Role of Maternal Dengue Virus Antibodies in the Development of Congenital Zika Syndrome

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To my parents

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List of abbreviations

ADE	Antibody dependent enhancement
Ae.	*Aedes*
AIC	Akaike information criterion
B. pertussis	*Bordetella pertussis*
C protein	Nucleocapsid protein
C. trachomatis	*Chlamydia trachomatis*
CHIKV	Chikungunya virus
CI	Confidence interval
CMV	Cytomegaly virus
CZS	Congenital Zika syndrome
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxynucleic acid
DSS	Dengue shock syndrome
E protein	Envelope protein
EDE	Envelope dimer epitope
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FLE	Fusion loop epitope
GBS	Guillain-Barre Syndrome
HLA	Human leucocyte antigen
HSV-1, HSV-2	Herpes simplex virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
INF	Interferon

L15	Leibovitz’s L-15 medium
mAbs	Monoclonal antibodies
MEM	Minimum Essential Medium
mL	Milliliter
MOI	Multiplicity of infection
n.s.	Not significant
NO	Nitric oxide
NS	Non-structural
NT, NT_{50}, NT_{90}	Neutralization titer, 50 or 90 % neutralization titer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
prM/M protein	Precursor membrane / membrane protein
PRNT	Plaque reduction neutralization test
PV-B19	Parvovirus B19
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RUBV	Rubella virus
Spp.	Species
STAT	Signal transducers and activator of transcription
T. pallidum, T. gondii	*Treponema*
UV	Ultra violet
VZV	Varicella zoster virus
WHO	World Health Organization
WNV	West Nile virus
ZIKV	Zika virus
µL	Microliter

1 . Introduction

1.1 Epidemiology of flaviviruses

Flaviviruses (family *Flaviviridae*, genus *Flavivirus*) are a large, heterogenic group of over 50 icosahedral, enveloped, positive sense, single stranded RNA viruses (Guarner and Hale, 2019). Flaviviruses account for the most prevalent arthropod-borne viruses causing disease in humans such as dengue fever, yellow fever, Japanese encephalitis and Zika fever (Guarner and Hale, 2019; Uno and Ross, 2018). The impact of these viruses on global health continues to rise as factors such as climate change and urbanization lead to the spread of vectors (Guarner and Hale, 2019).

Dengue virus (DENV) is the world's most abundant arthropod-borne viral pathogen in humans and has four serotypes (DENV 1 - 4). It infects near 390 million of 3.9 billion people each year who live in endemic areas, causing about 96 million clinical manifestations (Tremblay et al., 2019; WHO, 2020). While only a fraction of infected individuals develops symptomatic dengue disease, the virus is responsible for 10 to 20 thousand deaths annually (Tremblay et al., 2019). It is impossible to determine the exact date of the first dengue outbreak. The disease presents in such a variety of ways, though the first mentions date back to 992 before Christ in a medical encyclopedia from China (Salles et al., 2018). Before the 18th century came to an end, sporadic epidemics of diseases with a great resemblance to Dengue were reported in Asia and the Americas repeatedly leading to the hypothesis that the virus had spread over the (sub-) tropics during the 19th and 20th centuries (Salles et al., 2018). To date, about 70 % of DENV infections are reported in Asia though the Western Pacific, the Mediterranean, Africa and the Americas are also affected as seen in Fig. 1 (WHO, 2020). The number of cases annually is rising due to the spread of the disease into new areas, such as Europe, where autochthonous infections can be observed in many countries (WHO, 2020).

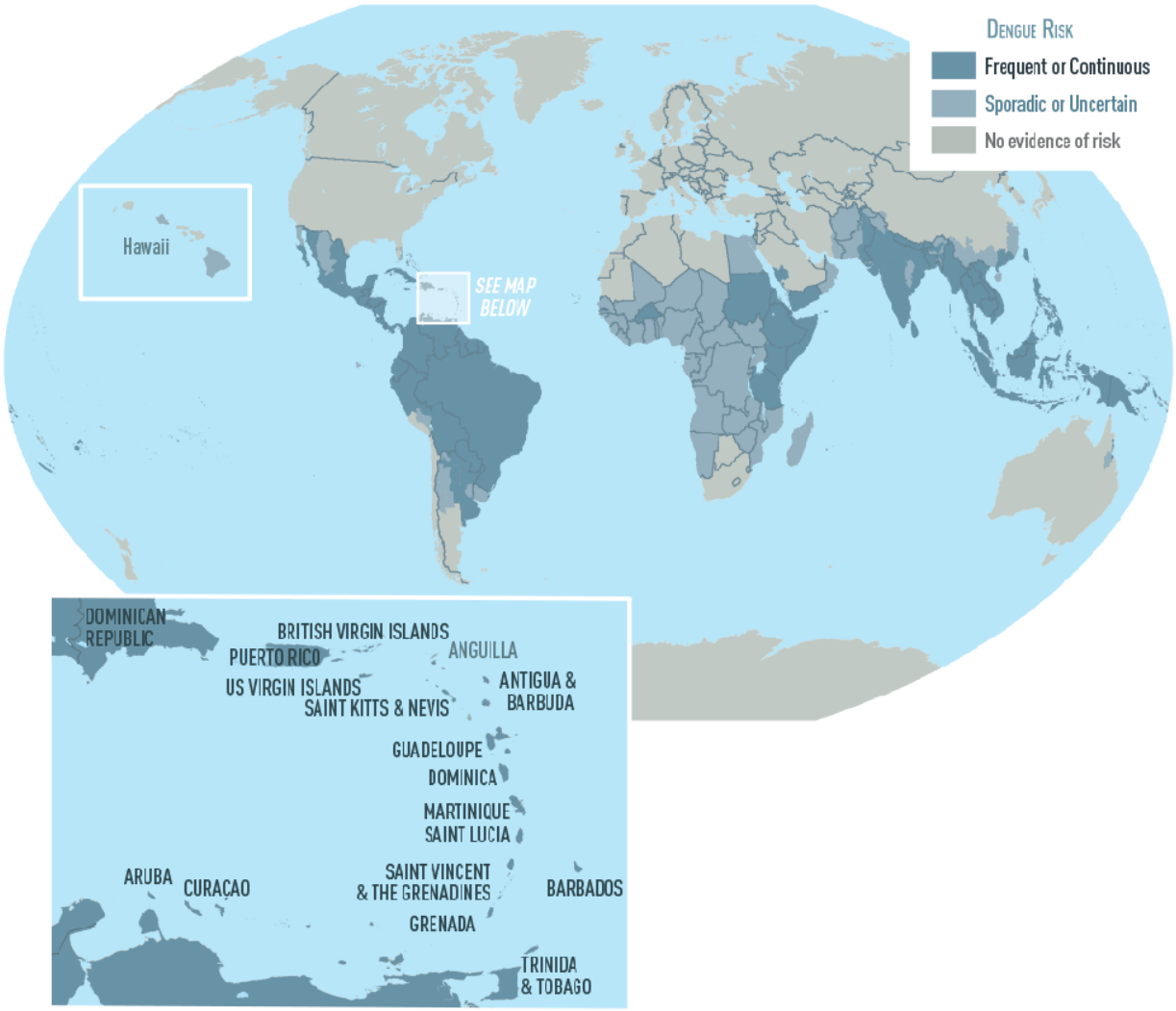

Fig. 1: Global distribution of DENV: This map illustrates areas with differing degrees of risk of DENV. Areas with a high risk of DENV are also areas with a greater risk of infection (figure from the CDC, 2020).

In South America specifically, a successful vector eradication was achieved in many countries during 1947 to 1970, only to have a reinfestation of the *Ae. aegypti* species during 1971 to 1999 and a surge of dispersion from 2000 to 2010 leading to a rise in DENV circulation (Salles et al., 2018). As this study pertains to women between the ages of 15-44, the outbreaks and presence of DENV after 1971 is of greater relevance. From 2011 to 2017 a drastic increase of about 30 % in dengue cases was observed in the Americas

in comparison to the 2001 to 2010 period totaling around 10,851,043 cases; this seven-year span contributed around 47 % of cases during the time span of 1980 to 2017 (Salles et al., 2018). In Brazil specifically, the endemo-endemic pattern of dengue outbreaks every three to five years was maintained through the end of the 20th century until 2010, when a switch to every two years occurred (Salles et al., 2018). DENV 2 and 3 were predominant in the early parts of the 21st century, with a change to DENV 1 predominance in 2009 in most of Brazil (Salles et al., 2018). In 2010 the first reports of DENV 4 surfaced, in 2011 a resurgence of DENV 1, and in 2015 DENV 2 (Salles et al., 2018). Notably, since 1990, 2015 had the highest recorded number of dengue cases, followed by 2016 (Salles et al., 2018). All four DENV serotypes are circulating in Brazil currently with a continuing predominance of DENV 1 (Salles et al., 2018).

Zika virus (ZIKV) was first isolated in 1947 from a nonhuman primate in Uganda and caused scattered human infections for about 50 years before the first larger outbreaks were recorded (Lazear and Diamond, 2016; Musso and Gubler, 2016). In 2007, the first epidemic of ZIKV was recorded in Yap State in the Western Pacific where an estimated 75 % of the population became infected in a short time span of four months (Lazear and Diamond, 2016). Shortly after, in 2013, a Zika epidemic was described in French Polynesia where an estimated 30,000 cases (about 11.5 % of the population) of Zika fever were reported. A small number of serious neurological complications, as well as non-vector-borne transmissions were recorded during the 2013 outbreak (Musso and Gubler, 2016). ZIKV continued to spread throughout the region though the exact number of cases is difficult to determine due to mild clinical presentations and overlapping endemicity with other flaviviruses. In February 2015 an outbreak was declared in the Americas for the first time, specifically in northeastern Brazil. By December 2015 there were 440,000 to 1,300,000 suspected ZIKV cases and confirmed autochthonous transmissions in states throughout Brazil (Musso and Gubler, 2016). The distribution of ZIKV infections as of 2019 can be seen in Fig. 2 (CDC, 2019).

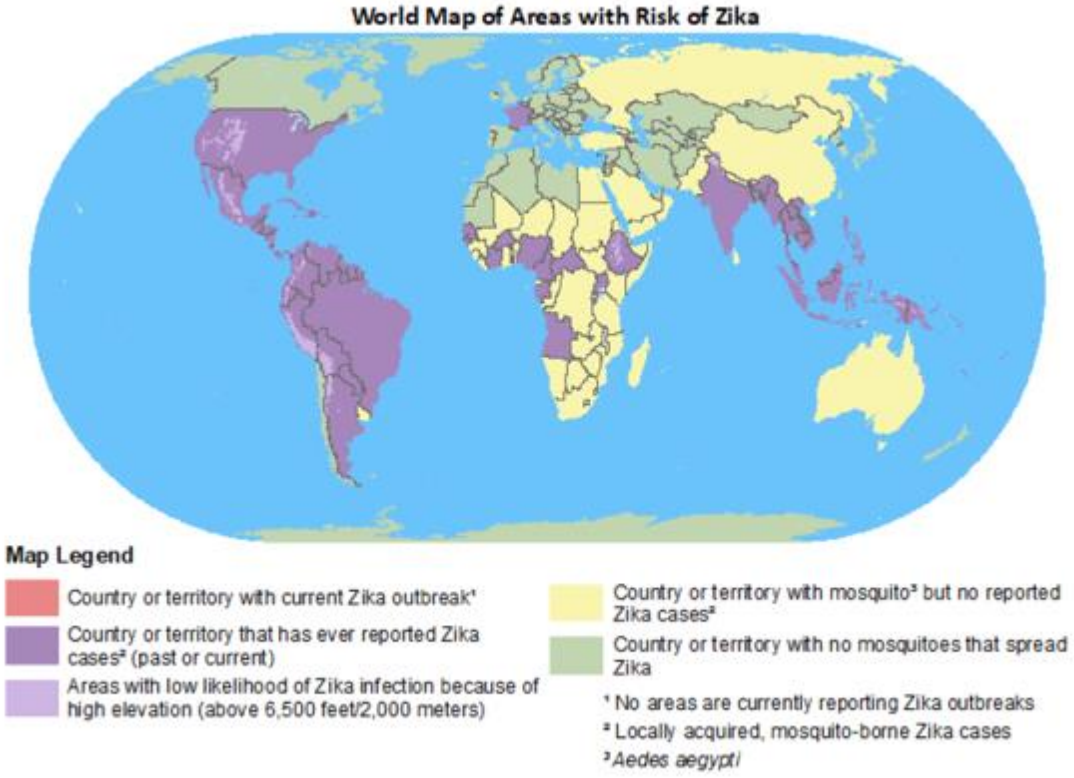

Fig. 2: Global distribution of ZIKV: This map illustrates the occurrence of ZIKV infection as of 2019 outlining areas which have recorded ZIKV cases currently or in the past, areas with a diminished risk of ZIKV infection due to high altitudes, areas in which *Aedes aegypti*, the main vector for ZIKV, can be found without reported ZIKV cases and areas with no evidence of the *Aedes aegypti* mosquito (figure from CDC, 2019).

1.2 Transmission

Arboviruses such as the flaviviruses have their respective vectors: for DENV and ZIKV these are the *Aedes* spp. of mosquitoes foremost *Aedes aegypti* followed by *Aedes albopictus* (Khetarpal and Khanna, 2016; Lazear and Diamond, 2016; WHO 2018; WHO, 2020). *Aedes* spp. are widespread throughout subtropical and tropical areas of the world as can be seen in Fig. 3 and suitable areas continue to grow as climate change progresses (Kamal et al., 2018). These mosquitoes are most active during the day and thrive in urban settings where they mostly breed in man-made habitats (WHO, 2018; WHO, 2020). The wide distribution of *Ae. aegypti* and *Ae. albopictus* in Latin America, particularly in urban areas, allowed such a rapid spread of ZIKV during the 2016 outbreak in the mostly ZIKV naïve population (Lazear and Diamond, 2016).

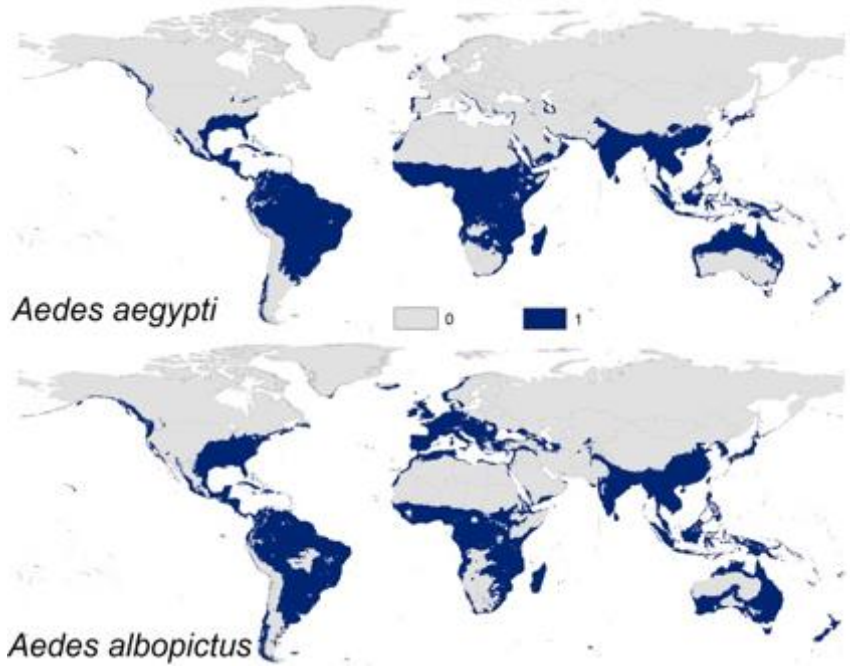

Fig. 3: Current *Ae. aegypt*i and *Ae. albopictus* global distribution: These maps show the distribution of the two main *Aedes* species. The areas are expected to expand as climate change continues; 0 = modeled as unsuitable for *Ae*. spp., 1 = modeled as suitable for *Ae*. spp. (Figure 2 from Kamal et al., 2018).

Arboviruses are transmitted from female mosquitoes to vertebrate hosts, in the case of DENV and ZIKV to primates (monkeys and humans), leading to viral replication in the host. The cycle is closed through host-to-mosquito transmission during the bite of a female *Aedes* mosquito at viremic stages (Lazear and Diamond, 2016; Musso and Gubler, 2016; WHO, 2020). Apart from vector transmission ZIKV has also been reported to be sexually transmitted with virus being detected in semen even when the blood tested negative (Lazear and Diamond, 2016; Musso et al., 2015). Furthermore, perinatal transmission was observed in French Polynesia where serum from mother and child, as well as the mother's breast milk tested positive for ZIKV RNA (Besnard et al., 2014). In Brazil, ZIKV RNA was found in amniotic fluid, blood and tissue samples from newborns with Congenital Zika Syndrome (CZS) suggesting that transplacental transmission also occurs. Although not published, there have been reports of transmissions through blood transfusion in Brazil.

As other arboviruses have been shown to transmit through transfusions, this mode of transmission should also be suspected for ZIKV (Musso and Gubler, 2016; Lazear and Diamond, 2016). Since the American epidemic starting in 2015, ZIKV has also reemerged in Africa, more specifically in Cape Verde in late 2015 with 5,000 reported cases. As Cape Verde is a popular tourist destination for Brazilians, it is believed that the virus was imported and able to spread through an abundant population of *Ae. aegypti* (Musso and Gubler, 2016). Furthermore, imported cases of ZIKV have been reported from parts of Europe, including Germany, France, Italy and Spain, baring the possibility of ongoing transmission through endemic *Ae. albopictus* (Musso and Gubler, 2016).

