



Impact of Zika virus infection during pregnancy following epidemic events in the Caribbean and in South East Asia

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*To Paul,
The champagne is in the fridge.*

Acknowledgments

I grew up with my parents telling me that education was life-long and that opportunities to learn were everywhere. But not even they – the most avid supporters of continuing education – would have envisaged that I would spend my thirty third birthday in Paris, France preparing for the oral defence of my thesis. My thesis is therefore dedicated to my parents and my brother, for their endless support and encouragement – two Masters degrees and one PhD later. *Vive l'éducation !*

I owe much of this to my thesis director, Professor Arnaud Fontanet. I will always be grateful for his expertise, his support and trust in my abilities and for always finding time for me, despite all the COVID-19-related demands on his time over the last two years. Thank you for taking a gamble on a non-epidemiologist from Sciences Po in 2015.

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It has been a privilege to work at the Institut Pasteur for the past five years – first as a Research Officer in the Centre for Global Health and now as a PhD candidate. I have particularly enjoyed the opportunity to work with the Epidemiology of Emerging Diseases Unit and am honoured that I have been able to make a small contribution to the impressive works of the Unit. I would especially like to thank Anna Funk, Laura Tondeur, Laura Schaeffer, Yoann Madec, Tiffany Charmet, Yusuke Shimakawa, Muriel Vray, Romulus Breban, Liem-Binh Luong Nguyen, and Tim Dub, for their expertise and support, as well as the endless encouragement from Marie-Laurence Meignan. Thank you to David Smith from the other epidemiology unit for the constant entertainment and *ad hoc* lessons in epidemiology.

My thesis describes the work of two studies that I have been involved in – one in the French Territories of the Americas, and one in Ho Chi Minh City, Vietnam. Both were very enriching experiences and ones that I know I will draw on throughout my career. I am very grateful to Professor Bruno Hoen for his leadership of the ZIKA-DFA cohort studies, to Benoît Tressières, Elvire Couchy and the CIC Antilles-Guyane team for their warm welcome as part of the study team, and to all the clinicians, nurses, midwives, lab and clinical research staff in each of study site for their invaluable contributions to the studies. I would like to thank Thao Thi Thanh Nguyen, Manh Huy Dao, Hang Thi Thu Pham, Quang Duy Pham, and Quang Chan Luong for the multidisciplinary and collaborative efforts in Vietnam, and Professor David Baud and Dr Leo Pomar for their expertise on adverse pregnancy and neonatal outcomes.

The COVID-19 pandemic has bought much of the world to a standstill over the past two years. But the emergence of SARS-CoV-2 follows on from a long list of emerging or re-emerging pathogens, including Zika virus in the Americas in 2015-2016. Each one has highlighted the impacts epidemics have not only on public health, but also on society and the economy. As an infectious disease epidemiologist, I am proud to contribute to the understanding of pathogens and the diseases they cause, in the hope that we continue to be better prepared for the arrival of the next novel pathogen.

Impact of Zika virus infection during pregnancy following epidemic events in the Caribbean and in South East Asia

Abstract

The impact of epidemic events of emerging infectious diseases tend to focus on acute disease outcomes. In the case of Zika virus (ZIKV) – a flavivirus from the *Flaviviridae* family and transmitted to humans primarily from *Aedes* mosquitoes (*africanus*, *aegypti* or *albopictus*) – the 2013-2014 epidemic in French Polynesia and the 2015-2016 ZIKV epidemic across the Americas and other regions of the world identified severe neurological complications associated with acute ZIKV infection. Epidemiological investigations largely focused on these severe neurological outcomes, including congenital malformations in fetuses and newborns and Guillain-Barré syndrome in adults. What remains unclear are the longer-term health outcomes following a ZIKV epidemic. This thesis describes and evaluates the health impacts of ZIKV infection in French Territories of the Americas and in Vietnam.

In French Territories of the Americas, namely Guadeloupe, Martinique and French Guiana, we conducted a population-based mother-child cohort study of women whose pregnancies overlapped with the 2016 ZIKV epidemic. In a first analysis, we assessed the abnormalities possibly associated with *in utero* ZIKV exposure up to 24 months of age in infants born to women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy. Among 555 infants born to women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy, we found the overall risk of adverse pregnancy and early childhood outcomes possibly related to *in utero* Zika virus exposure to be 15.7% (95%CI:12.8-19.0), distributed as follows: 3.6% (95%CI:2.3-5.6) severe sequelae or fatality; 2.7% (95%CI:1.6-4.5) major abnormalities; 9.4% (95%CI:7.1-12.2) mild abnormalities. We found the risk of severe sequelae or fatality to be higher when Zika virus infection occurred during the first trimester (7.0%), compared to the second (2.7%) or third trimester (1.4%) ($p=0.02$). Our study provides an important contribution to understanding the adverse outcomes of ZIKV infection on pregnancy and early childhood outcomes.

In the same mother-child cohort study in French Territories of the Americas, we conducted a second analysis to determine the impact of *in utero* ZIKV exposure on neurodevelopment at 24 months of age among toddlers who were born normocephalic to women who were pregnant during the 2016 ZIKV outbreak. We found that among 156 toddlers with and 79 toddlers without *in utero* ZIKV exposure, there were minimal differences apparent in neurodevelopment outcomes at 24 months of age. Our study indicates that, in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there does not appear to be an impact on longer term neurodevelopment outcomes attributable to *in utero* ZIKV exposure which may manifest after birth up to 24 months of age.

In Vietnam, we conducted a retrospective cohort investigation of women who had RT-PCR confirmed infection during pregnancy in Southern Vietnam in 2016-2017. Of the 68 pregnancies, 58 were live births and 10 were medically terminated. Four of the medical records from cases of fetal demise were able to be retrieved, of which one had abnormalities consistent with congenital ZIKV syndrome. Of the 21 children included in the study, 3 had microcephaly at birth. No other clinical abnormalities were reported and no differences in neurodevelopment were observed compared to a control group. Phylogenetic analysis revealed a clade within the Asian lineage and branch at the root of samples from the 2013-2014 French Polynesian outbreak. We were therefore able to demonstrate an intermediate evolutionary ZIKV strain of Asian lineage associated with adverse pregnancy outcomes. Further, as all but two women had serologic evidence of ZIKV infection, we were able to show the long-term antibody kinetics following RT-PCR confirmed ZIKV infection.

Taken together, these studies suggest that the birth defects associated with Zika virus infection during pregnancy are more severe and more frequent when infection occurs in the first trimester of pregnancy and that there appear to be few abnormalities that appear for the first time beyond the immediate post-natal period and into early childhood. Our studies in the French Territories of the Americas found low rates of vertical transmission of Zika virus, as well as low rates of congenital abnormalities, as compared to studies in Brazil, without an obvious explanation. Beyond vector control efforts – the primary preventive measure – reducing Zika-related abnormalities relies on women of childbearing age avoiding mosquito bites in areas where the virus is known to circulate in human and/or vector populations. Our studies also highlight the need for close monitoring of fetal development through ultrasonographic imaging throughout the antenatal period and thorough clinical examination in the immediate post-natal period in areas where Zika virus may circulate. Further preventive efforts, such as the advancement of vaccine candidates through clinical trials, are currently hindered due to limited ZIKV circulation, but ZIKV outbreaks and/or endemic circulation can nonetheless be expected in many tropical and sub-tropical areas of the world in which competent vector populations are established.

Resumé

Le virus Zika est un flavivirus du genre *Flaviviridae*. Il s'agit d'un virus transmis par les moustiques du genre *Aedes* (*africanus*, *aegypti* ou *albopictus*), qui a provoqué plusieurs épidémies importantes au cours des 10 dernières années : sur l'île de Yap en 2007, en Polynésie française en 2013-2014 ainsi que dans les Amériques en 2015-2016. Au cours d'une épidémie, la surveillance épidémiologique et la recherche clinique se concentrent généralement sur les infections sévères nécessitant une hospitalisation. Dans le cas du Zika, les issues défavorables de grossesse et les anomalies chez les fœtus liées à une infection au cours de la gestation ont été bien décrites. En revanche, les conséquences à plus long terme d'une infection pendant la grossesse sur le développement de l'enfant ont été peu évaluées alors que la plupart des régions touchées entrent dans les phases post-aiguës des épidémies. Le sujet de ma thèse porte sur l'investigation des conséquences à long terme des épidémies de virus Zika.

Dans les départements d'outre-mer, (à savoir, la Guadeloupe, la Martinique et la Guyane française) nous avons mené une étude de cohorte mère-enfant basée sur la population de femmes enceintes pendant l'épidémie du virus Zika de 2016. Dans une première analyse, nous avons évalué les anomalies pouvant être associées à une exposition *in utero* au virus Zika jusqu'à l'âge de 24 mois chez les nourrissons nés de femmes présentant une infection symptomatique au virus Zika confirmée par RT-PCR pendant la grossesse. Parmi 555 nourrissons et au cours du suivi jusqu'à 24 mois, nous avons trouvé que le risque global d'issues indésirables de la grossesse et de la petite enfance lié à l'exposition *in utero* au virus Zika était de 15,7 % (IC à 95 % : 12,8-19,0), réparti comme suit : 3,6 % (IC à 95 % : 2,3- 5,6) séquelles graves ou décès ; 2,7 % (IC à 95 % : 1,6-4,5) anomalies majeures ; 9,4 % (IC à 95 % : 7,1-12,2) anomalies légères. Nous avons constaté que le risque de séquelles graves ou de décès était plus élevé lorsque l'infection par le virus Zika survenait au cours du premier trimestre (7,0 %), par rapport au deuxième (2,7 %) ou au troisième trimestre (1,4 %) ($p = 0,02$). Notre étude apporte une contribution importante à la compréhension des conséquences neurologiques et développementales d'une infection par le virus Zika durant la grossesse.

Dans cette même étude de cohorte mère-enfant basée sur la population de femmes enceintes pendant l'épidémie de Zika de 2016, nous avons mené une deuxième analyse pour déterminer l'impact d'une exposition *in utero* au virus Zika sur le développement neurologique à 24 mois des enfants nés indemnes de microcéphalie. Nous avons constaté qu'entre les 156 enfants exposés au virus Zika *in utero* et 79 enfants sans exposition au virus Zika, les différences de développement neurologique à 24 mois étaient minimales. Notre étude est rassurante du point de vue de la santé publique et de la prise en charge clinique, car nos résultats indiquent qu'en l'absence d'anomalies congénitales ou de résultats échographiques anormaux au dernier trimestre de la grossesse ou à l'accouchement, l'exposition *in utero* au virus Zika n'aura pas de conséquences à plus long terme sur le développement de l'enfant.

Au Vietnam, nous avons mené une enquête de cohorte rétrospective chez des femmes ayant eu une infection par le virus Zika confirmée par RT-PCR pendant la grossesse dans le sud du Vietnam en 2016-2017. Sur les 68 grossesses, 58 menèrent à des naissances vivantes et 10 à des interruptions de grossesse. ont été interrompues pour des raisons médicales. Quatre des dossiers médicaux d'interruptions de grossesse ont été récupérés, seul l'un d'entre eux présentait des anomalies compatibles avec un syndrome associé à l'infection congénitale à virus Zika. Sur les 21 enfants nés vivants inclus dans l'étude, 3 avaient une microcéphalie à la naissance. Aucune autre anomalie clinique n'a été signalée et aucune différence de développement neurologique n'a été observée par rapport à un groupe témoin. L'analyse phylogénétique a révélé l'implication d'une souche ancestrale du Zika dont l'apparition précède l'épidémie de Polynésie française 2013-2014. Ceci confirmait donc la capacité des souches ancestrales du Zika à provoquer des issues défavorables en cas d'infection au cours de la grossesse. En outre, le suivi des femmes ayant développé une réponse sérologique suite à leur infection confirmée par RT-PCR, nous a également permis d'étudier la cinétique des anticorps spécifiques contre le virus Zika.

L'ensemble de ces études suggère que le risque d'anomalies congénitales sévères associées au virus Zika survient lors d'infections de la mère ayant eu lieu pendant le premier trimestre de la grossesse, et que les enfants nés bien portants de mères infectées pendant la grossesse sont peu à risque de complications sévères dans les deux années qui suivent. Nos études aux Antilles françaises ont documenté un faible risque de transmission congénitale du virus Zika, et un faible risque d'anomalies neurologiques congénitales chez les enfants, comparativement aux études menées au Brésil, sans que l'on puisse apporter d'explications formelles à ce résultat. La prévention des anomalies congénitales liées au virus Zika repose, au-delà de la lutte anti-vectorielle, sur une sensibilisation des femmes en âge de procréer aux risques liés aux piqûres de moustiques en cas de reprise de la circulation du virus. Il faut également s'assurer d'un suivi échographique régulier des grossesses en cas d'épidémie liée au virus Zika permettant une détection précoce des anomalies afin de permettre leur prise en charge dans les meilleures conditions. L'évaluation des candidats vaccins sera rendue très complexe en absence de reprise épidémique franche de la circulation du virus.

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Abbreviations

ADE	Antibody-dependent enhancement
ASQ	Ages and Stages Questionnaire
CNS	Central nervous system
CZS	Congenital Zika syndrome
DENV	Dengue virus
ELISA	Enzyme-linked immunosorbent assay
GBS	Guillain Barré syndrome
IFDC	French MacArthur Inventory Scales
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JEV	Japanese encephalitis virus
M-CHAT	Modified Checklist for Autism on Toddlers
MRI	Magnetic resonance imaging
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus
ZIKA-DFA-FE	Zika – Départements français d’Amérique – Femmes enceintes <i>Cohort study: Pregnancy consequences of Zika virus infection in French Overseas Territories in the Americas</i>
ZIKA-DFA-BB	Zika – Départements français d’Amérique – Bébés <i>Cohort study: Neonatal and pediatric consequences of Zika virus infection in French Overseas Territories in the Americas</i>

Chapter I

An introduction to Zika virus

Previously considered to cause sporadic human infection with only mild, self-limiting illness, Zika virus (ZIKV) caused several large outbreaks in Yap Island in 2007, in French Polynesia in 2013-2014 and the 2015-2016 epidemic across the Americas which resulted in large outbreaks in several regions of the world. This chapter describes vector-borne transmission of the virus, the global epidemiology of ZIKV in humans – from its discovery in 1947 in Uganda to the epidemic of 2015-2016 in several regions of the world – and an overview of the disease outcomes ZIKV infection can cause.

Zika virus

ZIKV is a flavivirus from the *Flaviviridae* genus, along with other mosquito-borne viruses, including dengue virus (DENV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), and West Nile virus (WNV). It is an enveloped, spherical, positive-stranded RNA virus with approximately 11 kb genome. It has three structural proteins (E, C and prM) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).¹ The structural proteins allow the assembly of infectious virions and facilitate entry into host cells.² The non-structural proteins ensure viral replication and evasion from host defense.³

Vector-borne transmission of Zika virus

Zika virus was first described in 1947, following isolation from a febrile rhesus monkey at a sentinel site in the Zika forest, near Entebbe, Uganda.⁴ The following year, in the same forest, the virus was isolated from a mosquito of the *Aedes africanus* species.⁵

ZIKV is now understood to be primarily a vector-borne virus and *Aedes aegypti* is considered the mosquito species that is most competent for vector-borne ZIKV transmission and the major driver of ZIKV epidemics.⁶ The vector competence of *Aedes albopictus* has also been established.⁶ *Aedes* species are particularly well established in urban settings in tropical and subtropical areas, accounting for areas in which approximately 3.6 billion of the world's population inhabit.⁷ Warmer temperatures and urbanization have favoured the geographic expansion of *Aedes* species, particularly poor water, sanitation and hygiene and the use of containers to store water in and around inhabited areas, which create an aquatic larval environment for mosquito reproduction.^{6,8,9} Over 20 mosquito species have been identified as being naturally infected by ZIKV and ZIKV has been isolated in *Culex*, *Anopheles* and *Mansonia* species. However, the vector competence of these species of mosquito, or the extent to which they contribute to mosquito-borne transmission of ZIKV to humans, is unclear.^{6,10-13}

Aedes mosquitoes become infected with ZIKV during a viremic blood meal.^{7,14} ZIKV must disseminate to the hemocoel, infect the salivary glands and be secreted into the saliva in order for the mosquito to

transmit the virus to a human through a bite and the injection of infectious saliva.⁶ Mosquitoes become infectious approximately 10 days following a blood meal from an infectious human and are thought to remain infectious until death.¹⁵

Emergence of Zika virus

Discovery of Zika virus

Following the discovery of the virus in 1947, the first ZIKV infections in humans were described in 1952, through a serological survey of participants in Uganda and Tanzania.⁴ The first signs of illness following ZIKV infection were described following experimental infection in 1956¹⁵ and natural infection in 1964 by an individual working in the Uganda Virus Research Institute, located in the Zika forest.^{16,17} Both reports described symptoms that included fever and maculopapular rash.

Circulation of ZIKV in Africa and Asia

Serological evidence continued to accumulate across the latter half of the 20th century, indicating the circulation of ZIKV in humans throughout Africa and Asia. This included serological studies identifying the presence of anti-ZIKV antibodies in East Africa,¹⁸ Egypt,¹⁹ Nigeria,²⁰⁻²² India,²³ Malaysia,²⁴ the Philippines,²⁵ Thailand.²⁶ However, the extensive cross-reactivity with related flaviviruses, including DENV and JEV particularly given the available tests at that time, makes interpretations as to the certitude of ZIKV circulation at this time more difficult. Nonetheless, ZIKV was first isolated from *Aedes aegypti* mosquitoes in 1966 in Malaysia,²⁷ with acute human infections first reported in the region in Central Java, Indonesia in 1977.²⁸ Further, more specific neutralization tests appear to confirm the widespread circulation of ZIKV in Africa and Asia across the latter half of the 20th century.¹⁴

ZIKV epidemics in the South Pacific

The first significant outbreak of ZIKV and the first description of transmission outside Africa or Asia occurred on the island of Yap, Micronesia from April to July, 2007.^{29,30} Using Zika virus RNA or a specific neutralizing antibody response, an infection attack rate was shown to be 73% (95%CI: 68-77) in the population.³¹

Six years later, a larger ZIKV epidemic occurred in French Polynesia, extending to New Caledonia, the Cook Islands, Easter Island and the rest of the South Pacific.³² As in Yap Island, the majority of patients presented with rash, low-grade fever, conjunctivitis, arthralgia and myalgia.^{31,33} However, the first neurological complications associated with ZIKV infection were observed in adults and in fetuses. This included Guillain-Barre syndrome in adults^{34,35} and a retrospective temporal association between ZIKV and microcephaly in fetuses.³⁶ In addition, non-vector-borne transmission of ZIKV was described:

perinatal transmission when two mothers and their newborns had RT-PCR confirmed ZIKV infection in serum samples,³⁷ sexual transmission when infectious viral particles were found in the semen of a ZIKV patient³⁸ and transmission via blood transfusion after viral RNA was found in asymptomatic blood donors.³⁹

ZIKV epidemic in the Americas

The largest epidemic of ZIKV to date occurred in 2015-2016 across the Americas and resulted in spread to other regions of the world. The exact introduction event is not clear, but it is thought to have occurred between 2013 and 2015, most probably from the Pacific.⁴⁰ ZIKV is understood to have become established initially in Bahia province in northeastern Brazil⁴¹ and hypotheses as to the introduction event first focused on the 2014 World Cup soccer tournament, held in June and July, and the 2014 International Va'a Canoe World Sprint competition, held in August.⁴² The large epidemic spread rapidly in Brazil, particularly the northern and southeast states, and across the Americas. This led to spread to other regions of the world and transmission continued across the Pacific. Transmission during the epidemic has been described as phasic, with a first phase of rapid, often asymptomatic, spread across the northern and southeast states of Brazil, followed by a decline in transmission, likely due to seasonal, temperature-related declines in mosquito populations, and then a third phase of widespread transmission across the Americas from August 2015, which resulted in the first autochthonous transmission events in areas in North America.⁴³

In Brazil, prior to 2015, fewer than 200 cases of microcephaly were reported annually, but from mid-2015 to January 2016, more than 4000 suspected cases of microcephaly were reported.⁴⁴ This increase in cases of microcephaly, concurrent with the ZIKV epidemic, particularly in northeastern states of Brazil led to the declaration of a national public health emergency in November 2015.⁴⁵ This was followed in February 2016 by the declaration by the World Health Organization that the clusters of microcephaly cases and other neurological disorders reported in Brazil, following a similar cluster in French Polynesia in 2014, temporally associated with ZIKV, constituted a Public Health Emergency of International Concern.⁴⁶

During this epidemic event, the virus re-emerged in parts of Asia and Africa, most often through travellers returning from areas with autochthonous, vector-borne transmission of ZIKV. Epidemics have been described in Singapore,⁴⁷ Vietnam,⁴⁸ Thailand,⁴⁹ India,⁵⁰ Cabo Verde,⁵¹ Angola,⁵² and Mali.⁵³ Surveillance and diagnostics capacities for ZIKV were enhanced in regions of the world in which competent mosquitos for sustaining ZIKV transmission are established. This has resulted in reports of circulation of ZIKV in different regions of the world, as well as the detection of adverse pregnancy and fetal outcomes.

As of 2021, circulation of ZIKV has dropped to low levels, with the epidemic across the Americas being brought to an end in 2016. The epidemics in Yap Island in 2008, French Polynesia in 2013-2015 and the islands of the Caribbean in 2015-2016, have shown that when ZIKV successfully invades, the reproduction number, the generation time and the connectivity of the population result in a high infection attack rate in the population.^{8,54} Mathematical modelling suggests that herd immunity derived from the 2015-2016 epidemic may delay the recurrence of large epidemics in areas that experienced ZIKV transmission by at least a decade and that the mean age of infection will fall in future epidemics as a result of the immunity acquired by older people.⁵⁴ Nonetheless, even in countries that were heavily affected during the 2015-2016 epidemic, some regions within the country that have established vector populations were spared from ZIKV transmission, leaving them immunologically susceptible to ZIKV epidemics.^{55,56} Further, when considering the geographic range of competent vector populations, much of the tropical and sub-tropical regions of the Americas, Africa, Southeast Asia, the Indian subcontinent, the Pacific Islands and Northern Australia are considered as being at risk for ZIKV outbreaks.⁵⁷⁻⁵⁹

While ZIKV is now considered to be an epidemic virus, in parts of Africa and Asia, ZIKV is understood to be endemic. Further, serological surveys in human populations in Africa and parts of Asia are consistent with endemic circulation, with repeated introduction into the human population from a sylvatic reservoir involving non-human primates,^{60,61} or ongoing transmission between humans via mosquitoes.^{20,26,62-64}

Phylogeny of Zika virus

Two major lineages of ZIKV, known as the African and Asian lineages, have now been identified. The African lineage derives from the first identification of the virus in Uganda in 1947. The Asian lineage was first identified in Asia in the 1950s and subsequently spread to the Pacific Islands in 2013-2014 and then to the Americas in 2015-2016. The 2015-2016 epidemic in the Americas was caused by a strain of the Asian lineage, commonly referred to as the American strain. The strain of the Asian lineage that had been and continues to circulate in Asia will be referred to in this thesis as the 'Asia lineage Asia strain.' The re-emergence of ZIKV in parts of Asia and Africa during the 2015-2016 epidemic concentrated largely in the Americas was often observed to be the introduction of the Asia lineage American strain.^{47,52,65} All human outbreaks of ZIKV to date have been the result of ZIKV strains of the Asian lineage.⁶⁶ The African ZIKV lineage has never been detected beyond the African continent, nor associated with epidemic transmission or disease in humans.⁶⁶ The African and Asian lineages of ZIKV have remained distinct from the 1940s onwards.⁶⁷

Disease associated with Zika virus infection

Detection of ZIKV RNA using nucleic acid amplification tests

Confirmation of acute ZIKV infection is based on detection of viral sequences by nucleic acid amplification tests, including real-time, reverse-transcriptase polymerase chain reaction (RT-PCR). ZIKV RNA can be detected in serum, urine and whole blood from the day of symptom onset up to 7 days post symptom onset, although persistence of ZIKV RNA has reliably been shown in urine and serum up to 20 days post symptom onset.^{68,69}

Confirmation of ZIKV infection in pregnancy outcomes remains unclear. ZIKV RNA has been detected in placental tissue, fetal brain tissue, amniotic fluid and fetal blood, but ZIKV viremia in these specimens has been found to be transient and as such, cannot yet be used reliably to confirm congenital ZIKV infection.^{70,71} Vertical transmission was estimated to occur in 18% of fetuses in French Guiana.⁷¹ While in Brazil, vertical transmission was shown to have occurred more frequently, but results between IgM and PCR had limited concordance and were not necessarily associated with infant abnormalities.⁷² Vertical transmission has been shown to occur in all trimesters of pregnancy, whether infection in the mother is symptomatic or asymptomatic.^{71,73-75}

Detection of anti-ZIKV antibodies using serological assays

IgM class antibodies are detectable approximately one week following infection and anti-ZIKV IgM antibodies may be detectable more than 2 months following infection.⁷⁶ IgG class antibodies develop following the IgM response and remain detectable up to 6 months,^{77,78} although the duration of persistence remains unclear.^{79,80} The neutralizing ability of IgG antibodies is specific to ZIKV in individuals without prior flavivirus infection. However, for individuals with prior flavivirus infection, particular the four serotypes of DENV, specificity of the IgG neutralizing antibodies may be reduced as a result of cross-reactivity.

Serology cannot be used to confirm acute ZIKV infection. In any event, the results of serology testing must be interpreted alongside several factors including symptoms suggestive of ZIKV infection, the epidemiological context, evidence of prior flavivirus infection, the type of serological assay used and the reliability of the results. At best, serology may be used to indicate probable ZIKV infection, and will often require a confirmatory neutralization test – for example, positive results from anti-ZIKV IgM tests should be confirmed with a neutralization test.

Clinical presentation of ZIKV infection

Serological studies post epidemic event indicate that the majority of ZIKV infections are asymptomatic.^{31,79,81-83} Among symptomatic ZIKV infections, clinical manifestations are usually mild and self-

limiting. The most common symptoms that have been reported from large cohorts of individuals with symptomatic ZIKV infection are: macular or maculopapular rash, pruritus, arthralgia, conjunctivitis, headache, myalgia and fever.^{73,74,85}

ZIKV infection and neurological complications in adults

During the epidemic in French Polynesia in 2013-2014, the first neurological complications associated with ZIKV infection were described. Oehler et al³⁴ described a case of Guillain Barré syndrome (GBS) occurring immediately following suspected ZIKV infection. This was followed by a case control study of 42 adult patients in French Polynesia during the ZIKV epidemic with GBS, characterized by acute motor axonal neuropathy and rapid evolution of disease. All cases had detectable neutralizing antibodies against ZIKV,³⁵ implicating ZIKV as a cause of GBS. Incidence of GBS increased in areas which highlighted the temporal and geographical association with the concurrent ZIKV epidemic in the Americas in 2015-2016.^{86,87} There is heterogeneity in the clinical and electrophysiological phenotype of cases of GBS following ZIKV infection, but ZIKV-related GBS generally appears to be similar to the classic GBS and appears to be characterized by rapidly progressive and symmetrical weakness of the limbs with sensory symptoms, often with facial palsy, reduced or absent tendon reflexes and high rates of respiratory dysfunction and ICU admission.⁸⁶

The spectrum of neurological complications caused by ZIKV infection likely extends beyond GBS. Case reports of encephalopathy,⁸⁸ encephalitis,⁸⁹ meningoencephalitis,⁹⁰ and myelitis⁹¹⁻⁹³ following RT-PCR confirmed ZIKV infection have also been published.

ZIKV infection and neurological complications in fetuses and infants

In contrast to the mature central nervous system, the developing fetal CNS appears to be more susceptible to the neurovirulent effect of ZIKV. Following the detection of an increase in cases of microcephaly in the northern state of Pernambuco in Brazil in late 2015, obstetricians identified ZIKV RNA in amniotic fluid and fetal tissues in fetuses and neonates with microcephaly.⁹⁴⁻⁹⁸ This was followed by evidence of transplacental transmission and neurotropism of ZIKV⁹⁹ and by case control studies that confirmed the causal link between ZIKV infection during pregnancy and microcephaly and other neurological abnormalities in fetuses.^{100,101} Experimental studies found that ZIKV is able to infect human neural progenitor cells,¹⁰² triggering apoptosis.¹⁰³

The spectrum of fetal abnormalities associated with ZIKV infection during pregnancy is not limited to microcephaly. The 2015-2016 epidemic in the Americas resulted in the first description of congenital Zika syndrome (CZS), a condition that is characterized by five distinguishing features: severe microcephaly with partially collapsed skull, cranial morphology and brain anomalies, including thin

cerebral cortices with subcortical calcifications, ventriculomegaly, corpus callosum anomalies; ocular anomalies, including macular scarring and focal pigmentary retinal mottling; congenital contractures, such as clubfoot, arthrogryposis; and marked early neurologic sequelae, including motor or cognitive disabilities, hyper- or hypotonia, swallowing dysfunction, vision or hearing impairment, epilepsy.¹⁰⁴ In addition, congenital ZIKV infection has been reported to be associated with eye findings in the absence of microcephaly or other brain anomalies, and with other abnormalities, including cardiac anomalies, digestive disorders and respiratory conditions.¹⁰⁵ Placentomegaly has been observed more frequently in ZIKV-infected placentae.¹⁰⁶ Further, fetal autopsies have found ZIKV in fetal and kidney tissues.¹⁰⁷

The 2015-2016 ZIKV epidemic allowed risk estimates risk of adverse pregnancy outcomes following ZIKV infection to be calculated. These ranged from 5% (95%CI:4-6) in a registry-based study in the United States of America,⁷⁵ 5% in a prospective cohort study of symptomatic women in the Brazilian Amazon,¹⁰⁸ 6% (95%CI:4-8) in a registry-based study in the United States of America,¹⁰⁹ 7% (95%CI:5-10)⁷⁴ and 13% (95%CI:9-18)⁷¹ in two prospective cohort studies in French Territories in the Americas, to 37%¹¹⁰ and 46% (95%CI:37-56)⁷³ in two prospective cohort studies of women from Brazil. Part of the heterogeneity in risk estimates across studies is likely attributable to differences in study designs, use of imaging and definitions of adverse events. Further, the risk of adverse pregnancy outcomes has been found to be greatest when ZIKV infection occurred in the first trimester compared to second or third trimesters.^{73-75,109}

ZIKV infection and neurological complications in infants

The description of abnormalities caused by ZIKV has largely focused on pregnancy and infant outcomes, and to a lesser extent, outcomes into infancy. Beyond birth, abnormalities have been described in infants exposed to ZIKV *in utero*. In infants with CZS, these include profound delays across all functional domains.¹¹¹ This includes cognitive and language delays,¹¹¹⁻¹¹³ motor impairment,^{111,112,114-117} epilepsy, hearing and vision losses and sleeping difficulties.^{114,116,118,119} oropharyngeal dysphagia,¹²⁰ postnatal onset hydrocephalus in infants with microcephaly at birth,¹²¹ electroencephalogram abnormalities in infants with microcephaly at birth,¹²² and diaphragmatic paralysis in infants with microcephaly and arthrogryposis from birth.¹²³⁻¹²⁵

The appearance of abnormalities in early infancy possibly related to ZIKV infection during pregnancy has also been described. This includes postnatal onset microcephaly in infants with a normal head circumference at birth. Rice et al¹²⁶ assessed follow-up care reports of 1450 with confirmed or possible Zika virus infection during pregnancy and found 20 (1%) with postnatal-onset of microcephaly. Pereira HVFS et al¹¹⁷ described 75 children with CZS, 15 of whom did not have microcephaly at birth, but who went on to develop microcephaly in early infancy.

ZIKV infection and development outcomes in early childhood

Adverse development findings in infants exposed *in utero* to ZIKV with and without CZS have been described in the United States of America,¹²⁶ Brazil^{112,127-129} and Colombia.¹³⁰

Rice et al¹²⁶ assessed follow-up care reports of 1450 infants with *in utero* ZIKV exposure with and without ZIKV associated birth defects aged up to 1 year of age or older and found 9% had at least one neurodevelopment abnormality possibly associated with *in utero* ZIKV exposure. Nielson-Saines et al¹²⁸ used the Bayley Scales of Infant and Toddler Development-III at 18 months of age and described the neurodevelopment outcomes of 146 infants born to women with rash and RT-PCR confirmed ZIKV infection during pregnancy. 41 (28.1%) infants were found to have abnormal neurodevelopment, with language being the area of neurodevelopment with the greatest proportion of infants with an abnormal outcome. Bertolli J et al¹¹² assessed development of 120 infants exposed *in utero* to ZIKV with and without adverse anthropometric findings at birth. In those with microcephaly at birth, 63% were found to have severe at 24 months of age; while in those without microcephaly at birth, 5% were found to have severe developmental delays at 24 months of age.

Several studies have combined neuroimaging investigations and assessments of neurodevelopment. Lopes Moreira ME et al¹²⁷ used the Bayley Scales of Infant and Toddler Development-III to assess 94 children who had also undergone neuroimaging between 12-18 months of age. 24 (25.5%) infants were found to have at least one abnormal neurodevelopment finding. Einspieler C et al¹²⁹ followed 56 infants without microcephaly at birth and who were born to women with RT-PCR positive ZIKV infection during pregnancy. 10 (17.9%) infants were found to have an adverse neurologic examination or neurodevelopment assessment at 12 months of age.

Phylogeny of Zika virus and disease outcomes

The differences in the pathogenicity of the two principal viral lineages are not fully understood.

The Asian lineage ZIKV has been identified as associated with all human outbreaks and adverse pregnancy or infant outcomes to date.⁶⁶ In contrast, while studies have demonstrated that ZIKV has circulated in Africa for since the mid-20th century, no case reports or studies have described the impact of the African lineage ZIKV on pregnancy or infant outcomes. *In vitro* and animal model studies of the African lineage point to increased transmissibility^{131,132} and pathogenicity,¹³³⁻¹³⁶ as compared to the Asian lineage. A comprehensive phenotypic characterisation of recently isolated ZIKV strains of African and Asian lineage found higher transmissibility in mosquitoes and higher lethality in both adult and fetal mice in African ZIKV strains than Asian strains, suggesting that African strains have a propensity to cause fetal loss rather than birth defects.¹³⁷

The comparative pathogenicity of the strains within the Asian ZIKV lineage are also not fully understood. The severe disease outcomes following ZIKV infection became apparent after the epidemics in French Polynesia in 2013-2014 and in the Americas in 2015-2016. Further, animal models show the American strains of Asian lineage to be more neurovirulent in mice compared to the ancestral Asian lineage Asia strain. Nonetheless, one case report of fetal microcephaly following congenital ZIKV infection with the Asian lineage Asia strain of ZIKV has been reported from Thailand,¹³⁸ as well as a report from Cambodia of two expatriate mothers who gave birth to infants with microcephaly in 2009-2011 at the time the Asian lineage Asia strain was circulating in the country.^{63,113} Both reports suggest that the Asian lineage Asia strain of ZIKV is able to cause adverse fetal outcomes.

One hypothesis as to the change in disease epidemiology and appearance of severe disease outcomes prior to the epidemic in French Polynesia is mutations in the virus, which may have increased its virulence. The prM protein, one of the three structural proteins of the ZIKV genome, has been revealed to harbour amino acid substitutions as strains evolve.¹³⁹ Specifically, a single serine-to-asparagine substitution has been identified in the prM protein, known as the prM S139N mutation.¹⁴⁰ Evolutionary phylogenetic analysis indicates that the mutation appeared prior to the epidemic in French Polynesia and was largely maintained during the epidemic in the Americas.¹⁴⁰ As such, the prM S139N mutation may be responsible for more severe disease outcomes.¹⁴¹ Another mutation, the A982V substitution in the NS1 protein has been suggested to have resulted in enhanced ZIKV infectivity in mosquitoes, which may in turn have increased transmissibility across the Americas in 2015-2016.¹⁴²

A second hypothesis, not mutually exclusive to the first, to explain the change in disease epidemiology derives from the introduction of a virus into an immunologically naïve population, facilitated by competent mosquito vectors.^{8,43} That is, serological evidence suggests low levels of ZIKV circulation in Asia across the 1950s, in contrast to the immunologically naïve population in the Americas.⁵⁶ The resulting epidemic may be such that the magnitude of the event provides sufficient statistical power to detect rare events,¹⁴³ particularly when considering that diagnostic and surveillance capacities for ZIKV and associated complications in Africa and Asia were previously limited.

Prevention of Zika virus infection

There are currently no available licensed vaccines for ZIKV infection, with evaluations of vaccine candidates unable to complete phase 2 and 3 clinical trials in the absence of ongoing ZIKV transmission.¹⁴⁴ In addition, despite several antiviral drugs showing *in vitro* activity against ZIKV,^{145,146} there are currently none that have replicated the same activity against ZIKV *in vivo*. As a result, prevention and control of ZIKV relies primarily on vector control and limiting ZIKV exposure by using topical insect repellents to reduce mosquito biting.

During the 2015-2016 epidemic, vector control included repellents, bednets and indoor residual spraying. In addition, efforts were launched to evaluate the use of *Wolbachia* bacteria in the biological control of mosquito populations. This involves the introduction of *Wolbachia* into *Aedes aegypti* mosquito eggs, rendering the eggs of the subsequent generation of *Aedes aegypti* non-viable. It also has shown to inhibit ZIKV infection and reduce the viral load in mosquito saliva.^{147,148}

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Objectives of the thesis

The following three chapters of the thesis describe the results of original research conducted in cohort studies in the French Territories in the Americas following the 2015-2016 ZIKV epidemic and in Vietnam following a ZIKV outbreak in 2016-2017.

Description of the ZIKA-DFA-FE / ZIKA-DFA-BB cohort studies in French Territories in the Americas

In 2015-2016 there was widespread circulation of ZIKV in the Caribbean, including French Territories in the Americas: Guadeloupe, Martinique and French Guiana. In the early stages of the epidemic, a cohort study was implemented to follow women who were pregnant during the ZIKV epidemic. This included women with molecular or serological evidence of ZIKV infection during pregnancy, as well as women who had no evidence of molecular or serological evidence of ZIKV infection during pregnancy. A second cohort study followed on from this, which involved the enrolment of neonates born to women who participated in the first cohort study. The children born to women who were pregnant during the 2015-2016 ZIKV epidemic are now into early childhood.

