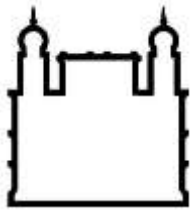

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CURSO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA EM SAÚDE E MEDICINA INVESTIGATIVA

TESE DE DOUTORADO

PERFIL CLÍNICO-LABORATORIAL E DA IMUNIDADE CELULAR DE PACIENTES COM INFECÇÃO AGUDA PELO VÍRUS ZIKA (ZIKV)

ANTONIO CARLOS DE ALBUQUERQUE BANDEIRA

Salvador – BA
2022

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Tese apresentada ao Curso de Pós- graduação em Biotecnologia em Saúde e Medicina Investigativa para obtenção do grau de Doutor.

Orientadora: Profa. Dra. Maria Fernanda Rios Grassi

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"PERFIL CLÍNICO-LABORATORIAL E DA IMUNIDADE CELULAR DE PACIENTES COM INFECÇÃO AGUDA PELO VÍRUS ZIKA (ZIKV)"

ANTONIO CARLOS DE ALBUQUERQUE BANDEIRA

FOLHA DE APROVAÇÃO

Salvador, 01 de outubro de 2021.

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BANDEIRA, Antonio Carlos de Albuquerque. **Perfil clínico-laboratorial e da imunidade celular de pacientes com infecção aguda pelo vírus zika (zikv)**. 2021. 86 f. Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) - Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2021.

RESUMO

Introdução: As manifestações clínicas e laboratoriais da infecção pelo vírus da Zika (ZIKV) eram desconhecidas quando a doença passou a ser identificada no Brasil em 2015. Os relatos clínicos eram baseados em pequenas séries de casos e o diagnóstico de ZIKV era baseado em técnicas sorológicas que apresentavam reação cruzada com anticorpos anti-vírus da Dengue (DENV). Além disso era necessário caracterizar a resposta imune celular em pacientes com quadros agudos de ZIKV, muito pouco estudada até o início do presente estudo. **Objetivo:** Descrever as manifestações clínicas, os achados laboratoriais, e a resposta imune celular de pacientes com infecção aguda pelo ZIKV. **Metodologia:** Foi realizado um estudo descritivo clínico de corte transversal com pacientes selecionados em um pronto- atendimento na cidade de Salvador. Foram avaliados sequencialmente os pacientes com quadro sugestivo de infecção viral aguda entre 27 de maio de 2015 e 31 de agosto de 2017. O diagnóstico de ZIKV foi baseado na positividade ao rt-PCR no sangue ou urina ou saliva. Os pacientes também foram avaliados para infecção por Dengue ou Chikungunya (CHIKV) através do rt-PCR. Foi aplicado questionário para coleta de informações clínicas e laboratoriais. A fim de avaliar a resposta imune celular específica ao ZIKV, células mononucleares do sangue periférico (PBMC) foram obtidas de alguns pacientes no momento da inclusão no estudo (fase 1, P1) e após 15 dias (fase 2, P2). A partir do PBMC, foi avaliada a produção de interferon- gama em resposta a peptídeos recobrindo as proteínas do ZIKV, DENV e CHIKV por ELISPOT. A avaliação da assinatura imunológica dos linfócitos T foi realizada por citometria de massa (Cytof), utilizando um painel de 40 anticorpos. **Resultados**: Foram incluídos 78 pacientes no estudo clínico, sendo 66,7% do sexo feminino e média de idade de 38 anos. Os achados clínicos mais frequentes foram mialgia, artralgia e febre baixa. A análise laboratorial demonstrou níveis normais de hematócrito, plaquetas e enzimas hepáticas. Foi proposto um algoritmo para diagnóstico clínico de infecção pelo ZIKV. No estudo da resposta imune celular foram incluídos 29 indivíduos: 11 infectados por ZIKV, 11 por CHIKV, e 7 doadores de sangue como controles. Três dos 11 pacientes com ZIKV (27,3%) tiveram uma resposta de células T detectável aos peptídeos do ZIKV (C, NS2A, NS4A e NS5). A magnitude média das respostas anti-ZIKV foi de 89 SFC / 10^6 PBMC [IQR: 79-156]. NS5 foi a proteína imunodominante que foi reconhecida na maioria dos respondendores (5/9, 55,6%). Para análise de linfócitos T CD4 + a proporção de células que expressaram IFN-gama foi significativamente maior em todas as subpopulações de TCM, memória efetora (TEM) (CD45RA-CD27 + CCR7-) e memória de transição (TTM) (CD45RA-CD27 + CCR7-) de pacientes na fase P1 em comparação a fase P2 e controles saudáveis (p <0,005). Para o perfil de linfócitos T CD8 +, expansão das subpopulações TN, TCM, TEM, TEMRA e menor proporção da subpopulação TTM foram observados nos pacientes na fase P1 em comparação a controles saudáveis. Os pacientes na fase P2 exibiram uma proporção menor das subpopulações TEM e TEMRA em comparação com pacientes na fase P1. **Conclusão**. Os pacientes com ZIKV apresentarm manifestações clínicas leves e inespecíficas, com uma resposta imune celular de baixa intensidade e dirigida predominantemente para antígenos não-estruturais.

Palavras-chave: Zika. Manifestações clínicas. Resposta imune celular. Elispot. Citometria de massa.

BANDEIRA, Antonio Carlos de Albuquerque. **Clinical, laboratory and cellular immunity features of patients with acute zika virus (zikv) infection**. 2021. 86 f. Thesis (Doctorate in Biotechnology in Health and Investigative Medicine) - Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2021.

ABSTRACT

Introduction: The clinical and laboratory manifestations of Zika virus (ZIKV) infection were unknown when the disease was identified in Brazil in 2015. Clinical reports were based on small case series and diagnosis was based on serological techniques that could confound with Dengue virus (DENV) infection. In addition, it was necessary to characterize the cellular immune response in patients with acute ZIKV conditions, which was not much studied until the beginning of the present study. **Objective**: To describe the clinical manifestations, laboratory findings and cellular immune response in patients with acute ZIKV infection. **Methodology**: A descriptive, cross-sectional clinical study was carried out with selected patients in an emergency room in the city of Salvador. Patients with symptoms suggestive of acute viral infection between May 27, 2015 and August 31, 2017 were sequentially evaluated. The diagnosis of ZIKV was based on rt-PCR positivity in blood or urine or saliva. Patients were also evaluated for Dengue or Chikungunya infection (CHIKV) using rt-PCR. A questionnaire was applied to collect clinical and laboratory information. In order to assess the specific cellular immune response to ZIKV, peripheral blood mononuclear cells (PBMC) were obtained from some patients at the time of study enrollment (phase/stage 1, P1) and after 15 days (phase/stage 2, P2). From the PBMC, interferon-gamma production was evaluated in response to peptides covering the proteins of ZIKV, DENV and CHIKV by ELISPOT. The evaluation of the immunological signature of T lymphocytes was performed by mass cytometry (Cytof), using a panel of 40 antibodies. **Results:** Seventy-eight patients were included in the clinical study, 66.7% female and mean age 38 years. The most frequent clinical findings were myalgia, arthralgia and low-grade fever. The laboratory analysis showed normal levels of hematocrit, platelets and liver enzymes. A clinical algorithm has been proposed for the clinical diagnosis of ZIKV infection. A smaller sample of 29 individuals was selected for studying the cellular immune response, which included: 11 infected by ZIKV, 11 by CHIKV, and 7 blood donors as controls. Three of 11 patients with ZIKV (27.3%) had a detectable T-cell response to ZIKV peptides (C, NS2A, NS4A and NS5). The mean magnitude of anti-ZIKV responses was 89 SFC / 106 PBMC [IQR: 79-156]. NS5 was the immunodominant protein that was recognized in most responders (5/9, 55.6%). For the analysis of CD4+ T lymphocytes, the proportion of cells that expressed IFN-gamma was significantly higher in all TCM subpopulations, effector memory (TEM) (CD45RA-CD27 + CCR7-) and transition memory (TTM) (CD45RA-CD27 + CCR7-) of patients in phase P1 compared to phase P2 and healthy controls ($p<0.005$). For the CD8 + T lymphocyte profile, expansion of the TN, TCM, TEM, TEMRA subpopulations and a lower proportion of the TTM subpopulation were observed in patients in stage P1 compared to healthy controls. Stage P2 patients exhibited a lower proportion of TEM and TEMRA subpopulations compared to patients in stage P1. **Conclusion.** Patients with ZIKV have mild and nonspecific clinical manifestations, with a low-intensity cellular immune response predominantly directed towards non-structural antigens.

Keywords: Zika. Clinical manifestations. Cellular immune response. Elispot. Mass cytometry.

SUMÁRIO

1 **INTRODUÇÃO**

Em dezembro de 2014 vinha atendendo inúmeros pacientes que estavam chegando ao ambulatório de doenças infecciosas não-HIV do hospital Couto Maia, secretaria de saúde do estado da Bahia, com quadro clinico de uma doença recentemente introduzida no estado da Bahia – febre Chikungunya. Eram pacientes provenientes de Feira de Santana, com quadros de artropatia que se arrastava já por alguns meses, e esses pacientes vinham referenciados para obtenção de tratamentos. Nessa mesma época eu atendia também no Hospital Santa Helena, em Camaçari, e no hospital Aliança em Salvador e comecei a perceber também a chegada de pacientes com quadro clínico compatível com dengue, de forma branda, porém com os marcadores sorológicos negativos tanto para Dengue quanto para Chikungunya. Esses pacientes apresentavam-se com rash cutâneo, febre variável, dor de cabeça e mialgias, além de transitória dor articular em alguns pacientes. A partir de janeiro de 2015 o número de casos de pacientes com esse padrão clínico começou a aumentar, e começaram a surgir indagações sobre a possibilidade de um surto de parvovirose B19 em várias localidades do nordeste. Eu estava bastante acostumado em atender pacientes com Dengue, pois em vários surtos da doença no estado da Bahia eu estava atendendo esses pacientes no Pronto-atendimento do Hospital Geral Roberto Santos e do Hospital Aliança. O quadro clínico, apesar de semelhante, tinha inúmeras diferenças em relação aos pacientes que se apresentavam naquele momento. Uma das diferenças importantes era a ausência de sangramentos nesses pacientes. Outras diferenças incluíam a presença do rash estendendo-se para a palma das mãos, o prurido associado, e também a quase ausência de alterações laboratoriais tais como aumento das enzimas hepáticas, plaquetopenia, ou hemoconcentração. Também era diferente o quadro clínico desses pacientes em relação a Febre Chikungunya: praticamente não havia comprometimento articular, e quando ocorria era leve e transitório, sem deixar sequelas articulares subsequentes. Além disso, os testes confirmatórios tanto para Dengue quanto para Chikungunya eram sempre negativos, assim como a sorologia para sífilis e o VDRL, testes que inicialmente solicitei para os pacientes pela presença de rash palmar. Em alguns desses pacientes foram solicitadas sorologias para Parvovirus B19 com resultados negativos, o que me fazia crer que estávamos diante de uma outra doença, até então desconhecida. Os casos foram crescendo tanto nas cidades de Camaçari quanto de Salvador e região metropolitana, assim como em diversos estados do Nordeste, e começam a circular notas de secretarias de saúde ora afirmando tratar-se de um surto de Parvovirus B19 ora de um surto de Dengue de sorotipo 4. Não me convenci em relação a essas explicações, e entre dezembro de 2014 e janeiro de 2015 propus ao Dr. Gubio

Campos, virologista no Instituto de Ciências da Saúde da Universidade Federal da Bahia, a buscarmos uma outra entidade nosológica para esses pacientes. Eu tinha certeza de que se tratava de outro vírus ainda não identificado. Coloquei para ele que deveríamos iniciar uma investigação para determinar a causa dessa doença, que me parecia uma provável arbovirose pela forma como ela estava se espalhando, pelas manifestações clínicas em parte semelhantes a Dengue, mas que deveríamos ampliar o espectro da busca de agentes se não encontrássemos respaldo em processos virais transmitidos por arbovirus. Assim, em 26 de março de 2015 25 pacientes atendidos na emergência do Hospital Santa Helena, em Camaçari, com quadro febril agudo compatível com processo viral, alguns com rash cutâneo, foram selecionados para participarem dessa investigação, posteriormente publicada no *Emerging Infectious Diseases*, e as amostras de sangue foram levadas para o laboratório de virologia do ICS para realização de procedimentos de biologia molecular que incluíram Dengue, Chikungunya, Mayaro, Oropouche, e por fim Zika virus.

Em março de 2015 os jornais do estado da Bahia já vinham expondo que uma doença misteriosa estava ocorrendo em Camaçari, Bahia. Em 25 de março de 2015 o R7 (canal da Record) afirma em seu site que "...Os pacientes apresentam pintas na pele e reclamam de coceiras e dores pelo corpo... Já foi descartada a possibilidade de se tratar de dengue, febre chikungunya, rubéola ou sarampo. Embora não tenha sido identificada, a doença não causa nenhum outro problema de saúde".

O professor Gubio Campos foi descartando inúmeras doenças através dos resultados de rt-PCR, porém quando as amostras foram amplificadas para Zika virus foram encontrados 8 resultados positivos dentre as 25 amostras. Em 28 de abril de 2015 parte desses resultados para Zika foram descobertos e Dr. Gubio Campos me enviou uma mensagem através do Whattsup para me informar dos resultados – "Analisei 06 amostras para Zika virus...A amostra (01) deu positiva para Zika vírus e os sintomas da literatura coincidem com os daqui. Vou a partir de amanhã analisar mais amostras e tenho certeza que o surto é Zika virus". Ali estava definida a causa da doença misteriosa que vinha desde o final de 2014 até o primeiro trimestre de 2015 sem explicação. No dia 29 de abril o professor Gubio Campos e Silvia Sardi anunciaram ao portal G1 a descoberta do vírus Zika. Nessa reportagem afirmava-se "A identificação do vírus foi realizada nesta semana, após a dupla de pesquisadores trabalhar por cerca de 20 dias em amostras de sangue de pacientes de Camaçari, cidade da região metropolitana de Salvador, por meio de uma técnica chamada RT- PCR, que amplifica o material genetico do virus, através de reagentes, aumentando o sinal deste material genético". E segue a reportagem afirmando que "A descoberta de Gúbio e Silvia derruba as duas hipóteses levantadas pela Vigilância

Epidemiológica e a Secretaria de Saúde de Camaçari, para explicar a doença. No último mês de março, os dois órgãos suspeitavam que os sintomas seriam causados por roséola ou parvovirus-B19".

No dia 01 de maio de 2015, assim que retornei de Copenhagem, fui à imprensa e complementei o anúncio da descoberta do vírus Zika. Nesse dia, em entrevista ao canal G1, a reportagem afirmava "...Com a identificação na Bahia de um novo vírus, o Zika, e o aumento de casos da doença, o infectologista Antônio Bandeira explica as diferenças com os sintomas da dengue. Suspeitos da doença foram identificados em 20 cidades baianas. Ela tem o mesmo mosquito transmissor da dengue, o aedes aegypti, mas o contágio pode ser feito por outros, a exemplo do aedes albopictus. Febre, diarreia, dores e manchas no corpo são sintomas. É o primeiro registro do vírus no país".

Tínhamos chegado à causa e anunciado imediatamente a descoberta. Passamos em seguida todas as informações que tínhamos, inclusive as alíquotas das amostras, para o Ministério da Saúde e para a Secretaria de Saúde do Estado da Bahia. Fizemos a notificação dos casos no sistema de vigilância epidemiológica, e durante a semana seguinte aguardando que o MS referendasse nossos achados, o que ocorreu no dia 14 de maio de 2015, quando o MS solicitou também que amostras do Rio Grande do Norte fossem testadas juntamente com as nossas.

Ao termos buscado as informações clínicas e laboratoriais desses 25 pacientes foi percebido que a etiqueta de um dos pacientes foi trocada com a de um paciente internado, e não conseguimos saber de que paciente atendido na emergência aquela amostra pertencia. Por isso, ao termos preparado a carta ("letter") ao *Emerging Infectious Diseases* (EID), tivemos que retirar esse caso (que fora positivo) e enviamos nossa amostra com 24 pacientes. Isso explica também porque o Ministério da Saúde anunciou em 14 de maio de 2015 as 8 amostras da Bahia, e no trabalho do EID a amostra fora somente de 7 positivos. Em seguida o EID nos solicitou que testássemos novamente todas as amostras para Dengue, e refizemos toda análise que mostrou somente sorologia para IgG em alguns pacientes porém sem nenhum caso de rt-PCR positivo para Dengue. Re-enviamos novamente para o EID o material com os testes realizados e o artigo foi aprovado. No entanto desde que fora confirmado o diagnóstico de Zika nesses pacientes busquei apoio nas publicações existentes sobre o quadro clínico da doença, sua patogênese, os principais marcadores laboratoriais de infecção, os testes diagnósticos existentes, assim como o tratamento e complicações dessa nova doença. E percebi a paucidade de publicações existentes sobre o vírus Zika até aquele momento. Havia muito poucos trabalhos clínicos, na verdade séries pequenas de casos até o surto nas Ilhas Yap, as manifestações clínicas descritas nos

artigos não necessariamente se enquadrando no que estávamos observando, e não havia nenhuma descrição até aquele momento sobre os marcadores laboratoriais dessa doença. Era necessário conhecer agora o espectro clínico da doença assim como seus principais marcadores laboratoriais. E assim surgiu a idéia de conhecer as características clínicas e laboratoriais da infecção pelo vírus Zika em um processo mais acadêmico que culminasse em uma Tese de Doutorado.

2 REVISÃO DE LITERATURA

O vírus Zika (ZIKV) surgiu recentemente como um novo patógeno no Brasil e na região das Américas, transmitido por mosquitos do gênero Aedes, e na sequência de uma grande expansão na região do Pacífico. (DUFF *et al.,* 2009; CAO-LORMEAU *et al.,* 2013). Essa expansão no Pacífico começa a ocorrer de forma mais intensa a partir de 2007, e estudando essa expansão na Micronésia, Duffy *et al.(*2009) revisaram prontuários médicos e conduziram estudo de vigilância prospectiva em um hospital e nos quatro centros de saúde em Yap para identificar pacientes com suspeita de doença pelo vírus Zika durante o período de 1º de abril a 31 de julho de 2007(DUFF *et al*., 2009). Basearam-se em evidências empíricas de pacientes atendidos para definirem paciente com suspeita de doença como tendo um início agudo de exantema macular generalizado ou erupção papular, artrite ou artralgia ou conjuntivite não purulenta. Pacientes com suspeita de doença foram solicitados a fornecer amostras de sangue durante a fase aguda (ou seja, dentro de 10 dias após o início dos sintomas) e durante a fase de convalescença (ou seja, 14 dias depois). Foi selecionada uma amostra de conveniência desses pacientes com o uso de um questionário padrão para se coletar informações sobre características demográficas, sinais e sintomas clínicos e a duração e gravidade da doença. Em somente 15 pacientes houve o diagnóstico de infecção aguda através de amplificação molecular (rt-PCR). Segundo Roth *et al*. (2014) a linhagem asiática do ZIKV reapareceu na Polinésia Francesa em outubro de 2013 e, desde então, causou grandes surtos na Nova Caledônia (1.400 casos confirmados), Ilhas Cook (mais de 900 casos) e Ilha de Páscoa[3]. Na Polinésia Francesa, a extrapolação dos 8.746 casos suspeitos notificados pela rede de vigilância sentinela permitiu inferir que mais de 30.000 consultas médicas ocorreram devido à propagação do vírus Zika em todo o arquipélago. Entre novembro de 2013 e fevereiro de 2014, o aumento da incidência de complicações neurológicas, incluindo 42 casos da síndrome de Guillain-Barré, foi uma característica única e preocupante do surto da Polinésia Francesa. Nessas mesmas áreas geográficas já circulavam de forma endêmica os vírus da Dengue (DENV) e da Chikungunya (CHIKV), com isso representando uma carga significativa para os serviços de saúde, e que incluem desafios clínicos significativos, em particular a síndrome de Guillain-Barré, meningoencefalite, manifestações autoimunes e, mais recentemente, anomalias congênitas. (ROTH *et al*., 2014; MUSSO *et al*., 2015; MUSSO *et al*., 2014; EUROPEAN, 2014; OEHLER *et al*., 2014; DIRECTION DE LA SANTÉ, 2014; OEHLER *et al*., 2015; OLIVEIRA *et al*., 2015).

Musso *et al.* (2015) reportam 1 caso de síndrome de Guillain-Barré (GBS) associada a

infecção pelo ZIKV em novembro de 2013 na Polinésia Francesa uma semana após infecção aguda por ZIKV confirmada. (MUSSO *et al.*, 2014). Inúmeros casos subsequentes de GBS foram identificados, correlacionando-se temporariamente com o surto de ZIKV, com estimativa da taxa de incidência de casos de GBS durante o surto de ZIKV de aproximadamente 20 vezes maior do que o esperado, dado o tamanho da população na Polinésia Francesa e as taxas de incidência estabelecidas de GBS anteriores (1-2 / 100.000 habitantes por ano). (MUSSO *et al*., 2014).

Desde o primeiro isolamento do ZIKV nas Américas em 26 de março de 2015, estima-se que 1,3 milhão de indivíduos foram infectados no nordeste do Brasil, com uma taxa de ataque estimada de 5,5 casos por 1000. (CAMPOS *et al*., 2015; CARDOSO *et al*. 2015).

Campos et al. (2015) descreveram 24 pacientes com quadro exantemático na cidade de Camaçari, com 7 pacientes tendo infecção aguda por ZIKV e 3 com infecção aguda pelo vírus Chikungunya (CHIKV), ambos através da técnica de rt- PCR no sangue. Nesse estudo pioneiro que identifica pela primeira vez a circulação do ZIKV na América Continental, os principais sintomas foram exantema cutâneo (“rash”), mialgia e febre em 85,7%, 57,1% e 43%, respectivamente (CAMPOS *et al*., 2015). A amostra foi selecionada em uma unidade de pronto- atendimento de um hospital privado em Camaçari (Hospital Santa Helena) com os pacientes mais sintomáticos naquele momento, e somente amostras de sangue foram utilizadas para a reação de cadeia de polimerase (rt-PCR).

Pela primeira vez houve uma apresentação dos principais achados laboratoriais gerais nesses pacientes que até então não tinha ocorrido, nem no surto das Ilhas Yap, nem anteriormente, não sendo descritas alterações significativas nos parâmetros hematológicos ou bioquímicos nos pacientes com ZIKV (CAMPOS *et al.*, 2015).

