Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study

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Summary

Background The incidence of microcephaly in Brazil in 2015 was 20 times higher than in previous years. Congenital microcephaly is associated with genetic factors and several causative agents. Epidemiological data suggest that microcephaly cases in Brazil might be associated with the introduction of Zika virus. We aimed to detect and sequence the Zika virus genome in amniotic fluid samples of two pregnant women in Brazil whose fetuses were diagnosed with microcephaly.

Methods In this case study, amniotic fluid samples from two pregnant women from the state of Paraíba in Brazil whose fetuses had been diagnosed with microcephaly were obtained, on the recommendation of the Brazilian health authorities, by ultrasound-guided transabdominal amniocentesis at 28 weeks' gestation. The women had presented at 18 weeks' and 10 weeks' gestation, respectively, with clinical manifestations that could have been symptoms of Zika virus infection, including fever, myalgia, and rash. After the amniotic fluid samples were centrifuged, DNA and RNA were extracted from the purified virus particles before the viral genome was identified by quantitative reverse transcription PCR and viral metagenomic next-generation sequencing. Phylogenetic reconstruction and investigation of recombination events were done by comparing the Brazilian Zika virus genome with sequences from other Zika strains and from flaviviruses that occur in similar regions in Brazil.

Findings We detected the Zika virus genome in the amniotic fluid of both pregnant women. The virus was not detected in their urine or serum. Tests for dengue virus, chikungunya virus, *Toxoplasma gondii*, rubella virus, cytomegalovirus, herpes simplex virus, HIV, *Treponema pallidum*, and parvovirus B19 were all negative. After sequencing of the complete genome of the Brazilian Zika virus isolated from patient 1, phylogenetic analyses showed that the virus shares 97–100% of its genomic identity with lineages isolated during an outbreak in French Polynesia in 2013, and that in both envelope and NS5 genomic regions, it clustered with sequences from North and South America, southeast Asia, and the Pacific. After assessing the possibility of recombination events between the Zika virus and other flaviviruses, we ruled out the hypothesis that the Brazilian Zika virus genome is a recombinant strain with other mosquito-borne flaviviruses.

Interpretation These findings strengthen the putative association between Zika virus and cases of microcephaly in neonates in Brazil. Moreover, our results suggest that the virus can cross the placental barrier. As a result, Zika virus should be considered as a potential infectious agent for human fetuses. Pathogenesis studies that confirm the tropism of Zika virus for neuronal cells are warranted.

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Introduction

Since 2015, Brazil has been facing a public health emergency regarding the dramatic increase in the number of newborn babies with microcephaly. Epidemiological data indicate that up to Jan 6, 2016, 4783 cases were reported in 21 states in the North, Northeast, South, and Southeast Regions of Brazil.[1] This incidence of microcephaly is 20 times higher than in previous years, reaching 99·7 per 100 000 livebirths, and including 76 deaths of neonates as of Jan 6, 2016.[1]

When diagnosed prenatally by ultrasound imaging, congenital microcephaly is a strong predictor of adverse neurological outcomes.[2] As defined by WHO, microcephaly occurs whenever the occipital frontal circumference of the head of the newborn child or fetus is 2 standard deviations smaller than the mean for the same age and sex.[3] A brain size that is significantly different to that in the normal range is an important risk factor for cognitive and motor delay.[4] Microcephaly is associated with several causes, including genetic disorders (eg, autosomal recessive microcephaly, Aicardi-Goutières syndrome, chromosomal trisomy, Rett syndrome, and X-chromosomal microcephaly); drug and chemical intoxication of the pregnant mother (eg, use of alcohol, cocaine, or antiepileptic drugs, lead or mercury intoxication, or radiation); maternal malnutrition; and transplacental infections with viruses or bacteria.[5] Maternal viral infections, including rubella,

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Research in context

Evidence before this study

Many cases of microcephaly in newborn babies in Brazil have occurred in regions where infections of Zika virus and other arboviruses have also been detected. We searched PubMed with the search terms "Zika", and "microcephaly" for articles published up to Feb 5, 2016. We found 11 articles that suggested a possible relation between Zika virus and microcephaly in neonates. A short case report by our group, reporting the ultrasound evidence of the two fetal microcephaly cases reported here, has been published previously. Our search found no other clear evidence that Zika virus could cross the placental barrier and infect the human fetus.

Added value of this study

This study presents the virological and genetic data implicating Zika virus in the two cases of fetal malformation that we described briefly in our previous case report. We used quantitative reverse transcription PCR and viral metagenomics technology applied to samples of amniotic fluid obtained from the two pregnant women carrying fetuses with microcephaly, and obtained sequences of the Zika virus genome. The study of these cases provides empirical evidence for the association between Zika virus infection during pregnancy and fetal microcephaly. Furthermore, we isolated the whole genome of Zika virus directly from the amniotic fluid of two pregnant women, and provided evidence to support previous findings indicating that the origin of the virus is French Polynesia.

