diagnostic Test (RDT) for ZIKV diagnosis for use with an automatic reader. The assay was designed to support the diagnosis of ZIKV in primary care centres in order to reduce the delay in the response to patients suspected to have ZIKV.

3.4.4.10 Indirect immunofluorescence test for Zika Virus detection

The indirect immunofluorescence test (IIFT) based on virus-infected cells offers an alternative sensitive screening assay for ZIKV antibodies. I evaluated the first commercial immunofluorescence test for detection of antibodies against Zika virus. It is based in a BIOCHIP combination, which aims to differentiate Zika, dengue and chikungunya antibodies in serum samples. It is produced by Euroimmun (Lubeck, Germany) and their evaluations report excellent specificity and sensitivity (>90%). The two assays evaluated are:

3- **IIFT Arbovirus Fever Mosaic 2.** Designed for specific detection of 6 pathogens, including ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV.

4- **IIFT Arbovirus Profile Mosaic 3.** Designed for specific detection of 10 pathogens, including the 6 above mentioned in the IIFT Mosaic 2 [ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV] but also Tick-Borne Encephalitis Virus (TBEV), Yellow Fever Virus (YFV), West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV).

The detailed protocol for both assays is shown in Chapter 2.

3.4.5 Molecular Diagnostic Assays

Molecular detection of pathogen RNA was performed for ZIKV and DENV though RT-PCR.

3.4.5.1 RNA extraction

The individuals in the ZIKV panel with clinical presentation indicative of recent ZIKV infection (during 2015 and 2016) were tested for detection of ZIKV nucleic acid material. RNA was extracted using the QIAamp Mini Elute (Qiagen, Brazil) following the manufacturer's instructions as described in Chapter 2.

3.4.5.2 RT-PCR

RT-PCR reactions were conducted for detection of specific RNA from ZIKV, DENV1, DENV2, DENV3 and DENV4. The primers and probes used for all the RT-PCRs and the RT-PCR thermomixer protocols are described in Chapter 2.

3.4.6 Calculation of ROC Curves, sensitivity and specificity

Diagnostic performance of the ELISAs was assessed by calculating the probability of a true positive (sensitivity), a true negative result (specificity) and by generating receiver operating characteristic (ROC) curves as described in Chapter 2 [123].

The optimal cut-off values for the ELISAs were investigated to maximize the combination of sensitivity and specificity. ROC curves provide graphical visualization of the sensitivity and specificity at all the cut-off values possible for a particular test. The larger the area under the curve, the better the assay performance. The sensitivity, specificity and accuracy of the assays was determined using MedCalc Statistical Software, version 16.2.0 [MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2016]). ROC curves were generated using PRISM GraphPad v7 (GraphPad Software, La Jolla, CA).

Samples with borderline antibody results were re-tested (when enough reagents and sample volume were available). If samples were still borderline, they were considered negative for calculation of sensitivity, specificity and ROC curves.

In this chapter, I will also be using the kappa statistic also known as Cohen's Kappa. Kappa is a tool that controls for random agreement factor. The Kappa statistic varies from 0 to 1, where:

0 = agreement equivalent to chance.
0.1 – 0.20 = slight agreement.

0.21 – 0.40 = fair agreement.

0.41 – 0.60 = moderate agreement.

0.61 – 0.80 = substantial agreement.

0.81 – 0.99 = near perfect agreement

1 = perfect agreement.

Kappa is described in further detail in Chapter 2. The Coefficient of Variation (CV) for the Green BOB assay was calculated as follows:

Coefficient of Variation (CV) = $\frac{Standard\ Deviation\ (SD)}{Mean\ of\ the\ 5\ Green\ Bob\ Ratio\ results}\ x\ 100$

3.4.7 Statistical methods and graphical representations

Statistics and figures were generated using PRISM GraphPad. A one way ANOVA with repeated measures was used to assess the differences among more than two groups. Non-parametric Mann–Whitney U tests were used to determine differences among two groups.

3.5 Results

3.5.1 Performance of diagnostic assays

3.5.1.1 Performance of the IgM NS1 ELISA:

Overall, sensitivity, specificity and accuracy of the IgM NS1 ELISA was 22.0% (95%CI 16.11-29.2), 96.4% (92.3-98.7) and 59.2% (53.9-64.4) respectively (Table 3.3).

Sensitivity varied relative to the number of days after onset of acute illness the samples were collected. Samples collected 1-5 (n=75); 6-13 (n=45) and ≥ 14 days (n=47) after acute illness exhibited the following sensitivity: 9.3%, 37.7% and 27.6% respectively (Table 3.3). Antibody levels were significantly lower in samples collected during day 1-5 following symptom onset compared with samples collected at early convalescence and late convalescence phase (Figure 3.4).

Specificity varied between patient groups. The lowest specificity was observed among patients with measles or rubella (87.5% with 95% CI of (77.3-97.8). A total of 6/166

specimens from Cohort 2 exhibited positive results for the IgM NS1 ZIKV assay: five measles and one hepatitis serum collected in 2011 and 2012.

3.5.1.2 Performance of the IgG NS1 ELISA:

Overall, sensitivity, specificity and accuracy of the IgG NS1 ELISA was 67.9% (95%CI 60.2-74.8), 77.6% (71.6-84.3) and 72.5% (67.6-77.4) respectively (Table 3.3).

Sensitivity varied relative to the number of days after onset of acute illness the samples were collected. Samples collected 1-5 (n=75); 6-13 (n=46) and ≥ 14 days (n=47) after acute illness exhibited the following sensitivity: 45.3%, 80.4% and 91.5% respectively (Table 3.3). Antibody levels were significantly lower in samples collected during day 1 to 5 following onset of symptoms compared with samples collected at early convalescence and late convalescence phase (Figure 3.4).

Specificity varied between patient groups. The lowest specificity was observed among subjects with DENV-3 (42.9% with 95% CI of 21.7-64.0). Due to this poor specificity, I tested an additional 52 specimens from a control population (Cohort 3) which exhibited a similar specificity of 63.5% (33/52 samples tested scored negatively).

3.5.1.3 Performance of the combined IgM and IgG NS1 ELISAs

The results obtained combining the IgM and IgG assay were calculated considering positive any sample that showed positive ratios with either of the IgM or IgG NS1 commercial ELISAs (as recommended by the manufacturers). Overall sensitivity increased to 72.2% (65.5-78.9), while specificity decreased to 74.6% (67.4-81.9) and showing over 25.4% of false positive and 27.8% of false negative results. In future outbreaks in Brazil, a proportion of the population will have already acquired ZIKV antibodies, and therefore, the IgG testing for ZIKV will not help distinguishing recent from past ZIKV exposures.

Table 3.3 Sensitivity, specificity and accuracy of the IgM NS1 and the IgG NS1 commercial ELISAs.
Data from ZIKV-positive cases served only for determining the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative subjects (cohort 2 and 3) serve only for determining the specificity and were not used for calculating the sensitivity. Equivocal Results were considered negative for the calculation. Non-flavivirus include the measles & rubella specimens, hepatitis and control population samples. DENV (all) includes all DENV samples (DENV 1-4). Abbreviations: CI, Confidence Interval, PPV., Positive Predictive Value., NPV., Negative Predictive Value., Days: number of days the sample was collected after symptom onset.

	IGM NS1 ELISA			IGG NS1 ELISA		
	Tested (n)	**% Sensitivity (95%CI)**	**% Specificity. (95% CI)**	**Tested (n)**	**% Sensitivity (95%CI)**	**% Specificity (95% CI)**
Zika Positive	**167**	**22.1 (15.8-28.4)**		**168**	**67.9 (60.8-74.9)**	
Zika (1-5 Days)	**75**	9.3 (2.7-15.9)		75	45.3(34.1-56.6)	
Zika (6-13 Days)	**45**	37.7 (23.6-51.9)		46	80.4 (68.9-91.9)	
Zika (>14 Days)	**47**	27.6 (14.8-40.4)		47	91.5 (83.5-99.5)	
Non ZIKV	**166**		**96.3 (93.5-99.2)**	**204**		**77.6 (71.0-84.3)**
DENV (all)	89		100.0(100.0-100.0)	89		76.40 (67.6-85.2)
DENV1	21		100.0(100.0-100.0)	21		95.2 (86.1-100.0)
DENV2	17		100.0(100.0-100.0)	17		76.4(56.3-96.6)
DENV3	21		100.0(100.0-100.0)	21		42.9(21.7-64.0)
DENV4	30		100.0(100.0-100.0)	30		86.6(74.5-98.8)
Yellow fever	19		100.0(100.0-100.0)	19		84.2(67.8-100.6)
Non-flavivirus	58		89.7 (81.8-97.5)	44		77.2(64.9-89.7)
Measles & Rubella	40		87.5 (77.3-97.8)	17		94.1 (82.9-105.3)
Hepatitis	13		92.3 (77.8-100.0)	13		69.2(44.14-94.3)
Other	5		100.0(100.0-100.0)	14		64.3(39.19-89.4)
Control Population(Cohort 3)	-			52		63.5 %(48.9-76.4)
Overall	**333**	**22.0 (16.11-29.2)**	**96.4 (92.3-98.7)**	**320**	**67.9(60.2-74.8)**	**77.6(71.2-84.3)**
PPV		**86.0(75.7-96.4)**			**77.0(70.2-83.8)**	
NPV		**55.2(49.4-60.9)**			**68.6(61.7-75.5)**	
Accuracy		**59.2(53.9-64.4)**			**72.5(67.6-77.4)**	

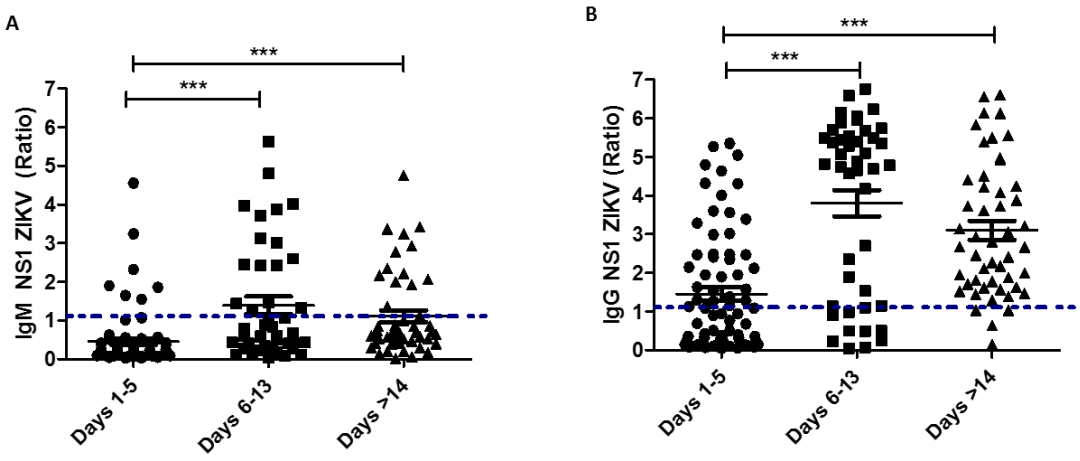


Figure 3.4 Antibody levels among the ZIKV specimens with the IgM and IgG NS1 ELISAs
Antibody levels observed for the acute (1-5), early-convalescent phase (6-13) and late convalescent-phase (>14) ZIKV specimens obtained with the IgM and IgG NS1 commercial assays. A) Antibody levels tested with the IgM NS1 Zika ELISA. B) Antibody levels tested with the IgG NS1 Zika ELISA. The dotted horizontal lines represent the cut-off value for each method which is Ratio=1.1. Dots over the dotted line represent positive samples. Results are shown in Ratios for each assay. ***=p-value<0.0005

3.5.1.4 Performance of the CDC MAC ELISA

The MAC-ELISA is a capture ELISA assay. Overall, sensitivity, specificity and accuracy of the CDC MAC ELISA was 62.4% (95%CI 53.3-70.9), 63.6% (53.9-72.6) and 63.0% (56.8-69.2) respectively (Table 3.4).

Sensitivity varied relative to the number of days after onset of acute illness the samples were collected. Samples collected ≤5days (n=65); 6-13 (n=39) and > 14 days (n=21) after acute illness exhibited the following sensitivity: 40.0%, 84.6% and 90.5% respectively (Table 3.4). ZIKV antibody titres detected by the MAC ELISA in samples collected 5 days from onset of symptom, or before, is significantly lower than samples collected at any later point (P-value <0.0005). (Figure 3.5).

Specificity varied between patient groups. The lowest specificity was observed among patients with DENV-2 (35.3% with 95% CI of (12.6-58.0). Due to limited availability of some of the reagents provided by CDC (these were non-purchasable), a smaller number of serum samples was tested with this assay (235 instead of 277 tested with the IgM μ-capture assay). Most representative samples with high volume were tested.

3.5.1.5 Performance of the IgM μ-capture ZIKV ELISA assay

The IgM μ-capture ELISA is a commercial ELISA that targets the NS1 region. Overall, sensitivity, specificity and accuracy of the IgM μ-capture ELISA was 31.7% (95%CI 24.7-38.8), 92.7% (87.9-97.6) and 56.0% (50.1-61.8) respectively (Table 3.4).

Sensitivity varied relatively to the number of days after onset of acute illness the samples were collected. Samples collected 1-5 (n=75); 6-13 (n=45) and > 14 days (n=47) after acute illness exhibited the following sensitivity: 10.7%, 53.3% and 44.7% respectively (Table 3.4). The titre of ZIKV antibodies detected by the IgM μ-capture assay among samples collected 5 days after symptom onset or before is significantly lower than samples collected at any later point (p-value<0.0005) (Figure 3.5).

Table 3.4 Sensitivity, specificity and accuracy of the MAC-ELISA and IgM µ-capture assays

Specificity values were calculated for each assay based on the cohorts 2 and 3. Data from ZIKV-positive cases served only for determining the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases (Cohort 2 and 3) serve only for determining the specificity and were not used for calculating the sensitivity. Equivocal results were considered negative for the calculation. Non-flavivirus include the measles & rubella specimens, hepatitis and control population samples. DENV (all) includes all DENV samples (DENV 1-4).

Abbreviations: CI, Confidence Interval, PPV: Positive Predictive Value, NPV. Negative Predictive Value, Days: number of days the sample was collected after symptom onset

	MAC ELISA			IgM µ-capture		
	Tested (n)	**% Sens. (95%CI)**	**% Spec. (95% CI)**	**Tested (n)**	**% Sens. (95%CI)**	**% Spec. (95% CI)**
Zika Positive	**125**	62.4(53.9-70.9)		**167**	31.7 (24.7-38.8)	
Zika (1-5 Days)	65	40.0(28.1-51.9)		75	10.7(3.7-17.7)	
Zika (6-13 Days)	39	84.6(73.3-95.9)		45	53.3(38.8-67.9)	
Zika (>14 Days)	21	90.5(77.9-103.0)		47	44.7(30.5-58.9)	
Non ZIKV	**110**		63.6 (54.7-72.6)	**110**		92.7 (87.9-97.6)
DENV (all)	88		61.3 (51.2-71.5)	79		92.4 (86.6-98.25)
DENV 1	21		52.3(31.0-73.7)	16		100.0(100.0-100.0)
DENV2	17		35.3(12.6-58.0)	16		100.0(100.0-100.0)
DENV3	21		61.9(41.1-82.7)	17		88.2(72.9-103.55)
DENV4	29		82.8(69.0-96.5)	30		86.6 (74.5-98.8)
Yellow fever	14		57.1 (31.2-83.1)	16		87.5(71.3-103.7)
Non Flavivirus	8		100.0(100.0-100.0)	15		100.0(100.0-100.0)
Measles & Rubella	8		100.0(100.0-100.0)	1		100.0(100.0-100.0)
Hepatitis	-			13		100.0(100.0-100.0)
Control population	-			1		100.0(100.0-100.0)
Overall	**235**	**62.4 (53.3-70.9)**	**63.6 (53.9-72.6)**	**277**	**31.7 (24.7-38.8)**	**92.7 (87.9-97.6)**
PPV	**66.1(57.6-74.6)**			**86.9(78.4-95.4)**		
NPV	**59.8(50.9-68.7)**			**47.2(40.6-53.9)**		
Accuracy	**63.0(56.8-69.2)**			**56.0(50.1-61.8)**		

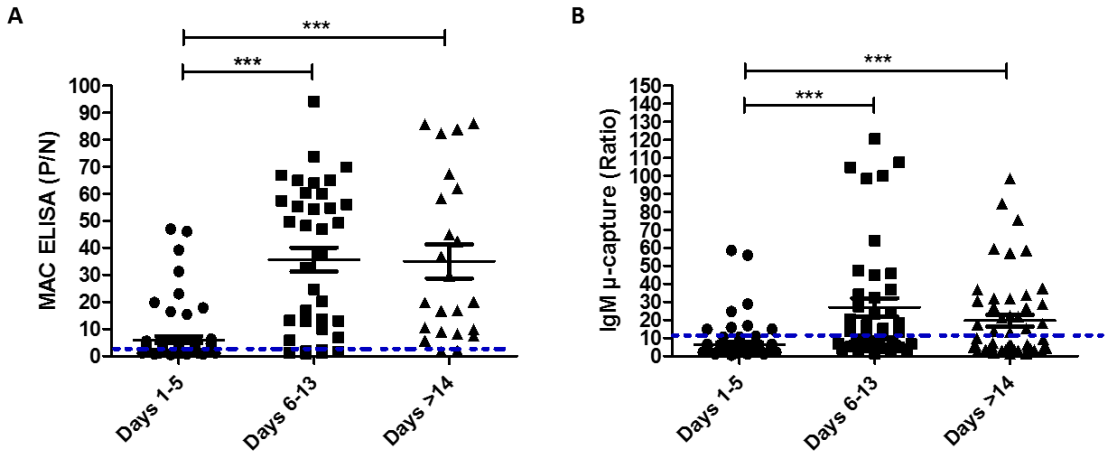


Figure 3.5 Antibody levels among the ZIKV specimens for MAC ELISA and the IgM µ-capture ELISAs
Antibody levels observed for the acute (1-5), early-convalescent phase (6-12) and late convalescent-phase (>13) ZIKV specimens for the MAC ELISA and the IgM µ-capture assays. A) Antibody levels tested with the MAC ELISA assays. B) Antibody levels tested with the IgM µ-capture assay. The dotted blue horizontal lines represent the cut-off value for each method. Black line in each group represents median for each population. Dots over the cut-off line represent positive samples. ***=p-value<0.0005

3.5.2 Comparing Assay performance

Overall, the highest accuracy was obtained with the commercial IgG NS1 ELISA followed by the MAC ELISA, IgM NS1 ELISA and IgM µ-capture assay (Table 3.5). The same volume of patient serum was required for all the serologic assays evaluated and all the assays needed to be kept refrigerated between 2 and 8 °C. The approximate time it took to complete one plate for each assay was calculated, measuring the average time of performing each assay in 3 or more independent assay runs.

Table 3.5 Comparison of ZIKV assays main features
Comparison of key assay features for IgM NS1 ZIKV ELISA, IgG NS1 ZIKV ELISA, CDC MAC ELISA and IgM μ-capture ZIKV ELISA. *Assay can also be performed with 4μl sample/patient.

Assay feature	IgM NS1 ZIKV ELISA (Euroimmun)	IgG NS1 ZIKV ELISA (Euroimmun)	CDC ZIKV MAC ELISA	IgM μ-capture ZIKV ELISA
Accuracy order	**3**	**1**	**2**	**4**
Accuracy [%,(95%CI)]	59.2(53.9-64.4)	72.5(67.6-77.4)	63.0(56.8-69.2)	56.0(50.1-61.8)
Immunoglobulin antibody detected	IgM	IgG	IgM	IgM
Antigen detected	ZIKV NS1	ZIKV NS1	ZIKV E/prM; flavivirus cross-reactive	ZIKV NS1 (HRP-conjugated)
Specimen type	Serum, plasma	Serum, plasma	Serum / CSF	Serum, plasma
Number of samples per plate	93	93 (Semi-quantitative) 91 (Quantitative)	28	93
Number of wells	96 wells (8-well strips per plate)	96 wells (8-well strips per plate)	96 wells (8-well strips per plate)	96 wells (8-well strips per plate)
Sample volume needed	10μl	10μl	10μl*	10μl
Sample Dilution	1:101	1:101	1:400	1:100
Time to perform assay (for 1 plate)	~2.5-3 h	~2.5-3 h	~4.5-5.30 h (in 3 different days)	~3.0-3.5 h

3.5.3 Investigating improved cut-offs for the South American population

As the overall accuracy of the assays was poor, I investigated whether the accuracy could be improved by modifying the established cut-offs suggested by the manufacturers. Manufacturers had set their cut-offs based on evaluations using samples mainly from travellers. For example, for the IgM μ-capture ELISA, the cut-off was defined using a panel of 13 ZIKV samples and 47 Non-ZIKV control samples from Europeans.

I performed ROC analyses for the IgM NS1 and IgM μ-capture ELISAs. I used both the ZIKV and the non-ZIKV specimens to generate the ROC curves in Figure 3.6 and the Table 3.6.

Current cut-offs established by the manufacturers set the threshold to Ratio of 1.1 (IgM NS1 assay) and Ratio of 1.15 or 11.5 Novagnost Units (IgM u-capture assay). The results showed that by lowering the cut-offs for both assays to 0.81 and 0.80 (respectively for the IgM NS1

assay and the IgM µ-capture), the sensitivity would improve to 29.3% and 37.7% respectively. However, specificity would fall to 92.2% and 82.2% respectively (**Figure 3.6**). Further analysis showed that the best accuracy (highest likelihood ratio) would be obtained with higher cut-offs of 2.31 and 2.84 (respectively). These cut-offs, though improving specificity, resulted in poorer sensitivities (13.2% and 16.8% respectively).

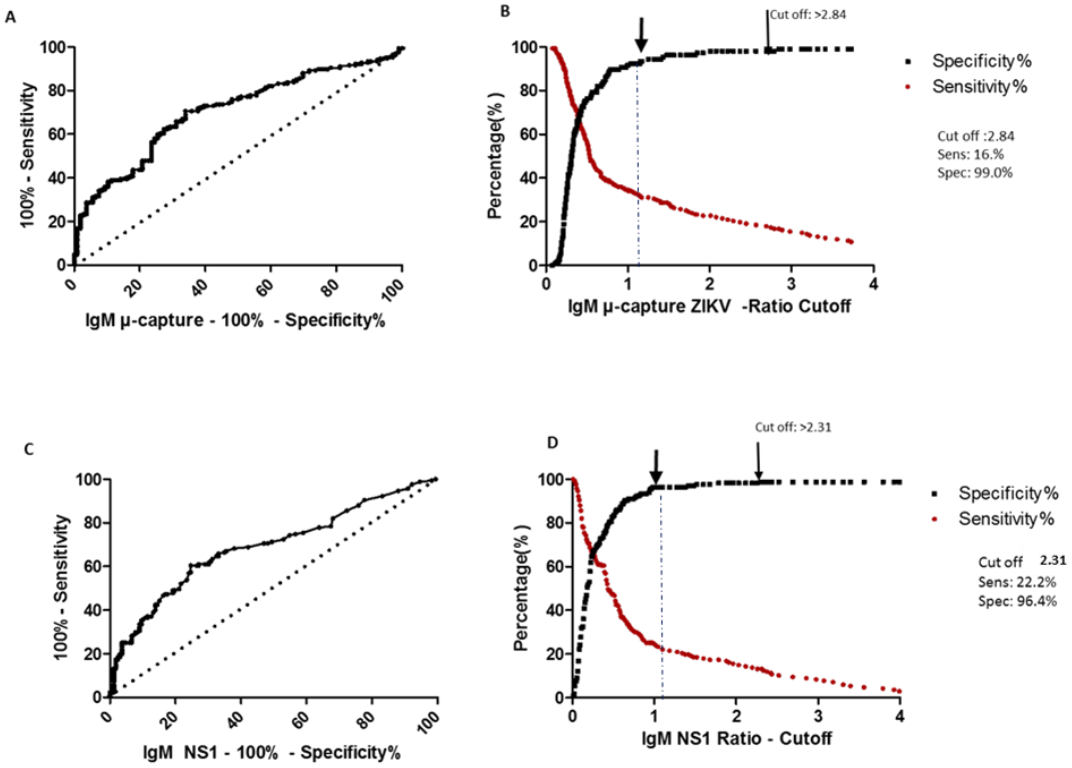


Figure 3.6 Receiver Operating Curve (ROC) analysis to compare sensitivity and specificity
ROC analysis comparing sensitivity and specificity for different cut-off values for the (A) IgM µ-capture assay and (C) IgM NS1 ZIKV ELISA performed on the results shown in Table 3.3 and Table 3.4. The area under the ROC Curve indicates the accuracy of the assay. Curves showing specificity and sensitivity values for each possible cut-off for the IgM µ-capture; (B) and IgM NS1 (D) Blue dotted line with an arrow on B and D indicates the cut-offs set by the manufacturers at 1.15 and 1.1 for the IgM µ-capture assay and IgM NS1 assay respectively. The second arrow to the right indicates cut offs that offer the highest likelihood ratios with the highest accuracy values for the study population. On the legend on the right side, indicated the Sens (Sensitivity) and Spec (Specificity) for the cut-off with the highest likelihood ratio.

Table 3.6 Sensitivity, specificity and likelihood ratio for each cut-off value
Sensitivity, specificity and likelihood ratios calculated for the IgM NS1 and IgM μ-capture ELISAs based on the ROC analysis performed above in Figure 3.6. *Current cut-off suggested by the manufacturer; ** Cut-off with the highest likelihood ratio. CI: Confidence Interval. **‡:** Suggested lower cut-off with improved sensitivity.

	Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
IgM NS1 ELISA	> 0.0100	100	97.82-100.0	0.6024	0.02-3.312	1.01
	> 0.1050	85.63	79.38-90.57	27.71	21.06-35.18	1.18
	> 0.8050‡	**29.34**	**22.56-36.87**	**92.17**	**86.98-95.76**	**3.75**
	> 1.000	24.55	18.23-31.80	96.39	92.30-98.66	6.79
	> 1.100*	**22.16**	**16.11- 29.22**	**96.39**	**92.30-98.66**	**6.13**
	> 2.030	14.97	9.93-21.30	98.19	94.81-99.63	8.28
	> 2.310**	**13.17**	**8.44-19.2**	**98.8**	**95.72-99.85**	**10.93**
	> 3.070	7.784	4.21-12.94	98.8	95.72-99.85	6.46
	> 4.025	2.395	0.66-6.02	98.8	95.72-99.85	1.99
	> 5.210	0.5988	0.02-3.29	100	97.80-100.0	1.01
IgM μ- capture ELISA	> 0.0800	99.4	96.71- 99.98	0	0.0 -3.420	0.99
	> 0.2005	92.22	87.06-95.79	12.26	6.695-20.06	1.05
	> 0.8075‡	**37.72**	**30.35-45.54**	**89.62**	**82.19-94.70**	**3.64**
	> 1.006	34.13	26.98-41.86	91.51	84.49-96.04	4.02
	> 1.093*	**32.93**	**25.87-40.62**	**92.45**	**85.67-96.69**	**4.36**
	> 1.505	28.14	21.47-35.61	96.23	90.62-98.96	7.46
	> 2.011	22.75	16.63-29.87	98.11	93.35-99.77	12.06
	> 2.844**	**16.77**	**11.44-23.31**	**99.06**	**94.86-99.98**	**17.77**
	> 3.125	14.97	9.93-21.30	99.06	94.86-99.98	15.87
	> 4.129	10.18	6.04-15.80	99.06	94.86-99.98	10.79
	> 5.912	5.988	2.91-10.74	99.06	94.86-99.98	6.35
	> 6.186	5.389	2.494-9.98	99.06	94.86-99.98	5.71

3.5.4 Investigating the biological diversity of the antibody responses to Zika virus infections

As I observed differences in ZIKV responses among patients, I studied the different human antibody responses to ZIKV infections analysing the variation in duration, strength, immunoglobulin type generated and dynamics. The duration of antibody response is the number of days that remain detectable above the assay threshold. The strength of antibody response is the magnitude of antibodies detected measured in the respective Units (such as Ratio for the Euroimmun assays or Panbio Units for the dengue assays). A better understanding of the biological variability of human antibody responses to ZIKV would support optimization and development of serologic assays for ZIKV diagnostics.

3.5.4.1 Biological diversity of Zika IgM responses among patients

In order to study the diversity in duration and magnitude of the antibody responses to ZIKV, I compared the titres obtained for four different patients for whom I had 5 or more sequential serum samples. I defined them as patient A and B (DENV naïve) and patient C and D (DENV immune). Sera had been collected at different time points from day 0 up to day 276 after symptom onset. Results for the IgM NS1 and IgM μ-capture assays are shown in Figure 3.7. Antibody titres showed high variation between patients. IgM levels were not detectable until day 25 post symptom onset for patient A. There was no obvious difference in antibody responses between the patients that were DENV Naïve (A and B) and DENV immune (C and D).

Only 27.6% (13/47) of the samples tested with the IgM NS1 assay and 44.7% (21/47) tested with the IgM μ-capture remained positive 14 days post symptom onset. IgM levels continued to decrease, with only 8%(2/25) and 20% (5/25) specimens positive at 27 days tested for the IgM NS1 and the IgM μ-capture assay respectively. No subjects tested had IgM positive results at day 90 post symptom onset.

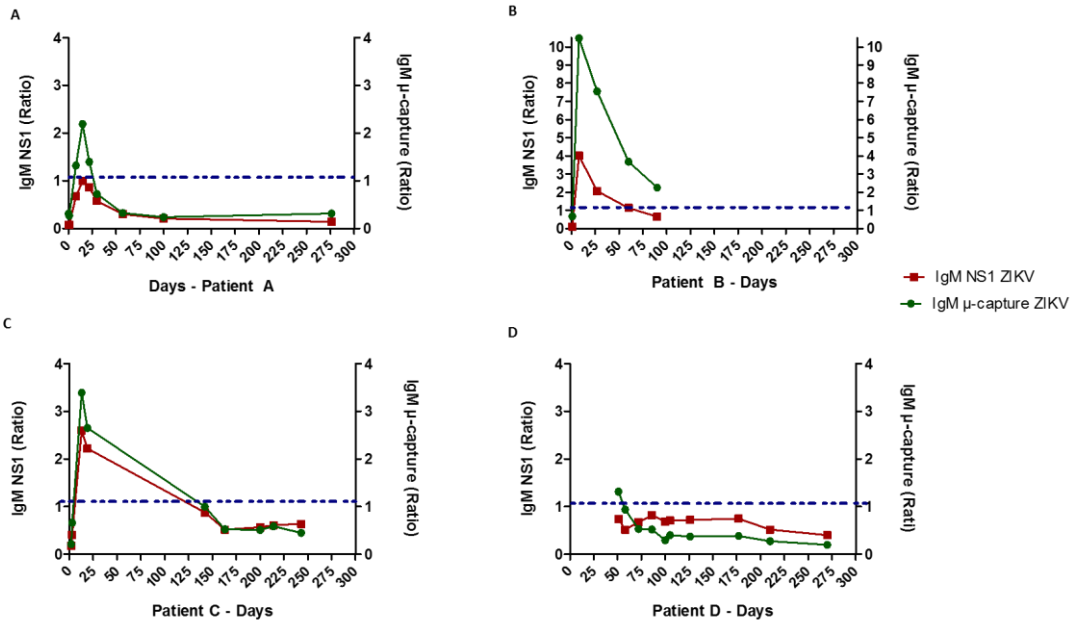


Figure 3.7 Biological diversity of ZIKV IgM antibody responses between patients.
Representation of the dynamics of IgM ZIKV antibody titres for four different ZIKV positive patients with five or more consecutive sequential samples collected between day 0 and day 276 and tested for IgM µ-capture and IgM NS1 ZIKV ELISA. A) Antibody dynamics for patient A; B) Antibody dynamics for patient B; C) Antibody dynamics for patient C. D) Antibody dynamics for patient D. Patients A and B are dengue naïve while C and D had previously been exposed to dengue. Blue dotted lines represent cut-off values for both assays. Points above the cut-off are positive results. Days=days sample was collected from symptom onset. First sample collected for each patient was PCR positive. Values expressed as ratios as recommended by the manufacturers. Green=IgM µ-capture assay; red= IgM NS1 ZIKV assay.

3.5.4.2 Biological variation of the strength of antibody responses

Then, I wanted to explore the different strengths of antibody responses found in those four patients (Figure 3.8). There was high variation in the magnitude or strength of the antibody responses found among the patients. Patient B showed a very strong antibody response to ZIKV measured with the IgM NS1 and IgM u-capture assay. The antibody ratios observed for patient B were of 4.1 for the IgM NS1 assay and 10.5 for the IgM µ-capture assay. The samples from patient B were tested three times to ensure the result was not a product of a technical error obtaining the same values with a very low Coefficient of Variation (CV) among replicates (<5%). Whether this may be related with the condition of this patient of being DENV immune, will need to be further investigated.

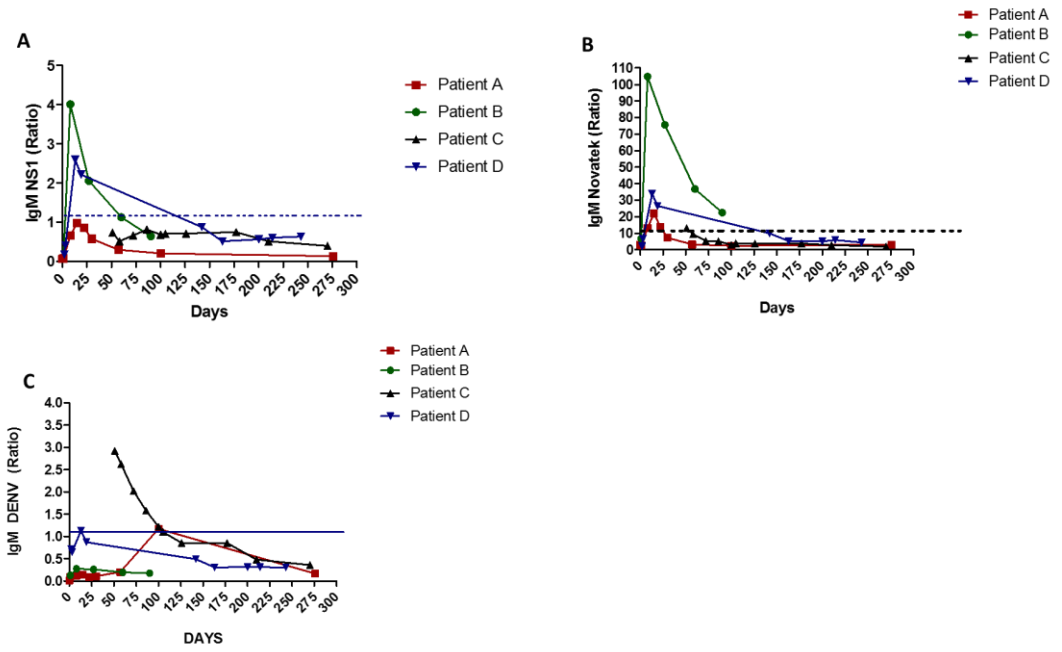


Figure 3.8 Biological diversity of strength of ZIKV antibody responses in 4 patients
Sequential results obtained for four different patients with five or more sequential serum samples collected for the following assays: A) IgM NS1 ZIKV, B) IgM µ-capture ZIKV C) IgM DENV. The blue dotted line represents cut-off for each assay. Points above cut-off are considered positive

3.5.4.3 Investigation of the diversity of the strength of the IgM ZIKV antibody responses

Then, I investigated the diversity in the strength of the antibody responses for the IgM NS1 and the IgM µ-capture assay for the ZIKV positive group and non-ZIKV groups (Figure 3.9). The histograms show the distribution of the number of samples tested that produced the same ratio result (organised in bins of ratios of 0.01). The distribution found was quite homogenous, highlighting that only a handful of samples showed very high ratios compared with the rest. In Figure 3.9 B and E the distribution of ratios obtained for the non-ZIKV group of patients is represented. Most of the control non-ZIKV samples have ratios close to the cut-off line. This suggest that a proportion of the samples that generated false positive results for this ELISAs had antibody titres close to the cut-off.

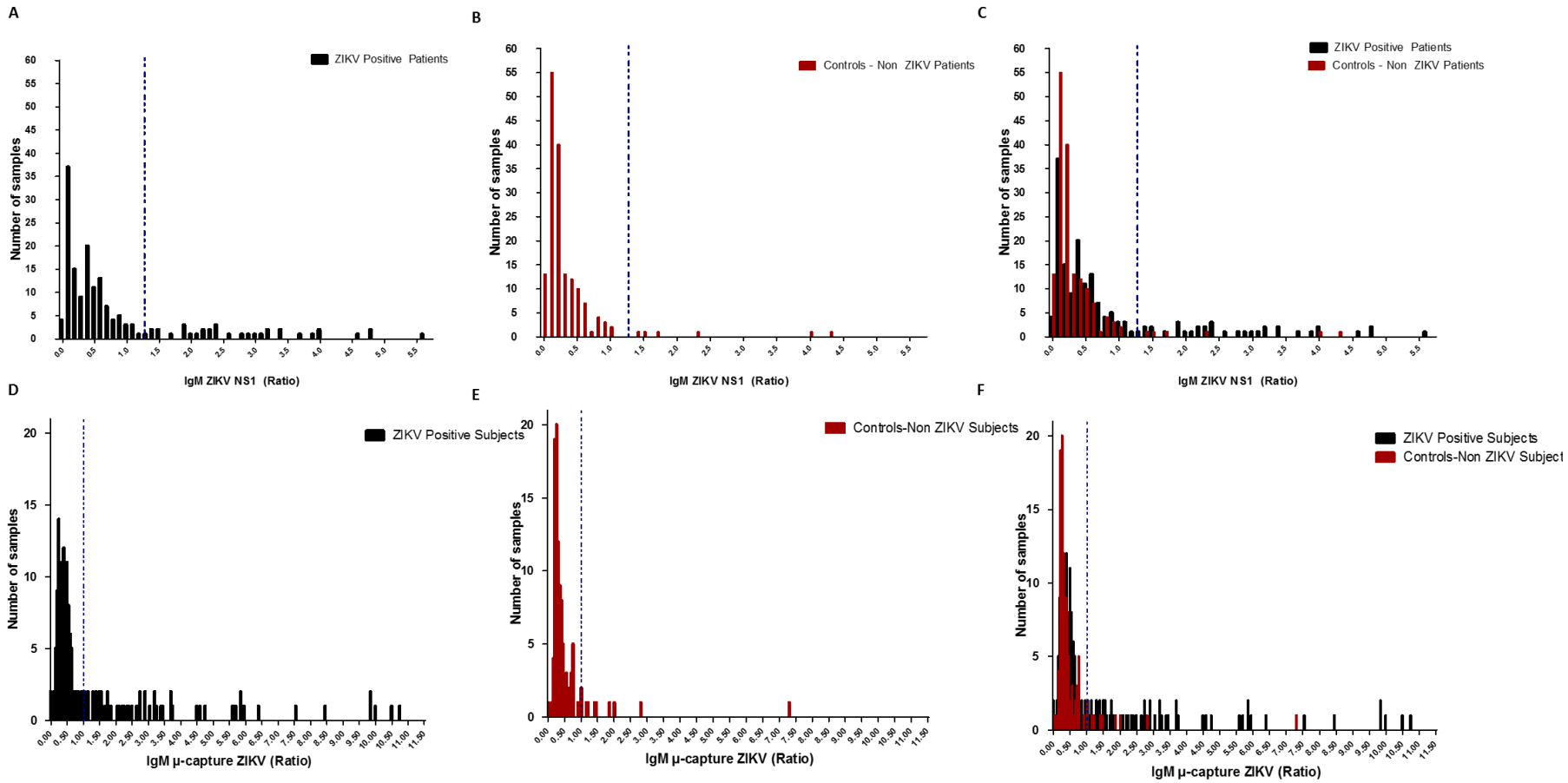


Figure 3.9 Histogram representing the distribution of IgM ZIKV antibody titres

Histogram showing the graphical representation of the distribution of the IgM ZIKV antibody responses. Y axes show number of samples and X axes represent the distribution of antibody titres among the population in bins of 0.01. A) Histogram for IgM NS1 ratio distribution among the ZIKV subjects (black); B) Histogram for IgM NS1 ratio distribution among the Controls – non- ZIKV subjects (in red); C) Histogram of the overlapping distribution of the ZIKV and non-ZIKV cases (Black and Red respectively). D) Histogram for IgM µ-capture ZIKV ELISA ratio distribution among the ZIKV subjects; E) Histogram for the antibody ratio distribution for the IgM µ-capture ZIKV ELISA for F) Histogram for IgM u-capture ZIKV ELISA ratio distribution among the ZIKV (black) and non-ZIKV (red) subjects. Blue dotted line represents the cut-off. Values to the right of the cut-off line are positive. Antibody titres are expressed as ratios for the IgM NS1 assay and as Novagnost® Units. IgM ZIKV NS1 ELISA = IgM ZIKV Euroimmun ELISA ; IgM µ-capture ELISA= IgM NovaTec ELISA (Novagnost®)

3.5.4.4 Investigation of the correlation between IgM antibody titres

Then, I investigated the relationship among the results obtained among the ZIKV assays and the DENV IgM and IgG assays. A highly significant correlation was found between the antibody levels observed with the IgM NS1 and the IgM μ-capture titres for each individual, with slightly higher titres on the IgM μ-capture assay (P-value <0.0001, R^2=0.69) (Figure 3.10A). Both assays target the NS1 region of the ZIKV antibodies, although using a different method. Consequently, it was not surprising to find that the results were highly correlated.

The results obtained for the correlations between IgM NS1 ZIKV and the IgM DENV and between the IgM μ-capture (NovaTec) assay and the IgM DENV ELISA are shown in Figure 3.10 B and Figure 3.10 C (no significant correlation).

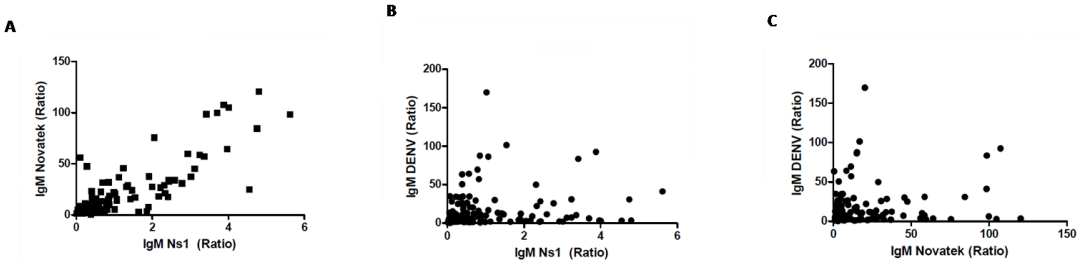


Figure 3.10 Correlations between antibody titres among ZIKV patients.
A) High correlation between the IgM NS1 and the IgM μ-capture (P-value <0.0001, R2=0.69) B) Correlation between IgM DENV antibodies and IgM NS1 ZIKV antibody titres and C) Correlation between IgM DENV and IgM μ-capture ZIKV antibody titres. IgM and IgG results expressed in Panbio Units. IgM NovaTec results are expressed in Novagnost Units. IgM ZIKV NS1 ELISA = IgM ZIKV Euroimmun ELISA; IgM NovaTec ELISA (Novagnost®) = IgM μ-capture ELISA.

3.5.4.5 Investigation of antibody responses among paired samples

One commonly used approach to define a patient's recent flaviviral infection is to see an increase in antibody titres between samples collected sequentially (paired samples). Paired samples are generally collected at acute-phase (1-5 days after symptom onset) and at early-convalescence (>6 days to 30 days) [157]. Consequently, I wanted to explore the dynamics between paired samples and therefore, the antibody response generated by the host immune response following ZIKV infection. I compared the antibody titres in paired sera collected at acute-phase (1-6 days) and early convalescent-phase (7-30 days) for the following four assays: IgM NS1 ZIKV ELISA, IgM μ-capture ZIKV ELISA, IgM MAC ELISA and IgG NS1 ZIKV ELISA (Figure 3.11). I classified the patients as high antibody responder if the increase in the antibody titre was ≥2.5. Low-responder if the ratio between antibody titres at convalescent-phase and acute phase was <2.5.

I aimed to detect if there was an increase in the antibody responses between the days following infection (before an antibody response is typically produced) and compare it with the antibody responses at early convalescence-phase when the antibody titres should already be detectable (as it has been shown with other flaviviruses). The number of subjects that were high responders for the IgM NS1 ZIKV ELISA, IgM μ-capture ZIKV ELISA, IgM MAC ELISA and IgG NS1 ZIKV ELISA was 31/53, 25/50, 39/45, 39/53 (respectively). Three of the samples could not be tested for the IgM μ-capture assay due to low sample volume available and eight samples were not tested with the IgM MAC ELISA due to low reagents volume available.

The wide range of antibody responses obtained within the ZIKV positive cohort highlights the enormous biological diversity found between different patients. Then, I also wanted to investigate the usefulness of these diagnostic assays when paired samples are collected. I observed that only 58.5% (31/53) of patients had a ≥2.5 fold increase in IgM titres from acute-phase to early-convalescence phase (Figure 3.11A). However, 41.9% (13/31) of those high responders, have titres below the detection cut-off level at their convalescent-phase sample. Similar patterns were observed with the IgM μ-capture assay.

Interestingly, the IgM MAC ELISA assay had the highest rate of IgM response with 86.7% (39/45) of the paired serum specimens tested producing ratio results of ≥2.5 (Figure 3.11C). However, as I found this assay had very low specificity, it remains to be understood the nature of that antibody change. I observed that the IgG NS1 assay had shown that 2/54 samples had a decay in their levels of IgG antibodies as shown in Figure 3.11D. Testing for these paired samples was repeated obtaining similar results (CV<5%). This may suggest that IgG antibodies may be decaying quickly in a proportion of patients thought the mechanism for this still remains to be understood.

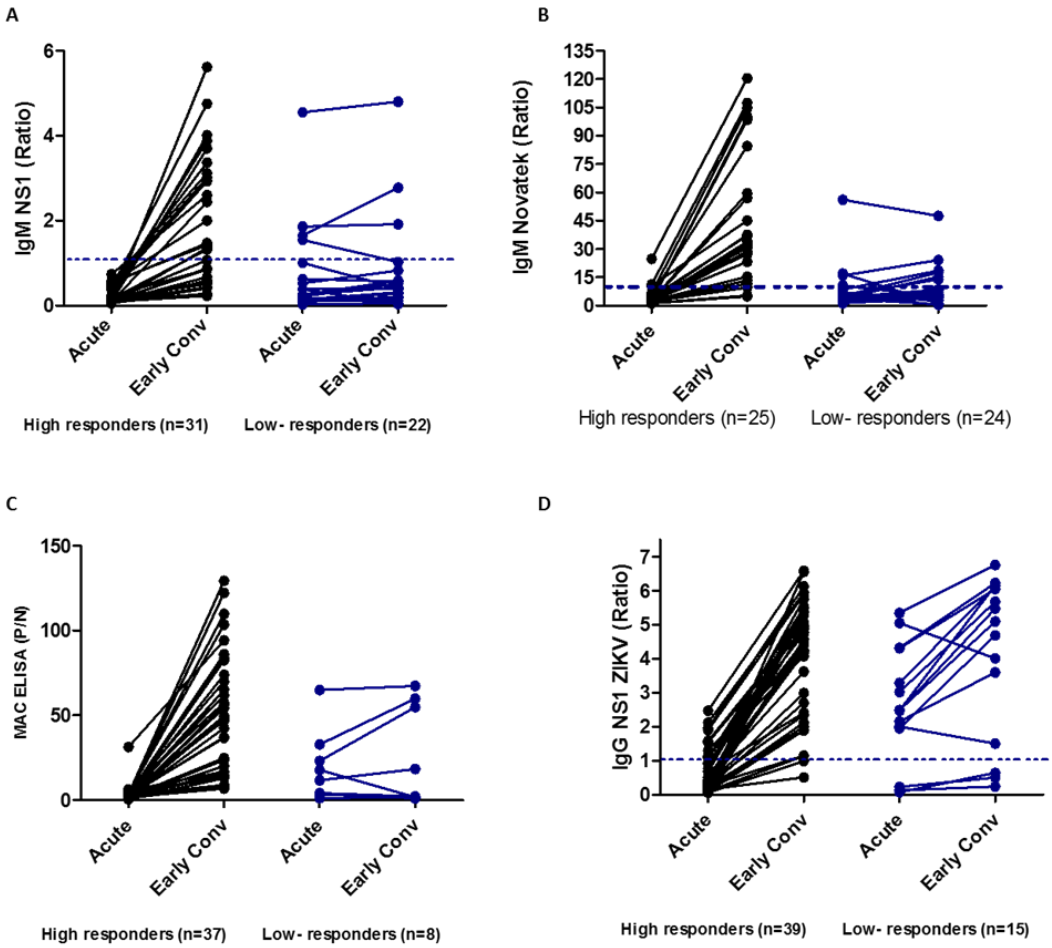


Figure 3.11 Dynamics of ZIKV antibodies in paired specimens
Antibody changes between paired samples collected at acute-phase (1-6 days) and early conv. (convalescent-phase)(6-30 days) in ZIKV positive patients using the following diagnostic methods: A) IgM NS1 ZIKV ELISA, B) IgM NovaTec/ μ-capture ZIKV ELISA, C) IgM MAC ELISA and D) IgG NS1 ZIKV ELISA. Blue dotted lines represent the cut-off for each assay. Points above the line are positive. Lines connecting acute to early-conv. sample represent the antibody titre change. Number of samples that were high or low responders included below each graph. High responders have an increase in their titre of ≥2.5 fold.