No período de descoberta do ZIKV uma questão que estava pouco clara era quando o vírus teria sido introduzido na Bahia. Pelo menos uma primeira resposta pôde ser apontada no estudo de Naccache et al com nossas amostras de pacientes com quadro agudo de ZIKV e rt-PCR-+ (NACCACHE *et al*., 2016). Nesse estudo, com 15 pacientes, foram sequenciados genomas do ZIKV em onze, e todos eles agruparam-se em torno de um Clade (NACCACHE *et al*., 2016). Essas linhagens do ZIKV passaram a compor o novo Clade C (NACCACHE *et al*., 2016). As análises filogenéticas desse novo Clade apontaram que o ZIKV possivelmente tenha sido introduzido na Bahia entre março-setembro de 2014 (NACCACHE *et al*., 2016). Essa estimativa é corroborada por estudos mais recentes em amostras coletadas em outubro de 2014 em Salvador, mostrando soropositividade ao ZIKV por ensaios sensíveis de IgG3 para NS1 em somente 7% da população amostrada, sugerindo baixa transmissão em período anterior a essa

data (RODRIGUEZ-BARRAQUER *et al*. 2019). Isso aponta para a entrada do ZIKV em Salvador em período não superior a 6 meses ao início do crescimento no número de casos a partir de janeiro de 2015 e que culminaria com o pico de casos em Salvador entre maio-junho de 2015. Nesse período Salvador experimentava uma dupla epidemia – pelos virus Zika e Chikungunya – inclusive com a documentação da ocorrência de pacientes coinfectados por esses virus nesse mesmo período, sem evidências de maiores complicações clínicas no entanto (SARDI *et al*., 2016). A ocorrência de coinfecção – Zika e Chikungunya – não tinha sido relatada na literatura até então, e as manifestações clínicas nesses pacientes não eram conhecidas. Nesse estudo um dos pacientes teve predomínio de manifestações articulares com quadro semelhante ao de CHIKV e o outro paciente apresentou conjuntivite, mialgias sem manifestações articulares, com apresentação mais próxima do esperado em ZIKV (SARDI *et al*., 2016).

A infecção pelo ZIKV normalmente causa uma infecção autolimitada semelhante à da Dengue, doença caracterizada por erupção cutânea, febre baixa, conjuntivite e arthralgia. (EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL, 2014). Muitos relatos sobre ZIKV na literatura descrevem manifestações clínicas com base em evidências empíricas, uma vez que o diagnóstico depende da detecção de vírus usando a reação de cadeia da polimerase com a transcriptase reversa (PCR) ou no teste de neutralização por redução de placa, ambas as quais normalmente não estão disponíveis na maioria dos laboratórios clínicos nas áreas afetadas. (DUFFY *et al.*, 2009). Mesmo em investigações conduzidas durante surtos, a maioria dos pacientes testou positivo apenas nos primeiros três dias de doença, com sensibilidade decrescente do PCR observada após este período inicial. (LANCIOTTI *et al,* 2008). Havia uma escassez de estudos avaliando o comportamento clínico e laboratorial da infecção aguda pelo ZIKV. A maioria dos estudos conta com número reduzido de pacientes com confirmação molecular, e algumas das principais manifestações clínicas têm sido imputadas a partir de estudos retrospectivos. Apesar do estudo de Sardi *et al.* (2016), muito pouco tem sido descrito em relação a coinfecções com o DENV ou CHKV. Uma compreensão abrangente dos parâmetros clínicos e laboratoriais específicos para cada uma dessas doenças tem sido necessária. Em março de 2015, o município de Salvador testemunhou um número crescente de pacientes adultos com sintomas semelhantes aos da Dengue em busca de atendimento médico em serviços de emergência de toda a cidade, e inúmeras hipóteses foram levantadas para explicar esse aumento súbito – infecção pelo virus da Dengue tipo 4, infecção pelo parvovirus B19 e assim por diante. (CARDOSO *et al.,* 2015). No entanto após a identificação e a divulgação da circulação do ZIKV no final de abril de 2015 esse grande aumento pôde ser esclarecido como

uma combinação de 2 surtos superpostos – por ZIKV e por CHIKV. (CARDOSO *et al.*, 2015).

Apesar da infecção pelo ZIKV vir sendo bastante desconhecida, quase não estudada até 2007, trabalhos anteriores à publicação do surto nas Ilhas Yap eram muito pobres em descrições clínicas e laboratorias, com menos de 12 trabalhos sobre ZIKV até 2007. Exemplo disso é o trabalho de Olson e Ksiazec (1981) na Indonésia em 1977-1978, publicado em 1981, partindo de 30 pacientes com quadros febris, em Klaten, Indonésia. (OLSON; KSIAZEC, 1981).

Os pacientes estavam em investigação para infecção por *Leptospira sp*, sendo triados para diversos flavivirus e alphavirus por reação de inibição de hemaglutinação. Aqueles positivos foram testados por pareamento sorológico com técnica de micro-neutralização com 7 pacientes tendo apresentado aumento de mais de 4 vezes nos títulos sorológicos para ZIKV. (OLSON; KSIAZEC, 1981). Os sintomas clínicos principais reportados eram febre alta, mal-estar, dor na região do estômago, tontura e fraqueza. Em nenhum desses pacientes foi descrito rash cutâneo, conjuntivite ou cefaléia. Entre os 7 pacientes com ZIKV todos apresentavam febre alta (OLSON; KSIAZEC, 1981), diferindo bastante dos estudos subsequentes com maior número de pacientes confirmados para infecção pelo ZIKV. Esse estudo tinha sido o maior em número de pacientes com ZIKV, com uma melhor documentação laboratorial até o surto de 2007 nas Ilhas de Yap. Nesse último estudo nas ilhas de Yap (DUFFY *et al*., 2009) os pacientes foram entrevistados por amostragem de conveniência e foi definido como caso confirmado uma de 2 condições: rt-PCR positivo ou combinação de IgM Zika + PRNT90 $\geq$ 20 + PRNTzika/PRNTdengue $\geq$ 4, sendo no entanto descritos sintomas relacionados a ZIKV em 49 pacientes, sendo que somente 15 pacientes tiveram o diagnóstico por confirmação molecular. (DUFFY *et al*., 2009). Nessa série de casos a presença de rash cutâneo, febre e artrite/artralgia foi reportada em 90%, 65% e 65% dos casos, respectivamente. Com o dobro do número de casos frente ao estudo de Olson e Ksiazec (1981), além de uma metodologia com maior especificidade para o diagnóstico de ZIKV, a apresentação clínica da infecção pelo ZIKV parecia apontar agora para comprometimento de pele, conjuntivite e artralgias (DUFFY *et al*., 2009). O surto de ZIKV em 2007 nas ilhas de Yap representa a divisão temporal entre um período anterior em que a infecção pelo ZIKV era vista como algo ocasional e restrito a descrição de poucos casos de uma zoonose rara, para um período em que a infecção pelo ZIKV passa a ser vista como um importante e crescente problema de Saúde Pública no mundo. Será, no entanto a partir de 2013, com a descrição do aumento de casos da síndrome de Guillain-Barré na Polinésia Francesa após o surto de ZIKV (CAO-LORMEAU *et al*., 2016) e, principalmente após 2015, com a descrição dos primeiros casos de microcefalia em bebês nascidos de mães infectadas pelo ZIKV (OLIVEIRA *et al*., 2016) que a infecção pelo ZIKV será declarada uma emergência

internacional em Saúde Pública e passará a integrar a lista de doenças a serem priorizadas e monitoradas no mundo.

Ficava, no entanto, a necessidade de melhor detalhamento do quadro clínico e laboratorial de pacientes com infecção confirmada por ZIKV, no intuito de agregar diversas informações previamente apontadas e responder à necessidade de diagnóstico dessa nova entidade. Qual seria o espectro de sinais e sintomas da infecção pelo ZIKV? Haveria outros sinais e sintomas não ainda bem esclarecidos? Sintomas inespecíficos tais como náuseas ou outros seriam frequentes como apontado pela pequena série de casos de Olson e Ksiazec (1981)?. Qual seria o comportamento dos exames laboratoriais inespecíficos na infecção pelo ZIKV? Seriam semelhantes aos do DENV? Quais tipos de alterações laboratoriais poderiam diferenciar ZIKV de outras arboviroses? Outra questão que se colocava era o comportamento da resposta imune ao ZIKV. Não havia em nenhum estudo prévio à descrição dos casos de ZIKV no Brasil por Campos et al a descrição do comportamento da resposta imune ao ZIKV, principalmente no que referia a resposta imune celular.

Posteriormente, Hamel et col, demonstraram que queratinócitos, células dendríticas e fibroblastos eram permissivas à infecção pelo ZIKV, provavelmente devido a interação com o receptor de fosfatidil-serina AXL. (HAMEL *et al*., 2018). Além disso, os autores passaram a relatar que interferons dos tipos I e II são capazes de impedir a infecção das células por esses virus. (INGONO; SHRESTA, 2018).

A resposta imune celular a ZIKV pode ser influenciada por exposições prévias a outros flavivirus. Reynolds et al mapearam epítopos de células CD4+ do envelope do ZIKV e de antígenos não-estruturais imunodominantes de HLA classe II em camundongos transgênicos HLA classe II. Em vários casos, células CD4 expostas ao ZIKV responderam a sequências homólogas de outros vírus, incluindo DENV1–4, WNV ou YFV (REYNOLDS *et al.*, 2018). Outro estudo mostrou melhora da resposta imune a ZIKV após infecção sequencial por DENV e ZIKV. (DELGADO *et al*., 2018). Nesse estudo foram analisadas as respostas de linfócitos T e B em relação ao ZIKV em doadores com ou sem infecção prévia por DENV. Usando células mononucleares (PBMCs) do sangue periférico de doadores que vivem em uma área endêmica na Colômbia, os autores identificaram por técnica de ELISPOT a maioria dos epítopos imunodominantes das células T do ZIKV nas proteínas não-estruturais (NS) NS1, NS3 e NS5. Também nesse estudo as análises de células T e B nos mesmos doadores revelaram uma resposta de células T mais intensa contra os peptídeos conservados entre o DENV e ZIKV, com um nível mais alto de anticorpos neutralizantes de ZIKV em doadores previamente imunes a DENV em comparação a doadores que nunca foram expostos ao DENV

(naîves).

Um outro dado importante foi que o efeito ADE (antibody mediated enhancement) foi menor naqueles com infecção sequencial por DENV e ZIKV quando comparado somente àqueles com DENV somente. Assim, esses dados sugerem que os indivíduos com infecção prévia por DENV apresentam respostas imunológicas melhoradas contra o ZIKV. (DELGADO *et al.,* 2018).

Em outro estudo Grifoni et al mostraram que as células T que foram previamente induzidas por infecção anterior com DENV, ou vacinação com a vacina tetravalente para DENV, reconhecem peptídeos do ZIKV, e que esta reatividade cruzada é explicada pela similaridade de sequência genética dos dois virus. (GRIFONI *et al.,* 2017). Esses peptídeos de ZIKV reconhecidos por células T de memória são idênticos ou altamente conservados tanto no DENV quanto no ZIKV. A exposição prévia ao DENV antes da infecção pelo ZIKV também influencia o momento e a magnitude da resposta das células T. Células T reativas ao ZIKV na fase aguda da infecção são detectadas mais cedo e em maior magnitude em pacientes previamente expostos ao DENV. Por outro lado, a frequência de células T reativas ao ZIKV continua a aumentar na fase de convalescença em doadores de células nunca expostos previamente ao DENV, porém cai em doadores pré-expostos ao DENV, sugerindo controle mais eficiente da replicação do ZIKV. A qualidade das respostas também é influenciada pela exposição anterior ao DENV, e células T CD8+ específicas para ZIKV de doadores já expostos previamente ao DENV mostram aumento da produção de Granzima B e de PD1, ao contrário de doadores nunca previamente expostos ao DENV. Nesse estudo, também foi apontado que as proteínas estruturais (E, prM e C) do ZIKV são os principais alvos das respostas de células T CD4+ e CD8+. (GRIFONI *et al.*, 2017).

El Sahly *et al*. (2019) em estudo envolvendo 45 pacientes estadunidenses com infecção confirmada por ZIKV encontrou resposta T CD4+ mais robusta em peptídeos de proteínas não estruturais (NS) 1, NS3 e NS5, enquanto as células T CD8+ responderam de forma mais intensa às proteínas do capsídeo e do envelope. Células T CD4 + antígeno-específicas produzindo IFN-γ, IL-2 e / ou TNF-α foram observadas contra todas as 10 proteínas do ZIKV. Proteínas estruturais do envelope (E) e do nucleocapsídeo (C) tiveram as maiores proporções de respondentes positivos (89%) enquanto apenas 2 dos 27 casos de ZIKV testados (7%) tinham células T CD4 + que responderam aos peptídeos NS2A. Também, a intensidade das respostas de células T CD4 + foram mais altas para as proteínas não-estruturais NS1, NS5 e NS3. (EL SAHLY et al., 2019) Em relação à resposta de células T CD8+ a produção de IFN-γ, IL-2 e / ou TNF-α também foram observadas para todas as 10

proteínas do ZIKV, mas, com NS3, NS5 e NS4B tendo as maiores proporções de respondentes positivos (89%, 82% e 100%, respectivamente). (EL SAHLY *et al.,* 2019).

Resultados semelhantes foram descritos em um relato de caso. (RICCIARDI et al., 2017). Usando técnicas de ELISPOT, as respostas de células T CD4 + específicas do ZIKV foram detectadas contra a proteína ZIKV-NS2A, e respostas de células T CD8 + foram detectados contra a proteína do envelope (E). (RICCIARDI *et al*., 2017).

No entanto, outros estudos com pequeno número de pacientes relataram respostas mais modestas com menos de <1% de células T CD4+ ou CD8+ produtoras de citocinas em resposta aos peptídeos virais. (LAI *et al*., 2018). No entanto forte ativação de células T CD8 + (15% a mais de 25% células T CD8 +) ocorreu precocemente e persistiu em paralelo com a presença do RNA viral, assim como ativação moderada de células T CD4 + (2% –5%). (LAI *et al.*, 2018).

Assim, faz-se necessário uma melhor caracterização da resposta imune celular para determinar a capacidade polifuncional dessas células, e uma possível assinatura fenotípica das subpopulações de células T CD4+ e CD8+.

3 OBJETIVOS

3.1 GERAL

Descrever os aspectos clínicos, laboratoriais e imunológicos de pacientes infectados pelo ZIKV.

3.2 ESPECÍFICOS

1. Caracterizar os principais sinais e sintomas clínicos e laboratoriais da infecção aguda pelo ZIKV;
2. Quantificar a proporção de linfócitos T específicos aos peptídeos de ZIKV, DENV e CHIKV utilizando ELISPOT;
3. Caracterizar o fenótipo, a função, a ativação e a produção de citocinas de linfócitos T CD4 e T CD8 pela avaliação multiparamétrica utilizando citometria de massa.

4 MATERIAIS E MÉTODOS

4.1 ARTIGO 1 - ZIKA VIRUS OUTBREAK, BAHIA, BRAZIL. Emerg Infect Dis. 2015; 21(10): 1885-1886.

A descoberta do ZIKV no Brasil possibilitou responder parte do primeiro objetivo dessa Tese.

Em 26 de março de 2015, amostras de soro foram obtidas de 24 pacientes do Hospital Santa Helena de Camaçari que receberam diagnóstico presuntivo de doença viral aguda exantemática por médicos do pronto-atendimento. Amostras de soro foram analisadas na Universidade Federal da Bahia com realização de RT-PCR para DENV, CHIKV, West Nile virus (WNV), vírus Mayaro e ZIKV. Para ZIKV foram utilizados primers para a região parcial do gene do envelope nas posições 1538- 1558 e 1902-1883, sequenciados os produtos e depositados no GenBank sob os números de acesso. KR816333 – KR816336. Todos os pacientes foram negativos para RT-PCR para DENV, Vírus Mayaro e WNV. Amostras de 7 (29,2%) pacientes foram positivas para ZIKV (fragmento de 369 pb) e de 3 (12,5%) pacientes para CHIKV (fragmento de 305 pb). Não houve detecção simultânea de ZIKV e CHIKV. A maioria (85,7%) dos pacientes positivos para ZIKV eram mulheres; idade mediana de 28 anos e nenhuma história de viagem internacional. Os pacientes positivos para ZIKV procuraram atendimento médico após uma média de 4 dias (variação de 1 a 5 dias) de história de erupção cutânea (85,7%), mialgias (57,1%), ou febre (43,0%).

Três pacientes tinham IgG positiva para DENV. A média da leucometria total foi de 3.750 células / mm^3 (intervalo de 2.790 células / mm^3 a 6.150 células / mm^3); a média da contagem de plaquetas foi de 180.000 / mm^3 (intervalo de 151.000–274.000 plaquetas / mm^3). A média da proteína C reativa foi de 16,3 mg / L (intervalo de 0,9 mg / L - 19,7 mg / L).

As sequências obtidas pertenciam à linhagem asiática e mostraram 99% de identidade com uma sequência de um isolado de ZIKV da Polinésia Francesa (KJ776791).

database used would require optimization with addition of reference spectra for the organism and its close relatives (e.g., *B. thailandensis*). *B. pseudomallei*, although different from other *Burkholderia* spp. in its pathogenicity and epidemiology, is not easily discriminated from *B. thailandensis* or *B. cepacia* complex by using phenotypic tests (*10*).

In summary, infection with *B. pseudomallei* should be considered in patients with pneumonia after travel to the Baja Peninsula in Mexico, and especially after an extreme weather event. Because of risk for transmission to laboratory workers and the potential for *B. pseudomallei* to be used for bioterrorism, clinical laboratories should perform only limited work up of suspected isolates before referring them to a public health laboratory for definitive identification.

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Zika Virus Outbreak, Bahia, Brazil

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To the Editor: Zika virus (ZIKV) is a mosquito-borne flavivirus related to yellow fever virus, dengue virus (DENV), and West Nile virus (WNV). It is a single-stranded positive RNA virus (10,794-nt genome) that is closely related to the Spondweni virus and is transmitted by many *Aedes* spp. mosquitoes, including *Ae. africanus*, *Ae. luteocephalus*, *Ae. hensilli*, and *Ae. aegypti*. The virus was identified in rhesus monkeys during sylvatic yellow fever surveillance in the Zika Forest in Uganda in 1947 and was reported in humans in 1952 (*1*).

In 2007, an outbreak of ZIKV was reported in Yap Island, Federated States of Micronesia (*2*). ZIKV also caused a major epidemic in the French Polynesia in 2013–2014 (*3*), and New Caledonia reported imported cases from French Polynesia in 2013 and reported an outbreak in 2014 (*4*).

A new challenge has arisen in Brazil with the emergence of ZIKV and co-circulation with others arboviruses (i.e., DENV and chikungunya virus [CHIKV]). We report ZIKV infection in Brazil associated with a recent ongoing outbreak in Camaçari, Bahia, Brazil, of an illness characterized by maculopapular rash, fever, myalgias/arthralgia, and conjunctivitis.

On March 26, 2015, serum samples were obtained from 24 patients (Table) at Santa Helena Hospital in Camaçari who were given a presumptive diagnosis of an acute viral illness by emergency department physicians. These patients were given treatment for a dengue-like illness, and blood samples were obtained for complete blood counts and serologic testing by using an ELISA specific for IgG and IgM against DENV.

Serum samples were analyzed at the Federal University of Bahia by reverse transcription PCR (RT-PCR) to detect DENV, CHIKV, WNV, Mayaro virus, and ZIKV. In brief, serum samples were subjected to RNA extraction by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse transcribed by using the SuperScript II Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) and subjected to PCRs specific for DENV (*5*) CHIKV (*6*), WNV (*7*) and Mayaro virus (*8*). A positive RT-PCR for a partial region of the envelope gene with primers ZIKVENF and ZIKVENVR (positions

Table. Characteristics of 24 patients with positive and negative results for infection with Zika virus, Brazil, 2015

Reverse transcription PCR result for Zika virus (no.)	Mean (SD) patient age, y	Patient sex, F/M	No. (%)			
			Rash	Fever	Myalgia	Headache
Positive (7)	33 (15)	6/1	6 (85.7)	3 (43)	4 (57.1)	3 (43)
Negative (17)	31 (8.5)	12/5	12 (70.6)	6 (35.3)	9 (53)	11 (64.7)

1538–1558 and 1902–1883, respectively) (*9*) was considered indicative of ZIKV infection. PCR products (362 bp) were sequenced at the ACTGene Analises Moleculares, Alvorada, Rio Grande do Sul (Porto Allegre, Brazil), and sequences were deposited in GenBank under accession nos. KR816333–KR816336.

All patients were negative by RT-PCR for DENV, Mayaro virus, and WNV. Samples from 7 (29.2%) patients were positive by RT-PCR for ZIKV (369-bp fragment) and from 3 (12.5%) patients for CHIKV (305-bp fragment). There was no simultaneous detection of ZIKV and CHIKV. Most (85.7%) patients positive for ZIKV were women; they had a median age of 28 years and no history of international travel. Patients positive for ZIKV sought medical care after a 4-day (range 1–5 days) history of rash, myalgias, arthralgias, or fever. Three patients had IgG against DENV, which is consistent with a previous DENV infection, and none of the 7 ZIKV-positive patients had a positive response for DENV.

Mean laboratory findings for patients with acute ZIKV infection were a leukocyte count of 3,750 cells/mm^3 (range 2,790 cells/mm^3–6,150 cells/mm^3) and a platelet count of 180,000 platelets/mm^3 (range 151,000 platelets/mm^3–274,000 platelets/mm^3). The mean C-reactive protein level was 16.3 mg/L (range 0.9 mg/L–19.7 mg/L). Sign and symptom duration was 1–5 days, and most patients had a maculopapular rash, myalgias, fever, and headache. Arthralgia was seen less frequently.

ZIKV infections were assessed by sequencing partial ZIKV envelope gene regions of isolates. Phylogenetic analysis rooted with Spondwei virus showed that ZIKV sequences obtained belonged to the Asian lineage and showed 99% identity with a sequence from a ZIKV isolate from French Polynesia (KJ776791) (*10*).

We report ZIKV infection in Brazil in association with an ongoing outbreak of an acute maculoexantematic illness. Although the patient population samples were not randomly selected, 42% (10/24) of the patients were positive for ZIKV (n = 7) or CHIKV (n = 3) and had maculopapular rash, fever, myalgias and headache. After detection of ZIKV in Bahia, many cases have been identified in other states (http://www.promedmail.org, archive no. 20152015602.343.1158).

