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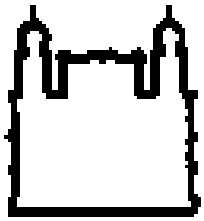
PERSISTÊNCIA DO VÍRUS CHIKUNGUNYA EM FLUIDOS CORPORAIS E FATORES DE RISCO PARA DOR CRÔNICA: UM ESTUDO DE COORTE EM UM CENTRO DE REFERÊNCIA PARA DOENÇAS FEBRIS AGUDAS NO RIO DE JANEIRO

EZEQUIAS BATISTA MARTINS

Rio de Janeiro
Junho de 2023



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Persistência do vírus chikungunya em fluidos corporais e fatores de risco para dor crônica: um estudo de coorte em um centro de referência para doenças febris agudas no Rio de Janeiro

Tese apresentada ao Instituto Oswaldo Cruz como parte dosrequisitos para obtenção do título de Doutor em Medicina.

Orientadores: Dr. Guilherme Amaral Calvet
Drª. Patrícia Brasil

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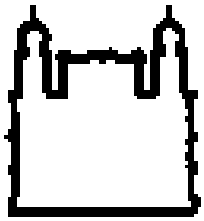

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Em memória de todas as pessoas que tiveram suas vidas ceifadas e negligenciadas pela pandemia de Covid-19.

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“...mesmo que o tempo e a distância digam não...”

INSTITUTO OSWALDO CRUZ

Persistência do vírus chikungunya em fluidos corporais e fatores de risco para dor crônica: um estudo de coorte em um centro de referência para doenças febris agudas no Rio de Janeiro

RESUMO

TESE DE DOUTORADO EM MEDICINA TROPICAL

Ezequias Batista Martins

A chikungunya é uma arbovirose transmitida por mosquitos do gênero *Aedes* spp., e o vírus chikungunya (CHIKV) é o agente causador da infecção. A doença causa febre de início súbito, intensas dores articulares e exantema. As manifestações articulares e musculoesqueléticas são as mais importantes, podendo perdurar por anos. Este é um estudo de coorte prospectivo que avaliou a presença e a duração do ácido ribonucleico (RNA) do CHIKV em sangue, urina, saliva, sêmen e secreções vaginais. Foram incluídos homens e mulheres (≥ 18 anos) com teste de reação em cadeia da polimerase da transcriptase reversa em tempo real para CHIKV (CHIKV rRT-PCR) positivo na fase aguda da doença. Dados clínicos e amostras foram coletados a cada 15 dias durante os primeiros dois meses, e uma coleta final foi realizada três meses após o recrutamento. O método de censura de intervalo de Kaplan-Meier e o modelo paramétrico de Weibull foram ajustados para estimar a mediana de persistência viral. As estimativas pontuais da mediana de persistência do RNA do CHIKV para cada fluido foram construídas usando intervalo de confiança (IC) de 95%. Uma análise multivariada foi realizada por modelo de regressão logística, usando *odds ratios* e IC de 95% associados, para determinar fatores de risco para dor articular crônica após três meses de infecção por CHIKV. Os fatores de risco associados à artralgia prolongada com $p < 0,10$ na análise univariada foram ajustados por idade, sexo e presença de artrose e inseridos em um modelo de regressão logística múltipla. No modelo final, as variáveis só foram mantidas se fossem estatisticamente significativas ($p<0,05$) ou se alterassem substancialmente os coeficientes de outras variáveis do modelo. O limite de significância foi de 5%. De abril a dezembro de 2019, 170 participantes foram selecionados. Destes, 152 foram incluídos no estudo. O RNA do CHIKV foi detectado em 80,3% (122/152) no soro, 30,3% (46/152) na saliva, 23,0% (35/152) na urina, 20,2% (20/99) na secreção vaginal e 14,3% (6/42) no sêmen. O tempo médio até a perda da detecção de RNA do CHIKV foi de 19,6 dias (IC 95%, 17,5-21,7) para soro, 25,3 dias (IC 95%, 17,8-32,8) para urina, 24,3 dias (IC 95%, 18,3-30,3) para saliva e 25,8 dias (IC 95%, 19,8-31,9) para secreção vaginal. A incidência de dor articular três meses após o início dos sintomas foi de 61,7% (66/107). Diarreia [OR Ajustado (AOR) 5,94, IC95% 1,67-21,13], CHIKV rRT-PCR positivo na urina até cinco dias após o início dos sintomas [AOR 5,87, IC95%1,01-34,10], CHIKV rRT-PCR positivo na saliva até cinco dias após o início dos sintomas [AOR 4,02, IC 95% 1,10-14,63] e dor articular intensa nos punhos [AOR 15,22, IC 95% 2,35-98,65] foram os fatores associados à dor articular crônica na análise multivariada. Este estudo contribuiu para o conhecimento da cinética viral do CHIKV pela detecção do RNA em todos os fluidos corporais estudados, incluindo secreções genitais, durante as fases aguda e subaguda da doença. Contribuiu também para evidenciar fatores associados à persistência de dor crônica em pacientes com infecção pelo CHIKV: diarréia, a detecção do CHIKV em fluidos corporais (saliva e urina) na fase inicial da doença e a dor crônica em punho.