1.3 Biology

Flaviviruses are single-strand, positive sense, enveloped RNA viruses. The non-segmented viral genome consists of about eleven kilobases (Lazear and Diamond 2016; Musso and Gubler, 2016; Tremblay et al., 2019). There are four DENV serotypes which share about 60 to 70 % of their entire amino acid sequence (Guzman and Harris, 2015). Notably, ZIKV is closely related to these four serotypes sharing about 43 % of amino acid identity in the viral polyprotein and in the ectodomain of the envelope protein, or E-protein (Lazear and Diamond, 2016). Between ZIKV and DENV a conservation of about 68 % can be observed in the non-structural proteins, which are the principle antigen targets of the T-cell response against DENV (Rivino and Lim, 2017). Notably, through phylogenetic analyses of the nonstructural proteins, ZIKV could be shown to cluster with encephalitic viruses in the flavivirus genus, but analyses of the E-protein showed a clustering with DENV (Barba-Spaeth et al., 2016). Monoclonal antibodies (mAbs) directed against the envelope dimer epitope (EDE) could be shown to efficiently neutralize ZIKV similarly to the effect on DENV, but mAbs directed against the fusion loop epitope (FLE) could not, although the antibodies were shown to bind to the highly conserved antigen (Barba-Spaeth et al., 2016).

Flaviviruses encode three structural proteins, the capsid (C) protein, the precursor membrane/membrane (prM/M), and the envelope (E), as well as seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Lazear and Diamond, 2016;

Mukhopadhyay et al., 2005; Musso and Gubler, 2016; Tremblay et al., 2019). While ZIKV and DENV particles show a notable similarity in cryo-EM structures, a relevant difference can be spotted in the E-protein which may interfere with access to an important site for antibody binding (Hasan et al., 2018; Sirohi et al., 2016).

Replication of flaviviruses occurs in the cytoplasm of infected cells where the viral RNA is translated into a polyprotein which is subsequently cleaved by both host and viral proteases to form the mature viral proteins (Lazear and Diamond, 2016; Mukhopadhyay et al., 2005; Musso and Gubler, 2016; Tremblay et al., 2019). The structural proteins make up the virus particle itself: the genome is encapsulated through the C-protein, the prM-protein may aid in the chaperoning of E-protein folding, and the E-protein carries the receptor-binding domain and is also involved in the membrane fusion of the virus during the infection of a cell (Mukhopadhyay et al., 2005). While ZIKV and DENV particles show a notable similarity in cryo-EM structures, a relevant difference can be spotted in carbon densities in the area surrounding the E-protein glycosylation site at Asn^{154} and Asn^{153} in ZIKV and DENV, respectively (Hasan et al., 2018; Sirohi et al., 2016). Interestingly, glycosylation at Asn^{154}, which is seen in ZIKV and WNV, has been associated to neurotropism of West Nile virus (WNV) (Hasan et al., 2018). For ZIKV, glycosylation at Asn^{154} may interfere with access to the fusion loop and surrounding areas of the E-protein which present an important site for antibody binding (Hasan et al., 2018).

As flaviviruses undergo maturation in the host cell the appearance and build-up of their outer surface varies (Mukhopadhyay et al., 2005). Upon entering the host cell through receptor-mediated endocytosis, the acidity of the endosome creates a structural change in the E-protein leading to the fusion of the virus with cell membranes (Mukhopadhyay et al., 2005). The C-protein associated with the viral RNA dissociates which allows the viral RNA to be translated as well as replicated (Mukhopadhyay et al., 2005). In the lumen of the ER immature viral particles are assembled containing E-, prM-, and C-proteins, as well as viral RNA; later the prM-protein is cleaved through host furin proteases in the *trans*-Golgi network to create mature virus particles which can leave the host cell through exocytosis (Mukhopadhyay et al., 2005). Often, only a partial cleavage of the prM-protein can be observed leading to virus particles with different amounts of prM-protein varying

from completely immature particles retaining almost all prM-proteins, to mosaic particles containing some cleaved M-proteins, to fully mature particles (Junjhon et al., 2010). In studies performed by Junjhon et al. (2010), 30-40 % of prM-protein was found to be present in extracellular DENV 2 particles. As seen in Fig. 4, in contrast to mature DENV, which displays a smooth surface, the immature virion has prM proteins extending over the surface leading to a “spiky” appearance (Mukhopadhyay et al., 2005; Zhang et al., 2003).

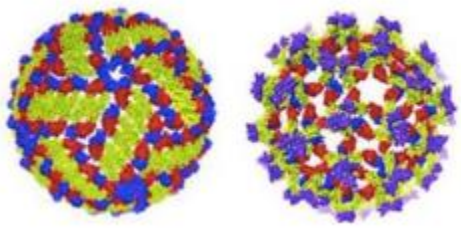

Fig. 4: Flavivirus mature and immature particles: The figure shows the structure of a mature flavivirus particle (left) and an immature flavivirus particle (right). The smooth outer surface of the mature virus is shown in stark contrast to the appearance of protruding arrangements of E and prM proteins in the immature virus particle (Figure 1 reprinted from Current Opinion in Virology, Vol 2, Pierson and Diamond, Degrees of maturity: the complex structure and biology of flaviviruses, 168-175, 2012 with permission from Elsevier, License Number 5245910910860.)

This difference in the amount of prM-proteins present on the virus could be important for interactions with antibodies. Two important groups of antibodies are those aimed at the E-dimer epitope (EDE), which joins two E-proteins together, and the fusion loop epitope (FLE), localized on a different part of the E-protein (Dejnirattisai et al., 2015). Monoclonal antibodies to both the FLE and the EDE neutralized all four dengue serotypes, but when the neutralization capacity was studied at different stages of virus maturation it was shown that EDE antibodies did not attach to immature virus particles retaining close to 100 % of prM, whereas FLE mAbs showed better binding to those particles with a higher amount of retained prM. Furthermore, both groups were shown to cause antibody dependent enhancement, but the FLE mAbs caused about a four- to eight-fold higher enhancement (Dejnirattisai et al., 2015). While there is not much published research on the detailed

process of ZIKV replication, the crystal structure of ZIKV has been shown to be similar to those of other flaviviruses supporting that a similar cycle of events is likely to occur during ZIKV infection (Dai et al., 2016). One notable difference in the structure of ZIKV is a positively charged patch found at the binding area of EDE antibodies which could influence how these antibodies attach to the virus particle (Dai et al., 2016).

1.4 Disease

A wide range of symptoms can be caused by flaviviruses making it difficult to distinguish which pathogen is the cause of illness. Symptoms can vary from asymptomatic to life-threatening, though the latter is rare (WHO, 2020; WHO, 2018). Both DENV and ZIKV infections usually present with flu-like symptoms such as fever, myalgia and arthralgia after about three to eight days following infection (Guzman and Harris, 2015; Lazear and Diamond, 2016; Musso and Gubler, 2016; Tremblay et al., 2019; WHO 2018; WHO, 2020). The differences in clinical presentation of these two viruses can be associated with differing preferences in the cell types which are infected (Guarner and Hale, 2019). While DENV displays tropism for endothelial cells, ZIKV is neurotropic as it can cross the blood-brain barrier and infect neurons and other cells in the brain (Guarner and Hale, 2019). Tab. 1 shows common symptoms of each disease, as well as possible complications.

Tab. 1: Overview of symptoms and complications of dengue fever and zika disease (Tremblay et al., 2019; WHO 2018; WHO 2020).

Virus	DENV	ZIKV
Symptoms	High fever (> 40 °C) Severe headaches Retroorbital pain Myalgia and arthralgia Nausea Vomiting Swollen glands Rash	Fever Rash Conjunctivitis Myalgia and arthralgia Headaches
Complications	Severe abdominal pain Persistent vomiting Tachypnoea Bleeding gums Fatigue Restlessness Hematemesis Increase in hematocrit with rapid decrease of platelet count	Guillain-Barré syndrome Neuropathy Myelitis CZS in newborns of mothers infected during pregnancy

1.4.1 Dengue

In 2009 the WHO introduced a new classification system for DENV with emphasis on the presence of warning signs (Tremblay et al., 2019). Dengue without warning signs pertains to individuals displaying high fever as well as two of the symptoms seen in Tab. 1 under DENV symptoms (Tremblay et al., 2019). Around three to five days following the onset of the abrupt fever, a macular-papular rash beginning on the trunk, before spreading to peripheral areas, can often be observed (Bäck and Lundkvist, 2013). The classification of dengue with warning signs, also known as dengue hemorrhagic fever (DHF), consists of the above-mentioned symptoms plus complications listed in Tab. 1. Complications in DENV infections arise largely through a cytokine-mediated heightened permeability of the capillaries (Guzman and Harris, 2015; Tremblay et al., 2019) which can result in bleeding and multiple organ failure (Tremblay et al., 2019). The severity of DHF is largely defined by the amount of plasma leakage, indicated by a rise in haematocrit of 20 % or the addition of ascites or pleural effusion, and can further be split into four varying grades (I – IV) with grade IV representing the most severe condition (Khetarpal and Khanna, 2016; Tremblay et al., 2019). Severe dengue, or dengue shock syndrome (DSS) is defined when

excessive plasma leakage, organ failure, or extensive loss of blood occur (Tremblay et al., 2019). While severe dengue is quite rare, observed in only 1 % of cases, the high mortality rate at a maximum of 20 % when untreated creates an important health concern (Uno and Ross, 2018). Heterotypic secondary DENV infections often lead to dengue fever (DF), but 2-3 % lead to dengue hemorrhagic fever (DHF) (Khetarpal and Khanna, 2016). As of yet, treatment remains symptomatic (Tremblay et al., 2019, WHO 2018; WHO 2020). The only approved vaccine against DENV does not show great efficacy and has even caused safety questions, particularly in seronegative individuals (Tremblay et al., 2019).

1.4.2 Zika

ZIKV usually presents as a self-limiting illness without severe complications as described above, but during the outbreak in French Polynesia a 20-fold higher rate of Guillain-Barre-Syndrome (GBS) was seen (Musso and Gubler, 2016). Additionally, in 2015-2016, during the outbreak in the Americas, a very high rate of microcephaly and other anomalies in neonates were observed (Oliveira et al., 2016; Schuler-Faccini et al., 2016). The WHO declared a global health emergency which led to a retrospective examination of the French Polynesian outbreak finding 17 central nervous system malformations in neonates with a temporal relationship to ZIKV (Musso and Gubler, 2016). To this day, there is no antiviral therapy for ZIKV and treatment remains symptom-oriented (Musso and Gubler, 2016).

In total, in 2015 and 2016 there were 1,673,272 reported cases of ZIKV, 2.5 % of which were in pregnant women; during the same time 1950 cases of infection-related microcephaly occurred, 70.4 % being in northeast region of Brazil (Oliveira et al., 2017). Many case reports and studies emerged describing different anomalies which became known as CZS (closer defined by Moore et al. (2017) through a review of available reports). CZS encompasses structural anomalies and functional impairment as a result of central and possibly peripheral nervous system damage (Moore et al., 2017). An important aspect was the differentiation to other congenital diseases and malformations, resulting in five common features: “severe microcephaly [more than 3 standard deviations under the norm], “thin cerebral cortices with subcortical calcifications, macular scarring and focal pigmentary retinal mottling, congenital contractures, and marked early hypertonia and symptoms of extrapyramidal involvement” (Moore et al., 2017). Retrospectively, when

asked about ZIKV infection during their pregnancy, 67.1 % of mothers who gave birth to children with CZS reported their infection to have been during the first trimester. A rash was the most common symptom described during pregnancy in 45.7 % whereas only 27.8 % reported fever (Oliviera et al., 2017). Of the women who described a rash, 77 % reported the appearance in the first, 18 % in the second, and 5 % in the third trimester (França et al., 2016). The head circumference of newborns measured differed with the expression of a rash during pregnancy with the highest deviation from the norm correlating with the rash in the first trimester, though brain damage was also detected in children who's mothers' reported rashes later in the pregnancy (França et al., 2016). About 30 % of children with a suspected *in utero* ZIKV infection showed significant retinal and optical nerve abnormalities (Lazear and Diamond, 2016). Case studies showed that ZIKV could be detected in fetal brain and placenta tissue after suspected ZIKV infection during the first trimester of pregnancy in women living in Brazil during the epidemic (Martines et al., 2016; Mlakar et al., 2016).

Even before the development of animal models to show the effect of ZIKV on the fetus, Rasmussen et al. (2016) determined substantial evidence to assume a causal link between prenatal ZIKV infections and congenital malformations such as microcephaly. Since then a great amount of evidence has come forth to further strengthen this statement. Cugola et al. (2016) showed that the ZIKV strain present in Brazil during the outbreak not only infected fetuses in a murine model, but also caused intra-uterine growth restrictions such as microcephaly. The virus can also be shown to infect human cortical progenitor cells leading to an increased rate of apoptosis, as well as infection of human brain organoids inducing results consistent to abnormalities found in children with CZS (Cugola et al., 2016). A further mouse model showed an accumulation of ZIKV in the placenta with a 1000-fold concentration when compared to maternal serum (Miner et al., 2016). The model also displayed infection of different trophoblasts within the placenta as well as vascular injury to fetal capillaries and small placental vessels all of which could lead to damage of the placental barrier making a fetal infection possible (Miner et al., 2016). Apart from murine models, non-human primate (NHP) models have also been developed to study the effect of ZIKV infection. Early infection with ZIKV during pregnancy in macaques resulted in neuropathology of the fetuses or in fetal loss congruent with findings in reported

human cases such as microcalcifications, hemorrhage and necrosis (Martinot et al., 2018). In 66 % of infected macaques significant pathologies in the placenta were observed (Martinot et al., 2018). Furthermore, vascular changes and neuroprogenitor cell dysfunction, in 66 % and 83 % of cases respectively, were also observed in the infants of the infected macaques (Martinot et al., 2018). Even after inoculation of pregnant macaques during the second and third trimesters of pregnancy significant brain lesions were seen in the fetus, as well as presence of ZIKV RNA in fetal tissues including the brain and eyes (Adams Waldorf et al., 2016; Coffey et al., 2018).

1.5 Diagnostics

Laboratory diagnostics of flavivirus infections can be divided into different groups depending on the biomarkers which are used: virus itself, products produced by the virus, or the immune response of the host (Muller et al., 2017). The long-established method of virus isolation, where blood from infected patients until five days after onset of symptoms is cultured on cells, is no longer method of choice (Muller et al., 2017). While the RT-PCR is specific and sensitive during the acute phase of disease, it requires particular laboratory equipment which is often not available in endemic areas (Muller et al., 2017). NS1 is secreted by flaviviruses and can be found in the blood of patients for around ten days making it an excellent target for diagnostic tests. Furthermore, as summarized by Muller et al. (2017) it has been shown to be a marker for the amount of virus present in patients and can be used to predict the advancement to severe disease in dengue. ELISA and strip tests used to detect NS1 have become the new standard for acute-phase dengue diagnosis as they require less specialized equipment while providing high specificity as well as sensitivity (Muller et al., 2017). A notable downside of the NS1 detection tests is the minimized capture of the antigen in secondary dengue infections because of the earlier activity of antibodies which react with the antigen leading to immune complexes which in turn are not detected by the tests (Muller et al., 2017).

Serological detection of flaviviruses focuses on immune response of the host and is therefore better for convalescence or past-acute infection. Possibilities as summarized by Muller et al. (2017) are: hemagglutination inhibition assays (HI), complement fixation tests,

dot-blot assays, Western blots, immunofluorescent antibody tests, ELISAs to detect IgM and IgG, and plaque reduction neutralization tests (PRNT). One important fact to keep in mind is the window of false-negative results in which the body has not yet produced antibodies against the infecting virus. IgM can be found about three to five days post infection, whereas IgG are not seen in the acute phase of disease during primary cases, although a steep rise in IgG titers can be detected about three days following a secondary infection (Muller et al., 2017). In areas where several flaviviruses circulate, serological diagnostics can be complicated through the cross-reactive nature of antibodies often aimed at the conserved E-protein (Muller et al., 2017). The neutralization assay is seen as the gold standard to measure the titers of anti-DENV-antibodies, though interlaboratory reproducibility is often low (Guzman and Harris, 2015). It is seen as the most specific serological test for flaviviruses, but remains time-consuming, often requires biosafety level 3 laboratories (depending on the virus), is expensive and depends on well trained staff for reliable results (Maeda and Maeda, 2013).

1.6 Immunological aspects

The antiviral immune response can be divided into an initial, unspecific innate response and a lagged adaptive response. During an infection with flaviviruses the importance of the innate immune system becomes apparent through the usually short and self-limiting characteristics of the illness (Ngono and Shresta, 2018). As summed up by Ngono and Shresta (2018), evidence pointing to the importance of the interferon type I system, consisting of IFN α and β, during flavivirus infection includes high serum levels of type I interferons in acute febrile portions of the disease, as well as experiments showing that certain interferon-stimulated genes hinder DENV and ZIKV infections. Flaviviruses have to implement methods to evade this strong innate antiviral defense in order to infect and cause illness in vertebrate hosts (Ngono and Shresta, 2018). Several DENV proteins, such as NS2A and NS5, have been shown to impede interferon type I antiviral effects through inhibition of important signaling pathways; it was anticipated that ZIKV uses similar evasion methods because of the similarity between the viruses (Ngono and Shresta, 2018). This anticipation was confirmed as NS5 was shown to be a robust antagonist in the signaling cascade involving interferon type I during ZIKV infection,

specifically of the type I interferon receptor preventing the necessary phosphorylation of signal transducers and activator of transcription (STAT 1 and 2) which in turn impedes the activation of interferon stimulated genes (Bowen et al., 2017; Hertzog et al., 2018; Kumar et al., 2016; Schilling et al., 2020). Release of type I interferons is prompted through cellular pattern recognition receptors which can activate transcription factors to induce interferon and cytokine production which in turn aid in the further coordination of innate and adaptive responses of the immune system (Ngono and Shresta, 2018).