Cases of infection with DENV, CHIKV, and ZIKV in Brazil and elsewhere will make diagnosis based on clinical and epidemiologic grounds unreliable. These issues show the need for laboratory confirmation of these arboviral infections. More studies are needed to address the effects of these concurrent arboviruses infections in Brazil.

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Figura 1 – Artigo: Zika Virus Outbreak, Bahia, Brazil
Fonte: Campos, Bandeira e Sardi (2015)

4.2 ARTIGO 2 - CLINICAL AND LABORATORY FINDINGS OF ACUTE ZIKA VIRUS INFECTION IN PATIENTS FROM SALVADOR DURING THE FIRST BRAZILIAN EPIDEMIC. *Braz J Infect Dis*. 2020 Sep-Oct;24(5):405-411. doi:10.1016/j.bjid.2020.08.005. Epub 2020 Sep. 14. PMID: 32941805.

A fim de caracterizar os aspectos clínicos e laboratoriais da infecção aguda pelo ZIKV foi elaborado o estudo que respondeu ao primeiro objetivo específico desta tese.

Foi realizado estudo descritivo transversal no serviço de pronto-atendimento do Hospital Aliança, Salvador, Bahia.

Todos os pacientes com 18 anos ou mais que procuraram o serviço com sintomas compatíveis com uma condição clínica infecciosa [sistêmica, respiratória, gastrointestinal (GI), urinária (ITU) ou pele / tecidos moles (SSTI)] foram triados e examinados por um médico de plantão. Os pacientes eram considerados elegíveis se apresentassem pelo menos um dos seguintes sintomas agudos (<7 dias): febre (relatada ou medida), mialgia, artralgia, erupção cutânea ou cefaleia. Durante o período inicial do estudo, de 27 de maio a 17 de junho de 2015, amostras de sangue foram coletadas para realizar o diagnóstico de ZIKV. De 19 de junho de 2015 até 31 de agosto de 2017, além de sangue, amostras de urina e saliva também foram coletadas. Todas as amostras foram submetidas à extração de RNA viral e amplificadas por reação em cadeia da polimerase de transcrição reversa (PCR) usando primers para ZIKV e CHIKV. Dengue foi diagnosticada usando um teste rápido para demonstrar a presença de anticorpos IgM e IgG e / ou antígeno NS1. Os pacientes eram incluídos no estudo se uma amostra de soro, urina ou salivas fosse positiva para a presença de ZIKV. Os testes moleculares foram realizados no Laboratório de Virologia da Universidade Federal da Bahia.

Durante o primeiro período epidêmico, de 27 de maio a 17 de junho de 2015, um total de 448 pacientes foram triados. Uma doença viral sistêmica aguda foi diagnosticada em 219 (48,9%) de 448 pacientes, com 29 pacientes (13,2%) testando positivo para a presença de ZIKV. Posteriormente, no segundo período do dos 215 pacientes elegíveis (22,8%) com doença febril sistêmica apresentaram teste positivo para a presença de ZIKV. A amostra final foi composta por 78/434 (18,0%) indivíduos infectados pelo ZIKV, triados durante todo o período do estudo. Cinquenta e dois pacientes eram do sexo feminino (66,7%), com média de idaded e 38 anos. Sessenta e seis pacientes apresentaram resultados positivos para a presença de ZIKV no soro, sendo dois pacientes também apresentando vírus na saliva e um na urina, sendo três pacientes positivos em saliva e urina. Além disso, oito pacientes sem detecção viral no soro apresentaram

positividade apenas na saliva, enquanto três foram positivos apenas na urina e um na saliva e na urina. Quatro pacientes (5,1%) estavam coinfetados com CHIKV no momento do diagnóstico de ZIKV: três testaram positivo no soro, enquanto o outro foi positivo apenas na saliva. Dos 67 pacientes infectados pelo ZIKV testados, um paciente (1,5%) apresentou IgM específico para Dengue e 13 pacientes (19,4%) apresentaram anticorpos IgG no soro para Dengue. Apenas um paciente (1,5%) apresentou resultado positivo para o antígeno Dengue NS1. O tempo médio entre o início dos sintomas e a coleta da amostra foi de três dias (variação de 1 a 10 dias). A maioria dos pacientes queixou-se de mialgia (74,4%), febre (61,5%), cefaleia (59,0%), erupção cutânea (51,3%) e artralgia (44,9%), consistentes com a apresentação clássica do ZIKV. Alguns pacientes também relataram outros sintomas bastante incomuns, tais como náuseas (23,1%), vômitos (12,8%), diarreia (12,8%), tontura (6,4%) e dor de garganta (6,4%).

Em relação aos achados laboratoriais, a maioria dos pacientes apresentava hemograma normal, enquanto hemoconcentração foi observada em apenas 5,3% e somente 1 paciente apresentou trombocitopenia. Os pacientes com ZIKV estudados não apresentavam níveis elevados de enzimas hepáticas e menos de 5% tinham níveis de AST acima de 100 U / L. Isso contrasta com os pacientes com Dengue que apresentam mais frequentemente hemoconcentração e trombocitopenia, além de elevações nas enzimas hepaticas durante o curso da doença.

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Original article

Clinical and laboratory findings of acute Zika virus infection in patients from Salvador during the first Brazilian epidemic

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ABSTRACT

Several major epidemics of Zika fever, caused by the ZIKA virus (ZIKV), have emerged in Brazil since early 2015, eventually spreading to other countries on the South American continent. The present study describes the clinical manifestations and laboratory findings of patients with confirmed acute ZIKV infection during the first epidemic that occurred in Salvador, Brazil. All included patients were seen at the emergency room of a private tertiary hospital located in Salvador, Brazil from 2015 through 2017. Patients were considered eligible if signs of systemic viral febrile disease were present. All individuals were tested for ZIKV and Chikungunya infection using PCR, while rapid test was used to detect Dengue virus antibodies or, alternatively, the NS1 antigen. A diagnosis of acute ZIKV infection was confirmed in 78/434 (18%) individuals with systemic viral febrile illness. Positivity was mainly observed in blood, followed by saliva and urine. Coinfection with Chikungunya and/or Dengue virus was detected in 5% of the ZIKV-infected patients. The most frequent clinical findings were myalgia, arthralgia and low-grade fever. Laboratory analysis demonstrated normal levels of hematocrit, platelets and liver enzymes. In summary, in acute settings where molecular testing remains unavailable, clinicians face difficulties to confirm the diagnosis of ZIKV infection, as they rely only on clinical examinations and conventional laboratory tests.

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Introduction

Zika virus (ZIKV) has recently emerged as a critical mosquito-borne pathogen, especially after outbreaks in the Eastern Pacific region.[1,2] Concurrent infections with Dengue (DENV) and Chikungunya (CHIKV) virus have posed a significant burden for health care services, and new clinical challenges have been associated with these infections, in particular Guillain-Barré Syndrome, meningoencephalitis, autoimmune manifestations and, more recently, congenital abnormalities.[3–10] Since the first isolation of ZIKV in the Americas on March 26, 2015, an estimated 1.3 million individuals have been infected in northeastern Brazil, with an estimated attack rate of 5.5 cases per 1000.[11,12]

ZIKV infection typically causes a self-limiting, Dengue-like, illness characterized by cutaneous rash, low-grade fever, conjunctivitis, and arthralgia.[6] Many reports on ZIKV in the literature describe clinical manifestations based on empirical evidence, since diagnosis is dependent either on virus detection using reverse transcriptase-polymerase chain reaction (PCR) or on plaque reduction neutralization testing, both of which are typically unavailable in most clinical laboratories in affected areas.[1] Even in investigations conducted during outbreaks, the majority of patients only tested positive in the first three days of illness, with decreasing sensitivity of PCR observed after this initial period.[13]

There is a paucity of clinical studies evaluating acute ZIKV infection in the literature and to date no case-series have extensively reported an in depth analysis of the clinical and laboratory abnormalities seen in patients with ZIKV as well as co-infections with DENV or CHKV. A comprehensive understanding of the clinical and laboratory parameters specific to each of these diseases is urgently needed.

As of March 2015, the municipality of Salvador, located in the state of Bahia-Brazil, has witnessed an increasing number of adult patients with Dengue-like symptoms seeking medical assistance at emergency departments throughout the city.[12] The present study aimed to describe the clinical manifestations and laboratory findings of patients with confirmed acute ZIKV infection seen at a tertiary care facility in Salvador from 2015 through 2017.

Methods

The present cross-sectional study was conducted at the emergency department of a 210-bed tertiary care facility of a private hospital at Salvador, Bahia, Brazil from May 27, 2015 through August 31, 2017. All patients aged 18 years or older who sought emergency services and presented symptoms compatible with an infectious clinical condition [systemic, respiratory, gastrointestinal (GI), urinary (UTI) or skin/soft tissue (SSTI)] were triaged by a nurse and then examined by an on-duty physician. Patients were considered eligible if they presented with at least one of the following acute (< 7 days) symptoms: fever (reported or measured), myalgia, arthralgia, rash or headache.

During the initial study period, from May 27 to June 17, 2015, all patients screened for infectious conditions were seen and oriented by a physician specialized in infectious diseases, and blood samples were collected to perform ZIKV diagnosis (Fig. 1). From June 19, 2015 until the end of the study, in addition to blood, urine and saliva samples were also collected. All samples were submitted to viral RNA extraction (Qiagen, USA) and amplified by reverse transcription polymerase chain reaction (PCR) using primers for CHIKV and ZIKV according to previously described protocols.[13–15] Dengue fever was diagnosed using a rapid test to demonstrate the presence of IgM and IgG antibodies and/or Dengue NS1 antigen (ECO Diagnostica, Brasil). Patients were selected if a serum, urine or saliva sample tested positive for the presence of ZIKV by molecular diagnosis. All molecular tests were done at the Virology Laboratory, Federal University of Bahia. Any individuals diagnosed with bacterial disease or infectious diseases other than ZIKV were excluded. Blood, urinary, and radiological exams were ordered at the discretion of the attending physician for diagnostic purposes.

The following demographic and clinical variables were evaluated: sex, age, location of residence, history of recent travel to a country with confirmed ZIKV circulation, time (in days) of onset of initial symptoms, rash, myalgia, arthralgia, headache, arthritis (including the involvement of specific joints), conjunctivitis, lymphadenopathy, nausea, diarrhea, dizziness, muscle weakness, axillary temperature, heart and respiratory rate, blood pressure, in addition to any other symptoms and/or abnormal signs. The laboratory parameters investigated were: complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CK). Lymphopenia was defined as the total lymphocyte count under 1000 per mm^3 and hemoconcentration as a hematocrit level above 47%.

The present study was conducted in accordance with the Good Clinical Practice guidelines and received approval from the Institutional Review Board of Oswaldo Cruz Foundation (protocol numbers 1.159.814 and 1.593.256/CAAE 55882016.6.0000.0040). Written informed consent was obtained from all patients who agreed to participate.

Results

During the first epidemic period from May 27 to June 17, 2015, a total of 448 patients were triaged. An acute systemic viral illness was diagnosed in 219 (48.9%) out of 448 patients (Fig. 1), with 29 (13.2%) testing positive for the presence of ZIKV on molecular testing. Subsequently, in the second period of the study, 49 out of 215 eligible patients (22.8%) with systemic febrile illness tested positive for the presence of ZIKV. The final sample comprised 78/434 (18.0%) ZIKV-infected individuals screened throughout the entire study period. Fifty-two patients were female (66.7%), with a mean age of 38 years. Using molecular diagnostics, 66 patients tested positive for the presence of ZIKV in serum, with two patients also presenting virus in saliva and one in urine, whereas three patients were positive in both saliva and urine. In addition, eight patients without viral detection in serum presented positivity in saliva only, while three were positive in urine only, and one in both saliva and urine (Fig. 2). Altogether, 12/49 (24.5%) of the

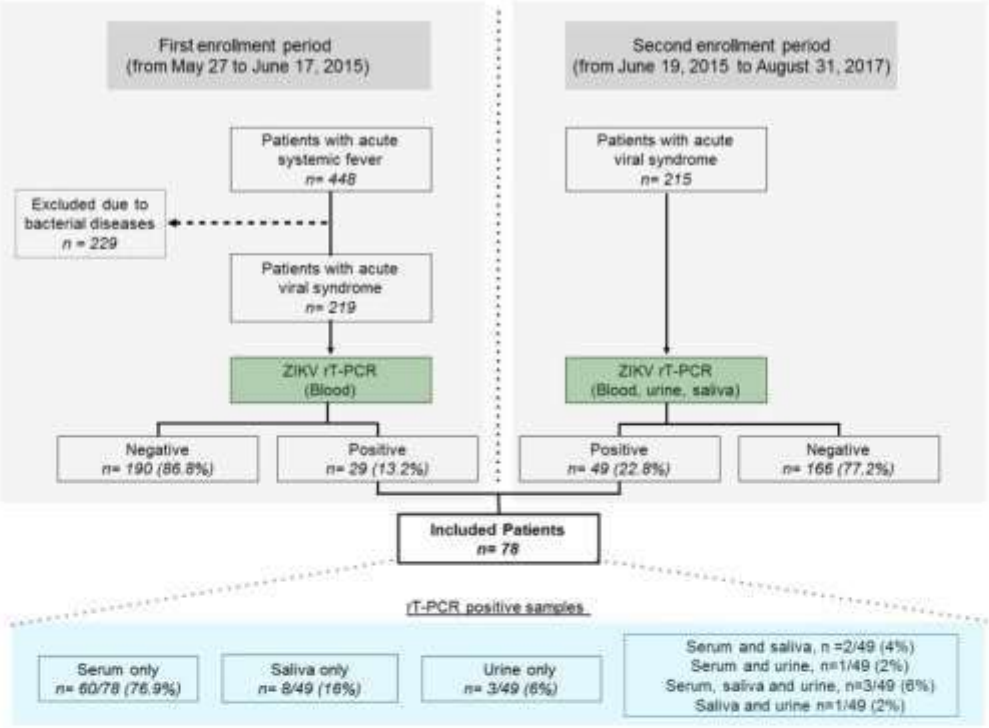

Fig. 1 – Flowchart detailing study design and results of PCR testing for ZIKV in biological fluids.

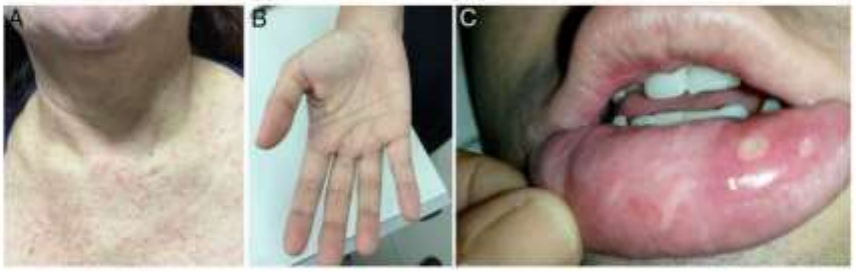

Fig. 2 – Mucocutaneous involvement during acute ZIKV infection (A) Maculopapular rash; (B) Palm rash; (C) Oral ulcers in a co-infected patient with ZIKV and CHIKV.

patients tested positive in saliva or urine (or both) and had no detectable levels of ZIKV in serum.

Four patients (5.1%) were co-infected with CHIKV at the time of the diagnosis of ZIKV: three tested positive for CHIKV in serum, whereas the other was positive only in saliva. Dengue rapid testing indicated that out of 67 ZIKV-infected patients tested, one patient (1.5%) presented DEN-specific IgM and 13 patients (19.4%) had IgG antibodies in serum. Just one patient (1.5%) tested positive for the presence of the Dengue NS1 antigen.

The median time between the onset of symptoms and sample collection was three days (range 1–10 days). The frequency of clinical signs and symptoms are shown in Table 1. Most patients complained of myalgia (74.4%), fever (61.5%),

Table 1 – Distribution of reported and observed signs and symptoms of acute Zika infection among 78 patients seen in an emergency room setting.

Symptoms/signs	N	(%)
Myalgia	58	(74.4)
Reported fever	48	(61.5)
Headache	46	(59.0)
Rash	39	(50.0)
Pruritic rash	25	(32.1)
Arthralgia	35	(44.9)
Nausea	18	(23.1)
Conjunctivitis	10	(12.8)
Diarrhea	10	(12.8)
Confirmed fever > 38.0 °C	8	(10.3)

Table 2 – Distribution of signs and symptoms in co-infected patients with acute Zika and Chikungunya.

Sex	Age	Max temperature (Celsius)	Polyarthralgia/ arhtritis	Rash/pruritic	Abdominal pain	Diarrhea
Female	39	38.0	Intense	Yes/Yes	Yes	Yes
Female	24	38.7	Intense	Yes/No	No	No
Female	50	38.0	Intense	Yes/No	No	No
Male	16	37.0	Mild	Yes/Yes	No	No

Table 3 – Laboratory markers among 78 patients with acute Zika infection seen in an emergency room setting.

Variable	Median (range) or positive/total
Hematocrit (%)	41 (31–49)
Total leukocyte (per mm^3)	5505 (1340–21,250)
Atypical lymphocytes (%)	0 (0–12)
Platelets (per mm^3)	217,000 (67,000–571,000)
AST (U/L)	25.0 (12.0–161.0)
ALT (U/L)	28.0 (13.0–249.0)
CRP (mg/dL)	1.8 (0.5–20.9)
CK (U/L)	74.5 (19.0–9585)
Dengue IgG	13/67

AST (aspartate aminotransferase), ALT (alanine aminotransferase), CRP (C-reactive protein). CK (creatine phosphokinase).

headache (59.0%), rash (51.3%) and arthralgia (44.9%), consistent with the classical presentation of ZIKV. Some patients also reported other rather unusual symptoms, such as nausea (23.1%), vomiting (12.8%), diarrhea (12.8%), dizziness (6.4%) and sore throat (6.4%).

At the time of admission in the emergency room, 39 patients (50.0%) presented with a cutaneous rash (Fig. 2A) and some with a palmar rash (Fig. 2B). Other less frequent clinical findings were conjunctivitis (10/78, 12.8%), cervical lymphadenopathy (9/78, 11.5%), body temperature >38 °C (8/78, 10.3%) and tachycardia, which was found in 8/78 patients (10.3%). It is worth noting that five patients had ankle edema, and four had edema and pain in their wrists and hands. Two of these patients were also found to be co-infected with CHIKV. Two patients presented painful bilateral inguinal lymphadenopathy. Oral ulcers were observed in one patient co-infected with CHIKV (Fig. 2C). The classical presentation of acute viral infection with fever, myalgia, arthralgia and rash was observed in only 13 patients (16.7%), while 19 patients (24.4%) presented with fever, myalgia and rash, but without arthralgia. The patient who tested positive for NS1 for DENV had a 4-day history of myalgia, rash, and headache, but was not tested for DENV by PCR.

Signs and symptoms in coinfected patients with Zika and Chikungunya are shown in Table 2. Articular involvement predominated in all patients with intense polyarthritis in three.

Laboratory findings of the included patients are presented in Table 3. A CBC was ordered for 76 patients. Four patients with ZIKV infection (5.3%) presented with hemoconcentration. Total leukocyte counts ranged from 1340 to 21,250 cells per mm^3 with a median of 5505 cells per mm^3. Ten patients (13.2%) had a total leukocyte count above 10,000 cells per mm^3, with two patients presenting leukocytosis with over 20,000 leukocytes per mm^3. The first, a 24 yr-old woman, reported fever and chills one day before admission, with no gastrointestinal, respiratory, urinary, or cutaneous symptoms. Her total leukocyte count was 20,210 cells per mm^3 with 1% atypical lymphocytes and 2% band forms. The second patient, a 23 yr-old pregnant woman at five weeks of gestation at the time of diagnosis, complained of a prior pruritic rash one day earlier, which lasted 24 h. She was taking corticosteroids at the time of her emergency room visit. Her total leukocyte count was 21,250 cells per mm^3, with 1% band forms and 11% atypical lymphocytes. She gave birth to a normal baby with no neurological abnormalities. Both patients presented negative blood cultures and both totally recovered a few days later. All patients were discharged without complications.

Lymphopenia (less than 1000 per mm^3) was observed in 26 patients (34.2%) and atypical lymphocytes (ranging from 1% to 12%) were found in 23 patients (30.3%). Twelve patients (15.8%) had a total monocyte count above 900 cells/mm^3, and four (5.3%) below 200 cells/mm^3. Three patients (3.9%) had band forms greater than 2%. Platelet count below 100,000 cells/mm^3 was seen in one patient (1.3%).

Results of C-reactive protein (CRP) were obtained for 71 patients, median 1.8 mg/dL (ranging from 0.5 to 20.9 mg/dL). In eight patients (11.3%), CRP levels were >5.0 mg/dL, and in the four >10.0 mg/dL. One of the latter patients was a 61 yr-old man with type II diabetes who presented with fever lasting four days, as well as chills and diarrhea. His CRP level was 20.9 mg/dL and total leukocyte count was 2800 cells/mm^3 (0% band forms and 0% atypical cells.) A transient elevation of liver enzymes was observed, evidenced by AST of 161 U/L and ALT of 249 U/L. The patient had no organ dysfunction. The second patient was a 21 yr-old man with four-day asthenia, arthralgia, fever, headache, and myalgia. His CRP level was 15.6 mg/dL, total leukocyte count was 6600 cells/mm^3, with 0% band forms and normal liver enzymes. Another patient was a 24 yr-old woman with 1-day history of fever and chills, with no other complaints. Her CRP value was 13.8 mg/dL and total leukocyte count was 20,210 with 2% band forms. She had normal liver enzyme levels (AST of 18 U/L and ALT of 23 U/L). Finally, the fourth patient was a 22 yr-old woman with a four-day history of myalgia and fever, who, after two days, began experiencing a non-productive cough. Her CRP level was 13.4 mg/dL, and total leukocyte count was 6940 cells/mm^3, with 0% band forms. All four patients recovered in a few days with an uneventful course. For all patients, blood cultures showed negative results.

AST and ALT liver enzyme levels, tested in 63 patients, showed a median value of 25 U/L (ranging from 12 to 161 U/L) and 28 U/L (ranging from 913 to 249 U/L), respectively. Only two patients (3.2%) had elevated AST levels above 100 U/L. CK levels were ordered for 24 patients. Four patients (16.7%) had values > 175 U/L and one patient had a CK of 9585 U/L. This patient was a 41 yr-old man with a three-day history of intense pain

in back, thighs, and calves. The presence of ZIKA was positive in saliva only.