INSTITUTO OSWALDO CRUZ

Chikungunya virus persistence in body fluids and risk factors for chronic pain: a cohort study at a reference center for acute febrile illnesses in Rio de Janeiro

ABSTRACT

PHD THESIS IN TROPICAL MEDICINE

Ezequias Batista Martins

Chikungunya is an arbovirus transmitted by mosquitoes of the genus *Aedes* spp., and the chikungunya virus (CHIKV) is the causative agent. The disease causes a sudden onset of fever, severe joint pain, and rash. Joint and musculoskeletal manifestations are the most important and can last for years. A prospective cohort study was conducted to assess the presence and duration of detectable levels of CHIKV ribonucleic acid (RNA) in blood, urine, saliva, semen, and vaginal secretions. Men and women (≥ 18 years) with a positive real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) test for CHIKV in the acute phase of the disease were included. After enrollment, clinical data and samples were collected every 15 days over the first two months, and a final collection was performed 3 months after recruitment. The Kaplan–Meier interval-censoring method and the parametric Weibull model were fitted to estimate the median time of viral persistence until the lack of CHIKV RNA detection among all body fluids. Punctual estimates of the median time of CHIKV RNA persistence for each fluid were estimated using a 95% confidence interval (CI). Multivariate logistic regression analysis was used to calculate odds ratios (ORs) and 95% CI for the association between variables and joint tenderness at the 3 months visit. Considering a statistical significance level of 10% for variable selection, the variables that showed significance in the univariate analysis and those that were clinically important (age, sex, and presence of arthrosis) were included in the multivariate analysis. Backward elimination (at the same level of significance) was performed for these variables. Only the variables that were statistically significant at the 5% level were included in the final model. From April to December 2019, 170 participants were screened. Of these, 152were enrolled in the study. CHIKVRNA was detected in 80.3% (122/152) of serum samples, 30.3% (46/152) of saliva samples, 23.0% (35/152) of urine samples, 20.2% (20/99) of vaginal secretion samples, and 14.3% (6/42) of semen samples. The median time until the loss of CHIKV RNA detection was19.6 days (95% CI, 17.5–21.7) in serum, 25.3 days (95% CI, 17.8–32.8) in urine, 23.1 days (95% CI, 17.9–28.4) in saliva, and 25.8 days (95% CI, 20.6–31.1) in vaginal secretion. The incidence of joint pain three months after the onset of symptoms was 61.7% (66/107). Diarrhea [adjusted odds ratio (AOR) 5.94, 95% CI: 1.67–21.13], severe joint pain in the wrists [AOR 15.22, 95% CI:2.35–98.65], and CHIKV rRT-PCR positivity up to 5 days post onset of symptoms in urine [AOR 5.87, 95% CI: 1.01–34.10] and saliva [AOR 4.02, 95% CI:1.10-14.63] were predictors of persistent chronic pain in the multivariate analysis. This study contributed to the knowledge of CHIKV viral kinetics by detecting RNA in all body fluids studied, including genital secretions, during the acute and convalescent phases of the disease. It also highlighted factors associated with the persistence of chronic pain in patients with CHIKV infection: diarrhea, the detection of CHIKV in body fluids (saliva and urine) in the initial phase of the disease and severe joint pain in the wrists.

