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1 **Title:** Zika virus IgM-specific based diagnostic is highly correlated with detection of

2 neutralising antibodies in neonates with congenital disease

3 **Running title**: Zika virus antibodies in neonates with microcephaly

4 **Authors**: Marli T. Cordeiro, Aggeu Magalhães Research Center, Fundação Oswaldo Cruz

5 (FIOCRUZ-PE), Recife, PE, Brazil; Carlos A. A. Brito, Department of Clinical Medicine,

6 Federal University of Pernambuco (UFPE), Recife, Brazil; Lindomar J. Pena, Priscila M. S.

7 Castanha, Laura H. V. G. Gil, Kennya G. S. Lopes and Rafael Dhalia, Aggeu Magalhães

8 Research Center, Fundação Oswaldo Cruz (FIOCRUZ-PE), Recife, PE, Brazil; Jucille A.

9 Meneses, Ana C. Ishigami and Luisa M. Mello, Department of Pediatrics, Instituto de

10 Medicina Integral Professor Fernando Figueira (IMIP), Recife, Brazil; Liciana X. E. Alencar

11 and Klarissa M. Guarines, Central Public Health Laboratory, Secretaria de Saúde do Estado

12 de Pernambuco, Recife, Brazil; Laura C. Rodrigues, Faculty of Epidemiology and Population

13 Health, London School of Hygiene and Tropical Medicine, London, England; Ernesto T. A.

14 Marques, Aggeu Magalhães Research Center, Fundação Oswaldo Cruz (FIOCRUZ-PE),

15 Recife, PE, Brazil, and Center for Vaccine Research and Department of Infectious Disease

16 and Microbiology, University of Pittsburgh, Pittsburgh, PA

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Corresponding Author (Pos-Publication): Ernesto T. A. Marques, Departamento de Virologia, Centro de Pesquisas Aggeu Magalhães-CPqAM, FIOCRUZ-PE, Av. Professor Moraes Rego, SN, Campus da UFPE, Cidade Universitária, Recife, PE, Brazil, CEP 50.740.465, Tel. 55-00-81-2101-2500. Email: marques@pitt.edu; emarques@cpqam.fiocruz.br

19 **ABSTRACT**

20 **Background.** Usually IgM serology is not sufficiently specific to confirm Zika infection.
21 However, since IgM does not cross the placenta it may be a good marker of infection in
22 neonates.

23 **Methods.** We tested blood from 42 mothers and neonates with microcephaly; and collected
24 cerebrospinal fluid (CSF) from 30 neonates. Molecular assays were performed for ZIKV,
25 DENV and CHIKV; ELISA-IgM and PRNT for ZIKV and DENV. No control neonates
26 without microcephaly were evaluated.

27 **Results.** In neonates, 38/42 (90.5%) were ZIKV-IgM positive, all 30 CSF were positive and
28 considering blood or CFS all 42 neonates were ZIKV-IgM positive. ZIKV-IgM results were
29 higher in CSF (median 14.9 [9.3-16.4]) than in serum (median 8.9 [2.1-20.6]) p=0.0003. All
30 ZIKV-IgM positive results in the neonates were confirmed by the presence of neutralising
31 antibodies (NAb). ZIKV-primary infection had NAb to ZIKV only; secondary
32 Flavivirus/ZIKV-infected cases had high titter of NAb to ZIKV; mothers median was 2,072
33 (232-12,980) and neonate 2,730 (398-12.980), p <0.0001; CSF median was 93 (40-578),
34 p<0.0001.

35 **Conclusion.** Detection of Zika IgM in the neonate is confirmatory of congenital Zika and in
36 the CSF of neurologic infection. Therefore, we recommend testing of Zika IgM in neonates
37 suspected of congenital Zika and PRNT in equivocal cases.

38 **Keywords:** Zika virus, microcephaly, congenital Zika, Anti-ZIKV IgM, ZIKV antibodies.