Women were recruited through antenatal attendance at the Gynaecology and Obstetrics services of hospitals in Point-à-Pitre and Basse-Terre in Guadeloupe, Fort-de France in Martinique, and Cayenne and Saint-Laurent du Maroni in French Guiana. There was no predetermined sample size for either the cohort of pregnant women or of their infants. Rather, the aim was to recruit the maximum number of women with ZIKV infection during pregnancy and retain their infants in the subsequent cohort study.

Description of the ZIKV outbreak investigation in Southern Vietnam

Enhanced epidemiological surveillance for ZIKV in Vietnam following the 2015-2016 ZIKV epidemic in the Americas resulted in the detection of 68 women living in Southern Vietnam who had a RT-PCR confirmed ZIKV infection during pregnancy. As above, there was no predetermined sample size for the retrospective cohort study. Rather, the aim was to describe the pregnancy outcomes for all 68 women and recruit the highest proportion of these women and their infants in the subsequent epidemiological and virological investigations.

Objectives of the studies

The developmental delays in early childhood of those with CZS have been described in the literature. However, descriptions of the developmental outcomes of children who were normocephalic at birth and had no adverse clinical findings at birth possibly related to congenital ZIKV exposure are scarce. This is important to understanding whether developmental delays may appear in the post-natal period, and by extension, to the clinical management into early childhood of infants born to women with ZIKV infection during pregnancy. As such, the second chapter of the thesis seeks to identify any differences

in neurodevelopment outcomes at 24 months of age attributable to *in utero* ZIKV exposure among those who were normocephalic and no adverse clinical findings at birth. Using a cohort study design, we are able to compare the neurodevelopment outcomes at 24 months of age in children with and without *in utero* ZIKV exposure.

The ZIKA-DFA-FE cohort study has previously provided an estimate of the risk of adverse pregnancy outcomes following ZIKV infection during pregnancy. However, these risk estimates have not yet been extended into adverse outcomes into early childhood, and descriptions of developmental delays in early childhood of those with CZS have also tended to be case series. The third chapter therefore seeks to estimate the risks of adverse pregnancy outcomes and adverse early childhood outcomes among women in the ZIKA-DFE-FE cohort study with symptomatic, RT-PCR confirmed Zika virus infection during pregnancy and their infants up to 24 months of age, including an assessment of severity of adverse outcomes. As above, a prospective cohort study design was used, with standardized clinical assessments during pregnancy and into early childhood.

The fourth chapter uses a multidisciplinary investigation to describe the outcomes of the pregnancies among the 68 women with RT-PCR confirmed ZIKV infection, the virological and phylogenetic analyses of the ZIKV strain implicated in the outbreak and the kinetics of the longer-term immune response to ZIKV infection.

Overall, the findings of these studies highlight the importance of epidemiological research both during an epidemic ZIKV event and into the post-acute phase. Together, these findings contribute to the accumulative body of literature on the impact of ZIKV infection on maternal, neonatal and early childhood health outcomes which is discussed in the final chapter of the thesis.

Chapter II

Consequences of *in utero* Zika virus exposure and adverse pregnancy and early childhood outcomes: a prospective cohort study

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Abstract

Background: Background: We aimed to describe adverse pregnancy outcomes among women who had symptomatic, RT-PCR confirmed ZIKV infection and early childhood outcomes among their infants.

Methods: We enrolled pregnant women with symptomatic, RT-PCR confirmed ZIKV infection in a prospective cohort study, and their infants in a prospective pediatric cohort study. We defined adverse pregnancy and early childhood outcomes based on selected neurologic, ophthalmologic, auditory, musculoskeletal and anthropometric abnormalities. We used RT-PCR and serologic tests to determine the ZIKV infection status of the child.

Results: Between March 10 and November 24, 2016, we enrolled 546 pregnant women with RT-PCR-confirmed ZIKV infection. The overall risk of adverse pregnancy and early childhood outcomes possibly related to *in utero* ZIKV exposure was 15.7% (95%CI:12.8-19.0), distributed as follows: 3.6% (95%CI:2.3-5.6) severe sequelae or fatality; 2.7% (95%CI:1.6-4.5) major abnormalities; 9.4% (95%CI:7.1-12.2) mild abnormalities. The risk of severe sequelae or fatality was higher when ZIKV infection occurred during the first trimester (7.0%), compared to the second (2.7%) or third trimester (1.4%) ($p=0.02$). Among the infants for whom ZIKV infection status could be determined, the vertical transmission rate was 3.0% (5/167) (95%CI:1.1-7.2).

Conclusion: Among pregnant women with symptomatic, RT-PCR confirmed ZIKV infection, severe or major pregnancy or early childhood outcomes were present in 6.3% of fetuses and infants. Severe outcomes occurred more frequently in fetuses and infants whose mothers had been infected in the first trimester.

Introduction

In utero ZIKV infection is known to cause congenital neurologic abnormalities, vision and hearing defects, motor dysfunction, and developmental delays.¹⁻⁷ Descriptions of abnormalities caused by *in utero* ZIKV infection have largely focused on pregnancy and neonatal outcomes. Abnormalities in early childhood have also been described, including developmental delays and anthropometric abnormalities, including microcephaly, occurring in the post-natal period.^{6,8} However, the risk of adverse outcomes in early childhood in infants born to women infected with ZIKV during pregnancy has not yet been well quantified.

The ZIKV epidemic in French Territories in the Americas (French Guiana, Guadeloupe and Martinique) began in early 2016. In a cohort study of 546 pregnant women with symptomatic, RT-PCR-confirmed ZIKV infection, the risk of adverse pregnancy outcomes was estimated to be 7.0%, with a greater risk when the ZIKV infection occurred during the first trimester (12.7%), compared to the second trimester (3.6%) or the third trimester (5.3%).⁹ A subsequent pediatric cohort study was established to follow the infants born to these women up to 24 months of age. Here, we present the outcomes up to 24 months of age of the infants born to women who had symptomatic RT-PCR confirmed ZIKV infection during pregnancy.

Methods

Study overview

To study the impact of *in utero* ZIKV exposure on pregnancy and early childhood outcomes, the ZIKA-DFA-FE (pregnant women) and ZIKA-DFA-BB (newborns) prospective cohort studies were implemented in Guadeloupe, Martinique, and French Guiana in 2016 during the ZIKV epidemic. A key component of the ZIKA-DFA-FE and ZIKA-DFA-BB cohort studies was the prospective and standardized follow-up, which lasted until the end of pregnancy in the case of the pregnant women cohort study, and until 24 months of age, in the case of the newborn cohort study.

Criteria for inclusion in analysis

In accordance with the guidelines of the French High Council for Public Health and the French National College of Gynaecologists and Obstetricians that were issued on July 28, 2015, and February 5, 2016, respectively, whenever a pregnant woman presented to an outpatient clinic or emergency department of a participating centre with symptoms consistent with acute ZIKV infection, she underwent a clinical examination, and blood and urine specimens were obtained to confirm a recent ZIKV infection.

Women were included in this analysis if they met all of the following criteria at the time of their enrolment in the ZIKA-DFA-FE cohort study: 1) ongoing pregnancy; 2) clinical symptoms consistent with acute ZIKV infection, with at least one symptom of pruritic rash, fever, conjunctival hyperemia, arthralgia, or myalgia; 3) laboratory confirmation by RT-PCR of recent ZIKV infection performed on a specimen of blood, urine, or both.⁹ All live-born infants born to women meeting the eligibility criteria described above were included in the analysis of adverse early childhood outcomes.

Cohort follow-up

All neonates/fetuses underwent routine clinical examination at the end of pregnancy. Live-born infants were eligible for inclusion in the pediatric cohort study if they were born at or after 35 weeks gestational age. Eligible infants included in the prospective pediatric cohort underwent further standardized clinical examinations at two, four, nine, 18 and 24 months of age. Additional examinations were offered between 4-9 months, 9-18 months and 18-24 months of age, if the infant had missed any previous clinical examination. Additional imaging (cerebral ultrasonography) evaluations and clinical (ophthalmologic and auditory) examinations were conducted primarily at birth, or at any point in the follow-up period if the additional imaging or examinations were not performed at birth. Further magnetic resonance imaging (MRI) was performed at any point in the follow-up period, upon indication of the attending pediatric clinician.

For more complete follow-up of those who declined participation in the pediatric cohort, or who were lost to follow-up across the 24-month follow-up period, medical registries in Guadeloupe, Martinique

and French Guiana were consulted to determine the infant's vital status at 24 months of age. For any death, the date and cause of death were recorded. Any abnormal developmental outcomes at 24 months of age that were noted in the medical records were also recorded.

Adverse early childhood outcomes

As *in utero* ZIKV infection has been associated with neurological, visual, auditory and anthropometric abnormalities^{1,3,5} and developmental delays,²⁻⁷ we considered the following abnormalities and adverse developmental outcomes at any time in the 24-month follow-up period, as defined by Rice et al:⁸

- Abnormal auditory examination which included otoacoustic emissions or automated auditory evoked potential assessments
- Abnormal ophthalmologic examination which included RetCam wide-field retinal imaging (Shuttle, Clarity Medical Systems, Pleasanton, USA) for fundus imaging and abnormalities in vitreous, choroid, retina and optic disc; or visual impairment documented by visual screening test
- Clinician-documented seizures, excluding febrile seizures
- Body tone abnormalities, hypertonia or hypotonia, suspicion or diagnosis of cerebral palsy
- Musculoskeletal or motor abnormalities, dyskinesia or dystonia, documented by the clinician
- Swallowing abnormalities, documented by instrumented or non-instrumented evaluation
- Post-natal onset microcephaly, defined as the two most recent head circumference measurements <3rd percentile for age and sex based on the World Health Organization's Child Growth Standards, or by a downwards trajectory of head circumference percentiles, with the most recent measurement <3rd percentile
- Possible neurodevelopmental delays, through administration of three pediatric neurodevelopment evaluation tools administered to a parent/legal guardian at the time of the 24-month pediatric consultation:¹⁰ a 30-item parent-reported screening test Ages and Stages Questionnaire-III (ASQ),¹¹ previously validated in France,¹² to identify toddlers at risk for developmental delay; a 23-item parent-reported screening test Modified Checklist for Autism on Toddlers (M-CHAT), to identify toddlers at risk for behaviour disorder;¹³ the French MacArthur-Bates Communicative Development Inventories (Inventaires français du développement communicatif - IFDC) to assess French language acquisition.¹⁴

In addition, we also considered abnormal transfontanelle cerebral ultrasound, and/or MRI at any time in the 24-month follow-up period.

ZIKV infection status

As all women had RT-PCR confirmed ZIKV infection during pregnancy, all infants were considered exposed to ZIKV *in utero*. A cord blood sample was collected at delivery, or a serum sample from the newborn within the first 10 days of life. In addition, a serum sample was collected during one of the routine clinical examinations after 9 months of age. These samples were used to determine the ZIKV infection status of the infant. An infant was considered to have had *in utero* ZIKV infection if ZIKV RT-PCR was positive at birth in the infant's blood or urine, in placenta or in amniotic fluid; or if anti-ZIKV IgM antibodies were detected (EuroImmun ELISA or in-house MAC ELISA in French Guiana¹⁵) in the cord blood or serum collected within the first 10 days of life; and/or if anti-ZIKV IgG antibodies were detected (EuroImmun ELISA or in-house MAC ELISA in French Guiana¹⁵) in the serum sample beyond 9 months of age and the date of birth was posterior to the end of the ZIKV epidemic: September 11, 2016 in French Guiana, 25 September 2016 in Guadeloupe, and 16 October 2016 in Martinique. An infant was considered as having no evidence of *in utero* ZIKV infection if the above tests were negative. If samples or results were missing or equivocal, the infant's ZIKV infection status was considered undetermined.

Statistical analysis

The characteristics of women and infants included and not included in the pediatric cohort study were compared using Student's t-test for continuous variables and Pearson's chi-squared test for categorical variables. Adverse pregnancy and early childhood outcomes were categorized based on severity and, in the case of early childhood outcomes, persistence, in consultation with the study's referent pediatrician, and compared by trimester of maternal ZIKV infection using Pearson's chi-squared test. For those included in the pediatric cohort study, adverse early childhood outcomes were also compared by the child's ZIKV infection status using Fisher's exact test for categorical variables due to the small number of participants in the group with evidence of ZIKV infection. For all statistical analyses, $p < 0.05$ was considered statistically significant. All statistical analyses were performed using R, version 3.6.1.

Ethics approval and consent to participate

The study received ethical approval from the Comité de Protection des Personnes Sud-Ouest et Outre Mer III (registration numbers: ZIKA-DFA-FE (pregnant women) study DC-2016-2636; ZIKA-DFA-BB (pediatric) study 2016-A00684-47). All study procedures were explained to and informed consent obtained from the pregnant woman and from a parent or legal guardian of the newborn before enrolment in the study. Both cohort studies are registered with ClinicalTrials.gov under the identifiers: NCT02916732 and NCT02810210.

Results

Between March 2, 2016 and November 24, 2016, 1152 pregnant women were assessed for possible acute ZIKV infection. Of these, 546 women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy were included in this analysis. From the 546 pregnant women, there were 555 fetuses or newborns (9 twin pregnancies). Of these, there were 28 (5.0%) pregnancy losses: spontaneous or voluntary abortions, medical terminations of pregnancy or stillbirths. Among the 527 live births, 320 were included in the prospective cohort study, of which 206 completed 24 months of follow-up. Additional information from medical registries was obtained at 24 months of age for 151 infants whose parent/guardians declined their inclusion in the prospective cohort study at birth and from 106 infants who were included in the pediatric cohort study but who were lost to follow-up during the 24-month study period (Figure 1).

Table 1 describes the characteristics of the 546 women and 555 fetuses/newborns included in this analysis. Supplementary Table 1 compares the maternal and neonatal characteristics of the women whose infant was included in the pediatric cohort study and of the women whose infant was not included in the pediatric cohort study. The only difference between the two groups was in the place of residence, with a greater proportion of infants included in Martinique and Guadeloupe, compared to French Guiana ($p<0.001$).

Adverse pregnancy outcomes

The risk of adverse pregnancy outcomes in this cohort has been previously published.⁹ Briefly, the overall risk of neurologic and ocular defects possibly associated with maternal ZIKV infection that were evident at birth was 7.0% (95%CI 5.0-9.5) and the risk of congenital Zika syndrome was 3.1%.⁹ Neurologic and ocular abnormalities were more common when maternal ZIKV infection had occurred during the first trimester (12.7%), compared to the second (3.6%) or third trimester (5.3%) ($p=0.001$).⁹

ZIKV infection status

Three of 252 infants tested had a positive RT-PCR ZIKV result at birth in a blood, urine, placenta or amniotic fluid sample. 169 cord blood or newborn serum samples collected during the first 10 days of life were available for anti-ZIKV serologic testing: 60 from infants born to women infected in the first trimester, 84 from infants born to women infected in the second trimester and 25 from infants born to women infected in the third trimester. None of these available samples were positive for anti-ZIKV IgM antibodies. Anti-ZIKV IgG antibodies were detected in 11 of 182 (6.0%) serum samples collected from infants after 9 months of age. According to the diagnostic criteria described in the Methods, we were able to classify five (1.6%) children as having evidence of ZIKV infection, 162 (51.9%) as having no evidence of ZIKV infection, and the remaining 153 (46.6%) as having

unknown/undetermined ZIKV infection status. Among the infants for whom ZIKV infection status could be determined, we found a vertical transmission rate of 3.0% (95%CI:1.1-7.2).

Adverse early childhood outcomes

Of the 320 infants who were included in the pediatric cohort study, 272 (85.0%) completed additional auditory examinations; 261 (81.6%) completed additional ophthalmologic examinations; 216 completed transfontanelle cerebral ultrasonography evaluations (67.5%); and 76 (23.8%) were referred for and completed an MRI examination. Table 2 shows the selected adverse early childhood outcomes at two, four, nine, 18 and 24 months of age by ZIKV infection status among those included in the pediatric cohort study. We did not consider neurodevelopmental delays as not all children completed the neurodevelopment assessment at 24 months of age. Overall, we found that across the 24-month follow-up period, three (60%) of those with *in utero* ZIKV infection, 43 (26.5%) of those with no *in utero* ZIKV infection, and 33 (23.1%) of those with unknown/undetermined status had at least one of the selected abnormalities possibly linked to *in utero* ZIKV exposure, with most abnormalities reported at the first visit at two months of age and not again at subsequent follow-up consultations.

Of the 151 infants not included in the pediatric cohort due to parents/guardians declining their participation, consultation of medical registries indicated that there were two deaths in early childhood: one due to severe perinatal asphyxia and occurring three weeks after birth; the other at 14 months of age, but the cause of death was not reported. With respect to selected abnormalities related to *in utero* ZIKV exposure, in non-standardized clinical assessments, three infants had vision impairment, and one had impaired hearing. Of the 106 infants who did not complete 24 months of follow-up as part of the pediatric cohort study, consultation of medical registries indicated that there had been no deaths during the follow-up period. With respect to selected abnormalities related to *in utero* ZIKV exposure, in non-standardized clinical assessments, vision impairment had been reported for three infants, and one infant had experienced seizures.

When considering adverse pregnancy and early childhood outcomes likely related to *in utero* ZIKV exposure (Table 3B), we found the overall risk to be 15.7% (95%CI:12.8-19.0): 3.6% (95%CI:2.3-5.6) severe sequelae or fatality; 2.7% (95%CI:1.6-4.5) major abnormalities; 9.4% (95%CI:7.1-12.2) mild abnormalities. Among the adverse pregnancy and early childhood outcomes likely related to *in utero* ZIKV exposure, we found the risk of severe sequelae or fatality to be higher when ZIKV infection had occurred during the first trimester (7.0%), compared to the second (2.7%) or third trimester (1.4%) ($p=0.02$).

Of the 15 children who had microcephaly detected at birth and the two children whose had microcephaly was detected in the post-natal period (Figure 2), one with evidence of ZIKV infection had persistent

microcephaly across the duration of follow-up. Importantly, 14 of the 16 children with anthropometric data available after birth showed resolution of microcephaly across the 24-month period of follow-up. Of these, one infant had recurrent and transient microcephaly between 9 and 18 months of age but which was no longer detectable at 24 months of age.

Discussion

We have been able to describe the risk of adverse pregnancy and early childhood outcomes during the ZIKV epidemic in Guadeloupe, Martinique and French Guiana in 2016. Among 546 women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy, we found an overall risk of fetal or infant neurological and ocular defects possibly associated with maternal ZIKV infection to be 7% (95%CI 5.0-9.5), and the risk of congenital Zika syndrome to be 3.1%.⁹ Among the 320 infants included in the pediatric cohort study, 79 (24.7%, 95%CI: 20.1-29.9) had at least one of the selected abnormalities possibly related to *in utero* ZIKV exposure in the post-natal period, but the majority of these were transient and mild abnormalities. When considering adverse pregnancy and early childhood outcomes likely related to *in utero* ZIKV exposure among the 555 fetuses and infants, we found the overall risk to be 15.7% (95%CI:12.8-19.0): 3.6% (95%CI:2.3-5.6) severe sequelae or fatal outcome; 2.7% (95%CI:1.6-4.5) major abnormalities; 9.4% (95%CI:7.1-12.2) mild abnormalities.

To our knowledge, this is the first study to quantify the risk of adverse pregnancy and early childhood outcomes, including an assessment of severity, using a standardized clinical assessment in a cohort of women with RT-PCR-confirmed ZIKV infection during pregnancy. The prospective mother-child cohort study design allowed us to follow the evolution of clinical conditions from time of maternal ZIKV infection to 24 months of age. The categorization of the severity and persistence of abnormalities allowed us to distinguish infants with single occurrence, mild abnormalities from those with severe sequelae and impaired early childhood development. We were also able to show severe sequelae or fatal outcomes to be more frequent when maternal ZIKV infection occurred in the first trimester (7.0%), compared to the second (2.7%) or third trimester (1.4%) ($p=0.02$). This is a particularly important consideration for clinical decisions regarding pregnancy management in the context of ZIKV infection.

Our results regarding the risk of selected abnormalities possibly related to *in utero* ZIKV exposure in the post-natal period are similar to those of the largest ZIKV *in utero* exposed pediatric cohort to date. Rice M et al⁸ found that among 1450 children over age of 1 year and born to women with ZIKV infection during pregnancy in the United States of America who had non-standardized follow-up care reported at any time after 14 days of life, 87 (6%) had at least one ZIKV-associated birth defect, and 203 (14%) had a ZIKV-associated birth defect, neurodevelopmental abnormality possibly associated with congenital ZIKV infection, or both. In contrast, risk estimates from Brazil remain higher. Among 130 children with antenatal exposure to ZIKV in Brazil, 45 had at least one abnormal finding on brain imaging, eye, neurological or neurodevelopmental assessments in the first year of life.¹⁶ In a separate cohort study in Brazil, Lopes Moreira ME et al¹⁷ followed 182 born to women with symptomatic RT-PCR confirmed ZIKV infection and offered systematic neuroimaging, complete eye examinations and assessments of brainstem auditory evoked responses to all children. Among those who underwent both

neurodevelopmental and neuroimaging assessments, 39/115 (34%) had abnormal findings, of which 20 were nonspecific hypersignal findings on MRI.

All infants included in our analysis were born to women with symptomatic, RT-PCR-confirmed ZIKV infection during pregnancy. As such, we assume that all infants were exposed to ZIKV *in utero*. With respect to ZIKV infection status, among those for whom *in utero* ZIKV infection status could be determined, we found a vertical transmission rate of 3.0% (5/167) (95%CI:1.1-7.2). Our estimate is close to the 4% estimate in 1450 children born to women enrolled in a registry-based study with confirmed or possible ZIKV infection during pregnancy in territories of the United States,⁸ as well as to the 7% estimate among 295 neonates born to women with confirmed or probable ZIKV infection during pregnancy in New York City, using ELISA immunoassay.¹⁸ However, the inclusion in these two studies of women with possible or probable ZIKV infection, rather than RT-PCR confirmed ZIKV infection, during pregnancy, likely underestimates the rate of vertical transmission. A recent estimate from 130 children with antenatal exposure in Brazil found 84 (65%) to have at least one positive result in sera or urine tested by RT-PCR and IgM assays.¹⁶ However, this percentage dropped to 33% beyond 3 months of age, and concordance between RT-PCR and IgM was only 52%. Further, the study design did not effectively exclude the possibility of postnatal infection, which likely overestimates the vertical transmission rate. The most comprehensive assessment of the rate of vertical transmission comes from systematic RT-PCR and serologic testing on multiple tissue samples, including amniotic fluid, fetal and neonatal blood, cerebrospinal fluid, urine and placenta, from 291 fetuses and newborns in French Guiana.¹⁹ This study found the rate of vertical transmission to be 26.1%, revised to 18% when placental samples were not considered because of the risk of contamination from maternal viremia, which is considerably higher than our own estimate. For those in our study with no evidence of ZIKV infection, or for whom ZIKV infection was not able to be determined, we are unable to infer whether this reflects: an absence of placental infection or *in utero* ZIKV infection; the waning of antibodies in early childhood; the inability of the developing fetal immune system to mount an immune response if infected early in the perinatal period; and/or an antibody response that is not easily detectable by the immunoassays used in this study. These findings highlight the need for further investigations into antibody kinetics in the perinatal and early childhood periods, and how these may correlate with the detection of abnormalities, in order to provide revised definitions of *in utero* ZIKV infection.

What remains unclear is the reason for lower rates of infection and abnormalities compared to other cohort studies, particularly those conducted in Brazil. It also remains unclear whether immunological differences may partially explain the apparent differences in disease outcomes following ZIKV infection. For example, secondary infection with a heterologous DENV serotype is well established as a risk factor for severe DENV disease, which may be explained by cross reactive antibodies that enhance DENV infection, known as antibody-dependent enhancement (ADE).^{20,21} For ZIKV, the role of prior

flavivirus infection, particularly the role of the closely related DENV, which shares antigenic similarities with ZIKV, remains unclear. Studies have shown that the prior DENV infection may protect against ZIKV.^{22,23} Other studies have found evidence for ADE *in vitro*,²⁴ but little *in vivo*,²⁵ or in epidemiological studies.²⁶ A reconciliation of the two hypotheses may lie in the timing of exposure to DENV and in the DENV serotype which may contribute to the differences observed in the strength of the relation between ZIKV infection and fetal abnormalities.²⁷ Our study involved participants living primarily in Guadeloupe and Martinique – French Territories in the Americas in which DENV is considered endemic, with co-circulation of more than one serotype²⁸ and where DENV seroprevalence exceeds 90%.²⁸ The extent to which flavivirus immunological differences may explain lower rates of infection and abnormalities in our study compared to other cohort studies warrants further investigation.

Underlying our findings is the assumption that all selected abnormalities were attributable to *in utero* ZIKV exposure. However, early childhood developmental trajectories are also influenced by genetic and environmental factors.^{29,30} This is a critical consideration as a separate comparison of toddlers from the same study population who were exposed to ZIKV *in utero* with those who were not exposed recently found that while 15.4% of *in utero* exposed toddlers had possible neurodevelopmental delay at 24 months of age, there were minimal differences compared to ZIKV-unexposed toddlers.¹⁰ Further, an analysis of infants born to women who had no evidence of prior ZIKV infection at the time of delivery found no statistically significant differences in the risk of neurological birth defects in live-born infants compared to infants born to women with ZIKV infection during pregnancy.³¹ Both studies highlight the importance of an appropriate control group to avoid overattributing adverse outcomes to perinatal ZIKV infection.

Our findings are limited by our inability to include in the pediatric cohort study all infants born to women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy and by the loss to follow-up from delivery to 24 months of age. However, the consultation of medical registries provided additional information as to the clinical status at 24 months of those not included in the pediatric cohort and those who were lost to follow-up. We are unable to determine the direction of possible selection bias among those who remained in the cohort study. That is infants with and without abnormalities declined to participate or were lost to follow-up. So, it is unclear whether the absence of those infants may have resulted in an artificially over- or under-estimated risk of adverse pediatric outcomes.

Overall, our findings provide an important contribution to understanding the risk of adverse pregnancy and early childhood outcomes following ZIKV infection during pregnancy. They provide evidence that will be helpful to clinical decisions regarding pregnancy management in the context of ZIKV infection. While many studies evaluating the impact of ZIKV infection during pregnancy have focused on adverse

pregnancy outcomes, our study highlights the need for continued follow-up of infants born to women with ZIKV infection in pregnancy into childhood.

Declarations

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Data sharing

All data relevant to the study are included in the article or in the supplementary information. Deidentified participant data may be made available upon reasonable request to the corresponding author.

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Tables and Figures

Figure 1. Inclusion of women with symptomatic RT-PCR confirmed ZIKV infection during pregnancy in ZIKA-DFA-FE cohort study and inclusion of liveborn infants in ZIKA-DFA-BB cohort study

Table 1. Characteristics of 546 women with RT-PCR confirmed ZIKV infection during pregnancy and 527 liveborn infants

Table 2. Selected adverse early childhood outcomes in 320 infants included in the ZIKA-DFA-BB cohort study across 24-month follow-up by ZIKV infection status

Table 3. Severity of adverse pregnancy and early childhood outcomes by trimester of maternal ZIKV infection

Figure 2. Evolution of microcephaly among 17 children included in the ZIKA-DFA-BB pediatric cohort study with microcephaly detected at birth or in the post-natal period

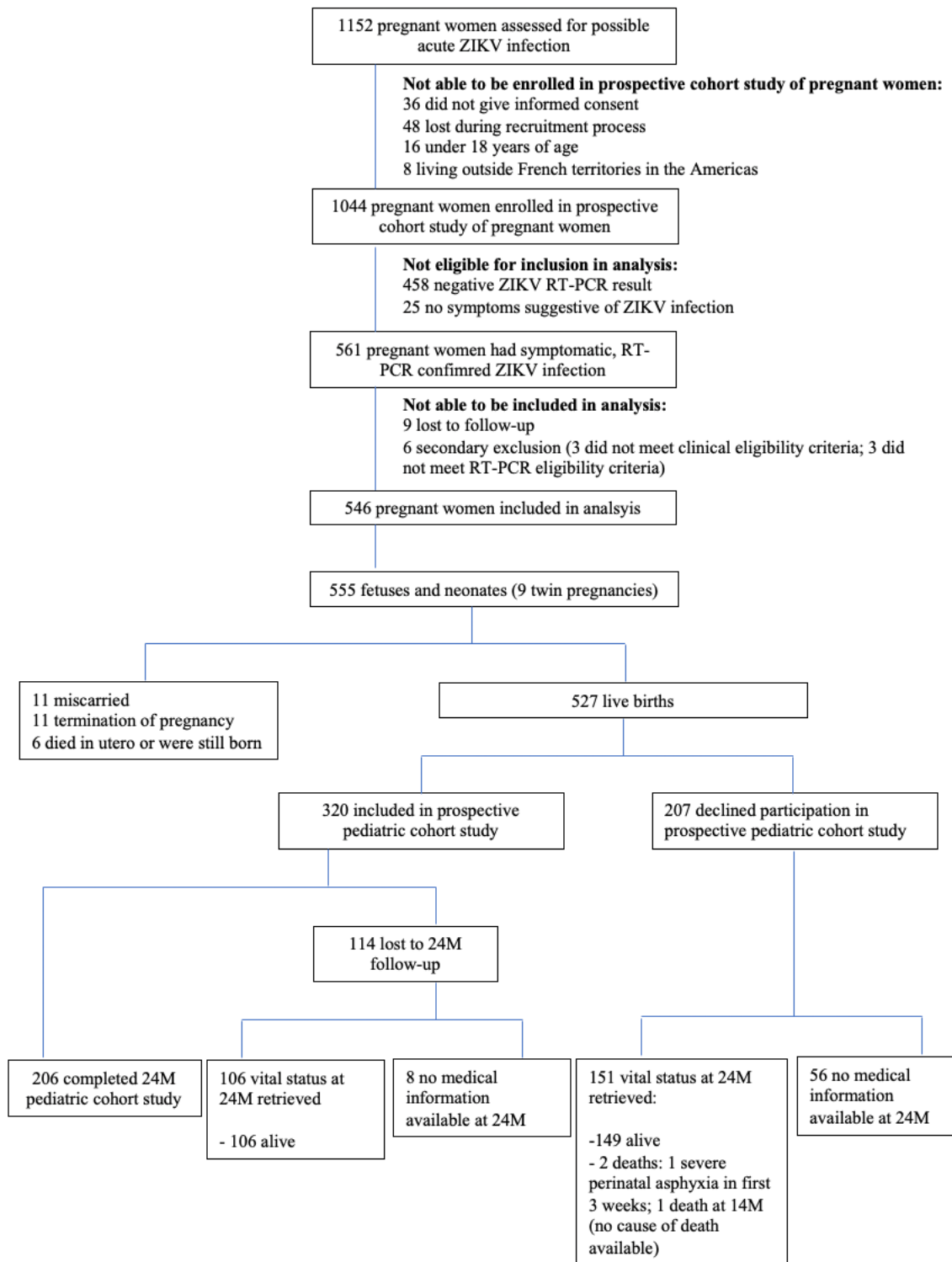


Figure 1. Inclusion of women with symptomatic RT-PCR confirmed ZIKV infection during pregnancy in ZIKA-DFA-FE cohort study and inclusion of liveborn infants in ZIKA-DFA-BB cohort study

Table 1. Characteristics of 1A. 546 women with RT-PCR confirmed ZIKV infection during pregnancy and 1B. 527 liveborn infants born to these women

1A. Maternal characteristics		N=546
Age at time of pregnancy (years)	Mean \pm SD	29.7 \pm 6.2
	Interquartile range	25.0 – 34.3
Occupation – n (%)		
	Student	23 (4.2)
	Self-employed/ business owner/ farmer	31 (5.7)
	Executive/ highly skilled worker	55 (10.1)
	Intermittent profession	56 (10.3)
	Salaried employee	181 (33.2)
	Not employed	188 (34.4)
	Unknown or declined to respond	12 (2.2)
Educational attainment of mother – n (%)		
	Primary	18 (3.3)
	Secondary	38 (7.0)
	Tertiary	60 (11.0)
	Unknown or declined to respond	429 (78.7)
Educational attainment of father – n (%)		
	Primary	11 (2.0)
	Secondary	33 (6.0)
	Tertiary	27 (4.9)
	Unknown or declined to respond	475 (87.0)
Residence – n (%)		
	Guadeloupe	245 (44.9)
	Martinique	277 (50.7)
	French Guiana	24 (4.4)
Previous pregnancies – n (%)*		
	0	131 (24.0)
	1	150 (27.5)
	2	128 (23.4)
	3+	137 (25.1)
Previous adverse pregnancy outcomes – n (%)		
	Congenital abnormalities	6 (1.1)
	Stillbirth	10 (1.8)
	Medical termination of pregnancy	10 (1.8)
Lifestyle practices during 2016-2017 pregnancy – n (%)		
	Alcohol consumption	2 (0.4)
	Drug use	6 (1.1)
	Smoking	23 (4.2)
	Use of mosquito repellents	445 (81.5)
	Use of larvicides	337 (61.7)
1B. Infant characteristics		N=527
Gestational age (weeks)	Mean \pm SD	38.7 \pm 1.9
Delivery type – n (%)		
	Cesarean	95 (18.0)
Sex – n (%)		
	Male	266 (50.5)
Birth weight (g)	Mean \pm SD	3112 \pm 530

Table 2. Selected adverse early childhood outcomes in 320 infants included in the pediatric cohort study across 24-month follow-up by ZIKV infection status

ZIKV infection status of child	Consultation at 2M				Consultation at 4 M				Consultation at 9M				Consultation at 18M				Consultation at 24M				Consultation at any time across 24M			
	N=262 (%)		N=261 (%)		N=235 (%)		N=208 (%)		N=206 (%)		N=310 (%)		N=206 (%)		N=206 (%)		N=206 (%)		N=206 (%)		N=310 (%)		N=310 (%)	
	+ve n=4	-ve n=134	UD n=124	+ve n=4	-ve n=138	UD n=119	+ve n=4	-ve n=128	UD n=103	+ve n=4	-ve n=130	UD n=74	+ve n=4	-ve n=144	UD n=58	+ve n=5	-ve n=162	UD n=143	+ve n=5	-ve n=162	UD n=143	+ve n=5	-ve n=162	UD n=143
Hearing loss or deficiency*	0 (0)	4 (3.0)	3 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.0)	1 (25.0)	0 (0)	0 (0)	0 (0)	0 (0.7)	1 (1.7)	1 (20.0)	5 (3.1)	5 (3.5)	1 (20.0)	5 (3.1)	5 (3.5)	1 (20.0)	5 (3.1)	5 (3.5)
Visual impairment**	1 (25.0)	8 (6.0)	3 (2.4)	0 (0)	1 (0.7)	2 (1.7)	1 (25.0)	0 (0)	1 (1.0)	1 (25.0)	0 (0)	1 (1.4)	0 (0)	2 (1.4)	1 (1.7)	2 (40.0)	11 (6.8)	8 (5.6)	2 (40.0)	11 (6.8)	2 (40.0)	11 (6.8)	2 (40.0)	11 (6.8)
Seizures, excluding febrile seizures	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.8)	1 (1.4)	0 (0)	1 (0.7)	2 (3.4)	0 (0)	1 (0.6)	2 (1.4)	0 (0)	1 (0.6)	0 (0)	1 (0.6)	0 (0)	2 (1.4)
Body tone, musculoskeletal or motor abnormalities	0 (0)	3 (2.2)	1 (0.8)	1 (25.0)	5 (3.6)	3 (2.5)	1 (25.0)	2 (1.6)	1 (1.0)	1 (25.0)	2 (1.5)	1 (1.4)	1 (25.0)	1 (0.7)	3 (5.2)	2 (40.0)	9 (5.6)	6 (4.2)	1 (25.0)	3 (5.2)	2 (40.0)	9 (5.6)	2 (40.0)	6 (4.2)
Swallowing abnormalities	0 (0)	12 (9.0)	6 (4.8)	0 (0)	0 (0)	1 (0.8)	-	-	-	-	-	-	-	-	-	0 (0)	12 (7.4)	7 (4.9)	-	-	0 (0)	12 (7.4)	-	7 (4.9)
Post-natal microcephaly	0 (0)	0 (0)	0 (0)	1 (25.0)	0 (0)	0 (0)	0 (0)	1 (0.8)	0 (0)	1 (25.0)	1 (0.8)	0 (0)	1 (25.0)	1 (0.7)	0 (0)	1 (20.0)	1 (0.6)	0 (0)	1 (20.0)	1 (0.6)	1 (20.0)	1 (0.6)	1 (20.0)	0 (0)
Any selected abnormality***	1 (25.0)	23 (17.2)	12 (9.7)	1 (25.0)	6 (4.3)	5 (4.2)	1 (25.0)	3 (2.3)	2 (1.9)	1 (25.0)	4 (3.1)	3 (4.0)	1 (25.0)	6 (4.2)	5 (8.6)	3 (60.0)	36 (22.2)	24 (16.7)	3 (60.0)	36 (22.2)	3 (60.0)	36 (22.2)	3 (60.0)	24 (16.7)
Imaging abnormality ⁺	2 (50.0)	11 (8.2)	10 (8.1)	0 (0)	1 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	-	-	-	-	-	-	2 (40.0)	12 (7.4)	10 (7.0)	-	-	2 (40.0)	12 (7.4)	-	10 (7.0)
Any selected abnormality, including imaging ⁺⁺	3 (75.0)	34 (25.4)	21 (16.9)	1 (25.0)	7 (5.1)	5 (4.2)	1 (25.0)	3 (2.3)	2 (1.9)	1 (25.0)	4 (3.1)	3 (4.1)	1 (25.0)	6 (4.2)	5 (8.6)	3 (60.0)	43 (26.5)	33 (23.1)	1 (25.0)	6 (4.2)	3 (60.0)	43 (26.5)	3 (60.0)	33 (23.1)

ZIKV infection status of child: +ve: *in utero* ZIKV infection confirmed; -ve: *in utero* ZIKV infection excluded; UD: *in utero* ZIKV infection confirmed unknown/undetermined

* Includes results of screening evaluation, and additional auditory examination

** Includes results of screening evaluation, and additional ophthalmologic examination

*** Any selected abnormality: Hearing loss or deficiency; Visual impairment; Seizures, excluding febrile seizures; Body tone, musculoskeletal or motor abnormalities; Swallowing abnormalities; Post-natal microcephaly

Table 3. Severity of 3A. adverse pregnancy and early childhood outcomes in 555 fetuses/infants by trimester of maternal ZIKV infection and of 3B. adverse pregnancy and early childhood outcomes related to maternal ZIKV infection in 555 fetuses/infants by trimester of maternal ZIKV infection

3A	Trimester of maternal ZIKV infection			All
Severity of adverse pregnancy and early childhood outcomes	1 N=158 (%)	2 N=257 (%)	3 N=140 (%)	N=555 (%)
Severe sequelae or fatality	24 (15.2)	11 (4.3)	2 (1.4)	37 (6.7)
Major abnormalities	7 (4.4)	9 (3.5)	8 (5.7)	24 (4.3)
Mild abnormalities	23 (14.6)	38 (14.8)	23 (16.4)	84 (15.1)
No abnormality reported	90 (57.0)	171 (66.5)	95 (64.1)	356 (64.1)
Unknown	14 (8.9)	28 (10.9)	12 (8.6)	54 (9.7)

3B	Trimester of maternal ZIKV infection			All
Severity of adverse pregnancy and early childhood outcomes related to maternal ZIKV infection*	1 N=158 (%)	2 N=257 (%)	3 N=140 (%)	N=555 (%)
Severe sequelae or fatality	11 (7.0)	7 (2.7)	2 (1.4)	20 (3.6)
Major abnormalities	3 (1.9)	8 (3.1)	4 (2.9)	15 (2.7)
Mild abnormalities	15 (9.5)	19 (7.4)	18 (12.9)	52 (9.4)
No abnormality reported	103 (65.2)	195 (75.9)	104 (74.3)	402 (72.4)
Unknown	26 (16.5)	28 (10.9)	12 (8.6)	66 (11.9)

*Selected adverse pregnancy and early childhood outcomes, with reclassification as follows: Miscarriages = unknown; stillbirths without abnormalities = no abnormality reported; non pathological findings for vision (retinal hemorrhages at birth) = no vision abnormality; non pathological imaging findings = no imaging abnormality.

