UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Pâmela Jóyce Previdelli da Conceição

Identificação da presença do vírus da dengue e do vírus Zika em amostras de soro e urina no município de Mirassol, São Paulo

São José do Rio Preto

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Financiadora CNPq – Proc. 440723/2016-7

Financiadora CAPES – Proc. 2046/2016

Orientadora: Profª. Drª. Cíntia Bittar Oliva

Coorientadora: Profª. Drª. Paula Rahal

São José do Rio Preto

2021

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Conceição, Pâmela Jóyce Previdelli da
Identificação da presença do vírus da dengue e do vírus Zika em amostras de soro e urina no município de Mirassol, São Paulo / Pâmela Jóyce Previdelli da Conceição. -- São José do Rio Preto, 2021
70 f. : il., tabs.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto
Orientadora: Cíntia Bittar Oliva
Coorientadora: Paula Rahal

1. Microbiologia. 2. Virologia. 3. Arbovírus. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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Financiadora CNPq – Proc. 440723/2016-7

Financiadora CAPES – Proc. 2046/2016

Comissão Examinadora

Profª. Drª. Cíntia Bittar Oliva
UNESP – Câmpus de São José do Rio Preto
Orientadora

Prof. Dr. Bruno Moreira Carneiro
UFR- Universidade Federal de Rondonópolis

Profª. Drª. Lívia Sacchetto Pengo
FAMERP – Faculdade de Medicina de São José do Rio Preto

São José do Rio Preto

07 de abril de 2021

DEDICATÓRIA

Dedico esse trabalho aos meus familiares e a todas as pessoas que me amam e me incentivaram desde o princípio, sem vocês eu nada seria.

AGRADECIMENTOS

A Deus pela vida e por ter me dado saúde e vitalidade para vencer os desafios e obstáculos da vida.

Aos meus familiares, Célia, Silvio, Paulo, Lu, Kelver, Layra, Marina e Nicolas, que tornam meus dias mais iluminados, que sempre me apoiaram e me incentivaram a não desistir dos meus sonhos, sempre compreendendo minha ausência em alguns momentos para realização do meu trabalho.

Ao meu namorado Samuel pelo amor, pela paciência, compreensão e por estar presente em todos os momentos da minha vida, me dando forças para continuar minha caminhada.

À minha orientadora Profa. Dra. Cíntia Bittar, pelos ensinamentos, por ter tido confiança no meu trabalho, pela paciência com que me ensinou e por sempre estar presente em todos os momentos, auxiliando no que fosse preciso.

À minha coorientadora Profa. Dra. Paula Rahal, por todos os ensinamentos, por ter me dado a oportunidade de fazer parte de sua equipe e por ter depositado sua confiança no meu trabalho.

Ao Instituto de Biociências, Letras e Ciências Exatas (UNESP/IBILCE) por ter cedido toda sua estrutura, tornando possível a realização do projeto e pelo corpo docente extremamente qualificado.

À UPA de Mirassol e a todos os funcionários pela parceria, por ter cedido sua estrutura para que esse projeto pudesse ser realizado da melhor forma possível.

À banca examinadora por terem aceito participar da minha banca.

Aos meus queridos companheiros Lucas e Bianca, por todos os ensinamentos preciosos, pelo compartilhamento de experiência, pela ajuda em todas as análises realizadas, por terem sempre estado juntos de mim nesse projeto.

Às minhas amigas e companheiras de casa Dayla e Gabi, pelas risadas, alegrias, pela troca de experiência e por terem feito parte do meu amadurecimento, tanto profissional, como pessoal.

Às minhas queridas amigas Tamara, Marilê, Bárbara, Nay e Ágata pelos momentos de risada, pela amizade, pelos maravilhosos ensinamentos e por sempre me apoiarem e me ajudarem.

Aos meus colegas do laboratório, por todos os ensinamentos, por sempre estarem dispostos a ajudar no que fosse preciso, que foram amigos e parceiros em todos os momentos, sejam eles profissionais ou pessoais, bons ou ruins.

A todos os meus familiares e amigos, sou muito grata por tudo.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

O presente trabalho foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

EPÍGRAFE

“Eu não posso fazer todo o bem que o mundo precisa, mas o mundo precisa de todo o bem que eu posso fazer” (Jana Stanfield)

RESUMO

As doenças transmitidas por vetores, tal como o *Aedes aegypti* causam um grande impacto na saúde pública. As arboviroses fazem parte desse grupo de doenças, afetando milhares de pessoas ao redor do mundo todos os anos. O vírus da dengue (DENV), um arbovírus do gênero *Flavivirus*, família *Flaviviridae,* é responsável por diversas epidemias e também por trazer complicações graves aos pacientes, que vão de quadros hemorrágicos a complicações orgânicas irreversíveis. Outro arbovírus do gênero *Flavivirus* de importância clínica é o Zika vírus (ZIKV), responsável pela epidemia no ano de 2015 no Brasil, trazendo consigo a Síndrome Congênita do Zika, que implicou no nascimento de centenas de crianças com microcefalia. Este estudo tem por objetivo avaliar a circulação de DENV e ZIKV no município de Mirassol-SP. Para este fim, foram coletadas amostras de voluntários presentes na Unidade de Pronto Atendimento de Mirassol (UPA-Mirassol) entre fevereiro de 2018 a abril de 2019. Os voluntários foram divididos em 2 grupos. Do primeiro grupo, composto por pacientes com suspeita clínica de arbovirose, foram coletadas amostras de soro e urina. O segundo grupo incluiu pacientes sem diagnóstico clínico de arboviroses e acompanhantes de pacientes da UPA, sendo coletadas apenas amostras de urina. Todos os voluntários preencheram questionários sobre a sintomatologia apresentada no momento da coleta. Após a extração do RNA, a investigação da presença dos vírus foi realizada por RT-qPCR. Ambas as amostras do grupo suspeito para arbovirose foram testadas para DENV e ZIKV enquanto as amostras de voluntários sem diagnóstico clinico para arbovirose e acompanhantes foram testadas apenas para ZIKV. Foram coletadas 697 amostras de urina dos indivíduos sem diagnóstico clínico de arboviroses e acompanhantes, dessas, 11,3% (79) foram positivas para ZIKV. Dentre os indivíduos positivos, 16,5% (13) eram acompanhantes e 83,5% (66) indivíduos sem diagnóstico clínico de arbovirose, sendo 59,5% (47) do gênero feminino. Do grupo de sintomáticos, foram coletadas 285 amostras de soro e 272 amostras de urina de 308 pacientes e desses 42,5% (131) foram positivos para DENV2, 0,3% (1) foi positivo para DENV1, 36% (111) foram positivos para ZIKV e 12,7% (39) estavam coinfectados com ZIKV/DENV2. Se apenas as amostras de soro tivessem sido usadas, a detecção de ZIKV teria caído para 24,7% (76/308). De todos os pacientes incluídos no estudo, apenas um tinha suspeita de infecção pelo ZIKV e os demais eram suspeitos de DENV de acordo com o diagnóstico clínico. Com isso, através do nosso estudo foi possível ver a importância da realização de um diagnóstico diferencial, utilizando amostras de soro e urina, diminuindo assim o número de casos positivos subnotificados.

Palavras-chave: Zika vírus. ZIKV assintomático. Urina. Sem sintomas clínicos de arbovirose.

ABSTRACT

Diseases transmitted by vectors, such as *Aedes aegypti,* have an impact on public health. Arboviruses are part of this group of diseases, affecting thousands of people around the world every year. The dengue virus (DENV), an arbovirus of the genus *Flavivirus*, family *Flaviviridae*, is responsible for several epidemics and for bringing complications to patients, ranging from hemorrhagic conditions to irreversible organic complications. Another arbovirus from the genus *Flavivirus* of clinical importance is the Zika virus (ZIKV), responsible for the 2015 epidemic in Brazil, bringing with it the Zika Congenital Syndrome, which caused the birth of a hundreds of children with microcephaly. This study aims to evaluate the circulation of DENV and ZIKV in the municipality of Mirassol-SP. For this purpose, samples were collected from volunteers in the Mirassol Emergency Care Unit (UPA-Mirassol) from February 2018 to April 2019. The volunteers were divided into two groups. From the first group, composed of patients with clinical hypothesis of arbovirusis, serum, and urine samples were collected. The second group included patients without a clinical diagnosis of arbovirusis and companions from patients attended at the UPA, with only urine samples collected. The volunteers completed questionnaires about the symptoms presented at the time of collection. After RNA extraction, the investigation of the presence of viruses was performed by RT-qPCR. Both samples from the group suspected for arbovirus infection were tested for DENV and, ZIKV while samples from volunteers without a clinical diagnosis for arbovirus and companions were tested only for ZIKV. Six hundred and ninety-seven urine samples were collected from individuals without a clinical diagnosis of arbovirosis and companions, of which 11.3% (79) were positive for ZIKV. Among positive individuals, 16.5% (13) are companions and, 83.5% (66) are individuals without a clinical diagnosis of arbovirosis, 59.5% (47) of whom are female. From the symptomatic group, 285 serum samples and 272 urine samples were collected from 308 patients, and from those, 42.5% (131) were positive for DENV2, 0.3% (1) was positive for DENV1, 36.4% (111) were positive for ZIKV and 12.5% (38) were co-infected with ZIKV / DENV2. If only serum samples had been used, ZIKV detection would have dropped to 24.7% (76/308). From all the patients included in the study only one was suspected of ZIKV infection and the remaining were suspected of DENV according to the clinical diagnosis. Thus, through our study, it was possible to see the importance of making a differential diagnosis using serum and urine samples so that there is a decrease in the number of underreported positive cases.

Keywords: Zika virus. ZIKV asymptomatic. Urine. No clinical symptoms of arboviruses.

SUMÁRIO

Capítulo I - Considerações gerais

1. INTRODUÇÃO

1.1 Arbovírus

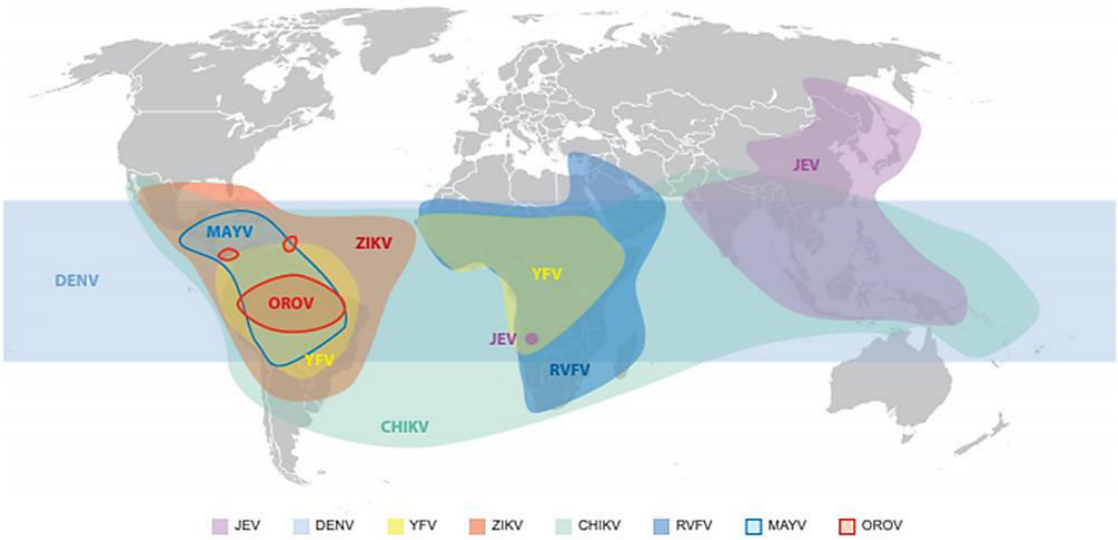
Os arbovírus tem sido um dos maiores motivos de preocupação em saúde pública mundial, atingindo todos os continentes, exceto o Antártico (WEAVER; REISEN, 2010; RUST, 2012; DONALISIO;FREITAS; ZUBEN, 2017; WEAVER, SCOTT C. et al., 2018) (Figura 1). Os arbovírus (*Arthropod-borne virus*) são um grupo de centenas de vírus que possuem diversas características em comum, sendo a principal, a transmissão por vetores artrópodes hematófagos, em sua maioria mosquitos (WEAVER; REISEN, 2010; RUST, 2012; WEAVER, SCOTT C. et al., 2018). Também pode-se destacar os vírus transmitidos por outros artrópodes hematófagos como carrapatos (WEAVER; REISEN, 2010; BARTÍKOVÁ et al., 2017; HOLBROOK, 2017). Os ciclos dos arbovírus variam de acordo com as condições ecológicas em que se encontram, sendo mantidos em ciclos urbanos e silvestres. No ciclo urbano ocorre a disseminação dos vírus através da picada do mosquito, que infecta o ser humano e no ciclo silvestre a transmissão ocorre entre mosquitos e primatas não humanos (WEAVER, SCOTT C. et al., 2018).

Os vírus mais conhecidos e importantes clinicamente são os que são transmitidos por mosquitos dos gêneros *Aedes* e *Culex* (WEAVER; REISEN, 2010; RUST, 2012). No Brasil, o vetor mais comum é o *Aedes aegypti*, no qual o seu ciclo de transmissão envolve apenas os mosquitos e os seres humanos, sem necessidade de manutenção em ciclos silvestres (WEAVER; VASILAKIS, 2009; LINDENBACH et al., 2013). Estudos também apontam o mosquito *Culex quinquefasciatus* como potencial vetor do ZIKV, pois foram encontrados mosquitos infectados no nordeste do Brasil (GUEDES et al., 2017). Assim como também, em testes laboratoriais, o *Aedes albopictus* demonstrou a sua capacidade de se infectar e disseminar o DENV (PANCETTI et al., 2015).

Os arbovírus de maior importância clínica pertencem as famílias *Togaviridae* (gênero *Alphavirus*), *Flaviviridae (*gênero *Flavivirus*), *Benyviridae, Reoviridae* e *Rhabdoviridae*. As manifestações clínicas das arboviroses podem se apresentar de forma assintomática, doença febril (DF) moderada a severa, erupções cutâneas e artralgia (AR), síndromes neurológicas (SN) e síndromes hemorrágicas (SH) (CLETON et al., 2012). Outros sintomas podem ser descritos, tais como hepatites, broncopneumonias e conjuntivites (CLETON et al., 2012). A doença febril geralmente apresenta sintomas moderados, tais como febre, dores de cabeça, mialgia, náuseas, vômito, dor retro orbital. As ARs apresentam exantema e artralgia (CLETON et al., 2012). Dentre as SNs pode-se apresentar meningites, mielites e/ou encefalites, além de alterações

comportamentais, paralisias e convulsões (CLETON et al., 2012). No caso das SHs ocorre uma baixa contagem das plaquetas, petéquias e episódios hemorrágicos, podendo causar a Síndrome do Choque da Dengue e óbito. A morbidade e a mortalidade envolvendo arboviroses aumenta à medida que vão ocorrendo as epidemias (CLETON et al., 2012).

Figura 1 - Mapa da distribuição das arboviroses emergentes ao redor do mundo. Abreviações: DENV: *Dengue virus*; YFV: *Yellow fever virus*; ZIKV: *Zika virus*; CHIKV: *Chikungunya virus*; JEV: *Japanese encephalitis virus;* RVFV: *Rift Valley fever virus*; MAYV: *Mayaro virus*; OROV: *Oropouche virus*.



Fonte: (WEAVER, S. C. et al., 2018)

Alguns fatores têm contribuído drasticamente para o aumento das arboviroses, tais como desmatamento, crescimento populacional exagerado, ocupação das áreas urbanas, mudanças climáticas, condições sanitárias precárias, podendo assim contribuir para a expansão dos insetos vetores e disseminação de alguns tipos de vírus (WEAVER; REISEN, 2010).

1.2 Gênero *Flavivirus*

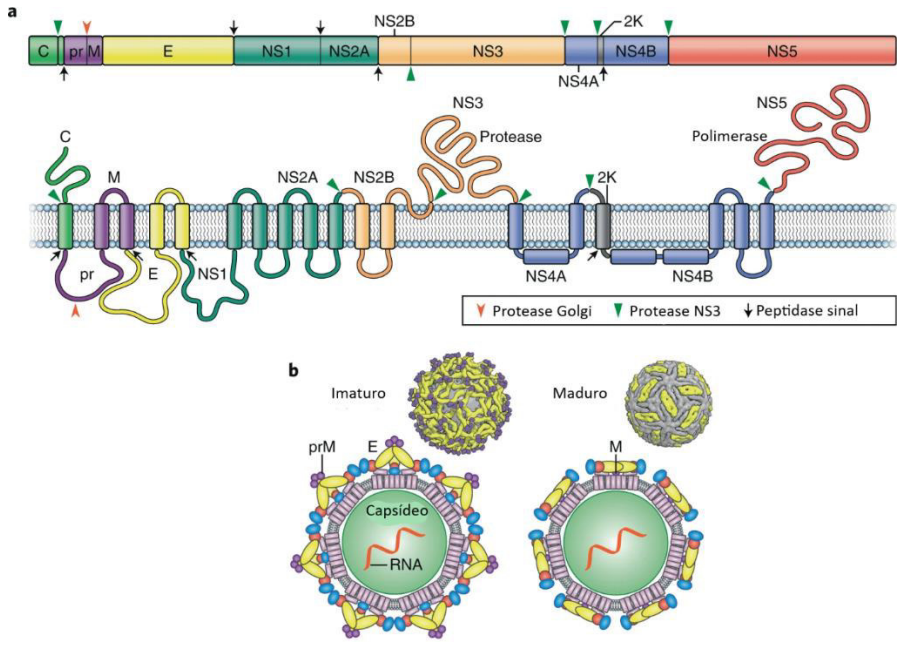
Os flavivirus são vírus de RNA pertencentes a família *Flaviviridae*, que acometem tanto humanos, quanto animais, podendo causar várias doenças, com sintomas leves ou graves, podendo levar a óbito (PIERSON; DIAMOND, 2020). O gênero *Flavivirus* compreendem mais de 53 espécies, sendo transmitidos por mosquitos e carrapatos (WEAVER; REISEN, 2010;

CLETON et al., 2012). Eles formam um dos gêneros pertencentes à família *Flaviviridae*, assim como os gêneros *Pestivirus* e *Hepacivirus* (CLETON et al., 2012).

A partícula viral dos flavivirus mede cerca de 40 a 60 nm de diâmetro, possuindo um capsídeo de formato icosaédrico, envolto por um envelope lipídico (LINDENBACH et al., 2013). Seu genoma é formado por um RNA fita simples de polaridade positiva, com aproximadamente 11kb, a partir do qual ocorre a tradução de uma única poliproteína, que é clivada por enzimas celulares e enzimas virais, formando as proteínas estruturais e as proteínas não estruturais (LINDENBACH et al., 2013). Dentre as proteínas estruturais, encontra-se a proteína C do capsídeo, as proteínas M e prM, e a proteína E que compõem o envelope viral e dentre as proteínas estruturais estão as proteínas NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 2) (LINDENBACH et al., 2013).