Adaptive immune responses are mediated through lymphocytes, specifically B- and T-cells. Through a complex activation system utilizing humoral and cell-mediated pathways, B and T-cells respectively are activated. In turn B-cells produce antigen specific antibodies, later also forming memory B-cells which can be activated more quickly during a second infection. T-cells aid in the immune response through a plethora of ways including cytotoxic T-cells, inducing activation of B-cells, as well as the secretion of different cytokines further activating the immune response (Lüllman-Rauch, 2006). The role of the adaptive immune response in DENV infections, whether playing a protective or a pathogenic role, continues to be a topic of discussion (Ngono and Shresta, 2018).

1.6.1 Antibody dependent enhancement

After a primary DENV infection lifelong immunity against that serotype and transitory immunity against heterotypic serotypes is produced (Guzman and Harris, 2015). If a second heterotypic infection develops within two years of the first infection there seems to be limited protection through cross-reactive antibodies leading to asymptomatic or mild illness (Anderson et al, 2014). Furthermore, after reviewing hospitalization rates during three different DENV outbreaks in Cuba, a much higher number of severe disease was recorded during the outbreak 20 years after the initial infection rather than the epidemic only four years later strengthening the hypothesis that partial cross protection of different DENV serotypes decreases over time (Guzmán et al, 2002).

*D*uring a secondary heterotypic DENV infection, circulating antibodies bind to, but do not neutralize, the virion and allow a Fcγ receptor-mediated entry into cells such as monocytes and macrophages; this process is known as antibody-dependent enhancement (ADE)

(Dejnirattisai et al., 2010; Guzman and Harris, 2015; Halstead and O'Rourke, 1977). Severe DF and DHF are more often seen in secondary DENV infections or in infants of dengue-immune mothers displaying a primary infection (Guzman and Harris, 2015). Not only does ADE lead to an increase in the number of infected cells through mediated uptake, it also curbs innate cellular immunity because immune complexes decrease the generation of reactive nitrogen radicals, INF-α and INF-β while causing a surge in IL-10 concentrations (Halstead, 2014).

Patients with DHF showed lower levels of NO, decreased transcription of IFN and greater IL-10 serum levels in comparison to those who suffered only from mild DF (Halstead, 2014). Antibodies produced from B-cells of previously DENV infected patients were examined for either a reaction to structural proteins using the whole DENV or non-structural ones utilizing NS1 (Dejnirattisai et al., 2010). Those antibodies which showed a reaction against the whole DENV, therefore to structural proteins, were shown to consist of anti-prM antibodies and anti-E antibodies with 60 % and 40 % respectively, and both show high cross reactivity to other DENV serotypes (Dejnirattisai et al., 2010). Specifically, poorly neutralizing and cross-reactive antibodies against the FLE of domain II of the E-protein and against the prM-protein seem to dominate the immune response to DENV (Ngono and Shresta, 2018). The most potent serotype specific antibodies against DENV have been found to be directed against domain III of the E-protein, although these tend to only make up a minority of all DENV-specific antibodies (Ngono and Shresta, 2018). As a whole, anti-prM antibodies were not shown to neutralize DENV infection, but rather displayed a 10 -to- 800-fold ADE and also lead to increased infectivity through partially immature virus displaying a greater portion of prM antigen (Dejnirattisai et al., 2010).

1.6.2 Original antigenic sin

Another theory proposed to explain the higher occurrence of severe disease in secondary heterotypic DENV infections is original antigenic sin. This concept was first studied in relation to antibodies, concluding that when a second challenge with a heterologous but cross-reactive antigen was undertaken, the new antibodies formed showed a superior reaction to the primary antigen rather than the current one (Fazekas de St Groth and Webster, 1966). This concept has since been applied to the T-cell responses. The reason

that original antigenic sin is proposed as a theory to explain DHF and DSS is through a massive activation and subsequent death of CD4+ and CD8+ T-cells with massive cytokine release resulting in plasma leakage (Mongkolsapaya et al., 2003). There have been various studies that support original antigenic sin in the development of DHF (Duangchinda et al., 2010; Rivino, 2018). On the other hand, there have been several studies published which push towards a protective role of T-cells during DENV infection (Rivino, 2018). Weiskopf et al. (2013) found no difference in the function or response of T-cells in secondary heterotypic infections of DENV, even though original antigenic sin could be observed, as the response showed affinity to conserved portions of the virus.

1.7 Goals and aims of this project

The 2015-2016 ZIKV epidemic in South America suggested an association between the virus and congenital malformations, known as CZS. While the epidemic spread throughout the continent, the highest incidence of CZS was observed in north-eastern Brazil, raising the question of cofactors affecting the risk of CZS. There are four subtypes of DENV (1-4) which are endemic in many parts of the world, including South America, especially Brazil. The genetic similarities between ZIKV and DENV raise the question of immune interaction, such as ADE and original antigenic sin which have been controversially discussed in the literature (Dejnirattisai et al., 2016; Pantoja et al., 2017). Nevertheless, as DENV is endemic in all Brazil, it would not explain the higher rate of CZS in north-eastern Brazil. Therefore, a DENV-mediated effect would require specific differences in past DENV exposure.

During the ZIKV outbreak in Brazil, serum from mothers, who gave birth to children with or without CZS, was obtained from Salvador, Brazil. These sera were tested for ZIKV antibody titers using ELISA, and those who tested positive were further tested for ZIKV neutralizing antibodies using PRNT (Moreira-Soto et al., 2017). Results showed significantly higher ZIKV neutralization titers in mothers who had given birth to children with CZS compared to those who had given birth to children without (Moreira-Soto et al., 2017). Reinforcing the relationship of high ZIKV antibody titers with the CZS, the sera were tested for CHIKV, DENV, RUBV, HSV-1, HSV-2, VZV, CMV, PV-B19, *C.*

trachomatis, B. pertussis, T. pallidum, and T. gondii showing no significant differences between the two groups, and only ZIKV was significantly associated through conditional logistic regression analyses (Moreira-Soto et al., 2018). As DENV is endemic in all of Brazil, the question arose whether specific differences in DENV exposure correlated to the probability of CZS.

We hypothesized that "*DENV antibodies present in the serum of mothers led to interaction with ZIKV particles, much like an infection with a different DENV serotype would lead to a higher viral load or protection depending on the different constellation of DENV antibodies present."*

The goals of this dissertation can be divided into two main parts:

1) The establishment and validation of DENV serotype-specific neutralization assays. The high rate of cross-reactivity among serotypes and other flaviviruses remains a hurdle to overcome when performing assays to determine antibody titers, especially in sera from endemic regions.

2) Determining the DENV serotype-specific antibody titers of Brazilian mothers who gave birth to children with or without CZS during the ZIKV epidemic in 2015- 2016.

   The results will then be analyzed for differences in exposure to DENV serotypes.

2 . Materials and methods

2.1 Materials

2.1.1 Cell lines

Tab. 2: Utilized cell lines and respective supplier

Cell line	Supplier
BHK-J	Kindly provided by Charles M. Rice, New York, USA
C6/36	University of Bonn Medical Centre, Institute of Virology
Vero B4 and E6/7	University of Bonn Medical Centre, Institute of Virology

2.1.2 Mediums

Tab. 3: Utilized Mediums and respective manufacturer

Product	Manufacturer
Carboxymethylcellulose sodium salt	Sigma Life Science
Fetal Bovine Serum Gold	GE Healthcare, PAA Laboratories GmbH
Dulbecco's Phosphate Buffered Saline (DPBS 1x)	gibco® by life technologies™
Leibovitz's L-15 Medium (1x) +L-Glutamine, + L-Amino Acids	gibco® by life technologies™
Dulbecco's Modified Eagle Medium	gibco® by life technologies™
Minimum Essential Medium	gibco® by life technologies™

2.1.3 Viruses

Tab. 4: Dengue virus strains used in propagation, plaque assays and plaque reduction neutralization tests and respective supplier.

Serotype	Strain	Genotype	Origin	Supplier
DENV 1	16007	II	Thailand	Kindly provided by Jonas Schmidt-Chanasit, of the Bernhard-Nocht Institute Hamburg, Germany
DENV 2	16681	Asian I	Thailand	
DENV 3	H87	V	Philippines	
DENV 4	H241	I	Philippines	

2.2 Methods

2.2.1 Study cohort

As is described in Pedroso et al. (2019) we used sera from Brazilian mothers attained after delivery from May 2015 through December 2016 in the maternity ward in the University of Bahia Climério de Oliveira. As is stated in our paper, the Institutional Research Ethics Board approved this study under protocol number 1.408.49. All participants of the study accepted participation in the above-named protocol. In our study, 29 mothers of children who gave birth to children with CZS (cases) and 108 mothers who gave birth to children without CZS (controls) all from Salvador, northeastern Brazil were included. Moreira-Soto et al. (2017) showed past ZIKV exposure through ELISA and PRNT. Age distribution analyzation was done by Carlo Fischer and is noted to be not significantly different between cases and controls (Pedroso et al., 2019).

2.2.2 CZS diagnosis

The diagnosis of CZS was carried out by attending gynecologists at the University of Bahia Climério de Oliveira using characteristics previously discussed in the introduction portion and outlined by Moore et al. (2017). The lead symptom was microcephaly being in this case defined as the cephalic circumference of the newborn measuring two standard deviations under the normal values for same aged neonates using intergrowth charts from the WHO along with clinical and imaging data (Pedroso et al., 2019). Other symptoms leading to the diagnosis of CZS included other neurologic birth defects such as intracranial calcifications, ventriculomegaly, dysgenesis of the corpus callosum, Dandy-Walker-like malformations, hydranencephaly, porencephaly, hydrocephalus, severe intracranial calcifications and reduced brain tissue (Moreira-Soto et al., 2018; Pedroso et al., 2019).

2.2.3 Cell culture

This study was done *in vitro* using cell cultures of Vero, BHK-J, and C6/36 cells. To utilize these cells in the following experiments and studies, a population of the individual cell types was cultured and grown in flasks. Vero cells were propagated in DMEM containing 1 % L-glutamine + 10 % FBS and BHK-J cells in MEM-medium containing 1 % L-glutamine

and 1 % NEAA with 7.5 % FBS, at 37 °C and 5 % CO_2. C6/36 cells required L15 medium + 5 % FBS at 28 °C. The cells were passaged in accordance to their growth, allowing a confluency of 80 – 90 % before the next passage. Cells were passaged by removing the supernatant from the flask. BHK-J and Vero cells were then washed once or twice, respectively, using PBS. To release cell adherence, the afore mentioned cell lines were trypsinized utilizing 1 mL of trypsin then incubated for 5 minutes at 37 °C + 5 % CO_2. Loss of cell adherence to the flask was evaluated using a microscope. Once the cells were seen to have lost adherence the correct medium for each cell line was added to the flask to stop the trypsin reaction. Cells were resuspended in the medium and either split in suitable ratios in a new flask onto which the respective medium was added to allow further growth of the cells, or the cells were counted (2.2.4) for use in following experiments. Adherent C6/36 cells were released using a cell scraper and 5 mL of fresh respective medium. Loss of cell adherence was evaluated using a microscope, and cells were resuspended as described above.

2.2.4 Seeding cells

To properly perform plaque assays and plaque neutralization tests which are described later, a confluent or nearly confluent cell monolayer with a determined cell count is required. After resuspension of cells utilizing the methods in 2.2.3, 50 µL of cell solution and 50 µL of trypan blue were combined into an Eppendorf tube and mixed well using a pipette. This solution was then inserted into a Neubauer counting chamber used to determine the number of cells in 1 mL of solution. To attain the number of cells, cells in four quadrants of the cell counter were averaged. This number was multiplied by the dilution of the cell suspension, which is 2 in this case because of the 1:1 dilution with trypan blue, and by the factor 10^4 resulting in the number of cells per mL of solution. The volume needed to attain the desired cell count for seeding according to plate or flask size was then calculated and multiplied by the number of wells or flasks needed. The resulting volume was the amount of cell suspension solution required to attain the desired number of cells per well or flask. The calculated cell suspension volume was then subtracted from the overall required volume for the flask or well in question, and a mastermix of cell solution and fresh medium was made and well mixed using a pipette. Depending on the

cell type and the size of the plate the number of cells used varies and is described in the individual protocols.

2.2.5 Virus propagation

To find the optimal protocol for viral propagation, three cell lines: Vero B4, BHK-J and C6/36 cells were seeded into 12- well-plates using $1.5*10^5$, $2*10^5$, and $4*10^5$ cells per well respectively on the day prior to infection. The wells were incubated at 37 °C and 5 % CO_2 for Vero B4 and BHK-J, and 28 °C for C6/36 cells overnight until cells were 80-90 % confluent. On the day of infection medium was removed from the cell monolayer and the cells were inoculated with DENV (Tab. 4) using a multiplicity of infection (MOI) of 0.01 in a total infection volume of 200 µL (virus + medium). An incubation at 37 °C and 5 % CO_2 for Vero B4 and BHK-J cells, and at 28 °C for C6/36 cells for one hour followed. Subsequently, the inoculum was removed and the wells were gently washed with PBS. Lastly, 2.5 mL of respective medium was added to each well and allowed to incubate under the before mentioned conditions. Each day for 7 days, 100 µL of supernatant was removed from each well and frozen at -80 °C for titration through plaque assays on Vero B4 cells (described later). To produce larger quantities of the virus stocks, C6/36 cells were seeded at $2.5*10^6$ cells per T75 flask and allowed to reach 80-90 % confluency on the day prior to infection. The following day, medium was removed from the cell monolayer, and the cells were inoculated with DENV (Tab. 4) using a MOI of 0.01 in a total infection volume of 3 ml (virus + L15). After one hour of incubation at 28 °C 9 ml of L15 + 2.5 % FBS were added. After 5 days at 28 °C the supernatant was collected and centrifuged at 1200 rpm for 10 minutes, pooled and mixed, then aliquoted and frozen at -80 °C.

2.2.6 Molecular biological methods

2.2.6.1 DENV serotyping and sequencing

The four DENV subtypes are well known to cross-react because of their close genetic and antigenic relations. The first step in assuring subtype specific tests was to identify each subtype of the virus. For this we used a reverse transcriptase polymerase chain reaction

(RT-PCR) with primers targeting a region in the conserved DENV NS5 gene, known as a PanFlavi PCR that amplifies all DENV serotypes (Crochu et al., 2004).

Using the NucleoSpin® RNA Isolation Kit viral RNA was isolated following the manufacturer's instructions (MACHEREY-NAGEL GmbH & Co. KG, 2011). Subsequently, the PCR was conducted as described by Crochu et al, 2004 according to the parameters shown in Tab. 5. The PCR product was visualized on a 2 % agarose gel containing 1 µL of ethidium bromide. The DNA band corresponding to 290 base pairs was excised under UV light, and the DNA was extracted using NucleoSpin® Extract II Kit (Clontech Laboratories Inc, 2011). The PCR products were sent to be sequenced at which point the genomic sequence was used to confirm the subtype of each virus.

Tab. 5: PanFlavi RT-PCR
a) PanFlavi RT-PCR Primers
b) PanFlavi RT-PCR Reaction
c) PanFlavi RT-PCR Cycle-Protocol

a

PF1S	TGYRTBTAYAACATGATGGG
PF2R-bis	GTGTCCCADCCDGCDGTRTC

b

H_2O	1.5 µL
IVT 2x RM	6.25 µL
BSA (1 mg/ml)	0.5 µL
Fwd (10 µM) PF1s	0.625 µL
Rev (10 µM) PF2Rbis	0.625 µL
IVT SS III OneStep RT-PCR Enzyme Mix	0.5 L µL
Template RNA	2.5 µL

c

50 °C	30’	
95 °C	3’	
94 °C	15’’	10 x
55 °C	20’’	
72 °C	30’’	
95 °C	15’’	40 x
50 °C	20’’	
72 °C	30’’	
72 °C	5’	
*Touch Down 0.5 °C per cycle		

2.2.7 Plaque assay

Plaque assay tests are based on the cytopathic effect of a virus. The virus causes infected cells to die, creating holes in the cell monolayer, known as plaques, which can be visually counted after staining of the remaining cells. For a consistent read-out of these tests, the best conditions to visualize the plaques should be used. Before determining Vero B4 cells

with a methylcellulose overlay to be optimal, experiments followed using three types of Vero cells (Vero E6/7, Vero B4 and Vero CCL81), and three overlays (agarose, avicell and methylcellulose). Each was done in 12 well-plates with $1.5*10^5$ cells per well following the protocol described below. All three overlays were in a 1:1 dilution with 2xMEM. Once optimized the following protocol was used.

