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MARIANA KIKUTI

VALIDAÇÃO DE TESTES DIAGNÓSTICOS PARA DENGUE, ZIKA E CHIKUNGUNYA

Salvador

2018

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VALIDAÇÃO DE TESTES DIAGNÓSTICOS PARA DENGUE, ZIKA E CHIKUNGUNYA

Tese apresentada ao Programa de Pós-Graduação em Saúde Coletiva, Instituto de Saúde Coletiva, Universidade Federal da Bahia, como requisito para obtenção do grau de Doutor em Saúde Pública.

Orientador: Prof. Dr. Guilherme de Sousa Ribeiro

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Validação de testes diagnósticos para Dengue, Zika, e Chikugunya.

A Comissão Examinadora abaixo assinada aprova a tese, apresentada em sessão pública ao Programa de Pós-Graduação do Instituto de Saúde da Universidade Federal da Bahia.

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LISTA DE TABELAS E FIGURAS

Artigo 1

Artigo 2

Artigo 3

Artigo 4

Artigo 5

LISTA DE SIGLAS E SÍMBOLOS

DENV – Vírus da Dengue

CHIKV – Vírus Chikungunya

ZIKV – Vírus Zika

ELISA – E*nzyme-linked immunoassays* (imunoensaio enzimático)

IgM – Imunoglobulina M

RT-PCR – Reação em cadeia da polimerase via transcriptase reversa

RNA – Ácido ribonucleico

NS1 – *Non-structural protein 1* (proteína não-estrutural 1)

RDT – *Rapid Diagnostic Test* (teste diagnóstico rápido)

IgG – Imunoglobulina G

AFI – *Acute Febrile Illness* (doença febril aguda)

CI – *Confidence interval* (intervalo de confiança)

IgMSC – *IgM-ELISA seroconversion* (soroconversão de IgM por ELISA)

SMEC – *São Marcos Emergency Center* (Unidade de Saúde de São Marcos)

PPV – *Positive Predictive Value* (valor preditivo positivo)

NPV – *Negative Predictive Value* (valor preditivo negativo)

IQR – *Interquartile range* (intervalo interquartílico)

EUSM – *Emergency Unir of São Marcos* (Unidade de Saúde de São Marcos)

PCR – *Polymerase Chain Reaction* (Reação em cadeia da polimerase)

TMB – *Tetramethylbenzidine* (tetrametilbenzidina)

PRNT – *Plaque reduction neutralization assay* (teste de neutralização por redução de placas)

MAC-ELISA – *Capture enzyme-linked immunosorbent assay* (imunoensaio enzimático de captura)

WHO – *World Health Organization* (Organização Mundial da Saúde)

STARD – *Standards for Reporting of Diagnostic Accuracy*

NA – *Not available* (não disponível)

GBS – *Guillain-Barré Syndrome* (Síndrome de Guillain-Barré)

LACEN-BA – Laboratório Central de Saúde Pública - Professor Gonçalo Moniz (*State's Central Laboratory of Public Health*)

AEI – *Acute exantematous illness* (doença exantemática aguda)

ECSA – *East-Central-South-African*

SUMÁRIO

RESUMO

Clinicamente, a maior parte das infecções pelos vírus da dengue (DENV), chikungunya (CHIKV) e Zika (ZIKV) se apresentam de forma semelhante, como uma doença febril ou exantemática, de curso agudo e auto-limitado. Desta forma, o diagnóstico depende de testes laboratoriais sensíveis e específicos. A correta identificação dessas doenças é importante não apenas para um manejo clínico adequado, como para atividades de vigilância, que incluem a rápida identificação de surtos e o planejamento de ações de prevenção. O objetivo desta tese foi avaliar a acurácia de uma série de testes diagnósticos para arboviroses. Na primeira avaliação, identificamos que um teste rápido para dengue, que combina a detecção do antígeno NS1 e de anticorpos IgM, apresentava uma baixa sensibilidade (47%) e uma alta especificidade (>87%) antes da introdução do ZIKV no Brasil. A avaliação em campo deste mesmo teste revelou uma sensibilidade ainda mais baixa (21%), embora a especificidade tenha permanecido elevada (>89%), mesmo após a introdução do ZIKV em Salvador. Em relação à performance de imunoensaios enzimáticos (ELISAs) para detecção de antígeno NS1 e de anticorpos IgM para DENV, observamos uma sensibilidade baixa em amostras de fase aguda (3% para o ELISA-NS1 e 17% para o ELISA-IgM), e em amostras de fase convalescentes (47% para o ELISA-IgM). Quanto a especificidade desses mesmos ensaios, ambos os ELISAs apresentaram boa performance em amostras de pacientes febris negativos para arboviroses (>87%) e em amostras de fase aguda de pacientes com ZIKV (100% para os dois ELISAs), mas a especificidade foi baixa no ELISA-IgM realizado em amostras de fase convalescente de pacientes com ZIKV (57%), demonstrando um alto grau de reações cruzadas. Já a avaliação da performance do ELISA-IgM para detecção de infecções por CHIKV revelou alta sensibilidade (93%) nas amostras de fase convalescente e alta especificidade (>89%) independente da fase da doença em que a amostra foi coletada. Resultados positivos nos ELISAs para CHIKV foram identificados em pacientes confirmados para dengue e provavelmente representam infecções prévias recentes por CHIKV ou co-inecções, dada a intensa transmissão destes arbovirus. Dentre os últimos testes avaliados, o ELISA-IgM para detecção de infecções por ZIKV apresentou baixa sensibilidade (<15%) e alta especificidade (>98%), enquanto que o ELISA-IgG para ZIKV apresentou alta sensibilidade (100%) e baixa especificidade (<50%), especialmente em pacientes com dengue secundária (7%). Esta série de avaliações reforça a complexidade do diagnótico sorológico em áreas de intensa co-circulação destas arboviroses.

Palavras-chave: dengue, Zika, chikugunya, arbovírus, epidemiologia, sensibilidade e especificidade.

ABSTRACT

Dengue (DENV), chikungunya (CHIKV) and Zika viruses (ZIKV) infections usually presents similarly as acute and self-limited exanthematous or febrile illness. Thus, diagnosis relies on sensitive and specific laboratory tests. A correct case identification is important not only for adequate clinical management but also for surveillance activities such as rapid identification of outbreaks and planning preventive actions. This thesis aimed to evaluate the accuracy of a series of diagnostic tests for arboviruses. In the first evaluation, we identified that a rapid dengue test, which combines the detection of NS1 antigen and IgM antibodies, had a low sensitivity (47%) and a high specificity (> 87%) before the introduction of ZIKV in Brazil. Field evaluation of this same test revealed an even lower sensitivity (21%), although specificity remained high (> 89%), even after the introduction of ZIKV in Salvador.Regarding the performance of enzyme immunoassays (ELISAs) for detection of NS1 antigen and IgM antibodies to DENV, we observed a low sensitivity in acute phase samples (3% for ELISA-NS1 and 17% for IgM ELISA), and in convalescent phase samples (47% for ELISA-IgM).As for the specificity of these same assays, both ELISAs showed good performance in arbovirus negative febrile patients (> 87%) and in acute phase samples from patients with ZIKV (100% for both ELISAs), but the specificity was low in ELISA-IgM in convalescent phase samples from patients with ZIKV (57%), demonstrating a high degree of cross-reactivity. On the other hand, the evaluation of the performance of the ELISA-IgM for the detection of CHIKV infections revealed high sensitivity (93%) in convalescent phase samples and high specificity (> 89%) independent of the stage of the disease in which the sample was collected. Positive results in ELISAs for CHIKV were identified in patients confirmed for dengue and probably represent recent previous infections by CHIKV or co-infections, given the intense transmission of these arboviruses. Among the last test evaluated, the ELISA-IgM for the detection of ZIKV infections showed low sensitivity (<15%) and high specificity (> 98%), whereas IgE ELISA for ZIKV presented high sensitivity (100%) and low specificity (<50%), especially in patients with secondary dengue (7%). This series of evaluations reinforces the complexity of the serological diagnosis in areas of intense co-circulation of these arboviruses.

Key words: dengue, Zika, chikugunya, arbovirus, epidemiology, sensitivity and specificity.

1. **Introdução**

Arboviroses são doenças transmitidas aos seres humanos por meio de vetores artrópodes. Dentre elas, as arboviroses transmitidas por mosquitos tem recentemente causado grande impacto na população mundial.

Estima-se que o vírus da dengue (DENV) seja responsável por cerca de 100 milhões de infecções sintomáticas por ano no mundo (BHATT et al., 2013). Nas Américas, o primeiro relato de dengue foi nos Estados Unidos na década de 1790, de onde se espalhou para a América Latina (WILLOQUET, 2009). Entre as décadas de 1940 e 1970, lançou-se através da Organização Mundial da Saúde (OMS) uma campanha de erradicação do mosquito vetor da dengue *Aedes aegypti* na América Latina, conseguindo a erradicação em 21 países. Apenas um genotipo viral (DEN-2) parecia continuar em circulação segundo evidências sorológicas de pacientes de Trinidad e Tobago, Cuba e Panamá, porém sem a ocorrência de epidemias (HALSTEAD, 2006). No Brasil, a reintrodução do vírus da dengue se deu na década de 1980, em Roraima (sorotipos DEN-1 e DEN-4), quando foi possível controlar a nível regional a sua transmissão (MACIEL; SIQUEIRA JÚNIOR; TURCHI MARTELLI, 2008). Em seguida, houve nova introdução também pelos portos do Rio de Janeiro (sorotipo DEN-1) em 1986, de onde foi possível sua disseminação para as demais regiões do país. Com a entrada no país de novos sorotipos foram registradas epidemias por todo o país (DEN-2 em 1990 e DEN-3 em 1994) (MACIEL; SIQUEIRA JÚNIOR; TURCHI MARTELLI, 2008; TEIXEIRA et al., 2005). Desde então, o Brasil tem reportado grandes epidemias de dengue, ultrapassando a barreira de 1 milhao de casos notificados nos anos de 2010, 2013, 2015 e 2016 (BRASIL, 2017).

O primeiro surto documentado pelo vírus chikungunya (CHIKV) fora da África ocorreu na década de 1950 na Ásia (WEAVER; FORRESTER, 2015). Em 2005, CHIKV se espalhou para as ilhas do pacífico causando grande epidemia na Ilha Reunión, onde estimou-se cerca de 300,000 pessoas infectadas (GÉRARDIN et al., 2008). Pequenos surtos foram descritos no norte da Itália e sul da França devido à importação do vírus por viajantes infectados (WEAVER; FORRESTER, 2015). Nas Américas, transmissão local de CHIKV só foi detectada em 2013 no Caribe, de lá se expandindo para o norte do México e

para o sul na maior parte da América do Sul (WEAVER; FORRESTER, 2015). No Brasil, CHIKV foi introduzido quase simultaneamente pelo Oiapoque, Amapá, e por Feira de Santana, Bahia, em Setembro de 2014 (NUNES et al., 2015). O país registrou mais de 3.000 casos notificados em 2014 em 5 estados brasileiros com transmissão autóctone, que rapidamente de disseminou para todo o país com números crescentes de casos notificados a cada ano (mais de 20.000 em 2015, de 30.000 em 2016 e mais de 270.000 em 2017) (BRAZIL, 2015a, 2016, 2017, 2018).

O vírus Zika (ZIKV) foi descoberto mais recentemente, em 1947, em amostras de sangue de macacos sentinela com episódios febris na Floresta Zika, na Uganda (DICK; KITCHEN; HADDOW, 1952). Casos esporádicos e evidência sorológica de infecções por Zika foram descritos na Ásia e África, entretanto o primeiro grande surto da doença ocorreu na ilha Yap em 2007, onde estima-se que cerca de 70% dos seus residentes tenham sido infectados (DUFFY et al., 2009; FAJARDO; CRISTINA; MORENO, 2016; WEAVER et al., 2016). Em 2013, um novo surto foi reportado na Polinésia Francesa com mais de 28.000 casos estimados (cerca de 11,5% da população) (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL (ECDC), 2014). No final de 2014, o ZIKV foi introduzido nas Américas pelo nordeste brasileiro, onde inicialmente se manifestou como uma doença exantemática de origem desconhecida, tendo sido identificado como surto de ZIKV apenas no início de 2015 (CAMPOS; BANDEIRA; SARDI, 2015; ZANLUCA et al., 2015). O número exato de casos de Zika que ocorreram durante esta epidemia é desconhecido, visto que a identificação etiológica e consequentemente notificação obrigatória só ocorreram mais tardiamente. Entretanto, a Vigilância Epidemiológica de Salvador, Bahia, identificou durante o período mais de 17,000 casos de doença exantemática de origem desconhecida (taxa de ataque de 5,5 casos por 1.000 habitantes) (CARDOSO et al., 2015a; PAPLOSKI et al., 2016a). Apesar de ter sido identificada a co-circulação de DENV, ZIKV e CHIKV dentre os indivíduos reportado, ZIKV foi definida como provável etiologia da maior parte dos casos devido às manifestações clínicas apresentadas. Desde então, a transmissão autóctone ocorre por praticamente toda as Américas (PAHO, 2017).

Clincamente, a maior parte das infecções pelos vírus da dengue (DENV), chikungunya (CHIKV) e Zika (ZIKV) se apresentam de forma semelhante como

uma doença febril ou exantemática, de evolução aguda e auto-limitada. As manifestações clínicas de infecções pelo DENV podem classificadar a doença como dengue (com ou sem sinais de alarme) e dengue grave. Após o período de incubação, a fase febril tem início abrupto e duração de 2 a 7 dias acompanhada geralmente de exantema, mialgia, artralgia e cefaleia. É frequente a presença de exantema máculo-papular e anorexia, náusea e vômitos, bem como manifestações hemorrágicas leves podem estar presentes. A fase crítica se inicia com a defervescência. Nesta fase pode ocorrer o aumento da permeabilidade vascular pode ocorrer, levando à perda plasmática significativa em 24 a 48 horas. Pacientes que melhoram após a fase febril são classificados como dengue não grave (dengue sem sinais de alarme). Pacientes que evoluem com extravasamento plasmático irão desenvolver sinais de alarme (dengue com sinais de alarme). Neste caso, se o extravasamento plasmático for crítico, o paciente pode evoluir à choque e, com o choque prolongado, apresentar hemorragias severas devido à coagulação intravascular disseminada e comprometimento grave de órgãos (dengue grave). Para os pacientes que superam a fase crítica, a fase de recuperação se dá pelas próximas 24 a 48 horas com a reabsorção do fluido extravascular e a involução dos sinais clínicos manifestados (BRASIL, 2011; WHO, 2009).

Em infeções por CHIKV, mialgia, cefaleia, náusea e fatiga também podem estar presentes durante a fase aguda da doença (BRAZIL, 2015b; HORCADA; DÍAZ-CALDERÓN; GARRIDO, 2015; KRUTIKOV; MANSON, 2016; WHO, 2008). Entretanto, a artralgia é o sintoma mais comumente descrito, tipicamente como poliartralgia simétrica, sendo que cerca de 35% dos pacientes reportam artralgia persistente ou recidivante, que podem evoluir para poliartrite crônica (WEAVER et al., 2018). Um aumento de casos de Síndrome de Guillain-Barré foi observado durante um surto de CHIKV na Polinésia Francesa, sugerindo que esse arbovírus também pode desencadear manifestações neurológicas severas (OEHLER et al., 2015).

Pacientes infectados por ZIKV também apresentam sinais e sintomas inespecíficos, como exantema, febre baixa, hiperemia conjuntival e prurido (CARDOSO et al., 2015b; DUFFY et al., 2009; MUSSO; GUBLER, 2016). Entretanto, o exantema maculopapular costuma estar presente em 90-100% dos casos (WEAVER et al., 2018) e foi acompanhado de prurido em mais de 90%

dos casos reportados em Salvador (CARDOSO et al., 2015a). A Zika chamou grande atenção mundial após causar epidemias que foram associadas a casos de Síndrome de Guillain-Barré na Polinésia Francesa e no Brasil (CAO-LORMEAU et al., 2016; PAPLOSKI et al., 2016a; ROSÁRIO et al., 2016) e do nascimento de crianças com microcefalia e outras malformações congênitas inicialmente percebido no Brasil e descrito retrospectivamente após o surto de Zika na Polinésia Francesa (CAUCHEMEZ et al., 2016; DE ARAÚJO et al., 2016; PAPLOSKI et al., 2016a; SCHULER-FACCINI et al., 2016).Desta forma, devido à similaridade nas manifestações clínicas mais típicas apresentadas, o diagnóstco diferencial destas arboviroses baseado apenas na avaliação clínica é difícil. Além disso, uma vez que DENV, ZIKV e CHIKV são transmitidos pelo mesmo vetor (mosquitos *Aedes spp.*), a co-circulação destes três vírus em regiões endêmicas não é incomum e o diagnóstico depende ainda mais de testes laboratoriais específicos para uma correta identificação de casos. Testes que detectam a presença de anticorpos, como immunoensaio enzimático (ELISA) e testes de neutralização, podem diferenciar entre alphavirus (CHIKV) e flavivírus (DENV e ZIKV) (WILDER-SMITH et al., 2017), entretanto, a interpretação de tais testes pode ser desafiadora devido à possibilidade de reações cruzadas (DENV e ZIKV são flavivirus e apresentam semelhanças estruturais entre si) e infecções sequenciais (DEJNIRATTISAI et al., 2016; DUFFY et al., 2009; LANCIOTTI et al., 2008). Desta forma, os testes diretos para detecção de infecção, como a transcrição reversa da polimerase (PCR) para detecção do material genético do vírus e ELISA para detecção da proteína não-estrutural 1 (NS1), são considerados mais confiáveis no diagnóstico diferencial. Apesar disso, há que se considerar outros fatores complicadores como o aumento do uso de vacinas contra dengue e febre amarela no caso dos testes sorológicos, além da baixa e rápida viremia de Zika no caso dos testes moleculares (MUSSO et al., 2016; WILDER-SMITH et al., 2017). A escolha do método diagnóstico utilizado deve considerar a fase da doença em que o paciente se encontra, bem como as vantagens e limitações de cada técnica (KAO et al., 2005). Os métodos diretos em geral são recomendados até o sétimo dia após o início dos sintomas, quando os pacientes ainda se encontram na fase de viremia. Já os indiretos, especificamente para detecção de anticorpos da classe IgM, são recomendados

a partir do quarto dia após o início dos sintomas, mas tipicamente persistem por 2-12 semanas (CENTERS OF DISEASE CONTROL, 2016).

A confirmação laboratorial é importante não apenas para um manejo clínico adequado, como também para atividades de vigilância, que incluem uma rápida identificação de surtos e planejamento de ações de prevenção. Embora ações de prevenção e controle possam ser feitas para os três arbovírus, como controle vetorial, uma identificação precisa da real carga de cada uma destas enfermidades pode guiar medidas mais específicas, como orientações à população sobre riscos gestacionais em momentos epidêmicos de ZIKV. No que tange ao manejo clínico, um teste ambulatorial capaz de identificar corretamente estas arboviroses pode direcionar o cuidado médico para hidratação e monitoramento para formas hemorrágicas no caso de infecções por DENV, acompanhamento gestacional e monitoramento para complicações neurológicas em infecções por ZIKV e monitoramento de quadros de artralgia para evitar sua evolução para artralgia persistente incapacitante nos casos de CHIKV. Desta forma, é imprescindível a avaliação da performance diagnóstica dos exames laboratoriais específicos para tais arboviroses, não apenas para validar a atuação definição de casos confirmados laboratorialmente como também para distinguir se tais exames são mais adequados para determinada população que para outra (como status imune prévio para flavivírus, ou se seu uso é mais adequado como exame de triagem ou como confirmatório).

Embora a proposta inicial desta tese tenha sido avaliar a performance diagnóstica de um teste rápido para dengue e suas implicações no manejo clínico de pacientes e potenciais impactos na vigilância em saúde, o cenário epidemiológico de ocorrência de arboviroses no Brasil sofreu grandes mudanças nos últimos 4 anos. Em Salvador, Bahia, local onde os estudos foram conduzidos, houve intensa circulação destes três arbovirus nos últimos anos (CARDOSO et al., 2015b; PAPLOSKI et al., 2016b). Dado esta situação, o escopo da presente tese foi ampliado com o intuito de também avaliar a performance de outros testes diagnósticos para detecção de infecções por DENV, CHIKV e ZIKV.

Para diagnóstico de dengue, nós avaliamos um teste rápido (SD Bioline Dengue Duo – Standard Diagnostics Inc, Korea) e ELISAs para detecção de NS1 (Panbio Dengue Early ELISA – Panbio, Australia) e IgM (Panbio Dengue IgM

Capture ELISA – Panbio, Australia). Disponível comercialmente, apenas dois testes rápidos para dengue são capazes de detectar tanto a presença do antígeno NS1 quanto de anticorpos da classe IgM (BLACKSELL et al., 2011; FRY et al., 2011; TRICOU et al., 2010; WANG; SEKARAN, 2010). São eles o kit da Biogate Laboratories Ltd. (Dengue Fever 3 in 1 Rapid Test) e o kit da Standard Diagnostics (SD Bioline Dengue Duo), apenas o último com acurácia relatada na literatura. O kit SD Bioline Dengue Duo apresentou sensibilidade de 46-98% 93 e especificidade de 84-100% (BLACKSELL, 2012; BLACKSELL et al., 2011; HUNSPERGER et al., 2014; SÁNCHEZ-VARGAS; SÁNCHEZ-MARCE; VIVANCO-CID, 2014; TRICOU et al., 2010). No que tange aos ELISAs avaliados, o ELISA-NS1 tem sensibilidade relatada na literatura de 45-91% (BESSOFF et al., 2008; BLACKSELL et al., 2008; MCBRIDE, 2009; PAN-NGUM et al., 2013) e enquanto que o ELISA-IgM de 54-93% (BLACKSELL et al., 2012; PAN-NGUM et al., 2013), a depender do padrão-ouro utilizado. A especificidade de ambos tem sido reportada como acima de 90% (BLACKSELL et al., 2008, 2012; PAN-NGUM et al., 2013).

Para diagnóstico de Chikungunya, nós avaliamos o ELISA para detecção de IgM (CHIKjj DetectTM IgM ELISA – Inbios, USA). Embora outros testes para detecção de anticorpos IgM contra CHIKV tambpem estejam disóníveis comercialmente, o teste avaliado apresentou sensibilidade de 100% e especificidade de 93-100% relatadas na literatura (JOHNSON et al., 2016). Já para o diagnóstico de ZIKV, nós avaliamos ELISAs para detecção de IgM (Anti-Zika Virus ELISA IgM – Euroimmun, Germany) e IgG (Anti-Zika Virus ELISA IgG – Euroimmun, Germany). Poucos kits comerciais para diagnóstico sorológico de ZIKV estão disponíveis até o momento, a maioria dos quais com raros ou nenhum estudo publicado em revistas científicas. Os kits escolhidos para serem avaliados na presente tese apresentam o maior número de publicações científicas, entretanto a maioria deles avalia sua acurácia em indivíduos de áreas não-endêmicas que retornam de áreas com circulação viral. A sensibilidade relatada na literatura foi de 29-87% para o ELISA-IgM e de 23% para o ELISA-IgG, e a especificidade de 81-100% e 47-100%, respectivamente (L'HUILLIER et al., 2017; STEINHAGEN et al., 2016)

Produtos de outras atividades desenvolvidas durante o doutoramento estão descritas como anexos da tese.

1.2 Objetivos da pesquisa

1.2.1 Objetivo Geral

Avaliar a acurácia de testes laboratoriais para diagnóstico de DENV, ZIKV e CHIKV em pacientes com sinais clínicos sugestivos de arboviroses na cidade de Salvador, Bahia.

1.2.2 Objetivos específicos

1. Estimar, em uma coleção de soros do período anterior a introdução do ZIKV e CHIKV na cidade de Salvador, a sensibilidade e especificidade de um teste rápido para diagnóstico de dengue.
2. Avaliar a sensibilidade e especificidade de um teste rápido para diagnóstico de dengue em pacientes febris em uma região endêmica para DENV, ZIKV e CHIKV.
3. Estimar a sensibilidade e especificidade de imunoensaios enzimáticos (*enzyme-linked immunoassays* - ELISAs) para diagnóstico de dengue em uma região endêmica para DENV e ZIKV.
4. Avaliar a sensibilidade e especificidade de um imunoensaio enzimático para detecção de anticorpos IgM contra CHIKV em uma região de intensa co-circulação de CHIKV, ZIKV e DENV.
5. Estimar a sensibilidade e especificidade de imunoensaios enzimáticos para diagnóstico de Zika por meio da detecção de anticorpos IgM e IgG.

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2. Artigo 1 – Accuracy of the SD Dengue Duo for rapid point-of-care diagnosis of dengue

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Abstract

Background: Rapid diagnosis tests (RDT) are easy to implement, give fast results, and could potentially assist the physician's diagnosis and medical treatment decisions. We evaluated the accuracy of a commercially available RDT for dengue in differentiating dengue among non-severe febrile patients from endemic areas.