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44 **INTRODUCTION**

45 Zika virus (ZIKV) was first detected in Northern Brazil in March 2015 in the serum of
46 patients with a dengue-like illness. Phylogenetic analysis demonstrated that the Brazilian
47 strain belonged to the Asian lineage and was highly related to a ZIKV isolate from French
48 Polynesia [1]. By the end of year, the virus had spread to 19 of the 27 Brazilian states [2] and
49 by April 2016 to 34 countries and territories in America. An epidemic of microcephaly started
50 in Brazil in October, six to seven months after health authorities confirmed ZIKV
51 transmission in the country; and it was declared a Public Health Emergency of International
52 Concern by the World Health Organization (WHO) in February 1st, 2016 [3].
53 Based on the growing evidence that ZIKV was the etiological agent of the microcephaly, as
54 well as other manifestation of zika syndrome [4-10], the causal link between ZIKV and
55 microcephaly was finally recognized [11]. The hesitancy to accept causation in the presence
56 of much epidemiological circumstantial evidence was related to the difficulty of laboratory
57 confirmation in affected infants.
58 Laboratory diagnosis of ZIKV is challenging. In an outbreak of ZIKV in Yap Island,
59 Federated States of Micronesia, in 2007, Lanciotti and colleagues [12] analysed serum
60 samples from ZIKV-infected patients by using serological assays, such as detection of IgM
61 antibodies by IgM-capture enzyme-linked immunosorbent assay (ELISA) and plaque
62 reduction neutralization test (PRNT) to diagnose and evaluate the extent of cross-reactivity
63 among flaviviruses. They found marked cross-reactivity with other flaviviruses, mainly in
64 patients where the ZIKV was the secondary flavivirus infection; an accurate diagnosis
65 required combining serologic data to ZIKV detection by molecular methods [12].
66 The current recommendation for routine diagnosis of ZIKV infection include detection of the
67 viral nucleic acid by reverse transcription polimerase chain reaction (RT-PCR), detection of
68 specific IgM antibodies by ELISA and PRNT for confirmation of positive IgM result and

69 exclusion of other flaviviruses [13,14]. The cross-reactivity of flavivirus antibodies is
70 accepted as the main challenge in the interpretation of serologic test results, especially when
71 the PRNT is not available [12-14]. Laboratory diagnosis is an essential tool for research and
72 for public health prevention measures; thus, validation of commercial kits and a better
73 understanding of the performance of currently available kits for detection of IgM antibodies is
74 an urgent priority to diagnose ZIKV infection.
75 In this study, we compared results of serologic testing (ELISA) for ZIKV-IgM, dengue virus
76 (DENV) IgM and IgG antibodies and of ZIKV and DENV neutralising antibodies in serum
77 from 42 mothers and their neonates born with microcephaly. The levels of ZIKV-specific
78 IgM and neutralising antibodies in the CSF samples of 30 neonates were also assessed.
79

80 **METHODS**

81 **Population, study settings and ethic issues**

82 From 21 to 30 October 2015 we collected samples from 42 neonates with microcephaly and
83 from their mothers. Neonates were born between September 5 and October 27, 2015, in
84 hospitals located in the Metropolitan Region of Recife, Pernambuco State, Brazil. These cases
85 constituted the very first of microcephaly identified in this outbreak. No control neonates
86 without microcephaly born in this period were included in this study. Microcephaly was
87 defined as neonatal occipitofrontal circumference ≥ 2 SD below the mean for gestational age
88 and sex of the new born at birth [15]. We conducted this investigation as part of the routine
89 epidemiologic and laboratory surveillance and as such, ethical committee approval was not
90 required. However, we did collect mother´s written informed consent to participate.
91