Using 6, 12, or 24 well plates, Vero B4 cells were seeded at $4*10^5$, $1.5*10^5$, or $8*10^4$ cells per well, respectively, and allowed to grow confluent overnight at 37 °C and 5 % CO_2. On the day of the titration, dilutions of the propagated virus were made in DMEM + 1 % FBS ranging from undiluted to 10^{-6}. The medium was then removed from the cells and 200 µL using 6 or 12-well-plates or 150 µL in 24-well-plates of virus dilution was added to each well. The cells were incubated with the virus dilutions for 1 hour at 37 °C and 5 % CO_2. Next, 3 mL, 1,5 mL or 1 mL of overlay was added to each well in 6, 12 or 24-well-plates, respectively, consisting of a 1:1 ratio of 1.5 % methylcellulose and 2xMEM in the 6 and 12-well-plates and 2.4. % methylcellulose and 2xMEM in the 24-well-plates. 6-well-plates were incubated at 37 °C and 5 % CO_2 for 7 days, 12-well-plates for 5 days (DENV 1 and 3) or 7 days (DENV 2 and 4), and 24-well-plates for 4 days (DENV 1, 2 and 3) or 5 days (DENV 4) before the overlay was removed and the plates were fixated using a 6 % formaldehyde solution. After at least 30 minutes of complete submersion in the formaldehyde, the plates were removed and gently rinsed off using distilled water. Following this, a crystal violet solution (Ampuwa® with 0.2 % crystal violet and 20 % ethanol) was added to each well so that the entire cell monolayer was submerged and allowed to stain for 5-10 minutes. The solution was then pipetted off, and the plates were again rinsed gently with distilled water to remove excess crystal violet. The plates were left at room temperature to air dry. Finally, the plaques were visually counted and used along with the viral dilution factor and the factor to attain 1 ml of virus dilution (in this case 5 because 200 µL of virus dilution were used) to determine the titer of the virus in plaque forming units (PFU) so if 200 µL of a viral dilution of 10^{-4} leads to a plaque count of 24 in a 6-well-plate, the resulting PFU = $24*10^4 * 5 = 1.2*10^6$ PFU/ml.

2.2.8 Plaque reduction neutralization test (PRNT)

Plaque reduction neutralization test (PRNT) assays utilize the cytopathic effect of the virus just like the before mentioned plaque assays. With the help of PRNT assays the titer of neutralizing antibodies present in serum can be determined. Different dilutions of serum are incubated with the same amount of virus before the virus-serum mixture is given to the cell monolayer. During this initial incubation neutralizing antibodies present in the serum bind to the virus hindering the infectivity of the virus resulting in a reduction of visualized plaques. Fig. 5 shows a schematic example of a PRNT assay.

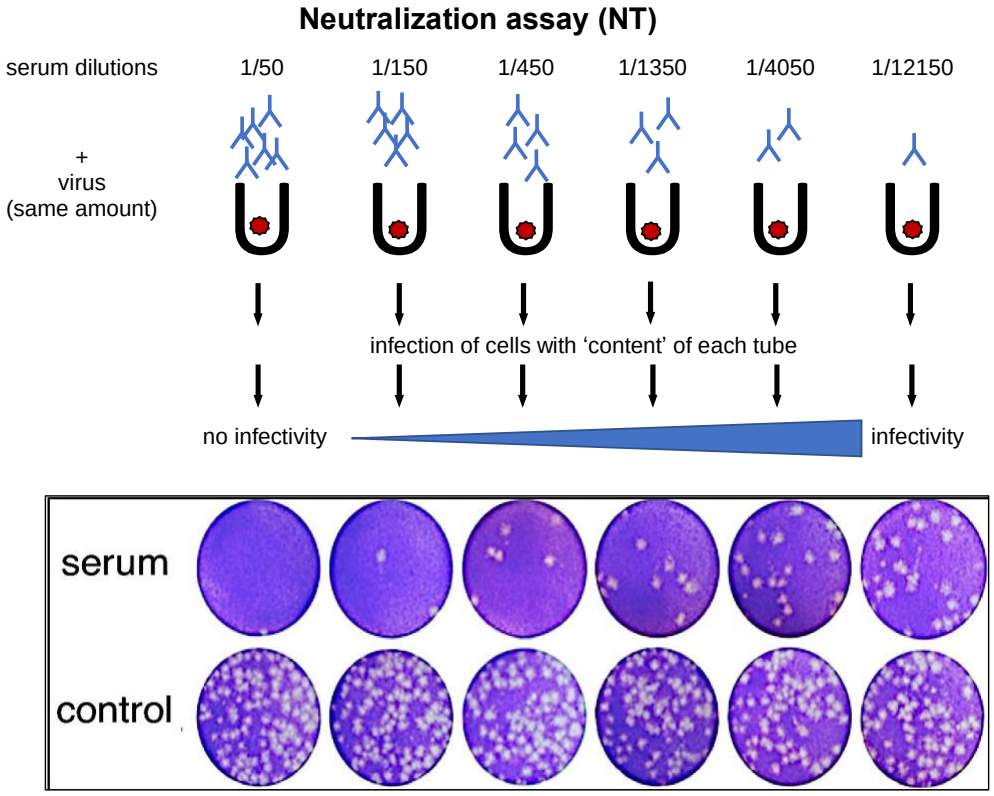

Fig. 5: PRNT schematic: Serum dilutions are allowed to incubate with virus before infection of the cell monolayer. Neutralizing antibodies, shown here using blue symbols, bind and neutralize the virus, shown here using the red symbols The serum-virus mixture is then allowed to incubate with the cell monolayer. After incubation and staining the plaque reduction caused by the neutralizing antibodies present in serum is visualized and compared to controls (containing only virus and no serum) as seen in the inlay. (adapted figure kindly supplied by Beate Kümmerer).

2.2.8.1 12-well-plates

Vero B4 cells were seeded at $1.5*10^5$ cells per well and allowed to grow confluent overnight at 37 °C and 5 % CO_2. For the neutralization test, six 3-fold serum dilutions starting at 1:50 were made with DMEM + 1% FBS. Virus dilutions were made to produce 60 plaques per well. In a round-bottom 96-well-plate 28 µL of the serum dilution was combined with 28 µL of the viral dilution and allowed to incubate for 1 hour at 37 °C and 5 % CO_2. Following incubation, medium was removed from the cells and 150 µL of DMEM + 1 % FBS was added to each well. 50 µL of the virus-serum mix was given into each well (DMEM + 1 % FBS for the control) and the plates were gently swayed. An incubation at 37 °C and 5 % CO_2 for 1 hour followed and then 1.5 ml of overlay (methylcellulose 1.5 % + 2xMEM in a 1:1 ratio) was added to each well. Plates were incubated at 37 °C and 5 % CO_2 for 5 days (DENV 1 and 3) or 7 days (DENV 2 and 4), then fixed, stained and plaques were counted as described above. The antibody titer was then determined using non-linear regression curves as described below.

2.2.8.2 24-well-plates

On the day of infection 3-fold dilutions of the sera were made starting at 1:50 and ending with 1:12150 using DMEM + 1 % FBS. Viral dilutions were made, using known titer of the virus, to produce 60 plaques per well for DENV 1, 2, and 3 and 80 plaques per well for DENV 4. Serum and virus were incubated as described above. The medium was then removed from each well containing cells, and 100 µL of DMEM + 1 % FBS was added to each well. Then 50 µL of the virus-serum mixture was added (50 µL of DMEM + 1 % FBS was used for the control) and the plates were gently swayed to allow for equal distribution. Cells and virus-serum mixtures were incubated for 90 minutes at 37 °C and 5 % CO_2, during which the plates were swayed 2-3 times to avoid drying of the cell monolayer. After incubation, the inoculum was removed and 1 ml methylcellulose 2.4 % + 2xMEM (1:1) was added to each well as an overlay. Plates were gently swayed and allowed to incubate at 37 °C and 5 % CO_2 for 4 days (DENV 1, 2, 3) or 5 days (DENV 4). Plates were fixed, stained, and plaques were counted as described. The antibody titer was then determined using non-linear regression curves as described below.

2.2.9 Statistical analyses

2.2.9.1 Interpolation of NT90

The goal of a PRNT is to attain the serum dilution or neutralization titer (NT) which sufficiently reduces the amount of plaques formed by the virus when compared to the uninhibited virus (control). Most often a NT_{50} is used, meaning the serum dilution at which the antibodies present have reduced plaque formation by 50 % when compared to the control. DENV subtypes are well known to show cross-reactivity, so we determined the NT_{90} value instead (90 % reduction) to allow for a more specific readout.

As only six serum dilutions were tested in the PRNT, a direct readout of the NT_{90} value was not always possible. The counted plaques were converted into percentages of the control and graphed against the log of the serum dilutions. Using the non-linear regression curve fit in GraphPad Prism 6 NT_{90} values were interpolated from the standard curve and verified to have $R^2 \geq 0.85$. The interpolated x-value was then used to calculate the NT_{90} titer through the formula: $1/ (10^{\text{interpolated value}}) = NT_{90}$ titer.

2.2.9.2 Statistical significance of different variables

To test statistical significance of attained data, we used bivariate comparison tests. As stated in our paper, p values were determined using two-tailed tests (Pedroso et al., 2019). Furthermore, power calculations were performed using OpenEpi (Sullivan et al., 2009) to attain 95 % CI.

2.2.9.3 Finding the best fit model

To compare different variables and their effect on the outcome of CZS, we utilized logistic regression models. For each of the 15 models, one variable was chosen to predict the binary outcome of case or control, coded as 1 or 0. The generalized linear model function of R 3.5.2 was used to model 15 variables. Using the bbmle package version 1.0.20 in R the Akaike Information Criterion, or AIC, the difference between the model and the best-fit model, or delta (AIC), and the Akaike weights were calculated to compare the different models. The likelihoods ratio and odds-ratio were also calculated for each model.

3 . Results

3.1 Method optimization

3.1.1 Verification of DENV strains

The strain and serotype of each of the utilized viruses was determined using a PanFlavi RT-PCR as described in Tab. 5. The PCR product of 290 bp was visualized in the agarose gel for all four DENV samples and subsequently excised out of the gel. The product was sent to be sequenced. After receiving the sequence, a length of 150 bp out of the NS5 region was evaluated using Nucleotide Blast® and alignment confirmed each of the four serotypes and strains seen in Tab. 4.

3.1.2 Viral propagation and growth curve

To quantify DENV antibody titers in sera, PRNT tests should be used. These tests require the incubation of infectious virus and sera. To obtain this infectious virus, it was first propagated and then titrated. To optimize titers, we grew DENV on three different cell lines: Vero B4, BHK-J, and C6/36 cells, each in duplicate. After infection with virus at MOI 0.01, supernatant was removed each day and titrated to calculate the PFU to produce a growth curve for each subtype of DENV on each cell line (Fig. 6). The optimal protocol for viral propagation was determined to be using C6/36 cells and allowing a growth period of 5 days. As C6/36 cells are derived from mosquitos, this cell line mimics best the natural life cycle of DENV and the incubation for 5 days offered the highest measured titer. The grown virus stocks with titers can be seen in Tab. 6.

Tab. 6: Virus stock titers

	Passage	Cell line	Days incubated	Date harvested	Titer (PFU/ml)
DENV 1	2	C6/36	5	04.05.17	$9.5*10^5$
DENV 2	2	C6/36	5	04.05.17	$1.2*10^6$
DENV 3	2	C6/36	5	04.05.17	$1.15*10^6$
DENV 4	2	C6/36	5	08.11.17	$6.5*10^4$

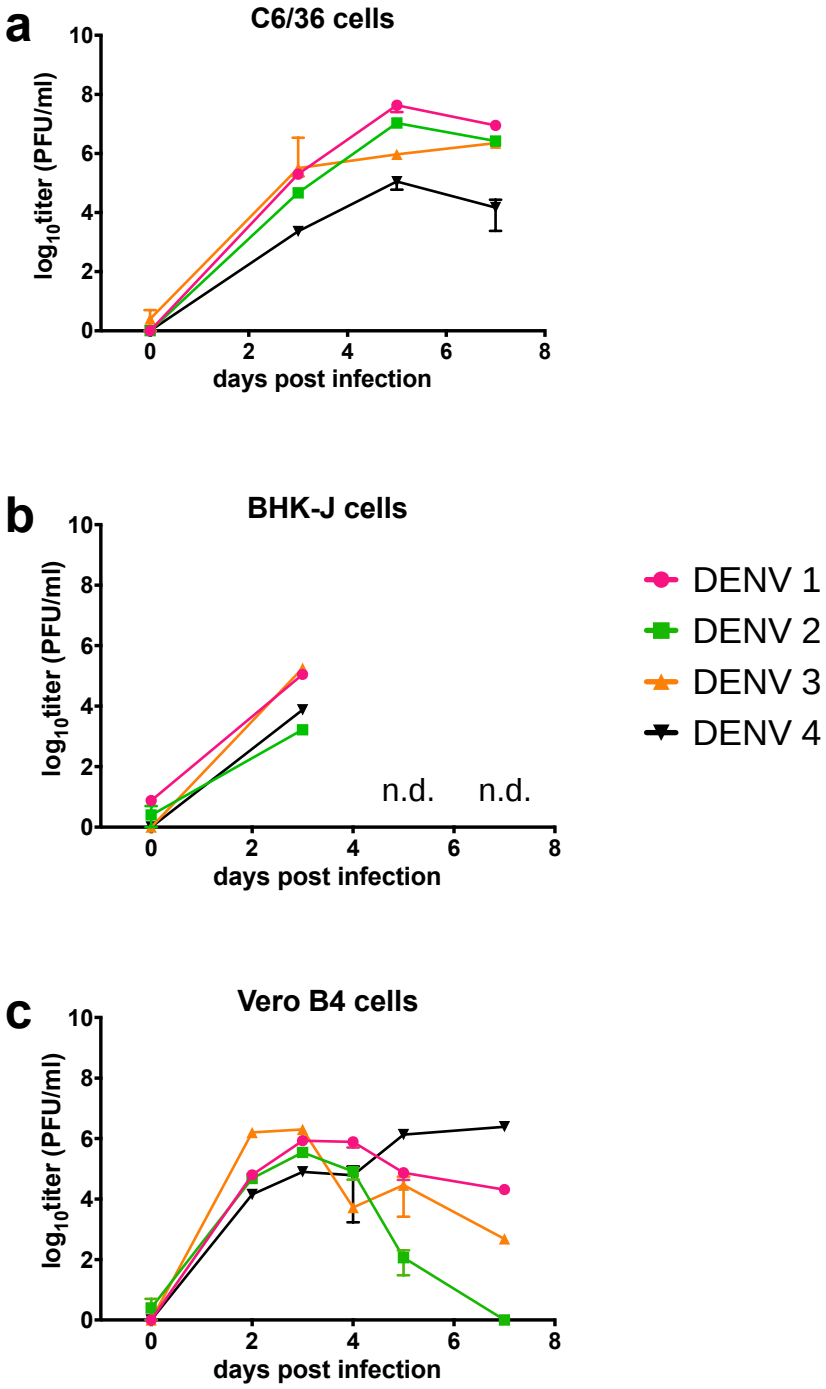

Fig. 6: DENV propagation: DENV serotypes 1-4 were propagated on cell lines with an MOI of 0.01 a) C6/36, b) BHK-J, and c) Vero B4. At the indicated time points samples were taken from the supernatant and the viral titers were determined on Vero B4 cells by plaque assay as plaque forming units (PFU). On days 5 and 7 no samples from the virus produced on BHK-J cells could be removed because the cell monolayer had become too dense for cell survival. n.d. = not determined; PFU= plaque forming units.

3.1.3 Plaque assay

3.1.3.1 Optimization of cell line, overlay and plaque number

Three cell lines (Vero E6/7, Vero B4 and Vero CCL81) and three overlays (agarose, methylcellulose and avicell) were compared in plaque assays to find the optimal protocol. The overlay was used after the virus was allowed to infect the cells for one hour to stop the spread of the virus into the supernatant, and therefore stopping the infection of further cells. This leads to clear plaque formation and makes the evaluation of the assay easier. The plaques formed on Vero B4 cells using a methylcellulose overlay provided the clearest readout as can be seen in Fig. 7. The amount of virus used was also optimized to result in the highest number of plaques while still allowing a good optic count of the

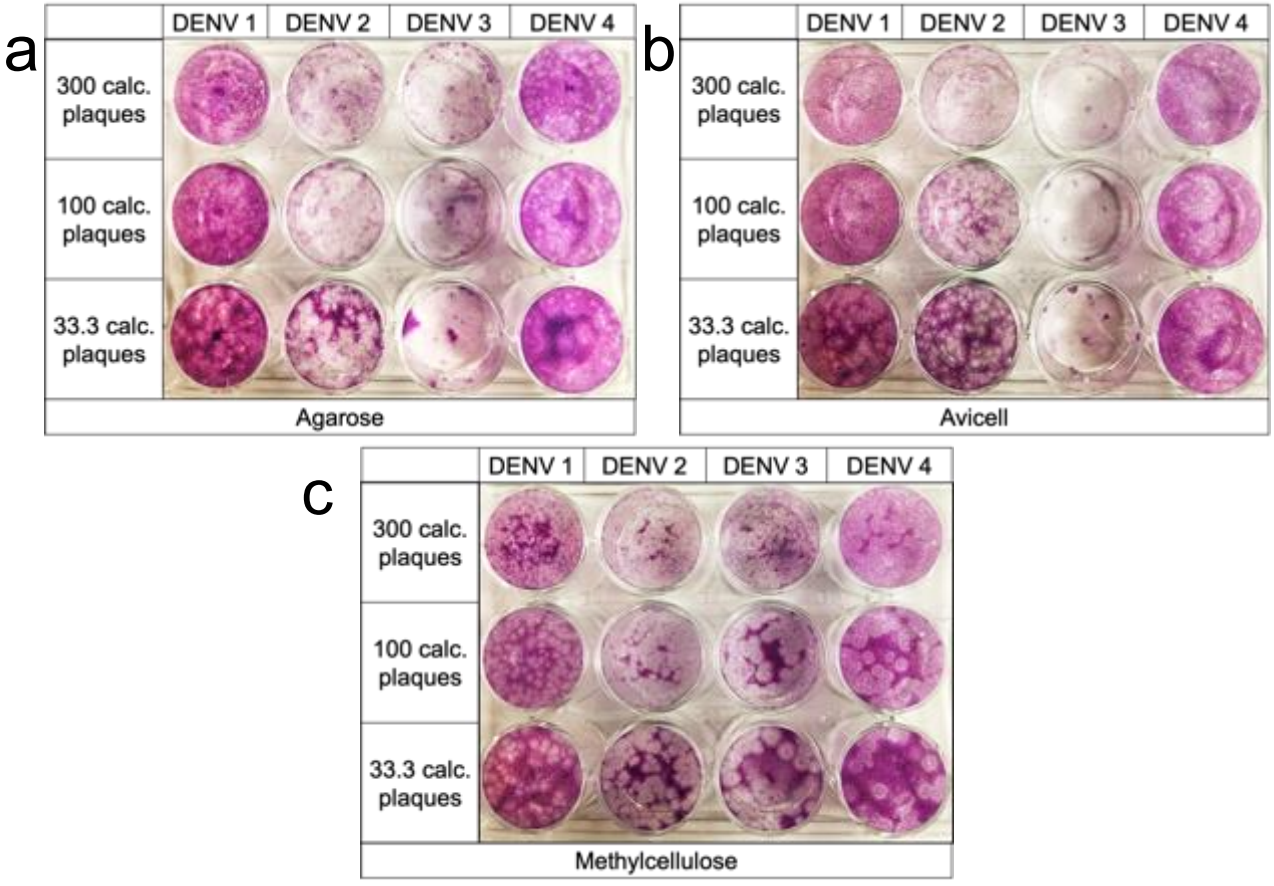

Fig. 7: Plaque assay optimization: Vero B4 cells infected with DENV 1-4 in different dilutions as labeled (calc. plaques = number of plaques calculated based on virus titer) and using different overlays being agarose (a), avicell (b) and methylcellulose(c) respectively. The clearest readout was attained using methylcellulose overlay.

individual plaques. For DENV 1, 2 and 3 the optimal plaque number per well was 60, whereas the optimal plaque number for DENV 4 was 100 plaques per well.

