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Características clínicas y epidemiológicas de casos probables de síndrome congénito por Zika y los niveles de anticuerpos anti-dengue, Tuxtla Gutiérrez, Chiapas, México

Víctor M. Cardenas Ayala[1], Jazmín Moreno Pérez[2], Karla Cabrera Besares [3], Iris F. Moreno Lara [4], Sabino Morales Arias[5], Ernesto Salvatierra Izaba [6], Victor De Dios Gómez[7], Pedro M. Palermo[8], Jeanette, Orbegozo[9], & Douglas M. Watts[10]

Abstract: Introduction: We previously found that the occurrence of congenital Zika syndrome was under-reported in Mexico. It was postulated that high dengue virus antibody levels found at the population-level in endemic countries might have contributed to the occurrence of the regional pandemic of Zika disease. A case series of suspected cases of congenital Zika syndrome in a maternity hospital in Tuxtla Gutierrez, Chiapas, Mexico was assembled to assess why they were not reported and to explore the hypothesis of dengue virus antibody-dependent enhancement of Zika disease. **Methods:** We used a quantitative approach to describe clinical and imaging records and used data from interviews of a total of 13 suspected cases of congenital Zika syndrome. We also quantitated dengue 1 and 2 antibodies using an 80% plaque reduction neutralization test of sera specimens obtained from the mothers of these 13 cases and compared them to those of a group of mothers who delivered normal newborns in the same hospital. **Results:** Only one of the suspected cases was laboratory-confirmed because appropriate specimens were not collected from the newborns as required by the case definition. We found 1) microcephaly, 2) hypoplasia/hypogeneses, thinning or absence of brain structures, 3) multiple birth defects, 4) calcifications, and cysts, 5) meningocele/encephalocele, and 6) hydrocephalus in 100 %, 76.9 %, 38.5 %, 38.5 %, 30.8 %, and 23.1 %, respectively of the case series. The cases clustered geographically, and 77 % occurred between May 2016 to March 2017 and recalled or were told by a doctor they had Zika fever. There was a four-fold increased risk of congenital Zika syndrome among those with dengue 1 antibody as compared to those with dengue 2

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antibodies (odds ratio = 3.6; 95 % confidence interval: 0.7, 20.5), reaching only borderline statistical significance. **Conclusions:** We found in the largest maternal facility of the capital of the State of Chiapas, in Mexico, that only 7.7 % of suspected cases were confirmed, and that the rather complex requirement of cerebrospinal fluid specimens or serological specimens of newborns for suspected cases of congenital Zika syndrome used during the pandemic

resulted in low sensitivity of the surveillance system. The finding of higher levels of dengue 1 than dengue 2 antibodies in cases than the referent population, requires further evaluation and may suggest a role for dengue antibody-dependent response in Zika disease.

Keywords: Zika Virus Infection; Epidemiologic studies; Congenital Abnormalities; Dengue Virus/immunol.

Resumen: Introducción: Previamente los autores habían encontrado evidencia de sub-notificación de la ocurrencia del síndrome congénito por Zika en México. Se ha postulado que niveles elevados de anticuerpos contra los virus del dengue a nivel poblacional en los países endémicos hubiese contribuido a la ocurrencia de la pandemia regional de enfermedad por Zika. Ensamblamos una serie de casos sospechosos de síndrome congénito por Zika en un hospital de maternidad en Tuxtla Gutiérrez, Chiapas, México, para evaluar por qué no fueron notificados y explorar la hipótesis de enfermedad por Zika incrementada por anticuerpos anti-dengue. **Métodos:** Utilizamos un enfoque cuantitativo para describir 13 casos sospechosos de síndrome congénito por revisamos registros clínicos e imágenes, entrevistas. También cuantificamos los niveles de anticuerpos para los virus dengue 1 y 2 en suero de las madres de los casos comparados con los de mujeres que tuvieron recién nacidos normales en el mismo hospital. **Resultados:** Solamente uno de los 12 casos sospechosos fue confirmado por laboratorio, porque en los demás no se recolectaron especímenes adecuados de los neonatos como lo requería la definición de casos. Encontramos 1) microcefalia, 2) hipoplasia y adelgazamiento de las estructuras cerebrales, 3) malformaciones múltiples, 4) calcificaciones o quistes, 5) meningocele/encefalocele, y 6) hidrocefalia en: 100 %, 76.9 %, 38.5 %, 38.5 %, 30.8 %, y 23.1 %, en ese orden entre los casos sospechosos. Los casos se aglutinaron geográficamente y 77 % ocurrieron entre Mayo del 2016 y Marzo del 2017, y sus madres recordaban que tuvieron o que un profesional de la salud les dijo que tuvieron fiebre por Zika. Encontramos un incremento de casi 4 veces en el riesgo de síndrome congénito por Zika para aquellos con altos niveles de anticuerpos anti-dengue 1 comparado con anticuerpos anti-dengue 2 (cociente de suertes = 3.6; intervalo de confianza del 95 %: 0.7, 20.5), alcanzando solamente una significancia estadística limítrofe. **Conclusiones:** Encontramos en el establecimiento de atención a la maternidad más grande en la capital de Chiapas, México, que solamente 7.7 % de los casos sospechosos de síndrome congénito por Zika fueron confirmados y que los relativamente complejos requerimientos de la definición de casos de muestras serológicas o de líquido cefalorraquídeo resultó en una baja sensibilidad del sistema de vigilancia. El hallazgo de niveles más altos de anticuerpos a dengue 1 que dengue 2 requiere más evaluación y pudiera sugerir un papel de la respuesta dependiente de anticuerpos al dengue en Zika.

