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RESEARCH ARTICLE

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# Epidemiological and clinical suspicion of congenital Zika virus infection: Serological findings in mothers and children from Brazil

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## Abstract

The emergence of Zika virus in the Americas has caused an increase of babies born with microcephaly or other neurological malformations. The differential diagnosis of Zika infection, particularly serological diagnosis, is an important but complex issue. In this study, we describe clinical manifestations of 94 suspected cases of congenital Zika from Bahia state, Brazil, and the results of serological tests performed on children and/or their mothers at an average of 71 days after birth. Anti-Zika immunoglobulin M (IgM) antibodies were detected in 44.4% and in 7.1% of samples from mothers and children, respectively. Nearly all the IgM, and 92% of immunoglobulin G positive results were confirmed by neutralization test. Zika specific neutralizing antibodies were detected in as much as 90.4% of the cases. Moreover, dengue specific neutralizing antibodies were detected in 79.0% of Zika seropositive mothers. In conclusion, Zika IgM negative results should be considered with caution, due to a possible rapid loss of sensitivity after birth, while the NS1-based Zika IgM enzyme-linked immunosorbent assay test we have used has demonstrated to be highly specific. In a high percentage of cases, Zika specific neutralizing antibodies were detected, which are indicative of a past Zika infection, probably occurred during pregnancy in this population.

**KEYWORDS**

congenital infection, diagnosis, flavivirus, microcephaly, neutralization test, serological tests

## 1 | INTRODUCTION

Zika virus (ZIKV) was first identified in 1947 in Africa, and then sporadically detected among humans in Africa and Asia, where it has likely been endemic for decades.[1] Starting from 2007, several large

**Abbreviations:** b.l., border line; CHIKV, chikungunya virus; DENV, dengue virus; EI, exanthematic illness; HSA-OSID, Santo Antônio Hospital of the Obras Sociais Irmã Dulce; ISS, Istituto Superiore di Sanità; PRNT, Plaque Reduction Neutralization Test; WHO, World Health Organization; ZIKV, Zika virus.

ZIKV outbreaks occurred, such as those in Micronesia and French Polynesia.[2] In March, 2015, autochthonous virus transmission was first detected in Brazil.[3] ZIKV has since then rapidly spread throughout South and Central America, and the Caribbean. These regions had already been interested, in the last decades, by a dramatic increase in dengue virus (DENV) circulation, with the simultaneous co-circulation of the four serotypes in several areas, and also by the emergence of other arboviruses, such as West Nile and chikungunya virus (CHIKV).[4-6]

Illness resulting from ZIKV infection is typically mild and self-limiting. The majority (approximately 80%) of ZIKV infections have been estimated to be asymptomatic.[2] However, since 2013, an increased incidence of neurological symptoms following ZIKV acute infection, including the Guillain Barré syndrome, has been reported.[7] Furthermore, the emergence of ZIKV in the Americas coincided with increased reports of babies born with microcephaly and brain and ocular malformations.[8-13] The causal association was acknowledged by the World Health Organization (WHO)[14] and by the US Centers for Disease Control and Prevention in April, 2016,[15] and further supported by accumulating evidence.[10,11,16-21]

Differential diagnosis of ZIKV infection, particularly the diagnosis of congenital ZIKV infection, as well as the screening of pregnant women for detection of ZIKV infection, are important but complex issues.[22] Molecular detection of the virus is the golden standard for differential diagnosis, but it is limited by the short or variable persistence of the virus in different biological fluids both of the mother and the fetus.[10,23-26] Serological diagnosis of ZIKV infection is challenging, mainly due to the high cross-reactivity between flaviviruses.[27-29] It includes an initial screening for anti-ZIKV immunoglobulin M (IgM), followed by confirmation using a neutralization test, the standard serological assay for distinguishing between different flaviviruses. NS1-based enzyme-linked immunosorbent assay (ELISA) IgM tests have demonstrated to be highly specific,[30] but their sensitivity might be limited among ZIKV infected patients with past flavivirus infections.[31,32] Moreover, while neutralization tests are highly specific in case of primary flavivirus infection, secondary flavivirus infections often stimulate the original antigenic sin phenomenon, leading to significant neutralizing antibody cross-reactivity between closely related flaviviruses.[26,33] However, it is not known whether these cross-reactive neutralizing antibodies are durable. Notably, pre-existing immunity to DENV might enhance infection with ZIKV, leading to increased disease severity, with possible implications also for the risk of development of fetal disease.[34-37]