Acredita-se que os flavivirus surgiram há cerca de 100.000 anos e que sua disseminação ocorreu por conta da migração e expansão das populações (PETTERSSON; FIZ-PALACIOS, 2014; HOLBROOK, 2017). A família *Flaviviridae* engloba três gêneros: os *Flavivirus*, *Pestivirus* e *Hepacivirus* (LINDENBACH, 2007). Com relação às doenças de maior impacto e importância clínica envolvendo os *Flavivirus* pode-se destacar a dengue, a febre amarela, a febre do Zika, a encefalite japonesa, a febre do Oeste do Nilo, dentre outras (LINDENBACH, 2007; CLETON et al., 2012).

Figura 2 - Representação do genoma e estrutura dos *Flavivirus* e de suas proteínas estruturais (C, M, PrM e E) e não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5).



Fonte: Adaptado de (PIERSON; DIAMOND, 2020)

1.3 O vírus da dengue (DENV)

O DENV é o vírus causador da doença de caráter agudo conhecida como dengue (KUHN et al., 2002; ZHANG et al., 2003). No Brasil, o DENV é representado por 4 sorotipos de ciclo urbano com características antigênicas distintas (DENV1, DENV2, DENV3 e DENV4) (HOLMES, 1998). Existe um potencial novo sorotipo de DENV em ciclo silvestre (DENV5), no qual o vírus permanece circulando em primatas não humanos, porém novos estudos precisam ser feitos para provar tal descoberta (NORMILE, 2013; MUSTAFA et al., 2015).

O DENV é transmitido por mosquitos hematófagos do gênero *Aedes*, tais como o *Aedes aegypti* e o *Aedes albopictus*, sendo o vetor mais comum o *Aedes aegypti*, no qual o seu ciclo de transmissão envolve os mosquitos e os seres humanos (WEAVER; VASILAKIS, 2009; ALTHOUSE et al.,2014). No Brasil, não foram registradas epidemias ou transmissão envolvendo o *Aedes albopictus*, embora em testes laboratoriais tenha sido demonstrada a sua capacidade de se infectar e disseminar o DENV (PANCETTI et al., 2015).

Diversos estudos usando primatas não humanos como modelos experimentais têm demonstrado que eles são potenciais hospedeiros de DENV. Além disso, sabe-se que o DENV2 possui ciclo silvestre na África e os quatro sorotipos circulam em primatas não humanos no Sudeste da Ásia (ALTHOUSE et al.,2014).

De acordo com a OMS, estima-se que ocorram ao redor do mundo mais de 390 milhões de infecções por DENV anualmente, do qual 50 milhões são de casos graves da doença, atingindo mais de 100 países, que se encontram em regiões tropicais e subtropicais, tais como África, Américas, sudeste da Ásia, Mediterrâneo oriental e Pacífico ocidental (OPAS; OMS, 2019). Apenas nas Américas, no ano de 2015 foram registradas 1181 mortes (OPAS; OMS, 2019).

As primeiras epidemias envolvendo dengue grave com hemorragias ocorreram em 1953 nas Filipinas e em 1958 na Tailândia, sendo esses países pertencentes às regiões do sudeste da Ásia e do Pacífico ocidental, regiões com os maiores surtos registrados (WHO, 2011).

No Brasil, o primeiro surto da doença ocorreu no estado de Roraima, no ano de 1981, no qual foi notada a circulação dos sorotipos 1 e 4 (OSANAI et al., 1983). O sorotipo 2 foi descrito pela primeira vez, no estado do Rio de Janeiro em 1990, no qual notou-se também os primeiros casos de Febre hemorrágica do dengue e o sorotipo 3 foi descrito no ano 2000, em Nova Iguaçu, Rio de Janeiro (NOGUEIRA et al., 1990; NOGUEIRA et al., 2001). No ano de 2015 ocorreu uma das maiores epidemias de DENV registradas no país, no qual foram

registrados na 52ª Semana Epidemiológica (período de 04/01/2015 a 02/01/2016) 1.649.008 possíveis casos da doença com registros confirmados de 863 óbitos (MS; SVS, 2016b).

No presente momento, o Brasil ainda continua passando por epidemias todos os anos, sendo considerado um país superendêmico, no qual o único meio de prevenção até o momento é a eliminação dos vetores (MS; SVS, 2019b).

A infecção pelo DENV pode ser desde assintomática até doença grave, com período de incubação de 2 a 7 dias podendo levar a óbito (PONTES; RUFFINO-NETTO, 1994). Estima-se que a cada 4 infecções causadas pelo DENV, uma seja assintomática, tendo importante papel na transmissão da doença, pois a viremia é a mesma dos casos sintomáticos (DUONG et al., 2015). Essa forma da doença pode ocorrer devido à baixa virulência ou devido ao tipo de resposta imunológica que o hospedeiro apresenta (SOUZA, 2016). Um estudo em Singapura mostrou casos de síndrome de Guillain-Barré em que os pacientes possuíam DENV assintomática, podendo haver relação entre a infecção pelo vírus e o quadro clínico neurológico. (UMAPATHI et al., 2016).

A maioria dos casos de dengue se apresenta de forma sintomática, ocorrendo em três fases clínicas diferentes: febril, crítica e de recuperação (MS; SVS, 2016a). Na fase febril um dos primeiros sintomas é a febre alta e abrupta (39-40°C), que pode durar de 2 a 7 dias, juntamente com cefaleia, mialgia, artralgia, dor retroorbital e adinamia (MS; SVS, 2016a). Posterior ao desaparecimento da febre, pode ocorrer o aparecimento de exantema, que ocorre em 50% dos casos, apresentando-se como do tipo maculo-papular, com ou sem prurido (MS; SVS, 2016a). Outros sintomas que podem aparecer nessa fase são vômito, náuseas, anorexia e diarreia. Passada essa fase, o paciente inicia o processo gradativo de recuperação e melhora do seu estado geral (MS; SVS, 2016a).

A fase crítica se divide em dengue com sinais de alarme e dengue grave e ela se inicia no período de defervescência, entre 3 e 7 dias após o início dos sintomas e é nessa fase que a doença pode evoluir para as formas mais graves, sendo de extrema importância que medidas de manejo clínico e observação eficientes sejam adotadas pelos profissionais de saúde (MS; SVS, 2016a). A dengue com sinais de alarme é assim denominada, por ser a fase em que o paciente irá apresentar sintomas que irão marcar o início de possível piora do paciente, que poderá evoluir para choque e óbito. Os sinais e sintomas dessa fase são vômitos persistentes, acúmulo de líquidos, dor abdominal intensa e constante, sangramento das mucosas, hipotensão postural e/ou lipotimia, hepatomegalia, letargia, irritabilidade e aumento do hematócrito. Todos esses sintomas indicam o aumento da permeabilidade vascular com posterior extravasamento de

plasma. Na forma grave, ao ocorrer o extravasamento de plasma, ocorre o choque ou acúmulo de líquidos, que leva à disfunção de órgãos vitais e hemorragias (MS; SVS, 2016a).

O choque ocorre após perda significativa de plasma e a evolução desse quadro ocorre de forma rápida, podendo levar o paciente a óbito entre 12 e 24 horas, com isso após esse quadro deve-se avaliar qualquer alteração hemodinâmica que venha a ocorrer nos pacientes, para impedir maior comprometimento (MS; SVS, 2016a). As hemorragias graves também são sinais de dengue grave, podendo ocorrer com ou sem choque, elas ocorrem em grande parte, em pacientes com distúrbios digestivos, tais como úlceras pépticas ou gastrites, ou em pacientes que fazem uso de determinados tipos de medicamentos, tais como alguns tipos de antiinflamatórios, anticoagulantes, entre outros (MS; SVS, 2016a). Além disso, também pode ocorrer dentro do quadro de dengue grave, as disfunções graves dos órgãos, que ocorrem com ou sem choque e extravasamento de plasma. Os órgãos acometidos são geralmente, órgãos do sistema hepático, sistema nervoso e sistema cardíaco, causando as hepatites, encefalites e miocardites, síndromes de Reye e Guillain-Barré, dentre outras (MS; SVS, 2016a).

Por fim, ocorre a fase de recuperação, no qual todo o conteúdo extravasado será reestabelecido ocasionando a melhora do paciente. Nessa fase ainda podem ser observados alguns sinais e/ou disfunções, assim como também o aparecimento de infecções bacterianas que podem contribuir para piora e óbito (MS; SVS, 2016a).

1.4 O vírus Zika (ZIKV)

O ZIKV causador da febre do Zika, assim como o DENV é um arbovírus do gênero *Flavivirus*, família *Flaviviridae*, transmitidos por mosquitos *Aedes aegypti* (KUNO; CHANG, 2007). O ZIKV foi isolado pela primeira vez de um macaco rhesus na floresta Zika, em Uganda (DICK et al., 1952; WHITE et al., 2016). As regiões do leste e oeste africano são consideradas endêmicas para ZIKV, porém no ano de 2007 ocorreu o primeiro surto de ZIKV fora do continente africado, na Ilha de Yap nos Estados Federados da Micronésia, no qual o vírus infectou 73% da população (DUFFY et al., 2009; PERKASA et al., 2016). Depois desse surto, o vírus se espalhou rapidamente e no ano de 2013 foi notificado novo surto na Polinésia Francesa (CAO-LORMEAU et al., 2014).

No Brasil, o primeiro caso autóctone de ZIKV ocorreu no ano de 2015, porém estudos sugerem que a introdução do ZIKV no país tenha ocorrido 1 ou 2 anos antes, por conta da Copa do mundo de futebol masculino que ocorreu em 2014 ou por conta de um torneio de canoagem que ocorreu em 2013 no Rio de Janeiro, no qual os participantes eram nativos de países em que

circulava o ZIKV (MUSSO et al., 2015; ZANLUCA et al., 2015; SALVADOR; FUJITA, 2016). No ano de 2015, os primeiros estados atingidos foram Bahia, Pernambuco e Rio Grande do Norte e médicos da região nordeste do estado começaram a relatar que as infecções por ZIKV coincidiam com o aumento no número de casos atípicos de microcefalia em recém-nascidos da região (CALVET et al., 2016; RASMUSSEN et al., 2016). Após esses achados, foi coletado o líquido amniótico e detectada a presença do RNA e anticorpos do ZIKV nesse fluido, mostrando uma possível transmissão via intrauterina (CALVET et al., 2016). Depois desses surtos foi declarado estado de emergência global, pela Organização Mundial de Saúde, no ano de 2016 (WHO, 2016b).

No ano de 2016 foram registrados até a Semana Epidemiológica 52 (03/01/2016 a 31/12/2016) 215.319 possíveis casos de ZIKV no país e 11.052 gestantes foram confirmadas com base em dados clínicos-epidemiológicos ou laboratorialmente (MS; SVS, 2016b). Em relação à Síndrome Congênita do Zika (*Congenital Zika Syndrome – CZS*), entre as Semanas Epidemiológicas SEs 45/2015 e 40/2019 (08/11/2015 a 05/10/2019) foram confirmados 3.474 casos, além de 743 casos prováveis e 615 inconclusivos. Esses casos distribuídos entre os anos investigados mostram um número de 954 casos em 2015, 1.927 em 2016, 369 em 2017, 178 em 2018 e 55 em 2019 (MS; SVS, 2020).

Em relação às manifestações clínicas, é visto que o ZIKV se apresenta em 80% dos casos de forma assintomática e quando doença sintomática, os sinais e sintomas do ZIKV são muito parecidos com os de outras arboviroses, pois os pacientes apresentam febre leve, cefaleia, erupções cutâneas maculopapulares, conjuntivite não purulenta, artralgia, mialgia e linfoadenopatia (DUFFY et al., 2009; SINGH et al., 2016). Além desses sintomas, eles também podem apresentar náusea, vômito, diarreia, vertigem, dor abdominal e queimação nas extremidades dos membros (SINGH et al., 2016; COLOMBO et al., 2017).

Além dos sintomas citados acima, o ZIKV pode trazer outras complicações, tais como distúrbios neurológicos, a *CZS* e a Síndrome de Guillain-Barré (SGB) (MS, 2015; CAO-LORMEAU et al., 2016). A *CZS* é identificada pela medida do perímetro cefálico menor, quando comparada com recém-nascidos normais (VILLAR et al., 2014). Os recém-nascidos com microcefalia desenvolvem diversas complicações, tais como dificuldade de desenvolvimento cognitivo, problemas de visão e audição, paralisia cerebral e retardo mental (DOLK, 1991; VILLAR et al., 2014; WHO, 2016a).

1.5 Diagnóstico diferencial

Atualmente no Brasil o diagnóstico e definição de caso suspeito de arboviroses é feito, na maioria dos casos, baseado em critérios clínicos-epidemiológicos, pois não são feitos exames específicos em todos os casos, com isso acaba ocorrendo uma subnotificação das arboviroses com importância clinica que circulam no país, prejudicando a contenção dessas doenças (MS; SVS, 2016c; 2019a). Quando são solicitados exames laboratoriais, na maioria das vezes, são solicitados os exames de métodos imunoenzimáticos, pois estes testes possuem um custo mais baixo, sendo mais aplicáveis quando existe um grande número de amostras (CASTELLANOS; CORONEL-RUIZ, 2014). No diagnóstico utilizando testes imunoenzimáticos, pode-se detectar o antígeno, sendo pesquisada a proteína NS1, que é extremamente conservada, assim como realizar a pesquisa de anticorpos IgG e IgM específicos (OSORIO et al., 2015). Esse método é eficiente, porém possui baixa especificidade, podendo apresentar reações cruzadas entre diferentes tipos de *Flavivirus*, dando resultados errôneos (PEELING et al., 2010). O teste mais indicado para a pesquisa de anticorpos de arbovirus é o teste de redução de neutralização de placas (PRNT). O PRNT é o padrão ouro na pesquisa sorológica de flavivirus, porém é um método inviável para aplicação nos serviços de saúde, por ser de alto custo, demorado, pois leva 7 dias para obter o resultado, e por necessitar de profissionais extremamente capacitados para realizar a técnica e interpretar corretamente os resultados. Além disso, dependendo do vírus é necessária a utilização de Sala de Nível de Biossegurança 3 (PETERSEN;JAMIESON; HONEIN, 2016; ROEHRIG et al., 2008).

Outros métodos utilizados são os métodos moleculares, onde é feita a detecção do ácido nucléico viral, por meio das técnicas de PCR convencional e PCR em tempo real, que são métodos de alta especificidade e sensibilidade, porém de alto custo (GURUKUMAR et al., 2009; PEELING et al., 2010; COLOMBO et al., 2018).

O diagnóstico laboratorial pode ser feito utilizando diferentes tipos de fluídos biológicos, tais como sangue, saliva, urina, sêmen, cordão umbilical, swab nasofaríngeo, líquido amniótico e secreção vaginal (KORHONEN et al., 2014; IANNETTA et al., 2017; PAZ-BAILEY et al., 2017). No caso do ZIKV a urina é um dos fluidos recomendados quando usada a técnica de RT-qPCR, pois sua viremia se mostra baixa, quando utilizadas amostras de soro (OZKURT; TANRIVERDI, 2017). Estudos mostram que a detecção de ZIKV em soro pode ser feita até 10 dias após aparecimento dos sintomas, enquanto que em urina esse período é muito maior, podendo chegar a semanas (ROZÉ et al., 2016; OZKURT; TANRIVERDI, 2017). No caso do DENV, o soro é o mais utilizado para realizar sua detecção, no qual o período de

viremia varia de 3 a 6 dias após início dos sintomas (KORHONEN et al., 2014). O sangue também é utilizado para realização do hemograma onde é feita a contagem de plaquetas, cujo seu monitoramento é de grande importância caso a doença tenda a evoluir para dengue grave (WHO, 1997). No caso da urina, o período da presença do vírus varia de 3 a 16 dias após início dos sintomas e no caso da saliva varia de 2 a 10 dias (HIRAYAMA et al., 2012; KORHONEN et al., 2014).

Assim, a análise da urina, onde a excreção é prolongada, aumenta o tempo em que o diagnóstico molecular é possível, sendo uma alternativa importante já que muitos pacientes demoram a procurar o serviço de saúde, e o fazem quando o vírus se torna indetectável no sangue (HIRAYAMA et al., 2012; KORHONEN et al., 2014; SHINOHARA et al., 2016; PAZ-BAILEY et al., 2017). Ainda, a utilização de amostras de urina permite a obtenção de um grande volume de fluido e por ser um método pouco invasivo podem ser usadas em casos em que a obtenção de amostras de sangue seja inviável, tais como no caso de coleta em recém nascidos e crianças, no caso de pacientes com quadros hemorrágicos e no caso de viajantes que retornam de áreas endêmicas, já que a persistência de ZIKV em urina é alta (POLONI et al., 2010; HIRAYAMA et al., 2012; KORHONEN et al., 2014; SHINOHARA et al., 2016).

2. OBJETIVOS

2.1 Objetivo Geral

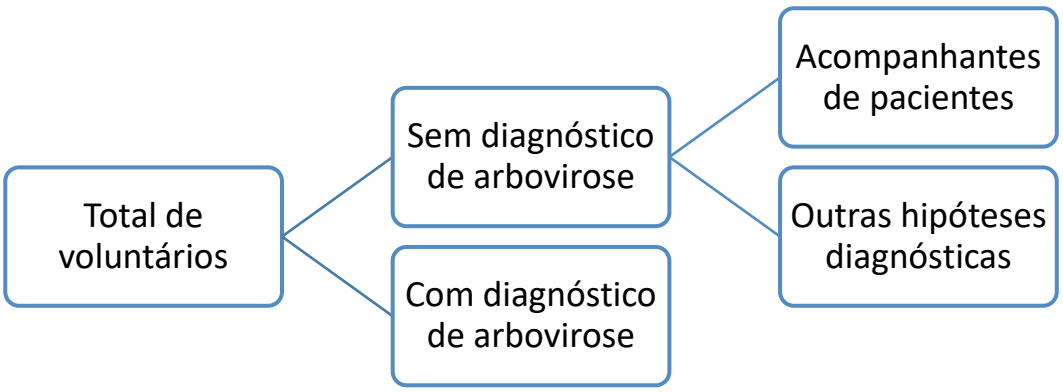
Investigar a presença do RNA viral de DENV e ZIKV em amostras de soro e urina de pacientes e seus acompanhantes na UPA do município de Mirassol-SP.

2.2 Objetivos Específicos

- Investigar molecularmente a presença de ZIKV em amostras de urina de pacientes assintomáticos e sem diagnóstico clinico para arboviroses;
- Investigar a presença de DENV e ZIKV em amostras de soro de pacientes sintomáticos;
- Investigar a presença de DENV e ZIKV em amostras de urina de pacientes sintomáticos;

3. MATERIAIS E MÉTODOS

Figura 3 - Representação dos grupos e subgrupos de pacientes



Fonte: (Autor)

3.1 Aspectos Éticos

O projeto foi aprovado pelo Comitê de Ética em Pesquisa do Instituto de Biociências, Letras e Ciências Exatas (IBILCE – UNESP) de São José do Rio Preto – SP (CAAE: 66489617.4.0000.5466). Todos os voluntários convidados a participar do estudo, assinaram o Termo de Consentimento Livre e Esclarecido (TCLE).