3.1.3.2 Optimization of plate size

Starting out, 12-well-plates were used for the plaque assays and the PRNTs. As plaque assays in all forms are labor intensive, the protocol was optimized for a 24-well-format. The additional wells per plate allowed a higher output of data per titration. As each well was smaller, it was of great importance to optimize the evaluation of the plaques. Different concentrations of methylcellulose were tested (1.5 %, 2.4 %, 3 %, and 4 %) with 2.4 % in a 1:1 ratio with 2xMEM producing optimal plaques for counting. The optimal incubation time for the cells after application of the overlay was determined to be four days for DENV 1, 2, and 3 and five days for DENV 4. This optimization was done by comparing plates incubated for three to seven days for plaques of the right size to ease the readout.

3.1.4 Plaque reduction neutralization test (PRNT)

3.1.4.1 DENV serotype specificity

To evaluate the specificity of the PRNT, sera used for interlaboratory testing with known DENV subtypes (summed up in Tab. 7) were analyzed using optimized conditions with 12-well-plates and a 1.5 % methylcellulose overlay. Being obtained from returning travelers, it is assumed that the person had exposure to only one dengue subtype, although the possibility of previous exposure cannot be completely ruled out.

NT_{50} and NT_{90} values were calculated for each of the interlaboratory sera. The results show that a greater subtype specificity is achieved through the interpolation of NT_{90} values which resulted in these values being utilized throughout the rest of the project. Each serum underwent PRNT in independent duplicates as to verify the results. Serum 350031 was shown to have an NT_{90} values of 1:19.5 and 1:29.5 against DENV 2 (Fig. 8), serum 350033 an NT_{90} value of 1:25.1 and 1:10.3 against DENV 2 (Fig. 9), and serum 350038 was shown to have a NT_{90} value of 1:12.2 and 1:14.7 against DENV 4 (Fig. 10). As the NT_{90} value of ≥1:10 was set as the positive cut off no substantial plaque reduction was seen against other DENV subtypes in the three above mentioned sera. Serum 350048

showed the highest NT_{90} value of 1:80.5 and 1:38.5 against DENV 2, and also NT_{90} of 1:14.5 and 1:20.9 against DENV 1 but showed no reduction against DENV 3 and DENV 4 (Fig. 11).

Tab. 7: Serum for subtype specificity information: Sera used for interlaboratory testing with antibodies against a defined dengue subtype

Serum	**DENV subtype**
350031	DENV 2 (20 months post infection)
350033	DENV 2
350038	DENV 4 (7 months post infection)
350048	DENV 2 (4-18 months post infection)

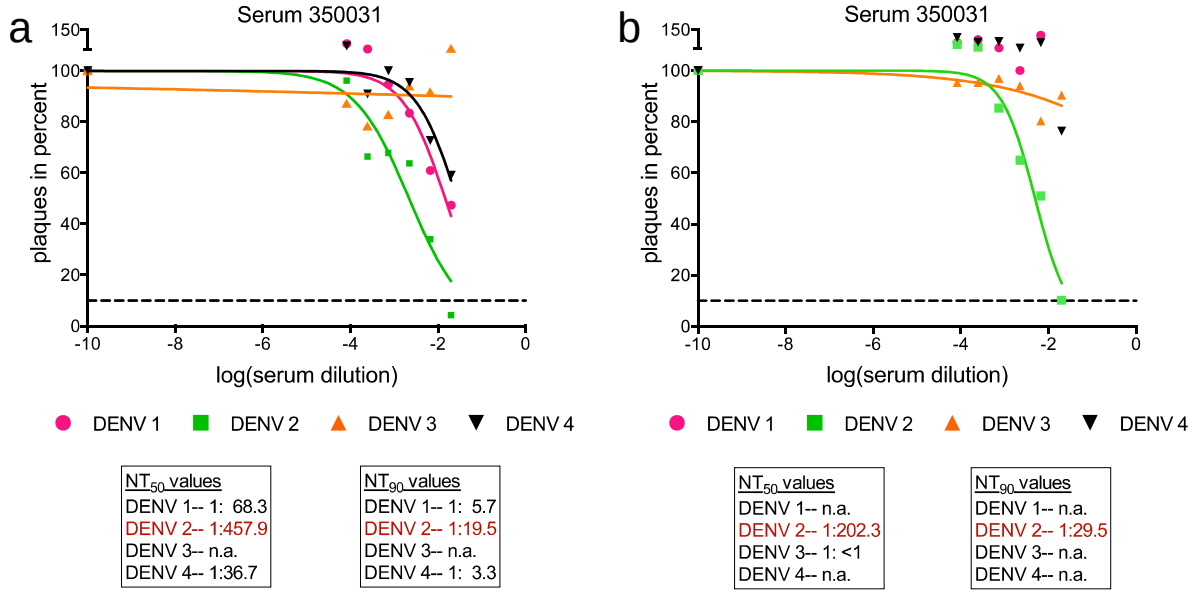

Fig. 8: Validation of PRNT subtype specificity: serum 350031
Serum with antibodies against DENV 2 20 months post infection is performed to validate subtype specific PRNT in independent duplicates (a and b) to verify results. Here the plaques formed in the PRNT with serum 350031 are plotted in relation to control virus not incubated with serum. The NT_{50} and NT_{90} values are interpolated as the value at which the non-linear regression line reaches a 50 % or 90 % reduction of plaques compared to control. 90 % reduction is visualized here by the dotted line. The inserted squares show the interpolated NT_{50} and NT_{90} values against each of the DENV serotypes. The cut-off for a positive titer was set at an NT_{90} value of ≥ 1: 10. DENV = dengue virus, n.a.= not applicable, in this case meaning no value could be interpolated.

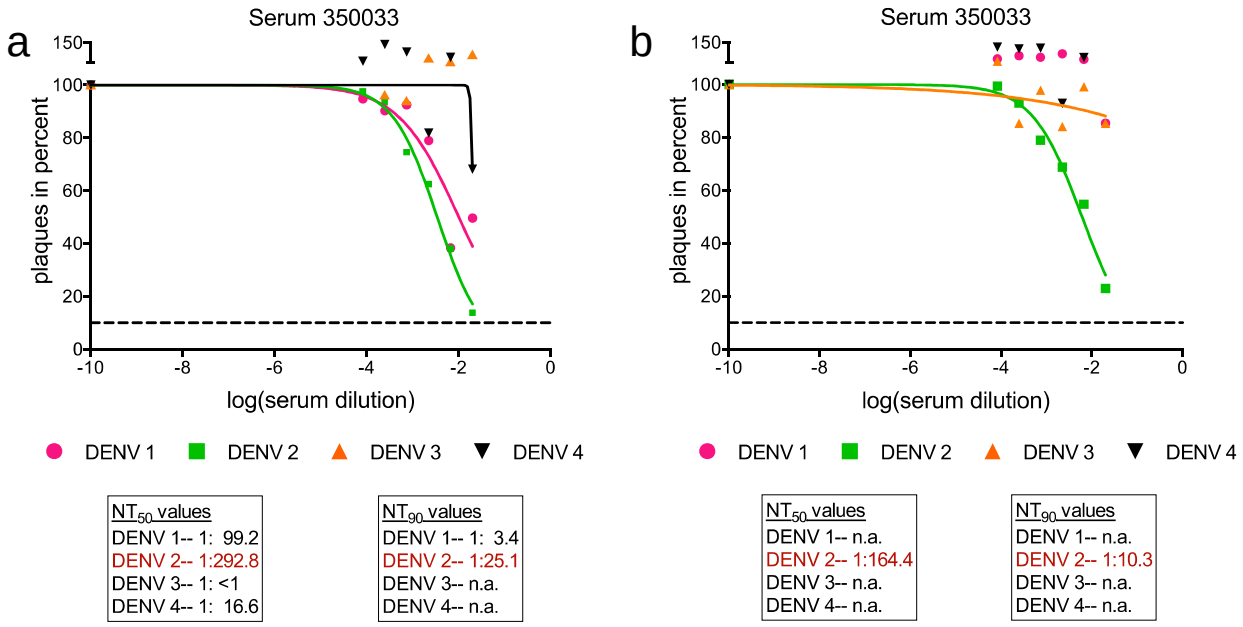

Fig. 9: Validation of PRNT subtype specificity: serum 350033
Serum with antibodies against DENV 2 is performed to validate subtype specific PRNT in independent duplicates (a and b) to verify results. Here the plaques formed in the PRNT with serum 350033 are plotted in relation to control virus not incubated with serum. The NT_{50} and NT_{90} values are interpolated as the value at which the non-linear regression line reaches a 50 % or 90 % reduction of plaques compared to control. 90% reduction is visualized here by the dotted line. The inserted squares show the interpolated NT_{50} and NT_{90} values against each of the DENV serotypes. The cut-off for a positive titer was set at an NT_{90} value of ≥ 1: 10. DENV = dengue virus, n.a.= not applicable, in this case meaning no value could be interpolated.

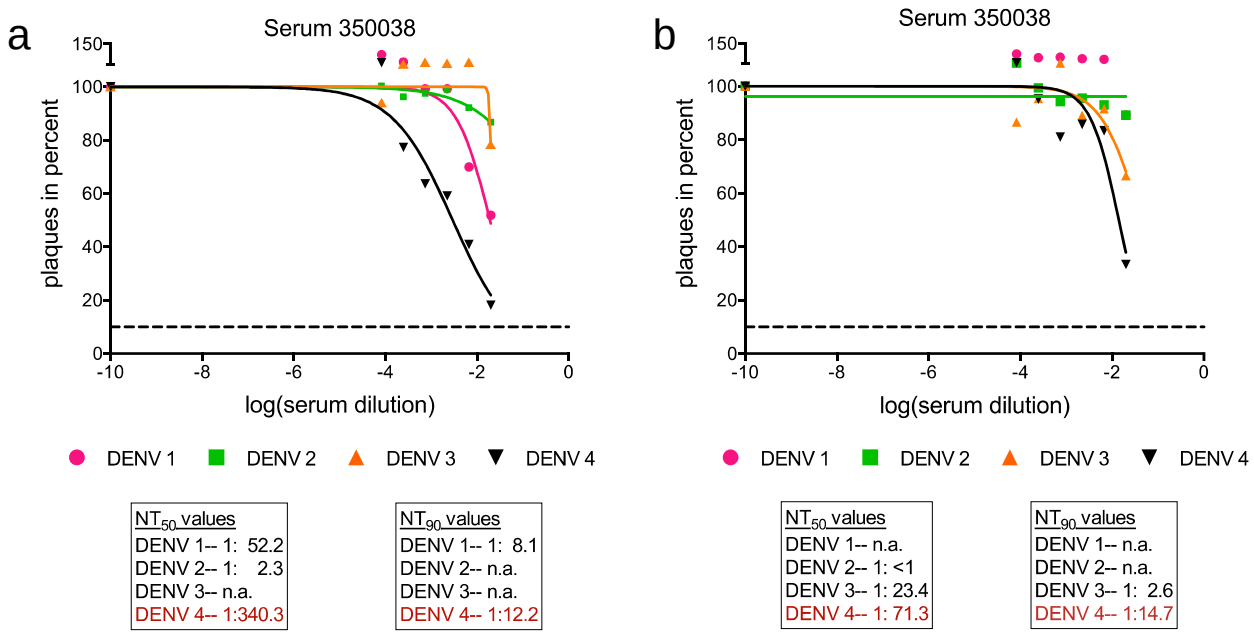

Fig. 10: Validation of PRNT subtype specificity: serum 350038
Serum with antibodies against DENV 4 seven months post infection is performed to validate subtype specific PRNT in independent duplicates (a and b) to verify results. Here the plaques formed in the PRNT with serum 350038 are plotted in relation to control virus not incubated with serum. The NT_{50} and NT_{90} values are interpolated as the value at which the non-linear regression line reaches a 50 % or 90 % reduction of plaques compared to control. 90 % reduction is visualized here by the dotted line. The inserted squares show the interpolated NT_{50} and NT_{90} values against each of the DENV serotypes. The cut-off for a positive titer was set at an NT_{90} value of ≥ 1: 10. DENV = dengue virus, n.a.= not applicable, in this case meaning no value could be interpolated.

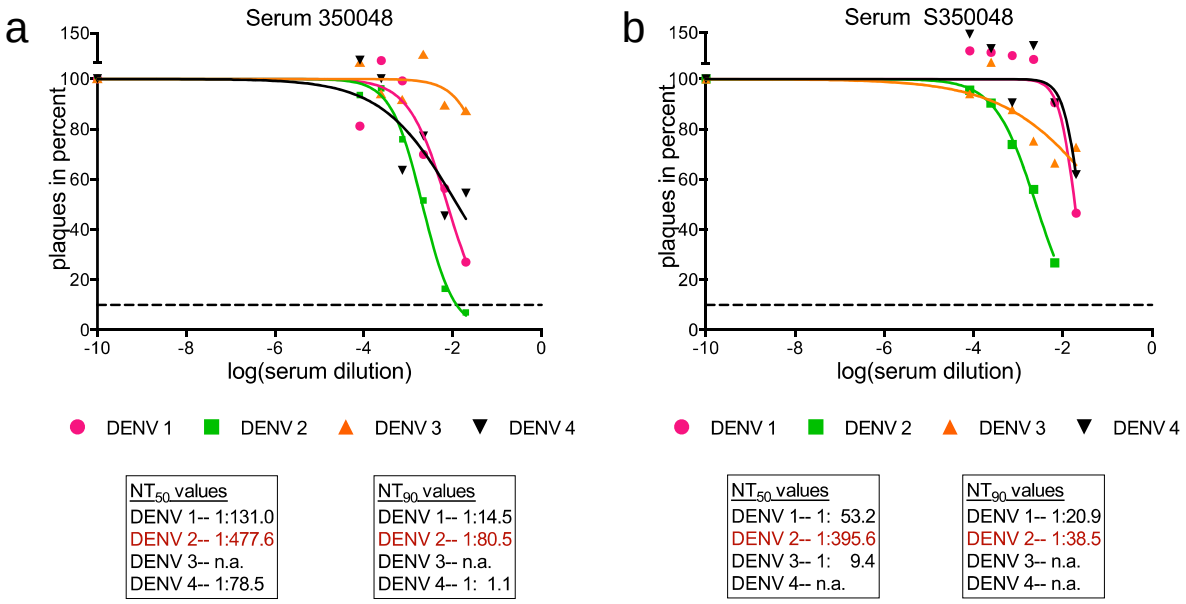

Fig. 11: Validation of PRNT subtype specificity: serum 350048
Serum with antibodies against DENV 2 four to 18 months post infection is performed to validate subtype specific PRNT in independent duplicates (a and b) to verify results. Here the plaques formed in the PRNT with serum 350048 are plotted in relation to control virus not incubated with serum. The NT_{50} and NT_{90} values are interpolated as the value at which the non-linear regression line reaches a 50 % or 90 % reduction of plaques compared to control. 90 % reduction is visualized here by the dotted line. The inserted squares show the interpolated NT_{50} and NT_{90} values against each of the DENV serotypes. The cut-off for a positive titer was set at an NT_{90} value of ≥ 1: 10. DENV = dengue virus, n.a.= not applicable, in this case meaning no value could be interpolated.