Palabras clave: Infección por virus Zika, Estudios epidemiológicos, Anomalías congénitas, virus dengue/inmunología

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1. Introduction

Since the first reported transmission of Zika virus (ZIKV) during May of 2015 in Brazil and the subsequent rapid spread of the virus through the Americas, a total of 3,270 cases of congenital Zika syndrome (CZS) were reported as of January 2018, including 2,952 (90.3 %) in Brazil only (Pan American Health Organization [PAHO], 2018) (Supplemental Table). The disproportionate higher reported occurrence of Zika fever and CZS cases in Brazil in comparison to other countries in the region is not well-understood.

One possible reason is the under-reporting of both Zika fever and CZS in several countries including Mexico (Hernández-Avila et al., 2018, Cárdenas et al., 2019). Based on the examination of existing death records with microcephaly as the underlying cause of death, we previously reported a 50 % excess of microcephaly deaths attributable to the Zika pandemic than the number of CZS fatalities reported by the existing surveillance system in Mexico (Cardenas et al., 2019).

At the outset of the investigation, it was unclear why such failure to report microcephaly cases and deaths and other malformations which characterize CZS would occur. One reason we considered was the complexity of the case definition established for surveillance in 2016 by the regional office of the World Health Organization, the Pan American Health Organization (PAHO). According to this definition, a confirmed case of CZS is a "[L]ive newborn who meets the criteria for a suspected case of congenital syndrome associated with confirmed evidence of Zika virus AND Zika virus infection was detected in specimens of the newborn, regardless of detection of other pathogens" (PAHO, 2016). A requirement for confirming Zika virus infection in newborns is the timely collection and testing of appropriate clinical specimens, which we suspected was not always possible due to the lack of manpower and resources, and therefore could explain the failure to confirm cases.

In support of such explanation, a retrospective review of 39 cases of CZS reported in the state of Espírito Santo, Brazil, from 2015 to 2016 showed that of the 39 cases diagnosed using the PAHO case definition, only 20.5 % (n=8) were diagnosed by using serological testing results (Mocelin et al., 2019).

In 2017 Halstead postulated that the Zika pandemic and the occurrence of CZS was taking place in areas where dengue was highly endemic. Halstead based on the analogy of the dengue antibody dependent enhanced (ADE) infection to explain subsequent severe dengue infections (i.e., hemorrhagic dengue fever) in dengue endemic areas, advanced the hypothesis that circulating dengue antibody in areas also affected by ZKV could possibly explain the occurrence of CZS and other manifestations of severe infection with the ZKV such as Guilliain Barré Syndrome (GBS) due to ADE response (Halstead, 2017).

However, one early cohort study based on Rio de Janeiro concluded that dengue virus antibodies were not associated with the occurrence of CZS (Halai et al., 2017). The negative finding of this study should not be surprising, as cohort studies need large sample study sizes to achieve enough statistical power to

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detect associations on rare outcomes (<10 %). In contrast, a large population-based case-control study conducted in Recife (Castanha et al., 2019) found an increased risk of microcephaly (odds ratio (OR)= 2.9]. The results of both studies were based on 50 % plaque reduction neutralization antibody endpoints (PRNT50), which is less specific than 80 or 90 % plaque reduction neutralization antibody endpoints (PRNT80 or PRNT90). A more recent study reported a significant increase (odds ratio [OR]= 3.3; 95 % Confidence Interval [CI] = [0.8, 13.5]) risk of CZS among those who had neutralizing antibody titers using PRNT90 to dengue virus 1 (DENV1), but the authors concluded that antibody to dengue 2 (DENV2) reduced the risk of CZS (Pedroso, 2019).

Other studies have addressed this issue through several experimental and observational designs with conflicting results. A systematic review disregarded individual studies that showed an elevated risk of Zika associated GBS (e.g., Cao-Lormeau et al., 2016, reported an OR = 6.0 (95 % CI 0.8, 269.5); P= 0.10) and the other human study was the small cohort study of CZS by Halai et al. (2017), and nevertheless the authors of the meta-analysis concluded that "no existing published human studies have offered high-quality measurement of both acute ZIKV and antecedent DENV infections" (Masel et al, 2019), and did not include the study of Recife mentioned above (Castanha et al., 2019). An assessment of the role of ADE on Zika infection used viral loads of DENV and ZKV in persons with and without previous dengue and reported absence of ADE effect on Zika infection but confirmed the presence of ADE in secondary DENV infection (Santiago et al., 2019). The authors acknowledged that they could "not rule out that enhancement of ZIKV may occur among other populations with a different history of prior infection with DENV" (Serrano-Collazo et al., 2020). Experimental studies in rhesus macaques (Santiago et al, 2020) and observational ecologic studies (Carvalho et al., 2020) suggest that the time elapsed between previous DENV infections and subsequent ZKV infection is related to the occurrence of CZS and GBS.

As an effort to explore the question of the possible role of DENV antibody as a risk factor for CZS, we conducted a case-referent study in the spring of 2019 at the end of the Zika pandemic in Tuxtla Gutierrez, (850,000 population), the capital of the State of Chiapas, Mexico. Chiapas has documented circulation of DENV1 since 1983 and DENV3 in 1995 (Zárate-Aquino et al., 1995). As in other dengue-endemic areas in Mexico (Gómez-Dantés et al., 2014), in Chiapas, DENV2 circulation was documented in 2000-2008. DENV1 predominated again in 2009 when a record 250,000 cases of dengue fever were reported. Since 2013 and through 2019, the DENV2 predominated.

An earlier timing of the ZIKV infection during pregnancy it has been associated with increased risk of CZS, doubling the risk of a birth defect of those infected during the last trimester (Shapiro-Mendoza et al., 2017). Similar findings had been documented for other viral infections during early pregnancy causing other congenital syndromes such as rubella (Gregg, 1941), but in CZS the risk was not circumscribed to infections early on during pregnancy.