3.2 Coleta e processamento das amostras

Todas as coletas de amostras foram realizadas na UPA do município de Mirassol-SP. A cidade de Mirassol está localizada a 20º25'S e 51º21'W, no estado de São Paulo, Brasil a cerca de 460 Km da capital de São Paulo e 15 Km de São José do Rio Preto. Tem uma população estimada de 59.824 habitantes e a densidade populacional é de 221,10 habitantes por Km2 com aproximadamente 97,9% de domicílio urbano. A cidade tem um clima tropical; o verão é mais chuvoso do que o inverno, com chuva entre novembro e abril. O nível de precipitação anual é de 1223 mm e a temperatura média anual de 22 ° C (IBGE, dados não publicados).

Os voluntários do estudo foram divididos em dois grupos, no qual o primeiro continha indivíduos com diagnóstico clínico de arbovirose e o segundo continha indivíduos sem diagnóstico clínico de arbovirose. O segundo grupo foi subdividido em mais dois grupos:

acompanhantes de pacientes e pacientes com outras hipóteses diagnósticas. Todos os indivíduos, exceto acompanhantes de pacientes, passaram por consulta médica na Unidade de Pronto Atendimento de Mirassol/SP (UPA – Mirassol), a única da cidade, no qual receberam o diagnóstico, baseado em dados clínicos-epidemiológicos, além de exames de rotina realizados a pedido do médico, caso necessário. Foram coletadas amostras de sangue e urina do primeiro grupo e do segundo grupo, apenas amostras de urina.

Após a realização das coletas, as amostras foram estocadas a 4ºC por no máximo 48 horas até o processamento. Todas as amostras foram encaminhadas ao Laboratório de Estudos Genômicos da UNESP - IBILCE, para serem processadas.

Todos os voluntários do estudo preencheram um questionário sobre sinais e sintomas, incluindo a duração em dias e os dados foram analisados para determinar os sintomas apresentados por pacientes infectados pelos vírus estudados.

3.2.1 Urina

As amostras de urina foram coletadas em frasco coletor universal, centrifugadas a 448 x g por 10 minutos em temperatura ambiente, utilizando tubos de centrifuga contendo um volume máximo de 40mL. Após o término da centrifugação, parte do sobrenadante foi descartado, restando 5 mL. Foi realizada a homogeneização do "*pellet"* e a urina foi filtrada em filtros de membrana de 0,45 nm, com auxílio de seringa, para evitar possível contaminação com outros microrganismos. As amostras foram armazenadas em ultrafreezer a -150°C.

3.2.2 Soro

As amostras de sangue foram coletadas usando tubo com gel separador ativador de coágulo, para separação do soro. Os tubos foram centrifugados a 1008 x g por 15 minutos. O soro foi separado e armazenado em tubos de criogenia em ultrafreezer a -150 ºC para a realização das etapas posteriores e o restante do sangue foi descartado.

3.3 Extração de RNA

A extração de RNA das amostras foi feita utilizando Trizol (Invitrogen™, Life Technologies®, EUA). Desta forma, foram adicionados 750 µL de solução monofásica de tiocianato de guanidina e fenol (TRIzol - Invitrogen™, Life Technologies®, EUA) à 250 µL amostra. Depois as amostras foram homogeneizadas com auxílio de um agitador para tubos do tipo vórtex e mantidas por 5 minutos a temperatura ambiente (TA) para permitir a completa

dissociação dos complexos de nucleoproteínas. Após esse tempo, foram adicionados 200 µL de clorofórmio às amostras, que foram homogeneizadas novamente por alguns segundos e incubadas por 3 minutos a TA. Em seguida, as amostras foram centrifugadas a 21952 x g por 15 minutos a 4°C, para separação das fases e o sobrenadante (fase aquosa superior) foi transferido para um novo tubo de 1,5 mL (o RNA total encontra-se nessa fase). Foram adicionados 500 µL de isopropanol absoluto (Merck Millipore®), responsável pela precipitação do RNA, homogeneizando por inversão 15 vezes e incubando à TA por 10 min. Depois as amostras foram centrifugadas a 21952 x g por 20 minutos a 4°C, formando um "*pellet*" do RNA precipitado. O sobrenadante foi descartado cuidadosamente adicionando 1mL de etanol 75% (Merck Millipore®), e agitando por inversão, para lavar o "*pellet*". Após esta etapa as amostras foram centrifugadas a 4°C por 5 minutos a 11200 x g e o sobrenadante foi descartado. O tubo contendo o RNA foi inserido no equipamento *Concentrator 5301* (*Eppendorf AG,* Hamburg, Germany) para secagem a vácuo por aproximadamente 5 minutos. Após seco, o "*pellet*" foi eluído em 20 µL de água tratada com o reagente dietil pirocarbonato (DEPEC - Sigma Aldrich®) e armazenado a -150°C.

3.4 Reação em Cadeia da Polimerase em Tempo Real (qPCR)

A investigação da presença ou ausência de RNA de DENV para os 4 sorotipos e do RNA de ZIKV foi realizada por PCR em tempo real (qPCR) *OneStep*, utilizando o kit *GoTaq® Probe 1-Step RT-qPCR System* (*Promega Corporation,* Madison, EUA) segundo as instruções do fabricante. Foram utilizados *primers* e sondas específicos para cada sorotipo de DENV e para ZIKV (Tabela 1) (JOHNSON;RUSSELL; LANCIOTTI, 2005; LANCIOTTI et al., 2008). As analises foram realizadas no equipamento *QuantStudio™ 12K Flex Real Time PCR System* (*Applied Biosystems™,* Foster City, EUA).

Tabela 1 - Sondas e Oligonucleotídeos iniciadores utilizados nas reações de OneStep-qPCR para detecção de ZIKV e DENV.

Nome	Sequência 5'-3'
ZIKV 1086	CCGCTGCCCAACACAAG
ZIKV 1162c	CCACTAACGTTCTTTTGCAGACAT
ZIKV 1107-SONDA	6FAM-AGCCTACCTTGACAAGCAGTCAGACACTCAA-BHQ1
DEN-1 F	CAAAAGGAAGTCGTGCAATA
DEN-1 C	CTGAGTGAATTCTCTCTACTGAACC
DEN-1 SONDA	FAM-CATGTGGTTGGGAGCACGC-BHQ1
DEN-2 F	CAGGTTATGGCACTGTCACGAT
DEN-2 C	CCATCTGCAGCAACACCATCTC
DEN-2 SONDA	HEX-CTCTCCGAGAACAGGCCTCGACTTCAA-BHQ1
DEN-3 F	GGACTGGACACACGCACTCA
DEN-3 C	CATGTCTCTACCTTCTCGACTTGTCT
DEN-3 SONDA	TR-ACCTGGATGTCGGCTGAAGGAGCTTG-BHQ2
DEN-4 F	TTGTCCTAATGATGCTGGTCG
DEN-4 C	TCCACCTGAGACTCCTTCCA
DEN-4 SONDA	Cy5-TTCCTACTCCTACGCATCGCATTCCG-BHQ-3

Fonte: Johnson *et al*., 2005; Lanciotti *et al*.

3.5 Análises estatísticas

As análises estatísticas foram realizadas utilizando o software StatsDirect Statistical Software (version 3.3.4, Merseyside, UK). Estatísticas descritivas foram calculadas para análise primária. No capítulo I foi utilizado o teste t não pareado para comparar a distribuição de idade entre indivíduos positivos e negativos para ZIKV. Os testes de associação entre o gênero e ZIKV foram analisados por meio da análise de ODDS Ratio e teste do Qui quadrado de Pearson, com nível de significância de 5%. No capítulo II, o teste de Kruskal-Wallis foi usado para comparar a idade por grupos de doenças (ZIKV, DENV, coinfecção e sem doença). O pós teste de Conover-Iman foi usado para comparações de pares entre os grupos. Os demais testes de associação em ambos os capítulos foram feitos por meio do teste de Qui quadrado de Pearson e quando os pré-requisitos não foram atendidos, foi usado o Teste Exato de Fisher.

4. REFERÊNCIAS

ALTHOUSE, B. M. *et al.* Viral kinetics of primary dengue virus infection in non-human primates: A systematic review and individual pooled analysis. *Virology* **452-453**, 237-246, doi:https://doi.org/10.1016/j.virol.2014.01.015 (2014).

BARTÍKOVÁ, P. et al. Tick-borne viruses. **Acta Virol,** v. 61, n. 4, p. 413-427, 2017. ISSN 0001-723X (Print) 0001-723x.

BRATHWAITE DICK, O. et al. The history of dengue outbreaks in the Americas. **Am J Trop Med Hyg,** v. 87, n. 4, p. 584-593, Oct 2012. ISSN 0002-9637.

CALVET, G. et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. **The Lancet Infectious Diseases,** v. 16, n. 6, p. 653-660, 2016. ISSN 1473-3099. Disponível em: < https://doi.org/10.1016/S1473-3099(16)00095-5 >. Acesso em: 2020/10/16.

CAO-LORMEAU, V.-M. et al. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. **The Lancet,** v. 387, n. 10027, p. 1531-1539, 2016/04/09/ 2016. ISSN 0140-6736. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0140673616005626 >.

CAO-LORMEAU, V. M. et al. Zika virus, French polynesia, South pacific, 2013. **Emerging infectious diseases,** v. 20, n. 6, p. 1085-1086, Jun 2014. ISSN 1080-6059 (Electronic)
1080-6040 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24856001 >.

CASTELLANOS, J. E.; CORONEL-RUIZ, C. Dengue disease diagnosis: A puzzle to be solved. **Revista de la Facultad de Medicina,** v. 62, p. 617-629, 2014. ISSN 0120-0011.

CLETON, N. et al. Come fly with me: review of clinically important arboviruses for global travelers. **J Clin Virol,** v. 55, n. 3, p. 191-203, Nov 2012. ISSN 1386-6532.

COLOMBO, T. E. et al. Clinical, laboratory and virological data from suspected ZIKV patients in an endemic arbovirus area. **Journal of Clinical Virology,** v. 96, p. 20-25, 2017/11/01/ 2017. ISSN 1386-6532. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1386653217302500 >.

COLOMBO, T. E. et al. Zika detection: comparison of methodologies. **Brazilian Journal of Microbiology,** v. 49, p. 144-147, 2018. ISSN 1517-8382.

DICK, G. et al. Zika virus (I). Isolations and serological specificity. v. 46, n. 5, p. 509-520, 1952. ISSN 0035-9203.

DOLK, H. The predictive value of microcephaly during the first year of life for mental retardation at seven years. **Dev Med Child Neurol,** v. 33, n. 11, p. 974-983, Nov 1991. ISSN 0012-1622 (Print)
0012-1622.

DONALISIO, M. R.; FREITAS, A. R. R.; ZUBEN, A. P. B. V. Arboviruses emerging in Brazil: challenges for clinic and implications for public health. **Revista de Saúde Pública,** v. 51, 2017. ISSN 0034-8910.

DUFFY, M. R. et al. Zika virus outbreak on Yap Island, federated states of Micronesia. v. 360, n. 24, p. 2536-2543, 2009. ISSN 0028-4793.
DUONG, V. et al. Asymptomatic humans transmit dengue virus to mosquitoes. **Proc Natl Acad Sci U S A,** v. 112, n. 47, p. 14688-14693, Nov 24 2015. ISSN 0027-8424.

GUEDES, D. R. et al. Zika virus replication in the mosquito Culex quinquefasciatus in Brazil. **Emerg Microbes Infect,** v. 6, n. 8, p. e69, Aug 9 2017. ISSN 2222-1751.

GURUKUMAR, K. R. et al. Development of real time PCR for detection and quantitation of Dengue Viruses. **Virol J,** v. 6, p. 10, Jan 23 2009. ISSN 1743-422x.

HIRAYAMA, T. et al. Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum. **J Clin Microbiol,** v. 50, n. 6, p. 2047-2052, 2012. ISSN 1098-660X0095-1137. Disponível em: < https://pubmed.ncbi.nlm.nih.gov/22442323 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3372171/ >.

HOLBROOK, M. R. Historical Perspectives on Flavivirus Research. **Viruses,** v. 9, n. 5, Apr 30 2017. ISSN 1999-4915.

HOLMES, E. C. Molecular epidemiology and evolution of emerging infectious diseases. **Br Med Bull,** v. 54, n. 3, p. 533-543, 1998. ISSN 0007-1420 (Print)0007-1420.

IANNETTA, M. et al. Persistent detection of dengue virus RNA in vaginal secretion of a woman returning from Sri Lanka to Italy, April 2017. **Euro Surveill,** v. 22, n. 34, Aug 24 2017. ISSN 1025-496X (Print) 1025-496x.

JOHNSON, B. W.; RUSSELL, B. J.; LANCIOTTI, R. S. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. **J Clin Microbiol,** v. 43, n. 10, p. 4977-4983, Oct 2005. ISSN 0095-1137 (Print) 0095-1137.

KORHONEN, E. M. et al. Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue. **J Clin Virol,** v. 61, n. 3, p. 353-358, Nov 2014. ISSN 1386-6532.

KUHN, R. J. et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. **Cell,** v. 108, n. 5, p. 717-725, Mar 8 2002. ISSN 0092-8674 (Print) 0092-8674.

KUNO, G.; CHANG, G. J. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. **Arch Virol,** v. 152, n. 4, p. 687-696, 2007. ISSN 0304-8608 (Print) 0304-8608 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17195954 >.

LANCIOTTI, R. S. et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. **Emerging infectious diseases,** v. 14, n. 8, p. 1232-1239, Aug 2008. ISSN 1080-6040 (Print) 1080-6040.

LINDENBACH, B., D; THIEL, H; RICE C.M. Flaviviridae: the viruses and their replication. In: KNIPE DM H. P. (Ed.). **Fields virology.** . 6. Philadelphia: Lippincott Williams & Wilkins 2007. p.1101-1152.

LINDENBACH, B. D. et al. Flaviviridae. In: KLUWER W. (Ed.). **Fields Virology**. 6. Philadelphia, PA 19103 USA, v.1, 2013. cap. 25, p.712. ISBN 1000217984826.

MARQUES, G. R. A. M. et al. Água de abastecimento público de consumo humano e oviposição de Aedes aegypti J Revista de Saúde Pública. v. 47, p. 579-587, 2013. ISSN 0034-8910. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0034-89102013000300579&nrm=iso >.

MS. Ministério da Saúde confirma relação entre vírus Zika e microcefalia. Brasilia, 2015. Disponível em: < https://www.gov.br/saude/pt-br/assuntos/noticias/ministerio-da-saude-confirma-relacao-entre-virus-zika-e-microcefalia >. Acesso em: 10/03/2020.

MS; SVS. **Levantamento Rápido de Índices para Aedes aegypti – LIRAa – para Vigilância Entomológica do Aedes aegypti no Brasil.** TRANSMISSÍVEIS D. D. V. D. D. Brasil: Ministério da Saúde 84 p. 2013.

MS; SVS. **Dengue: diagnóstico e manejo clínico: adulto e criança.** TRANSMISSÍVEIS D. D. V. D. D. Brasil: Ministério da Saúde 2016a.

MS; SVS. **Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 52, 2015**. Brasil: Ministério da Saúde. 47**:** 1-10 p. 2016b.

MS; SVS. **Nota Informativa - Procedimentos a serem adotados para a vigilância da Febre do vírus Zika no Brasil**. Brasil: Ministério da Saúde 2016c.

MS; SVS. **Guia de Vigilância em Saúde**. SERVIÇOS C.-G. D. D. D. E. E. Brasil: Ministério da Saúde**:** 740 p. 2019a.

MS; SVS. **Monitoramento dos casos de arboviroses urbanas transmitidas pelo Aedes (dengue, chikungunya e Zika) até a Semana Epidemiológica 12 de 2019 e Levantamento Rápido de Índices para Aedes aegypti (LIRAa)** TRANSMISSÍVEIS D. D. V. D. D. Brasilia, Brasil: Ministério da Saúde. 50 2019b.

MS; SVS. **Situação epidemiológica da síndrome congênita associada à infecção pelo vírus Zika em 2020: até a SE 25**. Brasil: Ministério da Saúde. 51**:** 45 p. 2020.

MUSSO, D. et al. Potential sexual transmission of Zika virus. **Emerg Infect Dis,** v. 21, n. 2, p. 359-361, Feb 2015. ISSN 1080-6040 (Print) 1080-6040.

MUSTAFA, M. S. et al. Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. **Medical Journal Armed Forces India,** v. 71, n. 1, p. 67-70, 2015/01/01/ 2015. ISSN 0377-1237. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0377123714001725 >.

NOGUEIRA, R. M. R. et al. Dengue virus type 3 in Rio de Janeiro, Brazil %J Memórias do Instituto Oswaldo Cruz. v. 96, p. 925-926, 2001. ISSN 0074-0276. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762001000700007&nrm=iso >.

NOGUEIRA, R. M. R. et al. Isolation of dengue virus type 2 in Rio de Janeiro %J Memórias do Instituto Oswaldo Cruz. v. 85, p. 253-253, 1990. ISSN 0074-0276. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02761990000200022&nrm=iso >.

NORMILE, D. Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. **Science,** v. 342, n. 6157, p. 415, Oct 25 2013. ISSN 0036-8075.

OPAS; OMS. **Folha informativa - Dengue e dengue grave**. Brasil: Ministério da Saúde 2019.

OSANAI, C. H. et al. [Dengue outbreak in Boa Vista, Roraima. Preliminary report]. **Rev Inst Med Trop Sao Paulo,** v. 25, n. 1, p. 53-54, Jan-Feb 1983. ISSN 0036-4665 (Print) 0036-4665.

OSORIO, L. et al. The use of rapid dengue diagnostic tests in a routine clinical setting in a dengue-endemic area of Colombia. **Memórias do Instituto Oswaldo Cruz,** v. 110, p. 510-516, 2015. ISSN 0074-0276.

OZKURT, Z.; TANRIVERDI, E. C. Global Alert: Zika Virus-an Emerging Arbovirus. **Eurasian J Med,** v. 49, n. 2, p. 142-147, Jun 2017. ISSN 1308-8734 (Print) 1308-8734.

PANCETTI, F. G. M. et al. Twenty-eight years of Aedes albopictus in Brazil: a rationale to maintain active entomological and epidemiological surveillance %J Revista da Sociedade Brasileira de Medicina Tropical. v. 48, p. 87-89, 2015. ISSN 0037-8682. Disponível em: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037-86822015000100087&nrm=iso >.

PAZ-BAILEY, G. et al. Persistence of Zika Virus in Body Fluids — Final Report. v. 379, n. 13, p. 1234-1243, 2017. Disponível em: < https://www.nejm.org/doi/full/10.1056/NEJMoa1613108 >.

PEELING, R. W. et al. Evaluation of diagnostic tests: dengue. **Nat Rev Microbiol,** v. 8, n. 12 Suppl, p. S30-38, Dec 2010. ISSN 1740-1526.

PERKASA, A. et al. Isolation of Zika virus from febrile patient, Indonesia. v. 22, n. 5, p. 924, 2016.