3.2 Sera from Brazilian mothers

The previously established DENV serotype specific PRNT assays were used to detect the antibody titers against DENV 1-4 in the sera of 29 Brazilian women who gave birth to children with CZS and 108 women who gave birth to children without CZS. The NT_{90} titer of each serotype was determined as described above. The results obtained ranged from neutralizing titers against none of the four DENV subtypes to neutralizing titers against all of the DENV subtypes. To show an example of the obtained data from the PRNT assays Fig. 12 displays the utilized PRNT and accompanying regression curve for 2 of the total 137 sera used in this project. In Fig. 12a serum which only truly inhibited DENV 2 leading to an NT_{90} of 1:45 against DENV 2 is seen. Fig. 12b exemplifies a serum for which similar NT_{90} titers were obtained for all four DENV serotypes. The latter result may indicate exposure to all serotypes, but it could also be the result of cross-reactivity after heterotypic dengue infection. The NT_{90} titers in Fig. 12b resulted in 1:71 against DENV 1, 1:189 against DENV 2, 1:156 against DENV 3, and 1:42 against DENV 4.

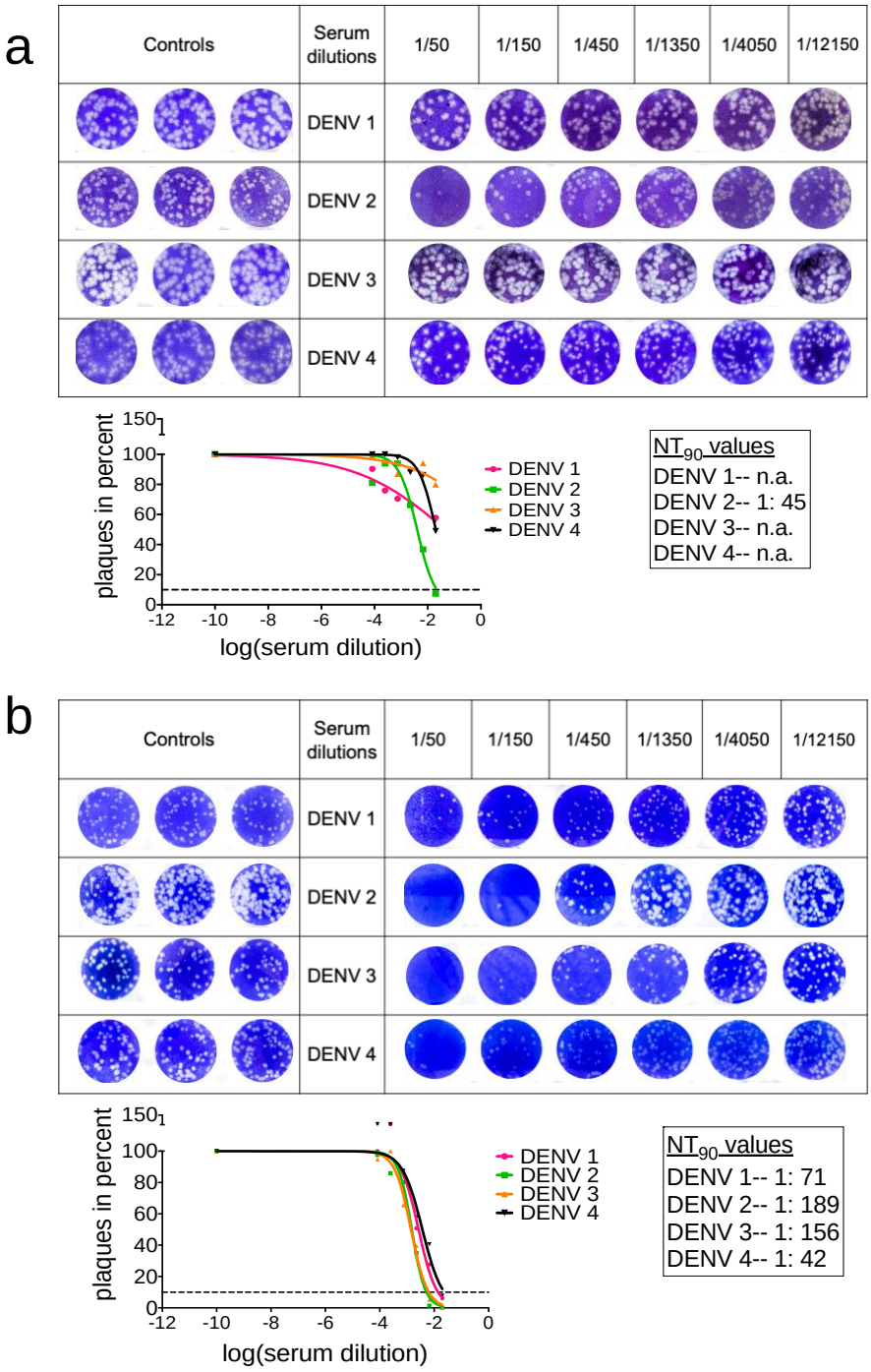

Fig. 12: PRNT assay and regression curve of serum: Serum dilutions were incubated with virus before infection of cells, after which an overlay of 2.4 % methylcellulose was added. Cells were fixed at 4 (DENV 1,2,3) or 5 (DENV 4) days post infection in formaldehyde solution and stained using crystal violet. The resulting plaques for differing dilutions of serum are seen in comparison to control plates incubated without serum. Below each PRNT are the NT_{90} regression curves made with GraphPad Prism. The dotted line represents the 90 % reduction of plaque formation in comparison to controls. The interpolated NT_{90} values are shown in the inserted squares. n.a. = not applicable, in this case meaning no NT_{90} value could be interpolated.

3.2.1 Magnitude of NT_{90} values

As noted in Pedroso et al., 2019, it has been described that low levels of pre-existing antibodies against DENV are a risk factor for severe disease (Katzelnick et al., 2017). To examine if this may be a factor contributing to CZS we evaluated the magnitude of the antibody titers against DENV in the cases and controls. Fig. 13a shows that the median reciprocal NT_{90} values against all DENV serotypes did not show a significant difference in magnitude. The controls showed a median reciprocal NT_{90} value of 61.4 with a 95 % confidence interval (95% CI) of 54.3- 73.1. The cases had a median reciprocal NT_{90} value of 56.5 with a 95 % confidence interval of 42- 79. To further investigate the effect of the magnitude of the antibody titers, we calculated the median reciprocal NT_{90} value of cases and controls against each individual serotype of DENV; Fig. 13b shows these results graphically. No significant difference was seen between cases and controls in the magnitude of antibody titers against the individual serotypes of DENV. The median reciprocal NT_{90} values against DENV 1 were 38 (95 % CI 17-81) for cases and 64.5 (95 % CI: 51.2-100.9) for controls resulting in a p-value of 0.076 using the Mann-Whitney-U test. Against DENV 2 the median reciprocal NT_{90} value for cases (95 % CI: 45-189) and 79 for controls was 102.6 (95 % CI: 79.1-133.6) resulting in a p-value of 0.628. The median reciprocal NT_{90} values against DENV 3 were 54 (95 % CI: 24-163) for cases and 44 (95 % CI: 31.3-53.2) for controls resulting in a p-value of 0.814. Against DENV 4 the median reciprocal NT_{90} value for cases was 73 (95 % CI: 24-92) and 51.9 for controls (95 % CI: 41.6-61.4) resulting in a p-value of 0.524.

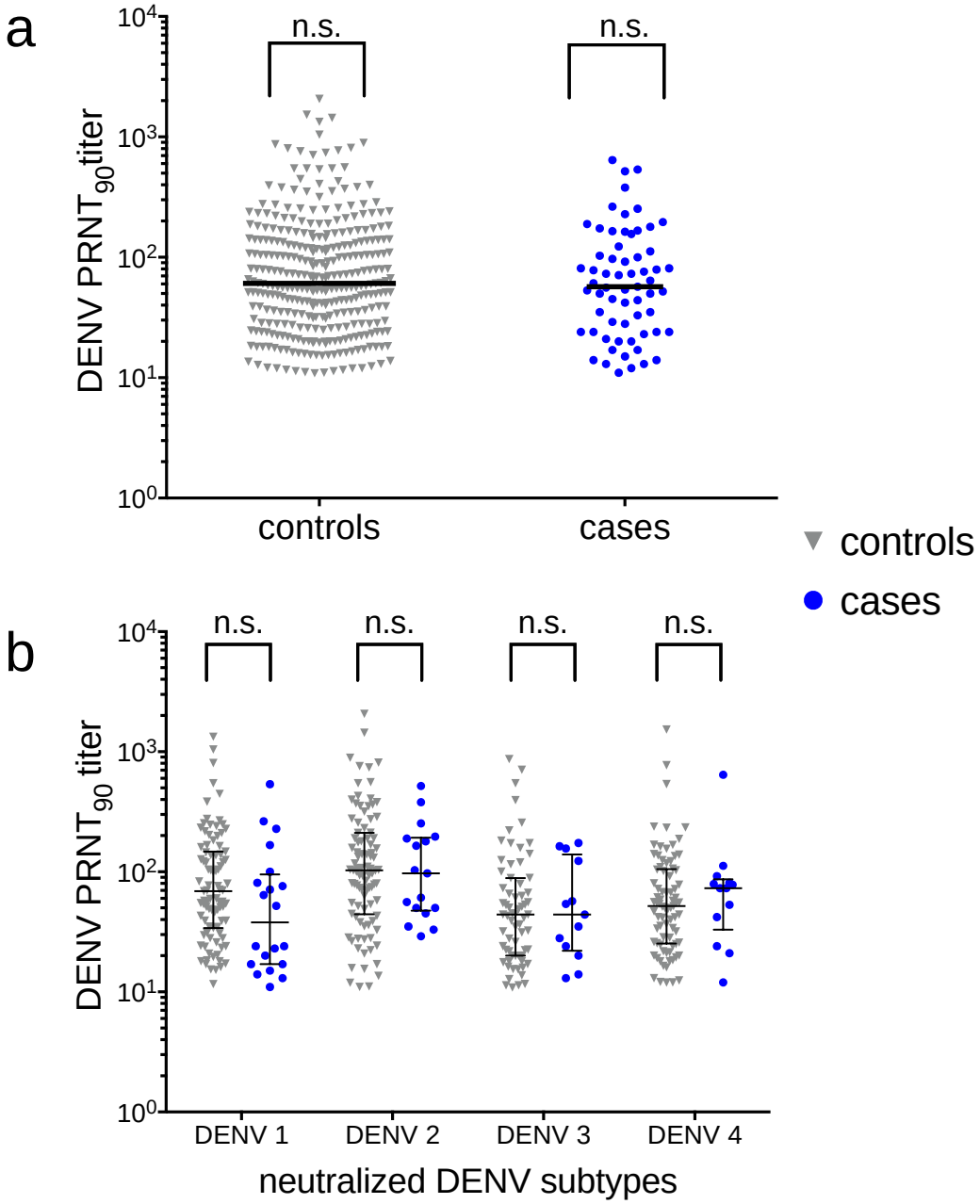

Fig. 13: Anti-DENV titers of ZIKV sera: a) NT_{90} values with median portrayed through black horizontal line of all cases and controls against all DENV serotypes. The median reciprocal NT_{90} value was 61.4 (95 % CI: 54.3- 73.1) for controls and 56.5 (95 % CI:42-79) for cases against all dengue serotypes. With a p-value of 0.3664 the difference evaluated by the Mann-Whitney-U test was not significant between cases and controls. b) Here the NT_{90} values of each individual dengue serotype are portrayed, with the median values marked through black lines. The Mann-Whitney-U calculations showed no statistical significance in the median values between cases and controls for any of the four dengue serotypes (adapted from Figure 3 from Pedroso et al., 2019). DENV = dengue virus, n.s. = not significant, $PRNT_{90}$ = 90 % plaque reduction neutralization test.

3.2.2 Seroprevalence

As no significant difference was seen between the magnitude of NT_{90} values of cases and controls, we further investigated the seroprevalence. In our study this means whether or not a serum showed neutralization of the challenging virus; the cut-off was determined at an $NT_{90} \geq 1:10$. When evaluating the cases against the controls in this regard, there was a significant difference. First of all, the overall prevalence of DENV antibodies, was significantly higher in controls compared to cases with 92 % of controls displaying neutralizing anti-DENV antibodies compared to only 66 % of cases resulting in a p-value of <0.001 using a chi-square test (Fig. 14a). Furthermore, the prevalence of antibodies against each individual DENV subtype was significantly higher in controls than in cases with a p value of <0.05 for each using a chi-square test, which is displayed in Fig. 14a. The number of sera neutralizing several subtypes was also significantly higher in controls when compared to cases as displayed in Fig. 14b. Furthermore, the difference of multitypic exposure was robust throughout all age groups as is shown in Fig. 15, showing that time in endemic areas did not lead to a difference in the exposure.

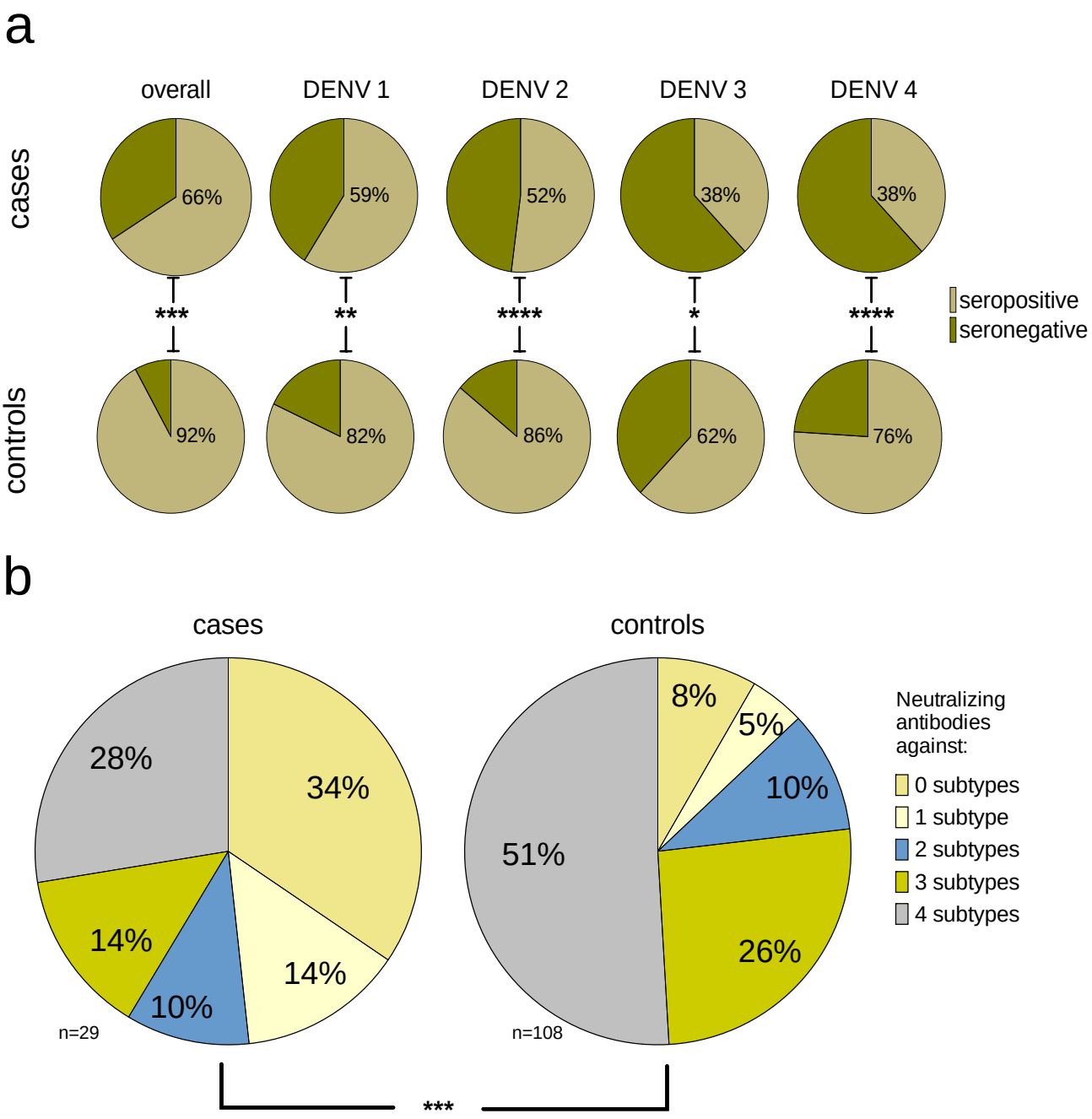

Fig. 14 : DENV seroprevalence of ZIKV sera: a) Seroprevalence of overall and dengue subtype specific antibodies in cases and controls. χ^2 tests were used to calculate statistical significance, and p values are represented through stars as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. b) The number of dengue serotypes neutralized by cases and controls. To calculate the statistical significance the Mann-Whitney-U test was used with a resulting ***p=0.0004 (adapted from Figure 4 from Pedroso et al., 2019).