Implications of all the available evidence

On the basis of our findings, Zika virus should be regarded as a possible causative agent in cases of microcephaly, especially during Zika virus outbreaks in endemic regions. Our work emphasises not only the importance of controlling the *Aedes aegypti* mosquito population while no vaccine or antiviral is available, but also the need for further studies to understand the mechanisms of immunopathogenicity that lead to congenital malformation due to Zika virus infection.

cytomegalovirus, herpes simplex, varicella zoster virus, HIV, and arboviruses such as chikungunya, have also been associated with microcephaly in neonates.[5,6]

Epidemiological evidence suggests that Zika virus infection of pregnant women in Brazil might be associated with the increasing numbers of congenital microcephaly cases reported in the country. Several mosquito species have been found to be naturally infected with Zika virus, including *Aedes africanus*, *Aedes luteocephalus*, *Aedes hensilli*, *Aedes polynesiensis*, *Aedes dalzielii*, *Aedes albopictus*, *Aedes apicoargenteus*, and *Aedes aegypti* among others, but little is known about their vector competence.[7–10] *A aegypti* is the overwhelmingly predominant mosquito species found in Brazil, and is also associated with other arboviruses already reported in Brazil, such as the dengue and chikungunya viruses.

Zika virus was first isolated from human beings in Nigeria[7] during studies undertaken in 1954. Further cases were reported in other African countries[11] (Uganda, Tanzania, Egypt, Sierra Leone, Gabon, Nigeria, Côte d'Ivoire, Cameroon, Senegal, and Central African Republic), in Asian countries (India, Pakistan, Malaysia, Philippines, Thailand, Cambodia, Vietnam, and Indonesia), in several islands of the Pacific region since 2007 (Federated States of Micronesia, Cook Islands, French Polynesia, New Caledonia, Guam, Samoa, Vanuatu, and Solomon Islands), and since about early 2015 in the Americas (Chile, Colombia, El Salvador, Guatemala, Mexico, Paraguay, Suriname, Venezuela, Canada, and the USA).[9–15] Outbreaks of Zika virus infection on Yap Island (in 2007) and in French Polynesia (2013–14), with further spread to New Caledonia, the Cook Islands, and Easter Island, have shown the propensity of this arbovirus species to spread outside its usual geographical range and to cause large outbreaks.

The first autochthonous cases of Zika virus in Brazil were confirmed in May, 2015.[16] Since then, as of Jan 6, 2015, 21 states have confirmed virus circulation, with a higher prevalence in the Northeast Region.[17] Reports of microcephaly incidence in Brazil geographically overlap with Zika virus reports; most of the mothers whose infants were diagnosed with microcephaly complained during their pregnancies of clinical manifestations, such as low-grade fever, headache, and cutaneous rashes, that might have been symptoms of Zika virus infection or infection with any other arbovirus species that is prevalent in the region.

In the face of this potential association between Zika virus infection and the increasing number of cases of microcephaly, the Brazilian Ministry of Health and WHO have recommended that pregnant women should take precautions to avoid contact with all potential vectors, since no specific antiviral treatment for Zika virus infection exists.[1]

Small fragments of the genome of the Zika virus strain circulating in Brazil have been sequenced and phylogenetic analysis has indicated that the virus is similar to the one that circulated in French Polynesia in 2013.[16,17] However, evidence linking the high incidence of microcephaly to the presence of Zika virus is scarce. In January, 2016, our group reported ultrasound image evidence of two cases of fetal microcephaly in women who had complained of Zika-like virus symptoms during pregnancy, and we reported brief preliminary PCR findings, confirming the presence of Zika virus in their amniotic fluid.[18] In this case study, we expand upon these previously reported findings, and describe how we used quantitative reverse transcription PCR (RT-qPCR) and

viral metagenomics to detect and sequence the Zika virus genome in the amniotic fluid samples of these two pregnant women with microcephalic fetuses.