Discussion

The results of the present study showed that during the recent ZIKV outbreak in the city of Salvador, Brazil, 18% of patients with acute viral syndrome were confirmed with ZIKV infection by molecular testing. The most frequently reported clinical symptoms, myalgia, fever, and low-grade fever, were mild and non-specific. Laboratory markers also did not show significant alterations. In addition, co-infection with CHIKV was present in 5% of the ZIKV-infected patients.

Our results corroborate those reported by researchers on Yap Island, who estimated that just one in six patients with ZIKV-confirmed infection presented supposedly classical symptoms of Zika fever, i.e. a combination of a rash, fever and conjunctivitis.[3] In addition, these symptoms were observed in just 26.2% of ZIKV-positive blood donors between 3 to 10 days after blood donation during the French Polynesia epidemic.[16] Our results are similar to findings reported in patients diagnosed with acute ZIKV infection in 2016 from Araraquara and Ribeirão Preto (São Paulo), as well as Rio de Janeiro, Brazil.[17–19] A similar distribution of sex, age, and clinical findings, such as myalgia, headache, and conjunctivitis, was found between these three studies and our results.

In contrast to the signs and symptoms of ZIKV infection described herein, DENV-infected patients more often present classical symptoms, which may aid in performing differential diagnosis in clinical settings. Indeed, during outbreaks of Dengue fever in Kenya and in Ecuador, almost all patients had fever, headache, arthralgia and myalgia. In each of these settings, 67% and 20% of patients required hospitalization, respectively, which is a very distinct outcome not seen in our patients.[18,20]

The moderate rate (18%) of confirmed ZIKV diagnosis in the patients considered herein is 10-fold higher than that reported by Silva et al., who confirmed ZIKV infection in 1.4% of febrile patients seen at a public health unit in Salvador between 2014 and 2016.[21] This discrepancy may be explained by the fact that these authors conducted surveillance for arboviral diseases for a longer period than our study, prior to the outbreak in Bahia in 2015.

Regarding laboratory findings, the majority of our patients presented normal CBC, while hemoconcentration was observed in 5.3% and only one patient presented thrombocytopenia. This contrasts to patients with Dengue fever, who more frequently experience hemoconcentration and thrombocytopenia. The ZIKV studied patients did not present non-specific elevated levels of liver enzymes, and less than 5% had AST levels higher than 100 U/L. In comparison to Dengue fever, these findings may help differentiate ZIKV-infected patients from those with DENV in an acute setting, and can provide indications of a clinical diagnosis in the absence of hemoconcentration and thrombocytopenia. A study conducted in midwestern Brazil observed differences in laboratory parameters among patients with Dengue and Zika infection. Azeredo et al. demonstrated that patients infected with DENV alone presented higher ALT levels compared to patients with acute ZIKV infection.[22] In South Australia, thrombocytopenia was seen in 49.0% of patients with Dengue fever compared to just 1.3% of the patients in the present study.[23]

Yan et al. presented simple clinical and laboratory assessments for distinguishing between Zika infection and Dengue fever.[24] Non-purulent conjunctivitis and normal monocyte and platelet counts were the main parameters used to establish a diagnosis of ZIKV.

With respect to coinfection, 5.1% of the ZIKV-infected patients also presented evidence of CHIKV infection. However, no differences in clinical or laboratory markers were seen in these coinfected patients. Pruritic rash which occurred in half of the coinfected patients could help in adding clinical suspicion of ZIKV infection in patients presenting with fever and polyarthralgia. Herein, 19.4% the studied patients presented serological evidence of past DENV infection, and just one patient had a positive Dengue NS1 test result. Of note, a false-positive DENV NS1 antigen test observed in the context of acute ZIKV infection might help to explain this result, as proposed by Gyurecha et al.[25]

Our study proposes that health professionals consider the following clinical manifestations when attempting to establish a diagnosis of acute ZIKV infection: non-specific acute symptoms, e.g. myalgia, arthralgia, headache; variable physical signs that may include a pruritic rash and fever; and lab results ranging from normal levels of hematocrit and platelets to mild liver enzyme levels. Although the intent of the present study was not to design a protocol for the differential diagnosis of ZIKV, the authors feel that a future investigation should take into account the above-mentioned variables.

With respect to ZIKV laboratory diagnostics, PCR positivity was mainly observed in blood samples (85% of cases), while saliva (29%) and urine (8%) positivity was lower than that reported in other studies.[26,27] It is important to consider that ZIKV RNA may remain present in urine samples for more than three weeks after the onset of symptoms,[28] which may help explain the discrepancy in urine analysis. Accordingly, it could be advantageous to simultaneously test multiple tissue types in order to more accurately reveal the true rate of virus detection.

The present study suffers from several limitations. Patients were screened at a hospital emergency service and some may have been discharged prior to recruitment, especially in the case of mild symptoms or fever. It was also conducted in a private hospital where the selected sample does not come from the general population with a bias towards a better-nourished population. However, since this study was conducted during the epidemic peak of ZIKV in northeastern Brazil, it was possible to include a significant number of patients with acute ZIKV infection. It is also important to note that during the epidemic peak period, PCR analysis for ZIKV was primarily conducted in blood samples, which may have negatively influenced the accurate identification of ZIKV-positive patients, especially in the early enrollment period. Although there were no control patients included herein, we are confident that no patients were misclassified, as this cross-sectional study aimed to describe clinical and laboratory characteristics exclusively in PCR-positive patients.

In conclusion, the diagnosis of ZIKV infection will remain a difficult task for clinicians in acute settings where molecular testing is not available, thus necessitating the reliance on clinical examination and laboratory tests. In addition to increasing access to rapid ZIKV and DENV diagnostic tests, more studies are also needed to provide clinicians with better algorithms based on symptoms and biomarkers to facilitate differential diagnoses with respect to other arboviral infections.

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Conflicts of interest

The authors declare no conflicts of interest.

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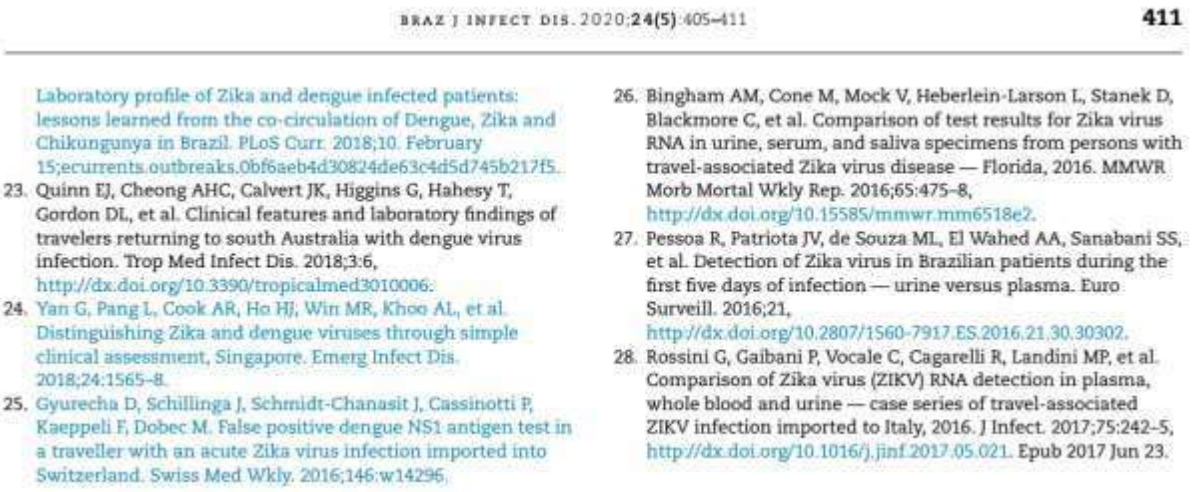

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Figura 2 – Artigo: Clinical and laboratory findings of acute Zika virus infection in patients from Salvador during the first Brazilian epidemic
Fonte: Bandeira et.al (2020)

4.3 ARTIGO 3 - PHENOTYPIC SIGNATURE OF T-LYMPHOCYTES FROM BRAZILIAN INDIVIDUALS INFECTED WITH ZIKV THROUGH MASS CYTOMETRY AND CROSS- REACTIVITY TO OTHER ARBOVIRUS. (Manuscrito em preparação)

Para caracterizar os aspectos imunológicos da infecção aguda pelo ZIKV, a resposta imune celular foi analisada em 11 pacientes com infecção pelo ZIKV e 11 pacientes com infecção aguda pelo CHIKV que participaram do processo de seleção do primeiro estudo, em dois períodos, na fase aguda e na fase de convalescência. Foram também incluídos 7 controles saudáveis (doadores de sangue) para fins de comparação da resposta imune. As amostras da fase aguda foram coletadas no momento da inclusão do paciente no estudo e as amostras da fase de convalescência foram coletadas após 15 dias. Este trabalho foi realizado em colaboração com Centre de Recherches en Immunologie et Maladies infectieuses (CIMI), Université Pierre et Marie Curie Paris. As células mononucleares do sangue periférico (PBMC) foram obtidas e enviadas para o CIMI com objetivo de realizar a quantificação dos linfócitos TCD8+ específicos produtores de IFN-γ e caracterização do perfil de linfócitos T por Cytof. A Cytof realiza a caracterização extensiva das célula através de uma plataforma de análise de alta tecnologia disponível em poucos centros de pesquisa no mundo. As células T foram caracterizados utilizando 40 parâmetros em um único tubo, a fim de definir as assinaturas de células T específicas ZIKV associadas com a expressão clínica da doença.

Os resultados são apresentados no manuscrito em preparação- artigo 3.

Phenotypic signature of T-lymphocytes from Brazilian individuals infected with ZIKV through mass cytometry and cross-reactivity to other arbovirus

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ABSTRACT

Keywords: Zika virus. Dengue virus. T-cell epitopes. Cross-reactivity. Mass cytometer.

INTRODUCTION

For decades, the infection with ZIKA virus (ZIKV) remained as a sporadic disease in humans restricted to areas from equatorial Africa and Asia. The first ZIKV epidemic occurred in the Yap Islands, with almost 70% of the population infected in 2007 [1] followed by scattered reports of cases in travelers returning from Africa or Southeast Asia [2,3]. However, from October 2013 when 10% of the French Polynesian population presented suggestive symptoms of infection the ZIKV has spread to other areas of the Pacific [4], including the Cook Islands, New Caledonia and Easter Island[5,6]. By March 2015, the first cases were detected in Brazil, initially in Bahia and soon thereafter in other regions of Brazil[7,8] . It is estimated that between 440,000

and 1,300,000 individuals had ZIKV infection during the pandemic [9].

The ZIKV epidemic in Brazil was associated with an increase in the number of newborns with microcephaly, leading the World Health Organization (WHO) to declare, on February 1, 2016, the ZIKV epidemic as a major public health emergency of international importance[10]. Another striking feature of the pandemic was the emergence of serious complications from ZIKV infection. Clusters of Guillain-Barré syndrome were first identified during the outbreak in French Polynesia and later in the Americas.

The pathogenesis of neurological complications associated with ZIKV infection is partially described. Elevated levels of inflammatory cytokines are markers of acute phase infection[11]. Particularly, elevated levels of CXCL10, IL-18 and IP-10 are associated with congenital ZIKV infection [12] and with the development of Guillan- Barré syndrome[13]. On the other hand, high levels of anti-inflammatory cytokines, especially IL-10, is found to be higher in patients without neurological complications[12].

The CD4 and CD8 T-lymphocytes play a central role in the control and elimination of virus-infected cells. It has been reported that the T-cell response against infections with flavivirus is mainly protective; however, a pathogenic response may also occur [14] For example, CD8 T-cells are critical for controlling the yellow fever virus in B-cell knockout mice[15]. On the other hand, the infiltration of CD8+ T- lymphocytes has been associated with tissue damage and neurological manifestations in mice infected with Japanese encephalitis virus or West Nile virus [16].

Regarding ZIKV, few studies have evaluated the specific T-cell response to viral antigens. A study involving American patients with confirmed ZIKV infection suggested that CD4+ T-cells recognized mainly structural proteins C and E while nonstructural proteins NS3, NS5 and NS4B were recognized mostly by CD8+ T-cells[17]. However, the magnitudes of the responses to structural proteins were higher by CD8+ T-cells [17].

Similar results were described in a case report, which looked at NS2-specific CD4 T cells and ZIKV envelope-specific CD8 T cells [18]. However, other studies report a more modest response to viral peptides with less than 1% of cytokine-producing CD4 or CD8 T-cells [19].

This study aims to describe the phenotypic signature of T-cell in individuals infected with ZIKV through mass cytometry and to characterize the response of T- cells to proteins of ZIKV and the cross-reactivity to proteins of others arbovirus.

METHODS

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and with French statutory and regulatory law and received approval from the Institutional Review Board of Oswaldo Cruz Foundation and the National Research Ethics Commission (CONEP), Brazil (protocol numbers 1.159.814 and 1.593.256/CAAE 55882016.6.0000.0040). All participants were adults and provided written informed consent for the collection of samples and subsequent analyses.

Patients & Controls

Peripheral blood samples were obtained from Brazilian patients seen in outpatient clinics in Salvador, Brazil, during the first ZIKV outbreak between March and December 2017. All patients were screened for ZIKV, CHIKV, and DENV infection by rt-PCR assays and serology markers. Patients with ZIKV were classified according to rt-PCR and/or serology results in the following groups: acute ZIKV infection (Group A), defined by positive ZIKV RT-PCR and negative serology (IgG) and subacute ZIKV infection patients (Group S), defined by both positive ZIKV RT-PCR and IgG. These patients were selected from a cohort described elsewhere[20].

Another group of patients with acute CHIKV infection based on rt-PCR positivity or clinical symptoms was included as a comparison group. They were all negative for ZIKV rt-PCR.

Healthy volunteers from Salvador (Brazil) and blood donors from the French blood bank (EFS, Pitié-Salpêtrière Hospital, Paris, France) were selected as controls. For some patients, serial blood samples were collected during the acute and the convaslecent phases. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient and cryopreserved in liquid nitrogen. Plasma was stored at - 80°C. A part of these samples was shipped to the CIMI Paris laboratory.

Serology

Serum samples were tested through IgG enzyme-linked immunosorbent assay (ELISA) for ZIKV (Euroimmun, Germany), DENV 1, 2, 3 and 4 serotypes (Focus Diagnostics, USA),

and CHIKV (Euroimmun, Germany).

RT-PCR Assays

Blood, urine and saliva samples were collected. All samples were submitted to PCR (AccessQuick™ System, Promega, USA) for ZIKV, CHIKV and DENV screening according to the manufacturer's recommendations[21,22].

Identification of predicted T-cell epitopes

Promiscuous T-cell epitopes restricted to human leukocyte antigen (HLA) class I were predicted by using the NetCTLpan 1.1 [3-5] prediction tool which has been benchmarked to be among one of the best performing publicly available prediction servers [6]. NetCTLpan 1.1 integrates prediction of peptide MHC class I binding and proteasomal C terminal cleavage by using artificial neural networks and antigen processing transport (TAP) efficiency by using weight matrix. The prediction values are calculated as a weighted average of the MHC, TAP and C terminal cleavage scores and as %-Rank to a set of 200.000 random natural peptides. In order to be more stringent, we used a threshold of 0.1t as rank threshold for strong binding peptides instead of the 0.5 value usually used. The prediction was done for nine amino acid long peptides. In order to predict the ZIKV epitopes we used the sequence of ZIKV Strain Natal RGN (GenBank Accession number: KU527068.1) which had been isolated from brain tissue from the fetus of a ZIKV-infected mother during the 2015 Brazilian epidemic [7]. We focused on the 103 HLA class I molecules representative of the Brazilian population (http://www.allelefrequencies.net/) (Supplementary Table S1). Predictions were carried out 98 out of the 103 Brazilian HLA class I molecules and excluded five HLA molecules (HLA-A*2301; HLA-A*6602; HLA-A*6603; HLA-B*1401; HLA-C*w0301) that represent only 9% of the Brazilian population. A total of 118 epitopes (nine amino acid peptides) were predicted in the different ZIKV protein (29 in NS5, 21 in NS3, 14 in NS4B, 13 in the envelope, 11 in NS1, 11 in NS2B, 10 in NS2A, 6 in the capsid and 3 in NS4A). Supplementary Table S2 summarizes the results of the predicted epitopes and the associated HLA restricted element.

Peptides

We synthesized 101 ZIKV 15-mer peptides overlapped by 11 aminoacids that included the 118 predicted epitopes and spanned the structural proteins [capsid (N=5), envelope (n=12

peptides)] and non-structural (NS) proteins [NS1 (n=11), NS2A (n=8), NS2B (n=10), NS3 (n=18), NS4A (n=3), NS4B (n=12), NS5 (n=22)] (Genecust, Boynes, France). Peptides were further reconstituted in 10% DMSO and organized in 9 pools, one for each ZIKV protein (Supplementary Table S3), Seven 9-10 mers immunodominant peptides from capsid, NS3, NS4B and polyprotein covering all four DENV serotypes) were synthesized (Genecust, Boynes, France), reconstituted in 10% DMSO, and tested in one pool (Supplementary Table S4). One hundred twenty 20-mer peptides overlapping by 10 amino-acids from the CHIKV covering envelope, capsid and non structural protein NSP1 were synthesized (Sigma-Genosys) and organized in 2 pools capsid + envelope (1 pool); NSP1 (1 pool) (Supplementary Table S4) [23].

Forty-eight 15-mer peptides overlapping by 11 amino-acids from the Yellow Fever Virus (YFV) covering envelope, non-structural (NS) proteins NS1, NS2, NS3, NS4 and NS5 and 1 peptide for capsid were synthesized (Eurogentec®), reconstituted in 10% DMSO and organized in 7 pools (one for each protein) used here in a superpool (Supplementary Table S5)[24].

One pool of 42 peptides (9-mers) (Epytop, Nîmes, France) covering the most common HLA-restricted immunodominant epitopes from Epstein-Barr Virus (EBV) was synthetized and used as control (Supplementary Table S6).

Interferon-gamma ELISpot assays

IFN-gamma produced by T-cells were analyzed by ELISPOT after stimulation by the 9 pools of ZIKV peptides Cryopreserved PBMCs with a median viability > 74% after thawing were incubated as described [24] in triplicate in 96-well flat-bottom plates (MSIPN4550, Millipore, St Quentin en Yvelines France), coated with anti-IFN-γ antibody (Diaclone, Besancon, France), with peptides at a final concentration of 2 μg/ml of each peptide in medium with 10% fetal calf serum for 18 h. Phytohemagglutinin (0.5 μg/ml) (Fisher Scientific, Illkirch, France) and medium alone were positive and negative controls, respectively. After incubation, cells were removed and biotinylated anti-IFN-γ mAb (Diaclone, Besancon, France) was added followed by streptavidin–alkaline phosphatase conjugate (Sigma-Aldrich, France) and chromogenic substrate (NBT/BCIP) (Sigma-Aldrich, France) before washing the plates with water and counting the number of spot-forming cells (SFC) Spots were counted using an AID-ELISpot reader (Autoimmun Diagnostika GmbH, Germany). Antigen-specific responses were considered positive when Spot Forming Cells (SFC)/10^6 PBMC were above 50 after

background subtraction.

Staining and mass cytometry acquisition

Forty monoclonal antibody clones were used for labeling PBMCs Primary antibody transition metal-conjugates were prepared with the MaxPAR antibody conjugation kit (Fluidigm) using the manufacturers recommended protocol. Following conjugation, antibodies were diluted to 100 x working concentration in Candor PBS Antibody Stabilization solution (Candor Bioscience GmbH) and stored at 4°C.

For each sample, PBMCs were thawed rapidly, incubated with 50 μM (final concentration) 127-IdU Cell-IDTM (Fluidigm) for 25 min at 37°C, followed by the addition of 2.5 μM Cisplatin Cell- IDTM (Fluidigm) for 5 min at 37°C. Cells were washed with Staining buffer (SB: 1xPBS with 0.5% bovine serum albumin and 0.02% sodium azide), and then stained with surface directed antibodies in 100 μL for 30 min at room temperature (RT). Cells were washed with SB, fixed for 15 min with Paraformaldehyde (Sigma Aldrich) at a final concentration of 2%, permeabilized with methanol (Sigma Aldrich) at 48C for 10 min and then intracellularly staining in 100 uL of SB for 60 min at RT. Cells were washed twice with SB and then incubated overnight in 2% PFA with 1:4000 the iridium intercalator (pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine; Fluidigm) at 4°C, and then frozen at -80°C.

Before acquisition, cells were thawed rapidly, resuspended in distilled- deionized water at 106 cells per mL and mixed with 4-Element EQ Beads (Fluidigm) and passed through a cell strainer cap with 35 mm pores (BD Biosciences). Cell events were acquired on the CyTOF-2 mass cytometer (Fluidigm) and CyTOF software version 6.0.626 (Fluidigm) at the "Plateforme de Cytométrie de la Pitié- Salpêtrière (CyPS)." Flow cytometry standard files produced by the CyTOF-2 were normalized with the MatLab Compiler software normalizer using the signal from the 4-Element EQ beads (Fluidigm) as recommended by the software developers.

Mass cytometry data analysis

At first, intact cells were gated on the basis of the DNA staining of the iridium intercalator, and then we selected singlets on Ir191 versus cell length plot. Live cells were selected before cell subset-specific gating.

For SPADE analysis (Cytobank), mass cytometry data were first singlet gated in

Cytobank using a cell length by DNA (Ir intercalator) gate. SPADE analysis was performed with the default configuration (10% of down-sampling and 50 target nodes) Clustering was performed on the studied cell markers Cell immunophenotype identification was performed manually based on the median expression of all markers in the individual clusters.

For viSNE (Cytobank), analysis samples were initially analyzed separately. Given that each condition provided a visually comparable response in all donors, samples from each treatment condition were subsequently concatenated into a single group before analysis, to facilitate the comparison among sample' groups.

Citrus (https://github.com/nolanlab/citrus) analysis was used to create a hierarchical clustering map, were circle size represents the number of cells in each cluster and arrows are displayed between the clusters to show their relationships.

RESULTS

Patients & Controls

Eleven patients had a diagnosis of ZIKA infection, seven in group A and four in group S. Eight patients were female (72.7%) and the median age was 39 years. Eleven patients (54.5% female, median age of 32 years) had a diagnosis of acute CHIKV infection (Table 1). Two of them were coinfected with DENV and six had a positive serology for ZIKV (IgG) consistent with past infection. Healthy controls comprised four donors from Brazil and three from France.