Then, I tried to further understand the differences among the high and low responders groups and compared their level of previous exposure to DENV infection (Figure 3.12). When comparing the results for the IgM NS1 Euroimmun assay I found that the non-responders group had significantly increased levels of IgG dengue antibodies in the acute-phase (P-value=0.005) (Figure 3.12A). Supporting this, I also observed significantly higher IgG dengue antibodies in the acute sample of patients that subsequently were positive for IgM antibodies measured with the IgM NS1 assay (P-value =0.011, Figure 3.12B). I conducted the same analysis for the high and low responders observed after using the MAC ELISA assay in Figure 3.12C and D. I observed non-significant differences in the IgG DENV titres between the low and high responders groups (measures obtained with the MAC ELISA assay).

However, I found a significant increase of IgG DENV antibodies among the subjects with IgM positive results with the MAC ELISA assay (p-value=0.0001).

All this highlights not only the high degree of biological diversity in the duration and magnitude of the antibody responses, but also that previous DENV infection has a role in modulating antibody responses to ZIKV. The response is correlated with a lower IgM ZIKV antibody response which could imply that the host is generating a "secondary immune response" in flavivirus immune individuals. This secondary immune response would be characterised by low or non-detectable IgM ZIKV production. This mechanism is common among DENV infections.

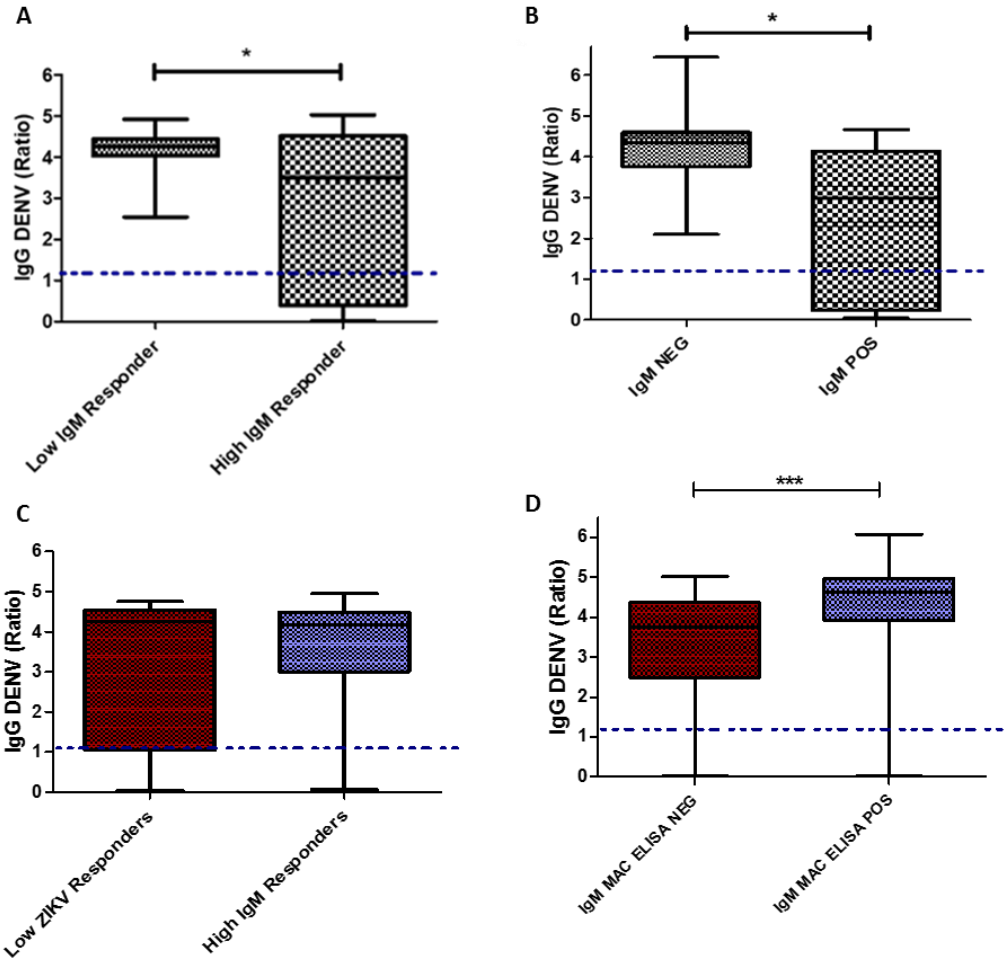


Figure 3.12 Investigating the differences in DENV antibodies among ZIKV patients
IgG DENV antibody levels comparing the following groups: A) Low IgM responder and High IgM responder with the IgM NS1 assay. High responders are individuals with >2.5 fold increase in antibodies between acute and convalescent serum. B) IgM NS1 ZIKV positive and negative with the IgM Euroimmun assay C) Low IgM responder and High IgM responder with the CDC MAC ELISA assay. High responders are individuals with >2.5 fold increase in antibodies between acute and convalescent serum. D) IgM MAC ELISA Positive and Negative individuals.
Box plot showing IgG dengue antibody titres measured in Ratio. (High IgM responders have ≥ 2.5fold increase of IgM between acute and convalescent sample measured with the IgM NS1 assay titres as shown in Figure 3.11A and C. * for statistically significant (p-value <0.05). Blue dotted lines represent the cut-off for each assay. Positive IgG DENV Ratio results above the cut-off line. The middle line in the box plots represents median value.

In Figure 3.13B I identified two different populations regarding their IgG NS1 ZIKV responses between 6-13 days. I had observed a population with low IgG NS1 ZIKV levels which had a median IgG ZIKV ratio of 0.94 (min: 0.05, max 2.7; n=16) and a population with high IgG antibody ratios of a median IgG ZIKV Ratio of 5.41 (min: 4.2; Max: 6.8; n=28). As I wanted to further understand the differences between those two populations of ZIKV patients I further investigated the DENV responses among those subjects (Figure 3.13). I observed that the group with high IgG NS1 ZIKV ratios had a significantly raised IgM DENV antibody titres (p-value=0.002) and significantly increased IgG DENV antibody titres (p-value=0.016).

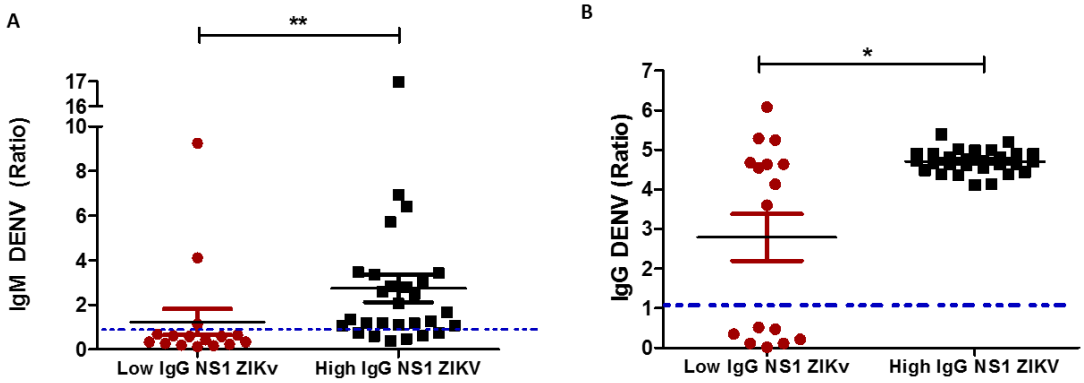


Figure 3.13 Differences in Dengue antibodies among ZIKV subjects with High and low IgG ZIKV antibodies (Day 6-13).
Differences among the two populations seen in Figure 3.4B (Day 6 to 13) showing significant differences in the Dengue antibody levels. A) Differences between the two groups for IgM DENV. B) Differences between the two groups for IgG DENV. * for statistically significant (p-value<0.05). **=p-value<0.005. Blue dotted lines represent the cut-off for each assay. Positive ratio results above the cut-off line. The middle line in each group represents median value.

3.5.5 Cross-reactivity of ZIKV and DENV serological assays

To investigate the potential of cross-reactivity between ZIKV and DENV antibodies, first I wanted to gain a better understanding of the relationship between production of IgM and IgG antibodies in each patient. This will help in better understanding the specificity challenges faced in the evaluation of the ZIKV ELISAs. I correlated the IgM NS1 ZIKV levels obtained for the cohort (both ZIKV patients and non-ZIKV patients) with the IgG ZIKV and IgG DENV levels (**Figure 3.14A and B**). There was a high proportion of IgM ZIKV negative patients which were IgG ZIKV positive. Interestingly, a high proportion of patients showed very high IgG DENV antibody levels. Thus, most IgM negative patients were IgG DENV and/or IgG NS1 ZIKV positive. However, the pattern for IgG ZIKV antibodies and IgG DENV antibodies in relation to IgM NS1 ZIKV antibodies was different. When I compared the correlation between

IgG ZIKV antibodies and IgG DENV antibodies, a low, but highly significant correlation was observed (Spearman R of 0.53, an r^2 of 0.26 and p-value of 0.0001) (Figure 3.14C).

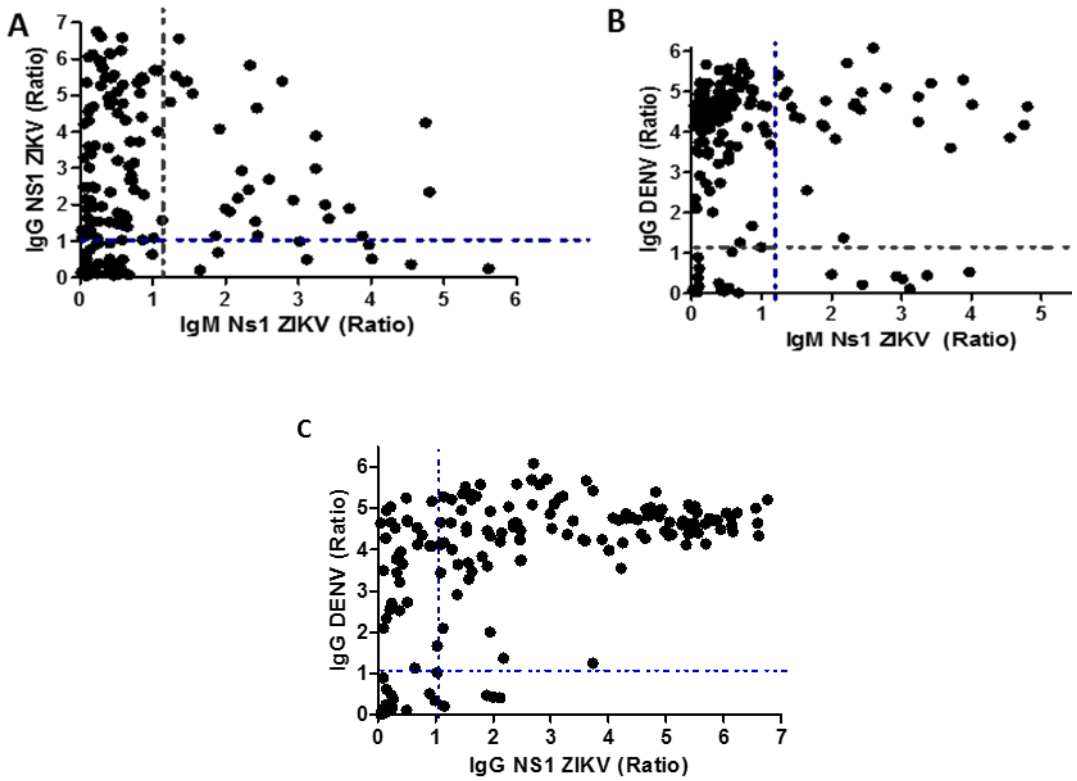


Figure 3.14. Scatterplot representing the relationship between IgM and IgG ZIKV and IgG DENV antibodies in ZIKV positive subjects

Scatterplots showing correlations between IgM ZIKV antibodies (IgM NS1 ELISA) and IgG ZIKV and IgG DENV antibodies. A) Scatterplot between IgM NS1 and IgG NS1 ZIKV antibody Ratios. B)Scatterplot showing IgM NS1 ZIKV antibody titres compared to IgG DENV antibody titres for ZIKV positive specimens (n=172). C) Scatterplot showing IgG NS1 ZIKV Ratio compared with IgG DENV antibody titres for ZIKV Positive specimens. Dotted line represent cut-off values for each assay. Points above and to the right side of the cut-off lines are positive.

Then, I also investigated the detection of dengue antibodies in paired acute-phase (1-5 days post symptom onset) and early convalescent-phase samples (6-30 days) among the ZIKV subjects (Figure 3.15). I observed that most ZIKV subjects exhibited significantly increased levels of IgM and IgG dengue antibodies in the convalescent-phase samples compared with the acute samples (p-value<0.0001 for IgM and p-value<0.0001 for IgG DENV). This was observed in 83% (47/57) of the ZIKV patients for the IgM DENV antibodies. Regarding the IgG DENV, 85% (46/54) of patients showed increased antibody titres at convalescent phase. Moreover, 42% (24/57) of the patients investigated showed conversion from a negative to a positive diagnosis for IgM dengue following ZIKV infection. Out of the seven subjects that were IgG DENV negative at acute phase, three converted to IgG DENV positive (Wilcoxon matched –pairs signed rank test).

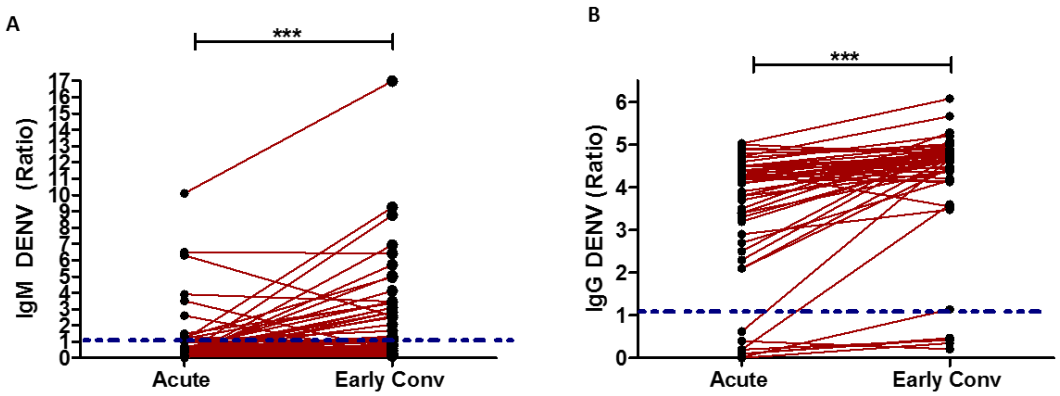


Figure 3.15 Increase of dengue antibody titres between acute and early convalescent-phase in ZIKV patients.

Graphical representation of the increase of IgM and IgG dengue antibody responses following ZIKV infections. Acute-phase (days 1-5 post symptom onset), early convalescence-phase (days 6-30 post symptom onset) of ZIKV positive subjects. A) IgM DENV ratio for acute and early convalescent ZIKV paired samples. B) IgG DENV antibody ratios for acute and early convalescence antibody ratios. Included only of paired acute-phase and convalescent-phase ZIKV positive subjects with results tested for both of their paired samples. Black lines represent the increase in antibody titres. Blue dotted lines represent cut-off values for each assay. ***= p-value<0.0001

I also compared the difference in ZIKV antibody production among patients IgG DENV positive and IgG DENV negative in acute-phase samples (1-5 days) and early convalescent-phase samples (6-30 days) (Figure 3.14). I observed that patients with negative IgG DENV antibodies (DENV naïve individuals) have lower production of IgM ZIKV antibodies at acute phase and slightly higher production at convalescent phase (non-statistically significant). This would follow a DENV secondary immune response pattern. In addition, I investigated the IgG NS1 ZIKV responses and found that patients with negative IgG DENV status (both at acute and at convalescent-phase) have significantly lower IgG ZIKV antibody levels ($p<0.005$) (Figure 3.16B). This may also suggest strong cross-reactivity especially among the IgG ZIKV antibodies and DENV assays.

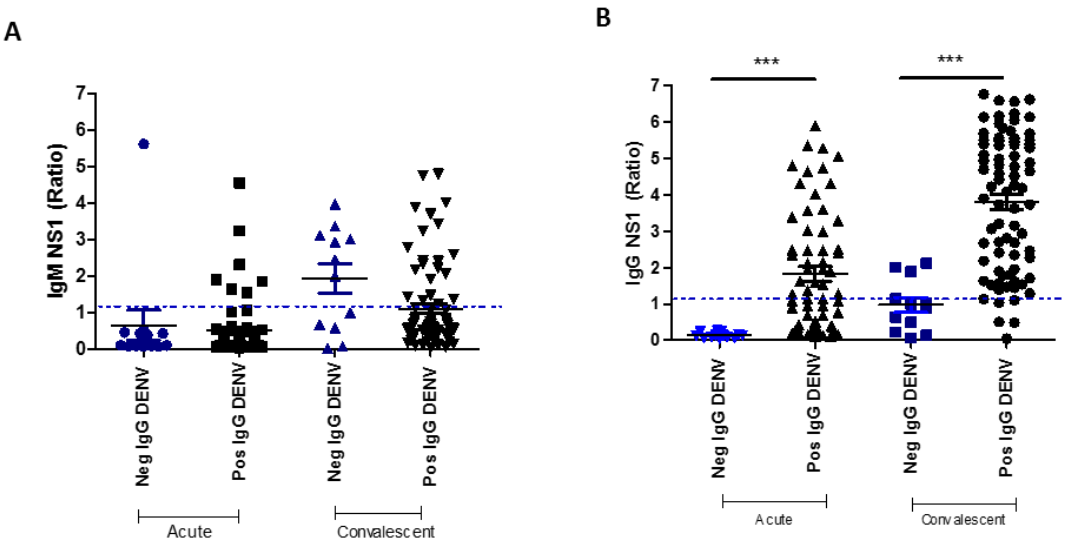


Figure 3.16 Production of IgM and IgG ZIKV antibodies in IgG DENV positive and negative specimens

Zika antibody titres for 4 groups: (i) Acute-phase samples (1 to 5 days) with negative IgG dengue status, (ii) Acute-phase samples with positive IgG dengue levels, (iii) Convalescent-phase samples (7 to 30 days) negative for IgG dengue and (iv) Convalescent-phase samples positive for IgG dengue. A) IgM NS1 ZIKV ELISA antibody ratio and B) IgG NS1 ZIKV ELISA antibody titres. Blue circles/squares represent specimens negative for IgG DENV. Antibody titres measured in Ratio. ***=p-value <0.0005. Blue dotted lines represent cut-off values for each of the assays (Ratio=1.1).

3.5.6 Evaluation and optimization of the ZIKV BOB assay

Specificity and sensitivity are presented in Table 3.7 as a percentage and 95% Confidence Interval (CI). The cut-off used to determine the positivity of each specimen was established at 50% inhibition as previously described in [116].

Overall, sensitivity, specificity and accuracy of the BOB assay was 54.2% (95%CI 45.3-63.1), 96.6% (96.6-96.7) and 77.7% (72.2-82.5) respectively (Table 3.7).

Sensitivity varied relative to the number of days after onset of acute illness that the samples were collected. Samples collected 1-5 (n=63); 6-13 (n=37) and > 14 days (n=20) after acute illness exhibited the following sensitivity: 28.6%, 78.4% and 90.0% respectively (Table 3.7 and Figure 3.17Table 3.3). Specificity varied between patient groups. The lowest specificity was observed among patients with DENV-3 (75.0%).

Table 3.7 Sensitivity, specificity and accuracy of the BOB Assay for detecting ZIKV-specific IgGs
Data from ZIKV-positive cases was only used to determine the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases were only used for determining the specificity and were not used for calculating the sensitivity. Abbreviations: CI, Confidence Interval, PPV., Positive Predictive Value, ZIKV, Zika virus, DENV: Dengue virus, Days: number of days the sample was collected after symptom onset NPV., Negative Predictive Value. Non-flavivirus include the measles & rubella specimens, hepatitis and general population samples. DENV (all) includes all DENV samples (DENV 1-4).

BOB Assay	Tested (n)	Positive (GBR>1.0)	Negative (GBR>1.0)	Sensitivity % (95% CI)	Specificity % (95% CI)
Zika Positive	120	65	55	54.17 (45.25-63.08)	
Zika (1-5 Days)	**63**	**18**	**45**	28.57 (17.42-39.73)	
Zika (6-13 Days)	**37**	**29**	**8**	78.38 (65.11-91.64)	
Zika (>14 Days)	**20**	**18**	**2**	90 (76.85-103.15)	
Non ZIKV	**149**	**5**	**144**		96.64 (96.62-96.67)
DENV (all)	95	5	90		94.74 (94.69-94.78)
DENV1	21	0	21		100 (100-100)
DENV2	16	0	16		100 (100-100)
DENV3	16	4	12		75 (74.79-75.21)
DENV4	31	1	30		96.77 (96.71-96.84)
Secondary DENV	11	0	11		100 (100-100)
Yellow fever	19	0	19		100 (100-100)
Non-flavivirus	34	0	34		100 (100-100)
Measles & Rubella	21	0	21		100 (100-100)
Other	14	0	14		100 (100-100)
Overall	**269**	**70**	**199**	**54.17 (45.25-63.08)**	**96.64 (96.62-96.67)**
PPV	**92.86 (84.39- 96.90)**				
NPV	**72.36 (68.26- 76.12)**				
Accuracy	**77.7 (72.24- 82.53)**				

A further representation of the results is shown in Figure 3.17. I observed that one ZIKV sample collected 31 days post symptom onset exhibited very low percentage of inhibition. Repeated testing provided similar results. I observed a trend of high percentage of inhibition in specimens collected >5 days following the onset of ZIKV symptom onset. Figure 3.17B shows five samples with a relatively high percentage of inhibition (50-75%) above the cut-off. Another seven samples, positive for DENV, Yellow Fever and other infections exhibited a percentage of inhibition just below the cut-off (between 40-50%).

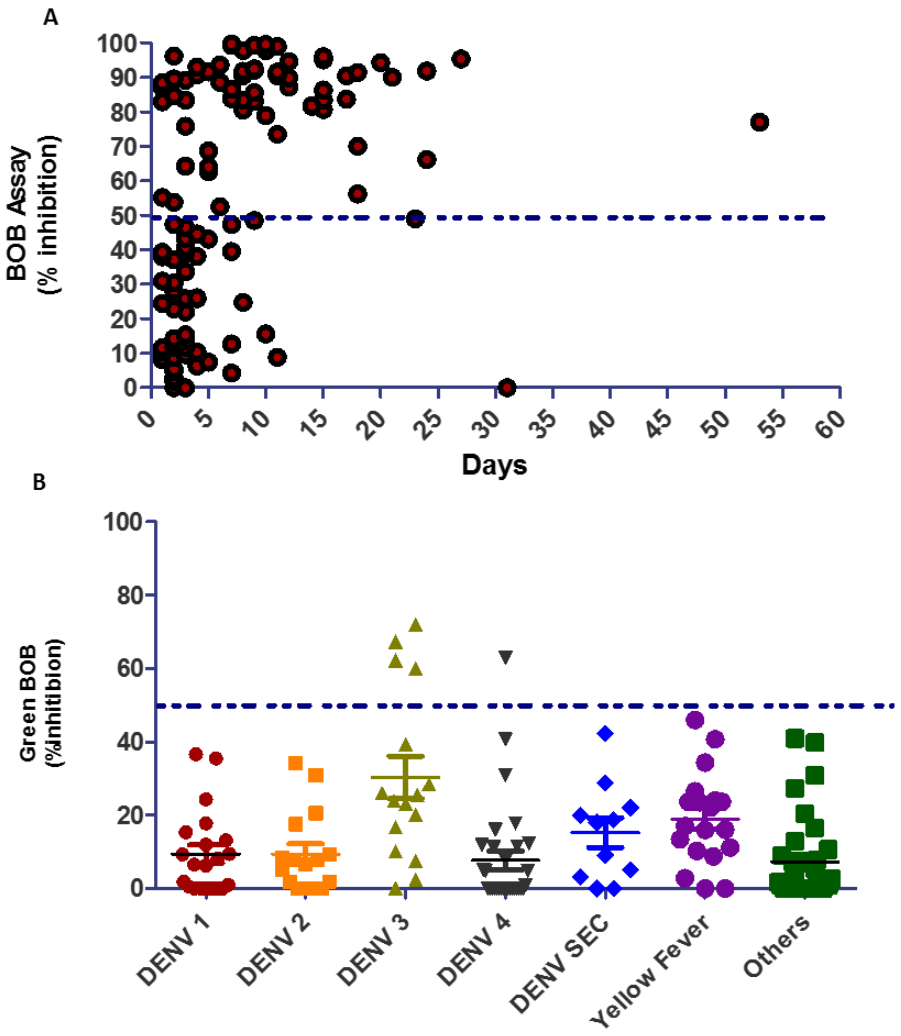


Figure 3.17 ZIKV NS1 Blockade-of-Binding (BOB) analysis of ZIKV Panel and Specificity Panel from Brazil.
Percentage of ZKA35 mAb binding inhibition by ZIKV and DENV serum/ samples in the BOB assay. A) Plotted are the BOB % of inhibition in ZIKV specimens (n=114); B) Plotted are the BOB % of inhibition in Specificity Panel (n=120) for DENV-1 to 4, Yellow fever and others. Others include measles, rubella, hepatitis, and non-infected individuals from before the ZIKV outbreak. Blue dotted line represents cut-off for the BOB Assay (Positive with % of inhibition >50.0%).

3.5.7 Evaluation and optimization of the ZIKV GREEN BOB assay

The Green BOB assay is a version of the BOB assay, with modifications to the protocol to reduce variability and the time to conduct the assay. I evaluated the modified version of the BOB assay and found that it was easier to perform, required less time and was easy to carry out in low resource laboratories, such as those found in countries most affected by the ZIKV epidemic. As the concept and monoclonal used in the assay were the same, I conducted a small study to confirm that the accuracy of the Green BOB assay was as good as that observed with the standard BOB assay.

3.5.7.1 Comparing the performance of BOB assay and modified GREEN BOB assay

To show consistency in performance between the modified and original BOB assay, I assessed the comparative ELISA results using 108 serum specimens from two well-defined ZIKV positives and ZIKV negative cohorts demonstrating good correlation levels. I compared the results obtained with the BOB assay and its modified version, the Green BOB assay observing highly significant correlations (Figure 3.18). Specimens tested with both assays, included 42 specimens from the ZIKV panel and 66 specimens from the specificity panel. I found a highly significant correlation in the reported antibody levels with an R^2 of 0.8 and a p-value <0.0001 (Figure 3.18).

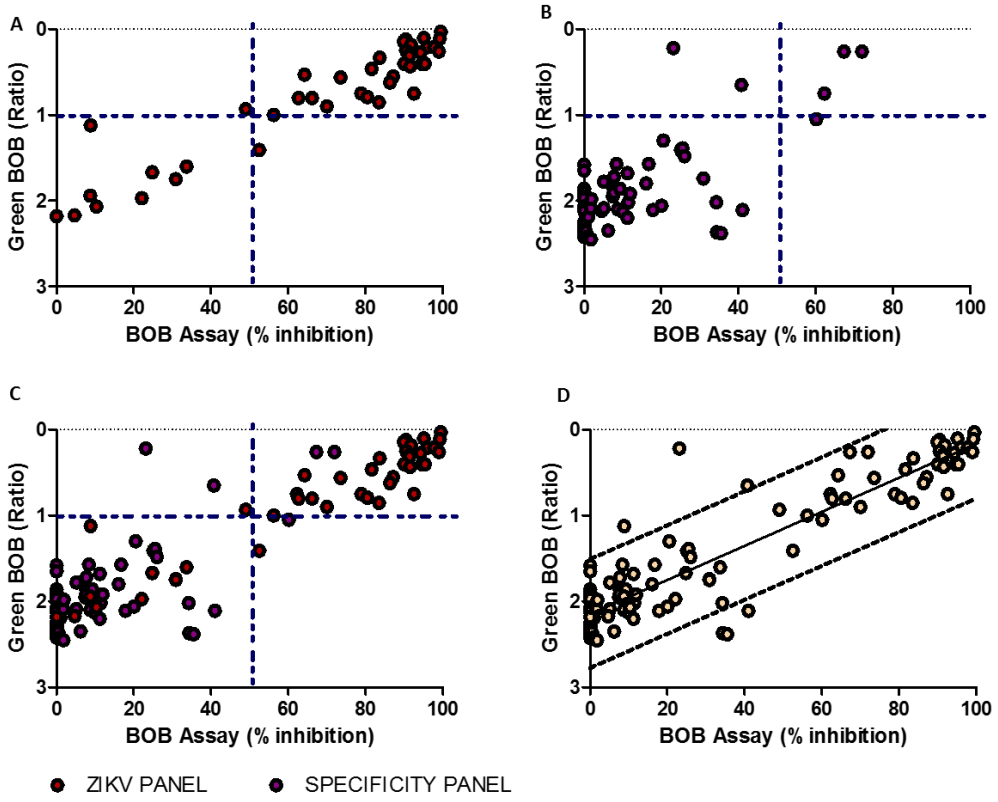


Figure 3.18 Correlation of the NS1 Blockade-of-Binding (BOB) assay and Green BOB
Agreement between Green BOB and BOB assay measured in Green BOB Ratio and percentage (%) of Inhibition respectively tested in a "bridging data" panel with n=108. A) Correlation of ZIKV Panel specimens (n=42). B) Correlation of the specificity panel which includes DENV, Yellow fever and others (n=66). C) Combines the ZIKV and the specificity panel (n=108). D) Correlation between the ZIKV BOB assay and the modified version GREEN BOB showing high correlation (Kappa coefficient >0.9). Dotted black inclined lines show the 95% prediction band. Black line shows linear regression (R^2 = 0.8). *Blue dotted lines show the cut-offs for each assay*. Green BOB Positive when Ratio <1.0. BOB assay Positive when % inhibition >50.0%.

Then, I conducted a 2x2 tables comparing the positive and negative agreement for both assays (Table 3.8 and Table 3.10). I also measured the Cohen's Kappa coefficient (κ). The Kappa coefficient is a statistical measure for interrater agreement which measures agreement for qualitative (categorical) items (such as a positive or negative diagnosis). It is

generally thought to be a more robust measure than simple calculations of the percentage of agreement and particularly useful when no gold standard method is available (like in this case).

I conducted the analyses with 2 cohorts: Cohort A, the cohort described above with n=108 individuals (including 42 ZIKV samples and 66 specimens from the specificity panel). We also conducted the same analyses with Cohort B, a selection of 98 of those samples by excluding the 10 ZIKV specimens that were collected during the first week following symptom onset. This is because antibody responses are typically detectable 6 days or more following the onset of symptoms. However, as shown in Chapter 3 antibody may be detectable earlier.

The analyses with the whole cohort (n=108) gave an overall positive agreement of 0.93 (95% CI: 0.87 - 0.99) and overall negative agreement was 0.97 (95% CI: 0.93 - 1.00) (Table 3.9). The Kappa Coefficient of agreement was 0.90 (95% CI: 0.81 - 0.99).

Table 3.8 Agreement between Green BOB and BOB Assay using the panel with n=108 (Cohort A)
2x2 table comparing agreement scores for both assays. Green BOB cut-off (positive if Green BOB Ratio <1.0). NS1 BOB assay cut-off (positive if % inhibition >50%)

		Green BOB		
		Positive	**Negative**	**Total**
BOB Assay	**Positive**	34	2	36
	Negative	3	69	72
Total		37	71	108

Table 3.9 Agreement between Green BOB and BOB assay (n=108) (Cohort A)
Table showing positive agreement, negative agreement, overall agreement and Kappa Coefficient. Results show 95% CI.

Positive Agreement	Negative Agreement	Overall Agreement	Kappa
0.93	0.97	0.95	0.90
(95% CI: 0.87 - 0.99)	(95% CI: 0.93 - 1.00)	(95% CI: 0.90 - 0.98)	(95% CI: 0.81 - 0.99)

The analyses with the subset of the cohort (n=98) showed a higher positive agreement of 0.96 (95% CI: 0.90 - 0.99) and overall negative agreement of 0.97 (95% CI: 0.94 - 1.00) (Table 3.10, Table 3.11). The Kappa coefficient of agreement was 0.91 (95% CI: 0.82 - 1.00).

These results demonstrated the high degree of correlation in the performance of the two BOB assays and that the Green BOB assay was suitable to use for detection of ZIKV antibodies in Brazilian patients following acute infection (after day 6 from symptom onset).

Table 3.10 Agreement between Green BOB and BOB Assay using the panel with n=98 (Cohort B)
2x2 Table comparing agreement scores for Green BOB and NS1 BOB Assay. Sample selection excludes 10 ZIKV samples collected <7 days from symptom onset compared to Table 3.9. Green BOB cut-off (positive if Green BOB Ratio <1.0). NS1 BOB assay cut-off (positive if % inhibition >50%)

		Green BOB		
		Positive	Negative	Total
BOB Assay	Positive	31	1	32
	Negative	3	63	66
Total		34	64	98

Table 3.11 Agreement between Green BOB and BOB assay (n=98) (Cohort B)
Table showing positive agreement, negative agreement, overall agreement and Kappa Coefficient. Results show 95% CI.

Positive Agreement	Negative Agreement	Overall Agreement	Kappa
0.94 (95% CI: 0.88 - 1.00)	0.97 (95% CI: 0.94 - 1.00)	0.96 (95% CI: 0.90 - 0.99)	0.91 (95% CI: 0.82 - 1.00)

3.5.7.2 Determination of the most appropriate cut-off for the Green BOB assay in the Brazilian population

Receiver Operating Curve (ROC) is a fundamental tool for diagnostic test evaluation. ROC analysis were generated to determine the most appropriate cut-off for the Green BOB in the Brazilian population using Cohort A and Cohort B (Table 3.9). The area under the ROC curve is shown in Table 3.12. The results showed that the area of the ROC Curve is 0.88 when calculated with Cohort A (n=108) and 0.933 when calculated with Cohort B (n=98).

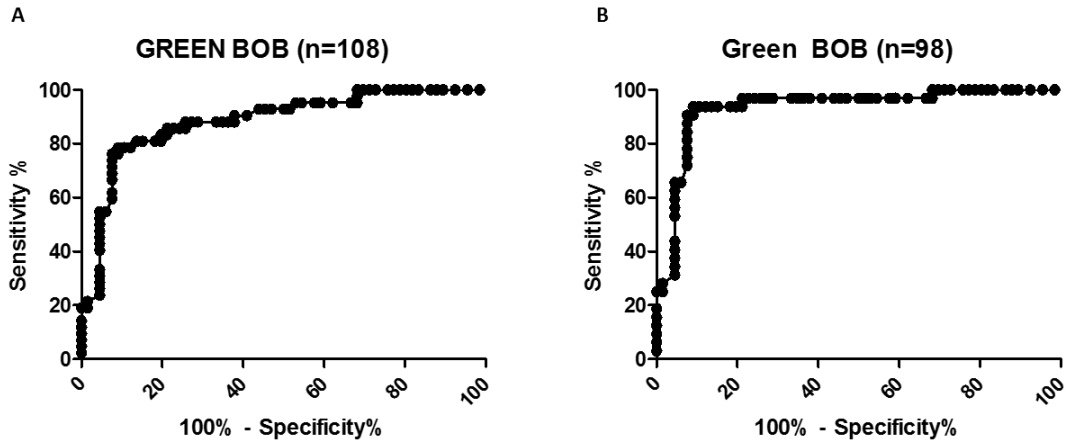


Figure 3.19 ROC Curves evaluating the performance of the Green BOB assay
Receiving Operating Curves (ROC) assessing the performance of the Green BOB assay and measured with A) Cohort A with 108 specimens showing the sensitivity and specificity of the Green BOB assay in the Brazilian population. B) ROC curve for cohort B (n=98) showing the sensitivity and specificity of the Green BOB assay with ZIKV specimens collected >7 days post symptom onset (n=32) and DENV specimens from before 2013 (n=66). Axes show percentages (%) Area under the curve and confidence intervals are shown in Table 3.12.

Table 3.12 ROC CURVE for GREEN BOB assay
Cohort A (n=108) and Cohort B (n=98) including only ZIKV specimens collected > 6 days post symptom onset

AREA ROC CURVE	ROC (n=108)	ROC (n=98)
Area	0.8838	0.9337
Std. Error	0.034	0.02832
95% confidence interval	0.8172 to 0.9505	0.8782 to 0.9892
P value	< 0.0001	< 0.0001
Controls (NON ZIKV SAMPLES)	66	66
Patients (ZIKV SAMPLES)	42	32

I further analysed the specificity and sensitivity of the Green BOB assay using different assay cut-cut-offs when testing Cohort A and Cohort B (Table 3.13 and

Table 3.14 respectively). As it is a blockade-of-binding assay, low binding correlates with a higher ZIKV antibody concentration. Our results show that a cut-off of 1.0 offers high likelihood ratios of 10.06 and 11.96, respectively for both cohorts. The sensitivity and specificity it produced when testing Cohort A are 76.2 and 92.4 respectively, and improved to 90.6 and 92.4 when testing Cohort B (Figure 3.20). Lower cut-offs result in significantly lower sensitivity percentages and higher cut-offs reduce the specificity to less than 92%. Therefore, I believe that using a cut-off of 1.0 to determine the positive and negative specimens in the Brazilian population will be the optimal threshold. Using a Green BOB Ratio of 1.0 offer specificities of over 92% and sensitivity of >76% that rises to >90% when testing samples collected > 7 days after symptom onset (Table 3.10).

Table 3.13 Specificity, sensitivity and likelihood ratio for different cut-offs for the Green BOB Assay

Number of Specimens included (n=108). Underlined the cut-off with the best balance of specificity and sensitivity and high likelihood.

Cut-off	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.2350	19.05	8.601-34.12	98.48	91.84-99.96	12.57
< 0.2550	21.43	10.30-36.81	98.48	91.84-99.96	14.14
< 0.3200	30.95	17.62-47.09	95.45	87.29-99.05	6.81
< 0.3650	33.33	19.57-49.55	95.45	87.29-99.05	7.33
< 0.5400	47.62	32.00-63.58	95.45	87.29-99.05	10.48
< 0.5550	50.00	34.19-65.81	95.45	87.29-99.05	11
< 0.5900	52.38	36.42-68.00	95.45	87.29-99.05	11.52
< 0.6350	54.76	38.67-70.15	95.45	87.29-99.05	12.05
< 0.7000	54.76	38.67-70.15	93.94	85.20-98.32	9.04
< 0.7700	59.52	43.28-74.37	92.42	83.20-97.49	7.86
< 0.7950	61.9	45.64-76.43	92.42	83.20-97.49	8.17
< 0.8250	66.67	50.45-80.43	92.42	83.20-97.49	8.8
< 0.8750	69.05	52.91-82.38	92.42	83.20-97.49	9.11
< 0.9150	71.43	55.42-84.28	92.42	83.20-97.49	9.43
< 0.9700	73.81	57.96-86.14	92.42	83.20-97.49	9.74
< 1.030	76.19	60.55-87.95	92.42	83.20-97.49	10.06
< 1.085	76.19	60.55-87.95	90.91	81.26-96.59	8.38
< 1.210	78.57	63.19-89.70	90.91	81.26-96.59	8.64
< 1.345	78.57	63.19-89.70	89.39	79.36-95.63	7.41
< 1.400	78.57	63.19-89.70	87.88	77.51-94.62	6.48
< 1.445	80.95	65.88-91.40	86.36	75.69-93.57	5.94
< 1.575	80.95	65.88-91.40	81.82	70.39-90.24	4.45
< 1.675	85.71	71.46-94.57	78.79	66.98-87.89	4.04
< 1.905	88.1	74.37-96.02	65.15	52.42-76.47	2.53
< 1.975	92.86	80.52-98.50	56.06	43.30-68.26	2.11
< 2.095	95.24	83.84-99.42	42.42	30.34-55.21	1.65
< 2.285	100	91.59-100.0	24.24	14.54-36.36	1.32

Table 3.14. Table showing the sensitivity, specificity for each cut-off used for Green BOB assay
Number of specimens included *(n=98).* Underlined the cut-off with the best balance of specificity and sensitivity and high likelihood.

Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Likelihood ratio
< 0.2350	25	11.46-43.41	98.48	91.84-99.96	16.5
< 0.2550	28.13	13.75-46.75	98.48	91.84-99.96	18.56
< 0.3200	40.63	23.70-59.36	95.45	87.29-99.05	8.94
< 0.3650	43.75	26.36-62.34	95.45	87.29-99.05	9.63
< 0.5900	62.5	43.69-78.90	95.45	87.29-99.05	13.75
< 0.6350	65.63	46.81-81.43	95.45	87.29-99.05	14.44
< 0.7000	65.63	46.81-81.43	93.94	85.20-98.32	10.83
< 0.7700	71.88	53.25-86.25	92.42	83.20-97.49	9.49
< 0.7950	75	56.59-88.54	92.42	83.20-97.49	9.9
< 0.8250	78.13	60.03-90.72	92.42	83.20-97.49	10.31
< 0.8750	81.25	63.56-92.79	92.42	83.20-97.49	10.72
< 0.9150	84.38	67.21-94.72	92.42	83.20-97.49	11.14
< 0.9650	87.5	71.00-96.49	92.42	83.20-97.49	11.55
< 1.025	90.63	74.98-98.02	92.42	83.20-97.49	11.96
< 1.085	90.63	74.98-98.02	90.91	81.26-96.59	9.97
< 1.210	93.75	79.19-99.23	90.91	81.26-96.59	10.31
< 1.345	93.75	79.19-99.23	89.39	79.36-95.63	8.84
< 1.400	93.75	79.19-99.23	87.88	77.51-94.62	7.73
< 1.445	93.75	79.19-99.23	86.36	75.69-93.57	6.88
< 1.525	93.75	79.19-99.23	84.85	73.90-92.49	6.19
< 1.575	93.75	79.19-99.23	81.82	70.39-90.24	5.16
< 1.660	93.75	79.19-99.23	78.79	66.98-87.89	4.42
< 1.760	96.88	83.78-99.92	74.24	61.99-84.22	3.76
< 1.975	96.88	83.78-99.92	56.06	43.30-68.26	2.2
< 2.075	96.88	83.78-99.92	46.97	34.56-59.66	1.83
< 2.185	100	89.11-100.0	31.82	20.89-44.44	1.47
< 2.355	100	89.11-100.0	13.64	6.430-24.31	1.16

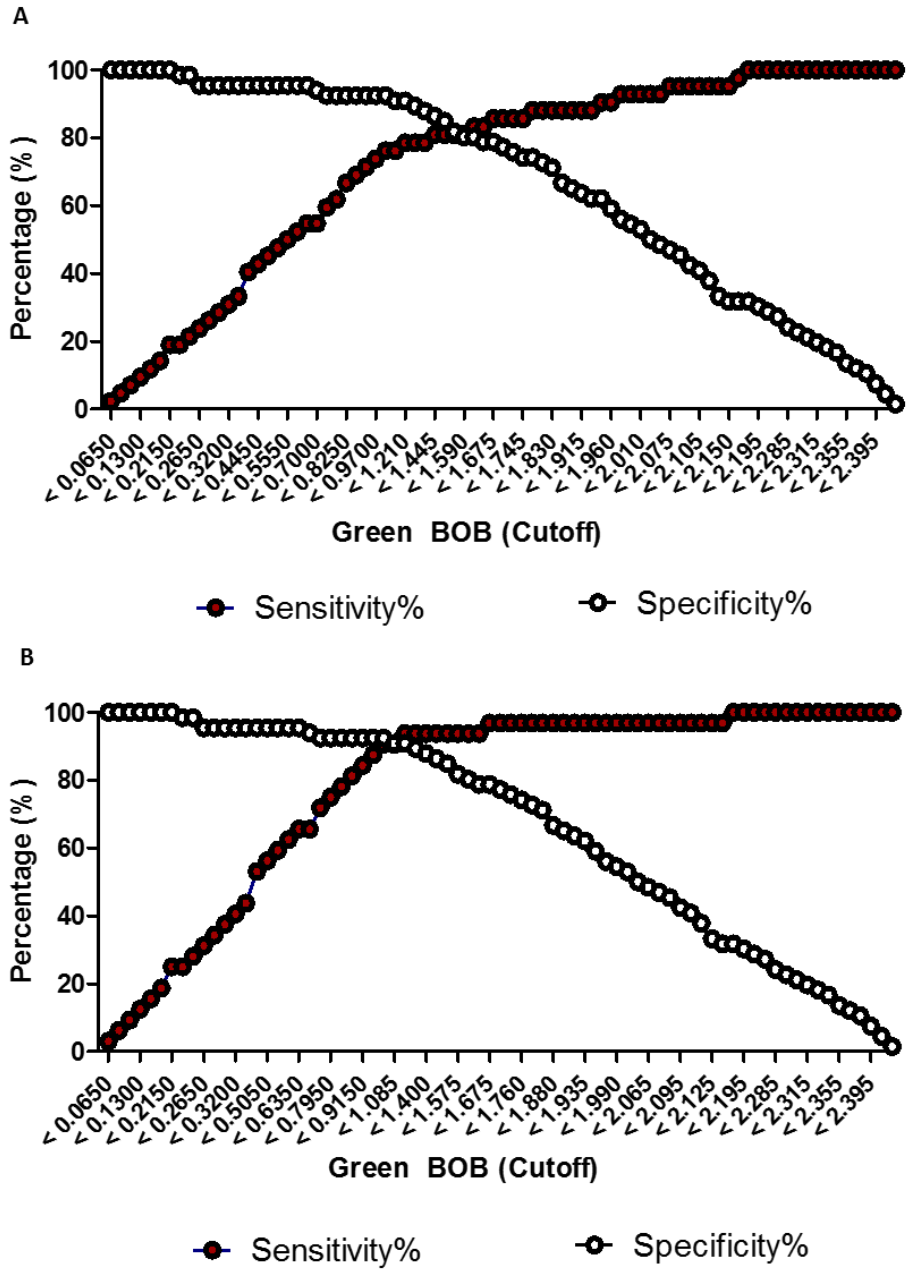


Figure 3.20 Sensitivity and specificity of various cut-off levels in the Green BOB assay
Results are based on the ROC analysis shown in Figure 3.19. A) Sensitivity and Specificity for Cohort A (n=108) which includes n=42 ZIKV specimens. B) Sensitivity and Specificity for Cohort B (n=98) which includes n=32 ZIKV specimens. Cohort B includes only ZIKV specimens collected > 7 days from symptom onset.

3.5.7.3 Performance of the Green BOB assay

As discussed above, a cut-off of 1.0 was used to determine the positivity of each specimen (ratio <1 for ZIKV positive individuals). Overall, sensitivity, specificity and accuracy of the BOB assay was 73.3% (95%CI 63.9-82.6), 92.1% (92.0-92.2) and 82.1% (75.3-87.7) respectively (Table 3.15).

Sensitivity varied relative to the number of days after onset of acute illness when samples were collected. Samples collected 1-5 (n=18); 6-13 (n=22) and > 14 days (n=46) after acute illness exhibited the following sensitivity: 22.2%, 72.7% and 93.5% respectively (Table 3.15 and Figure 3.21). The lowest specificity was observed among patients with DENV-3 with 68.8% (95% CI of 68.52-68.98).

Table 3.15 Sensitivity, specificity and accuracy of the GREEN BOB Assay
Data from ZIKV-positive cases was only used to determine the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases were only used for determining the specificity and were not used for calculating the sensitivity. Abbreviations: Sens., Sensitivity, Spec., Specificity, CI, Confidence Interval, PPV., Positive Predictive Value., Days: number of days the sample was collected after symptom onset NPV., Negative Predictive Value. Equivocal Results were considered negative for the calculation. Non-flavivirus include the measles & rubella specimens, hepatitis and general population samples. DENV (all) includes all DENV samples (DENV 1-4). ZIKV Positive if Green BOB Ratio (GBR) <1.0.