The main aim of the present work is to describe the results of serological tests performed on serum samples collected from children with a suspected congenital ZIKV infection and/or from their mothers. Overall, we have analyzed 94 suspected cases of congenital ZIKV infection from Bahia state, an area which has been heavily affected by the ZIKV epidemic.[38,39] Clinical manifestations, together with bioimaging findings, are also described.

## 2 | MATERIALS AND METHODS

### 2.1 | Studied population and samples

In this study, the cases ($n = 94$) were recruited among children referred to the pediatric neurology service of Santo Antônio Hospital of the Obras Sociais Irmã Dulce (HSA-OSID) in Salvador City, north-eastern Brazil, starting from November 2015. In all the cases, the informed consent was obtained from each child's parent or guardian. The children were born in the period between March 2015 and February 2016. For most of the cases, a maternal history of exanthematic illness (EI)[38] and/or of contact during pregnancy with relatives who received an EI diagnosis during the arbovirus infection outbreak, was reported. Serological tests for rubella, toxoplasmosis, and cytomegalovirus, were performed during pregnancy. General clinical examination and Computed Tomography Scan of the skull were performed for all the children. The contour curves of the children's growth and cephalic perimeter were determined in accordance with the percentiles established by WHO.[40] All serum samples, both of children and/or of their mothers, were collected during the child first visit at the pediatric neurology service. The age of the children at the time of sample collection ranged from 3 to 331 days after birth, with a mean of 71.0 ± 67.5 standard deviation, and a median of 53 days. Overall, 160 serum samples from the 94 cases were analyzed (90 samples from the mothers and 70 samples from the children): for 66 cases, samples were obtained both from the children and from the mothers, while for 24 cases only from the mothers, and in four cases only from the children.

### 2.2 | Serological assays

Serological assays were performed by the Italian National Reference Laboratory for Arboviruses of the Istituto Superiore di Sanità (ISS). The IgM and IgG antibodies against ZIKV were detected using commercial ELISA systems (Anti-ZIKV IgM/IgG ELISA, DiaPro, Diagnostic Bioprobes s.r.l, Sesto San Giovanni, MI, Italy). Absorbance was measured at 450 nm using an ELISA reader, according to manufacturer's instructions. Sample optical density readings were compared with reference cut-off optical density readings to determine results. Index values more than 1.1 for ZIKV were considered presumptive for the presence of IgM/IgG antibodies, while values between 0.9 and 1.1 were considered as border line (b.l.). Both Elisa IgM and IgG tests were performed for all serum samples, both from the mothers and the children. Plaque Reduction Neutralization test (PRNT) was carried out in six-well tissue culture plates with VERO cell monolayers (approximately 70% confluence). The following viruses were used: serotype 2 DENV (NGB strain), a CHIKV strain isolated from a patient during the 2007 Italian outbreak,[41] and the ZIKV H/PF/2013 strain of the Asian genotype (kindly provided by Dr Isabelle Leparc-Goffart of the French National Reference Center on Arboviruses in Marseille).[42] Sera were diluted 1:20 in serum-free maintenance medium, heat-inactivated, and tested in duplicate. Equal volumes (100 µL) of DENV/CHIKV/ZIKV dilution containing approximately 80 Plaque Forming Units (PFU), and serum