IFN-gamma production by T-cells in response to ZIKV peptides

Three out of 11 ZIKV-infected patients (27.3%) had a detectable positive T-cell response to ZIKV peptides (one from group A and two from group S) (Table 2). In addition, five out of 11 patients (45.5%) with acute CHIKV infection also had a detectable positive T-cell response to ZIKV peptides (two of them presented a positive IgG for ZIKV and one was coinfected with DENV) (Table 3). Only one healthy blood donor from France had a positive T-cell response to ZIKV peptides Among acute ZIKV-infected patients (group A) one patient responded to NS5 (87 SFC/10^6 PBMC) and NS4A (53 SFC/10^6 PBMC). Two patients from group S responded to ZIKV-peptides – one responded to NS5 (90 SFC/10^6 PBMC) and the other to NS5 (60 SFC/10^6 PBMC), NS2A (140 SFC/10^6 PBMC) and C (85 SFC/10^6 PBMC).

Among CHIKV-infected patients one patient responded to NS2A (85 SFC/10^6 PBMC)

and NS3 (100 SFC/10^6 PBMC); one patient responded to NS2B (67 SFC/10^6 PBMC) and NS3 (100 SFC/10^6 PBMC) and; each of the other 3 patients responded to just one peptide – NS1 (90 SFC/10^6 PBMC) and NS5 (205 and 655 SFC/10^6 PBMC, respectively).

The median magnitude of anti-ZIKV responses in all of the responders was 89 SFC/10^6 PBMC [IQR: 79-156] (Table 2). The NS5 was the immunodominant protein that was recognized in five responders (three from ZIKV-infected and two from CHIKV-infected patients, respectively). This immunodominance is in accordance with our predictive study of T-cell epitopes described in Table 2. The median magnitude of T-cells specific for NS5 was 87 SFC/10^6 PBMC (IQR: 60-90) with a trend toward increasing values in CHIKV-infected patients with ZIKV positive serology (430 SFC/10^6 PBMC, IQR 205-655) as compared to ZIKV infected patients (groups A and S) (less than 100 SFC/10^6 PBMC). The NS2A and NS4A peptide pools were recognized in two patients, the capsid in one, and no patient responded to the envelope pools.

T-cell Immune responses to DENV detected in IFN-gamma-ELISpot assay

Specific T-cell responses to DENV were detected in four ZIKV patients (4/11, 36.4%), in one patient with acute CHIKV infection (1/8, 12.5%) (1187 SFC/10^6 PBMC) and in one healthy blood donnor from France (1/7, 14.3%) (67 SFC/10^6 PBMC) (Table 2). The median magnitude of T-cells specific for DENV among ZIKV patients was 401.5 SFC/10^6 PBMC [IQR: 73.2-720.0]. A response to both ZIKV (NS5) and DENV peptides was identified in two patients from Group S, from 1 patient with acute CHIKV infection (NS2B and NS3), and from the healthy blood donor from France (NS3).

Cross-reactivity between ZIKV and DENV

The percentage of homology between the ZIKV and DENV peptides varied from 28% to 85% according to the evaluated protein. A median homology of 77% was observed between the NS1 sequences between ZIKV and the DENV serotypes. A lower homology was observed between the sequences of the NS2B and NS2A proteins and the DENV serotypes (57% and 45%, respectively). In addition, we also identified a high homology between NS3 and NS5 with DENV peptides, with medians of 85% and 77%, respectively. (Supplementary Table S7).

T-cell Immune responses to CHIKV, yellow fever virus (YFV) and Epstein-Barr virus (EBV) detected in IFN-gamma ELISpot assay

With respect to CHIKV peptides, detectable responses were observed in four out of eleven (36.4%) patients with CHIKV. NSP1 pool was recognized in three patients (median 60 SFC/10^6 PBMC, IQR 57-85) and Capsid + Env pool in two patients (median 98.5 SFC/10^6 PBMC, IQR 67-130) (Table 2).

Regarding the response to YFV peptides, four patients out of 22 tested (18.2%) had detectable responses: three infected with ZIKV from group S and one patient with acute CHIKV (Table 2). The median response was 369 SFC/10^6 PBMC [IQR: 151-755].

The responses against EBV were assessed in 29 subjects and 20 out of them responded (69.0%) to EBV peptides (median 209 SFC/10^6 PBMC, IQR 83-509) (Table 2).

Mass cytometry data analysis

Regarding the differentiation profile of CD4+ T-lymphocytes, an expansion of the T-cell central memory sub-population (TCM) (CD45RA-CD27+CCR7+) was observed in ZIKV-infected patients in acute (P1) and convalescence (P2) phases, compared to healthy controls (Figure 1B). The proportion of cells that expressed IFN- gamma was significantly higher in naïve (TN), TCM, transition memory (TTM) (CD45RA-CD27+CCR7-) in ZIKV-infected patients in phase P1 and P2 compared to healthy controls ($p<0.005$) (Figure 1C). A higher expression of IFN-gamma was also observed in effector memory (TEM) (CD45RA-CD27+CCR7-) and RA effector memory (TEMRA) (CD45RA+CD27-CCR7-) in ZIKV-infected patients in phase P2 compared to healthy controls ($p<0.005$) (Figure 1C). In addition, a greater expression of activation (HLA-DR and CD38) and exhaustion (PD1 and CTLA-4) markers was observed in the TCM, TTM and TEM subsets in ZIKV-infected patients in phase P1 compared to healthy controls. The Ki67, marker of proliferation, was more expressed in TN, TCM, TTM and TEM subsets in ZIKV-infected patients in phase P1 compared to healthy controls (Figure 1C). There was also a higher expression in ZIKV-infected patients in phase P2 compared to healthy controls in TN and TCM subsets. In TEMRA subset was observed a higher frequency of Ki67 in ZIKV-infected patients in phase P2 compared to phase P1 ($p<0.005$) (Figure 1C).

Regarding the CD8+ T-lymphocyte profile, an expansion of the TN, TCM, TEM, TEMRA subsets and a lower proportion of the TTM subset were observed in the ZIKV-infected

patients in phase P1 compared to healthy controls (Figure 2B). Patients in phase P2 exhibited a lower proportion of the TEM and TEMRA subsets compared to patients in phase P1 (Figure 2B). A higher expression of IFN-gamma was detected in the TN and TTM subsets of ZIKV-infected patients in phase P1 and P2 compared to healthy controls (Figure 2C). In addition, ZIKV-infected patients in the phase P1 or P2 showed a higher expression of activation markers (HLA-DR and CD38/HLA-DR) in the TTM, TEM and TEMRA subsets when compared to healthy controls (Figure 2C). The expression of the proliferation marker Ki67 was higher in the TN, TTM and TEMRA subsets of ZIKV-infected patients in phase P1 compared to healthy controls. ZIKV-infected patients in phase P1 and P2 showed a higher expression of CTLA-4 in the TN, TCM and TTM subsets compared to healthy controls (Figure 2C). ZIKV-infected patients in phase P2 showed more expression of CD38 and CD38/HLA-DR compared to phase P1 ($p<0.05$) (Figure 2C).

DISCUSSION

The present study indicated that acute ZIKV infection induced a cellular immune response in approximatively 30% of patients. This response was predominantly directed agaisnt the NS5 protein of ZIKV. The response was of low intensity, appeared in the early stage of the disease, and tended to be of greater magnitude in patients with evidence of past ZIKV infection. NS2A and NS4A proteins were also recognized by two of the five responders with a low response.

Grifoni et al showed that T cells that were induced by previous infection with DENV, or vaccination with the tetravalent vaccine for DENV, recognize ZIKV peptides, and that this cross-reactivity is explained by the similarity of the genetic sequence of the two viruses [25]. We showed a strong similarity of the genetic sequences between DENV and ZIKV with the non-structural proteins NS3 and NS5 among the other proteins. We had 4 ZIKV infected patients recognizing DENV peptides and the two of them that also recognized ZIKV peptides belonged to the subacute group. This was in contrast to the 2 patients in the acute ZIKV group that recognized DENV peptides but not ZIKV peptides with both showing a positive serology for DENV. The recognition of ZIKV peptides were not necessarily related to DENV peptide recognition.

Another point made by Grifoni et al is about the quality of responses being influenced by previous exposure to DENV, and CD8+ T cells specific for ZIKV from donors previously exposed to DENV show increased production of Granzyme B and PD1, unlike donors never

previously exposed to DENV [25]. In our study we showed a significant proportion of PD1+CD4+ T-cells in early acute ZIKV infection when compared to healthy controls. PD1 is an exhaustion marker of T-lymphocytes that was found mainly in CD4+ TCM, TEM and TTM cells but we could not relate it to previous DENV exposure.

The cellular immune response to ZIKV may be influenced by previous exposures to other flaviviruses. The NS5 protein of ZIKV presents an average of 77% of homology compared with the 4 DENV serotype. Reynolds et al mapped epitopes of CD4+ T-cells from the ZIKV envelope and HLA class II immunodominant non- structural antigens in HLA class II transgenic mice[26]. In several cases, CD4+ T-cells exposed to ZIKV responded to homologous sequences from other viruses, including DENV1–4, WNV or YFV [26]. When we analysed the 3 responders to ZIKV peptides inside the ZIKV groups we found DENV response in one patient with previous antibodies to DENV but not in the seronegative patient. That may indicate that peptide recognition may again not necessarily cross-react in either direction.

Similar to our results, a previous study in Colombia involving ZIKV-infected patients with or without previous DENV infection found that the regions of NS1, NS3 and NS5 proteins of ZIKV were immunodominant to T-cells responses[27].

A study involving American patients with confirmed ZIKV infection suggested that CD4+ T-cells recognized mainly structural proteins C and E while nonstructural proteins NS3, NS5 and NS4B were recognized mostly by CD8+ T-cells [17].

In addition, a higher proportion of CD8+ T-cells producing IFN-γ, IL-2, and/or TNF in response to NS3, NS5 e NS4B protein was detected in individuals with previous or acute ZIKV infection [17] Thus, the CD8+ T-cells immune response was predominantly directed against non-structural ZIKV proteins.

NS3 and NS5 were the predominantly proteins eliciting a ZIKV peptide recognition (7 out of the 8 reactive patients) by ELISPOT in our study.

In early acute ZIKV infection we observed a high proportion of IFN-gamma- producing CD4+ and CD8+ T-cells, activated CD4+ and CD8+ T-cells, CD4+ T-cells in exhaustion and CD8+ T-cells in proliferation in accordance with the acute insult by ZIKV and a trend towards reducing activation and proliferation in the convaslence phase.

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Disclosure

The authors declare no financial or commercial conflict of interest.

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Table 1 - Age and sex distribution among ZIKV and CHIKV infected patients

Subjetcs	**N**	**Female (%)**	**Age (years)***
ZIKV-infected			39.0
Acute (RT-PCR + / IgG/M -)	07	85.7	39.0
Subacute (RT-PCR + / IgG/M +)	04	50.0	36.5
CHIKV-infected	11	54.5	32.0

* Median.

Fonte: Elaborado pelo autor

Table 2 - ELISpot responses to Zika, Chikungunya, Dengue, Yellow fever and EBV viruses

Group	ELISpot results (SFC/10^6 PBMC)													
	ZIKV									DENV	CHIKV		YFV	EBV
	C	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5		C + E	NSP1		
ZIKV-INFECTION (n=11)														
Acute (RT-PCR+ / IgG-) (n=07)														
A1	5	20	0	10	10	5	0	10	5	**60**	30	0	10	20
A2	0	10	7	0	0	0	7	17	10	**113**	7	13	50	**213**
A3	0	0	0	0	0	0	3	0	3	27	0	0	20	**83**
A4	0	7	23	13	0	10	0	23	3	18	18	33	18	**290**
A5	17	30	10	27	17	30	**53**	13	**87**	37	30	23	27	17
A6	0	0	0	40	0	0	0	0	10	0	0	0	0	**70**
A7 *	13	7	7	23	3	3	10	13	13	30	30	47	40	17
Subacute (RT-PCR+ / IgG+) (n=04)														
S1	10	30	20	10	0	10	0	20	**90**	**690**	35	25	**455**	**365**
S2	40	43	30	30	7	47	13	27	23	30	23	17	**283**	**67**
S3	**85**	20	10	**140**	0	0	0	20	**60**	**730**	35	0	**855**	**1955**
S4	0	0	0	0	0	15	0	0	20	0	15	0	5	**110**
ACUTE CHIKV INFECTION (n=11)														
C1**	50	20	**90**	10	30	50	0	0	30	ND	**130**	**85**	ND	**55**
C2	3	20	3	0	0	10	3	0	7	0	40	**57**	10	**830**
C3	0	3	7	20	**67**	**117**	0	0	47	**1187**	**67**	47	**107**	**83**
C4§	0	0	13	3	0	0	0	10	0	0	0	27	40	47
C5**§	0	0	0	0	0	0	0	0	0	0	0	0	0	3
C6§	ND	30	20	0	ND	0	0	ND	**205**	ND	25	**60**	ND	**925**
C7§	ND	ND	ND	ND	ND	ND	ND	ND	**655**	ND	25	25	ND	35
C8§	7	0	13	13	3	10	0	0	17	7	20	0	7	**290**
C9§***	5	5	0	0	0	0	0	0	40	0	0	10	0	0
C10***	0	10	5	5	5	10	5	5	10	7	25	40	0	**205**
C11***	15	40	20	**85**	15	**100**	0	0	50	0	5	0	40	20
HEALTHY CONTROLS (n=07)														
HC1	0	0	0	0	0	0	0	0	0	0	0	0	ND	**90**
HC2	0	15	0	5	0	15	5	15	0	5	5	0	5	**365**
HC3	0	0	0	0	0	0	0	0	0	0	0	0	0	**770**
HC4	0	17	0	3	0	3	13	3	0	0	3	8	8	**88**
HC5	0	0	0	0	0	3	0	43	50	0	0	0	50	**557**
HC6	20	17	7	43	13	**67**	27	17	40	**67**	3	50	0	10
HC7	0	0	0	3	10	33	0	3	0	0	0	17	10	**57**

* RT-PCR positive to CHIKV
** RT-PCR positive to DENV
***Acute CHIKV infection based on clinical grounds
§ Positive serology (IgG) to ZIKV

Antigen-specific responses were considered positive when Spot Forming Cells (SFC)/10^6 PBMC were above 50 after background subtraction C: Capsid; E: Envelope
ND: not done; pos: positive; neg: negative
Fonte: Elaborado pelo autor

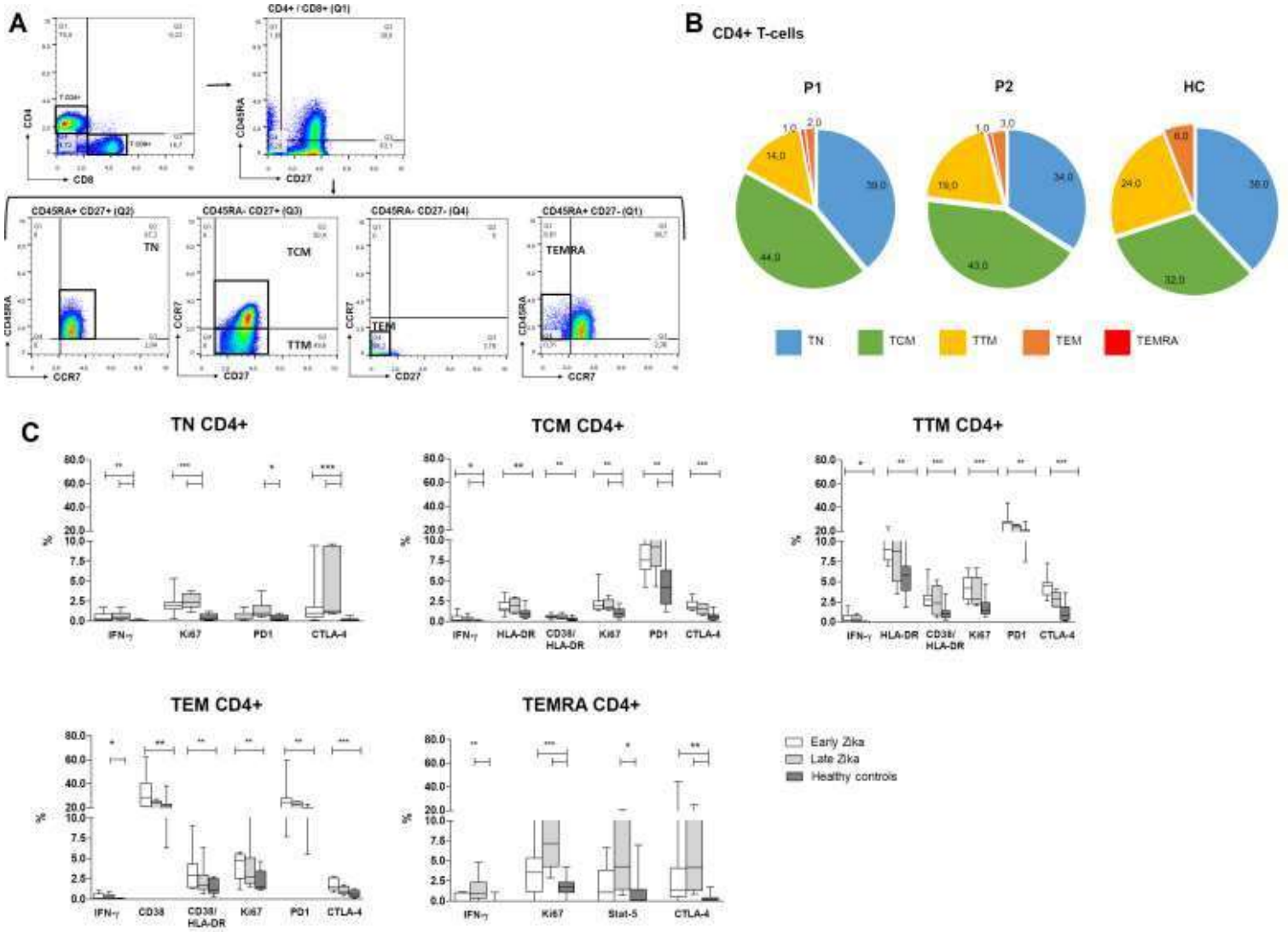

Figure 3 - CD4+ T-cells from ZIKV-infected patients in acute (P1, n=18) and convaslecent phases of infection (P2, n=7) and healthy controls (n=10). **A.** Phenotypic characteristics of selected CD4+ T-cells (CD4+CD8-) and Naive (TN) (CD45RA+CD27+CCR7+), central memory (TCM) (CD45RA-CD27+CCR7+), past memory (TTM) (CD45RA-CD27+CCR7-), effector memory (TEM) (CD45RA- CD27+CCR7-) and RA effector memory (TEMRA) (CD45RA+CD27-CCR7-) subpopulations. **B.** TN, TCM, TTM, TEM and TEMRA subpopulations in ZIKV- infected patients in acute (P1) and convaslecent (P2) phases and healthy controls (HC). **C.** IFN-gama expression, proliferation (Ki67), activation (HLA-DR, CD38 / HLA- DR, pStat-5) and exhaustion (PD1 and CTLA-4) markers in CD4+ T-cell subpopulations. Data are expressed as median and interquartile range. Significant when $p < 0.05$. *Kruskal-Wallis test, followed by Dunn's post-test was performed to compare three groups. #Wilcoxon test was performed to comprare ZIKV-infected patients in acute and convalesce phase.

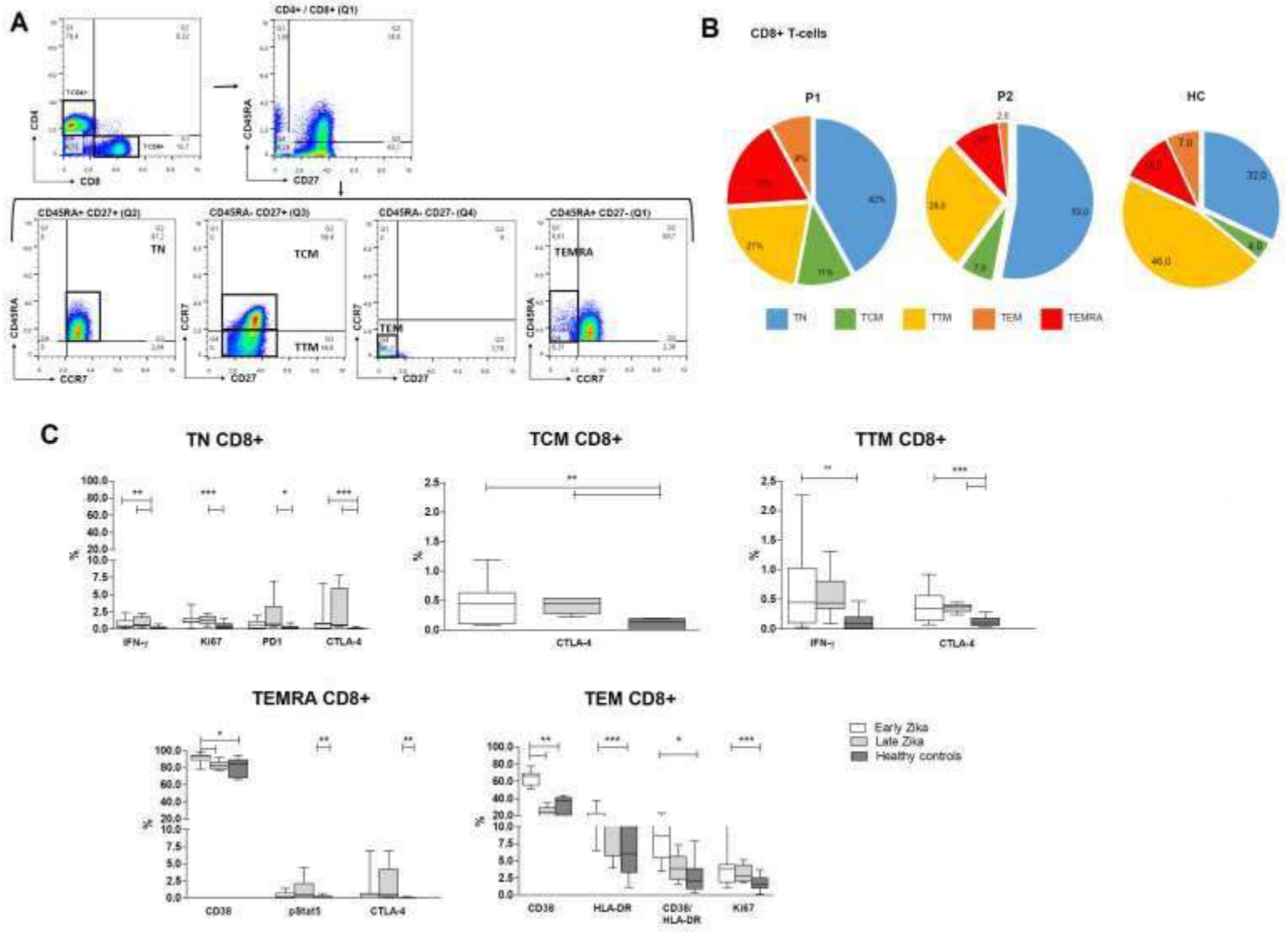

Figure 3 - CD8+ T-cells from ZIKV-infected patients in acute (P1, n=18) and convaslecent phases of infection (P2, n=7) and healthy controls (n=10). A. Phenotypic characteristics of selected CD8+ T-cells (CD4-CD8+) and Naive (TN) (CD45RA+CD27+CCR7+), central memory (TCM) (CD45RA-CD27+CCR7+), past Memory (TTM) (CD45RA-CD27+CCR7-), effector memory (TEM) (CD45RA- CD27+CCR7-) and RA effector memory (TEMRA) (CD45RA+CD27-CCR7-) subpopulations. B. TN, TCM, TTM, TEM and TEMRA subpopulations in ZIKV- infected patients in acute (P1) and convaslecent (P2) phases and healthy controls (HC). C. IFN-gama expression, proliferation (Ki67), activation (HLA-DR, CD38 / HLA- DR, pStat-5) and exhaustion (PD1 and CTLA-4) markers in CD8+ T-cell subpopulations. Data are expressed as median and interquartile range. Significant when $p < 0.05$. *Kruskal-Wallis test, followed by Dunn's post-test was performed to compare three groups. # Wilcoxon test was performed to comprare ZIKV-infected patients in acute and convalesce phase.