GREEN BOB	*Tested (n)*	*Positive (GBR<1.0)*	*Negative (GBR>1.0)*	*% Sens. %, (95% CI)*	*% Spec. %, (95% CI)*
Zika Positive	`86	63	23	73.26 (63.9-82.61)	
Zika (1-5 Days)	18	4	14	22.22 (3.02-41.43)	
Zika (6-13 Days)	22	16	6	72.73 (54.12-91.34)	
Zika (>14 Days)	46	43	3	93.48 (86.34-100.61)	
Non Zika	**76**	**6**	**70**		92.11 (92.04-92.17)
DENV (all)	60	5	55.00		91.67 (91.6-91.74)
DENV1	11	0	11		100 (100-100)
DENV2	13	0	13		100 (100-100)
DENV3	16	5	11		68.75 (68.52-68.98)
DENV4	20	0	20		100 (100-100)
Yellow fever	6	1	5		83.33 (83.04-83.63)
Non-flavivirus	10	0	10		100 (100-100)
Measles & Rubella	3	0	3		100 (100-100)
Other	7	0	7		100 (100-100)
Overall	**162**	**69**	**93**	**73.26 (63.9-82.61)**	92.11 (92.04-92.17)
PPV	**91.30 (82.82 - 95.81)**				
NPV	**75.27 (68.07 - 81.29)**				
Accuracy	**82.1 (75.31 - 87.67)**				

A better visualization of the results obtained is shown in Figure 3.21. I observed a trend of high seropositivity in specimens collected just a week after the onset of ZIKV symptoms. Six ZIKV negative samples exhibited a high percentage of inhibition ratio values (Ratio 0.2- Ratio

0.9) above the threshold. Another 2 (ZIKV negative) samples exhibited a percentage of inhibition just under the cut-off (between 1.1 and 1) (Figure 3.21 B).

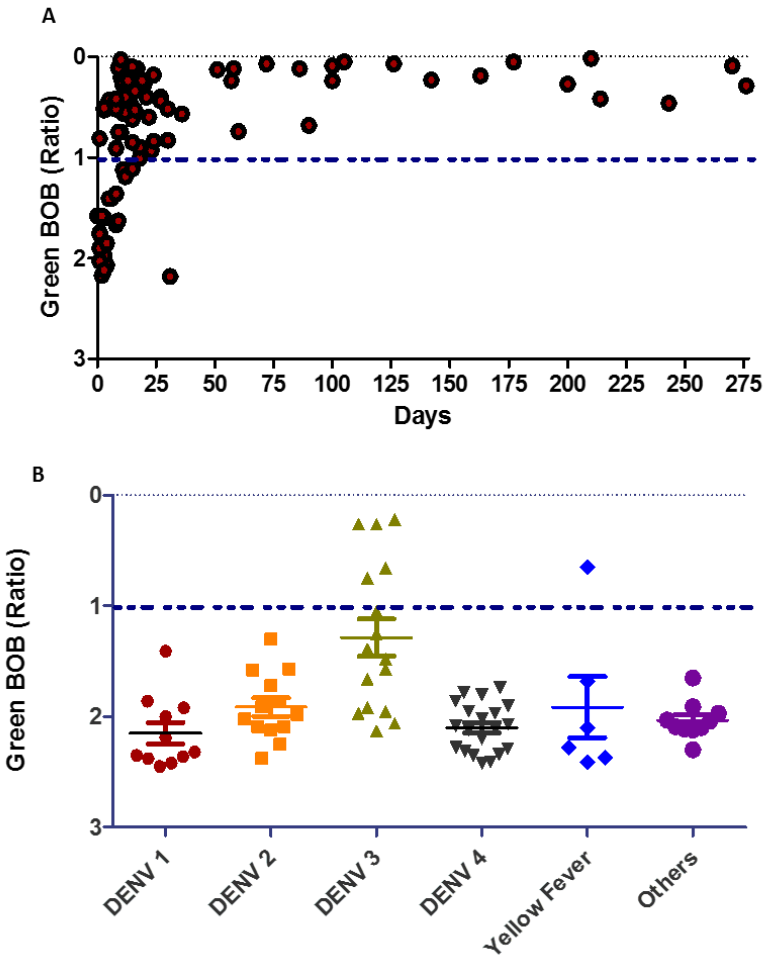


Figure 3.21 Performance of ZIKV GREEN BOB Assay with ZIKV Panel and Specificity Panel
Total testing (n=234). Ratio of ZKA35 mAb binding Green BOB assay by ZIKV Panel (n=86) and specificity panel (n=76) including DENV, yellow fever and other serum samples. A) Plotted are the Green BOB Ratios in ZIKV specimens (n=114); B) Plotted are the Green BOB ratios in specificity panel (n=120) for DENV-1 to 4, Yellow fever and others. Others include measles, rubella, hepatitis, and non-infected individuals from before the ZIKV outbreak. Blue dotted line represents cut-off for the Green BOB Assay (Ratio <1.0).

3.5.7.4 Evaluation of the reproducibility of GREEN BOB assay

Then, I further investigated the reproducibility and repeatability of the Green BOB assay. This is fundamental to ensure the assay is suitable for large scale testing. In order to estimate this, I selected 6 specimens that represented a mix of true positives, true negatives, false positives and specimens with antibody ratios close to the cut-off. Each sample was tested 5 times in independent experiments. The 6 Samples selected have the following characteristics:

- Sample 1: ZIKV Positive specimen (PCR Positive subject) with a low positive

- Sample 2: ZIKV Positive specimen (PCR Positive subject) with a high positive (Ratio <0.5)
- Sample 3: DENV 4 specimen from before 2013 (Sensitivity Panel). Negative IgG ZIKV
- Sample 4: DENV 4 DENV 4 specimen from before 2013 (Sensitivity Panel). Negative IgG ZIKV
- Sample 5: DENV 3 specimen from 2008– False positive ZIKV results with multiple assays.
- Sample 6: DENV 3 specimen from 2008– Negative antibody levels but close to borderline.

Results are shown in Figure 3.22 and the coefficient of variation (CV) is shown in Table 3.16. The CV found across all the samples was always <20% with 2.7 and 18.2% as a min and maximum CV identified. The findings demonstrate that there is a high reproducibility of this assay.

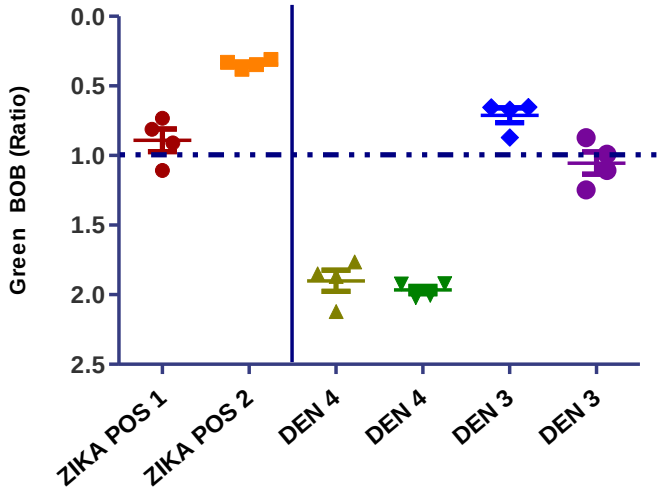


Figure 3.22 Reproducibility of Green BOB Assay
The characteristics of the samples tested are described in Table 3.16. Blue dotted line represents cut-off value for Green BOB assay (Positive if Ratio <1.0).

Table 3.16 Green BOB reproducibility for 6 different specimens

Reproducibility Sample ID	Sample characteristics	Green BOB Coefficient of Variance (CV)
1	ZIKV Borderline (collected in 2015)	18.2%
2	ZIKV Positive (collected in 2015)	8.8%
3	DENV- 4 ; ZIKV antibody negative with Euroimmun assays (collected in 2012)	8.1%
4	DENV- 4 ; ZIKV antibody negative with Euroimmun assays (collected in 2012)	2.7%
5	DENV- 3; False ZIKV positive. ZIKV antibody positive with Green BOB and Euroimmun assays (collected in 2008)	15.0%
6	DENV- 3; ZIKV negative antibody levels close to cut-off (collected in 2008)	15.3%

3.5.8 Evaluation and optimization of PRNT

As the plaque reduction neutralizing test (PRNT) is currently the gold standard assay for the serological differentiation of flavivirus infections, I worked on developing and adapting PRNT protocols for detection of ZIKV antibodies. As establishing PRNTs is a complex technique, optimization of the cell culture and virus culture techniques were needed.

3.5.8.1 Optimising ZIKV PRNTs as diagnostic assays

I initially adapted a protocol developed for JEV PRNTs and optimised it for accurate detection of ZIKV in the Institute of Infections and Global Health in Liverpool with the objective of a later optimization of the assay in Brazil.

$PRNT_{50}$ was defined as the serum dilution factor that could inhibit 50% of the input virus. Optimization assays described here were conducted with the study population described in 5.4.2.2. A detailed protocol to perform the PRNTs is described in section 2.2.7.

I evaluated two different staining techniques; crystal violet and neutral red (Figure 3.23). (Protocols 1 and 2 in Appendix). ZIKV plaques did not appear to be contained as well when using neutral red. This meant, that they were easier to count and distinguish individual plaques when using crystal violet. Both protocols exhibited similar results with no obvious differences in number of plaques observed. Example of this two staining techniques are shown in Figure 3.23.

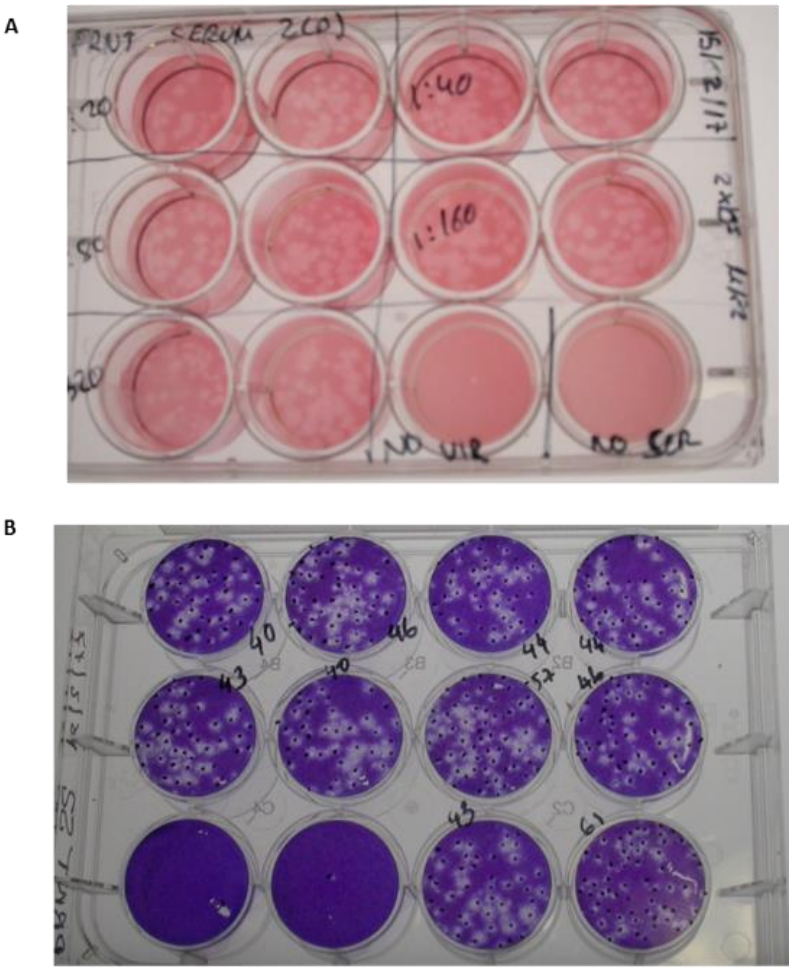


Figure 3.23 Optimization of ZIKV PRNTs
Testing and optimizing PRNT assay for Zika virus diagnosis using Brazilian ZIKV strain: PE-243. A) PRNT ZIKV assay using Protocol 1 for sample Liv14 (appendix 3). B) Selection of PRNT ZIKA assay results using Protocol 2 (Appendix 3) for sample Liv14. Two negative controls included: No virus control (with serum) and no serum control, neither virus. ZIKV Titres with $PRNT_{50}$<20 and thus, ZIKV negative.

Then, further optimizations were conducted using 24 and 14-well plates and to determine the most appropriate viral titre to use (Figure 3.24). Serum dilutions were included for each specimen tested as the $PRNT_{50}$ was estimated calculating the serum dilution that produces a 50% reduction in the number of plaques formed compared to the number formed on wells with only ZIKV virus (no serum added). The cut-off used with > 50% reduction at a serum dilution of 1:100 had been previously established in preliminary studies in Brazil and was expressed in ZIKV titres [119].

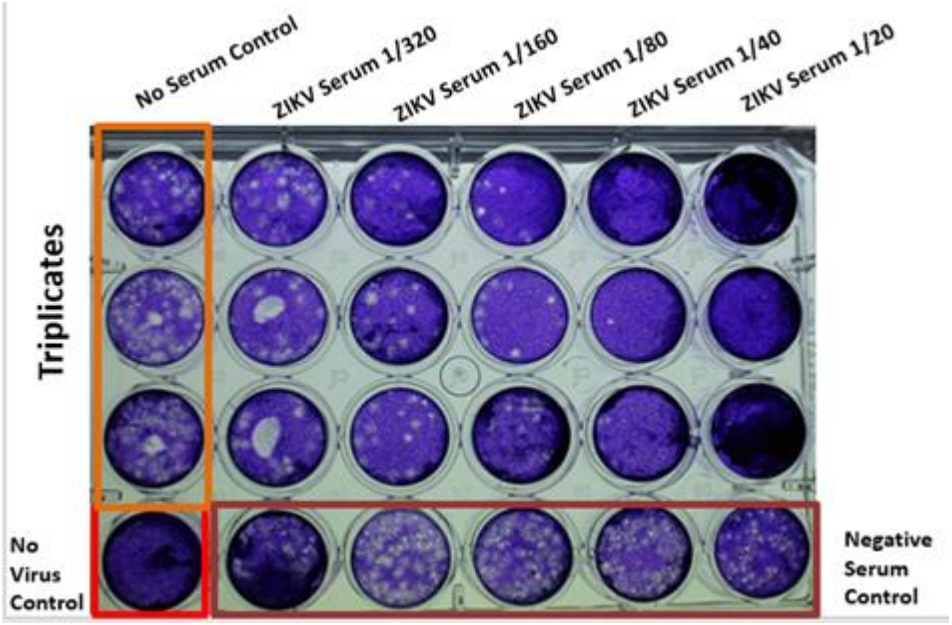


Figure 3.24 Further optimization of PRNTs for ZIKV diagnosis in a ZIKV infected patient
ZIKV Positive specimen collected at 50 days post symptom onset from a traveller that returned to UK. $PRNT_{50}$ >250. Controls used indicated in the figure: no serum control, no virus control and negative serum control. PRNTs performed using Brazilian ZIKV Strain PE-243

Then, once the PRNT assays offered easy-to-read and reproducible results, I evaluated the 16 specimens from the Liverpool cohort (Figure 3.25). Two of the 16 specimens tested exhibited ZIKV positive PRNT titres (cut-off $PRNT_{50}$, dilution of 1/100). The known ZIKV positive specimen had high levels of neutralization detected (ZIKV titre >1280 with $PRNT_{50}$, at a serum dilution of 1:100). In addition, the specimen OX08, a subject with extensive exposure to the four dengue serotypes and with no history of recent travel to South America, also showed very high neutralization values ($PRNT_{50}$ ZIKV titre >1280) (Figure 3.25B). All the other 14 specimens tested exhibited negative results with no neutralization titres observed ($PRNT_{50}$ with ZIKV titres <100). Assays were conducted with 30 to 50 plaques per control well.

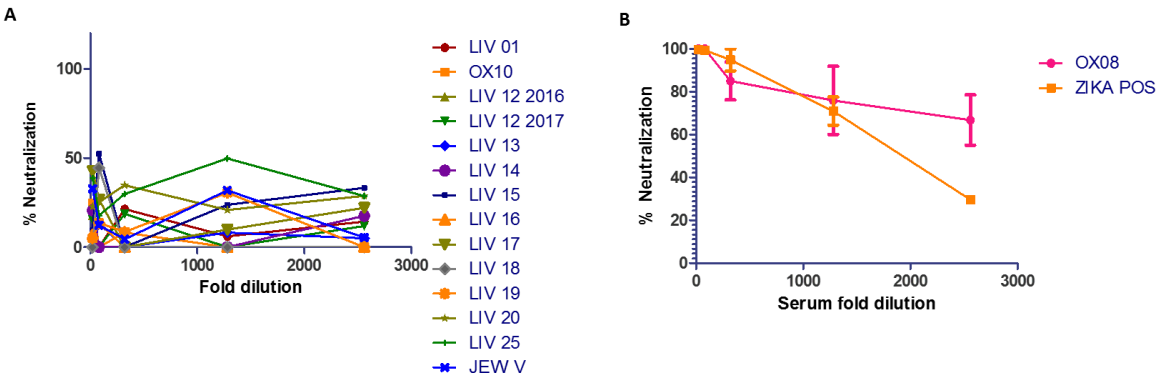


Figure 3.25 ZIKV PRNT results for the Liverpool cohort (n=16)
The optimised ZIKV PRNT assay results of testing performed in Liverpool with 16 specimens. PRNTs performed using Brazilian ZIKV Strain PE-243 A) ZIKV PRNT Neutralization for the 14 subjects that scored negatively (ZIKV Titres <100). B) ZIKV PRNT neutralization for the 2 subjects that scored positively (ZIKV titres >1280). Graphs show % of neutralization and serum fold dilution time-points. Positive specimens were tested in duplicate.

3.5.8.2 Evaluation of ZIKV PRNTs in the Brazilian population

Then, we evaluated the specificity, sensitivity and accuracy of PRNT assays in Brazil using a similar protocol, adapted to the Brazilian equipment and strains (full description in methods). We evaluated the assay with the same panel of sera as the panel used for the ELISA assay evaluations.

3.5.8.3 Performance of PRNT assay in the Brazilian population

Overall, sensitivity, specificity and accuracy of the PRNT assay was 68.18% (95%CI 60.83-75.54), 96.08% (96.04-96.17) and 79.3% (73.8 – 84.09) respectively (Table 3.17). Sensitivity varied relative to the number of days after onset of acute illness the samples were collected. Samples collected 1-5 (n=63); 6-13 (n=43) and > 14 days (n=48) after acute illness exhibited the following sensitivity: 34.9%, 88.37% and 93.8% respectively (Table 3.17, Figure 3.26 and Figure 3.27).

The lowest specificity was observed among patients with DENV-3 (75.0% with 95% CI of (74.79-75.21). Only 4/102 specimens from Cohort 2 exhibited positive results for the PRNT assay. The assay also showed highly reproducible results as shown in Appendix 3.

As shown in Figure 3.26, cut-off established at ZIKV Titres >100 with $PRNT_{50}$ to limit cross-reactivity and improve specificity. This cut-off had been evaluated and established in independent evaluations conducted by the team in Fiocruz Recife (Data not published).

Table 3.17 Sensitivity, specificity and accuracy of the PRNTs

Data from ZIKV-positive cases served only for determining the sensitivity and was not used for the specificity calculation. Similarly, data from ZIKV-negative cases serve only for determining the specificity and were not used for calculating the sensitivity. Abbreviations: Sens., Sensitivity, Spec., Specificity, CI, Confidence Interval, PPV, Positive Predictive Value, Days: number of days the sample was collected after symptom onset NPV, Negative Predictive Value. Equivocal Results were considered negative for the calculation. Non-flavivirus include the measles & rubella specimens, hepatitis and general population samples. DENV (all) includes all DENV samples (DENV 1-4).

PRNTs	Tested (n)	Positive (GBR<1.0)	Negative (GBR>1.0)	% Sens.	% Spec.
Zika Positive (All)	154	105	49	68.18 (60.83-75.54)	
Zika (1-5 Days)	63	22	41	34.92 (23.15-46.69)	
Zika (6-13 Days)	43	38	5	88.37 (78.79-97.95)	
Zika (>14 Days)	**48**	45	3	93.75 (86.9-100.6)	
Non ZIKV (All)	**102**	**4**	**98**		96.08 (96.04-96.12)
DENV (all)	71	4	67		94.37 (94.31-94.42)
DENV1	14	0	14		100 (100-100)
DENV2	12	0	12		100 (100-100)
DENV3	16	4	12		75.00 (74.79-75.21)
DENV4	29	0	29		100 (100-100)
Yellow fever	15	0	15		100 (100-100)
Non-flavivirus	15	0	15		100 (100-100)
Measles & Rubella	0				
Other	16	0	16		100 (100-100)
Overall	**256**	**109**	**147**	**68.18 (60.83-75.54)**	**96.08 (96.04-96.12)**
PPV	**96.33 (90.90 - 98.57)**				
NPV	**66.67 (61.27 - 71.66)**				
Accuracy	**79.3 (73.81 - 84.09)**				

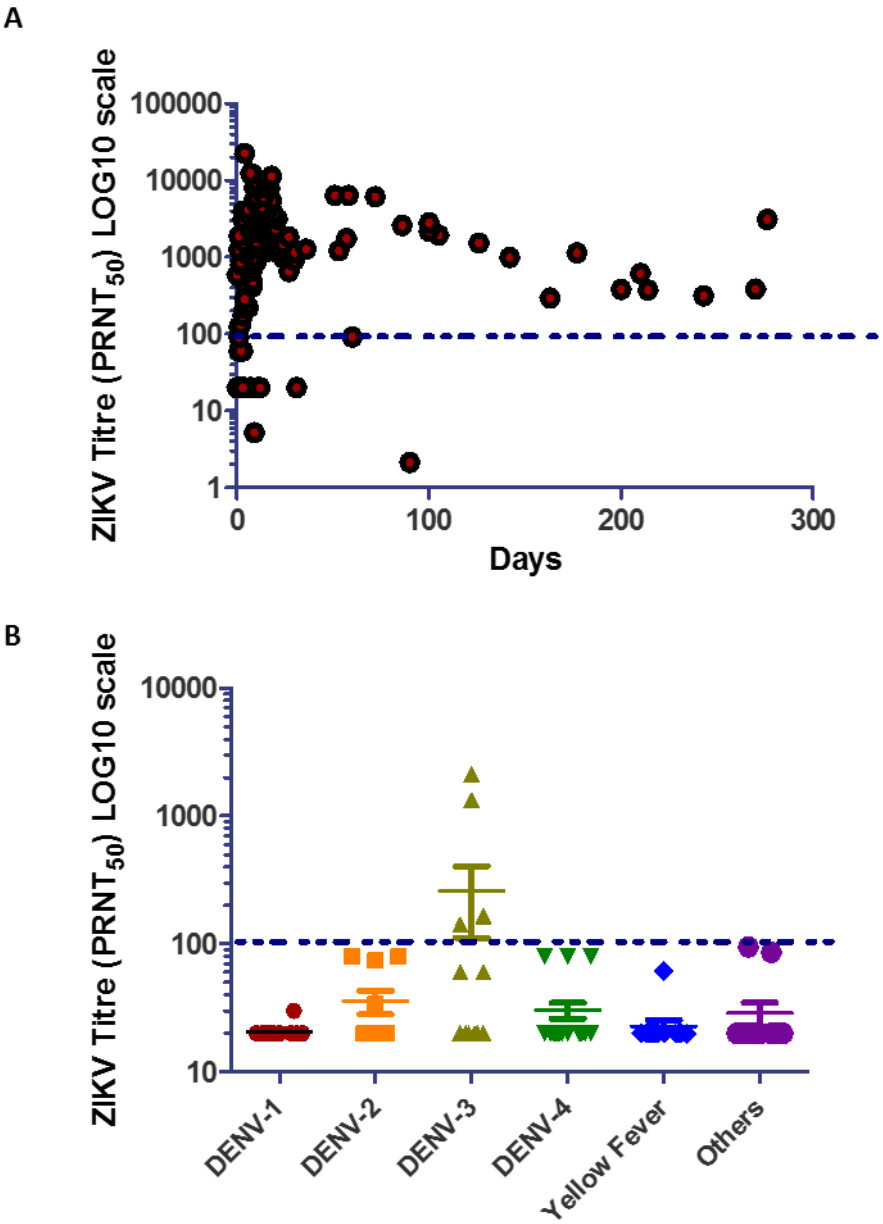


Figure 3.26 ZIKV $PRNT_{50}$ analysis of ZIKV Panel and specificity panel from Brazil
ZIKV titre for $PRNT_{50}$. Y Axes shown in Log_{10} . PRNTs performed using Brazilian ZIKV Strain PE-243. A) $PRNT_{50}$ in ZIKV specimens (n=154); B) $PRNT_{50}$ in Specificity Panel (n=102) for DENV-1 to 4, Yellow fever and others. Others include measles, rubella, and hepatitis. Samples with titre shown as 20 represents a ZIKV titre of <20. Blue dotted line represents cut-off at 1:100 dilution (Positive with $PRNT_{50}$ ZIKV Titres>100).

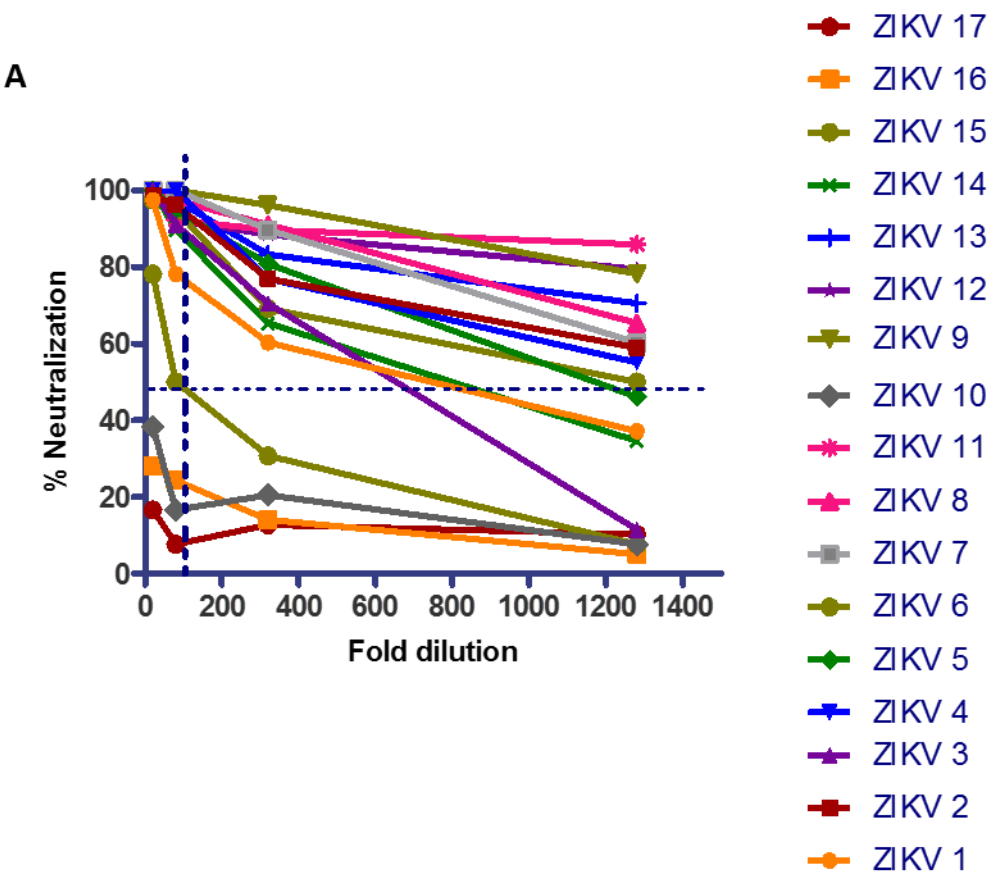


Figure 3.27 ZIKV $PRNT_{50}$ Neutralization for a subset of ZIKV positive specimens
Graph showing % of Neutralization of ZIKV antibodies in a subset of 17 specimens from the Brazilian ZIKV panel. The percentage of neutralization measures the difference between the concentration of serum to reduce the number of plaques by 50% compared to the control (serum free virus) which correlates with how much antibody is present. Cut-off ($PRNT_{50}$ at dilution 1/100). 14/17 specimens showed in this graphic scored positive. Blue dotted lines shows $PRNT_{50}$ cut-off line (Horizontal) and Fold Dilution at 1:100 dilution. Positive above cut-off line for dilution 1:100.

3.5.9 Further investigation of other methods to detect ZIKV antibodies

The following methods were also investigated for ZIKV diagnosis:

- Immunofluorescent assays (IFA)
- A Point of Care Test (POCT) for ZIKV IgG and IgM detection

3.5.9.1 Evaluation of Immunofluorescence assays for ZIKV antibody detection

Immunofluorescence assays (IFA) have been used for decades for the diagnosis of flaviviruses. IFA are techniques read with a fluorescence microscope. I evaluated the first commercial indirect immunofluorescence assay (IFA) for specific detection of ZIKV antibodies. This commercial IFA assay was developed by the company Euroimmun and consists of a Biochip technology that detects immunoglobulin G (IgG) and IgM antibodies against a range of pathogens. I conducted two small evaluations of two of their assays:

IIFT Arbovirus Fever Mosaic 2. Designed for specific detection of 6 pathogens including ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV.

IIFT Arbovirus Profile Mosaic 3. Designed for specific detection of 10 pathogens including the 6 above mentioned in the IIFT Mosaic 2 [ZIKV, DENV (serotypes 1, 2, 3 and 4) and CHIKV] but also Tick-Borne Encephalitis Virus (TBEV), Yellow Fever Virus (YFV), West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV).

Our preliminary analyses to test the protocol, fluorescence conditions and controls is shown in Figure 3.28. As shown, the cells used for CHIKV staining are significantly different from the cells used for the flavivirus pathogens. The positive and negative controls were shown to work well offering good fluorescent images.

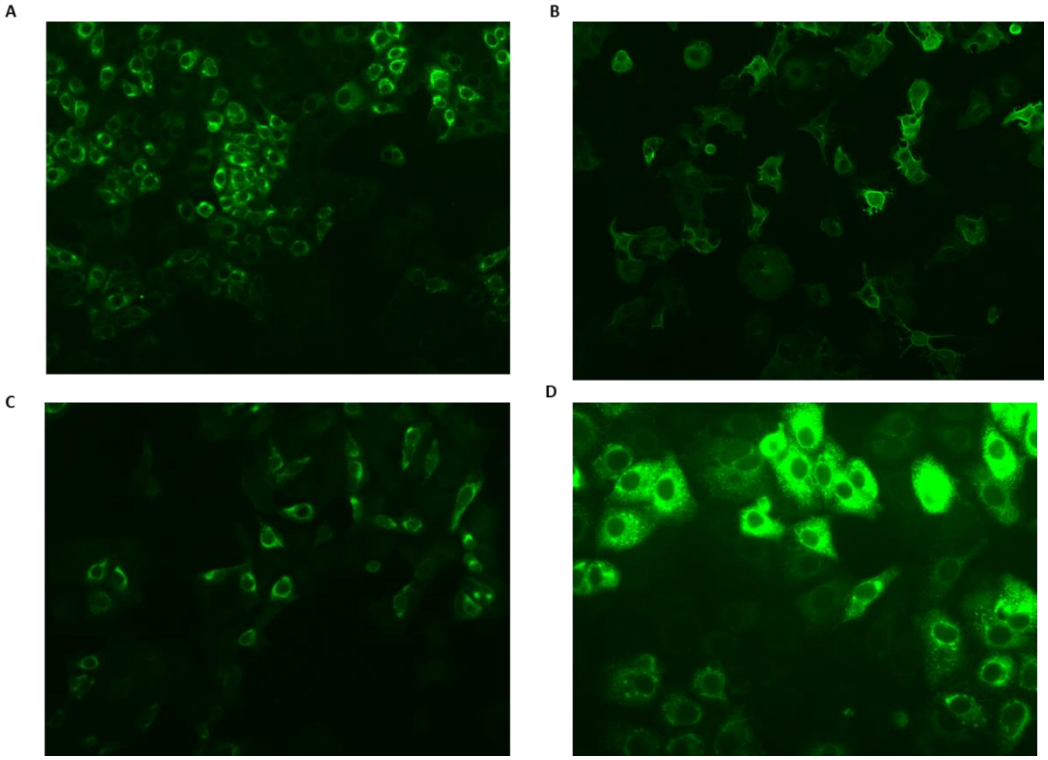


Figure 3.28 IIFT 2 Mosaic results examples of results found
A.)DENV; B) CHIKV Positive Control; C) DENV ; D) ZIKV positive. 40x amplification.

I tested the Arbovirus profile III with 13 specimens that were selected because of their unique characteristics. This assay allows for simultaneous testing of five specimens per slide (plus a positive and negative control). The results obtained and a summary of the characteristics of the samples are shown in (Table 3.18). The results obtained for specimen number 9, a DENV-3 positive sample collected in 2008 are shown in Figure 3.29 as an example. The results

showed a poor overall performance of the assay. The assay was specific for the differentiation of CHIKV antibodies and flavivirus antibodies, however, for both the ZIKV specimens (collected in 2016) as well as the DENV specimens (collected before the ZIKV outbreak in 2008 and 2012), an intense non-specific cross-reactive antibody response is shown with positive detection of antibodies against all flaviviruses including DENV (serotypes 1, 2, 3 and 4), TBEV, YFV, WNV and JEV. Due to the low prevalence of TBE, WNV and JEV in Brazil, this finding of positive antibodies for all three pathogens in 10/13 specimens tested highlights the low specificity for flavivirus detection of the assay when testing ZIKV or DENV specimens. Unfortunately, the positive control provided for the assay was just a control for CHIKV with no positive controls for the other pathogens. The assay also showed specific detection of YFV antibodies (three specimens tested).

Table 3.18 Summary results IIFT Arbovirus Profile III

Thirteen specimens were tested in 3 slides (5 samples per slide + Positive and Negative controls). Each biochip detects 10 different pathogens: ZIKV, DENV1 to 4, CHIKV, Tick-Borne Encephalitis (TBE), West Nile Virus (WNV), Japanese Encephalitis Virus (JEV) and Yellow Fever Virus (YFV). A symbol of + represents a positive result. A symbol of – represents a negative result. Year of sample collection is shown. Year. = Year of sample collection.

Sample ID.	Sample (year.)	ZIKV	DENV-1	DENV-2	DENV-3	DENV-4	CHIKV	TBE	WNV	JEV	YFV
0	Positive Control	-	-	-	-	-	+	-	-	-	-
0	Negative Control	-	-	-	-	-	-	-	-	-	-
1	ZIKV sample (2016)	+	+	+	+	+	-	+	+	+	+
2	ZIKV sample (2016)	+	+	+	+	+	-	+	+	+	+
3	ZIKV sample (2016)	+	+	+	+	-	-	+	+	+	+
4	ZIKV sample (2016)	+	+	+	+	+	-	+	+	+	+
5	ZIKV sample (2016)	+	+	+	+	+	-	+	+	+	+
6	Yellow Fever vaccination (2007)	-	-	-	-	-	-	-	-	-	+
7	Yellow Fever vaccination (2007)	-	-	-	-	-	-	-	-	-	+
8	Yellow Fever vaccination (2007)	-	-	-	-	-	-	-	-	-	+
9	DENV-3 (2008)	+	+	+	+	+	-	+	+	+	+
10	DENV-3 (2008)	+	+	+	+	+	-	+	+	+	+
11	DENV-3 (2008)	+	+	+	+	+	-	+	+	+	+
12	DENV-4 (2012)	+	+	+	+	+	-	+	+	+	+
13	DENV-4 (2012)	+	+	+	+	+	-	+	+	+	+

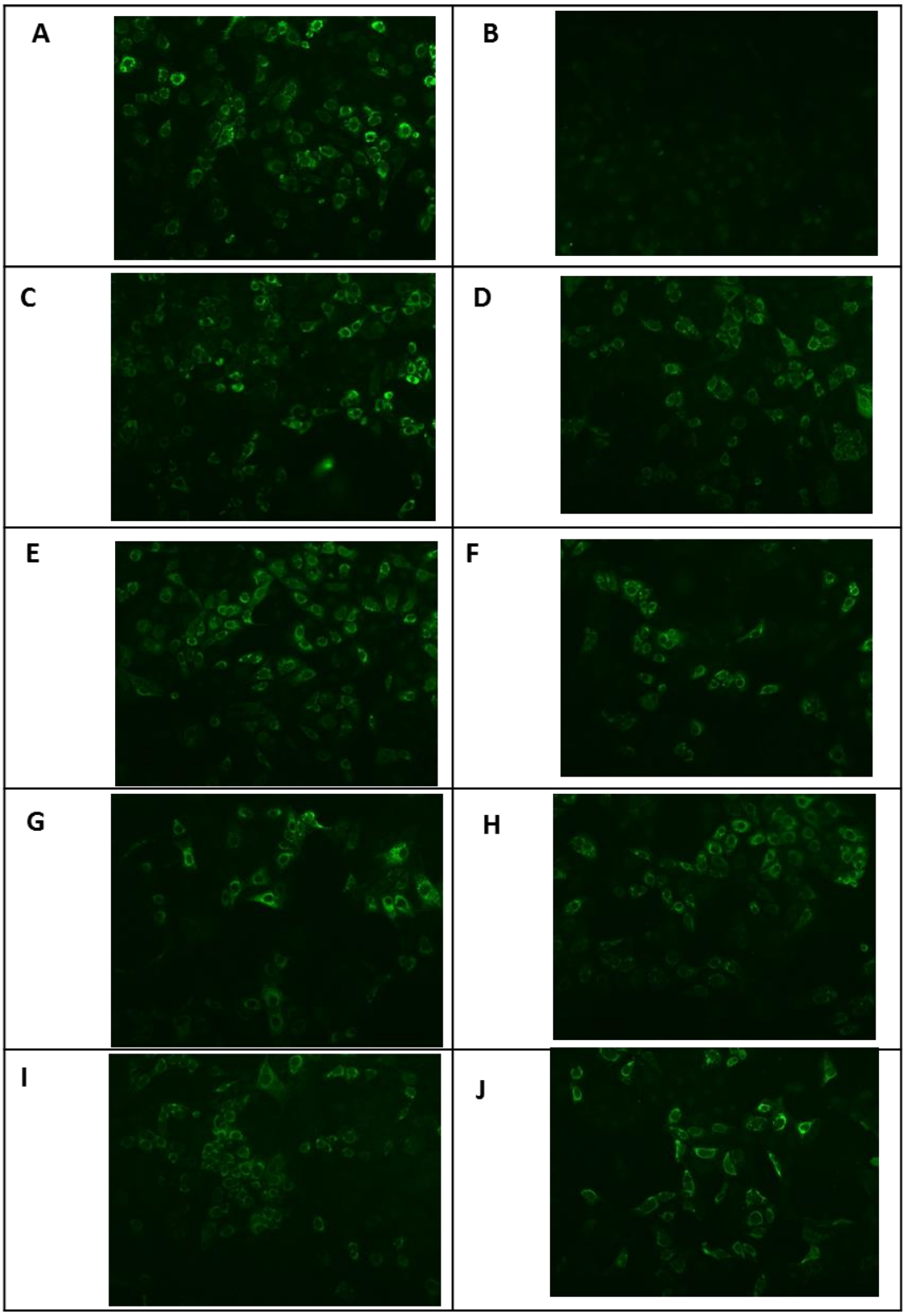


Figure 3.29 IIFT Arbovirus Profile III results for a DENV-3 specimen collected in 2008
Immunofluorescent results obtained by light microscopy for a specimen collected in 2013. Images collected at 20X are shown for: A) ZIKV, B) CHIKV, C) DENV-1, D) DENV-4, E) DENV-2, F) DENV-3, G) Tick-Borne Virus (TBE), H) Yellow Fever Virus, I) West Nile Virus (WNV), J) Japanese Encephalitis Virus (JEV). Results show the specimen is negative for CHIKV antibodies, but there is a positive reaction for all the other pathogens.

I then tested a simpler version of the IFA assay, the IIFT Mosaic 2: which is meant to target just ZIKV, CHIKV and DENV-1 to 4. I tested three cohorts of specimens collected in 2013 (n=23), in 2014 (n=45) and in 2015 (n=60). Results obtained are shown in Table 3.19. An example of specimen tested with this IIFT Arbovirus Mosaic III assay collected in 2013 with negative ZIKV diagnoses (as per IgM and IgG Euroimmun assay) but positive IgG DENV antibodies tested with the Panbio ELISA is shown in Figure 3.28. The results showed high specificity for detection of CHIKV antibodies. I also showed a large population cross-reactive response between the flaviviruses tested (ZIKV, and the 4 serotypes of DENV). This test in the population highlights the poor performance of this IFA assay with particularly low specificity to distinguish flavivirus infections. Even specimens classified as DENV naïve (with no IgG DENV antibodies as tested with the Panbio ELISA, showed an antibody response with the IIFT assay highlighting the poor performance.

Table 3.19 Antibody prevalence identified with the IIFT Mosaic 2 assay. Prevalence of antibody detection results for three cohorts from 2013, 2014 and 2015 are shown.

Substrate	2013			2014			2015		
	Tested (n)	Positive (n)	Prevalence (%)	Tested (n)	Positive (n)	Prevalence (%)	Tested (n)	Positive (n)	Prevalence (%)
ZIKV	23	23	100	45	44	97.78	60	58	96.67
DENV-1	23	23	100	45	44	97.78	60	58	96.67
DENV-2	23	23	100	45	44	97.78	60	58	96.67
DENV-3	23	23	100	45	44	97.78	60	58	96.67
DENV-4	23	23	100	45	40	88.89	60	39	65.00
CHIKV	23	0	0	45	0	0.00	60	2	3.33

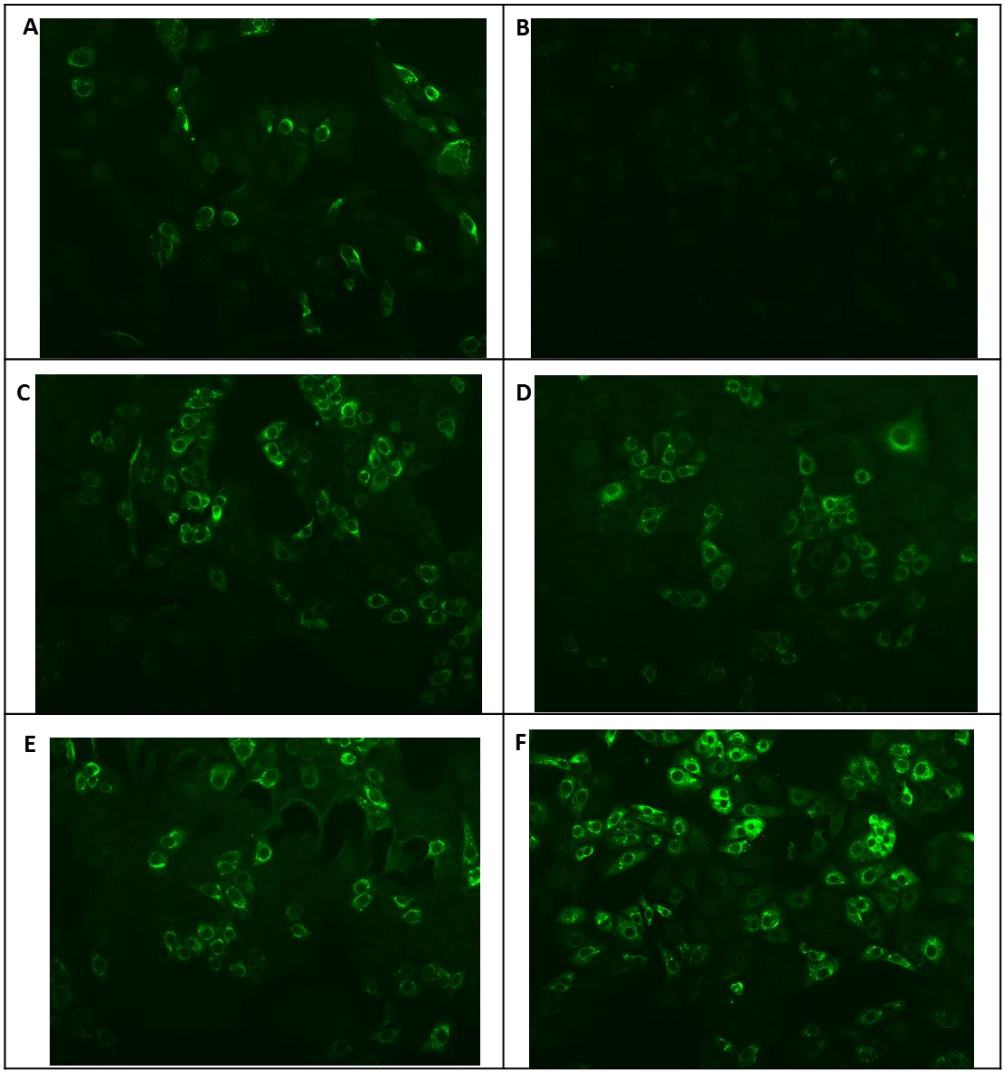


Figure 3.30 IIFT Arbovirus Mosaic 2 results for a specimen collected in 2013
Immunofluorescent results obtained by light microscopy for specimen number 9, a DENV-3 specimen collected in 2008. Images collected at 20X are shown for: A) ZIKV, B) CHIKV, C) DENV-1, D) DENV-4, E) DENV-2, F) DENV-3. Results show the specimen is negative for CHIKV antibodies, but there is a positive reaction for all the other pathogens.

3.5.10 A Point of Care Test (POCT) for ZIKV IgG and IgM detection

A preliminary study was conducted to assess the accuracy of a rapid diagnostic assay developed in Brazil TRDPP ZIKA IgM and IgG (Biomanguinhos, Rio de Janeiro, Brazil). A total of 30 Samples were tested for the TRDPP ZIKA IgM & IgG following the manufacturer's instructions. The samples tested were classified as follows:

- Sensitivity Panel: 16 known ZIKV positive cases from 3 different subjects (8 sequential specimens from one subject, 7 sequential serum samples from another individual and 1 specimen from other). The first specimen sample from these subjects was ZIKV PCR Positive.

- Specificity Panel: 14 flavivirus positive samples collected before the ZIKV outbreak in Brazil (2013 or before). All the specimens chosen had shown false positive results with the IgG Euroimmun Assay.

Results obtained are summarised in Table 3.20. The ZIKV IgM RDT assay showed a very high specificity (100%) while very low sensitivity [6.25(95% CI of -5.61-18.11)]. The ZIKV IgG RDT assay showed higher sensitivity values with 68.75% (95 % CI 46.04-91.46) but lower specificity 64.29% (95% CI of 64.03-64.54). Combined analysis of the IgM and IgG results did not improve the specificity or sensitivity of the assay.

Even though this evaluation was conducted with a very small number of samples, the results show a very poor overall accuracy for both the IgM and IgG assays with 50.00% (95% CI of 31.30 - 68.70) and 66.67% (95% CI of 47.19 - 82.71), respectively. These preliminary results highlight the issues of the use of this assay in the Brazilian population.

Table 3.20 Evaluation of the specificity and sensitivity of the TRDPP ZIKA IgM and IgG assay
Sens.=Sensitivity, Spec=Specificity, DENV=Dengue Virus. Ratio expressed in the DTT Units. Pos=Positive; Neg=Negative

		ZIKV IgM RDT				ZIKV IgG RDT			
	Tested (n)	Pos (Ratio>28)	Neg (ratio <28)	Sens. % (95% CI)	Spec. % (95% CI)	Pos (ratio>28)	Neg (ratio<28)	Sens. % (95% CI)	Spec. % (95% CI)
Zika Positive	16	1	15	6.25(-5.61-18.11)		11	5	68.75(46.04-91.46)	
Zika (1-5 Days)	4	0	4	0(0-0)		1	3	25(-17.44-67.44)	
Zika (6-13 Days)	2	1	1	50(-19.3-119.3)		0	2	0(0-0)	
Zika (>14 Days)	**10**	0	10	0(0-0)		10	0	100(100-100)	
Non ZIKV	**14**	**0**	**14**		100(100-100)	5	9		64.29(64.03-64.54)
DENV	10	**0**	10		100(100-100)	4	6		60(59.7-60.3)
Yellow fever	3	**0**	3		100(100-100)	1	2		66.67(66.13-67.2)
Hepatitis	1	**0**	1		100(100-100)	0	1		100(100-100)
Overall	**30**	**1**	**29**	6.25(-5.61-18.11)	100(100-100)	16	14	68.75(46.04-91.46)	64.29(64.03-64.54)

3.6 Discussion

To the best of my knowledge, this chapter represents the first comprehensive evaluation of different methods to detect ZIKV antibodies in the Brazilian population. The results in this chapter highlight the challenging scenario currently present in endemic regions for serological diagnosis of ZIKV on acute and early convalescent subjects.