PETERSEN, L. R.; JAMIESON, D. J.; HONEIN, M. A. Zika Virus. **N Engl J Med,** v. 375, n. 3, p. 294-295, Jul 21 2016. ISSN 0028-4793.

PETTERSSON, J. H.; FIZ-PALACIOS, O. Dating the origin of the genus Flavivirus in the light of Beringian biogeography. **J Gen Virol,** v. 95, n. Pt 9, p. 1969-1982, Sep 2014. ISSN 0022-1317.

PIERSON, T. C.; DIAMOND, M. S. The continued threat of emerging flaviviruses. **Nature Microbiology,** v. 5, n. 6, p. 796-812, 2020/06/01 2020. ISSN 2058-5276. Disponível em: < https://doi.org/10.1038/s41564-020-0714-0 >.

POLONI, T. R. et al. Detection of dengue virus in saliva and urine by real time RT-PCR. **Virol J,** v. 7, p. 22-22, 2010. ISSN 1743-422X. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/20105295 https://www.ncbi.nlm.nih.gov/pmc/PMC2835670/ >.

PONTES, R. J.; RUFFINO-NETTO, A. J. R. D. S. P. Dengue em localidade urbana da região sudeste do Brasil: aspectos epidemiológicos. v. 28, p. 218-227, 1994. ISSN 0034-8910.

RASMUSSEN, S. A. et al. Zika Virus and Birth Defects — Reviewing the Evidence for Causality. v. 374, n. 20, p. 1981-1987, 2016. Disponível em: < https://www.nejm.org/doi/full/10.1056/NEJMsr1604338 >.

ROEHRIG, John T .; HOMBACH, Joachim; BARRETT, Alan DT. Diretrizes para testes de neutralização por redução de placa de anticorpos humanos contra o vírus da dengue. **Imunologia viral** , v. 21, n. 2, pág. 123-132, 2008.

ROZÉ, B. et al. Zika virus detection in urine from patients with Guillain-Barré syndrome on Martinique, January 2016. **Euro Surveill,** v. 21, n. 9, p. 30154, 2016. ISSN 1025-496x.

RUST, R. S. Human Arboviral Encephalitis. **Seminars in Pediatric Neurology,** v. 19, n. 3, p. 130-151, 2012/09/01/ 2012. ISSN 1071-9091. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1071909112000186 >.

SALVADOR, F. S.; FUJITA, D. M. Entry routes for Zika virus in Brazil after 2014 world cup: New possibilities. **Travel Med Infect Dis,** v. 14, n. 1, p. 49-51, Jan-Feb 2016. ISSN 1477-8939.

SHINOHARA, K. et al. Zika fever imported from Thailand to Japan, and diagnosed by PCR in the urine. **Journal of Travel Medicine,** v. 23, n. 1, 2016. ISSN 1195-1982. Disponível em: < https://doi.org/10.1093/jtm/tav011 >. Acesso em: 7/28/2020.

SINGH, R. K. et al. Zika virus - emergence, evolution, pathology, diagnosis, and control: current global scenario and future perspectives - a comprehensive review. **Vet Q,** v. 36, n. 3, p. 150-175, Sep 2016. ISSN 0165-2176.

SOUZA, L. J. D. **Dengue, Zika e Chikungunya - Diagnóstico, Tratamento e Prevenção**. 1ª. 2016. ISBN 9788584110674.

UMAPATHI, T. et al. Asymptomatic dengue infection may trigger Guillain-Barre syndrome. **J Peripher Nerv Syst,** v. 21, n. 4, p. 375-377, Dec 2016. ISSN 1085-9489.

VILLAR, J. et al. International standards for newborn weight, length, and head circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project. **Lancet,** v. 384, n. 9946, p. 857-868, Sep 6 2014. ISSN 0140-6736.

WEAVER, S. C. et al. Zika, Chikungunya, and Other Emerging Vector-Borne Viral Diseases. **Annu Rev Med,** v. 69, p. 395-408, Jan 29 2018. ISSN 1545-326X (Electronic) 0066-4219 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28846489 >.

WEAVER, S. C. et al. Zika, Chikungunya, and Other Emerging Vector-Borne Viral Diseases. v. 69, n. 1, p. 395-408, 2018. Disponível em: < https://www.annualreviews.org/doi/abs/10.1146/annurev-med-050715-105122 >.

WEAVER, S. C.; REISEN, W. K. J. A. R. Present and future arboviral threats. v. 85, n. 2, p. 328-345, 2010. ISSN 0166-3542.

WEAVER, S. C.; VASILAKIS, N. Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. **Infection, Genetics and Evolution,** v. 9, n. 4, p. 523-540, 2009/07/01/ 2009. ISSN 1567-1348. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1567134809000367 >.

WHITE, M. K. et al. Zika virus: An emergent neuropathological agent. **Ann Neurol,** v. 80, n. 4, p. 479-489, Oct 2016. ISSN 0364-5134 (Print) 0364-5134.

WHO. **Dengue hemorrhagic fever: diagnosis, treatment, prevention and control**. Geneva: World Health Organization 1997.

WHO. **Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever.** New Delhi, India: World Health Organization**:** 212 p. 2011.

WHO. **Avaliação de bebês com microcefalia no contexto do vírus Zika:** 3 p. 2016a.

WHO. WHO to convene an International Health Regulations Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations. 2016b. Disponível em: < https://www.who.int/en/news-room/detail/28-01-2016-who-to-convene-an-international-health-regulations-emergency-committee-on-zika-virus-and-observed-increase-in-neurological-disorders-and-neonatal-malformations >.

ZANLUCA, C. et al. First report of autochthonous transmission of Zika virus in Brazil. v. 110, n. 4, p. 569-572, 2015. ISSN 0074-0276.

ZHANG, Y. et al. Structures of immature flavivirus particles. **Embo j,** v. 22, n. 11, p. 2604-2613, Jun 2 2003. ISSN 0261-4189 (Print)
0261-4189.

Capítulo II – Artigo I: Detection of Zika virus in urine from individuals unsuspected of arboviral infection

Detection of Zika virus in urine from individuals unsuspected of arboviral infection

Pâmela Jóyce Previdelli da Conceição[a], Lucas Rodrigues de Carvalho[a], Bianca Lara Venâncio de Godoy[b], Mauricio Lacerda Nogueira[c], Ana Carolina Bernardes Terzian[c], Moacir Fernandes de Godoy[d], Marília Freitas Calmon[a], Cintia Bittar [a], Paula Rahal[a].

a. Department of Biology, São Paulo State University—UNESP, São José do Rio Preto, São Paulo, Brazil.
b. Department of Molecular Biology, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.
c. Department of Dermatological, Infectious and Parasitic diseases, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.
d. Department of Cardiology and Cardiovascular Surgery, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.

Pâmela Jóyce Previdelli da Conceição and Lucas Rodrigues de Carvalho share first authorship. Cintia Bittar and Paula Rahal share last authorship.

Corresponding Author Information:
Cintia Bittar
Department of Biology, Instituto de Biociências, Letras e Ciências Exatas - IBILCE/UNESP
Rua Cristóvão Colombo, 2265, 15054-000 - São José do Rio Preto, SP, Brazil
e-mail: cibittar@gmail.com

Abstract

Purpose: Studies show that 80% Zika virus (ZIKV) infections are asymptomatic. Thus the present study tested urine samples from volunteers, unsuspected of arboviral infection, which attended an Emergency Care Unit (UPA) in Brazil from March 2018 to April 2019.

Methods: The volunteers were divided into two groups. The first is composed of patients who have not been clinically diagnosed with arboviruses by the attending physician. This group was subdivided into two subgroups: patients with and without arbovirus-related symptoms. The second group consisted of companions of patients treated at the UPA and was also subdivided into two subgroups: totally asymptomatic individuals and those who had symptoms related to arbovirus. RNA from urine samples was extracted followed by RT-qPCR for ZIKV.

Results: We found that 11% (79/697) of the samples were positive for ZIKV. Among the positive individuals, 16.5% (13) were companions, of which 61.5% (n = 8/13) were totally asymptomatic and 38.5% (n = 5/13) reported symptoms that could be suggestive of arbovirus. In addition, 83.5% (66) were patients without a clinical diagnosis of arbovirus, of which 47% (n = 31/66) belonged to the subgroup of patients without symptoms related to arbovirus and 53% (n = 35/66) to the subgroup of patients with arbovirus-related symptoms.

Conclusion: Here we show how urine can be an effective noninvasive sample to detect ZIKV infection. This study also highlights the importance of ZIKV molecular diagnosis in order to aid public health surveillance and prevent congenital Zika syndrome and other ZIKV-associated diseases.

Keywords: Zika virus, ZIKV asymptomatic, urine, no clinical symptoms of arboviruses.

1. Introduction

Arboviruses, transmitted by hematophagous arthropods such as *Aedes aegypti,* cause important impact on public health and big economic losses every year [1]. Although Zika virus (ZIKV), an arbovirus from the *Flavivirus* genus, was first isolated in 1947 from a rhesus monkey in the Zika Forest, Uganda, only after decades it became an important health issue worldwide [2]. Following its discovery human infections were sporadic being reported in Africa and Asia [3]. The first ZIKV outbreak occurred in Yap Island, federated states of Micronesia in 2007, it is estimated that the virus reached 73% of the population [4]. The virus spread and in 2013 an outbreak was reported in French Polynesia [5]. In Brazil, the first autochthonous case of ZIKV occurred in 2015 [6]. During the epidemic, an increase in microcephaly cases was reported by the state of Pernambuco which was later confirmed to be associated to Zika virus infection [7, 8]. Besides, there was also an increase in the number of cases of Guillain Barré syndrome in Brazil and other countries [9]. After this outbreak, the World Health Organization (WHO) declared a state of global emergency in 2016 [10].

So far, all 27 states Brazilian confirmed the circulation of ZIKV. Between 2015 to 2020 a total of 3535 cases of congenital Zika syndrome were confirmed in the country, with the northeast and southeast regions being the most affected [11]. The infection can by asymptomatic in approximately 80% of the cases [4]. When symptoms are present they resemble the ones caused by other arbovirus, like Dengue virus. [4]. After confirmation of autochtonous transmission, only severe cases are laboratory-analyzed, such as pregnant women, patients with signs of neurological disease and death, the remaining cases are confirmed based on clinical and epidemiological data [12]. The clinical symptoms defined by the Brazilian Ministry of Health for a clinical diagnosis of Zika infection are pruritic maculopapular rash with two or more symptoms, such as fever, conjunctival hyperemia without secretion and pruritus, polyarthralgia, or periarticular edema [12].

Several fluids can be used for the diagnosis of ZIKV, such as serum, saliva, urine, umbilical cord, nasopharyngeal swab, amniotic fluid and vaginal secretion [13]. Urine is recommended for the diagnosis of ZIKV using RT-qPCR, as it is a noninvasive sample and the virus can be detected for a longer period of time when compared to serum samples [13]. Studies show that the presence of the virus in urine can be observed approximately 10 to 20 days, after the onset of symptoms, while in serum this period is shorter, approximately 3 to 7 days.[14, 15]. The same can be noticed for other types of flaviviruses, such as Dengue virus and West Nile virus [14, 16, 17].

Here we investigate the presence of ZIKV in urine samples from patients without clinical diagnosis of arboviruses attended in an Emergency Care Unit in the city of Mirassol, located in the northwestern region of the São Paulo state, Brazil. We also evaluated healthy volunteers that were accompanying patients attended at the healthcare facility, for the presence of the virus.

2. Methods

2.1 Population

This study included two groups of volunteers. The first is composed by patients that attended the Emergency Care Unit (UPA) from the city of Mirassol, SP, Brazil and where not clinically diagnosed with arbovirus infection by the attending physician. This group was subdivided into two subgroups: patients with and without symptoms related to arboviruses. The second comprised companions of patients who were being attended at the healthcare facility and was also subdivided into two groups: completely asymptomatic individuals and those that disclosed symptoms related to arboviruses (Fig. 1). Samples were collected from March 2018 to April 2019. All volunteers invited to participate on the study signed a written consent form. This study was approved by the Research Ethics Committee of the São Paulo University State, Institute of Biosciences, Letters and Exact Sciences (UNESP-IBILCE) of São José do Rio Preto –SP (CAAE: 66489617.4.0000.5466).

The city of Mirassol is located at 20º25'S and 51º21'W, in the state of São Paulo, Brazil about 460 Km from the capital of São Paulo and 15 Km of São José do Rio Preto. It has an estimated population of 59,824 inhabitant and the population density is 221, 10 inhabitants per Km^2 with approximately 97.9% of urban household. The city has a tropical climate (Köppen-Geiger); with rainy summers and dry winters. The annual precipitation level is 1223 mm and average annual temperature of 22°C (IBGE, unpublished data).

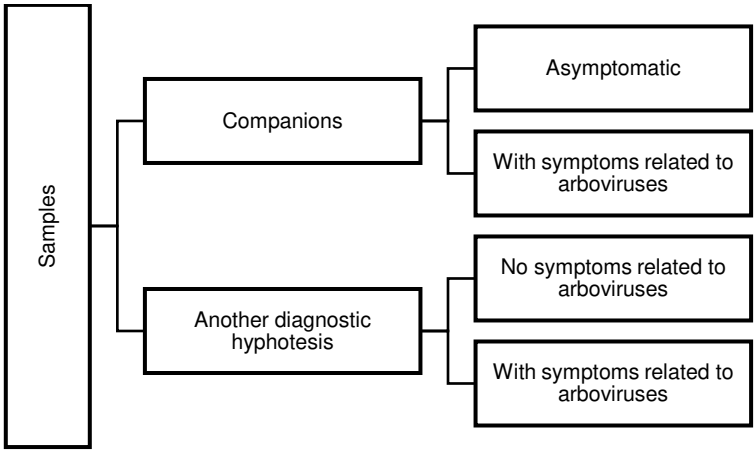


Fig. 1 Divisions of the groups and subgroups described in the study

2.2 Evaluation of symptoms

All volunteers participating in the study, including companions, received a questionnaire to assess possible symptoms. The questionnaire contained questions about the clinical complaints that led the patients to the UPA, including the type of symptoms and duration.

According to the Brazilian Ministry of Health patients infected with arbovirus may present several symptoms [18]. A suspected case of dengue fever should show symptoms of fever, between two and seven days, with two or more of the following symptoms: nausea, vomiting, exanthema, arthralgia, headache, retro-orbital pain, rash or positive loop test, and leukopenia [18]. In the case of ZIKV, they can present pruritic maculopapular rash with two or more of the following signs and symptoms, fever, conjunctival hyperemia without secretion and itching, polyarthralgia or periarticular edema. Suspected infection by the Chikungunya virus should be considered in the event of a sudden onset of fever above 38.5 ° C and severe joint pain, with or without inflammatory signs, with cannot be explained by other diseases. Volunteers were classified as having symptoms related to arbovirus infection when reported having one or more of the symptoms described above [18].

2.3 Samples

Patients were asked to provide urine samples that were stored at 4°C up to 48h until processing**.** The urine samples were collected in a universal collection bottle and received a specific code for each volunteer. The tubes were centrifuged at 448 x g for 10 minutes at room

temperature. After the end of centrifugation, part of the supernatant was discarded, leaving 5 mL. The *pellets* were ressuspended and samples were filtered with a 0.45 nm PES syringe filter in order to eliminate possible contaminants such as other microorganisms. The samples were stored in an ultra-freezer at -150°C.

2.4 RNA extraction and ZIKV detection

Viral RNA extraction was performed using 250 µL of urine sample using monophasic solution of guanidine thiocyanate and phenol (TRIzol - Invitrogen™, Life Technologies®, USA) according to the manufacturer's instructions. Viral detection was performed by RT-qPCR as proposed by Lanciotti (2008) using *GoTaq® Probe 1-Step RT-qPCR System* (*Promega Corporation,* Madison, USA) [19]. The reaction was carried out on *QuantStudio™ 12K Flex Real Time PCR System* (*Applied Biosystems™,* Foster City, USA).

2.5 Statistical analysis

Statistical analyses were performed using StatsDirect Statistical Software (version 3.3.4, Merseyside, UK). The Unpaired t test was used to compare the age distribution between positive and negative individuals for ZIKV. The association tests between the gender and laboratory-confirmed ZIKV were analyzed using ODDS Ratio analysis, Pearson's chi square test with a significance level of 5% and, when the prerequisites were not met, we used the Fisher's exact test. The association between the other variables and laboratory-confirmed ZIKV was also made using Pearson's chi square test.

3. Results

Samples were collected from 697 volunteers and were transported to the Genomic Studies Laboratory from São Paulo State University (UNESP) to carry out all analyzes. Of the studied population, 69.6% (485) of the individuals were female and 30.4% (212) were male. In addition, 15.8% (110) individuals belonged to the group of companions and 84.2% (587) to the group of patients without clinical diagnosis of arboviruses. The age of all individuals varied from 0 to 97 years, with median of 43 years.

From the 697 urine samples analyzed, 11.3% (79) were positive for ZIKV. Regarding gender, 40.5% (32) were male, 59.5% (47) were female and among these women, one was pregnant. The age of infected individuals varied from 3 to 97 years of age, with a median of 44

years (Table 1). We observed an association between gender and ZIKV infection, although the p value was borderline (0.0523), an ODDS ratio showed significance, that is, the chance of a man being positive for ZIKV is 65.7% greater than the chance of a woman being positive for ZIKV (OR= 1.657; CI95% 1.023-2.681). No association was observed for any age group.

Table 1 Characteristics of individuals with laboratory-confirmed ZIKV

Characteristics of individuals		**ZIKV**					
		Negative		**Positive**		**Total**	
		(n=618)	%	(n=79)	%	n=697	%
Gender	**Female**	438	70.9	47	59.5	485	69.6
	Male	180	29.1	32	40.5	212	30.4
Age range	**0-19 years**	98	15.9	12	15.2	110	15.8
	20 -59 years	374	60.5	51	64.6	425	61
	60 years or over	143	23.1	16	20.2	159	22.8
	No data	3	0.5	0	0	3	00.4
Group	**Companions**	97	15.7	13	16.5	110	15.8
	Another Diagnostic hypothesis	521	84.3	66	83.5	587	84.2

Among the positive individuals, 16.5% (13) were companions and 83.5% (66) were patients without clinical diagnosis of arboviruses (Fig. 2).

Among the ZIKV-positive companions, 61.5% (n= 8/13) were totally asymptomatic and 38.5% (n= 5/13) reported symptoms that could be suggestive of arbovirus infection (Fig. 2). Despite not having a medical consultation at the UPA, the companions reported feeling some minor symptoms, such as muscle pain (80%; n= 4/5), itchy skin (40%; n= 2/5), nausea (20%; n= 1/5) and vomiting (20%; n= 1/5), however, since this symptoms were mild they felt no need to seek health service.

In the ZIKV-positive group with clinical diagnosis of other diseases, 47% (n= 31/66) belonged to the subgroup of patients without symptoms related to arboviruses and 53% (n= 35/66) to the subgroup of patients with symptoms related to arboviruses. Patients positive for ZIKV were diagnosed with various diseases by the attending physician as can be seen in Table 2. The predominant diagnostic hypothesis was disease of the respiratory system (15.49%) followed by multisystemic and urinary system diseases (Table 2). The individuals were classified as multisystemic when they presented symptoms related to two or more systems.