Methodology/Principal Findings: We randomly selected samples from 354 acute febrile illness (AFI) patients (246 dengue case and 108 non-dengue cases according to RT-PCR, NS1-ELISA, and IgM-ELISA), 73 community healthy controls and 73 blood donors to blindly test using SD Dengue Duo RDT, which simultaneously detect NS1 protein and IgM antibodies. Sensitivity of the RDT was assessed by RDT component (NS1, IgM, and a combination of both). Overall sensitivity of the RDT was 38.6%, 13.8%, and 46.8% for

the NS1, IgM, and a combination of both components, respectively. A higher sensitivity was found in secondary infections (compared to primary infections) in either components of the RDT, in fewer days of onset symptoms for the NS1 component, and in greater days of symptoms for the IgM component. Sensitivity remained high regardless of days of symptoms when considering a combination of NS1 and IgM components of the RDT. Specificity ranged from 87.7% to 98.6% depending on control group and RDT component assessed. Overall, specificity was higher in blood donors control group and lower in the community control group.
Conclusions/Significance: The RDT showed an overall high specificity and low sensitivity, suggesting this may be a more useful test to rule in than to rule out dengue diagnosis in non-severe outpatients presenting AFI.

Introduction

Dengue is estimated to infect 390 million people per year, of which an estimated 96 million are symptomatic cases (BHATT et al., 2013). Dengue virus (DENV) symptomatic infections may cause a wide spectrum of acute clinical manifestations, from a self-limiting, mild disease of non-specific symptoms, such as fever, myalgia, and headache, to a severe disease evolving with plasma leakage and hemorrhage (WHO, 2009). Due to the low specificity of the clinical presentation, early laboratory confirmation may allow timely fluid replacement, critical to prevent disease complications and death (TEIXEIRA et al., 2005).

Several laboratory exams for dengue diagnosis are commercially available, including molecular tests for detection of DENV RNA by reverse transcriptase polymerase chain reaction (RT-PCR), enzyme linked immunosorbent assays (ELISAs) for detection of the non-structural 1 (NS1) protein of DENV during the viremic phase and for detection of IgM antibodies after the viremic phase, among others. The choice of the best diagnostic method needs to take into consideration the timing of sample collection during the clinical course of disease, as well as other advantages and limitations of each technique, such

as accuracy, cost, and the need for specialized personels and equipment (KAO et al., 2005). However, molecular tests and ELISAs are typically time-consuming and require laboratory structure not commonly found in outpatient health units. Thus, they are of limited use for confirming or rejecting the physician's clinical impression and often do not help guiding medical treatment.

Rapid tests, which are easy to implement and give fast results, could potentially assist the physician's diagnosis and medical treatment decisions. Yet, they generally present lower sensitivity and specificity than the other recommended tests, and, therefore, their results have to be interpreted with caution (WHO, 2009). There is a wide range of commercially available rapid diagnostic tests (RDT) for dengue that focus primarily on the detection of antibodies of IgM or IgG class, detection of the NS1 DENV antigen, or in a combination of them. Although previous studies have evaluated the accuracy of these tests, few were conducted in populations where they have the greatest potential to be applied in practice, such as for differentiation of dengue among non-severe febrile patients from endemic areas.

This study aimed to evaluate retrospectively the diagnostic performance of a commercial rapid diagnostic test (RDT) among ambulatory febrile patients in a dengue-endemic area. Among the many rapid tests for dengue diagnosis commercially available, the SD Dengue Duo test was selected for evaluation because of its capability to detect simultaneously the NS1 antigen and IgM/IgG antibodies, and because it has been used in public laboratories for dengue diagnosis during epidemics in some Brazilian municipalities.

Methods

Study design. To evaluate the RDT accuracy we selected a random n of representative serum samples of patients with acute febrile illnesses (AFI) enrolled in a community-based enhanced surveillance study for dengue between January 2009 and December 2011. The AFI surveillance was performed in a public outpatient health unit, located in the Pau da Lima community, Salvador, northeast Brazil. Pau da Lima presents an

endemic transmission of dengue, with an estimated incidence of 21 and 70 cases per 10,000 inhabitants in 2009 and 2010, respectively (KIKUTI et al., 2015). The surveillance enrolled patients fulfilling the following inclusion criteria: age of five years or more, reported fever or measured axillary temperature ≥37.8°C of up to 21 days of duration, and household address within the study area in the community.

We collected acute-phase (at enrollment) and convalescent-phase (≥ 15 days post enrollment) blood samples from participants. Sera were stored in aliquots at -20°C and -70°C for serologic and molecular testing, respectively. All acute-phase sera samples were tested for dengue by NS1- and IgM-ELISA, and all convalescent-phase sera underwent IgM-ELISA. The ELISAs kits used were commercialized by Panbio Diagnostics, Brisbane, Australia, and the assays were performed according to manufacturer's instructions. Acute-phase sera from patients who were positive by NS1- or by IgM-ELISA in either the acute- or the convalescent-phase sera were also tested by reverse transcriptase polymerase chain reaction (RT-PCR) (LANCIOTTI et al., 1992) to identify the serotype responsible for the infection. A dengue case was defined as an AFI patient with a positive NS1 ELISA, an IgM-ELISA seroconversion (a negative result in the acute-phase IgM-ELISA followed by a positive result in the convalescent-phase IgM-ELISA), or a positive DENV RT-PCR. Dengue confirmed cases also had their acute-phase serum samples tested by IgG-ELISA (Panbio Diagnostics, Brisbane, Australia) to determine the type of infection (primary, defined by a negative result in the IgG-ELISA; versus secondary, defined by a positive result in the IgG-ELISA). A non-dengue case was defined as an AFI patient without any laboratory evidence of dengue infection in all of performed tests.

Since Pau da Lima community is a dengue endemic area, serum samples from participants from a survey in the same community were also included as presumably healthy community controls. This serosurvey recruited inhabitants of >5 years from randomly selected households within Pau da Lima community from January to June 2010. Blood samples were drawn and sera were stored in aliquots at -20°C for serologic testing. NS1-, IgM- and IgG-ELISAs for dengue were performed in the selected samples.

Lastly, serum samples from volunteer blood donors from Salvador metropolitan area were included as an additional group of negative control. Blood donors samples

were collected in December 2013, after they have undergone the donation's health triage, which included age (16 to 69 years), weight (> 50kg), and health restrictions (volunteers diagnosed with Hepatitis, pregnant or breastfeeding women, risky behavior for blood borne infectious diseases, and fever in the 15 days prior to blood donation). Blood donors' sera were stored in aliquots at -20°C for serologic testing. To assure they did not have a dengue infection, dengue NS1-, IgM- and IgG-ELISAs were performed in their serum samples.

Sample selection. From the collection of serum sample collection available, we randomly selected: i) 246 acute-phase blood serum samples from dengue cases detected during the AFI enhanced surveillance; ii) 108 acute-phase blood serum samples of non-dengue AFI controls (participants without any laboratory evidence of dengue infection in the acute- or convalescent-phase serum sample); and iii) 73 serum samples from Pau da Lima serosurvey (community controls). In addition, a fourth group with all 73 volunteer blood donors serum available were included in the analysis (healthy controls) (Figure 1). All the 500 serum samples tested by the RDT were stored at -20°C until and have never been thawed before. Sample size was determined based on an average of 85% sensitivity and 95% specificity of the RDT (BLACKSELL et al., 2011; OSORIO et al., 2010; TRICOU et al., 2010; WANG; SEKARAN, 2010) with 5% precision and 95% confidence interval.

Blinding. To assure blinding of the RDT readers, the 500 selected samples were shuffled and re-identified with a sequential number. Readers had no access to the dataset containing the key code of the samples and did not participate in the re-identification process. Two independent blinded members of the study team entered the RDT results into a dataset, which were then validated to assure data quality and minimize bias. The complete dataset containing the all the laboratory results for each patients' sample and the key identifications were only available for data analysis after all laboratory analysis were completed and inserted in the database.

Laboratory testing. SD Dengue Duo RDT testing was performed according to the manufacturer's instruction on all 500 samples, and results were interpreted by three independent blinded readers previously trained by a manufacturer's representative. Briefly, we added 100 µL of serum in the NS1 component and 10 µL of serum and 4 drops of the buffer solution in the IgM/IgG component. Reading was performed in a bright area within 15-20 minutes. Each component of the RDT test was considered positive for a component if it showed two positive bands (the band of the test component and of the internal control), negative if it presented only one band (internal control band) and invalid if the internal control band was absent. Results were interpreted and recorded independently by three different readers. The most frequent reading result was considered the final test result. Each sample was tested just once, unless the RDT presented an invalid result. If so, the test was repeated according to the same protocol, and the most frequent result between the tree readers in the repetition test was considered final. All tests were stored at the health unit at room temperature in a closed room with air conditioning (Salvador has mean annual temperature of 25.3°C and relative humidity of 80.9%) (INSTITUTO NACIONAL DE METEOROLOGIA, 2014).

We also evaluated test-to-test reproducibility by randomly selecting 50 samples (10%) and repeating the testing protocol. In addition, we randomly selected 30 patients of all laboratory-confirmed dengue cases who had a negative IgM result in the RDT and who had a convalescent-phase serum sample available, and tested this sample with the RDT. To investigate false positive results in the RDT, after finishing the 500 RDT testing, we performed RT-PCR (LANCIOTTI et al., 1992) in serum samples (stored at -70ºC) of all non-dengue AFI controls who had a positive result in either the NS1- or IgM-RDT component to assure they had been correctly classified as a non-dengue AFI case. Likewise, to assure that community and blood donors control groups did not include an asymptomatic dengue infection during the time of sample collection, we performed NS1- and IgM-ELISAs on their -20ºC serum samples. RT-PCR was not performed because these control groups did not have serum samples stored at -70ºC.

Statistical analysis. We used frequencies and means plus standard deviation to describe sociodemographic and clinical characteristics of the patients from which serum samples were included in this study. RDT sensitivity was assessed for the IgM and the NS1 test component, both separately and in combination. RDT sensitivity was also calculated by type of dengue infection (primary or secondary), DENV serotype, number of days of symptoms (0-2, 3-4, and ≥5), and laboratory confirmation criteria. RDT specificity was assessed for each control group (non-dengue AFI, community controls, and blood donors). Additionally, sensitivity and specificity by RDT component were also assessed against its analogous reference test (NS1-, IgM- or IgG-ELISA). Inter-operator agreement between RDT readers and test-to-test reproducibility were calculated using global agreement (overall concordant results divided by total tested) and Kappa. Confidence intervals at 95% were calculated for all accuracy measurements. Invalid results on the RDT or equivocal results on the ELISAs were excluded from the analysis.

Ethics statement. This study was designed and conducted according to the "Evaluation of diagnostic tests for dengue Guidelines" (PEELING et al., 2010) and was approved by the Research Ethics Committee at the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation. All adult subjects provided written informed consent. Participants <18 years old who were able to read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis.

Results

Characteristics of the study participants. Dengue and non-dengue AFI patients whose samples were selected for this study had similar age distribution, but were younger than the community controls and the blood donor controls (Table 1). The four tested groups were mostly comprised of female participants, with the exception of the blood donor group. Dengue and non-dengue AFI patients reported similar frequencies of headache (>80%), prostration (>75%) and myalgia (>70%). However, the following manifestations were more frequent among dengue cases than among non-dengue AFI controls: retro-

orbital pain (59.8% vs. 42.6%; *p*=0.001), arthralgia (46.3% vs. 32.4%; *p*=0.01), and rash (21.5% vs. 12.0%; *p*=0.02). Most of studied patients did not have a clinical suspicion recorded in their medical chart (Table 1). Even though, a suspicious of dengue was made for 15.9% of the dengue confirmed cases and for 1.9% of the non-dengue AFI controls. The most common suspicious for the non-dengue AFI controls were upper respiratory tract infections (16.7%).

Sensitivity and specificity. Of the 500 tested samples, only 2 (0.4%) needed to be retested to achieve a valid result (both in the NS1 and IgM/IgG components). Sensitivities for the NS1 and IgM components of the RDT were 38.6% (95%CI 32.5 – 45.0) and 13.8% (95%CI 3.9 – 18.8), respectively (Table 2). However, considering a positive result in either the NS1 or the IgM component of the RDT, the RDT sensitivity showed a non-significant increase to 46.8% (95%CI 40.4 – 53.2).

Sub-analyses of the RDT sensitivity revealed that some differences among the confirmed dengue cases, such as the type of infection, the infecting serotype, the number of days with symptoms at the time of testing, and even the laboratory criteria used for case confirmation might influence the test performance (Table 2). The sensitivities for the NS1 and IgM RDT components, as well as for them combined, were significantly higher among secondary DENV infections than among primary DENV infections. In addition, the sensitivity of the NS1 component was statistically greater for DENV4 infections when compared to DENV2 infections (61.2% vs 46.0%). As expected, the NS1 RDT sensitivity decreased, while the IgM RDT sensitivity increased with the increase in the number of days of symptoms at acute-phase sample collection. Consequently, the overall RDT sensitivity (considering both components) reduced from about 48% when the test was used in the first 4 days after disease onset to 37.9% when used in patients with ≥5 days of symptoms, but this difference was not statistically significant. Lastly, we found that the reference laboratory criteria used for dengue confirmation influenced the sensitivity of the RDT components. The NS1 RDT component had greater sensitivity (ranging 88.6% and 94.4%) when the dengue case had a positive NS1-ELISA as one of the confirmation criteria. In contrast, the IgM RDT component presented low sensitivity regardless of the dengue confirmation criteria, but it had a poor performance (sensitivities bellow <10.0%)

when the dengue case had an IgM-ELISA seroconversion between the acute- and the convalescent-phase serum samples as one of the confirmation criteria.

The RDT specificity evaluated in combination of both NS1 and IgM components were 94.4% for the non-dengue AFI controls, 87.7% for the community healthy controls, and 95.9% for the blood donor controls (Table 3). Specificity of the NS1 component ranged from 97.3% to 98.6% and was higher than the specificity for the IgM-component, which ranged from 90.4% to 97.3%. Among blood donors, all 73 samples were negative by NS1-ELISA, whereas IgM-ELISA was positive for 7 samples, negative (none of which were positive in the IgM-RDT) for 61 samples and equivocal for 5. The community control group presented a positive NS1-ELISA result in 2 samples (none of them were positive in the NS1-RDT) and a positive IgM-ELISA in 16 samples (2 of which were positive in the IgM-RDT). Acute-phase samples of all non-dengue AFI and community controls with a positive result in either NS1- or IgM-RDT component were tested by DENV RT-PCR and presented negative results.

Comparing the results obtained for the RDT's components to their corresponding ELISAs results we found that the sensitivity of the NS1 component was 88.0% (95%CI 78.4 – 94.4), whereas IgM and IgG sensitivities were 29.5% and 30.8%, respectively. Specificities for all the three components were >90% (Table 4).

Of the 30 dengue confirmed patients with a negative IgM-RDT result in the acute-phase sample that were randomly selected to have their convalescent-phase serum sample tested by the RDT, 20 were NS1-RDT negative and 10 were NS1-RDT positive in the acute sample. Their RDT results for the convalescent sample were all negatives for the NS1 component, and 13 (43%) positives for the IgM component. Thus, altogether, the evaluation of these 30 paired sera resulted in a sensitivity of 76.7% (23 out of 30).

Reproducibility. Test-to-test reproducibility with the 50 randomly selected samples revealed a global agreement of 94.0% (kappa=0.81) for the NS1-RDT component, 96.0% (kappa=0.81) for the IgM-RDT component, and 86.0% (kappa=0.68) for the IgG component. Inter-operator agreement in visual interpretation of the test ranged from 96.0% to 98.8% (kappa ranging from 0.87 to 0.96) for the NS1-RDT component, 94.4%

to 97.8% (kappa 0.63 – 088) for the IgM-RDT, and 87.0% to 94.0% (kappa 0.67 – 0.86) for the IgG-RDT.

Discussion

In this study, we evaluated the diagnostic performance of the SD DENGUE DUO, a commercial diagnosis test for rapid dengue detection, among febrile patients from an outpatient health unit in a dengue endemic region, as well as among three different types of controls. Although the test presented good levels of specificity (88%-96%), its overall sensitivity (combining positivity in either the NS1 or the IgM component) was of only 46.8%. The low sensitivity was associated with a suboptimal performance of both the NS1 and the IGM components of the test in detecting dengue cases early and late in the course of the disease, respectively.

Previous hospital-based studies evaluations of this RDT found much greater sensitivities, ranging between 75.5% - 97.5% (ANDRIES et al., 2012; BLACKSELL et al., 2011; GAN et al., 2014; OSORIO et al., 2010; TRICOU et al., 2010; VICKERS et al., 2015; WANG; SEKARAN, 2010). The distinct characteristics between the study patients whose samples were evaluated in our study and those evaluated in other studies may explain the observed differences in the measured sensitivities. In our study, dengue confirmed cases were outpatients with mild disease. In contrast, majority of the other studies were conducted on hospitalized patients, which usually have greater disease severity and duration, consequently influencing the intensity of inflammatory and immune response. Even though, a previous evaluation of this RDT in Cambodia, enrolling children hospitalized due to a febrile illness of no clear source, also showed an overall low sensitivity (57.8%) (CARTER et al., 2015). Alternative explanations for the distinct estimated accuracies include differences in frequency of primary and secondary infections, in circulating DENV serotypes, and in reference methods used to diagnose dengue infections.

Some studies of this RDT found higher sensitivity among primary infections (ANDRIES et al., 2012; HUNSPERGER et al., 2009; TRICOU et al., 2010), suggesting

that DENV IgG antibodies present in secondary infections may form immune complexes with viral antigens, such NS1, reducing the sensitivity of the NS1 component of the RDT (VICKERS et al., 2015). In addition, the lower IgM production in secondary dengue infections would reduce the sensitivity of the IgM component of the test (GUZMAN et al., 2010). We, however, found a higher sensitivity among secondary infections than in primary infections, regardless of the RDT component being evaluated, similar to studies from Colombia (OSORIO et al., 2010) and Sri Lanka (BLACKSELL et al., 2011). Conversely to what had been postulated before, it is possible that secondary DENV infections boost a faster and DENV-specific IgM immune response that improve the test sensitivity. However, other studies found no significant difference in this RDT sensitivity according to the infection type (GAN et al., 2014; SÁNCHEZ-VARGAS; SÁNCHEZ-MARCE; VIVANCO-CID, 2014; VICKERS et al., 2015) and a potential influence of the type of infection in the sensitivity of this particular RDT remains controversial.

Although our small number of confirmed dengue cases presenting with ≥5 days of symptoms underpowered our capacity to detect statistical differences in the RDT sensitivity according to disease duration, our findings suggest that the test may have a poorer performance when applied late on the course of disease. Supporting evidence for that include the sustained low sensitivity of the IgM component of the test (lower than 30%) even for samples collected from patients with ≥5 days of symptoms and for samples that tested positive on the IgM-ELISA.

We defined a dengue case by a combination of RT-PCR, NS1-ELISA or IgM-ELISA seroconversion to ensure high confidence that the AFI patients really had an ongoing dengue infection and to provide the diverse laboratory scenario of dengue confirmed cases. In addition, we randomly selected the 246 dengue confirmed cases from all the dengue cases confirmed during a surveillance study to detect dengue cases among AFI patients. Thus, the proportion of samples fulfilling the different combinations for the used confirmation criteria should be representative of the reality encountered in dengue endemic regions. This choice revealed to be of great relevance, because we found that the accuracy of the RDT was largely influenced by the reference diagnostic method used. As an example, when considering only the samples from patients with a

positive result in the NS1-ELISA, we found that the NS1 component of the RDT achieved a high sensitivity (88.0%). However, the overall sensitivity for the NS1 RDT component was of 38.6% because only 73 (29.7%) of our 246 confirmed dengue patients had a positive result in the NS1 ELISA. As we did in our evaluation, it is critical that studies testing the accuracy of diagnostic methods use serum samples from representative cases instead of a convenient serum sample collection.

The specificity of the RDT was high, regardless of the control group. However, the community controls presented a lower RDT specificity compared to other groups, which may be explained by recent dengue infections in this randomly selected representatives from a region of endemic dengue transmission (KIKUTI et al., 2015), as IgM for dengue may persist for up to 2 or 3 months (GUZMAN et al., 2010). This was evidenced by the fact that the 7 samples presenting a false positive RDT result were also positive in the IgM-ELISA. When we evaluated the RDT on 30 convalescent sample of dengue confirmed patients who had a negative IgM-RDT result in the acute-phase sample, we detected 13 additional dengue cases, giving a combined sensitivity of 76.7% (10 cases detected by the NS1 RDT component in the acute-phase sera plus 13 cases detected by the IgM RDT component in the convalescent-phase sera). This increase in the sensitivity occurred because IgM levels are usually detected after 5-7 days post onset of fever, peaking at around 3 weeks (GUZMAN et al., 2010), and therefore may not yet be present at the acute-phase serum sample. Thus, paired sample testing should be encouraged if the RDT returns a negative result in an acute-phase sample. Test-to-test reproducibility was considered very good for the NS1 and the IgM RDT components, and good for the IgG component (BYRT, 1996), while the visual interpretation of RDT results showed good to excellent agreements between independent readers.

Some limitations of this study need to be acknowledged. First, samples were not tested for other flaviviruses, such as yellow fever (YFV) and Zika (ZIKV) viruses, which have antigenic similarities to DENV and could potentially produce serological cross-reactions. However, YFV transmission did not occur in Salvador before 2016, when an epizootic outbreak was first detected (PAPLOSKI et al., 2018). In addition, the large ZIKV epidemics that recently reached Brazil, having the northeast region as the epicenter, only

started in late 2014 and, in Salvador, it peaked in May of 2015, with over 17,000 reported cases (PAPLOSKI et al., 2016). According to phylogenetic studies ZIKV was introduced to Brazil in 2013 (FARIA et al., 2016; MUSSO, 2015). Thus, although possible, it is unlikely that the subjects providing blood samples for our study have had a ZIKV infection, because community control samples were collected in 2010, the AFI samples from dengue and non-dengue cases were collected between 2009 and 2011, and only the blood donors were obtained in 2013. Secondly, RT-PCR for dengue diagnosis was only systematically performed on AFI patients presenting a positive result for dengue in the NS1- or IgM-ELISA, but in none of the control groups. However, to verify for potential misclassification bias among the non-dengue AFI control group, we tested their acute-phase serum sample by RT-PCR whenever the RDT gave a positive result and none of the tested samples were RT-PCR positive.

In conclusion, this RDT showed high specificity, indicating that AFI patients with a positive result in either in the NS1 or the IgM component of the test can be considered dengue cases. However, it presented a low sensitivity and, therefore, AFI patients with a negative RDT result should not have a dengue diagnosis discarded. In this case, performing additional diagnostic tests and/or obtaining a convalescent-phase blood sample to test might help achieve a more accurate result. Still, prospective studies in outpatient health units are needed to evaluate the impact of using this RDT in proper disease clinical management and in case reporting.

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Table 1. Characteristics of patients included in the sera panel for SD DENGUE DUO Rapid Test evaluation.

	Dengue AFI (n=246)	Non-dengue AFI (n=108)	Community's healthy controls (n=73)	Blood donors (n=73)
Median age (IQR)	18 (10 - 31)	17 (10 - 30)	25 (14 - 37)	33 (27 - 41)
Male (%)	42.3	44.4	37.0	64.4
Median days of symptoms (IQR)	2 (2 - 4)	3 (1 - 4)	-	-
Clinical manifestations				
Headache	214 (87.0%)	91 (84.3%)	-	-
Prostration	195 (79.3%)	82 (75.9%)	-	-
Myalgia	187 (76.0%)	77 (71.3%)	-	-
Retro-orbital pain	147 (59.8%)	46 (42.6%)	-	-
Arthralgia	114 (46.3%)	35 (32.4%)	-	-
Rash	53 (21.5%)	13 (12.0%)	-	-
Vomiting	74 (30.1%)	30 (27.8%)	-	-
Diagnosis at hospital chart				
None	144 (58.5%)	56 (51.9%)	-	-
With a clinical suspicion[a]				
Dengue	39 (15.9%)	2 (1.9%)	-	-
Upper respiratory tract infections	16 (6.5%)	18 (16.7%)	-	-
Indetermined viral disease	28 (11.4%)	7 (6.5%)	-	-
Gastrointestinal tract infections	4 (1.6%)	2 (2.9%)	-	-
Urinary tract infections	2 (0.8%)	1 (0.9%)	-	-
Pneumoniae	1 (0.4%)	1 (0.9%)	-	-
Sinusitis	0 (0%)	3 (2.8%)	-	-
Leptospirosis	3 (1.2%)	2 (1.9%)	-	-
Tonsilitis	10 (4.1%)	12 (11.1%)		

[a]Some patients presented more than one clinical suspicion recorded at hospital chart

Table 2. Sensitivity of SD DENGUE DUO Rapid Diagnostic Test (RDT) according to type of infection, infecting serotype, days post onset of symptoms and reference tests confirmation criteria.