Table 3 - RT-PCR and serology to Zika, Chikungunya and Dengue viruses

Group	RT-PCR[1]			Serology (IgG)[1]		
	ZIKV	CHIKV	DENV	ZIKV	CHIKV	DENV
ZIKV-INFECTION (n=11)						
Acute (RT-PCR+ / IgG-) (n=07)						
A1	**pos**	neg	ND	neg	neg	pos
A2	**pos**	neg	neg	neg	neg	pos
A3	**pos**	neg	ND	neg	neg	pos
A4	**pos**	neg	neg	neg	neg	neg
A5	**pos**	neg	neg	neg	neg	neg
A6	**pos**	neg	neg	neg	ND	ND
A7 *	**pos**	**pos**	ND	neg	ND	ND
Subacute (RT-PCR+ / IgG+) (n=04)						
S1	**pos**	neg	ND	**pos**	ND	ND
S2	**pos**	neg	neg	**pos**	pos	pos
S3	**pos**	neg	neg	**pos**	neg	pos
S4	**pos**	neg	neg	**pos**	neg	neg
ACUTE CHIKV INFECTION (n=11)						
C1**	neg	**pos**	**pos**	neg	neg	pos
C2	neg	**pos**	neg	neg	neg	pos
C3	neg	**pos**	neg	neg	ND	ND
C4§	neg	**pos**	ND	**pos**	ND	ND
C5**§	neg	**pos**	**pos**	**pos**	ND	ND
C6§	neg	**pos**	ND	**pos**	neg	pos
C7§	neg	**pos**	ND	**pos**	neg	pos
C8§	ND	**pos**	ND	**pos**	neg	pos
C9§***	neg	neg	neg	**pos**	ND	ND
C10***	neg	neg	neg	neg	ND	ND
C11***	neg	neg	neg	neg	ND	ND
HEALTHY CONTROLS (n=07)						
HC1#	ND	ND	ND	neg	neg	pos
HC2	ND	ND	ND	neg	neg	pos
HC3	ND	ND	ND	neg	neg	pos
HC4	ND	ND	ND	neg	neg	neg
HC5	ND	ND	ND	ND	ND	ND
HC6	ND	ND	ND	ND	ND	ND
HC7	ND	ND	ND	ND	ND	ND

* RT-PCR positive to CHIKV
** RT-PCR positive to DENV
***Acute CHIKV infection based on clinical grounds
§ Positive serology (IgG) to ZIKV
ND: not done; pos: positive; neg: negative
Fonte: Elaborado pelo autor

Tabela 4 - Supplementary Table S1: HLA present in Brazil and Frequency in the population

HLA A	% In the population	HLA B	% In the population	HLA C	% In the population
A*0201	20,7	B*3501	9,0	Cw-0401	22,2
A*0301	9,7	B*4403	7,2	Cw-0701	11,5
A*2301	6,6	B*5101	6,6	Cw-1601	7,5
A*0101	5,8	B*0702	6,3	Cw-0602	6,8
A*3001	5,1	B*5301	5,6	Cw-0304	6,6
A*1101	4,4	B*1402	4,7	Cw-0802	5,6
A*2402	4,4	B*4402	4,3	Cw-0702	5,0
A*6801	3,2	B*5201	3,8	Cw-0501	4,7
A*3402	3,1	B*1801	3,6	Cw-1203	3,9
A*3601	3,1	B*0801	3,0	Cw-0303	3,8
A*6802	2,9	B*5801	3,0	Cw-0202	3,6
A*7401	2,7	B*4201	2,9	Cw-1701	3,2
A*3002	2,6	B*1501	2,7	Cw-0210	2,3
A*2601	2,4	B*1510	2,5	Cw-1801	2,3
A*3301	2,0	B*4901	2,5	Cw-1402	2,0
A*0202	1,9	B*1503	2,0	Cw-1502	2,0
A*2902	1,7	B*4001	2,0	Cw-0102	1,3
A*3201	1,7	B*8101	2,0	Cw-1202	1,1
A*3101	1,5	B*5703	1,8	Cw-1505	1,1
A*0205	1,4	B*4501	1,4	Cw-1602	0,9
A*6602	1,2	B*3801	1,3	Cw-0704	0,7
A*2901	1,0	B*4002	1,3	Cw-0804	0,7
A*8001	0,9	B*5501	1,1	Cw-0103	0,2
A*3004	0,7	B*5701	1,1	Cw-0301	0,2
A*0302	0,5	B*5802	1,1	Cw-0302	0,2
A*2403	0,5	B*1302	0,9	Cw-0705	0,2
A*2501	0,5	B*1401	0,9	Cw-0707	0,2
A*3303	0,5	B*1516	0,9	Cw-0813	0,2
A*0222	0,3	B*2705	0,9	Cw-1704	0,2
A*0234	0,3	B*3503	0,9		
A*6603	0,3	B*3508	0,9		
A*7403	0,3	B*4004	0,9		
A*6602	0,2	B*5001	0,9		
A*0102	0,2	B*0705	0,7		
A*0211	0,2	B*1517	0,7		
A*6901	0,2	B*3504	0,7		
A*7402	0,2	B*3701	0,7		

Tabela 5 - Supplementary Table S2: Peptide sequence for Zika virus with predicted epitope and restriction element

Protein	Amino Acid Sequence	Predicted Epitope	Restriction element
Capsid	VA**RVSPFGGLK**RLPA	RVSPFGGLK	**HLA-A*03:01; HLA-A*03:02; HLA-A*11:01**
	G**LKRLPAGLLL**GHGP	KRLPAGLLL	**HLA-B*27:05**
	HGP**RMVLAILAFL**R	RMVLAILAF/MVLAILAFL	**HLA-C*07:01; HLA-C*07:05; HLA-B*15:01;HLA-B*15:03; HLA-C*03:02;HLA-C*07:02; HLA-C*07:07;HLA-C*16:01/HLA-A*68:02; HLA-A*69:01**
	AI**LAFLRFTAI**KPSL	LAFLRFTAI	**HLA-B*08:01; HLA-B*52:01; HLA-C*01:02; HLA-C*07:04; HLA-C*18:01**
	A**AMLRIINAR**KEKKR	AMLRIINAR	**HLA-A*74:01; HLA-A*74:02;HLA-A*74:03;HLA-A*31:01**
Envelope	SN**MAEVRSYCY**EASI	MAEVRSYCY	**HLA-A*01:01;HLA-A*01:02**
	QPEN**LEYRIMLSV**HG	LEYRIMLSV	**HLA-B*13:02; HLA-B*45:01, HLA-B*49:01;HLA-B*50:01;HLA-B*52:01**
	EPRT**GLDFSDLYY**LT	GLDFSDLYY	**HLA-A*01:01;HLA-A*36:01; HLA-A*80:01**
	GLD**FSDLYYLTM**NNK	FSDLYYLTM	**HLA-A*01:01**
	WLVH**KEWFHDIPL**PW	KEWFHDIPL	**HLA-B*40:01; HLA-B*40:02; HLA-B*40:04; HLA-B*37:01; HLA-B*50:01; HLA-B*13:02; HLA-B*38:01**
	KEALV**EFKDAHAKR**Q	EFKDAHAKR	**HLA-A*33:01; HLA-A*33:03; HLA-A*32:01**
	V**SYSLCTAAFTFTK**I	SYSLCTAAF/CTAAFTFTK	**HLA-A*24:03; HLA-A*24:02/HLA-A*03:02; HLA-A*11:01; HLA-A*34:02; HLA-A*68:01; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
	A**ETLHGTVTV**EVQYA	ETLHGTVTV	**HLA-A*68:02; HLA-A*69:01**
	NSK**MMLELDPPF**GDS	MMLELDPPF	**HLA-C*07:02; HLA-C*07:01; HLA-C*07:05**
	IVIGV**GEKKITHHW**H	GEKKITHHW	**HLA-B*44:02**
	KR**MAVLGDTAW**DFGS	MAVLGDTAW	**HLA-B*53:01; HLA-B*58:01**
	VLG**DTAWDFGSV**GGA	DTAWDFGSV	**HLA-A*68:02**
NS1	HPD**SPRRLAAAV**KQA	SPRRLAAAV	**HLA-B*07:02; HLA-B*07:05; HLA-B*42:01; HLA-B*55:01**
	SVSR**MENIMWRSV**EG	MENIMWRSV	**HLA-B*45:01**
	RGPQ**RLPVPVNEL**PH	RLPVPVNEL	**HLA-C*01:02**
	FV**RAAKTNNSF**VVDG	RAAKTNNSF	**HLA-B*15:03;HLA-C*03:02; HLA-C*07:01; HLA-C*12:02; HLA-C*12:03; HLA-C*14:02**
	CP**LEHRAWNSF**LVED	LEHRAWNSF	**HLA-B*18:01**
	GFGV**FHTSVWLKV**RE	FHTSVWLKV	**HLA-B*38:01**

	NDT**WRLKRAHLI**EMK	WRLKRAHLI	**HLA-B*27:05**
	EMKT**CEWPKSHTL**WA	CEWPKSHTL	**HLA-B*37:01; HLA-B*13:02; HLA-B*38:01; HLA-B*40:02**
	SDLI**IPKSLAGPL**SH	IPKSLAGPL	**HLA-B*07:02; HLA-B*07:05**
	RSTT**ASGRVIEEW**CC	ASGRVIEEW	**HLA-B*57:01; HLA-B*58:01**
	E**CTMPPLSFR**AKDGC	CTMPPLSFR	**HLA-A*33:03; HLA-A*33:01; HLA-A*68:01; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
NS2A	STS**MAVLVAMIL**GGF	MAVLVAMIL	**HLA-B*67:02**
	AK**LAILMGATF**AEMN	LAILMGATF	**HLA-C*07:03**
	FKV**RPALLVSFI**FRA	RPALLVSFI	**HLA-B*42:04**
	PALL**VSFIFRANW**TP	VSFIFRANW	**HLA-B*57:01; HLA-B*57:03; HLA-B*57:04; HLA-B*58:02; HLA-B*58:05**
	GDL**MVLINGFAL**AWL	MVLINGFAL	**HLA-C*08:01; HLA-C*08:03**
	VVP**RTDNITLAI**LAA	RTDNITLAI	**HLA-A*32:01; HLA-A*32:02; HLA-A*32:03**
	LPFYMALGLTAVPLV	LPFYMALGL/MALGLTAVP/YMALGLTAV	**HLA-B*81:01; HLA-B*81:02; HLA-B*81:03; HLA-B*42:01; HLA-B*42:04; HLA-B*42:05; HLA-B*51:04;HLA-B*55:03; HLA-B*55:04; HLA-B*67:01; HLA-B*81:05; HLA-B*82:01;HLA-B*82:02/HLA-A*33:03/HLA-A*02:03**
	SGK**RSWPPSEVL**TAV	RSWPPSEVL	**HLA-B*57:02; HLA-B*57:03; HLA-B*57:05**
NS2B	SWPP**SEVLTAVGL**IC	SEVLTAVGL	**HLA-B*40:01; HLA-B*40:04**
	LL**IVSYVVSGK**SVDM	IVSYVVSGK	**HLA-A*34:02; HLA-A*34:03; HLA-A*34:04**
	PM**REIILKVVL**MTIC	REIILKVVL	**HLA-B*40:01; HLA-B*40:02**
	VVL**MTICGMNPI**AIP	MTICGMNPI	**HLA-A*25:02; HLA-A*25:03; HLA-A*25:04; HLA-A*68:02; HLA-A*69:01; HLA-C*15:02; HLA-C*15:03; HLA-A*34:05; HLA-C*08:01; HLA-C*08:03; HLA-C*12:02; HLA-C*15:05; HLA-C*17:01; HLA-C*17:02; HLA-C*17:03; HLA-C*17:04; HLA-C*17:05**
	TIC**GMNPIAIPF**AAG	GMNPIAIPF	**HLA-B*15:03**
	M**NPIAIPFAA**GAWYV	NPIAIPFAA	**HLA-B*54:01; HLA-B*54:02**
	AIP**FAAGAWYVY**VKT Y	FAAGAWYVY/IPFAAGAW	**HLA-B*18:04; HLA-B*35:01; HLA-B*35:05; HLA-B*46:01; HLA-B*46:02; HLA-B*46:04; HLA-B*46:05; HLA-B*53:03; HLA-B*56:03; HLA-C*02:03; HLA-C*02:04; HLA-C*02:05; HLA-C*03:02; HLA-C*05:04; HLA-C*06:03; HLA-C*12:02; HLA-C*12:03; HLA-C*12:04; HLA-C*12:05; HLA-C*15:04; HLA-C*16:01; HLA-C*16:02; HLA-C*16:04; HLA-A*29:03; HLA-A*29:04; HLA-B*15:02; HLA-B*35:03; HLA-B*46:03; HLA-B*53:01; HLA-B*53:02; HLA-B*53:05; HLA-B*83:01;HLA-C*01:04; HLA-C*07:01; HLA-C*07:03; HLA-C*15:02; HLA-C*15:03; HLA-A*29:01; HLA-A*29:02; HLA-C*06:02; HLA-C*07:02; HLA-C*14:05/HLA-B*35:01; HLA-B*56:03; HLA-B*83:01; HLA-B*35:05; HLA-B*53:03**

	W**YVYVKTGKR**SGALW	YVYVKTGKR	**HLA-A*33:03**
	KRSG**ALWDVPAPK**EV	ALWDVPAPK*	**HLA-A*03:02; HLA-A*03:07; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
	KG**ETTDGVYRV**MTRR	ETTDGVYRV*	**HLA-A*68:02; HLA-A*69:01**
NS3	TD**GVYRVMTRRLL**GS	GVYRVMTRR/YRVMTRRLL	**HLA-A*74:01; HLA-A*74:02; HLA-A*74:03; HLA-A*31:01/HLA-B*27:05**
	T**RRLLGSTQV**GVGVM	RRLLGSTQV	**HLA-B*27:05**
	HTM**WHVTKGSALR**SG	WHVTKGSAL/HVTKGSALR	**HLA-B*15:10; HLA-B*14:01; HLA-B*14:02/HLA-A*33:03; HLA-A*68:01; HLA-A*33:01; HLA-A*34:02**
	PYWG**DVKQDLVSY**CG	DVKQDLVSY	**HLA-A*25:01; HLA-A*26:01**
	VIKNGS**YVSAITQGR**	YVSAITQGR	**HLA-A*68:01**
	FEPS**MLKKKQLTV**LD	MLKKKQLTV	**HLA-B*08:01**
	EAI**KTRLRTVI**LAPT	KTRLRTVIL	**HLA-A*30:01**
	MG**EAAAIFMTA**TPPG	EAAAIFMTA	**HLA-A*68:02**
	AAIF**MTATPPGTR**DA	MTATPPGTR	**HLA-A*68:01; HLA-A*34:02; HLA-A*33:03**
	RDA**FPDSNSPIM**DTE	FPDSNSPIM	**HLA-B*35:03; HLA-B*35:04; HLA-B*35:08**
	EVPE**RAWSSGFDW**VT	RAWSSGFDW	**HLA-B*57:01; HLA-B*57:03; HLA-B*58:01, HLA-B*58:02, HLA-B*15:16**
	HSG**KTVWFVPSVR**NG	KTVWFVPSV/TVWFVPSVR	**HLA-A*69:01/HLA-A*33:03; HLA-A*68:01; HLA-A*33:01; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03; HLA-A*31:01; HLA-A*34:02**
	SR**KTFETEFQK**TKHQ	KTFETEFQK	**HLA-A*74:01; HLA-A*74:02; HLA-A*74:03; HLA-A*03:02, HLA-A*11:01**
	I**SEMGANFKA**DRVID	SEMGANFKA	**HLA-B*45:01**
	AGP**MPVTHASAA**QRR	MPVTHASAA	**HLA-B*55:01; HLA-B*42:01**
	WLEA**RMLLDNIYL**QD	RMLLDNIYL	**HLA-C*07:04**
	EQR**KTFVELMKR**GDL	KTFVELMKR	**HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
	GD**LPVWLAYQV**ASAG	LPVWLAYQV	**HLA-B*55:01; HLA-B*51:01**
NS4A	AQLP**ETLETIMLL**GL	ETLETIMLL	**HLA-A*69:01; HLA-A*68:02**
	IG**KMGFGMVTL**GASA	KMGFGMVTL	**HLA-A*32:01**
	G**TVSLGIFFV**LMRNK	TVSLGIFFV	**HLA-A*69:01; HLA-A*68:02**
NS4B	IDL**RPASAWAIY**AAL	RPASAWAIY	**HLA-B*35:01; HLA-C*14:02; HLA-B*35:08; HLA-C*06:02; HLA-C*07:07**
	PA**SAWAIYAALTTF**I	SAWAIYAAL/AIYAALTTF	**HLA-C*01:02; HLA-C*01:03; HLA-C*07:01; HLA-C*07:02; HLA-C*07:04; HLA-C*08:04; HLA-C*08:13/ HLA-A*32:01; HLA-B*15:01**

	TF**ITPAVQHAVTTSY**	ITPAVQHAV/VQHAVTTSY	**HLA-A*69:01/HLA-B*15:01; HLA-B*15:03**
	VLF**GMGKGMPFY**AWD	GMGKGMPFY	**HLA-A*80:01**
	MPF**YAWDFGVPL**LMI	YAWDFGVPL	**HLA-C*08:04; HLA-C*08:13; HLA-C*01:02; HLA-C*03:03; HLA-C*03:04; HLA-C*07:01; HLA-C*07:02; HLA-C*08:02; HLA-C*15:02; HLA-C*15:05; HLA-C*16:01; HLA-C*17:01; HLA-C*17:04; HLA-C*01:03; HLA-C*03:02; HLA-C*05:01; HLA-C*07:04; HLA-C*07:05; HLA-C*07:07**
	AI**ILLVAHYMY**LIPG	ILLVAHYMY	**HLA-A*29:01; HLA-A*29:02**
	YM**YLIPGLQAA**AARA	YLIPGLQAA	**HLA-A*02:05**
	DID**TMTIDPQVEK**KM	MTIDPQVEK	**HLA-A*34:02; HLA-A*68:01; HLA-A*11:01**
	IAV**AVSSAILSR**TAW	AVSSAILSR	**HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
	W**GEAGALITA**ATSTL	GEAGALITA	**HLA-B*45:01**
	GAL**ITAATSTLW**EGS	ITAATSTLW	**HLA-B*15:16; HLA-B*57:01; HLA-B*58:01; HLA-B*15:17; HLA-B*57:03; HLA-B*58:02**
	RGS**YLAGASLIY**IVT	YLAGASLIY	**HLA-A*29:01; HLA-A*29:02; HLA-A*80:01; HLA-C*02:02; HLA-C*02:10**
NS5	NQ**MSALEFYSYK**KSG	MSALEFYSY/SALEFYSYK	**HLA-A*01:02; HLA-A*01:01; HLA-A*29:01; HLA-A*29:02; HLA-A*30:04; HLA-A*36:01; HLA-C*16:02; HLA-A*30:02; HLA-B*15:17; HLA-C*16:01/HLA-A*34:02; HLA-A*03:02; HLA-A*11:01; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03**
	L**VERGYLQPY**GKVID	VERGYLQPY	**HLA-B*18:01; HLA-B*44:02; HLA-B*50:01**
	WNIV**RLKSGVDVF**HM	RLKSGVDVF	**HLA-B*15:01**
	CDI**GESSSSPEV**EEA	GESSSSPEV	**HLA-B*45:01**
	CPYTS**TMMETLERL**Q	TMMETLERL	**HLA-A*02:02; HLA-A*02:11; HLA-A*02:22; HLA-A*02:01; HLA-A*02:05**
	RLQ**RRYGGGLVR**VPL	RRYGGGLVR	**HLA-B*27:05**
	RNSTH**EMYWVSGAK**S	EMYWVSGAK	**HLA-A*34:02**
	IR**SEHAETWFF**DENH	SEHAETWFF	**HLA-B*44:02; HLA-B*44:03; HLA-B*18:01**
	HAET**WFFDENHPY**RT	WFFDENHPY	**HLA-A*29:01; HLA-A*29:02; HLA-A*30:04**
	Y**RTWAYHGSY**EAPTQ	RTWAYHGSY	**HLA-A*30:02; HLA-A*30:04; HLA-B*15:17; HLA-A*29:01; HLA-A*29:02; HLA-A*32:01; HLA-A*80:01; HLA-B*15:16; HLA-A*01:02; HLA-A*74:01; HLA-A*74:02; HLA-A*74:03; HLA-C*01:03; HLA-C*03:02; HLA-C*06:02; HLA-C*07:01; HLA-C*07:07; HLA-C*14:02**
	G**IAMTDTTPY**GQQRV	IAMTDTTPY	**HLA-B*35:01; HLA-C*02:02; HLA-C*02:10; HLA-C*03:02; HLA-C*12:02; HLA-C*12:03; HLA-C*16:01; HLA-C*16:02; HLA-B*35:08**