Table 2 Diagnostic hypothesis from volunteers in the group of patients without clinical diagnosis of arbovirus infection

Diagnosis	*ZIKV (+)*	*ZIKV (-)*	*% positive*	*Total*
Multisystemic diseases	13	89	12.75	102
Cardiovascular diseases	3	25	10.71	28
Digestive disorder	10	82	10.87	92
Diseases of the reproductive system	1	14	6.67	15
Musculoskeletal disorders	6	63	8.70	69
Diseases of the nervous system and sense organs	1	22	4.35	23
Diseases of the respiratory system	11	60	15.49	71
Disease cutaneous system	0	3	0	3
Disease of the urinary system	21	162	11.48	183
No data				1
Total	66	520	12.69	587

Thus, altogether from the 79 ZIKV-positive individuals, 49.4% (39) had no symptoms suggestive of arboviruses and 50.6% (40) presented symptoms that could be associated to arbovirus infection (Fig. 2). We observed that the signs and symptoms reported by individuals with ZIKV infection were myalgia (32.9%; n=26), fever (19%; n=15), nausea (19%; n=15), vomiting (17.7%; n=14), retro orbital pain (15.2%; n=12), arthralgia (12.7%; n=10), itchy skin (10.1%; n=8), intolerance to light (9%; n=7), rash (2.5%; n=2) and red eyes (2.5%; n=2) (Table 3).

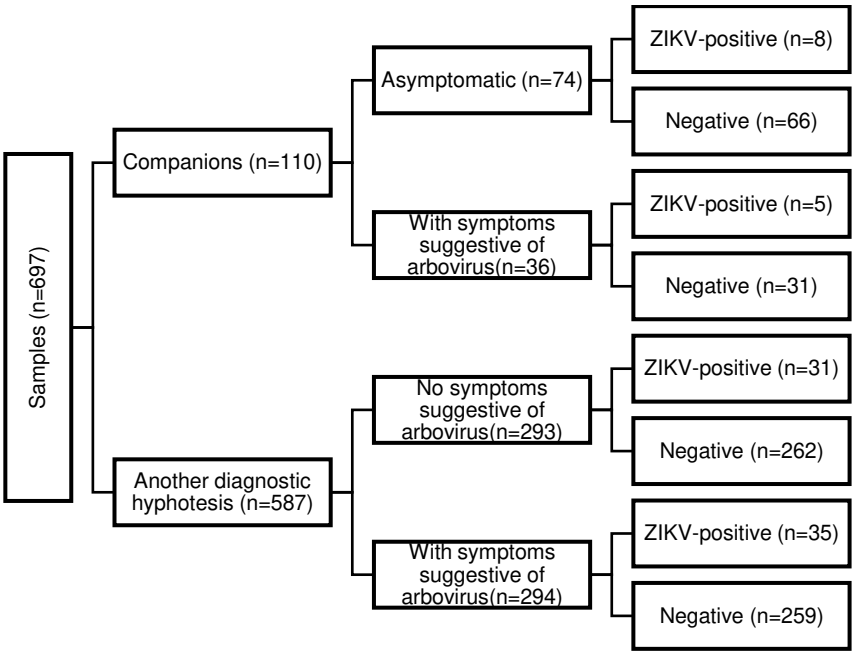


Fig. 2 Representation of the number of samples and ZIKV-positive individuals and their respective groups and subgroups

Table 3 Symptoms reported by and ZIKV-positive and ZIKV-negative individuals

Symptoms	**ZIKV**			
	Positive (n=79)		**Negative (n=618)**	
	n	**%**	**n**	**%**
Fever	15	19	123	19.9
Nausea	15	19	132	21.4
Vomiting	14	17.7	91	14.7
Red eyes	2	2.5	26	4.2
Retro-orbital pain	12	15.2	62	10.0
Intolerance to light	7	9	27	4.4
Itchy skin	8	10.1	43	7.0
Rash	2	2.5	10	1.6
Myalgia	26	32.9	139	22.5
Arthralgia	10	12.7	91	14.7

The monthly distribution of ZIKV-positive cases, as well as the number of samples collected are presented in Fig. 3. Positive cases were around 10% from April through September 2018. After a decrease in October, the percentage of positive cases rose in the following months reaching its peak in January 2019. There was significantly more cases of ZIKV infection in December (16%; n=8/79), January (31.6%; n=25/79) and February (20.8% n=11/79) which corresponds to summer in the Southern hemisphere, being the warmest and wettest season (p-value < 0,001).

Fig 3 Distribution of samples collected and ZIKV-positive cases during the year.

In fact, when analyzing temperature and precipitation over the year (Fig. SI1), an increase in both is observed in the months where ZIKV cases rise, but there was no significant correlation between such data and the percentage of positivity for ZIKV. According to The Integrated Agrometeorological Information Center (CIIAGRO), from September 2018, there is a gradual increase in the number of days of rain, which decreases with the arrival of autumn 2019 and the same is observed for the temperature, which as expected increases in spring and summer (Supplementary Fig. 1) [20].

4. Discussion

Zika virus expansion throughout the Americas highlights the importance of surveillance and monitoring of emerging diseases. Several studies show that ZIKV infection is a self-limiting and asymptomatic disease, in 80% of cases [21, 4]. Symptomatic patients have non-characteristic symptoms, which make differential clinical diagnosis a challenge, often confused with other arbovirus infection, non-viral diseases, or even flu [22-25]. In Brazil, ZIKV is diagnosed based on clinical and epidemiological data, hardly being confirmed by laboratory tests, making it more difficult to monitor the real circulation of the virus [21]. In this study, we

collected urine samples from asymptomatic individuals located in a municipality with a history of arbovirus circulation, due to the importance of more effective monitoring of ZIKV infection and the need to present up-to-date data on the circulation of the virus in this region. The city of Mirassol, located in the northwest of the state of São Paulo is 15 Km from the city of São José do Rio Preto, a hyperendemic city for Dengue fever [26, 27]. Studies in São José do Rio Preto, showed the co-circulation of CHIKV, DENV and ZIKV, and confirmed cases of the Saint Louis encephalitis virus (SLEV) [28, 29, 22].

Here we analyzed urine samples from 697 individuals, either asymptomatic or without clinical diagnosis of arbovirus infection, over a period of 14 months. We found that 11.3% (79/697) of the volunteers analyzed were positive for ZIKV. Although the number of women positive for ZIKV is higher, the chance of a man being positive for ZIKV is 65.7% greater than that of a woman. This is explained since the population in our study is composed mainly of women (69.6%). Other studies also show to have higher number of participants from the female gender and also report a higher number of cases of ZIKV infection in this population. [22, 25, 30, 31]. However, those studies did not perform any statistical analysis to normalize for the disparity of the number of volunteers from each gender. Women are known to most frequently seek medical attention and this fact can be explained by socio-cultural factors [32]. In addition, most of the volunteers in our study were companions of patients that were being attended at the health care unit, also for social-cultural reasons this task is more frequently performed by women.

We divided the individuals into two groups, companions and patients without clinical diagnosis of arboviruses, in order to investigate asymptomatic circulation of the virus. We also divided these individuals into totally asymptomatic and those with and without symptoms suggestive of arbovirus infection. Regarding subjects that reported symptoms related to arbovirus infection, ZIKV-positive individuals disclosed myalgia, fever, nausea, vomiting, retro-orbital pain, arthralgia, itchy skin, intolerance to light, rash and red eyes. Although 50.6% of the ZIKV-positive individuals presented one or more of these symptoms, them alone were not sufficient for them to be clinically diagnosed with ZIKV or other arboviral disease. According to the Brazilian Ministry of Health the definition of a ZIKV suspect case includes a pruritic maculopapular rash with two or more of the following signs and symptoms, fever, conjunctival hyperemia without secretion and itching, polyarthralgia or periarticular edema [18]. Other studies have also reported ZIKV detection associated with fever, rash, non-purulent conjunctivitis, myalgia and headaches in ZIKV-positive patients [33]. One study has also reported the presence of cough, vomiting, nausea and sore throat [22]. Since most of these

subjects were attending the emergency care unit for other complaints and, myalgia was the most observed symptom it is unclear if the other symptoms disclosed are related to the ZIKV infection or not. Zika virus infection is characterized majorly as asymptomatic [4]. In fact, we found that 49.4% (39/79) of the subjects enrolled in this study did not have symptoms suggestive of arbovirus infection and 10.1% (8/79) were completely asymptomatic.

It is interesting that among patients with other diagnostic hypotheses 15% of patients with respiratory system disease had ZIKV. This finding is important, as studies show that arbovirus symptoms can be easily confused with flu symptoms [24, 23, 22]. In addition, the months when arboviruses are circulating, there is also the circulation of other viruses, such as the *Influenza virus* [34, 35].

We observed the increase in the number of ZIKV cases as of September, when spring begins, and the decrease in the number of cases with the beginning of autumn. The seasonality of the vector explains this behavior since the months with increased temperature, relative humidity and rainfall helps dissemination of the vector and the increase of breeding [36]. Countries in tropical and subtropical regions are more susceptible to the spread of diseases caused by arboviruses, due to the ideal conditions for proliferation of vector mosquitoes [36]. Other factors that contribute to the proliferation of vectors are: population growth and urbanization without adequate infrastructure, deforestation, soil management and also climate change, which negatively impact the risk of increase and transmission of arbovirus [36].

To date in Brazil, ZIKV outbreaks were caused by Asian strain circulation of the disease, but recent studies show the circulation of the African strain in primates and mosquitoes in the southeastern region of the country [37-40]. Although the real risk of the of a spillover of the African strain is yet to be determine, surveillance strategies are crucial to prevent epidemics of new strains of ZIKV in the country [37].

In conclusion, our study showed the importance of better investigation of ZIKV cases, through specific laboratory tests of urine samples from unsuspected individuals. Our data also showed the occurrence of asymptomatic infection of ZIKV. The diagnosis based only on clinical and epidemiological data is not ideal, especially for the diagnosis of ZIKV, which is a disease that does not present specific symptoms and can be confused with several diseases. All these factors make it difficult to investigate and correctly diagnose the disease, causing underreporting of real ZIKV cases. Moreover, these factors prevent the municipality from taking the necessary measures to contain the disease and avoid clinical complications, especially for pregnant women.

Acknowledgements

We thank the city of Mirassol and the director and staff of the Emergency Care Unit of Mirassol that allowed us to conduct this study. We also thank all the participants that contributed to this study.

Funding

This study was financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001 and Grant number 2046/2016; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Grant number 440723/2016-7 and São Paulo Research Foundation (FAPESP) – Grant Number 2014/22198-0.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

The Committee Ethics from São Paulo University State – UNESP approved this study (CAAE: 66489617.4.0000.5466).

Consent to participate

All volunteers signed an informed consent form before participating in the study.

5. References

1. Weaver SC, Reisen WKJAr. Present and future arboviral threats. 2010;85(2):328-45.

2. Dick G, Kitchen S, Haddow AJTotrsotm, hygiene. Zika virus (I). Isolations and serological specificity. 1952;46(5):509-20.

3. Hayes EB. Zika virus outside Africa. Emerging infectious diseases. 2009;15(9):1347-50. doi:10.3201/eid1509.090442.

4. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS et al. Zika virus outbreak on Yap Island, federated states of Micronesia. 2009;360(24):2536-43.

5. Cao-Lormeau VM, Roche C, Teissier A, Robin E, Berry AL, Mallet HP et al. Zika virus, French polynesia, South pacific, 2013. Emerging infectious diseases. 2014;20(6):1085-6. doi:10.3201/eid2006.140138.

6. Zanluca C, Melo VCAd, Mosimann ALP, Santos GIVd, Santos CNDd, Luz KJMdIOC. First report of autochthonous transmission of Zika virus in Brazil. 2015;110(4):569-72.

7. Turchi Martelli CM, Albuquerque MdFP, Araújo TV, Barkokebas A, Bezerra LCA, Braga C et al. Microcephaly in Infants, Pernambuco State, Brazil, 2015. 2016;22(6).

8. MS. Ministério da Saúde confirma relação entre vírus Zika e microcefalia. Ministério da Saúde, Brasilia. 2015. https://www.gov.br/saude/pt-br/assuntos/noticias/ministerio-da-saude-confirma-relacao-entre-virus-zika-e-microcefalia. Accessed 10/03/2020.

9. Campos GS, Bandeira AC, Sardi SI. Zika Virus Outbreak, Bahia, Brazil. Emerging infectious diseases. 2015;21(10):1885-6. doi:10.3201/eid2110.150847.

10. WHO. WHO to convene an International Health Regulations Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations. 2016. https://www.who.int/en/news-room/detail/28-01-2016-who-to-convene-an-international-health-regulations-emergency-committee-on-zika-virus-and-observed-increase-in-neurological-disorders-and-neonatal-malformations.

11. MS, SVS. Situação epidemiológica da síndrome congênita associada à infecção pelo vírus Zika em 2020: até a SE 25. Brasil: Ministério da Saúde; 2020. p. 45.

12. MS. Procedimentos a serem adotados para a vigilância da Febre do vírus Zika no Brasil. 2016.

13. Paz-Bailey G, Rosenberg ES, Doyle K, Munoz-Jordan J, Santiago GA, Klein L et al. Persistence of Zika Virus in Body Fluids — Final Report. 2017;379(13):1234-43. doi:10.1056/NEJMoa1613108.

14. Gourinat A-C, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M. Detection of Zika virus in urine. Emerging infectious diseases. 2015;21(1):84-6. doi:10.3201/eid2101.140894.

15. Shinohara K, Kutsuna S, Takasaki T, Moi ML, Ikeda M, Kotaki A et al. Zika fever imported from Thailand to Japan, and diagnosed by PCR in the urine. Journal of Travel Medicine. 2016;23(1). doi:10.1093/jtm/tav011.

16. Hirayama T, Mizuno Y, Takeshita N, Kotaki A, Tajima S, Omatsu T et al. Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum. Journal of clinical microbiology. 2012;50(6):2047-52. doi:10.1128/JCM.06557-11.

17. Barzon L, Pacenti M, Franchin E, Pagni S, Martello T, Cattai M et al. Excretion of West Nile virus in urine during acute infection. J Infect Dis. 2013;208(7):1086-92. doi:10.1093/infdis/jit290.

18. MS. GUIA DE VIGILÂNCIA EM SAÚDE. 3 ed. Brasília: Ministério da Saúde; 2019. p. 740.

19. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerging infectious diseases. 2008;14(8):1232-9. doi:10.3201/eid1408.080287.

20. CIIAGRO. Centro Integrado de Informações Agrometeorológicas. Instituto Agronômico, São Paulo. http://www.ciiagro.sp.gov.br/ciiagroonline/. Accessed 14 de outubro 2020.

21. Haby MM, Pinart M, Elias V, Reveiz L. Prevalence of asymptomatic Zika virus infection: a systematic review. Bull World Health Organ. 2018;96(6):402-13d. doi:10.2471/blt.17.201541.

22. Colombo TE, Estofolete CF, Reis AFN, da Silva NS, Aguiar ML, Cabrera EMS et al. Clinical, laboratory and virological data from suspected ZIKV patients in an endemic arbovirus area. Journal of Clinical Virology. 2017;96:20-5. doi:https://doi.org/10.1016/j.jcv.2017.09.002.

23. Perdigão ACB, Ramalho ILC, Guedes MIF, Braga DNM, Cavalcanti LPG, Melo MELd et al. Coinfection with influenza A(H1N1)pdm09 and dengue virus in fatal cases %J Memórias do Instituto Oswaldo Cruz. 2016;111:588-91.

24. Chacon R, Clara AW, Jara J, Armero J, Lozano C, El Omeiri N et al. Influenza illness among case-patients hospitalized for suspected dengue, El Salvador, 2012. 2015;10(10):e0140890.

25. Fernanda Estofolete C, Terzian ACB, Parreira R, Esteves A, Hardman L, Greque GV et al. Clinical and laboratory profile of Zika virus infection in dengue suspected patients: A case series. Journal of Clinical Virology. 2016;81:25-30. doi:https://doi.org/10.1016/j.jcv.2016.05.012.

26. Colombo TE, Vedovello D, Pacca-Mazaro CC, Mondini A, Araújo JP, Cabrera E et al. Dengue virus surveillance: Detection of DENV-4 in the city of São José do Rio Preto, SP, Brazil. Acta Tropica. 2016;164:84-9. doi:https://doi.org/10.1016/j.actatropica.2016.09.004.

27. Mondini A, Bronzoni RVdM, Cardeal ILS, Santos TMILd, Lázaro E, Nunes SHP et al. Simultaneous infection by DENV-3 and SLEV in Brazil. Journal of Clinical Virology. 2007;40(1):84-6. doi:https://doi.org/10.1016/j.jcv.2007.06.007.

28. Mondini A, Cardeal ILS, Lázaro E, Nunes SH, Moreira CC, Rahal P et al. Saint Louis Encephalitis Virus, Brazil. Emerging Infectious Disease journal. 2007;13(1):176. doi:10.3201/eid1301.060905.

29. Estofolete CF, Terzian ACB, Colombo TE, de Freitas Guimarães G, Ferraz HCJ, da Silva RA et al. Co-infection between Zika and different Dengue serotypes during DENV outbreak in Brazil. J Infect Public Health. 2019;12(2):178-81. doi:10.1016/j.jiph.2018.09.007.

30. Brasil P, Calvet GA, Siqueira AM, Wakimoto M, de Sequeira PC, Nobre A et al. Zika Virus Outbreak in Rio de Janeiro, Brazil: Clinical Characterization, Epidemiological and Virological Aspects. PLoS neglected tropical diseases. 2016;10(4):e0004636. doi:10.1371/journal.pntd. 0004636.

31. Sharp TM, Quandelacy TM, Adams LE, Aponte JT, Lozier MJ, Ryff K et al. Epidemiologic and spatiotemporal trends of Zika Virus disease during the 2016 epidemic in Puerto Rico. PLoS neglected tropical diseases. 2020;14(9):e0008532-e. doi:10.1371/journal.pntd.0008532.

32. Levorato CD, Mello LMd, Silva ASd, Nunes AA. Fatores associados à procura por serviços de saúde numa perspectiva relacional de gênero %J Ciência & Saúde Coletiva. 2014;19:1263-74.

33. Cerbino-Neto J, Mesquita EC, Souza TML, Parreira V, Wittlin BB, Durovni B et al. Clinical Manifestations of Zika Virus Infection, Rio de Janeiro, Brazil, 2015. Emerging infectious diseases. 2016;22(7):1318-20. doi:10.3201/eid2207.160375.

34. MS, SVS. Monitoramento dos casos de influenza no Brasil, Semanas Epidemiológicas 1 a 32 de 2019. Brasil: Ministério da Saúde; 2019. p. 1-9.

35. MS, SVS. Influenza: Monitoramento até a Semana Epidemiológica 28 de 2018. Brasil: Ministério da Saúde; 2018.

36. Araújo RAF, Uchôa NM, Alves JMB. Influência de Variáveis Meteorológicas na Prevalência das Doenças Transmitidas pelo Mosquito Aedes Aegypti %J Revista Brasileira de Meteorologia. 2019;34:439-47.