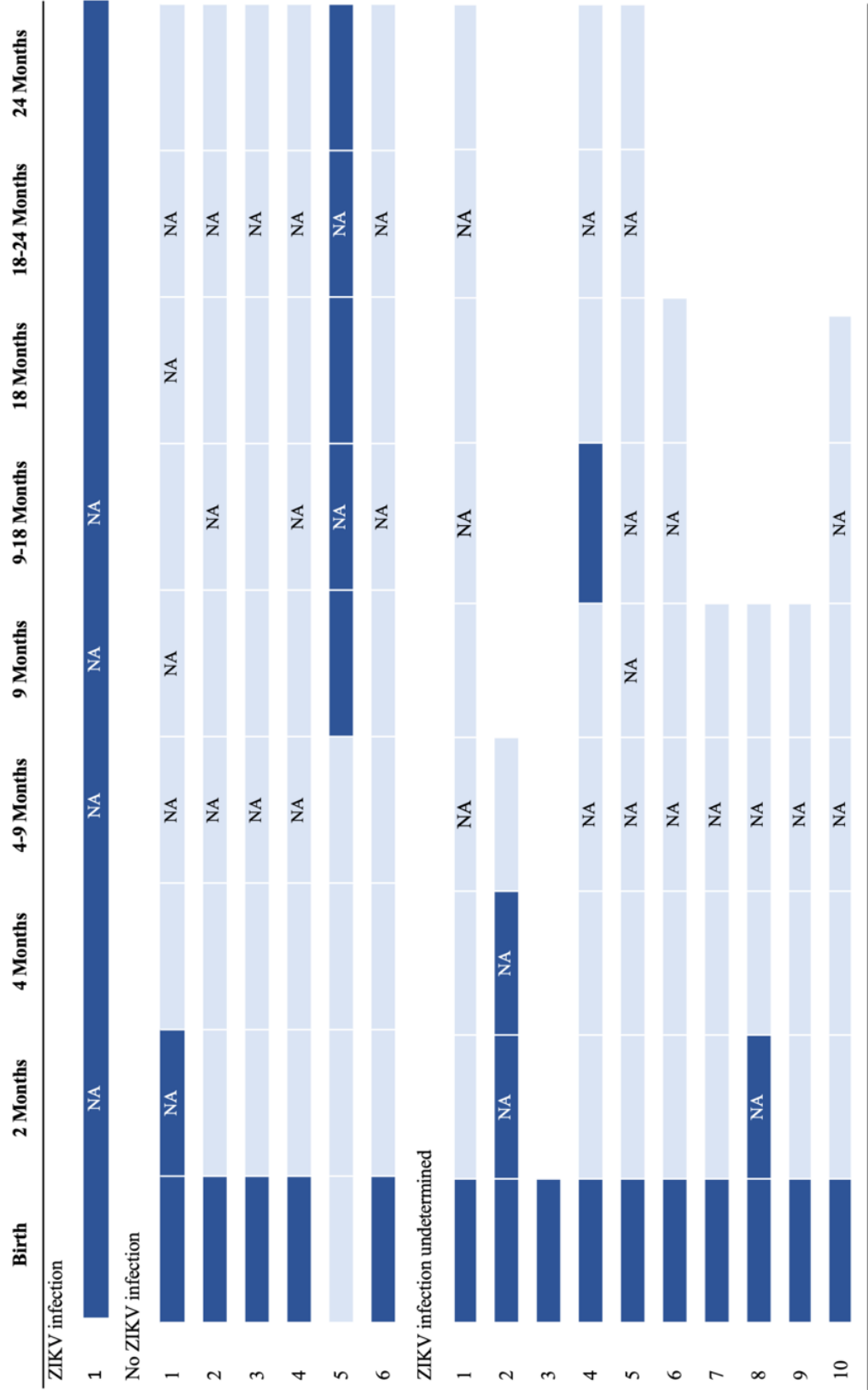


Figure 2. Evolution of microcephaly among 17 children with microcephaly at birth or onset in post-natal period

Legend: dark blue = microcephaly; light blue = absence of microcephaly; NA = pediatric consultation not conducted, no information on head circumference available

Consequences of *in utero* Zika virus exposure and adverse pregnancy and early childhood outcomes: a prospective cohort study

Supplementary Material

Supplementary Table 1. A. Maternal and B. Neonatal characteristics of 315 women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy who included their infant in pediatric cohort study and 203 women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy who declined participation of their infant in the pediatric cohort study

A. Maternal characteristics		Mothers of infants included in pediatric cohort study N=315 (%)	Mothers of infants not included in pediatric cohort study N=203 (%)	p-value
Age at time of pregnancy (years)				
	Mean \pm SD	30.0 \pm 6.4	29.1 \pm 5.9	0.10
	Interquartile range	25.4 – 34.7	24.8 – 33.4	
Occupation – n (%)				0.20
	Student	12 (3.8)	9 (4.8)	
	Self-employed/ business owner/ farmer	15 (4.8)	16 (6.9)	
	Executive/ highly skilled worker	31 (9.8)	19 (10.4)	
	Intermittent profession	33 (10.5)	18 (10.0)	
	Salaried employee	120 (38.1)	57 (26.4)	
	Not employed	97 (30.8)	79 (39.3)	
	Unknown or declined to respond	7 (2.2)	5 (2.2)	
Educational attainment of mother – n (%)				
	Primary	18 (5.7)	-	
	Secondary	38 (12.1)	-	
	Tertiary	60 (19.0)	-	
	Unknown or declined to respond	199 (63.2)	-	
Educational attainment of father – n (%)				
	Primary	11 (3.5)	-	
	Secondary	33 (10.5)	-	
	Tertiary	27 (8.6)	-	
	Unknown or declined to respond	244 (77.5)	-	
Residence – n (%)				<0.001
	Guadeloupe	139 (44.1)	98 (45.9)	
	Martinique	175 (55.6)	85 (45.0)	
	French Guiana	3 (1.0)	20 (9.1)	
Parity – n (%)*				0.97
	0	75 (23.8)	52 (25.6)	
	1	86 (27.3)	53 (26.1)	
	2	73 (23.2)	46 (22.7)	
	3+	81 (25.7)	52 (25.6)	
Previous adverse pregnancy outcomes – n (%)				
	Congenital abnormalities	4 (1.3)	2 (0.9)	1
	Stillbirth	7 (2.2)	1 (0.9)	0.23
	Medical termination of pregnancy	3 (1.0)	6 (3.0)	0.17
Lifestyle practices during 2016-2017 pregnancy – n (%)				
	Alcohol consumption	0 (0)	1 (0.9)	0.82
	Drug use	3 (1.0)	3 (1.3)	0.89
	Smoking	11 (3.5)	12 (5.3)	0.28
	Use of mosquito repellents	259 (82.7)	165 (81.6)	0.94
	Use of larvicides	193 (62.3)	123 (65.2)	0.87
B. Neonatal characteristics		Included in pediatric cohort study N=320 (%)	Not included in pediatric cohort study N=207 (%)	p-value
Gestational age (weeks)				
	Mean \pm SD	39.0 \pm 1.4	38.4 \pm 2.4	0.002

Delivery type – n (%)*	Cesarean	60 (18.8)	35 (17.8)	0.81
Sex – n (%)	Male	163 (50.9)	103 (48.9)	0.95
Birth weight (g)	Mean	3159 ± 466	3039 ± 612	0.02

Supplementary Table 2. Categorization of severity of abnormalities

Severe sequelae or fatal outcome
Not liveborn or post-natal death
Multiple and persistent sequelae, abnormal imaging
Multiple and persistent sequelae, normal imaging
Isolated and persistent non-febrile seizures
Spina bifida at birth (alive at 24M, no abnormalities reported)
Club foot at birth (alive at 24M, no abnormalities reported)
Club foot at birth (no information after birth)
Major abnormalities
One selected abnormality and abnormal imaging
Two isolated selected abnormalities, normal imaging
Two isolated selected abnormalities, no imaging information
Microcephaly at birth, lost to follow up (no information after birth)
Isolated and persistent post-natal onset microcephaly
Isolated, single occurrence non-febrile seizures (at 24M)
Mild abnormality
Isolated abnormal imaging (+ transient swallowing abnormality at 2M)
Isolated eye abnormality (+ transient swallowing abnormality at 2M)
Single isolated abnormality
Abnormal imaging (no other abnormality)
Isolated microcephaly at birth (+ transient swallowing abnormality at 2M)
No abnormality reported
Transient swallowing abnormality at 2M
Non-pathological imaging finding
No abnormalities reported
Unknown
Unknown (no abnormality information available)

Chapter III

***In utero* Zika virus exposure and neurodevelopment at 24 months in toddlers normocephalic at birth: a cohort study**

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Abstract

Background: *In utero* exposure to Zika virus (ZIKV) is known to be associated with birth defects. The impact of *in utero* ZIKV exposure on neurodevelopmental outcomes in early childhood remains unclear. The objective of this study was to determine the impact of *in utero* ZIKV exposure on neurodevelopment at 24 months of age among toddlers who were born normocephalic to women who were pregnant during the 2016 ZIKV outbreak in French Territories in the Americas.

Methods: We conducted a population-based mother-child cohort study of women whose pregnancies overlapped with the 2016 ZIKV epidemic in Guadeloupe, Martinique and French Guiana. Infants were included in this analysis if maternal ZIKV infection during pregnancy could be determined; the newborn had a gestational age ≥ 35 weeks; no abnormal transfontanelle cerebral ultrasound findings after delivery or no abnormal ultrasound findings on the last ultrasound performed during the third trimester of the mother's pregnancy; an absence of microcephaly at birth; and the parent completed the 24-month neurodevelopment assessment of the infant at 24 months (± 1 month) of age. ZIKV exposure of the toddler was determined by evidence of maternal ZIKV infection during pregnancy. Neurodevelopment assessments included the Ages and Stages Questionnaire (ASQ) for five dimensions of general development – communication, gross motor, fine motor, problem solving, and personal-social skills; the Modified Checklist for Autism on Toddlers (M-CHAT) for behaviour; and the French MacArthur Inventory Scales (IFDC) for French language acquisition.

Results: Between June 2018 and August 2019, 156 toddlers with and 79 toddlers without *in utero* ZIKV exposure completed neurodevelopment assessments. Twenty-four (15.4%) ZIKV-exposed toddlers and 20 (25.3%) ZIKV-unexposed toddlers had an ASQ result below the reference -2SD cut-off ($P=0.10$) for at least one of the five ASQ dimensions.

Conclusion: In one of the largest population-based cohorts of *in utero* ZIKV-exposed, normocephalic newborns to date, there were minimal differences apparent in neurodevelopment outcomes at 24 months of age compared to ZIKV-unexposed toddlers at 24 months of age.

Background

Zika virus (ZIKV) infection during pregnancy is understood to cause neurological complications in the fetus, collectively known in its most severe form as congenital Zika syndrome (CZS).¹ The 2015-2016 ZIKV epidemic enabled risk of adverse pregnancy outcomes associated with ZIKV infection during pregnancy to be estimated.²⁻⁵ However, adverse pregnancy outcomes and CZS do not cover the full spectrum associated with *in utero* ZIKV exposure, and the characterization and risk of developmental outcomes beyond birth remain unclear.⁶ The newborns who were born to women whose pregnancy occurred during the ZIKV epidemic are now in early childhood and the development of these young children needs to be assessed, as well as the risk for abnormal development attributable to *in utero* ZIKV exposure.

Adverse neurodevelopment findings in infants exposed *in utero* to ZIKV with and without CZS have recently been documented in the United States of America⁷, Brazil⁸⁻¹¹, and Colombia.¹² Yet, within these prospective studies assessing neurodevelopment outcomes, there has been minimal use of comparative and appropriate control groups. This is a critical consideration, particularly given that neurodevelopment in early childhood is known to be influenced by both genetic and environmental factors, including substance abuse during pregnancy, maternal age,¹³ sociodemographic determinants,¹⁴ prematurity or low birth weight, maternal and early infant nutritional status,¹⁵ and sex of the infant in the case of language acquisition.¹³

To study the impact of *in utero* ZIKV exposure on pregnancy and early child development outcomes, the ZIKA-DFA-FE (pregnant women) and ZIKA-DFA-BB (newborns) prospective cohort studies were established in Guadeloupe, Martinique, and French Guiana in 2016. The dynamics of the 2016 ZIKV epidemic in the three French Territories were such that they enable the ZIKA-DFA-BB cohort study to closely examine the impact of *in utero* ZIKV exposure on early child outcomes beyond birth. Here, we present the neurodevelopment assessment results at 24 months of age. The objective of the study was to compare the neurodevelopment outcomes to toddlers who were exposed to ZIKV *in utero* with those who were not exposed to estimate the impact of *in utero* ZIKV exposure on neurodevelopmental outcomes at 24 months.

Methods

All women whose pregnancy overlapped with the 2016 ZIKV epidemic in Guadeloupe, Martinique or French Guiana who sought antenatal care at one of the participating hospitals were invited to participate in the ZIKA-DFA-FE prospective cohort study of pregnant women, for which the recruitment and ZIKV testing strategies has been previously described.³

ZIKA-DFA-BB was a prospective cohort study of toddlers born to women whose pregnancies overlapped with the 2016 ZIKV epidemic in French Territories in the Americas: Guadeloupe, Martinique, and French Guiana. Toddlers were followed up in routine pediatric consultation until 24 months of age.

Inclusion in analysis

Toddlers were included in this analysis if all of the following criteria were met: maternal ZIKV infection during pregnancy could be determined; the newborn had a gestational age of 35 weeks or more; the newborn had no abnormal transfontanelle cerebral ultrasound findings after delivery up to 2 months of age or, in the absence of a transfontanelle cerebral ultrasound after delivery, no abnormal ultrasound findings on the last ultrasound performed during the third trimester of the mother's pregnancy; the newborn had an absence of microcephaly at birth, defined as a head circumference above -2SD below the mean according to sex and gestational age on the INTERGROWTH-21st chart; and the parent completed the neurodevelopment assessment of the toddler at 24 months (± 1 month) of age.

Exposure assessment

In utero ZIKV exposure was determined by RT-PCR or serological evidence of ZIKV infection in serum and /or urine samples from the mother collected during pregnancy. An toddler was considered exposed to ZIKV if the mother had RT-PCR (RealStar Zika Virus RT-PCR Kit 1.0, Altona Diagnostics) positive result for ZIKV in blood, urine or both at any stage during her pregnancy; if the toddler had anti-ZIKV IgM (EuroImmun ELISA or in-house MAC-ELISA in French Guiana¹⁶) in the cord blood or blood taken within the first 10 days of life; or if the toddler had anti-ZIKV IgG (EuroImmun ELISA or in-house MAC-ELISA in French Guiana¹⁶) in the blood beyond 12 months of age and the date of birth was posterior to the end of the ZIKV epidemic: 11 September 2016 in French Guiana, 25 September 2016 in Guadeloupe, and 16 October 2016 in Martinique.

Anti-ZIKV kinetic studies have shown that anti-ZIKV IgG antibodies appear rapidly after infection and remain detectable up to 6 months with 100% sensitivity.¹⁷⁻¹⁹ Therefore, a negative serology for anti-ZIKV IgG in mothers at the time of delivery has a 100% negative predictive value for ZIKV infection in the mother during pregnancy and was used to define toddlers as unexposed to ZIKV *in utero*.

Neurodevelopment assessment

Three pediatric neurodevelopment evaluation tools were used to assess neurodevelopment and administered to a parent/legal guardian at the time of the 24-month pediatric consultation of the toddler. All questionnaires were administered in French.

The 30-item parent-reported screening test Ages and Stages Questionnaire-III (ASQ), previously validated in France,²⁰ was used to identify toddlers at risk for developmental delay across five dimensions: communication, gross motor, fine motor, problem solving, and personal-social skills.²¹ Each dimension consists of six questions, with possible responses: ‘yes’ (10 points) if the child performs the behaviour, ‘sometimes’ (5 points), and ‘not yet’ (0 points). A score for each dimension was calculated by adding the points from each dimension and each dimensional score reflected the child’s ability within that dimension. Abnormal ASQ outcomes were described as a dimension score below validated cut-off values, set at 2SD below the mean using reference norms.²¹

The 23-item parent-reported screening test Modified Checklist for Autism on Toddlers (M-CHAT) was used to identify toddlers at risk for behaviour disorder.²² A total score was calculated by adding the points from the screening test, with low risk defined as a total score of 2 or lower, medium risk 3-7 points, and high risk 8 points or above. A positive M-CHAT screen was considered as having a total score of 3 or above.

The French MacArthur-Bates communicative development inventories (Inventaires français du développement communicatif - IFDC) was used to assess French language acquisition for which the parent identified from a validated list of 100 words the words that the toddler says spontaneously.²³ Total word count was calculated as the sum of the words the toddler says spontaneously. Abnormal IDFC outcomes were described as total word count below validated 10th percentile thresholds derived from a reference population.²³

Statistical analysis

The maternal and newborn characteristics of the *in utero* ZIKV exposed and ZIKV unexposed toddlers were compared using Student’s *t*-test for continuous variables and chi-squared test for categorical variables. Missing values for questions within each ASQ dimension were replaced by the average of the other questions in that same dimension.

Univariable and multivariable logistic regression models were used to evaluate the association between *in utero* ZIKV exposure and abnormal ASQ score per dimension.

For all statistical analyses, $P < 0.05$ was considered statistically significant. All statistical analyses were performed using R, version 3.6.1.

Results

From June 2018 to August 2019, 1180 newborns were enrolled in the ZIKA-DFA-BB cohort study. Of these, 572 were excluded from analysis: ZIKV infection status of the mother could not be determined (360 newborns), or it could not be excluded that maternal ZIKV infection preceded the start of the pregnancy (38 newborns), or the newborn had congenital abnormalities at birth (21 newborns), or the newborn had a head circumference Z score of -2SD or lower at birth (27 infants), or the newborn had no ultrasound findings available from the third trimester of the mother's pregnancy (96 newborns), or the newborn had an abnormal transfontanelle cerebral ultrasound findings at birth up to 2 months of age (34 newborns). Of the remaining 604 newborns, 247 completed the 24-month neurodevelopment assessment (169 toddlers born to women with confirmed ZIKV infection during pregnancy, 81 toddlers born to women with no ZIKV infection during pregnancy). A further 15 toddlers were excluded from the analysis as the neurodevelopment assessment was not administered within ± 1 month of the toddlers 24 months of age. We were therefore able to describe the results of the neurodevelopment assessment at 24 months for 235 toddlers: 156 *in utero* ZIKV exposed toddlers and 79 toddlers ZIKV unexposed toddlers (Figure 1).

Table 1 shows the maternal and newborn characteristics of the 235 toddlers by ZIKV exposure status. Of those included in the analysis, no abnormalities associated with ZIKV exposure were reported in routine pediatric consultation at birth. Additional file 1: Supplementary Table 1 shows the RT-PCR and serological evidence of maternal ZIKV infection. Comparisons between ZIKV exposed and ZIKV unexposed toddlers indicated a lower maternal age ($P=0.01$), higher maternal education ($P=0.04$) and higher paternal education ($P=0.04$) in the ZIKV unexposed; a higher proportion of toddlers from Guadeloupe in the ZIKV exposed group and a higher proportion of toddlers from Martinique in the ZIKV unexposed group ($P<0.001$); higher parity in the ZIKV exposed ($P=0.04$); and greater use of mosquito repellents in the ZIKV exposed group ($P=0.05$).

Comparisons between the toddlers included in the analysis ($N=235$) and those who were excluded because they did not complete the neurodevelopment assessment ($N=369$) revealed higher completion of the questionnaire in Martinique and lower completion of the questionnaire in French Guiana in exposed ($P<0.001$) and unexposed groups ($P<0.001$); higher completion rate of questionnaire in toddlers born to women with lower parity in the unexposed groups ($P<0.001$); and higher completion rate in toddlers born to women who did not consume alcohol during pregnancy ($P=0.04$) (data not shown).

Table 2 shows the mean ASQ results per dimension by ZIKV exposure status, as well as the number of toddlers in each group with a score below the -2SD cut-off. The communication dimension showed a statistically significant difference between the two groups, with the ZIKV exposed having higher mean

communication score (49.5 vs 44.0; $P=0.01$) and a smaller proportion of toddlers falling below the -2SD cut-off value (8.3% vs 20.3%; $P=0.02$). The dimensions most frequently scoring below the threshold in the ZIKV exposed and unexposed groups were communication and personal-social-skills.

Additional file 1: Supplementary Table 2 shows the number of ASQ dimensions below the -2SD cut-off value by ZIKV exposure. Twenty-four (15.4%) ZIKV-exposed toddlers and 20 (25.3%) ZIKV-unexposed toddlers had an ASQ result for at least one of the five ASQ dimensions below the reference -2SD cut-off ($P=0.10$).

Further analyses among the ZIKV exposed toddlers demonstrated no difference in the proportion of toddlers with at least one ASQ dimension below the -2SD cut-off value by trimester of pregnancy during which ZIKV infection occurred for the women who had ZIKV infection determined by RT-PCR ($P=0.67$).

In univariable logistic regression analyses shown in Table 3, *in utero* ZIKV exposure and sex of the toddler were associated with an ASQ score for communication below the -2SD cut-off. In multivariable regression analysis, shown in Table 4, *in utero* ZIKV exposure and sex of the toddler were identified as independent predictors of an ASQ score for communication below the -2SD cut-off.

Additional file 1: Supplementary Table 3 shows the M-CHAT behaviour disorder risk by ZIKV exposure status. There was no difference observed in behaviour disorder screening risk between ZIKV-exposed and ZIKV unexposed toddlers ($P=0.15$).

Additional file 1: Supplementary Table 4 shows language acquisition of 233 francophone toddlers by ZIKV exposure status. There were no observed differences in mean language acquisition ($P=0.36$), nor in the proportion of toddlers below the 10th percentile threshold ($P=0.53$). Further stratification by sex and by the number of languages spoken in the household did not identify any differences by *in utero* ZIKV exposure status.

Discussion

In one of the largest population-based, mother-child cohorts of *in utero* ZIKV-exposed normocephalic at birth to date, we found no apparent differences in neurodevelopment outcomes compared to ZIKV-unexposed toddlers at 24 months of age.

We found 24 (15.3%) toddlers with *in utero* ZIKV exposure at risk of neurodevelopment delay using the ASQ neurodevelopment screening tool. This is largely comparable to neurodevelopmental findings in other prospective ZIKV cohort studies published to date. Lopes Moreira et al⁸ used the Bayley Scales of Infant and Toddler Development-III to assess 94 children who had also undergone neuroimaging between 12-18 months of age. Twenty-four (25.5%) toddlers were found to have at least one abnormal neurodevelopment finding. Considering only the 59 with normal imaging for comparison with our findings, the number with abnormal neurodevelopment was 13 (13.8%). Einspieler et al¹⁰ followed 56 toddlers without microcephaly at birth and who were born to women with RT-PCR positive result for ZIKV during pregnancy. Ten (17.9%) toddlers were found to have an adverse neurologic exam or neurodevelopment assessment at 12 months of age.

Among *in utero* ZIKV exposed infants, irrespective of abnormalities at birth, Rice et al⁷ assessed follow-up care reports of 1450 toddlers with *in utero* ZIKV exposure with and without ZIKV-associated birth defects aged 1 year of age or older and found 9% had a least one neurodevelopment abnormality possibly associated with *in utero* ZIKV exposure. Nielson-Saines et al⁹ used the Bayley Scales of Infant and Toddler Development-III at 18 months of age and described the neurodevelopment outcomes of 146 toddlers born to women with rash and RT-PCR ZIKV infection during pregnancy. Forty-one (28.1%) toddlers were found to have abnormal neurodevelopment, with language having the greatest proportion of toddlers with an abnormal outcome. In this respect, we have limited our analyses to those toddlers who were normocephalic at birth, defined by an absence of microcephaly at birth, and by an absence of abnormal transfontanelle cerebral findings after delivery or of ultrasound findings during the third trimester of the mother's pregnancy. The results of assessments of the toddlers excluded from our analysis with CZS or abnormal ultrasound findings will be described in future publications.

The important contribution of our study is the comparison of neurodevelopment outcomes of toddlers with *in utero* ZIKV exposure with toddlers who were known to have no *in utero* exposure to ZIKV. Of the 79 toddlers born to women with no evidence of ZIKV infection at the time of delivery, we found 20 (25.3%) to have an abnormal neurodevelopment finding using the ASQ neurodevelopment assessment tool. Further, there were no differences between ZIKV-exposed and ZIKV-unexposed toddlers for behaviour disorder screening risk ($P=0.15$), nor for language acquisition, whether by mean ($P=0.36$) or the proportion below the 10th percentile threshold ($P=0.53$). These findings underscore the pertinence of a comparative control group for determining the risk of adverse outcomes - whether during pregnancy

or into early childhood – which may be attributable to ZIKV infection. Previous findings of prospective ZIKV cohort studies have attributed abnormal neurodevelopment in early childhood solely to *in utero* ZIKV exposure. Our findings would suggest that this attribution may not necessarily be warranted and that other factors which are known to be associated with neurodevelopment in early childhood need to be accounted for when determining these risk estimates.

While we found that communication and personal-social skills to be those most frequently below the -2SD threshold in the ZIKV exposed and the ZIKV unexposed, we also found in univariable and multivariable logistic regression analyses that *in utero* ZIKV exposure was an independently protective factor against an abnormal ASQ dimension score for communication. The strength of this association is such that the likelihood that this is a spurious finding is minimal. Further, the multivariable logistic regression analysis shown in Table 4 indicates that the *in utero* ZIKV exposure protective effect is not confounded. Recall bias is unlikely, as the statistically significant difference between ZIKV exposed and ZIKV unexposed was only observed for communication and not across the other ASQ dimensions, as would perhaps be expected if the parent/legal guardian – aware of the ZIKV exposure status of the toddler – responded more positively to the questionnaire in the *in utero* ZIKV exposed group compared to the ZIKV unexposed group. Our finding is further strengthened by the fact that the multivariable logistic regression model identified other risk factors for neurodevelopment in early childhood that have been previously identified, including parity and sex of the toddler.^{13,24} This suggests that the ASQ screening tool itself is an appropriate tool in this setting, that it has performed well in identifying risk factors for those at risk of abnormal neurodevelopment and that the communication finding may be influenced by a factor which has not been quantified as part of our analyses, such as parent stimulation in early childhood, or eagerness from the parents of ZIKV-exposed toddlers to emphasize attainment of developmental milestones, more so than perhaps the parents of the ZIKV unexposed toddlers.

The results of our study are strengthened by the study design. In addition to the inclusion of a comparative control group, as described above, the ZIKA-DFA-FE and ZIKA-DFA-BB cohort studies were conducted in an unselected population-based cohort, in that both the exposed and unexposed groups were derived from the same source population. While we cannot rule out that self-selection in the cohort study may have occurred, the external validity of the findings is nonetheless strengthened by the study design. The prospective nature of the ZIKA-DFA-FE pregnancy cohort, followed by the ZIKA-DFA-BB pediatric cohort enabled us to capture acute ZIKV infection during pregnancy – the majority by RT-PCR – and then to follow development outcomes into early childhood.

One limitation of our study is the loss to follow-up of toddlers from delivery to 24 months of age. However, maintaining toddlers in an extended follow-up has proved challenging for almost all ZIKV pediatric cohorts to date.^{7,9} Healthy toddlers are generally more difficult to maintain in an extended

follow-up cohort study. At this stage, we cannot exclude a selection bias that may have resulted in toddlers who were lost to follow-up in the control group had normal neurodevelopment and their loss to follow up may have artificially inflated the proportion of toddlers in the control group with abnormal neurodevelopment findings. However, the comparison of medical consultations outside routine pediatric consultations did not differ between the ZIKV exposed and the ZIKV unexposed, as would be expected if the control group was somehow different in terms of non-ZIKV related disease status compared to the ZIKV exposed group. Further, one of the principal reasons for the loss to follow up and non-completion of the neurodevelopment assessment was the unavailability of the neurodevelopment assessment in each of the study sites by the time of the 24-month visit. Finally, the use of developmental screening tools, rather than a neurodevelopment and/or behavior evaluation diagnostic tool. The comparative ease-of-use of the screening tools and budget-related constraints related to the requirement of a trained psychologist or psychotherapist led to the decision to use the various screening tools. Nonetheless, the ASQ-III tool has been widely used in studies to assess the neurodevelopment of infants and toddlers.²⁵⁻³¹

Conclusions

Overall, we found 15.3% of toddlers exposed to ZIKV *in utero* to have abnormal neurodevelopment findings at 24 months of age, a finding largely consistent with the results of other cohort studies published to date. However, when comparing this result with the result of toddlers not exposed to ZIKV *in utero* in our cohort study, we found no statistically significant difference. Therefore, in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there would not appear to be an impact on longer term neurodevelopment outcomes attributable to *in utero* ZIKV exposure which may manifest after birth up to 24 months of age. However, it is important that these neurodevelopment assessments are continued into early childhood.

Declarations

Ethics approval and consent to participate

The study received ethical approval from the Comité de Protection des personnes Sud-Ouest et Outre Mer III (registration number: 2016-A00684-47). All study procedures were explained to and informed consent obtained from a parent or legal guardian of the newborn before enrolment in the study. The study is registered with ClinicalTrials.gov under the identifier: NCT02810210.

Competing interests

The authors declare that they have no financial or non-financial competing interests.

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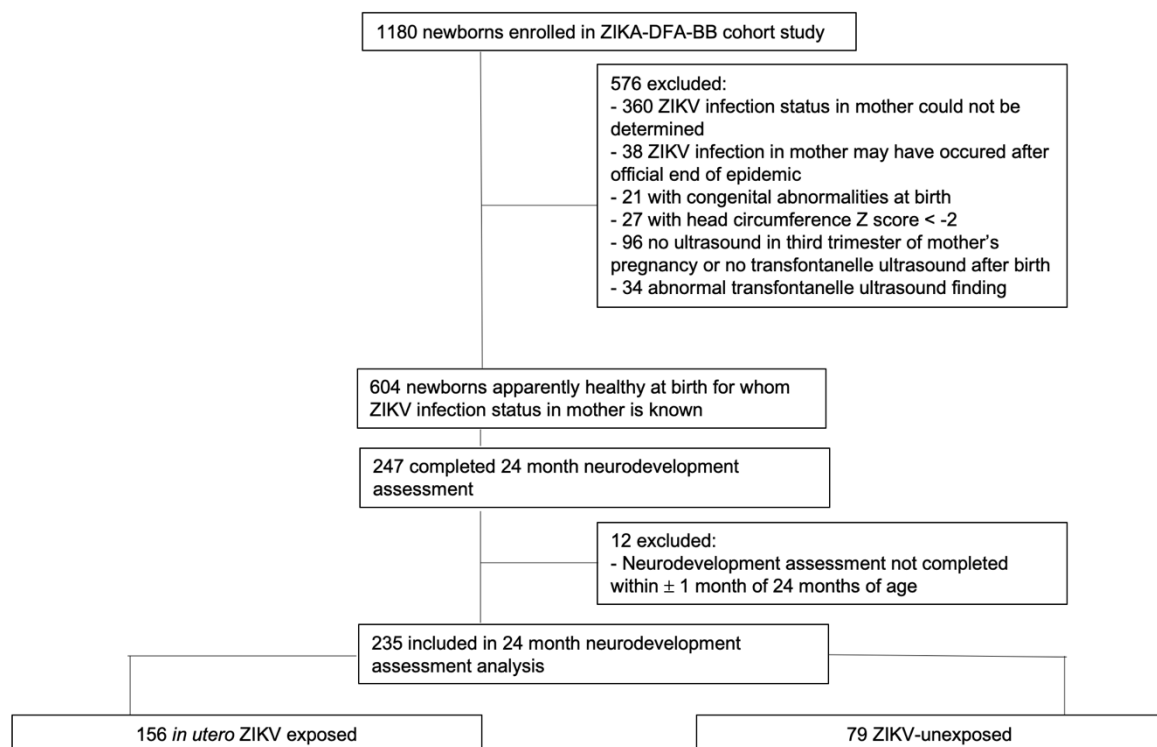


Figure 1. Toddlers from ZIKA-DFA-BB prospective cohort study in Guadeloupe, Martinique and French Guiana included in analysis

Table 1. Maternal and newborn characteristics of 235 toddlers included in analysis by ZIKV exposure status

	<i>In utero</i> ZIKV exposure (N=156)	ZIKV unexposed (N=79)	P value
Maternal characteristics			
Age at time of pregnancy (years)			
Mean \pm SD	30.7 \pm 6.3	28.6 \pm 6.0	0.01
Interquartile range	26 – 36	25 – 33	
Occupation – n (%)			<0.001
- Student	5 (3.2)	3 (3.8)	
- Self-employed/ business owner/ farmer	11 (7.1)	2 (2.5)	
- Executive/ highly skilled worker	22 (14.1)	10 (12.7)	
- Intermittent profession	10 (6.4)	24 (30.4)	
- Salaried employee	60 (38.5)	16 (20.2)	
- Not employed	47 (30.1)	24 (30.4)	
- Unknown or declined to respond	1 (0.6)	0 (0)	
Educational attainment of mother – n (%)			0.04
- Primary	30 (19.2)	7 (8.9)	
- Secondary	47 (30.1)	31 (39.2)	
- Tertiary	66 (42.3)	39 (49.4)	
- Unknown or declined to respond	13 (8.3)	2 (2.5)	
Educational attainment of father – n (%)			0.04
- Primary	22 (14.1)	4 (5.1)	
- Secondary	39 (25.0)	28 (35.4)	
- Tertiary	32 (20.5)	22 (27.8)	
- Unknown or declined to respond	62 (39.7)	25 (31.6)	
Residence – n (%)			<0.001
- Guadeloupe	88 (56.4)	12 (15.1)	
- Martinique	65 (41.7)	63 (79.7)	
- French Guiana	3 (1.9)	4 (5.1)	
Parity – n (%)			0.05
0	64 (41.0)	41 (51.9)	
1	51 (32.7)	27 (34.2)	
2	25 (16.0)	3 (3.9)	
3+	16 (10.3)	8 (10.1)	
Previous adverse pregnancy outcomes – n (%)			
- Congenital abnormalities	0 (0)	0 (0)	-
- Stillbirth*	6 (3.8)	0 (0)	0.18
- Medical termination of pregnancy*	1 (0.6)	0 (0)	1
Lifestyle practices during 2016-2017 pregnancy – n (%)			
- Alcohol consumption*	1 (0.6)	0 (0)	1
- Drug use*	1 (0.6)	1 (1.4)	1
- Smoking*	5 (3.2)	5 (6.8)	0.38
- Use of mosquito repellents*	116 (75.3)	47 (61.0)	0.04
- Use of larvicides*	91 (59.5)	44 (59.5)	1
Newborn characteristics			
Gestational age (weeks)			
Mean \pm SD	39.1 \pm 1.3	39.1 \pm 1.5	0.84
Delivery type – n (%)			
Caesarean	29 (18.6)	8 (10.1)	0.13

- Guadeloupe	17/88 (19.3)	2/12 (16.7)	1
- Martinique	10/64 (15.6)	5/63 (7.9)	0.29
- French Guiana	2/3 (66.7)	1/4 (25.0)	0.49**
Sex – n (%)			
Male	70 (44.9)	40 (50.6)	0.49
Birth weight (g)			
Mean	3176 ±476	3196 ±451	0.75
Medical consultations since birth – n (%)*			
- Hospitalisation >1 day	33 (21.1)	17 (21.5)	1
- Emergency room consultation	84 (54.5)	52 (65.8)	0.13
- Osteopath consultation	28 (18.1)	13 (16.7)	0.93
- Psychomotor therapist consultation	3 (2.0)	1 (1.3)	1
- Psychologist consultation	1 (0.6)	0 (0)	1
- Speech therapist consultation	5 (3.2)	0 (0)	0.26
- Chest physiotherapist consultation	44 (28.2)	29 (35.4)	0.32
- Physiotherapist consultation	6 (3.9)	2 (2.6)	0.89

* Data are missing for stillbirth = 1; medical termination of pregnancy = 1; alcohol consumption = 6; drug use = 6; smoking = 6; mosquito repellent use = 4; larvicide use = 8; delivery type = 1; emergency room consultation = 2; osteopath consultation = 2; psychomotor consultation = 3; psychologist consultation = 1; speech therapist consultation = 2; physiotherapist consultation = 2.