dilutions, were mixed, and incubated overnight at 4°C. Subsequently, VERO cells plates were infected with 200 µL/well of virus-serum mixtures in duplicate. After 1 hour incubation at 37°C and 5% $CO_2$, the inocula were aspirated and the wells were overlayed with a mixture of one part 2% Gum Tragacanth and one part of supplemented medium (2× minimal essential medium, 2.5% inactivated fetal calf serum and 2% 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]). The plates were incubated at 37°C and 5% $CO_2$ for 2 (CHIKV), 7 (DENV), 4 (ZIKV) days, and then were stained with 1.5% crystal violet. A titration of CHIK/DEN/ZIK viruses with three dilutions in duplicate (the working dilution, 1:2 and 1:8 dilutions) was performed in each assay and used as a control for the assay. Reciprocal of the serum dilution more than or equal to 20 that gave an 80% reduction of the number of plaques (PRNT80 ≥ 20) was considered as positive. Reciprocal of the serum dilution more than or equal to 20 that gave a 50% reduction of the number of plaques (PRNT50 ≥ 20) was considered as b.l. PRNT for ZIKV was performed in all mothers' serum samples. It was also performed in a subset (46/70) of children' samples, to assess that ZIKV infection in the mothers had been before the birth of the infants: indeed, because of the transplacentar transfer of maternal antibodies, lack of antibodies in children samples was interpreted as the mothers having acquired the infection after the birth of the infant. PRNTs for DENV and CHIKV were performed in all mothers' serum samples.

## 3 | RESULTS

### 3.1 | Epidemiological and clinical features

All the patients lived in Bahia, Brazil. For 93 of the 94 cases (98.9%), birth took place in the hospital and only in one case at home. 50/94 (53.2%) children were born by normal delivery, while 44/94 (46.8%) after a cesarean section. Among the children, 57/94 (60.6%) were female and 37/94 (39.4%) male. Among the mothers who were tested during pregnancy for other possible congenital infections, 50/56 were IgG positives and 1/56 IgM positive for cytomegalovirus, 30/56 IgG and 3/56 IgM positives for toxoplasmosis, and 31/45 IgG positives for rubella. Overall, these seroprevalence data are comparable with data available from the same area.[12,43] The clinical findings at birth were: the cephalic perimeter varied between 20.5 and 36.5 centimeters, with a mean of 30.3 + 2.7 standard deviation; microcephaly was observed in 73/94 (77.6%) of the cases. In the first neurological examination, facial skull disproportion, irritability, hypertonia, and global hyperreflexia were observed. The main abnormalities observed in transfontanelar ultrasonography or computed tomography scan of the skull scan were: microcephaly, ventriculomegaly, malformations (lissencephaly, cerebellar hemisphere hypoplasia, agenesis of corpus callosum, and cerebellar vermis), and diffuse encephalic calcifications. The main aspects in clinical and bioimaging findings are listed in Table 1. For 5/94 (5.3%) children bioimaging exams were not performed.

In 70 of the 94 cases, the mother reported an EI occurring during pregnancy. In 22 of the 94 cases the mother did not develop any symptom during pregnancy, but in eight of these cases the father or other relatives reported an EI occurring during pregnancy. Finally, in two of the 94 cases, information on exposure to ZIKV infection during pregnancy was not available. The period of pregnancy in which the mothers were exposed to infection was also investigated. In 39 of the 70 (55.7%) cases the ZIKV-like infection happened in the first trimester of pregnancy; in 22 of the 70 (31.4%) it occurred in the second trimester; in nine of the 70 (12.9%) cases it occurred during the third trimester.