37. Kasprzykowski JI, Fukutani KF, Fabio H, Fukutani ER, Costa LC, Andrade BB et al. A recursive sub-typing screening surveillance system detects the arising of the ZIKV African lineage in Brazil: Is there risk of a new epidemic? 2020.

38. Faria NR, da Silva Azevedo RdS, Kraemer MU, Souza R, Cunha MS, Hill SC et al. Zika virus in the Americas: early epidemiological and genetic findings. 2016;352(6283):345-9.

39. Alencar J, Mello C, Marcondes C, Guimarães AÉ, Toma H, Bastos A et al. Natural infection and vertical transmission of two flaviviruses (Yellow fever and Zika) in mosquitoes in primary forests in the Brazilian state of Rio de Janeiro (Diptera: Culicidae). 2019:688713.

40. de Almeida PR, Ehlers LP, Demoliner M, Eisen AKA, Girardi V, De Lorenzo C et al. Detection of a novel African-lineage-like Zika virus naturally infecting free-living neotropical primates in Southern Brazil. 2019:828871.

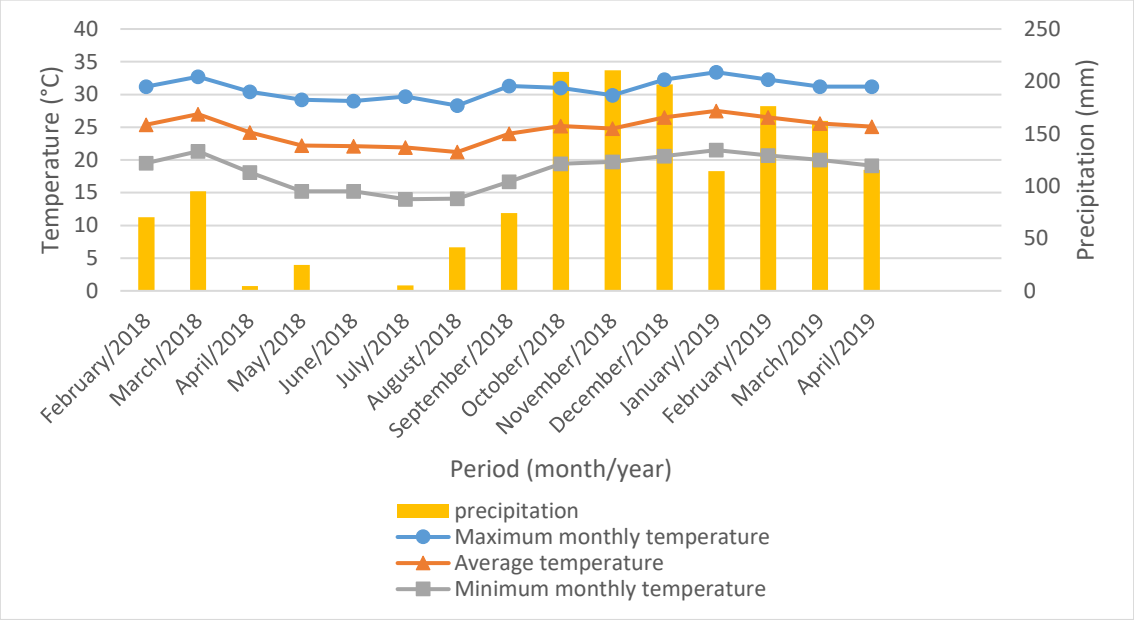


Fig. SI1 Precipitation index, maximum temperatures, average and minimum from February 2018 to April 2019 in the city of Mirassol. Data source: Adapted from CIIAGRO ONLINE/2020

Capítulo III – Artigo II: DENV-2 and ZIKV co-infection detected by testing serum and urine in southeastern Brazil

DENV-2 and ZIKV co-infection detected by testing serum and urine in southeastern Brazil

Pâmela Jóyce Previdelli da Conceição[a], Lucas Rodrigues de Carvalho[a], Bianca Lara Venâncio de Godoy[b], Mauricio Lacerda Nogueira[c], Ana Carolina Bernardes Terzian[c], Moacir Fernandes de Godoy[d], Marília Freitas Calmon[a], Cintia Bittar [a], Paula Rahal[a].

a. Department of Biology, São Paulo State University—UNESP, São José do Rio Preto, São Paulo, Brazil.
b. Department of Molecular Biology, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.
c. Department of Dermatological, Infectious and Parasitic diseases, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.
d. Department of Cardiology and Cardiovascular Surgery, Medical School of São José do Rio Preto – FAMERP, São José do Rio Preto, São Paulo, Brazil.

Pâmela Jóyce Previdelli da Conceição and Lucas Rodrigues de Carvalho share first authorship. Cintia Bittar and Paula Rahal share last authorship.

Corresponding Author Information:
Cintia Bittar
Department of Biology, Instituto de Biociências, Letras e Ciências Exatas - IBILCE/UNESP
Rua Cristóvão Colombo, 2265, 15054-000 - São José do Rio Preto, SP, Brazil
e-mail: cibittar@gmail.com

Abstract

Purpose: The *Aedes aegypti* mosquito-borne diseases cause a great impact in public health in Brazil. In this study, we investigated the presence of Zika virus (ZIKV), Dengue virus (DENV) in serum and urine samples from symptomatic patients who were attended at an Emergency Care Unit in located in a city in the northwestern region of the state of São Paulo, from February 2018 to April 2019.

Methods: Serum and urine samples were collected from volunteers suspected of arbovirus infection. After extraction of viral RNA, the viral detection was performed by real time RT-qPCR (One-Step RT-qPCR).

Results: A total of 308 volunteers participated in the study, and we collected a total of 287 blood samples and 273 urine samples. From the 308, 36.0% (111/308) had ZIKV, 42.5% (132/308) had DENV2, and 0.3% (1/308) had DENV1.When evaluating possible co-infection, we saw that 12.7% of the volunteers were co-infected with ZIKV/DENV2. If only serum samples had been used; ZIKV detection would have dropped to 24.7% (76/308). From all the patients included in the study only one was suspected of ZIKV infection and the remaining were suspected of DENV according to the clinical diagnosis. Symptoms that were most reported for both viruses were myalgia, fever, arthralgia and retro-orbital pain.

Conclusion: By testing serum and urine samples, we increased the detection of both viruses and detected considerable levels of ZIKV / DENV-2 co-infection when compared to other studies. Also, we detected an unnoticed ZIKV outbreak in the city. These findings show the importance of the molecular diagnosis of arboviruses to aid public health surveillance and management strategies.

1. Introduction

The arboviruses cause an impact on public health, affecting thousands of people around the world every year [1, 2]. Among the most important arboviruses is dengue virus (DENV), an arbovirus of the *Flavivirus* genus, *Flaviviridae* family, represented by four urban serotypes with distinct antigenic characteristics (DENV1, DENV2, DENV3, and DENV4), and one sylvatic serotype (DENV5) [3-5]. DENV infection can range from asymptomatic to severe disease that can lead to death [6]. When symptomatic, dengue fever presents different clinical aspects, occurring in three clinical phases: febrile, critical, and recovery [7].

Another *Flavivirus* that has become clinically important in the last decade is Zika virus (ZIKV) [8]. Although it was first isolated from a rhesus monkey in the Zika forest in Uganda in 1947, few reports were made of ZIKV infection in humans until an outbreak that started in 2015 through the spread of an Asian lineage that affected several countries [9, 10]. After the outbreak in French Polynesia, there was also an increase in the number of patients with Guillain-Barré syndrome, linking the disease to the ZIKV outbreak [11]. In Brazil, the first autochthonous case of ZIKV occurred in 2015 in the states of Bahia, Pernambuco, and Rio Grande do Norte. At the same time, a rise in cases of microcephaly in newborns of the region was also observed and researchers suggested a link with ZIKV infection [12-15]. After proving that ZIKV was the cause of microcephaly, other studies have also shown its relationship with congenital Zika syndrome (CZS) [16]. In 2016 the World Health Organization declares a global state of emergency [17].

The transmission of DENV and ZIKV occurs mainly by the bite of mosquitoes of the genus *Aedes*, such as *A. aegypti* and *A. albopictus*, the most common being the first [1]. However, other transmission types have also been reported, such as sexually transmitted, blood transfusion, and perinatal, being more common in the case of ZIKV [18, 19]. Currently, Brazil still goes through arbovirus epidemics every year and is considered a super-endemic country for dengue, in which the only means of prevention so far is the elimination of vectors [20].

The laboratory diagnosis of arboviruses can be made using several types of samples, such as serum, saliva, urine, umbilical cord, nasopharyngeal swab, amniotic fluid, and vaginal secretion [15, 21-25]. In Brazil, the diagnosis is made based on clinical and epidemiological data. In some cases, when DENV is suspected, rapid diagnostic tests are used [26]. These tests are widely used, simple to perform, and are based on the research of the NS1 antigen and IgG and IgM antibodies [26]. However, specificity and sensitivity vary depending on the stage of

the disease. It is also common to experience false positive or false negative results due to cross-reactions with other *Flavivirus*, causing the patient to be incorrectly diagnosed [26].

In this study, we investigated the presence of arboviruses ZIKV and DENV, in serum and urine samples from patients with a clinical diagnosis of arbovirus treated at the Emergency Care Unit (UPA) in the city of Mirassol, located in the northwest region of the state of São Paulo Brazil.

2. Methods

2.1 Population

A prospective study was carried out, in which all volunteers included in this study were considered suspected of arboviral infection by the attending physician at the Emergency Care Unit (UPA) in the city of Mirassol-SP, Brazil. The clinical diagnosis is based on the criteria stablished by the Brazilian Health Ministry. For a case to be suspected of ZIKV, the patient needs to have a pruritic maculopapular rash with two or more of the following symptoms: fever, periarticular edema or polyarthralgia, conjunctival hyperemia without secretion and itching [27]. In the case of DENV, the patient needs to have a fever, between two and seven days, with two or more of the following symptoms: nausea, vomiting, exanthema, myalgia, arthralgia, headache, retro-orbital pain, rash or positive tourniquet test, and leukopenia [27]. Also, patients must reside or be in areas endemic or with *Aedes aegypti* infestation [27].

Blood and urine samples were collected from February 2018 to April 2019. The Research Ethics Committee of the São Paulo University State, Institute of Biosciences, Letters and Exact Sciences (UNESP-IBILCE) of São José do Rio Preto –SP approved this study (CAAE: 66489617.4.0000.5466), and all volunteers signed the Informed Consent to be part of the study. All included patients completed questionnaires about the type and duration of the symptoms (days).

2.2 Samples

Samples were collected and stored at 4 °C for 48 hours until processing. Blood samples were collected in a tube with a gel separator with a coagulation activator to separate the serum. The tubes were centrifuged at 1008 x g for 15 minutes. Serum was separated and stored in cryogenic tubes at -150 °C.

The urine samples were collected in a universal collector bottle at any time of the day, since it was collected at the time of the consultation at the UPA. Urine samples were centrifuged

at 448 x g for 10 minutes at room temperature. After the centrifugation, part of the supernatant was discarded, leaving 5mL in the tubes. Subsequently, the pellet was homogenized with the remaining 5 mL, and filtered with a 0.45 nm PES syringe filter for the elimination of other microorganisms. Finally, urine samples were stored in an Ultra-Low Temperature Freezer -150°C (MDF model - C2156VANC, Sanyo).

2.3 RNA extraction and Real-time PCR

The isolation of RNA from the samples was done using a monophasic solution of guanidine thiocyanate and phenol (TRIzol - Invitrogen™, Life Technologies®, USA) and 250 µL of urine or serum samples according to the manufacturer's instructions. The investigation of the presence of the four serotypes of DENV and ZIKV was carried out by RT-qPCR using *GoTaq® Probe 1-Step RT-qPCR System* (*Promega Corporation,* Madison, USA). Specific primers and probes were used for each serotype of DENV and for ZIKV [28, 29]. The analyses were carried out on *QuantStudio™ 12K Flex Real Time PCR System* (*Applied Biosystems™,* Foster City, USA).

2.4 Statistical analysis

The software used to perform the statistical analysis was the StatsDirect Statistical Software (version 3.3.4, Merseyside, UK). Descriptive statistics were calculated for primary analysis. The Kruskal-Wallis test was used to compare age by disease groups (ZIKV, DENV, co-infection and, without disease). Conover-Iman test was used for pairwise comparisons between groups. The association tests between the gender and ZIKV, DENV and, co-infection were analyzed using Pearson's chi square test, and Fisher's exact test. Difference with p-value ≤ 0.05 was considered.

3. Results

This study was conducted from February 26, 2018, to April 11, 2019, and 308 patients were included, of which 59.7% (184/308) were female. Regarding age, most women (47.3%; n=87/184) ranged from 30 to 59, while for men, most patients (44.3%; n=54/124) were in the 0 to 29 age range (Table 1). The age of all individuals varied from 3 to 83 years, with median of 33 years. From the 308 patients, 80.8% (249/308) collected blood and urine samples, 12.3% (38/308) collected only blood, and 7.8% (24/308) collected only urine. Overall, a total of 287 blood samples and 273 urine samples were collected. The analysis of the clinical reports

revealed 307 patients were clinically diagnosed with DENV, and only 1 patient was diagnosed with ZIKV.

Table 1 Epidemiological data for 308 symptomatic patients for arboviruses

	Female (n=184)	**Male (n=124)**	**Total (n=308)**
0 - 29 years	34.8% (64)	44.4% (55)	38.6% (119)
30 - 59 years	47.3% (87)	30.6% (38)	40.6% (125)
> 60 years	8.1% (15)	11.3% (14)	9.4% (29)
No data	9.8% (18)	13.7% (17)	11.4% (35)

3.1 *Zika vírus*

From the 308 patients included in this study, 36.0% (111/308) had ZIKV infection. Of these, 56.8% (63/111) were female. The age of infected individuals varied from 4 to 69 years, with median of 30 years, upper quartile of 49 years and, lower quartile of 15 years. There was no significant association between gender and ZIKV positive test (p value= 0.4683).

Among the patients with ZIKV infection, 32.4% (36/111) had ZIKV only in the serum, 36.0% (40/111) only in the urine, and 31.5% (35/111) in both fluids. ZIKV was detected in 24.7% (71/287) of the serum samples and in 27.5% (75/273) of the urine samples. The only patient who was diagnosed with ZIKV tested positive for the virus.

The months with the highest number of collections of symptomatic patients in 2018 were March (n= 45/308), April (n= 27/308) and May (n= 26/308). Then, we observed a decrease in the number of collections from June to January 2019. The higher rates of suspected cases were observed in February (n = 57/308), March (n = 57/308) and April (n = 39/308).

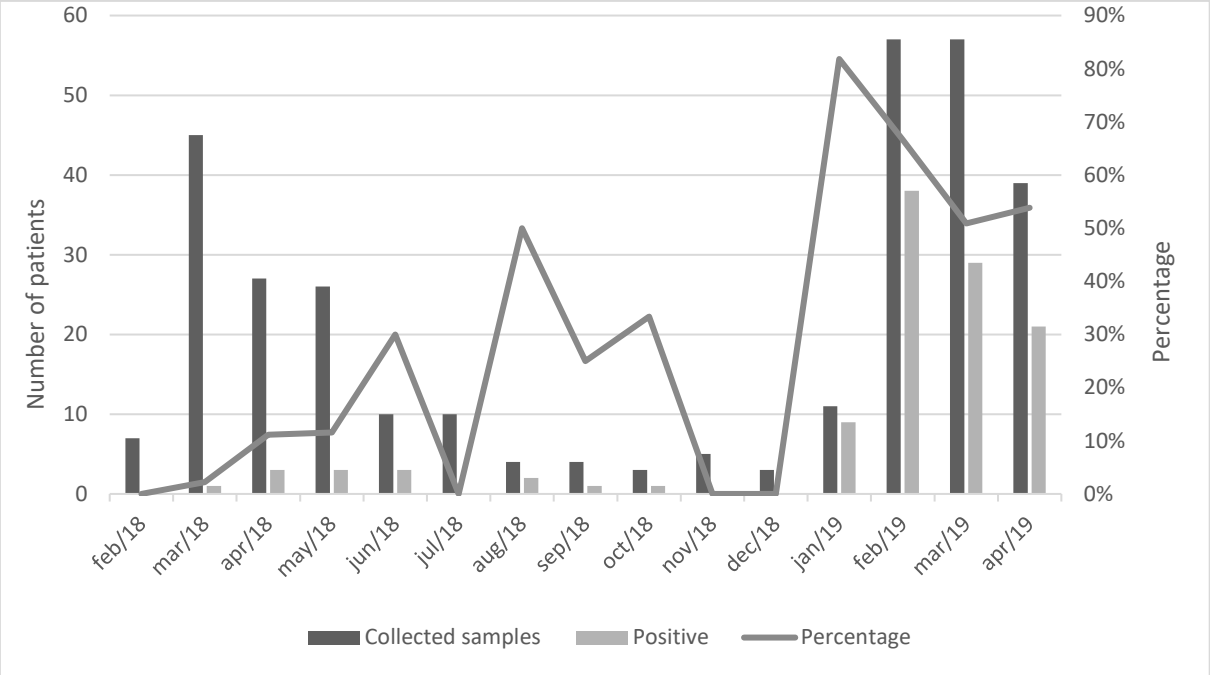


Fig 1 Monthly distribution of number of patients and ZIKV-positive cases during the year.

The number of positive samples for ZIKV remained constant from April/2018 to June/2018, with 3 positive cases each month. The percentage of positive cases increased with 82% (n = 9/11) in January/2019, 67% (n = 38/57) in February/2019, 51% (29/57) in March/2019 and, 54% (n = 21/39) in April/2019 (Fig 1).

Regarding symptoms, myalgia (73%), fever (65.7%) and, arthralgia (61.3%) were the most reported by ZIKV positive patients (Table 2). The number of days of symptoms are shown in Supplementary Fig. 2.

Table 2 Symptoms reported by patients positive for ZIKV.

Symptoms	***ZIKV***				
	Positive (n=111)		**Negative (n=197)**		**Total (n=308)**
	n	**%**	**n**	**%**	**N (%)**
Fever	73	65.7	131	66.5	204 (66.2)
Nausea	57	51.4	99	50.2	156 (50.6)
Vomiting	20	18	41	20.8	61 (19.8)
Red eyes	27	24.3	42	21.3	69 (22.4)
Retro-orbital pain	65	58.6	103	52.3	168 (54.5)
Intolerance to light	37	33.3	59	29.9	96 (31.2)
Itchy skin	49	44.1	86	43.7	135 (43.8)
Rash	44	39.6	91	46.2	135 (43.8)
Myalgia	81	73	138	70.1	219 (71.1)
Arthralgia	68	61.3	110	55.8	178 (57.8)

3.2 *Dengue virus*

When analyzing samples for DENV, from the 308 symptomatic patients, 42.5% (132/308) were positive for DENV2, and 0.3% (1/308) were positive for DENV1. Among these positive patients, 59.1% (78/132) were female. The age of DENV positive patients ranged from 6 to 74 years, with median of 35 years, upper quartile of 50 years and, lower quartile of 21 years. There was no significant association between gender and DENV-positive.