ÍNDICE

ÍNDICE DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

Anti-CHIKV-IgM	Anticorpos Anti-Chikungunya do tipo Imunoglobulina M
Anti-CHIKV-IgG	Anticorpos Anti-Chikungunya do tipo Imunoglobulina G
Anvisa	Agência Nacional de Vigilância Sanitária
AOR	Odds ratios ajustado (do inglês: *adjusted odds ratio*)
CHIK	Chikungunya
CHIKV	Vírus chikungunya (do inglês: *chikungunya virus*)
Ct	Do inglês *Cycle threshold*
ECSA	Leste-Centro-Sul Africano, de sigla ECSA (do inglês, *East-CentralSouth African*)
DENV	Vírus dengue (do inglês: *dengue virus*)
DFA	Doenças Febris Agudas
FIOCRUZ	Fundação Oswaldo Cruz
IC	Intervalo de confiança
IIQ	Intervalo interquartil
IOL	Linhagem do Oceano Índico (do inglês:*Indian Ocean Lineage*)
IQR	do inglês: *interquartile range*
INI	Instituto Nacional de Infectologia Evandro Chagas
K-M	Kaplan Meier
LapClinDFA	Laboratório de Pesquisa Clínica em Doenças Febris Agudas
NAAT	Teste de amplificação de ácido nucleico (do inglês: *nucleic acid amplification test*)
OMS	Organização Mundial da Saúde
OPAS	Organização Pan-Americana da Saúde
OR	do inglês:*odds ratio*
PCR	Proteína C reativa
REDCap	Do inglês: Research Eletronic Data Capture
RNA	Ácido ribonucleico (do inglês: *ribonucleic acid*)
rRT-PCR	Reação em cadeia da polimerase da transcriptase reversa em tempo real (do inglês: *real time reverse transcription polymerase* chain reaction)
VHS	Velocidade de hemossedimentação
ZIKV	Vírus zika (do inglês: *zika virus*)

1. INTRODUÇÃO

1.1 Revisão Teórica e o Estado da Arte sobre o Vírus Chikungunya

1.1.1 Aspectos Históricos da Chikungunya

A Chikungunya (CHIK) é uma doença viral, incluída no grupo das arboviroses emergentes no território brasileiro. É uma doença infecciosa, causada pelo vírus Chikungunya (CHIKV), um *Alphavirus* de genoma RNA, da família *Togaviridae* (1).

São conhecidos três genótipos do CHIKV: Oeste Africano, Asiático e Leste Centro Sul Africano (ECSA). Também foi identificada uma linhagem descendente do genótipo ECSA, denominada Linhagem Oceano Índico – IOL (2). No território brasileiro foi confirmada a presença dos genótipos Asiático e ECSA, introduzidos, respectivamente, nas regiões norte e nordeste (3,4). No estado do Rio de Janeiro, em 2014 e 2015, foi identificado o genótipo Asiático em casos importados (5). Subsequentemente, no ano de 2016, foi descrita a circulação do genótipo ECSA em casos autóctones no Rio de Janeiro (6).

A transmissão do vírus é feita através da picada de fêmeas de insetos-vetores do gênero *Aedes*; predominando em áreas urbanas, *Aedes aegypti* e nas áreas rurais ou silvestres, *Aedes albopictus*. Devido a uma mutação genética no genótipo ECSA, foi possível a adaptação do vírus ao vetor *Aedes albopictus* (7).

A palavra “Chikungunya” é oriunda da língua Makonde, um dos idiomas utilizados na Tanzânia (swahili), e significa “aqueles que se dobram”. O nome faz referência à postura dos pacientes infectados, pela intensa artralgia, na primeira epidemia documentada na Tanzânia (leste do continente africano), entre os anos de 1952 e 1953 (8). Posteriormente, a doença tem sido relatada em surtos e epidemias nos continentes asiático e africano. Em 2005, nas Ilhas Reunião, um terço da população foi infectada, onde foram documentados 244.000 casos e 203 mortes por complicação da doença (9). Nas Américas, o vírus foi identificado pela primeira vez em outubro de 2013, na Ilha de Saint-Martin, região do Caribe. Posteriormente foi isolado em outras ilhas do Caribe e países da América do Sul (10).

Desde que o CHIKV foi isolado pela primeira vez na Tanzânia em 1952, a doença passou a ser relacionada a surtos localizados na Ásia e na África, representada por centenas de milhares de casos (8). Em 2005, uma nova cepa de

CHIKV, que provavelmente se originou na costa do Quênia, espalhou-se para as ilhas do Oceano Índico e da Índia (9,10). Desde então, o CHIKV espalhou-se pelo mundo, causando grandes surtos no sudeste e leste da Ásia, ou por meio de casos importados em viajantes que retornaram a regiões não endêmicas da Europa e América do Norte (10). A Figura 1.1 mostra a cronologia dos surtos e epidemias de CHIK no mundo, de 1952 a 2013, período que antecede a chegada da doença no Brasil.