** Fisher's exact test

Table 2. ASQ results of 235 toddlers included in analysis by ZIKV exposure status

	<i>In utero</i> ZIKV exposure (N=156)		ZIKV unexposed (N=79)		Comparison of means	Comparison of n below cut-off
	Mean (\pm SD)	n below -2SD cut-off (%)	Mean (\pm SD)	n below -2SD cut- off (%)	P value	P value
Communication	49.5 \pm 12.6	13 (8.3)	44.0 \pm 16.0	16 (20.3)	0.01	0.02
Gross motor	55.9 \pm 7.6	5 (3.2)	53.4 \pm 8.6	3 (3.8)	0.04	1
Fine motor	52.7 \pm 9.0	4 (2.6)	52.7 \pm 8.5	4 (5.1)	0.96	0.54
Problem solving	48.0 \pm 12.2	8 (5.1)	47.4 \pm 10.2	2 (2.5)	0.68	0.56
Personal-social	48.6 \pm 9.8	11 (7.1)	46.7 \pm 10.1	8 (10.1)	0.16	0.57

Table 3. Univariable logistic regression model predicting ASQ dimension result below -2SD cut-off for 235 toddlers included in analysis by ZIKV exposure status

Characteristic	OR (95% CI) of ASQ dimension below -2SD cut-off						
	Communication	P value	Gross motor	P value	Fine motor	P value	Problem solving
ZIKV exposure							
Exposed	0.36 (0.2-0.8)	0.01	0.84 (0.2-3.6)	0.81	0.49 (0.1-2.0)	0.32	2.08 (0.4-10.0)
Unexposed	1		1		1	1	1
Sex							
Male	1		1		1		1
Female	0.35 (0.2-0.8)	0.01	1.49 (0.4-6.4)	0.59	0.88 (0.2-3.6)	0.85	0.36 (0.1-1.4)
Education of mother							
Primary	1	0.57	1	0.65	1	0.65	1
Secondary	0.76 (0.3-2.3)		0.70 (0.1-4.4)		0.70 (0.1-4.4)		0.30 (0.1-1.9)
Tertiary	0.54 (0.2-1.6)		0.34 (0.1-2.5)		0.34 (0.1-2.5)		0.45 (0.1-2.1)
Unknown	1.29 (0.3-6.0)		1.25 (0.1-14.9)		1.25 (0.1-14.9)		0.81 (0.08-8.5)
Education of father							
Primary	1	0.28	1	0.55	1	0.65	1
Secondary	0.52 (0.2-1.6)		0.56 (0.1-3.6)		0.70 (0.1-4.4)		0.56 (0.09-3.6)
Tertiary	0.27 (0.07-1.1)		0.23 (0.02-2.6)		0.34 (0.05-2.5)		0 (0)
Unknown	0.43 (0.1-1.3)		0.28 (0.04-2.1)		1.25 (0.1-14.9)		0.73 (0.1-4.0)
Languages spoken in household							
One	1		1		1		1
More than one	2.00 (0.8-4.9)	0.13	0.98 (0.2-4.2)	0.98	0.58 (0.1-2.4)	0.44	2.43 (0.5-11.7)
Parity	1	0.08	1	0.75	1	0.44	1
0	0.65 (0.2-1.8)		0.89 (0.2-5.5)		0.53 (0.1-2.8)		0.26 (0.03-2.3)
1	1.29 (0.4-4.4)		2.62 (0.4-16.5)		0 (0)		1.54 (0.3-8.4)
2							
Personal-social							
Exposed							
Unexposed							
Sex							
Male							
Female							
Education of mother							
Primary							
Secondary							
Tertiary							
Unknown							
Education of father							
Primary							
Secondary							
Tertiary							
Unknown							
Languages spoken in household							
One							
More than one							
Parity							
0							
1							
2							

3+	3.19 (1.1-9.3)	1.48 (0.2-14.9)	0.87 (0.1-7.8)	1.82 (0.3-10.0)	1.73 (0.4-7.1)
Birth weight					
Low birth weight	0.98	9.88 (2.1-45.9)	0.003	0	0.51
Normal	1	1	1	1	1

Table 4. Multivariable logistic regression model predicting ASQ dimension result below -2SD cut-off for 235 toddlers included in analysis by ZIKV exposure status

Characteristic	Adjusted OR (95% CI) of ASQ dimension below -2SD cut-off					
	Communication	P	value	Gross motor	P	value
ZIKV exposure						
Exposed	0.26 (0.1-0.6)	0.004		0.71 (0.1-4.0)	0.70	
Unexposed	1			1		
Sex						
Male	1			1		
Female	0.35 (0.1-0.9)	0.03		1.73 (0.3-8.8)	0.51	
Education of mother						
Primary	1	0.71		1	0.87	
Secondary	0.68 (0.2-2.5)			0.82 (0.1-7.3)		
Tertiary	0.57 (0.1-2.3)			0.39 (0.03-5.0)		
Unknown	1.65 (0.3-9.4)			1.22 (0.1-25.8)		
Education of father						
Primary	1	0.55		1	0.75	
Secondary	0.47 (0.1-2.2)			1.35 (0.1-15.9)		
Tertiary	0.33 (0.1-1.9)			0.57 (0.02-15.7)		
Unknown	0.40 (0.1-1.5)			0.51 (0.05-4.9)		
Languages spoken in household						
One	1			1		
More than one	2.15 (0.8-5.7)	0.12		0.64 (0.13-3.2)	0.58	
Parity						
0	1	0.10		1	0.85	
1	0.59 (0.2-1.8)			0.95 (0.1-6.8)		
2	1.15 (0.3-5.0)			2.25 (0.2-22.0)		
3+	3.22 (1.0-11.0)			0.67 (0.03-14.1)		
Problem solving						
1	1			1		
2	1.73 (0.3-9.9)	0.54		1		
3	0.34 (0.1-1.5)	0.16		1		
4	1			1		
5	0.28 (0.04-2.1)			0.81 (0.1-5.4)		
6	0.81 (0.1-5.4)			1.05 (0.1-12.6)		
7	1			1		
8	0.52			1		
9	1.16 (0.2-7.3)			1		
10	1.53 (0.3-9.5)			1		
11	9.54 (1.3-69.6)			1		
Personal-social						
1	1			1		
2	0.57 (0.1-2.7)			0.66 (0.1-6.1)		
3	0.24 (0.04-1.6)			0 (0)		
4	0.18 (0.04-0.9)			0.8 (0.1-5.6)		
5	1			1		
6	1.01 (0.4-2.9)			1		
7	1			1		
8	0.92 (0.3-3.0)			1		
9	0.58 (0.1-4.3)			1		
10	1.14 (0.2-6.0)			1		

Birth weight								
Low birth weight	0.35 (0.1-2.5)	0.30	10.21 (1.7-61.3)	0.01	2.3 (0.2-30.1)	0.52	0 (0)	0.82 (0.1-6.4)
Normal	1		1		1		1	0.85

Supplementary Material

Additional file 1: Supplementary Table 1. RT-PCR and serological evidence of maternal ZIKV infection during pregnancy of 235 infants included in this analysis

	N
Symptomatic women with RT-PCR positive ZIKV infection during pregnancy	
- ZIKV infection during first trimester	46
- ZIKV infection during second trimester	51
- ZIKV infection during third trimester	16
Asymptomatic women with RT-PCR positive ZIKV infection during pregnancy	
- ZIKV infection during first trimester	1
- ZIKV infection during second trimester	3
- ZIKV infection during third trimester	0
Women with serological evidence of acute ZIKV infection during pregnancy	42
Women with no serological evidence of ZIKV infection at the end of pregnancy	79

Additional file 1: Supplementary Table 2. ASQ dimensions below -2SD cut-off value of 235 infants included in this analysis by ZIKV exposure

Number of ASQ dimensions below -2SD cut-off	<i>In utero</i> ZIKV exposure (N=156)	ZIKV unexposed (N=79)	P value
			0.22
0	132 (84.6)	59 (74.7)	
1	15 (9.6)	12 (15.1)	
2	5 (3.2)	4 (5.1)	
3	2 (1.3)	3 (3.8)	
4	0 (0)	1 (1.3)	
5	2 (1.3)	0 (0)	

Additional file 1: Supplementary Table 3. M-CHAT behaviour disorder risk of 235 infants included in analysis by ZIKV exposure status

	<i>In utero</i> ZIKV exposure (N=156)	ZIKV unexposed (N=79)	P value
			0.15
No risk	118 (75.6)	52 (65.8)	
At risk	38 (24.4)	27 (34.1)	

Additional file 1: Supplementary Table 4. IFDC language acquisition of 233 francophone infants included in analysis by ZIKV exposure status

	<i>In utero</i> ZIKV exposure (N=154)			ZIKV unexposed (N=79)			Comparison of means	Comparison of n below cut-off
	N	Mean (\pm SD)	n below 10 th percentile cut-off (%)	N	Mean (\pm SD)	n below 10 th percentile cut-off (%)		
Word count	154	54.6 \pm 32.0	45 (29.2)	79	50.5 \pm 33.3	27 (34.2)	0.36	0.53
Stratification								
Sex								
- Male	69	51.7 \pm 32.0	24 (34.8)	41	44.3 \pm 32.9	18 (45.0)	0.25	0.39
- Female	85	57.0 \pm 32.0	21 (24.7)	39	56.9 \pm 32.9	9 (23.1)	0.98	1
Languages spoken in household								
- One	54	60.5 \pm 32.7	13 (24.1)	31	52.0 \pm 32.5	8 (25.8)	0.25	1
- More than one	100	51.4 \pm 31.3	32 (32.0)	48	49.5 \pm 34.1	19 (39.6)	0.74	0.47

Chapter IV

Maternal and neonatal outcomes related to Zika virus in pregnant women in Southern Vietnam: an epidemiological and virological prospective analysis

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Abstract

Background: In 2016-2017, 68 women in Southern Vietnam had RT-PCR confirmed Zika virus (ZIKV) infection during pregnancy. We report here the outcomes of the pregnancies and the virological analyses related to this outbreak.

Methods: We collected clinical and epidemiological information from the women who were enrolled in the study. Medical records related to the pregnancy in 2016-2017 were retrieved for those who were not able to be enrolled in the study. Children born to women with ZIKV infection during pregnancy were also enrolled. Serum samples were evaluated for presence of ZIKV antibodies. Phylogenetic analyses were performed on Zika virus genomes sequenced from the 2016-2017 serum samples.

Findings: Of the 68 pregnancies, 58 were livebirths and 10 were medically terminated. Four of the medical records from cases of fetal demise were able to be retrieved, of which one was consistent with congenital ZIKV infection. Of the 58 women with a livebirth, 21 participated in the follow-up investigation. All but two women had serologic evidence of ZIKV infection. Of the 21 children included in the study (mean age: 30.3 months), 3 had microcephaly at birth. No other clinical abnormalities were reported and no differences in neurodevelopment were observed compared to a control group. Phylogenetic analysis revealed a clade within the ZIKV Asian lineage and branch at the root of samples from the 2013-2014 French Polynesian outbreak. The prM S139N mutation was not observed.

Interpretation: We have been able to demonstrate a clade within the ZIKV Asian lineage implicated in adverse pregnancy outcomes in Southern Vietnam.

Introduction

Prior to the epidemics of 2007 in Yap Island, of 2013-2014 in French Polynesia and of 2015-2016 across the Americas, Zika virus (ZIKV) was understood to have extensive geographic distribution across Africa and Asia.¹ At that time, the clinical presentation of ZIKV infection was understood to be restricted to mild, self-limiting disease.¹

However, severe disease outcomes following ZIKV infection became apparent after the epidemics in French Polynesia in 2013-2014 and in Latin America in 2015-2016. The first severe neurologic complications associated with ZIKV infection, including Guillain-Barre syndrome in adults and microcephaly in fetuses and infants, were identified in French Polynesia^{2,3}, followed by the additional congenital malformations in fetuses and infants associated with *in utero* ZIKV infection in Brazil.⁴ ZIKV is now known to cause abnormalities in fetuses and infants exposed to the virus *in utero* including microcephaly, and congenital Zika syndrome (CZS). CZS comprises cranial morphology and brain anomalies, congenital contractures, ocular anomalies and marked early sequelae.⁴⁻⁹

Within the ZIKV Asian lineage, the comparative infectivity and pathogenicity, including the ability to cause severe disease, beyond the French Polynesia and Latin America epidemics remain unclear.¹⁰⁻¹² One hypothesis as to the change in disease epidemiology and the appearance of severe disease outcomes prior to the epidemic in French Polynesia is a mutation in the virus, which may have increased its virulence. The prM S139N mutation on the ZIKV genome has been identified and phylogenetic analysis suggests it appeared before the outbreak in French Polynesia, and as such, may be responsible for more severe disease outcomes.^{10,12} Improved diagnostic techniques and enhanced surveillance in other regions of the world since the epidemic in Latin America have shown continued circulation of the virus in South East Asia.

Such enhanced surveillance in Vietnam identified circulation of ZIKV in Southern Vietnam in 2016-2017.¹³ During this time, 68 pregnant women had ZIKV infection confirmed by reverse transcription polymerase chain reaction (RT-PCR) performed at the Pasteur Institute, Ho Chi Minh City. We report here an overview of the outcomes of the pregnancies and the development of the children born to the mothers with RT-PCR confirmed ZIKV infection during pregnancy and on the phylogenetic analysis of the ZIKV implicated in this outbreak.

Methods

Epidemiological investigation

Pregnant women

ZIKV surveillance in Vietnam identified 68 pregnant women with ZIKV infection confirmed by RT-PCR performed on a blood sample collected by the Pasteur Institute, Ho Chi Minh City. At the time, women were informed of the result of ZIKV testing and the serum samples were stored at -70°C at the Pasteur Institute, Ho Chi Minh City.

The samples were retrieved in 2019 and all women were contacted to participate in a follow-up epidemiological investigation. Those who were able to be contacted were invited to participate in the study. For those who were not able to be contacted, medical records at the hospital where the women had sought antenatal care, or where a medical termination of the pregnancy had been conducted following fetal demise, were retrieved for information related to the pregnancy, including the results of any ultrasonographic examinations, in accordance with local ethical regulations.

Once women were enrolled, they were interviewed by a trained member of the study personnel from Pasteur Institute, Ho Chi Minh City using a structured questionnaire. This covered sociodemographic information, including age, ethnicity, residence, and lifestyle factors. The participants were also asked about clinical information related to the pregnancy in 2016-2017: symptoms of ZIKV infection in 2016-2017, results of TORCH (toxoplasmosis, other [syphilis, human immunodeficiency virus infection (HIV)], rubella and cytomegalovirus) infection testing during pregnancy, where available, hospitalizations and/or medications taken during pregnancy, obstetrical history and the outcome of the pregnancy. Participants were encouraged to bring health records to the interview. The health records of both the mother and the infant were reviewed, as well as the results of any ultrasonographic evaluations during pregnancy in 2016-2017.

In addition, a 3mL blood sample was collected to evaluate the long term ZIKV antibody response, as well as the presence of antibodies of related viruses: dengue virus (DENV) and Japanese encephalitis virus (JEV).

Children born to women with confirmed ZIKV infection during pregnancy

The children born to women with confirmed ZIKV infection during pregnancy were also invited to participate. The women completed a questionnaire of behalf of the child which covered clinical information such as anthropometric measurements at birth and abnormalities at birth and into early childhood, extracted from the child's personal health record. Microcephaly at birth was defined as moderate when the head circumference Z score at birth was between -2 and -3, and severe when the head circumference Z score at birth was below -3 based on gestational age and sex according to

INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk>). Microcephaly at birth was further defined as proportionate if the infant was also small for gestational age at delivery, and disproportionate if not.

All children were referred to Children's Hospital Number 1, Ho Chi Minh City for clinical examination by a pediatrician. Further eye examination by fundoscopy was conducted. An auditory screening examination was performed, followed by otoacoustic emissions (OAE), auditory brainstem response (ABR) and auditory steady-state response (ASSR) tests. Hearing of the child was considered normal if OAE, ABR and ASSR were within normal ranges.

In addition, a 3mL blood sample was collected from the child to evaluate the long term ZIKV antibody response, as well as the presence of antibodies of related viruses: DENV and JEV.

For assessment of neurodevelopment, a trained member of the study personnel, blinded to the *in utero* ZIKV exposure status of the child, assessed all children using the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III). These scales provide scores for three major development domains: motor, cognition and language. The normal range for each domain is 100 ± 15 ; scores below 85 indicate developmental delay. Children with scores in the normal range in all three domains were considered developmentally normal; children with any score below 85 in any of the three domains were considered as having a developmental delay.

Control group

To investigate the role of *in utero* ZIKV exposure on neurodevelopment outcomes in early childhood, a control group of children (n=21) were recruited among children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City. Those eligible for inclusion in the control group self-reported no known ZIKV infection in the mother during pregnancy and were matched for age (within 1 month) and sex to the children born to women with confirmed ZIKV infection during pregnancy.

A trained member of the study personnel, blinded to the *in utero* ZIKV exposure status of the children, conducted the neurodevelopment assessment using the Bayley-III, as described above.

Laboratory evaluations

Laboratory tests included RT-PCR for the detection of ZIKV with Trioplex reagents. The testing procedure followed the primer and probe sequences, as described previously.¹⁴

The serum samples collected from the women and children as part of the prospective investigation were tested for anti-ZIKV IgM and IgG using a commercial enzyme-linked immunosorbent assay (ELISA)

(Euroimmun, Germany) according to the manufacturer's instructions, as well as anti-ZIKV neutralizing antibodies, using a plaque reduction neutralization test (PRNT), the details of which are provided in the Supplementary material. Serum samples were considered to be seropositive if ZIKV PRNT₅₀ was positive and the ratio of ZIKV to DENV titres was higher than 2:1. Serum samples were considered likely positive if ZIKV PRNT₅₀ was positive but the ratio of ZIKV to DENV titres was less than 2:1. Anti-ZIKV antibodies were considered to have persisted if serum samples were positive in 2020.

The stored samples from 2016-2017 were re-tested by nucleic acid amplification, followed by genomic sequencing and phylogenetic analysis. The methodology is described in the Supplementary material.

Ethics considerations

The study received approval from the Institutional Review Board at Pasteur Institute, Ho Chi Minh City, Vietnam and Children's Hospital Number 1 (Reference numbers: 21/GCN-PAS, 28 June 2019, 2097/QD-BVND1, 5 September 2019). All study procedures were explained to and informed consent obtained from all eligible participants, and from a parent or legal guardian in the case of the children, by a trained member of study personnel before enrolment in the study.

Results

Between March 2016 and November 2017, 68 pregnant women were referred from antenatal care to Pasteur Institute, Ho Chi Minh City for ZIKV diagnosis and had a RT-PCR confirmed ZIKV infection. Eighteen women had a positive test during the first trimester of pregnancy, 31 during the second trimester, 18 during the third trimester, and one woman for whom the trimester of pregnancy was unknown.

Of the 68 women with a RT-PCR confirmed ZIKV infection during pregnancy, 58 were livebirths and 10 pregnancies were medically terminated. Four medical records related to the pregnancy from the cases of fetal demise were able to be retrieved; six were unable to be retrieved. Attempts were made to contact all 58 women whose pregnancy resulted in a livebirth. Twenty-one women were able to be contacted and agreed to participate in the investigation, the remaining 37 were unable to be contacted. Of these, the medical records related to the pregnancy of 14 women were able to be retrieved; 23 medical records were unable to be retrieved. The 21 children born to the 21 women who agreed to participate in the study were also enrolled in the study. In addition, 21 children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City and who were matched for age and sex were recruited as a control group (see Figure 1).

Epidemiological investigation

Fetal demise pregnancy outcomes

Of the 10 cases of fetal demise, the medical records of four cases were able to be retrieved, one of which has been previously described.¹⁵ Briefly, maternal ZIKV infection was confirmed on 30 March 2016, one day after the onset of rash, conjunctivitis and fatigue. Fetal demise was reported on 5 April 2016 during routine ultrasonographic examination at 8 weeks gestational age. RT-PCR testing on the fetus and a sample of the placenta was positive for ZIKV.

In the second case, maternal ZIKV infection was confirmed on 14 November 2016, three days after the onset of rash. Routine ultrasonographic examination at 6-8 weeks gestational age reported fetal demise and medical termination was performed on 26 November. No fetal or placental samples were collected or tested for ZIKV infection.

In the third case, maternal ZIKV infection was confirmed on 14 January 2017, three days after the onset of rash. Routine ultrasonographic examination in the 8th week of gestation reported fetal demise and medical termination was performed on 17 January 2017. RT-PCR testing on a placental sample was positive for ZIKV.

In the fourth case, routine ultrasonographic examination at the end of the second trimester on 16 March 2017 identified fetal microcephaly and intrauterine growth restriction (IUGR). TORCH testing was negative on 6 January 2017, but maternal RT-PCR ZIKV testing on 17 March 2017 was positive. At the time of the medical termination on 21 April 2017, fetal weight was 1000g at 28·5 weeks gestation.

Liveborn pregnancy outcomes

Of the 58 livebirths, 21 women were able to be contacted and recruited into the study. The remaining 37 were unable to be contacted, but the medical records of 14 women, specific to the pregnancy in 2016-2017, were able to be retrieved. Among these 14 livebirths, three reported abnormalities at the time of delivery, of which two were consistent with *in utero* ZIKV exposure. For these three livebirths, consultation of medical records indicates that maternal ZIKV, HIV and Rubella (IgM) tests were performed during pregnancy. All three had positive ZIKV, negative HIV and negative Rubella (IgM) test results.

In the first case, maternal ZIKV infection was confirmed by RT-PCR on 8 November 2016, following the onset of rash and fever on 29 October 2016. IUGR was noted during pregnancy and the infant was born at 36 weeks' gestation on 28 November 2016 with low birth weight and moderate proportionate microcephaly.

In the second case, maternal ZIKV infection was confirmed by RT-PCR on 15 December 2016, following onset of symptoms on 12 December. The infant was born at 35 week's gestation on 19 March 2017 with low birth weight and severe disproportionate microcephaly.

In the third case, maternal ZIKV infection was confirmed by RT-PCR on 7 May 2017 following the onset of symptoms on 3 May 2017. The infant was born at 39 week's gestation on 17 October 2017 with low birth weight, moderate disproportionate microcephaly, a left-tilted neck, edema of the right collarbone, and club foot.

Among the 21 infants that were liveborn and included in the study, three had moderate disproportionate microcephaly at birth. No other abnormalities were reported. The characteristics of the 21 women and the 21 children recruited into the study are described in Table 1.

TORCH testing was not routinely conducted for most pregnancies, although all tests during pregnancy were negative when conducted (Supplementary Table 1). Of the prospective blood samples collected between March and July 2020 from 20 of the 21 women who had RT-PCR confirmed ZIKV infection in 2016-2017, all but two samples were seropositive (Table 2). In contrast, all but one of the 21 blood

samples collected from the children were serologically negative for anti-ZIKV antibodies; one blood sample was likely anti-ZIKV seropositive (Table 3).

All clinical assessments of the children were normal. The mean age at the time of the assessment was 30.8 ± 2.6 months. All 15 and 19 children who underwent auditory and fundoscopic examinations, respectively were normal.

For neurodevelopment assessment, 21 children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City were recruited as a control group. Three children (14.3%) born to women with RT-PCR confirmed infection had a developmental delay: 2 in language domain and 1 in motor domain (Table 4). One of the two children with a delay in the language domain was also one of the three children who had moderate disproportionate microcephaly at birth. One child in the control group had a developmental delay in cognitive domain. However, in comparison with the control group, the difference was not statistically significant ($P=0.60$).

Phylogenetic analysis

We combined ZIKV genomic sequences sampled from pregnant women in 2016-2017 in Vietnam with ZIKV sequences available from around the world in public databases to perform spatio-temporal analysis of ZIKV spread, via phylogenetic tree reconstruction, dating and phylogenetic inference. The results are consistent with current knowledge of ZIKV circulation (Figure 2). Asian, French Polynesian and South American sequences clustered in the phylogenetic tree, as expected. The samples of the pregnant women from Vietnam form a clade, potentially likely corresponding to a single introduction, however the bootstrap support of this clade (bootstrap value: 49) does not exclude the possibility of multiple introductions. Nonetheless, the hypothesis of a single introduction is also in agreement with the phylogenetic inference which places the root of the well supported (bootstrap value: 87) parent clade in Vietnam. The Vietnamese sequences belong to the Asian lineage and branch at the root of French Polynesian samples (bootstrap value: 87), which is also consistent with current knowledge of ZIKV circulation.¹²

Among the 3 additional sequences in the clade we have identified in Vietnam, 1 has been reported in Japan and 2 in China. The Japanese sample is known to be an importation from Vietnam in 2016,¹⁶ which is in accordance to our phylogenetic tree (tip annotated with a green star in Figure 2). Among the two sequences from China, one has been submitted in July 2019 with little information on its origins, and the other has been sampled from a person returning from Myanmar.¹⁷

S139N Mutation

Of the ZIKV samples that we sequenced, five had sufficient coverage to infer the genotype at this position. We found that our samples do not harbour the prM S139N mutation. Although we cannot exclude the presence of the S139N mutation in samples with low coverage, it is likely that this mutation was not present at the time of introduction of the virus to Vietnam and did not emerge in Vietnam.

Dating and geographical origin of outbreaks

Known sampling dates of public sequences and of samples from the pregnant women in Vietnam allowed us to estimate the date of each internal node of the phylogenetic tree with Least Squares Regression method. The date of introduction to Vietnam was estimated between October 2004 (lower 95%CI value for the most recent high-confident (marginal probability: 1.0) non-Vietnamese ancestor (Thailand)) and January 2011 (upper 95%CI value of the common ancestor of all the Vietnamese sequences, bootstrap value: 87), for which the location was confidently (marginal probability: 0.96) estimated as Vietnam, as shown in Figure 2. The virus introduction to Thailand is estimated around 1999 (see Supplementary Material), with an introduction to Singapore around 2015. The Vietnamese clade is estimated to have been introduced to South America around 2013 via French Polynesia (2011). While our phylogeographic analysis was shown to be quite robust against sampling variations (see Supplementary Material), it cannot estimate locations that were not present in our dataset (e.g. Myanmar as an intermediate in the Vietnam-to-China introduction¹⁷). Therefore, we cannot dismiss the possibility of non-direct introduction from Thailand to Vietnam (via intermediate, non-sampled locations) or from Vietnam to French Polynesia

Though the dates may display some variability in the confidence intervals, the general scenario aligns with current literature,¹⁸ and the outbreak in Vietnam is estimated to have emerged around March 2009 [December 2006 – August 2010].

Discussion

We have been able to document the outcomes of pregnancies in women with RT-PCR confirmed ZIKV infection between March 2016 and November 2017 and identify abnormalities in the fetuses associated with maternal ZIKV infection. Further, phylogenetic analysis of the viral genomes enables us to identify a clade within the ZIKV Asian lineage implicated in the outbreak.

Of the 10 pregnancies that were medically terminated, available medical records for 4 of these pregnancies indicate abnormalities associated with maternal ZIKV infection in three fetuses. Of these, two had positive ZIKV placental samples, confirming congenital ZIKV infection. An additional 3 neonates not included in the follow up investigation were identified as having microcephaly, low birth weight and other complications at birth associated, of which 2 have abnormalities likely associated with maternal ZIKV infection. Among the further 21 neonates included in the follow up investigation, 3 had moderate disproportionate microcephaly at birth, although no other abnormalities were reported. These findings allow us to infer the pathogenicity, including an ability to cause adverse fetal outcomes, of the clade within the ZIKV Asian lineage implicated in the outbreak.

The phylogenetic analysis conducted on the stored samples from 2016 reveal a clade within the Asian lineage and branch at the root of samples from the 2013-2014 French Polynesian outbreak. Our data suggest a potential single introduction, estimated to have occurred between October 2004 and January 2011. However, we cannot exclude an alternative hypothesis of several, later introductions into Vietnam. In any case, the introduction event appears to be many years before infections were detected in the pregnant women included in our study in Southern Vietnam. We are also unable to infer from our study whether the infections detected in pregnant women were the result of an outbreak of considerable magnitude across a period of 20 months, or the result of low but sustained levels of endemic circulation, as has been documented in Thailand.¹⁹ However, the available epidemiological surveillance data from 2016, showing an increase in ZIKV infections in Southern Vietnam in the last quarter of the year¹³, as well as the absence of evidence of ZIKV infection in children, would be more consistent with an epidemic event.

The prM S139N mutation on the ZIKV genome may be responsible for more severe disease outcomes identified in French Polynesia and Latin America.^{10,12} Importantly, our samples do not harbour this mutation. Our findings are consistent with the finding that the Asian lineage of ZIKV is able to cause adverse fetal outcomes, including microcephaly. A case report of fetal microcephaly following congenital ZIKV infection with the Asian lineage of ZIKV has previously been reported from Thailand.¹¹ An alternative hypothesis as to the change in disease epidemiology may be another mutation that occurred in Asia and allowed secondary spread to French Polynesia and Latin America, and/or the introduction of the virus in an immunologically naive population.

Anti-ZIKV antibody kinetic studies have shown that anti-ZIKV IgG antibodies appear rapidly after RT-PCR confirmed infection and remain detectable up to 6 months.²⁰⁻²² What remains unclear is the longer-term antibody response to ZIKV infection. In French Polynesia, seroprevalence dropped from 49% between February-March 2014 at the end of the ZIKV outbreak to 22% in September-November 2015.²³ In contrast, in Florida, United States of America, 62 Miami residents with confirmed ZIKV infection in 2016 were found to have neutralizing antibodies 12-19 months after infection,²⁴ although direct comparison with seroprevalence findings in French Polynesia is limited by differences in immunoassays used in each study. In our study, we have been able to show the persistence of specific anti-ZIKV neutralizing antibodies beyond 3 years following RT-PCR confirmed infection. All but two of the 21 women included in the follow up investigation had persistent anti-ZIKV antibodies. The absence of a decline in antibodies may also be influenced by the endemic circulation of ZIKV,²⁵ which, in contrast to islands in the Pacific, may maintain anti-ZIKV antibody titres.

Congenital ZIKV infection has been demonstrated by nucleic acid amplification based diagnostic tests on placenta and fetal samples.²⁶ What remains unclear is the diagnosis of congenital infection using serology.^{26,27} There is an assumption, derived from antibody kinetics for other congenital infections including HIV, that maternal IgG may be able to be detected in the neonate at birth, but these antibodies wane during the first year of life. As a result, any anti-ZIKV IgG detected in the child beyond 12 months of age can be assumed to have been mounted *in utero* in response to congenital ZIKV infection. In our study, we found only 1 of the 21 toddlers included in the follow up to have detectable anti-ZIKV neutralizing antibodies. We are unable to infer whether the absence of detectable anti-ZIKV neutralizing antibodies in the other children reflects an absence of congenital ZIKV infection, the waning of antibodies in early childhood or the inability of the immune system of the fetus to mount an immune response if infected early in the perinatal period. Further longitudinal investigations on antibody kinetics in infected mothers and their infants are needed.

While our study offers important contributions to the understanding of the relative pathogenicity of the Asian lineage of ZIKV and long-term antibody kinetics following RT-PCR confirmed ZIKV infection, our findings are limited by the fact that not all 68 women could be followed up as part of the prospective study. Although we do not find any adverse development outcomes in the 21 children born to women with confirmed ZIKV infection during pregnancy, we cannot rule out the existence of selection bias, in that among those who did not participate in the follow up may have been those with developmental delays. The main reason for not participating in the follow up study was our inability to contact the women to invite them to participate in the study. This is likely the result of deliveries in Southern Vietnam primarily take place in the large maternity wards of hospitals in Ho Chi Minh City, with women from rural areas returning home after delivery. A further limitation is the lack of systematic

TORCH testing during pregnancy, however the retrospective data collection of pregnancy information meant that we were restricted to the routine practices of clinicians in Vietnam.

In conclusion, we have been able to demonstrate a clade within the ZIKV Asian lineage has been able to cause adverse pregnancy outcomes among women who were infected during pregnancy in Southern Vietnam. We are also able to demonstrate persistence of anti-ZIKV antibodies in the women more than four years after RT-PCR confirmed infection. As the follow-up was incomplete, we cannot draw conclusions as to the impact of congenital ZIKV infection on development outcomes in early childhood.

Declarations

Data sharing

Sequence and phylogenetic analysis workflows are publicly available on GitHub.

ZIKV genome sequences of high quality are available on GenBank, under the accession numbers: MW915410, MW915411, MW915412, MW915413, MW915414, MW915415.

All consensus sequences are available on the GitHub repository, on the release v0.1

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Figures and Tables

Figure 1. Inclusion in analysis

Table 1. Characteristics of women with RT-PCR confirmed ZIKV infection (N=21)

Table 2: Results of prospective serologic testing* in women with RT-PCR confirmed ZIKV infection during pregnancy in 2016/2017

Table 3: Results of prospective serologic testing* in children born to women with RT-PCR confirmed ZIKV infection during pregnancy in 2016-2017

Table 4: Comparison of children born to women with ZIKV confirmed infection during pregnancy (N=21) and control group of infants attending routine immunization visits at Pasteur Institute, Ho Chi Minh City.