	Samples (n=)	NS1 RDT Positive (n=)	NS1 RDT Sensitivity % (95% CI)	IgM RDT Positive (n=)	IgM RDT Sensitivity % (95% CI)	NS1 or IgM RDT Positive (n=)	NS1 or IgM RDT Sensitivity % (95% CI)
Overall	246	95	38.6 (32.5 - 45.0)	34	13.8 (9.8 - 18.8)	115	46.8 (40.4 - 53.2)
Type of infection[a]							
Primary	45	12	26.7 (14.6 - 41.9)	2	4.4 (0.5 - 15.2)	14	31.1 (18.2 - 46.7)
Secondary	199	81	40.7 (33.8 - 47.9)	31	15.6 (10.8 - 21.4)	99	49.8 (42.6 - 56.9)
Serotype							
DENV1	18	10	55.6 (30.8 - 78.5)	2	11.1 (1.4 - 34.7)	11	61.1 (35.8 - 82.7)
DENV2	113	52	46.0 (36.6 - 55.7)	18	15.9 (9.7 - 24)	61	54.0 (44.4 - 63.4)
DENV4	49	30	61.2 (46.2 - 74.8)	7	14.3 (5.9 - 27.2)	34	69.4 (54.6 - 81.8)
Days of symptoms							
0 to 2	125	54	43.2 (34.4 - 52.4)	8	6.4 (2.8 - 12.2)	60	48.0 (39.0 - 57.1)
3 to 4	88	35	39.8 (29.5 - 50.8)	17	19.3 (11.7 - 29.1)	42	47.7 (37.0 - 58.7)
≥5	29	5	17.2 (5.9 - 35.8)	8	27.6 (12.7 - 47.2)	11	37.9 (20.7 - 57.7)
Confirmation criteria							
RT-PCR	58	8	13.8 (6.2 - 25.4)	15	25.9 (15.3 - 39.0)	20	34.5 (22.5 - 48.1)
IgMSC[b]	63	2	3.2 (0.4 - 11.0)	6	9.5 (3.6 - 19.6)	8	12.7 (5.7 - 23.5)
RT-PCR and NS1	36	34	94.4 (81.3 - 99.3)	8	22.2 (10.1 - 39.2)	34	94.4 (81.3 - 99.3)
RT-PCR and IgMSC	52	19	36.5 (23.6 - 51.0)	4	7.7 (2.1 - 18.5)	21	40.4 (27.0 - 54.9)
RT-PCR, NS1 and IgMSC	35	31	88.6 (73.3 - 96.8)	0	0.0 (0.0 - 10.0)	31	88.6 (73.3 - 96.8)

[a]According to IgM-ELISA in the acute sample (IgM negative: primary infection; IgM positive: secondary infection)

[b]IgMSC: IgM-ELISA seroconversion

Table 3. Specificity of SD DENGUE DUO Rapid Diagnostic Test (RDT) components according to control group.

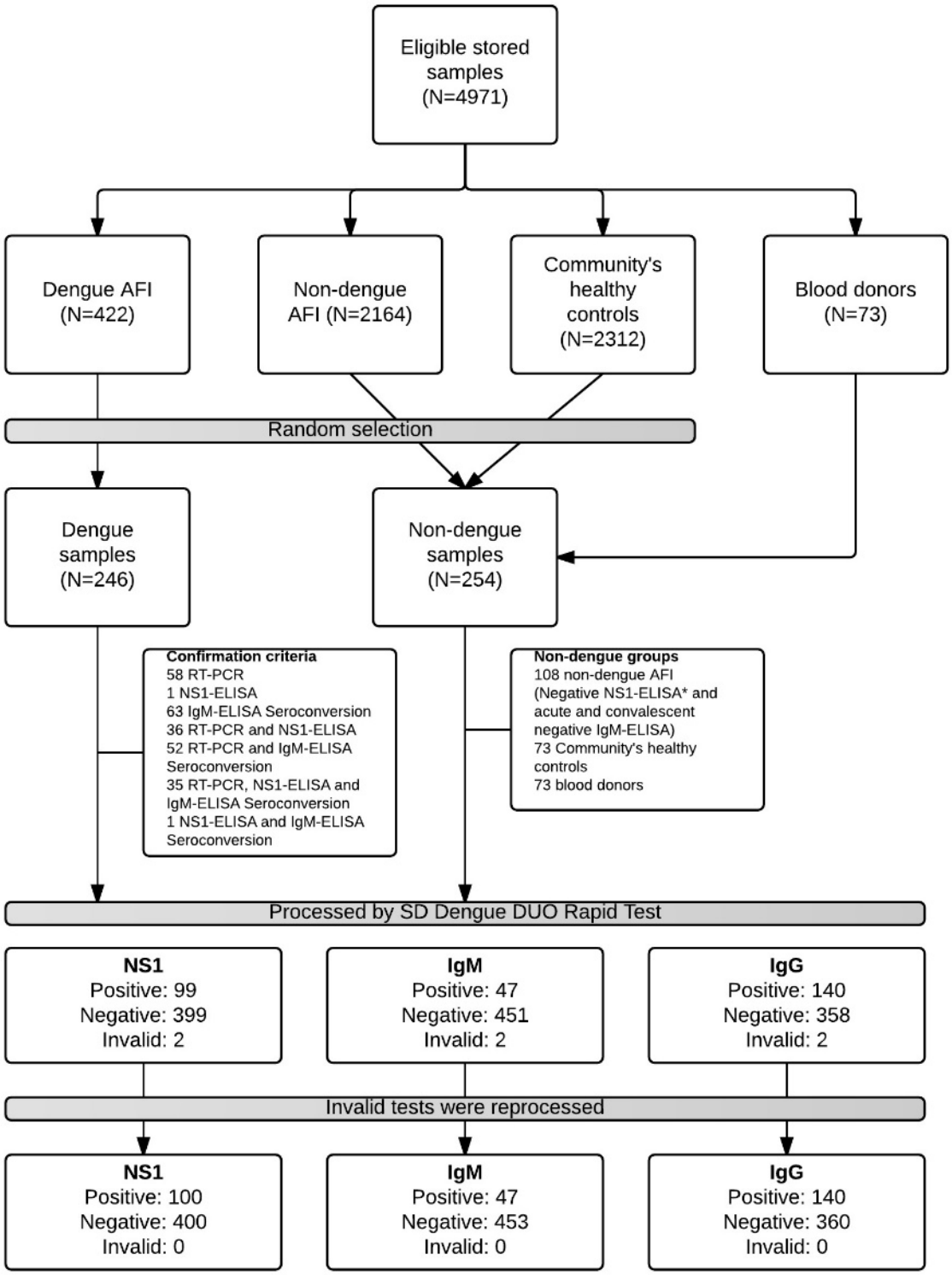
	False Positive (n=)	True Negative (n=)	Specificity % (95% CI)
Non-dengue AFI			
NS1 RDT	2	106	98.2 (93.5 - 99.8)
IgM RDT	4	104	96.3 (90.8 - 99.0)
NS1 or IgM RDT	6	102	94.4 (88.3 - 97.9)
Community's healthy controls			
NS1 RDT	2	71	97.3 (90.5 - 99.7)
IgM RDT	7	66	90.4 (81.2 - 96.1)
NS1 or IgM RDT	9	64	87.7 (77.9 - 94.2)
Blood donors			
NS1 RDT	1	72	98.6 (92.6 - 100.0)
IgM RDT	2	71	97.3 (90.5 - 99.7)
NS1 or IgM RDT	3	70	95.9 (88.5 - 99.1)

Table 4. Accuracy of SD DENGUE DUO Rapid Diagnostic Test (RDT) components compared to its correspondent ELISAs.

	RDT + / ELISA +	RDT - / ELISA +	RDT + / ELISA -	RDT - / ELISA -	Sensitivity % (95% CI)	Specificity % (95% CI)
NS1	66	9	32	377	88.0 (78.4 - 94.4)	92.2 (89.1 - 94.6)
IgM	26	62	20	380	29.3 (20.3 - 40.2)	95.0 (92.4 - 96.9)
IgG	105	236	0	71	30.8 (25.9 - 36.0)	100.0 (94.9 - 100.0)

[a]Equivocal results on ELISAs were excluded from analysis

Figure 1. Sample selection and SD DENGUE DUO testing.



3. Artigo 2 – Prospective field evaluation a rapid dengue diagnosis test in a region endemic for dengue, Zika, and Chikungunya

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Introduction

Dengue is one of the most important arbovirus, with an estimated 390 million new infections per year worldwide (BHATT et al., 2013). In the Americas, over two million dengue cases were reported in 2016, of which Brazil alone accounted for almost 1.5 million (PAHO, 2017). Dengue virus (DENV) infections may be asymptomatic or cause a wide spectrum of clinical manifestations, from self-limiting mild disease with non-specific symptoms such as acute fever, myalgia, and headache, to severe disease with plasma leakage and hemorrhage (WHO, 2009). Although clinical management is relatively simple, timely interventions are key in preventing complications and may influence disease lethality (TEIXEIRA et al., 2005; WHO, 2012). An opportune diagnosis also

provides key information for epidemiological surveillance, allowing rapidly detection of outbreaks and prompt response.

Laboratory diagnosis of dengue comprise the isolation of virus, detection of viral RNA, or of viral antigens (such as the non-structural protein NS1) during the acute-phase of disease, or the detection of anti-DENV IgM and IgG antibodies later in the course of disease (WHO, 2012). However, the choice of the dengue diagnostic method to be used should consider in addition to the timing of the disease clinical course, other factors that may pose advantages and limitations of each technique, such as accuracy, cost, necessity of specialized personnel or equipment, workload, and time delay to obtain a result (KAO et al., 2005). Many of the commonly used dengue tests, such as RT-PCR, viral isolation, and enzyme linked immunosorbent assay (ELISA) for IgM, IgG, or NS1 detection, are time-consuming, or require laboratory structure not commonly found in basic health units, hampering a timely diagnosis that could contribute in the clinical decision and patient care.

Rapid tests are practical, ease of use, and offer rapid results. Thus, they have appealing potential to be implemented in outpatient and hospital care units. However, rapid tests generally have lower sensitivity and specificity compared to recommended reference tests and, therefore, their results should be interpreted with caution and reliable serological test are preferred in case confirmation (WHO, 2009). As of 2014, the Brazilian Ministry of Health considers results of rapid tests for NS1 detection as a laboratory confirmation criterion for dengue fever (BRAZIL, 2013). However, few prospective, field studies have evaluated the performance of dengue rapid tests in differentiating dengue of other febrile illnesses at point-of-care health units (ANDRIES et al., 2012a; GAN et al., 2014; SÁNCHEZ-VARGAS; SÁNCHEZ-MARCE; VIVANCO-CID, 2014). Field-evaluations are important to determine both the tests' performance and their clinical utility in a local context and in the target population (PEELING et al., 2010).

This study evaluated the diagnostic performance of a rapid diagnosis test (RDT) for dengue, capable of simultaneal detection of the non-structural NS1 protein and IgM antibodies (SD Dengue Duo). Our aim was to determine the accuracy in which this RDT can discriminate dengue among febrile patients attending an emergency, outpatient

health unit in a region of concomitant transmission of DENV, Zika (ZIKV), and chikungunya (CHIKV) viruses.

Methods

Study design. Between June 2015 to June 2016, a prospective, enhanced surveillance study to monitor for arboviral infections among acute febrile illness has been conducted in the sole public outpatient health unit (São Marcos Emergency Center [SMEC]; 38°26'09"W, 12°55'32"S) of Pau da Lima community, in Salvador, Northeast Brazil. Dengue incidence in the neighborhood was estimated at 21 and 70 per 10,000 inhabitants in 2009 and 2010, respectively (KIKUTI et al., 2015). The unit mainly serves the local residents of Pau da Lima, but also attend patients coming from several regions of metropolitan Salvador.

The surveillance recruited patients ≥ 6 months old attending SMEC in business hours (Monday to Friday, 07h30 to 16h00) and reporting fever or presenting axillary temperature ≥ 37.8°C for up to 7 days plus two or more of the following: headache, retroorbital pain, myalgia, arthralgia, prostration or exanthema (BRASIL, 2009). Patients who consent participation were subjected to acute-phase blood sampling (at the time of recruitment). SD Dengue Duo RDT test was performed immediately after acute-phase blood sample collection and results were timely handed to the attending physician. A convalescent-phase (>15 days after recruitment) blood sampling was schedule, either at SMEC or at patient's household.

RDT Testing. A research team, blinded regarding physician's clinical suspicion and following the manufacturer's instruction, performed the SD Dengue Duo RDT testing. Briefly, whole blood was added in both the NS1 test component (100 µL) and the IgM/IgG component (10 µL plus 4 drops of the buffer solution). Reading was performed in a bright area within 15-20 minutes by two trained independent readers. Each component of the RDT test (NS1 and IgM/IgG) was considered positive if it showed two bands (the positive test band and the internal control band), negative if it presented only the internal control

band, and invalid if the internal control band was absent. Results by each reader were recorded separated and then compared. If results were discrepant, both readers openly re-interpreted the RDT until a consensus result was found. No repetitions were made if the RDT presented an invalid result. RDT testing result was immediately handed to the attending physician and to the patient. Before being used, all tests had been stored at the health unit at room temperature in a closed room with air conditioning (Salvador has mean annual temperature of 25.3°C and relative humidity of 80.9%) (INSTITUTO NACIONAL DE METEOROLOGIA, 2014).

Reference tests. Acute- and convalescent-phase samples were processed daily and store in -20°C and -70°C aliquots. Acute-phase samples were tested by dengue NS1- and IgM-ELISA (Panbio Diagnostics, Brisbane, Australia), as well as by RT-PCR for DENV detection (LANCIOTTI et al., 1992). Convalescent-phase samples were tested by IGM-ELISA. A dengue case was defined as a patient presenting a positive NS1- or IgM-ELISA (either acute- or convalescent-phase), or a positive RT-PCR, as defined by the Brazilian Ministry of Health (BRASIL, 2016). A non-dengue case was defined as an AFI patient without any laboratory evidence of dengue infection according to the reference tests. Additionally, acute-phase serum samples were also tested by IgM-ELISA for Chikungunya (CHIKV) (Inbios International Inc., Washington, USA) and if the tests yielded negative results, the respective convalescent-phase serum sample underwent the same test to investigate seroconversion. Acute-phase samples were also tested by RT-PCR for CHIKV (EDWARDS et al., 2007) and ZIKV (BALM et al., 2012) for specificity evaluation purposes.

Statistical analysis. Participants were described regarding demographic and clinical characteristics. Dengue RDT results were described according to dengue confirmation criteria. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were assessed by RDT component against dengue AFI cases and non-dengue AFI controls as defined by the reference tests. Sensitivity was also calculated by DENV serotype, days of symptoms, and laboratory confirmation criteria. Confidence

intervals at 95% were calculated for all accuracy measurements. Agreement between RDT and its correspondent ELISA was calculated using global agreement (overall concordant results divided by total tested) and Kappa. For combined NS1- and IgM-RDT assessment, a positive result was considered if either component yielded a positive result, a negative result was defined as NS1- and IgM-RDT negative, and samples with missing or invalid results for either component were excluded from the analysis. This study was design according to the WHO guidelines for evaluation of diagnosis tests for dengue and the Standards for Reporting of Diagnostic Accuracy (STARD) (BOSSUYT et al., 2003; PEELING et al., 2010).

Ethics statement. This project was approved by the Research Ethics Committee at the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, the Brazilian National Council for Ethics in Research. All adult subjects provided written informed consent. Participants <18 years old who were able to read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis. However, dengue is a notifiable disease and confirmed cases of dengue were reported to the competent authorities. The results of the RDT were provided to the attending physicians and patients in a timely manner for clinical decision making.

Results

A total of 582 AFI patients were recruited, of which 126 (21.7%) had reference test results compatible with our dengue case classification criteria. 81 of these were confirmed by either acute- or convalescent-phase IgM-ELISA, 25 by RT-PCR, 16 by IgM-ELISA seroconversion and 5 by both RT-PCR and IgM-ELISA seroconversion (Table 1). Most common clinical signs and symptoms presented were headache, prostration, and myalgia (Table 1). A suspected diagnosis was not frequently registered at the medical chart, but dengue and chikungunya were more frequent diagnosis registered (Table 1).

A total of 580/582 (99.5%) of the AFI patients had an RDT test result. The remaining 2 patients without RDT results did not have sufficient blood sample collected

for both the reference testing and RDT testing, and reference tests were prioritized. NS1-RDT tested invalid in 23 samples, whereas the IgM/IgG component of the RDT was invalid on only 3 of the samples tested. In total, 7 samples were NS1-RDT positives, 64 were IgM-RDT positives and 322 were IgG-RDT positives. Sensitivity, specificity, and predictive values of the NS1- and IgM-RDT components and combination of both are described in Table 2. The NS1-RDT component had the lower sensitivity, positive predictive value, and negative predictive value (1.7%, 28.6% and 78.6%, respectively), and presented the higher specificity (98.9%).

Serotype identification among confirmed dengue AFI cases was possible in only 29 of the confirmed AFI cases, of which 2 were DENV1, 13 were DENV3 and 14 were DENV4. NS1-RDT did not detect any of them, whereas IgM-RDT detected one DENV1 and two DENV4 cases (Table 3). Overall sensitivity of the RDT considering both NS1 and IgM components or considering each component separately did not varied according to days past onset of symptoms at the moment of testing or by laboratory confirmation criteria (Table 3). Global agreement between NS1-RDT and NS1-ELISA was 98.9%, between IgM-RDT and acute-phase sample IgM-ELISA was 79.4% (kappa 0.111), and between IgG-RDT and acute-phase IgG-ELISA was 63.6% (kappa 0.138), excluding invalid and equivocal results from ELISAs and RDT. Intra-rater global agreement was 98.5% (kappa 0.830) for the NS1 component, 97.8% (kappa 0.892) for the IgM component and 96.6% (kappa 0.931) for the IgG component.

Among the 582 AFI patients recruited, 213 had laboratory evidence of Chikungunya infection (87 by RT-PCR, 57 by RT-PCR and IgM-ELISA seroconversion, 42 by acute-phase IgM-ELISA, and 27 by IgM-ELISA seroconversion). Among those, 66/213 were also classified as a dengue case according to dengue reference laboratory testing and 36/213 were NS1- and/or IgM-RDT positives. Dengue RDT specificity among Chikungunya confirmed patients was 85.7% (95%CI 78.8 – 91.1).

RT-PCR for Zika virus detection was positive in 2 of the 571 tested AFI patients, and both were also considered dengue cases (one by dengue IgM-ELISA seroconversion, and one by acute- and convalescent-phase dengue IgM-ELISA positive

as well as a positive RT-PCR for DENV). Only the later was tested by the RDT, which yielded a negative result in the NS1 component and positive result in the IgM component.

Discussion

Overall the RDT assessed presented poor sensitivity and positive predictive value, good specificity, and fair negative predictive value. However, considering rapid tests are quick and easy diagnose methods for point of care and bedside use, its ideal characteristics would be to correctly distinguish dengue from diseases with similar clinical manifestations and to be highly sensitive during the acute stage of infection (PEELING et al., 2010). Our results indicate that a negative result in the RDT does not rule out dengue infection, thus it may not be appropriate as a screening method. Low sensitivity of this RDT was also found in a Cambodian study with febrile hospitalized children, where a sensitivity of 58% was found when combining NS1 and IgM components (CARTER et al., 2015). However, many other studies found better performance, from 75.5% to 97.5% (ANDRIES et al., 2012b; BLACKSELL et al., 2011; GAN et al., 2014; OSORIO et al., 2015; TRICOU et al., 2010; VICKERS et al., 2015; WANG; SEKARAN, 2010). This difference may be explained due to a more severe and typical clinical manifestation of the patients whose samples were used to evaluate this test diagnosis performance on those studies or even differences in circulating DENV serotypes, in incidences of primary and secondary infections, and differences in reference methods used to diagnose dengue infections.

Sensitivity of the RDT did not change substantially according to days post onset of symptoms when sample was collected for testing. We would expect, however, NS1 component's sensitivity to decrease over time whereas IgM component's sensitivity should increase, as NS1 is can be detected in the bloodstream up to 9 days after onset of illness, whereas IgM is initially detectable between 3 to 5 days post onset of symptoms (PEELING et al., 2010). Possible reasons why this phenomenon was not observed in our study are the few cases identified by the RDT, thus power of such analysis was limited, and the fact that majority of the patients included in the study sought medical care with less than 5 days of symptoms, so in the very early stages of disease.

We observed a high frequency of dengue cases throughout the study period (20%). Salvador experienced the introduction and epidemic transmission of ZIKV and CHIKV in early 2015 (CARDOSO et al., 2015, 2017). Although we identified a high frequency of CHIKV infections among our febrile patients, only 2 ZIKV cases were identified. Since most patients presented to the health unit with less than four days of onset of symptoms, which favors diagnosis with direct and more specific techniques such as RNA detection, ZIKV laboratory diagnosis is difficult because viremia tends to be low in magnitude (MUSSO et al., 2016). More than half of all dengue cases were defined due to either an acute- or convalescent-phase IgM-ELISA positive without seroconversion. Although cross-reaction with ZIKV may be present, additional analysis considering as dengue cases only those with a positive NS1-ELISA or RT-PCR showed similar results (Appendix Table 1).

The evaluation of this RDT as a point-of-care diagnosis tool for patients presenting symptoms compatible with dengue infection in a dengue, Zika, and Chikungunya endemic region revealed a poor performance as a screening test, due to low specificity and positive predictive value. However, the test presented high specificity and fair negative predictive value, which would be useful to rule out dengue infections.

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Table 1. Demographic and clinical characteristics of acute febrile illness (AFI) patients and dengue confirmation criteria

	AFI patients (n=582)
Age - median (IQR)	29 (19 - 40)
Male - n/N (%)	274/579 (47.3%)
Days of symptoms - median (IQR)	2 (1 - 4)
Clinical manifestations - n/N (%)	
Headache	537/585 (92.3%)
Prostration	524/581 (90.2%)
Myalgia	519/581 (89.3%)
Retro-orbital pain	406/575 (70.6%)
Arthralgia	478/582 (82.1%)
Rash	167/579 (28.8%)
Vomiting	162/581 (27.9%)
Diagnosis registered at medical chart* - n (%)	297/571 (52.0%)
Dengue	119/286 (41.6%)
Zika	39/297 (86.9%)
Chikungunya	27/297 (9.1%)
Undetermined exanthematic disease	9/297 (3.0%)
Upper respiratory tract infections	15/297 (5.1%)
Undetermined viral disease	140/297 (47.1%)
Urinary tract infections	5/297 (1.7%)
Gastrointestinal tract infections	9/297 (3.0%)
Tonsilitis	7/297 (2.4%)
Pneumoniae	2/297 (0.7%)
Sinusitis	3/297 (1.0%)
Leptospirosis	2/297 (0.7%)
Dengue confirmation crateria	
RT-PCR positive	25/582 (4.3%)
IGM-ELISA seroconversion	16/582 (2.8%)
RT-PCR positive and IgM-ELISA seroconversion	4/582 (0.7%)
IgM-ELISA positive without seroconversion	81/582 (13.9%)
Non-dengue AFI**	456/582 (78.4%)

IQR: Interquartile range

*Diagnosis registered at medical chart are not mutually exclusive

Table 2. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of SD DENGUE DUO Rapid Diagnostic Test (RDT).

	True Positive (n=)	False Negative (n=)	False Positive (n=)	True Negative (n=)	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
NS1 RDT	2	118	5	432	1.7% (0.2 - 5.9)	98.9 (97.4 - 99.6)	28.6 (3.7 - 71.0)	78.6 (74.9 - 81.9)
IgM RDT	23	102	41	411	18.4 (12.0 - 26.3)	90.9 (87.9 - 93.4)	35.9 (24.3 - 48.9)	80.1 (76.4 - 83.5)
NS1 or IgM RDT	25	96	46	392	20.7 (13.8 - 29.0)	89.5 (86.2 - 98.2)	35.2 (24.2 - 47.5)	80.3 (76.5 - 83.8)

Table 3. Sensitivity of SD DENGUE DUO Rapid Diagnostic Test (RDT) according to type of infection, infecting serotype, days post onset of symptoms and reference tests confirmation criteria.

	NS1 RDT TP[a]/Diseased (n/N)	**NS1 RDT Sensitivity % (95% CI)**	**IgM RDT TP[a]/Diseased (n/N)**	**IgM RDT Sensitivity % (95% CI)**	**NS1 or IgM RDT TP[a]/Diseased (n/N)**	**NS1 or IgM RDT Sensitivity % (95% CI)**
Serotype						
DENV1	0/2	0.0 (0.0 - 84.2)	0/2	0.0 (0.0 - 84.2)	0/2	0.0 (0.0 - 84.2)
DENV3	0/13	0.0 (0.0 - 24.7)	2/13	15.4 (1.9 - 45.5)	2/13	15.4 (1.9 - 45.5)
DENV4	0/13	0.0 (0.0 - 24.7)	2/14	14.3 (1.8 - 42.8)	3/13	23.1 (5.0 - 53.8)
Days of symptoms						
0 to 2	1/54	1.9 (0.1 - 9.9)	11/58	19.0 (9.9 - 31.4)	12/54	22.2 (12.0 - 35.6)
3 to 4	1/46	2.2 (0.1 - 11.5)	7/47	14.9 (6.2 - 28.3)	8/47	17.0 (7.7 - 30.8)
≥5	0/20	0.0 (0.0 - 16.8)	5/20	25.0 (8.7 - 49.1)	5/20	25.0 (8.7 - 49.1)
Confirmation criteria						
RT-PCR	1/24	4.2 (0.1 - 21.1)	3/25	12.0 (2.6 - 31.2)	4/24	16.7 (4.7 - 37.4)
IgMSC[b]	0/13	0.0 (0.0 - 24.7)	4/15	26.7 (7.8 - 55.1)	4/13	30.8 (9.1 - 61.4)
RT-PCR and IgMSC	0/4	0.0 (0.0 - 60.2)	1/4	25.0 (0.6 - 80.6)	1/4	25.0 (0.6 - 80.6)
IgM postitive without IgMSC	1/79	1.3 (0.03 - 6.9)	15/81	18.5 (10.8 - 28.7)	16/80	20.0 (11.9 - 30.4)

Appendix Table 1. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of SD DENGUE DUO Rapid Diagnostic Test (RDT) components considering only NS1-ELISA and RT-PCR as reference laboratory testing.