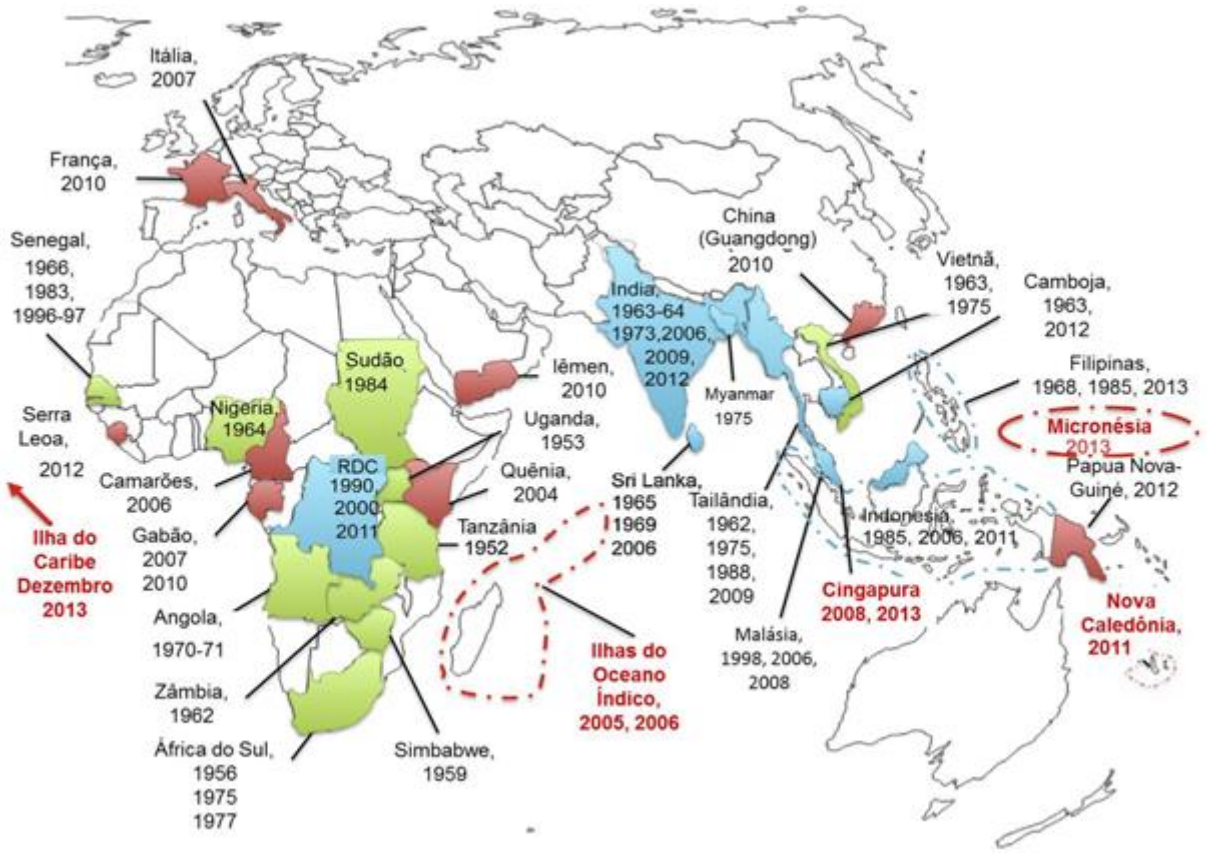

Figura 1.1: Epidemias e surtos históricos da Febre Chikungunya no mundo, no período de 1952 a 2013. As cores sinalizam a cronologia: verde (antigo), azul (antes e após 2004) e vermelho (após 2004). Adaptado de Rougeron et al., 2015 (11).

A suscetibilidade de mosquitos em regiões não endêmicas, como Austrália e América do Norte, e a ocorrência de surtos autóctones na Itália e na França, mostraram que o CHIKV não poderia ser considerado um problema isolado de países tropicais (10).