Among the patients with DENV infection, 72% (95/132) had DENV only in the serum, 3.7% (5/132) only in the urine, and 24.2% (32/132) in both fluids. DENV2 was present in 43.9% (126/287) of the serum samples and, 0.3% (1/287) were positive for DENV1. Only DENV2 was detect in 13.3% (37/273) of the urine samples.

In 2018, the highest percentage of cases of DENV infection, in relation to the number of symptomatic patients per month was in March (66.7%; n = 30/45), April (70.4%; n = 19/27), and May (65.4%; n = 18/26). As of June, there was a drop in the number of collections and positive cases. The months of October (n = 0/3), November (20%; n = 1/5), and December (n = 0/3) had the lowest number of positive cases in the study (Fig. 2). In January/2019 (55%;n = 6/11), there was a peak of positive cases, but these cases decreased again in the following months (February (31.6%; n = 19/57), March (19.3%; n = 11/57) and, April (23%; n=9/39)) (Fig 2).

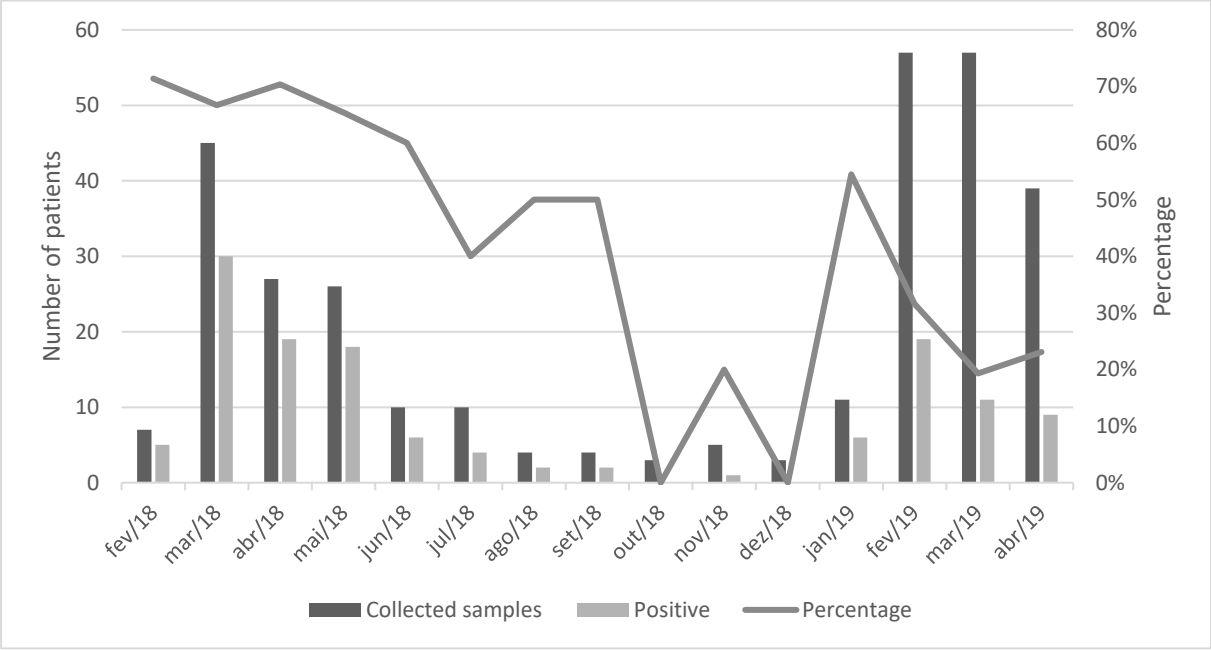


Fig 2 Monthly distribution of number of patients and DENV-positive cases during the year.

The symptoms that were most reported by patients positive for DENV were myalgia (73.5%; n=97/132), fever (68.9%; n=91/132) and, retro-orbital pain (57.6%; n=76/132) (Table 3). Supplementary Fig. 1 shows the number of days of symptoms.

Table 3 Symptoms reported by patients positive for DENV.

Symptoms	**DENV**				
	Positive (n=132)		**Negative (n=176)**		**Total (n=308)**
	n	**%**	**n**	**%**	**N (%)**
Fever	91	68.9	113	64.2	204 (66.2)
Nausea	59	44.7	97	55.1	156 (50.6)
Vomiting	19	14.4	41	23.3	60 (19.5)
Red eyes	33	25	36	20.5	69 (22.4)
Retro-orbital pain	76	57.6	92	52.3	168 (54.5)
Intolerance to light	37	28	59	33.5	96 (31.2)
Itchy skin	64	48.5	71	40.3	135 (43.8)
Rash	65	49.2	70	39.8	135 (43.8)
Myalgia	97	73.5	122	69.3	219 (71.1)
Arthralgia	70	53	108	61.4	178 (57.8)

3.3 Co-infection ZIKV-DENV2

Co-infection ZIKV-DENV2 was observed in 12.7% (39/308) of the patients. This corresponds to 35.1% (39/111) of patients positive for ZIKV and 29.8% (39/131) of patients positive for DENV2. If only serum samples had been analyzed, the percentage would have dropped to 11.4% (n = 35). Table 4 shows the fluids were each virus was detected.

Table 4 Co-infection data for ZIKV and DENV2.

	ZIKV		**DENV2**	
Patients (n=39)	Serum	Urine	Serum	Urine
3	-	+	+	+
1	+	-	+	+
10	+	+	+	-
1	+	+	-	+
16	+	-	+	-
8	-	+	+	-

The main symptoms reported by the co-infected patients were myalgia (66.7%; n=26/39), fever (66.7%; n=26/39), and retro-orbital pain (59%; n=23/39) (Table 5).

Table 5 Symptoms reported by patients with ZIKV-DENV2 co-infection.

Symptoms	Co-infection	
	Positive (n=39)	
	n	%
Fever	26	66.7
Nausea	15	38.5
Vomiting	3	7.7
Red eyes	10	25.6
Retro-orbital pain	23	59
Intolerance to light	10	25.6
Itchy skin	15	38.5
Rash	14	35.9
Myalgia	26	66.7
Arthralgia	20	51.3

Statistical analysis

The Kruskal-Wallis test is the non-parametric test used to compare three or more independent samples. It indicates whether there is a difference between at least two of these samples. This test used to compare the ZIKV, DENV, co-infection and, without disease groups, based on age, showed that there was a difference between the groups. When rejecting the null hypothesis, we performed the Conover-Iman test to compare two groups in the Kruskal-Wallis test. We saw that the age distribution was different [X^2 (3) = 8.377; $p < 0.05$] between groups without disease vs. only with DENV ($p = 0.0413$), co-infection vs. only with ZIKV ($p = 0.0431$), and between groups without disease vs. co-infection ($p = 0.0152$).

4. Discussion

Among all the existing arbovirus species, 150 are responsible for causing disease in humans, causing a major impact on public health [30]. Besides, the expansion of ZIKV throughout the Americas, which is already endemic to DENV, makes the countries face the co-circulation of different arboviruses of clinical importance [31]. This situation highlights the importance of surveillance and monitoring of emerging diseases.

Of the 308 patients analyzed, 42.5% were positive for DENV2, 0.3% for DENV1, 36.0% were positive for ZIKV, and 33.8% were negative. When comparing our data with data from the literature, the lowest percentage of positives for DENV was 15.5% and the highest 78% [32-34]. For ZIKV, the lowest percentage was 22.2%, and the highest was 50.7% [32, 34]. The studies cited also had high percentages of patients with negative results, the highest being 77.2% [32, 34]. When using urine samples together with the serum, we noticed an increase in the percentage of infected people. If only serum samples had been used, the percentage of ZIKV would have dropped to 24.7% (76/308). The percentage of DENV would also drop to 40.9% (126/308).

Regarding the temporal distribution of ZIKV and DENV, it can be noted that in 2018 there was a greater number of cases of DENV and few cases of ZIKV. In 2019, starting in January, there is an increase in the number of samples collected suspected of arbovirus. In the same period, the number of DENV decreased with 55% of cases in January, 31.6% in February, 19.3% in March, and 23.1% in April. This occurs at the same time that the number of positive samples for ZIKV begins to increase, with 82% of cases in January, 67% in February, 51% in March, and 54% in April.

In this study we also report that 12.7% (39) of the patients were co-infected with ZIKV/DENV2, corresponding to 35.1% of patients ZIKV-positive and 29.8% of patients DENV-positive. When comparing our data with other studies, we saw that the percentage of co-infection is higher than the one described by other authors. In 2015 in Pernambuco, there was a percentage of 2.6% co-infected [35]. Two studies of the year 2016 in São José do Rio Preto, a city 14.9 km from Mirassol reported a percent of 0.9% [32, 34]. One of the reasons we found higher rates of co-infection might be related to testing not only serum but also urine, while the cited authors only tested the former. In our population, if we have tested only serum, we would have a co-infection rate of 11.5%, which is still higher than what has been reported in literature. Another reason can be related to when the study was carried out. Our study was performed in 2018 and 2019. Official reports of ZIKV infection decreased and consequently studies doing differential molecular diagnosis for ZIKV also declined [36-38].

Here, we detected an outbreak of DENV-2 in 2018 and the beginning of an increase of ZIKV in the same year. Studies show the possibility of transmitting DENV, ZIKV and Chikungunya (CHIKV) simultaneously and others show that mosquitoes co-infected with ZIKV and DENV, preferentially transmit ZIKV [39, 40]. Co-infections are still poorly studied, but they commonly appear in places where there are outbreaks and can also be explained by exposure to large numbers of mosquitoes [41].

Regarding the fluids used, we noticed that 36.0% (40/111) of the patients had ZIKV detected only in the urine. When analyzing the days of symptoms, we noticed that the majority had recent symptoms for a maximum of 7 days. Studies show that the detection of ZIKV in urine is prolonged however, in our study; it was not possible to follow these patients for further tests to describe the detection kinetics of ZIKV in the urine [21, 42]. Only 3.7% (5/132) had DENV2 only in the urine and 72% (95/132) in the serum. We also analyzed the days of symptoms and, most of them had been experiencing symptoms for a maximum of 7 days, with rare exceptions. These findings agree with other studies that detection rates of 50% or more are evident between days 6 to 16 for urine samples and days 0 to 7 for serum samples [43]. Mizuno et.al showed that DENV is detected in urine in prolonged periods after the disappearance in the serum (Supplementary Fig. 1 and Fig. 2) [44].

Among the patients with DENV, the most reported symptoms were myalgia (73.5%), fever (68.9%) and, retro-orbital pain (57.6%). In the case of ZIKV, the main symptoms reported were myalgia (73%), fever (65.7%) and, arthralgia (61.3%), in line with other studies, which also show the appearance of these symptoms [34, 45, 46]. Co-infected patients also reported the same symptoms: myalgia (66%), fever (66%), and retro-orbital pain (58%). Through these data, we show how the clinical diagnosis is unable to differentiate infection by DENV and ZIKV. In fact, according to clinical evaluation, only one of the 308 patients was suspected of ZIKV infection, the remaining patients were diagnosed with DENV. We must also emphasize the difficulty in diagnosing ZIKV, which is mainly asymptomatic, with mild signs and symptoms, with the low and short viremia [42]. Besides, national guidelines for the diagnosis of DENV and ZIKV do not include co-infections, making it more difficult for the physician to diagnose and understand the dynamics of co-infections [7, 47]. Although general treatment for both arbovirus are similar, this situation raises concerns when considering the clinical complications that ZIKV can cause for pregnant women, and also the risk of Guillain-Barré syndrome [7, 48]. When it comes to surveillance, the adopted clinical diagnosis is also a problem. As we report here ZIKV was circulating in the city where the study was carried out in 2018 and 2019 corresponding to considerable rates of the arbovirus suspected cases. However, if we did not perform this study only one case of ZIKV would be notified. These factors highlight the importance of specific molecular tests in order to identify the viruses that are causing outbreaks. This might help contain the emergence of other arbovirus and aid the clinical management of the ones that are already circulation.

Here we report the prevalence, in symptomatic patients, of ZIKV and DENV from February 2018 to April 2019 in a city in southeastern Brazil. By doing molecular test we detected an unnoticed ZIKV outbreak in the city. Also, by testing serum and urine samples, we increased the detection of both viruses and detected considerable levels of ZIKV / DENV-2 co-infection, when compared to other studies. Thus, we show the importance of molecular diagnosis to reduce the number of underreported cases aiding public health surveillance and strategies to contain virus spread.

Acknowledgements

We thank the city of Mirassol and the director and staff of the Emergency Care Unit of Mirassol that allowed us to conduct this study. We also thank all the participants that contributed to this study.

Funding

This study was financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001 and Grant number 2046/2016; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Grant number 440723/2016-7 and São Paulo Research Foundation (FAPESP) – Grant Number 2014/22198-0.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

The Committee Ethics from São Paulo University State – UNESP approved this study (CAAE: 66489617.4.0000.5466).

Consent to participate

All volunteers signed an informed consent form before participating in the study.

5. References

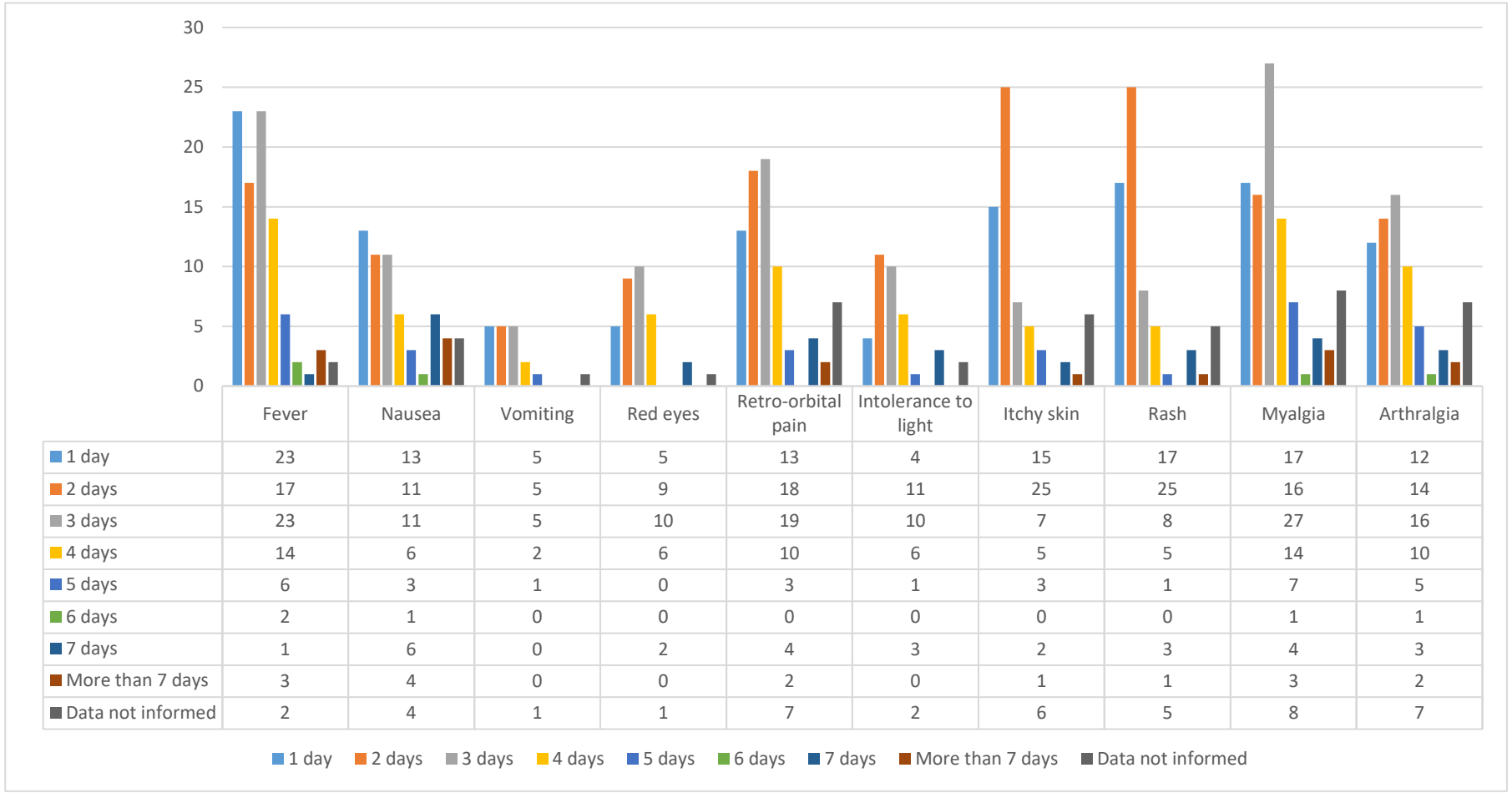
1. Weaver SC, Reisen WKJAr. Present and future arboviral threats. 2010;85(2):328-45.
2. Cleton N, Koopmans M, Reimerink J, Godeke GJ, Reusken C. Come fly with me: review of clinically important arboviruses for global travelers. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2012;55(3):191-203. Epub 2012/07/31. doi: 10.1016/j.jcv.2012.07.004. PubMed PMID: 22840968.