**TABLE 1** Main clinical and bioimaging findings in children

| Clinical aspects | *n* = 94 |
|---|---|
| Microcephaly | |
| With closed fontanelle and facial skull disproportion | 68/94 (72.3%) |
| With open fontanelle | 05/94 (5.3%) |
| Without Microcephaly | 21/94 (22.3%) |
| Hypertonia and global hyperreflexia | 64/94 (68.1%) |
| Bioimaging aspects | *n* = 94 |
| Lissencephaly/pachygyria | 32/94 (34.0%) |
| Dysgenesis/agenesis of corpus callosum | 13/94 (13.8%) |
| Hydrocephalus | 49/94 (52.1%) |
| Encephalic calcifications (diffuse or periventricular) | 58/94 (61.7%) |
| Without bioimaging examination | 05/94 (5.3%) |

### 3.2 | Serological laboratory findings in mothers and children

The results of ZIKV ELISA IgM and IgG tests, and PRNTs for ZIKV, DENV, and CHIKV in mothers and children serum samples are shown in Table 2. IgM antibodies specific for ZIKV were detected in 44.4% (24 plus 16 b.l./90) of the mothers, and only in 7.1% (2 positive plus 3 b.l./70) of the children. IgG antibodies specific for ZIKV were detected in 96.7% (87/90) of the mothers (median index value:

**TABLE 2** Serological laboratory findings in mothers and children

| | Mothers, positives/tested (%) | Children, positives/tested (%) |
|---|---|---|
| ELISA IgM ZIKV | 24 + 16b.l./90 (44.4%) | 2 + 3b.l./70 (7.1%) |
| ELISA IgG ZIKV | 87/90 (96.7%) | 62/70 (88.6%) |
| PRNT ZIKV | 81/90 (90.0%) | 39/46 (84.8%) |
| PRNT DENV | 71/90 (78.9%) | |
| PRNT ChikV | 9/90 (10.0%) | |
| PRNT ZIKV + Denv | 64/81 (79.0%) | |
| PRNT ZIKV + Denv + ChikV | 7/81 (8.6%) | |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; DENV, dengue virus; IgG, immunoglobulin G; GPRNT, plaque reduction neutralization test; ZIKV, Zika virus.

11.4; range: 1.1-12.6), and 88.6% (62/70) of the children (median index value: 8.8; range: 1.1-12.2). ZIKV-specific neutralizing antibodies were detected in 90.0% (81/90) of the mothers and 84.8% (39/46) of tested children: overall, as much as 90.4% (85/94) of the cases were positive for ZIKV in PRNT (PRNT80 > 20), in samples from the mother, the child, or both. All but one of the 45 (97.8%) samples with a ZIKV ELISA IgM positive/b.l. result were confirmed by PRNT for ZIKV. Of the ZIKV ELISA IgG positive mothers, 7/87 (8.0%) were considered as possible cross-reactions against different flaviviruses, as they were ZIKV PRNT negative ($n = 2$) or b.l. ($n = 5$), while all seven were DENV PRNT positives. Of the ZIKV ELISA IgG positive children tested in PRNT for ZIKV, 3/41 (7.3%) were considered as possible cross-reactions toward different flaviviruses, as they were ZIKV PRNT negative ($n = 2$) or b.l. ($n = 1$) while their mothers were DENV PRNT positives. Considering the ZIKV ELISA IgG indexes, 95.9% (71/74) of the mothers and 100% (34/34) of the children with an ELISA index > 5.5 were confirmed by PRNT for ZIKV, in agreement with results from the literature.[44-46] Results of PRNT for ZIKV were very similar in mother-child pairs (tested pairs $n = 42$): overall, concordant results were obtained for 39/42 (92.9%) tested pairs (for 36/42 pairs both the mother and the child showed a positive PRNT80 > 1:20 result, 1/42 both b.l. PRNT50 > 1:20, 2/42 both negative). In 2/42 pairs the mother showed a b.l. PRNT50 > 1:20 result for ZIKV while the child showed a negative result. Finally, in only one case the mother showed a positive PRNT80 > 1:20 result for ZIKV while her child showed a b.l. PRNT50 > 1:20 result.