No Brasil, os primeiros casos autóctones foram notificados nas cidades de Oiapoque (Amapá) e Feira de Santana (Bahia), em setembro de 2014 (12). Nesta época, foi isolado o genótipo Asiático no Oiapoque e o genótipo ECSA em Feira de Santana (13). As condições climáticas favoráveis do Brasil proporcionaram uma rápida expansão do vírus, principalmente pela abundância dos seus principais vetores. No território brasileiro, o *Aedes aegypti* pode ser encontrado em mais de 4.000 municípios e o *Aedes albopictus*, em 3.285 municípios (14,15). Fatores como: alta dispersão vetorial, amplo fluxo de pessoas e suscetibilidade da população à infecção, transformaram esta arbovirose em um grave problema de saúde pública no Brasil (16-18). A Figura 1.2 mostra a dispersão do CHIKV no Brasil, a partir de 2014, originada em dois pontos distintos no norte e nordeste brasileiro.

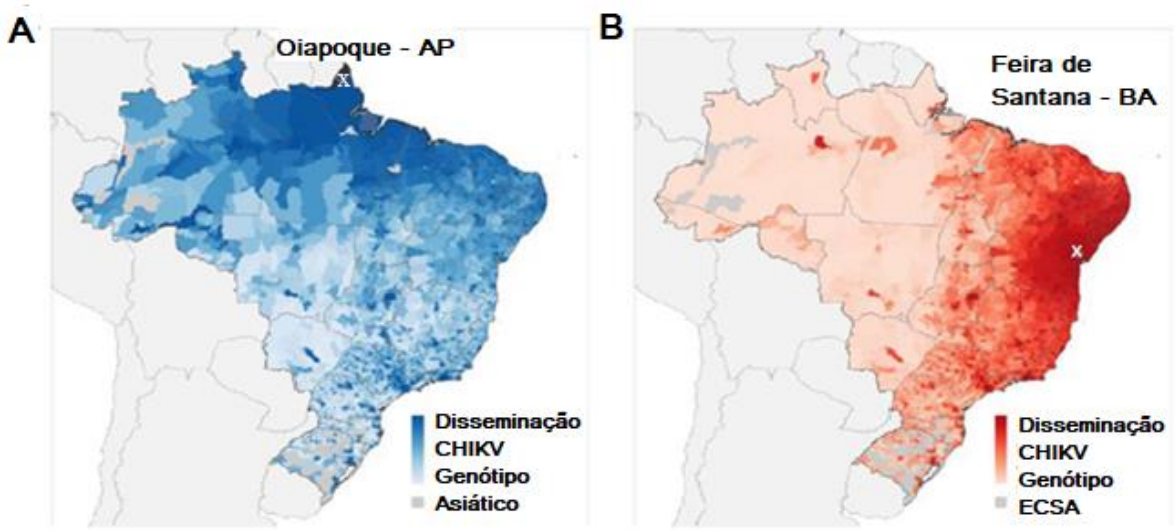

Figura 1.2: Risco de propagação do vírus chikungunya no Brasil, a partir dos casos nos municípios de Oiapoque (A) e Feira de Santana (B) em 2014. Adaptado de Nunes et al.,2015 (19).

Os principais reservatórios do CHIKV em períodos epidêmicos são os humanos. Em períodos interepidêmicos alguns vertebrados têm sido implicados como potenciais reservatórios, tais como: primatas, roedores, pássaros e outros pequenos mamíferos. Dada à distribuição dos vetores nas Américas, toda a região é

suscetível à introdução e propagação do vírus, o que torna necessária a implantação e o aprimoramento das ações de vigilância do vírus no Brasil (18).

Faz-se necessária uma maior elucidação sobre fatores relevantes que justifiquem o progressivo aumento de casos da CHIK, independente dos períodos climáticos. Os estudos de fatores que predispõem a transmissibilidade e a dispersão viral poderão auxiliar em medidas eficazes de controle e prevenção de agravamento dos casos (20). Devido ao destaque desta doença, inclusive no cenário internacional, e a sua relevância para a Saúde Pública, ampliou-se a investigação de infecção aguda por CHIKV entre os casos em que o diagnóstico de dengue foi descartado (21).

