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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 sequalae [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 $\pm$ 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 $PRNT_{50}$ was positive and the ratio of ZIKV to DENV titers was higher than 2:1. Serum samples were considered likely positive if ZIKV $PRNT_{50}$ 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/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.

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, 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 fe-

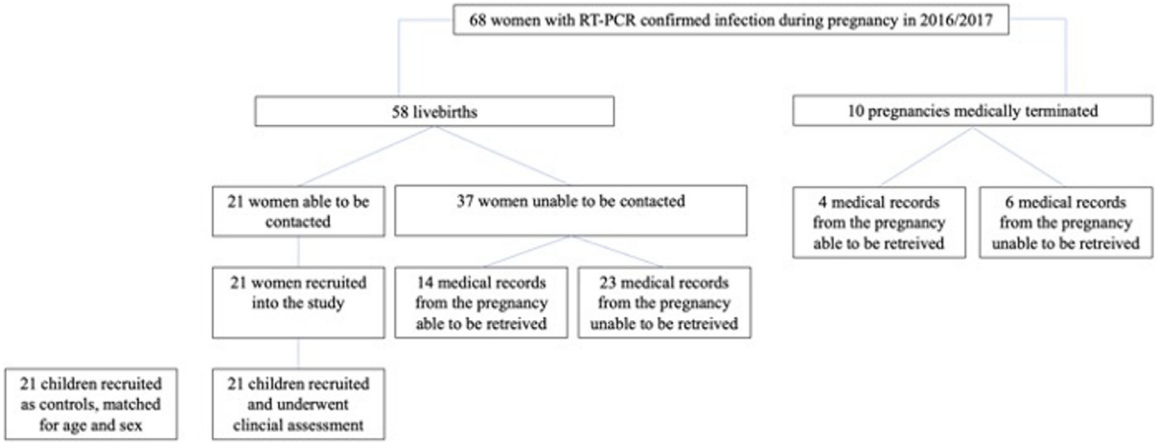

Fig. 1. Inclusion in analysis.

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 (±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 (±SD) time from date of birth to time of blood collection for serologic testing: 30.8 ± 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 ± 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).

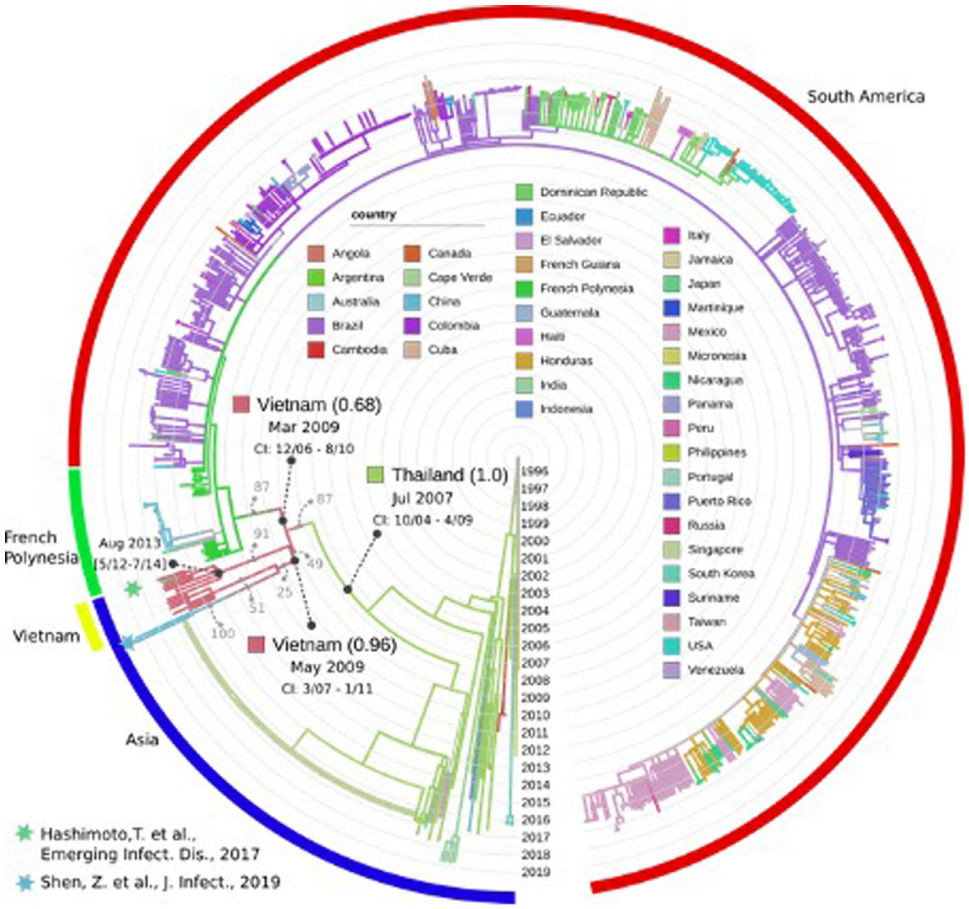

Fig. 2. Time-scaled phylogenetic tree of Vietnamese ZIKV sequences. Time-scaled phylogenetic tree of Vietnamese ZIKV sequences (pink branches under the yellow ribbon) along public ZIKV sequences, with inferred geographical information. External colour strips indicate the known strains of ZIKV: Asian 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.

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