Interestingly, as much as 78.9% (71/90) of the tested mothers were seropositive for DENV by PRNT; moreover, 79.0% (64/81) of ZIKV seropositive mothers were also seropositive for DENV. Anti CHIKV specific antibodies were detected in 10.0% (9/90) of the mothers (Table 2).

We subsequently evaluated serological tests results obtained with mothers' and/or children' serum samples with respect of CDC case classification criteria,[47] which were approved in June, 2016. All the children in this study fully met the CDC clinical and epidemiological criteria for Zika disease, congenital, but none met the laboratory criteria, since none of them have had a serum sample collected within 2 days of birth. With respect of the mothers, they also all met the CDC clinical (ie, complication of pregnancy: neonate with congenital microcephaly, congenital intracranial calcifications, other structural brain or eye abnormalities, or other congenital central nervous system-related abnormalities including defects such as clubfoot or multiple joint contractures) and epidemiological criteria for Zika disease, noncongenital, and were classified as follow on the base of laboratory findings: 6/90 confirmed cases (ZIKV IgM positive/b.l. plus ZIKV PRNT positive and DENV PRNT negative), and 33/90 probable cases (ZIKV IgM positive/b.l. plus ZIKV PRNT positive and also PRNT positive for DENV). However, for 2/33 probable and 1/6 confirmed cases, an infection by cytomegalovirus or toxoplasmosis, possibly occurred during pregnancy, could not be excluded. Overall, because of the detection of ZIKV-specific neutralizing antibodies in mothers and/or children samples, a ZIKV infection, possibly occurred during pregnancy, cannot be excluded for as much as 90.4% (85/94) of the study cases.

## 4 | DISCUSSION

The diagnosis of suspected congenital Zika syndrome is based on clinical maternal history of EI, and/or exposure to arbovirus infection during pregnancy, and on clinical and laboratory data, and requires the exclusion of other causes of congenital infection. In this study, we have performed a serological investigation on serum samples obtained from children with a suspected ZIKV congenital infection and/or from their mothers. Samples were collected at the time of children examination at the pediatric neurology service of HSA-OSID, which did not occur immediately after birth in most cases. All the children were born during the neonatal microcephaly epidemic peak; their clinical presentation was highly suggestive of a ZIKV congenital infection in most cases. Besides microcephaly, other alterations have been observed in this children population by clinical neurological examination and bioimaging of the brain, which have been already described in the literature for ZIKV congenital infection cases.[48] The predominance of female patients observed in this study needs further investigation, considering that the demographic data of live births in the state of Bahia, Brazil, for the year 2015, correspond, in greater number, to the masculine gender.[49]

With respect to IgM detection, we used a commercial, NS1-based, ELISA test (Diapro). This test is routinely used at the Italian National Reference Laboratory for the diagnosis and surveillance of arbovirus infections, and has been evaluated through the comparison with another commercial, NS1-based, ELISA test (Euroimmun) and with PRNT and molecular tests, and assessed to be highly sensitive and specific for the diagnosis of imported, acute, ZIKV infections (manuscript in preparation). We then performed PRNTs for ZIKV, DENV, and CHIKV. At the Italian National Reference Laboratory, samples of patients not exposed to arboviruses in their own country, such as travelers, are routinely tested: samples able to reduce the 80% of plaques at a dilution of 1:10 are usually considered as positive, while samples able to reduce the 50% of plaques at a dilution of 1:10 are considered as b.l.[50] For this population of Brazilians, we chose a higher cut off, considering as positive those samples able to reduce the 80% of plaques at a dilution of 1:20.