1.1.2 O Vírus Chikungunya

O CHIKV é do gênero *Alphavirus*, da família *Togaviridae*. Existem 29 espécies de *Alphavirus*, classificados em oito complexos antigênicos distintos (*Barmah Forest, Eastern Equine, Encephalitis, Middelburg, Ndumu, Semliki Forest, Venezuelan Equine Encephalitis e Western Equine Encephalitis*). No complexo "***Semliki Forest***" estão os arbovírus de importância médica, como o CHIKV, o vírus O'Nyong Nyong e o vírus Mayaro, entre outros (22).

O CHIKV é uma pequena partícula envelopada, de 60 a 70 nanômetros de diâmetro, com formato esférico, que possui um capsídeo icosaédrico envolvido por um envelope lipídico. O genoma viral é constituído por um RNA de fita simples, de polaridade positiva, cujo material genético codifica duas poliproteínas, uma estrutural e uma não estrutural, que após clivadas dão origem a nove proteínas maduras. Destas, três formam o envelope viral (E1, E2 e E3), uma o capsídeo (C), e outras quatro estão envolvidas no processo de replicação viral (23). A Figura 1.3 mostra as principais estruturas deste vírus.

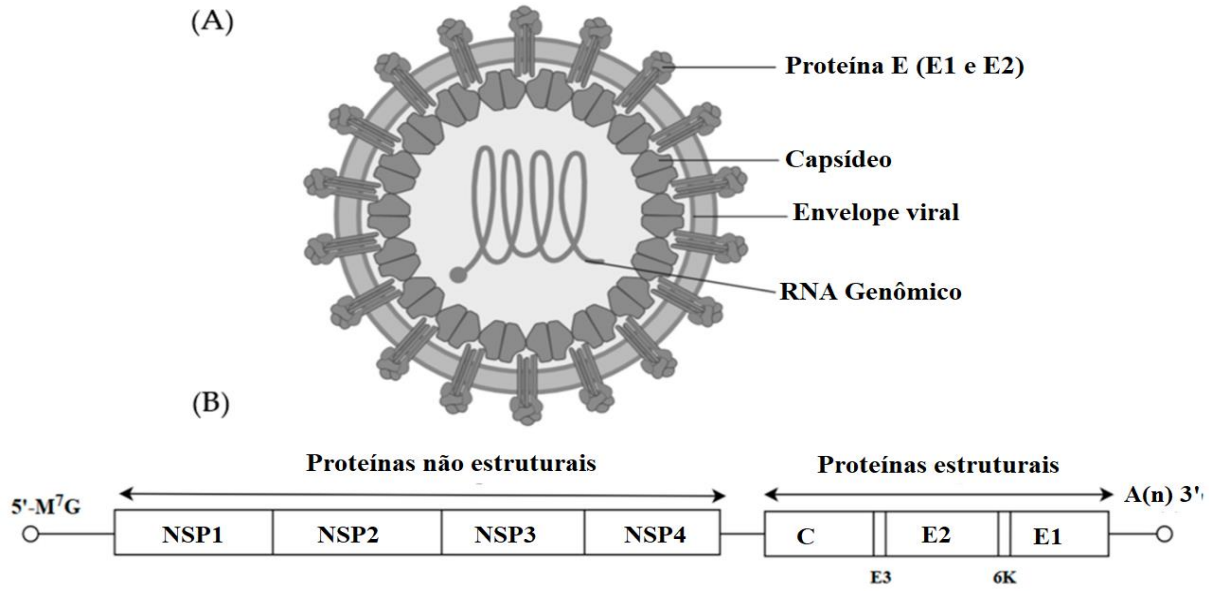

Figura 1.3: (A) A estrutura do alphavírus representando a posição da proteína E (E1 e E2), proteína do capsídeo e o RNA genômico. (B) A estrutura do genoma do alphavírus esquematizado nas regiões não traduzidas 5' e 3'. As caixas representam as proteínas não estruturais (NSP1, NSP2, NSP3 e NSP4) e as proteínas estruturais (CP, E3, E2, 6K e E1). Adaptado de Mandary et al., 2019 (24).

1.1.3 Aspectos Epidemiológicos da Chikungunya

A distribuição geográfica do CHIKV tem influência de vários fatores e está relacionada com o ciclo biológico dos vetores no ambiente e as relações entre os vetores e o vírus. O clima, e particularmente a temperatura e a precipitação, têm um efeito significativo na distribuição e abundância de diferentes espécies de mosquitos (25,26). O aumento da temperatura global reduz o tempo de desenvolvimento das larvas dos insetos, promove um aumento de vetores adultos e flutuações naturais de temperatura durante o dia e a noite, e também influencia na competência do vetor para transmitir doenças. Como resultado, o período de incubação extrínseco é também reduzido, favorecendo a possibilidade de maior transmissão viral nos países com clima tropical e subtropical (27,28).