Methods

Case histories

The first case in our study was of a 27-year-old woman in her first pregnancy, from an inner city in the state of Paraíba, in the Northeast Region of Brazil (patient 1). Her prenatal care was uneventful until early September, 2015, when, at 18 weeks of gestation, the woman developed a cutaneous rash with itching of the hands and back. On the basis of her clinical status, she was diagnosed at an emergency service unit with allergic reaction, and was prescribed intravenous hydrocortisone. The next day, her symptoms worsened as she developed a fever and myalgia. She had a normal fetal ultrasound at 16 weeks. The patient had not travelled outside the state of Paraíba during the previous few years, and she had not had contact with any ill individuals. She had no immunodeficiency or autoimmune diseases. At 21 weeks of gestation, a further ultrasound indicated a fetal microcephaly diagnosis with moderate ventriculomegaly and partial agenesis of the cerebellar vermis. A third ultrasound done at 27 weeks confirmed the microcephaly diagnosis with relevant dilation of ventricles, asymmetry of hemispheres, and hypoplastic cerebellum with complete absence of the cerebellar vermis. The patient was healthy and stable during the ultrasound and amniocentesis procedures. Results of all laboratory examinations showed no diabetes and blood-pressure-related disorders. Additionally, the patient did not report taking any medication (other than hydrocortisone), recreational drug use, alcohol consumption, or smoking during the pregnancy. Patient 1 is still being monitored by the physicians in our group. At 40 weeks of gestation the fetus presented microcephaly with calcification areas and head circumference of 29 cm assessed by ultrasonography before birth. The baby was born at 40 weeks of gestation and had an actual head circumference of 30 cm.

The second case in our study was of a 35-year-old woman in her first pregnancy, also from the state of Paraíba (patient 2). The patient, with no relevant past medical history, sought care when she developed mild Zika virus disease-like symptoms at 10 weeks of gestation. She was prescribed symptomatic treatment. A morphological ultrasound at 22 weeks of gestation revealed mild hypoplasia of the cerebellar vermis. The fetal head circumference on the 22nd week of gestation was below the 10th percentile. A second ultrasound done at 25 weeks of gestation revealed more severe hypoplasia of the cerebellar vermis, enlargement of the posterior fossa, and microcephaly, yielding a head circumference below the third percentile. The brain parenchyma was normal. The patient was healthy and stable during the ultrasound and amniocentesis procedures. All the laboratory examinations showed no evidence of diabetes or blood-pressure-related disorders. Additionally, she did not report taking any medication, recreational drug use, alcohol consumption, or smoking during the pregnancy. Patient 2 is still being monitored by the physicians in our group. She delivered on Feb 3, 2016, and the neonate presented severe ventriculomegaly, microphthalmia, cataract, and severe arthrogryposis in the legs and arms.

Sample collection

Following Brazilian health public recommendations, amniocentesis was done at gestational week 28 in both women to investigate the cause of microcephaly. Ultrasound-guided transabdominal amniocentesis was done and about 5 mL of amniotic fluid was aspirated and immediately stored at –80°C.

Viral metagenomics and sequence analysis

A 0·5 mL sample of the amniotic fluid from each patient was filtered through 0·45 μm filters to remove residual host cells. The samples were then centrifuged at 21 130 × g and 15 000 rpm (rotor FA-45–24–11, Eppendorf, Hamburg, Germany) for 90 min at 4°C to concentrate virus particles. Pelleted virus particles were treated with deoxyribonuclease and ribonuclease A at 37°C for 90 min according to previously reported protocols.[19] RNA was isolated using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany), omitting carrier RNA. Double-stranded cDNA libraries were prepared using the TruSeq Stranded Total RNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA). Library size distribution was assessed using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the High Sensitivity DNA Kit (Agilent). Accurate quantification of the libraries was accomplished with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA). Paired-end sequencing (2 × 210 bp) was done using a MiSeq sequencing system (Illumina).

The sequences obtained were preprocessed using the PRINSEQ software to remove reads smaller than 35 bp and sequences with scores of lower quality than a Phred quality score of 20. Fast length adjustment of short reads (FLASH) software was used to merge and extend the paired-end Illumina reads using the default parameters, with a maximum overlap of 400 bp. The extended reads were analysed with basic local alignment search tool (BLAST), against the Human Transcriptome Database (RefSeq, Annotation Release 107; 162 916 sequences), with e-value cutoff of 1e-5, to remove human RNA sequences. Non-human reads were analysed against all GenBank viral genomes (65 052 sequences), and reads that were similar to the Zika virus were collected and used for genomic assembly. The Zika virus genome (Brazil strain) was assembled de novo using the CAP3 assembly software,

using the parameters overlap length cutoff (-o) of 16, and overlap percent identity cutoff (-p) of 85. The Atlas genome was constructed using BRIG (BLAST Ring Image Generator) software. We used the Zika virus genome sequence H/PF/2013 (KJ776791.1) as the reference. This strain was isolated in French Polynesia, and we compared it with a strain from Uganda, MR 766 (accession: NC_012532.1), another strain isolated in Senegal, ArD157995 (accession: KF383118), and our assembled Zika virus genome.