Assays that detect IgG antibodies for ZIKV are key to indicating the presence of recent or past infection. As the 2015/2016 outbreak was the first ZIKV epidemic in South America and no further significant epidemics of ZIKV have occurred, identifying an accurate IgG diagnostic assay became a priority to be able to conduct accurate seroepidemiological studies. Moreover, identifying an easy-to-perform, highly reproducible assay that could be used to conduct large-scale studies in a cost-effective manner, became a fundamental need for use in Brazil [158].

3.6.1 Compared performance of the serological assays

The commercial IgG and IgM ZIKV NS1 ELISAs and the MAC ELISA were the first commercial diagnostic assays approved by the National Health Surveillance Agency of Brazil (ANVISA). The IgG NS1 Euroimmun assay showed the highest accuracy with 72.5% and the highest NPV with 68.6%. The poor specificity observed with the IgG NS1 Euroimmun assay indicates that this assay provides 22.4% of false positive results in flavivirus exposed populations. Moreover, users must be aware, 19.6% of the ZIKV positive samples collected between 6 to 14 days (median) post-illness onset were IgG negative. Missed positive diagnosis of ZIKV cases measured with the IgG NS1 assay increased to 65% when samples were collected acutely between 0 to 5 days post-illness onset. The commercial IgM μ-capture assay showed the highest PPV of 86.9 and could potentially be used as a rule-in method for recent ZIKV infection for patients presenting after acute phase.

When I started this research work, there was no published validation study available that had evaluated serological assays. To date (as of July 2018), there are few published validation studies of commercial serological assays using well-characterised samples, but none from Brazil, the country that was impacted by ZIKV the most. Moreover, none of the published studies use serum collections from before the 2015-2016 epidemic, which was when ZIKV spread widely across the Americas.

My findings evaluating these assays in a highly flavivirus exposed population contrast with recently published studies conducted using traveller populations which reported much higher sensitivity (of over 90%) for the commercial IgM and IgG Euroimmun (NS1) assays

[106, 136, 159]. Their evaluation using mainly European travellers' samples also found a high specificity. However, it has also been reported that the specificity of the IgM NS1 assay may be low among malaria positive subjects [137]. Surprisingly, it was also reported a very high specificity for the IgG NS1 assay of 90% among Canadian travellers [14] which is much higher to the specificity levels that I obtain of 77.6%, with the test showing even lower specificity, in my study, for subjects previously infected with DENV-2.

A recently published multicentre study conducted in North America by Basile *et al.* (2018) includes evaluations performed in three different laboratories, reported sensitivity and specificity for the IgM NS1 assay of 53.1% and 97.0% (respectively) [160]. In the results presented in this chapter, similar levels of specificity (96.4) were observed while a much lower sensitivity of 22% (which is 31% less). This study also found much lower sensitivity for the IgG NS1 Euroimmun assay of 34.4% compared to 67.9%. However, they report specificity values of 94.2%, which are much higher than the specificity values that the found in this study at 77.6%. This evidence demonstrates the relevance of testing in populations with previous flavivirus exposure. All these suggest that among DENV immune populations (such as Brazil), the IgG NS1 assay has a much higher sensitivity because the specificity is also much lower due to cross-reactivity with antibodies such as DENV or yellow fever. This also reinforces the claim that the IgM and IgG NS1 Euroimmun assays have poor performance among the Brazilian population and its use for routine diagnostics should be revised.

The IgM μ-capture ELISA which has recently been approved by the FDA and became the recommended method by ANVISA (Brazil) in 2017, is currently being used across the Americas. However, appropriate validation with samples from South America is needed. To date, only two publications have evaluated this assay both with samples from North America, which reported low specificity values ranging from 66% (study conducted in Canada) to 97.6% (study conducted in the USA) [14] [160].

The CDC recommends the use of an IgM antibody capture enzyme-linked immunosorbent assay (MAC ELISA), which is based on the CDC FDA-emergency-use-authorization protocol. Nevertheless, it has recently recognised the existence of multiple limitations on the use of this assay and related serologic assays for ZIKV diagnosis due to potential false-positive and false-negative test results [161]. Differences among the assays accuracies observed are probably linked to differences between travellers and the Brazilian population [162]. Moreover, recent results now in Nicaragua and the USA have suggested this assay has low specificity (around 82 to 85%) and full evaluations are needed [163, 164]. Based on this

results, I believe it is not appropriate to use exclusively specimens from travellers for validation of serological assays for flaviviral diagnostics.

The cut-off levels established for this commercial assays were optimised mainly using samples from travellers. For example, the cut-offs for the IgM and IgG NS1 Euroimmun ELISAs were established with testing of 29 ZIKV positive samples and over 600 non ZIKV controls from German blood donors or travellers (as reported in their manual). Similarly, the IgM μ-capture was validated using a panel of samples from the US and North America. I suggest the performance of optimizations for the South American population and recommend adjusting the cut-off assay values to flavivirus-exposed populations in order to improve assay accuracy. Nevertheless, this is a compromise and the overall performance of the commercial assays is still poor. I believe that my findings reinforce the limited capacity for these assays to discriminate between infections caused by closely related flaviviruses.

Cross-reaction seems to be one of the most challenging problems around the diagnosis of flaviviral infections. Recent studies have suggested that the epitopes in the E region may not be as specific as the NS1 region of the flaviviruses [165]. In these results I observed a very high degree of false positives with the MAC ELISA assay. Specificity improved as the cut-off was raised, but sensitivity was then lowered. As expected, the in-house MAC ELISA assay required several additional steps when compared to the commercial ELISAs with over five hours required distributed in over at least two days, compared to around three to four hours for the commercial assays. Moreover, the CDC MAC-ELISA needs site-specific optimization of the dilutions used for some of the reagents leading to potential variability among sites.

Regarding the key features of the kit and taking into account the assay length and number of samples that can be processed per plate, both the IgM and IgG Euroimmun NS1 assays have shown to be the easiest to perform and most high throughput methods among the methods evaluated in this chapter.

Analysis of the performance of the assays demonstrated that the four of them performed quite poorly. Serological testing remains the primary method for diagnosis of infections following acute phase, even though the cross-reactive nature of antibodies elicited during flavivirus infections. Therefore, I tried to better understand the host immunological response following ZIKV infections in order to identify the factors influencing in the poor performance of the commercial assays.

3.6.2 Investigations of the biological diversity of the ZIKV antibody response

The investigations in this chapter highlight the great biological variation in the immunological responses to ZIKV infection regarding timing, duration and magnitude of the antibody responses. The IgM ELISA assays showed overall poor performance and especially quite low levels of sensitivity. The lower sensitivity found among acute specimens was expected as IgM responses generally take 2 to 5 days to be generated by most flaviviruses [166].

Findings in samples collected at later time points from acute illness also showed low IgM detection. This was not expected. Also there appears to be a fall in antibody detection in samples collected after day 27 (8%) compared with samples collected between day 6 and 14 post symptom onset (32.6%).

This suggest that the window for ZIKV IgM detection may be much smaller than the 12 weeks estimated previously [28, 130]. This could also in part reflect that for a proportion of the subjects, the IgM antibody titres may not be detectable using the cut-off established by the manufacturers.

I have shown that there is also a considerable proportion of individuals with low IgM response while having significantly increased IgG dengue titres (p-value <0.05). This may be due to a potential secondary flavivirus immune response as it has been previously described for sequential dengue infections [167, 168]. My results suggest that following ZIKV infection in subjects that had previously been infected by other flavivirus, the production of IgM antibodies may not exist or the IgM titre may not be detectable while a high number of IgG flavivirus antibodies that increase during the first days following acute infection may be generated. See diagram with the suggested antibody dynamics for ZIKV secondary flaviviral infections in Figure 3.31. This has crucial implications for vaccine development and implementation strategies. I have seen that the production of IgG antibodies overlaps to a large degree between IgG ZIKV and IgG DENV suggesting that the human immune system is generating IgG antibodies that could neutralize not only ZIKV but also Dengue and possibly other flaviviruses.

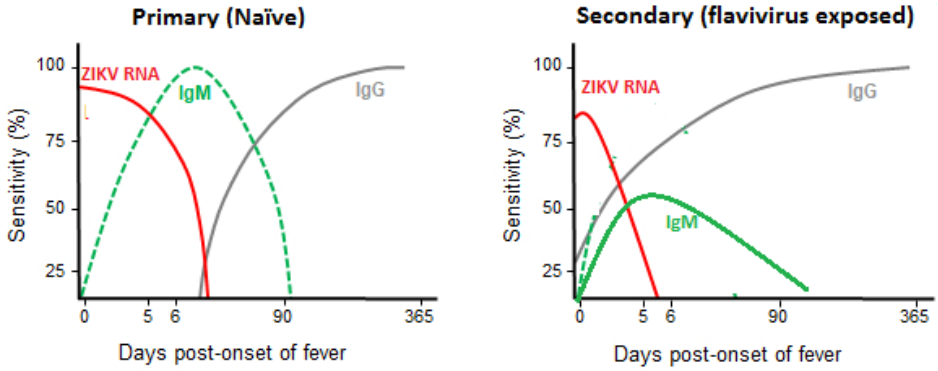


Figure 3.31 Diagram representing suggested ZIKV antibodies production
ZIKV antibody dynamics following ZIKV infection in flavivirus naïve and flavivirus exposed. Image adapted from diagram in: https://www.arigobio.com/dengue-virus.

In addition, I have demonstrated that human antibody responses after ZIKV infection are highly cross-reactive to dengue increasing the challenges of diagnosis of DENV in ZIKV exposed areas. This also emphasises the potential anamnestic antibody responses generated by the body and the challenge for current methods to diagnose acute and convalescent dengue infections. Dengue assays should be re-validated as their accuracy to distinguish among flavivirus may be limited following the spread of ZIKV. Moreover, recent epidemics of yellow fever in Brazil and an increase in vaccine coverage will pose a new challenge to this already highly exposed flavivirus region. This may be due to lack of specificity of the IgM and IgG DENV assays. It may also reflect that following ZIKV infection, non-specific flavivirus antibodies are generated by the body.

It has been recently suggested that the DENV cross-reactivity may drive antibody-dependent enhancement (ADE) of ZIKV infection [169]. My results suggest that antibody dependent enhancement (ADE) may occur in humans following ZIKV infection in previously infected flavivirus individuals. ADE occurs as a result of a secondary flaviviral infections has become the focus of several research projects [170]. It has been suggested that ADE may be a phenomena associated with the most severe presentations following dengue infection: Dengue haemorrhagic fever cases (DHF). This phenomenon is the cause of disease-enhancing activity of cross-reactive antibodies. Our results suggest that in DENV immune individuals, ZIKV responses are characterized for low IgM levels and raised IgG ZIKV antibodies. This is a similar response as the observed among subjects with a secondary dengue infection.

For individuals presenting after 5 days from onset of symptoms, The Pan American Health Organization (PAHO) currently recommends ELISA testing for both ZIKV and DENV [171] and the CDC recommends both nucleic acid testing and IgM testing [130]. A low rate of false

positive results is crucial for ZIKV diagnosis as the risk of a misdiagnosis can be devastating for the populations at risk such as pregnant women or individuals suffering from neurological complications such as Guillain-Barré Syndrome (GBS). Nevertheless, there is a critical need for further assay evaluations in South American populations that can inform public health authorities towards designing the most appropriate guidelines for laboratory diagnosis of recent ZIKV infection. This highlights the challenge that will be faced in future flavivirus outbreaks interpreting IgG antibodies.

3.6.3 Cross-reactivity between ZIKV and DENV assays

In this Chapter I have shown the enormous challenge that the ZIKV epidemic represents for serological diagnosis of DENV [172]. It was recently suggested that another dengue assay that is also based on NS1 antigen detection (the SD Dengue Due test) was giving false-positive results to Dengue in ZIKV positive travellers from Switzerland. These results provide further support for the idea that the Dengue Panbio assays are giving false positive dengue results in ZIKV infected patients. However, appropriate understanding of this mechanism should be investigated with a well-prepared panel of samples which includes a good number of ZIKV infected patients with no evidence or risk of potential prior DENV infection. Within the study cohort, and due to the high exposure of DENV in the population (already before the ZIKV outbreak), identifying a cohort of patients with those characteristics is challenging.

Another possibility that may explain this high extent of positive IgM and IgG DENV results in the ZIKV cohort is that flavivirus immune individuals may recognise quickly a new ZIKV infection and may generate initially flavivirus responses (similar to DENV) to fight the virus instead of ZIKV specific antibodies.

3.6.3.1 Evaluation of the BOB and Green BOB assay

I contributed to the multi-country evaluation of the BOB assay described in Balmaseda *et al.,* (2017). Our findings in Brazil were integrated with parallel evaluations in Nicaragua, Italy and the UK team leading the publication [116]. The results showed that the BOB assay had a sensitivity of 91.8% (145/158) and an overall specificity of 95.5% (152/171). In this chapter, I have shown that the BOB assay showed high sensitivity of 90.0% (76.85-103.15) with ZIKV specimens collected following acute infection (>14days post symptom onset) in the population of Rio de Janeiro. The sensitivity of the assay for the sera collected during the first days post symptom onset was lower [54.2% (45.3-63.1)]. This might be related to a later production of NS1-reactive antibodies or to an inhibitory effect by circulating NS1. As the secreted NS1 is highly immunogenic during flavivirus infection, the role of NS1 during ZIKV

infection is currently being investigated to better understand the exact effect and mechanism [173].

The NS1 BOB assay was evaluated in several countries (including UK, Nicaragua and Italy) and similar ranges of sensitivity and specificity were identified [116]. This demonstrates that the assay is sufficiently robust to be established in different laboratories (even from developing regions) and in multiple countries. This is particularly relevant in the context of this epidemic as the populations most affected by ZIKV are also endemic to various other flaviviruses and are generally in developing countries.

I have also shown that the modified version of the BOB assay, the Green BOB assay has a high correlation with a Kappa coefficient of 0.90 (0.81-0.99). With the threshold cut-off at a ratio of 1.0, I found that this assay offers even higher sensitivity than the BOB assay, overall at 73.26% (63.9-82.6) and just slightly lower specificity with 92.1% (92.0-92.7). The Green BOB also showed higher accuracy than the IgG Euroimmun commercial assay. In addition, another benefit of the Green BOB compared with the BOB assay is that it is quicker, highly reproducible and easier to establish requiring just under 5 hours to be completed compared with two days of the BOB assay.

3.6.4 Evaluation of PRNTs

The evaluation of the PRNT assay showed the highest specificity (96.1%), PPV (96.33%) and accuracy (79.3%). Moreover, PRNTs also exhibited the highest sensitivity in specimens collected after day 5 post symptom onset (88.37%). A few sera showed low level of reaction (under the $PRNT_{50}$ cut-off), but only 4/102 specimens showed false positive cross-reactions and interestingly, they were all DENV-3 specimens. PRNT assays measure only functional Zika antibodies.

Our results suggest that it is appropriate that PRNTs should be considered the “gold standard” technique for ZIKV serological diagnosis. However, PRNT is a time-consuming, tedious and labour-intensive technique and therefore, not suitable for large scale surveys in low-resource countries. PRNTs has been used in combination with other assays, such as the ZIKV Euroimmun ELISAs, to compensate for the low specificity of the ELISAs. Moreover, it generally lacks of reproducibility results, though our evaluations showed a high reproducibility in our testing. The findings suggest PRNTs are an adequate method to be performed after day 5 post symptom onset, which is when the RT-PCR becomes less sensitive as viremia levels decrease. The WHO also recommends serologic testing for ZIKV 7 days post symptom onset. PRNTs are designed to detect only functional specific antibody responses,

however, previous reports from Micronesia, indicate that ZIKV PRNTs can show certain levels of cross-reactivity with other flaviviruses. [122]. Moreover, in many settings, PRNTs may not be logistically viable as they are labour intensive and results may take 7 to 12 days [174].

3.6.5 Evaluation of IFA assays

I also evaluated other assay formats for the detection of ZIKV IgG antibodies that have been used for other flaviviruses. IFA methods had been used for DENV for decades [175], but IFA techniques generally lack reliable standardisation and results are very dependent on the subjectivity and therefore expertise of the observer. The advantage of the IIFT evaluated here is that the process can be automated and a large number of samples can be tested at the same time. In our evaluations, even though the ZIKV substrate was combined with other substrates as a BIOCHIP mosaic, enabling potential cross-reactive antibodies or relevant differential diagnostic parameters to be investigated in parallel, the high degree of cross-reactive responses made very difficult to distinguish which flavivirus was causing the antibody response. Our results showed that overall, the performance was very poor with very low specificity. Thus as cross-reactivity is common in patients with secondary flavivirus infections, I believe that these IFA techniques are most useful for patients in Europe or North America, in non-epidemic countries. For example, it could be used to screen travellers returning from epidemic regions with no prior flavivirus exposure. Further studies should be carried out to investigate if the specificity improves in populations with fewer endemic flaviviruses. Currently, there is only one study recently published from Spain evaluating the accuracy of the IFA methods and they identified a sensitivity of 96.8% and specificity of 72.5% [176].

3.6.6 Evaluation of a POCT assay

POCT for ZIKV diagnosis has great potential to help provide diagnostics for ZIKV in the community. The TR DPP© ZIKV IgM and IgG POCT has been approved for use in the USA. However, this rapid assay showed very poor overall performance among the Brazilian population with a sensitivity as low as 6.25% (5.6-18.11) and 68.7% (46.0-91.5) for the IgM and IgG TR DPP assay respectively. The specificity identified was 100% for the IgM assay and 64.3% for the IgG assay. In order to fully determine the accuracy levels, a further evaluation should be performed involving a larger number of samples to determine the exact sensitivity and specificity of this POCT. Unfortunately, this preliminary evaluation seems to suggest that this assay may not be accurate enough to be used in the Brazilian population. However, international groups have reported the development of a POCT assays with improved accuracy, with specificity and sensitivity of 85% and these should be a priority in the

international research agenda [177]. The WHO has determined that the ideal rapid POCT for ZIKV diagnoses should be affordable, sensitive, specific, user-friendly, rapid to perform and robust. It would be ideal for use in primary health centres. A POCT for Zika virus RNA amplification have been recently proposed, but there has not been a systematic investigation of POCT for ZIKV antibodies yet [178, 179]. POCT are simple tests, easy to be performed that increase the likelihood that the patient would receive better immediate clinical management.

3.6.7 Strengths and limitations of the investigations in this chapter and future plans

In this chapter, I present an evaluation conducted using a large, well characterised, panel of samples from Brazil; the country that has been the most devastated by ZIKV. I received serum samples from four different laboratories to conduct these studies. In addition, I included in this study a high proportion of sequential samples with invaluable information about the dynamics of antibody responses to ZIKV.

Several limitations should be acknowledge for the research carried out in this chapter. Firstly, due to limitations on reagents availability and low sample volume for some specimens, not all samples were tested on all assays. Secondly, there is no IgM or IgG gold standard method, which makes the interpretation of the specificity and sensitivity of the assays investigated challenging. Lacking gold standard methods and standardised controls is a major challenge that I tried to overcome using strict definitions for the subjects included in the study. Thirdly, the low sensitivity observed in a few of the commercial IgM NS1 and IgM μ-capture assays may be a consequence of low production of IgM antibodies instead of poor performance of the assays. Moreover, in the panel of ZIKV individuals, I only had serum samples from before the ZIKV infection for one of the subjects (patient A). A more meaningful analysis of the influence of previous IgG DENV antibodies in serum could have been performed if more samples from those subjects were available.

Fourthly, ZIKV may have arrived in Rio de Janeiro in 2013 instead of 2015, as it was initially thought when we designed and started this study. Recently, a small study performed with 210 subjects showing 3 positive cases of ZIKV in 2013 [180] in Rio de Janeiro, however, the increase on reported febrile illness and the epidemic only started in mid-2015 as shown in the epidemiological reports from the Council of Rio de Janeiro [181]. In this study, only 15/190 in Cohort 2 were collected in 2013. I re-calculated the specificity of the assays using

only specimens from prior 2013 obtaining very similar ranges (62.9% for the IgM NS1 Euroimmun compared to 63.6%).

Several limitations were encountered in the investigations described in this chapter. The main challenge was the low numbers of samples available for the comparison between the BOB and the Green BOB assay, which was 108 specimens. Unfortunately, limited assay availability and serum sample volume precluded more testing. Moreover, not having a gold standard IgG diagnostic assay makes it challenging to understand if the lower sensitivity obtained during the first 14 days following symptom onset may be due to lack of antibodies response or a lower sensitivity of the assay. The evaluations of the IFA and POCT assays were conducted on smaller number of specimens, however, as the performance of the assays was so poor with the preliminary evaluation, I concluded that a larger evaluation was not a priority.

3.6.8 Further work

Further studies to better understand the performance of this assays with even larger datasets are recommended including more ZIKV positive sequential samples (over 270 days post symptom onset). Moreover, it would be beneficial to include more than four patients with five or more sequential samples available in order to better understand the dynamics of antibody responses to ZIKV. Future plans involve datasets with specimens collected over a year post symptom onset and with a wider range of positive antibodies to other infections such as malaria, St Louis encephalitis, WNV.

Additionally, it would also be relevant to test an international quality control sample panel to ensure that the assays that we evaluated for ZIKV diagnosis are offering similar results across various laboratories.

In this chapter, I have described the optimization and evaluation of a ZIKV assay, the Green BOB assay that has shown over 90% specificity and sensitivity. Consequently, the use of this assay in a larger population, while conducting seroprevalence studies, became my next objective. Further quality assurance for the BOB and the Green BOB ZIKV assays should be carried out, including an additional internal quality control that can measure precision of the assay. Also, an external quality control panel set should be prepared and tested across different laboratories. Finally, an algorithm suggesting the appropriate ZIKV testing for suspected patients taking into account the challenges of cross-reactivity and the overall sensitivity and specificity obtained with the different assays evaluated should be performed. Further evaluations investigating ZIKV PRNT performance using larger panels of samples

should be conducted. Additionally, studies to develop faster PRNT methods should be undertaken.

3.6.9 Conclusions

My findings in this chapter demonstrated that the development of specific serological tests for ZIKV diagnosis in populations with chronic heterologous flavivirus exposure will be challenging [105]. Moreover, this chapter highlights the relevance of evaluating commercial and in-house diagnostic assays as well as published protocols before using them in clinical settings, particularly in flavivirus exposed populations.

I have shown poor sensitivity in the IgM NS1 and IgM μ-capture Zika assays and poor specificity in the MAC ELISA and IgG NS1 Zika assay. Overall performances were quite poor. Additionally, I have shown a considerable variation in the IgM antibody responses to ZIKV both in the strength and duration of the response. Moreover, I demonstrated strong cross-reactivity levels between Zika and dengue antibody responses. Our results suggest that ZIKV immune responses may be influenced by the variable exposure to dengue. I have also shown the need to improve our understanding of the serologic response to ZIKV infection and delineate the impact of previous dengue infection.

The findings presented in this chapter highlight the importance of diagnostic test validation using samples appropriate to the local population. To the best of my knowledge, this chapter represents the first systematic evaluation to assess the performance of the currently used serological assays to diagnose Zika virus infection in Brazil. The poor performance of the assays emphasizes the need for accurate diagnostic assays which will be particularly valuable for managing patients that present late following acute infection, such as exposed pregnant women or those with neurological complications, and identifying at-risk patient groups.

The PRNT assay exhibited the highest sensitivity and accuracy across all the assays evaluated. They should remain to be considered the “gold standard” method for ZIKV serological diagnosis, however, they are time-consuming and laborious, which limits their applications. Both the Blockade-of-binding (BOB) and the Green BOB assays have a high specificity and sensitivity among the Brazilian population. Their results have a high correlation and both methods demonstrated good accuracy. The IFA and POCT ZIKV diagnostic methods investigated showed poor performance due to mainly the high levels of cross-reactivity in sera from individuals previously infected with related flaviviruses. Accurate assays to detect

Zika IgG are essential for confirmation of cases and investigating congenital Zika infection as well as for seroprevalence studies.

Together, the results in this chapter highlight the urgent need for novel or improved high-throughput serological assays for diagnosis of ZIKV following acute infection. A series of novel methods to detect Zika virus have been developed for both molecular and serological diagnosis [182].

Chapter 4.

Investigating the association between Zika virus and neurological disease in Brazil

Chapter 4: Investigating the association between Zika virus and neurological disease in Brazil

4.1 Overview

When I arrived at the Zika virus (ZIKV) Reference Laboratory, Fiocruz, Rio de Janeiro, it soon became apparent that understanding the role of ZIKV in neurological disease was a major public health concern. Neurologists working in public hospitals in Rio related how there was an upsurge in the number of new Guillain-Barré Syndrome (GBS) and other acute neurological cases when the ZIKV outbreak occurred. My laboratory received specimens for ZIKV diagnosis from hospitals across the city. Thus, as a member of the Brain Infections Group, I was motivated to try and use the ZIKV diagnostic serological and molecular assays to unravel the mystery around the increase in neurological diseases in Rio and other regions of Brazil. I performed the diagnostic testing of samples from neurologic patients received at the reference laboratory. The work presented in this chapter was conducted during 2016.

4.2 Introduction

The World Health Organisation (WHO) declared ZIKV a public health emergency not simply because of the increasing number of congenital malformation cases. They also recognised ZIKV may be associated with an increase in other neurological disorders, particularly GBS. This association was based on the carefully conducted case-control studies performed during the French Polynesian ZIKV outbreak in 2013-2014. The studies identified a 20-fold increase in the incidence of GBS [50] [183].

During my first months working in Rio de Janeiro, reports of an increased incidence of GBS started appearing [184] [185]. Other flaviviruses, such as dengue virus (DENV), Japanese encephalitis (JE) or West Nile virus (WNV) were known to cause both peripheral and central nervous system (CNS) neurological disease [186, 187]. Therefore, it was not unreasonable to hypothesise that Zika virus also caused neurological disease. In addition, similar neurological complications had been reported in association with Chikungunya virus (CHIKV) infection in other continents [188] [189]. However, since CHIKV had first been recognised in Brazil in 2013, no reports existed from South America. Therefore, CHIKV, as well as ZIKV infection, could potentially be associated with the increased incidence of neurological complications in Brazil.

4.2.1 Arboviral diagnostic challenges in neurological patients

The Investigation of the association between arboviral infection and neurologic disease faces several challenges. Elucidating the pathogen causing the neurological disease based on clinical features remains a considerable challenge due to the wide range of aetiologies (viral and bacterial) and the lack of specificity of symptoms and signs [190]. Neurologic infections affecting the CNS may only show evidence of infection in the cerebrospinal fluid (CSF). CSF surrounds and interacts with the brain and spinal cord. CSF has a much closer relationship to the CNS compared to other body fluids, e.g. serum, saliva or urine, and hence a higher chance of harbouring the infecting pathogen [100]. Molecular methods (such as pathogen-specific polymerase chain reaction [PCR]) are considered one of the most accurate methods for diagnosing viral infections. However, during the natural course of infection, presence of virus in the bloodstream (viremia) may last less than 7 days (as shown with ZIKV, CHIKV and WNV) [191-193]. This is a major challenge for diagnosing the cause of neurological complications. Neurological symptoms generally develop after acute illness, typically 1 to 60 days later. In addition, patients may not present to hospital until days after the onset of neurological symptoms [194]. Consequently, a large proportion of neurological subjects are investigated for viral infection using specimens collected more than 7 days after the onset of acute illness. With samples frequently collected outside the recommended time-frame for using molecular methods, arboviral diagnosis is dependent on detection of anti-viral IgM or IgG antibody responses. However, due to variability in the body's antibody response to infection and the potential for detection of cross-reactive antibodies, the results of antibody testing need to be interpreted with caution.

4.2.2 Central and peripheral neurological infections

An understanding of the different neurological syndromes associated with ZIKV is central to investigating the spectrum of neurological disease. I summarise the different types of neurological syndromes below.

The nervous system has two components (Figure 4.1):

1) The central nervous system (CNS). The CNS consists of the brain and spinal cord. It gathers information from all over the body and coordinates activity.

2) The peripheral nervous system (PNS). The PNS consists of the nerves and ganglia outside the brain and spinal cord. The PNS connects the CNS to the limbs and organs.

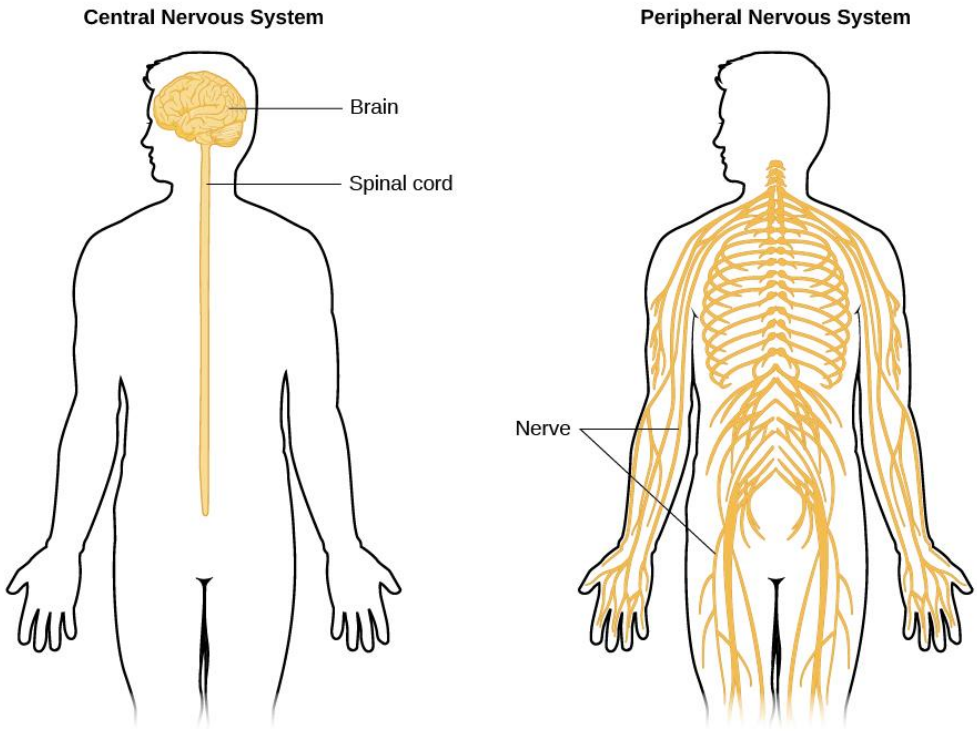


Figure 4.1 The nervous system in the human body
It is made up of two major parts: the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). Adapted from: https://courses.lumenlearning.com/parts-of-the-nervous-system

CNS infections are generally classified according to the localization where the infection takes place. Thus, the most relevant ones can be defined as follows:

- Infection of the meninges, which are the three membranes that envelop the brain and spinal cord is called **meningitis**. Meningitis is characterised by the onset of fever, headache, neck stiffness, and photophobia over a period of hours to days [195].
- Infection in the brain is called **encephalitis** is characterised by brain parenchymal inflammation, and generally alteration in mental conscience, ranging from lethargy to coma.
- Infection in the spinal cord results in **myelitis** which is characterised by the inflammation of the spinal cord with symptoms including fever, headache, and paraparesis or paralysis.

Infection in the CNS may be limited to a single anatomic compartment or can involve multiple sites leading to diseases that are called: meningo-encephalitis, encephalo-myelitis, acute disseminated encephalomyelitis (ADEM), Myeloradiculitis, etc. [196].

PNS disease associated with infection can cause severe neurological injury, either due to direct effects of the microbe or due to secondary immune activation [197]. Infections in the PNS can cause motor neuron dysfunction or sensory neuro dysfunction. Motor neuron dysfunction results in muscle weakness or paralysis while sensory neuron dysfunction results in abnormal or lost sensation. Some disorders are progressive and fatal including GBS, peripheral neuropathy and sensory polyneuropathy. GBS is an immune-mediated neurological syndrome characterised by the acute onset of varying degrees of weakness in limbs or cranial nerve-innervated muscles. This is generally associated with decreased or absent deep tendon reflexes and a characteristic profile in the CSF and in electrodiagnostic studies [198]. Infection in the nerve roots at its connection to the spinal column is called radiculitis.

4.3 Aims

As the ZIKV outbreak was turning into a pandemic in 2016, it became evident there was a need to better understand the potential neurological sequelae caused by this flavivirus. Thus, I took the opportunity, through our collaborative diagnostic work in the reference Zika Fiocruz laboratory, to conduct a retrospective study. The objective was to investigate the association between ZIKV and neurological complications in children and adults in Rio de Janeiro. I also wanted to understand the usefulness of the ZIKV diagnostic assays in a population at risk. The key objectives addressed in this chapter were:

1. Use the evaluated diagnostic assays to investigate the neurologic disease population in Brazil
2. Better understand the association between ZIKV and neurological disease in Brazil
3. Describe the clinical spectrum of acute neurological manifestations associated with ZIKV infection and its outcome in Brazil

As sub-objectives, we also aimed to:

4. Understand the association between other arboviruses and neurological disease in Brazil
5. Describe the clinical spectrum of acute neurological manifestations associated with arboviral infection and its outcome
6. Describe the frequency of Zika, chikungunya and dengue Viruses and the rate of co-infections among patients suffering from acute neurology

4.4 Materials and Methods

We studied patients over the age of 12 months who had developed a new neurological condition associated with suspected Zika virus infection, whose samples had been submitted to the Flavivirus Reference Laboratory of the Instituto Oswaldo Cruz (Fiocruz), Rio de Janeiro.

4.4.1 Study population

We studied patients admitted to 11 hospitals in the city of Rio de Janeiro. The list of hospitals involved in the study can be found in Appendix 4. Patients recruited had presented to these hospitals between the 1st of November 2015 and the 1st of June 2016. Subjects who had presented with an acute neurological condition associated with a suspected Zika virus infection, as identified by fever, arthralgia or rash illness in the preceding three months were investigated. The ZIKV epidemic required urgent investigation of these cases and we used three different approaches to identify patients:

1) Using the Fiocruz laboratory database, we identified 24 patients retrospectively who had had cerebrospinal fluid (CSF)
2) Using the Fiocruz laboratory database, we identified 5 patients retrospectively who had had serum and/or urine collected in the context of neurological disease, sent to the laboratory for Zika virus diagnostics;
3) Physicians from our study hospitals identified six further patients, whose CSF sample was not on the database and whose serum and/or urine request forms did not have any clinical indication (Figure 4.2).

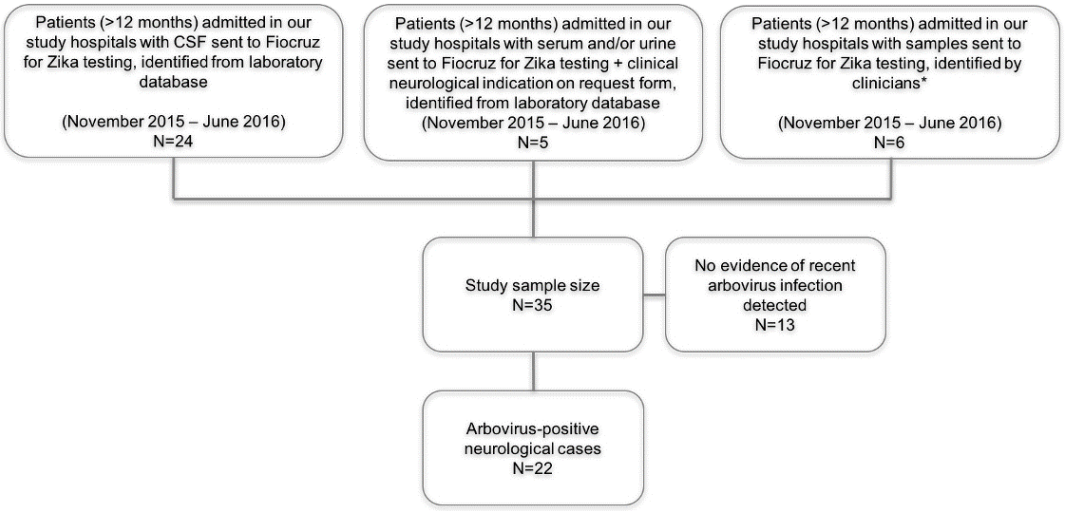


Figure 4.2 Flowchart study population included in the study
Study subjects were patients with neurological disease associated with suspected Zika virus infection. *These patients did not appear in the laboratory database search because their CSF sample was not recorded on the database and no clinical information was included in request forms for serum and/or urine. They were identified by the clinicians who had previously managed their care in our study hospitals. Flowchart adapted from Mehta#, Soares#, Medialdea-Carrera# *et al*. (2018). #=first co-authors.

Clinical information was obtained from case notes and discussion with the patients' clinicians. It was documented using a standardised case report forms by a member of the study team. The information obtained included demographics, past medical history, admission history, examination, investigations, diagnosis and management. Investigations included brain and spine imaging for patients with suspected CNS infection, and nerve conduction studies with or without electromyography for those with peripheral disease. Nerve conduction study results were reviewed by an independent expert neurophysiologist to ensure consistency. The Brighton criteria were used to indicate the level of certainty for diagnosing GBS and similar criteria were applied for radiculitis, encephalitis, myelitis, and meningitis (see these criteria and sources in appendix). We determined whether patients had peripheral nervous system disease (GBS, radiculitis), CNS disease (encephalitis, myelitis, meningitis) or both.

4.4.2 Diagnostic testing

An expanded protocol based on the interim recommendations from the WHO for laboratory testing for Zika virus was followed [129]. I performed all the arboviral laboratory investigations at the Fiocruz Flavivirus Laboratory. I tested the CSF, serum, and urine samples available for evidence of Zika, chikungunya, and dengue virus infection. We considered detection of viral RNA and/or IgM-specific antibody in the CSF as evidence of recent CNS infection as previously described [73]. IgM antibody in the serum or RNA in the serum or urine was taken as evidence of systemic infection.

4.4.2.1 Handling of specimens

All serum, CSF and urine specimens were kept at -80 ^{o}C and repeated cycles of freeze-thaw were avoided. Specimens tested were labelled with an anonymous identification code and stored appropriately. When possible, aliquots were prepared to avoid the freeze-thaw cycles.

4.4.2.2 Molecular diagnostic testing for ZIKV, DENV and CHIKV

RNA was extracted using the Qiamp Mini Elute Virus Spin Kit from Qiagen as described in chapter 2. The CSF, serum, and urine samples were tested by quantitative RT-PCR for detection of Zika, chikungunya, and dengue virus RNA as described in chapter 2.

4.4.2.3 Serological diagnostic testing for ZIKV, DENV and CHIKV

In order to choose the most appropriate assay for detection of ZIKV antibodies, we followed the results described in Chapter 3. As the IgM Euroimmun assay showed the best specificity, serum IgM antibodies to Zika virus NS1 antigen were measured using that commercial ELISA. I followed the manufacturer's protocol. CSF IgM was measured using the CDC MAC ELISA based on the CDC emergency use authorization protocol. I followed the instructions described in chapter 2. As recommended by the CDC, I used a modified ratio of clinical sample [CSF]:diluent of 1:50. This modification had already been validated for CSF in the Fiocruz laboratories.

IgG antibodies to ZIKV were measured using the IgG NS1 ZIKV ELISA assay from Euroimmun following the manufacturer's protocol. We wanted to quantify the IgG antibody titre response in our neurological patients. The IgG antibody titres were calculated quantitatively using the standard curve obtained by point-to-point plotting of the extinction values of the three calibration sera against the corresponding units. A specimen was considered IgG positive if the result was ≥ 22 relative units (RU)/ml, equivocal if ≥16 and <22RU/ml and negative if <16RU/ml.

The IgM and IgG antibodies to chikungunya virus were measured using the Euroimmun CHIKV ELISAs (Euroimmun, Lübeck, Germany), according to the manufacturer's protocol. The IgG antibody titres were calculated quantitatively using the standard curve obtained by point-to-point plotting of the extinction values of the three calibration sera against the corresponding units. A specimen was considered IgG positive if the result was ≥ 22 relative units (RU)/ml, equivocal if ≥16 and <22RU/ml and negative if <16RU/ml.

Serum and CSF IgM and IgG antibodies to dengue virus were measured using the commercial ELISAs (Panbio, Brazil) and the results were calculated in Panbio Units. These units were calculated according to the manufacturer's instructions and > 11 units were defined as a positive result, 9–11 units were defined as an equivocal result and < 9 units was defined as a negative result.

4.4.3 Ethics statement

The study protocol was approved by the Comitê de Ética em Pesquisa do Instituto Nacional de Infectologia Evandro Chagas (reference 59254116.0.1001.5262). All the patients' CSF, serum and urine specimens were anonymised. Patient-identifying information was removed from all databases.

4.5 Results

4.5.1 Characteristics of the study population

A total of 35 patients fulfilled the inclusion criteria to the study. The median age was 51.5 (range 17-84) years and there was an equal distribution between male and female in the study population with 17 and 18 subjects respectively. The medical neurological diagnosis provided by the responsible physician are shown in Table 4.1. The neurological diagnostic criteria used by the clinicians are summarised in the appendix. Of the patients included in the study, 34.3% (12/35) were diagnosed with Guillain-Barré Syndrome (GBS), 14.3% (5/35) had encephalitis and another 14.3% (5/35) had myeloradiculitis. A more detailed breakdown of the medical presentation, clinical symptoms and details of the disease can be found in the manuscript: Mehta, Soares, Medialdea-Carrera *et al.* (2018).

Table 4.1 Summary diagnosis of patients recruited with neurological complication from suspected arboviral infection.
*Ona patient presented with a clinical presentation of encephalitis and myositis
Evidence of recent arboviral infection determined by the presence of ZIKV, CHIKV or DENV RNA and/or IgM

Diagnosis	Number of patients with suspected recent arboviral infection (n=35)	Number of patients with confirmed recent arboviral infection (n=22)
Guillain-Barré Syndrome (GBS)	12	7
Encephalitis	5*	4*
Myeloradiculitis	5	4
Myelitis	4	3
Encephalo-myelitis (I,I)	2	2
Miller Fisher Syndrome	1	0
Neuromyelitis optica	1	0
Facial nerve palsy	1	0
Encephalomyeloradiculitis	1	0
Radicular pain	1	0
Meningo-encephalo-myelitis	1	1
ADEM	1	1

4.5.2 Body fluids available from study subjects

All the body fluids samples sent to Fiocruz for routine testing were used in our examinations including serum, CSF and urine. CSF specimens were available for 94.2% (33/35) of study subjects. Serum specimens were available for 74.3% (26/35) of study subjects. Urine specimens were available for 77.1% (27/35) of study subjects. Two sequential serum samples were available for 17.1% (6/35) of the study subjects. Sample volumes were low in most cases, particularly the CSF samples. Completing the molecular and serological testing for ZIKV, CHIKV and DENV was not possible in all subjects due to low volumes. ZIKV testing was prioritised.

4.5.3 Laboratory diagnosis of neurological patients

Specific detection of viral RNA or positive IgM antibody to the virus were considered evidence for recent arboviral infection.

We identified a total of 22 study subjects with evidence of recent ZIKV, CHIKV and/or dengue virus infection. A summary of the molecular and serological results for our study subjects can be found in Table 4.2. Antibody titres and number of days between arboviral symptom onset and sample collection are described in Table 4.3.

Table 4.2 Evidence of arboviral infection in patients presenting with acute neurological disease.

Patient results ordered by date of admission. Key: "+" =positive, "-" =negative, "na" =sample not available or inadequate volume; PCR=polymerase chain reaction; CSF=cerebrospinal fluid; Chik=chikungunya; CNS=virus detected in central nervous system, Syst=virus detected systemically (i.e. outside CNS) only. †These patients had PCR evidence of Zika virus infection, with serological evidence of dengue infection potentially secondary to cross-reactivity.*Preliminary information of this patient was published recently in Soares, Medialdea *et al.* (2016). Table amended from Mehta[#], Soares[#], Medialdea-Carrera[#] *et al.* (2018). [#]=first co-authors

Patient	Zika					Chikungunya					Dengue					Virological Diagnosis
	CSF PCR	CSF IgM	Serum PCR	Serum IgM	Urine PCR	CSF PCR	CSF IgM	Serum PCR	Serum IgM	Urine PCR	CSF PCR	CSF IgM	Serum PCR	Serum IgM	Urine PCR	
1*	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	Zika-CNS +/- Dengue-CNS †
2	-	+	-	-	-	-	-	-	+	-	-	+	-	+	-	Zika-CNS or Dengue-CNS or both, Chik-Syst
3	-	+	+	-	+	-	-	-	-	-	-	-	-	+	-	Zika-CNS +/- Dengue-Syst †
4	+	+	-	-	na	+	-	+	-	na	-	-	-	-	na	Zika/Chik-CNS
5	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	Zika-CNS
6	na	na	-	-	-	na	na	-	+	-	na	na	-	+	-	Chik/Dengue-Syst
7	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	Zika-CNS or Dengue-Syst or both
8	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	Chik-CNS
9	-	+	na	na	na	+	+	na	na	na	-	-	na	na	na	Zika/Chik-CNS
10	-	+	-	+	+	-	-	-	-	-	-	na	-	-	-	Zika-CNS
11	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	Chik-CNS
12	-	-	na	na	-	+	+	na	na	-	-	-	na	na	-	Chik-CNS
13	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	Chik-CNS
14	-	-	na	na	na	+	-	na	na	na	-	-	na	na	na	Chik-CNS
15	-	-	na	na	na	+	-	na	na	na	-	-	na	na	na	Chik-CNS
16	+	+	na	na	-	+	+	na	na	-	-	-	na	na	-	Zika/Chik-CNS
17	na	na	-	-	+	na	na	+	-	-	na	na	-	-	-	Zika/Chik-Syst
18	-	-	na	na	+	+	+	na	na	-	-	-	na	na	-	Chik-CNS, Zika-Syst
19	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	Chik-CNS, Dengue-Syst
20	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	Chik-Syst
21	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	Chik-Syst
22	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Zika-CNS

Table 4.3 Immunological assays and days between infection and sample collection for patients with evidence of arboviral infection presenting with neurological disease, ordered by date of admission

Key: "na" =sample not available/not enough volume; "-" =negative; CSF=cerebrospinal fluid. Units and cut-off values: Zika CSF IgM (P/N = ratio of patient optical density to negative control value); Pos: P/N >3. Zika serum IgM and chikungunya IgM (ratio = patient sample/cut-off value); Pos: ratio ≥1·1. Zika IgG and Chikungunya IgG (relative units/ml, RU/ml as described by the manufacturer); Pos: RU/ml ≥22. Dengue IgM and IgG (Panbio Units, PU = ratio of patient optical density to cut-off value x10); Pos: PU >11. Table amended from Mehta*, Soares*, Medialdea-Carrera* *et al.* (2018). *=first co-authors.

Patient	Days between infection and sample collection			Zika				Chikungunya				Dengue			
	CSF	Serum	Urine	CSF IgM (P/N)	CSF IgG (RU/ml)	Serum IgM (Ratio)	Serum IgG (RU/ml)	CSF IgM (Ratio)	CSF IgG (RU/ml)	Serum IgM (Ratio)	Serum IgG (RU/ml)	CSF IgM (PU)	CSF IgG (PU)	Serum IgM (PU)	Serum IgG (PU)
1	11	11	11	5.5	-	-	111.5	-	-	-	-	21.1	63.2	23.8	60.5
2	25	25	25	35.6	-	-	>200	-	-	1.2	-	17.7	16.0	43.6	62.7
3	4	6	4	7.1	-	-	>200	-	-	-	-	-	21.8	13.7	66.2
4	20	20	na	3.1	40.3	-	>200	-	-	-	-	-	15.1	-	69.4
5	23	20	20	27.7	-	-	>200	-	-	-	-	-	na	-	60.5
6	na	33	33	na	na	-	>200	na	na	1.2	124.0	na	na	47.7	65.0
7	18	18	19	41.1	369.3	1.2	55.4	-	-	-	-	-	21.6	12.2	62.3
8	29	29	29	-	-	-	>200	-	-	-	-	-	-	-	57.1
9	10	na	na	8.7	155.8	na	na	2.5	92.0	na	na	-	-	na	na
10	15	15	70	25.1	na	1.5	na	-	na	-	-	na	na	-	55.2
11	26	26	26	-	-	-	114.3	1.1	-	1.6	-	-	32.6	-	57.7
12	12	na	12	-	-	na	na	3.1	75.8	na	na	-	23.8	na	na
13	6	9	9	-	-	-	40.6	-	na	6.9	73.2	-	na	-	60.0
14	31	na	na	-	-	na	na	-	-	na	na	-	-	na	na
15	7	na	na	-	-	na	na	-	-	na	na	-	-	na	na
16	23	na	23	3.5	67.5	na	na	3.0	56.3	na	na	-	17.1	na	na
17	na	71	72	na	na	-	22.0	na	na	-	-	na	na	-	59.3
18	23	na	23	-	37.2	na	-	1.5	35.9	na	na	-	16.0	na	na
19	13	13	13	-	151.6	-	>200	1.8	58.4	4.4	112.5	-	25.3	14.1	64.5
20	35	25	36	-	-	-	-	-	-	6.8	89.2	-	-	-	53.6
21	72	72	72	-	-	-	86.4	-	-	2.1	-	-	-	-	54.3
22	66	60	60	4.5	-	-	24.6	-	-	-	-	-	-	-	65.7

4.5.4 Diagnosis of Zika Virus

Twelve subjects had evidence of recent ZIKV infection

4.5.4.1 Molecular detection of Zika virus

I used the protocol described by Lanciotti *et al.* (2008) to perform ZIKV RT-PCR [122]. Specificity of the ZIKV RT-PCR assay was assessed in the Fiocruz laboratory using an external quality control (QC) panel of specimens. Reflecting previous reports, the assay exhibited over 97% specificity [159] [199]. ZIKV RNA was detected in eight subjects; two CSF, one serum and six urine samples.