Figure 2: Time-scaled phylogenetic tree of Vietnamese ZIKV sequences

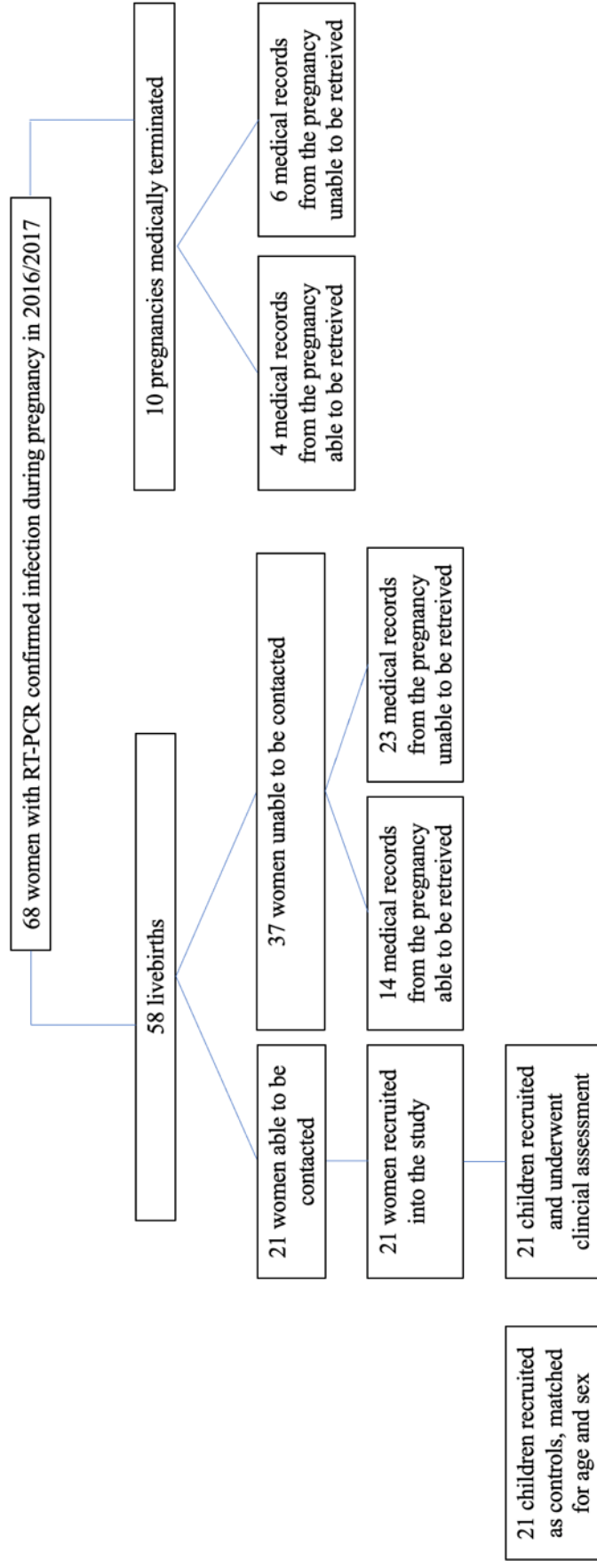


Figure 1. Inclusion in analysis

Table 1. Characteristics of women with RT-PCR confirmed ZIKV infection (N=21)

Maternal characteristics		N (%)
Age at time of conception (median, range)		30 (21-42)
Occupation		
	Highly qualified professional, Manager	7 (33·3)
	Artisan, Merchant, Business owner	3 (14·3)
	Housewife	5 (23·8)
	Labourer, Factory worker	5 (23·8)
	Employee	1 (4·8)
Residence		
	Urban	13 (61·9)
	Rural	8 (38·1)
Parity (at time of 2016-2017 pregnancy)		
	0	7 (33·3)
	1	10 (47·6)
	2	3 (14·3)
	3	1 (4·8)
Previous adverse pregnancy outcomes		
	Congenital abnormalities	0 (0)
	Stillbirth	1 (4·8)
	Miscarriage	4 (19·1)
	Medical termination	1 (4·8)
Lifestyle practices during 2016-2017 pregnancy		
	Alcohol consumption	5 (23·8)
	- Weekly alcohol consumption	2 (9·5)
	- Occasional alcohol consumption	3 (14·3)
	Drug use	0 (0)
	Smoking	0 (0)
Trimester of ZIKV infection		
	First	6 (28·6)
	Second	9 (42·9)
	Third	6 (28·6)
Signs and symptoms of ZIKV infection during pregnancy		
	Rash	17 (81·0)
	Fever	9 (42·9)
	Itching	5 (23·8)
	Limb swelling	4 (19·1)
	Myalgia	3 (14·3)
	Arthralgia	3 (14·3)
	Headache	3 (14·3)
	Conjunctival hyperemia	1 (4·8)
	Bleeding	1 (4·8)
	Pain behind eyes	0 (0)
	Petechiae	0 (0)
Neonate characteristics		
Gestational age (mean, range) (n=16)		38·7 (37-40)
Sex		
	Male	6 (28·6)
Head circumference Z score (mean, IQR) (n=13)		-0·66 (-1·1, 0·3)
	Normal	10 (76·9)
	Abnormal (head circumference Z score <-2)	3 (23·1)

Table 2: Results of prospective serologic testing* in women with RT-PCR confirmed ZIKV infection during pregnancy in 2016/2017

	ZIKV PRNT	JEV PRNT	DENV1 PRNT	DENV2 PRNT	DENV3 PRNT	DENV4 PRNT	ZIKV IgG	ZIKV IgM	ZIKV serologic status
1	160	80	10	40	0	0	Positive	Negative	Positive
2	1280	80	40	0	0	0	Negative	Negative	Positive
3	80	0	80	20	10	20	Positive	Negative	Likely positive
4	640	80	80	80	1280	10	Positive	Negative	Likely positive
5	-	-	-	-	-	-	-	-	NA
6	640	0	80	20	0	0	Positive	Negative	Positive
7	80	20	40	10	0	0	Negative	Negative	Positive
8	80	20	80	80	40	20	Borderline	Negative	Likely positive
9	1280	80	80	40	80	80	Positive	Negative	Positive
10	160	0	10	0	0	0	Positive	Negative	Positive
11	0	20	20	10	40	10	Negative	Negative	Negative
12	160	0	80	80	40	0	Positive	Negative	Positive
13	0	20	20	20	40	0	Positive	Negative	Negative
14	160	0	40	20	0	0	Borderline	Negative	Positive
15	10240	40	40	640	80	80	Positive	Negative	Positive
16	2560	0	40	160	40	10	Positive	Negative	Positive
17	160	0	320	80	40	10	Positive	Negative	Likely positive
18	1280	160	40	20	20	0	Positive	Negative	Positive
19	160	0	160	0	0	80	Positive	Negative	Likely positive
20	320	20	20	160	80	0	Positive	Negative	Positive
21	2560	0	80	20	20	10	Positive	Negative	Positive

*Mean (\pm SD) time from date of RT-PCR ZIKV testing to time of blood collection for serologic testing: 36.0 ± 1.7 months

Table 3: Results of prospective serologic testing* in children born to women with RT-PCR confirmed ZIKV infection during pregnancy in 2016-2017

	ZIKV PRNT	JEV PRNT	DENV1 PRNT	DENV2 PRNT	DENV3 PRNT	DENV4 PRNT	ZIKV IgG	ZIKV IgM	ZIKV serologic status
1	0	0	0	0	0	0	Negative	Borderline	Negative
2	0	0	0	0	0	0	Negative	Negative	Negative
3	0	80	0	10	0	0	Negative	Negative	Negative
4	0	0	0	0	80	0	Negative	Negative	Negative
5	0	0	0	0	0	0	Negative	Negative	Negative
6	20	0	160	1280	2560	40	Positive	Negative	Inconclusive
7	0	0	20	0	0	0	Negative	Negative	Negative
8	0	0	0	0	0	0	Borderline	Negative	Negative
9	0	0	0	0	0	0	Negative	Negative	Negative
10	0	0	0	0	0	0	Negative	Negative	Negative
11	0	0	20	0	0	0	Negative	Negative	Negative
12	0	0	0	0	0	40	Negative	Negative	Negative
13	0	20	0	0	0	0	Negative	Negative	Negative
14	0	0	20	0	0	0	Negative	Negative	Negative
15	0	0	0	0	0	0	Negative	Negative	Negative
16	0	0	0	0	0	0	Negative	Negative	Negative
17	0	0	10	0	0	0	Negative	Negative	Negative
18	0	0	0	0	0	0	Negative	Negative	Negative
19	0	0	0	0	0	0	Negative	Negative	Negative
20	0	0	0	0	0	0	Negative	Negative	Negative
21	0	0	0	0	0	0	Negative	Negative	Negative

*Mean (\pm SD) time from date of birth to time of blood collection for serologic testing: 30.8 ± 2.6 months

Table 4: Comparison of children born to women with ZIKV confirmed infection during pregnancy (N=21) and control group of infants attending routine immunization visits at Pasteur Institute, Ho Chi Minh City.

	Children born to women with ZIKV confirmed infection during pregnancy (N=21)	Control group of children attending routine immunization visit (N=21)
Age at the time of assessment (mean, SD)	30.8 ± 2.6	30.5 ± 2.8
Height (mean, SD)	90.4 ± 5.9	88.7 ± 5.8
Weight (mean, SD)	13.3 ± 2.5	13.6 ± 2.3
Eye examination (n=19)*		
Normal	20 (100%)	-
Abnormal	0 (0.0%)	-
Auditory test (n=15)**		
Normal	20 (100%)	-
Abnormal	0 (0.0%)	-
Neurodevelopment+		
Normal (>85)	18 (85.7%)	20 (95.2%)
Abnormal (<85)	3 (14.3%)	1 (4.8%)
Language	2	0
Motor	1	0
Cognitive	0	1

*Eye examination by fundoscopy

** Auditory screening examination was performed, followed by otoacoustic emissions (OAE), auditory brainstem response (ABR) and auditory steady-state response (ASSR) tests. Hearing of the child was considered normal if OAE, ABR and ASSR were within normal ranges.

+ Neurodevelopment assessed using the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III).

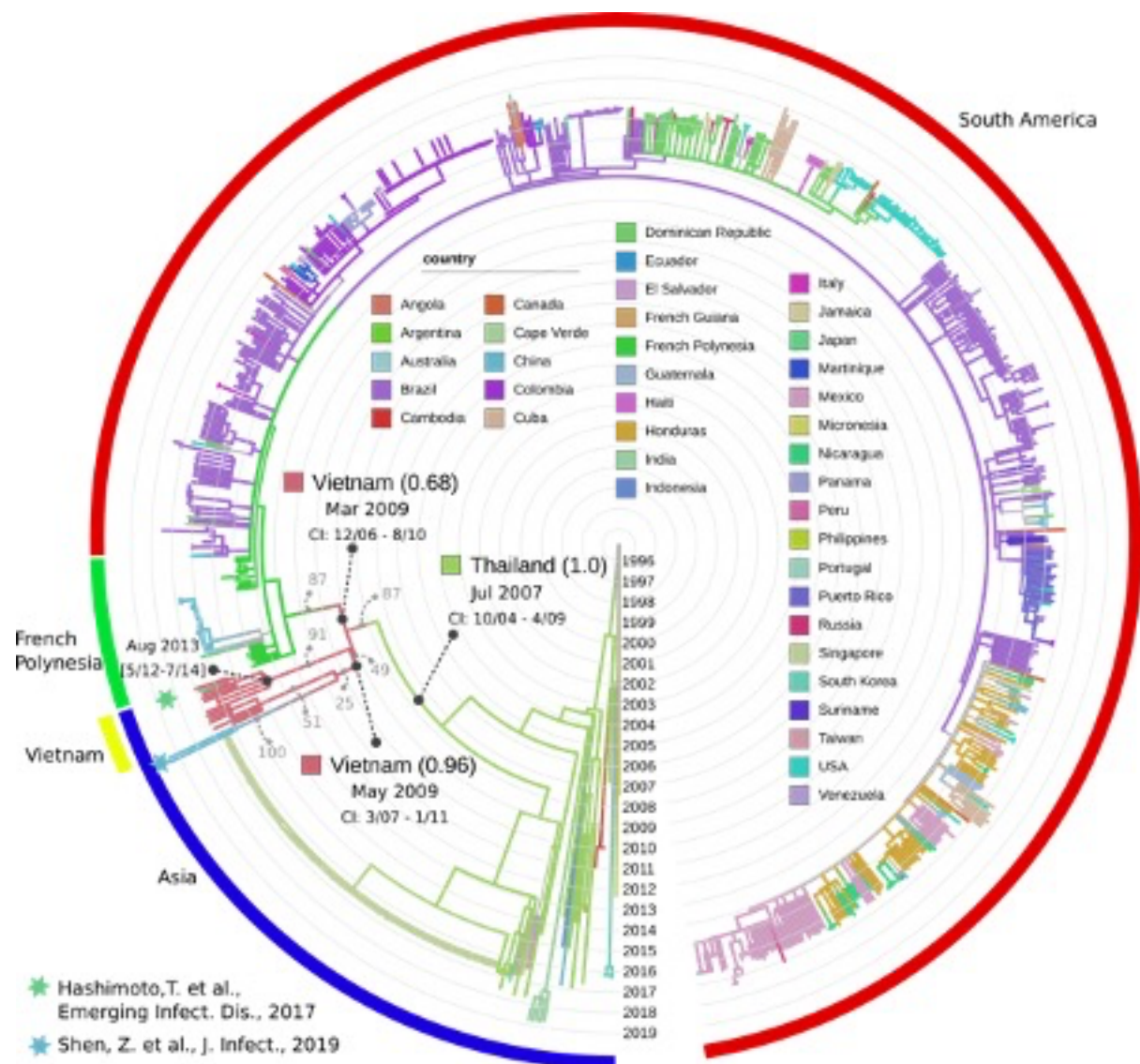


Figure 2: Time-scaled phylogenetic tree of Vietnamese ZIKV sequences

Time-scaled phylogenetic tree of Vietnamese ZIKV sequences (pink branches under the yellow ribbon) along public ZIKV sequences, with inferred geographical information. External colour strips indicate the known strains of ZIKV: Asian in blue (includes sequences mostly sampled in Asia), French Polynesian in green, and South American (sequences mostly but not only from South America) in red. Branch colours indicate their inferred geographical origin. For example, the geographical origin of the clade containing Vietnamese, French Polynesian and South American strains is inferred as Vietnam (marginal probability is shown in parenthesis) and its date is estimated as March 2009 (95%CI in parenthesis). The parent node is inferred as Thailand and its date is estimated as July 2007. Temporal predictions are obtained with LSD2,²⁸ geographic predictions - with PastML²⁹ (MPPA+F81), the visualisation is performed with iTOL.³⁰ The bootstrap supports of the branches around the Vietnamese cluster are shown in grey.

Supplementary Material

Supplementary Table 1: Available results of TORCH testing* in women with RT-PCR confirmed ZIKV infection during pregnancy in 2016-2017

Positive result on any TORCH test during pregnancy		N (%)
Toxoplasmosis	Tested during pregnancy	2 (9.5)
	Positive	0
Syphilis	Tested during pregnancy	4 (19.1)
	Positive	0
HIV	Tested during pregnancy	19 (90.4)
	Positive	0
Rubella IgM	Tested during pregnancy	14 (66.7)
	Positive	0
Cytomegalovirus	Tested during pregnancy	3 (14.3)
	Positive	0

* In Vietnam, maternal rubella IgM testing is routinely performed in the first 16 weeks of pregnancy. As of 2018, all pregnant women are also tested for HIV and syphilis during pregnancy. Testing for other TORCH pathogens is only performed if the fetal abnormalities are detected, or if the woman has symptoms suggestive of infection.

ZIKV plaque reduction neutralization test

PRNT of DENV, ZIKV and JEV were performed using DENV1 (4316/17 strain), DENV2 (5227/17 strain), DENV3 (VL6A.073 strain), DENV4 (3253/17 strain), ZIKV (3015/16 strain), JEV (6194/14 strain) strains. The ZIKV strain was detected from the first case of Zika virus in Vietnam, which has been previously published.¹

PRNT of DENV, ZIKV and JEV were performed in duplicate using 12-well plate (Corning Costar, Sigma, USA). Baby hamster kidney (BHK-21) cells were seeded at a density of 2.5×10^5 cells/well and cultured at 37 °C for 24 hours. Serum samples were heat inactivated at 56°C for 30 min before testing. PRNT started with 1:10 dilution and the maximum dilution was 20,480. Serial two-fold dilutions of serum with Eagle's minimum essential medium (EMEM) (Sigma, USA) supplemented 2% fetal bovine serum (FBS) (Gibco) were prepared. Viruses were diluted with EMEM 2% FBS to obtain a virus infectivity concentration of 30-150 plaque forming units. 60 µL of diluted serum sample was mixed with 60 µL prepared virus. The mixtures were incubated at 37 °C for 1 hour, and 50 µL mixture was added onto BHK cells, followed by incubation at 37 °C in 5% CO₂ for 1 hour. After that, we added 1.5 – 2mL EMEM/1.5% methylcellulose into each well, then continued incubating at 37°C in 5% CO₂ for several days until the plaques were observed. We used formaldehyde 4% for fixing cells in 1 hour, then stained with Methylene blue 6X buffer. Plaque numbers were counted and PRNT₅₀ was defined as the highest serum dilution which reduced the number of plaques by 50%.

Nucleic acid amplification methodology

Clinical samples were extracted using EZ1 device and EZ1 Virus Mini kit v2.0 (Qiagen) following manufacturer's instructions by mixing 200 μ L of clinical sample with 200 μ L of VXL lysis buffer (elution volume: 60 μ L). Twelve overlapping amplicons were produced using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Thermo Fisher Scientific) and specific primers (Supplementary Table 2). Reaction mixture contained 12.5 μ L of buffer 2X mixed with 200nM of primers, 3 μ L of viral nucleic acid and nuclease free water up to 8 μ L. Thermal profiles used were 50°C for 30 minutes, 94°C for 2 minutes, 45 cycles at 94°C for 15 seconds and 56°C for 30 seconds and 68°C for 3 minutes ending by a step of 68°C for 5 minutes. PCR products were pooled in equimolar proportions. After Qubit quantification using Qubit® dsDNA HS Assay Kit and Qubit 2.0 fluorometer (ThermoFisher Scientific), amplicons were fragmented (sonication) into 200bp fragments. Libraries were built adding barcode, for sample identification, and primers to fragmented DNA using AB Library Builder System (ThermoFisher Scientific). To pool equimolarly the barcoded samples, a quantification step by real time PCR using Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) was performed. An emulsion PCR of the pools and loading on 520 chip was conducted using the automated Ion Chef instrument (ThermoFisher). Sequencing was performed using the S5 Ion torrent technology v5.12 (Thermo Fisher Scientific) following manufacturer's instructions.

Supplementary Table 2. ZIKV Primers

Name	Sequence	Forward/Reverse	Working concentration
ZIKV- 1Sbis3	AGTTGTTGATCTGTGTGAGTCAG	Sens 1	10 μ M
ZIKV-epidemic-947R	AATCAGCAGTATCATGACCAAGT	Reverse 1	10 μ M
ZIKV-epidemic-889S	TTAGCAGCAGCTGCCATCGC	Sens 2	10 μ M
ZIKV-epidemic-1893R	GGTACACARGGAGTATGACACG	Reverse 2	20 μM
ZIKV-epidemic-1795S	GCTGGAGCTCTGGAGGCTG	Sens 3	10 μ M
ZIKV-epidemic-2753R	ATCCCACAACGACCGTCAGTT	Reverse 3	10 μ M
ZIKV-epidemic-2718S	GGAGCTCAACGCAATCCTGGA	Sens 4	10 μ M
ZIKV- 3844Rbis	TGTCCAATTAGCTCTGAAGATG	Reverse 4	10 μ M
ZIKA_3581S_camille	AGTGCTTGTGATTCTGCTCATGGT	Sens 5	10 μ M
ZIKV-epidemic-4581R	GTACCACGCTCCAGCTGCA	Reverse 5	10 μ M
ZIKV-epidemic-4535S	TGGTCCTGATGACCATCTGTG	Sens 6	10 μ M
ZIKV-epidemic-5610R	GGTGTCCATAATTGGTGAGTTG	Reverse 6	10 μ M
ZIKV-epidemic-5432S	TACTACAGCCAATYAGAGTCC	Sens 7	20 μM
ZIKV-epidemic-6545R	CGAGGTTGTCAATGGCTTCCT	Reverse 7	10 μ M
ZIKV-epidemic-6404S	CGAGGTGGATGGAYGCCAGAG	Sens 8	20 μM
ZIKA_7511R_camille	CACAAAGTGGAAGTTGCSGCTGT	Reverse 8	20 μM
ZIKV-7328S	ACGGCAGCTGGCATCATGAAG	Sens 9	10 μ M
ZIKV-epidemic-8243R	TGCTGGTGTATGGGCACAACA	Reverse 9	10 μ M
ZIKV-epidemic-8166S	AGAAGCACGGACGCTCAGAG	Sens 10	10 μ M
ZIKV-epidemic-9171R	CATCCAGTGATCCTCGTTCAAG	Reverse 10	10 μ M
ZIKV-epidemic-8963S	CAGTGAAGCTGTGAACGATC	Sens 11	10 μ M
ZIKV-epidemic-10338R	GTGGATAGGTARTCCATGTAC	Reverse 11	20 μM
ZIKV-epidemic-10248S	TCTCATAGGGCACAGACCGC	Sens 12	10 μ M
ZIKV-epidemic-10670R	TCCCTCTTCTGGAGATCCAC	Reverse 12	10 μ M

Genomic sequencing methodology

We first assessed the quality of the sequencing using FastQC v0.11.18 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v1.9.² We assigned reads to taxonomic levels using Kraken v2.0.8-beta and the database minikraken2_v2_8GB_201904_UPDATE.³ It resulted in an estimation of the percentage of ZIKV reads in each sample ranging from 0% to 99% (see Supplementary Table 2). We then mapped the reads on a reference ZIKV sequence (GenBank accession KJ776791.2 as in Theys K et al⁴) using Minimap v2.17⁵ and generated bam files with samtools v1.10.⁶ This resulted in 0 to 517K mapped reads depending on the sample (see Supplementary Table 2).

From these mapping files, we built consensus sequences using bcftools⁶ (commit f27f849), replacing lowly covered (<50x) regions with Ns. We then computed a multiple sequence alignment of all consensus sequences using mafft v7.453⁷ with options ‘--memsave --retree 1 --maxiterate 0 --add --keeplength’ in order to keep the numbering of reference sequence sites and to minimize the number of inserted gaps which is compatible with downstream phylogenetic analysis. The full workflow is implemented in Nextflow⁸ and is publicly available on the github repository:

https://github.com/evolbioinfo/zika_Vietnam

To estimate read coverage along the genome, we visualized bam files with IGV⁹ (Supplementary Figure 1), which showed that i) among the 15 samples, 4 had an homogeneous coverage on almost all positions, one sample had no coverage at all, and others have some parts of the genome covered and ii) the sequences that are covered at position 139 do not harbour the S139N mutation (see Supplementary Figure 2).

Supplementary Table 3: Quality control of sequences*

Sample Name	K Reads Mapped	% Zika virus	Length	K Seqs
222_IonXpress_003	32.9	21.2%	212 bp	153.7
3015_IonXpress_030	517.2	99.0%	245 bp	520.5
3015u_IonXpress_001	1.9	2.5%	200 bp	77.1
7040_IonXpress_033	297.6	80.0%	249 bp	369.8
7938_IonXpress_048	3.3	2.4%	202 bp	138.1
8546_IonXpress_034	153.8	58.0%	228 bp	263.8
8607_IonXpress_035	158.8	69.5%	241 bp	227.5
8628_IonXpress_066	31.3	21.6%	246 bp	143.3
8711_IonXpress_002	120.1	36.5%	230 bp	326.8
9453_IonXpress_078+	0.0	0.0%	230 bp	1860.0
IonXpress_003+	0.1	0.0%	236 bp	330.3
IonXpress_004	242.8	79.5%	234 bp	297.5
IonXpress_039	180.4	63.9%	217 bp	281.2
IonXpress_040	511.9	95.8%	251 bp	527.2
IonXpress_041	41.7	94.9%	243 bp	43.5

*Sequences are maternal sequences collected in 2016-2017 at the time of RT-PCR confirmed ZIKV infection in Southern Vietnam.

+Sequences with 0% ZIKV were not used in the phylogenetic analyses below.

Phylogenetic analysis

We combined sequences with those downloaded from GenBank¹⁰ (on 11 August 2020 for the organism “Zika virus” with a sequence length between 8,000 and 14,000 nucleotides). We aligned these sequences against the reference KJ776791⁴ using MAFFT⁷ (same options as above), and extracted metadata for host, country and collection date with Entrez programming utilities,¹¹ and for subtype (African or Asian) using Genome detective.¹² We then filtered out all the sequences for non-human hosts. This led to a multiple sequence alignment of 759 samples (including 13 samples from Vietnam).

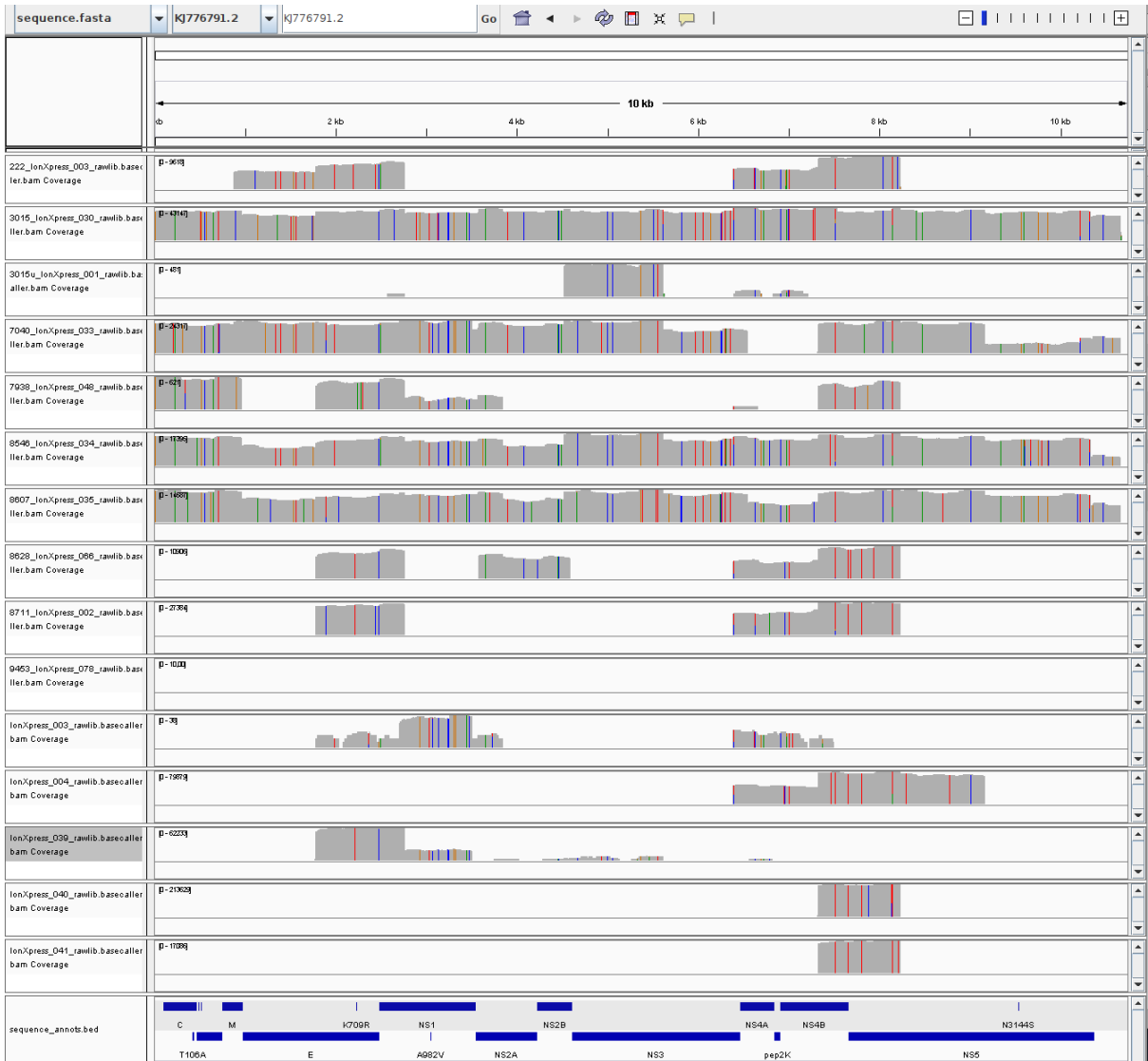
We partitioned the alignment into two groups: (1) codon positions 1 and 2, and (2) codon position 3 (based on annotations from (Theys K, et al⁴), as the third position mutates faster. We then reconstructed phylogenetic trees using RAxML-NG¹³ (v0.9.0, evolutionary models GTR+G6+FO+IO for each of the partitions, Felsenstein bootstrap¹⁴ support values calculated on 100 bootstrap trees) and IQ-TREE 2¹⁵ (v2.0-rc1, same models) and kept the best tree in terms of likelihood (recalculated on the obtained tree topologies with IQ-TREE 2): RAxML-NG tree was selected (loglikelihood of -63,692 vs -63,712 for IQ-TREE 2 tree). The tree was then rooted with the African sequences as outgroup (MN025403, KU963574, and HQ234500, which were then removed). The rooted tree was dated with LSD2¹⁶ (v1.6.5, strict molecular clock with outlier removal: ‘-c -s 10269 -f 1000 -l 0 -e 3 -u 0 -U 0 -m 249’) using tip sampling dates: nine sequences were marked as temporal outliers (MH157208, MF098770, MN577544, MK269355, KY325478, MK269356, KU744693, KY241700, and KY241712), the substitution rate was estimated to be 5.9 (95%CI: 4.7 - 7.0) x 10⁻⁴ substitutions per site per year. LSD2 tree implements the Gaussian model closely related to the Langley-Fitch molecular-clock model, which is robust to uncorrelated violations of the molecular clock. To further assess the robustness of LSD2 date estimates, we also dated the tree with the uncorrelated relaxed molecular clock (implemented in TreeTime¹⁷, v0.6.3, ‘--sequence-length 10269 --keep-root --confidence --covariation --branch-length-mode input --relax 1 0 --clock-filter 3’): The results were very similar to those obtained by LSD2 (see Supplementary Table 4). The root-to-tip regression plot generated by TreeTime indicated the presence of the temporal signal in the data (see Supplementary Figure 3)

We reconstructed ancestral characters for countries on the dated tree using PastML¹⁸ (v1.30, MPPA+F81 with options ‘--smoothing --resolve_polytomies’). To assess the robustness of the phylogeographic reconstruction with respect to the sampling bias, we repeated PastML analysis on 4 subsampled trees. Each subsampled tree was obtained from the dated tree by pruning its tips in order to keep up to 15 samples per country: for countries with no more than 15 samples, all the samples were kept, for the others 15 samples were randomly selected. The results obtained were compatible with the full tree reconstruction, though less resolved (see Supplementary Figure 4): for instance the marginal probabilities of the clade containing Vietnamese, French Polynesian and South American strains were inferred as Vietnam (marginal probability of 0.68, marginal probability of the second most probable country, Thailand, was 0.17) on the full tree, but on the subsampled tree the marginal probability of Vietnam for this node was lower (0.55-0.59), suggesting a higher doubt with Thailand (0.21-0.23). Note however, that subsampling cannot solve the issue of potentially missing intermediate locations (e.g. between Thailand, Vietnam and French Polynesia): without having samples from a country in the data set, phylogeographic inference cannot infer it as potential ancestral location.

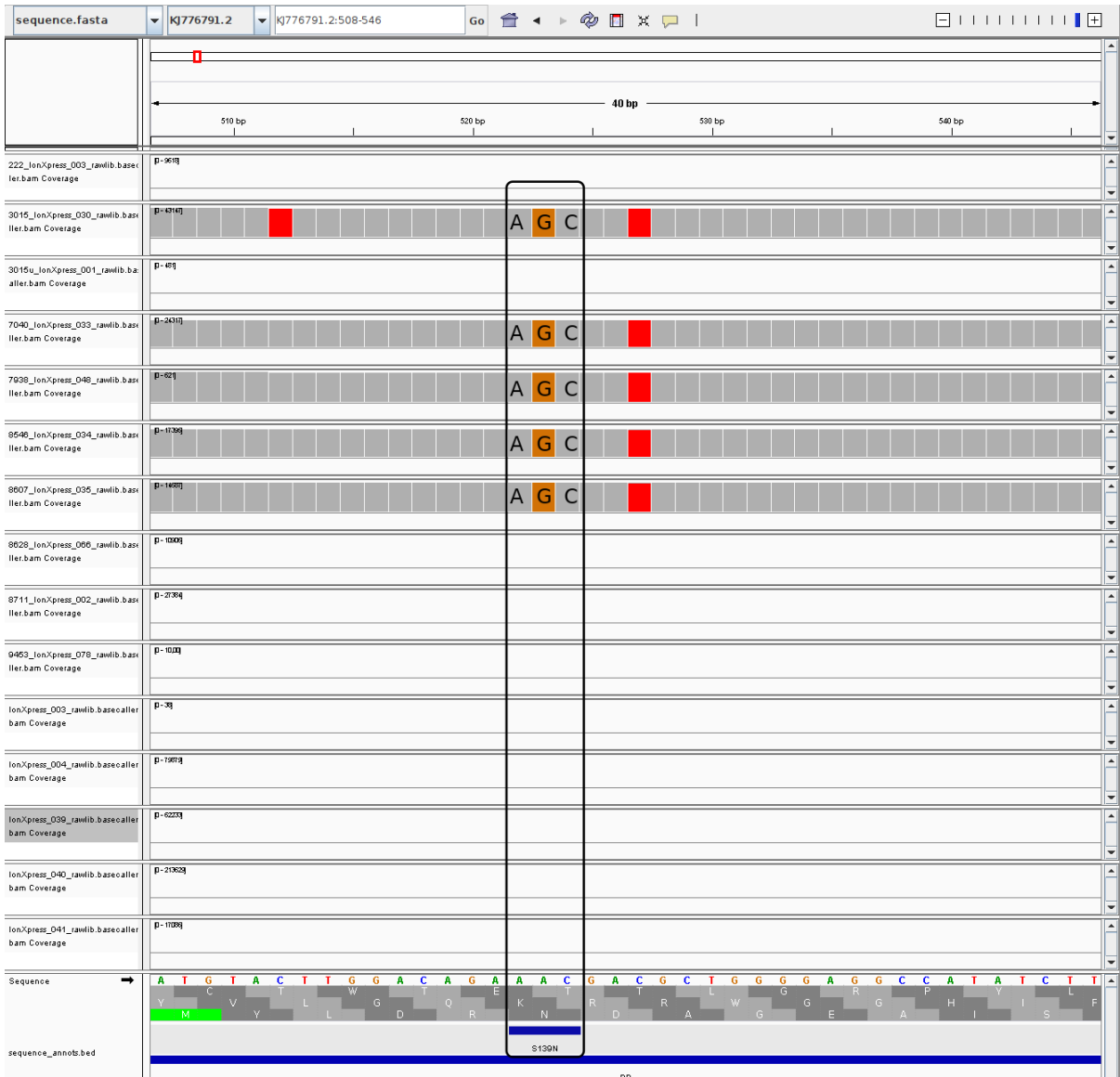
The phylogenetic analysis workflow is implemented in Snakemake¹⁹ and is publicly available on:

https://github.com/evolbioinfo/zika_Vietnam. Along with the tree reconstruction, dating and phylogeography tools mentioned above, we used gotree (v0.4.1a, github.com/evolbioinfo/gotree) and ETE3²⁰ for basic tree manipulations (format conversion, pruning, etc.).

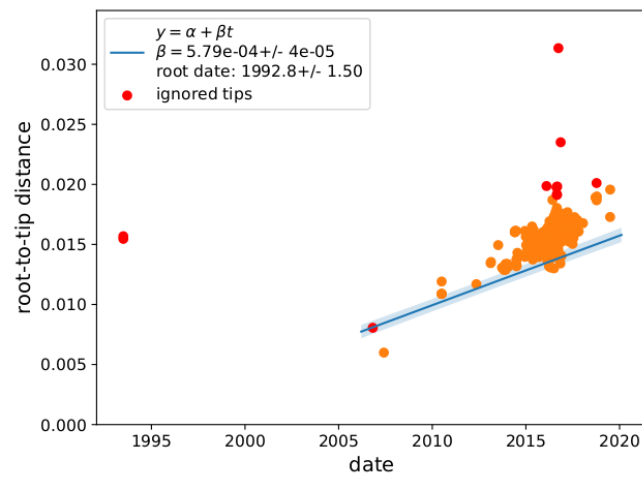
Supplementary Figure 1: Sample coverage



Supplementary Figure 2: S139N mutation

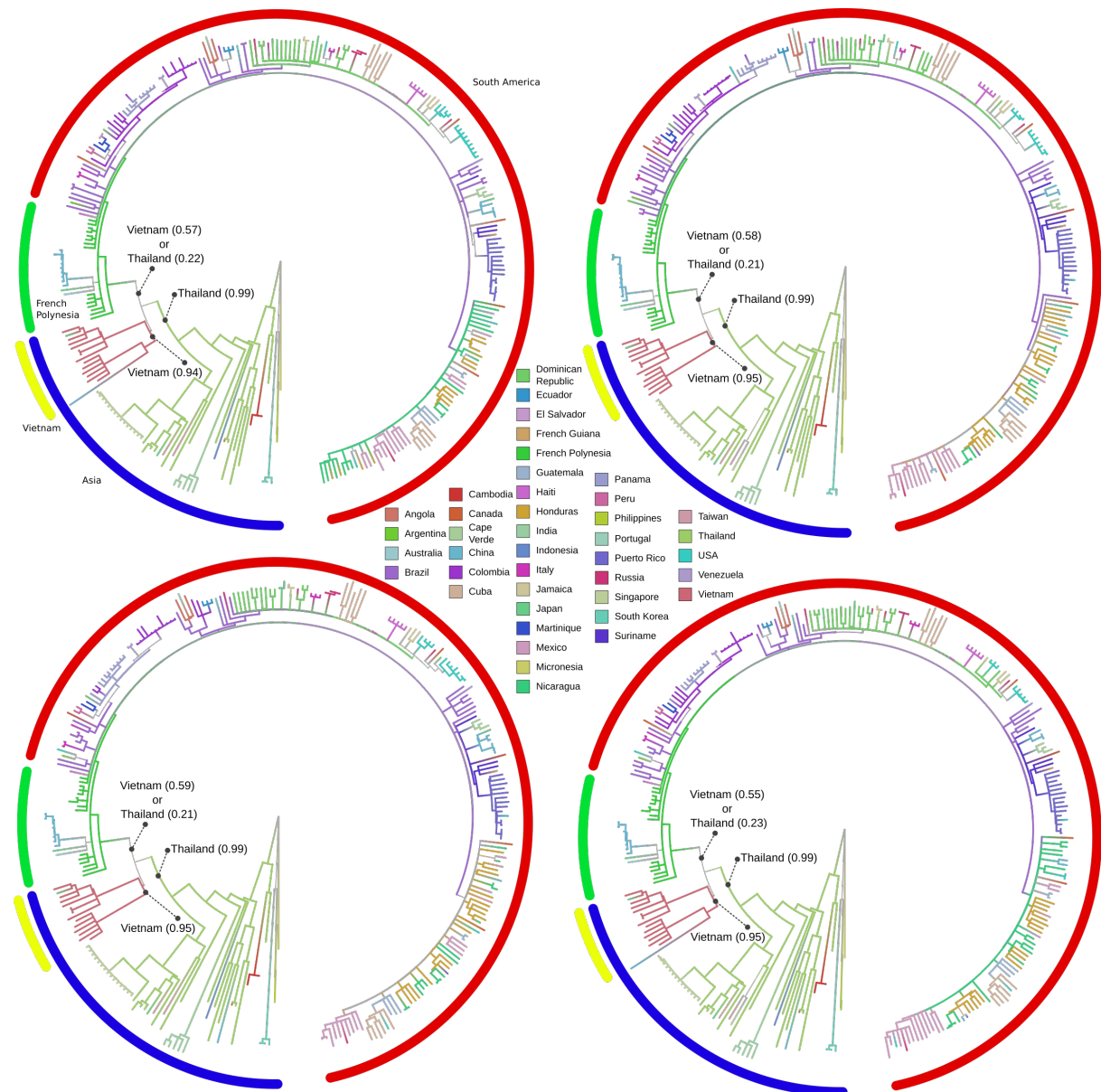


Supplementary Figure 3: Root-to-tip regression calculated with TreeTime¹⁷.



The ignored tips correspond to temporal outliers (more than 3 interquartile ranges from regression) or tips with imprecise dates.

Supplementary Figure 4: Phylogeographic reconstruction on subsampled time-scaled phylogenetic trees



Each subsampled tree was obtained from the dated tree by pruning its tips in order to keep 15 randomly selected samples per country (or all for countries with less than 15 samples). For the locations inferred around the Vietnamese cluster their marginal probabilities are shown in parentheses.

Marginal probabilities are shown in parentheses.