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We aimed in this study to describe the clinical and epidemiologic characteristics of probable and confirmed CZS in the study area and to explore the hypothesis that pre-existing DENV antibodies posed an increased risk for the occurrence of CZS.

2. Population and Methods

2.1 Study population

In 2016-2018 one of us (KC) implemented a protocol for active case finding of suspected CZS among newborns and assembled a case series of CZS in the main maternity facility serving the Tuxtla Gutierrez metropolitan area, the Pascacio Gamboa regional hospital (120 beds) with a 30-bed maternity unit where over 10,000 births are delivered every year.

2.2 CZS case definition

The working case definition of a suspected case of CZS was that of the Mexican Health Secretariat (Mexican Ministry of Health [MoH]), General Directorate of Epidemiology, and PAHO "as the presence of [M]icrocephaly: head circumference below -2 standard deviations for gestational age and sex, measured at 24 hours post-partum according to the standardized reference; OR Other congenital malformation of the central nervous system" (PAHO, 2016). We used the Inter-Growth 21st as Standard Population for the head circumference (Papageorghiou et al.,2018).

2.2 Data collection on cases

We abstracted all available medical records, including imaging documents on file from the cases and after obtaining written informed consent, we interviewed the parents of the cases and assessed the achievements of milestones using guidelines of the USA Centers for Disease Control and Prevention's (CDC) National Center on Birth Defects and Developmental Disabilities, tracker (CDC, 2021). Images available on records from ultrasound (US), including transfontanellar ultrasound (TUS), magnetic resonance imaging (MRI) and computer tomography scan (CT) were evaluated by two independent radiologists (IFML and SAM). In addition, we obtained a 5 mL venous blood specimen from the mother of the suspected CZS cases. A laboratory-confirmed case was based on the confirmation of Zika virus infection in the newborn. In the absence of laboratory confirmation, we relied on the detailed clinical description, including imaging data based on medical records and our assessment of child development at our follow-up visit.

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2.3 Comparison group and data collection

To compare the prevalence of dengue antibodies with an unaffected sample of the population, we sought and obtained consent from mothers who delivered a newborn not affected by CZS in the same hospital as referent group from March 27 to April 2, 2019. The prevalence of dengue antibodies in the referent group is representative of the population from which the cases originated. Specifically, women in this referent group were those who delivered a singleton neonate with head circumference within the normal range for their gestational age and fetal sex, and who provided written informed consent. Women in the referent group were asked the same questions about risk factors as cases, and to provide a 5 mL venous blood sample. Serum specimens were separated and transported frozen from Tuxtla Gutierrez, Chiapas to El Paso, Texas, to the laboratory of one of us (DMW) in the University of Texas at El Paso (UTEP). We transported 13 specimens from cases and 39 from controls, but laboratory data from four controls were missing, leaving only 48 individuals for the analysis (13 cases and 35 cases).

2.4 Lab testing

At the UTEP lab, three of the co-authors (PEP, JO and DMW) tested the control and suspected case specimens blindly for ZIKV IgG antibody and for DENV1 and DENV2 IgG antibodies using an enzyme immunosorbent assay (ELISA) (Ansari et al., 1993; Innis et al., 1989, Morrison et al., 2010). The protocol for this study was reviewed and approved by the IRBs of University of Arkansas for Medical Sciences (Protocol 229125), the Chiapas Secretary of Health, and the UTEP.

2.5 Statistical Analysis

As an exploratory study, the aim was to assess the reasons for under-reporting as the total number of reported cases in the study population was limited. We examined the available records for imaging evidence of brain abnormalities, and the distribution of cases by time and place. The assessment of cluster used a purely temporal analysis (disregarding place of residence), as the catchment population of the hospital is not well defined, and instead look for clustering of cases by months, using a discrete Poisson distribution scan statistic with minimum temporal cluster of one month and maximum temporal cluster including 20 % percent of study period available in SaTScan (Kulldorff, 1997). We examined the distribution of DENV1 and DENV2 antibodies adding 1 to each report of the final dilution (PRNT80 +1) and transformed in the log10 scale. We estimated the crude odds ratio (OR) and 95 % confidence interval (CI) for the association between case-control status using both the presence of previously validated cutoff levels of antibodies to DENV1 (1:80) or DENV2 (1:60), and an empirical cutoff of 1:640 for high levels of DEN1 antibodies based on the median of the distribution in the controls, and a fourfold ratio of DENV1 to DENV2 antibodies. We calculated OR for high levels of DEN1 antibodies adjusted for maternal age and maternal education (as a proxy of socioeconomic status) and regular use of bed nets for the overall association. Fisher exact mid-P values were calculated, and exact logistic regression analysis was conducted to estimate the OR and its 95 % CI. Log transformed titers were compared using the Mann-

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Whitney test for the shape of the distribution of antibodies in the two groups. A two-sided P-value <0.05 was considered statistically significant. All of the analyses were done using SAS version 9 (SAS Institute Inc., Cary, NC, USA) GraphPad Prisma 8 (San Diego, CA, USA), SaTScan (Calverton, MD, USA), Microsoft Excel (Redmond, WA, USA) and Epi-Info (Atlanta, GA, USA).