	True Positive (n=)	False Negative (n=)	False Positive (n=)	True Negative (n=)	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
NS1 RDT	1	27	5	432	3.6 (0.1 - 18.4)	98.9 (97.4 - 99.6)	16.7 (0.4 - 64.1)	94.1 (91.6 - 96.1)
IgM RDT	4	25	41	411	13.8 (3.9 - 31.7)	90.9 (87.9 - 93.4)	8.9 (2.5 - 21.2)	94.3 (91.7 - 96.3)
NS1 or IgM RDT	5	23	46	392	17.9 (6.1 - 36.9)	89.5 (86.2 - 92.2)	9.8 (3.3 - 21.4)	94.5 (91.8 - 96.5)

4. Artigo 3 – Diagnostic performance of dengue enzyme-linked immunoassays (ELISAs) in an endemic region for dengue and Zika viruses.

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Introduction

Dengue is one of the most important arboviral disease, causing large epidemics with over 390 million estimated infections yearly worldwide, of which 96 million are expected to be symptomatic (BHATT et al., 2013). Dengue virus (DENV) is a flavivirus that causes self-limiting non-severe acute febrile exanthematous illness. Typical clinical manifestations may also be accompanied by myalgia, arthralgia and headache (WHO, 2009). Although minority of cases evolve with severe manifestations, infected patients may present plasma leakage and hemorrhage leading to shock and even death (WHO, 2009).

DENV diagnosis comprise the detection of virus genetic material or viral antigen (such as the non-structural protein NS1) during the acute-phase of disease or the detection of anti-dengue virus IgM and IgG antibodies levels on later stages of infection

(WHO, 2012). Since molecular detection is still a relatively expensive technique, DENV diagnosis often relies on serological test, especially considering the burden of an epidemic transmission. However, Zika virus (ZIKV, also a flavivirus) infections emerged recently worldwide, making flavivirus serology even more difficult due to antigenic cross-reactions (DEJNIRATTISAI et al., 2016; DUFFY et al., 2009; LANCIOTTI et al., 2008). Differentiation between DENV and ZIKV infections may be particularly important among pregnant women, in which a ZIKV infection can result in congenital malformations (BRASIL et al., 2016; DE ARAÚJO et al., 2016; RASMUSSEN et al., 2016).

In the present study we aimed to evaluate the diagnostic sensitivity and specificity of commercially available enzyme-linked immunoassays (ELISAs) for detection of DENV non-structural protein 1 (NS1) antigen and DENV IgM antibodies in blood samples of febrile patients with DENV and ZIKV infections confirmed by RT-PCR.

Methods

Study site

The study was carried out in Pau da Lima, a slum community in Salvador, Northeast Brazil. Salvador has experienced dengue epidemics endemically since 1995, with approximately 7,000 cases reported each year between 2011 and 2016 (BRASIL, 2017a; TEIXEIRA et al., 2001). Pau da Lima community also have historically high transmissions of DENV, with annual incidences of 21.3 to 70.2 cases per 10,000 inhabitants (KIKUTI et al., 2015). In early 2015, a large outbreak of exanthematous illness occurred in Salvador with over 17,000 reported cases, and was latter attributed to Zika virus (CARDOSO et al., 2015a; PAPLOSKI et al., 2016). From then on, the Northeast remains as one of the regions most affected by ZIKV transmission in Brazil, with incidences of 132.0 and 9.2 cases per 100,000 inhabitants in 2016 and 2017, respectively (BRASIL, 2017b). Other arbovirus also co-circulate in Salvador, such as Chikungunya virus (CHIKV) which transmission began in late 2014 with few reported cases, although a large epidemics appeared to have occurred only in mid 2015 (CARDOSO et al., 2015b).

Surveillance for acute febrile illness.

From September 2014 to March 2015, we recruited acute febrile illness (AFI) patients attending to the solely public health unit that serves Pau da Lima community (Emergency Unit of São Marcos, EUSM) with the follow inclusion criteria: having ≥ 6 months old, residing in Pau da Lima study site, presenting to the EUSM with reported or measured fever (≥ 37,8°C) up to 7 days of duration plus two or more signs/symptoms compatible with dengue (headache, retroorbital pain, myalgia, arthralgia, prostration or exanthema)(BRASIL, 2009). From April 2015 to July 2016, the surveillance used more inclusive recruitment criteria which comprised AFI patients ≥ 6 months old with reported or measured fever up to 7 days of duration, regardless of additional signs/symptoms and place of residency. Patients who consent participation had acute-phase (at enrollment) and convalescent-phase (~15 days after enrollment) blood sample drawn for arbovirus diagnosis. Demographic and clinical characteristics data were obtained by a structured standard questionnaire applied to patients at enrollment and by chart review.

Laboratory testing for arboviral diseases.

All acute-phase serum samples aliquots which were previously unthawed and stored at -70°C were submitted to RNA extraction and tested by RT-PCR for DENV, ZIKV, and CHIKV detection. Briefly, viral RNA was extracted using the Maxwell® 16 Total RNA Purification kit (Promega, Wisconsin, USA) or QIAmp® Viral RNA mini kit commercial kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Subsequently, RT-PCR (Access RT-PCR kit- Promega, Wisconsin, USA) was performed separately on the extraction product using specific primers to identify DENV (LANCIOTTI et al., 1992), ZIKV (BALM et al., 2012) and CHIKV (EDWARDS et al., 2007).

All acute-phase serum samples were tested by DENV NS1- and DENV IgM- (both by Panbio Diagnostics, Brisbane, Australia). All convalescent-phase samples were tested by DENV IgM-ELISA (Panbio Diagnostics, Brisbane, Australia). ELISA reading was performed by automated microplate reader (TECAN, Maennedorf, Switzerland). Readers

were blinded regarding clinical suspicion during testing. All serological tests were performed according to manufacturer's instruction.

Data analysis.

Patients included in the study were described according to their demographic and clinical characteristics. Arboviral infection responsible for the acute febrile illness event that led to health care seeking was defined based on DENV and ZIKV RT-PCR results as the standard reference test. A non-arboviral AFI case was defined if a negative result on RT-PCR for all three tested arbovirus was found. Positivity of each serological test in acute-phase sample, in convalescent-phase sample and seroconversion were described, when applicable, according to arboviral infection. Sensitivity, specificity, and their respective 95% confidence intervals were calculated for each serological dengue test being evaluated. Equivocal results were excluded from the analysis.

Ethic statement.

This project was approved by the Research Ethics Committee at the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, the Brazilian National Council for Ethics in Research. All adult subjects provided written informed consent. Participants <18 years old who were able to read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis. However, dengue, Zika and Chikungunya are notifiable diseases and confirmed cases of dengue were reported to the competent authorities.

Results

A total of 973 AFI patients were recruited. Of those, we were able to obtain an acute-phase serum sample from 938 and convalescent-phase serum sample from 438 patients. Paired samples were obtained from 428 of them. RT-PCR was performed in acute-phase samples of 915 patients (97.6%) for DENV and CHIKV diagnosis, and of

914 patients (97.4%) for ZIKV diagnosis. We identified 32 DENV cases and 13 ZIKV cases. A negative RT-PCR result for all three arboviruses was found for 718 patients. A total of 139 CHIKV cases, 12 co-infections of DENV and CHIKV, and one co-infection of DENV and ZIKV were identified but excluded from the analysis.

Clinical manifestations were similar among DENV, ZIKV, and non-arboviral AFI cases. However, pruritus (69.2% vs 34.4%, *p*=0.02) was more frequently reported by ZIKV than DENV cases (Table 1). Although clinical suspicion was not frequently reported in medical charts of these patients, only 17.7% of DENV cases had a correct probable diagnosis recorded. Additionally, DENV cases sought medical assistance later after onset of symptoms than ZIKV cases (median of 4 days of symptoms at recruiting) (Table 1).

Dengue IgM-ELISA yielded a positive result in the acute-phase serum sample of 16.7% DENV cases (Table 2). It also yielded positive in convalescent-phase serum sample of 47.1% of them. However, among all non-dengue cases, DENV IgM-ELISA positivity in the acute-phase sample was observed in 0% ZIKV cases, and 10.7% non-arboviral AFI cases, whereas positivity in convalescent-phase serum was observed in 42.9% and 12.7% of them, respectively. Thus, DENV IgM-ELISA presented an overall sensitivity of 16.7% in acute-phase samples and 47.1% in convalescent-phase samples (Table 3). Additionally, only 35.7% of DENV cases were detected by DENV IgM-ELISA seroconversions. Specificity was higher in acute-phase blood samples of ZIKV cases (100.0%) and among IgM-ELISA seroconversions in non-arboviral AFI cases (92.7%) (Table 3). Dengue NS1-ELISA presented a positive result in only one PCR confirmed DENV case yielding a sensitivity of 3.2%. Specificity of DENV NS1-ELISA was 100.0% among ZIKV cases and 99.9% among non-arboviral AFI cases (Table 3).

Discussion

Since the study was carried out in an outpatient health unit where patients usually present with mild disease, clinical manifestations of patients presenting with AFI were overall very similar, with exception of pruritus which was more frequently reported among ZIKV cases.

DENV IgM-ELISA had low sensitivity, especially in acute-phase serum samples (16.7%). However, since RT-PCR of the acute-phase serum sample was used as case definition, all arboviral cases identified were in the viremia phase of disease during acute-phase blood collection. Thus, a lower sensitivity of IgM detection in the acute-phase sample was to be expected. Nevertheless, the use of a direct highly sensitive and specific diagnosis test such as RT-PCR as a reference test for case definition ensures a correct identification of these arboviruses given the possibility of co-infections and cross-reactions in serological tests. Still, even when a convalescent-phase sample and seroconversion among paired samples were evaluated, sensitivity was <50%, granted a convalescent-phase serum sample was only possible to obtain in about half our patients. Dengue NS1 detection also performed poorly in the acute-phase sample testing, with less than 4% sensitivity.

Overall, specificity of serological tests evaluated among non-arboviral AFI case were good (>84%). False-positive results among the PCR Negative groups could be explained by low viremia or health care seek after the viremia period, in which case our reference test would not be able to detect such arboviral case. ZIKV cases tested positive in 42.9% of the convalescent-phase sera DENV-IgM testing. This highlights the complexity of IgM-based serodiagnosis when both these arboviruses co-circulate, especially given that DENV IgM-ELISA of the convalescent-phase serum samples was capable of correctly identify only half of DENV cases. The percentage of false-positives observed in our study if much higher than observed in a previous study in which 19% of ZIKV cases from Araraquara, Brazil, also detected by RT-PCR in plasma and/or urine, cross-reacted with DENV IgM-ELISA in the convalescent-phase serum samples (FELIX et al., 2017). Cross-reactivity between both arboviruses have been reported to occur particularly when a ZIKV infection occurs after a prior flavivirus infection (LANCIOTTI et al., 2008), which is the case of the majority of Salvador's population. Although IgM detection may also represent a recent DENV infection, the fact that none of the tested ZIKV cases presented a positive result in the acute-phase sample leads us to believe that these results are a consequence of cross-reactions between ZIKV neutralizing antibodies and the DENV IgM-ELISA evaluated.

In conclusion, in a scenario of high transmission of multiple and related arbovirus such as DENV and ZIKV, serologic diagnosis is complex as it may represent cross-reactions or recent flavivirus infections. Since clinical manifestations are similar, clinical diagnosis is also not reliable. Thus, molecular detection remains the most trustworthy diagnosis method to distinguish between these arboviral diseases in such endemic areas, albeit the limitations of cost.

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Table 1. Demographic and clinical characteristics of Dengue, Zika and non- arboviral acute febrille illness (AFI) cases, Salvador - Brazil, 2014 - 2016.

	Dengue (n=32)	Zika (n=13)	Non-abroviral AFI* (n=718)
Age - median (IIQ)	31 (14.5 - 39.5)	20 (15 - 38)	27 (18 - 38)
Gender (M) - n/N (%)	16/32 (50.0%)	6/13 (46.2%)	332/714 (46.5%)
Days of symptoms - median (IIQ)	4 (3 - 6)	2 (2 - 3)	3 (2 - 4)
Clinical manifestations - n/N (%)			
Headache	29/31 (93.6%)	12/13 (92.3%)	645/717 (90.0%)
Prostration	28/32 (87.5%)	12/13 (92.3%)	620/717 (86.5%)
Retro-orbital pain	21/31 (67.7%)	9/13 (69.2%)	456/711 (64.1%)
Myalgia	25/31 (80.7%)	11/13 (84.6%)	589/714 (82.5%)
Arthralgia	20/32 (62.5%)	7/13 (53.9%)	474/718 (66.0%)
Vomiting	9/32 (28.1%)	1/13 (7.8%)	201/718 (28.0%)
Rash	13/32 (40.6%)	9/13 (69.2%)	240/718 (33.4%)
Pruritus	11/32 (34.4%)	9/13 (69.2%)	245/718 (34.1%)
Clinical suspicion of dengue - n/N (%)	3/17 (17.7%)	1/6 (16.7%)	82/316 (26.0%)

*PCR negative for Dengue, Zika and Chikungunya

Table 2. Samples description and Dengue enzyme-linked immunoassays (ELISA) results according to RT-PCR as reference test.

	Dengue (n=32)	Zika (n=13)	Non-abroviral AFI* (n=718)
Paired samples - n/N (%)	17/32 (53.1%)	7/13 (53.9%)	330/718 (46.0%)
Days between acute and convalescent sample - median (IIQ)	18 (12 - 40)	28 (19 - 43)	18 (12 - 32)
Positive Dengue IgM-ELISA in acute sample - n/N (%)	5/30 (16.7%)	0/13 (0%)	76/708 (10.7%)
Positive Dengue IgM-ELISA in convalescent sample - n/N (%)	8/17 (47.1%)	3/7 (42.9%)	41/322 (12.7%)
Dengue IgM-ELISA seroconversion† - n/N (%)	5/14 (35.7%)	3/7 (42.9%)	21/289 (7.3%)
Positive Dengue NS1-ELISA in acute sample - n/N (%)	1/31 (3.2%)	0/13 (0%)	1/685 (0.2%)

*Acute febrile illness (AFI) patients with PCR negative results for Dengue, Zika and Chikungunya

†For patients with paired samples and acute-phase IgM-ELISA negative

Table 3. Sensitivity and specificity of Dengue enzyme-linked immunoassays (ELISA).

		Specificity % (95%CI)	
	Sensitivity % (95%CI)	Zika	Non-arboviral AFI*
Dengue IgM-ELISA in acute sample	16.67% (5.64 - 34.72)	100.00% (75.29 - 100.00)	89.27% (86.75 - 91.45)
Dengue IgM-ELISA in convalescent sample	47.06% (22.98 - 72.19)	57.14% (18.41 - 90.10)	87.27% (83.13 - 90.71)
Dengue IgM-ELISA seroconversion†	35.71% (12.76 - 64.86)	57.14% (18.41 - 90.10)	92.73% (89.11 - 95.45)
Dengue NS1-ELISA in acute sample	3.23% (0.08 - 16.70)	100.00% (75.29 - 100.00)	99.85% (99.19 - 100.00)

*Acute febrile illness (AFI) patients with PCR negative results for Dengue, Zika and Chikungunya

†For patients with paired samples and acute-phase IgM-ELISA negative

5. Artigo 4 – Evaluation of a commercially available chikungunya virus immunoglobulin M detection ELISA in an endemic region for chikungunya, dengue and Zika viruses.

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Abstract

Acute- and convalescent-phase blood collections from acute febrile illness patients were performed and sera was tested by RT-PCR for chikungunya (CHIKV), dengue and Zika virus. Using RT-PCR as gold standard, we evaluated the diagnostic performance of the the CHIKjj Detect IgM-capture ELISA kit (Inbios). We found a high sensitivity and fair positive predictive value of the CHIKV IgM-ELISA specially in the convalescent-phase sera (94.4% and 67.1%, respectively). Specificity and negative predictive values were high (>80%) regardless of acute- or convalescent-phase. Dengue cases yielding a false positive result may represent recent or sequential infection in the context of intense arboviral transmission.

Introduction

Chikungunya virus (CHIKV) is an alphavirus transmitted by the *Aedes* mosquitoes (WHO, 2008). Transmission was initially restricted to few outbreaks and sporadic cases in Africa and Asia, but caused large outbreaks in India and Southeast Asia in early 2000s and in the Caribbean in 2013 (ZELLER; VAN BORTEL; SUDRE, 2016). It now represents a great burden in Latin America, with over 351,334 reported cases in 2016 alone (PAHO, 2017). Brazil experienced a large epidemics in 2016 with over 260,000 reported cases, of which more than 80% occurred in the northeast region (BRASIL, 2017). In Salvador, northeast Brazil, where flavivirus also transmitted by the *Aedes* mosquitoes such as dengue (DENV) and Zika virus (ZIKV) occurs endemically since 1995 and 2015 (CARDOSO et al., 2015; TEIXEIRA et al., 2001), respectively, CHIKV was introduced in 2014, causing a larger outbreak in mid-2015 where approximately 70% of all sera of suspected patients sent for CHIKV diagnosis was positive (CARDOSO et al., 2017).

Clinical manifestations are unspecific, usually presenting as a self-limiting non severe acute febrile exanthematous illness, however arthralgia is the most prominent symptom (WHO, 2008). In areas where arboviruses with similar clinical manifestations co-occur, laboratory confirmation is essential to differentiate CHIKV from DENV and ZIKV infections, as well as to exclude other rheumatologic disorders to provide appropriate management.

Here we evaluated the diagnostic performance of commercially available enzyme-linked immunoassays (ELISAs) for detection of CHIKV-specific IgM antibodies in sera of febrile outpatients from Salvador, Brazil.

Methods

Surveillance for acute febrile illness.

From September 2014 to July 2016, we recruited acute febrile illness (AFI) patients attending to the solely public health unit that serves Pau da Lima community (Emergency Unit of São Marcos, EUSM) with the follow inclusion criteria: having ≥ 6 months old and presenting to the EUSM with reported or measured fever (≥ 37,8°C) up to 7 days of duration. Patients who consent participation had acute-phase (at enrollment) and

convalescent-phase (≥15 days after enrollment) blood sample drawn for arbovirus diagnosis. Demographic and clinical characteristics data were obtained by a structured standard questionnaire applied to patients at enrollment and by chart review.

Reference laboratory testing for arboviral diseases.

All acute-phase serum samples aliquots which were previously unthawed and stored at -70°C were submitted to RNA extraction and tested by RT-PCR for DENV, ZIKV, and CHIKV detection. Briefly, viral RNA was extracted using the Maxwell® 16 Total RNA Purification kit (Promega, Wisconsin, USA) or QIAmp® Viral RNA mini kit commercial kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Subsequently, RT-PCR (Access RT-PCR kit- Promega, Wisconsin, USA) was performed separately on the extraction product using specific primers to identify DENV (LANCIOTTI et al., 1992), ZIKV (BALM et al., 2012) and CHIKV (EDWARDS et al., 2007). CHIKV, DENV, and ZIKV cases were defined if a positive result on each arboviral-specific RT-PCR was found. A PCR negative case was defined if a negative result on RT-PCR for all three tested arbovirus was found.

Detection of CHIKV-specific IgM antibodies by ELISA

Sera was tested using the CHIKjj Detect IgM-capture ELISA kit (Inbios, Washington, USA). All acute-phase sera were tested, as well as convalescent-phase sera from patients who had a negative CHIK IgM-ELISA result in the acute-phase. Tests were performed according to manufacturer's instruction. Briefly, positive, cut-off and negative controls provided by the kit as well as sera to be tested were diluted and added to microtiter well pre-coated with capture anti- human IgM. Plate was submitted to several incubations (37°C for 30 minutes with CHIKV antigen, 37°C for 30 minutes with enzyme conjugate, and in the dark at room temperature for 10 minutes with TMB substrate) interspaced by washes and finalized with the addition of a stop solution. ELISA reading was performed by automated microplate reader at 450nm (TECAN, Maennedorf, Switzerland). Whenever the ratio in the optical density obtained with the patients' serum

and the calibrator was ≥0.9 to <1.1 testing was repeated. Final ratios of <1.0 were interpreted as negative results and ≥1.1 were considered positive.

Data analysis.

Patients included in the study were described according to their demographic and clinical characteristics. Sensitivity and positive predictive value considering positivity of serological testing in acute- and convalescent-phase samples as well as IgM seroconversion were calculated for CHIKV cases. Specificity and negative predictive value were calculated considering DENV, ZIKV, and PCR negative control groups. 95% confidence intervals were calculated for all accuracy measurements. Detailed positivity by control group was also described.

Ethic statement.

This project was approved by the Research Ethics Committee at the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation, the Brazilian National Council for Ethics in Research. All adult subjects provided written informed consent. Participants <18 years old who were able to read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis. However, dengue, Zika and Chikungunya are notifiable diseases and confirmed cases were reported to the competent authorities.

Results

A total of 973 AFI patients were recruited. RT-PCR was performed in acute-phase samples of 915 patients (97.6%) for DENV and CHIKV diagnosis, and of 914 patients (97.4%) for ZIKV diagnosis. We identified 139 CHIKV cases, 32 DENV cases and 13 ZIKV cases. Co-infections were also identified (12 DENV and CHIKV, and one DENV and ZIKV co-infections) but were excluded of this analysis. The remaining 718 samples presented a negative RT-PCR result for all three arboviruses.

Arthralgia was more frequently reported by CHIKV cases (95.7%) compared to DENV and ZIKV cases (62.5% and 53.9%, respectively, Table 1). Although clinical suspicion was not frequently reported in the medical charts of these patients, only 16.5% of CHIKV cases had a correct probable diagnosis recorded. CHIKV cases also sought medical assistance earlier after onset of symptoms (median of one day post onset of symptoms) and convalescent-phase blood collection was also earlier than the control groups patients (median 19 days post onset of symptoms; Table 1).

Sensitivity of the chikungunya IgM-ELISA was higher in convalescent- than in acute-phase sera (93.4% versus 3.7%; Table 2). However, although convalescent-phase sera testing also had a higher predictive value than acute-phase sera, probability of being a CHIKV case given a positive result in the chikungunya IgM-ELISA was less than 70%. Overall specificity was high in both acute- and convalescent-phase sera, as well as negative predictive values (Table 2). Among DENV cases, chikungunya IgM-ELISA yielded positive result in 13.3% (4/30) of the acute-phase samples, in 6.7% (1/15) of the convalescent-phase sera, and seroconversion was observed in 7.1% (1/14). None of the ZIKV cases yielded a positive result for chikungunya IgM-ELISA in either acute- or convalescent-phase sera. Among the PCR negative control group, 10.9% (77/708) of the acute-phase samples and 9.8% (28/287) of convalescent-phase sera yielded positive, and 9.8% (28/286) seroconversions were observed.

Discussion

Although it is difficult to differentiate DENV, ZIKV and CHIKV infections clinically, we observed a higher frequency of arthralgia in CHIKV cases, in accordance to the main manifestations of such infections described in the literature (HUA; COMBE, 2017). CHIKV IgM diagnosis test of the acute-phase sera performed poorly. However, since RT-PCR of the acute-phase serum sample was used as case definition, all arboviral cases identified were in the viremia phase of disease during acute-phase blood collection. Thus, a lower sensitivity of IgM detection in the acute-phase sample was to be expected. For convalescent-phase sera and seroconversions, however, IgM-ELISA was highly sensitive (>90%).

Although overall specificities were also good (~90%), false-positive results were present, mainly on PCR negative control group and DENV cases. False-positive results among the PCR Negative groups could be explained by low viremia, in which case our reference test would not be able to detect such CHIKV case. However, since majority of patients had acute-phase blood collection in very early stage of disease, it is unlikely that another gold-standard serological test such as PRNT would greatly improve CHIKV case detection. Since DENV and CHIKV belong to separate viral families, false positive results among DENV cases likely represents a recent DENV infection prior to the CHIKV infection identified in our study or a subsequent infection between acute- and convalescent-phase blood collection since Salvador experienced a high transmission period of all three arbovirus during our study period (CARDOSO et al., 2015, 2017).