Supplementary Table 4: Dates and CI estimates for the largest country clusters obtained by LSD2 and TreeTime

Country	LSD2 date [CI]	TreeTime date [CI]
Brazil	18 Mar 2013 [28 Jun 2012-15 Aug 2013]	19 May 2012 [03 Mar 2012-19 Dec 2012]
China	06 Sep 2015 [27 Apr 2012-30 Jan 2016]	02 Feb 2015 [28 May 2012-16 Jul 2015]
Colombia	09 Apr 2014 [02 Apr 2013-13 Sep 2014]	26 Aug 2013 [26 Jan 2013-17 Jan 2014]
Dominican Republic	30 May 2014 [17 Dec 2013-30 May 2014]	01 Oct 2013 [20 Jul 2013-31 Jan 2014]
French Polynesia	02 Dec 2011 [14 Nov 2010-18 Nov 2012]	26 Aug 2011 [18 Mar 2011-27 May 2012]
Haiti	30 May 2014 [17 Dec 2013-30 May 2014]	09 Apr 2014 [16 Feb 2014-18 May 2014]
Honduras	30 Jan 2014 [15 Mar 2013-25 Jun 2014]	20 Sep 2013 [09 Apr 2013-24 Feb 2014]
India	20 Apr 2017 [13 Sep 2015-08 Aug 2018]	23 Oct 2016 [01 Mar 2016-17 May 2017]
Puerto Rico	11 Jan 2015 [21 Jun 2014-01 May 2015]	13 Sep 2014 [12 Jun 2014-14 Jan 2015]
Singapore	28 Jun 2015 [17 Sep 2014-15 Jan 2016]	14 Jun 2015 [28 Nov 2014-20 Nov 2015]
Thailand	29 Aug 1999 [16 Oct 1988-21 Feb 2002]	13 Apr 2002 [01 Jan 1990-05 Nov 2003]
Vietnam	07 Mar 2009 [17 Dec 2006-22 Aug 2010]	16 Apr 2009 [09 Nov 2007-02 May 2010]

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Chapter V

Quantifying the impact of Zika infection on health outcomes in pregnancy and early childhood

The three preceding chapters of the thesis describe the results of original research conducted in cohort studies in the French Territories in the Americas following the 2015-2016 ZIKV epidemic and in Vietnam following a ZIKV outbreak in 2016-2017. The findings of these studies highlight the importance of epidemiological research both during an epidemic event and in the post-acute phase and contribute to the cumulative literature on the impact of ZIKV infection on health outcomes. This chapter places these three original research publications in the broader body of scientific evidence on ZIKV infection and describes the implications for ongoing research studies and the implications for future research on ZIKV.

Implications of the original research studies in the broader context of Zika virus literature

The second chapter of the thesis describes the results of a population-based mother-child cohort study of women whose pregnancies overlapped with the 2016 ZIKV epidemic in Guadeloupe, Martinique and French Guiana. The objective of this study was to determine the impact of *in utero* ZIKV exposure on neurodevelopment at 24 months of age among toddlers who were born normocephalic to women who were pregnant during the 2016 ZIKV outbreak. We found that among 156 toddlers with and 79 toddlers without *in utero* ZIKV exposure, there were minimal differences apparent in neurodevelopment outcomes at 24 months of age compared to ZIKV-unexposed toddlers at 24 months of age, as assessed by the Ages and Stages Questionnaire (ASQ) for five dimensions of general development – communication, gross motor, fine motor, problem solving, and personal-social skills; the Modified Checklist for Autism on Toddlers (M-CHAT) for behaviour; and the French MacArthur Inventory Scales (IFDC) for French language acquisition. Twenty-four (15.4%) ZIKV-exposed toddlers and 20 (25.3%) ZIKV-unexposed toddlers had a neurodevelopment result below the reference -2SD cut-off ($P=0.10$) for at least one of the five dimensions of the ASQ assessment. There were no differences observed in M-CHAT behaviour disorder screening risk between ZIKV-exposed and ZIKV unexposed toddlers, nor in French language acquisition. Our findings indicate that, in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there does not appear to be an impact on longer term neurodevelopment outcomes attributable to *in utero* ZIKV exposure which may manifest after birth up to 24 months of age.

The third chapter describes the adverse pregnancy and pediatric outcomes possibly associated with *in utero* ZIKV exposure up to 24 months of age in infants born to women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy from the same mother-child cohort study in French Territories in the Americas. Among 546 women with symptomatic, RT-PCR confirmed ZIKV infection during pregnancy, we found an overall risk of fetal or infant neurological and ocular defects possibly associated with maternal ZIKV infection to be 7% (95%CI 5.0-9.5), and the risk of CZS to be 3.1%.

When considering adverse pregnancy and early childhood outcomes likely related to *in utero* ZIKV exposure among the 555 fetuses and infants, we found the overall risk to be 15.7% (95%CI:12.8-19.0); 3.6% (95%CI:2.3-5.6) severe sequelae or fatal outcome; 2.7% (95%CI:1.6-4.5) major abnormalities; 9.4% (95%CI:7.1-12.2) mild abnormalities. We found the risk of severe sequelae or fatality to be higher when ZIKV infection occurred during the first trimester (7.0%), compared to the second (2.7%) or third trimester (1.4%) ($p=0.02$).

The fourth chapter describes a study conducted following a separate outbreak of ZIKV in Vietnam. This study contributes to the understanding of older lineages of ZIKV and their pathogenicity. We conducted a retrospective cohort investigation of women who had RT-PCR confirmed infection during pregnancy in Southern Vietnam in 2016-2017. Of the 68 pregnancies, 58 were livebirths and 10 were medically terminated. Four of the medical records from cases of fetal demise were able to be retrieved, of which one had abnormalities consistent with congenital ZIKV infection. Of the 21 children included in the study, 3 had microcephaly at birth. No other clinical abnormalities were reported and no differences in neurodevelopment were observed compared to a control group. Phylogenetic analysis demonstrated a clade within the Asian lineage and branch at the root of samples from the 2013-2014 French Polynesian outbreak associated with adverse pregnancy outcomes. Further, as all but two women had serologic evidence of ZIKV infection, we were able to show the long-term antibody kinetics following RT-PCR confirmed ZIKV infection.

Our two studies in French Territories of the Americas contribute to the understanding and risk of adverse outcomes in early childhood following maternal ZIKV infection during pregnancy. Among infants with CZS at birth, a number of studies have demonstrated the persistent adverse developmental outcomes into early childhood. Wheeler AC et al¹ described the development outcomes of 121 children with CZS diagnosed at birth in Brazil and found 106 (87.6%) to 118 (97.5%) children to have profound developmental delays between 2-3 years of age across the various functional domains, with a mean developmental age of 2-4 months, as assessed by the Bayley Scales of Infant and Toddler Development. Periera HVFS et al² described the neurological assessment of 75 children with CZS diagnosed at birth in Brazil and found that the 23 children with neuromuscular syndrome, compared to the 48 children with corticospinal syndrome and the 4 children with isolated dyskinetic signs, had worse gross motor function, manual abilities and communication outcomes between 2-4 years of age. Bertolli J et al³ described the functional outcomes at 24 months of age of 43 children in Brazil who met anthropometric and laboratory criteria for CZS and found severe motor, auditory and visual impairment in early childhood. Satterfield-Nash A et al⁴ described the development outcomes of 19 children with microcephaly at birth and evidence of ZIKV infection and found persistent microcephaly into early childhood, non-febrile seizures, sleeping difficulties and eating or swallowing challenges. 13 (68.4%) had impaired hearing and 11 (57.9%) had impaired vision beyond 24 months of age. A review of 8

publications from Brazil reporting motor development and epilepsy outcomes in infants with CZS found 54% had epilepsy and 100% had motor abnormalities.⁵

In addition, a number of studies have demonstrated adverse neurodevelopment outcomes in early childhood among those exposed to ZIKV *in utero*, but without CZS at birth. Rice ME et al⁶ assessed follow-up care reports of 1450 toddlers with *in utero* ZIKV exposure with and without ZIKV-associated birth defects aged 1 year of age or older and found 9% had a least one neurodevelopment abnormality possibly associated with *in utero* ZIKV exposure. Sobral da Silva PF et al⁷ used the Bayley Scales of Infant and Toddler Development-III and neurological examination to evaluate 235 ZIKV exposed and 39 controls up to 42 months of age and although 9% had mild or moderate cognitive delay, there was no considerable increased risk compared to controls. Nielson-Saines K et al⁸ also used the Bayley Scales of Infant and Toddler Development-III at 18 months of age and described the neurodevelopment outcomes of 146 toddlers born to women with rash and RT-PCR ZIKV infection during pregnancy. Forty-one (28.1%) toddlers were found to have abnormal neurodevelopment, with language having the greatest proportion of toddlers with an abnormal outcome. Lopes Moreira ME et al⁹ used the Bayley Scales of Infant and Toddler Development-III to assess 94 children who were exposed to ZIKV *in utero* and who had also undergone neuroimaging between 12-18 months of age. Twenty-four (25.5%) toddlers were found to have at least one abnormal neurodevelopment finding. Considering only the 59 with normal imaging, 13 (13.8%) had abnormal neurodevelopment. Einspieler C et al¹⁰ followed 56 toddlers without microcephaly at birth and who were born to women with RT-PCR positive result for ZIKV during pregnancy. Ten (17.9%) toddlers were found to have an adverse neurologic exam or neurodevelopment assessment at 12 months of age.

The two studies conducted in the French Territories of the Americas make two important contributions to the literature described above. Firstly, they add to the literature that congenital abnormalities are more frequent and more severe in when ZIKV infection occurs earlier in pregnancy. However, compared to studies in Brazil, we found a low rate of congenital abnormalities related to *in utero* ZIKV exposure, as well as a low rate of ZIKV vertical transmission. Secondly, that the large majority of complications following *in utero* ZIKV exposure are detectable in the pre-natal or immediate post-natal period, or in other words, in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there does not appear to be an impact on longer term neurodevelopment outcomes attributable to *in utero* ZIKV exposure which may manifest after birth up to 24 months of age. The ZIKV epidemic in the Americas was particularly traumatic for expectant mothers due to the severe fetal abnormalities reported initially from Brazil and the uncertainties around the risk of adverse pregnancy and early childhood outcomes following ZIKV infection during pregnancy. In this respect, the findings of our two studies are particularly relevant for clinicians managing pregnancies of women who become infected with ZIKV during pregnancy. They reinforce

the need for close monitoring of fetal development through ultrasonographic imaging throughout the antenatal period and thorough clinical examination in the immediate post-natal period in areas where ZIKV may circulate. They also indicate that in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there should be limited concern as to adverse development outcomes manifesting in the post-natal period.

Our two studies in the French Territories of the Americas highlight the strength of the population-based prospective cohort study design. For estimating the risk of adverse pregnancy and early childhood outcomes, there was no predetermined sample size for the recruitment of pregnant women, and the centralised antenatal and maternal health services in Guadeloupe, Martinique and French Guiana maximised the number of pregnant women who were enrolled in the population-based cohort study. This likely explains why previous risk estimates of adverse pregnancy outcomes from this cohort study¹¹ align well with those of similar population-based cohort studies¹² those from registry-based studies in the United States of America.¹³ This study design minimises selection bias that are likely present in other studies that, for example, derive a cohort study from women with ZIKV infection who are referred to specialised antenatal care when diagnosed with ZIKV infection during pregnancy. Such selection bias likely increases the likelihood of more severe cases, and as a result, likely overestimates the risk of adverse pregnancy and early childhood outcomes.

Studies conducted to date have limited use of an appropriate control group and tend employ cross-sectional assessments at one point in early childhood, which do not allow for clinical evolution of abnormalities to be described. Our first study extended beyond those with *in utero* ZIKV exposure, to include infants born to women who were pregnant during the 2016 ZIKV epidemic, irrespective of ZIKV infection status during pregnancy. Such study design enabled us to quantify the adverse neurodevelopment outcomes in those without *in utero* ZIKV exposure, thus allowing appropriate comparison to those with *in utero* ZIKV exposure. This study design permits a more refined estimate of the adverse outcomes attributable to *in utero* ZIKV exposure that case series or cohort studies restricted to those with *in utero* ZIKV exposure do not allow.

Our third study provides an important contribution to the understanding of the pathogenicity of the ZIKV Asian lineage that predates the strains responsible for the epidemics in French Polynesia and across the Americas. Importantly, the prM S139N mutation – thought to be responsible for more severe disease outcomes identified in French Polynesia and the Americas¹⁴ – was not detectable in our samples. This allows us to make inferences as to the relative pathogenicity based on clinical outcomes described above, and secondly, to define more precisely the emergence of the prM S139N mutation, after the introduction to Vietnam. That is, we identified a strain that predates those implicated in the epidemics in French Polynesia in 2013-2014 and in the Americas in 2015-2016. Alongside a previous publication

from Thailand,¹⁵ our study provides counter evidence to the hypothesis that the prM S139N mutation may be responsible for the more severe outcomes observed during the epidemics in French Polynesia and across the Americas. The evidence is currently not sufficient to entirely discount the role of the prM S139N mutation in the occurrence of severe fetal abnormalities, however it may not be the only explanation.

It also provides insights into the epidemic potential of ZIKV Asian lineage. Largely as a result of the 2015-2016 ZIKV epidemic, surveillance and diagnostics capacities for ZIKV were enhanced in regions of the world in which mosquitos competent for sustaining ZIKV transmission are well established. In this study, epidemiological surveillance detected 68 women as having a RT-PCR confirmed ZIKV infection during pregnancy. The introduction event appears to be many years before infections were detected in the pregnant women, with the virus likely circulating before the subsequent infections in pregnant women. In contrast to more recent outbreaks in Singapore,¹⁶ India,¹⁷ Angola¹⁸ and Cabo Verde¹⁹ for which outbreaks have been linked to travel-related importation of the virus, the clade within the ZIKV Asian lineage predated those implicated in the epidemics in French Polynesia and the Americas and was able to either cause a substantial epidemic event, or maintain endemic transmission. Indeed, we are unable to infer from our study whether the infections detected in pregnant women were the result of an outbreak of considerable magnitude across a period of 20 months, or the result of low but sustained levels of endemic circulation, consistent with the findings of a two-year observational study of ZIKV infection in the human population in Thailand in which persistent circulation across the country.²⁰ This signals that circulation of ZIKV Asian lineage, even those that predate those implicated in the more severe epidemics in French Polynesia and the Americas should not be considered low-risk.²¹

Our study also provides an important contribution to the understanding of maternal anti-ZIKV antibody kinetics. The persistence of anti-ZIKV antibodies is contested: in French Polynesia, seroprevalence dropped from 49% between February-March 2014 at the end of the ZIKV outbreak to 22% in September-November 2015.²² In contrast, in Florida, United States of America, 62 Miami residents with confirmed ZIKV infection in 2016 were found to have neutralizing antibodies 12-19 months after infection.²³ In our study, we have been able to show the persistence of specific anti-ZIKV neutralizing antibodies in women beyond 3 years following RT-PCR confirmed ZIKV infection, with all but two of the 21 women included in the follow up investigation demonstrating persistent anti-ZIKV antibodies.

However, none of our studies have been able to provide reliable estimates of the rate of vertical ZIKV transmission. The most detailed investigation on vertical transmission to date using multiple tissue samples, including amniotic fluid, fetal and neonatal blood, cerebrospinal fluid, urine and placenta, from 291 fetuses and newborns in French Guiana found the rate of vertical transmission to be 26.1%, which was revised to 18% when placental samples were not considered because of the risk of

contamination from maternal viremia.¹² In this study, the sensitivity of IgM in neonatal samples for the diagnosis of vertical transmission was approximately 80%.¹² In Brazil, using broader definitions of infection, which included viral or IgM detection in serum and urine samples across the first year of life, vertical transmission from women with RT-PCR infection during pregnancy was shown to have occurred frequently, with 84 of 130 (65%) infants have at least one positive result.²⁴ However, results between IgM and RT-PCR had limited concordance and were not necessarily associated with infant abnormalities. In the French Territories of the Americas, we found a vertical transmission rate of 3.0%. In Vietnam, we found all but one of the 21 blood samples collected from the children were negative for anti-ZIKV antibodies at a mean age of 30.8 ± 2.6 months. These results may be explained, in part, by organizational challenges resulting in a lack of systematic sampling of cord blood at delivery and newborn serum, and the sensitivity of the IgM tests used. Accounting for the sensitivity of IgM, as derived from Pomar L et al,¹² we can conceivably revise the vertical transmission rate from our study to 3.8% (95%CI: 1.4-9.0), but this remains well short of the 18% estimate from Pomar L et al.¹² Another explanation for why such few infants had detectable immune responses in early childhood in our cohort studies in both the French Territories of the Americas and in Vietnam lies in a recent cohort study of 98 infants born to women with RT-PCR confirmed ZIKV infection during pregnancy. Using repeated serum sampling to understand the anti-ZIKV neutralizing antibody response, the study found that, beyond 18 months of age, all but one infant displayed a marked decline in anti-ZIKV neutralizing antibodies, even when stratified by trimester of infection or evidence of congenital ZIKV infection.²⁵ These initial findings would indicate that there may be no long-lasting antibody response in infants born to women who are infected during pregnancy and therefore no marker of *in utero* ZIKV transmission using antibody detection in early childhood.

Finally, all three of our studies are hampered by the loss to follow-up. We were able to resolve some of the loss to follow-up in the studies in the French Territories of the Americas and in Vietnam through consultation of medical registries to determine the infant's vital status. However, maintaining toddlers in an extended follow-up has proved challenging for almost all ZIKV pediatric cohorts to date.^{6,8} In all of our studies, we were unable to determine the direction of possible selection biases. Healthy toddlers are generally more difficult to maintain in an extended follow-up cohort study, but we observed in the second study that a small number of infants with neurological and anthropometric abnormalities at birth were also lost to follow-up.

To summarise, much of the research in the acute phase of ZIKV epidemics has focused on the immediate severe disease outcomes, notably adverse pregnancy outcomes and severe neurological conditions. These three original research studies have been conducted following an acute ZIKV epidemic and contribute to understanding the longer-term health impacts of ZIKV infection. We are able to show that the most severe disease outcomes in children are detectable in the acute phase of an

epidemic and may persist into the early childhood, and that there appear to be few abnormalities that appear beyond the immediate post-natal period. We are able to show the persistence of serological markers of infection in pregnant women, however, our studies provide limited clarification as to the understanding of vertical transmission and antibody persistence into early childhood.

Ongoing clinical research efforts to address unanswered questions

The consideration of the results of these three original research publications in the broader context of ZIKV literature highlights several unanswered questions. Specifically, the rate of adverse pediatric outcomes in those with *in utero* ZIKV exposure compared to those without *in utero* ZIKV exposure; the persistence of adverse pediatric outcomes into childhood in those with CZS; the rate of vertical ZIKV transmission; explanations as to the differences in adverse outcomes in different populations; and the likelihood for further ZIKV outbreaks.

Several ongoing pediatric cohort studies may be able to provide some answers. In French Territories of the Americas, further analyses of the prospective mother-child cohort study may allow for adverse pediatric outcomes attributable to congenital ZIKV exposure to be quantified. That is, inclusion in the mother-child cohort study extends beyond those with RT-PCR confirmed ZIKV infection during pregnancy, and thus allows comparison of adverse early childhood outcomes in those with and those without *in utero* ZIKV exposure up to 24 months of age. Recent analysis at the time of delivery of infants born to women who had no evidence of prior ZIKV infection has recently found no statistically significant differences in the risk of neurological birth defects in liveborn infants compared to infants born to women with ZIKV infection during pregnancy.²⁶ Similar analysis using the same categorisation of severity of abnormalities as described in our second study could be conducted on adverse early childhood outcomes among ZIKV exposed and ZIKV unexposed children to quantify the proportion of abnormalities that may be attributable to *in utero* ZIKV infection.

Beyond the French Territories of the Americas, several pediatric cohort studies of children exposed to ZIKV *in utero* are ongoing and will be able to describe abnormalities beyond 24 months of age. This will likely include descriptions as to the clinical evolution of those with CZS at birth across childhood. The current evidence as to the persistence of impaired development across multiple domains of functioning in those with CZS at birth and into early childhood would suggest that educational attainment will likely be compromised as children reach school age. Children with such complex medical needs will likely have high ongoing health care requirements across multiple domains and chronic functional impairment or delays.²⁷ The impact of CZS is not only restricted to clinical outcomes for the child – there are also the caregiving and associated economic impacts of the family, and the uncertainty as to the outcomes for the child to consider.²⁷

Further data as to the rate of vertical transmission may come from the Zika in infants and pregnancy (ZIP) study, conducted by the National Institute of Health.²⁸ In this multi-country observational study, pregnant women are recruited during the first trimester of pregnancy and followed prospectively, with regular RT-PCR and serological testing. In addition, large longitudinal cohort studies of pregnant women that use highly sensitive serological assays to discriminate flavivirus infection are needed in areas of endemic circulation of flaviviruses in order to better understand the role of prior flavivirus infection on ZIKV pathogenicity. However, the main challenge facing the studies that followed pregnant women and for which recruitment began from the end of 2016 or other efforts to understand prior flavivirus infection on ZIKV pathogenicity is the decline in ZIKV transmission. As of 2021, circulation of ZIKV has dropped to low levels, with the epidemic across the Americas being brought to an end in 2016. A reduction the number of infections translates to a reduction in the number of adverse events, thus hindering ongoing and future studies.

Understanding the rate of vertical transmission and the extent to which this reflects the risk of adverse pregnancy or fetal outcomes will be increasingly important for the clinical management of pregnancies in areas of ongoing or future ZIKV circulation. Revised estimates of the rate of vertical transmission may be addressed through secondary data analyses of existing cohort data. Small case series or cohort studies may not be able to describe rates of vertical transmission, but the strength of pooled analyses lies in increased sample size and statistical power to detect rare events. These analyses may also be able to provide more refined estimates of adverse pediatric outcomes in early childhood. Several data harmonization efforts are underway across pregnant women cohort studies participating in the ZIKAlliance research consortium.²⁹ In addition, an individual patient data meta-analysis is also underway, led by the World Health Organization. This will use a combined dataset from individual cohort studies to estimate the relative and absolute risk of adverse pregnancy, fetal, infant and child outcomes, using Bayesian and frequentist methods to account for sources of uncertainty in each of the risk estimates.³⁰ The challenge in harmonizing variables that are common to each dataset is substantial, as many studies were implemented rapidly at the beginning of the ZIKV epidemic and were designed to collect data that reflected the local epidemiological situation. Further variability would be expected in the performance of the various tools used to diagnose ZIKV infection, as well as differences in diagnostic criteria and clinical outcomes across sites. An example of such variability can be observed in the risk estimates of adverse pregnancy outcomes among women with ZIKV infection during pregnancy. In a registry-based study in the United States of America, the risk was estimated to be 5% (95%CI:4-6);¹³ 5% in a prospective cohort study of symptomatic women in the Brazilian Amazon;³¹ 6% (95%CI:4-8) in a registry-based study in the United States of America;³² 7% (95%CI:5-10)¹¹ and 13% (95%CI:9-18)¹² in two prospective cohort studies in French Territories in the Americas. However, risk estimates were found to be substantially higher in two prospective cohort studies of women from Brazil: 37% and 46% (95%CI:37-56), respectively.^{33,34} Part of the heterogeneity in risk estimates across

studies is likely attributable to differences in study designs and methods of recruitment (hospital-based cohort studies versus registry-based cohort studies), the use of imaging to define clinical outcomes, as well as the definitions of adverse events. Secondary analyses that are able to account for and resolve heterogeneity in studies may be able to provide more refined estimates of adverse pregnancy, fetal, infant and child outcomes.

It also remains unclear whether immunological differences may explain the apparent differences in disease outcomes following ZIKV infection. Our studies in French Territories of the Americas were conducted in areas in which DENV is considered endemic and in which seroprevalence exceeds 90%.³⁵ Secondary infection with a heterologous DENV serotype is well established as a risk factor for severe DENV disease, which may be explained by cross reactive antibodies that enhance DENV infection, known as antibody-dependent enhancement (ADE).^{36,37} For ZIKV, the role of prior flavivirus infection, particularly the role of the closely related DENV which shares antigenic similarities with ZIKV, remains unclear. Studies have found the prior DENV infection may protect against ZIKV.^{38,39} Other studies have found evidence for ADE *in vitro*,⁴⁰ but little *in vivo*,⁴¹ or in epidemiological studies.⁴² A reconciliation of the two hypotheses may lie in the timing of exposure to DENV serotype and the DENV serotype which may contribute to the differences observed in the strength of the relation between ZIKV infection and fetal abnormalities.^{43,44} Further investigation is needed into immunological differences from prior flavivirus epidemics in areas of Brazil that were severely affected by ZIKV, as compared to the French Territories of the Americas, in which lower rates of infection and abnormalities were observed, for example. This may also be able to be explored in secondary analyses of pooled data from cohort studies.

The final unanswered question concerns the current population susceptibility to further ZIKV epidemics. Our study in Vietnam indicates that circulation of ZIKV strains that predate the strains from French Polynesia and the Americas should not be considered low-risk.²¹ Studies in Thailand²⁰ and Fiji⁴⁵ have shown low level multi-year transmission of ZIKV. In contrast, several serosurveys conducted after the 2015-2016 outbreak in the Americas suggest that in areas where the virus circulated, high levels of population immunity brought transmission to an end. In Martinique, seroprevalence among asymptomatic blood donors reached 42.2%;⁴⁶ in Managua, Nicaragua, seroprevalence among adults was found to be 56% (95%CI: 53.1-59.6);⁴⁷ in tropical areas of Bolivia, seroprevalence among blood donors was found to reach 39% (95%CI: 30-48);⁴⁸ in northeastern Brazil, seroprevalence in subpopulations of Salvador was found to be 63.3% (95%CI: 59.4-66.8).⁴⁹ This is supported by mathematical modelling which suggests that the levels of population immunity in areas that experienced ZIKV transmission may delay the recurrence of large epidemics by at least a decade and that the mean age of infection will fall in future epidemics as a result of the immunity acquired by older people.⁵⁰ Nonetheless, even in countries that were heavily affected during the 2015-2016 epidemic, some regions

within the country that have established vector populations were spared from ZIKV transmission, presumably leaving them immunologically susceptible to ZIKV epidemics.^{51,52} This includes areas which have experienced prior flavivirus epidemics, such as regions of Bolivia,⁴⁸ as well as other regions of the world that had limited travel-related transmission linked to the 2015-2016 epidemic, including the Indian subcontinent.²¹ These regions are undoubtedly susceptible to large outbreaks in the human population if the virus is introduced into the established vector population. Indeed, mathematical modelling suggests that due to little or no existing ZIKV immunity, a ZIKV epidemic in India could result in more than 450 million infections.⁵³ Recent mathematical modelling jointly fitted to flavivirus surveillance data in Fiji suggests seasonal variation in transmission and the time of introduction of ZIKV into the population determine subsequent ZIKV outbreak dynamics.⁵⁴

Related to the question of future circulation of ZIKV is the apparent contradiction of the ZIKV African lineage. To date, despite seemingly favourable climatic conditions, urban populations and established ZIKV-competent vector populations, no outbreaks in human populations or severe disease outcomes following ZIKV infection with ZIKV African lineage have been reported, suggesting that the ZIKV African may be a less transmissible and/or less pathogenic lineage compared to the ZIKV Asian lineage counterparts.⁴⁴ However, animal and *in vitro* studies suggest higher transmissibility⁵⁶ and higher pathogenicity⁵⁷⁻⁶⁰ of African ZIKV strains compared to ZIKV Asian counterparts. The current hypothesis is that the increased pathogenicity of the ZIKV African lineage manifests as fetal loss, rather than as fetal abnormalities, which is why reports of fetal complications following ZIKV infection have not been reported in areas in which ZIKV African lineage is understood to circulate.⁵⁵ Further, the subspecies of *Aedes aegypti* that is predominant across the African continent – *Aedes aegypti formosus* – has been shown experimentally to be less susceptible to ZIKV infection, compared to the globally invasive subspecies – *Aedes aegypti aegypti* – which preferentially bites humans and establishes in urban settings compared to more forest-dwelling *Aedes aegypti formosus*.⁵⁵ Ongoing monitoring of *Aedes aegypti* populations is needed, as well as epidemiological surveillance of ZIKV infection in areas in which *Aedes aegypti* populations are established and surveillance of adverse pregnancy outcomes. In addition, molecular epidemiologic investigations are needed to explore other possible mutations that may distinguish ZIKV lineages and strains, as well as investigations into pathogen-specific markers of adverse fetal outcomes.

Preparedness for future Zika virus epidemics and related clinical research

These unanswered questions point to what is needed to prevent further ZIKV epidemics, as well as what clinical research is needed in the event ZIKV re-emerges in previously affected areas, or emerges in new geographic areas.

The primary measure to prevent ZIKV infection, and by extension ZIKV epidemics, currently lies in continuing vector control efforts. This includes measures taken by individuals to reduce opportunities for mosquito biting through the use of insect repellent, bednets, and screens for windows and doors. It also includes public health measures to control vector populations, such as improving water, sanitation and hygiene, removing stagnant water near households, as well as novel technologies such as the introduction of *Wolbachia* bacteria to *Aedes aegypti* populations. This has shown to inhibit ZIKV infection and reduce the viral load in mosquito saliva.^{61,62} *Wolbachia* bacteria also renders the eggs of the subsequent generation of *Aedes aegypti* non-viable. The benefits of vector control for ZIKV are two-fold, in that efforts to control vector populations for reducing ZIKV transmission will also prevent outbreaks of other *Aedes aegypti*-borne diseases, including DENV, YFV and CHIKV.

The presence of the *Aedes aegypti* populations in many tropical and sub-tropical areas of world highlights the need for preparedness for further ZIKV epidemics. Enhanced surveillance of ZIKV infections and associated fetal complications must be maintained, not only in areas of previous epidemic events, but also in areas with established competent vector populations. WHO does not currently recommend universal ZIKV testing of pregnant women.⁶³ Rather, it recommends counselling pregnant women about personal protection measures to avoid ZIKV infection during pregnancy and for close monitoring of fetal development through ultrasonographic imaging throughout the antenatal period and thorough clinical examination in the immediate post-natal period.⁶³ The United States of America Centers for Disease Control and Prevention (CDC) has included birth defects potentially related to ZIKV infection during pregnancy to birth defect surveillance efforts.⁶⁴ The implementation of prenatal ZIKV screening programmes, as is currently done for TORCH agents, has recently been evaluated, identifying the utility of such an approach in areas of active ZIKV circulation.⁶⁵ However, this evaluation also highlighted the key barriers to the implementation of such screening, including, but not limited to, performance of current diagnostics, lack of effective and safe antiviral treatments, and current capacities to provide social services, particularly when termination of pregnancy is not a legal option.⁶⁵

The lack of data and the uncertainties around the rate of vertical transmission, as well as how this may correlate to ZIKV-related abnormalities, highlights the need for further studies to refine the diagnostic criteria for congenital ZIKV infection, and whether serology can be used in the immediate post-natal period, as the window for viral and IgM detection appears to be narrow. This may need to expand beyond humoral immune response and include studies which quantify cellular immune responses – studies that are currently sparse.^{66,67}

The current lack of ZIKV circulation poses a problem for comprehensive perinatal sampling studies to refine rates of vertical ZIKV transmission. It also prevents clinical trials of ZIKV vaccine candidates advancing beyond phases II/III. ZIKV vaccine candidates that are in preclinical and clinical trials

include nucleic acid vaccines, inactivated vaccines, viral-vectored vaccines and attenuated vaccines.⁶⁸ Many of these vaccines have been shown to induce high levels of neutralizing antibodies in mice and non-human primates,⁶⁹⁻⁷¹ although the neutralizing antibody and cellular immune responses, as well as the surrogates of protection against severe fetal abnormalities or against ZIKV infection, ideally lasting through years of reproduction, need to be determined in further clinical trials.⁷² Vaccine platforms have different considerations that also need to be assessed in clinical trials – live attenuated vaccine generate infectious particle may cause reversion of virulent forms; viral-vectored vaccines may be less effective due to pre-existing immunity to the vectored virus; inactivated vaccines may require multiple doses, posing the risk of ADE.⁶⁸ Support from vaccine producers and donors has waned considerably following the decline in ZIKV transmission, presumably driven by uncertainty over future epidemics and use scenarios or target populations.⁷² Yet, the success of comprehensive childhood vaccination programmes on congenital rubella syndrome⁷³ should serve as a goal for future ZIKV vaccination efforts.

For research in the acute and post-acute period of an epidemic, it is therefore important that well designed epidemiological studies are conducted that are able to assess the impact of an infectious disease on health outcomes. The organization required to conduct such studies is not negligible; it requires well prepared teams on site, ready to initiate studies in the early stages of an infectious disease outbreak and maintain studies beyond the acute phase of an outbreak. In the context of the studies in the French Territories of the Americas, the clinical research teams involved in the prospective mother-child cohort study drew on experiences conducting clinical research during previous arbovirus epidemics: DENV in 2013 and CHIKV in 2014.

In future ZIKV epidemics, addressing many of the unknowns in child development will require more complete follow-up in pediatric cohorts. Losses to follow-up in cohort studies of pregnant women during the 2015-2016 epidemic were very low, presumably linked to the uncertainty at the time as to the rate of adverse pregnancy outcomes following ZIKV infection, and to the WHO deceleration of a Public Health Emergency of International Concern. As the epidemic came to an end and the ending of the Public Health Emergency of International Concern, interest in the virus has waned, as has the concern related to adverse outcomes in infants beyond birth. This may explain the losses to follow-up in our original research publications.

Conclusion

Following the emergence, or re-emergence in the case of ZIKV, initial epidemiological surveillance focuses primarily on those with severe disease requiring hospitalisation. Research in the acute phase of such an epidemic event is not able to capture the longer-term health impacts of a disease. As a result, it is important that assessments of health outcomes continue in the post-acute phase of an epidemic to quantify the broader spectrum of complications and adverse outcomes associated with an epidemic event, such as ZIKV. Birth defects associated with ZIKV infection during pregnancy are more severe and more frequent when infection occurs in the first trimester of pregnancy and there appear to be few abnormalities that appear for the first time beyond the immediate post-natal period and into early childhood. Our studies in the French Territories of the Americas found low rates of vertical transmission of Zika virus, as well as low rates of congenital abnormalities, as compared to studies in Brazil, without an obvious explanation. Further studies are needed to understand the rate of vertical transmission and adverse pediatric outcomes, immunological differences that may explain differences in severity of disease outcomes, as well as efficacy of ZIKV vaccine candidates. Such studies are currently on hold due to limited ZIKV circulation, but ZIKV outbreaks and/or endemic circulation can nonetheless be expected in many tropical and sub-tropical areas of the world in which competent vector populations are established.

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Curriculum Vitae

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Professional Overview

PhD candidate in infectious disease epidemiology at the Institut Pasteur, Paris, France.

Education

PhD in Epidemiology: Impact of exposure to Zika virus during pregnancy on the development of infants in French territories in the Americas Sorbonne Université, France Institut Pasteur, France	2018 –
Master of Public Health (M2): Epidemiology and Biostatistics Sorbonne Université, France	2018
Master of International Development: Global Health and Environment Sciences Po Paris School of International Affairs, France	2016
Bachelor of Applied Science with First Class Honours: Nutrition University of Otago, New Zealand	2013
Bachelor of Arts with First Class Honours: French Language and Literature University of Otago, New Zealand Université Sorbonne Nouvelle Paris III, France	2012

Professional Experience

Consultant, World Health Organization Consultant epidemiologist to MERS-CoV and COVID-19 Technical Lead	2018 –
Research Officer, Institut Pasteur Centre for Global Health	2016 – 2018
Visiting Researcher, Geneva University Hospitals Infection Prevention and Control Department	2018
Intern, Institut Pasteur Centre for Global Health	2015
Research Assistant, International Food Policy Research Institute Ethiopian Country Office	2014

Awards

Boursière d'Excellence: Université de Genève	2018
Bourse du gouvernement français for post-graduate study in France	2014
New Zealand-France Friendship Fund Academic Excellence Scholarship for post-graduate study in France	2014
University of Otago prize for the highest level of academic attainment in the Fourth Year for the degree of Bachelor of Applied Sciences with Honours	2013
University of Otago prize for the best dissertation in Human Nutrition	2013
University of Otago Department of Languages Prix d'Excellence (Fourth Year)	2012

List of Scientific Publications: 2018 – 2021

Coronavirus Disease 2019

1. Lefèvre B, Tondeur L, Madec Y, **Grant R**, Lina B, van der Werf S, Rabaud C, Fontanet A. Beta SARS-CoV-2 variant and BNT162b2 vaccine effectiveness in long-term care facilities in France. *Lancet Healthy Longev*. 2021. doi: 10.1016/S2666-7568(21)00230-0.
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7. Fontanet A, **Grant R**, Greve-Isdahl M, Sridhar D. Covid-19: Keeping schools as safe as possible. *BMJ*. 2021;372:n524.
8. **Grant R**, Dub T, Andrianou X, Nohynek H, Wilder-Smith A, Pezzotti P, Fontanet A. SARS-CoV-2 population-based seroprevalence studies in Europe: A scoping review. *BMJ Open*. 2021;11(4):e045425.

9. Fontanet A, Tondeur L, **Grant R**, Temmam S, Madec Y, Bigot G, Grzelak L, Cailleau I, Besombes C, Ungeheuer MN, Renaudat C, Perlazza BL, Arowas L, Jolly N, Fernandes Pellerin S, Kuhmel L, Staropoli I, Huon C, Chen KY, Crescenzo-Chaigne B, Munier S, Charneau P, Demeret C, Bruel T, Eloit M, Schwartz O, Hoen B. SARS-CoV-2 infection in schools in a northern French city: a retrospective serological cohort study in an area of high transmission, France, January to April 2020. *Euro Surveill.* 2021;26(15):2001695.
10. Fafi-Kremer S, Bruel T, Madec Y, **Grant R**, Tondeur L, Grzelak L, Staropoli I, Anna F, Souque P, Fernandes-Pellerin S, Jolly N, Renaudat C, Ungeheuer MN, Schmidt-Mutter C, Collongues N, Bolle A, Velay A, Lefebvre N, Mielcarek M, Meyer N, Rey D, Charneau P, Hoen B, De Seze J, Schwartz O, Fontanet A. Serologic responses to SARS-CoV-2 infection among hospital staff with mild disease in eastern France. *EBioMedicine.* 2020;59:102915.