3. Results

3.1 Case series and descriptive epidemiology by time and place

A total of 17 cases met the definition of a probable case of CZS among live births. In addition, one fetal death and one stillbirth had microcephaly among other malformations that were present in women who were thought to have Zika during pregnancy. Three cases were outside the reach of the study team, including one immigrant from El Salvador whose mother had Zika while in transit in Tuxtla Gutierrez, but had moved to the US-Mexico border. Of the remaining 13 cases included in the study (Table 1), a cerebrospinal fluid specimen was obtained from only one, who was confirmed as a case of CZS (record 19 in Table 1). We observed that there were no dedicated skilled staff to obtain blood or cerebrospinal fluid specimens from neonates to meet the short stay of physiologic puerperium in the hospital. One probable case (record 18 in Table 1) did not meet the PAHO definition as it occurred in a stillbirth, but it was included as all of the medical records were available to rule in as a probable case of CZS.

As shown in the epidemic curve in Figure 1, there was a cluster of 6 (Observed or O) of the 13 suspect cases of CZS in the second semester of 2016, particularly on December 2016 when four cases occurred (colored in red in Figure 1), compared to 1.7 expected (E) for a O/E ratio of 3.6, with a borderline statistically significant P-value of 0.06. As shown in the spot map in Figure 2, they also seemed to cluster geographically in the northern foothills of the metropolitan area of Tuxtla Gutierrez and the vicinity of the airport, which are areas of lowest socioeconomic status, but lack of denominator data for the catchment population of the hospital, prevented formal assessment of the geographic clustering.

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Figure 1

Probable cases of Congenital Zika Syndrome by date of birth, Pascasio Gamboa Hospital, Tuxtla Gutierrez, Chiapas, Mexico, 2016-2018.

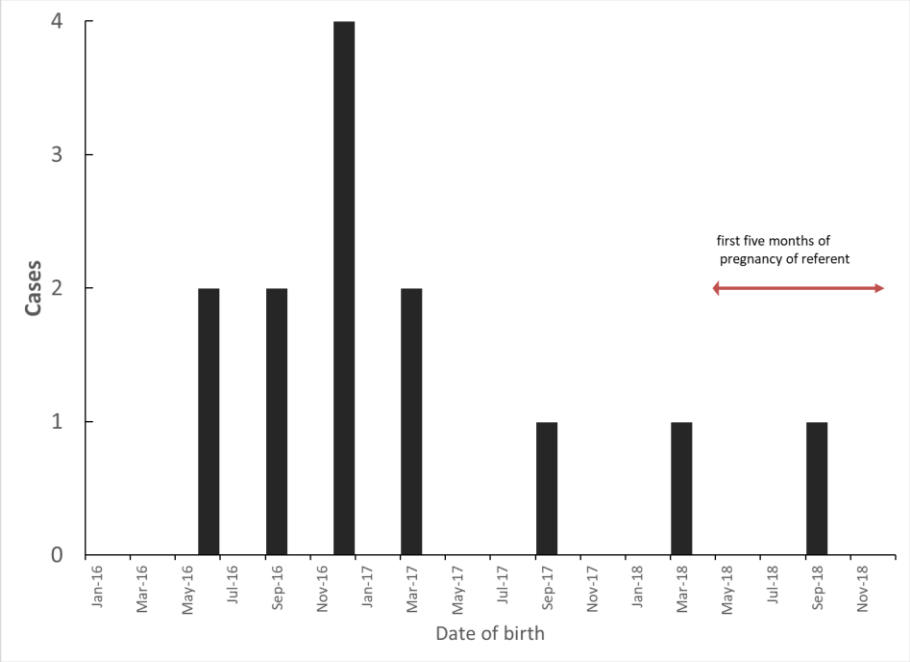
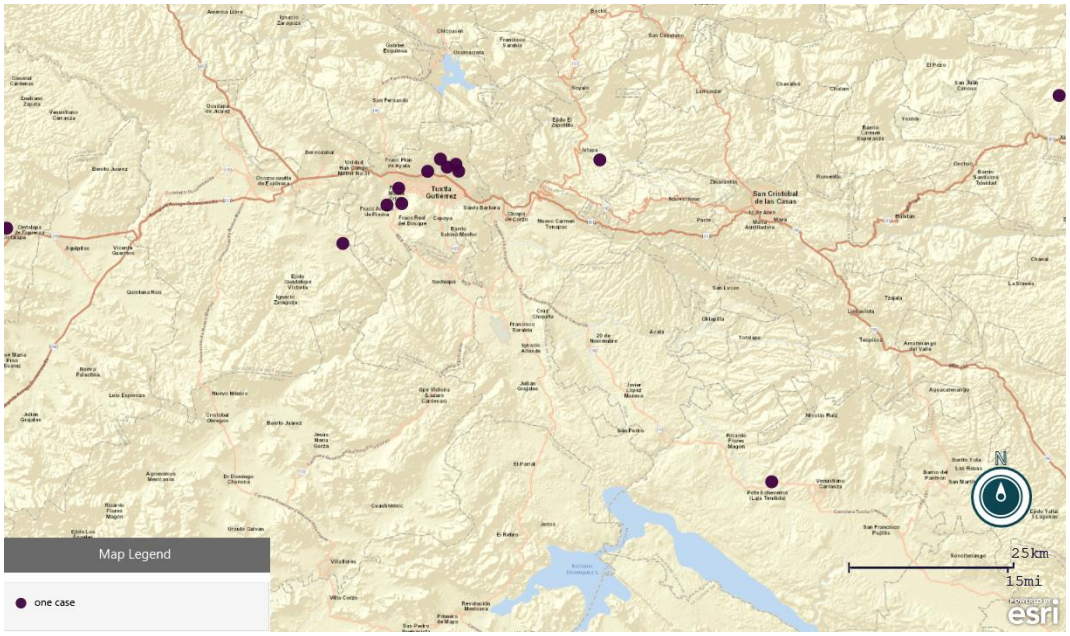


Figure 2

Cases of suspect congenital Zika syndrome seen at the Pascacio Gamboa Hospital by place of residence, Chiapas, Mexico, 2016-2018