Phylogenetic analysis

Phylogenetic reconstruction was completed using both maximum likelihood and Bayesian inference methods on alignments of the envelope and NS5 regions of the polyprotein coding sequence. The best choice of substitution model used in the maximum likelihood and Bayesian inference analyses was determined with the likelihood-ratio test, implemented using HyPhy software. The generalised time-reversible (GTR) model with gamma-distributed evolutionary rates (G) and invariable sites (I), GTR+G+I, was chosen. We undertook maximum likelihood analysis with PhyML 3.0 phylogeny software, using the approximate likelihood-ratio test as a means of assigning statistical significance to internal branches. Bayesian inference was run on MrBayes 3.2 software with default Markov chain Monte Carlo (MCMC) algorithm settings—ie, two independent runs with four chains each were sampled every 500th generation until 1 000 000 samples were obtained. 25% of the MCMC samples were discarded as a burn-in step. Chain convergence was measured by the Gelman-Rubin statistic, using the potential scale reduction factor, or PSRF, which was close to 1 for all parameters. Maximum likelihood and Bayesian inference topologies were identical. We therefore report the results from the maximum likelihood analysis.

To investigate recombination breakpoints along the Zika virus genome, a sliding window strategy was implemented using an in-house script. By building a stand-alone BLAST database containing all reference flavivirus genomes, we scanned the Zika virus genome every 50 bp regions and registered their BLAST hits using a cutoff e-value of 0·0001. We did genome-wide multiple alignments using the Multi-LAGAN algorithm as implemented in the VISTA database. Phylogeny of whole genomes was also inferred by maximum likelihood and Bayesian inference methods.

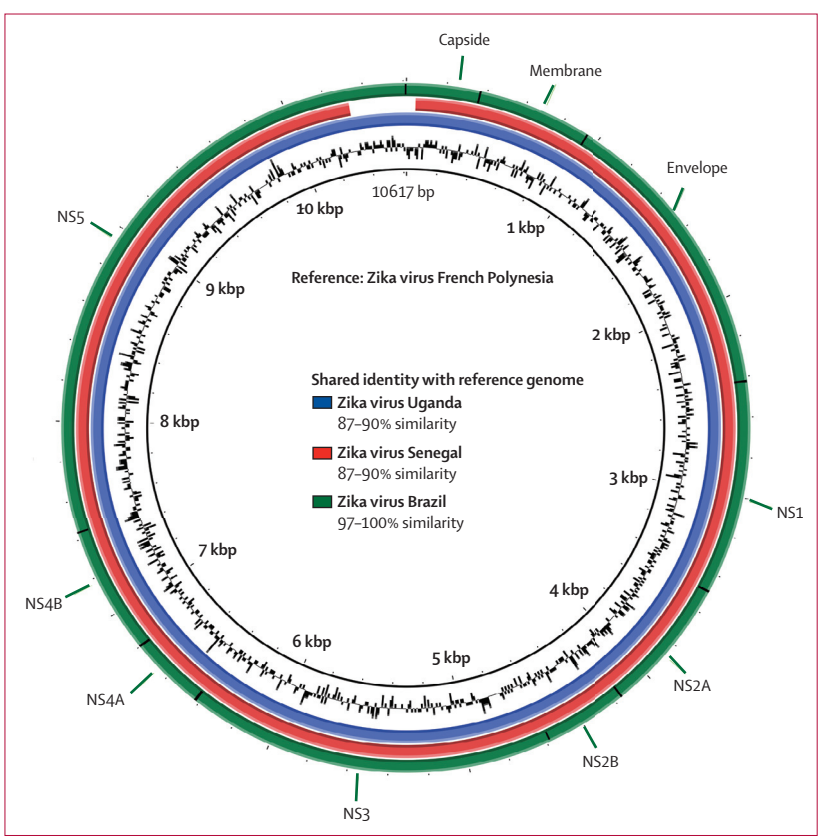

***Figure 1*: Comparative genome BLAST Atlas diagram of Zika virus**
The green outer circle corresponds to the complete Brazilian Zika virus genome isolated from the amniotic fluid of patient 1. 10 793 bases were sequenced. The red circle corresponds to the Senegal (KF383118.1) strain of Zika virus and the blue circle corresponds to the Uganda strain (NC_012532.1). The percentage deviation in GC content between the Brazilian Zika virus and the reference Zika virus is represented along the Zika virus genome by the varying heights of the black bars. The innermost (black) circle corresponds to the reference genome (French Polynesia, KJ776791.1). Genome shared identity between each strain and the reference genome are shown as percentages. BLAST=basic local alignment search tool.

Role of the funding source

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

Results

Serum, urine, and amniotic fluid samples from both patients (all taken at week 28 of gestation) were tested for dengue virus, chikungunya virus, and Zika virus. The RT-qPCR for dengue virus[20] and the RT-qPCR for chikungunya virus[21] were negative in all samples. The RT-qPCRs for Zika virus[22] confirmed Zika virus infection in the amniotic fluids of patients 1 and 2, but were negative in urine and serum samples in both patients. Serology tests of serum, urine, and amniotic fluid samples using anti-dengue-virus IgM, anti-dengue-virus IgG, anti-chikungunya-virus IgM, and anti-chikungunya-virus IgG yielded negative results by ELISA. However, ELISA for Zika virus was positive for anti-Zika-virus IgM in amniotic fluid, and negative in serum and urine in both patients 1 and 2. TORCH (toxoplasmosis, HIV, syphilis, measles, rubella, cytomegalovirus, and herpes simplex) panels of both women were also negative, as well as specific HIV, syphilis, cytomegalovirus, and parvovirus B19 screens.