3. Normile D. Tropical medicine. Surprising new dengue virus throws a spanner in disease control efforts. Science (New York, NY). 2013;342(6157):415. Epub 2013/10/26. doi: 10.1126/science.342.6157.415. PubMed PMID: 24159024.
4. Holmes EC. Molecular epidemiology and evolution of emerging infectious diseases. British medical bulletin. 1998;54(3):533-43. Epub 1999/05/18. doi: 10.1093/oxfordjournals.bmb.a011708. PubMed PMID: 10326282.
5. Mustafa MS, Rasotgi V, Jain S, Gupta V. Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. Med J Armed Forces India. 2015;71(1):67-70. Epub 2014/11/24. doi: 10.1016/j.mjafi.2014.09.011. PubMed PMID: 25609867.
6. Pontes RJS, Ruffino-Netto A. Dengue em localidade urbana da região sudeste do Brasil: aspectos epidemiológicos %J Revista de Saúde Pública. 1994;28:218-27.
7. MS, SVS. Dengue: diagnóstico e manejo clínico: adulto e criança. In: Transmissíveis DdVdD, editor. 5 ed. Brasil: Ministério da Saúde; 2016.
8. Kuno G, Chang GJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. Arch Virol. 2007;152(4):687-96. Epub 2007/01/02. doi: 10.1007/s00705-006-0903-z. PubMed PMID: 17195954.
9. Dick G, Kitchen S, Haddow AJTotrsotm, hygiene. Zika virus (I). Isolations and serological specificity. 1952;46(5):509-20.
10. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, federated states of Micronesia. 2009;360(24):2536-43.
11. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastère S, Valour F, et al. Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. 2014;19(9):20720. doi: doi:https://doi.org/10.2807/1560-7917.ES2014.19.9.20720.
12. Musso D, Cao-Lormeau VM, Gubler DJ. Zika virus: following the path of dengue and chikungunya? The Lancet. 2015;386(9990):243-4. doi: 10.1016/S0140-6736(15)61273-9.
13. Zanluca C, Melo VCAd, Mosimann ALP, Santos GIVd, Santos CNDd, Luz KJMdIOC. First report of autochthonous transmission of Zika virus in Brazil. 2015;110(4):569-72.
14. Rasmussen SA, Jamieson DJ, Honein MA, Petersen LR. Zika Virus and Birth Defects — Reviewing the Evidence for Causality. 2016;374(20):1981-7. doi: 10.1056/NEJMsr1604338. PubMed PMID: 27074377.
15. Calvet G, Aguiar RS, Melo ASO, Sampaio SA, de Filippis I, Fabri A, et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. The Lancet Infectious Diseases. 2016;16(6):653-60. doi: 10.1016/S1473-3099(16)00095-5.
16. Sarno M, Aquino M, Pimentel K, Cabral R, Costa G, Bastos F, et al. Progressive lesions of central nervous system in microcephalic fetuses with suspected congenital Zika virus syndrome. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2017;50(6):717-22. Epub 2016/09/20. doi: 10.1002/uog.17303. PubMed PMID: 27644020.
17. WHO. WHO to convene an International Health Regulations Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations 2016. Available from: https://www.who.int/en/news-room/detail/28-01-2016-who-to-convene-an-international-health-regulations-emergency-committee-on-zika-virus-and-observed-increase-in-neurological-disorders-and-neonatal-malformations.
18. Blitvich BJ, Magalhaes T, Laredo-Tiscareño SV, Foy BD. Sexual Transmission of Arboviruses: A Systematic Review. 2020;12(9):933. PubMed PMID: doi:10.3390/v12090933.
19. Chen LH, Wilson ME. Update on non-vector transmission of dengue: relevant studies with Zika and other flaviviruses. Tropical diseases, travel medicine and vaccines. 2016;2:15.

Epub 2017/09/09. doi: 10.1186/s40794-016-0032-y. PubMed PMID: 28883959; PubMed Central PMCID: PMCPMC5530933.
20. MS, SVS. Monitoramento dos casos de arboviroses urbanas transmitidas pelo *Aedes* (dengue, chikungunya e Zika), Semanas Epidemiológicas 1 a 22, 2020. In: Transmissíveis DdVdD, editor. Brasilia, Brasil: Ministério da Saúde; 2020.
21. Paz-Bailey G, Rosenberg ES, Doyle K, Munoz-Jordan J, Santiago GA, Klein L, et al. Persistence of Zika Virus in Body Fluids — Final Report. 2017;379(13):1234-43. doi: 10.1056/NEJMoa1613108. PubMed PMID: 28195756.
22. Iannetta M, Lalle E, Musso M, Carletti F, Scorzolini L, D'Abramo A, et al. Persistent detection of dengue virus RNA in vaginal secretion of a woman returning from Sri Lanka to Italy, April 2017. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2017;22(34). Epub 2017/09/01. doi: 10.2807/1560-7917.Es.2017.22.34.30600. PubMed PMID: 28857045; PubMed Central PMCID: PMCPMC5753441.
23. Shinohara K, Kutsuna S, Takasaki T, Moi ML, Ikeda M, Kotaki A, et al. Zika fever imported from Thailand to Japan, and diagnosed by PCR in the urine. Journal of Travel Medicine. 2016;23(1). doi: 10.1093/jtm/tav011.
24. Korhonen EM, Huhtamo E, Virtala AM, Kantele A, Vapalahti O. Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2014;61(3):353-8. Epub 2014/09/23. doi: 10.1016/j.jcv.2014.08.021. PubMed PMID: 25242312.
25. Andries AC, Duong V, Ly S, Cappelle J, Kim KS, Lorn Try P, et al. Value of Routine Dengue Diagnostic Tests in Urine and Saliva Specimens. PLoS neglected tropical diseases. 2015;9(9):e0004100. Epub 2015/09/26. doi: 10.1371/journal.pntd.0004100. PubMed PMID: 26406240; PubMed Central PMCID: PMCPMC4583371 following competing interests: PB is employed by GlaxoSmithKline. This does not alter our adherence to all PLOS NTDs policies on sharing data and materials.
26. Borges HCBG, Adati MC, Vigo DC, de Mendonça VF, Issobe MA, dos Santos FB, et al. Avaliação dos testes rápidos para diagnóstico da dengue no Brasil. Vigilância Sanitária em Debate: Sociedade, Ciência & Tecnologia (Health Surveillance under Debate: Society, Science & Technology) – Visa em Debate. 2021;9(1):82-90. doi: 10.22239/2317-269x.01451.
27. MS, SVS. Guia de Vigilância em Saúde. In: Serviços C-GdDdEe, editor. 3 ed. Brasil: Ministério da Saúde; 2019. p. 740.
28. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerging infectious diseases. 2008;14(8):1232-9. Epub 2008/08/06. doi: 10.3201/eid1408.080287. PubMed PMID: 18680646; PubMed Central PMCID: PMCPMC2600394.
29. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. Journal of clinical microbiology. 2005;43(10):4977-83. Epub 2005/10/07. doi: 10.1128/jcm.43.10.4977-4983.2005. PubMed PMID: 16207951; PubMed Central PMCID: PMCPMC1248506.
30. Lopes N, Nozawa C, Linhares REC. Características gerais e epidemiologia dos arbovírus emergentes no Brasil %J Revista Pan-Amazônica de Saúde. 2014;5:55-64.
31. Holbrook MR. Historical Perspectives on Flavivirus Research. Viruses. 2017;9(5). Epub 2017/05/05. doi: 10.3390/v9050097. PubMed PMID: 28468299; PubMed Central PMCID: PMCPMC5454410.

32. Estofolete CF, Terzian ACB, Colombo TE, de Freitas Guimarães G, Ferraz HCJ, da Silva RA, et al. Co-infection between Zika and different Dengue serotypes during DENV outbreak in Brazil. J Infect Public Health. 2019;12(2):178-81. Epub 2018/10/12. doi: 10.1016/j.jiph.2018.09.007. PubMed PMID: 30301701.
33. Colombo TE, Vedovello D, Pacca-Mazaro CC, Mondini A, Araújo JP, Cabrera E, et al. Dengue virus surveillance: Detection of DENV-4 in the city of São José do Rio Preto, SP, Brazil. Acta Tropica. 2016;164:84-9. doi: https://doi.org/10.1016/j.actatropica.2016.09.004.
34. Colombo TE, Estofolete CF, Reis AFN, da Silva NS, Aguiar ML, Cabrera EMS, et al. Clinical, laboratory and virological data from suspected ZIKV patients in an endemic arbovirus area. Journal of Clinical Virology. 2017;96:20-5. doi: https://doi.org/10.1016/j.jcv.2017.09.002.
35. Pessôa R, Patriota JV, Lourdes de Souza M, Felix AC, Mamede N, Sanabani SS. Investigation Into an Outbreak of Dengue-like Illness in Pernambuco, Brazil, Revealed a Cocirculation of Zika, Chikungunya, and Dengue Virus Type 1. Medicine. 2016;95(12):e3201. Epub 2016/03/26. doi: 10.1097/md.0000000000003201. PubMed PMID: 27015222; PubMed Central PMCID: PMCPMC4998417.
36. MS, SVS. Monitoramento dos casos de arboviroses urbanas transmitidas pelo Aedes (dengue, chikungunya e Zika) até a Semana Epidemiológica 12 de 2019 e Levantamento Rápido de Índices para Aedes aegypti (LIRAa) In: Transmissíveis DdVdD, editor. Brasilia, Brasil: Ministério da Saúde; 2019.
37. MS, SVS. Monitoramento dos casos de dengue, febre de chikungunya e doença aguda pelo vírus Zika até a Semana Epidemiológica 49 de 2018. In: Transmissíveis DdVdD, editor. Brasilia, Brasil: Ministério da Saúde 2018.
38. MS, SVS. Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 52, 2016. In: Transmissíveis DdVdD, editor. Brasilia, Brasil: Ministério da Saúde; 2017.
39. Vogels CBF, Rückert C, Cavany SM, Perkins TA, Ebel GD, Grubaugh ND. Arbovirus coinfection and co-transmission: A neglected public health concern? PLoS biology. 2019;17(1):e3000130. Epub 2019/01/23. doi: 10.1371/journal.pbio.3000130. PubMed PMID: 30668574; PubMed Central PMCID: PMCPMC6358106.
40. Chaves BA, Orfano AS, Nogueira PM, Rodrigues NB, Campolina TB, Nacif-Pimenta R, et al. Coinfection with Zika Virus (ZIKV) and Dengue Virus Results in Preferential ZIKV Transmission by Vector Bite to Vertebrate Host. The Journal of Infectious Diseases. 2018;218(4):563-71. doi: 10.1093/infdis/jiy196 %J The Journal of Infectious Diseases.
41. Carrillo-Hernández MY, Ruiz-Saenz J, Villamizar LJ, Gómez-Rangel SY, Martínez-Gutierrez M. Co-circulation and simultaneous co-infection of dengue, chikungunya, and zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border. BMC infectious diseases. 2018;18(1):61. Epub 2018/02/01. doi: 10.1186/s12879-018-2976-1. PubMed PMID: 29382300; PubMed Central PMCID: PMCPMC5791178.
42. Gourinat A-C, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M. Detection of Zika virus in urine. Emerging infectious diseases. 2015;21(1):84-6. doi: 10.3201/eid2101.140894. PubMed PMID: 25530324.
43. Hirayama T, Mizuno Y, Takeshita N, Kotaki A, Tajima S, Omatsu T, et al. Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum. Journal of clinical microbiology. 2012;50(6):2047-52. Epub 2012/03/21. doi: 10.1128/JCM.06557-11. PubMed PMID: 22442323.
44. Mizuno Y, Kotaki A, Harada F, Tajima S, Kurane I, Takasaki T. Confirmation of dengue virus infection by detection of dengue virus type 1 genome in urine and saliva but not in plasma.

Transactions of the Royal Society of Tropical Medicine and Hygiene. 2007;101(7):738-9. Epub 2007/04/10. doi: 10.1016/j.trstmh.2007.02.007. PubMed PMID: 17418320.
45. Cerbino-Neto J, Mesquita EC, Souza TML, Parreira V, Wittlin BB, Durovni B, et al. Clinical Manifestations of Zika Virus Infection, Rio de Janeiro, Brazil, 2015. Emerging infectious diseases. 2016;22(7):1318-20. Epub 2016/07/15. doi: 10.3201/eid2207.160375. PubMed PMID: 27070847.
46. Karkhah A, Nouri HR, Javanian M, Koppolu V, Masrour-Roudsari J, Kazemi S, et al. Zika virus: epidemiology, clinical aspects, diagnosis, and control of infection. Eur J Clin Microbiol Infect Dis. 2018;37(11):2035-43. Epub 2018/09/01. doi: 10.1007/s10096-018-3354-z. PubMed PMID: 30167886.
47. MS, SVS. Nota Informativa - Procedimentos a serem adotados para a vigilância da Febre do vírus Zika no Brasil. Brasil: Ministério da Saúde; 2016.
48. MS, SVS. ZIKA ABORDAGEM CLÍNICA NA ATENÇÃO BÁSICA. Brasil2016.



	Fever	Nausea	Vomiting	Red eyes	Retro-orbital pain	Intolerance to light	Itchy skin	Rash	Myalgia	Arthralgia
1 day	23	13	5	5	13	4	15	17	17	12
2 days	17	11	5	9	18	11	25	25	16	14
3 days	23	11	5	10	19	10	7	8	27	16
4 days	14	6	2	6	10	6	5	5	14	10
5 days	6	3	1	0	3	1	3	1	7	5
6 days	2	1	0	0	0	0	0	0	1	1
7 days	1	6	0	2	4	3	2	3	4	3
More than 7 days	3	4	0	0	2	0	1	1	3	2
Data not informed	2	4	1	1	7	2	6	5	8	7

Fig. S1 Symptoms, in days, reported by patients positive for DENV.

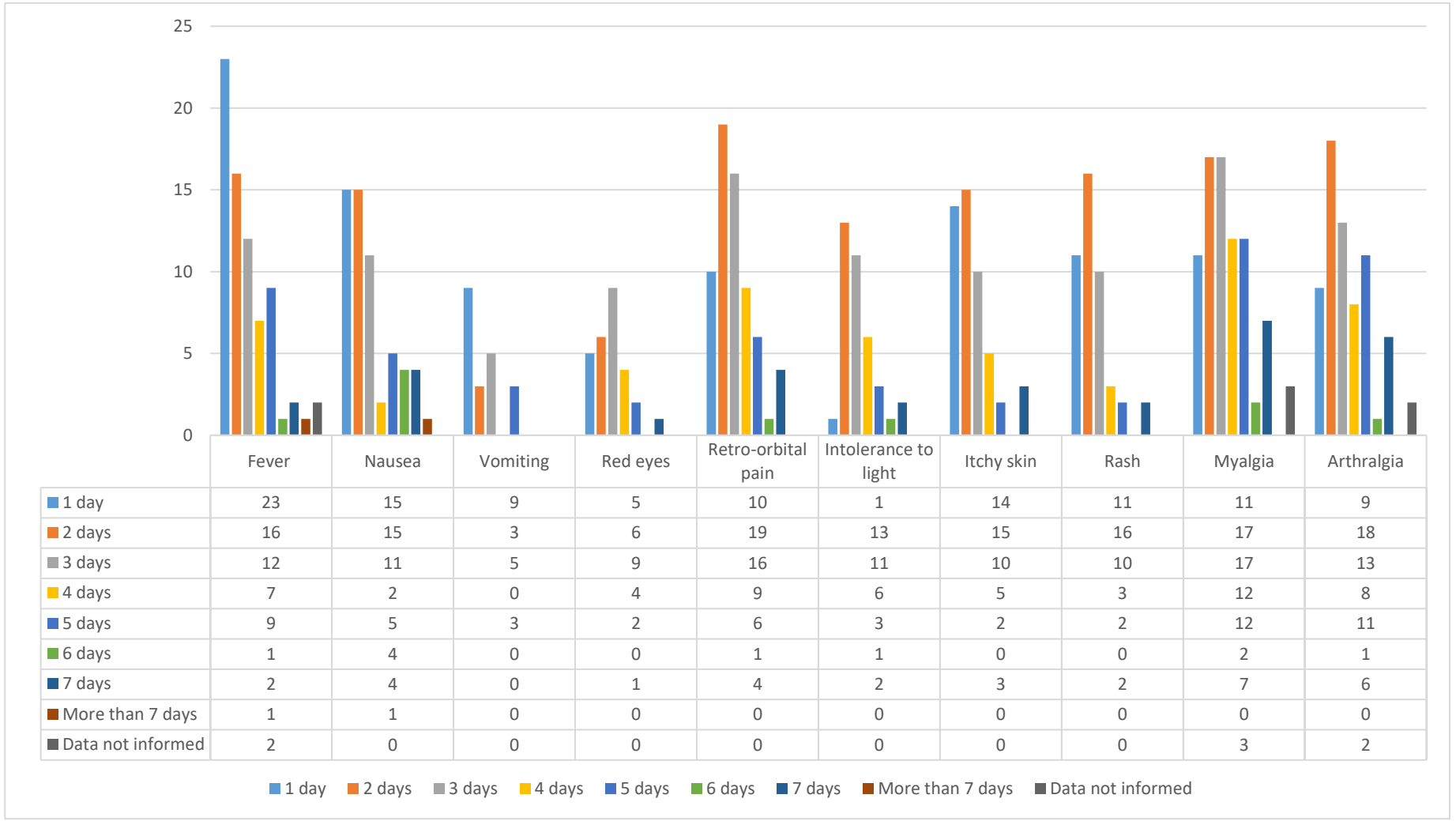


Fig. S2 Symptoms, in days, reported by patients positive for ZIKV.

Capítulo IV - Conclusões

1. CONCLUSÕES

Entre fevereiro de 2018 e abril de 2019, foram coletadas amostras de 2 grupos: sintomáticos e sem diagnóstico clínico de arboviroses. Do grupo de sintomáticos foram coletadas 287 amostras de sangue e 273 amostras de urina de 308 pacientes. Já do grupo sem diagnóstico clínico de arboviroses, foram coletadas 697 amostras de urina.

Grupo de pacientes sintomáticos.

Dos 308 pacientes sintomáticos, 36% (111) tinham ZIKV, 42,5% (131) tinham DENV2 e 0,3% (1) tinha DENV1. Também avaliamos coinfecção e vimos que 12,7% dos pacientes estavam coinfectados com ZIKV/DENV2. Todos esses pacientes foram diagnosticados com base em dados clínicos e epidemiológicos, sendo que 307 receberam o diagnóstico de DENV e apenas 1 recebeu o diagnóstico de ZIKV. Também foi possível avaliar a importância da utilização das amostras de urina, pois e tivéssemos utilizado apenas amostras de soro, o percentual de positivos para ZIKV, cairia de 36% para 24,7%. Além disso, dentre todos esses pacientes positivos, tanto para ZIKV, DENV e ambos os vírus, vimos que os sintomas mais relatados foram mialgia, febre, artralgia e dor retroorbital.

Grupo de pacientes sem diagnóstico clínico de arboviroses.

No grupo de pacientes sem diagnóstico clínico de arboviroses, detectamos a presença de ZIKV nas amostras de urina de 79 indivíduos, sendo de assintomáticos e pacientes com e sem sintomas sugestivos de arboviroses. Dentre os pacientes que possuíam sintomas sugestivos de arboviroses, os principais sintomas apresentados foram mialgia, febre, náusea e vômito. Ao analisar as causas que levaram os voluntários do grupo de indivíduos sem diagnóstico clínico de arboviroses a irem até a UPA, pode-se notar que a maioria dos pacientes infectados com ZIKV, apresentou infecções do sistema respiratório, doenças multissistêmicas e infecção do trato urinário. Ao analisarmos a circulação temporal de ZIKV e DENV em Mirassol, por meio de amostragem mensal, notamos que em 2018 havia um maior número de casos de DENV e poucos casos de ZIKV e que esse quadro foi se invertendo no início de 2019, no qual tivemos maior número de casos de ZIKV ao mesmo tempo em que ocorre a diminuição dos casos de DENV.

Por fim, destacamos a importância de uma melhor investigação da circulação de ZIKV e DENV na cidade e na região de Mirassol. Em nosso estudo ficou evidente a necessidade de um diagnóstico diferencial, pois o diagnóstico baseado apenas em dados clínicos-epidemiológicos não é o ideal, por conta das arboviroses serem doenças com sintomas inespecíficos, que podem ser confundidas entre si, além de serem confundidas com outras

doenças não virais, causando uma subnotificação dos reais casos dessas doenças. Outro ponto importante é a utilização de outros fluidos, tal como a urina, principalmente no caso do ZIKV. Além disso, os dados obtidos nesse estudo são importantes para desenvolvimento de medidas preventivas, campanhas de vigilância e medidas mais eficazes de eliminação dos criadouros do vetor, com o intuito de prever e impedir novos surtos da doença na região da cidade de Mirassol.