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3.2 Clinical and imaging characteristics of probable cases of CZS

Also, as shown in the case listing in table 1, all but one of the 13 probable cases of CZS had Z-scores for head circumference taken 24 hours post-partum below 2 standard deviations of the sex-gestational age specific InterGrowth 21st population, but the child (record 19 in Table 1) with apparent normal head circumference was virologically confirmed as CZS and was diagnosed with microcephaly by ultrasound before developing hydrocephalus, which would explain the normal Z-score at birth. As documented in table 1, in all but three instances, or 76.9 % of the suspected CZS cases, the images suggested hypoplasia, or hypogenesis, or thinning of brain structures or frank absence of brain structures. In the three cases without these defects, only images from US were available. In five cases (38.5 %) multiple birth defects were present, including four with clubfoot, three had arthrogryposis. Another five cases (38.5 %) had calcification or cysts; four cases (30.8 %) had myelomeningocele or encephalocele, and 3 (23.1 %) had hydrocephalus. One child had Dandy Walker malformation. Five (41.7 %) of the 12 live births with conditions consistent with CZS had died within 2 years of birth. There were scannable documents for 10 of the 13 suspected cases of CZS, microcephaly was noted in all but one patient who had ventriculomegaly still the head circumference Z-score was -3.3 consistent with this condition. The supplemental figures show the imaging findings with comments from two of us (IML and SAM).

At the home visits to collect blood specimens from the mothers of the cases, we found among the nine evaluable children with probable CZS (Table 2) that all but one (88.9 %) had significant deficits in the achievement of developmental milestones, whether movement/physical, or cognitive and communication spheres. Only two of the seven patients who survived at least one year were able to stand, but one of them only while holding to a chair. One was blind and deaf, two had recurrent seizures, and one of these two also had inconsolable crying. All but three of the mothers of probable cases of CZS in our case series (77 % or 10/13), during our interviewed recalled having an episode consisting of fever and rash or being told by a doctor that they had ZKV fever.

The lack of trained staff to draw venous blood or cerebrospinal fluid from newborns was the main reasons given for not submitting specimens to the national reference laboratory in Mexico City, where PCR and antibody assays were available for confirmation of CZS diagnosis and reporting. However, based on the clinical and epidemiological evidence we conclude these 13 cases were probable cases of CZS.

3.3 Comparison of dengue antibodies in mothers of CZS cases with a sample of puerperal women

We assessed if there was any association between being a case or a member of the comparison group with select socio-demographic variables and risk factors for vector borne diseases at the individual level, and there was only an indication of increased risk for younger maternal age, less maternal education, and

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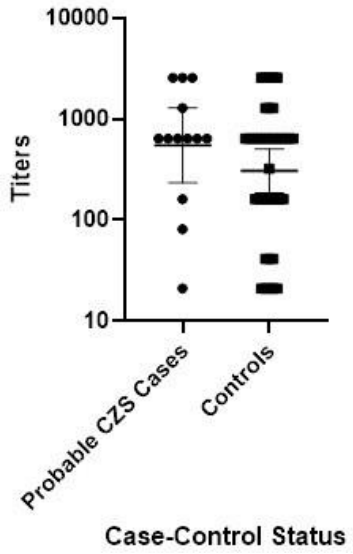
decreased risk for self-reported use of bed nets, although none of them were statistically significant (Table 3).

As shown in Table 4, all mother of probable suspected cases of CZS had Zika antibody by ELISA and confirmed by PRNT80. Also, a large proportion of controls (88.6 %) had antibodies to ZKV. We found that the levels of antibody to either DENV1 or DENV2 did not differ significantly, but particularly the point estimate of the geometric mean (log10) of DENV1 was larger in probable cases of CZS (1: 548.7) than in controls (1: 363.2) (Table 4 and Figure 3). The dot plot with box plot in Figure 3, also shows that 10 of the 13 cases had levels of DENV1 above the 75th percentile of the distribution of the controls (i.e., 1: 640). The levels of neutralizing antibody to DENV2 were higher among controls than cases. However, none of these differences were statistically significant. Most mothers of probable cases of CZS (76.9 %) and controls (82.9 %) had neutralizing antibody levels consistent with infections to both DENV1 and DENV2. A profile consistent with predominant DENV1 was found in 2 cases and 1 control, predominant DENV2, in no instance among cases and one control. Neither DENV1 or DENV2 in the specimens of mothers of 1 probable CZS case (7.7 %) and 4 controls (11.4 %). Using established cutoff values in other Latin American population (DENV1 or DENV2 antibody titers ≥80) we did not find any difference. However, the exact odds ratio for a ratio >10 for DENV1 to DENV2 antibody titers was elevated (OR=3.6 95 % CI: 0.7, 20.5), although only borderline statistically significant (mid P-value=0.052).

Figure 3

Distribution of neutralizing antibodies against dengue 1 serotype by CZS case-control status, Tuxtla Gutierrez, Mexico.



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4. Discussion

We found some evidence indicating that the under reporting of CZS cases through the surveillance system established under the PAHO guidelines was at least partly due to the complexity of the requirements of blood or CSF specimens from neonates. Lacking trained personnel such as neonatologist on call to collect blood specimens of suspect CZS cases in highly dengue-endemic areas which tend to be poor urban areas, was the most likely factor that could explain that only one suspected case of CZS was laboratory-confirmed among a pool of thirteen suspected cases.

4.1 Under-reporting of CZS

The clinical, imaging and developmental features of cases of CZS in Middle-America has seldom been reported. Clinically, and by imaging, the cases are similar to those described elsewhere as CZS, although only one was laboratory-confirmed. Epidemiologically, the cases clustered in time and in space. The case-fatality has also seldom been reported: almost 42 % had died within 2 years of life. Among cases of CZS that were evaluated at follow-up, 89 % had significant developmental limitations.