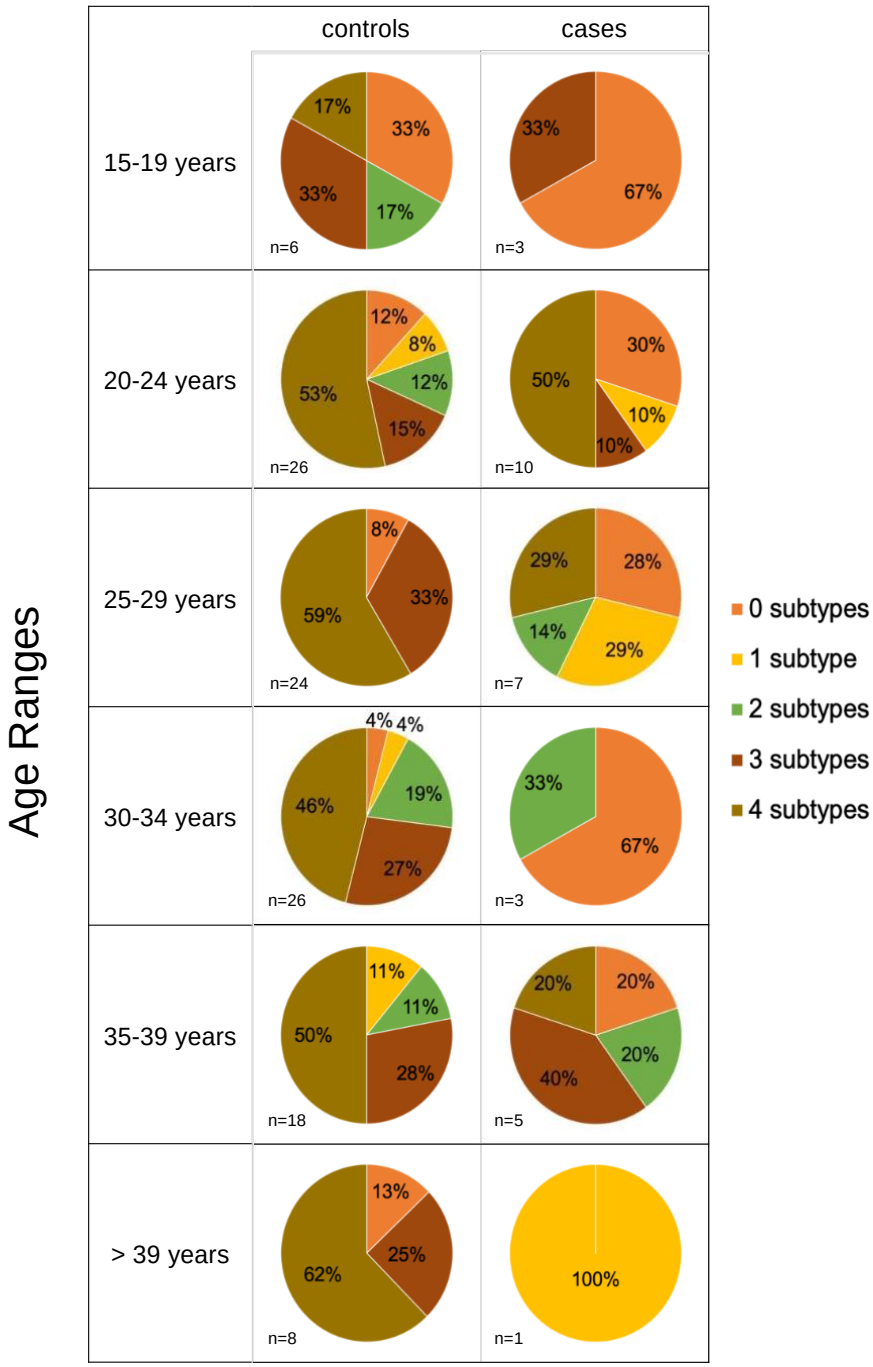

Fig. 15 : Seroprevalence divided into different age groups. The difference in multitypic DENV exposure, determined through an $NT_{90} \geq 1:10$ in DENV subtype specific PRNT is depicted for different age groups (adapted from Figure 4 from Pedroso et al., 2019). n = number of samples

3.2.3 Best fit model for correlations of known variables

To better compare the different factors that could be affecting CZS, we used general linearized model analyses. Both factors that showed a significant difference between cases and controls in bivariate comparisons, as well as factors that did not were evaluated including serotype neutralization, the number of serotypes neutralized, neutralization titers, and age. Each individual factor was plugged into a logistic regression to see the effect it had on the development of CZS. As can be seen in the Tab. 8, the highest correlation was seen when considering the neutralization of two or more DENV serotypes with an Akaike information criterion (AIC) value of 130.4, or the overall number of neutralized serotypes with an AIC of 130.6. Regarding the development of CZS, an 84.2 % risk reduction was seen when neutralizing antibodies against two or more serotypes were present, and a 42.3 % risk reduction per increase of neutralized serotype was seen. Magnitude of the neutralization titers, age, and the neutralization of one DENV serotype had high AIC values of >140 and therefore not showing a good correlation to development of CZS.

Tab. 8: Comparison of different models used to aid in the identification of factors effecting the development of CZS: different models are ordered based on the calculated AIC, with lower values representing models with a better quality. P-values of the different models were calculated using likelihood ratio tests. AIC= Akaike Information Criterion, ΔAIC = difference of calculated AIC – best model, AW = Akaike Weight, CI= Confidence Interval, DENV = dengue virus, $PRNT_{90}$ = 90% plaque reduction neutralization test (Pedroso et al. 2019).

Model	Predictor scale	AIC	ΔAIC	AW	Odds ratio (95% CI)	p value
Neutralization of ≥2 DENV serotypes	binary	130.4	0	0.2812	0.158 (0.062-0.395)	<0.0001
No. of neutralized serotypes	ordinal, 5 ranks	130.6	0.2	0.2590	0.577 (0.432-0.763)	<0.0001
DENV-4 neutralization	binary	131.4	0.9	0.1750	0.192 (0.078-0.499)	<0.0001
DENV-2 neutralization	binary	131.5	1.1	0.1657	0.170 (0.068-0.423)	<0.0001
Neutralization of ≥3 DENV serotypes	binary	133.0	2.6	0.0771	0.210 (0.086-0.492)	<0.0001
Neutralization of ≥1 DENV serotypes	binary	134.8	4.4	0.0319	0.170 (0.060-0.477)	<0.0001
DENV-1 neutralization	binary	139.1	8.6	0.0038	0.298 (0.122-0.733)	0.009
DENV-3 neutralization	binary	140.3	9.9	0.0020	0.368 (0.154-0.844)	0.018
Neutralization of 4 DENV serotypes	binary	140.5	10.1	0.0018	0.361 (0.139-0.852)	0.020
Anti-DENV-2 $PRNT_{90}$ titer	continuous	141.8	11.4	<0.001	0.998 (0.990-1.000)	0.043
Anti-DENV-1 $PRNT_{90}$ titer	continuous	142.8	12.4	<0.001	0.997 (0.990-1.000)	0.079
Neutralization of 1 DENV serotype	binary	143.2	12.8	<0.001	3.328 (0.776-13.477)	0.101
Age of mothers when giving birth	continuous	143.2	12.8	<0.001	0.953 (0.896-1.010)	0.136
Anti-DENV-3 $PRNT_{90}$ titer	continuous	144.2	13.8	<0.001	0.996 (0.990-1.000)	0.195
Anti-DENV-4 $PRNT_{90}$ titer	continuous	144.9	14.5	<0.001	0.998 (0.990-1.000)	0.326

4 . Discussion

The 2015-2016 Zika epidemic led to a rise in microcephaly cases, particularly in northeastern Brazil. Genetic similarities between the emerging ZIKV, and longtime endemic DENV raised the question if previous DENV exposure and resulting antibodies played a role in the development of CZS. Using the base of cross-reactive antibodies found between different DENV serotypes and a higher rate of severe dengue disease in heterologous secondary DENV infections, we wanted to investigate whether a difference in dengue history was seen between ZIKV positive mothers who gave birth to children with or without CZS. To evaluate previous DENV exposure in our samples, we established and optimized a serotype-specific PRNT. Applying the established DENV PRNTs to sera from a cohort of women who gave birth during the Zika epidemic, we found that heterotypic DENV antibodies played a protective rather than a detrimental role in the development of CZS. Other factors evaluated such as the magnitude of the DENV antibody titers, antibodies against specific DENV serotypes and age did not seem to play an important role.

4.1 Establishment and validation of serotype-specific antibody tests

The PRNT used in this work showcases the gold standard of DENV serotype-specific diagnostics. The optimization of DENV propagation in mosquito C6/36 cells allowed for similarities to the vector life cycle of DENV. We were able to increase the output of the assays by adapting protocols to use plates with a larger number of wells, allowing a higher throughout of sera in a shorter time period. Furthermore, the serotype-specificity of our tests was established through interlaboratory standard control sera. This highlighted the lower prevalence of DENV 3 and 4 in our samples which, as shown by the phylogenetic analysis (Pedroso et al., 2019), were introduced into Brazil later than DENV 1 and 2.

While continuing to be time-intensive and vulnerable to many factors, a validated serotype-specific DENV test remains important in the field, especially in regards to current and future potential evaluation of vaccines. Being a virus with such a large endemic area

and causing many hospitalizations every year, an efficient vaccine is of great public health importance. Furthermore, in respect to sub-neutralizing titers often reported in relation to DENV, and potential ADE, a method which not only measures the presence of antibodies, such as an ELISA or Western blot, but the neutralizing capabilities of these antibodies is of high relevance. According to the WHO guidelines published in 2007, “the PRNT measures the biological parameter of *in vitro* virus neutralization” and remains “the most serologically virus-specific test among flaviviruses, and serotype-specific test among DENV”. Alternative test forms must be evaluated for validity against the gold-standard of the PRNT (WHO, 2007). Carpp et al. (2020) were able to show that the titers elicited by the current tetravalent DENV vaccine CYD-TDV or Dengvaxia® were comparable when measured with ELISA-based microneutralization and PRNT assays. The advantage of this ELISA-based microneutralization test is the higher throughput, smaller amounts of required serum, and a greater objectivity through a spectrophotometric readout. A move away from the PRNT to a less cumbersome alternative, while yielding similarly accurate results would be a great advancement for flavivirus diagnostics and research. However, for now, the PRNT remains the gold standard test.

4.2 Factors affecting CZS development

4.2.1 Interplay of immunity against DENV and ZIKV

While we saw no significant effect of DENV antibody titer magnitudes on the development of CZS in our cohort, Moreira-Soto et al. (2017) reported a significantly higher ZIKV antibody titer in cases compared to controls in our cohort. Our findings showing that multitypic DENV immunity seemed to protect from development of CZS, could also hint that multitypic immunity could aid against the development of a high viral load in patients. A further interplay of DENV and ZIKV immunity was shown in two prospective studies. In Nicaragua, children presenting with DENV immunity prior to ZIKV infection were less likely to develop a symptomatic ZIVK infection, though the rate of viral infection remained the same (Gordon et al., 2019). A study done in Salvador, Brazil showed a reduction in ZIKV infections and symptoms in persons with existing DENV antibodies (Rodriguez-Barraquere et al., 2019). The study showed that a higher IgG antibody titer against DENV

NS1, as well as neutralizing antibody titers in PRNT correlated with a lower ZIKV infection rate in the study population (Rodriguez-Barraquere et al., 2019).

Furthermore, it was shown that DENV-immune nonhuman primates were able to clear a ZIKV infection more quickly than those which were DENV-naïve (Pantoja et al., 2017). The previously DENV-infected nonhuman primates showed a shorter viremia after being challenged with ZIKV almost three years after primary DENV infection, a time after which the transient cross-protection induced by a primary DENV infection is believed to have subsided (Pantoja et al., 2017). In turn, higher ZIKV viremia has been shown to be associated with CNS invasion of ZIKV and a longer duration of ZIKV viremia has been reported with fetal abnormalities (Meaney-Delman et al., 2016; Osuna et al., 2016). Therefore, Pantoja et al., 2017, suggested that pregnant women with previous DENV exposure may be less likely to show CNS invasion of ZIKV possibly protecting the fetuses from the development of CZS. These results support the outcome of our investigation that past multitypic DENV infections play a protective role in ZIKV infection.

While our results support an interplay between DENV and ZIKV immunity in one direction, it is plausible that the interaction works similarly when virus immunity is introduced in reverse order. It is hypothesized, that through acquired herd immunity against ZIKV, the transmission of ZIKV was reduced in Salvador, Brazil during the outbreak (Netto et al., 2017; Rodriguez-Barraquere et al., 2019). In addition, the number of DENV cases was reduced during the ZIKV outbreak further supporting the hypothesis of immune interactions between the two closely related flaviviruses (Ribeiro et al., 2018). It is possible, that recent immunity against ZIKV could in turn provide immunity against DENV infection. The recurrence of a high number of DENV cases in the Americas in 2019 suggests that the cross-protection offered by a prior ZIKV infection is only temporary (Ribeiro et al., 2020).

The hypothesis of viral interference in the host leading to a fall in DENV infections during the ZIKV outbreak is rebuked by showing the ability of coinfection in *Ae. aegypti* mosquitoes with up to three arboviruses, specifically CHIKV, ZIKV and DENV, as well as coinfection of humans in Brazil with DENV and ZIKV (Chaves et al., 2018; Estofolete et

al., 2019; Rückert et al., 2017). Interestingly, in coinfections with ZIKV and DENV in the mosquitoes, ZIKV was able to produce higher titers and better infect the salivary glands (Chaves et al., 2018). Furthermore, when transmission of the coinfected mosquitoes was studied, mice were more likely to be infected with ZIKV than DENV (Chaves et al., 2018). The topic of coinfection and potential viral interference gains importance as the vector for these and many other diseases spread due to urbanization and global warming, though it does not seem to play an important role in the question of interplay between ZIKV and DENV in mosquitoes or humans.

The effect of DENV antibodies and immunity on the probability of developing CZS remains controversial. A heterogenous picture has been painted in studies published since the ZIKV outbreak regarding potential protective or detrimental effects of prior DENV immunity on the discourse of ZIKV infection. Several studies show ADE occurring in vitro when ZIKV infection occurs in the presence of DENV antibodies. In one study, ZIKV replication and infection rate were intensified in placenta explants in the presence of sera containing antibodies against DENV 1 – 4 (Hermanns et al., 2018). The researchers further hypothesized not only that the ADE seen was Fc-receptor-dependent, because the cells showing the most substantial ADE correlated to the cells with the highest density of Fc-receptor bearing cells, but also that the recorded ADE was specific to DENV antibodies and ZIKV interaction as it did not occur under the influence of YFV antibodies (Hermanns et al., 2018). In another study, serum attained from DENV immune pregnant women from Recife, Brazil was shown to enhance ZIKV ADE in FcyRII receptor displaying human cells *in vitro* (Castanha et al., 2017). Interestingly, the authors here also discussed that ZIKV infections were not associated with CZS in Asia, where DENV has been hyperendemic for several decades and suggested that, since many pregnant women in their study were immune only to DENV 3, having heterotypic DENV exposure may lead to a different interaction with ZIKV (Castanha et al., 2017). Lastly, Dejnirattisai et al. (2016) showed that cross-reactive human DENV antibodies led to ADE of ZIKV *in vitro*, but that these antibodies were not able to neutralize the virus. When diving deeper, these authors found that DENV directed against the fusion loop led to ADE, whereas EDE mAb with less avidity to ZIKV were able to protect from ADE of ZIKV (Dejnirattisai et al., 2016).

Yet, similar to the results we found showing that multitypic DENV antibodies present in the serum of pregnant women correlated with a reduced risk of developing CZS, *in vitro* studies showed sera from some secondary DENV infections could neutralize ZIKV (Priyamvada et al., 2016; Swanstrom et al., 2016). Primary DENV infections on the other hand could not, underscoring the potential importance of multitypic DENV immunity (Priyamvada et al., 2017; Swanstrom et al., 2016). Swanstrom et al. (2016) interestingly reported, that only some of the secondary sera showed the capability of neutralizing ZIKV infection, leading them to question if this may be based on previous DENV or ZIKV infections or if the potently cross-neutralizing EDE1 antibodies dominate in some individuals (Swanstrom et al., 2016). DENV immune sera, shown to most likely be the result of numerous past DENV infections, displayed a strong neutralizing effect on ZIKV in PRNT test (Priyamvada et al., 2016). The ADE of ZIKV infection through DENV exposed human sera was also tested, showing varying results depending on concentration and most likely the individual make-up of present antibodies (Priyamvada et al., 2016).

In regards to an association with CZS and prior DENV antibodies, a review published by Petzold et al. (2021) describes a protective or unaffecting role for DENV antibodies during ZIKV infections in retrospective *in vivo* studies in humans. The protective role was seen in the cohort used in our study showing that DENV exposure did not correlate with higher ZIKV antibody titers. Rather, it showed that DENV antibodies were associated with CZS not occurring, underlining our showing of a potential protective role for heterotypic maternal DENV antibodies against CZS (Moreira-Soto et al., 2017; Moreira-Soto et al., 2018; Pedroso et al., 2019). A prospective study of pregnant women testing positive for ZIKV showed no correlation of adverse outcomes of the pregnancy, defined as fetal death or clinically abnormal macroscopic or imaging results, with the existence of DENV IgG antibodies, nor did DENV immune mothers present a higher viral load of ZIKV (Halai et al., 2017). A case-control study also showed a similar rate of previous DENV infections detected through PRNT in ZIKV-positive mothers who gave birth to children with or without birth defects (Castanha et al., 2019). Campos et al. (2018) also stated that there was no association found with the distribution of DENV and the reported microcephaly cases in their study of reported infections during the ZIKV epidemic.

In contrast, an enhancement of ZIKV infection with microcephaly was reported in an *in vivo* study when DENV antibodies were present (Petzold et al., 2021). Here, ZIKV and DENV naïve mouse dams were compared with dams having cleared a DENV 2 infection 3 weeks prior to pregnancy and also with dams inoculated with passive monoclonal antibody against DENV 2 (Rathore et al., 2019). The fetuses of both groups of dams with previous DENV exposure displayed a significantly smaller head circumference after ZIKV infection during the third trimester of pregnancy supporting that existing maternal DENV antibodies increase the severity of the ZIKV infection leading a microcephaly (Rathore et al., 2019). While noting that the differences in the immune systems of mice and humans leads to the need for further investigations, the authors suggest, that a link possibly between ADE of ZIKV infection caused by maternal DENV antibodies and the severity of CZS should not be ruled out (Rathore et al., 2019).