Virus particles were filtrated and concentrated from the amniotic fluid samples. After cellular contaminants and human sequences were eliminated, 288 904 sequences

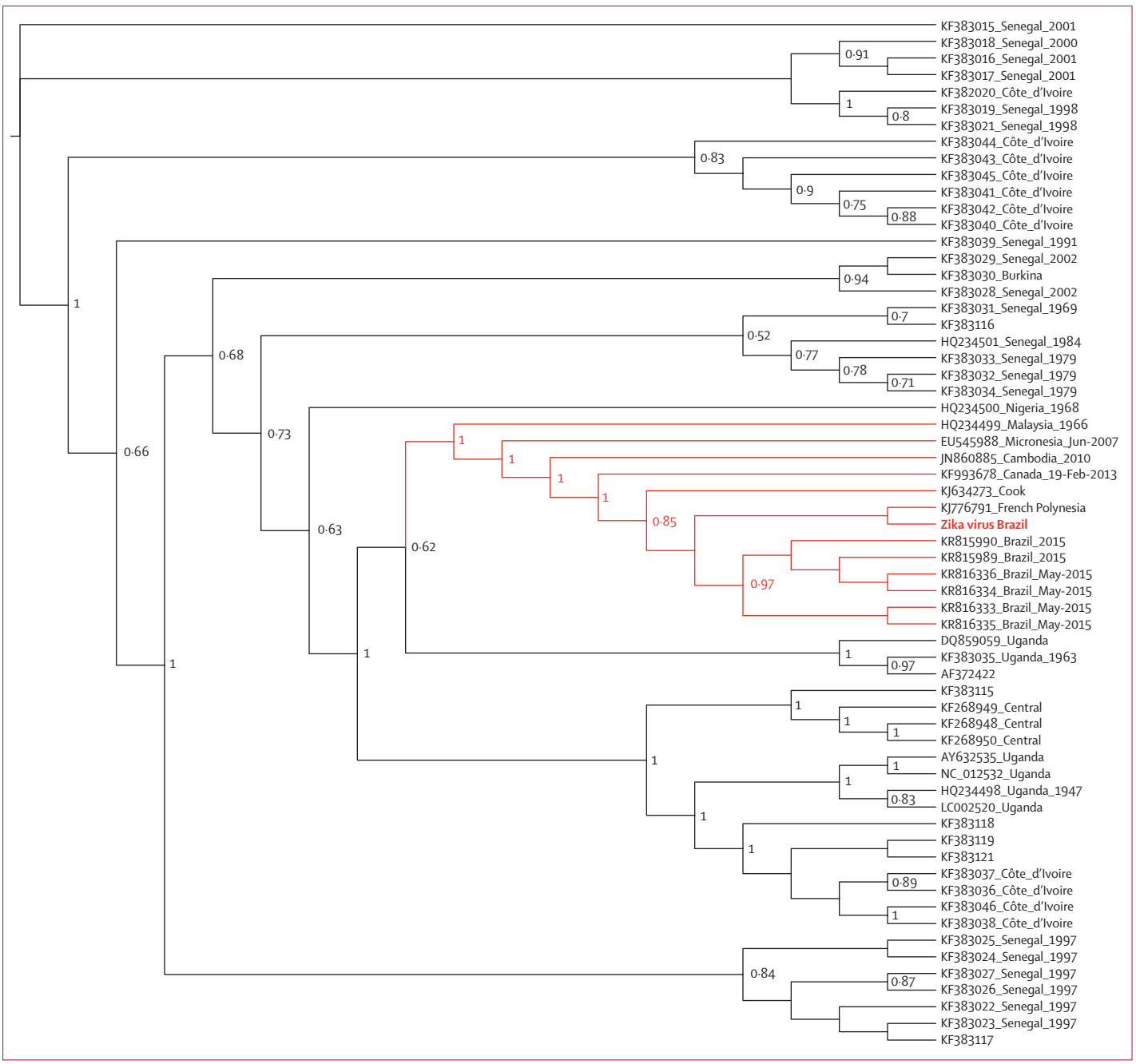

***Figure 2*: Maximum likelihood topologies of envelope genomic region from Brazilian Zika virus**

Brazilian Zika virus (in red text) isolated from the amniotic fluid of patient 1, whose fetus was diagnosed with microcephaly, was compared with previously released sequence data. Approximate likelihood-ratio test support values greater than 0·5 are shown at nodes. Zika virus Brazil shares the same origin as those of Asian, Pacific, and American lineages (red branches). For most strains, the country of isolation is shown, in some cases followed by the date of isolation. Burkina=Burkina Faso. Central=Central African Republic. Cook=Cook Islands.