Previous studies demonstrating the diagnostic performance of the evaluated CHIKV IgM-ELISA kit are scarce. One demonstrated high sensitivity (100%) and specificity (93-100%, in two different reference labortories) in panel of serum samples from CHIKV in-house positive cases and controls for DENV, o'nyong-nyong, Mayaro, Venezuelan equine encephalitis, and North American eastern equine encephalitis (JOHNSON et al., 2016). Here, we were able to evaluate test performance in all febrile outpatients recruited in the context of intense arboviral transmission. Other alphaviruses were not included in differential diagnosis testing because even though few human sporadic cases have been described to occur in Brazil, such as Venezuelan and easter equine encephalitis and Mayaro viruses, including in other states of the northeast region (LOPES; NOZAWA; LINHARES, 2014), no evidence of their circulation in Salvador have been described.

In a scenario of high transmission of multiple arbovirus such as DENV, ZIKV and CHIKV, serologic diagnosis is complex as it not only may represent cross-reactions but also be confused by recent arboviral infection. Still, CHIKV IgM-ELISA evaluated showed a high specificity and negative predictive value, but as expected may be more useful in convalescent-phase sera to identify CHIKV infections.

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Table 1. Demographics, clinical characteristics, and serum sample availability for acute febrile illness patients included in the study according to RT-PCR result.

Characteristics	PCR+ Chikungunya (n=139)	PCR+ Dengue (n=32)	PCR+ Zika (n=13)	PCR Negative* (n=718)
Demographic				
Age†	36 (22 - 46)	31 (14.5 - 39.5)	20 (15 - 38)	27 (18 - 38)
Gender (M) - n/N (%)	78/139 (56.1%)	16/32 (50.0%)	6/13 (46.2%)	332/714 (46.5%)
Clinical Manifestations				
Headache - n/N (%)	130/139 (93.5%)	29/31 (93.6%)	12/13 (92.3%)	645/717 (90.0%)
Retro-orbital pain - n/N (%)	101/138 (73.2%)	21/31 (67.7%)	9/13 (69.2%)	456/711 (64.1%)
Mialgia - n/n (%)	134/139 (96.4%)	25/31 (80.7%)	11/13 (84.6%)	589/714 (82.5%)
Arthralgia - n/N (%)	133/139 (95.7%)	20/32 (62.5%)	7/13 (53.9%)	474/718 (66.0%)
Poliarthralgia‡ - n/N (%)	126/133 (94.7%)	20/20 (100%)	7/7 (100%)	457/474 (96.4%)
Symetric§ - n/N (%)	118/126 (93.7%)	20/20 (100%)	7/7 (100%)	439/457 (96.1%)
Swollen joints - n/N (%)	57/139 (41.0%)	11/32 (34.4%)	4/13 (30.8%)	148/718 (20.6%)
Emesis - n/N (%)	30/139 (21.6%)	9/32 (28.1%)	1/13 (7.8%)	201/718 (28.0%)
Rash - n/N (%)	32/139 (23.0%)	13/32 (40.6%)	9/13 (69.2%)	240/718 (33.4%)
Pruritus - n/N (%)	19/139 (13.7%)	11/32 (34.4%)	9/13 (69.2%)	245/718 (34.1%)
Clinical suspicion - n/N (%)	79/139 (56.8%)	17/32 (53.1%)	6/13 (46.2%)	316/718 (44.0%)
Dengue - n/N (%)	42/79 (53.2%)	3/17 (17.7%)	1/6 (16.7%)	82/316 (26.0%)
Zika - n/N (%)	9/79 (11.4%)	5/17 (29.4%)	3/6 (50.0%)	30/316 (9.5%)
Chikungunya - n/N (%)	13/79 (16.5%)	0/17 (0%)	0/6 (0%)	17/316 (5.4%)
Exantematous illness - n/N (%)	3/79 (3.8%)	1/17 (5.9%)	0/6 (0%)	10/316 (3.2%)
Blood sample collection				
Acute-phase sample	139/139 (100%)	32/32 (100%)	13/13 (100%)	718/718 (100.0%)
Time between symptoms onset and sample collection†	1 (1 - 3)	4 (3 - 6)	2 (2 - 3)	3 (2 - 4)
Convalescent-phase sample	65/139 (46.8%)	17/32 (53.1%)	7/13 (53.8%)	330/718 (46.0%)
Time between symptoms onset and sample collection†	19 (14 - 33)	21 (16 - 42)	30 (20 - 44)	22 (15 - 35)

*RT-PCR negative for Dengue, Zika and Chikungunya.

† Median (interquartile range)

‡ >1 joint involved.

§ At least one pair of joint with symetric arthralgia.

Table 2. Diagnostic performance of the chikungunya IgM-ELISA in the acute- and convalescent-phase sera, and according to seroconversions.

	VP	FP	FN	VN	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)
Chikungunya IgM-ELISA in acute sample	5	77	132	644	3.7% (1.2 - 8.3)	89.3% (86.8 - 91.5)	6.1% (2.0 - 13.7)	83.0% (80.2 - 85.6)
Chikungunya IgM-ELISA in convalescent sample	57	28	4	266	93.4% (84.1 - 98.2)	90.5% (86.5 - 93.6)	67.1% (56.0 - 76.9)	98.5% (95.3 - 99.6)
Chikungunya IgM-ELISA seroconversion*	57	28	4	265	93.4% (84.1 - 98.2)	90.4% (86.5 - 93.6)	67.1% (56.0 - 76.9)	98.5% (96.2 - 99.6)

*For patients with paired samples and acute-phase IgM-ELISA negative.

6. Artigo 5 – Diagnostic performance of commercial IgM and IgG enzyme-linked immunoassays (ELISAs) for diagnosis of Zika virus infection

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Abstract

Serologic detection of Zika virus (ZIKV) infections is challenging because of antigenic similarities among flaviviruses. We used sera from 14 febrile patients with RT-PCR-confirmed ZIKV infection to evaluate the sensitivity of commercial ZIKV IgM and IgG enzyme-linked immunoassay (ELISA) kits. To estimate the specificity of the kits, we used sera from RT-PCR-confirmed dengue cases and blood donors, both of which were collected before ZIKV epidemics in Brazil (2009-2011 and 2013, respectively). The ZIKV IgM-ELISA sensitivity was 0.0% (0/14) and 12.5% (1/8) for acute- and convalescent-phase sera, respectively, while its specificity was 100.0% (58/58) and 98.3% (58/59) for acute- and convalescent-phase sera of dengue patients, and 100.0% (23/23) for blood donors. The ZIKV IgG-ELISA sensitivity was 100.0% (6/6) on convalescent-phase sera from RT-PCR confirmed ZIKV patients, while its specificity was 27.3% (15/55) on convalescent-phase sera from dengue patients and 45.0% (9/20) on blood donors' sera. The ZIKV IgG-ELISA specificity among dengue confirmed cases was much greater among patients with primary dengue (92.3%; 12/13), compared to secondary dengue cases (7.1%; 3/42). In summary, in a setting of endemic dengue transmission, the ZIKV IgM-ELISA had high specificity, but poor sensitivity. In contrast, the ZIKV IgG-ELISA

showed low specificity, particularly for patients previously exposed to dengue infections. Our findings suggest that this ZIKV IgM-ELISA is not useful in confirming a diagnosis of ZIKV infection in suspected patients, whereas the IgG-ELISA is more suitable for ZIKV diagnosis among travelers, who reside in areas free of flavivirus transmission, rather than for serosurveys in dengue-endemic areas.

Introduction

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus that received global attention when large epidemics occurring in the Americas in 2015 were associated with congenital syndromes in newborns (BRASIL et al., 2016). The virus was initially isolated in Uganda, in 1947 (DICK; KITCHEN; HADDOW, 1952), but only during the last decade were the first outbreaks detected, affecting 1% (~900 people) of the Yap Island population, in 2007 and 7% (~19.000 inhabitants) of the French Polynesia population, in 2013 (CAO-LORMEAU et al., 2014; DUFFY et al., 2009). In 2015-16, ZIKV caused an explosive outbreak in Brazil, when over 309,000 suspected cases were reported and 500,000-1.5 million infections estimated (DE OLIVEIRA et al., 2017; RIBEIRO; KITRON, 2016). The virus spread though the Americas, and by March 2017, the World Health Organization (WHO) had reported 47 countries/territories with autochthonous ZIKV transmission (WORLD HEALTH ORGANIZATION, 2017) .

Acute-phase clinical manifestations of ZIKV infections are usually mild and self-limited, or absent. Non-specific signs and symptoms may include exanthema, low-grade fever, conjunctivitis, and arthralgia (CARDOSO et al., 2015; DUFFY et al., 2009; MUSSO; GUBLER, 2016). However, following ZIKV outbreaks in French Polynesia and Brazil, increases in cases of Guillain-Barré syndrome in adults and in congenital abnormalities in newborns were reported (CAO-LORMEAU et al., 2016; PAPLOSKI et al., 2016). Subsequent case-control and cohort studies have confirmed ZIKV infection during pregnancy as an unequivocal cause of congenital neurological abnormalities (BRASIL et al., 2016; DE ARAÚJO et al., 2016).

Due to the similarity in the initial clinical presentation between ZIKV infections and other infectious diseases, especially with other arboviral diseases that usually co-occur

in tropical and sub-tropical regions, such as dengue (DENV) and chikungunya (CHIKV), clinical diagnosis is challenging, and often depends on laboratory confirmation. However, ZIKV laboratory diagnosis is also difficult because viremia tends to be low in magnitude (MUSSO et al., 2016). In addition, ZIKV presents structural similarities with other flaviviruses, especially DENV, resulting in cross-reactive antibodies (DUFFY et al., 2009; LANCIOTTI et al., 2008). Consequently, serological tests for ZIKV may cross-react with DENV, particularly when pre-existing immunity to DENV is present (DEJNIRATTISAI et al., 2016; FELIX et al., 2017; LANCIOTTI et al., 2008; PRIYAMVADA et al., 2016).

A correct differential diagnosis between ZIKV and other arboviral infections is critical to alert patients and clinicians for potentially evolving complications, as well as to guide appropriate supportive care (i.e. fluid replacement for dengue patients at risk of hemorrhagic disease). In addition, an accurate ZIKV laboratory diagnosis can inform surveillance activities, which include detection and monitoring of virus circulation, estimation of disease burden, guiding preventive and control actions to interrupt virus transmission, and informing pregnant women on the risk of a gestational infection. In this study, we evaluated the diagnostic sensitivity of commercial enzyme-linked immunoassays (ELISAs) (Euroimmun, Lübeck, Germany) for detection of ZIKV IgM and IgG antibodies in sera of febrile patients with ZIKV infections confirmed by RT-PCR. We also assessed the specificity of these kits in sera of both dengue-confirmed patients and blood donors.

Methods

Study site and design

This study was conducted and reported according to the STARD (Standards for Reporting of Diagnostic Accuracy) guideline (BOSSUYT et al., 2015). The performance of the IgM- and IgG-ELISAs was evaluated using serum collections from clinical-epidemiological studies conducted in Salvador, northeast Brazil. Salvador has experienced DENV transmission since 1995, when an outbreak of DENV-2 first occurred. Following this outbreak, the other three DENV serotypes were introduced in the city, leading to periodic DENV epidemics and establishment of endemic transmission

(TEIXEIRA et al., 2001). Between 2011-2016, approximately 7,000 dengue cases per year were reported in Salvador (BRASIL, 2017), but the actual disease burden has been estimated to be at least 12 times greater (SILVA et al., 2016). In early 2015, Salvador faced a large outbreak of acute exanthematous illness later attributed to ZIKV, with over 17,000 reported cases (CARDOSO et al., 2015; PAPLOSKI et al., 2016).

Most of the serum samples used for our diagnostic test evaluation were obtained from an acute febrile illness (AFI) enhanced surveillance study that we conducted in a public emergency health unit of Salvador, between January 2009 and August 2016. Details were been previously described (KIKUTI et al., 2015). Briefly, the surveillance study enrolled patients of age 5 years or older presenting to the emergency unit with reported fever or measured axillary temperature ≥37.8°C of up to 7 days of duration. Self-reported data on days of symptoms and clinical characteristics were obtained from participants at enrollment.

We collected blood samples from participants at study enrollment (acute-phase sample) and ≥15 days post-enrollment (convalescent-phase sample). Sera were stored in aliquots at -20°C and -70°C for serologic and molecular testing, respectively. Acute-phase samples were tested for DENV by NS1- and IgM-ELISA (Panbio Diagnostics, Brisbane, Australia) and convalescent-phase samples by IgM-ELISA (Panbio Diagnostics, Brisbane, Australia). ELISAs performers were blinded regarding clinical manifestations and clinicians' diagnosis suspicion.

Between 2009 and 2014, acute-phase samples that yielded a positive result in either ELISA underwent RNA extraction (Maxwell® 16 Total RNA Purification kit by Promega, Wisconsin, USA, or QIAmp® Viral RNA mini kit by Qiagen, Hilden, Germany) and RT-PCR (AccessQuick RT-PCR System, Promega, Madison,WI) for dengue typing (LANCIOTTI et al., 1992). Beginning in 2014, however, all acute-phase samples underwent RT-PCRs for DENV (LANCIOTTI et al., 1992), ZIKV (BALM et al., 2012) and CHIKV (EDWARDS et al., 2007), regardless of serological tests results, and RT-PCR performers/readers were blinded regarding probable diagnosis. Dengue IgG-ELISA (Panbio) was performed for all acute-phase serum samples of dengue-confirmed cases; those with a negative result were considered a primary dengue infection, whereas those with a positive result were considered secondary dengue infections.

Additionally, we obtained sera from presumably health volunteer blood donors at the State Blood Donation Center located in Salvador during December 2013. Collection of blood was performed after the regular screening process (which, among others, excludes donors reporting fever in the previous 15 days). Blood donors' sera were stored in both -20° and -70°C and subsequently tested for DENV with the same NS1-, IgM- and IgG-ELISA kits.

Sample selection

To determine the sensitivity of the ZIKV IgM- and IgG-ELISA (EUROIMMUN, Lübeck, Germany), we tested the available serum for the 14 patients enrolled during the surveillance study who had a ZIKV infection confirmed by RT-PCR (recruited between May and July, 2015). Acute- and convalescent-phase serum samples (14 and 8 sera, respectively) were tested by the ZIKV IgM-ELISA kit, and these patients' convalescent-phase samples were tested by the ZIKV IgG-ELISA. We did not test the acute-phase samples of the Zika cases with the IgG-ELISA because these samples were collected early during disease course (<7 days of illness), when IgG-ELISA testing is not recommended.

To determine the specificity of the ZIKV IgM- and IgG-ELISA in a group of confirmed dengue patients, we selected from RT-PCR-positive dengue cases detected during the AFI surveillance study the first 20 cases positive for each DENV serotype. We selected the first 20 confirmed cases of each DENV type to ensure that there was no ZIKV transmission in Salvador at the time such that there was no chance of a prior ZIKV infection. The DENV1-confirmed patients were detected from April 2009 to May 2011; the DENV2 from February 2009 to February 2010; and DENV4 from October 2010 to April, 2011. DENV-3 patients were not selected for testing because we confirmed only a very small number of such cases during the study period. We also determined the specificity of the ZIKV IgM- and IgG-ELISA in randomly selected sera from 23 blood donors. The initially planned number of samples from DENV-confirmed patients and blood donors (143 samples: 60 DENV acute- and convalescent-phase samples each and 23 blood donor samples) was defined to provide 95% confidence for an estimated specificity of 95% and

a precision of ~3.5%. All available ZIKV-confirmed serum samples were used to estimate the test sensitivity.

Zika IgM- and IgG-ELISA

The ZIKV IgM-ELISA (El 2668-9601 M) and ZIKV IgG-ELISA (El 2668-9601 G) by Euroimmun (Lübeck, Germany) were performed according to the manufacturer's instructions for semiquantitative interpretations. Although assay performers were not blinded to group identification of tested samples (ZIKV, DENV, or blood donors), reading was performed by automated microplate reader (TECAN, Maennedorf, Switzerland). Briefly, diluted patient sera were incubated in 96-wells coated with recombinant ZIKV NS1 protein. Whenever the tested sample contains specific IgM/IgG antibodies, they bind to the ZIKV NS1 protein, and a second incubation with anti-human IgM/IgG (enzyme conjugate) catalyzes a reaction detected by optical absorbance. Whenever the ratio in the optical density obtained with the patients' serum and the calibrator is <0.8 the result is interpreted as negative; when it is between ≥0.8 to <1.1 the result is equivocal; and when it is ≥1.1 the result is considered positive. Kits were provided with positive and negative controls that also underwent internal quality control by parameters provided by the manufacturer.

Data analysis

The AFI patients with a RT-PCR-based diagnosis of ZIKV and DENV were characterized according to demographic characteristics (sex and age) and clinical manifestations at study enrollment. We calculated the sensitivity and specificity of ZIKV IgM- and IgG-ELISA tests, with respective 95% confidence intervals, using the samples from RT-PCR-confirmed cases of ZIKV infection as the reference group of true positives for sensitivity estimation, and the samples from RT-PCR-confirmed dengue cases, as well as blood donors as reference groups of true negatives for specificity estimation. The decision to use RT-PCR as the gold standard for ZIKV or DENV infections among febrile patients relied on the higher degree of certainty of the agent responsible for the infection when compared to serological tests (which are more susceptible to cross-reactions). We also calculated the specificity among dengue-confirmed cases stratified by the infecting

DENV type and by the type of infection (primary versus secondary). Samples with equivocal ELISA results were excluded from the accuracy analysis.

Ethical Approvals

This project was approved by the Research Ethics Committee at the Gonçalo Moniz Institute, Oswaldo Cruz Foundation. All adult subjects provided written informed consent. Participants <18 years old who could read provided written assent following written consent from their parent or guardian. All study data were anonymized before analysis.

Results

Participants Characteristics

The 14 RT-PCR-confirmed Zika cases had a median age of 22.5 years and 42.9% were male. The most frequent clinical manifestations were headache (92.9%), myalgia (85.7%), retro-orbital pain (71.4%), rash (71.4%), and pruritus (71.4%) (Table 1). Median time between onset of symptoms and acute-phase serum samples of Zika cases was 2.5 days. 8 of these patients had a convalescent-phase serum sample available, which were collected with a median of 27 days post onset of symptoms (Table 1). None of the Zika cases was positive for DENV on the NS1-ELISA. However, the DENV IgM-ELISA was positive for the convalescent-phase serum of 3 patients and in both acute- and convalescent-phase samples of one patient.

The 60 RT-PCR-confirmed dengue cases had a median age of 18 years and 51.6% were male. The most frequent clinical manifestations were headache (96.7%), myalgia (76.7%), retro-orbital pain (56.7%), and arthralgia (46.7%) (Table 1). Contrasting with the Zika cases, only 10.0% of the dengue cases presented with rash. Dengue cases had a median time between onset of symptoms and acute-phase serum sample collection of 2 days. 60 dengue cases had a convalescent-phase serum sample available, which were collected with a median of 27 days post onset of symptoms (Table 1). The RT-PCR-positive dengue cases were also positive by NS1-ELISA (10 patients), by NS1- and IgM-ELISA in the acute-phase sera (6 patients), by IgM-ELISA seroconversion between acute-

and convalescent-phase sera (23 patients), and by NS1-ELISA and IgM-ELISA seroconversion (21 patients). The median age for the 23 blood donors was 32 years and 69.6% were male. All blood donors tested negative by dengue NS1- and IgM-ELISA, and 20 (87.0%) tested positive by DENV IgG-ELISA. For the Zika and dengue cases, the blood sample availability for ZIKV IgM- and IgG-ELISAs testing are also summarized in Table 1.

ZIKV IgM-ELISA performance

None of the 14 acute-phase samples of RT-PCR-confirmed Zika cases yielded a positive result in the ZIKV IgM-ELISA (0% sensitivity for acute-phase samples), whereas one of 8 convalescent-phase samples was positive (12.5% sensitivity for convalescent-phase samples; Table 2). Zika IgM-ELISA yielded a positive result in only one of the 140 non-Zika sera tested (58 acute- and 59 convalescent-phase samples from dengue cases and 23 blood samples from donors; 99.3% specificity overall) (Table 2). None of the blood donors and acute-phase sera from dengue cases tested positive in the ZIKV IgM-ELISA (100% specificity in both groups). Among the convalescent-phase sera from dengue cases, only one (from a patient with a primary infection with DENV-2) tested positive in the ZIKV IgM-ELISA (98.3% specificity for convalescent-phase samples).

Zika IgG-ELISA performance

All 6 convalescent-phase samples from RT-PCR-confirmed Zika cases tested with the ZIKV IgG-ELISA were positive, yielding 100% sensitivity (Table 3). Among convalescent-phase sera from confirmed dengue cases, ZIKV IgG-ELISA yielded false-positive results in 40 (66.7%) samples, true negative results in 15 (25.0%) samples, and equivocal results in 5 (8.3%) samples (which were excluded from further analysis). Among the 23 sera from blood donors, 11 (47.8%) were false positive in the ZIKV IgG-ELISA, 9 (39.1%) were true negatives, and 3 (13.0%) generated equivocal results (and were excluded from further analysis).

Overall, the specificity of the ZIKV IgG-ELISA for the 75 samples with valid results was 32.0%. The specificity for convalescent-phase samples of dengue patients was 27.3%, ranging for 5.9% in DENV-4 infections to 45.0% in DENV-1 infections (Table 3).

The specificity was much lower for patients diagnosed secondary DENV infections (7.1%) compared to those with primary DENV infections (92.3%). Specificity of the Zika IgG-ELISA among blood donors was 45.0%. All 11 blood donors' samples for which the ZIKV IgG-ELISA returned a false positive result had anti-dengue IgG antibodies, as did 6 of the 9 true negative samples.

Discussion

In this study we evaluated the diagnostic performance of commercial IgM- and IgG-ELISAs for serodiagnosis of Zika virus infection. We used a set of sera collected from AFI patients prospectively enrolled in an enhanced surveillance study designed to detect arboviral infections, rather from a reference laboratory serum sample collection. Thus, we were able not only to characterize the clinical manifestations of the patients providing the sera tested, but also to evaluate the accuracy of the tests in a group of patients' samples that represent a realistic epidemiologic situation. Of note, clinical manifestations of RT-PCR-confirmed Zika and dengue AFI patients were similar, with the exception of rash, which was most commonly reported among Zika patients, reinforcing the difficulties in clinically differentiating these arboviral infections.

We found a high specificity for the Euroimmun ZIKV IgM-ELISA among both dengue-infected and blood donor control groups. Specificity among dengue controls was not affected by infecting serotype or by primary versus secondary infection. However, the sensitivity of the ZIKV IgM-ELISA was low for either acute- or convalescent-phase serum samples of Zika febrile patients (0.0% and 12.5%, respectively). As the time elapsed between Zika symptoms onset and acute-phase serum collection was short (median 2.5 days), a low IgM detection in the acute-phase sample was expected because the antibody response may not have yet developed in many of these patients. However, even for the convalescent-phase serum samples, collected on average 3-6 weeks after disease onset, the ZIKV IgM-ELISA yielded a low sensitivity. This low performance has also reported for RT-PCR-confirmed Zika samples from residents from other ZIKV- and DENV-endemic areas (Suriname, Dominican Republic and Colombia), where Euroimmun Zika IgM-ELISA detected only 6 of 19 evaluated samples, yielding a sensitivity of 31.6% (STEINHAGEN

et al., 2016). Interestingly, the same study reported sensitivity of 87.5% among RT-PCR-confirmed Zika samples from returning travelers, suggesting that previous flavivirus infections may alter ZIKV antibody responses. Although reports of cross-reactivity of ZIKV-infected patients in DENV-IgM assays have been reported (LANCIOTTI et al., 2008), our data suggest that this problem is minimal, as observed with previous diagnosis evaluation studies of this test (ESBROECK et al., 2016; HUZLY; HANSELMANN; PANNING, 2016; STEINHAGEN et al., 2016).

Although recent studies suggest a high specificity of this Zika IgG-ELISA against dengue infections in returning travelers (ESBROECK et al., 2016; HUZLY; HANSELMANN; PANNING, 2016; LUSTIG et al., 2017; STEINHAGEN et al., 2016), we have demonstrated an overall lower performance in a dengue-endemic region. This may be due to a greater frequency of secondary DENV infections in the population we studied (78.3%), in which specificity was 7.1% contrasting with 92.3% in primary infections. The low specificity of the Zika IgG-ELISA among our blood donor control group may be also explained by the high frequency (87.0%) of DENV IgG antibodies observed in this group. We also found a large difference in the ZIKV IgG-ELISA specificity among DENV-confirmed samples according to DENV serotype, varying from 5.9% in DENV4-infected patients to 45.0% in DENV1-infected patients. This difference is also partially explained by the prior dengue-immune status of these patients, since 100% of DENV4-infected patients had secondary infections, contrasting with 65% of DENV1-infected patients. Although ZIKV IgG antibodies were detected in all 6 convalescent-phase samples of Zika cases, the same samples were also positive for dengue IgG antibodies, making it impossible to exclude cross-reactions.