It is unquestionable that thousands of cases of CZS went under-reported through Latin America based on the number of cases reported in Brazil, and the findings of the best surveillance system in place in Puerto Rico, which found that 5 % of the offspring of ZIKV infected pregnant women had at least one birth defect (Shapiro- Mendoza, 2017). Based on the study of birth defects in Puerto Rico, and the estimate of 26 % of ZIKV infection in the island (Quandelacy et al., 2021) there should have been 364 cases of CZS (or approximately 28,000 pregnancies times 0.26 the proportion infected with ZKV, times 0.05 the proportion expected with at least one birth defect -CZS). A scientific paper estimated that 180 cases of microcephaly due to Zika were bound to occur in Puerto Rico (Ellington et al., 2016). Whether we use 364 or 180, these figures are considerably larger than the reported 47 confirmed cases of CZS in Puerto Rico (PAHO, 2018). Therefore, even in jurisdictions with ample support such as Puerto Rico, there has been considerable under-reporting. Given the detailed clinical, radiological and the epidemiological evidence we gathered, we can draw the inference that 12 unconfirmed cases included in this case series were at least probable cases of CZS.

4.2 Dengue and ADE in severe Zika infection

The virologic surveillance in place in Chiapas is rather limited, but there is evidence that most DENV types circulating back to the late 1970s were DENV1 and DENV2, although their genotypes continue to change (Rico- Hesse, 2003). The largest recent epidemics of 2009 and 2012 were caused by DENV1 and DENV2, and possibly explain the antibody profile in our study population. It has been postulated that high levels

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of dengue antibodies protect from severe dengue, while intermediate values increase the risk of severe dengue (Katzelnick et al., 2016).

Our findings are overall in agreement with some, but not all of the previous epidemiologic studies: A study of microcephaly in Recife found a three-fold increased risk for any previous dengue infection (Castanha, 2019). Also is consistent with the findings of another Brazilian study which reported a three-fold increased risk for infection by DENV1 but a decreased risk with DENV2 infection (Pedroso et al., 2019). The interpretation of the authors of the latter study was that "[their] results suggest that multitypic DENV infection may protect from, rather than enhance, development of CZS", choosing to ignore the increased odds of CZS with DENV1 of 3.3 (95 % CI =0.8– 13.5) (Pedroso et al., 2019, Table). The findings of the only cohort study are inconsistent with those of two published two case-control studies, but it is based on only 122 pregnancies of women infected with Zika infection, and interesting 47 % of them had adverse pregnancy outcomes (OR = 0.8). It would not be surprising that an increased risk of CZS among pregnant women is only associated with had high levels of neutralizing antibodies to the most common DENV, that is DENV1, but not all DENV. Our study did not evaluate the titers of neutralizing antibodies to DENV3. High levels of dengue antibody to the predominant serotype, could be indicative of secondary infections. The biological basis for the association between DENV antibody and risk of severe Zika infection has strong support by experimental data including the induction by DENV-specific antibodies in ZIKV-infected pregnant mice of 1) placental damage, 2) fetal growth restriction and 3) fetal resorption (Rathore et al., 2019). Studies in fetal mice demonstrated that DENV-specific antibodies in ZIKV-infected pregnant mice enhanced vertical ZIKV transmission and resulted in a severe microcephaly-like syndrome, which was dependent on the neonatal Fc receptor (Brown et al., 2019). Our findings suggested that circulating antibodies to dengue could have played a role in the occurrence of CZS and other severe manifestations of ZKV infection in Mexico and other countries in Latin America during the regional pandemic that caused almost 3,000 cases of CZS cases in Brazil alone, consistent with the hypothesis postulated by Halstead (2017). This hypothesis does not take away the central role of ZKV infection as the etiologic agent of CZS, but rather adds to the understanding of the contributing factors to the occurrence of CZS and possibly other associated pathologies such as GBS.

4.3 Limitations

The series of probable cases of CZS in this study was limited to one hospital and therefore, may not be entirely representative of all cases in the catchment area of the maternity hospital. We did not include the maternity hospitals of the Mexican Social Security Institute, the largest pre-paid (by employers and employees) healthcare organization or smaller public health organizations or private hospitals, which tend to serve a relatively more affluent populations than the hospitals under the Ministry of Health. Also, the case-referent study has several limitations. For one, the women in the referent group delivered normal newborns in early 2019, and they were not at-risk of having a newborn with CZS. However, the purpose

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of the comparison is to have a referent to compare the levels of dengue antibody. Undoubtedly their titers of DENV antibody were representative of the levels of infection of the underlying cohort from which the CZS cases arose. On the other hand, the close phylogenetic relation between ZKV and DENV underlying the cross-reactivity would have worked towards a dilution of the observed association. We only tested for two of the four different DENV serotypes, and all four have been reported in Chiapas. If any systematic error is present, we argue would be a conservative bias, that is biasing the results towards the null value (i.e., OR=1).

Another important limitation is the limited sample size (13 cases and 35 controls) which has a statistical power of only 20 % to detect an OR of 3 if the prevalence of dengue infection among controls was 80 % as suggested in our data. A larger study of size 300 (75 cases and 225 controls) would be needed to achieve an 80 % statistical power to detect an OR of 3.

5. Conclusions

The data indicates the need of adequate public health surveillance case definitions that are specific and simple to implement during pandemics such as the ZKV pandemic. The use of complex definitions as the one used since 2016 by the PAHO to monitor the ZKV epidemic and the most serious consequence of ZKV infection, CZS and GBS, comes at the high price of lack of credibility and loss of the trust of the public. The preliminary data is inconclusive but suggest the need of larger studies to test the hypothesis that circulating dengue antibody leads to ADE severe Zika infections (SGB and CZS) using at least 75 cases and testing for all evidence of dengue virus infection using highly specific neutralization tests.