92 **Sample collection and processing**

93 We collected blood from 42 mothers and neonates and CSF from 30 of the neonates;
94 specimens were sent to the Department of Virology at Fiocruz Pernambuco, under
95 refrigeration. Blood samples were spun down for 10 minutes at 1500 g and serum was stored
96 at –80º C. CSF samples were frozen at –80º C without processing. We carried out molecular
97 diagnostic assays for ZIKV, DENV and chikungunya virus (CHIKV) in the neonate
98 specimens (serum and CSF). Serologic test were performed for DENV and ZIKV in all
99 samples.
100
101 **Molecular assays**
102 Serum and CSF were tested by RT-PCR or, real-time RT-PCR (qRT-PCR) for detection of
103 ZIKV, DENV and CHIKV. Viral RNA was extracted using the QIAamp Viral RNA Mini Kit
104 (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed
105 for ZIKV using previously described methods with minor modifications [16] in which two
106 steps RT-PCR was used (cDNA synthesis followed by the PCR) instead of one step protocol.
107 For DENV a well-established RT-PCR protocol was employed [17]; for CHIKV, the qRT-
108 PCR was performed according to Lanciotti et al [18].
109
110 **Serologic assays**
111 DENV IgM and IgG antibodies - Serum samples from mothers and neonates were first tested
112 for dengue IgM and IgG antibodies detection using the commercial kits: Dengue IgM-capture
113 ELISA and Dengue IgG capture ELISA (PANBIO, Inverness Medical Innovations Australia
114 Pty Ltd), following the manufacturer´s guidelines.
115
116 ELISA for ZIKV and DENV**-** Serum and CSF were tested by IgM antibody capture ELISA
117 using the protocol [12] and reagents provided by the Centers for Disease Control and

118 Prevention (CDC, Fort Collins, CO, EUA). Samples were tested in parallel with ZIKV and
119 DENV antigens to investigate cross-reactivity; reagents were previously tittered and diluted
120 as appropriated. Briefly, plates were coated with 75μl of goat anti-human IgM (Kirkegaard
121 and Perry Laboratories, USA) in carbonate/bicarbonate (pH 9.6) and incubated overnight at 4°
122 C. After blocking with PBS pH 7.2, 5% non-fat dry-milk /0.05% tween 20, for 30 minutes at
123 room temperature (RT) and washing (done after each step), 50μl of serum samples diluted
124 1/400 in PBS pH 7.2, 0.05% Tween 20 and CSF undiluted; negative (pooled flavivirus-
125 negative serum) and positive (CDC humanized 6B6C-1 pan-flavivirus) controls, were added
126 and incubated at 37°C for 1 hour. Viral Zika antigen (CDC Vero E6 derived, inactivated
127 ZIKV antigen; Normal antigen (CDC Vero E6 derived, mock-infected normal antigen) and
128 dengue antigen: mixture of the four serotypes (Evandro Chagas Institute, Brazil) were added
129 to each sample (50μl) and incubated overnight at 4°C. Detecting antibody conjugate
130 (Horseradish peroxidase conjugated monoclonal antibody 6B6C-1, CDC) diluted in blocking
131 buffer was added: incubation for 1 hour at 37°C. The substrate TMB (3,3'5, 5'
132 tetramethylbenzidine base, Becton Dickson, USA) was added; after 10 minutes incubation at
133 RT the reaction was stopped with 1NH2SO4 solution and the optical density (OD) read at 450
134 nm. The ratio (P/N) was calculated as follows: Mean OD of the test sample reacted on viral
135 antigen (P) divided by the Mean OD of the negative control reacted on viral antigen (N). P/N
136 value <2.0 was negative; >3.0 was positive; and between 2.0 and 3.0 was considered
137 equivocal. If the sample showed some degree of cross-reactivity it was considered ZIKV-IgM
138 positive only when the Zika P/N was, at least, twice higher than the dengue P/N.
139
140 Plaque Reduction Neutralization Test – PRNT50 was performed following a modified
141 protocol previously described elsewhere [19]. Briefly, tests were carried out on Vero cells
142 seeded at the density of 300,000 cells/mL using 24-well plates. The assay was performed after