Cross-reactivity of the Euroimmun ZIKV IgM- and IgG-ELISA tests against pathogens other than DENV was not assessed in our study. Although high specificities of the IgM- and IgG-ELISA test against DENV, yellow fever (ESBROECK et al., 2016), tick-borne encephalitis virus, and hepatitis C virus (HUZLY; HANSELMANN; PANNING, 2016) have been reported, cross-reactivity with malaria infections has been described (ESBROECK et al., 2016; SCHWARZ et al., 2017). Another limitation of our study was the fact that the ZIKV RT-PCR was not performed on the acute-phase samples of the dengue cases to ensure the absence of co-infection. However, considering that our

dengue case selection specifically included patients with infections that occurred prior to 2012, it is unlikely that they had a dengue and Zika co-infection or a prior ZIKV infection. ZIKV introduction into Brazil was estimated to have occurred between late 2012 and early 2013 (AYLLÓN et al., 2017; FARIA et al., 2016; PASSOS et al., 2017). Therefore, prior ZIKV infections in the group of blood donors included in this study cannot be completely ruled out because their serum samples were obtained in December 2013; but, as the ZIKV spread in Salvador was clearly recognized during a large outbreak in 2015, it is improbable that these individuals had had ZIKV infections before 2015.

The strengths of our study were the use of the RT-PCR for confirmation both ZIKV and DENV infections, and the availability of acute- and convalescent-phase serum samples from our patients. The use of RT-PCR as a confirmation criterion for Zika cases in a region in which both ZIKV and DENV transmission are endemic assures a more accurate distinction between these flaviviruses serodiagnosis. However, RT-PCR diagnosis is only possible in the initial phase of infection, when detection of IgM and IgG antibodies is unlikely. The availability of paired sera provided a unique opportunity to perform RT-PCR diagnosis, as well as to evaluate the performance of commercial ZIKV IgM- and IgG-ELISAs in detection of antibodies in acute- and convalescent-phase samples.

In conclusion, our study revealed a high specificity but low sensitivity of the Euroimmun ZIKV IgM-ELISA in an endemic region for both dengue and Zika, as well as a high specificity of the Euroimmun ZIKV IgG-ELISA in DENV primary infections contrasting with low specificity in DENV secondary infections. This suggests that this IgG-ELISA may be more suitable for Zika diagnosis in travelers returning from non-endemic areas than for serosurveys in endemic areas. Further studies with larger well-characterized samples are needed to assess the diagnostic accuracy not only of this test, but of several others serological tests being developed for ZIKV detection.

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Table 1. Demographics, clinical characteristics, and serum sample availability for 14 Zika and 60 dengue cases confirmed by RT-PCR.

Characteristics	Zika cases (N=14)	Dengue cases (N=60)
	Number (%) or median (interquartile range)	
Demographic		
Male	6 (42.9)	31 (51.7)
Age	22.5 (15-41)	18 (10-34)
Clinical Manifestations		
Headache	13 (92.9)	58 (96.7)
Myalgia	12 (85.7)	46 (76.7)
Retro-orbital pain	10 (71.4)	34 (56.7)
Arthralgia	8 (57.1)	28 (46.7)
Vomit	2 (14.3)	12 (20.0)
Rash	10 (71.4)	6 (10.0)
Pruritus	10 (71.4)	NA
Prior dengue infection status		
Primary infection	-	13 (21.7)
Secondary infection	-	47 (78.3)
Blood sample collected		
Acute-phase sample	14 (100)	60 (100)
Time between symptoms onset and sample collection	2.5 (2-4)	2 (1-3)
Convalescent-phase sample	8 (57.1)	60 (100)
Time between symptoms onset and sample collection	27 (18.5-43.5)	27 (19.5-57.5)
Sera tested by ZIKV ELISA*		
ZIKV IgM-ELISA in acute-phase sera	14 (100)	58 (96.7)
ZIKV IgM-ELISA in convalescent-phase sera	8 (57.1)	59 (98.3)
ZIKV IgG-ELISA in convalescent-phase sera	6 (42.9)	60 (100)

NA. Not available.

***** Some patients did not have their acute- or convalescent serum sample tested for ZIKV because of insufficient volume of sera.

Table 2. Performance of the Euroimmun ZIKV IgM-ELISA kit.

Performance according to the type of serum samples	**ZIKV IgM-ELISA result**		**% (95% CI)**
Sensitivity	**True Positive**	**False Negative**	
Acute-phase RT-PCR-confirmed ZIKV samples	0	14	0.0 (0.0-23.2)
Convalescent-phase RT-PCR-confirmed ZIKV samples	1	7	12.5 (0.3-52.7)
Specificity	**True Negative**	**False Positive**	
Acute-phase RT-PCR-confirmed DENV samples	58	0	100.0 (93.8-100.0)
DENV-1 samples	19	0	100.0 (82.4-100.0)
DENV-2 samples	19	0	100.0 (82.4-100.0)
DENV-4 samples	20	0	100.0 (83.2-100.0)
Primary DENV infection samples	13	0	100.0 (75.3-100.0)
Secondary DENV infection samples	45	0	100.0 (92.1-100.0)
Convalescent-phase RT-PCR-confirmed DENV samples	58	1	98.3 (90.9-100.0)
DENV-1 samples	20	0	100.0 (83.2-100.0)
DENV-2 samples	18	1	94.7 (74.0-99.9)
DENV-4 samples	20	0	100.0 (83.2-100.0)
Primary DENV infection samples	12	1	92.3 (64.0-99.8)
Secondary DENV infection samples	46	0	100.0 (92.3-100.0)
Blood donors samples	23	0	100.0 (85.2-100.0)

Table 3. Performance of the Euroimmun ZIKV IgG-ELISA kit.

Performance according to the type of serum samples	**ZIKV IgG-ELISA result**		**% (95% CI)**
Sensitivity	**True Positive**	**False Negative**	
Convalescent-phase RT-PCR-confirmed ZIKV samples	6	0	100.0 (54.1-100.0)
Specificity	**True Negative**	**False Positive**	
Convalescent-phase RT-PCR-confirmed DENV samples*	15	40	27.3 (16.1-41.0)
DENV-1 samples	9	11	45.0 (23.1-68.5)
DENV-2 samples	5	13	27.8 (9.7-53.5)
DENV-4 samples	1	16	5.9 (0.2-28.7)
Primary DENV infection samples	12	1	92.3 (64.0-99.8)
Secondary DENV infection samples	3	39	7.1 (1.5-19.5)
Blood donors samples**	9	11	45.0 (23.1-68.5)

* Of the 60 convalescent-phase serum samples from dengue cases tested with the ZIKV IgG-ELISA, 5 presented equivocal results and were not included in this analysis.

** Of the 23 serum samples from blood donors tested with the ZIKV IgG-ELISA, 3 presented equivocal results and were not included in this analysis.

7. Conclusões

O novo cenário epidemiológico no Brasil no que tange às arboviroses trouxe grandes dificuldades diagnósticas visto que a sintomatologia apresentada por casos clínicos de DENV, ZIKV e CHIKV são em geral inespecíficos e o diagnóstico laboratorial enfrenta o desafio das reações cruzadas entre flavivírus relacionados (DENV e ZIKV). O estudo de acurácia dos testes diagnósticos específicos é crucial para entendermos como a intensa circulação destes três arbovírus afeta a identificação de casos, imprescindível para um manejo clínico adequado e para orientar ações de vigilância e controle.

Neste trabalho, descrevemos a acurácia de diversos testes diagnósticos para DENV, ZIKV e CHIKV. A partir de uma base de dados laboratoriais, descrevemos também a ocorrência de CHIKV e DENV em Salvador. Identificamos uma baixa sensibilidade (47%) de um teste rápido para dengue mesmo combinando a detecção de antígeno NS1 e anticorpos IgM, embora a especificidade tenha sido alta (>87%) antes da introdução do ZIKV na cidade, indicando que pacientes com doença febril aguda com um resultado positivo no teste rápido podem ser considerados casos de dengue, enquanto que um resultado negativo no teste não exclui a doença e uma amostra de sangue de fase convalescente por ser necessária para a confirmação diagnóstica. A avaliação a campo deste mesmo teste revelou uma sensibilidade ainda mais baixa (21%) em pacientes com sintomas compatíveis com dengue, embora a especificidade tenha permanecido alta (>89%) mesmo após a introdução do ZIKV em Salvador. O teste apresentou, portanto, baixo potencial para ser utilizado como exame de triagem. Considerando-se que o Ministério da Saúde considera o resultado de testes rápidos que detectam NS1 para diagnóstico de dengue para definição de casos confirmados laboratorialmente desde 2014, é importante ressaltar que devido à baixa sensibilidade do teste avaliado, outros exames devem ser recomendados para afastar a doença em caso de um resultado negativo no teste rápido.

Considerando a performance de imunoensaios enzimáticos (ELISAs) para detecção de antígeno NS1 e anticorpos IgM para DENV, observamos uma baixa sensibilidade no geral, sendo de 3% para o ELISA-NS1 e 17% para o ELISA-IgM (ambos na amostra de fase aguda), 47% para o ELISA-IgM na amostra de fase convalescente e 36% para

soroconversões de ELISA-IgM. Já no que tange especificidade, ambos os ELISAs apresentaram boa performance (>87%) em amostras de pacientes febris não arbovirus, entretanto a especificidade em pacientes com ZIKV foi de 100% para os ELISAs da amostra de fase aguda (NS1 e IgM) e 57% para ELISA-IgM da amostra de fase convalescente e soroconversões de ELISA-IgM, demonstrando uma alta frequência de reações cruzadas. Dado o contexto de co-circulação de DENV e ZIKV e alta frequência de reações cruzadas, é necessário repensar a atual classificação de caso de dengue confirmado laboratorialmente, que inclui como critério de confirmação um resultado positivo no ELISA-IgM. Desta forma, pode-se estar superestimando o número de casos de dengue em detrimento da identificação de casos de Zika. Este resultado também traz implicações práticas para o serviço, visto que um diagnóstico incorreto pode comprometer a conduta clínica para com o paciente em alguns casos. Em casos confirmados de dengue, por exemplo, o profissional médico pode direcionar o cuidado médico para hidratação e monitoramento para formas hemorrágicas, enquanto que em casos de Zika pode optar por acompanhamento gestacional e monitoramento para complicações neurológicas mais intenso.

Já a avaliação da performance do ELISA-IgM para detecção de infecções CHIKV revelou alta sensibilidade (93%) nas amostras de fase convalescente e soroconversões, e alta especificidade (>89%) independente da fase da doença em que a amostra foi coletada. Ainda assim, o ELISA-IgM para diagnóstico de CHIKV avaliado mostrou alta especificidade e valor preditivo negativo, entretanto pode ser mais útil em amostras de soros de fase convalescente. Em um cenário de alta transmissão de múltiplos arbovírus, como DENV, ZIKV e CHIKV, o diagnóstico sorológico é complexo, pois pode representar não só reações cruzadas como também ser confundido por infecções recentes por outros arbovírus. O profissional de saúde deve ter esta limitação em mente, e considerar a sintomatologia apresentada e histórico do paciente ao interpretar resultados de exames sorológicos em um cenário de intensa transmissão destes arbovírus.

O ELISA-IgM para detecção de infecções por ZIKV se mostrou com baixa sensibilidade (<15%) e alta especificidade (>98%), enquanto que o ELISA-IgG para ZIKV apresentou alta sensibilidade (100%) e baixa especificidade (<50%), especialmente em pacientes com dengue secundária (7%). Isto sugere que este ELISA-IgG pode ser mais

adequado para o diagnóstico de Zika em viajantes de áreas não-endêmicas que retornam de áreas onde há circulação viral do que para soroinquéritos em áreas endêmicas. Além destas implicações práticas para a pesquisa e vigilância, estes resultados reforçam a dificuldade diagnóstica do ZIKV, visto que diagnóstico molecular continua sendo o método de diagnóstico mais confiável para distinguir infecções por DENV de infecções por ZIKV em áreas endêmicas, entretanto além das limitações de custo e acessibilidade desta técnica as infecções por ZIKV tendem a apresentar viremia mais baixa e curta, limitando a oportunidade diagnóstica.

Estudos de acurácia de testes laboratoriais em populações de áreas endêmicas para DENV, ZIKV e CHIKV são escassos e imprescindíveis para uma adequada estratégia de detecção de casos para a vigilância e pesquisa científica. Embora as medidas de controle para as três arboviroses estudadas sejam em sua maioria direcionadas ao controle vetorial, é de suma importância a elaboração de uma estratégia diagnóstica para identificação de casos para que se possa conhecer a tendência de transmissão de cada uma delas e assim programar medidas de controle e prevenção específicas. Um bom exemplo foram as medidas de prevenção de orientação à população sobre os riscos gestacionais durante períodos de alta transmissão de ZIKV. Por fim, a correta identificação de casos traz inúmeros benefícios para a pesquisa, uma vez que a descoberta do ZIKV foi relativamente recente e ainda há muito o que se descobrir a respeito da sua transmissão, patogênese e diagnóstico.

8. Anexos

8.1 Anexo 1 – Unrecognized emergence of chikungunya virus during a Zika virus outbreak in Salvador, Brazil

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Abstract

Background - Chikungunya virus (CHIKV) entered Brazil in 2014, causing a large outbreak in Feira de Santana, state of Bahia. Although cases have been recorded in Salvador, the capital of Bahia, located ~100 km of Feira de Santana, CHIKV transmission has not been perceived to occur epidemically,

largely contrasting with the Zika virus (ZIKV) outbreak and ensuing complications reaching the city in 2015.

Methodology/Principal Findings - This study aimed to determine the intensity of CHIKV transmission in Salvador between November 2014 and April 2016. Results of all the CHIKV laboratory tests performed in the public sector were obtained and the frequency of positivity was analyzed by epidemiological week. Of the 2,736 tests analyzed, 456 (16.7%) were positive. An increasing in the positivity rate was observed, starting in January/2015, and peaking at 68% in August, shortly after the exanthematous illness outbreak attributed to ZIKV.

Conclusions/Significance - Public health authorities and health professionals did not immediately detect the increase in CHIKV cases, likely because all the attention was directed to the ZIKV outbreak and ensuing complications. It is important that regions in the world that harbor arbovirus vectors and did not experience intense ZIKV and CHIKV transmission be prepared for the potential co-emergence of these two viruses.

Introduction

Chikungunya virus (CHIKV), an arbovirus transmitted by *Aedes* mosquitoes, can cause a clinical disease that resembles dengue and other arboviral infections (COUDERC; LECUIT, 2015). Acute-phase signs and symptoms include fever, myalgia and rash, but severe arthralgia that may become chronic is the main hallmark of the disease (COUDERC; LECUIT, 2015). An increase in Guillain-Barré Syndrome (GBS) was also observed during a CHIKV outbreak in French Polynesia, indicating that this arbovirus may be associated with severe neurological outcomes (OEHLER et al., 2015). Clinical diagnosis is difficult, especially where co-circulation of other arbovirus such as dengue (DENV) and Zika (ZIKV) viruses occurs. In addition, as sequential arboviral infections and even co-infections could play a role in severe clinical manifestations, there is a need of a better understanding of multiple arboviruses transmission dynamics (RODRIGUEZ-MORALES; VILLAMIL-GÓMEZ; FRANCO-PAREDES, 2016).

Brazil has been in the spotlight for arbovirus transmission, especially since epidemics of Zika virus (ZIKV) in early 2015 (CAMPOS; BANDEIRA; SARDI, 2015; CARDOSO et al., 2015; ZANLUCA et al., 2015) were followed by outbreaks of GBS in adults and microcephaly in newborns (COSTA et al., 2016; PAPLOSKI et al., 2016). During January-September 2016 (up to epidemiological week 37), Brazil recorded 200,465 ZIKV cases, 236,287 CHIKV cases and 1,438,624 DENV cases (BRAZIL, 2016a). Oropouche and Mayaro virus infections have been identified sporadically in the country, restricted so far to the North and Midwest region (AZEVEDO, RAIMUNDA et al., 2009; COIMBRA et al., 2007; PINHEIRO et al., 1981; VASCONCELOS et al., 2009; VIEIRA et al., 2015), whereas Yellow Fever occurs endemically in the Amazon region with occasional transmission in the Midwest, South and Southeast regions, with 322 reported cases from July 2014 to June 2015 in Brazil (BRAZIL, 2015a). Salvador, the largest city in the northeastern region of Brazil, and the capital of Bahia State, was one of the cities most affected by ZIKV. While there was widespread occurrence of ZIKV cases in Salvador, transmission of CHIKV appeared to have been much less intense, to the extent that an outbreak was not detected by local health authorities, as during the period an outbreak of acute exantematous illness (AEI) attributed to ZIKV occurred over 14,000 AEI cases were reported in contrast to 58 CHIKV cases reported in Salvador (CARDOSO et al., 2015). This is intriguing, since CHIKV has caused large outbreaks in most places where it is introduced (BALLERA et al., 2015; FELDSTEIN et al., 2016; PETERSEN; POWERS, 2016; VAN GENDEREN et al., 2016), and CHIKV cases were first detected in Brazil in May 2014, in Feira de Santana, a city located approximately 100 km north of Salvador (TEIXEIRA et al., 2015). In Feira de Santana, CHIKV reached outbreak levels, with 4,088 reported cases in 2015 (an incidence of 668.0 cases/100,000 inhabitants) (BRAZIL, 2016b). In contrast, in Salvador, 1,240 CHIKV cases were reported in 2015 (an incidence of 42.7 cases/100,000 inhabitants, more than an order of magnitude lower) (BRAZIL, 2016b).

However, CHIKV transmission in Salvador may have been masked by the ZIKV, GBS and microcephaly outbreaks, which prevailed in the media and got much of the attention of health professionals. Here we describe the results of an investigation aiming to determine the intensity of transmission of CHIKV in

Salvador, Brazil, during the period of occurrence of ZIKV, GBS and microcephaly outbreaks.

Methods

In collaboration with the Salvador Secretary of Health and the State's Central Laboratory of Public Health (LACEN-BA), we retrospectively analyzed all the serum sample results from Salvador patients that were tested for CHIKV at LACEN-BA between November 4, 2014 and April 19, 2016. Other than research and private laboratories, LACEN-BA is the sole public health laboratory in the State of Bahia capable of performing arbovirus diagnosis. It receives samples from patients suspected of arboviral disease from public health units throughout the state. The decision as to which patients' serum samples are collected and sent for CHIKV testing is made by the attending physician, and follows the Brazilian guidelines for CHIKV suspicion, which is defined by sudden onset of fever (>38.5°C) and arthralgia or intense arthritis in residents or visitors of endemic or epidemic areas (BRAZIL, 2015b).

Serum samples collected through the fifth day of onset of symptoms were tested by CHIKV IgM ELISA (MARTIN et al., 2000) or by CHIKV RT-PCR (LANCIOTTI et al., 2007), while those collected more than 5 days after onset were only tested using CHIKV IgM ELISA. CHIKV IgM ELISA testing was performed during the whole study period, whereas CHIKV RT-PCR was performed for samples from November 2014 to December 2015. Additionally, during the AEI outbreak attributed to ZIKV (CARDOSO et al., 2015), samples sent to LACEN-BA due to AEI symptoms were also tested for CHIKV. We considered a sample positive for CHIKV if it tested positive by either ELISA or RT-PCR.

We constructed an epidemiological curve by epidemiological week of the date of serum collection, plotting the absolute and relative frequency of CHIKV detection. The percentage of positive samples was smoothed using a 5-week moving average, wherein the count of events for a given week was averaged with

the values in the 2 previous and 2 following weeks to reduce week-to-week variation.

This investigation was performed using de-identified secondary laboratory data obtained by routine activities of the Epidemiological Surveillance Office/Municipal Secretariat of Health from Salvador, Bahia, Brazil. The study was approved by the Salvador Secretariat of Health and the Oswaldo Cruz Foundation Ethics Committee.

Results

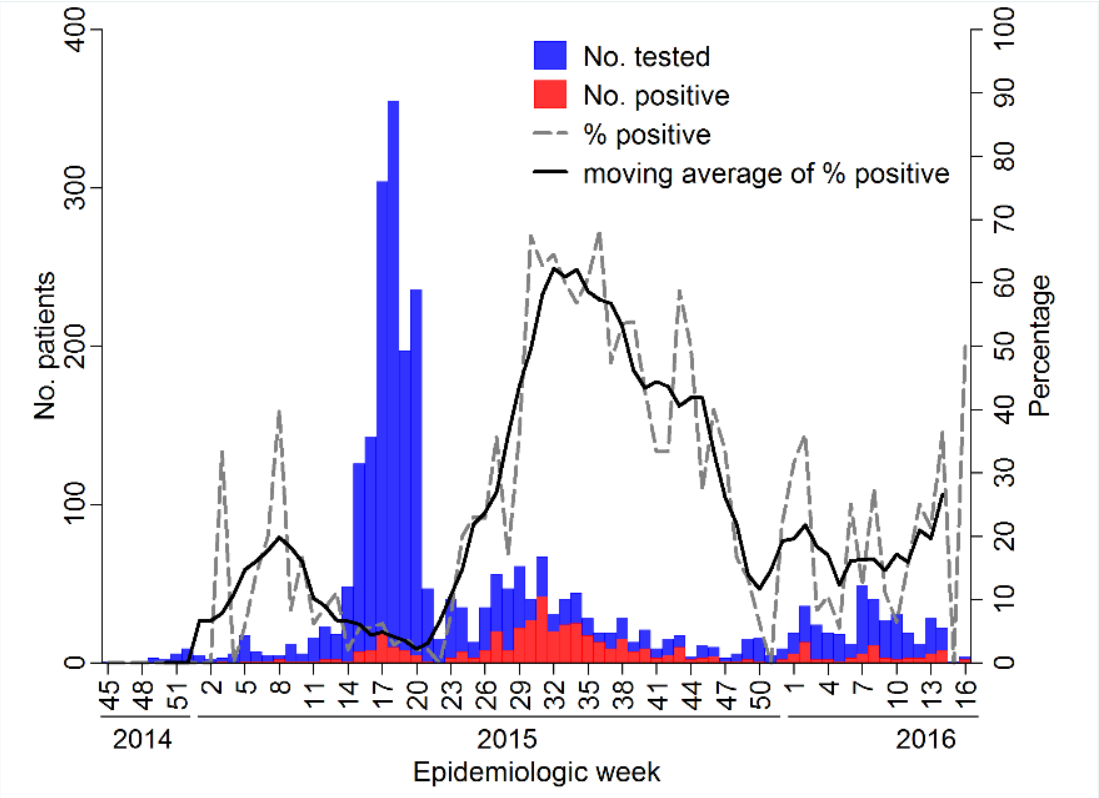
Of 3,042 serum samples from Salvador patients received by LACEN-BA for CHIKV testing during the study period, 2,656 (87%) were tested only using ELISA, 49 (2%) were tested only by RT-PCR, 31 (1%) were tested by both methods, and for 306 (10%) no result was available (either due to an insufficient sample or because it has not yet been tested). Samples that were not tested by at least one method (306) were excluded from analysis, resulting in a final count of 2,736 analyzed serum samples.

In total, 456 (16.7%) of the 2,736 samples analyzed during the study period were positive for CHIKV. Of the samples tested by RT-PCR 45% (36/80) were positive and of those tested by ELISA, 15.7% (422/2,687) were positive. The first laboratory-confirmed chikungunya case detected through this study occurred in week 3 (January 23) of 2015, and the positivity rate increased steadily until week 8 (February 22-28) (Figure 1). This trend was then interrupted and reversed, coinciding with the vast increase in number of samples tested, reaching more than 350 samples during week 18 (May 3-9) of 2015. The positivity rate then increased again, starting in week 20 (May 17), and peaking during weeks 26-47 (June 28-November 28) of 2015, when more than 25% (up to 68% at week 36, September 6-12, 2015) of the tested samples were positive for CHIKV. The percentage of CHIKV positive samples from Salvador remained at levels of ~10-20% through the rest of 2015 and the first weeks of 2016.

Additionally, during the study period a total of 3,328 samples were tested for DENV (from December 2014 to April 2016) using IgM or NS1 ELISA (Panbio

Diagnostics, Brisbane, Australia), of which 599 were positive. Of the 2,736 samples tested for CHIKV, 1,126 were also tested for DENV (from December/2014 to April/2016) and 158 (14.0%) were positive. Concomitant positivity for DENV and CHIKV was found for 37 samples (36 CHIKV positivity by ELISA and 1 by RT-PCR). A total of 129 samples were tested for ZIKV (from April/2015 to April/2016) by real-time RT-PCR (LANCIOTTI et al., 2008), four of which were positive. Laboratory testing for both CHIKV and ZIKV was performed for 58 samples (collected between the end of July 2015 and April 2016), none of which were positive for ZIKV (three of the 58 were positive for CHIKV by IgM ELISA).

Figure 1. Epidemiological curve of absolute and relative frequencies of Chikungunya detection by epidemiological week, Salvador, Brazil.



Discussion

The increase in the frequency of CHIKV positive laboratory results among Salvador patients during 2015 suggest that the intensity of CHIKV transmission in the city followed the same temporal pattern observed for the laboratory exams, with CHIKV transmission likely peaking in August, shortly after the exanthematous illness outbreak attributed by excess to ZIKV only (CARDOSO et al., 2015). Although Salvador established a surveillance for CHIKV detection following Feira de Santana's outbreak in 2014 (SALVADOR, 2015), the virus' introduction and subsequent spread in the city was not promptly noticed by the health authorities, because their main focus was on the AEI outbreak attributed to ZIKV, which affected about 14,000 people over a two month period (PAPLOSKI et al., 2016). Additionally, due to the overwhelming demand, especially during the AEI outbreak, laboratory testing was not performed in a timely manner. Therefore, the health authorities were only informed of the increase of CHIKV cases retrospectively.