A further ecological study discussed by Petzold et al. (2021) showed an interesting jump from protective qualities of previous DENV exposure against microcephaly for up to six years after infection, while pointing to a detrimental effect when the time lag between previous DENV outbreaks was over seven years (Carvalho et al., 2020). The authors hypothesize, that this large window is due to fluctuating DENV antibody levels, offering cross-protection against ZIKV at high concentrations, and moving into dangerously low levels possibly leading to an ADE effect when the antibody levels wean (Carvalho et al., 2020). As heterotypic DENV immunity provides a longer cross-protective effect, the results discussed by Carvalho et al. (2020) show support for our findings that multitypic maternal DENV exposure may aid in the protection against CZS. Carvalho et al. (2020), however do underline, that while previous DENV exposure and resulting antibodies may play a role in the development of CZS in the form of microcephaly, this factor alone cannot explain the stark differences in microcephaly rates of northeastern Brazil in comparison to other areas also effected by the ZIKV epidemic. Underlining this statement, is the results published in our paper of the phylogenetic study of DENV in northeastern Brazil in comparison to other areas of the country showing no unique DENV infection pattern in northeastern Brazil (Pedroso et al., 2019). Looking into the time period since the comeback of the *Ae. aegypti* in Brazil in 1976, analyses exposed little genetic differences in the envelope-NS1 junction, which is often used in genome-based serotyping, of DENV

strains present in northeastern Brazil when compared to other areas (Pedroso et al., 2019). As previous exposure to DENV was not found to be significantly different in northeastern Brazil compared to other areas of Brazil, other factors affecting the development of CZS must be further explored.

One hypothesis, to explain the surge in neurological complications associated with ZIKV, is that an attenuated version of the virus is leading to birth defects rather than fetal death (Aubry et al., 2021). Early abortions are less prone to be caught by public health surveillance than for instance CZS, inferring that the neurological problems caused by ZIKV may in fact not be novel, but rather less severe in the strain which caused the 2015-2016 ZIKV outbreak in the Americas as here fetal infection caused malformations instead of abortion (Lambrechts, 2021). While this offers a possible explanation as to the emergence of CZS so many decades after the initial discovery of the ZIKV, it does not shed light on the unequal geographical distribution of CZS cases in endemic areas.

4.2.2 Other factors possibly affecting CZS development

Apart from immune responses, a socio-economic factor seems to play into the probability of CZS developing during pregnancy. Possibly, the more densely populated, poverty-stricken areas in northeastern Brazil lead to a higher exposition of mosquitoes and thus to arbovirus-associated infections (Campos et al., 2018). In Recife, a city in northeastern Brazil, the prevalence of microcephaly was higher in areas with worse socio-economic conditions (Souza et al., 2018). Malnutrition is a common problem in bad socio-economic situations and protein-rich foods are sometimes hard to attain. The most prominent cause for immune deficiency in the world is protein malnutrition (Losada-Barragán et al., 2017; Revillard and Cozon, 1990). Protein deficiency has been shown to affect the immune system in a plethora of ways, including a reduction of lymphoid cells and a reduced T-cell response (Chandra, 1997). While it was shown that antibody responses remained sufficient when T-cells were not relevant in the equation, the affinity of antibodies was decreased in patients with protein deficiency (Chandra, 1997). Furthermore, protein malnutrition of pregnant mice was shown to enhance the susceptibility to CZS (Barbeito-Andrés et al., 2020). The ZIKV load in maternal spleens was increased, and the viral clearance took longer in malnourished mice compared to controls (Barbeito-Andrés et al.,

2020). The fetuses of the protein deficient mice infected with ZIKV also showed greater intrauterine growth restriction and flawed neurogenesis when compared to well-nourished controls (Barbeito-Andrés et al., 2020). Human data also showed a positive correlation between areas with a high prevalence of undernourishment and areas reporting high incidences of microcephaly, as well as 40 % of interviewed mothers of children born with CZS reporting decreased protein consumption (Barbeito- Andrés et al., 2020).

Overall, although the immune answer may not rely solely or predominantly on antibodies in an DENV or ZIKV infection, the presence of DENV immunity may aid in the reduction of the severity of a ZIKV infection.

4.2.3 Immune response mediation

Our results do not show an association of cross-protection against CZS with the magnitude of DENV antibody titers, rather an association with present heterotypic DENV antibodies. These results hint to the potential importance of heterotypic DENV immunity seen in post-secondary infections (Olkowski et al., 2013; Sierra et al., 2012). In a longitudinal study done in Peru, authors showed that the risk of developing disease after infection with DENV was significantly reduced in patients who had previous heterologous DENV exposure, meaning that their serum contained antibodies against at least two different serotypes before infection (Olkowski et al., 2013). A study in Cuba also showed, that tertiary and quaternary DENV infections elicited a milder expression of cytokines while secondary infections showed a higher expression (Sierra et al., 2012). Higher expression of these cytokines is often associated with severe disease. (Sierra et al., 2012).

As the actual level of DENV antibody titers in our study did not show a relation to the development of CZS, other parts in the immune system, for instance T-cells, may be mediating cross-protection resulting in reduced risk of CZS. A murine study showed, that DENV viral load was significantly increased in CD8+ T-cell depleted mice in comparison to controls (Yauch et al., 2009). Furthermore, the authors were also able to show immunization of mice with dominant CD8+ T-cell epitopes lead to an increase in the clearance of DENV (Yauch et al., 2009). Similarly, T-cell response peaks were linked to time of ZIKV viremia reduction in a nonhuman primate study, underlining the importance

of, in this case CD8+, T-cells in immune responses against ZIKV infection (Dudley et al., 2016). Given the genetic similarity between ZIKV and DENV, a cross-reactive effect, similar to the one seen in antibody response, could be hypothesized to occur in the T-cell response. Several studies support this hypothesis of T-cell cross-protection occurring between DENV and ZIKV. A CD8+ T-cell mediated response led to a reduction of ZIKV infections in mice which were previously immunized with DENV/ZIKV cross-reactive epitopes (Wen et al., 2017a). By also conducting the study of a ZIKV challenge in peptide immunized mice, which were CD8+ T-cell depleted, the authors were able to display T-cell dependency because ZIKV infection levels measured were much higher compared to non-T-cell depleted mice (Wen et al., 2017a). Furthermore, in DENV immune pregnant mice a depletion of DENV cross-reactive CD8+ T-cells lead to a boost in ZIKV infection in both placenta and fetus (Regla-Nava et al., 2018). An immunization inducing the production of the cross-reactive CD8+ T-cells also lead to a reduction of ZIKV infection leading to fetus weight and size comparable to control groups (Regla-Nava et al., 2018). T-cell analyzed from DENV-immune maternal spleens subsequent to ZIKV challenge displayed a larger and more polyfunctional cross-reactive epitope specific CD8+ T-cell response (Regla-Nava et al., 2018). Pantoja et al., (2017) also showed that macaques previously exposed to DENV displayed a greater percent of CD8+ T-cell activation when later challenged with ZIKV further supporting an enhancing rather than hindering effect of prior DENV exposure. It would be interesting to evaluate the importance of cross-reactive T-cell responses in the development of CZS in the future.

Associations have also been made in regard to the genetic variability of HLA genes having either a protective or detrimental role in the infection with and progression of dengue disease (Loke et al., 2001). A case-control study carried out in Vietnam established that alterations in the HLA-A gene, which showed to be vital for conserved and serotype-specific DENV epitopes, were important in the development of severe dengue (Loke et al., 2001). HLA alleles associated with a more severe disease show a weaker CD8+ response, whereas a stronger response with more multifunctional CD8+ T-cells was observed with HLA alleles associated with less susceptibility to severe dengue (Weiskopf et al., 2013; Loke et al., 2001). Weiskopf et al. (2013) also discussed the possibility that a combination of certain HLA alleles with sub-neutralizing, cross-reactive antibodies could

play a role in the development of severe dengue. Seeing the importance of having certain HLA alleles in relation to severe DENV raises the question if this may also have an impact on the development of CZS.

The CD-8+ T-cell response is mainly aimed at nonstructural proteins, whereas the antibody and CD-4+ T-cell response during a DENV infection is mostly aimed at structural proteins (Rivino et al., 2013; Weiskopf et al., 2013). The genetic similarity between DENV subtypes and the ZIKV is greater in nonstructural proteins compared to structural ones (Wen and Shresta, 2019). The current tetravalent DENV vaccine CYD-TDV, also known as Dengvaxia®, utilizes a yellow fever backbone with incorporated DENV E- and prM-proteins to elicit the formation of antibodies (Rivino and Lim, 2017). It was shown to strengthen CD8+ T-cell responses in DENV immune persons, but could not activate the production of these immune response cells in DENV naïve individuals (Harenberg et al., 2013). This vaccine has been seen to be problematic in DENV naïve individuals because of an increased observance of hospitalization due to dengue underscoring the hypothesis that other immune mediated responses, such as T-cell response, may be vital to acquire multitypic DENV immunity (Sridhar et al., 2018). The problems seen with the Dengvaxia® led both the WHO and the European Medicines Agency to recommend that only DENV-seropositive individuals to be vaccinated (Dengue vaccine: WHO position paper, September 2018 – Recommendations, 2019; Wilder-Smith, 2020).

Second generation dengue vaccines are currently undergoing final stages of testing. These vaccines incorporate, at least partially, the nonstructural proteins of dengue therein having a greater ability to elicit T-cell responses, as well as antibody responses (Wilder-Smith, 2020). The Takeda vaccine, also known as DENVax, utilizes an attenuated DENV 2 backbone and incorporates the prM and envelope proteins of DENV 1 ,3, and 4, while the NIH vaccine incorporates full length attenuated DENV 1, 3, and 4 serotypes as well as a chimeric virus, where prM and envelope proteins of the DENV 2 are replaced in DENV 4 (Wilder-Smith, 2020). Another benefit of these second-generation vaccines is the dosing. The Takeda vaccine requires two doses at a three-month interval, and the NIH vaccine, known as TV003/TV005, would only require one dose, which compared to the Dengvaxia®'s (three doses at six-month intervals) show a vast improvement in the ability

of distribution alone (Wilder-Smith, 2020). Phase three results from the Takeda vaccine show a good efficacy regardless of the serostatus of the individual, although little to no efficacy was seen against DENV 3 in seronegative individuals (Biswal et al., 2020). Even a single dose of the NIH vaccine was shown to evoke a potent and multifunctional CD8+ T-cell response, and also induce highly conserved epitopes which are believed to have the ability to react to DENV divergent from the exact strains used in the vaccine (Weiskopf et al., 2015).

Altogether, the immune response to both DENV and ZIKV seems complex with many factors such as virus strain, prior infection status, age, host genetic factors, and the state of the individual immune system intertwining (Ngono and Shresta, 2018). Vaccine candidates against ZIKV are also being developed, with the most promise seen in DNA vaccines with those coding for E and prM-E proteins displaying an induction of neutralizing antibodies and T-cell response providing protection against ZIKV challenge in both mice and nonhuman primates (Abbink et al., 2016; Elong Ngono and Shresta, 2019; Larocca et al., 2016; Griffin et al., 2017). Apart from DNA based vaccines, a plethora of other approaches are also being investigated (Lunardelli et al., 2021). mRNA vaccines have shown to provide a robust antibody answer in mice and nonhuman primates, and protect from transmission of the virus from dams to fetus in mice (Jagger et al., 2019; Pardi et al., 2017). A vaccine using an inactivated whole ZIKV also led to immunity in mice and rhesus monkeys and has been shown safe and effective in three clinical trials so far with the results of studies done in endemic areas still to come (Abbink et al., 2016; Larocca et al., 2016; Lunardelli et al., 2021). Live attenuated ZIKV vaccines are also being considered and tested producing immunity, seroconversion und T-cell responses in mice (Zou et al., 2018). Chimeric-attenuated vaccine approaches utilizing DENV, adenovirus, CHIKV and measles as bases are also showing promising results in studies (Cox et al., 2018; Lunardelli et al., 2021; Nürnberger et al., 2019; Prow et al., 2018; Xie et al., 2017). Both live attenuated vaccines and mRNA vaccines were able to prevent ZIKV transmission *in utero* which is an exciting prospect in relation to the development of CZS (Richner et al., 2017b).

The question arising in some studies, is the evidence of ZIKV ADE of subsequent DENV infections (Wen and Shresta, 2019). When mice were injected with antibodies against ZIKV, either developed in other mice post ZIKV infection or through ZIKV vaccination, a greater disease severity was seen in following DENV infections (Stettler et al., 2016). Furthermore, pups born with maternally attained ZIKV antibodies developed severe dengue disease, whereas those born naïve to the virus did not (Fowler et al., 2018). As this could implicate devastating complications in dengue endemic areas, an emphasis should be laid to circumvent these adverse complications. One option could be to curtail cross-reactivity in antibodies through mutations to the conserved fusion-loop of the E-protein, as shown in a modified ZIKV mRNA vaccine (Richner et al., 2017a). Another idea could be to develop vaccines with a robust T-cell response as this may play an important role in DENV vaccine development (Wen and Shresta, 2019).

Although not many studies have been done to evaluate the cross-reactivity of a T-cell response in ZIKV immune sera challenged with DENV, two studies in mice show promising cross-reactivity, with one study assessing the result of this cross-reactivity and seeing cross-protection (Huarong et al., 2017; Wen et al., 2017b). The effect of a potential ZIKV vaccine must therefore be evaluated not only for the ability to protect against ZIKV infection and CZS development, but also possible adverse interactions with the closely related DENV.

4.3 Limitation of work

This work has limitations in the aspect of sample numbers. A larger number of samples would have led to more robust statistical results. It is often difficult to attain a greater number of samples during an epidemic in countries with a struggling health system. The minimal volume of sera which were available to test was a further limitation as it led to the incapability to rerun tests to validify results. While a rerun of tests would have been optimal to minimize disturbance factors, the fact that the utilized PRNT tests were all performed by one individual strengthens the results as PRNT tests are prone to variability depending on the person performing them. The often asymptomatic clinical presentation of ZIKV infections further adds to the difficulty of finding and verifying mothers who were infected

during pregnancy. While the antibody titers were clearly able to determine an immune response to the ZIKV, the time of infection could often not be determined. CZS has been linked to ZIKV infections more commonly during the first trimester (Mendes et al., 2020). This effect could not be studied with our samples. Through intensive TORCH screenings of the samples used, several other agents causing fetal abnormalities could be excluded (Moreira-Soto et al., 2018), although there could be further factors playing into the fetal abnormalities which were not studied.

In conclusion, the full picture of the reason, why CZS develops in some cases but is spared in others, is still not fully understood. The role of multitypic DENV immunity in protecting against CZS is supported by our data, as well as several studies. It seems that while the presence of DENV antibodies does not exacerbate the development of CZS, they do not seem to account for the full picture of protection leading to the hypothesis of other immune mediating factors of importance, such as T-cells. Furthermore, the effect of other factors, such as socio-economic standing need to be further examined to determine their role in the equation. This complexity is an important factor to keep in mind, especially in regards to future vaccination efforts against both DENV and ZIKV.

5 . Summary

The Zika virus (ZIKV) epidemic and sharp increase in the number of microcephaly cases in the Americas in 2015-2016 led to the documentation of congenital zika syndrome (CZS) in children born to mothers who were infected with ZIKV during pregnancy. CZS encompasses several structural anomalies and functional impairment caused by damage to the nervous system. A cluster of CZS appeared in northeastern Brazil leading to the question of potential cofactors affecting the development of the syndrome. ZIKV is a flavivirus showing antigenic similarity to the dengue virus (DENV) subtypes endemic in many parts of the world, including northeastern Brazil. DENV is known to mediate complicated immune responses with a higher rate of severe disease reported in secondary infections with a subtype differing from the initial infection. Although not completely understood, antibody dependent enhancement is one theory explaining this phenomenon. Here the antibodies against the primary infecting subtype do not fully neutralize the secondary heterotypic virus and rather ease the uptake of viral particles into certain cells. As multiple antibody epitopes are preserved between DENV subtypes and ZIKV we hypothesized, that specific DENV antibodies present in maternal blood could affect the development of CZS during maternal ZIKV infection. Serum from a cohort of Brazilian women infected with ZIKV, who gave birth to children either with CZS or without CZS, was tested for DENV subtype-specific antibodies to elude if a difference in DENV exposure existed between the two groups. To carry out these subtype-specific tests, plaque reduction neutralization tests were established and validated.

Our results show that antibodies against two or more DENV subtypes were significantly more prevalent in mothers who gave birth to children without CZS compared to those who gave birth to children with CZS. As antibody responses against two or more DENV subtypes leads to immunity against all subtypes, maternal DENV immunity may rather lead to a reduced risk in developing CZS during maternal ZIKV infection. As the magnitude of DENV antibody titers did not correlate with the risk of developing CZS and as T-cells have been shown to play an important role in the DENV immune response, both humoral and cellular DENV immunity, which would include T-cell response *in vivo,* may also play

a vital role in the development of CZS. DENV immunity and its role in CZS plays an important role especially in the development of vaccines for endemic areas. A wholesome immune response should be elicited by DENV vaccines as to not only protect against heterotypic infection, but possibly also aid in reducing the risk of CZS. The development of CZS seems to be multifactorial including factors such socioeconomic standing, which need to be investigated further. Our findings did not show an exacerbation of CZS through the presence of DENV antibodies, but they also do not seem to account for the full immunological picture. The complexity of the immune response to these viruses becomes increasingly important as changes in climate and urbanization are leading to a further spread of vectors carrying the disease throughout the world.

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