Zika Virus Disease

1. **Grant R**, Nguyen TTT, Dao MH, Pham HTT, Piorkowski G, Pham TDT, Cao TM, Huynh LTK, Nguyen QH, Vien LDK, Lemoine F, Zhukova A, Hoang DTN, Nguyen HT, Nguyen NT, Le LB, Ngo MNQ, Tran TC, Le NNT, Nguyen MN, Pham HT, Hoang TTD, Dang TV, Vu AT, Nguyen QNT, de Lamballerie X, Pham QD, Luong QC, Fontanet A. Maternal and neonatal outcomes related to Zika virus in pregnant women in Southern Vietnam: An epidemiological and virological prospective analysis. *Lancet Reg Health West Pac.* 2021;11:100163.
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3. **Grant R**, Fléchelles O, Tressières B, Dialo M, Elenga N, Mediamolle N, Mallard A, Hebert JC, Lachaume N, Couchy E, Hoen B, Fontanet A. In utero Zika virus exposure and neurodevelopment at 24 months in toddlers normocephalic at birth: a cohort study. *BMC Med.* 2021;19(1):12.

Middle East Respiratory Syndrome

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Other

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2. Gildas Boris H, Idrissa D, Marie Louise S, Cheikh T, Aliou BM, Fatoumata DS, **Grant R**, Raymond B, Diamilatou T, Vincent R, Oumar F, Abdoulaye S, Muriel V. Identification of Pathogens Potentially Associated with Non-Malarial Fever in Children: A Pilot Study in Peri-Urban Dakar, Senegal. *Am J Trop Med Hyg*. 2021;104(4):1335-1341.
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Thesis-related Publications

RESEARCH ARTICLE

Open Access



In utero Zika virus exposure and neurodevelopment at 24 months in toddlers normocephalic at birth: a cohort study

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Abstract

Background: In utero exposure to Zika virus (ZIKV) is known to be associated with birth defects. The impact of in utero ZIKV exposure on neurodevelopmental outcomes in early childhood remains unclear. The objective of this study was to determine the impact of in utero ZIKV exposure on neurodevelopment at 24 months of age among toddlers who were born normocephalic to women who were pregnant during the 2016 ZIKV outbreak in French territories in the Americas.

Methods: We conducted a population-based mother-child cohort study of women whose pregnancies overlapped with the 2016 ZIKV epidemic in Guadeloupe, Martinique, and French Guiana. Infants were included in this analysis if maternal ZIKV infection during pregnancy could be determined, the newborn had a gestational age ≥ 35 weeks, there were no abnormal transfontanelle cerebral ultrasound findings after delivery or no abnormal ultrasound findings on the last ultrasound performed during the third trimester of the mother's pregnancy, there was an absence of microcephaly at birth, and the parent completed the 24-month neurodevelopment assessment of the infant at 24 months (± 1 month) of age. ZIKV exposure of the toddler was determined by evidence of maternal ZIKV infection during pregnancy. Neurodevelopment assessments included the Ages and Stages Questionnaire (ASQ) for five dimensions of general development—communication, gross motor, fine motor, problem solving, and personal-social skills; the Modified Checklist for Autism on Toddlers (M-CHAT) for behavior; and the French MacArthur Inventory Scales (IFDC) for French language acquisition.

Results: Between June 2018 and August 2019, 156 toddlers with and 79 toddlers without in utero ZIKV exposure completed neurodevelopment assessments. Twenty-four (15.4%) ZIKV-exposed toddlers and 20 (25.3%) ZIKV-unexposed toddlers had an ASQ result below the reference – 2SD cut-off ($P = 0.10$) for at least one of the five ASQ dimensions.

Conclusion: In one of the largest population-based cohorts of in utero ZIKV-exposed, normocephalic newborns to date, there were minimal differences apparent in neurodevelopment outcomes at 24 months of age compared to ZIKV-unexposed toddlers at 24 months of age.

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Trial registration: ClinicalTrials.gov, [NCT02810210](https://clinicaltrials.gov/ct2/show/study/NCT02810210). Registered 20 June 2016.

Keywords: Zika virus, Emerging infectious diseases, Neurodevelopment, Pediatrics, Epidemiology

Background

Zika virus (ZIKV) infection during pregnancy is understood to cause neurological complications in the fetus, collectively known in its most severe form as congenital Zika syndrome (CZS) [1]. The 2015–2016 ZIKV epidemic enabled the risk of adverse pregnancy outcomes associated with ZIKV infection during pregnancy to be estimated [2–5]. However, adverse pregnancy outcomes and CZS do not cover the full spectrum associated with in utero ZIKV exposure, and the characterization and risk of developmental outcomes beyond birth remain unclear [6]. The newborns who were born to women whose pregnancy occurred during the ZIKV epidemic are now in early childhood and the development of these young children needs to be assessed, as well as the risk for abnormal development attributable to in utero ZIKV exposure.

Adverse neurodevelopment findings in infants exposed in utero to ZIKV with and without CZS have recently been documented in the USA [7], Brazil [8–11], and Colombia [12]. Yet, within these prospective studies assessing neurodevelopment outcomes, there has been minimal use of comparative and appropriate control groups. This is a critical consideration, particularly given that neurodevelopment in early childhood is known to be influenced by both genetic and environmental factors, including substance abuse during pregnancy, maternal age [13], sociodemographic determinants [14], prematurity or low birth weight, maternal and early infant nutritional status [15], and sex of the infant in the case of language acquisition [13].

To study the impact of in utero ZIKV exposure on pregnancy and early child development outcomes, the ZIKA-DFA-FE (pregnant women) and ZIKA-DFA-BB (newborns) prospective cohort studies were established in Guadeloupe, Martinique, and French Guiana in 2016. The dynamics of the 2016 ZIKV epidemic in the three French territories were such that they enable the ZIKA-DFA-BB cohort study to closely examine the impact of in utero ZIKV exposure on early child outcomes beyond birth. Here, we present the neurodevelopment assessment results at 24 months of age. The objective of the study was to compare the neurodevelopment outcomes to toddlers who were exposed to ZIKV in utero with those who were not exposed to estimate the impact of in utero ZIKV exposure on neurodevelopmental outcomes at 24 months.

Methods

All women whose pregnancy overlapped with the 2016 ZIKV epidemic in Guadeloupe, Martinique, or French

Guiana who sought antenatal care at one of the participating hospitals were invited to participate in the ZIKA-DFA-FE prospective cohort study of pregnant women, for which the recruitment and ZIKV testing strategies have been previously described [3].

ZIKA-DFA-BB was a prospective cohort study of toddlers born to women whose pregnancies overlapped with the 2016 ZIKV epidemic in French territories in the Americas: Guadeloupe, Martinique, and French Guiana. Toddlers were followed up in routine pediatric consultation until 24 months of age.

Inclusion in analysis

Toddlers were included in this analysis if all of the following criteria were met: maternal ZIKV infection during pregnancy could be determined; the newborn had a gestational age of 35 weeks or more; there were no abnormal transfontanelle cerebral ultrasound findings after delivery up to 2 months of age or, in the absence of a transfontanelle cerebral ultrasound after delivery, no abnormal ultrasound findings on the last ultrasound performed during the third trimester of the mother's pregnancy; there was an absence of microcephaly at birth, defined as a head circumference above $-2SD$ below the mean according to sex and gestational age on the INTERGROWTH-21st chart; and the parent completed the neurodevelopment assessment of the toddler at 24 months (± 1 month) of age.

Exposure assessment

In utero ZIKV exposure was determined by RT-PCR or serological evidence of ZIKV infection in serum and/or urine samples from the mother collected during pregnancy. A toddler was considered exposed to ZIKV if the mother had RT-PCR (RealStar Zika Virus RT-PCR Kit 1.0, Altona Diagnostics) positive result for ZIKV in blood, urine, or both at any stage during her pregnancy; if the toddler had anti-ZIKV IgM (EuroImmun ELISA or in-house MAC-ELISA in French Guiana [16]) in the cord blood or blood taken within the first 10 days of life; or if the toddler had anti-ZIKV IgG (EuroImmun ELISA or in-house MAC-ELISA in French Guiana [16]) in the blood beyond 12 months of age and the date of birth was posterior to the end of the ZIKV epidemic: 11 September 2016 in French Guiana, 25 September 2016 in Guadeloupe, and 16 October 2016 in Martinique.

Anti-ZIKV kinetic studies have shown that anti-ZIKV IgG antibodies appear rapidly after infection and remain

detectable up to 6 months with 100% sensitivity [17–19]. Therefore, a negative serology for anti-ZIKV IgG in mothers at the time of delivery has a 100% negative predictive value for ZIKV infection in the mother during pregnancy and was used to define toddlers as unexposed to ZIKV in utero.

Neurodevelopment assessment

Three pediatric neurodevelopment evaluation tools were used to assess neurodevelopment and administered to a parent/legal guardian at the time of the 24-month pediatric consultation of the toddler. All questionnaires were administered in French.

The 30-item parent-reported screening test Ages and Stages Questionnaire-III (ASQ), previously validated in France [20], was used to identify toddlers at risk for developmental delay across five dimensions: communication, gross motor, fine motor, problem solving, and personal-social skills [21]. Each dimension consists of six questions, with possible responses: “yes” (10 points) if the child performs the behavior, “sometimes” (5 points), and “not yet” (0 points). A score for each dimension was calculated by adding the points from each dimension and each dimensional score reflected the child’s ability within that dimension. Abnormal ASQ outcomes were described as a dimension score below validated cut-off values, set at 2SD below the mean using reference norms [21].

The 23-item parent-reported screening test Modified Checklist for Autism on Toddlers (M-CHAT) was used to identify toddlers at risk for behavior disorder [22]. A total score was calculated by adding the points from the screening test, with low risk defined as a total score of 2 or lower, medium risk 3–7 points, and high risk 8 points or above. A positive M-CHAT screen was considered as having a total score of 3 or above.

The French MacArthur-Bates communicative development inventories (Inventaires français du développement communicatif (IFDC)) was used to assess French language acquisition for which the parent identified from a validated list of 100 words the words that the toddler says spontaneously [23]. Total word count was calculated as the sum of the words the toddler says spontaneously. Abnormal IFDC outcomes were described as total word count below validated 10th percentile thresholds derived from a reference population [23].

Statistical analysis

The maternal and newborn characteristics of the in utero ZIKV-exposed and ZIKV-unexposed toddlers were compared using Student’s *t* test for continuous variables and chi-squared test for categorical variables. Missing values for questions within each ASQ dimension were replaced by the average of the other questions in that same dimension.

Univariable and multivariable logistic regression models were used to evaluate the association between in utero ZIKV exposure and abnormal ASQ score per dimension.

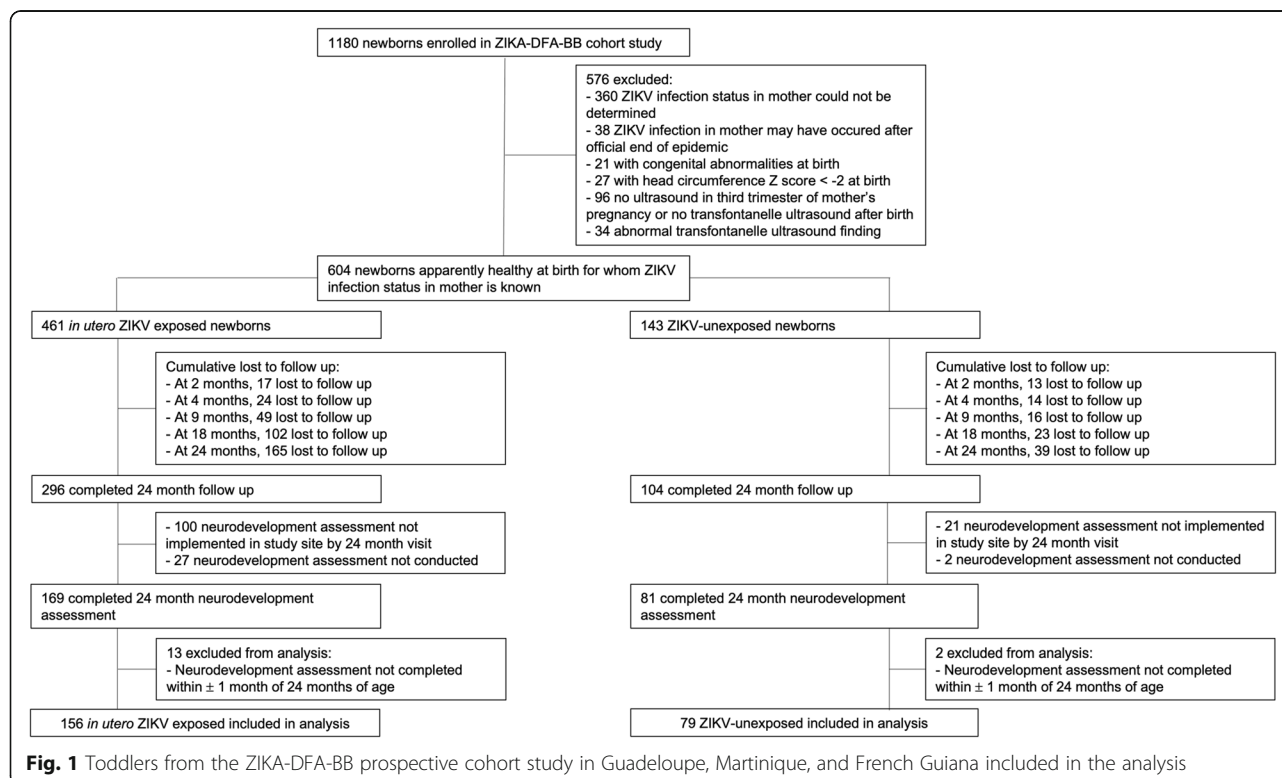
For all statistical analyses, $P < 0.05$ was considered statistically significant. All statistical analyses were performed using R, version 3.6.1.

Results

From June 2018 to August 2019, 1180 newborns were enrolled in the ZIKA-DFA-BB cohort study. Of these, 572 were excluded from analysis: ZIKV infection status of the mother could not be determined (360 newborns), or it could not be excluded that maternal ZIKV infection preceded the start of the pregnancy (38 newborns), or the newborn had congenital abnormalities at birth (21 newborns), or the newborn had a head circumference Z score of $-2SD$ or lower at birth (27 infants), or the newborn had no ultrasound findings available from the third trimester of the mother’s pregnancy (96 newborns), or the newborn had an abnormal transfontanelle cerebral ultrasound findings at birth up to 2 months of age (34 newborns). Of the remaining 604 newborns, 247 completed the 24-month neurodevelopment assessment (169 toddlers born to women with confirmed ZIKV infection during pregnancy, 81 toddlers born to women with no ZIKV infection during pregnancy). A further 15 toddlers were excluded from the analysis as the neurodevelopment assessment was not administered within ± 1 month of the toddlers 24 months of age. We were therefore able to describe the results of the neurodevelopment assessment at 24 months for 235 toddlers: 156 in utero ZIKV-exposed toddlers and 79 ZIKV-unexposed toddlers (Fig. 1).

Table 1 shows the maternal and newborn characteristics of the 235 toddlers by ZIKV exposure status. Of those included in the analysis, no abnormalities associated with ZIKV exposure were reported in routine pediatric consultation at birth. Additional file 1: Supplementary Table 1 shows the RT-PCR and serological evidence of maternal ZIKV infection. Comparisons between ZIKV-exposed and ZIKV-unexposed toddlers indicated a lower maternal age ($P = 0.01$), higher maternal education ($P = 0.04$), and higher paternal education ($P = 0.04$) in the ZIKV unexposed; a higher proportion of toddlers from Guadeloupe in the ZIKV-exposed group and a higher proportion of toddlers from Martinique in the ZIKV-unexposed group ($P \leq 0.001$); higher parity in the ZIKV exposed ($P = 0.04$); and greater use of mosquito repellents in the ZIKV-exposed group ($P = 0.05$).

Comparisons between the toddlers included in the analysis ($N = 235$) and those who were excluded because they did not complete the neurodevelopment assessment ($N = 369$) revealed higher completion of the questionnaire in Martinique and lower completion of the questionnaire in French Guiana in exposed ($P \leq 0.001$) and



unexposed groups ($P \leq 0.001$); higher completion rate of the questionnaire in toddlers born to women with lower parity in the unexposed groups ($P \leq 0.001$); and higher completion rate in toddlers born to women who did not consume alcohol during pregnancy ($P = 0.04$) (data not shown).

Table 2 shows the mean ASQ results per dimension by ZIKV exposure status, as well as the number of toddlers in each group with a score below the $-2SD$ cut-off. The communication dimension showed a statistically significant difference between the two groups, with the ZIKV exposed having a higher mean communication score (49.5 vs 44.0; $P = 0.01$) and a smaller proportion of toddlers falling below the $-2SD$ cut-off value (8.3% vs 20.3%; $P = 0.02$). The dimensions most frequently scoring below the threshold in the ZIKV-exposed and ZIKV-unexposed groups were communication and personal-social skills.

Additional file 1: Supplementary Table 2 shows the number of ASQ dimensions below the $-2SD$ cut-off value by ZIKV exposure. Twenty-four (15.4%) ZIKV-exposed toddlers and 20 (25.3%) ZIKV-unexposed toddlers had an ASQ result for at least one of the five ASQ dimensions below the reference $-2SD$ cut-off ($P = 0.10$).

Further analyses among the ZIKV-exposed toddlers demonstrated no difference in the proportion of toddlers with at least one ASQ dimension below the $-2SD$ cut-off value by trimester of pregnancy during which ZIKV infection occurred for the women who had ZIKV infection determined by RT-PCR ($P = 0.67$).

In univariable logistic regression analyses shown in Table 3, in utero ZIKV exposure and sex of the toddler were associated with an ASQ score for communication below the $-2SD$ cut-off. In multivariable regression analysis, shown in Table 4, in utero ZIKV exposure and sex of the toddler were identified as independent predictors of an ASQ score for communication below the $-2SD$ cut-off.

Additional file 1: Supplementary Table 3 shows the M-CHAT behavior disorder risk by ZIKV exposure status. There was no difference observed in behavior disorder screening risk between ZIKV-exposed and ZIKV-unexposed toddlers ($P = 0.15$).

Additional file 1: Supplementary Table 4 shows language acquisition of 233 francophone toddlers by ZIKV exposure status. There were no observed differences in mean language acquisition ($P = 0.36$), nor in the proportion of toddlers below the 10th percentile threshold ($P = 0.53$). Further stratification by sex and by the number of languages spoken in the household did not identify any differences by in utero ZIKV exposure status.

Discussion

In one of the largest population-based, mother-child cohorts of in utero ZIKV-exposed normocephalic at birth to date, we found no apparent differences in neurodevelopment outcomes compared to ZIKV-unexposed toddlers at 24 months of age.

Table 1 Maternal and newborn characteristics of 235 toddlers included in the analysis by ZIKV exposure status

	In utero ZIKV exposure (N = 156)	ZIKV unexposed (N = 79)	P value
Maternal characteristics			
Age at time of pregnancy (years)			
Mean ± SD	30.7 ± 6.3	28.6 ± 6.0	0.01
Interquartile range	26–36	25–33	
Occupation—n (%)			< 0.001
- Student	5 (3.2)	3 (3.8)	
- Self-employed/business owner/farmer	11 (7.1)	2 (2.5)	
- Executive/highly skilled worker	22 (14.1)	10 (12.7)	
- Intermittent profession	10 (6.4)	24 (30.4)	
- Salaried employee	60 (38.5)	16 (20.2)	
- Not employed	47 (30.1)	24 (30.4)	
- Unknown or declined to respond	1 (0.6)	0 (0)	
Educational attainment of mother—n (%)			0.04
- Primary	30 (19.2)	7 (8.9)	
- Secondary	47 (30.1)	31 (39.2)	
- Tertiary	66 (42.3)	39 (49.4)	
- Unknown or declined to respond	13 (8.3)	2 (2.5)	
Educational attainment of father—n (%)			0.04
- Primary	22 (14.1)	4 (5.1)	
- Secondary	39 (25.0)	28 (35.4)	
- Tertiary	32 (20.5)	22 (27.8)	
- Unknown or declined to respond	62 (39.7)	25 (31.6)	
Residence—n (%)			< 0.001
- Guadeloupe	88 (56.4)	12 (15.1)	
- Martinique	65 (41.7)	63 (79.7)	
- French Guiana	3 (1.9)	4 (5.1)	
Parity—n (%)			0.05
0	64 (41.0)	41 (51.9)	
1	51 (32.7)	27 (34.2)	
2	25 (16.0)	3 (3.9)	
3+	16 (10.3)	8 (10.1)	
Previous adverse pregnancy outcomes—n (%)			
- Congenital abnormalities	0 (0)	0 (0)	–
- Stillbirth*	6 (3.8)	0 (0)	0.18
- Medical termination of pregnancy*	1 (0.6)	0 (0)	1
Lifestyle practices during 2016–2017 pregnancy—n (%)			
- Alcohol consumption*	1 (0.6)	0 (0)	1
- Drug use*	1 (0.6)	1 (1.4)	1
- Smoking*	5 (3.2)	5 (6.8)	0.38
- Use of mosquito repellents*	116 (75.3)	47 (61.0)	0.04
- Use of larvicides*	91 (59.5)	44 (59.5)	1
Newborn characteristics			
Gestational age (weeks)			
Mean ± SD	39.1 ± 1.3	39.1 ± 1.5	0.84

Table 1 Maternal and newborn characteristics of 235 toddlers included in the analysis by ZIKV exposure status (Continued)

	In utero ZIKV exposure (N = 156)	ZIKV unexposed (N = 79)	P value
Delivery type—n (%) [*]			
Cesarean	29 (18.6)	8 (10.1)	0.13
- Guadeloupe	17/88 (19.3)	2/12 (16.7)	1
- Martinique	10/64 (15.6)	5/63 (7.9)	0.29
- French Guiana	2/3 (66.7)	1/4 (25.0)	0.49**
Sex—n (%)			
Male	70 (44.9)	40 (50.6)	0.49
Birth weight (g)			
Mean	3176 ± 476	3196 ± 451	0.75
Medical consultations since birth—n (%) [*]			
- Hospitalization > 1 day	33 (21.1)	17 (21.5)	1
- Emergency room consultation	84 (54.5)	52 (65.8)	0.13
- Osteopath consultation	28 (18.1)	13 (16.7)	0.93
- Psychomotor therapist consultation	3 (2.0)	1 (1.3)	1
- Psychologist consultation	1 (0.6)	0 (0)	1
- Speech therapist consultation	5 (3.2)	0 (0)	0.26
- Chest physiotherapist consultation	44 (28.2)	29 (35.4)	0.32
- Physiotherapist consultation	6 (3.9)	2 (2.6)	0.89

^{*}Data are missing for stillbirth = 1; medical termination of pregnancy = 1; alcohol consumption = 6; drug use = 6; smoking = 6; mosquito repellent use = 4; larvicide use = 8; delivery type = 1; emergency room consultation = 2; osteopath consultation = 2; psychomotor consultation = 3; psychologist consultation = 1; speech therapist consultation = 2; physiotherapist consultation = 2

^{**}Fisher's exact test

We found 24 (15.3%) toddlers with in utero ZIKV exposure at risk of neurodevelopment delay using the ASQ neurodevelopment screening tool. This is largely comparable to neurodevelopmental findings in other prospective ZIKV cohort studies published to date. Lopes Moreira et al. [8] used the Bayley Scales of Infant and Toddler Development-III to assess 94 children who had also undergone neuroimaging between 12 and 18 months of age. Twenty-four (25.5%) toddlers were found to have at least one abnormal neurodevelopment finding. Considering only the 59 with normal imaging for comparison with our findings, the number with abnormal neurodevelopment was 13 (13.8%). Einspieler et al. [10] followed 56 toddlers without microcephaly at birth and who were born to women with RT-PCR positive result

for ZIKV during pregnancy. Ten (17.9%) toddlers were found to have an adverse neurologic exam or neurodevelopment assessment at 12 months of age.

Among in utero ZIKV-exposed infants, irrespective of abnormalities at birth, Rice et al. [7] assessed follow-up care reports of 1450 toddlers with in utero ZIKV exposure with and without ZIKV-associated birth defects aged 1 year of age or older and found 9% had a least one neurodevelopment abnormality possibly associated with in utero ZIKV exposure. Nielsen-Saines et al. [9] used the Bayley Scales of Infant and Toddler Development-III at 18 months of age and described the neurodevelopment outcomes of 146 toddlers born to women with rash and RT-PCR ZIKV infection during pregnancy. Forty-one (28.1%) toddlers were found to have abnormal

Table 2 ASQ results of 235 toddlers included in the analysis by ZIKV exposure status

	In utero ZIKV exposure (N = 156)		ZIKV unexposed (N = 79)		Comparison of means	Comparison of n below the cut-off
	Mean (± SD)	n below – 2SD cut-off (%)	Mean (± SD)	n below – 2SD cut-off (%)	P value	P value
Communication	49.5 ± 12.6	13 (8.3)	44.0 ± 16.0	16 (20.3)	0.01	0.02
Gross motor	55.9 ± 7.6	5 (3.2)	53.4 ± 8.6	3 (3.8)	0.04	1
Fine motor	52.7 ± 9.0	4 (2.6)	52.7 ± 8.5	4 (5.1)	0.96	0.54
Problem solving	48.0 ± 12.2	8 (5.1)	47.4 ± 10.2	2 (2.5)	0.68	0.56
Personal-social	48.6 ± 9.8	11 (7.1)	46.7 ± 10.1	8 (10.1)	0.16	0.57

Table 3 Univariable logistic regression model predicting ASQ dimension result below the $-2SD$ cut-off for 235 toddlers included in the analysis by ZIKV exposure status

Characteristic	OR (95% CI) of ASQ dimension below – 2SD cut-off									
	Communication	P value	Gross motor	P value	Fine motor	P value	Problem solving	P value	Personal-social	P value
ZIKV exposure										
Exposed	0.36 (0.2–0.8)	0.01	0.84 (0.2–3.6)	0.81	0.49 (0.1–2.0)	0.32	2.08 (0.4–10.0)	0.36	0.67 (0.3–1.8)	0.41
Unexposed	1		1		1		1		1	
Sex										
Male	1		1		1		1		1	
Female	0.35 (0.2–0.8)	0.01	1.49 (0.4–6.4)	0.59	0.88 (0.2–3.6)	0.85	0.36 (0.1–1.4)	0.15	0.62 (0.2–1.6)	0.31
Education of mother										
Primary	1	0.57	1	0.65	1	0.65	1	0.58	1	0.15
Secondary	0.76 (0.3–2.3)		0.70 (0.1–4.4)		0.70 (0.1–4.4)		0.30 (0.1–1.9)		1.20 (0.2–6.5)	
Tertiary	0.54 (0.2–1.6)		0.34 (0.1–2.5)		0.34 (0.1–2.5)		0.45 (0.1–2.1)		1.44 (0.3–7.1)	
Unknown	1.29 (0.3–6.0)		1.25 (0.1–14.9)		1.25 (0.1–14.9)		0.81 (0.08–8.5)		6.36 (1.0–39.6)	
Education of father										
Primary	1	0.28	1	0.55	1	0.65	1	0.13	1	0.12
Secondary	0.52 (0.2–1.6)		0.56 (0.1–3.6)		0.70 (0.1–4.4)		0.56 (0.09–3.6)		0.49 (0.14–1.7)	
Tertiary	0.27 (0.07–1.1)		0.23 (0.02–2.6)		0.34 (0.05–2.5)		0 (0)		0.25 (0.05–1.1)	
Unknown	0.43 (0.1–1.3)		0.28 (0.04–2.1)		1.25 (0.1–14.9)		0.73 (0.1–4.0)		0.20 (0.05–0.8)	
Languages spoken in the household										
One	1		1		1		1		1	
More than one	2.00 (0.8–4.9)	0.13	0.98 (0.2–4.2)	0.98	0.58 (0.1–2.4)	0.44	2.43 (0.5–11.7)	0.27	1.00 (0.4–2.7)	0.99
Parity										
0	1	0.08	1	0.75	1	0.44	1	0.31	1	0.89
1	0.65 (0.2–1.8)		0.89 (0.2–5.5)		0.53 (0.1–2.8)		0.26 (0.03–2.3)		1.01 (0.3–3.0)	
2	1.29 (0.4–4.4)		2.62 (0.4–16.5)		0 (0)		1.54 (0.3–8.4)		0.93 (0.2–4.7)	
3+	3.19 (1.1–9.3)		1.48 (0.2–14.9)		0.87 (0.1–7.8)		1.82 (0.3–10.0)		1.73 (0.4–7.1)	
Birth weight										
Low birth weight	1.02 (0.2–4.7)	0.98	9.88 (2.1–45.9)	0.003	2.02 (0.2–17.5)	0.52	0		1.70 (0.4–8.1)	0.51
Normal	1		1		1		1		1	

Table 4 Multivariable logistic regression model predicting ASQ dimension result below the $-2SD$ cut-off for 235 toddlers included in the analysis by ZIKV exposure status

Characteristic	Adjusted OR (95% CI) of ASQ dimension below – 2SD cut-off									
	Communication	P value	Gross motor	P value	Fine motor	P value	Problem solving	P value	Personal-social	P value
ZIKV exposure										
Exposed	0.26 (0.1–0.6)	0.004	0.71 (0.1–4.0)	0.70	0.47 (0.1–2.2)	0.33	1.73 (0.3–9.9)	0.54	0.63 (0.2–1.8)	0.40
Unexposed	1		1		1		1		1	
Sex										
Male	1		1		1		1		1	
Female	0.35 (0.1–0.9)	0.03	1.73 (0.3–8.8)	0.51	0.83 (0.2–3.7)	0.81	0.34 (0.1–1.5)	0.16	0.60 (0.2–1.7)	0.33
Education of mother										
Primary	1	0.71	1	0.87	1	0.64	1	0.52	1	0.10
Secondary	0.68 (0.2–2.5)		0.82 (0.1–7.3)		0.34 (0.04–2.8)		0.28 (0.04–2.1)		1.16 (0.2–7.3)	
Tertiary	0.57 (0.1–2.3)		0.39 (0.03–5.0)		0.32 (0.03–2.9)		0.81 (0.1–5.4)		1.53 (0.3–9.5)	
Unknown	1.65 (0.3–9.4)		1.22 (0.1–25.8)		1.37 (0.1–19.7)		1.05 (0.1–12.6)		9.54 (1.3–69.6)	
Education of father										
Primary	1	0.55	1	0.75	1	0.36	1	0.17	1	0.11
Secondary	0.47 (0.1–2.2)		1.35 (0.1–15.9)		1.05 (0.1–15.2)		0.66 (0.1–6.1)		0.57 (0.1–2.7)	
Tertiary	0.33 (0.1–1.9)		0.57 (0.02–15.7)		0 (0)		0 (0)		0.24 (0.04–1.6)	
Unknown	0.40 (0.1–1.5)		0.51 (0.05–4.9)		1.11 (0.1–13.4)		0.8 (0.1–5.6)		0.18 (0.04–0.9)	
Languages spoken in the household										
One	1		1		1		1	0.29	1	0.99
More than one	2.15 (0.8–5.7)	0.12	0.64 (0.13–3.2)	0.58	0.39 (0.1–1.8)	0.23	2.4 (0.4–12.9)		1.01 (0.4–2.9)	
Parity										
0	1	0.10	1	0.85	1	0.33	1	0.27	1	0.94
1	0.59 (0.2–1.8)		0.95 (0.1–6.8)		0.39 (0.1–2.3)		0.19 (0.02–1.8)		0.92 (0.3–3.0)	
2	1.15 (0.3–5.0)		2.25 (0.2–22.0)		0 (0)		0.77 (0.1–6.3)		0.58 (0.1–4.3)	
3+	3.22 (1.0–11.0)		0.67 (0.03–14.1)		0.43 (0.1–6.1)		1.60 (0.3–10.5)		1.14 (0.2–6.0)	
Birth weight										
Low birth weight	0.35 (0.1–2.5)	0.30	10.21 (1.7–61.3)	0.01	2.3 (0.2–30.1)	0.52	0 (0)		0.82 (0.1–6.4)	0.85
Normal	1		1		1		1		1	

neurodevelopment, with language having the greatest proportion of toddlers with an abnormal outcome. In this respect, we have limited our analyses to those toddlers who were normocephalic at birth, defined by an absence of microcephaly at birth, and by an absence of abnormal transfontanelle cerebral findings after delivery or of ultrasound findings during the third trimester of the mother's pregnancy. The results of assessments of the toddlers excluded from our analysis with CZS or abnormal ultrasound findings will be described in future publications.

The important contribution of our study is the comparison of neurodevelopment outcomes of toddlers with in utero ZIKV exposure with toddlers who were known to have no in utero exposure to ZIKV. Of the 79 toddlers born to women with no evidence of ZIKV infection at the time of delivery, we found 20 (25.3%) to have an abnormal neurodevelopment finding using the ASQ neurodevelopment assessment tool. Further, there were no differences between ZIKV-exposed and ZIKV-unexposed toddlers for behavior disorder screening risk ($P = 0.15$), nor for language acquisition, whether by mean ($P = 0.36$) or the proportion below the 10th percentile threshold ($P = 0.53$). These findings underscore the pertinence of a comparative control group for determining the risk of adverse outcomes—whether during pregnancy or into early childhood—which may be attributable to ZIKV infection. Previous findings of prospective ZIKV cohort studies have attributed abnormal neurodevelopment in early childhood solely to in utero ZIKV exposure. Our findings would suggest that this attribution may not necessarily be warranted and that other factors which are known to be associated with neurodevelopment in early childhood need to be accounted for when determining these risk estimates.

While we found communication and personal-social skills to be those most frequently below the $-2SD$ threshold in the ZIKV exposed and the ZIKV unexposed, we also found in univariable and multivariable logistic regression analyses that in utero ZIKV exposure was an independently protective factor against an abnormal ASQ dimension score for communication. The strength of this association is such that the likelihood that this is a spurious finding is minimal. Further, the multivariable logistic regression analysis shown in Table 4 indicates that the in utero ZIKV exposure protective effect is not confounded. Recall bias is unlikely, as the statistically significant difference between ZIKV exposed and ZIKV unexposed was only observed for communication and not across the other ASQ dimensions, as would perhaps be expected if the parent/legal guardian—aware of the ZIKV exposure status of the toddler—responded more positively to the questionnaire in the in utero ZIKV-exposed group compared to the ZIKV-

unexposed group. Our finding is further strengthened by the fact that the multivariable logistic regression model identified other risk factors for neurodevelopment in early childhood that have been previously identified, including parity and sex of the toddler [13, 24]. This suggests that the ASQ screening tool itself is an appropriate tool in this setting, that it has performed well in identifying risk factors for those at risk of abnormal neurodevelopment, and that the communication finding may be influenced by a factor which has not been quantified as part of our analyses, such as parent stimulation in early childhood, or eagerness from the parents of ZIKV-exposed toddlers to emphasize attainment of developmental milestones, more so than perhaps the parents of the ZIKV-unexposed toddlers.

The results of our study are strengthened by the study design. In addition to the inclusion of a comparative control group, as described above, the ZIKA-DFA-FE and ZIKA-DFA-BB cohort studies were conducted in an unselected population-based cohort, in that both the exposed and unexposed groups were derived from the same source population. While we cannot rule out that self-selection in the cohort study may have occurred, the external validity of the findings is nonetheless strengthened by the study design. The prospective nature of the ZIKA-DFA-FE pregnancy cohort, followed by the ZIKA-DFA-BB pediatric cohort, enabled us to capture acute ZIKV infection during pregnancy—the majority by RT-PCR—and then to follow development outcomes into early childhood.

One limitation of our study is the loss to follow-up of toddlers from delivery to 24 months of age. However, maintaining toddlers in an extended follow-up has proved challenging for almost all ZIKV pediatric cohorts to date [7, 9]. Healthy toddlers are generally more difficult to maintain in an extended follow-up cohort study. At this stage, we cannot exclude a selection bias that may have resulted in toddlers who were lost to follow-up in the control group had normal neurodevelopment and their loss to follow-up may have artificially inflated the proportion of toddlers in the control group with abnormal neurodevelopment findings. However, the comparison of medical consultations outside routine pediatric consultations did not differ between the ZIKV exposed and the ZIKV unexposed, as would be expected if the control group was somehow different in terms of non-ZIKV-related disease status compared to the ZIKV-exposed group. Further, one of the principal reasons for the loss to follow-up and non-completion of the neurodevelopment assessment was the unavailability of the neurodevelopment assessment in each of the study sites by the time of the 24-month visit. Finally, we used developmental screening tools, rather than neurodevelopment and/or behavior evaluation diagnostic tools. The comparative ease-of-use of the screening tools and budget-related constraints related to the requirement of a trained psychologist or psychotherapist led to the decision to use the various screening

tools. Nonetheless, the ASQ-III tool has been widely used in studies to assess the neurodevelopment of infants and toddlers [25–31].

Conclusions

Overall, we found 15.3% of toddlers exposed to ZIKV in utero to have abnormal neurodevelopment findings at 24 months of age, a finding largely consistent with the results of other cohort studies published to date. However, when comparing this result with the result of toddlers not exposed to ZIKV in utero in our cohort study, we found no statistically significant difference. Therefore, in the absence of congenital abnormalities or abnormal ultrasound findings in the final stages of pregnancy or at delivery, there would not appear to be an impact on longer term neurodevelopment outcomes attributable to in utero ZIKV exposure which may manifest after birth up to 24 months of age. However, it is important that these neurodevelopment assessments are continued into early childhood.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12916-020-01888-0>.

Additional file 1: Supplementary Table 1. RT-PCR and serological evidence of maternal ZIKV infection during pregnancy of 235 infants included in this analysis. **Supplementary Table 2.** ASQ dimensions below -2SD cut-off value of 235 infants included in this analysis by ZIKV exposure. **Supplementary Table 3.** M-CHAT behavior disorder risk of 235 infants included in analysis by ZIKV exposure status. **Supplementary Table 4.** IFDC language acquisition of 233 francophone infants included in analysis by ZIKV exposure status.