4.5.4.2 Serological detection of Zika virus IgM antibodies

As described in the methods, detection of Zika IgM antibodies was based on two different methods:

1. In serum - IgM anti-ZIKV NS1 Euroimmun ELISA (measured as ratio, ratio>1.1 positive).
2. In CSF – IgM ZIKV MAC ELISA (measured in P/N ratio)

Two subjects were anti-ZIKV IgM positive in sera. Both subjects also exhibited positive anti-ZIKV IgM in the CSF.

Ten subjects were anti-ZIKV IgM positive in CSF.

The IgM MAC ELISA had previously shown poor specificity of 70/110 (Sensitivity 62.4 [Chapter 3]). Therefore, to support the IgM result, we gathered additional evidence for ZIKV infection. Six of the patients were ZIKV positive via RT-PCR in either CSF, serum or urine. One subject (Patient #7) was anti-ZIKV IgM positive in serum (Euroimmun assay).

Three subjects (patients #2, #9 and #22) exhibited anti-ZIKV IgM in the CSF, but had no other evidence of recent ZIKV infection based on RT-PCR or anti-ZIKV IgM testing. MAC ELISA testing for these subjects was repeated 3 times in separate experiments. A consistent positive result was obtained. The strength of the evidence supporting a recent ZIKV infection in these patients is explored in the discussion.

4.5.4.3 Serological detection of Zika virus IgG antibodies

ZIKV IgG antibodies were detected in 17/22 study subjects with evidence of recent arboviral infection. Positive ZIKV IgG antibodies were detected in six CSF (6/19 CSF tested) and 14 sera (14/16 sera tested). Five of these patients were ZIKV negative by RT-PCR or IgM testing.

These five patients also had evidence of DENV or CHIKV infection. Again, evidence for these patients having a true recent ZIKV infection is explored in the discussion.

4.5.5 Diagnosis of Chikungunya Virus

Sixteen subjects had evidence of recent CHIKV infection.

4.5.5.1 Molecular detection of Chikungunya virus

Samples from fourteen subjects exhibited a positive CHIKV PCR; 11 CSF, 6 sera and 3 urine samples.

Thirteen of these subjects also had a positive anti-CHIKV IgM or IgG response.

One subject (**patient #8)** had positive CHIKV PCR in CSF but negative IgM/IgG test result. RT-PCR was repeated in three separate experiments and RNA extraction was performed twice to confirm the result. The specimens from patient #8 were collected 29 days post symptom onset, when antibodies should be detectable (chikungunya antibodies are generally detectable after 7 days post symptom onset [200]).

4.5.5.2 Serological detection of chikungunya virus IgM antibodies

Eleven subjects exhibited a positive anti-CHIKV IgM response; six in CSF, seven in sera. Two subjects were positive in CSF and sera. In both subjects (patient #11 and patient #19) the IgM CHIKV antibody ratio was higher in sera.

4.5.5.3 Serological detection of chikungunya virus IgG antibodies

Eight subjects exhibited a positive anti-CHIKV IgG response; five in CSF, four in sera. One subject was positive in both. All subjects were also positive for anti-CHIKV IgM testing.

4.5.6 Diagnosis of dengue virus

Six subjects had evidence of recent DENV infection based on positive PCR or IgM testing. All subjects, where serum was available (16/22), showed evidence of past DENV infection based on a positive anti-DENV IgG response.

4.5.6.1 Molecular detection of dengue virus

Multiplex RT-PCR assay for DENV-1, DENV-2, DENV-3 and DENV-4 was conducted for all the specimens from the 35 study subjects. Dengue virus RNA was not found in any of the samples.

4.5.6.2 Serological detection of DENV IgM antibodies

Six subjects exhibited a positive anti-DENV IgM response; two CSF and six sera. Five subjects were positive for anti-ZIKV IgM testing. All six subjects were positive for anti-DENV IgG testing.

4.5.6.3 Serological detection of DENV IgG antibodies

All subjects, where serum was available (16/22), exhibited a positive anti-DENV IgG response. Ten subjects also exhibited a positive anti-DENV IgG response in CSF.

4.5.7 Interpreting laboratory diagnostic results

In order to interpret the arboviral laboratory diagnostic results, we considered a patient positive for recent arboviral infection if viral RNA and/or IgM antibodies were detected in at least one patient's specimen. IgG antibodies were not deemed as evidence of recent infection. We classified the laboratory results for each arbovirus independently into two categories: CNS infection and systemic infection. We considered detection of viral RNA and/or IgM-specific antibody in the CSF as evidence of recent CNS infection. We considered previously IgM antibody in the serum, or RNA in the serum or urine as evidence of systemic infection. Evidence for recent ZIKV, CHIKV and DENV infection are shown in Table 4.4.

We identified 12 patients with evidence of recent arboviral co-infection; five patients with CHIKV and ZIKV; two with CHIKV and DENV; 4 with potential ZIKV and DENV and 1 with potential ZIKV, DENV and CHIKV.

Table 4.4 Virological evidence for ZIKV, CHIKV and DENV recent infection in study subjects
Recent infection was determined by the presence of RNA or IgM in CSF, serum and/or urine specimens.

Virological Diagnosis	*Number of Subjects (n)*	*Proportion (%)*
Recent arboviral infection	22	62.9
Recent ZIKV infection	12	34.3
ZIKV CNS infection	10	28.6
ZIKV Systemic infection	2	5.7
Recent CHIKV infection	16	45.7
CHIKV CNS infection	11	31.4
CHIKV Systemic infection	5	14.3
Recent DENV infection	6	17.1
DENV CNS infection	2	5.7
DENV Systemic infection	4	11.4
Arboviral co-infections:	12	34.3
ZIKV and CHIKV infection	5	14.3
ZIKV and DENV infection	4	11.4
CHIKV and DENV infection	2	5.7
ZIKV, CHIKV and DENV infection	1	2.9

Twelve study subjects 12/35 (34.3%) had evidence of recent infection with at least two arboviral infections. The virological results are represented in a Venn diagram (Figure 4.3).

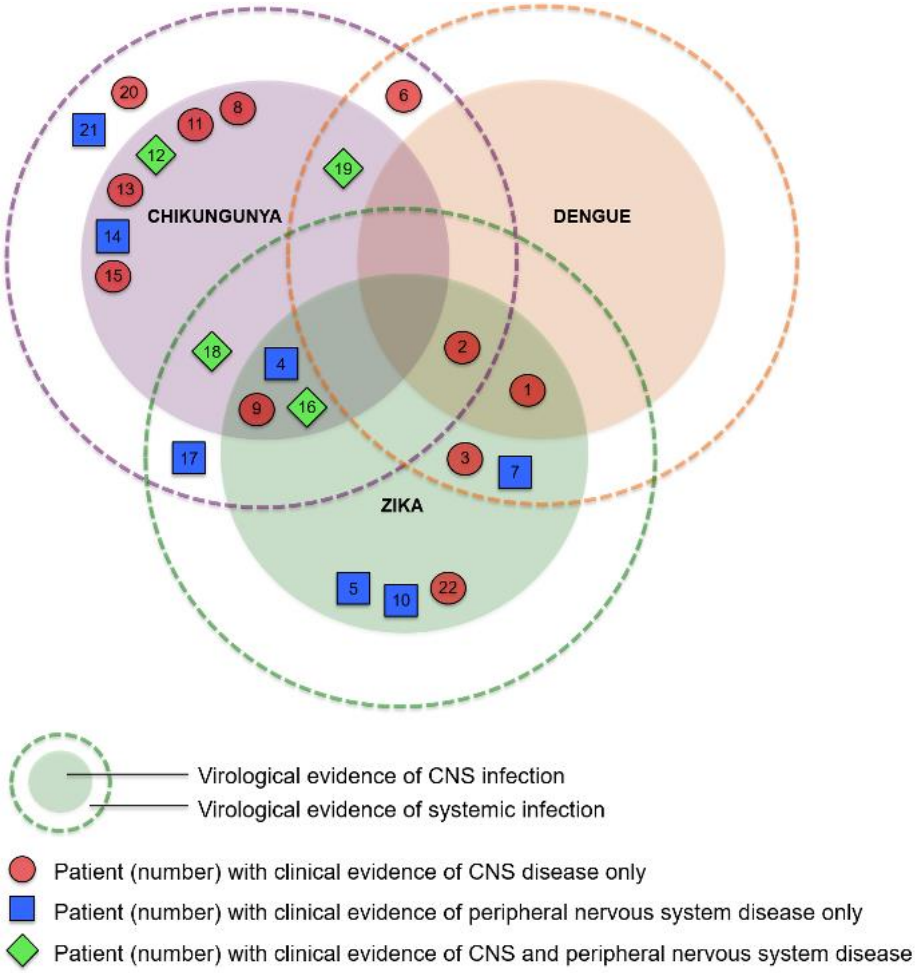


Figure 4.3 Venn Diagram summarizing virological evidence for arboviral positive patients
Showing the virological evidence for 22 patients. Observed CNS or systemic infection with Zika, chikungunya and/or dengue, and clinical presentation with CNS or peripheral nervous system disease. Figure amended from Mehta*, Soares*, Medialdea-Carrera* *et al.* (2018). *=first co-authors.

4.5.8 Relevance of arboviral laboratory diagnosis in the clinical context

The diagnostic laboratory investigations were considered together with the clinical information available for the 22 subjects with evidence of recent arboviral infections (Table 4.5). All 22 patients were Brazilian nationals with no recent travel history, who lived in 18 different districts of Rio de Janeiro. Eleven (50.0%) of subjects were female and the median age was 51.5 years (IQR of 35 - 64.5). The median number of days between rash-fever like onset and neurological disease was: 5.5 days with (IQR of 2.5 - 11.5).

Patients presented with a range of neurological syndromes affecting the CNS, peripheral nervous system, or both. The twelve (29%) patients with evidence of recent Zika virus infection presented with a wide range of neurological syndromes including GBS (n=4), GBS-variant (n=1), encephalitis (n=2), encephalomyelitis (n=2), myelitis (n=1) and myeloradiculitis (n=2). The 16 patients with evidence of recent CHIKV infection, presented with GBS (n=3),

GBS-variant (n=1), encephalitis (n=2), myelitis (n=2), meningo-encephalo-myelitis (n=1), myeloradiculitis (n=4), encephalo-myelitis (n=1) and ADEM (n=1). The 6 patients with evidence of recent DENV infection presented with GBS (n=1), encephalitis (n=1), myeloradiculitis (n=1), myelitis (n=2) and encephalomyelitis, (n=1). The patient with positive detection of ZIKV, CHIKV and DENV had myelitis. The remaining 13 patients (37%), with no evidence of a recent Zika, chikungunya, or dengue virus infection presented with GBS (6/13) or CNS disease (7/13).

Regarding patient progress:

Eight of the 22 patients (four with confirmed Zika infection) required admission to an intensive care unit; six needed intubation (including half the GBS patients). Ten patients were treated with intravenous immunoglobulin, two with corticosteroids, and seven with both. Four encephalitis patients received acyclovir as presumptive treatment for herpes simplex virus. Four patients developed hospital-acquired infections, including ventilator-associated pneumonia and osteomyelitis secondary to immobility.

Patient Outcome:

There was outcome data on 21 patients. Fourteen patients (six with confirmed Zika) improved. One patient with evidence of Zika +/- dengue infection deteriorated rapidly and died.

Table 4.5 Clinical features of patients presenting with neurological disease associated with Zika, chikungunya and/or dengue virus infection

Patients ordered by date of admission, (n=22) Days between rash-fever and neuro: interval between onset of infection and neurological illness. Chik=chikungunya; CNS=virus detected in central nervous system, Syst=virus detected systemically (i.e. outside CNS) only; GBS=Guillain-Barré syndrome, ADEM=acute disseminated encephalomyelitis; IVIG=intravenous immunoglobulin; ICU=intensive care unit admission. For neurological diagnoses, the levels of diagnostic certainty are indicated I-III (highest to lowest), as per the Brighton and other criteria (appendix). Table modified from published table in Mehta*, Soares*, Medialdea-Carrera* *et al.* (2018). *=first co-authors.

Patient ID number	Sex	Age	Virological Diagnosis	Days rash-fever to Neuro	Neurological Diagnosis (levels of certainty)	Progress and Outcome
1	F	47	Zika-CNS +/- Dengue-CNS	4	Encephalitis (I)	ICU; intubated; patient rapidly deteriorated and died
2	F	59	Zika-CNS or Dengue-CNS or both, Chik-Syst	7	Myelitis (I)	Developed pulmonary oedema on IVIG; responded to steroids, mRS 3 at 4 months
3	M	26	Zika-CNS +/- Dengue-Syst	1	Encephalo-myelitis (I,I)	ICU; intubated; improved, mRS 1 at 4 months
4	M	34	Zika/Chik-CNS	12	GBS variant (facial diplegia with paraesthesia)	Improved, full recovery at 2 months
5	F	41	Zika-CNS	5	GBS (II)	Improved (extent unknown)
6	F	30	Chik/Dengue-Syst	27	Myelitis (II)	Improved (extent unknown)
7	F	66	Zika-CNS or Dengue-Syst or both	13	GBS (I)	ICU; intubated; improved at 1 week (extent unknown)
8	M	20	Chik-CNS	0	Myelitis (I)	Improved, mRS 2 at 3 weeks
9	F	80	Zika/Chik-CNS	5	Encephalo-myelitis (I,I) with subclinical meningitis	ICU; developed sacral osteomyelitis; improved, mRS 4 at 2 months
10	M	38	Zika-CNS	10	GBS (II)	ICU; intubated, ventilator-associated pneumonia; improved (extent unknown)
11	M	76	Chik-CNS	0	Meningo-encephalo-myelitis (III,I,III)	Unknown outcome
12	M	63	Chik-CNS	2	Myeloradiculitis (II)	ICU, intubated (after fall and head injury); no improvement; mRS 5 at 2 months
13	F	51	Chik-CNS	6	Encephalitis (I)	Improved, full recovery at 2 months
14	M	45	Chik-CNS	29	GBS (II)	ICU; improved mRS 3
15	M	84	Chik-CNS	4	Encephalitis (I), Myositis	Ventilator-associated pneumonia; no improvement, mRS 5 at 6 weeks
16	M	65	Zika/Chik-CNS	0	Myeloradiculitis (I)	No improvement at 3 weeks
17	F	19	Zika/Chik-Syst	41	GBS (II)	No improvement at 3 weeks
18	F	56	Chik-CNS, Zika-Syst	4	Myeloradiculitis (II)	No improvement, mRS 5 at 1 month
19	M	62	Chik-CNS, Dengue-Syst	8	Myeloradiculitis (I)	Improved, mRS 2 at 1 month
20	M	17	Chik-Syst	16	ADEM	Improved, mRS 2 at 3 weeks
21	F	67	Chik-Syst	7	GBS (I)	ICU; intubated; no improvement, mRS 5 at 2 weeks
22	F	52	Zika-CNS	0	Encephalitis*	Improved, mRS 0 at 2 months

4.6 Discussion

In this chapter, I describe the arboviral diagnostic investigations conducted in a group of 35 patients with a variety of acute neurological syndromes and with suspected recent ZIKV infection. The laboratory investigations shown here demonstrate the challenging scenario for diagnosing ZIKV following acute arboviral infection in at-risk patient groups.

I confirmed recent arboviral infection in 62.86% (22/35) of the patients investigated. Our findings suggest that the full spectrum of neurological complications associated with Zika virus infection may be much wider than what was initially hypothesised.

4.6.1 Zika virus infection and neurological disease

Among the patients investigated 34.3% (12/35) had evidence of recent ZIKV infection. The patients exhibited a range of neurological disorders including GBS, encephalitis, encephalomyelitis, meningo-encephalo-myelitis or myeloradiculitis.

4.6.1.1 Serological detection of Zika virus IgM antibodies

Deciding which antibody test was most appropriate for diagnosing recent ZIKV infection in these patients was challenging. At the time these laboratory investigations were performed, few diagnostic assays for ZIKV detection were available. Ideally, antibody assays with very high specificity are used to avoid potential false positive results. Unfortunately, the IgM Euroimmun ELISA, which had shown high specificity (>90%) in sera, had not been validated for use with CSF samples. Instead, we used the MAC ELISA assay which had been validated for CSF [115]. However, because I had previously shown that the MAC ELISA assay exhibited low specificity in sera (Chapter 3), this assay was not used to test sera. I attempted to validate the IgM Euroimmun assay for use in CSF. Unfortunately, due to very low CSF volumes and limited reagents (prioritised for patient testing), this evaluation could not be completed.

Three patients with positive IgM ZIKV antibodies in the CSF had no other evidence of recent ZIKV infection. The additional serological and molecular results for these patients are discussed below:

- **Patient #2.** Although, the patient had raised anti-DENV IgM (and IgG) antibody levels in CSF, the titre for anti-DENV IgM was almost half that for ZIKV (17 and 31 respectively). Similarly, the patient had a higher titre for anti-ZIKV IgG than anti-DENV IgG in serum. Samples had been collected 25 days post-acute illness. Taken together, the results suggest the positive MAC ELISA anti-ZIKV IgM result in CSF is likely to indicate recent ZIKV infection.

- **Patient #9:** The patient exhibited high anti-ZIKV IgG titres in CSF (155.8 RU/ml). CSF was negative for DENV via RT-PCR, IgM and IgG testing. No serum or urine samples were available. The CSF was collected 10 days after acute illness. Overall, the data suggests the positive anti-ZIKV IgM in CSF indicate a likely recent ZIKV infection.
- **Patient #22** Dengue IgM testing was negative in CSF and serum. Both ZIKV and DENV IgG testing was positive in serum. Serum and urine specimens were collected 60 days and CSF 66 days post-acute illness. The high serum anti-DENV IgG titres (compared to anti-ZIKV) suggests the weak positive anti-ZIKA IgM in CSF may either reflect recent ZIKV infection or a cross-reactive antibody response to recent dengue infection.

4.6.1.2 Serological detection of Zika virus IgG antibodies

We identified ZIKV IgG antibodies in 17/22 of the study subjects with evidence of recent arboviral infection. Five of those patients had no evidence of ZIKV RNA or ZIKV IgM antibodies detected. The ZIKV evidence for each of those subjects is summarised as follows:

Patient #6. The patient exhibited high IgG ZIKV titres (>200 RU/ml) in serum. However, the serum and urine specimens available for this subject were collected 33 days post arboviral symptom onset and no other evidence of recent ZIKV infection was found. This patient also showed high ratios of IgM and IgG antibodies against DENV (PU=47.7 and PU=65.0 respectively). This suggests that the positive anti-ZIKA IgG in serum may either reflect recent ZIKV infection or a cross-reactive antibody response to recent dengue infection.

Patient #8. The patient exhibited high IgG ZIKV titres (>200 RU/ml) in serum. CSF, urine and serum specimens were collected 29 days post rash-fever onset and no other indication of ZIKV infection was found. IgG DENV antibodies were also detected in the serum sample, but no IgM dengue antibodies were found. This suggests that the positive anti-ZIKA IgG antibodies detected in the serum may reflect either reflect a ZIKV infection or a cross-reactive antibody response to a previous dengue infection.

Patient #11. The patient exhibited high IgG ZIKV titres in serum (114.3 RU/ml) but also high IgG DENV titres in both CSF and serum (32.6 and 57.7 PU respectively). CSF, serum and urine specimens were collected 26 days post rash-fever onset. As suggested above for Patients #6 and #8, the positive anti-ZIKA IgG in serum may reflect either recent ZIKV infection or a cross-reactive antibody response to infection.

Patient #13. The patient exhibited moderate IgG ZIKV titres (40.6 RU/ml) and high IgG DENV titres (60.0 PU) in serum. CSF, serum and urine were collected 6, 9 and 9 days respectively post symptom onset. The positive anti-ZIKA IgG in serum may either reflect ZIKV infection or a cross-reactive antibody response to infection.

Patient #21. The patient exhibited moderate IgG ZIKV titres (86.4 RU/ml) and IgG DENV titres (54.3 PU). CSF, serum and urine for this patient were collected 72 days post arboviral symptom onset and the IgM responses may have already declined. The positive anti-ZIKA IgG in serum may either reflect ZIKV infection or a cross-reactive antibody response to infection.

Of the 13 patients with no evidence of recent arbovirus infection, serum Zika virus IgG was detected in four subjects (30.8%). However, with no other evidence of recent arboviral infection, their viral diagnoses could not be confirmed. Moreover, this high number of IgG ZIKV results among our cohort suggested high exposure to ZIKV in the population.

4.6.2 Chikungunya virus infection and neurological disease

The arboviral diagnostic testing described in this chapter suggests an association between CHIKV infection and a spectrum of CNS and PNS disease. Patients had only been recruited if they were suspected to have had recent ZIKV symptoms. These findings highlight the high number of CHIKV infected cases among ZIKV suspected patients. These findings highlight the challenge faced by clinicians to differentiate arboviral infection based on clinical presentation.

4.6.2.1 Serological detection of chikungunya antibodies

Two of the patients with IgM antibodies detected in their serum had no further CHIKV RNA or IgM detected in their specimens. The strength of the evidence is discussed below:

Patient #2: The patient exhibited a low titre of IgM CHIKV antibodies (1.2 ratio – cut-off at 1.1). The CSF, serum and urine specimens were collected 25 days post arboviral symptom onset. Not detecting specific CHIKV RNA in such specimens would be expected, however, IgG CHIKV antibodies should be detectable. The specimen was re-tested 3 times in separate experiments. Whether this IgM CHIKV positive result may represent a false positive result should be investigated further.

Patient #6: In addition to the positive IgM CHIKV antibodies, the patient exhibited very high specific CHIKV IgG antibodies in serum (RU/ml=124.0) suggesting this result may reflect a recent CHKV infection.

4.6.3 Laboratory evidence suggesting recent arboviral co-infection

Chikungunya virus belongs to the *Togaviridae* family which is distinct from ZIKV or DENV (the *Flaviviridae* family) [201]. Therefore, cross-reactive antibody responses between these viral families are not expected [202].

Positive evidence for 2 recent arboviral infections may mean both infections occurred simultaneously or one followed the other. In each patient, it is difficult to identify which virus was associated with the patients' clinical presentation, or whether more than one virus may have been involved. Moreover, we could not determine if the outcome may be worse when more than one arbovirus has been present.

To interpret the virological diagnosis when evidence for both ZIKV and DENV exists, we have investigated the potential serological cross-reactivity between both flaviviruses. In chapter 3, we showed that the IgM and IgG DENV ELISA assays showed false positive results in ZIKV infection patients due to significant cross-reactivity with Zika virus. Thus, the potential dual evidence of ZIKV and DENV needs to be interpreted with caution as it may reflect a single-flavivirus infection.

4.6.4 Zika virus laboratory findings in the context of recent evidence

When these laboratory investigations took place, there was only a preliminary epidemiological report from the French Polynesian outbreak indicating that there has been an increase in GBS and other neurological manifestations associated with Zika virus [50]. However, prior flaviviral exposure mainly to DENV made interpretation of the arboviral findings challenging due to serological cross-reactivity [203].

These shreds of evidence increased the international concerns and several more reports were published over the next few months, including the description of a wider range of neurological conditions that seemed associated with Zika such as myelitis and meningoencephalitis [55, 204]. Recently, a new study from our ZikaPLAN colleagues from Colombia has shown a strong temporal association between GBS and ZIKV. They report the detection of ZIKV RNA in 17 patients [51].

In addition, several serious complications associated with ZIKV have been reported such as neuropathies, uveitis, thrombocytopenia and arthritis [205, 206]. A few case reports have described Zika virus-associated neurological complications such as: myelitis [55], encephalitis (such as our work published here [207]), meningoencephalitis [204], acute disseminated encephalomyelitis [208, 209], and the GBS-variant, Miller-Fisher syndrome [210]. This

accumulating evidence suggests that the spectrum of neurological disease associated with Zika virus may be wider than what was thought when we started our investigations. Moreover, recent publications have identified co-infections of Zika and chikungunya virus leading occasionally to death. In one case report of an immunosuppressed adult, ZIKV RNA persisted for 275 days. Fatal outcome occurred 310 days post ZIKV and CHIKV co-infection onset [211].

4.6.5 Chikungunya virus laboratory findings in the context of recent evidence

When we started this investigation, neurological disease associated with CHIKV infection, had only been reported in India or South-East Asia [188, 189, 212-214]. In the recent few months, in addition to our work, several new reports have also been published reporting neurological complications associated with CHIKV in South America [49]. However, most neurological infections caused by arboviruses are still misdiagnosed or undiagnosed [215].

4.6.6 Study limitations

The investigations described in this chapter have several limitations inherent to its design.

4.6.6.1 Limitations of current diagnostics

The Zika diagnostic assays used have several limitations many of which are shown in the work presented in Chapter 3.

Molecular testing was not always useful as most samples were not taken early in the onset of disease. Moreover, it has been shown recently that ZIKV RT-PCR assays may exhibit better accuracy in other body fluids, such as saliva or whole blood [192]. Future studies should use these approaches to validate these findings. Thus, diagnoses of study subjects relied heavily on serological testing.

Another limitation is the lack of accurate serological assays to distinguish between ZIKV and DENV infection to enable accurate case classification.

Although samples were always kept at -80 ^{o}C following receipt at the Fiocruz Laboratory, potential mishandling of the specimens in the hospitals following collection may have led to degradation of RNA in the samples and consequently to potential false negative RT-PCR results.

4.6.6.2 Limitations of study design:

Samples for diagnostic testing were collected when the patient was admitted to the neurological wards which meant that the samples were not always taken early enough for the detection of potential viremia.

The study was not designed to include patients with minor neurological complications that would not necessarily be admitted to hospital. Therefore, whether arboviruses may be associated with mild neurological conditions such as cranial nerve palsy is not yet known.

There were very low sample volumes for a proportion of patients which complicated the laboratory investigations. Moreover, it would have been very interesting to collect sequential samples to compare ZIKV antibody levels. Future studies should include this, but was not possible during the emergency response to the Zika epidemic.

Secondary dengue infections are associated with more severe dengue disease, and some have postulated that prior dengue may predispose to more severe Zika infection. This study could not investigate this correlation due to the high proportion of patients with prior exposure to DENV.

Moreover, this study was not designed to identify neurological complications due to asymptomatic arboviral infections, as one of the inclusion criteria was including cases where prior ZIKV infection was clinically suspected.

Finally, I want to highlight the challenge of conducting this study in Rio de Janeiro, Brazil, in the middle of a major crisis of the public hospitals. During the time of the study, several hospitals were not admitting neurological patients due to insufficient resources. Therefore, a proportion of not-so-severe patients that otherwise may have been admitted to the study hospitals, may have been missed.

4.6.6.3 Limitations of interpreting the study results

The results identified in this chapter need to be interpreted with care. Linking rare neurological diseases to infections that affected thousands of people in Brazil such as DENV, ZIKV or CHIKV can be challenging. Defining the causal relationship between a particular pathogen and neurological complications can be difficult [216]. To determine causality, detecting the virus in brain biopsy material is considered a “gold standard” technique, however, it is impractical in most cases. Granerod *et al.* (2010) suggest the hierarchical criteria shown in Figure 4.4**.** The interpretation of our results was conducted following broadly these criteria with stronger evidence of the link between neurological disease and ZIKV, CHIKV and or DENV when the organism/antibodies were found in a sterile site such as the CSF compared to when they were found in urine or serum.

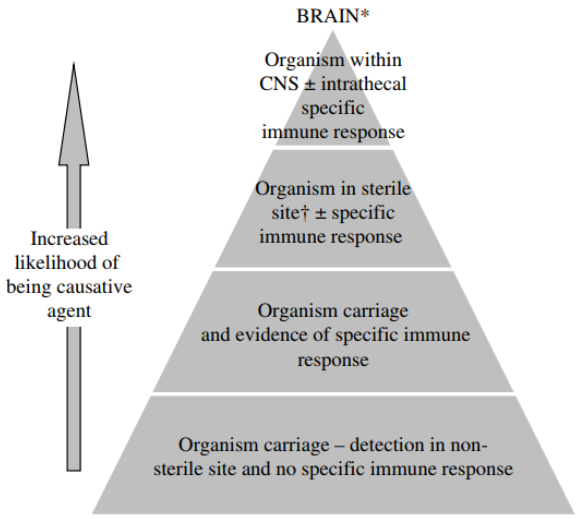


Figure 4.4 Diagram showing the hierarchy of diagnostic findings for defining the aetiology of brain infections
Hierarchy of diagnostic tests proposed by Granerod *et al*. (2010) to define the causal relationship between a microbe and encephalitis. Image adapted from Granerod *et al.,* (2010) [216].

Using paired patient samples, collected sequentially after illness (a few days difference) to look for an increase in anti-viral antibody tires over time can be a way to measure the occurrence of microbe-specific immune responses. This measurements add support to a raised antibody response and (particularly for IgG) allows for temporally linking these responses to the patient's course of the illness. Unfortunately, few paired samples were available from the study population (n=4). Observing a raised anti-viral antibody response in the CSF rather than serum, supports the response being triggered by infection in the brain or CNS. This information can be strengthened by comparing antibody tires in CSF: serum (also known as antibody index). A higher level in the CSF supports intra-thecal generated antibodies (generated within the CSF space) as opposed to antibody crossing into the CSF from the blood stream. Testing the intrathecal antibody production has been done with other neurological complications [217, 218]. Again, this index has not been measured in this study due to limited availability of paired CSF/serum samples taken at the same time-point.

4.6.7 Future perspective

As explained above, case series studies such as this one have several limitations inherent to its study type. Thus, to further confirm the causality of an association such as the one described in this chapter, prospective case-control studies are recommended. Consequently, at the end of 2016, we started a prospective case-control study in a total of 11 hospitals in two cities in Brazil: Rio de Janeiro and Recife. We used the lessons learnt from this

retrospective case series to design and establish a study designed to determine the association between ZIKV, CHIKV and neurological disease.

4.6.8 Implications for arboviral diagnosis of our findings

The association between Zika virus and neurological disease has important implications for diagnosing arboviruses. Given the findings presented in this chapter, testing for Zika virus should be routine for all new, unexplained neurological presentations in Zika virus-endemic regions. By doing this, it will be possible to explore further evidence for causality and aid in determining the epidemiology of Zika virus-associated neurological disease.

Moreover, my findings suggest that clinicians and public health officials must look beyond GBS in order to understand fully the disease burden of Zika virus. Additionally, this study highlights the need to investigate patients with acute neurological syndromes for other co-circulating arboviruses, particularly chikungunya and dengue virus.

These findings also highlight that there are still important questions concerning ZIKV and neurological sequelae. Although it seems to be relatively rare, their spectrum, incidence and risk factors are unknown and there is a clear need for more research.

In the future, guidelines directed to neurologists and routine laboratories should be produced in order to recommend appropriate antibody and molecular diagnostic testing to arboviral infections. Moreover, as these diseases are a potential threat to all in contact with the mosquito in endemic regions. Therefore, we suggest that regional clinicians incorporate routine testing for Zika and chikungunya viruses in patients presenting with unexplained neurology.

4.6.9 Conclusions

The results presented in this chapter highlight the challenging scenario of interpreting ZIKV serological and molecular diagnoses in populations where other arboviruses are also circulating. Our findings add to the growing body of evidence to support the hypothesis that there is a wide spectrum of neurological disease associated with Zika virus infection, including both CNS and PNS complications. We have also identified cases of chikungunya virus-associated neurology and the potential for dual co-infections leading to neurologic disease.

CHAPTER 5

Investigating the seroprevalence of Zika Virus in Rio de Janeiro

Chapter 5: Investigating the seroprevalence of Zika Virus in Rio de Janeiro

5.1 Overview Chapter 5

Following the ZIKV outbreak in 2015-2016, understanding the prevalence of ZIKV in the Brazilian population became an urgent public health priority. It was challenging due to the lack of accurate serology methods detect exposure to Zika virus, which were not susceptible to cross-reactivity with dengue virus. The high proportion of asymptomatic cases meant that estimating the total number of individuals exposed to ZIKV based on clinical recognition during the epidemics would grossly underestimate population exposure.

After completing the work in chapter 3, which investigated, evaluated and identified good methods for specific detection of IgG ZIKV antibodies, we started identifying populations that would help answer the following questions: How many people have been infected with ZIKV in Rio de Janeiro? What proportion of this population is ZIKV naïve? This information is key to prediction of the timing and size of future epidemics, vaccination strategies and informing physicians, public health experts and the general population of the potential risks of ZIKV.

5.2 Introduction

Since their emergence in the Americas in 2015 and 2013 respectively, explosive outbreaks of Zika virus (ZIKV) and chikungunya virus (CHIKV) have affected millions [219, 220]. Both are arthropod-borne viruses (arboviruses) transmitted mainly via *Aedes aegypti* mosquitos that cause rash-fever and arthralgia during the acute phase of infection. ZIKV and CHIKV infections have also been associated with severe complications both in children and adults. ZIKV can cause severe congenital malformation in neonates born to mothers infected with the virus during pregnancy. Zika virus has also been associated with peripheral and central nervous system disorders in adults [221].

CHIKV has been associated with a wide range of severe complications such as central and peripheral nervous system disorders in adults and children [49], congenital neonatal encephalitis [222], and complications of cardiovascular and respiratory systems as well as an increase in morbidity and mortality [223].

Molecular studies suggest ZIKV may have arrived in Rio de Janeiro as early as April 2013 [180]. However the first rapid rise in clinical cases was reported in 2015 [224]. Over 68,000 notified

cases were reported in Rio in 2016 [225]. Despite its devastating impact, the exact prevalence of ZIKV in Brazil has not been estimated.

Transmission of CHIKV in Rio de Janeiro was first reported in December 2015 [226]. The state of Rio de Janeiro, Brazil, experienced an CHIKV epidemic at the end of 2015, with over 15,000 notified cases reported in 2016 [225]. Between January 2015 and October 2016, 2,170 cases of ZIKV or CHIKV were confirmed by the Regional Reference Laboratory in Rio de Janeiro.

Given the high proportion of asymptomatic infections among ZIKV and CHIKV exposed individuals [203], serological testing is the only way to understand the scale of exposure across a population.

5.2.1 Previous seroprevalence studies of ZIKV

Since 1952, there have been several seroprevalence studies examining ZIKV exposure in different populations. A summary of previous studies is shown in Table 5.1. Past studies have used a variety of assays, including hemagglutination inhibition (HI) tests, neutralization tests, hemagglutination assays or ELISAs [220]. As discussed previously, in view of the lack of accurate assays, the results from past studies should be interpreted with caution. Nevertheless, the available evidence indicates that ZIKV is endemic in most of the African continent (East, Central, West and South) and several Asian countries [220].

Since the 2015-2016 outbreak, there have been four published estimates of ZIKV seroprevalence in the Americas, with only two in Brazil. Gallian *et al*. (2017) conducted a study in Martinique (a French-Administered-territory overseas), analysing over 400 blood donors. They reported a ZIKV seroprevalence of 13.5% in March 2016, which increased to 42.2% in June 2016, following the Martinique outbreak [227]. Villaroel and colleagues reported ZIKV prevalence ranging between 0 to 39% among blood donors from across different areas of Bolivia [228]. The study by Busso *et al.* (2017), based uniquely on RT-PCR and IgM ELISA results (with no IgG testing), reported a prevalence of acute ZIKV infection <1%. Netto *et al.* (2017), conducted a study in Salvador (Brazil). They reported a seroprevalence of 63.3% among a population cohort which included pregnant females, HIV patients and University staff members (n=633). Interestingly, they suggested low socioeconomic status may be a risk factor for ZIKV infection.

Table 5.1 Reports of main ZIKV seroprevalence studies in adults and children

	Author & year of publication	Country/Location	Period	Population examined and numbers	ZIKV Seroprevalence	Methods	Observations
AFRICA							
1	Dick (1952) [2]	**Uganda**	1951-1952	99 subjects	6.1%	Neutralization assay	Serological diagnosis methods not highly accurate. Results may be biased due to cross-reactivity.
2	Geser, Henderson *et al.* (1970) [229]	**Kenya**	November 1966 to April 1968.	2698 subjects	3.3% (Central Nyanza) 1.3% Kitui District 52% Malindi District	Hemagglutination test A small minority also PRNTs	Cross-reactivity with other flaviviruses. Very premature serological diagnostic methods
3	Fagbami, (1979) [230]	**Oyo State, Nigeria**	1971 to 1975	10,778 subjects	31%	Hemagglutination-inhibition tests	40% of the ZIKV positive cases were also positives for other flaviviruses
4	Robin and Mouchet (1975) [9]	**Sierra Leone**	1972	899 children (0-14 years old)	6.9%	Hemagglutination-inhibition Complement fixation tests	Cross-reactivity with other flaviviruses such as yellow fever. Very premature serological diagnostic methods

5	Saluzzo, Gonzalez *et al*. (1981) [11]	**Central African Republic**	1979	459 subjects	12.85%	Hemagglutination test	High arboviral presence. (89% population positive for at least one arboviral antibody) Cross-reactivity with other flaviviruses. Very premature serological diagnostic methods
6	Fokam, Levai *et a*l. (2010) [231]	**Cameroon**	2008-2009*	102 febrile patients (negative for malaria and typhoid fever)	11.4%	Serological methods. No further description	Potential cross-reactivity
7	O.A. Babaniyi (2014) [232]	**North-Western Provinces of Zambia**	2012-2013	3625 subjects	6.1% ZIKV	IgM and IgG ZIKV ELISA	Originally a Yellow Fever Study
8	Gake, Vernet *et al.* (2017) [233]	**Cameroon**	August to October 2015	Cameroonian blood donors: 1084 subjects	Overall Study: 5% ZIKV 10% in Douala 7.7% in Bertoua	Anti-NS1 IgG ELISA (Euroimmun) Positives confirmed by PRNT	Suggested existence of a local (peri-) sylvatic cycle of ZIKV transmission
9	Seruyange, Gahutu *et al*. (2018) [234]	**Sweden and Rwanda**	2015	874 subjects from Rwanda 270 Swedish blood donors	1.4 % prevalence in Rwanda 0.0 % prevalence in Sweden	ZIKV RT-PCR IgG ZIKV NS1 ELISA (Euroimmun)	High percentage of ZIKV susceptible individuals in Rwanda. High risk for a potential ZIKV epidemic.
EUROPE							

10	Loconsole, Metallo *et al*. (2018) [235]	**Southern Italy**	March 2015 to June 2017	156 travellers	1.3%	IgM and IgG ZIKV ELISA (Euroimmun)	Suggested international travellers to undergo serological surveillance Risk of introduction of emergent pathogens such as ZIKV in new areas
CENTRAL AND SOUTH AMERICA							
11	Netto, Moreira-Soto *et al.* (2017) [236]	**Salvador, North-eastern of Brazil**	2015-2016	633 individuals (pregnant, HIV patients and University staff members)	63.3 %	IgG ZIKV Euroimmun PRNT for confirmation	Sociodemographic data revealed a higher ZIKV burden in low SES populations
12	M.O. Cassaraa (2017) [237]	**Sao Paulo, Brazil**	April 2016 to December 2016.	759 patients	1 IGM Positive/759 **<0.1% (Acute infection)**	IgM ZIKV ELISA ZIKV RT-PCR	**Defined by the authors as a seroprevalence study, however, no IgG Testing was performed.**
13	Gallian, Cabie *et al*. (2017) [227]	**Martinique**	January 19 to June 10, 2016	4129 blood donors Sub cohort: 400 subjects for Antibody testing	13.5%(in March) 42.2% (in June)	ZIKV RT- PRC IgG ZIKV ELISA (Euroimmun)	Highlights importance of testing in blood banks
14	Saba Villarroel, Nurtop *et al.* (2018) [228]	**Bolivia**	December 2016 to April 2017	Bolivian Blood Donors: 814 subjects	Tropical areas: Beni: 39%; Santa Cruz de la Sierra: 21.5% Non Tropical areas: 0%	IgG ZIKV Euroimmun PRNT for positives or equivocal	ZIKV spread not limited by previous DENV immunity Cases were geo-localised in a wide range of urban areas No differences in seroprevalence related to gender or age-groups could be identified.

OCEANIA							
15	Duffy, Chen *et al*. (2009) [12]	**Yap Island**	2007	557 subjects from 173 households	74.3%	IgM ZIKV	18.9% of individuals were negative for IgM ZIKV, though, reported being symptomatic during outbreak
16	Aubry, Teissier *et al.* (2017) [203]	**French Polynesia**	2014-2015	Cluster 1 (Remote islands): 196 subjects Cluster 2 (main islands): 476 schoolchildren Cluster 3: 700 subjects (most inhabited archipelago)	49% (General population) 66% (Schoolchildren)	IgG indirect CHIKV ELISA (recombinant antigen-based) Microsphere immunoassay (MIA),	Estimated asymptomatic to symptomatic ratio of 1:1

5.2.2 Previous seroprevalence studies of CHIKV

Since 2004 CHIKV infection has been spreading across several tropical and sub-tropical areas of Africa, Asia, Europe, the Pacific and the Americas [238]. The Brazilian Ministry of Health declared a total of 20,661 and 271,824 notified CHIKV cases in 2015 and 2016 respectively, based on reports from over half of the municipalities [239]. Due to the magnitude of this outbreak, CHIKV has had a major public health impact in Brazil.

Few seroprevalence studies have been conducted in the American continent. A study in Puerto Rico in 2014-2015, with 1,232 blood donors, reported CHIKV seroprevalence at 23.5% [240]. A seroprevalence of 48.1% and 41.9% was reported in Guadeloupe and Martinique islands respectively, based on data from 9,506 blood donors tested via CHIKV IgG assays [219]. A study in Saint Martin reported a seroprevalence of 16.9%, based on a convenience sample of 203 individuals. Thirty-nine percent of subjects testing positive were reported to be asymptomatic [241]. In Nicaragua, a study based on random sampling of an urban population, reported a seroprevalence of 32.8% [242]. Interestingly, in the latter study, they also found a positive correlation between vector density and seroprevalence.

In Brazil, only two seroprevalence studies have been conducted. Cunha *et al.* (2017) reported a seroprevalence of 20.0% in April 2016, based on a cohort of 120 individuals from a rural community in Brazil [243]. In Salvador (Brazil), the seroprevalence for CHIKV during 2015-2016 has been estimated to be around 7% [236].

5.2.3 Challenges in ZIKV and CHIKV population serology: serological testing

Limited seroprevalence studies are being conducted across the Americas. The seroprevalence of ZIKV and CHIKV in metropolitan Rio de Janeiro remains unknown. One roadblock to accurate seroprevalence data, is the lack of a specific IgG ZIKV test. In Chapter 3, I demonstrated that IgG assays, approved by the Brazilian National Agency (ANVISA), were not specific. I also demonstrated the BOB Assay, developed by HUMABs, showed improved specificity (90-96%) when tested in our Brazilian population and in other cohorts worldwide [116]. Additionally, I also demonstrated that a simplified version of the BOB assay, the GREEN BOB, exhibited even better specificity and sensitivity.

Therefore, I chose the GREEN BOB assay to estimate the seroprevalence of Zika in the adult population in Rio. For estimating CHIKV seroprevalence, I used the Euroimmun CHIKV IgG assay, which has previously been reported to have high accuracy [121].

5.2.4 Challenges in population serology: understanding socioeconomic impact

Understanding the impact of socioeconomic status (SES) in ZIKV and CHIKV exposure is essential to the design of education and vector control campaigns. One of the key factors that measure SES is the Human Development Index (HDI). The United Nations Development Program (UNDP) defines HDIs as "*a summary measure of average achievement in key dimensions of human development: a long and healthy life, being knowledgeable and have a decent standard of living. The HDI is the geometric mean of normalised indices for each of the three dimensions*" [244]. HDIs measure life expectancy, education and per capita income indicators. The HDI was created to emphasize that people and their capabilities "*should be the ultimate criteria for assessing the development of a country, not economic growth alone*".

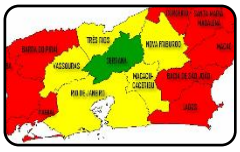
5.2.5 Geographical divisions in the State of Rio de Janeiro

The State of Rio de Janeiro is the third most populous State in Brazil, located in the Southeast region and with a predominantly tropical climate. The geographical divisions of the State of Rio de Janeiro can be summarised as follows in the following diagram:

State of Rio de Janeiro

Mesoregions in the State of Rio de Janeiro

- Baixadas Litoraneas (Costal mesoregion)
- Centre Fluminense
- Northeast Fluminense
- North Fluminense
- South Fluminense
- Metropolitan Area of Rio de Janeiro



Microregions in the Metropolitan Area of Rio de Janeiro

- Itaguaí
- Macacu-Caceribu
- Serrana
- Vassouras
- Rio de Janeiro

Areas within the City of Rio de Janeiro

- North
- South
- Centre
- West

Within the State of Rio de Janeiro, there are 6 mesoregions, 18 microregions and 92 municipalities. Within the municipality of Rio de Janeiro, there are 4 main areas: North, South Zone (which includes Copacabana and Ipanema), Centre and West.

Rio the Janeiro is one of the most populous cities in South America and one of the most frequented tourist destinations for decades. In 2016, Rio hosted the XXXI Olympic Games. Rio de Janeiro is highly connected via trains, roadways and air with all the regions in Brazil and the Americas. Moreover, the main transmitter of ZIKV, DENV and CHIKV, the mosquito *Aedes aegypti*, is generally found in high densities throughout most of the State of Rio de Janeiro [245]. The hot-wet season tends to occur between January and May and field entomologic studies have shown that the city's *Aedes aegypti* population tends to peak in the same period too [153].

5.2.6 Arboviruses in Rio de Janeiro

It has been suggested that two epidemic waves of ZIKV affected Rio de Janeiro during the first half of 2015 and 2016. This coincides with the seasonality described in the peak of mosquito activity in the State of Rio the Janeiro (generally between the end of December and May) [184]. Meanwhile, it has been suggested that only one wave of CHIKV affected Rio de Janeiro prior to the end of 2016 [226]. Dengue virus has circulated widely across Rio since 1986 causing outbreaks of relatively large magnitude every three to four years [153]. DENV is endemic in Rio de Janeiro with cases occurring throughout the year, however, there is a strong seasonality trend with a majority of cases coinciding with the hot-wet season between January and May [246].

Among the most used seroepidemiological study designs to understand the spread of an epidemic are cross-sectional and longitudinal cohort studies [247]. In this chapter, I conducted a cross-sectional and a longitudinal retrospective serological evaluation using samples already collected to estimate the spread of ZIKV in Rio de Janeiro.

5.3 Aims of this Chapter

Two linked seroprevalence studies were conducted: a cross-sectional study and a longitudinal study. The main objectives of the cross-sectional study can be summarized as follows:

1- Estimate the point seroprevalence of Zika and chikungunya viruses in the adult population of the metropolitan region of Rio de Janeiro, Brazil, following the arbovirus season Jan-April 2016.
2- Investigate the association between demographic, socioeconomic and geographic factors and the seroprevalence of ZIKV and CHIKV

The main objectives of the longitudinal study were:

3- To investigate the temporal changes in prevalence for Zika virus following each arbovirus season in the first half of 2015 and 2016 in the population Rio de Janeiro
4- To investigate the temporal changes in prevalence for chikungunya virus and dengue virus between 2013 and 2016
5- To investigate for an association between the patterns of change in prevalence of Zika, chikungunya and dengue viruses, given they are spread by the same vector.

To the best of my knowledge, I am reporting the first seroprevalence study for ZIKV and CHIKV in Rio de Janeiro, one of the world regions most impacted by the ZIKV.

5.4 Materials and Methods

We conducted a cross-sectional survey to estimate the prevalence of Zika and chikungunya virus infection following the 2016 arbovirus season (January-June) in the metropolitan region of Rio de Janeiro (n=1001).