143 serial dilution of serum (inactivated 30 minutes at 56º C) and CSF (1/20 to 1/20,480) and
144 incubation of 100 PFU challenge viruses, previously tittered. The assay was carried out using
145 viruses isolated in the State of Pernambuco: ZIKV (Brazil-PE243/2015); DENV-1 (BR-
146 PE/97-42735), DENV-2 (BR-PE/95-3808), DENV-3 (BR-PE/02-95016) and DENV-4 (BR-
147 PE/12-008), molecularly characterized. Neutralising antibody titters were determined by
148 using a PRNT with a 50% cut-off value (PRNT50); ZIKV and DENV serotype-specific
149 antibodies titters were calculated using a four-parameter non-linear regression.
150
151 **Data preparation and analysis** - The medians values were calculated using Wilcoxon test
152 for pared samples. DENV serotype-specific and ZIKV neutralising antibodies titters
153 (PRNT50) were estimated using a four-parameter non-linear regression. Statistical analysis
154 was performed using Graph Pad Prism, version 6.0e.
155 Classification of immunological response: Primary-ZIKV infection was considered present if
156 neonate/mother had a negative DENV-IgG and a positive ZIKV-IgM confirmed by a PRNT
157 positive to ZIKV only; Secondary Flavivirus/ZIKV infection was confirmed if the neonate
158 had positive DENV-IgG, positive ZIKV-IgM, PRNT positive to ZIKV and to any DENV
159 serotype. We present results for all 42 mother-neonate pairs in the text, and for the 30 in
160 which the neonate had a CSF sample in the tables.
161
162 **RESULTS**
163 Mothers were young (age 15 – 38 years), 13 were < 20 years old (31%); 21 (50% [20 to 29
164 years]) and 8 (19%) from 30 to 38. Twenty-six of 42 mothers (62%) reported rash during
165 pregnancy; 23 in the first trimester and three in the second trimester; fever was reported only
166 by 12 of 26 mothers with rash. Thirteen of 42 (31%) denied any symptoms consistent with
167 viral infection during pregnancy.

168 ZIKV, DENV and CHIKV viral genomes were not detected in serum or in CSF samples by
169 RT-PCR and/or qRT-PCR.
170 None of the 42 mother-neonate paired serum samples had IgM antibodies to DENV;
171 however, 83.3% of the mothers and their babies had DENV-IgG antibodies in serum, in the
172 remaining 16.7% (7/42) DENV-IgG was negative; these results were confirmed by PRNT.
173 In serum, 13 of 42 mothers (31%) had ZIKV-IgM antibodies; of the remaining mothers, 29/42
174 (69%) were negative.
175 In neonates, 38/42 (90.5%) of serum were positive for ZIKV-IgM antibodies; the remaining
176 four of 42 (9.5%) samples were negative. Importantly, specific ZIKV-IgM antibodies were
177 detected in all 30 samples of CSF obtained from neonates with microcephaly.
178 Results of the serologic testing for the sub-set of 30 mother-neonate pairs in which the
179 neonate had CSF collected are shown in the tables. Table 1 and 3 present results for the pairs
180 where ZIKV would be a primary flavivirus infection (eg those without DENV IgG in serum)
181 and tables 2 and 4 for those where ZIKV would be the secondary infection (for pairs with
182 DENV-IgG in serum).
183 Table 1 presents results of DENV-IgG, ZIKV-IgM and DENV-IgM in seven mother/neonate
184 pairs without DENV-IgG in serum (primary ZIKV infection). IgM antibody response in
185 primary ZIKV–infected neonates was specific for ZIKV in six of seven (85.7%) CSF tested.
186 In one (case 12), ZIKV-IgM antibodies cross-reacted with dengue; case 21 had a monotypic
187 ZIKV-IgM response in CSF (P/N 15.6) but IgM result was equivocal in serum (P/N 2.1)
188 (Table 1).
189 Table 2 shows results of 23 mother-neonate pairs in which ZIKV would be a secondary
190 flavivirus infection: of these, 11 mothers (47.8%) were positive for ZIKV-IgM. Among
191 neonates, ZIKV-IgM antibodies were detected in all 23 CSF samples and in 21 serum samples
192 (91.3%). A monotypic ZIKV-IgM antibody response was present in CSF in 18 samples