Abbreviations

ASQ: Ages and Stages Questionnaire; CZS: Congenital Zika syndrome; IFDC: French MacArthur Inventory Scales; IgG: Immunoglobulin G; IgM: Immunoglobulin M; M-CHAT: Modified Checklist for Autism on Toddlers; RT-PCR: Reverse transcriptase polymerase chain reaction; ZIKV: Zika virus

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Authors' contributions

Professors Fontanet and Hoen conceived and designed the study and wrote the first version of the manuscript. Dr. Flechelles conceived and designed the study and supervised the cohort study. Drs. Diallo, Elenga, Mediamolle, Mallard, Herbert, and Lauchaux assessed toddlers in pediatric consultation. Ms. Couchy coordinated the pediatric consultations for the study. Mr. Tressières performed statistical analyses on the data collected. Ms. Grant performed statistical analyses on the data collected and wrote the first version of the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study received ethical approval from the Comité de Protection des personnes Sud-Ouest et Outre Mer III (registration number: 2016-A00684-47). All study procedures were explained to and informed consent obtained from a parent or legal guardian of the newborn before enrolment in the study. The study is registered with ClinicalTrials.gov under the identifier: NCT02810210.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no financial or non-financial competing interests.

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Research paper

Maternal and neonatal outcomes related to Zika virus in pregnant women in Southern Vietnam: An epidemiological and virological prospective analysis

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ABSTRACT

Background: In 2016–2017, 68 women in Southern Vietnam had RT-PCR confirmed Zika virus (ZIKV) infection during pregnancy. We report here the outcomes of the pregnancies and the virological analyses related to this outbreak.

Methods: We collected clinical and epidemiological information from the women who were enrolled in the study. Medical records related to the pregnancy in 2016–2017 were retrieved for those who were not able to be enrolled in the study. Children born to women with ZIKV infection during pregnancy were also enrolled. Serum samples were evaluated for presence of ZIKV antibodies. Phylogenetic analyses were performed on Zika virus genomes sequenced from the 2016–2017 serum samples.

Findings: Of the 68 pregnancies, 58 were livebirths and 10 were medically terminated. Four of the medical records from cases of fetal demise were able to be retrieved, of which one was consistent with congenital ZIKV infection. Of the 58 women with a livebirth, 21 participated in the follow-up investigation. All but two women had serologic evidence of ZIKV infection. Of the 21 children included in the study (mean age: 30.3 months), 3 had microcephaly at birth. No other clinical abnormalities were reported and no differences in neurodevelopment were observed compared to a control group. Phylogenetic analysis revealed a clade within the ZIKV Asian lineage and branch at the root of samples from the 2013–2014 French Polynesian outbreak. The prM S139N mutation was not observed.

Interpretation: We have been able to demonstrate a clade within the ZIKV Asian lineage implicated in adverse pregnancy outcomes in Southern Vietnam.

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Research in context

Evidence before this study

Two major lineages of ZIKV have now been identified: the African and Asian lineages. During the ZIKV epidemics in French Polynesia (2013–2014) and the Americas (2015–2016), ZIKV has demonstrated an ability to cause severe disease outcomes, including congenital Zika syndrome in fetuses and infants and Guillain Barré syndrome in adults. We searched PubMed for publications on adverse fetal outcomes in Asia associated with ZIKV infection. We identified several case reports of ZIKV-associated microcephaly from Vietnam, Thailand and Cambodia. Only one reported the genomic sequencing analysis which found the Asian lineage to be the cause of fetal microcephaly in Thailand, indicating the pathogenicity of the Asian lineage beyond the epidemics in French Polynesia and the Americas.

Added value of this study

We conducted a multidisciplinary investigation into the outcomes of 68 pregnancies with RT-PCR confirmed ZIKV infection in 2016–2017. Through genomic sequencing and phylogenetic analysis, we have been able to identify a clade within the ZIKV Asian lineage. Sequence analysis suggests that the clade was likely introduced between October 2004 and January 2011, prior to the epidemics in French Polynesia and the Americas. We are able to describe adverse pregnancy outcomes, including fetal abnormalities. We also report the persistence of anti-ZIKV antibodies in the women beyond three years post-infection.

Implications of all the available evidence

Our study offers important contributions to the understanding of the relative pathogenicity of the Asian lineage of ZIKV, beyond what has been described previously, and of the longer-term kinetics of anti-ZIKV antibodies following RT-PCR confirmed ZIKV infection. Surveillance of ZIKV infection, particularly in pregnant women, needs to be maintained in countries across Asia.

Introduction

Prior to the epidemics of 2007 in Yap Island, of 2013–2014 in French Polynesia and of 2015–2016 across the Americas, Zika virus (ZIKV) was understood to have extensive geographic distribution across Africa and Asia [1]. At that time, the clinical presentation of ZIKV infection was understood to be restricted to mild, self-limiting disease [1].

However, severe disease outcomes following ZIKV infection became apparent after the epidemics in French Polynesia in 2013–2014 and in Latin America in 2015–2016. The first severe neurologic complications associated with ZIKV infection, including Guillain-Barre syndrome in adults and microcephaly in fetuses and infants, were identified in French Polynesia [2,3], followed by the additional congenital malformations in fetuses and infants associated with *in utero* ZIKV infection in Brazil [4]. ZIKV is now known to cause abnormalities in fetuses and infants exposed to the virus *in utero* including microcephaly, and congenital Zika syndrome (CZS). CZS comprises cranial morphology and brain anomalies, congenital contractures, ocular anomalies and marked early sequelae [4–9].

Within the ZIKV Asian lineage, the comparative infectivity and pathogenicity, including the ability to cause severe disease, beyond the French Polynesia and Latin America epidemics remain unclear [10–12]. One hypothesis as to the change in disease epidemiology

and the appearance of severe disease outcomes prior to the epidemic in French Polynesia is a mutation in the virus, which may have increased its virulence. The prM S139N mutation on the ZIKV genome has been identified and phylogenetic analysis suggests it appeared before the outbreak in French Polynesia, and as such, may be responsible for more severe disease outcomes [10,12]. Improved diagnostic techniques and enhanced surveillance in other regions of the world since the epidemic in Latin America have shown continued circulation of the virus in South East Asia.

Such enhanced surveillance in Vietnam identified circulation of ZIKV in Southern Vietnam in 2016–2017 [13]. During this time, 68 pregnant women had ZIKV infection confirmed by reverse transcription polymerase chain reaction (RT-PCR) performed at the Pasteur Institute, Ho Chi Minh City. We report here an overview of the outcomes of the pregnancies and the development of the children born to the mothers with RT-PCR confirmed ZIKV infection during pregnancy and on the phylogenetic analysis of the ZIKV implicated in this outbreak.

Methods*Epidemiological investigation**Pregnant women*

ZIKV surveillance in Vietnam identified 68 pregnant women with ZIKV infection confirmed by RT-PCR performed on a blood sample collected by the Pasteur Institute, Ho Chi Minh City. At the time, women were informed of the result of ZIKV testing and the serum samples were stored at -70 °C at the Pasteur Institute, Ho Chi Minh City.

The samples were retrieved in 2019 and all women were contacted to participate in a follow-up epidemiological investigation. Those who were able to be contacted were invited to participate in the study. For those who were not able to be contacted, medical records at the hospital where the women had sought antenatal care, or where a medical termination of the pregnancy had been conducted following fetal demise, were retrieved for information related to the pregnancy, including the results of any ultrasonographic examinations, in accordance with local ethical regulations.

Once women were enrolled, they were interviewed by a trained member of the study personnel from Pasteur Institute, Ho Chi Minh City using a structured questionnaire. This covered sociodemographic information, including age, ethnicity, residence, and lifestyle factors. The participants were also asked about clinical information related to the pregnancy in 2016–2017: symptoms of ZIKV infection in 2016–2017, results of TORCH (toxoplasmosis, other [syphilis, human immunodeficiency virus infection (HIV)], rubella and cytomegalovirus) infection testing during pregnancy, where available, hospitalizations and/or medications taken during pregnancy, obstetrical history and the outcome of the pregnancy. Participants were encouraged to bring health records to the interview. The health records of both the mother and the infant were reviewed, as well as the results of any ultrasonographic evaluations during pregnancy in 2016–2017.

In addition, a 3mL blood sample was collected to evaluate the long term ZIKV antibody response, as well as the presence of antibodies of related viruses: dengue virus (DENV) and Japanese encephalitis virus (JEV).

Children born to women with confirmed ZIKV infection during pregnancy

The children born to women with confirmed ZIKV infection during pregnancy were also invited to participate. The women completed a questionnaire of behalf of the child which covered clinical information such as anthropometric measurements at birth and abnormalities at birth and into early childhood, extracted from the

child's personal health record. Microcephaly at birth was defined as moderate when the head circumference Z score at birth was between -2 and -3, and severe when the head circumference Z score at birth was below -3 based on gestational age and sex according to INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk>). Microcephaly at birth was further defined as proportionate if the infant was also small for gestational age at delivery, and disproportionate if not.

All children were referred to Children's Hospital Number 1, Ho Chi Minh City for clinical examination by a pediatrician. Further eye examination by fundoscopy was conducted. An auditory screening examination was performed, followed by otoacoustic emissions (OAE), auditory brainstem response (ABR) and auditory steady-state response (ASSR) tests. Hearing of the child was considered normal if OAE, ABR and ASSR were within normal ranges.

In addition, a 3 mL blood sample was collected from the child to evaluate the long term ZIKV antibody response, as well as the presence of antibodies of related viruses: DENV and JEV.

For assessment of neurodevelopment, a trained member of the study personnel, blinded to the *in utero* ZIKV exposure status of the child, assessed all children using the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III). These scales provide scores for three major development domains: motor, cognition and language. The normal range for each domain is 100 ± 15 ; scores below 85 indicate developmental delay. Children with scores in the normal range in all three domains were considered developmentally normal; children with any score below 85 in any of the three domains were considered as having a developmental delay.

Control group

To investigate the role of *in utero* ZIKV exposure on neurodevelopment outcomes in early childhood, a control group of children ($n = 21$) were recruited among children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City. Those eligible for inclusion in the control group self-reported no known ZIKV infection in the mother during pregnancy and were matched for age (within 1 month) and sex to the children born to women with confirmed ZIKV infection during pregnancy.

A trained member of the study personnel, blinded to the *in utero* ZIKV exposure status of the children, conducted the neurodevelopment assessment using the Bayley-III, as described above.

Laboratory evaluations

Laboratory tests included RT-PCR for the detection of ZIKV with Trioplex reagents. The testing procedure followed the primer and probe sequences, as described previously [14].

The serum samples collected from the women and children as part of the prospective investigation were tested for anti-ZIKV IgM and IgG using a commercial enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Germany) according to the manufacturer's instructions, as well as anti-ZIKV neutralizing antibodies, using a plaque reduction neutralization test (PRNT), the details of which are provided in the Supplementary material. Serum samples were considered to be seropositive if ZIKV PRNT₅₀ was positive and the ratio of ZIKV to DENV titers was higher than 2:1. Serum samples were considered likely positive if ZIKV PRNT₅₀ was positive but the ratio of ZIKV to DENV titers was less than 2:1. Anti-ZIKV antibodies were considered to have persisted if serum samples were positive in 2020.

The stored samples from 2016–2017 were re-tested by nucleic acid amplification, followed by genomic sequencing and phylogenetic analysis. The methodology is described in the Supplementary material.

Ethics considerations

The study received approval from the Institutional Review Board at Pasteur Institute, Ho Chi Minh City, Vietnam and Children's Hospital Number 1 (Reference numbers: 21/GCN-PAS, 28 June 2019, 2097/QĐ-BVND1, 5 September 2019). All study procedures were explained to and informed consent obtained from all eligible participants, and from a parent or legal guardian in the case of the children, by a trained member of study personnel before enrolment in the study.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation or writing of the manuscript. The corresponding author has full access to the data in the study and had final responsibility for the decision to submit for publication.

Results

Between March 2016 and November 2017, 68 pregnant women were referred from antenatal care to Pasteur Institute, Ho Chi Minh City for ZIKV diagnosis and had a RT-PCR confirmed ZIKV infection. Eighteen women had a positive test during the first trimester of pregnancy, 31 during the second trimester, 18 during the third trimester, and one woman for whom the trimester of pregnancy was unknown.

Of the 68 women with a RT-PCR confirmed ZIKV infection during pregnancy, 58 were livebirths and 10 pregnancies were medically terminated. Four medical records related to the pregnancy from the cases of fetal demise were able to be retrieved; six were unable to be retrieved. Attempts were made to contact all 58 women whose pregnancy resulted in a livebirth. Twenty-one women were able to be contacted and agreed to participate in the investigation, the remaining 37 were unable to be contacted. Of these, the medical records related to the pregnancy of 14 women were able to be retrieved; 23 medical records were unable to be retrieved. The 21 children born to the 21 women who agreed to participate in the study were also enrolled in the study. In addition, 21 children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City and who were matched for age and sex were recruited as a control group (see Fig. 1).

Epidemiological investigation

Fetal demise pregnancy outcomes

Of the 10 cases of fetal demise, the medical records of four cases were able to be retrieved, of which has been previously described [15]. Briefly, maternal ZIKV infection was confirmed on 30 March 2016, one day after the onset of rash, conjunctivitis and fatigue. Fetal demise was reported on 5 April 2016 during routine ultrasonographic examination at 8 weeks gestational age. RT-PCR testing on the fetus and a sample of the placenta was positive for ZIKV.

In the second case, maternal ZIKV infection was confirmed on 14 November 2016, three days after the onset of rash. Routine ultrasonographic examination at 6–8 weeks gestational age reported fetal demise and medical termination was performed on 26 November. No fetal or placental samples were collected or tested for ZIKV infection.

In the third case, maternal ZIKV infection was confirmed on 14 January 2017, three days after the onset of rash. Routine ultrasonographic examination in the 8th week of gestation reported fe-

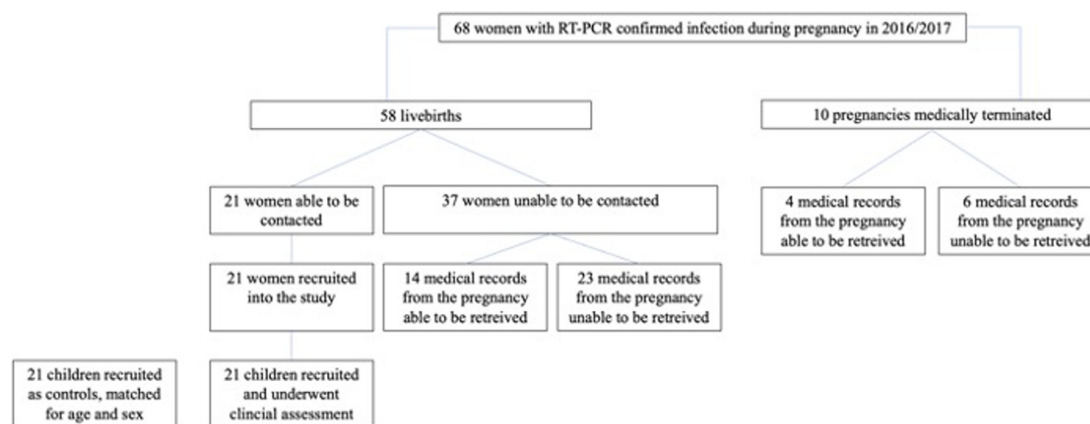


Fig. 1. Inclusion in analysis.

tal demise and medical termination was performed on 17 January 2017. RT-PCR testing on a placental sample was positive for ZIKV.

In the fourth case, routine ultrasonographic examination at the end of the second trimester on 16 March 2017 identified fetal microcephaly and intrauterine growth restriction (IUGR). TORCH testing was negative on 6 January 2017, but maternal RT-PCR ZIKV testing on 17 March 2017 was positive. At the time of the medical termination on 21 April 2017, fetal weight was 1000g at 28.5 weeks gestation.

Liveborn pregnancy outcomes

Of the 58 livebirths, 21 women were able to be contacted and recruited into the study. The remaining 37 were unable to be contacted, but the medical records of 14 women, specific to the pregnancy in 2016–2017, were able to be retrieved. Among these 14 livebirths, three reported abnormalities at the time of delivery, of which two were consistent with *in utero* ZIKV exposure. For these three livebirths, consultation of medical records indicates that maternal ZIKV, HIV and Rubella (IgM) tests were performed during pregnancy. All three had positive ZIKV, negative HIV and negative Rubella (IgM) test results.

In the first case, maternal ZIKV infection was confirmed by RT-PCR on 8 November 2016, following the onset of rash and fever on 29 October 2016. IUGR was noted during pregnancy and the infant was born at 36 weeks' gestation on 28 November 2016 with low birth weight and moderate proportionate microcephaly.

In the second case, maternal ZIKV infection was confirmed by RT-PCR on 15 December 2016, following onset of symptoms on 12 December. The infant was born at 35 week's gestation on 19 March 2017 with low birth weight and severe disproportionate microcephaly.

In the third case, maternal ZIKV infection was confirmed by RT-PCR on 7 May 2017 following the onset of symptoms on 3 May 2017. The infant was born at 39 week's gestation on 17 October 2017 with low birth weight, moderate disproportionate microcephaly, a left-tilted neck, edema of the right collarbone, and club foot.

Among the 21 infants that were liveborn and included in the study, three had moderate disproportionate microcephaly at birth. No other abnormalities were reported. The characteristics of the 21 women and the 21 children recruited into the study are described in Table 1.

TORCH testing was not routinely conducted for most pregnancies, although all tests during pregnancy were negative when conducted (Supplementary Table 1). Of the prospective blood samples collected between March and July 2020 from 20 of the 21 women who had RT-PCR confirmed ZIKV infection in 2016–2017, all but

Table 1

Characteristics of women with RT-PCR confirmed ZIKV infection (N = 21).

Maternal characteristics	N (%)
Age at time of conception (median, range)	30 (21–42)
Occupation	
Highly qualified professional, Manager	7 (33.3)
Artisan, Merchant, Business owner	3 (14.3)
Housewife	5 (23.8)
Labourer, Factory worker	5 (23.8)
Employee	1 (4.8)
Residence	
Urban	13 (61.9)
Rural	8 (38.1)
Parity (at the time of 2016–2017 pregnancy)	
0	7 (33.3)
1	10 (47.6)
2	3 (14.3)
3	1 (4.8)
Previous adverse pregnancy outcomes (prior to 2016–2017 pregnancy)	
Congenital abnormalities	0 (0)
Stillbirth	1 (4.8)
Miscarriage	4 (19.1)
Medical termination	1 (4.8)
Lifestyle practices during 2016–2017 pregnancy	
Alcohol consumption	5 (23.8)
- Weekly alcohol consumption	2 (9.5)
- Occasional alcohol consumption	3 (14.3)
Drug use	0 (0)
Smoking	0 (0)
Trimester of ZIKV infection	
First	6 (28.6)
Second	9 (42.9)
Third	6 (28.6)
Signs and symptoms of ZIKV infection during pregnancy	
Rash	17 (81.0)
Fever	9 (42.9)
Itching	5 (23.8)
Limb swelling	4 (19.1)
Myalgia	3 (14.3)
Arthralgia	3 (14.3)
Headache	3 (14.3)
Conjunctival hyperemia	1 (4.8)
Bleeding	1 (4.8)
Pain behind eyes	0 (0)
Petechiae	0 (0)
Neonate characteristics	
Gestational age (mean, range) (n=16)	38.7 (37–40)
Sex	
Male	6 (28.6)
Head circumference Z score (mean, IQR) (n=13)	-0.66 (-1.1, 0.3)
Normal	10 (76.9)
Abnormal (head circumference Z score <-2)	3 (23.1)

Table 2

Results of prospective serologic testing* in women with RT-PCR confirmed ZIKV infection during pregnancy in 2016/2017

	ZIKV PRNT	JEV PRNT	DENV1 PRNT	DENV2 PRNT	DENV3 PRNT	DENV4 PRNT	ZIKV IgG	ZIKV IgM	ZIKV serologic status
1	160	80	10	40	0	0	Positive	Negative	Positive
2	1280	80	40	0	0	0	Negative	Negative	Positive
3	80	0	80	20	10	20	Positive	Negative	Likely positive
4	640	80	80	80	1280	10	Positive	Negative	Likely positive
5	-	-	-	-	-	-	-	-	NA
6	640	0	80	20	0	0	Positive	Negative	Positive
7	80	20	40	10	0	0	Negative	Negative	Positive
8	80	20	80	80	40	20	Borderline	Negative	Likely positive
9	1280	80	80	40	80	80	Positive	Negative	Positive
10	160	0	10	0	0	0	Positive	Negative	Positive
11	0	20	20	10	40	10	Negative	Negative	Negative
12	160	0	80	80	40	0	Positive	Negative	Positive
13	0	20	20	20	40	0	Positive	Negative	Negative
14	160	0	40	20	0	0	Borderline	Negative	Positive
15	10240	40	40	640	80	80	Positive	Negative	Positive
16	2560	0	40	160	40	10	Positive	Negative	Positive
17	160	0	320	80	40	10	Positive	Negative	Likely positive
18	1280	160	40	20	20	0	Positive	Negative	Positive
19	160	0	160	0	0	80	Positive	Negative	Likely positive
20	320	20	20	160	80	0	Positive	Negative	Positive
21	2560	0	80	20	20	10	Positive	Negative	Positive

* Mean (\pm SD) time from date of RT-PCR ZIKV testing to time of blood collection for serologic testing: 36.0 \pm 1.7 months.**Table 3**

Results of prospective serologic testing* in children born to women with RT-PCR confirmed ZIKV infection during pregnancy in 2016–2017.

	ZIKV PRNT	JEV PRNT	DENV1 PRNT	DENV2 PRNT	DENV3 PRNT	DENV4 PRNT	ZIKV IgG	ZIKV IgM	ZIKV serologic status
1	0	0	0	0	0	0	Negative	Borderline	Negative
2	0	0	0	0	0	0	Negative	Negative	Negative
3	0	80	0	10	0	0	Negative	Negative	Negative
4	0	0	0	0	80	0	Negative	Negative	Negative
5	0	0	0	0	0	0	Negative	Negative	Negative
6	20	0	160	1280	2560	40	Positive	Negative	Inconclusive
7	0	0	20	0	0	0	Negative	Negative	Negative
8	0	0	0	0	0	0	Borderline	Negative	Negative
9	0	0	0	0	0	0	Negative	Negative	Negative
10	0	0	0	0	0	0	Negative	Negative	Negative
11	0	0	20	0	0	0	Negative	Negative	Negative
12	0	0	0	0	0	40	Negative	Negative	Negative
13	0	20	0	0	0	0	Negative	Negative	Negative
14	0	0	20	0	0	0	Negative	Negative	Negative
15	0	0	0	0	0	0	Negative	Negative	Negative
16	0	0	0	0	0	0	Negative	Negative	Negative
17	0	0	10	0	0	0	Negative	Negative	Negative
18	0	0	0	0	0	0	Negative	Negative	Negative
19	0	0	0	0	0	0	Negative	Negative	Negative
20	0	0	0	0	0	0	Negative	Negative	Negative
21	0	0	0	0	0	0	Negative	Negative	Negative

* Mean (\pm SD) time from date of birth to time of blood collection for serologic testing: 30.8 \pm 2.6 months.

two samples were seropositive (Table 2). In contrast, all but one of the 21 blood samples collected from the children were serologically negative for anti-ZIKV antibodies; one blood sample was likely anti-ZIKV seropositive (Table 3).

All clinical assessments of the children were normal. The mean age at the time of the assessment was 30.8 \pm 2.6 months. All 15 and 19 children who underwent auditory and fundoscopic examinations, respectively were normal.

For neurodevelopment assessment, 21 children attending routine immunization visits at Pasteur Institute, Ho Chi Minh City were recruited as a control group. Three children (14.3%) born to women with RT-PCR confirmed infection had a developmental delay: 2 in language domain and 1 in motor domain (Table 4). One of the two children with a delay in the language domain was also one of the three children who had moderate disproportionate microcephaly at birth. One child in the control group had a developmental delay in cognitive domain. However, in comparison with the control group, the difference was not statistically significant ($P = 0.60$).

Phylogenetic analysis

We combined ZIKV genomic sequences sampled from pregnant women in 2016–2017 in Vietnam with ZIKV sequences available from around the world in public databases to perform spatio-temporal analysis of ZIKV spread, via phylogenetic tree reconstruction, dating and phylogenetic inference. The results are consistent with current knowledge of ZIKV circulation (Fig. 2). Asian, French Polynesian and South American sequences clustered in the phylogenetic tree, as expected. The samples of the pregnant women from Vietnam form a clade, potentially likely corresponding to a single introduction, however the bootstrap support of this clade (bootstrap value: 49) does not exclude the possibility of multiple introductions. Nonetheless, the hypothesis of a single introduction is also in agreement with the phylogenetic inference which places the root of the well supported (bootstrap value: 87) parent clade in Vietnam. The Vietnamese sequences belong to the Asian lineage and branch at the root of French Polynesian samples (boot-

Table 4

Comparison of children born to women with ZIKV confirmed infection during pregnancy ($N = 21$) and control group of infants attending routine immunization visits at Pasteur Institute, Ho Chi Minh City.

	Children born to women with ZIKV confirmed infection during pregnancy ($N = 21$)	Control group of children attending routine immunization visit ($N = 21$)
Age at the time of assessment (mean, SD)	30.8 ± 2.6	30.5 ± 2.8
Height (mean, SD)	90.4 ± 5.9	88.7 ± 5.8
Weight (mean, SD)	13.3 ± 2.5	13.6 ± 2.3
Eye examination ($n=19$)*		
Normal	20 (100%)	-
Abnormal	0 (0.0%)	-
Auditory test ($n=15$)**		
Normal	20 (100%)	-
Abnormal	0 (0.0%)	-
Neurodevelopment ⁺		
Normal (>85)	18 (85.7%)	20 (95.2%)
Abnormal (<85)	3 (14.3%)	1 (4.8%)
Language	2	0
Motor	1	0
Cognitive	0	1

* Eye examination by fundoscopy

** Auditory screening examination was performed, followed by otoacoustic emissions (OAE), auditory brainstem response (ABR) and auditory steady-state response (ASSR) tests. Hearing of the child was considered normal if OAE, ABR and ASSR were within normal ranges.

⁺ Neurodevelopment assessed using the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III).

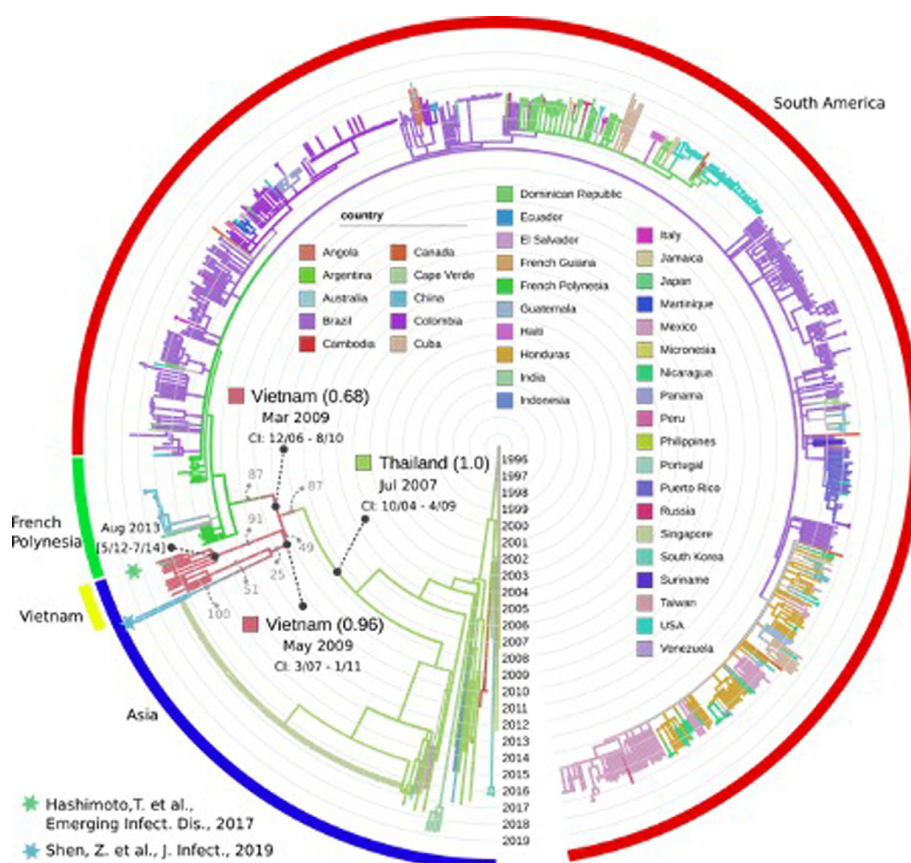


Fig. 2. Time-scaled phylogenetic tree of Vietnamese ZIKV sequences. Time-scaled phylogenetic tree of Vietnamese ZIKV sequences (pink branches under the yellow ribbon) along public ZIKV sequences, with inferred geographical information. External colour strips indicate the known strains of ZIKV: Asian in blue (includes sequences mostly sampled in Asia), French Polynesian in green, and South American (sequences mostly but not only from South America) in red. Branch colours indicate their inferred geographical origin. For example, the geographical origin of the clade containing Vietnamese, French Polynesian and South American strains is inferred as Vietnam (marginal probability is shown in parenthesis) and its date is estimated as March 2009 (95%CI in parenthesis). The parent node is inferred as Thailand and its date is estimated as July 2007. Temporal predictions are obtained with LSD2 [28], geographic predictions - with PastML [29] (MPPA+F81), the visualisation is performed with iTOL [30]. The bootstrap supports of the branches around the Vietnamese cluster are shown in grey.

strap value: 87), which is also consistent with current knowledge of ZIKV circulation [12].

Among the 3 additional sequences in the clade we have identified in Vietnam, 1 has been reported in Japan and 2 in China. The Japanese sample is known to be an importation from Vietnam in 2016, [16] which is in accordance to our phylogenetic tree (tip annotated with a green star in Fig. 2). Among the two sequences from China, one has been submitted in July 2019 with little information on its origins, and the other has been sampled from a person returning from Myanmar [17].

S139N mutation

Of the ZIKV samples that we sequenced, five had sufficient coverage to infer the genotype at this position. We found that our samples do not harbour the prM S139N mutation. Although we cannot exclude the presence of the S139N mutation in samples with low coverage, it is likely that this mutation was not present at the time of introduction of the virus to Vietnam and did not emerge in Vietnam.

Dating and geographical origin of outbreaks

Known sampling dates of public sequences and of samples from the pregnant women in Vietnam allowed us to estimate the date of each internal node of the phylogenetic tree with Least Squares Regression method. The date of introduction to Vietnam was estimated between October 2004 (lower 95%CI value for the most recent high-confident (marginal probability: 1.0) non-Vietnamese ancestor (Thailand)) and January 2011 (upper 95%CI value of the common ancestor of all the Vietnamese sequences, bootstrap value: 87), for which the location was confidently (marginal probability: 0.96) estimated as Vietnam, as shown in Fig. 2. The virus introduction to Thailand is estimated around 1999 (see Supplementary Material), with an introduction to Singapore around 2015. The Vietnamese clade is estimated to have been introduced to South America around 2013 via French Polynesia (2011). While our phylogeographic analysis was shown to be quite robust against sampling variations (see Supplementary Material), it cannot estimate locations that were not present in our dataset (e.g. Myanmar as an intermediate in the Vietnam-to-China introduction) [17]. Therefore, we cannot dismiss the possibility of non-direct introduction from Thailand to Vietnam (via intermediate, non-sampled locations) or from Vietnam to French Polynesia.

Though the dates may display some variability in the confidence intervals, the general scenario aligns with current literature [18], and the outbreak in Vietnam is estimated to have emerged around March 2009 [December 2006 – August 2010].

Discussion

We have been able to document the outcomes of pregnancies in women with RT-PCR confirmed ZIKV infection between March 2016 and November 2017 and identify abnormalities in the fetuses associated with maternal ZIKV infection. Further, phylogenetic analysis of the viral genomes enables us to identify a clade within the ZIKV Asian lineage implicated in the outbreak.

Of the 10 pregnancies that were medically terminated, available medical records for 4 of these pregnancies indicate abnormalities associated with maternal ZIKV infection in three fetuses. Of these, two had positive ZIKV placental samples, confirming congenital ZIKV infection. An additional 3 neonates not included in the follow up investigation were identified as having microcephaly, low birth weight and other complications at birth associated, of which 2 have abnormalities likely associated with maternal ZIKV infection. Among the further 21 neonates included in the follow up investigation, 3 had moderate disproportionate microcephaly at

birth, although no other abnormalities were reported. These findings allow us to infer the pathogenicity, including an ability to cause adverse fetal outcomes, of the clade within the ZIKV Asian lineage implicated in the outbreak.

The phylogenetic analysis conducted on the stored samples from 2016 reveal a clade within the Asian lineage and branch at the root of samples from the 2013–2014 French Polynesian outbreak. Our data suggest a potential single introduction, estimated to have occurred between October 2004 and January 2011. However, we cannot exclude an alternative hypothesis of several, later introductions into Vietnam. In any case, the introduction event appears to be many years before infections were detected in the pregnant women included in our study in Southern Vietnam. We are also unable to infer from our study whether the infections detected in pregnant women were the result of an outbreak of considerable magnitude across a period of 20 months, or the result of low but sustained levels of endemic circulation, as has been documented in Thailand [19]. However, the available epidemiological surveillance data from 2016, showing an increase in ZIKV infections in Southern Vietnam in the last quarter of the year [13], as well as the absence of evidence of ZIKV infection in children, would be more consistent with an epidemic event.

The prM S139N mutation on the ZIKV genome may be responsible for more severe disease outcomes identified in French Polynesia and Latin America [10,12]. Importantly, our samples do not harbour this mutation. Our findings are consistent with the finding that the Asian lineage of ZIKV is able to cause adverse fetal outcomes, including microcephaly. A case report of fetal microcephaly following congenital ZIKV infection with the Asian lineage of ZIKV has previously been reported from Thailand [11]. An alternative hypothesis as to the change in disease epidemiology may be another mutation that occurred in Asia and allowed secondary spread to French Polynesia and Latin America, and/or the introduction of the virus in an immunologically naive population.

Anti-ZIKV antibody kinetic studies have shown that anti-ZIKV IgG antibodies appear rapidly after RT-PCR confirmed infection and remain detectable up to 6 months [20–22]. What remains unclear is the longer-term antibody response to ZIKV infection. In French Polynesia, seroprevalence dropped from 49% between February–March 2014 at the end of the ZIKV outbreak to 22% in September–November 2015 [23]. In contrast, in Florida, United States of America, 62 Miami residents with confirmed ZIKV infection in 2016 were found to have neutralizing antibodies 12–19 months after infection [24], although direct comparison with seroprevalence findings in French Polynesia is limited by differences in immunoassays used in each study. In our study, we have been able to show the persistence of specific anti-ZIKV neutralizing antibodies beyond 3 years following RT-PCR confirmed infection. All but two of the 21 women included in the follow up investigation had persistent anti-ZIKV antibodies. The absence of a decline in antibodies may also be influenced by the endemic circulation of ZIKV [25], which, in contrast to islands in the Pacific, may maintain anti-ZIKV antibody titres.

Congenital ZIKV infection has been demonstrated by nucleic acid amplification based diagnostic tests on placenta and fetal samples [26]. What remains unclear is the diagnosis of congenital infection using serology [26,27]. There is an assumption, derived from antibody kinetics for other congenital infections including HIV, that maternal IgG may be able to be detected in the neonate at birth, but these antibodies wane during the first year of life. As a result, any anti-ZIKV IgG detected in the child beyond 12 months of age can be assumed to have been mounted *in utero* in response to congenital ZIKV infection. In our study, we found only 1 of the 21 toddlers included in the follow up to have detectable anti-ZIKV neutralizing antibodies. We are unable to infer whether the absence of detectable anti-ZIKV neutralizing antibodies in the other

children reflects an absence of congenital ZIKV infection, the waning of antibodies in early childhood or the inability of the immune system of the fetus to mount an immune response if infected early in the perinatal period. Further longitudinal investigations on antibody kinetics in infected mothers and their infants are needed.

While our study offers important contributions to the understanding of the relative pathogenicity of the Asian lineage of ZIKV and long-term antibody kinetics following RT-PCR confirmed ZIKV infection, our findings are limited by the fact that not all 68 women could be followed up as part of the prospective study. Although we do not find any adverse development outcomes in the 21 children born to women with confirmed ZIKV infection during pregnancy, we cannot rule out the existence of selection bias, in that among those who did not participate in the follow up may have been those with developmental delays. The main reason for not participating in the follow up study was our inability to contact the women to invite them to participate in the study. This is likely the result of deliveries in Southern Vietnam primarily take place in the large maternity wards of hospitals in Ho Chi Minh City, with women from rural areas returning home after delivery. A further limitation is the lack of systematic TORCH testing during pregnancy, however the retrospective data collection of pregnancy information meant that we were restricted to the routine practices of clinicians in Vietnam.

In conclusion, we have been able to demonstrate a clade within the ZIKV Asian lineage has been able to cause adverse pregnancy outcomes among women who were infected during pregnancy in Southern Vietnam. We are also able to demonstrate persistence of anti-ZIKV antibodies in the women more than four years after RT-PCR confirmed infection. As the follow-up was incomplete, we cannot draw conclusions as to the impact of congenital ZIKV infection on development outcomes in early childhood.

Contributors

AF, RG, QCL, QDP designed the investigation.

AF, RG, QCL, QDP, TTTN developed the study questionnaire.

TTTN, LDKV, MNN conducted the epidemiological investigation and maintained the database.

TDTP, HTN, NTT, LBL, MNQN, TCT, NNTL, HTP, TTDH, TVD, ATV, QNTN collected clinical data and performed clinical assessments.

MHD, HTTP, TMC, LTKH, QHN, DTNH conducted the virological analyses.

GP, XDL oversaw the virological analyses and interpreted the results.

FL, AZ performed the phylogenetic analysis and interpreted the results.

RG, FL, AZ, GP, TTTN, QDP wrote the first version of the manuscript.

All authors critically reviewed and approved the final version of the manuscript.

Data sharing

Sequence and phylogenetic analysis workflows are publicly available on GitHub.

ZIKV genome sequences of high quality are available on GenBank, under the accession numbers: MW915410, MW915411, MW915412, MW915413, MW915414, MW915415.

All consensus sequences are available on the GitHub repository, on the release v0.1

Declaration of Competing Interest

All authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.lanwpc.2021.100163](https://doi.org/10.1016/j.lanwpc.2021.100163).

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