The interruption of the ascending trend in CHIKV positivity in weeks 8-20 corresponds to the peak of the AEI outbreak attributed to ZIKV in Salvador (April 19–May 23, 2015) (PAPLOSKI et al., 2016). This probably accounts for the increase in the number of samples tested, given that suspected AEI cases were also tested for CHIKV. Despite the high number of samples tested, the frequency of CHIKV detection was relatively low (<10.0%), supporting the hypothesis that the AEI outbreak was associated mostly with ZIKV infections (CARDOSO et al., 2015). Thus, the low frequency of CHIKV detection during the AEI outbreak period should be interpreted with caution, since testing likely included primarily patients with AEI, rather than CHIKV suspected cases. Unfortunately, we cannot distinguish samples from this period that were sent to be tested because of a clinical suspicion of CHIKV from samples that were tested because of AEI. It is likely that the increase in CHIKV cases in Salvador in 2015 started before the first cases of AEI were detected, but the epidemiological curve lagged because testing targeted mostly AEI patients during this period. This possibility is supported by the fact that the first laboratory confirmed CHIKV cases were from January 2015.

This finding also suggests that the CHIKV transmission in Salvador was less explosive than the 2015 ZIKV outbreak (over 17,000 reported cases in nearly 10 weeks) (7), but, in contrast, was of longer duration and may have resulted in established endemic transmission, given that the percentage of CHIKV positive samples from Salvador remained at levels of ~10-20% through the rest of 2015 and the first weeks of 2016. It is also possible that the CHIKV outbreak reported here is under-estimated, while the ZIKV outbreak is over-estimated (i.e., all severe manifestations observed in Salvador were attributed almost entirely to ZIKV circulation). Even though the number of people infected by both viruses was certainly under-estimated given how surveillance of cases was assembled, health-seeking behavior and the general perception that AEI was a self-limited mild disease.

Salvador was one of the epicenters of ZIKV, GBS and microcephaly outbreaks in Brazil during 2015. A causal relation between ZIKV and the congenital disorders outbreaks has been established (BRASIL et al., 2016; RASMUSSEN et al., 2016). In French Polynesia, a case-control investigation also pointed to a link between prior ZIKV infection and GBS development (CAO-LORMEAU et al., 2016), supporting a relation between the outbreaks of ZIKV and GBS in Brazil. However, CHIKV has also been previously related with GBS in both French Polynesia and Réunion Island (LEBRUN et al., 2009; OEHLER et al., 2015). Thus, our findings of intense CHIKV transmission in Salvador between June and November co-occurring with the period of the GBS outbreak in the city (PAPLOSKI et al., 2016), support a possible connection between CHIKV infections and GBS development in Salvador.

Some limitations need to be acknowledged. First, the majority of the samples were tested only using IgM-based serology, and thus cross-reaction to other alphavirus has to be considered. However, although the occurrence of other alphavirus such as Mayaro have been described in the North and Midwest regions of Brazil (AZEVEDO, RAIMUNDA et al., 2009; COIMBRA et al., 2007), there is no evidence for their circulation in Salvador. Second, our epidemiological curve is based on the time when a sample was taken from the patient, which might not necessarily represent the time of infection, especially as CHIKV

infections may result in chronic clinical manifestations and serum samples may have been collected for diagnosis a long time after disease onset. In this case, IgM antibodies would no longer be present and IgG-ELISA would be more appropriate. Also, although RT-PCR for acute-phase samples and IgM detection in paired samples would provide a more accurate diagnosis (PAHO, 2014), a different algorithm for CHIKV testing was adopted due to limited resources. Third, this study included only cases that sought healthcare and whose attending physician requested laboratory testing for either CHIKV or differential diagnosis of an AEI, thus underestimating CHIKV cases. Additionally, with syndromic surveillance it is not possible to define accurately the etiology of cases, therefore laboratory testing is essential. In this study, we tried to address this limitation by analyzing the available laboratory results for all patients tested for CHIKV in Salvador, and our results are supported by previously published data showing that the AEI outbreak in Salvador that peaked in May was mainly due to ZIKV (CARDOSO et al., 2015). Yet, community-based studies using serological tests are needed to help better ascertain the intensity of CHIKV transmission, and there is an urgent need for ZIKV serological tests to accurately assess the intensity of ZIKV transmission. Fourth, CHIKV outbreaks in Feira de Santana appear to have occurred in two waves, the first in June-December 2014 and the second starting at January-2015 (RODRIGUES FARIA et al., 2016). Since the first patients from Salvador to be tested for CHIKV infection were not tested until November 2014, we might have missed any earlier CHIKV transmission in Salvador during the first wave of transmission in Feira de Santana. Lastly, both viral isolation and genome sequencing are not routinely performed by LACEN-BA; thus, detailed information on strains responsible for this outbreak was not available. However, other studies have identified CHIKV infections in Bahia associated with the East-Central-South African (ECSA) strain (RODRIGUES FARIA et al., 2016; SARDI et al., 2016).

Our findings reinforce the need for a better understanding of the co-circulation of these arboviruses. In such a setting, with high intensity of transmission of more than one arbovirus, co-infections may be common, as these viruses have the same vector, future studies are needed to better understand the role of sequential and co-infections in the severity of clinical manifestations. Failing of detect the co-circulation of other arbovirus in a timely fashion hampers

the ability to implement actions to prevent and treat severe or chronic manifestations that may elapse, such as incapacitating chronic arthralgia in the case of CHIKV infections, for example. In addition, the unrecognized co-circulation of other arboviruses could partially explain the occurrence of other severe outcomes in the region, such as GBS. Even with regard to microcephaly, Brazil authorities are now set to explore the country's peculiar distribution of Zika-related microcephaly (BUTLER, 2016). The concentration of such cases in the Northeast, where all three arboviruses have been co-circulating needs to be considered (together with other risk factors). As co-circulation of arboviruses is likely occurring in several other tropical cities, researchers, physicians, and public health professionals must consider CHIKV as a differential diagnosis together with DENV and ZIKV when studying arbovirus transmission and disease, while examining suspected case patients and when performing surveillance in Brazil and elsewhere.

Clinical differential diagnosis between these arbovirus is difficult, as observed by Salvador's experience during the AEI outbreak when ZIKV, DENV and CHIKV were co-circulating (CARDOSO et al., 2015), are three capable of causing AEI. In such a scenario, syndromic surveillance can provide a rough estimate of disease transmission, but a syndromic laboratory assessment approach testing all patients with non-specific arboviral disease symptoms for DENV, ZIKV and CHIKV (such as multiplex testing), should be considered, if possible.

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8.2 Anexo 2 – Reduction in dengue cases following an outbreak of Zika virus: Does immunity following Zika virus infection cross-protect against dengue?

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Zika (ZIKV) and dengue viruses (DENV) are closely related flaviviruses with immunological interactions and identical urban, mosquito-borne transmission cycles (DEJNIRATTISAI et al., 2016). Therefore, the recent introduction of ZIKV in the Americas and large-scale exposure of a uniformly naïve population may potentially

impact future DENV transmission. This hypothesis remains untested, largely because sufficient epidemiological data are not yet available from affected locations. We explored this hypothesis in Salvador, the fourth largest city in Brazil (population, 2·9 million), where extensive transmission of DENV1-4 (KIKUTI et al., 2015; TEIXEIRA et al., 2001) occurred prior to the introduction and spread of ZIKV in early 2015 (CARDOSO et al., 2015).

We have been conducting continuous enhanced surveillance of dengue among acute febrile illness (AFI) patients in an urban slum community of Salvador (population, 76,352) since Jan/2009 (KIKUTI et al., 2015), except for Sep/2013 to Sep/2014 and Aug-Sep/2016. Prior to 2015, the frequency of DENV RT-PCR-positivity followed a pattern of annual peaks, occurring in the second and/or third quarter of each year (Figure 1a). A much smaller peak occurred in 2015 during the ZIKV epidemic, but was not observed in 2016 and 2017, when DENV RT-PCR positivity was ~0%. We observed a significant decrease in the frequency of DENV RT-PCR-positive cases among AFI outpatients from 25·0% (484 of 1,937) before the ZIKV outbreak (Jan/2009 to Mar/2015) to 3·2% (43 of 1,334) afterwards (Apr/2015 to May/2017) (P<0·001) (Appendix 1).

We initiated routine testing for ZIKV and chikungunya virus (CHIKV) in September, 2014. Of 1,407 patients tested for ZIKV, 14 (1·0%) were confirmed by RT-PCR. We detected the first ZIKV case in May 2015 and observed a peak in Apr-Jun/2015 (3·9% of 285 tested patients). In contrast to what was observed for dengue cases, a large Chikungunya outbreak occurred at the surveillance site in 2015. The frequency of CHIKV RT-PCR or IgM-ELISA positive cases among AFI outpatients significantly increased from 6·5% to 19·5% after the Zika epidemic (P=0·004) (Appendix 1), indicating that there were sufficient environmental conditions for arboviral transmission following the Zika epidemic.

To complement enhanced surveillance data, we analyzed citywide data from 40,904 suspected dengue patients for which serum was tested between Jan/2009 and May/2017. The overall DENV positivity rate by serological and/or virological methods declined from 31·2% before to 8·0% after the ZIKV outbreak (P <0·001) (Figure 1b and Appendix 1). Positivity for ZIKV was highest in Apr-Jun/2015 (30·0% of 10 tested patients), and then declined to nearly 0% (Jul/2015-May/2017) (Figure 1b). Two large

peaks of CHIKV positivity were observed after the ZIKV outbreak, in Jul-Sep/2015 (47·3% positive rate) and in Oct-Dec/2016 (39·4% positivity).

Our findings, indicating lower frequencies of confirmed dengue over more than two years since early 2015, lower than at any other low transmission period analyzed, raised the hypothesis of an inhibitory role for the 2015 ZIKV outbreak on DENV transmission and/or for its impact on symptomatic infections. Because DENV serological tests, especially IgM ELISA, cross-react with ZIKV-immune sera (LANCIOTTI et al., 2008), it is possible that some citywide cases diagnosed as dengue since 2015 were actually Zika. Nonetheless, we found a downward trend in dengue cases. Although we found low frequency of ZIKV-confirmed cases during the ZIKV epidemic, there is a consensus that the Northeast region of Brazil, where Salvador is located, was the epicenter for the 2015 ZIKV epidemic. ZIKV diagnosis faces the challenges of lack of a specific serologic test and installed laboratory capacities to timely respond to the emergence of outbreaks. Still, ZIKV was the most likely cause for the acute exanthematous illness outbreak for which ~15,000 cases were reported in Salvador by mid-2015 (CARDOSO et al., 2015).

ZIKV, DENV, and CHIKV have mostly identical transmission cycles involving human amplification and transmission by *Aedes aegypti* mosquitoes. In our study, the proportion of CHIKV-positive tests was highest in the second half of 2015 and throughout 2016. The persistence of an elevated positivity rate for CHIKV, an alphavirus with no antigenic similarities to ZIKV and DENV flaviviruses, suggests that the reduction in DENV infections was likely unrelated to a lower efficiency in the shared *Ae. aegypti*-borne transmission cycle (such as lower vector populations related to weather or control activities), or to other non-immunological factors.

Although temporal associations do not prove causation, the strength and consistency from both enhanced surveillance and citywide data, together with the observed maintenance of high CHIKV positivity after the ZIKV outbreak, suggest that ZIKV infections induce cross-protection against DENV. Prospective studies will be critical to evaluate the subsequent risk of dengue following ZIKV exposure and to determine whether the putative DENV protection elicited by ZIKV infections is long-lasting or may wane over time. More specific serologic assays, such as for neutralizing antibodies, may help to demonstrate this effect conclusively. If such further studies

confirm our findings, they may have direct implications for epidemiological surveillance, immunological investigation of pathogenesis, and for vaccine development and evaluation.

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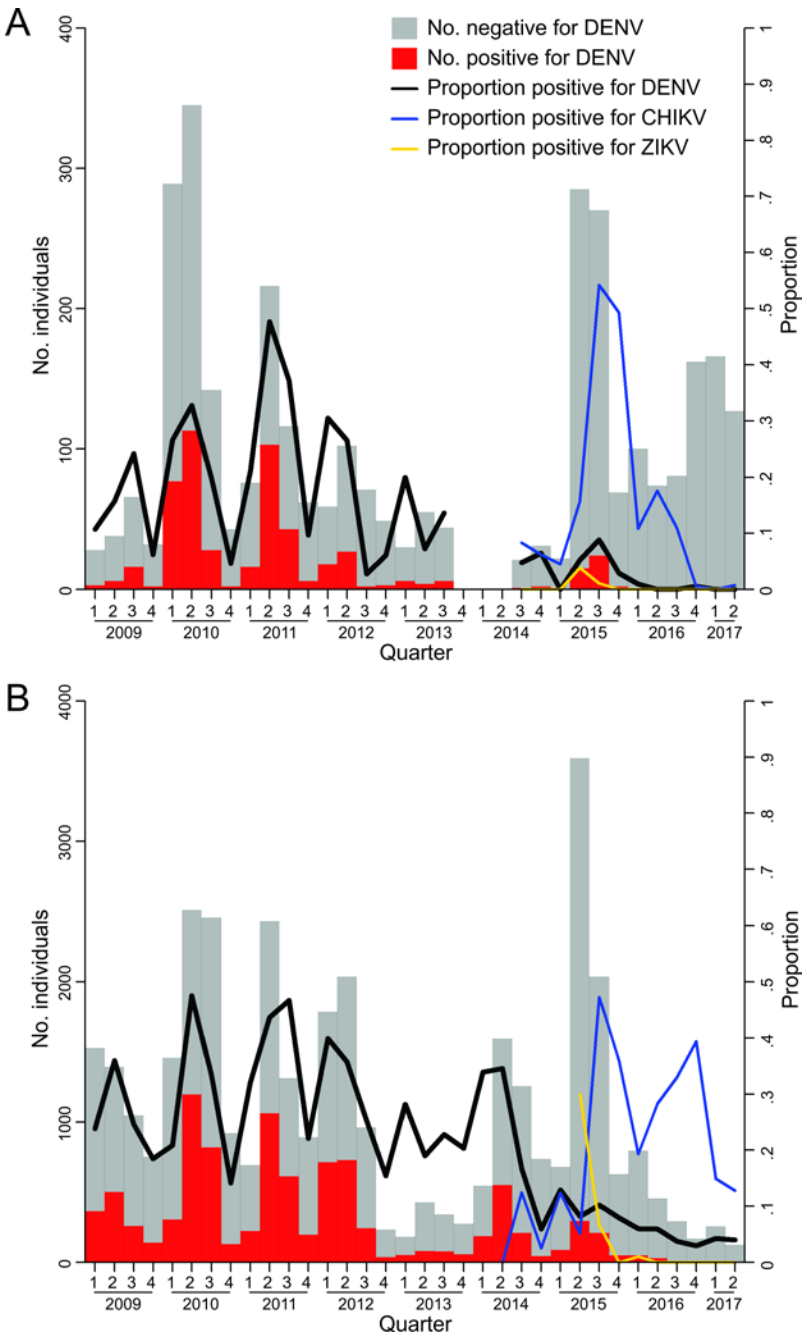
Figure 1: Quarterly frequency of laboratory-confirmed dengue in Salvador, Brazil, for the period before the appearance of ZIKV (January 2009 - March 2015) and thereafter (April 2015 - May 2017).

A) Dengue cases among acute febrile illness (AFI) patients enrolled during enhanced surveillance confirmed by RT-PCR;

B) Dengue cases among suspected dengue patients from all of Salvador confirmed by RT-PCR, IgM or NS1 ELISA, or viral isolation.

Figures also show the frequency of laboratory-confirmed cases of chikungunya by RT-PCR or IgM ELISA and of Zika by RT-PCR.



Appendix Table 1. Frequency of dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) virus infections among acute febrile illnesses (AFI) patients enrolled in an enhanced surveillance study and among citywide suspected DENV cases, before (January 2009 to March 2015) and after (April 2015 to May 2017) the ZIKV outbreak in Salvador, according the test performed.

Laboratory tests performed*	Before ZIKV outbreak		After ZIKV outbreak		P value
	Number Tested	Number Positive (%)	Number Tested	Number Positive (%)	
AFI enhanced surveillance					
Acute-phase sample DENV RT-PCR[1, 2]	1,937	484 (25.0)	1,334	43 (3.2)	<0.001
Acute-phase sample CHIKV RT-PCR[2, 3]	74	0 (0.0)	1,334	154 (11.5)	0.002
Acute- or convalescent-phase sample CHIKV IgM-ELISA[4]	77	5 (6.5)	842	173 (20.5)	0.003
Any CHIKV testing	78	5 (6.4)	1,347	263 (19.5)	0.004
Acute-phase sample ZIKV RT-PCR[2, 5]	73	0 (0.0)	1,334	14 (1.0)	0.38
Citywide arboviral diagnosis					
Any DENV testing	28,446	8,865 (31.2)	8,364	665 (8.0)	<0.001
DENV NS1-ELISA[6]	18,498	3,805 (20.6)	4,987	68 (1.4)	<0.001
DENV IgM-ELISA[6]	14,437	5,690 (39.4)	3,913	602 (5.4)	<0.001
DENV viral isolation[7]	5,095	1,015 (19.9)	152	4 (2.6)	<0.001
DENV RT-PCR[1]	682	484 (71.0)	121	21 (17.4)	<0.001
CHIKV IgM-ELISA[8]	231	18 (7.8)	4,156	881 (21.2)	<0.001
CHIKV RT-PCR[9]	83	1 (1.2)	53	37 (69.8)	<0.001
ZIKV RT-PCR[10]	0	0 (0.0)	456	6 (1.3)	NA

*Some patients had their serum samples tested by more than one method. References for the laboratory tests used are referenced in the footnote. DENV NS1-ELISA and DENV IgM-ELISA were performed for samples collected within 5 and >5 days after onset of symptoms, respectively.

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DPS- days post onset of symptoms
NA- not available

8.3 Anexo 3 – Congenital brain abnormalities during a Zika virus epidemic in Salvador, Brazil

(Submetido à Eurosurveillance)

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Abstract

We investigated clinical and epidemiological aspects of the outbreak of congenital brain abnormalities (CBAs) that followed the Zika virus (ZIKV) epidemic in Brazil, and evaluated the accuracy of different head circumference screening criteria in predicting CBAs. Suspected microcephaly cases from Salvador, Brazil had a diagnosis of CBA confirmed or excluded based on intracranial imaging studies. Of the 365 investigated cases, 166 (45.5%) had CBAs. The most common findings were intracranial calcifications (85.9%) and ventriculomegaly (66.9%). Prevalence of CBAs peaked in December 2015 (2.24 cases/100 live births). Cases of CBAs were significantly more likely to have been born pre-term and to mothers who had clinical manifestations of arboviral infection during pregnancy. None of the head circumference screening criteria performed optimally in predicting CBAs. This study highlights the magnitude of neurological consequences of the ZIKV epidemic and suggests that gestational

symptoms compatible with ZIKV infection should be combined with imaging studies for efficient detection of suspect CBAs during ZIKV epidemics.

Introduction

Early in 2015, large outbreaks of acute exanthematous illness were detected in several states in northeastern Brazil. By April 2015, Zika virus (ZIKV) was identified as the etiology of the illness (CAMPOS; BANDEIRA; SARDI, 2015; ZANLUCA et al., 2015). Seven to eight months after the epidemic peak, an increase in newborns with microcephaly was noted in northeastern Brazil (PAPLOSKI et al., 2016) and promptly gathered global attention due to a possible link between gestational ZIKV infection and microcephaly. Since then, evidence for a causal association between in utero exposure to ZIKV and microcephaly and other neurological complications has emerged (BRASIL et al., 2016b; DE ARAÚJO et al., 2016; RASMUSSEN et al., 2016). The constellation of clinical manifestations of congenital ZIKV infection may be referred to as “congenital Zika syndrome” (MIRANDA-FILHO et al., 2016).

In light of the surge of microcephaly cases, the Brazilian Ministry of Health (BMoH) declared a national public health emergency in November, 2015 and initiated a surveillance program for identification of suspected cases (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015a). All health facilities were required to report suspected microcephaly cases (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015a, 2015b, 2016) and encouraged to report spontaneous abortions and stillbirths in women with a history of a rash during pregnancy, in a national reporting system. Although head circumference was immediately adopted as the primary criterion for screening cases suspected of congenital abnormalities by Zika, not all children with neurological impairment due to ZIKV present with microcephaly at birth (VAN DER LINDEN et al., 2016a). Therefore, it is important to understand how well the criteria used to detect microcephaly can predict the congenital brain alterations of ZIKV.

Here, we describe the characteristics of the cases with congenital brain abnormalities (CBAs) confirmed by intracranial imaging studies among the reported cases of suspected microcephaly in Salvador, Brazil. We also identified clinical

manifestations during pregnancy that were associated with CBAs, and evaluated the accuracy of different screening criteria based on head circumference for predicting CBAs.

Methods

Suspected microcephaly case definition for mandatory reporting

From November 17 to December 11, 2015, the Brazilian Ministry of Health (BMoH) defined suspected microcephaly cases as newborns with head circumference measures ≤ 33 cm for term (≥ 37 weeks) or < 3rd percentile of the Fenton Preterm Growth Chart for preterm (< 37 weeks) (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015a) and required mandatory reporting of newborns fulfilling this case definition. This criterion was also applied to cases that were retrospectively identified during this period. From December 12, 2015 to March 12, 2016, suspected cases definition was updated to newborns with head circumference ≤ 32 cm for term and the previous criteria for preterm newborns were maintained (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015b). Lastly, on March 13, 2016, the suspected microcephaly definition was changed to newborns with head circumference < 3rd percentile of the World Health Organization (WHO) Child Growth Standards for term and < 3rd percentile of the INTERGROWTH-21st standards for preterm (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2016). Regardless of head circumference, reporting of spontaneous abortions, stillbirths or pregnancies with any detected alterations in the fetal central nervous system in women with self-reported history of rash during pregnancy was also encouraged, but not mandatory (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015b, 2016).

Investigation of reported cases suspected of microcephaly

Salvador, the third largest city in Brazil, was one of the northeastern cities most affected by the microcephaly epidemics (PAPLOSKI et al., 2016). The Salvador Centers for Information and Epidemiologic Surveillance (CIES) is the branch of the Municipal Secretary of Health in charge of the investigation of the reported suspected cases. Investigations were performed by reviewing medical records for intracranial imaging studies. In addition, mothers of reported suspect microcephaly cases were interviewed

about clinical manifestations during pregnancy using a standardized questionnaire (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2016). After concluding the investigation, CIES updated the national reporting system with the obtained information.

Image-confirmed brain abnormality case definition

In the present study, we analyzed data on suspected microcephaly cases investigated by CIES up to September 13, 2016. According to the availability of data on prenatal or postnatal intracranial imaging studies, the reported cases were classified as either investigated or not investigated. Investigated cases whose intracranial ultrasound, computed tomography, or magnetic resonance imaging results reported intracranial calcifications, ventriculomegaly, dysgenesis or agenesis of the corpus callosum, lissencephaly, cerebellar abnormalities, or anencephaly were classified as confirmed CBA cases. Reports of hydrocephalus or colpocephaly were consolidated as ventriculomegaly. Suspected microcephaly cases that underwent imaging studies and did not exhibit any of the previous findings were excluded from consideration as CBA cases.

Statistical analysis

Epidemiological curve of the temporal distribution of the suspected microcephaly cases stratified according to the confirmation status was constructed by epidemiological week of the date of birth. Records with incomplete information on date of birth were excluded from the epidemiological curve. Prevalence of CBA per month was calculated dividing the number of imaging confirmed cases by the monthly number of live births from mothers resided in Salvador. To estimate the average annual prevalence of CBA, we divided the CBA prevalence calculated for the complete study period by the number of months in the study period and multiplied the result by 12. Live birth data were obtained at the National Birth Registration System (SINASC) (DATASUS, 2016).

Clinical characteristics were compared between imaging confirmed and excluded cases of CBA using frequencies and medians. Two-tailed Fisher Exact test and odds ratios with 95% confidence intervals were used to test for differences in the frequencies of gestational characteristics of mothers of confirmed and excluded CBA cases. Wilcoxon rank test was used to test for difference in maternal age between confirmed and excluded

CBA cases. In order to investigate whether the imaging detected CBAs varied according to the presumptive timing of infection during pregnancy, the frequencies of each imaging abnormality in confirmed cases were compared according to the presence and timing of exanthema during pregnancy.