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8. Annexes

Annex 1

Table 1. Clinical data (mother and infant) of 13 children with presumed Zika virus congenital infection seen at General Regional Hospital Pascacio Gamboa, Tuxtla Gutierrez, 2016-2019

Patient No	Sex	DOB/End of Pregnancy	US-1	US-2	Mothers Age (years)	Trimester of pregnancy with rash		Phase of microcephaly diagnosis	Head circumference at birth (cm)	Z-score circumference at birth	Status/age at follow-up visit	Zika virus in CSF	Findings on US/TFUS/MRI/CT	Other findings (Histopathology, Labs) and follow-up
1	Girl	1/2018	29	-	19	Unknown	37	Pregnancy	28.5	-2.9	Died 59 days.	NA	Microcephaly (-4 SD), herniation of the orbital fat to the skull by US, giant occipital-parietal encephalocele, hypoplastic corpus callousum and absence of ventricles by MRI	Myelomeningocele, brainstem dysplasia, microcephaly
4	Girl	7/2018	12	13	32	Unknown	36	Birth	29.5	-3.2	Died 44 day.	NA	Microcephaly, hypoplastic left ventricle by US; hypoplastic corpus callosum by TFUS (Supp. Fig. 1 a-b)	Cleft palate, heart defects (hypoplastic right heart, tricuspid atresia and ventricular septum defect) by echocardiography (Supp. Fig. 1 c)
6	Girl	8/2016	21	27	41	First	33	Pregnancy	27	-3.6	Alive- 2 years	NA	Microcephaly detected by US at 28 weeks of gestation (Suppl. Fig. 2 a), fetal growth restriction and oligohydramnios by US; cysts germinal matrix, mild hydrocephalus, cystic encephalomalacia and cortical thinning by TFUS	At 32 months of age was able to stand but could not walk. Could babble but not speak more than 2 words.
7	Boy	8/2016	23	26	38	Unknown	39	Pregnancy	29	-3.6	Alive – 2years	NA	Hydrocephalus and microcephaly, right predominant ventriculomegaly by US (Suppl. Fig 3a); hydrocephalus (Evans 0.53, Suppl. Fig. 3 b); cysts germinal matrix and parenchymal cysts by TFUS (Suppl. Fig 3 c-d); basal ganglia calcifications, cortical thinning of both gyri and sulci by CT	Unable to walk, inconsolable crying, clubfoot, seizures, VIH (-), VDRL (-), CMV IgM (-), CMV IgG (+), Rubella IgG (+), Rubella IgM (-), Toxoplasma (-)

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8	Girl	10/2016	30	32	17	First	40	At birth	29	-3.8	Died at 1 year.	NA	Fetal growth retardation and severe oligohydramnios by US; microcephaly with absence of the septum pellucidum; intraventricular hemorrhage at the foramina of Monro by TFUS; hypoplasia of corpus callosum, brachycephaly, Dandy-Walker malformation, (Suppl. Fig. 4 a), thinning of sulci and gyri (Suppl. Fig. 4 b), in CT	Bronchopneumonia and disseminated intravascular coagulation on death certificate. Was able to say mom and dad at 8 months of age.
9	Girl	10/2016	5	11	21	Unknown	35	At birth	27	-3.3	Alive -2 years	NA	Severe oligohydramnios and enlargement of lateral ventricles by US (Suppl. Fig. 5); hypoplastic corpus callosum, lissencephaly and thin cortex by TFUS	Unable to walk, or seat, inconsolable crying, hip dysplasia, strabismus. VIH (-) VDRL (-)
11	Boy	2/2017	30	31	22	Unknown	40	Pregnancy	27	-4.6	Alive -2 years	NA	Fetal growth retardation and absence of midline structures and hypogenesis of frontal bone and nasal septum by US; hypogenesis of corpus callosum and basal ganglia calcifications by TFUS	Unable to walk, blind, deaf, able to babble at 4 months of age.
12	Boy	7/2017	23	30	30	Unknown	41	Pregnancy	28	-4.8	Alive	NA	Microcephaly (Suppl. Figure 6) and oligohydramnios by US	Mute
13	Boy	5/2016	35	36	24	Unknown	37	Pregnancy	27	-5.0	Died at 2 years	NA	Microcephaly and myelomeningocele (Suppl. Fig 7 a and b), fetal growth retardation and polyhydramnios by US	Seizures, multiple arthrogryposis, microcephaly in death certificate
16	Boy	11/2016	14	24	30	First	35	Pregnancy	28	-2.9	Died at 47 days	NA	Microcephaly and clubfoot by US;	Multiple arthrogryposis, CT documented

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													Ventriculomegaly with dangling choroid plexus, encephalic calcifications, and abnormal slits in the brain by US; periventricular calcifications; ventriculomegaly, y with periventricular calcifications; thinning of sulci and gyri by CT	microcephaly, craniosynostosis, CMV (-), Toxoplasma (-), Rubella (-)
17	Girl	1/2017	-	-	23	First	39	Pregnancy	30	-2.8	Alive	NA	Microcephaly by TFUS	Clubfoot.
18	Boy	5/2016	18	19	31	Unknown	38	Pregnancy	25	-5.4	Stillbirth	NA	Microcephaly with Occipital encephalocele and overlapping cranial sutures and bell-shaped chest and polycystic kidney by US	--
19	Girl	9/2016	6	35	23	Unknown	37	Pregnancy	32	0.5	Alive	Positive	Strawberry shaped head, microcephaly, ventriculomegaly, hydrocephalus (Suppl Fig 8 a - c), hypogenesis of corpus callosum, obliteration of cisterna magna (Supp. Fig. 8 d) sacral meningocele (Suppl. Fig. 8 e), by US	Clubfoot, hip dysplasia. HSV IgG (+) IgM (-), Chikungunya (-) DENV NS1 (-)