QV**MSMVSSWLW**KELG	MSMVSSWLW/SMVSSWLWK	**HLA-B*57:01; HLA-B*57:03; HLA-B*58:01; HLA-B*58:02; HLA-B*15:16; HLA-B*53:01; HLA-B*15:17/HLA-A*03:01; HLA-A*03:02; HLA-A*11:01**
E**KEWKTAVEA**VNDPR	KEWKTAVEA	**HLA-B*45:01**
AK**GSRAIWYMW**LGAR	GSRAIWYMW	**HLA-B*57:01; HLA-B*58:02**
RAIW**YMWLGARFL**EF	YMWLGARFL	**HLA-C*07:04; HLA-C*07:01; HLA-C*07:02; HLA-C*07:05**
GARF**LEFEALGFL**NE	LEFEALGFL	**HLA-B*40:01; HLA-B*37:01; HLA-B*40:02; HLA-B*40:04**
IIK**YTYQNKVVKVL**R	YTYQNKVVK/YQNKVVKVL	**HLA-A*34:02/HLA-B*15:10**
QVVT**YALNTFTNL**VV	YALNTFTNL	**HLA-C*07:04; HLA-C*08:04; HLA-C*08:13; HLA-C*01:02; HLA-C*03:03; HLA-C*03:04; HLA-C*07:02; HLA-C*08:02; HLA-C*18:01; HLA-C*04:01; HLA-C*05:01; HLA-C*15:05; HLA-C*17:01; HLA-C*17:04**
EV**LEMQDLWLL**RRSE	LEMQDLWLL	**HLA-B*37:01; HLA-B*40:01; HLA-B*40:04; HLA-B*13:02; HLA-B*38:01; HLA-B*40:02; HLA-B*44:03; HLA-B*45:01; HLA-B*49:01**
T**QEWKPSTGW**DNWEE	QEWKPSTGW	**HLA-B*44:02; HLA-B*44:03**
A**KSYAQMWQLLYF**HR	KSYAQMWQL/SYAQMWQLL/YAQMWQLLY/AQMWQLLYF	**HLA-B*57:03; HLA-B*57:01; HLA-A*32:01; HLA-B*15:16; HLA-B*58:01/HLA-C*04:01/HLA-A*01:02; HLA-A*29:01; HLA-A*29:02; HLA-A*36:01; HLA-A*01:01; HLA-A*30:04; HLA-A*80:01; HLA-A*30:02/ HLA-B*15:03**
K**YMDYLSTQVRYL**GE	YMDYLSTQV/YLSTQVRYL	**HLA-A*02:01; HLA-A*02:02; HLA-A*02:11; HLA-A*02:22; HLA-A*02:34/HLA-A*02:02; HLA-A*02:05; HLA-A*02:22**

Fonte: Elaborado pelo autor

Tabela 6 - Supplementary Table S3: Peptide sequence for Zika virus

Protein	Sequence
Capsid	VARVSPFGGLKRLPA
	GLKRLPAGLLLGHGP
	HGPIRMVLAILAFLR
	AILAFLRFTAIKPSL
	AAMLRIINARKEKKR
Envelope	SNMAEVRSYCYEASI
	QPENLEYRIMLSVHG
	EPRTGLDFSDLYYLT
	GLDFSDLYYLTMNNK
	WLVHKEWFHDIPLPW
	KEALVEFKDAHAKRQ
	VSYSLCTAAFTFTKI
	AETLHGTVTVEVQYA
	NSKMMLELDPPFGDS
	IVIGVGEKKITHHWH
	KRMAVLGDTAWDFGS
	VLGDTAWDFGSVGGA
NS1	HPDSPRRLAAAVKQA
	SVSRMENIMWRSVEG
	RGPQRLPVPVNELPH
	FVRAAKTNNSFVVDG
	CPLEHRAWNSFLVED
	GFGVFHTSVWLKVRE
	NDTWRLKRAHLIEMK
	EMKTCEWPKSHTLWA
	SDLIIPKSLAGPLSH
	RSTTASGRVIEEWCC
	ECTMPPLSFRAKDGC
NS2A	STSMAVLVAMILGGF
	AKLAILMGATFAEMN
	FKVRPALLVSFIFRA
	PALLVSFIFRANWTP
	GDLMVLINGFALAWL
	VVPRTDNITLAILAA
	LPFVMALGLTAVRLV
	SGKRSWPPSEVLTAV
NS2B	SWPPSEVLTAVGLIC
	LLIVSYVVSGKSVDM
	PMREIILKVVLMTIC
	VVLMTICGMNPIAIP
	TICGMNPIAIPFAAG
	MNPIAIPFAAGAWYV
	AIPFAAGAWYVYVKT
	WYVYVKTGKRSGALW
	KRSGALWDVPAPKEV
	KGETTDGVYRVMTRR

Protein	Sequence
NS3	TDGVYRVMTRRLLGS
	TRRLLGSTQVGVGVM
	HTMWHVTKGSALRSG
	PYWGDVKQDLVSYCG
	VIKNGSYVSAITQGR
	FEPSMLKKKQLTVLD
	EAIKTRLRTVILAPT
	MGEAAAIFMTATPPG
	AAIFMTATPPGTRDA
	RDAFPDSNSPIMDTE
	EVPERAWSSGFDWVT
	HSGKTVWFVPSVRNG
	SRKTFETEFQKTKHQ
	ISEMGANFKADRVID
	AGPMPVTHASAAQRR
	WLEARMLLDNIYLQD
	EQRKTFVELMKRGDL
	GDLPVWLAYQVASAG
NS4A	AQLPETLETIMLLGL
	GTVSLGIFFVLMRNK
	IGKMGFGMVTLGASA
NS4B	IDLRPASAWAIYAAL
	PASAWAIYAALTTFI
	TFITPAVQHAVTTSY
	VLFGMGKGMPFYAWD
	MPFYAWDFGVPLLMI
	AIILLVAHYMYLIPG
	YMYLIPGLQAAAARA
NS5	DIDTMTIDPQVEKKM
	IAVAVSSAILSRTAW
	WGEAGALITAATSTL
	GALITAATSTLWEGS
	RGSYLAGASLIYIVT
	NQMSALEFYSYKKSG
	LVERGYLQPYGKVID
	WNIVRLKSGVDVFHM
	CDIGESSSSPEVEEA
	CPYTSTMMETLERLQ
	RLQRRYGGGLVRVPL
	RNSTHEMYWVSGAKS
	IRSEHAETWFFDENH
	HAETWFFDENHPYRT
	YRTWAYHGSYEAPTQ
	GIAMTDTTPYGQQRV
	QVMSMVSSWLWKELG
	EKEWKTAVEAVNDPR
	AKGSRAIWYMWLGAR
	RAIWYMWLGARFLEF
	GARFLEFEALGFLNE
	IIKYTYQNKVVKVLR
	QVVTYALNTFTNLVV
	EVLEMQDLWLLRRSE
	TQEWKPSTGWDNWEE
	AKSYAQMWQLLYFHR
	KYMDYLSTQVRYLGE

Fonte: Elaborado pelo autor

Tabela 7 - Supplementary Table S4: Peptide sequence for Dengue and Chikungunya viruses

Peptide sequence for Dengue virus	
Protein Dengue	**Sequence**
Capsid	VTLLVLIPTV
Capsid	AFIAFVRF
NS3	MIIVDEAHF
NS4B	NIQTAINQV
POLYPROTEIN	DPASIAARGY
POLYPROTEIN	TPEGIIPALF
POLYPROTEIN	DTTPFGQQR

Fonte: Elaborado pelo autor

Tabela 8 - Peptide sequence for Chikungunya virus

Protein	Sequence	Protein	Sequence	Protein	Sequence
	MDPVYVDIDADSAFLKALQR		MEFIPTQTFYNRRYQPRPWA		STKDNFNVYKATRPYLAHCP
	DSAFLKALQRAYPMFEVEPR		YNRRYQPRPWAPRPTIQVIR		ATRPYLAHCPDCGEGHSCHS
	AYPMFEVEPRQVTPNDHANA		PRPTIQVIRPRPRPQRQAGQ		DCGEGHSCHSPIALERIRNE
	RQVTPNDHANARAFSHLAIK		RPRPQRQAGQLAQLISAVNK		PIALERIRNEATDGTLKIQV
	RAFSHLAIKLIEQEIDPDST		LAQLISAVNKLTMRAVPQQK		ATDGTLKIQVSLQIGIKTDD
	IEQEIDPDSTILDIGSAPAR		LTMRAVPQQKPRRNRKNKKQ		SLQIGIKTDDSHDWTKLRYM
	ILDIGSAPARRMMSDRKYHC		PRRNRKNKKQRQKKQAPQND		SHDWTKLRYMDSHTPADAER
	RMMSDRKYHCVCPMRSAEDP		RQKKQAPQNDPKQKKQPPQK		DSHTPADAERAGLLVRTSAP
	VCPMRSAEDPERLANYARKL		PKQKKQPPQKKPAQKKKKPG		AGLLVRTSAPCTITGTMGHF
	ERLANYARKLASAAGKVLDR		KPAQKKKKPGRRERMCMKIE		CTITGTMGHFILARCPKGET
	ASAAGKVLDRNISEKIGDLQ		RRERMCMKIENDCIFEVKHE		ILARCPKGETLTVGFTDSRK
	RNISEKIGDLQAVMAVPDAE		ENDCIFEVKHEGKVMGYACL		LTVGFTDSRKISHTCTHPFH
	AVMAVPDAETPTFCLHTDVS		GKVMGYACLVGDKVMKPAHV		ISHTCTHPFHHEPPVIGRER
	PTFCLHTDVSCRQRADVAIY	**Capsid**	GDKVMKPAHVKGTIDNADLA		HEPPVIGRERFHSRPQHGKE
	CRQRADVAIYQDVYAVHAPT		KGTIDNADLAKLAFKRSSKY		FHSRPQHGKELPCSTYVQST
	YQDVYAVHAPTSLYHQAIKG		KLAFKRSSKYDLECAQIPVH		LPCSTYVQSTAATAEEIEVH
	SLYHQAIKGVRVAYWIGFDT		DLECAQIPVHMKSDASKFTH		AATAEEIEVHMPPDTPDRTL
	RVAYWIGFDTTPFMYNAMAG		MKSDASKFTHEKPEGYYNWH		MPPDTPDRTLMTQQSGNVKI
	TPFMYNAMAGAYPSYSTNWA		EKPEGYYNWHHGAVQYSGGR		MTQQSGNVKITVNGQTVRYK
	AYPSYSTNWADEQVLKAKNI		HGAVQYSGGRFTIPTGAGKP		TVNGQTVRYKCNCGGSNEGL
	DEQVLKAKNIGLCSTDLTEG		FTIPTGAGKPGDSGRPIFDN		CNCGGSNEGLTTTDKVINNC
	GLCSTDLTEGRRGKLSIMRG		GDSGRPIFDNKGRVVAIVLG		TTTDKVINNCKIDQCHAAVT
	RRGKLSIMRGKKMKPCDRVL		KGRVVAIVLGGANEGARTAL	**E2**	KIDQCHAAVTNHKNWQYNSP
	KKMKPCDRVLFSVGSTLYPE		GANEGARTALSVVTWNKDIV		TNHKNWQYNSPLVPRNAELG
	FSVGSTLYPESRKLLKSWHL		SVVTWNKDIVTKITPEGAEEW		LVPRNAELGDRKGKIHIPFP
	SRKLLKSWHLPSVFHLKGKL				RKGKIHIPFPLANVTCRVPK
	PSVFHLKGKLSFTCRCDTVV				LANVTCRVPKARNPTVTYGK
NSP1	SFTCRCDTVVSCEGYVVKRI				ARNPTVTYGKNQVTMLLYPD
	SCEGYVVKRITISPGLYGKT				KNQVTMLLYPDHPTLLSYRN
	TISPGLYGKTTGYAVTHHAD				HPTLLSYRNMGQEPNYHEEW
	TGYAVTHHADGFLMCKTTDT				GQEPNYHEEWVTHKKEVTLT
	GFLMCKTTDTVDGERVSFSV				VTHKKEVTLTVPTEGLEVTW
	VDGERVSFSVCTYVPATICD				VPTEGLEVTWGNNEPYKYWP
	CTYVPATICDQMTGILATEV				GNNEPYKYWPQMSTNGTAHG
	DQMTGILATEVTPEDAQKLL				MSTNGTAHGHPHEIILYYYE
	TPEDAQKLLVGLNQRIVVNG				HPHEIILYYYELYPTMTVVI
	GLNQRIVVNGRTQRNTNTMK				ELYPTMTVVIVSVASFVLLS
	RTQRNTNTMKNYLLPVVAQA				VSVASFVLLSMVGTAVGMCV
	KNYLLPVVAQAFSKWAKECR				MVGTAVGMCVCARRRCITPY
	FSKWAKECRKDMEDEKLLGI				CARRRCITPYELTPGATVPF
	DMEDEKLLGIRERTLTCCCL				ELTPGATVPFLLSLLCCVRT
	RERTLTCCCLWAFKKQKTHT				LLSLLCCVRTTKA
	WAFKKQKTHTVYKRPDTQS				
	VYKRPDTQSIQKVPAEFDSF				
	IQKVPAEFDSFVVPSLWSSG				
	VVPSLWSSGLSIPLRTRIKW				
	SIPLRTRIKWLLSKVPKTDL				
	LLSKVPKTDLIPYSGDAKEA				
	IPYSGDAKEARDAEKEAEEE				
	RDAEKEAEEEREAELTREAL				
	REAELTREALPPLQAAQDDV				
	PPLQAAQDDVQVEIDVEQLE				
	VQVEIDVEQLEDRAGA				

Fonte: Elaborado pelo autor

Tabela 9 - Supplementary Table S5: Peptide sequence for Yellow-fever

Protein Yellow-fever	Sequence
Env	AHCIGITDRDFIEGV
	GITDRDFIEGVHGGT
	PAEARKVCYNAVLTH
	RKVCYNAVLTHVKIN
	YNAVLTHVKINDKCP
	LTHVKINDKCPSTGE
	WREMHHLVEFEPPHA
	HHLVEFEPPHAATIR
	EFEPPHAATIRVLAL
	PKGAPCKIPVIVADD
	PCRIPVIVADDLTAA
	PVIVADDLTAAINKG
	KINDKCPSTGEAHLA
	KCPSTGEAHLAEENE
	WQSGSGGVWREMHHL
	SGGVWREMHHLVEFE
	ADDLTAAINKGILVT
	TAAINKGILVTVNPI
	NKGILVTVNPIASTN
	LVTVNPIASTNDDEV
	NPIASTNDDEVLIEV
	STNDDEVLIEVNPPF
	DEVLIEVNPPFGDSY
NS1	GEIHAVPFGLVSMMI
	TRVYMDAVFEYTIDC
NS2	PLVALTLTSYLGLTQ
	LTQPFLGLCAFLATR
	SLALVGAALHPFALL
	VGAALHPFALLLVLA
	LHPFALLLVLAGWLF
NS3	SGREVIDAMCHATLT
	VIDAMCHATLTYRML
	MCHATLTYRMLEPTR
	PSEPWNTGHDWILAD
	WNTGHDWILADKRPT
	VLVDEGRKVAIKGPL
	LAECARRRLRTLVLA
	ARRRLRTLVLAPTRV
	HGLDVKFHTQAFSAH
	GEAMDTISVFLHSEE
NS4	IVMLFILAGLLTSGM
	IGCAMLHWSLILPGI
	SLLWNGPMAVSMTGV
NS5	TDTTPFGQQRVFKEK
	AKGSRAIWYMWLGAR
	GARYLEFEALGFLNE
	CVVRPIDDRFGLALS
Capsid	LRKVKRVVASLMRGL

Fonte: Elaborado pelo autor

Tabela 10 - Supplementary Table S6: Peptide sequence for EBV virus

Protein	Sequence
BMLF1	GLCTLVAML
BZLF1	EPLPQGQLTAY
	RAKFKQLL
EBNA1	HPVGEADYFEY
EBNA3A	RLRAEAQVK
	RYSIFFDY
	RPPIFIRRL
	VPAPAGPIV
	FRLGRAYGL
	LEKARGSTY
	YPLHEQHGM
	QAKWRLQTL
	AYSSWMYSY
	VFSDGRVAC
	SVRDRLARL
EBNA3B	AVFDRKSDAK
	IVTDFSVIK
	TYSAGIVQI
	VEITPYKPTW
	GQGGSPTAM
	HRCQAIRKK
	RRARSLSAERY
	AVLLHEESM
EBNA3C	LLDFVRFMGV
	QPRAPIRPI
	EGGVGWRHW
	LRGKWQRRYR
	RRIYDLIEL
	HHIWQNLL
	KEHVIQNAF
	FRKAQIQGL
	QNGALAINTF
	EENLLDFVRF
LMP2	SSCSSCPLSKI
	TYGPVFMCL
	LLWTLVVLL
	PYLFWLAAI
	IEDPPFNSL
	RRRWRRLTV
	VMSNTLLSAW
	LTAGFLIFL
	CLGGLLTMV

Fonte: Elaborado pelo autor

Tabela 11 - Supplementary Table S7: Percentages of homology between ZIKV protein and the 4 DENV serotypes

ZIKV		Homology for DENV Serotypes				Median homology for the 4 DENV serotypes
Protein	**Amino Acid Sequence**	**1**	**2**	**3**	**4**	
Capsid	VA**RVSPFGGLK**RLPA	46%	46%	40%	55%	46%
	G**LKRLPAGLLL**GHGP	60%	60%	62%	53%	60%
	HGPI**RMVLAILAFL**R	87%	87%	87%	80%	87%
	AI**LAFLRFTAI**KPSL	73%	73%	73%	60%	73%
	A**AMLRIINAR**KEKKR	67%	58%	75%	58%	63%
Median homology for each DENV serotype		67%	60%	73%	58%	63%
	SN**MAEVRSYCY**EASI	60%	67%	53%	85%	64%
	QPEN**LEYRIMLSV**HG	87%	87%	87%	87%	87%
	EPRT**GLDFSDLYY**LT	80%	73%	80%	80%	80%
	GLD**FSDLYYLTM**NNK	73%	73%	80%	67%	73%
	WLVH**KEWFHDIPL**PW	93%	93%	93%	93%	93%
	KEALV**EFKDAHAKR**Q	80%	80%	87%	73%	80%
	V**SYSLCTAAFTFTK**I	60%	60%	60%	67%	60%
	A**ETLHGTVTV**EVQYA	80%	73%	80%	80%	80%
	NSK**MMLELDPPF**GDS	67%	80%	67%	80%	74%
	IVIGV**GEKKITHHW**H	60%	60%	73%	67%	64%
	KR**MAVLGDTAW**DFGS	100%	100%	100%	100%	100%
	VLG**DTAWDFGSV**GGA	93%	93%	93%	100%	93%
Median homology for each DENV serotype		80%	77%	80%	80%	80%
NS1	HPD**SPRRLAAAV**KQA	80%	87%	67%	73%	77%
	SVSR**MENIMWRSV**EG	73%	80%	73%	73%	73%
	RGPQ**RLPVPVNEL**PH	40%	40%	47%	67%	44%
	FV**RAAKTNNSF**VVDG	60%	53%	67%	53%	57%
	CP**LEHRAWNSF**LVED	67%	73%	67%	73%	70%
	GFGV**FHTSVWLKV**RE	93%	93%	93%	87%	93%
	NDT**WRLKRAHLI**EMK	80%	87%	87%	87%	87%
	EMKT**CEWPKSHTL**WA	93%	87%	87%	93%	90%
	SDLI**IPKSLAGPL**SH	73%	93%	93%	73%	83%
	RSTT**ASGRVIEEW**CC	80%	93%	87%	93%	90%
	E**CTMPPLSFR**AKDGC	80%	80%	73%	73%	77%
Median homology for each DENV serotype		74%	79%	76%	77%	77%
NS2A	STS**MAVLVAMIL**GGF	62%	50%	44%	78%	56%
	AK**LAILMGATF**AEMN	60%	67%	47%	47%	54%
	FKV**RPALLVSFI**FRA	60%	47%	60%	53%	57%

	PALL**VSFIFRANW**TP	40%	27%	40%	40%	40%
	GDL**MVLINGFAL**AWL	0%	47%	53%	67%	50%
	VVP**RTDNITLAI**LAA	0%	0%	40%	53%	20%
	LPFVMALGLTAVRLV	0%	0%	67%	0%	0%
	SGK**RSWPPSEVL**TAV	0%	0%	0%	0%	0%
Median homology for each DENV serotype		28%	30%	44%	42%	45%
NS2B	SWPP**SEVLTAVGL**IC	73%	73%	73%	73%	73%
	LL**IVSYVVSGK**SVDM	73%	80%	73%	80%	77%
	PM**REIILKVVL**MTIC	47%	47%	53%	67%	50%
	VVL**MTICGMNPI**AIP	67%	67%	60%	80%	67%
	TIC**GMNPIAIPF**AAG	53%	60%	47%	60%	57%
	M**NPIAIPFAA**GAWYV	53%	73%	47%	60%	57%
	AIP**FAAGAWYVY**VKT	47%	60%	47%	67%	54%
	W**YVYVKTGKR**SGALW	60%	60%	60%	89%	60%
	KRSG**ALWDVPAPK**EV	0%	0%	0%	0%	0%
	KG**ETTDGVYRV**MTRR	0%	0%	0%	0%	0%
Median homology for each DENV serotype		47%	52%	46%	58%	57%
NS3	TD**GVYRVMTRRLL**GS	79%	64%	64%	73%	69%
	T**RRLLGSTQV**GVGVM	73%	67%	67%	67%	67%
	HTM**WHVTKGSALR**SG	73%	73%	73%	73%	73%
	PYWG**DVKQDLVSY**CG	73%	80%	73%	73%	73%
	VIKNGS**YVSAITQGR**	73%	73%	60%	73%	73%
	FEPS**MLKKKQLTV**LD	67%	73%	67%	73%	70%
	EAI**KTRLRTVI**LAPT	93%	87%	93%	93%	93%
	MG**EAAAIFMTA**TPPG	100%	93%	100%	100%	100%
	AAIF**MTATPPGTR**DA	93%	87%	93%	80%	90%
	RDA**FPDSNSPIM**DTE	67%	80%	73%	67%	70%
	EVPE**RAWSSGFDW**VT	100%	93%	93%	100%	97%
	HSG**KTVWFVPSVR**NG	87%	80%	87%	87%	87%
	SR**KTFETEFQK**TKHQ	93%	80%	87%	80%	84%
	I**SEMGANFKA**DRVID	100%	100%	100%	93%	100%
	AGP**MPVTHASAA**QRR	93%	100%	93%	93%	93%
	WLEA**RMLLDNIYL**QD	73%	73%	73%	80%	73%
	EQR**KTFVELMKR**GDL	93%	93%	93%	100%	93%
	GD**LPVWLAYQV**ASAG	93%	93%	93%	100%	93%
Median homology for each DENV serotype		85%	83%	82%	84%	85%
NS4A	AQLP**ETLETIMLL**GL	93%	86%	100%	93%	93%
	IG**KMGFGMVTL**GAS	67%	64%	67%	73%	67%
	G**TVSLGIFFV**LMRNK	40%	60%	40%	60%	50%
Median homology for each DENV serotype		67%	70%	69%	75%	67%
NS4B	IDL**RPASAWAIY**AAL	73%	80%	73%	80%	77%