5.4.1 Study Location

The study was conducted in the metropolitan region of Rio de Janeiro, south-east Brazil. Rio de Janeiro is one of 27 federal units in Brazil. It has a total area of 43,696.1 km^2 and a total population of 16,718,956 (at December 2016). It is the second largest state economically in Brazil. It has a Human Development index (HDI) of 0.778, the third highest in the country. Rio de Janeiro is composed of 92 municipalities. The municipalities are grouped into six meso-regions: Metropolitan area of Rio de Janeiro, Baixada, Centro Fluminense, Northeast Fluminense, North Fluminense and South Fluminense. The majority of the subjects in our study were residents of the Metropolitan area of Rio de Janeiro. All the samples were processed at the Flavivirus Reference laboratory.

5.4.2 Study Design

The study had two subject cohorts, forming two study arms; (i) the cross-sectional study examined the largest cohort (n=1001) and (ii) the longitudinal study examined a cohort (n=121) followed across four years. The study design and collection dates for the longitudinal and cross-sectional cohorts are shown in Figure 5.1.

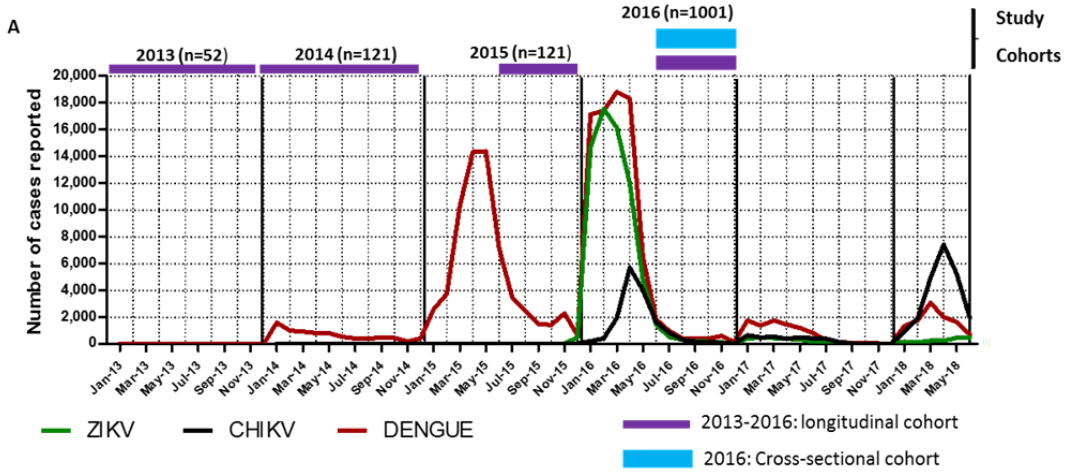


Figure 5.1 Diagram study design: sample collection of longitudinal and cross-sectional cohorts

Sample collection for the cross-sectional study was conducted from June 2016 to December 2016. These months were chosen, because they are outside the reported arboviruses season, in the metropolitan region of Rio de Janeiro. The cross-sectional cohort was designed to estimate the point prevalence of Zika and chikungunya virus. The longitudinal survey was designed to investigate the pattern of conversion over the years 2013, 2014, 2015 and 2016.

5.4.3 Sample selection

Serum was obtained from individuals referred to the Viral Hepatitis Clinic at IOC. Subjects were individuals with either confirmed hepatitis C, hepatitis B virus infection, suspected hepatitis or companions of the subjects.

Serum samples were processed at the Reference Viral Hepatitis Laboratory at IOC. All subjects with serum specimens taken at the hepatitis clinic service from the 1st of June 2016 to the 31st of December 2016 were included (n=1001).

Key demographic and clinical data were collected, including; date of birth, sex, age, clinical condition, address, postcode and pregnancy status. The participant's race/ethnicity was self-reported into the classifications recommended by the Brazilian Institute of Geography and

Statistics (IBGE), which are the categories used in the Brazilian census. As recommended, they were categorized as black, brown (mixed race), white, indigenous, yellow (or of Asian ancestry) or not reported.

5.4.4 Study population – Longitudinal Cohort

A subset of 121 subjects from the cross-sectional cohort (n=1001) formed the longitudinal cohort. As shown in Figure 5.1, during the first half of 2015 there was an arbovirus epidemic in the State of Rio de Janeiro, though there was not a significant outbreak during 2014. As I wanted to understand the change in prevalence following the two arboviral seasons, the subjects included in this longitudinal arm had had a serum specimen collected between the 1st of June 2015 and the 31st of December 2015 which is after the 2015 wave. Subjects included in the longitudinal cohort also had samples collected in 2014. Moreover, serum specimens collected in 2013 were also available for 52 individuals.

5.4.5 Ethics Statement

This study was approved by the Ethics Committee of FUNDAÇÃO OSWALDO CRUZ, Brazil (FIOCRUZ/IOC, number 2.476.733). The study protocol was followed according to the ethical guidelines of the 1975 Declaration of Helsinki. Identifiable information was removed from serum samples prior testing.

5.4.6 Laboratory diagnostic testing

All samples were tested for Zika and Chikungunya virus IgG antibodies.

Blood samples were collected by venepuncture and serum was separated by centrifugation (3000 rpm/5 min). The serum was stored at -20 ^{o}C until use. All specimens were tested by enzyme immunoassays for Zika and chikungunya IgG antibodies.

5.4.6.1 Serological testing of ZIKV and definition of ZIKV exposure

To investigate the seroprevalence for ZIKV, we used the ELISA assay that offered the highest levels of specificity and sensitivity as shown in Chapter 3: the in-house block of binding ELISA assay (Green BOB) with a Zika-specific monoclonal anti3ody.

The interpretation of results was done as follows: subjects with IgG ZIKV Ratios ≤1.0 were considered positive. Subjects with IgG ZIKV ratios >1.0 were considered negative for ZIKV IgG.

5.4.6.2 Serological testing of CHIKV and definition of CHIKV exposure

The IgG anti-CHIKV enzyme-linked immunosorbent assay (ELISA) from Euroimmun (Lubeck, Germany) was performed following the manufacturer's instructions. Further details in Chapter 2.

5.4.7 Secondary sources of arboviral incidence data

Zika, chikungunya and dengue viruses are mandatory reportable diseases in Brazil as declared by the National Brazilian Ministry of Health. The reporting system is dependent on the notifications of all the suspected cases attending health facilities (both public and private) and who fulfil clinical and epidemiological criteria (laboratory confirmation was not a requirement). A suspected case for an arboviral infection was defined as illness in a subject in an area of known arbovirus transmission, (ZIKV, DENV, or CHIKV) or there is a known *Aedes* mosquitoes infestation and the patient has the following symptoms:

Zika virus infection: presence of rash, conjunctivitis, exanthema and/or fever. Symptoms should not be explained by other conditions.

Chikungunya infection: sudden onset of fever (>38.5 ^{o}C) and arthralgia or acute arthritis. Symptoms should not be explained by other conditions.

Dengue infection: sudden onset of fever (>38.5 ^{o}C), arthralgia, headache and or rash. Symptoms should not be explained by other conditions.

Data for the figures (Figure 5.3 and Figure 5.5) showing the number of notified cases of suspected arborviral infection over time (epidemiological curves) was obtained from the Sistema Nacional de Informacao do Ministério da Saúde (SINAN). SINAN is the Brazilian Ministry of Health's National Information System for entering and processing national data (http://portalsinan.saude.gov.br/dados-epidemiologicos-sinan). I also obtained data on reported arboviral cases in the metropolitan region of Rio de Janeiro from the Secretary of Health Vigilance in Rio de Janeiro. This unit has monitored all individuals presenting with exanthemous diseases since 2001 [225].

5.4.8 Spatial and sociodemographic data:

Spatial and sociodemographic factors were mapped to participants. The home address of each participant was linked to a global map position (by latitude and longitude). Each participant was also linked to a human development unit (HDU). HDUs are a summary score encompassing different socio-demographic factors, including education health, income, population and demography [248]. HDU's were mapped to participants based on the census

data from the Instituto Brasileiro de Geografia e Estatística (IBGE). The area of the metropolitan region of Rio de Janeiro was divided into socioeconomically homogenous areas. A minimum of 400 permanent households as well as socioeconomic homogeneity were taken into account and values for each of the HDUs were obtained from the *Atlas do Desenvolvimento Humano no Brasil* (Brazilian Human development Atlas - http://atlasbrasil.org.br/2013/consulta) with data from census in 2010 from the United Nations Development Programme (UNDP).

5.4.8.1 Socioeconomic and sociodemographic data

A total of 228 indicators of Socioeconomic Status (SES) and sociodemographic status were matched to our study subjects according to their HDU (n=1000). Information was not available for 1 subject for which exact postcode of residence was unknown. A range of indicators of SES, income, health, education and income were investigated in detail and their definition is described in Chapter 2.

Socioeconomic status was measured with the Human Development Index (HDI). The HDI was developed by the United Nations as a metric to assess the social and economic development levels of countries (Further details described in Chapter 2). As recommended, the different population groups are classified as:

- HDI-Very high (0.85-1)
- HDI - High (0.75-0.85)
- HDI- Medium (0.650-0.75)
- HDI Low (0.550-0.650)
- HDI Very Low (0.2-0.550)

5.4.8.2 Spatial analyses

Geographic analysis and map figures were generated using ARC Map and ARCGIS v10 (ESRI, Redlands, CA, USA) and Google Maps (Google, CA). Baseline data were created using the ArcGIS Basemap OpenStreetMap service and the Database of Global Administrative Areas (GADM, https://gadm.org/).

5.4.9 Data analysis and Statistical analysis

All data was analysed with GraphPad Prism v5 software (GraphPad Software, La Jolla, CA) and Microsoft Excel 2013. The prevalence of ZIKV and CHIKV are expressed as percentages. Shapiro-Wilk normality tests were conducted. The IgG antibody titres for CHIKV and ZIKV (expressed as ratios) were analysed by the Mann-Whitney U test or by One-way analysis of variance (ANOVA). Differences in proportions among categorical data was examined using a

two-way contingency table with statistical significance assessed by Fisher's exact test. Odds ratio (OR) were calculated to measure association between an exposure and an outcome. Fisher's exact test was used in the analysis of contingency tables (2x2 tables).

5.5 Results

5.5.1 Characteristics of the study population – Cross sectional study

A summary of the key demographic characteristics of the study population such as sex, age, ethnicity, and clinical state is shown in Table 5.2. The proportion of female/male was equally represented in our population with 50.75% and 49.25% respectively. All age groups composing the population of the metropolitan region of Rio de Janeiro were represented in our study with a majority being adults between 41-60 years old (44.2% of our cohort) and the median age being 53 years old. Only 13 (1.3%) subjects included in our study were under 18 years old. We included individuals representing the different ethnic groups in the Brazilian population including 182 (18.2%) black, 476 (47.6%) brown, 315 (31.47%) white and 7 (0.7%) of other ethnic groups. Ethnic groups included in the "other" category include indigenous and yellow (Asian origin) ethnic groups. Ethnic information was not reported or not available for 21 subjects. Six subjects included in the study reported being pregnant at the moment of serum collection (during 2016).

Study subjects were classified into 5 groups regarding their SES measured in Human Development Index (HDI). Categories following the UNDP groups were: very low HDI, low HDI, medium HDI, high HDI and very high HDI. Subjects included in the study had a median HDI of 0.71 (IQR 0.48-0.79).

Table 5.2 Demographic characteristics of cross-sectional study population
Summary of key demographic characteristics of cross-sectional cohort from the metropolitan region of Rio de Janeiro. Samples were collected from 1st June 2016 – 31st Dec 2016. *HDI demographic data not available for one subject. Other ethnicity groups include: Yellow and indigenous.

Characteristics	Cross-Sectional Cohort (n)	Cross-Sectional Cohort (%)
Total	1001	
Female	508	50.75
Male	493	97.05
Age		
Total (Median, IQR)	53.0 (41.0-63.0)	
0-20	21	2.10
>21-40	219	21.88
>41-60	442	44.16
>60	319	31.87
Ethnicity		
Black	182	18.18
Brown/Mixed	476	47.55
White	315	31.47
Other	7	0.70
Unknown	21	2.10
Clinical Description		
Hepatitis B (HBV)	24	2.4
Hepatitis C (HCV)	161	16.1
Date of Sample Collection		
Jun-16	135	13.49
Jul-16	158	15.78
Aug-16	105	10.49
Sep-16	151	15.08
Oct-16	161	16.08
Nov-16	165	16.48
Dec-16	125	12.49
Human Development Index (HDI)		
HDI (Median - IQR)*	0.71 (0.48-0.79)	
HDI-Very high (0.85-1)	84	8.4
HDI - High (0.75-0.85)	257	25.7
HDI- Medium (0.650-0.75)	293	29.3
HDI Low (0.550-0.650)	261	26.1
HDI Very Low (0.2-0.550)	105	10.5

5.5.2 Geographic characteristics and spread of the study population

A majority of study subjects were residents of the municipality of Rio de Janeiro (n=627) coming from over 150 different neighbourhoods. A total of 374 subjects lived in 32 other municipalities within the metropolitan region of Rio de Janeiro (Table 5.3). The exact postcode of residence [which is called “Código de endereco postal” (CEP)] was known for study subjects and latitude and longitude was extrapolated. Geographical distribution for each study subject is shown in Figure 5.2.

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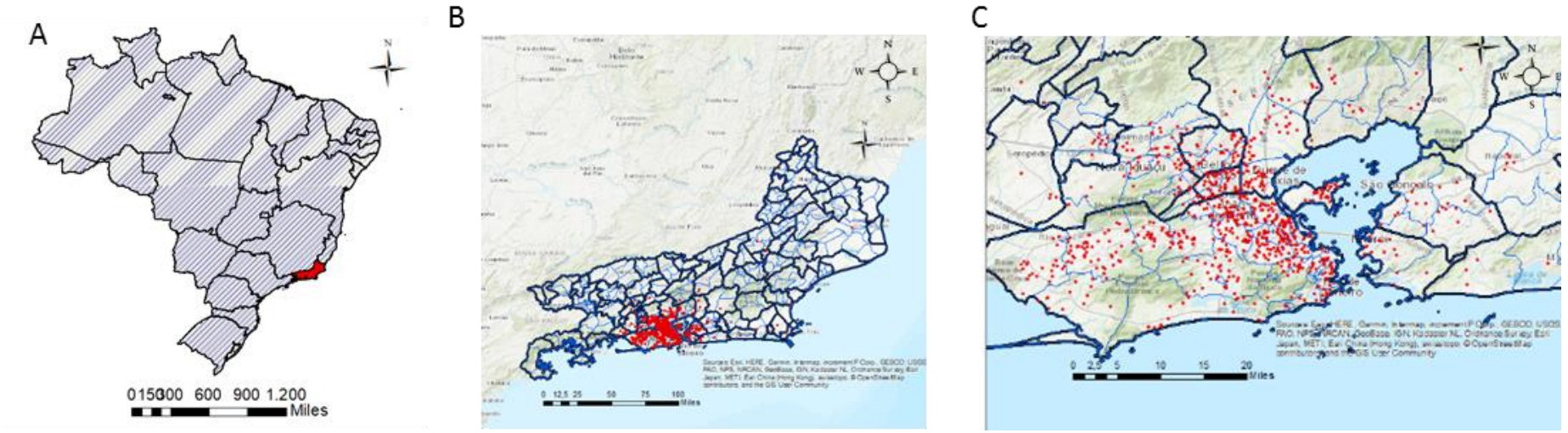

Figure 5.2 Geographical Distribution of the cross-sectional cohort from the State of Rio de Janeiro, Brazil

Geographical distribution of the cross-sectional cohort (n=1001) included in the investigations. Red circles represent residence of each one of the subjects investigated. (A) Map of Brazil highlighting the State of Rio de Janeiro where the study was conducted. (B) Map of the State of Rio de Janeiro showing most of the study subjects included come from the city of Rio de Janeiro or surrounding municipalities. (C) Distribution of cases across the municipality of Rio de Janeiro. Exact coordinates were not available for one subject from the municipality of Rio de Janeiro. Maps generated with ARCGis with GADM basemap. Ruler below represents distance in miles.

Table 5.3 Summary geographical distribution of study subjects in the cross-sectional cohort
Distribution of study subjects regarding area of residency within the state of Rio de Janeiro and description of municipality of precedence. *Percentage of study subject's distribution in proportion to the 374 subjects with residence outside the Municipality of Rio de Janeiro.

Geographical Area	Number of subjects	Percentage (%)
Residing in the State of Rio de Janeiro	**1001**	
Residing in the municipality of Rio de Janeiro	**627**	**62.64**
Residing Municipality outside the city of Rio de Janeiro	**374**	**37.36**
Angra Dos Reis	1	0.27*
Araruama	5	1.34*
Belford Roxo	34	9.09*
Cachoeira De Macacu	1	0.27*
Campo Dos Goytacazes	1	0.27*
Cantagalo	1	0.27*
Cassemiro De Abreu	3	0.80*
Duque De Caxias	96	25.67*
Engenho Paulo Frontin	1	0.27*
Guapimirim	3	0.80*
Iguaba Grande	3	0.80*
Itaborai	6	1.60*
Itaguai	9	2.41*
Japeri	2	0.53*
Macae	2	0.53*
Macuco	2	0.53*
Magé	17	4.55*
Marica	5	1.34*
Mesquita	9	2.41*
Miguel Pereira	2	0.53*
Nilópolis	5	1.34*
Niteroi	15	4.01*
Nova Iguaçu	46	12.30*
Paracambi	1	0.27*
Petropolis	1	0.27*
Queimados	11	2.94*
Rio Das Ostras	1	0.27*
São Gonçalo	20	5.35*
São João De Meriti	57	15.24*
Sao Pedro Da Aldeia	2	0.53*
Saquarema	3	0.80*
Seropédica	6	1.60*
Teresopolis	2	0.53*

5.5.3 Reported temporal spread of ZIKV, CHIKV and DENV in Rio de Janeiro

In order to better understand the seroprevalence of ZIKV and also CHIKV and DENV, I analysed the number of cases reported of ZIKV, DENV and CHIKV reported by the government of Rio de Janeiro between the 1st of January 2014 and the 31st of December

2016. An epidemiological curve with the cases notified by the Health Secretariat of Rio de Janeiro during this period is shown in Figure 5.3.

There were 39 and 65 notified cases of CHIKV in 2014 and 2015 (respectively), across the State of Rio de Janeiro. All of them were reported to have been acquired outside of Brazil. Therefore, the first reported native (autochthonous) case of CHIKV was reported in 2016. Figure 6.3 shows there were three major peaks (two Dengue and one Zika) of notification for suspected new arboviral infection cases during 2015-2016 in Rio. Start of study collection, in June 2016, coincided with the decline of the last major Zika outbreak in Rio.

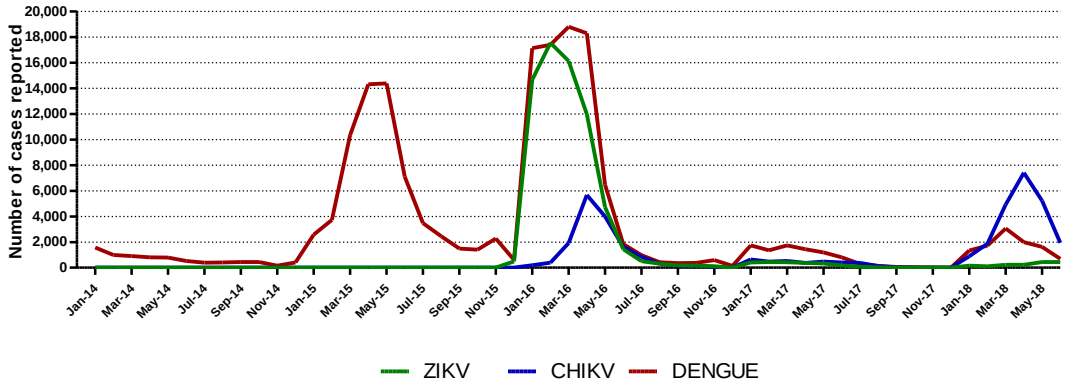

Figure 5.3. Epidemiological curve showing total reported cases of ZIKV, DENV and CHIKV
Data from the State of Rio de Janeiro between January 2014 and June 2018 in the Estate of Rio de Janeiro (Brazil).Data provided by the Subsecretaria de Vigilância em Saúde/Superintendência de Vigilância Epidemiológica e Ambiental from the Goberno do Rio de Janeiro. Clinical cases that are probable but not laboratory confirmed are also included. Total population in the State of Rio de Janeiro by December 2016: 16.550.024

5.5.4 Seroprevalence of ZIKV and CHIKV in the metropolitan region of Rio de Janeiro

Overall, the seroprevalence of ZIKV was 39.46% with 395 individuals positive for IgG ZIKV (Table 5.4 and Figure 5.4). CHIKV IgG seroprevalence was 14.99% with 150 positive subjects. The median IgG ZIKV ratio found was 1.30 and a histogram showing the frequency of distribution of ratios across the 1001 subjects is shown in Figure 5.4.

The cut-off used for the ZIKV detection with the IgG ZIKV assay was established in our previous investigations in Chapter 3. As a large proportion of specimens showed ratios close to the cut-off, we investigated the effect in the overall prevalence of ZIKV if a different cut-off was be used (Table 5.5). The lowest cut-off (0.6) showed better specificity while the highest cut-off (1.0) showed improved sensitivity.

Table 5.4 Seroprevalence of ZIKV and CHIKV in the metropolitan region of Rio de Janeiro

Age is expressed in years. Ethnicities are self-reported. HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; Antibody titres are shown as the median of the observed ratio of IgG CHIKV and IgG ZIKV respectively.

Characteristics	Cross-Sectional Cohort (n)	Cross-Sectional Cohort (%)	ZIKV Positives (n)	ZIKV Prevalence (%)	ZIKV Prevalence (95% CI)	ZIKV Antibody Ratio (Median)	CHIKV Positives (n)	CHIKV Prevalence (%)	CHIKV Prevalence (95% CI)	CHIKV Antibody titre (Median)
Total	1001		395	39.46	36.4-42.6	1.30	150	14.99	12.9-17.33	0.15
GENDER										
Female	508	50.75	200	39.37	35.1-43.8	1.27	81	15.94	13.01-19.39	0.14
Male	493	97.05	195	39.55	35.2-44	1.33	69	14	11.2-17.35	0.16
AGE										
Median (CI)	53.0 (41.0-63.0)									
0-20	21	2.1	6	28.57	11.3-52.2	1.64	2	9.52	1.45-30.12	0.11
>21-40	219	21.88	86	39.27	32.8-46.1	1.31	27	12.33	8.57-17.39	0.15
>41-60	442	44.16	154	34.84	30.4-39.5	1.43	61	13.8	10.88-17.34	0.16
>60	319	31.87	149	46.71	41.1-52.3	1.07	60	18.81	14.89-23.47	0.14
ETHNICITY										
Black	182	18.18	74	40.66	33.5-48.2	1.19	33	18.13	13.17-24.41	0.15
Brown/Mixed	476	47.55	203	42.65	38.2-47.2	1.21	75	15.76	12.75-19.31	0.15
White	315	31.47	108	34.29	29.4-40.3	1.48	39	12.38	9.16-16.51	0.15
Other	7	0.7	2	28.57	3.7-71	1.92	0	0	0-40.44	0.07
Unknown	21	2.1	6	28.57	11.3-52.2	1.22	3	14.29	4.14-35.48	0.15
CLINICAL STATUS										
Hepatitis B (HBV)	24	2.4	6	25.00		1.61	2	8.33	1.16-27	0.15
Hepatitis C (HCV)	161	16.08	67	41.61		1.21	26	16.15	11.21-22.66	0.13
Pregnant	6	0.6	3	50.00		0.77	0	0	0-44.28	0.16

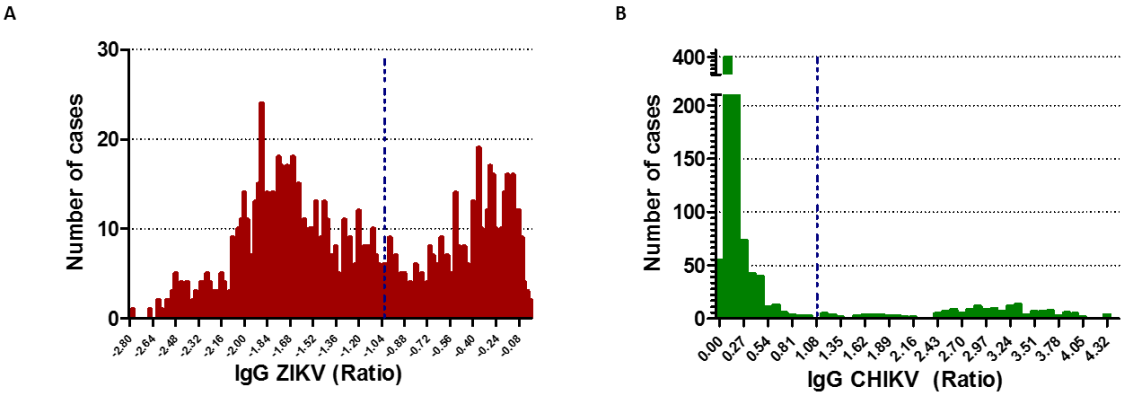

Figure 5.4 Frequency of distribution of the ZIKV and CHIKV IgG antibody titres
Distribution of ratios in the cross-sectional cohort (n=1001) for A) IgG ZIKV, measured in IgG Green BOB ratio, values under 1.0 are considered positive; B) IgG CHIKV, IgG antibody titres measured in ratio. Cut off at 1.1 and ratio> 1.1 are positive. Values to the right are positive. Blue dotted line shows cut-off for both assays.

Table 5.5 Investigation of the effect of modifying the assay cut-off in the ZIKV prevalence
Examining the difference in seroprevalence estimates if different cut-offs for the Green BOB assay were used. Cut-offs selected from Chapter 3. Proportion of ZIKV cases across the whole population of 1001 subjects shown in percentage.

ZIKV Cut-off determination	ZIKV Positives (n)	ZIKV Negatives (n)	ZIKV Prevalence (%)
Cut off at 0.6	292	709	29.17
Cut off at 0.7	327	674	32.67
Cut off at 0.87	360	641	35.96
Cut off at 1.0	395	606	39.46

No statistical difference was observed for the prevalence of ZIKV and CHIKV among individual months during sample collection. (Figure 5.5). The proportion of CHIKV positive results was lower in December 2016 (10 positive subjects, non-significant) while the lowest proportion for ZIKV positive results was in June 2016 (45 positive subjects). I compared the number of ZIKV and CHIKV positives with the number of notified arboviral cases in the metropolitan region of Rio de Janeiro during the same period with no significant difference identified (Figure 5.5).

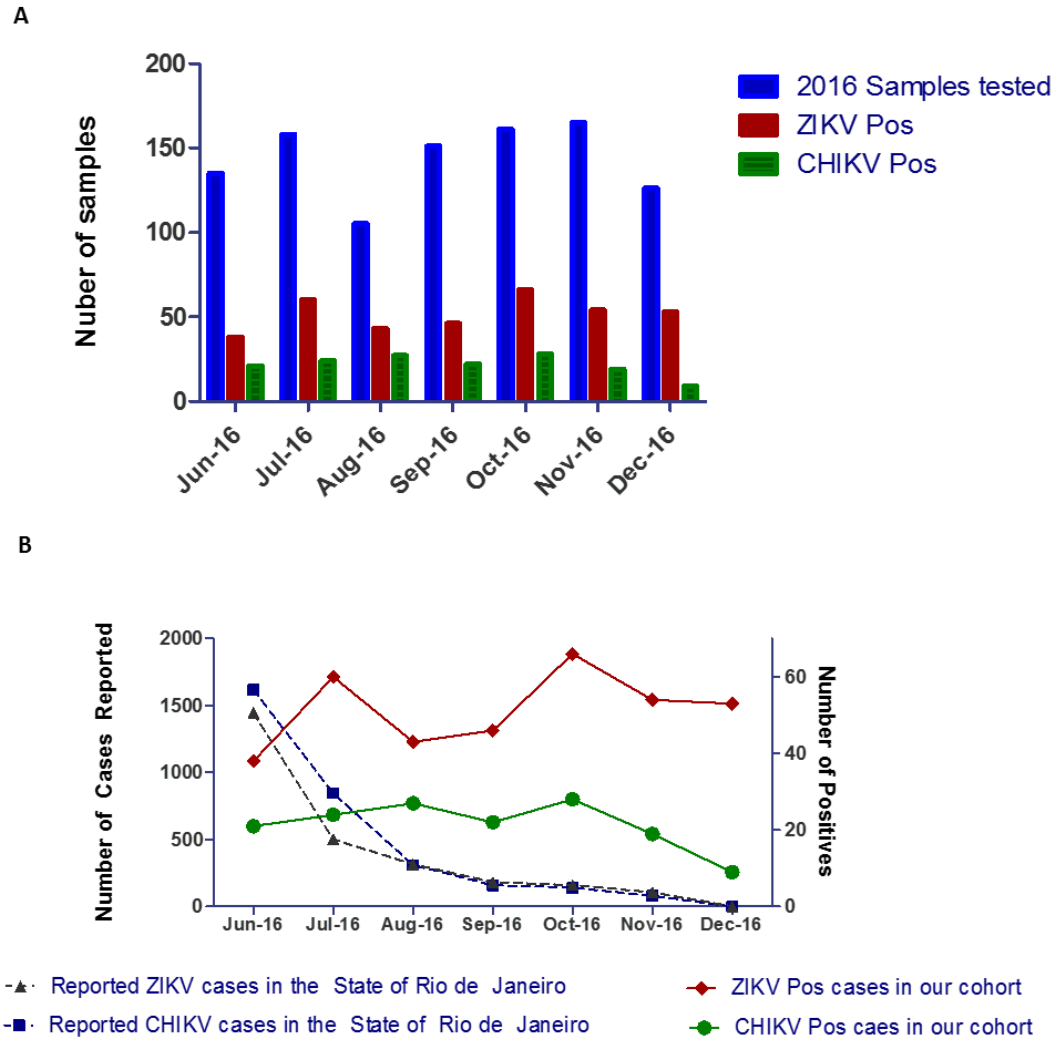

Figure 5.5 Impact on the ZIKV and CHIKV prevalence compared with the month of sample collection
A) Positive results for ZIKV and CHIKV obtained from samples collected from Jun-2016 until Dec 2016 (n=1001) compared with the total samples collected each month. Red bar for ZIKV positives. Green bar for CHIKV positives. B) ZIKV and CHIKV prevalence in our population compared with the overall number of cases reported to the Health Secretariat in the metropolitan region of Rio de Janeiro (confirmed and suspected) (shown as blue and grey dotted lines for CHIKV and ZIKV respectively).

5.5.5 Geographical distribution of ZIKV prevalence

No significant differences were identified in the geographical distribution of study subjects and spread of ZIKV positive cases (Figure 5.6). Within the city, a higher concentration of negative cases was identified in the South Zone of Rio de Janeiro.

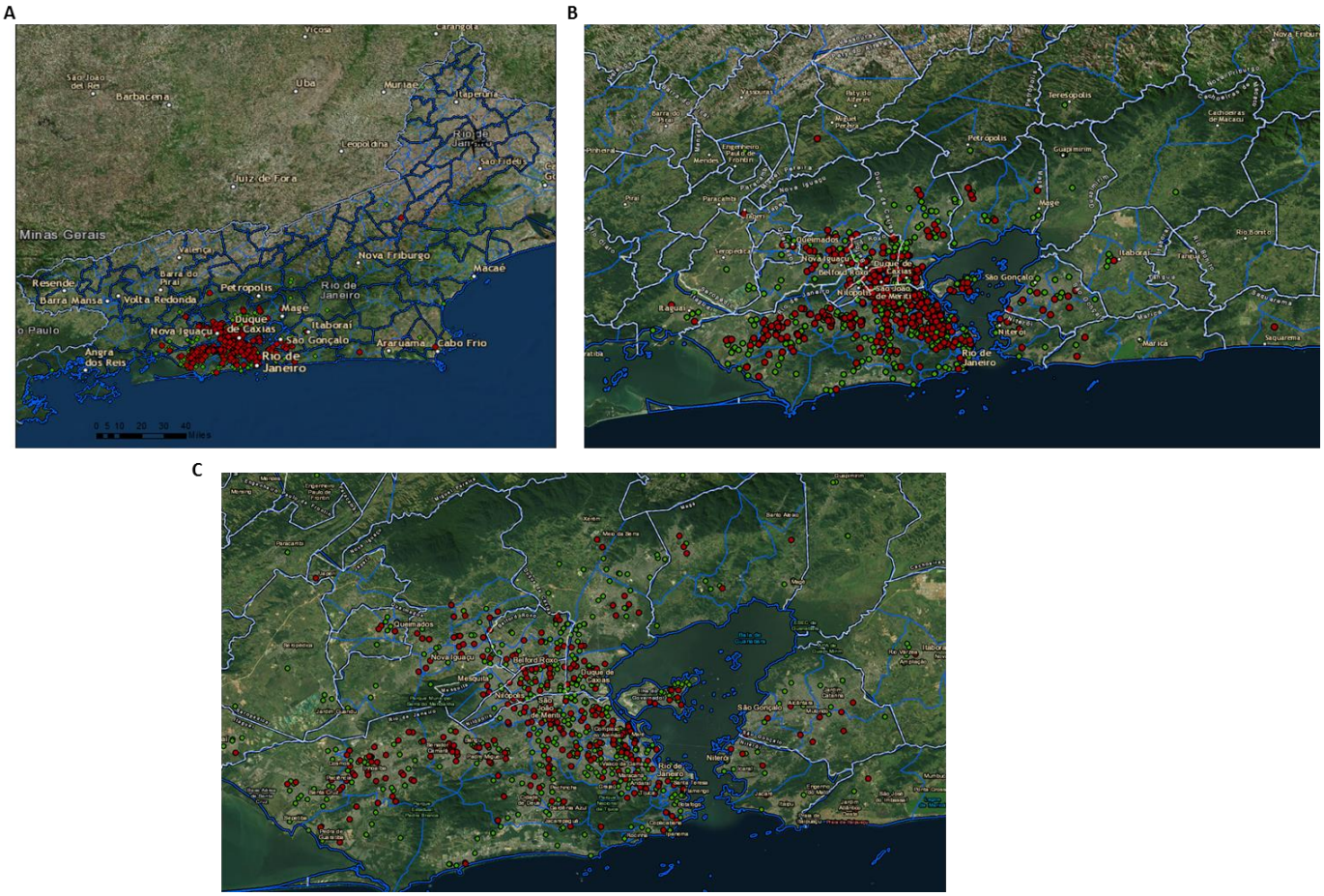

Figure 5.6 Map of showing geographical distribution of ZIKV cases in the cross-sectional cohort

Distribution of total samples tested (1001) in A) State of Rio de Janeiro. B) Municipality of Rio de Janeiro and neighbouring municipalities. C) Municipality of Rio de Janeiro.
Red Circles = Subjects positive for IgG ZIKV. Green Circles= Subjects negative for IgG ZIKV. Scale bar indicates miles. The exact address was unknown for 1 subject from the municipality of Rio de Janeiro.

5.5.6 Reporting of ZIKV and CHIKV cases in the State of Rio de Janeiro

The Health Secretariat of Rio de Janeiro started reporting cases of ZIKV in January 2016. Clinically suspected cases become a notifiable disease from November 2015. Knowing that in the State of Rio de Janeiro during 2015 and 2016 there were 15,324 ZIKV and 67,727 CHIKV notified cases, it is possible to estimate that only around 0.25% and 2.70% of subjects infected with ZIKV and CHIKV respectively were reported to the health surveillance system.

5.5.7 Demographic patterns of ZIKV and CHIKV spread

I aimed to further investigate the demographic characteristics and potential risk factors for ZIKV and CHIKV infections. The seroprevalence for the different populations regarding sex, age, ethnicity and other clinical features can be found in Table 5.4.

5.5.7.1 Variation of ZIKV and CHIKV prevalence with ethnicity

The following self-defined ethnic groups and categorized according to the IGBE census categories included in the study [black, brown, white, others (indigenous and Asian) and unknown] are shown in Table 5.4 and Figure 5.7.

ZIKV prevalence was higher among the population self-defined as brown or mixed race compared to the other ethnic groups (p-value=0.052, Odds Ratio (OR)=1.29). The prevalence of ZIKV among the white population was of 34.29% compared to 40.66% and 42.65% for the black and brown ethnic populations respectively. I identified a significantly higher IgG ZIKV ratio in the white group compared with black and brown (p-value of 0.031 and 0.008 respectively). As explained above, the higher IgG ZIKV ratios reflect less IgG ZIKV antibodies in serum and therefore, represent a larger proportion of negative ZIKV cases.

CHIKV prevalence was higher among the ethnic group black compared with the other ethnic groups (non-significant). The prevalence of CHIKV was higher among black individuals with 18.3% compared to 15.8% in the brown/mixed ethnic group and 12.4% of the white population. There was no significant difference in the IgG CHIKV ratio among the different ethnic populations.

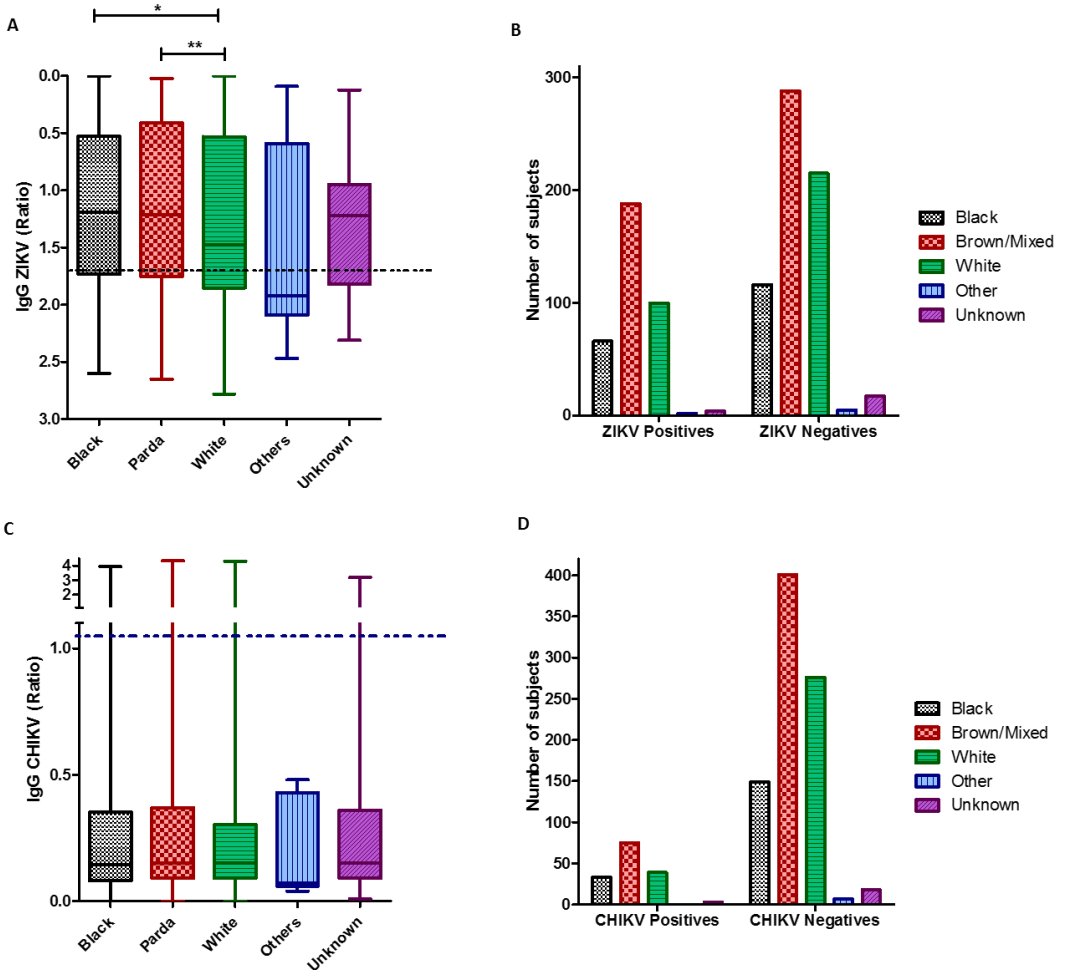

Figure 5.7 Ethnic differences in the seroprevalence of ZIKV and CHIKV
ZIKV and CHIKV antibody ratios and number of positives according to each ethnic group for the study population (n=1001) including black, brown, white, others and unknown. A) IgG ZIKV ratios for each ethnic group and B) number of ZIKV positives and negatives found among each group. C) IgG CHIKV ratios for each ethnic group. D) Number of CHIKV positives and negatives found among each group. Blue dotted line represents cut off for each assay and results above the line are positive. Boxplots show medians, 25th and 75th percentiles (box length) and min and max values (whiskers).

5.5.8 Variation of ZIKV and CHIKV prevalence with Human Development Index (HDI)

We matched the location of residence of our study population to the HDI correspondent and investigated. We observed that the subjects with very low and low HDI status had a higher prevalence of 42.86% and 48.25% respectively than any other population group of ZIKV compared with subjects with highest HDI Table 5.6. The lowest seroprevalence was found among subjects with very high HDI with a prevalence of 26.67% of ZIKV infection. The prevalence of ZIKV infection was significantly lower in the group with high HDI compared to the very low, low and medium HDI group (FE. P-value=<0.001, OR 2.16).

Similar patterns were found regarding CHIKV prevalence. The highest prevalence was found among subjects with very low HDI (20.2%) compared with just 6.6% among the group with very high HDI.

CHIKV prevalence was significantly lower among the group with very high HDI compared to the very low, low and medium HDI group (FE. P-value=0.008, OR 2.77). When compared the group with very low HDI and very high HDI the prevalence was significantly increased for the lowest socioeconomic group (p-value=0.008, OR 3.55)

Table 5.6 Variation of ZIKV and CHIKV prevalence with Human Development Index (HDI)
Socioeconomic status (SES) is measured in Human Development Index (HDI) from Very Low to Very High.

	Zika Virus				Chikungunya Virus			
Characteristics	Positives (n)	Prevalence (%)	Prevalence (95% CI)	Ratio (Median)	Positives (n)	Prevalence (%)	Prevalence (95% CI)	Antibody titre (Median)
Total	395	39.46	36.4-42.6	1.3	150	14.99	12.9-17.33	0.15
SES								
Very Low HDI	36	42.86	32.11-54.13	1.16	17	20.24	12.25-30.41	0.16
Low HDI	124	48.25	42-54.54	1.07	45	17.51	13.07-22.72	0.16
Medium HDI	119	40.61	34.94-46.48	1.34	43	14.68	10.83-19.25	0.15
High HDI	88	33.72	28-39.8	1.43	38	14.56	10.51-19.43	0.15
Very High HDI	28	26.67	18.51-36.19	1.59	7	6.67	2.72-13.25	0.11

I also found that the very low, low and medium HDI populations had significantly lower IgG ZIKV ratios (and therefore higher levels of ZIKV antibodies) than the population group with very high HDI with p-values of 0.0056, 0.0002 and 0.072 respectively (Figure 5.8). I also observed significantly increased levels when comparing the low HDI group and high HDI group (p-value =0.0189).

Similar patterns were observed when investigating the CHIKV prevalence (Figure 5.8B). All the population groups had significantly higher CHIKV antibody titres than the group with very high HDI with p-values of 0.041, 0.0013, 0.0073 and 0.0448 for the very low, low, medium and high HDI group. In Figure 5.8C we have shown how the exposure to ZIKV and CHIKV decreases remarkably with lower HDI status.

To better understand the low seroprevalence observed for ZIKV and CHIKV, I further investigated the group with very high HDI. The geographic distribution of subjects from the group with very high HDI is shown in Figure 5.9. I observed that all the cases except one from this group were located within the municipality of Rio de Janeiro. ZIKV exposed individuals, resident in the city of Rio de Janeiro, were mainly located in the South Zone of the city in

neighbourhoods such as Botafogo, Flamengo, Copacabana, Ipanema, which are the richest areas of the city.

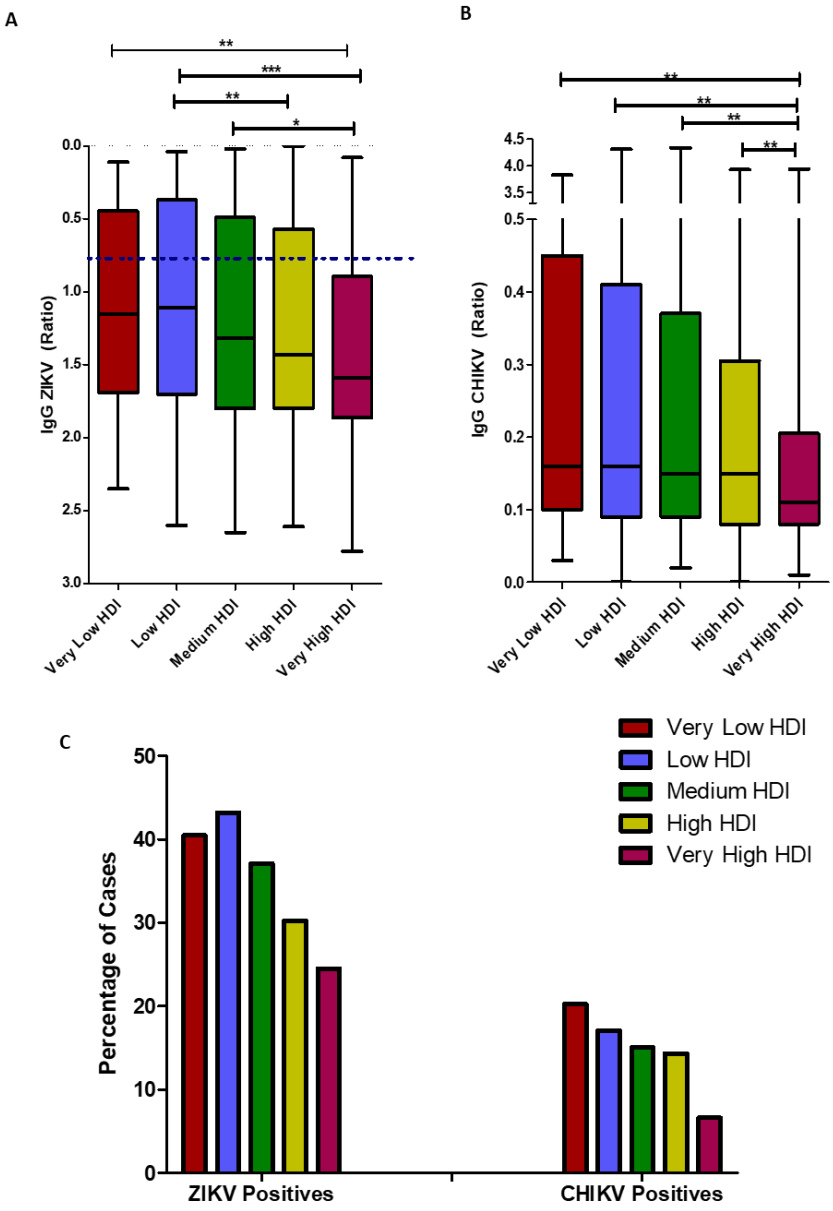

Figure 5.8 Variation of ZIKV and CHIKV prevalence with human development index (HDI)
Association of HDI group and ZIKV and CHIKV status. A) IgG ZIKV ratio observed for each of the HDI population groups. B) IgG CHIKV ratio observed for each of the HDI population groups. C) Prevalence of ZIKV and CHIKV among each population group.
Blue dotted line represents cut off. Points above that line are positive.*=p-value<0.05; **-p-value<0.005; ***=p-value<0.0005. Boxplots show medians, 25th and 75th percentiles (box length) and min and max values (whiskers).