showed similarity with virus sequences through BLAST analysis of the GenBank viral genome database. 683 sequences matched the Zika virus, comprising 167 143 bp, used to assemble the genome. Two different fragments corresponding to Zika virus genome positions 1641–1763 and 6466–6566 were sequenced from samples of patient 2. Metagenomic analysis of samples of patient 1 covered 10 793 bases of the Zika virus genome with 19×coverage. The complete sequence (10 793 nucleotides) was deposited at the GenBank database (accession number ID: KU497555).

Figure 1 shows the whole Zika virus genome isolated from the amniotic fluid of patient 1 with viral gene annotation. The Brazilian Zika virus shares 97–100% of

its genomic identity with the Zika virus sequence KJ776791.1 isolated in French Polynesia. The comparison with African strains such as NC_012532.1 (Zika virus Uganda) and KF383118.1 (Zika virus Senegal) yielded 87–90% identity. The proportion of GC content in the Brazilian Zika virus was 51·2% (figure 1).

We compared the viral sequences from patient 1 with previously released sequence data from Zika virus outbreaks in Asia and Africa and other flaviviruses, including dengue virus serotypes 1–4, West Nile virus, and yellow fever virus. Phylogenetic analyses were done using the coding region for the envelope (figure 2) and NS5 genes (figure 3). The geographical origin of the Brazilian Zika virus strain could not be determined because of sampling limitations, but Brazilian Zika virus is probably more closely related to French Polynesia sequences than to African strains. Maximum likelihood and Bayesian inference methods applied to the alignment of the envelope and NS5 regions of the polyprotein coding sequence yielded identical estimates of phylogenetic topologies. In both envelope (figure 2) and NS5 (figure 3) genomic regions, the new genome