193 among the 20 cases; all demonstrated low levels of cross-reactive IgM as shown by a dengue
194 P/N value in the equivocal range (2.0 – 3.0).
195 Presence of ZIKV-IgM in CSF and in serum was concordant in 95.6%. Among the 30 CSF
196 results with ZIKV-IgM, there were 24 with monotypic responses and only six cross-reacted
197 with DENV. Figure 1A shows ZIKV-IgM results in CSF (median 14.9 [9.3-16.4]) and in sera
198 (median 8.9 [2.1-20.6]) p=0.0003.
199 A subset of the suspected ZIKV-infected neonates for whom paired serum and CSF
200 specimens had been collected was analysed by using PRNT50 to search for specific ZIKV and
201 DENV neutralising antibodies and to evaluate the extent of cross reactivity to related
202 flavivirus.
203 Table 3 shows the PRNT50 results for the seven primary ZIKV infection cases. Mother and
204 neonate serum and CSF samples had specific neutralising antibodies only for ZIKV,
205 confirming the ELISA results for ZIKV-IgM presented in the Table 1. PRNT50 results also
206 showed that the neutralising antibody response among primary ZIKV–infected patients was
207 highly specific.
208 All 18 secondary ZIKV-infected cases (mothers and neonates) (Table 4), had high titter of
209 neutralising antibodies to ZIKV; for the mothers, the median was 2,072 (range 232-12,980)
210 and for the neonate the median was 2,730 (range 398-12.980), p <0.0001; in CSF the
211 neutralising antibodies titter was lower than in serum. In CSF, the median was 93 (range 40-
212 578), p<0.0001 (Figure 1B). Presence of neutralising antibodies to DENV serotypes in serum
213 of the neonates was similar to the mother. However, in CSF samples, the neutralising
214 antibodies were exclusively to ZIKV. There was a remarkably good concordance between
215 anti-ZIKV-IgM results in CSF and serum of neonates with the PRNT results.
216 As for neutralising antibodies for DENV, 11 had for DENV-3 and DENV-4; four for DENV-
217 4; one for DENV-3 and one case to all DENV serotypes. Of seven mothers with negative

218 serology for DENV, 28.6% had ZIKV-IgM antibodies in serum; of 23 mothers who were
219 positive for DENV, 11 (47.8%) had ZIKV-IgM antibodies in serum.
220

221 **DISCUSSION**

222 It is known that IgM antibodies to DENV can be detected at least 90 days after the onset of
223 symptoms [20], but it is not known how long ZIKV-IgM antibodies remain detectable after
224 infection, in blood and in cerebrospinal fluid of newborns with microcephaly.
225 Based on the fact that 23 of 26 mothers that reported rash indicated that the rash was in the
226 first trimester of pregnancy, it is likely that Zika infection would have occurred at least six
227 months prior birth. Although, we cannot be sure of when the infections have occurred, it is
228 possible that ZIKV-IgM antibodies would remain longer than 90 days; prospective studies are
229 needed to elucidate that.
230 Surprisingly, none of the mothers in this study reported presence of rash in the third trimester
231 of pregnancy; it is possible that 31% of them experienced asymptomatic Zika infection.
232 There is evidence that the virus can persist for long time in the central nervous system (CNS)
233 of foetuses [6], however, we could not detect ZIKV RNA in the neonate CSF samples.
234 In our study, the specimens that were ZIKV-IgM positive and DENV-IgM negative by ELISA
235 were initially considered as a presumptive recent ZIKV infection; and they were all confirmed
236 by the PRNT. We found that 90.5% and 97% of the neonates had ZIKV-IgM antibodies in
237 serum or in CSF, respectively; indicating that the embryo or the foetal brain were exposed.
238 In the ZIKV epidemic in Yap State, Micronesia, secondary flavivirus–infected patients had a
239 higher degree of serologic cross-reactivity with other flaviviruses than primary
240 flavivirus/ZIKV–infected patients [12]. This was much less marked in the neonates in our
241 study; serologic cross-reactivity with DENV was observed in only five of 23 secondary cases
242 (21.7%) and only one (14.3%) among our seven ZIKV primary cases. Antibody response to