Imaging confirmed and excluded CBA cases were used to assess the accuracy of different head circumference microcephaly screening criteria for prediction of CBAs. The microcephaly screening criteria evaluated were those adopted by the BMoH from 1- November to December 12, 2015 (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015a); 2- from December 12, 2015 to March 12, 2016 (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2015b); and 3- from March 13, 2016 to the present (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2016); 4- the recommended by the Pan American Health Organization (< -2 SD of the Fenton Preterm Growth Chart according to sex and gestational age for preterm newborns and < 3rd percentile of the WHO Child Growth Standards according to sex for term newborns) (PAN AMERICAN HEALTH ORGANIZATION, 2016); 5- the Fenton Preterm Growth Chart (< -2 SD according to sex and gestational age) (FENTON; KIM, 2013); 6- the INTERGROWTH-21st standards (< -2 SD according to sex and gestational age) (VILLAR et al., 2016); and 7- the WHO Child Growth Standards for term newborns (< -2 SD according to sex for term newborns) (WHO, 2016). Some of the criteria require detailed information on gestational age (weeks and days) but gestational age was recorded in full weeks in our dataset. We used the number of weeks plus 0 days in such cases. Accuracy of the criteria in predicting imaging confirmed CBAs was assessed by calculating sensitivity, specificity, positive and negative predictive values, and their respective 95% confidence intervals. Records with no information on head circumference, gestational age, or with late reporting (>28 days post birth) without a precise date on which head circumference was measured were excluded from the accuracy analysis. Data were analyzed with Stata 14 (STATACORP, 2015).

Ethics statement

This investigation was performed using de-identified secondary data obtained by routine activities of the Epidemiological Surveillance Office/Municipal Secretariat of

Health from Salvador, Bahia, Brazil. The Salvador Secretariat of Health and the Oswaldo Cruz Foundation Ethics Committee approved the study and granted a waiver of signed informed consent.

Results

By September 13, 2016, Salvador CIES had received 650 reports of suspected microcephaly cases, which were born between April, 2015 and July, 2016. Of these, review of medical records to retrieve results of intracranial imaging studies was completed for 365 cases. Among those, 166 (45.5%) had imaging findings consistent with a diagnosis of CBA, while 199 (54.5%) did not. The epidemiological curve of the temporal distribution of reported cases (built for 631 cases with available data on date of birth) peaked between week 47 of 2015 (November 22-28) and week 4 of 2016 (January 24-30, 2016) for both the suspected microcephaly reported cases and the imaging-confirmed CBA cases (Figure 1). Prevalence of imaging-confirmed CBA was 1.22 cases per 100 live births in November 2015, 2.24 cases per 100 live births in December 2015, and 1.55 cases per 100 live births in January 2016. Prevalence of imaging-confirmed CBA for the whole study period (April, 2015 to July, 2016) was 0.34 per 100 live births and the one-year adjusted annual prevalence of image-confirmed CBA was estimated as 0.26 per 100 live births. The last CBA case confirmed by imaging studies during the study period was born in epidemiological week 15 of 2016 (April 10-16).

Male sex was more frequent among the imaging confirmed CBA cases (45.8%) than among excluded cases (32.7%) (P = 0.01) (Table 1). Confirmed cases were more likely to have been born pre-term (<37 weeks) (OR 6.62; 95% CI 3.35 – 13.79) and had a lower head circumference median (30 cm; interquartile range [IQR]: 28 – 31) compared to excluded cases (32 cm; IQR: 31 – 32) (P < 0.001). Confirmed cases also had a broader head circumference range (21.5 – 42 vs. 29 – 36). The most frequent CBAs observed among confirmed cases were intracranial calcifications (86.1%) and ventriculomegaly (66.9%) (Table 1). Agenesis of the corpus callosum (12.3%), dysgenesis of the corpus callosum (11.5%), lissencephaly (10.2%), cerebellar abnormalities (5.4%), and anencephaly (1.8%) were identified in minority of patients. In terms of other findings, arthrogryposis was found in 6.6% of the confirmed cases, but in none of the excluded

cases. Oligohydramnios, intrauterine growth restriction, subependymal cysts, and auditory and ophthalmological abnormalities were also more frequent among the confirmed than the excluded cases (Table 1).

Maternal age at birth and type of gestation (single vs. multifetal) were not associated with imaging confirmation of CBAs (Table 2). Frequency of exanthema during pregnancy among mothers of children in the confirmed group was 73.4%. Among those, 70% had the rash during the first trimester, 22% the second trimester, and 9% during the third trimester. Mothers of children in the confirmed CBA group were more likely to have had exanthema during pregnancy (OR 5.04; 95% CI 3.12 – 8.19) than mothers of children whose diagnosis of CBA was excluded, especially in the first trimester (OR 2.91; 95% CI 1.42 – 5.97) when compared to the second and third trimester. All other symptoms commonly observed during arboviral infections were more frequent during pregnancy on the mothers of children in the confirmed group (Table 2).

When the confirmed cases were classified according to the timing of maternal rash during pregnancy (first, second, third trimester or no rash), there were no statistically significant differences in the frequency of CBAs, nor in the frequency of arthrogryposis, oligohydramnios, and intrauterine growth restriction, between the four groups. An exception was observed for calcifications, that were more frequently present when exanthema occurred during first trimester than when compared with mothers who did not present exanthema during pregnancy (*P*=0.02) (Table 3).

Among the different head circumference criteria used to screen for microcephaly, the first criterion adopted by the BMoH from November, 2015 to December, 2015 was the one with the highest sensitivity (83.6%) and lowest specificity (7.3%) in predicting the presence of CBAs (Table 4). On the other hand, the INTERGROWTH-21st standards had the lowest sensitivity (63.4%) and highest specificity (72.4%). Positive predictive value was the highest for the INTERGROWTH-21st standards (63.9%) and the lowest for the criterion adopted by BMoH from December 2015 to March 2016 (41.1%). Negative predictive value was the highest for the WHO Child Growth Standards (77.8%) and the lowest for the BMoH criterion used between November and December 2015 (36.1%).

Discussion

In this study, we described a high prevalence of confirmed CBAs in Salvador, as high as 2.2% of the live births in December 2015. Unfortunately, we did not have information on serological or virological ZIKV testing, which would allow ascertaining the etiology for such outbreak. However, the prevalence of image-confirmed CBA estimated for the study period adjusted for one year was 52 times higher than the estimated baseline prevalence of microcephaly in the Northeast region (average of 5 cases per 100,000 live births per year, between 2000 and 2014) (MARINHO et al., 2016). Further, the peak of births of babies with microcephaly occurred 30-33 weeks after the peak of ZIKV epidemic in Salvador (PAPLOSKI et al., 2016), and this is consistent with the growing body of evidence suggesting that the first trimester of pregnancy is the period when ZIKV infections pose the highest risk of adverse fetal outcome (CAUCHEMEZ et al., 2016; KLEBER DE OLIVEIRA et al., 2016; PAPLOSKI et al., 2016). Taken together, it is reasonable to assume that most of the imaging confirmed cases in this study were due to congenital ZIKV infection.

As we only considered cases with specific neuroimaging findings as confirmed cases, we certainly underestimated cases of congenital ZIKV infection. Several suspected cases had not been investigated by the time we analyzed the data and the imaging modality most commonly used was prenatal or postnatal intracranial ultrasound, which is not an optimal modality to detect abnormalities of the corpus callosum and cerebral cortex. In addition, suspected microcephaly cases were reported based on birth head circumference, which could be well within normal limits in some cases of congenital ZIKV infection (VAN DER LINDEN et al., 2016a). Although reporting of spontaneous abortions, stillbirths and fetuses presenting alterations in the central nervous system was also encouraged, allowing us to confirm a few cases with normal or large head circumference at birth, we could not evaluate whether there was an increase in abortions and stillbirths in Salvador during the study period. On the other hand, some cases counted as confirmed could be due to other causes such as congenital cytomegalovirus infection or genetic disorders, but the number of these cases is expected to be small, considering the baseline rate of microcephaly before the epidemic.

Similar increase in microcephaly cases is now being reported in other locations where ZIKV epidemics have occurred, such as Colombia, where the prevalence of microcephaly also increased around 6 months after the peak of ZIKV transmission. However, the microcephaly prevalence reported in Colombia peaked in July 2016 at 17.7 cases per 10,000 live births, much lower than observed in Salvador (CUEVAS et al., 2016). Potential reasons for this difference may include, variable intensity levels of ZIKV transmission, differences in circulating ZIKV strains and different case definitions and surveillance criteria. Further, co-circulation of other arboviruses (dengue and chikungunya, for example), differences in mosquito control measures, and prior exposure to yellow fever vaccination could be contributing factors (BUTLER, 2016; CUEVAS et al., 2016). Additionally, Brazil was the first country in the Americas to experience a large outbreak of ZIKV and to detect an increase in microcephaly cases, and this allowed other countries as Colombia to issue recommendations for delaying pregnancies, which might have resulted in decreased risk of congenital abnormalities associated with ZIKV infection during pregnancy (CUEVAS et al., 2016).

Female newborns were overrepresented among the reported cases who had a CBA diagnosis excluded. This finding may be due to the application of the same head circumference screening criteria for reporting boys and girls suspected of microcephaly until March 12, 2016 (period during which 85% of the suspect cases had been reported), since head circumference of girls tend to be smaller than boys at the same gestational age. We also found that the frequency of preterm births among the confirmed CBA cases was significantly greater than in discarded cases. Although this finding suggests that congenital Zika syndrome could be associated with preterm birth, we could not determine from the available data whether the early births were natural in their occurrence or due to a medical decision in the presence of fetal anomalies and distress.

The most frequent imaging findings among the confirmed cases were intracranial calcifications and ventriculomegaly. Although these findings are not specific for congenital Zika syndrome, they have been frequently observed among laboratory confirmed cases of congenital Zika syndrome (BRASIL et al., 2016a; DE FATIMA VASCO ARAGAO et al., 2016; HAZIN et al., 2016; MELO et al., 2016). Anencephaly has not been previously reported among laboratory-confirmed cases of congenital ZIKV infection, and further

studies are warranted to determine if it is part of the spectrum of the congenital Zika syndrome. Auditory and ocular manifestations were present in about 20% of the confirmed cases. However, they were also found in lower frequencies among the suspected microcephaly cases with normal intracranial imaging studies. Since these manifestations have been linked to congenital ZIKV infection (DE PAULA FREITAS et al., 2016; LEAL et al., 2016), it is important to further investigate whether ZIKV infection can cause auditory or ocular lesions in the absence of structural malformations in the brain and to monitor for long-term consequences in ZIKV-exposed babies born with no alterations in imaging studies.

Frequency of exanthema during pregnancy among mothers of children in the confirmed group was 73.4%. It has been previously estimated that only 20% of the ZIKV infections are symptomatic (DUFFY et al., 2009), but other studies have shown similarly high frequencies of exanthema among mothers who gave birth to children with congenital Zika syndrome (FRANÇA et al., 2016; MIRANDA-FILHO et al., 2016; VENTURA et al., 2016). Recall bias and the surveillance system associating a rash during pregnancy as a marker for microcephaly risk may have accounted for the high proportion of symptomatic women in this series. However, both confirmed and excluded CBA cases originated from the same reported dataset and only 36.0% of the mothers of children in the excluded group reported a rash. Therefore, it is likely that symptomatic ZIKV infection during pregnancy truly pose a higher risk of CBAs. Similar findings of an association of rash during pregnancy with increased CBA risk was previously reported in Brazil (FRANÇA et al., 2016; MIRANDA-FILHO et al., 2016; VENTURA et al., 2016), although absence of such association had been noted in the US (HONEIN et al., 2017).

Among mothers of children in the confirmed group who reported a history of rash during pregnancy, 70% had the rash during the first trimester, but, in addition, 22% had rash during the second trimester and 9% during the third trimester. Data linking ZIKV infection in the second and third trimester to congenital malformations are still scarce (BRASIL et al., 2016a; FRANÇA et al., 2016), and our findings reinforce that second and third trimester infections may also lead to congenital Zika syndrome with CBAs.

Head circumference-based criteria were primarily used during the epidemic of congenital ZIKV infection. These can be easily applied in any clinical setting and do not

require any special equipment. Although microcephaly in general is a risk factor for developmental delay, according to the U.S. National Collaborative Perinatal Project, only 11% of children with microcephaly (head circumference ≤ -2SD) at birth had IQ≤70 at 7 years of age (DOLK, 1991), suggesting that microcephaly has a relatively low specificity in predicting poor neurodevelopmental outcome, at least in the general population. Further, cases with congenital ZIKV infection without microcephaly at birth have been reported (VAN DER LINDEN et al., 2016b). On the other hand, the severity of neuroimaging during the neonatal period has been shown to have a good prognostic value in symptomatic congenital cytomegalovirus infection (ALARCON et al., 2016), which shares many clinical and radiological similarities to congenital ZIKV infection. Therefore, it is important to evaluate the accuracy of different microcephaly criteria in predicting CBAs. In this regard, none of the criteria performed particularly well. Overall, the INTERGROWTH-21st standards had a better performance, with sensitivity, specificity and positive and negative predictive values over 60%. A significantly low specificity of the criteria used by the BMoH until March 12, 2016 was also noted. There is only one small prior study with 31 cases that used imaging findings consistent with congenital infection as the reference criteria for sensitivity estimation of different head circumference criteria (VICTORA et al., 2016). Our study revealed lower sensitivity and much lower specificity compared to this prior study. Combined with relatively low positive and negative predictive values, these data clearly demonstrate the limitations of head circumference in accurately identifying children with CBA during ZIKV epidemics.

Novel screening methods for congenital Zika syndrome during ZIKV epidemics that incorporate additional parameters to head circumference are urgently needed in order to detect the maximum number of affected children and fetuses for further specific health assistance, without compromising specificity. Prenatal and postnatal intracranial ultrasound triage performed by experienced ultrasonographers may be an efficient approach during outbreaks, especially for women who experienced symptoms consistent with ZIKV infection during pregnancy. However, the importance of clinical examination and follow up of newborns, as well as development of better serological and molecular tests, cannot be understated.

Our study highlights the magnitude of neurological consequences of the ZIKV epidemic in Salvador, Brazil, further delineates congenital Zika syndrome, and identifies limitations of screening for congenital Zika syndrome based on head circumference that was performed during the recent epidemics. Follow-up studies of children with and without microcephaly or congenital abnormalities, who were exposed to ZIKV in utero are needed to fully understand the full spectrum of congenital Zika syndrome.

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Figure 1. Epidemic curve of the reported cases suspected of microcephaly per week of birth by status of congenital brain abnormality confirmation based on intracranial imaging studies.

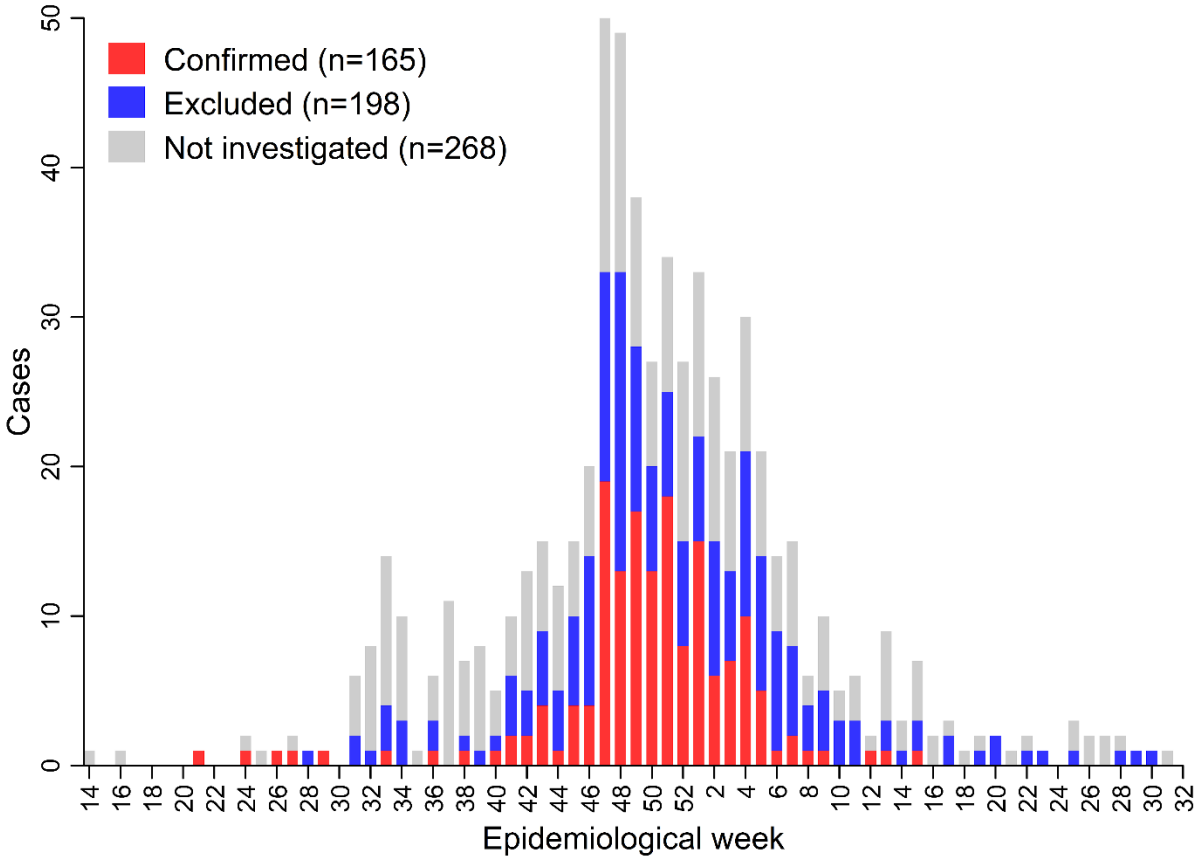

Table 1. Clinical characteristics of suspected microcephaly cases.

Clinical characteristics	Congenital brain abnormalities diagnosis	
	Confirmed (N=166)	Excluded (N=199)
	n (%)	
Male sex	76 (45.8%)	65 (32.7%)
Gestational age at birth		
<37 weeks	54/162 (33.3%)	13/185 (7.0%)
37 - 42 weeks	108/162 (66.7%)	172/185 (93.0%)
Head circumference (cm), median (IQR)[*]	30.0 (28.0 – 31.0)	32.0 (31.0 – 32.0)
Head circumference (cm), min – max[*] [†]	21.5 - 42.0	28.0 - 36.0
Intracranial imaging performed		
Ultrasound	136 (81.9%)	192 (96.5%)
Computed tomography	53 (31.9%)	9 (4.5%)
Magnetic resonance imaging	17 (10.2%)	4 (2.0%)
Image findings consistent with congenital malformations		
Intracranial calcifications	143 (86.1%)	0 (0.0%)
Ventriculomegaly	111 (66.9%)	0 (0.0%)
Agenesis of the corpus callosum	20 (12.1%)	0 (0.0%)
Dysgenesis of the corpus callosum	19 (11.5%)	0 (0.0%)
Lissencephaly	17 (10.2%)	0 (0.0%)
Cerebellar abnormalities[‡]	9 (5.4%)	0 (0.0%)
Anencephaly	3 (1.8%)	0 (0.0%)
Other image findings		
Oligohydramnios	17 (10.2%)	7 (3.5%)
Intrauterine growth restriction	14 (8.4%)	2 (1.0%)
Subependymal cysts	16 (9.6%)	7 (3.5%)
Arthrogryposis	11 (6.6%)	0 (0.0%)
Auditory abnormalities[§]	20 (19.2%)	10 (11.5%)
Ophthalmological abnormalities[¶]	14 (20.3%)	3 (4.5%)
Death[#]	6 (3.6%)	0 (0.0%)

IQR: Interquartile range.

[*]Data on head circumference were not available for two confirmed cases. Data on head circumference of 3 confirmed cases and 8 discarded cases were excluded due to a late measurement without a precise date (>28 days post birth).

[†]Some of the patients suspected of congenital brain abnormalities had a head circumference size greater than the screening limits used to detect microcephaly because reporting cases of spontaneous abortions, stillbirths, or pregnancies with any detected alterations in the fetal central nervous system in women with self-reported history of rash during pregnancy was also encouraged, but not mandatory

[‡]Cerebellar vermis agenesis or cerebellar hypoplasia.

[§]Data on auditory abnormalities were available for 104 confirmed cases (86 by auditory screening method, 3 by Brainstem Evoked Response Audiometry test [BERA] and 15 by both) and for 87 discarded cases (79 by auditory screening method, 3 by Brainstem Evoked Response Audiometry test [BERA] and 5 by both).

Table 2. Maternal clinical characteristics of suspected microcephaly cases.

Characteristics	Congenital brain abnormalities diagnosis		Odds Ratio (95% CI)	P-value
	Confirmed (N=166)	Excluded (N=199)		
	n/N (%)			
Mother's age, median (IQR)*	26 (21 - 32)	25 (21 - 31)	-	0.38
Type of gestation				
Multifetal	5/163 (3.1%)	4/195 (2.0%)	1.51 (0.32 - 7.74)	0.54
Single	158/163 (96.9%)	191/195 (98.0%)	1	
Symptoms during pregnancy				
Fever	66/155 (42.3%)	42/187 (22.5%)	2.56 (1.56 - 4.21)	<0.001
Exanthema	118/161 (73.3%)	68/193 (35.2%)	5.04 (3.12 - 8.19)	<0.001
Trimester of exanthema†				
First trimester	75/108 (69.4%)	25/57 (43.9%)	2.91 (1.42 – 5.97)	0.001
Second or third trimester	33/108 (30.6%)	32/57 (56.1%)	1	
Pruritus	72/133 (54.1%)	36/143 (25.2%)	3.51 (2.05 – 6.04)	<0.001
Arthralgia	60/135 (44.4%)	31/143 (21.7%)	2.89 (1.66 - 5.06)	<0.001
Myalgia	51/135 (37.8%)	25/142 (17.6%)	2.84 (1.58 - 5.17)	<0.001
Headache	48/133 (36.1%)	25/142 (17.6%)	2.64 (1.46 - 4.83)	0.001
Retro-orbital pain	24/133 (18.1%)	6/142 (4.2%)	4.99 (1.89 – 15.38)	<0.001
Conjunctival hyperemia	21/133 (15.8%)	9/142 (6.3%)	2.77 (1.16 - 7.14)	0.01

IQR: Interquartile range; 95% CI: 95% confidence interval.
*Data on mother's age were not available for 9 confirmed and 4 excluded cases.
†Data on trimester of exanthema were not available for 10 confirmed and 11 excluded cases.

Table 3. Imaging findings of confirmed congenital brain abnormalities cases, according to the timing of maternal exanthema.

Congenital brain abnormalities	Pregnancy trimester of exanthema			
	First (N=75)	**Second (N=24)**	**Third (N=9)**	**Without exanthema (N=43)**
	n (%)			
Intracranial calcifications	68 (90.7%)	23 (95.8%)	7 (77.8%)	32 (74.4%)
Ventriculomegaly	48 (64.0%)	18 (75.0%)	8 (88.9%)	28 (65.1%)
Agenesis of the corpus callosum	11 (14.7%)	3 (12.5%)	2 (22.2%)	4 (9.3%)
Dysgenesis of the corpus callosum	8 (10.7%)	5 (20.8%)	0 (0.0%)	5 (11.6%)
Lissencephaly	6 (8.0%)	4 (16.7%)	2 (22.2%)	4 (9.3%)
Cerebellar abnormalities*	6 (8.0%)	1 (4.2%)	0 (0.0%)	1 (2.3%)
Arthrogryposis	6 (8.0%)	2 (8.3%)	0 (0.0%)	2 (4.7%)
Oligohydramnios	12 (16.0%)	1 (4.2%)	0 (0.0%)	3 (7.0%)
Intrauterine growth restriction	9 (12.0%)	3 (12.5%)	0 (0.0%)	1 (2.3%)
Subependymal cysts	7 (9.3%)	1 (4.2%)	1 (11.1%)	6 (14.0%)

*Cerebellar vermis agenesis or cerebellar hypoplasia.

Table 4. Accuracy of different head circumference screening criteria in detecting congenital malformations.

Criteria	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Fenton Preterm Growth Chart	67.7% (59.0 - 75.5)	60.6% (52.9 - 67.9)	56.6% (48.5 - 64.4)	71.1% (63.2 - 78.3)
INTERGROWTH-21st standards	63.4% (54.7 - 71.6)	72.4% (65.1 - 78.9)	63.9% (55.1 - 72.1)	72.0% (64.7 - 78.5)
WHO Child Growth Standards	75.5% (66.2 - 83.3)	54.2% (46.3 - 61.9)	51.0% (42.9 - 59.0)	77.8% (69.2 - 84.9)
Brazilian MoH (Nov. to Dec. 12, 2015)	83.6% (76.4 - 89.3)	7.3% (3.9 - 12.1)	41.3% (35.5 - 47.3)	36.1% (20.8 - 53.8)
Brazilian MoH (Dec. 12, 2015 to Mar. 12, 2016)	79.3% (71.6 - 85.7)	11.2% (7.0 - 16.7)	41.1% (35.2 - 47.2)	40.8% (27.0 - 55.8)
Brazilian MoH (Mar. 13, 2016 to present)	68.6% (60.2 - 76.1)	55.3% (47.7 - 62.7)	54.5% (46.9 - 62.1)	69.2% (61.0 - 76.7)
Pan American Health Organization recommendation	73.6% (65.5 - 80.7)	40.2% (33.0 - 47.8)	49.0% (42.1 - 56.0)	66.1% (56.4 - 74.9)

95% CI: 95% confidence interval; WHO: World Health Organization; MoH: Ministry of Health; PPV: Positive predictive value; NPV: Negative predictive value.