	PA**SAWAIYAALTTF**I	73%	80%	73%	73%	73%
	TF**ITPAVQHAVTTSY**	60%	73%	60%	60%	60%
	VLF**GMGKGMPFY**AW	53%	60%	53%	67%	57%
	MPF**YAWDFGVPL**LMI	53%	53%	53%	60%	53%
	AI**ILLVAHYMY**LIPG	80%	73%	73%	73%	73%
	YM**YLIPGLQAA**AARA	60%	60%	60%	60%	60%
	DID**TMTIDPQVEK**KM	67%	73%	73%	80%	73%
	IAV**AVSSAILSR**TAW	53%	60%	53%	47%	53%
	W**GEAGALITA**ATSTL	47%	47%	47%	40%	47%
	GAL**ITAATSTLW**EGS	60%	60%	60%	53%	60%
	RGS**YLAGASLIY**IVT	83%	92%	83%	83%	83%
Median homology for each DENV serotype		64%	68%	63%	65%	60%
NS5	NQ**MSALEFYSYK**KSG	80%	60%	73%	60%	67%
	L**VERGYLQPY**GKVID	73%	67%	67%	87%	70%
	WNIV**RLKSGVDVF**HM	73%	93%	87%	87%	87%
	CDI**GESSSSPEV**EEA	80%	73%	87%	87%	84%
	CPYTS**TMMETLERL**Q	80%	67%	73%	73%	73%
	RLQ**RRYGGGLVR**VPL	87%	80%	87%	87%	87%
	RNSTH**EMYWVSGAK**S	73%	80%	73%	87%	77%
	IR**SEHAETWFF**DENH	80%	73%	67%	73%	73%
	HAET**WFFDENHPY**RT	80%	80%	73%	80%	80%
	Y**RTWAYHGSY**EAPTQ	73%	73%	73%	93%	73%
	G**IAMTDTTPY**GQQRV	93%	93%	93%	93%	93%
	QV**MSMVSSWLW**KELG	60%	80%	67%	67%	67%
	E**KEWKTAVEA**VNDPR	67%	73%	53%	73%	70%
	AK**GSRAIWYMW**LGAR	100%	100%	100%	100%	100%
	RAIW**YMWLGARFL**EF	100%	100%	100%	100%	100%
	GARF**LEFEALGFL**NE	100%	100%	100%	100%	100%
	IIK**YTYQNKVVKVL**R	80%	80%	80%	87%	80%
	QVVT**YALNTFTNL**VV	73%	73%	73%	80%	73%
	EV**LEMQDLWLL**RRSE	33%	20%	47%	40%	37%
	T**QEWKPSTGW**DNWEE	80%	80%	73%	73%	77%
	A**KSYAQMWQLLYF**HR	93%	87%	87%	87%	87%
	K**YMDYLSTQVRYL**GE	67%	58%	60%	58%	59%
Median homology for each DENV serotype		78%	77%	77%	81%	77%

Fonte: Elaborado pelo autor

5 DISCUSSÃO

Ao compararmos as manifestações clínicas do primeiro relato do ZIKV no Brasil (7 pacientes) com o estudo transversal (2º. estudo) - que incluiu 78 pacientes – podemos observar que as principais manifestações foram mialgia, cefaleia, febre (reportada ou medida) e erupção cutânea (rash).

A presença de rash cutâneo variou de 85,7% para 50,0% do 1º. estudo em relação ao segundo, que pode ser explicado pelo tamanho amostral maior no 2º. estudo, com estimativa possivelmente mais próxima do esperado.

Em relação a sintomas inespecíficos tais como náuseas ou vômitos, na publicação de Olson e Ksiazec (1981) ocorrem em 14,3% dos pacientes versus 23,1% no 2º estudo aqui presente.

Diarréia foi reportada em 12,8% no 2º estudo aqui presente versus 42,8% no estudo de Olson e Ksiazec (1981).

Assim, sintomas inespecíficos gastrointestinais podem ocorrer na infecção aguda pelo ZIKV e representam mais um rol de sintomas a serem suspeitados em pacientes de áreas endêmicas que chegam a unidades de pronto-atendimento com exantema, febre e conjuntivite seca.

O diagnóstico da infecção pelo ZIKV nessas unidades de pronto-atendimento (UPA) requer assim uma atenção clínica minuciosa, passando por questionar diversos sintomas inespecíficos como mialgia, cefaleia, artralgia, febre reportada (“febre interna”), além de náuseas, dor na barriga, diarreia, além de coceira no corpo e nos olhos.

Esses sintomas juntamente com um exame físico que mostre pelo menos um dos seguintes sinais – linfadenopatia cervical posterior, exantema cutâneo maculoeritematoso difuso ou máculopapular, pruriginoso ou não, e discreta conjuntivite seca – compõem as variáveis clínicas mais importantes para o diagnóstico de infecção pelo ZIKV.

Assim, os principais sintomas clínicos da infecção pelo ZIKV mostram-se pouco intensos, raramente levando os pacientes a serem internados, além de pouco específicos. São sugestivos de um processo infeccioso sistêmico brando, autolimitado, não-grave, e que requerem elevada suspeição clínica para seu diagnóstico.

No entanto o sequenciamento metagenômico (mNGS) de amostras de 15 pacientes com infecção pelo ZIKV, entre abril de 2015 a janeiro de 2016, identificou coinfecções com o CHIKV em 2 casos (13,3%) (SARDI *et al*., 2016). A proporção de coinfecções por CHIKV em nosso 2o.

estudo foi menor (5,1%) do que esse estudo de Sardi *et al*.(2016). Isso possivelmente se deveu ao fato de que no 2o. trabalho que compõe essa TESE o período amostral foi até 2017, diferentemente do período de maior transmissibilidade tanto do ZIKV quanto do CHIKV que abrangeu o período mais crítico de ambas transmissões. De toda forma foi bastante significativo que entre 5,1% e 13,3% de pessoas infectadas pelo ZIKV estavam coinfectadas com CHIKV em período de transmissão vetorial em potencial para ambos vírus. Isso aponta para a necessidade de sempre se considerar a possibilidade de coinfecções em comunidades submetidas a transmissão vetorial por diversos arbovirus de forma simultânea. Em nosso estudo (2^o.) os pacientes coinfectados com CHIKV apresentavam intensa artralgia/artrite, alguns com aftas bucais, o que apontou para a predominância do quadro de CHIKV sobre o de ZIKV diferentemente do estudo anterior em que 1 paciente tinha apresentado sintomas articulares e ou outro não (BANDEIRA *et al*., 2020). Nesse sentido a ocorrência de artralgia severa é um marcador da possibilidade não somente de CHIKV mas da possiblidade também de coinfeção CHIKV-ZIKV em pacientes de áreas endêmicas. Não foi observado em nenhum dos dois estudos com pacientes coinfectados uma maior prevalênica da síndrome de Guillain-Barré, nem de quadros neurológicos inflamatórios tais como encefalites, meningoencefalites ou mieloradiculites (SARDI *et al.,* 2016; BANDEIRA *et al*., 2020). No entanto a amostra de coinfectados (7 pacientes agregando os 2 estudos) ainda não pode ser considerada suficientemente adequada para uma inferência definitiva dos riscos de quadros neurológicos em pacientes coinfectados, havendo necessidade de mais estudos nessa população específica.

Juntamente com esse quadro clínico pouco específico, e autolimitado, os exames laboratoriais de triagem de pacientes com síndrome febril aguda, ajudam de forma limitada a diferenciar pacientes com ZIKV em relação a outros pacientes com processos virais agudos. Fica claro que a leucometria global mantém-se em níveis normais ou ligeiramente baixos como apontam a média do 1^o estudo e a mediana do 2^o estudo, em 3.750 e 5.505 leucócitos/mm^3, respectivamente, assim como os valores médios de plaquetas, das provas de atividade inflamatória tais como a proteína C reativa, e até mesmos os das enzimas hepáticas. Para todas essas variáveis laboratoriais os resultados esperados na infecção aguda pelo ZIKV geralmente encontram-se dentro da faixa normal ou ligeiramente alterados. São resultados com muito pouco valor preditivo positivo ou negativo para a infecção aguda pelo ZIKV.

No entanto algumas alterações laboratoriais podem ajudar a diferenciar a infecção pelo ZIKV em relação a Dengue.

Em relação ao hemograma não se espera hemoconcentração na infecção pelo ZIKV tanto por seus mecanismos fisiopatogênicos quanto pelo evidenciado no 2o. estudo aqui presente. A

mediana de hematócrito no 2º estudo foi de 41% com amplitude que não ultrapassou 49%. Uma das consequências da infecção pelo DENV é a hemoconcentração, especialmente naqueles pacientes que evoluem com sinais de alarme. As taxas globais de leucócitos tiveram mediana em 5.505 por mm^3, variando de discrete leucopenia a poucos casos de leucocitose franca, com linfócitos atípicos podendo restar presentes (variação de 0%-12%). Esse dado também ajuda na diferenciação em relação a doenças bacterianas febris em pacientes atendidos nas unidades de pronto-atendimento. A taxa de plaquetas mostrou-se normal na imensa maioria dos pacientes, com mediana de 217.000 por mm3, e somente 1,3% dos pacientes com infecção pelo ZIKV apresentaram taxas menores do que 100.000 por mm^3. Esse último dado aponta para uma diferença significativa quando comparado aos valores de plaquetas na Dengue. É bastante comum encontrarmos valores abaixo de 100.000 por mm^3 em pacientes com infecção pelo DENV. Em um estudo indiano 70% dos pacientes com dengue clássica apresentavam plaquetas abaixo de 100.000 por mm^3 (JAYASHREE *et al*., 2011), contrastando bastante com os pacientes com infecção pelo ZIKV.

Em relação aos parâmetros bioquímicos os pacientes no 2º estudo mostraram valores de proteína C reativa (PCR) com mediana de 1,8mg/dL, compatível com processos virais inespecíficos, porém muito pouca alteração nas enzimas hepáticas. Somente 3,2% dos pacientes no 2º estudo mostraram elevações de AST (TGO) maior do que 100 U/L, bastante diferente do esperado em pacientes infectados pelo DENV em que até 65% dos pacientes apresentam elevações nas aminotransferases (SOUZA *et al*., 2004).

Agregando as evidências laboratoriais aos sinais e sintomas clínicos previamente discutidos, podemos apontar que os pacientes com ZIKV apresentam-se com leucograma normal ou discretamente leucopênicos, sem hemoconcentração, com discreta elevação da atividade inflamatória medida pela proteína C reativa, com plaquetas e enzimas hepáticas normais.

Com essa combinação de sinais/sintomas clínicos e laboratoriais temos ferramentas para diferenciar um paciente com Zika em relação ao paciente com Dengue ou Chikungunya.

Na infecção pelo CHIKV espera-se maior comprometimento articular, muito frequentemente poliarticular, progressivo, com edema periarticular e restrição a movimentação e dor. Na infecção pelo DENV espera-se febre alta, exantemas mais intensos e morbiliformes, plaquetopenia, hemoconcentração e elevação das aminotransferases.

Na tabela abaixo é proposto um algoritmo facilitador para se identificar os pacientes com infecção pelo ZIKV (Tabela 1). Agregando as informações clínicas com os achados laboratoriais é possível a estratificação de pacientes com maior probabilidade de infecção pelo ZIKV. Assim,

a presença de pelo menos 2 sinais/sintomas clínicos com ao menos 2 critérios laboratoriais compatíveis com ZIKV baseados no 2º estudo aponta para alta probabilidade de ZIKV frente as outras arboviroses, devendo o caso ser tratado como ZIKV. Na ausência da coleta para exames laboratoriais (ou somente 1 achado laboratorial compatível) associado a pelo menos 2 sinais/sintomas clínicos deve ser considerada a possiblidade de ZIKV se há circulação ativa do vírus na região. Menos de 2 sinais/sintomas clínicos tornam menor a probabilidade de infecção pelo ZIKV e nessa situação a avaliação deve ser individual caso a caso pelo profissional médico. Em todas as situações de suspeita o caso deve ser notificado e coletadas amostras para confirmação etiológica do ZIKV (seja através do rt-PCR ou por sorologia).

Tabela 12 - Algoritmo para diagnóstico clínico-laboratorial da infecção pelo ZIKV

Critérios Clinicos	**Critérios laboratoriais**	**Interpretação**
Exantema máculoeritematoso, papular ou em placas **Conjuntivite não-exudativa** **Linfonodomegalia cervical posterior** **Mialgia** **Artralgia leve e transitória** **Náuseas** **Cefaléia** **Febre (medida ou reportada)**	**Ausência hemoconcentração** **Plaquetas > 100.000/mm^3** **AST/ALT < 100 U/L**	Presença de manifestação cutânea* + ≥ 1 critério clínico + ≥ 2 critérios laboratoriais: Considerar e tratar como ZIKV Pelo menos 2 critérios clínicos com 1 critério laboratorial ou sem possibilidade de coleta laboratorial: Considerar como caso provável de ZIKV e tratar se epidemiologia compatível < 2 critérios clínicos independente dos critérios laboratoriais: Avaliação individual do risco. Considerar outros diagnósticos.

* Exantema máculoeritematoso, papular ou em placas

Fonte: Elaborado pelo autor

Ao analisarmos os resultados da resposta imune celular fica também evidente a baixa prevalência de reconhecimento dos peptídeos do ZIKV, ao redor de 30%, em pacientes com infecção aguda pelo ZIKV. E mesmo aqueles que mostram reconhecimento pelas células T dos peptídeos de ZIKV os valores de ELISPOT mostram magnitude baixa como evidenciado pela mediana de 89 SFC/10^6 PBMC para os respondedores. A produção de interferon-gama, apesar de estatisticamente mais elevada nos pacientes com infecção aguda pelo ZIKV, é relativamente baixa, e se relaciona aos sintomas leves e às discretas alterações laboratoriais.

Por isso é muito difícil o diagnóstico da infecção pelo ZIKV por critérios clínicos ou laboratoriais. Isso contrasta com a infecção pelo DENV em que os pacientes apresentam rash morbiliforme, febre alta, sangramentos e frequentes alterações laboratoriais como plaquetopenia ou elevação das enzimas hepáticas. Da mesma forma, a infecção pelo CHIKV afeta tão frequentemente as articulações, levando a uma artropatia subaguda ou crônica com características semelhantes, e o diagnóstico sorológico é relativamente tão confiável, que o diagnóstico é relativamente direto.

6 CONSIDERAÇÕES FINAIS

Isso tem implicações muito importantes. Do ponto-de-vista clínico a infecção pelo ZIKV se comporta como uma infecção viral leve, inespecífica e autolimitada, confundindo-se com outras infecções virais. Dessa forma é muito frequentemente subdiagnosticada, e assim termina sendo subnotificada, e sua real incidência sendo subdimensionada.

Do ponto-de-vista da vigilância epidemiológica e das políticas de saúde pública, a infecção pelo ZIKV tende a ser subrepresentada e o investimento em seu controle e redução bastante subdimensionado.

A repercussão imediata é a falsa percepção de que o vírus não circula mais tanto nas cidades, com isso não havendo necessidade em se produzir políticas públicas para se melhorar o diagnóstico por um lado, e o controle vetorial da doença por outro.

Sem ferramentas diagnósticas específicas como o rt-PCR é impossível a real estimativa das taxas de incidência dessa doença e seu impacto.

E sem se ter o real conhecimento da incidência da Zika não haverá motivação para se alertar as mulheres em idade reprodutiva e as gestantes do risco em potencial de se contaminarem durante a gestação.

Somente com o investimento em técnicas diagnósticas precisas e com custos acessíveis podemos melhorar o diagnóstico da Zika, aumentar o número de casos notificados, informar melhor aos gestores do perigo real que a circulação do vírus representa para as mulheres em idade reprodutiva, e assim criar políticas de saúde que alertem para a presença do vírus, que divulguem formas de prevenção individual para essa população vulnerável, e que ajam no meio ambiente reduzindo a transmissão vetorial.

Esse é o principal desafio a ser enfrentado a partir das evidências geradas pelos 3 estudos clinicos e imunológicos que fazem parte dessa Tese, e que juntamente com os outros trabalhos científicos publicados nesse período, ajudam a melhor entender a dinâmica da infecção pelo ZIKV.

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APÊNDICES

Apêndice A – Artigo publicado: Management of infection by the zika virus

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REVIEW Open Access

Management of infection by the Zika virus

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Apêndice B – Artigo publicado: Neonatal encephalitis due to chikungunya vertical transmission: first report in Brazil

IDCases 5 (2016) 57–59

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Case study

Neonatal encephalitis due to Chikungunya vertical transmission: First report in Brazil

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Apêndice C – Artigo publicado: Distinct zika virus lineage in salvador, Bahia, Brazil

DISPATCHES

Distinct Zika Virus Lineage in Salvador, Bahia, Brazil

Samia N. Naccache,[1] Julien Thézé,[1] Silvia I. Sardi, Sneha Somasekar, Alexander L. Greninger, Antonio C. Bandeira, Gubio S. Campos, Laura B. Tauro, Nuno R. Faria, Oliver G. Pybus, Charles Y. Chiu

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Apêndice D – Artigo publicado: Coinfections of zika and chikungunya viruses in Bahia, Brazil, identified by metagenomic next- generation sequencing

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

Coinfections of Zika and Chikungunya Viruses in Bahia, Brazil, Identified by Metagenomic Next-Generation Sequencing

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Journal of Clinical Microbiology September 2016 Volume 54 Number 9

A**pêndice E** – Artigo publicado: The expanding spectrum of modes of transmission of zika virus: a global concern

Rodriguez-Morales *et al.*
Ann Clin Microbiol Antimicrob (2016) 15:13
DOI 10.1186/s12941-016-0128-2

Annals of Clinical Microbiology and Antimicrobials

EDITORIAL Open Access

The expanding spectrum of modes of transmission of Zika virus: a global concern

Alfonso J. Rodriguez-Morales[1,2*], Antonio Carlos Bandeira[3] and Carlos Franco-Paredes[4,5]

Apêndice F – A**rtigo publicado: First detection of chikungunya virus in breast milk**

The Pediatric Infectious Disease Journal • Volume 36, Number 10, October 2017

FIRST DETECTION OF CHIKUNGUNYA VIRUS IN BREAST MILK

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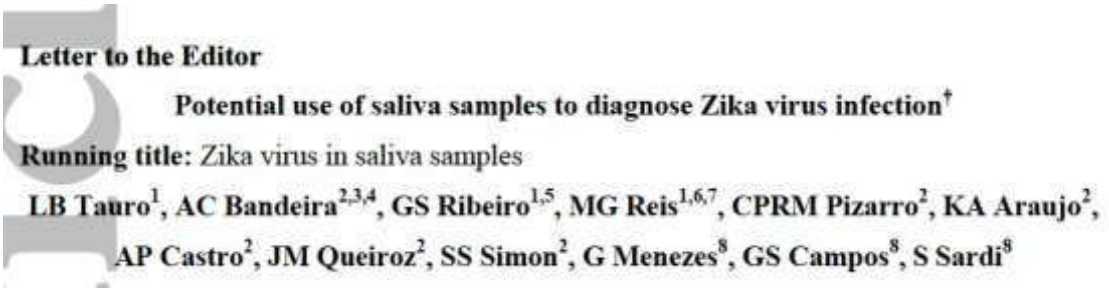
Verônica França Diniz Rocha, MD,‡ Juarez Pereira Dias, MD,§
Rejane Hughes Carvalho, PhD, and Silvia Ines Sardi, PhD**

Abstract: Chikungunya virus (CHIKV) has never been detected in human breast milk. This is a brief report of CHIKV infection in a breastfeeding woman of a 3-month-old baby. The mother's CHIKV-RT PCR was positive in serum, urine and milk. The baby's CHIKV serology and reverse transcription polimerase chain reaction (RT-PCR) were negative. The detection of CHIKV in milk raises clinical and epidemiologic questions.

Key Words: chikungunya, breast milk, chikungunya infection

Apêndice G – Artigo publicado: Potential use of saliva samples to diagnose zika virus infection



Letter to the Editor

Potential use of saliva samples to diagnose Zika virus infection†

Running title: Zika virus in saliva samples

LB Tauro[1], AC Bandeira[2,3,4], GS Ribeiro[1,5], MG Reis[1,6,7], CPRM Pizarro[2], KA Araujo[2], AP Castro[2], JM Queiroz[2], SS Simon[2], G Menezes[8], GS Campos[8], S Sardi[8]

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Apêndice H - Artigo publicado: Chikungunyavirus infection associated with encephalitis and anterior uveitis

Ocular Immunology and Inflammation

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Chikungunya Virus Infection Associated with Encephalitis and Anterior Uveitis

Verônica França Diniz Rocha, Adriano Hasler Principe de Oliveira, Antonio Carlos Bandeira, Silvia Ines Sardi, Rodrigo Freaza Garcia, Samuel de Araújo Magalhães, Camila Alves Sampaio & Gubio Campos Soares

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Apêndice I – Artigo publicado: detection of oropouche virus in saliva and urine samples of febrile patients in Salvador, Bahia, Brazil

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Detection of Oropouche virus in saliva and urine samples of febrile patients in Salvador, Bahia, Brazil

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Apêndice J – Artigo publicado: New challenge for zika virus infection: human reservoirs?

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New Challenge for Zika Virus Infection: Human Reservoirs?

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Apêndice L – Artigo publicado: Nk cell responses in zika virus infection are biased towards cytokine-mediated effector functions

NK Cell Responses in Zika Virus Infection Are Biased towards Cytokine-Mediated Effector Functions

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