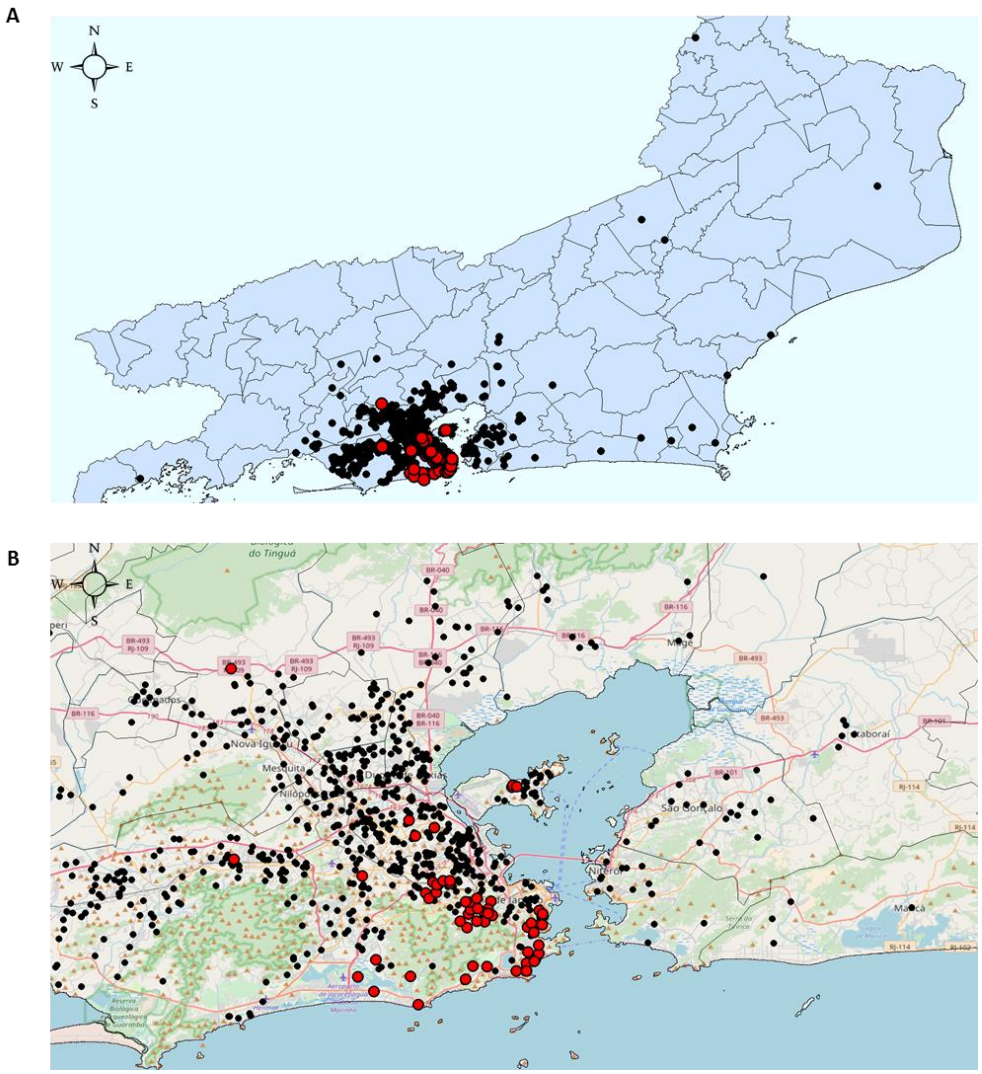

Figure 5.9 Map of Rio de Janeiro showing geographical distribution of subjects with very high HDI status

Maps showing the geographical distribution of subjects belonging to the very high HDI population group. This map does NOT show positive and negative infected cases. Red circles represent subjects with very high HDI (n=106). Black circles represent subjects from the other population groups: very low HDI, low HDI, medium HDI and high HDI. A) Map of the State of Rio de Janeiro. B) Map of the municipality of Rio de Janeiro. Maps generated with ARC GIS using GADM baselines.

5.5.9 Variation of ZIKV and CHIKV prevalence and socioeconomic status

I initially conducted a Shapiro-Wilk normality test for each socioeconomic indicator studied (defined in Chapter 2). All of the indicators studied did not follow a normal distribution (p value<0.00001). ZIKV positive subjects showed that they represented significantly lower socioeconomic status in 20/22 key representative indicators investigated

In Figure 5.10, I showed that low SES measured with HDI, income index, longevity index and education index were significantly associated with higher prevalence of ZIKV with p-values of <0.0001, 0.0002, <0.0001 and <0.0001 respectively.

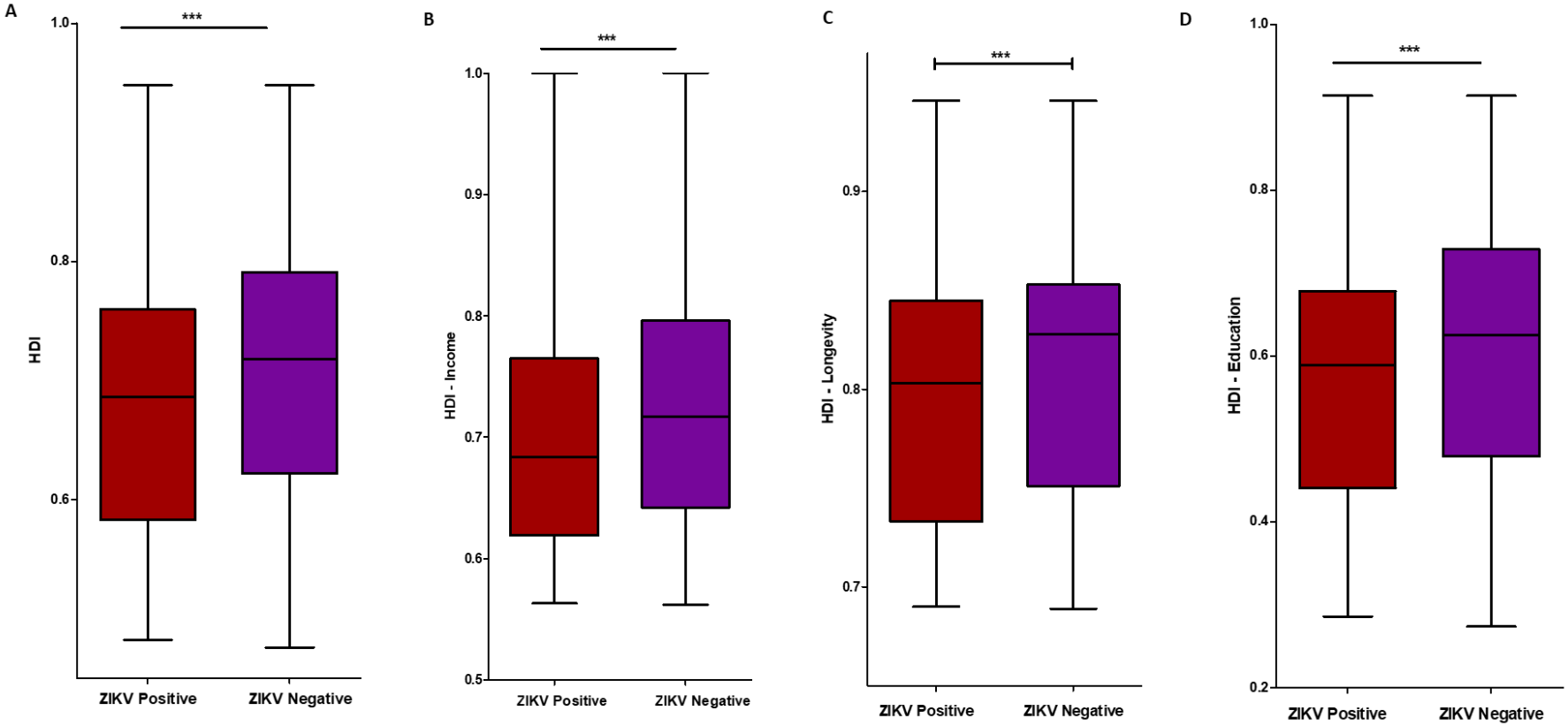

Figure 5.10 Association of HDI index of socioeconomic status and ZIKV exposure.
Sociodemographic indicators differ significantly between ZIKV exposed and unexposed subjects. A) Human Development Index (HDI). B) Human Development Index – Income index; C) HDI- Index of longevity; D) HDI-Index of Education. Boxplots show medians, 25th and 75th percentiles (box length) and min and max values (whiskers). Red plots: ZIKV Positive groups. Purple plots: ZIKV Negative groups. ***=p-value<0.001

5.5.10 Association between the prevalence of ZIKV and CHIKV

I wanted to explore further the relationship between ZIKV and CHIKV prevalence in the study population given they are spread through the same vector. There was a significantly increased proportion of subjects with CHIKV IgG antibodies and also ZIKV IgG antibodies compared to non-ZIKV positive subjects (FE. P-value=<0.001, OR=2.04). A total of 81 subjects were positive both for IgG ZIKV and IgG CHIKV which is 54.0% of all CHIKV infected subjects and 20.50% of all ZIKV infected subjects. A significantly higher prevalence of CHIKV in ZIKV-exposed individuals was identified (p-value <0.05). I identified a higher proportion of females exposed to both ZIKV and CHIKV (8.86%) than males (7.3%). Gender distribution among the ZIKV only, CHIKV only and no exposure groups were equally distributed.

I identified a higher proportion of black subjects among the population group exposed to both ZIKV and CHIKV with 10.99% of black individuals being exposed to both. A high proportion of brown subjects positive for ZIKV only (34.24%) was identified, while white, others and unknown populations had the highest proportions of subjects ZIKV and CHIKV negative with 58.41%, 71.43% and 71.43% respectively.

5.5.11 Variation of ZIKV and CHIKV prevalence with geographic distribution

The variation of ZIKV and CHIKV prevalence with geographic region is shown in

Table 5.7, Table 6.9 and *Table* 5.9 for the different mesoregions in the State of Rio de Janeiro, microregions and areas within the city of Rio respectively.

I showed that ZIKV and CHIKV prevalence varied geographically and clustered in the urban area of Rio de Janeiro. The prevalence of ZIKV was higher in the metropolitan area of Rio de Janeiro (39.65%) than outside the metropolitan area (29.17%). The municipalities with the highest prevalence were Queimados (5/11, 45.4%), Mesquita (5/9, 55.6%), Nova Iguazu (22/46, 47.83%) and Sao Joao de Mereti (29/57, 50.88%). Within the city of Rio de Janeiro, the exposure was over double in the South Zone (6/31, 19.4%) (which includes Copacabana and Ipanema) when compared to the West Area (85/209, 40.67%).

The prevalence of CHIKV outside the mesoregion of Rio de Janeiro (n=24) was lower (4.17%) than in the rest of the population (14.9%). The municipalities with the highest prevalence of CHIKV infection were Nilópolis (40.0%), Guapirim (33.3%) and Nova Iguacu (30.43%) compared with an overall average prevalence of 14.99%. Within the city of Rio de Janeiro, the South Zone is the region with the lowest prevalence of CHIKV 0/31 (0%).

Table 5.7 Summary prevalence of ZIKV and CHIKV across the different Mesoregions

Mesoregion	Municipality	Number of subjects	Percentage (%)	ZIKV Positives (n)	ZIKV Prevalence (%)	CHIKV Positives (n)	CHIKV Prevalence (%)
State of Rio de Janeiro	**All**	**1001**		395	39.46	150	14.99
Baixadas Litoraneas (Costal mesoregion)	**Araruama**	5	1.34	1	20.00	0	0
	Cassemiro De Abreu	3	0.8	0	0.00	0	0
	Iguaba Grande	3	0.8	1	33.33	0	0
	Rio Das Ostras	1	0.27	1	100.00	0	0
	Sao Pedro Da Aldeia	2	0.53	0	0.00	1	50
	Saquarema	3	0.8	2	66.67	0	0
Centre Fluminense	**Cantagalo**	1	0.27	0	0.00	0	0
	Macuco	2	0.53	1	50.00	0	0
Northeast Fluminense	**Macae**	2	0.53	1	50.00	0	0
North Fluminense	**Campo Dos Goytacazes**	1	0.27	0	0.00	0	0
South Fluminense	**Angra Dos Reis**	1	0.27	0	0.00	0	0
Total Outside Metropolitan Area of Rio de Janeiro		**24**	**2.4**	**7**	**29.17**	**1**	**4.17**
Metropolitan Area of Rio de Janeiro	**Rio de Janeiro**	627	62.64	244	38.92	86	13.72
	Belford Roxo	34	9.09	15	44.12	3	8.82
	Cachoeira De Macacu	1	0.27	0	0.00	0	0
	Duque De Caxias	96	25.67	30	31.25	18	18.75
	Engenho Paulo Frontin	1	0.27	0	0.00	0	0
	Guapimirim	3	0.8	0	0.00	1	33.33
	Itaborai	6	1.6	3	50.00	0	0
	Itaguai	9	2.41	4	44.44	0	0
	Japeri	2	0.53	1	50.00	0	0
	Magé	17	4.55	7	41.18	0	0
	Marica	5	1.34	3	60.00	0	0
	Mesquita	9	2.41	5	55.56	3	33.33
	Miguel Pereira	2	0.53	1	50.00	0	0
	Nilópolis	5	1.34	1	20.00	2	40
	Niteroi	15	4.01	7	46.67	1	6.67
	Nova Iguaçu	46	12.3	22	47.83	14	30.43
	Paracambi	1	0.27	0	0.00	0	0

	Petropolis	1	0.27	0	0.00	0	0
	Queimados	11	2.94	5	45.45	3	27.27
	São Gonçalo	20	5.35	9	45.00	1	5
	São João De Meriti	57	15.24	29	50.88	15	26.32
	Seropédica	6	1.6	1	16.67	1	16.67
	Teresopolis	2	0.53	0	0.00	1	50
Total Metropolitan Area of Rio de Janeiro		**976**	**97.5**	**387**	39.65	**149**	**15.27**

Table 5.8. Prevalence of ZIKV and DENV in the different microregions within the metropolitan area of Rio de Janeiro

	Municipality/Region	Number of subjects (n)	Percentage (%)	ZIKV Positives (n)	ZIKV Prevalence % (CI)	CHIKV Positives (n)	CHIKV Prevalence % (CI)
State of Rio de Janeiro	All	1001		395	39.46(36.4-42.6)	150	14.99(12.8-17.3)
Total Outside Metropolitan Area of Rio de Janeiro		24	2.4	7	29.17(12.6-51.1)	1	4.17(0.1-21.1)
Total Metropolitan Area of Rio de Janeiro		976	97.5	388	39.75(36.7-42.9)	149	15.27(13.1-17.7)
Metropolitan Area of Rio de Janeiro - (Not Microregion of Rio de Janeiro)	Itaguai	9	2.41	4	44.44(13.7-78.8)	0	0(0-33.6)
	Seropédica	6	1.6	1	16.67(0.4-64.1)	1	16.67(0.4-64.1)
	Cachoeira De Macacu	1	0.27	0	0(0-97.5)	0	0(0-97.5)
	Petropolis	1	0.27	0	0(0-97.5)	0	0(0-97.5)
	Teresopolis	2	0.53	0	0(0-84.2)	1	0(0-97.5)
	Engenheiro Paulo de Frontin	1	0.27	0	0(0-97.5)	0	0(0-97.5)
	Miguel Pereira	2	0.53	1	50(1.3-98.7)	0	0(0-84.2)
	Paracambi	1	0.27	0	0(0-97.5)	0	0(0-97.5)
	Queimados	11	2.94	5	45.45(16.7-76.6)	3	27.27(6-61)
	São Gonçalo	20	5.35	9	45(23.1-68.5)	1	5(0.1-24.9)
Metropolitan Area of Rio de Janeiro - Microregion of Rio de Janeiro	Belford Roxo	34	9.09	15	44.12(27.2-62.1)	3	8.82(1.9-23.7)
	Duque De Caxias	96	25.67	30	31.25(22.2-41.5)	18	18.75(11.5-28)
	Guapimirim	3	0.8	0	0(0-70.8)	1	33.33(0.8-90.6)
	Itaborai	6	1.6	3	50(11.8-88.2)	0	0(0-45.9)
	Japeri	2	0.53	1	50(1.3-98.7)	0	0(0-84.2)
	Magé	17	4.55	7	41.18(18.4-67.1)	0	0(0-19.5)
	Marica	5	1.34	3	60(14.7-94.7)	0	0(0-52.2)
	Mesquita	9	2.41	5	55.56(21.2-86.3)	3	33.33(7.5-70.1)
	Nilópolis	5	1.34	1	20(0.5-71.6)	2	40(5.3-85.3)
	Niteroi	15	4.01	7	46.67(21.3-73.4)	1	6.67(0.2-31.9)
	Nova Iguaçu	46	12.3	22	47.83(32.9-63.1)	14	30.43(17.7-45.8)
	São João De Meriti	57	15.24	30	52.63(39-66)	15	26.32(15.5-39.7)
Metropolitan Area of Rio de Janeiro - Municipality of Rio de Janeiro	**All Rio de Janeiro**	627	62.64	244	38.92(35.1-42.9)	86	13.72(11.1-16.7)
	Central	51	8.13	18	35.29(22.4-49.9)	8	15.69(7-28.6)
	South	31	4.94	6	19.35(7.5-37.5)	0	0(0-11.2)
	West	209	33.33	93	44.5(37.6-51.5)	28	13.4(9.1-18.8)
	North	335	53.43	127	37.91(32.7-43.3)	50	14.93(11.3-19.2)

Table 5.9 Prevalence of ZIKV and DENV in the different areas within the city of Rio de Janeiro

Distribution in the city of Rio de Janeiro (Metropolitan Area of Rio de Janeiro, Municipality of Rio de Janeiro)

Area in the city of Rio de Janeiro	Number of subjects	Percentage (%)	ZIKV positive(n)	ZIKV Prevalence (%, CI)	CHIKV positive (n)	CHIKV Prevalence (%,CI)
All	**627**	**62.64**	**223**	**35.57(31.8-39.5)**	**86**	**13.72(11.1-16.7)**
Central	51	8.13	14	27.45(15.9-41.7)	8	15.69(7-28.6)
South	31	4.94	6	19.35(7.5-37.5)	0	0(0-11.2)
West	209	33.33	85	40.67(33.9-47.7)	28	13.4(9.1-18.8)
North	335	53.43	118	35.22(30.1-40.6)	50	14.93(11.3-19.2)

Different geographical spread of ZIKV and CHIKV was displayed in Figure 5.11. As shown, a majority of the positive ZIKV cases are concentrated in the North and West areas of Rio de Janeiro. The spread of ZIKV is also increased across the other regions outside the city of Rio de Janeiro.

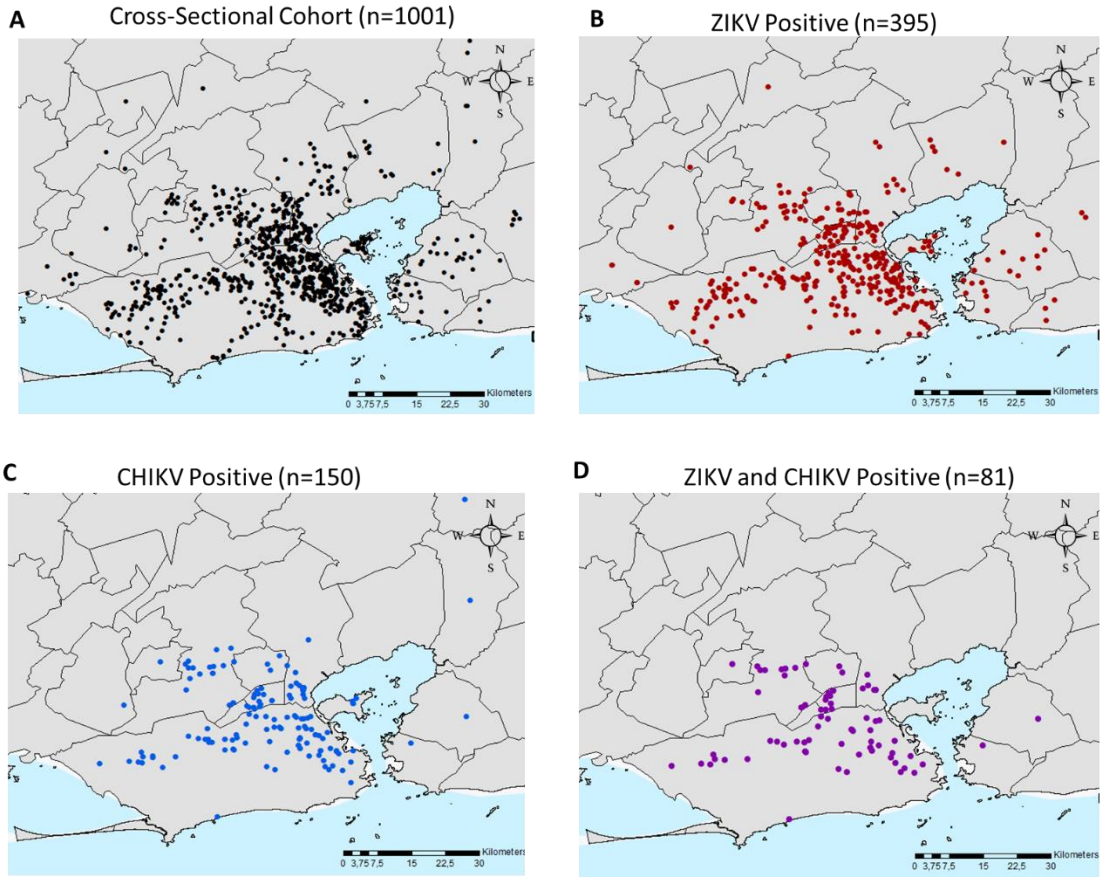

Figure 5.11 Spatial distribution of cross-sectional cohort and exposure to ZIKV and CHIKV
Maps showing geographical spread of cases across the metropolitan region of Rio de Janeiro. A) Spread of ZIKV and CHIKV cases in the cross-sectional cohort (n=1001). A) Geographical distribution of the cases; Location of each case shown as a black circle; B) Geographical distribution of ZIKV positives (n=381). Residence of each ZIKV case shown as a purple circle; C) Geographical spread of CHIKV positives (n=150). Residence of each case shown as a red circle; D) Geographical distribution of ZIKV positives, CHIKV positives and subjects negatives for both.

5.5.12 The longitudinal Cohort: Characteristics of the study population

The longitudinal seroprevalence study is comprised of a total of 121 Individuals from the metropolitan region of Rio de Janeiro studied retrospectively during four years (Brazil). Individuals were residents from different regions within the metropolitan region of Rio de Janeiro and most of them were based in the metropolitan area of Rio de Janeiro. I studied a total of 415 serum specimens collected in 2013 (n=52), 2014 (n=121), June to December 2015 (n=121) and June to December 2016 (n=121). The main demographic characteristics are described in Table 5.10. All the subjects were >21 with a median age of 55 y (IQR=45-64). The study population were 55.37% female (67/121). The main ethnicities present in the Brazilian population were represented with 53.72% (65/121) brown, 28.93% (35/121) white and 15.70% (19/121) black.

Table 5.10. Demographic characteristics of the longitudinal study population
Summary of key demographic characteristics of the longitudinal cohort (n=121). Study subjects from the metropolitan region of Rio de Janeiro. Key characteristics defined: gender, age, ethnicity and other clinical descriptions. Samples were collected from 1st June 2016 – 31st Dec 2016. Other ethnicity groups include: yellow and indigenous.

Characteristics	Longitudinal Cohort (n)	Longitudinal Cohort (%)
Total	121	
Female	67	55.37
Male	54	44.63
Age		
Total (Median, IQR)	55 (45.0-64.0)	
0-20	0	0.00
>21-40	22	18.18
>41-60	57	47.11
>60	42	34.71
Ethnicity		
Black	19	15.70
Brown/Mixed	65	53.72
White	35	28.93
Other	0	0.00
Unknown	2	1.65
Human Development Index (HDI) *		
HDI (Median - IQR)*	0.609 (0.49-0.638)	0.609 (0.49-0.638)
HDI-Very high (0.85-1)	12	9.92
HDI - High (0.75-0.85)	98	80.99
HDI- Medium (0.650-0.75)	11	9.09
HDI Low (0.550-0.650)	0	
HDI Very Low (0.2-0.550)	0	

5.5.13 Geographic characteristics and spread of the longitudinal study population

The study population lived in the metropolitan region of Rio de Janeiro in 13 different municipalities within the State of Rio de Janeiro (Table 5.11). Eighty nine subjects were residents of Rio de Janeiro with the majority [49/121 (40.5%)] coming from the North area.

Table 5.11 Area of residence of the study population in the longitudinal cohort (n=121)

	Geographical Area	Number of subjects	Percentage (%)
	Residing in the State of Rio de Janeiro	**121**	
	Residing Municipality outside the city of Rio de Janeiro	**32**	**26.45**
Municipality outside Rio de Janeiro	Belford Roxo	7	**5.79**
	Duque De Caxias	7	**5.79**
	Magé	2	**1.65**
	Mesquita	1	**0.83**
	Miguel Pereira	1	**0.83**
	Nilópolis	1	**0.83**
	Niteroi	1	**0.83**
	Nova Iguaçu	4	**3.31**
	Queimados	2	**1.65**
	São Gonçalo	2	**1.65**
	São João De Meriti	2	**1.65**
	Seropédica	2	**1.65**
Municipality of Rio de Janeiro	**Residing in the municipality of Rio de Janeiro**	**89**	**73.55**
	Central	7	7.87
	South	0	
	West	33	27.27
	North	49	40.50

The exact geographic spread of the study population in the longitudinal cohort is shown in Figure 5.12.

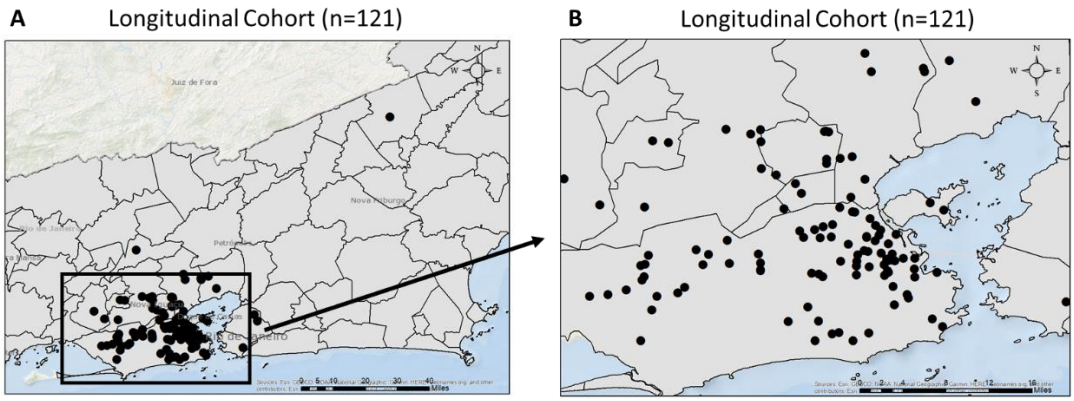

Figure 5.12 Geographical distribution of longitudinal cohort (n=121)
Geographical distribution of study subjects in the longitudinal cohort. A) Map of the State of Rio de Janeiro; B) Map of the metropolitan area of Rio de Janeiro.

5.5.14 Seroprevalence of ZIKV and CHIKV in the metropolitan region of Rio de Janeiro between 2013 and 2016 – Longitudinal Study

The spread of ZIKV, CHIKV and DENV in Rio de Janeiro between 2013 and 2016 was analysed. The prevalence of ZIKV in the longitudinal population (n=121) was 13.46%, 11.57%, 25.62% and 36.36% in 2013, 2014, 2015 and 2016 respectively (Table 5.12 Figure 5.13). These results correlate with the two waves of the epidemic estimated in Rio de Janeiro and misreported as suspected DENV in 2015 as previously described Figure 5.1 [184].

The antibody prevalence for chikungunya virus in the longitudinal cohort (n=121) was 1.92%, 3.31%, 4.13% and 14.05% for 2013, 2014, 2015 and 2016 respectively.

The antibody prevalence of dengue virus in the longitudinal cohort was 90.38%, 86.78%, 92.56% and 94.21% for 2013, 2014, 2015 and 2016 respectively. As shown in Chapter 3, the dengue serological assays are not highly specific and some of this positive results may be actually false positive Zika cases (due to antibody cross-reactivity).

Table 5.12 Changes in prevalence of ZIKV, DENV and CHIKV (2013-2016)

Prevalence measured during 2013, 2014, 2015 and 2016. Positive and negative scores calculated as: Green BOB assay Ratio >1.0 (negative) to Green BOB assay Ratio < 1.0 (Positive). Change in prevalence calculated as a difference in the population prevalence.

	2013 (n=52)	Change 2013-14	2014 (n=121)	Change 2014-15	2015 (n=121)	Change 2015-16	2016 (n=121)
ZIKV negatives (n)	45		107		90		77
ZIKV positives (n)	7		14		31		44
ZIKV prevalence (%)	13.46	-1.89	11.57	14.05	25.62	10.74	36.36
CHIKV negative (n)	51		117		116		104
CHIKV positive (n)	1		4		5		17
CHIKV prevalence (%)	1.92	1.38	3.31	0.83	4.13	9.92	14.05
DENV negative (n)	5		16		9		7
DENV positive (n)	47		105		112		114
DENV prevalence (%)	90.38	-3.61	86.78	5.79	92.56	1.65	94.21

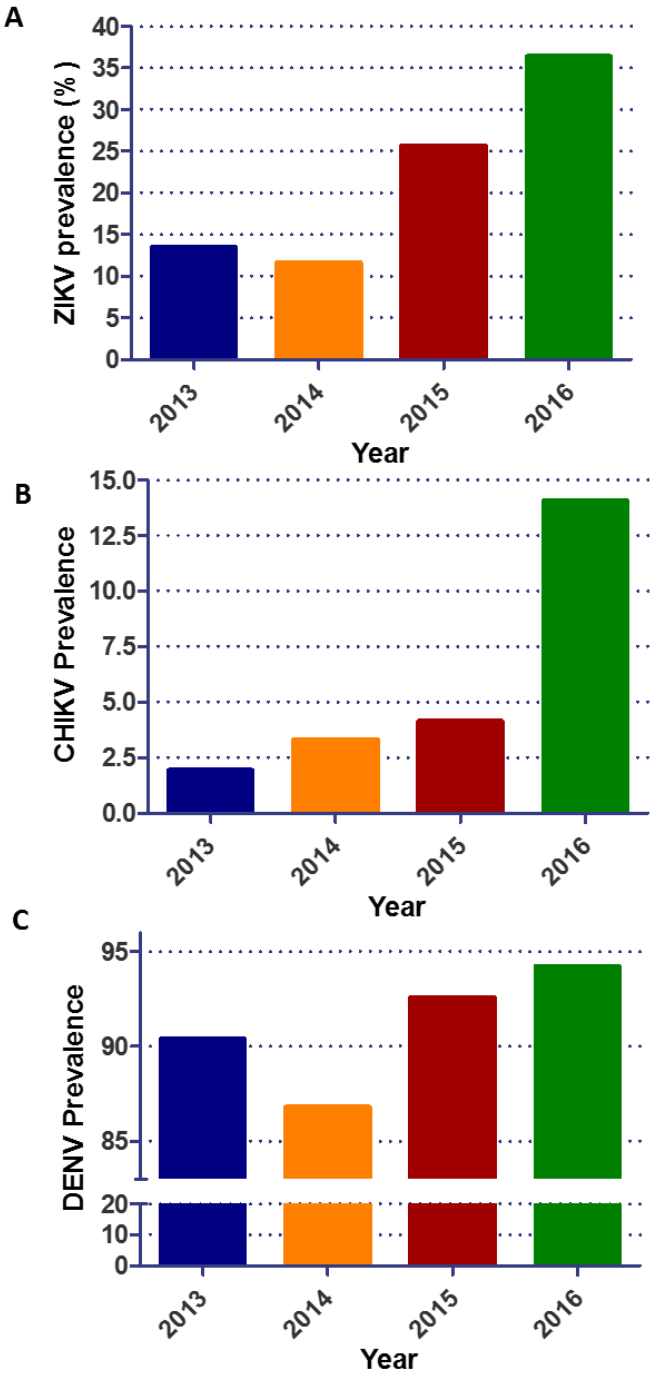

Figure 5.13 Changes in the prevalence of ZIKV, DENV and CHIKV in the longitudinal cohort
Represented prevalence for 2013 (n=52), 2014 (n=121), 2015 (n=121) and 2016 (n=121). Prevalence shown in %. A) ZIKV prevalence, B) CHIKV Prevalence, C) DENV Prevalence. Of note, Y axes in two sections to show differences between years. Column colours: Blue =2013, Orange = 2014, Red=2015 and Green = 2016. Note, Y axe varies for ZIKV, CHIKV and DENV in the figures A, B and C respectively.

5.5.15 Geographical spread of ZIKV and CHIKV in the longitudinal cohort

In order to investigate the changes in the spread of ZIKV and CHIKV across the population in Rio de Janeiro, I represented the geographical distribution of positive and negative cases in

the longitudinal study cohort (n=121) (Figure 5.14). A proportional distribution of positive and negative cases across the different regions of Rio de Janeiro was observed.

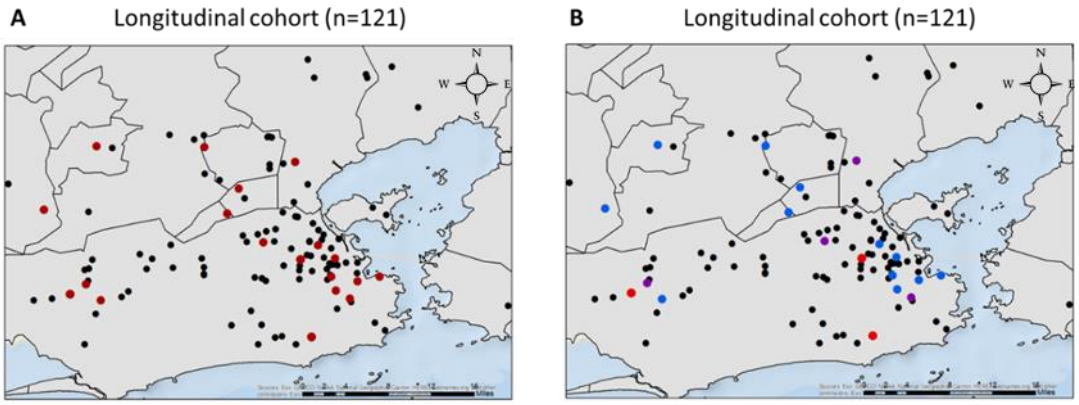

Figure 5.14 Geographical spread of ZIKV positive and negative cases in the longitudinal cohort
Maps showing the residence of study subjects in the longitudinal cohort (n=121) in the Metropolitan area of Rio de Janeiro: A) Positive ZIKV as red circles, negatives for CHIKV as black. B) Map showing the different ZIKV new cases each year: ZIKV positives in 2014 (purple circles), ZIKV positives in 2015 (red circles), ZIKV positives in 2016 (Blue circles), subjects consistently negative across 2013 to 2016 (Black circles).

In order to better understand the yearly spread of positive ZIKV cases across the city of Rio de Janeiro between 2013 and 2016 and particularly following the two epidemic waves, I investigated the areas where new positive cases appeared in 2015 and 2016 Figure 5.15. The results suggest that ZIKV may have spread around the urban areas in 2015 with an increase in the spread to areas further away from the centre of Rio de Janeiro after the second wave of the epidemic. Positive cases spread across different municipalities with ZIKV being detected in Belford Roxo (3), Duque de Caxias (3), Mesquita (1), Nilópolis (2), Nova Iguacu (2), Queimados (1), Sao Goncalo (2), São João de Mereti (1), Seropédica (1) and the municipality of Rio de Janeiro (29).

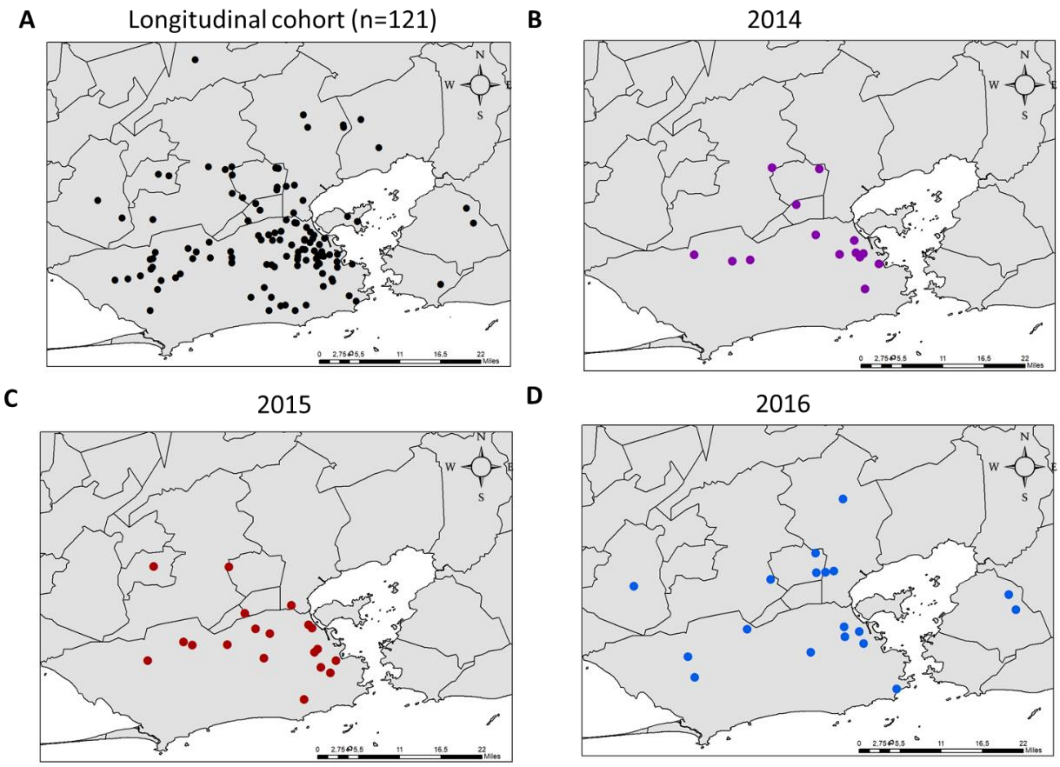

Figure 5.15 Geographical spread of Zika virus positive cases in Rio de Janeiro.
Maps of the Metropolitan Area of Rio de Janeiro showing: A) All cases represented (n=121) black circles; B) all positive ZIKV cases in 2014 (purple circles); C) New positive ZIKV cases in 2015 (red circles); D) New positive ZIKV cases in 2016 (Blue circles). C and D show uniquely patients that were newly positive.

New cases of CHIKV spread across different municipalities following the arbovirus wave at the beginning of 2016 identifying positive individuals across various municipalities within the metropolitan region of Rio de Janeiro including São João de Mereti (1), Queimados (1) Nova Iguacu (1), Seropédica (1) Nilópolis (1), Duque de Caxias (1) and the municipality of Rio de Janeiro (11). Among the positive cases in this municipality of Rio de Janeiro, 7/11 were from the North area, 2/11 from the central area and 2/11 from the West area (Figure 5.16).

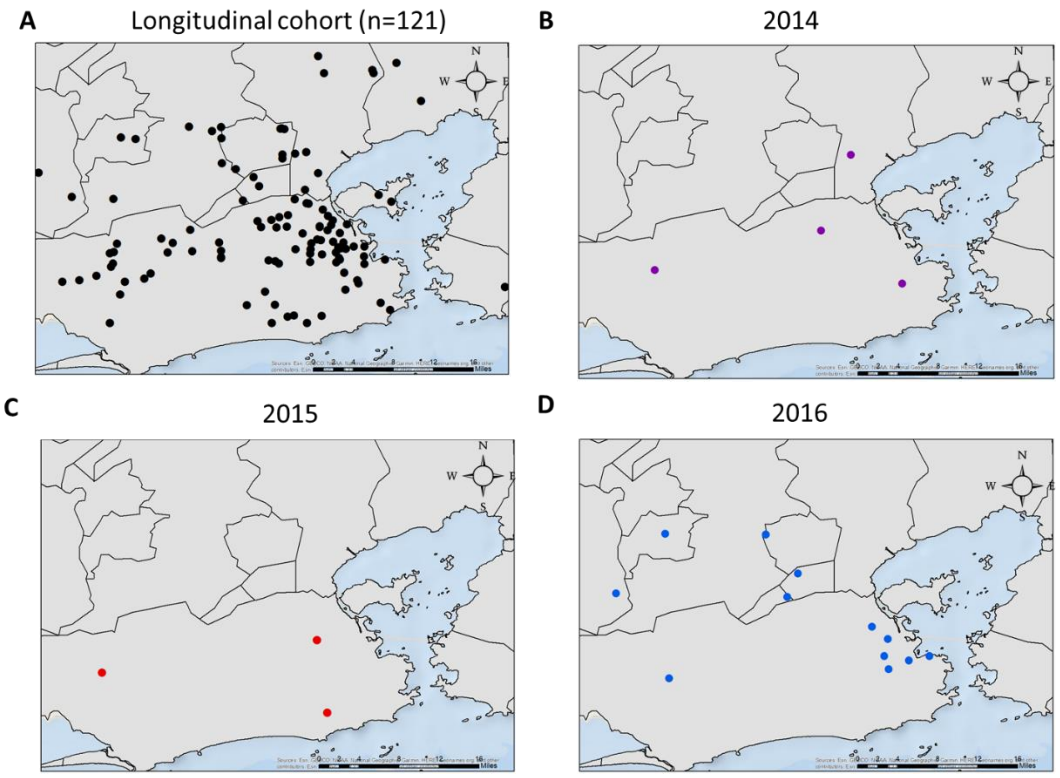

Figure 5.16 Geographical spread of chikungunya virus positive cases in Rio de Janeiro
Maps of the Metropolitan Area of Rio de Janeiro showing: A) All cases represented (n=121) black circles; B) all positive CHIKV cases in 2014 (purple circles); C) New positive CHIKV cases in 2015 (red circles); D) New positive CHIKV cases in 2016 (Blue circles). C and D show only cases that were newly positive.

5.5.16 Estimation of the prevalence of ZIKV in the longitudinal cohort by modifying the Zika assay cut-off

The investigation of the impact of modifying the Zika assay cut-off in the ZIKV prevalence estimated for 2013, 2014, 2015 and 2016 is shown in Table 5.13. In chapter 3, I established that the most appropriate cut-off for the Green BOG ZIKV assay was 1.0. I showed that modifying the assay cut-off had a major impact in the results showing that the range of ZIKV prevalence in 2015 may be between 13.2% and 25.6% while in 2016 it may vary between 24.0 and 36.4% (using an assay cut-off of <0.6 and <1.0 respectively).

In addition, I also compared the prevalence found in the longitudinal study population (n=121) with the different assay cut-offs compared with the prevalence values in the cross-sectional cohort (n=1001). I showed increased rates of positivity in the larger cross-sectional study population. The rates of change in prevalence also varied with the use of different cut-offs. Using the cut-off of 0.8 for the Green BOB assay, our results may suggest that both ZIKV epidemic waves in 2015 and 2016, respectively, may have had similar sizes with a rate of change in prevalence of exactly 11.6% for both seasons.

Table 5.13 ZIKV prevalence estimates with different cut-off values for the GREEN BOB assay
Estimation of the ZIKV prevalence by using different cut-off values for the Green BOB assay according to cut-off options discussed in Chapter 3. Highest assay sensitivity and specificity was shown to be at cut-off 1.0.

Longitudinal Cohort (Year)	Number of subjects (n)	Green BOB Cut-off <=0.6	Green BOB Cut-off <=0.8	Green BOB Cut-off <=1
2013	52	3.8% (2/52)	11.5% (6/52)	**14.5% (7/52)**
2014	121	5.0% (6/121)	8.3% (10/121)	**11.6% (14/121)**
2015	121	13.2% (16/121)	19.8% (24/121)	**25.6% (31/121)**
2016 (longitudinal cohort)	121	24.0% (29/121)	31.4% (38/121)	**36.4% (44/121)**
2016 (cross-sectional cohort)	1001	29.17% (292/1001)	34.7% (347/1001)	**39.8% (398/1001)**
Zika prevalence change rates				
Change 2013-14		1.10%	-3.20%	**-1.90%**
Change 2014-15		8.30%	11.60%	**14.00%**
Change 2015-16		10.80%	11.60%	**10.80%**

5.5.17 Investigation of the antibody dynamics for ZIKV, CHIKV and DENV

Then, I aimed to further investigate the prevalence of ZIKV, DENV and CHIKV during 2013/2014, 2014/2015 and 2015/2016. The antibody dynamics of ZIKV show high variation in the titres of annual sample collections and highlights the challenge of interpreting the ratios with a high number of results close to the cut-off value. The antibody changes in the ZIKV positive cases in 2013, 2014 and 2015 are shown in Figure 5.17. Among the 7/52 study subjects ZIKV positive in 2013, four of them remain positive throughout 2014, 2015 and 2016 whilst the other three had their ratios decreased in 2014 and remain negative throughout 2015 and 2016. All six cases had very high IgG dengue antibody titres in 2013 (ranging between 5.09 and 5.64). One of the cases with negative change in antibody titres, has also had a significant drop in the dengue antibody titres between 2013 and 2014. A total of eleven study subjects had a change in antibody titres to ZIKV positive in 2015. Nine out of eleven remained positive during 2015 and 2016, while two (8.2%) (out of 11) had the IgG Green BOB ratio dropped negative in 2015 and 2016. Finally, a total of 18 subjects seroconverted to ZIKV positive in 2015. Five (5/18) became ZIKV negative in 2016, however, four of them had ratios very close to the cut-off point (1.0-1.2) while 1/5 had very high Green BOB ratios (2.09) which correlates to very low levels of IgG ZIKV antibodies in the serum sample. IgG testing was repeated for those individuals for which false positive/negative results were suspected. A total of 18/121 subjects had a positive ZIKV diagnosis in 2016 while being negative during

2013, 2014 and 2015. These results highlight a high biological diversity of antibody responses.

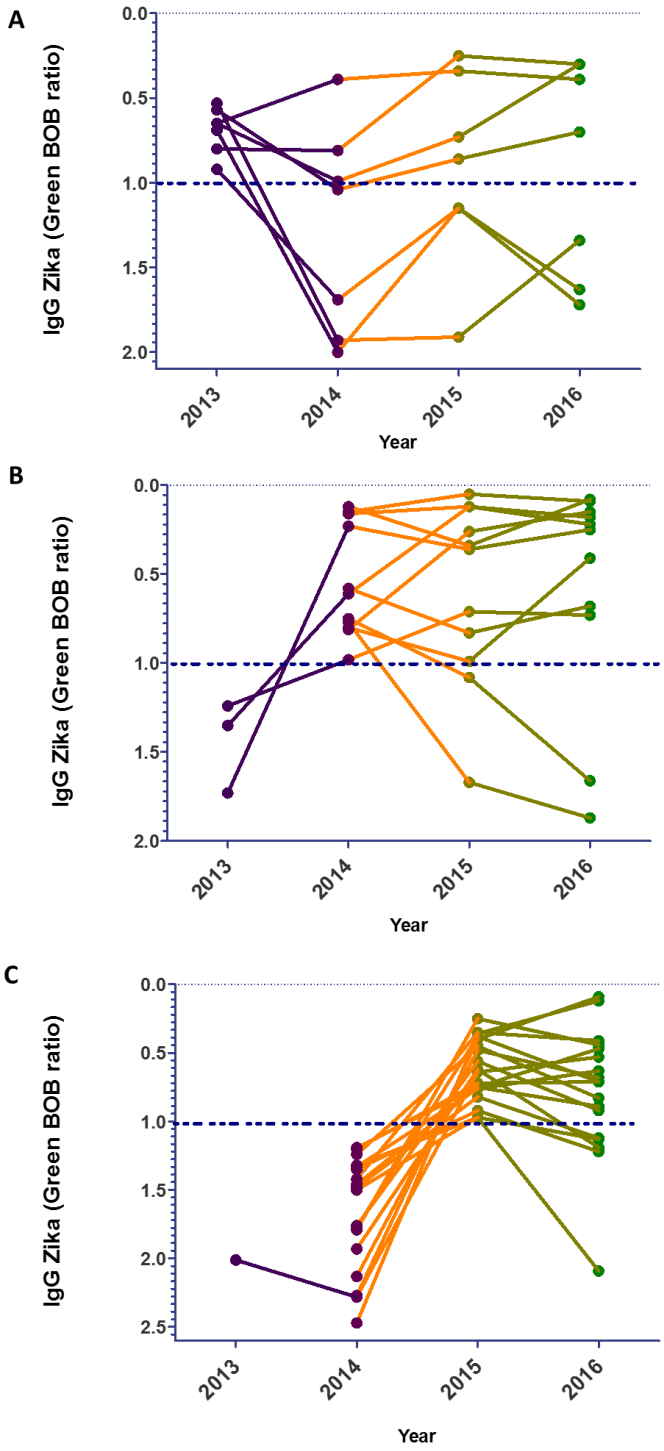

Figure 5.17 Investigation of the ZIKV positive cases in in 2013, 2014 and 2015
ZIKV positive cases in the longitudinal cohort. Antibody titre dynamics (measured in Green BOB Ratio) for the new ZIKV positive cases in: A) ZIKV Positive cases in 2013; B) ZIKV Positive cases in 2014 (and not positive in 2013); C) ZIKV positive cases in 2015 (and not positive previously).