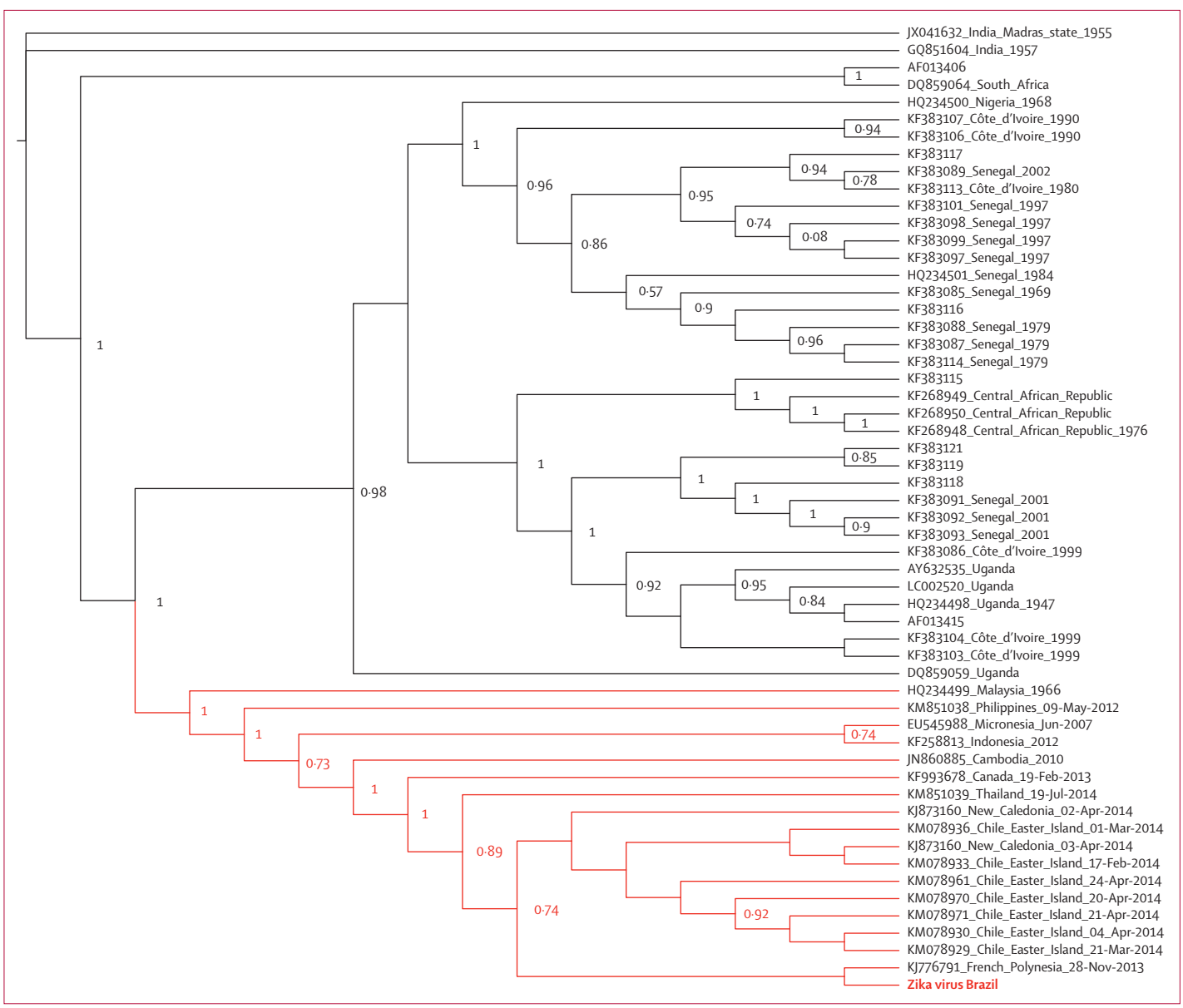

***Figure 3*: Maximum likelihood topologies of NS5 genomic region from Brazilian Zika virus**

Brazilian Zika virus (in red text) isolated from the amniotic fluid of patient 1, whose fetus was diagnosed with microcephaly, was compared with previously released sequence data. Approximate likelihood-ratio test support values greater than 0·5 are shown at nodes. Zika virus Brazil shares the same origin as those of Asian, Pacific, and American lineages (red branches). For most sequences, the country of isolation is shown, in some cases followed by the date of isolation.

clustered with sequences from North and South America, southeast Asia, and the Pacific.

The geographical and chronological distributions of Zika virus lineages also indicate that southeast Asian strains could have remained genetically isolated from African strains for about 50 years, because Malaysian sequence HQ234449, collected in 1966, is the sister group of the remaining New World and Pacific strains. This pattern was further confirmed by the genomic distance between the newly sequenced Brazilian Zika virus and the Ugandan Zika virus genome available in GenBank (ZIKV LC002520; figure 4).

We assessed the possibility of recombination events between the Zika virus and other flaviviruses by scanning the Zika virus genome every 50 bp using as references the genomes from dengue virus serotypes 1–4, West Nile virus, yellow fever virus, and chikungunya virus (an alphavirus that is transmitted by the same vector). The sliding window strategy with local alignments of genomic fragments ruled out the hypothesis that the newly sequenced Brazilian Zika virus genome is a recombinant strain with other mosquito-borne flaviviruses. All genomic regions consistently presented best hits and significant e-values with previously reported Zika virus genomes, ruling out the hypothesis of genomic recombination.

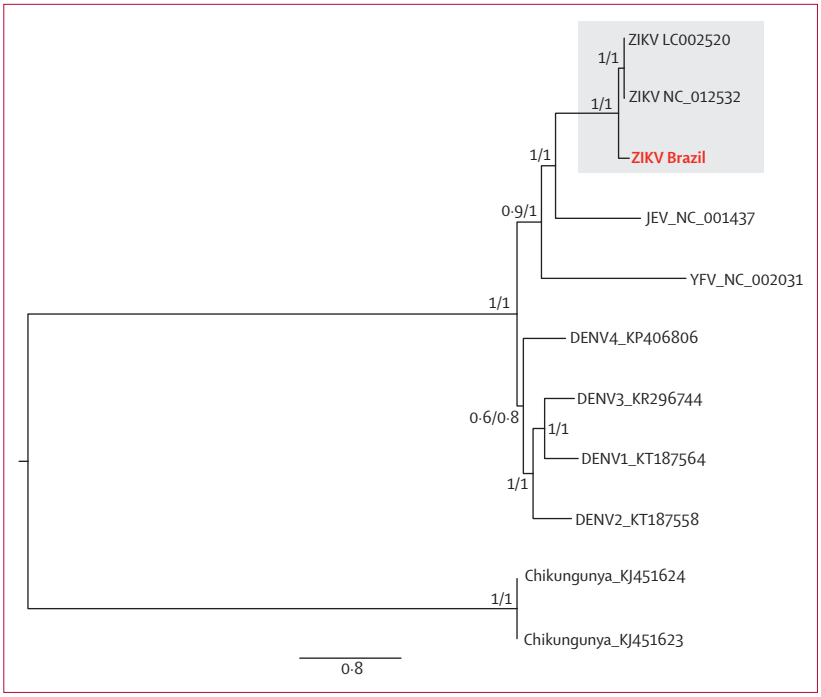

Figure 4: **Maximum likelihood phylogeny of Brazilian Zika virus, other Flaviviridae genomes, and an alphavirus genome**

Brazilian Zika virus (in red) was isolated from the amniotic fluid of patient 1, whose fetus was diagnosed with microcephaly. Approximate likelihood-ratio test and Bayesian inference support values are shown at nodes. Chikungunya is an alphavirus; all other viruses are from the Flaviviridae family. DENV=dengue virus. JEV=Japanese encephalitis virus. YFV=yellow fever virus. ZIKV=Zika virus.

