Short Communication

Cross reactivity of commercial anti-dengue immunoassays in patients with acute Zika virus infection†

Running title: DENV/ZIKV serological cross reactivity

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Abstract

Several countries have local transmission of multiple arboviruses, in particular, dengue and Zika viruses, which have recently spread through many American countries. Cross reactivity among Flaviviruses is high and present a challenge for accurate identification of the infecting agent. Thus, we evaluated the level of cross reactivity of anti-dengue IgM/G Enzyme-Linked Immunosorbent Assays (ELISA) from three manufacturers against 122 serum samples obtained at two time-points from 61 patients with non-dengue confirmed Zika virus infection. All anti-dengue ELISAs cross reacted with serum from patients with acute Zika infection at some level and a worrisome number of seroconversion for dengue IgG and IgM was observed. These findings may impact the interpretation of currently standard criteria for dengue diagnosis in endemic regions.

Keywords: serological tests; dengue virus; Zika virus; cross reaction.

Introduction

Dengue fever is caused by four phylogenetically related dengue viruses (DENV1-4) and represents a major public health problem in tropical and subtropical countries [Gubler, 1998]. Although DENV has been reported in America for many decades, there has been a dramatic increase in the number and severity of dengue cases in the Americas in the last three decades [San Martín et al., 2010]. Brazil is endemic for DENV and the co-circulation of the four serotypes caused larger and more severe outbreaks recently. Consequently, dengue seroprevalence nowadays in Brazil is high in some regions. For example, in Rio de Janeiro and Recife 90% of the blood donors are seropositive [Sabino et al., 2016]. Laboratory diagnosis of acute dengue is usually made by NS1 antigen capture tests and/or IgM detection on convalescent samples. When molecular tests are available, Real time PCR is used for diagnosis, determination of the viral load and virus typing.

As many countries in the Americas, Brazil has also concomitant transmission of other arboviruses that produce febrile illness with similar and confounding symptoms. Since 2015, at least 9 arboviruses that cause disease in humans have been detected in Brazil: Dengue virus, Yellow Fever, Saint Louis encephalitis virus, Mayaro, Oropouche, West Nile (although there is only one report of infection in humans so far), and more recently, Zika virus and Chikungunya virus [Figueiredo, 2015].

In 2015, Zika virus (ZIKV) was introduced in Brazil causing a large epidemic in the northeast region of the country [Campos et al., 2015]. The virus rapidly spread to other regions and also other countries on the American continent (https://www.cdc.gov/ zika/geo/active-countries.html). Given the recent spread of ZIKV and the evidence of serious Zika-associated congenital malformations in pregnant women, efforts have been made to develop sensitive and specific serologic assays to detect anti-ZIKV antibodies. But, due to serological cross reactivity among flaviviruses, current anti-ZIKV antibody

assays do not reliably distinguish zika from other flavivirus infections [Buathong et al., 2015; Dejnirattisai et al., 2016; Priyamvada et al., 2016; Vorou, 2016]. Therefore, in areas where DENV and ZIKV circulate simultaneously, to diagnose ZIKV infection is a challenge and accurate diagnostic tests that distinguish between flaviviruses are critical for both clinical and surveillance purposes.

If, on the one hand, the cross reactivity between DENV and ZIKV in serological tests against ZIKV is well known [Buathong et al., 2015; Dejnirattisai et al., 2016; Lanciotti et al., 1994; Priyamvada et al., 2016], on the other the potential impact of such phenomenon between these two viruses in DENV commercially available Enzyme-Linked Immunosorbent Assays (ELISA) kits has not been adequately emphasized. Herein, we tested the cross reactivity of sera from patients with confirmed ZIKV infection against three distinct commercial anti-dengue immunoglobulin M and G ELISAs.

Material and Methods

We evaluated three ELISA commercial tests for DENV IgG and IgM: the Focus Dengue Virus IgG DxSelect™ and DengueVirus IgM Capture DxSelect™ (FOCUS DIAGNOSTICS, Cypress, CA, USA), Euroimmun Anti-Dengue Virus ELISA IgG and IgM (EUROIMMUN AG, Luebeck, Germany) and Panbio Dengue IgG and IgM capture ELISA (Panbio, Brisbane, Australia).

Serum samples were obtained in 2015/2016 from 61 adult patients with acute ZIKV infection during a ZIKV outbreak in Araraquara, a 200.000 inhabitants city of São Paulo State, Brazil. ZIKV infection was confirmed by detection of ZIKV RNA either in plasma or urine (or both) during the first week after symptoms onset (1 to 7 days). Diagnosis was made through Real time PCR using the protocol developed by Lanciotti et al [Lanciotti et al., 2008] employing their second set of primers and probe (ZIKV-1086/1162c). Serum samples were collected from the same patients at two time-points: up to 7 days after zika symptoms- named DAO-7, and $\geq$ 14 days after the onset - DAO-14. The average days of symptoms from the DAO-7 group was 4 (range 1-7 days) and from DAO-14 was 17.7 (range 14-32

days). Concomitant dengue infection was excluded by Platelia DENV-NS1 Ag (Bio-Rad, Marnes-la-Coquete, France). Seventeen out of the sixty-one subjects (27%) declared past dengue, though no test for dengue antibodies detection other than those executed in this study was performed to confirm it. To get a picture of the actual dengue prevalence in Araraquara serum was collected from an unrelated population (n=73) of randomly selected adults from 1-28th October, 2016. The tests were performed using the Focus DENV IgG DxSelectTM.

All procedures were performed according to the terms agreed by the Institutional Review Board from the Hospital das Clínicas, University of Sao Paulo (CAPPesq - Research Projects Ethics Committee).