With respect of the mothers, our ZIKV ELISA IgM test results (44.4% positives/b.l.) are comparable to those reported by Cordeiro et al.[51] Conversely, the percentage of ZIKV IgM positive results among children (7.1%) in our study is much lower than the 27.0% of positivity reported by de Araújo et al,[12] and 90.5% reported by Cordeiro et al.[51] We think it is unlikely that this difference is due to the different methods used to detect IgM antibodies, since the results obtained with the mothers' samples with the same test are comparable to those already reported in the literature. Conversely, we hypothesize that it may be due to the different mean age of children at the time of sampling (71.0 ± 67.5 standard deviation days, while both in Cordeiro and in de Araújo studies, samples had been collected from neonates few days after birth). Thus, IgM negative results in children should be considered with caution due to a possible rapid loss of sensitivity of this test after birth. This stresses

the importance, when possible, of timing collection of samples, both from newborns and from their mothers, as well as of collecting different types of samples other than serum, such as cerebrospinal fluid.[12,51]

The specificity of serological tests in the context of co-circulation of different arboviruses is still a matter of discussion.[26,33] The main limitation of our study is the lack of a control population (healthy children, matched by date of delivery, and area of residence), so that definitive conclusions cannot be drawn from our laboratory data. However, the prevalence of ZIKV-specific IgG and neutralizing antibodies in our population was comparable to those reported among mothers of neonates born with microcephaly in the same area and in the same period.[12,52] The high DENV seroprevalence we have observed, and the lower prevalence for CHIKV compared to DENV and ZIKV, are also comparable with data available from the literature.[52-54]

Finally, in agreement with other evidence,[30,51] NS1-based ELISA IgM test showed good specificity in this population, and nearly all positive results were confirmed by PRNT. Moreover, 92% of positive results obtained by the ELISA IgG test were confirmed by PRNT.

With respect to the specificity of neutralization tests, ZIKV cross-reactive neutralizing antibodies induced by DENV infection have been reported not to be durable,[55] suggesting that neutralization tests for ZIKV should be sufficiently specific in late convalescent-phase sera. Efforts to define ZIKV neutralization tests cut off able to distinguish past ZIKV infections from immunity to previous flavivirus infections, as well as toward the standardization of PRNTs, are needed.

Overall, PRNT seems to be from our data the more sensitive and specific test for the diagnosis of past ZIKV infection, even if it cannot be used to determine timing of infection. At the moment, the presence in mothers and/or in children samples of ZIKV-specific neutralizing antibodies should be interpreted, in our opinion, as a possible ZIKV infection during pregnancy.

In this population, a high percentage of ZIKV PRNT positive mothers were also DENV PRNT positive (79.0%). Whether a previous DENV immunity may represent an additional risk factor for the development of ZIKV infection associated congenital syndrome is another important matter of discussion: however, again, only the analysis of a control population (mothers of healthy children), and also DENV and other arboviruses seroprevalence studies in areas of ZIKV circulation, will allow to draw conclusions from our and others' data.

While the possibility of some infants without apparent clinical findings at birth, but who may have complications from congenital ZIKV infection, has been documented,[56] guidance for testing and case definition for children visited not immediately after birth are not yet defined.[57,58] Although ZIKV-specific neutralizing antibody titers have been shown to be significantly higher in mothers of children with microcephaly than in mothers of children born without microcephaly, suggesting the potential utility of maternal antibody titers to corroborate congenital ZIKV infection,[43] an absolute titer threshold suggestive of congenital infection has not been indicated.

In conclusion, the evidence of congenital infection in a large proportion of cases of suspected ZIKV-associated malformations cannot be definitely proved. Our data confirm the difficulty of an accurate retrospective diagnosis of ZIKV congenital infection and the urgent need of further evaluation of available serological tests, as well as the development of innovative tools.

## 5 | DECLARATIONS

### 5.1 | Ethics approval and consent to participate

The study was submitted and approved in accordance with the endorsement of the research ethics committee number 2.254.083. In all the cases, the informed consent was obtained from each child's parent or guardian. In this study, no experiments involving recruitment of humans, nor animals, have been performed. No data attributable to individual patients are presented in this manuscript.

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### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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