Discussion

Detection of the Zika virus genome and anti-Zika-virus IgM in the amniotic fluid of pregnant women with microcephalic fetuses has not been previously reported in detail in the scientific literature. This finding shows that the Zika virus can cross the placental barrier and, possibly, infect the fetus. A previous report[23] suggested that fragments of Zika virus genome were identified in saliva, breastmilk, urine, and serum of two mothers and their newborn babies within 4 days of delivery. However, our group is the first, to our knowledge, to isolate the whole genome of Zika virus directly from the amniotic fluid of a pregnant woman before delivery, supporting the hypothesis that Zika virus infection could occur through transplacental transmission.

Some neglected tropical diseases have well known neurological effects. Many distinct clinical syndromes, from mild fever and arthralgia to severe haemorrhagic and encephalitic manifestations, are known to be associated with flavivirus infections.[24] Other severe neurological complications such as Guillain-Barré syndrome have been reported in patients infected with Zika virus.[25] Two key properties allow these viruses to affect the neural system: the ability to enter the CNS (neuroinvasiveness) and the capacity to infect neural cells through a process known as neurovirulence. A connection between Zika virus infections and poor CNS outcomes remains presumptive, and is based on a temporal association. New studies should be done to investigate whether the Zika virus can infect either neurological precursor cells or final differentiated cells.

Congenital microcephaly is a descriptive diagnosis. It can be caused by various factors, such as genetic disorders, exposure to chemicals, brain injury, consumption of teratogenic drugs, and intrauterine infections.[26] Here, we focused on viral infection to explain these two cases of microcephaly. However, other possible causes or contributing factors should continue to be pursued as new cases arise in Brazil.

In these two patients, fetal microcephaly was detected early during gestation and a severe outcome was expected. Ultrasound tests revealed the presence of malformation, including ventriculomegaly and cerebellar hypoplasia. Fetal brain malformation can often result from viral infections during pregnancy. Cytomegalovirus infection occurring before 18 weeks of gestation is frequently associated with lissencephaly with a thin cerebral cortex, cerebellar hypoplasia, and ventriculomegaly, among other malformations.[27] However, in the two cases presented here, serological and RT-PCR tests for cytomegalovirus were negative, ruling out cytomegalovirus infection. The viral metagenomic approach used here does not exclude either DNA or RNA viruses; nevertheless, no cytomegalovirus sequence was identified in the amniotic fluid in our analyses. An increase in the incidence of CNS malformations in fetuses and neonates was reported after a Zika virus outbreak in French Polynesia; however, the occurrence of

microcephaly associated with these previous outbreaks was not documented.[1]

Our previous image findings[18] and our results shown here of the presence of viral genomic material in both patients, several weeks after the acute phase of Zika virus disease, suggest that the intrauterine viral load results from persistent replication. In turn, this persistence could be partly explained by the reduced immune system response of the fetus, as described in the pathogenesis of congenital cytomegalovirus.[28,29]

The Zika virus could have undergone several recombination events, and the recurrent loss and gain of the N-linked glycosylation site in the E protein could be related to mosquito-cell infectivity.[30] We found no evidence of recombination events in the Zika virus genomes that we tested. The role of recombination in Zika virus virulence warrants further study.

Our results provide insight into the origin of the Zika virus circulating in Brazil, and suggest that a causal relation might exist between Zika virus infection and fetal microcephaly. New studies coordinated by the Brazilian Ministry of Health and other institutions are underway to further test this hypothesis, and hopefully elucidate the cellular and molecular mechanisms of Zika virus infection.

We recommend that Zika virus infection should be regarded as a possible causative agent in cases of microcephaly, especially during Zika virus outbreaks in endemic regions. Early diagnosis of Zika virus infection, supportive care, symptomatic treatment, and referral of children with microcephaly to specialised care are all necessary measures to improve neurodevelopment of affected children.

Contributors

AMBdF, RSA, AT, and FLT designed the study. ASOM did the ultrasound and collected the amniotic fluid samples. SAS, AF, ESMA, RSA, PCdS, MCLdM, and LdO did the laboratory studies. AMBdF, RMRN, FBdS, RSA, CGS, AT, FLT, DAT, PB, and IdF analysed the data. GC, AMBdF, and RSA wrote and edited initial drafts. All authors reviewed the final draft.

Declaration of interests

We declare no competing interests.

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