Results

All commercial tests presented cross reactivity with anti-Zika Immunoglobulin G and M at some level (Table 1). According to the kit used, DENV-IgM positivity ranged from 4.9% to 16.4% in samples from DAO-7 and from 13.1% to 37.7% in the DAO-14 samples. Yet, 19 patients who were IgM negative in DAO-7 "seroconverted" by Focus, five by Euroimmun and 10 by PanBio. Therefore, after fourteen days of symptoms, DENV-IgM positivity reached 37.7% considering the kit with the highest positivity. A small proportion of inconclusive results was also observed. Three and two samples with less than 7 days of symptoms had inconclusive results for IgM in Euroimmun and PanBio respectively (one of them in both kits), and after 14 days, eight patients were inconclusive in Euroimmun and three in PanBio IgM test. The Focus ELISAs manufacturer suggests considering results as either positive or negative, with no gray zone of optic densities that could be assigned as inconclusive results.
The reactivity of IgG ELISA was similar across the kits. IgG positivity in samples from DAO-7 was high, ranging from 62.3% to 68.8% according to the test. Critically, all patients were positive in the DAO-14 in all three tests, with DENV-IgG seroconversion observed in 37.7% (23/61) of patients according to Focus, 21.3% by Euroimmun (13/61) and 26.2% (16/61) by PanBio (Table 1).

The city of Araraquara is endemic for dengue since 2008 thus it is expected that a proportion of the patients from this study is actually positive for DENV-IgG. In fact, by investigating a sub-population of adults sampled on the same geographic region in October 2016, the DENV-IgG seropositivity was 53.4% (39/73). Hence, considering that an average of ≈53.0% of the ZIKV infected individuals analyzed in the present study may have experienced past dengue infection, the remaining proportion of participants that reacted for IgM and/or IgG and all seroconversions may reflect cross reactivity with anti-ZIKV IgG and/or IgM.

Discussion

The time of appearance of IgG differs between patients experiencing primary dengue infection and those with secondary or tertiary infection (http://www.cdc.gov/dengue /clinicallab/laboratory.html). In a primary infection by a flavivirus, IgG can be detected at very low titers already at the end of the first week of illness, and the titers increase over weeks. In contrast, in a secondary infection IgG is detected nearly at the same time that IgM emerges and may react with immunoglobulins produced against other flaviviruses. Therefore, cross reaction between ZIKA and DENV is expected, particularly in cases that ZIKV is 'secondary'. What is not expected however, is the percentage of cross reaction observed even in patients that seems not to have experienced past dengue infections, since they tested negative for anti-dengue IgG in samples obtained in the first week of ZIKV infection (see Table 1 for the percentage of seroconversion).

Serological cross reactivity between ZIKV and DENV has been demonstrated by others albeit at a lower level [Lanciotti et al., 2008] and in fewer individuals with confirmed Zika infection [Cabral-Castro et al., 2016] than reported here. The unexpected proportion of dengue IgG and IgM seroconversion as well as the large number of dengue IgM positivity in acute and convalescent serum samples from patients with non-dengue acute ZIKV infection is worrisome and may substantially impact the interpretation of current standard criteria for dengue diagnosis. The detection of dengue IgG

antibodies in 100% of convalescent samples is also concerning since it directly impacts on dengue seroprevalence studies. Moreover, this cross reaction is particularly critical for differential diagnosis of DENV and ZIKV infection in pregnant women.

Our results also raise a concern about the potential effect of this antibody cross reaction on the pathogenesis of both flavivirus infections. It has been demonstrated that cross reactivity with DENV antibodies increases the probability of antibody-dependent enhancement (ADE) in a subsequent infection with ZIKV [Dejnirattisai et al., 2016]. Apparently, the dominance of the antibodies that respond to the original antigen (original antigenic sin) can promote ADE not only in secondary dengue infections, but also in Zika-post-dengue infections. Since we (and others) have demonstrated high level of antibodies cross reactivity, it is necessary to further evaluate the implications of such non-specificity on the severity of both DENV/ZIKV and ZIKV/DENV sequential infections.

In sum, we showed an elevated number of probable artefactual seroconversions in all anti-dengue assays evaluated in this study, thus confirming that the commercially available ELISAs to detect dengue IgG and/or IgM have low specificity when used for patients infected by ZIKV. Although the kits behave slightly different in terms of specificity, possibly due to the inherent variations regarding to the peptides or recombinant proteins included in the kits, the 100% reactivity in all kits of dengue IgG observed in zika-convalescent serum samples creates a new concern on how to interpret seroprevalence studies of flaviviruses in endemic areas for two or more viruses.

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Table 1. Results of serologic testing of 122 ZIKV+ samples using three commercially available ELISAs against DENV IgM and IgG.

	IgM			IgG		
ELISA kit companies	<7 days (DAO-7)	>14 days (DAO-14)	Seroconversion	<7 days (DAO-7)	>14 days (DAO-14)	Seroconversion
Focus Diag.	7/61 (11,5%)	23/61 (37,7%)	19/61 (31%)	38/61 (62,3%)	61/61 (100%)	23/61 (37.7%)
Euroimmun AG	10/61 (16,4%)	8/61 (13,1%)	5/61 (8%)	42/61 (68,8%)	61/61 (100%)	13/61 (21.3%)
Panbio	3/61 (4,9%)	12/61 (19,6%)	10/61 (16.3%)	41/61 (67,2%)	61/61 (100%)	16/61 (26.2%)
Combined data	14/61 (22.5%)	30/61 (49%)	22/61 (36%)	44/61 (72,1%)	61/61 (100%)	23/61 (37.7%)

Seroconversions: those with IgM or IgG negative at baseline that turn into positive at follow up.

Combined data: Consider samples that tested positive in ELISA from at least one company.