The CHIKV antibody dynamics in the longitudinal cohort were investigated (Figure 5.18). I studied the antibody changes in the CHIKV positive cases in 2013, 2014 and 2015. Only one subject (1/52) showed IgG CHIKV positive ratio results in 2013, with decreased antibody levels in 2014 and increased during 2015 and 2016 (positive CHIKV detection). Another four subjects (4/121) showed positive IgG CHIKV levels in 2015. Another 2/121 subjects exhibited positive chikungunya antibody titres in 2015. One of them remained with similar IgG antibody titres during 2016 (IgG titres of 1.27 in 2015 and 2.225 in 2016), while the other had a drop off in antibody levels from 1.34 to 0.82. A total of 12/121 subjects had a positive CHIKV diagnosis in 2016 while being negative during 2013, 2014 and 2015. A better understanding of the dynamics of antibody responses following CHIKV infection would be needed to further interpret these results.

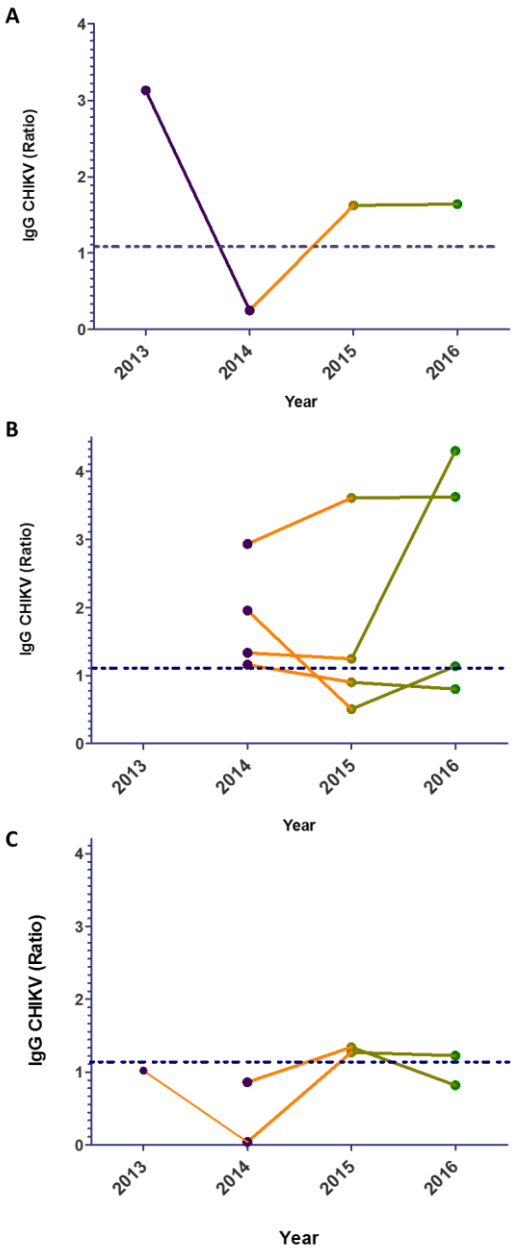

Figure 5.18 Antibody rates of the CHIKV positive cases in 2013, 2014 and 2015
Antibody titre dynamics (measured with the IgG CHIKV Euroimmun ELISA) for the positive cases in the longitudinal cohort (n=121): A) CHIKV positive cases in 2013; B) CHIKV positive cases in 2014 (and not positive in 2013); C) CHIKV positive cases in 2015 (not positive previously).

5.6 Discussion

Given the increasing spread of ZIKV, CHIKV and DENV in South America, understanding the geographic spread as well as the prevalence, socioeconomic impact of the outbreaks, and the burden of these arboviruses has become a crucial need in America, particularly in Brazil. Arboviral seroprevalence in Rio de Janeiro The "gold standard" method to measure the impact in the population is through serosurveys [111].

To the best of my knowledge, in this chapter I present the first serosurvey for Zika and chikungunya viruses in Rio de Janeiro, and the second study of this kind for Brazil.

5.6.1 Seroprevalence of ZIKV and CHIKV in Rio de Janeiro compared with other regions

I describe that the ZIKV infection rate in the metropolitan region of Rio de Janeiro was of 35.96% by the end of 2016. My findings contrast with seroprevalence rates found previously in other regions, such as the Yap Island (74.3%), French Polynesia (66%) or Salvador in Brazil (63.3%). Similar rates were found in Beni, a tropical area of Bolivia, with a seroprevalence of 39% [228].

I also demonstrate an infection rate of 14.99% for CHIKV in Rio de Janeiro. This is a much lower prevalence than reported rates in other regions in the Americas such as Guadalupe and Martinique which have an estimated CHIVK seroprevalence of 48.1% and 41.99% respectively. These CHIKV prevalence rates are similar to the reported infection rate found in a rural community in the Northeast of Brazil [243]. On the other side, these CHIKV prevalence levels are higher than reported by Netto *et al*. (2017) of 7% [236].

The potential factors contributing to this differential spread of Zika and Chikungunya viruses in Rio de Janeiro are still unclear. It could be linked with differences on the date and mode of spread of the viruses into Rio. Recent phylogenetic reconstructions together with molecular evidence suggest that ZIKV probably arrived in Rio de Janeiro in 2013 though, it was not until late 2015, when linked to an upsurge in clinical cases, it spread across the State and other regions of Brazil [224]. Thus, ZIKV arrived for the first time in Rio de Janeiro up to two years before than CHIKV (2013 and 2015 respectively).

The different prevalence may also be linked to differences in climate, environment or vector conditions as well as the capacity of the mosquitoes to transmit the strains of ZIKV and CHIKV circulating among the population in Rio de Janeiro. It has been shown that *Aedes* mosquitoes

can be co-infected by ZIKV and CHIKV and transmit both pathogens at the same time [249]. The rate of spread of ZIKV compared to CHIKV may be associated with viral properties that affect transmission [250]. Non-vector transmission routes may also play a role. There is now increasing evidence that sexual transmission of ZIKV was crucial during the ZIKV outbreak in Brazil [251].

Differences between the prevalence found in this study and the other publications in South America may also be explained by variations in the timing of the serosurvey. This study was conducted from June to December 2016 which is just after the end of the outbreak of ZIKV and CHIKV in Rio. Variation in the environmental climates of the different study sites may also play a part. Recent mathematical modelling studies both in the Americas and Asian countries stress the importance of the relationship between temperature, rainfall and increase in arboviral notifications [252].

The diagnostic assays used for the different studies vary. Consequently, the specificity and sensitivity results do too. For example, in the study in Salvador published by Netto *et al*. (2017) and the study in Bolivia published by Villaroel *et al.* (2018), they use the IgG NS1 Euroimmun assay to detect ZIKV infections [228, 236]. Netto et al. (2017) conducted some confirmatory testing for a proportion of the study subjects through PRNTs, however, Villaroel et al. (2018) only used the IgG NS1 assay. In Chapter 3, I demonstrated that this method had a much lower specificity and sensitivity than the assay that we used, the ZIKV Green BOB assay.

The findings highlight the challenging scenario of the Brazilian surveillance system when dealing with these arboviruses. I identified that around 0.25% and 2.70% of subjects infected with ZIKV and CHIKV respectively were reported to the health surveillance system. It has also now been demonstrated that there were already ZIKV cases during 2015, but were not detected by the government system. Prior to the end of 2015, there were no systems in place for diagnosing ZIKV routinely. In addition, the large percentage of asymptomatic cases and the fact that most symptomatic presentations are mild could have contributed to the underreporting of cases. For Dengue, it has been recognised that for each case reported, there are another 12 cases not reported to the Brazilian Ministry of Health's National Information System [162].

5.6.2 Variation of ZIKV and CHIKV exposure with socioeconomic status

My findings showed no significant differences between the prevalence of ZIKV and CHIKV among female and male. Similar results have been reported in other studies of dengue exposure in Brazil [253] [254].

I also identified a significant association between higher prevalence of both ZIKV and CHIKV among the older group (>60 years) than the younger population (p-value <0.05). The youngest population group was found to have the lowest seroprevalence for both ZIKV and CHIKV with only 23.8% and 9.5% of prevalence respectively. In previous studies on DENV prevalence in Brazil, the authors found that older age was associated with higher risk of DENV infection [254]. Similar results have been observed in other flavivirus seroprevalence studies in Peru, Singapore and Vietnam [255, 256] [257]. However, this correlation between age and DENV infection is related to the fact that DENV has circulated for the decades in those regions. However, both ZIKV and CHIKV have only arrived in the Americas in the last few years. Thus, this increased prevalence of ZIKV and CHIKV infection among the older population may be related to lower access to mosquito-bite preventive information campaigns or vector control strategies. Further studies involving larger numbers of individuals from different age groups equally distributed among high and low socioeconomic areas should be performed.

The findings suggest an association between ethnicity and arbovirus exposure. The brown ethnic population had the highest prevalence rate of ZIKV (39.5%), while the black population had the highest prevalence rate of CHIKV with 18.1%. Potential individual risk factors should be taken into account. It is likely that these results reflect socioeconomic differences among the ethnic groups. The socioeconomic inequality among different ethnic groups and races in Brazil has recently been described [258]. Different rates of prevalence of flavivirus infections among ethnic groups have been reported in Laos [259] and Singapore. Interestingly, two studies in Brazil have examined the relationship between ethnicity and prevalence to dengue infection. They both found white ethnicity had a higher risk for Dengue Haemorrhagic Fever than those defined as Afro-Brazilian or African ethnicity [260, 261].

5.6.3 Higher prevalence of ZIKV and CHIKV in low socioeconomic populations

My results suggest that ZIKV prevalence is increased in low socioeconomic populations. Similar patterns were observed for CHIKV. The prevalence of CHIKV was higher among the most deprived population group compared to the most privileged group. Further investigations analysing over 22 socioeconomic indicators confirm these findings. I have

shown that there is a significant relationship between low income, education levels and health and a higher prevalence of Zika virus infection. The analysis of all these indicators highlights a very relevant message that suggests an impact of low SES on the risk of acquiring arboviral infection. Similar results were described by Netto *et al.* (2017) in Salvador, Brazil [236].

My findings are comparable with other studies conducted across Brazil that investigated the association between low SES and flavivirus infections. Braga *et al.* (2010) found significantly higher DENV infection prevalence in lower socioeconomic groups in the Northeast of Brazil [254]. They analysed a population of 2,833 subjects and identified that the prevalence of DENV in the deprived, intermediate and high socioeconomic areas varied from 91.1%, 87.4% 74.3% respectively. Moreover, the risk of being infected by DENV was over three times higher in children up to 5 years living in deprived areas compared with areas with high socioeconomic status. Siqueira *et al.* (2004*)* also found a higher prevalence of DENV infection in population with lower incomes [253]. In addition, a study conducted by Vasconcellos *et al.* (1998) in Ceará, Brazil also showed that low socioeconomic status was significantly associated with higher rates of dengue virus infection [262]. Similar studies have also been conducted for CHIKV infection showing that low SES is a risk factor for chikungunya infections [263, 264].

Interestingly, our results showing that individuals with high SES have lower ZIKV and CHIKV infection rate may suggest that this proportion of the society may be a reservoir of susceptible individuals for future epidemics of both arboviruses. Whether this group represents a big enough proportion of the population for another ZIKV re-emergence of an epidemic remains to be seen.

My findings suggest that ZIKV and CHIKV control strategies should be revised and a better understanding of the high heterogeneity in the distribution of both infections among the different areas is needed. In this study, I showed that the most privileged group had a significantly lower prevalence of CHIKV and ZIKV. Previous studies have suggested that differences in arboviral prevalence between the privileged and groups with lower socioeconomic status can be attributed to a large extent due to the fact that deprived communities would have worse housing infrastructure and a lower proportion of apartment owners which is an indirect measure of low SES [254].

This variation in prevalence among socioeconomic groups which belong to different Human Development Units (HDUs) or neighbourhoods is unlikely to be explained by climate, because

local temperature, rainfall, humidity and altitude are similar amongst most areas across the city of Rio de Janeiro and the State with most of the region having a tropical climate. Moreover, most subjects in our study came from closely related areas within the municipality of Rio de Janeiro. This relationship may also be explained by higher infestation rates of *Aedes* mosquitoes in low socioeconomic areas and HDUs within Rio de Janeiro. Further research investigating this relationship should be conducted to better understand the results of our study. Nevertheless, I believe that these data is already a valuable piece of information for suggesting alternative control strategies to be taken within the city of Rio de Janeiro and in other regions affected by ZIKV in Brazil. Some of this strategies include intensifying the vector control efforts in less privileged areas.

5.6.4 The longitudinal study: Prevalence of ZIKV

I describe that two waves of ZIKV infection took place in Rio de Janeiro between 2014 and June 2015 and between December 2015 and June 2016. Interestingly, this finding suggests that the first wave between 2014 and 2015 had a larger impact on the study population with 14.1% increase in prevalence rates compared to 10.7% after the second wave [184]. These results correlate with the previously described evidence that two epidemic waves of ZIKV took place in the State of Rio de Janeiro during the first half of 2015 and the first half of 2016 [184]. Zika virus disease was not a notifiable condition in Rio de Janeiro until October 22th of 2015. These results suggest a high number of underreported ZIKV cases during 2015 (which were probably reported as suspected DENV infections) [226] [265].

No significant difference was identified in the geographical spread of Zika virus during the study years, though I identified an increase in the proportion of ZIKV positive cases outside the municipality of Rio de Janeiro in 2016, compared with the positive cases detected in 2014 and 2015.

I detected seven study subjects positive for ZIKV in 2013. The results may represent a false-positive DENV case. On the other hand, those subjects may had been infected with Zika virus during 2013 or before as recent research detected ZIKV positive PCR cases in Rio de Janeiro already in April of 2013.

5.6.5 The longitudinal study: prevalence of CHIKV

I have also shown that there was a change in prevalence of CHIKV of 9.9% between 2015/2016 reaching a prevalence of 14.1% in 2016. Even though some CHIKV may have circulated in Rio de Janeiro prior the end of 2015, our results suggest that the outbreak

spread during the first half of 2016. Interestingly, the increase of DENV prevalence correlates with the increase of ZIKV cases.

Our findings coincide with the large increase in number of cases of suspected CHIKV that were reported to the Health Department in the State of Rio de Janeiro and with the observed wave of CHIKV cases [226]. It is noteworthy that a small number of subjects (5/121) were IgG CHIKV positive before 2015, including one subject positive in 2013 (1/52). This suggests CHIKV may have arrived in Rio de Janeiro before it was detected. The high changes in prevalence obtained in the population in 2016 correlates with the wave of reported confirmed and suspected CHIKV cases in Rio de Janeiro from the end of 2015 until June 2016. The CHIKV prevalence levels described are higher than the seroprevalence reported in other regions in South America [236]. In 2018, a new epidemic wave of CHIKV spread in Rio de Janeiro with almost 6.600 notified cases from January to the 13th of August 2018 [266]. As the proportion of susceptible individuals is quite high (over 80%), I hypothesise that future CHIKV outbreaks may erupt in Rio de Janeiro during the next few years.

5.6.6 Interpretation of the prevalence rates of ZIKV and CHIKV in the metropolitan region of Rio de Janeiro

Some of my findings may be associated with the climate conditions in the State of Rio de Janeiro during 2013 until 2016. They may have played a role in the reproduction and multiplication of the mosquito vector and having consequently an effect in the length and attack rate of the 2015 and 2016 outbreaks of ZIKV and CHIKV [267] [268]. Moreover, I have also highlighted the differences in estimates that would be achieved if the Zika virus Green BOB assay cut-off was modified. The results obtained should be interpreted with caution and the prevalence observed should be considered an estimation of the prevalence in the region studied.

Overall, these findings contrast with the results identified in previous seroprevalence studies, which estimate that following an epidemic of the above-mentioned arboviruses, ZIKV prevalence ranges 60-70%, CHIKV prevalence ranges between 60-80% and DENV prevalence ranges between 0 to 100% [111]. However, Fritzell *et al.* (2018*)* recognise that the overall seroprevalence rates for ZIKV, DENV and CHIKV identified in a total of 147 studies conducted between 2000 and 2018 are characterised by high degree of variation and heterogeneity.

5.6.7 Understanding arboviral antibody dynamics: 2013-2016

In this chapter, I have shown a high degree of biological diversity among the antibody dynamics analysed in the longitudinal cohort between 2013 and 2016. These results highlight the challenge in the detection of IgG antibodies in an extensively flavivirus exposed population even when using highly specific diagnostic assays. They also highlight the challenge of interpreting IgG Green BOB ratios and the potential for false positives or false negatives in the assay results obtained.

It has recently been suggested that the IgG CHIKV ELISA assays may offer a small proportion of false positive results due to its similar antigenic properties with other important human alphaviruses such as O'nyong-nyong virus, Mayaro virus or Ross River viruses [269]. It is noteworthy that a high frequency of Mayaro IgM antibodies were reported only last year (2017) in areas of Northern and Central Brazil [270]. Therefore, additional testing using different assays or techniques such as PRNTs should be conducted on the positive CHIKV cases identified in 2013, 2014 and 2015 to confirm the prevalence rates in the metropolitan region of Rio de Janeiro. Moreover, a better understanding of the travel history of the study subjects that scored positively for IgG CHIKV in 2013-2015 should be investigated to understand the potential exposure to other alphaviruses in the study population.

The complex dynamics observed in the changes in IgG dengue antibodies highlights the enormous challenge of interpreting the flavivirus burden in a population where the four serotypes of dengue have circulated for the last 30 years and with the recent introduction of ZIKV in the last few years. The changes in antibody titres in the study population suggests that re-infections may have occurred among a proportion of cases in the longitudinal cohort. Alternatively, it has been suggested that DENV, ZIKV and CHIKV may persist and be shed in body fluids for months after symptom onset [28, 211, 271]. The effect that this may have in the production of specific IgG antibodies still remains to be elucidated.

Finally, these results offer some light to the understanding regarding the time to loss of immunity among CHIKV and/or ZIKV infected individuals. This findings suggest that in a small proportion of cases, antibody titres may decay to lower levels (below the cut-off value) for both ZIKV and CHIKV in less than a year.

All this demonstrates how important it is to use reliable diagnostic assays for differentiating between exposures when multiple arboviruses co-circulate. The ability of seroprevalence surveys to provide true estimates of prevalence in South America is a major public health challenge.

5.6.8 Strengths of this chapter

I believe that there are multiple strengths in the research conducted in this chapter. Firstly, even though we investigated the prevalence of ZIKV and CHIKV in a non-randomized cohort of subjects, we have included a large sample of over 1000 well-characterized subjects. A large number of individuals has allowed us to analyse and identify the different patterns associated with gender, age, ethnicity, and socioeconomic factors. The multidisciplinary approach that we have used to understand prevalence rates in our populations is a useful way to visualize the spread of infections. I have used geospatial data such as the coordinates (latitude and longitude) for each subject and I have generated maps that can show the spread of the disease. Moreover, matching the exact location of residence from our study population to the correspondent HDU and therefore to a large socioeconomic database developed by UNDU has provided a bigger insight into the impact that these arboviruses have caused in Brazil. I believe that all these assets have allowed us for a better understanding of the complexity of ZIKV infection differential population exposure.

Another strength of our study is the use of a well-defined very specific and sensitive IgG diagnostic method to detect IgG Zika virus antibodies, the Green BOB assay. The previous work carried out in Chapter 3 demonstrated that this was a highly sensitive and specific method for diagnosis of ZIKV exposure.

Another relevant addition is that I believe this study has crucial implications for public health in the State of Rio de Janeiro, Brazil. These findings will aid in the determination of risks factors for future outbreaks of CHIKV and ZIKV in Rio de Janeiro as well as for designing better vector control approaches and adequate vaccine strategies.

5.6.9 Limitations of this chapter

This chapter has some important limitations inherent to its design:

Study Subjects:

This study was designed for testing a convenience laboratory sample from a diverse population in Rio de Janeiro. However, this sample may not be representative of the whole population of the State. There is a large risk of selection bias among the study participants as all of the subjects attended a Clinic in the Reference Hepatitis Centre of the IOC in Rio de Janeiro. As a result of this, a majority of the subjects come from areas less than 50 kilometres far from this clinic. Moreover, it is likely that we have an underrepresentation of subjects

from the privileged strata of the society as subjects with high socioeconomic status generally attend private clinics, instead of public health centres in Brazil.

Moreover, a proportion of the subjects included in our study were Hepatitis positive cases with the potential confounding factors that this may imply. Additionally, our study subjects were mainly adults with only 21 individuals under 20 years old. In the future, a randomized serosurvey including a representative population in terms of age, gender, ethnicity and socioeconomical status form different geographical areas should be conducted to improve rigorousness and accuracy in the prevalence estimates observed.

Serological testing:

A major challenge from this study was using an accurate ZIKV serological assay. The high levels of cross-reactive antibodies across different flaviviruses in Brazil may have affected the accuracy of our study. The work conducted in Chapter 3 aimed to identify the most accurate assay. Unfortunately, the Green BOB assay is not a widely established assay yet. An important limitation may be the risk of false positive and false negative diagnosis. A multi-centre evaluation would be beneficial to better understand the specificity and sensitivity of this blockade-of-binding method. Moreover, it is unknown the period of persistence of ZIKV and CHIKV IgG antibodies following infection.

It would have been desirable to perform PRNT assays to confirm the prevalence rate with the method that is considered the gold standard assay, however, given its large population screened, it was not feasible to use this assay.

Study Design:

This study did not manage to respond to one of the most urgent questions around ZIKV infections: the proportion of asymptomatic cases. A different study design should be applied to try to determine the exact percentage of asymptomatic Zika infections in the population.

A major limitation of the investigations carried out in this chapter is the nature of the study population included in the longitudinal cohort, which was a convenience sample comprised of 121 individuals.

Further seroprevalence studies would ideally follow the WHO recommendations for seroprevalence studies [272], which advise on the design of serosurveys, including randomly recruited individuals from representative clusters of the population studied. Unfortunately,

these type of studies are expensive and difficult to perform because they require even major logistical resources, including a large workforce and diagnostic assay resources [111].

Spatial analysis:

A limitation of the analysis of the spatial spread of ZIKV, CHIKV and DENV is that the data that I had available (which was based in the study subject's postcodes) does not allow us to know if the cases of recent ZIKV and CHIKV are clustered. Further work should involve analysis of clustering over time to better understand the pattern of spread of these viruses. Potential bias may occur in the case of subjects who may have not had a permanent residence or who may have travelled to other regions or who may work or study in a different area. Further information related to the location where study subjects spend their daytime and evenings should be collected to achieve a more complete understanding of the spread. Moreover, this analysis lack environmental information which would allow for the interpretation of the vector ecology and location of clusters.

Socioeconomic and sociodemographic analysis:

Interpreting the effects of multiple SES factors on the prevalence of Zika and Chikungunya viruses is very challenging because most socioeconomic and sociodemographic indicators are interlinked. Moreover, we have used the respective HDU based on the postcode of each subject to estimate their SES, which is not an accurate measure. This is important because it is known that individual-level determinants related to location where study subjects spend time would provide a much more accurate information to understand the spread of this viruses. Therefore, our SES analysis should be interpreted with great care and further work interpreting these associations should be conducted to better understand the epidemic infection and attack rates in less privileged strata of the society.

Other limitation factors:

Another limitation of this study is the lack of entomological data associated to the area of residence of the study subjects. This would provide a much better understanding of the environmental risks of these arboviral infections which are transmitted mainly by Aedes mosquitoes. This information would allow to obtain a better understanding regarding the host-pathogen dynamics. However, implementing entomological studies alongside to seroprevalence studies is challenging as reported by Fritzell *et al.* (2018*) [111]*. Only 10% of the seroprevalence studies investigating ZIKV, DENV or CHIVK prevalence conducted between 2000 and 2017 had set up an entomological survey.

Moreover, during 2015/2016 extensive vector control and prevention programs were conducted in some regions in Rio de Janeiro involving breeding site elimination. It would have been extremely interesting to have data available regarding the areas where these programs took place to assess the effect that this intervention may have had.

5.6.10 Implications of the seroprevalence studies for further work

There still remain gaps in the knowledge of the geographic distribution of these arboviruses worldwide. Indeed, more seroprevalence studies are needed, particularly of ZIKV. However, conducting these studies is a challenging task due to the need for careful study design to avoid bias. The data presented in this chapter is relevant for the following:

1. I have managed to facilitate an estimation of the proportion of adults who are immunologically naïve and thus at risk of future infection. This will have a major impact in designing appropriate public health measures, including vaccination strategies.

2. These results facilitate an estimation of the proportion of people exposed to these new arboviral epidemics, giving perspective regarding the global burden of the rates of complications of arboviral infections particularly to the congenital and neurological complications.

3. These results will also help building models of prediction of future arboviral epidemics during the next years in the State of Rio de Janeiro. In addition, this findings may be applicable to estimate the prevalence in other cities with similar climatic conditions and Aedes infestation rates in South America such as Belo Horizonte (Brazil) or Fortaleza (Brazil).

4. In the future, I seek to generate further modelling projections using these findings in order to estimate the basic reproduction number of ZIKV and CHIKV. I also want to estimate the critical population immunity threshold in order to predict the number of years that may be needed before another large outbreak of ZIKV or CHIKV occurs as a result of susceptible individuals being replaced due to birth or migration.

5. More prevention strategies against ZIKV and CHIKV should be implemented targeting populations belonging to the lower strata of the society, in addition to vector control strategies and information about the risks of sexual transmission.

5.7 Conclusions

In summary, in this chapter, I summarize the results of two linked seroepidemiological studies: a cross-sectional and a longitudinal study.

With the cross-sectional study, I have estimated for the first time the seroprevalence of Zika and Chikungunya viruses in the metropolitan region of Rio de Janeiro after the 2015-2016 epidemic at 39.5% and 15.0% respectively. I have observed an association between patterns of infection, age and ethnicity. Investigations on the sociodemographic data have shown a higher prevalence of ZIKV in lower socioeconomic population groups. Moreover, there was an increased prevalence of Chikungunya virus in subjects also infected by ZIKV.

With the longitudinal study, I have given estimates to the prevalence of ZIKV, CHIKV and DENV from 2013 to 2016 in the population of Rio de Janeiro. I have also shown the enormous biological diversity in antibody responses found in the study subjects and I have hypothesised the implications of this seroprevalence findings. ZIKV and CHIKV spread across Brazil becoming a serious public health concern and affecting especially the poorest communities which have a more precarious healthcare system. The burden of ZIKV and CHIKV viruses to the public health system in Brazil, as well as to the affected families, is still very significant.

Finally, my results have shown that population immunity may have played a role in the decrease of the outbreak of Zika. Nonetheless, these data show that a majority of the population is still naïve to Zika and Chikungunya viruses and future outbreaks may occur in Brazil in the following years. My results have significant implications for guiding the design of vector control strategies, as well as, better public health responses and vaccine development.

CHAPTER 6

Discussion

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
- Marie Curie

Chapter 6: Discussion

6.1 Overview Chapter 6

During 2015-16, Brazil suffered the largest epidemic of Zika virus (ZIKV) ever reported and which affected millions of people. Even though the Government of Brazil declared the end of the Zika emergency in 2017, the devastating impact of the epidemic still remains to be fully understood [273]. The burden caused to the Brazilian population and other affected countries will continue for years and probably generations to come.

6.2 My thesis in the context of previous work

Significant advancements have been made over the past two years with respect to the understanding of Zika, the flavivirus that in 2015-2016 spread widely across South America spreading fear and uncertainty. We now understand much more about the virus' biology , the role in pathogenesis [274], the routes of transmission [27], the clinical manifestations, and the devastating congenital sequelae associated with neonatal infection [221]. This thesis contributes to the literature providing improved understanding and key research findings of three interlinked areas: serological diagnoses of Zika virus, the association between ZIKV and neurological disease and the seroprevalence of Zika virus in Brazil.

6.2.1 Diagnosis of Zika Virus

Since the outbreak exploded in Brazil, significant strides have been made rapidly in the area of Zika virus diagnosis. This speed with which novel diagnostic techniques and assays were developed reflects the international collaborative efforts and intense dedication of both researchers and diagnostic assay developers.

Over the last few months, more assays have become available, the United States (US) Food and Drug Administration (FDA) has now issued an authorisation for the use of five ZIKV diagnostic tests [275].

As new scientific evidence is generated, those assays may require further review and revision. However, our findings highlight the acute need for validation of any potential Zika diagnostic assay and/or surveillance tools in local populations. International efforts have been focussed on conducting quality assessments for ZIKV molecular testing in Brazil [276]. Some multi-centre studies investigating serological assays have been conducted in different US. Territories [277]. However, there remains a need for further peer-reviewed studies

assessing the performance of ZIKV serological assays in clinical settings. Moreover, a wide range of novel Zika virus assays have been recently being developed including:

Novel ZIKV antibody detection assays: Some examples include: novel assays detecting ZIKV specific IgA antibodies (Euroimmun), [278], microfluidic Paper-based Analytical Devices (μPAD) for flavivirus antibody detection (including Zika virus Detection) [279], Reporter Virus Neutralisation Test (RVNT) which uses a luciferase-tag to directly mark the neutralising antibodies [280], Multiplex Microsphere Immunoassays (MIA), which allow the addition of one or more antigens to augment the sensitivity and specificity of the ZIKV detection [281].

In spite of all these new advances, Plaque Reduction Neutralisation Tests (PRNTs) remains the "gold standard" technique. PRNT can suffer from some cross-reactivity, however, this can be overcome by setting a suitable cut-off for the population. As PRNTs are a labour-intensive, time-consuming technique that requires skilled personnel with experience handling live viruses and the appropriate equipment including incubators and safety hoods. Consequently, PRNTs are not extensively used across South America [282]. Currently, they are used to confirm ZIKV infection by comparing the fold rise in PRNT titre against a battery of flaviviruses endemic to a given area and also in a comparison of paired acute- and convalescent-phase serum specimens [105]. Novel techniques using neutralisation assays that require fewer days to perform are one of the most promising options to improve these assays [283].

6.2.2 Zika virus and neurological disease

In this thesis, I presented a retrospective cohort study investigating the association between Zika virus and neurological disease in a group of patients from Rio de Janeiro. This evidence which was published in here [284], contributes to the growing body of work supporting the increased risk of neurological complications in ZIKV exposed individuals. The WHO Zika Causality Working Group published a report assessing 36 studies that investigated the link between ZIKV and GBS. They identified that the odds of having had a recent Zika virus infection were up to over 30 times higher in patients with GBS than those without [43]. This expert group concluded that Zika virus was a trigger to Guillain-Barré syndrome, however, the exact mechanism and risk factors are yet to be elucidated.

Case reports and preliminary studies have suggested a clear relationship between ZIKV infection and certain neurological conditions such as meningoencephalitis, myelitis [208], encephalomyelitis [209] or meningoencephalitis [204]. However, only longitudinal prospective case-control studies, as well as experimental studies using either *in vitro* models

or animal models, will contribute to better understand the role of the virus and the pathogenesis of these disorders [285, 286]. In view of the new evidence accumulating, and as more data surfaces, the appropriate health authorities will need to ensure the general public, as well as physicians, are aware of the potential Zika virus associated dangers.

6.2.3 Seroprevalence of Zika Virus

Data on Zika virus prevalence rates is essential in order to understand the geographical distribution of this flavivirus as well as their contribution to global morbidity and mortality.

Currently, very few seroprevalence studies have been published from data from South America trying to understand the population exposure to Zika virus. The only two published studies are from Bolivia [228] and from Salvador, Brazil [236]. The results from Beni in Bolivia are similar to the ranges of ZIKV infection that we have identified with 39% ZIKV seropositivity while the results identified in Salvador (Northeast of Brazil) show a higher percentage of ZIKV exposure with a prevalence of over 60% in the population.

We demonstrated that lower social strata have a higher prevalence of ZIKV infection. This is something that had also been suggested by Netto *et al.* (2017) [236]. However, the prominence of socioeconomic inequalities in health in Brazil has been widely claimed [287, 288]. Brazil is the fifth most populous country in the world with over 208 million inhabitants [289], and is one of the countries in the world with the highest levels of income inequality [290]. It has been reported in Brazil that individuals with the lowest socioeconomic status report worse health, more functional limitations, fewer doctor visits and higher hospitalisation rates than wealthier individuals [291]. Thus, identifying that lower SES have a higher prevalence of arboviral infections could be expected and our results will inform health authorities to conduct further education campaigns targeting the most vulnerable populations.

6.3 Limitations of the research

Several shortcomings can be recognised in this thesis. Some of these limitations are inherent to the study design of the research carried out. Other limitations were due to the timing of my PhD, which was unavoidably associated with the urgent requirement of novel research as a result of it coinciding with the emergence of ZIKV outbreak that spread rapidly across the Americas. The diagnostic evaluations carried out would have been more powerful if more sera specimens could have been included. Our study investigating the association between Zika virus exposure and Zika virus infection would have been much more powerful if larger

numbers of patients could have been recruited as well as matched controls. The study was conducted in a period in which the State of Rio de Janeiro was undergoing a terrible political turmoil and the health system was deteriorating. This led to public health workers not being paid, medicines not being available at the hospitals, and various hospitals of the city of Rio de Janeiro having to close. The impact that this socioeconomic context may have had in our study is unknown. However, in order to improve this and as a response to that we decided to start a prospective case-control study based in Rio de Janeiro and Recife and funded by ZikaPLAN (the Zika Preparedness Latin American Network), which is an EU Consortium "to address research challenges posed by the Zika outbreak and build a sustainable response capacity in Latin America".

Finally, several limitations can be identified in the seroprevalence studies. The study was designed for testing a convenience sample, even though, it captured a diverse population in Rio de Janeiro. Further seroprevalence studies including random sampling with selected households would be needed. This would be a cost-effective method for obtaining better seroprevalence estimates that are representative of the target population. Finally, interpreting the effects of multiple SES factors in Zika and chikungunya exposure is very challenging because most socioeconomic and sociodemographic indicators are interlinked. Therefore, the socioeconomic analysis should be interpreted with great care and further work interpreting these associations should be conducted to better understand the epidemic infection and attack rates in less privileged strata of the society.

6.4 The importance of this research

I believe that the findings of this thesis will redound to the benefit of society considering that Zika virus, as emergent zoonotic infection has played and continues to play a major role in public health both in Brazil and worldwide. The impact of these results should be analysed in the context of this international Zika virus crisis.

There are multiple implications of my findings for governments and health institutions. With the key findings in mind, proposed recommendations have been formulated through written communications in peer review journals, several conference presentations and through ZikaPLAN (EU consortium). Health institutions across Brazil need to acknowledge that the diagnostic assays that are currently being used may provide false positive and false negative diagnostic results. Our findings will allow the development of some guidelines for ZIKV diagnostic testing, including a diagnostic algorithm that could account for the potential cross-

reactivity between Zika virus and other flaviviruses. Industries and private stakeholders will also benefit from the knowledge gained about diagnostic assays for ZIKV diagnosis.

The knowledge gained of the association between Zika virus and several neurological syndromes will aid neurologists and other health specialists in preparation for this. When patients present with acute and novel neurological syndromes in ZIKV exposed areas, or travellers coming from areas where current Zika virus epidemics are occurring, local services must be aware of the need for testing the patients not only for DENV but also for ZIKV and CHIKV.

I believe that our findings will have an impact to other experts in the field and public health professionals. My results advise and contribute to future study designs and provide a deeper understanding of ZIKV immunity in order to develop and chose better diagnostic assays that will accurately distinguish ZIKV infection in flavivirus exposed populations. Furthermore, research bodies should continue to promote research and development of new advances to better understand, control and improve our understanding of ZIKV pathogenesis and mechanisms, in order to keep developing knowledge that will better help control future outbreaks and avoid major damage to the general public.

However, most relevant of all, I believe that this thesis represents a contribution for the whole society, and above all, the populations that have been affected by Zika virus in Brazil. Throughout all of my PhD I have tried to communicate my research findings widely. Not only have they been communicated to scientific experts and government bodies through scientific publications and conference presentations, but also to wider audiences including to the patient groups affected.

6.5 Future research directions

ZIKV diagnostics is a fast-moving field. As shown by Kurani *et al*. (2018), a total of 19 novel ZIKV in vitro diagnostic (IVD) test were quickly developed and released for clinical diagnostic purposes [292]. New diagnostic technologies that make a quicker and more accurate identification of the causative agents in cases of suspected arboviral infection, will not only contribute to better individual patient care but also reduce the anxiety caused by not knowing if you have been exposed to ZIKV. The development of rapid point of care diagnostics could revolutionise ZIKV diagnostics allowing for a fast diagnosis at primary health centres.

Moreover, in the coming months, it will be essential to compile the available evidence and develop diagnostic algorithms and recommendations for diagnostic testing through guidelines. It is advised to employ serological diagnostics alone or in conjunction with other laboratory techniques to provide the most accurate diagnosis as possible. Moreover, the development of the large multi-centric case-control study in Rio and Recife that we started already at the end of 2016, will help to address the questions around neurology associated with Zika virus needed.

6.6 Future prospects

The findings presented in this thesis highlight that it has become evident that there is a need for well-organised public health responses combining efforts of the public health teams, the community organisations and research institutions to ensure that ZIKV is being tackled in the correct way.

Zika virus diagnostics

The future prospects of ZIKV diagnostics are quite optimistic as the research has moved rapidly to gain a better understanding of ZIKV immune responses and develop improved diagnostic assays. Currently, nearly 15 different companies worldwide have worked on developing new potential diagnostic tests, without taking into account the tens of research groups from public institutions also working on that. My results emphasise the need for increased governmental funding for optimising novel more accurate diagnostics.

Vaccination against ZIKV

There have been enormous efforts to develop novel vaccines against ZIKV and currently, there are three groups conducting human phase I trials to test their newly developed vaccines in a small percentage of the population. It will require some years of research and it will be challenging to develop vaccines that are completely safe and with high efficacy. It is been suggested that vaccines should be safe during pregnancy, to be able to apply them to gestating females only, as it is done with other pathogens. Findings like the ones presented in this thesis will help to influence Zika virus management including antiviral drugs, new markers of disease, the most appropriate prevention and vaccination strategies.

6.7 Reflections

This thesis has been an exciting research challenge and "life adventure". My PhD has had a multidisciplinary focus due to the urgent nature of the questions that needed to be addressed. However, I feel that by taking this multidisciplinary approach to my research, I have manged to contribute even more to this public health crisis. Working in international teams with multiple collaborators has allowed us to address more issues and gain even more knowledge and understanding.

The findings presented in this thesis will help in future ZIKV epidemics. However, unfortunately, the impact that the 2015-2016 epidemic had on the Brazilian population will last for generations. Our results showed that another ZIKV epidemic may occur in Brazil in the coming years. Thus, the Brazilian public health authorities need to be prepared to respond to it. I believe it is utterly necessary to develop vaccination campaigns across South America (once they are available), as well as prevention campaigns. The prevention campaigns to control the spread of the vector should target the most vulnerable parts of the society which have been shown to be at higher risk.

This PhD has been much more than a few manuscripts and a (very long) thesis. This PhD has represented three years of learning, hard work and multiple public health challenges that have taught me countless life lessons and had made me become a much better researcher and person. I hope that many of the conclusions drawn from this thesis could be applied in future ZIKV outbreaks, but also, future epidemics of other emergent infections.

Final conclusions

In the last three years, Zika virus has caused a devastating impact worldwide causing a major international public health crisis. In this thesis, I present the first systematic evaluation assessing the performance of the currently available serological assays to diagnose ZIKV infection in the Brazilian population. We assessed 6 ELISA assays for IgM and IgG detection of ZIKV specific antibodies, a neutralisation assay, the PRNT, two immunofluorescence assays and one point-of-care-test. My results show that the PRNTs remain to be the most accurate technique with specificity levels of 96%. This evaluation highlights the urgent need for improvement and validation of Zika diagnostic assays and surveillance tools in South American populations. Moreover, we have shown that Zika virus is associated with a wide range of neurological manifestations, including Guillain-Barré Syndrome and central nervous system disease. Also, our results measure for the first time the seroprevalence of Zika and

chikungunya viruses in the metropolitan region of Rio de Janeiro. Results have shown that a majority of the population in Rio de Janeiro is still naïve to Zika and Chikungunya virus and future outbreaks may occur in the coming years. Finally, we have shown that low socioeconomic populations have a significantly higher prevalence of ZIKV and CHIKV compared to high socioeconomic groups.

7 Appendix

Appendix 1. Neurologic diagnostic criteria

In here below can be found the neurologic diagnostic criteria used in our cohort to determine the diagnoses of the clinical cohort for:

1- Guillain-Barré syndrome: adapted from *Sejvar et al*. (2011), *Hadden et al. (*1998), *Hadden & Cornblath* et al. (1998) and *Sejvar & Kohl* et al. (2010). See criteria and levels of certainty of diagnosis in Table 7.1

2- Encephalitis adapted from *Granerod et al*. (2010), *Venkatesan et al.* (2013), *Granerod, Ambrose et al*. (2010) and *Venkatesan, Tunkel et al*. (2013) – See criteria and levels of certainty of diagnosis in Table 7.2.

   a. adapted from Granerod et al. 2010, Venkatesan et al. 2013)[293, 294]

3- Myelitis: adapted from the Transverse Myelitis Consortium Working Group Criteria, (2002) and the Transverse Myelitis Consortium Working Group (2002). See criteria and levels of certainty of diagnosis in Table 7.3.

   a. (adapted from Transverse Myelitis Consortium Working Group Criteria, 2002.)[295]

4- Meningitis: adapted from *McGill et al*. (2016) and *McGill et al*. (2016). See criteria and levels of certainty of diagnosis in Table 7.4.

   a. (adapted from McGill F, Heyderman RS, Michael BD, *et al.* 2016)[296]

5- Acute Disseminated Encephalomyelitis (ADEM): adapted from the clinical definition proposed by *Krupp et al.*, (2007) [297]

6- Radiculitis: based on clinical features and electrophysiological studies by Wilbourn & Aminoff (1998) [298]

7- Myositis based on clinical features and electrophysiological studies

8- Facial diplegia with paraesthesia (GBS variant): adapted from the *Dimachkie & Barohn* (2013) [299]

Table 7.1 Diagnostic criteria for Guillain-Barré Syndrome

Level 1	Level 2	Level 3	Level 4
[ ] Bilateral and flaccid weakness of the limbs AND [ ] Absence of an alternative diagnosis for weakness AND [ ] Decreased or absent deep tendon reflexes in affected limbs AND [ ] Monophasic illness pattern with weakness nadir between 12 hours and 28 days, followed by clinical plateau			Suspected GBS with no other diagnosis apparent, but does not fulfil level 3 criteria
[ ] CSF total white cell count < 50 cells/mm^3 OR [ ] If CSF results unavailable, electrophysiological findings consistent with GBS			
[ ] CSF protein level above laboratory normal value AND CSF total white cell count < 50 cells/mm^3 AND [ ] Electrophysiological findings consistent with GBS			
Classify → [ ] Level 1	[ ] Level 2	[ ] Level 3	[ ] Level 4

Table 7.2 Diagnostic criteria for encephalitis

Level 1	Level 2	Level 3
[ ] Acute or sub-acute (<4 weeks) alteration in consciousness, cognition, personality or behaviour persisting for more than 24 hours **[ ] Absence of an alternative diagnosis for symptoms**		
[ ] New onset seizure OR **[ ] New focal neurological signs** OR **[ ] Fever (≥ 38ºC)** OR **[ ] Movement disorder (includes: Parkinsonism, oromotor dysfunction etc.)** OR **[ ] EEG consistent with encephalopathy +/- epileptiform features**		
[ ] CSF total white cell count > 5 cells/mm^3 OR **[ ] Neuroimaging compatible with encephalitis**		
Classify → [ ] Level 1	**[ ] Level 2**	**[ ] Level 3**

Table 7.3 Diagnostic criteria for myelitis

Level 1	Level 2	Level 3	Diagnosis unclear
[ ] Acute onset of weakness or sensory disturbance of upper and/or lower limbs **[ ] Absence of an alternative diagnosis for symptoms**			
[ ] Brisk reflexes or extensor plantar response OR **[ ] Bladder or bowel dysfunction** OR **[ ] Clearly defined sensory level**			
[ ] Absence of extra-axial compressive aetiology by neuroimaging (MRI or CT myelography) AND **[ ] Absence of flow voids on the surface of the spinal cord suggestive of arteriovenous malformation** (MRI)			
[ ] CSF total white cell count > 5 cells/mm^3 OR **[ ] MRI changes consist with myelitis**			
Classify →[ ]Level 1	**[ ] Level 2**	**[ ] Level 3**	**[ ] Diagnosis unclear**

Table 7.4 Diagnostic criteria for meningitis

Level 1	Level 2	Level 3
[ ] Absence of an alternative diagnosis for symptoms AND **[ ] Neck stiffness** OR **[ ] Kernig's sign positive (with the hip and knee at 90° angles, subsequent extension of the knee causes pain)** OR **[ ] Brudzinsky's sign positive (involuntary lifting of the legs on lifting the patient's head from the examining couch)**		
[ ] Fever (≥ 38ºC)		
[ ] CSF total white cell count > 5 cells/mm^3 OR **[ ] Meningeal enhancement seen on contrast enhanced CT or MRI**		
Classify → [ ] Level 1	**[ ] Level 2**	**[ ] Level 3**

Appendix 2. Hospitals in Rio de Janeiro involved in the recruitment of neurological patients

List of Hospitals involved in the recruitment of neurological patients

Hospitals:

1. Hospital Federal dos Servidores do Estado
2. Hospital Universitário Pedro Ernesto
3. Instituto de Pesquisa Clínica Evandro Chagas (IPEC)
4. Hospital do Andaraí
5. Hospital Geral de Bonsucesso
6. Hospital Barra D'or
7. Hospital de Clínicas de Niterói
8. Hospital Bangu D'Or
9. Hospital Icaraí
10. Hospital Badim
11. Hospital São Vicente de Paulo

Appendix 3: Further supporting data of the ZIKV PRNT assays

1- Protocol for the two different staining techniques used in the ZIKV PRNTs evaluated:

a. Staining with Crystal Violet

After 5-6 days of incubation, the semi-solid medium is discarded. Then, the cell monolayer was fixed and stained for 20-30 minutes with a solution of crystal violet-formaldehyde prepared by dissolving 1.3 gm of crystal violet (Sigma Aldrich) in 50 mL of 95% isopropyl alcohol (Sigma Aldrich), 300 mL of 37% formaldehyde (Sigma Aldrich) and distilled water. Then, plaques were counted

b. Staining with Neutral Red

Neutral red staining was performed following a two-overlay approach. A secondary overlay with neutral red was prepared including 4% Neutral Red (Sigma Aldrich). 0.5ml per well of this secondary overlay were added. Plates were allowed to solidify for 15 minutes and incubated for 5-6 days. Development of plaques can be done "real time" without stopping the reaction with this technique as there is no need to fix the cells for counting the plaques.

2- Reproducibility of the ZIKV PRNT assays performed in Brazil

Reproducibility of the ZIKV PRNT assays performed in Brazil was assessed measuring three samples 6 times as show in Table 9.5.

Table 7.5 Analysis Reproducibility PRNTs
CV = Coefficient of Variation

	Repeat 1	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Repeat 6**	
	ZIKV titer	ZIKV titer	ZIKV titer	ZIKV titer	ZIKV titer	ZIKV titer	**CV (%)**
Sample ZIKV Negative	<20	<20	<20	<20	<20	<20	**0**
Sample ZIKV Positive	830.2	843.1	843.2	809	817.4	866.4	**4.15**
DENV CONTROL (Negative)	<20	<20	<20	<20	<20	<20	**0**

Appendix 4 : Publications arising indirectly as a result of this PhD

Brasil, P., Pereira, JP, Moreira, E.,Nogueira, RMR., **Medialdea-Carrera, Raquel** et al., (2016) Zika Virus Infection in Pregnant Women in Rio de Janiro. N Engl J Med; 375:2321-2334

Soares, C. Brasil, P., **Medialdea-Carrera, Raquel**, Sequeria, P. et al. (2016) Fatal encephalitis associated with Zika virus infection in an adult. Journal of Clin Virology 83: 63-65

Balmaseda, A., K. Stettler, **R. Medialdea-Carrera**, D. Collado, X. Jin, J. V. Zambrana, S. Jaconi, E. Cameroni, S. Saborio, F. Rovida, E. Percivalle, S. Ijaz, S. Dicks, I. Ushiro-Lumb, L. Barzon, P. Siqueira, D. W. G. Brown, F. Baldanti, R. Tedder, M. Zambon, A. M. B. de Filippis, E. Harris and D. Corti (2017). "Antibody-based assay discriminates Zika virus infection from other flaviviruses." Proc Natl Acad Sci U S A **114**(31): 8384-8389.

Mehta, R., Soares, C.N., **Medialdea-Carrera, Raquel**, et al.(2018) The spectrum of neurological disease associated with Zika and chikungunya viruses in adults in Rio de Janeiro, Brazil: A case series. PLOS NTD, 12 (2).

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