US=ultrasound; TFUS=transfontanellar ultrasound; CsF=cerebrospinal fluid; MRI=magnetic resonance imaging; CT=computer tomography scan, NA=not available

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Annex 2

Table 2. Early development of 9 of the 13 children with probable congenital Zika syndrome, Tuxtla Gutierrez, Chiapas, 2019 7

ID	Z-score of Head Circumference	Age at follow-up |evaluation	Movement/Physical (months normal)						Language/Cognitive (months normal)					
			Held head up w/o support (4)	Rolls over (6)	Seats with support (5)	Seats without support (6)	Crawls (9)	Stands (9)	Turns head toward sound (2)	Begins to smile at people (2)	Follow things with eyes and recognize people at a distance (2)	Babbling (4)	Says 'mama' and 'papa' (9)	Tries to say words spoken (12)
1	-2.9	Deceased 2 mos.	NA						1	NA	NA			
4	-3.2	Deceased 7 mos.	4	6	6				1	4	7	5	6	
6	-3.6	Alive 32 mos.	4	18	12	30	Not then	12	6 - 8	2 - 3	2 - 3	Not then	24	Not then
7	-3.6	Alive 31 mos.	Was Not Evaluated											
8	-3.8	Deceased 12 mos.	Did not	Did not	Did not	Did not	Did not	Did not	4	7	2	Did not	7-8	Did not
9	-3.3	Alive 29 mos.				Not then		Not then				4	9	Did not
11	-4.6	Alive 24 mos.	Not then	12	Not then	Not then	Not then	Not then	1	4	9 - 10	4	Not then	Not then
12	-4.8	Alive 20 mos.				9		9	Not then				Not then	Not then
13	-5.0	Deceased 26 mos.					Did not							
16	-2.9	Deceased 1 mo.												
17	-2.8	Alive 26 mos.	5	14	11	12	12	Not then	NA	NA	NA	10	9	NA
18	-5.4	Stillbirth	Was not Evaluated											
19	0.5*	Alive 30 mos.						Not then						

*Had hydrocephalus

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Annex 3

Table 3. Characteristics of cases of probable congenital Zika syndrome and their controls, Hospital Pascacio Gamboa, 2017-2019

Characteristics		Cases	(%)	Controls	(%)	OR (95% CI)
Zika antibody titer by $PRNT_{80}$						
	Present	13	100.0	31	88.6	2.1 (0.3, ∞)
	Absent	0		4		1
	Total	13		35		*P*-value = 0.4
Fetal sex						
	Female	9	69.2	20	57.1	1.7 (0.4, 6.3)
	Male	4		15		1
	Total	13		35		*P*-value = 0.5
Maternal age						
	21+	11	84.6	20	57.1	4.4 (0.9, 22.9)
	16-20	2		15		1
	Total	13		35		*P*-value = 0.1
Maternal education						
	<9 grade	12	92.3	25	65.7	6.2 (0.7, 53.4)
	10+ grade	1		13		1
	Total	13		35		*P*-value = 0.08
Periconceptional folic acid supplementation						
	Yes	2	15.4	3	8.6	1.9 (0.3, 13.2)
	No	11		32		1
	Total	13		35		*P*-value = 0.6
Use water containers						
	Yes	13	100.0	33	86.8	2.5 (0.4, ∞)
	No	0		5		1
	Total	13		35		*P*-value = 0.3
Regular bed net use						
	Yes	6	46.2	20	57.1	0.7 (0.2, 2.5)
	No	7		15		1
	Total	13		35		*P*-value = 0.7

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Annex 4

Table 4. Characteristics of cases of probable congenital Zika syndrome and their controls, Hospital Pascacio Gamboa, 2017-2019

Titers	Cases	(95% CI)	Controls	(95% CI)	t-test *P*-value
DENV1 Geometric mean (log_{10})	548.7	(234.3, 1285.0)	363.2	(219.1, 603.7)	0.38
DENV2 Geometric mean (log_{10})	119.0	(30.0, 471.6)	161.7	(75.1, 348.4)	0.68
DENV1 >1:80 or DENV2 >1:80 $PRNT_{80}$ Ab	Cases	(%)	Controls	(5)	Exact odds ratio (95% CI)
Present	12	92.3	31	88.6	1.5 (0.1, 82.5)
Absent	1		4		1
Total	13		35		*P*-value = 1.0
DENV1 $PRNT_{80}$ Ab	Cases	(%)	Controls	(%)	Exact odds ratio (95% CI)
640+	10	76.9	21	60.0	2.2 (0.5, 14.6)
<640	3		14		1
Total	13		35		*P*-value = 0.2
Ratio of DENV1 to DENV2 $PRNT_{80}$ Ab	Cases	(%)	Controls	(%)	Exact odds ratio (95% CI)
≥10	5	38.5	5	14.3	3.6 (0.7, 20.5)
<10	8		30		1
Total	13		35		*P*-value=0.08
Ratio of DENV1 to DENV2 ≥10$PRNT_{80}$ Ab Adjusted for					Exact odds ratio (95% CI)
Maternal age					2.7 (0.5, 15.8)
Maternal education					2.9 (0.5, 16.71)
Periconceptional folic acid supplementation					3.6 (0.7, 20.5)
Use water containers					3.1(0.8, 18.0)
Bed net use					3.3 (0.6, 19.8)
Use of water containers and bed net use					5.5 (0.7, 70.8)

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