243 ZIKV was monotypic in 78.3% (18/23) of the secondary flavivirus/ZIKV infection cases. The
244 most probable is that IgM does not cross the placental barrier, at least to a significant degree,
245 and since these samples were collected in the perinatal period these neonates, probably, have
246 not been directly exposed to other flaviviruses, except for ZIKV in uterus. Moreover, the IgM
247 in the CSF indicate the presence of antigen specific B-cell in the brain and high specificity in
248 the IgM capture ELISA. In contrast, the neonates had maternal IgG and hence also the anti-
249 DENV-IgG; for neonates IgG based diagnostic has low specificity.
250 Consistently with findings in Yap study, our PRNT results have shown higher titters of
251 neutralising antibody to ZIKV, however, in contrast to the Yap study [12], in our study cross-
252 reactivity among the two flaviviruses, ZIKV and DENV was not observed, although both
253 viruses are circulating in our region. In Pernambuco State, yellow fever vaccination is not
254 compulsory, the region is not at risk for sylvatic yellow fever virus (YFV), and as none of the
255 mothers were vaccinated against YFV this virus was not tested in the PRNT.
256 Neutralising antibody responses to DENV were mainly to DENV-3 and DENV-4 (only one
257 mother presented antibodies to DENV-1 and DENV-2 and one to all four serotypes). As the
258 mothers were young these findings reflect the epidemiological scenario of dengue circulation
259 in Pernambuco State in the last 30 years, with DENV-1 introduced in 1987, DENV-2 in
260 1994,and DENV-3 in 2002; DENV-3 predominated until DENV-4 introduction in 2010 [19-
261 23].
262 Our study adds important evidence that supports the etiological relationship between ZIKV
263 infection of the CNS and microcephaly. We found that 97% of the microcephalic infants had
264 ZIKV-positive IgM in the CNS. Since IgM does not normally cross either the placenta or the
265 blood-brain barrier, detection of virus-specific IgM in the CSF is a significant diagnostic
266 finding, indicating that the neonate had a CNS ZIKV infection during pregnancy [23].

267 Detection of neutralising antibodies to ZIKV in the neonates CSF samples corroborates the
268 ZIKV-IgM positive results and indicates that detection of ZIKV-IgM in the neonate blood or
269 CSF is a good diagnostic tool. The relevance of this study is that these were the first cases of
270 microcephaly identified in Recife, state of Pernambuco, in September 2015 that sparked the
271 investigation for the cause of the outbreak. Despite this study lacks control neonates samples
272 for comparison, our findings is another piece of evidence that suggest that the brain
273 abnormalities were a consequence of Zika congenital infection. Moreover, based on these
274 results we conclude that identification of anti-Zika IgM in the neonate serum during the
275 perinatal period is confirmatory of congenital Zika. Therefore, we recommend for testing anti-
276 Zika IgM in neonates suspected of congenital Zika, and that the PRNT could be employed to
277 elucidate equivocal cases.
278
279 **Conflict of interest.** None.

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284
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363 **Figure Legends**

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365 **Figure 1A - New born ZIKV- IgM results in serum and cerebrospinal fluid (CSF)**

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367 **Figure 1B - Maternal serum sample, new born serum and CSF samples and ZIKV PRNT**

368 **results.**

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