Segundo dados da Organização Mundial da Saúde (OMS), a CHIK é mais prevalente na África e na Ásia, contudo a frequência de casos está aumentando progressivamente em alguns países da Europa e na Região das Américas. Mais de 2 milhões de casos foram relatados desde o ano de 2005, em todo o mundo. A CHIK já foi diagnosticada em mais de 110 países na Ásia, África, Europa e Américas (29).

A Figura 1.4 mostra a distribuição mundial do CHIKV até o ano de 2022, identificando regiões com maior risco de transmissão da doença.

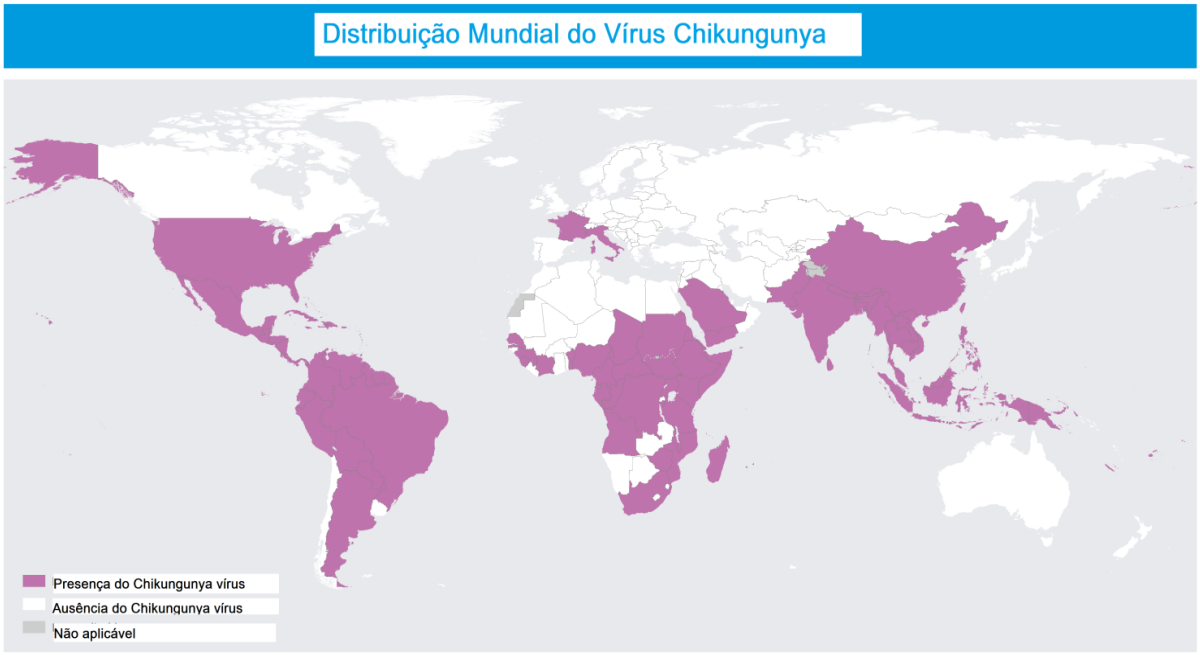

Figura 1.4: Distribuição mundial do vírus chikungunya. Fonte: OMS, 2022 (30).

Segundo dados da Organização Pan-Americana da Saúde (OPAS), para todo o continente americano, a América do Sul registrou o maior número de casos de arboviroses, sendo o Brasil apontado como o país com o maior número de casos notificados em toda a América Latina e Caribe, nas últimas décadas (31).

O Boletim Epidemiológico de janeiro de 2023, emitido pelo Ministério da Saúde do Brasil, mostrou que a taxa de incidência de CHIK no Brasil é de 81,8 casos por 100.000 habitantes, representando um aumento de 32,4% em comparação aos casos registrados em 2019 e de 78,9% para os casos registrados em 2021, para o mesmo período analisado (32). Estes dados confirmam que a doença continua sendo um relevante problema de saúde pública no Brasil.

A série histórica de arboviroses no continente americano mostrou que a maioria dos casos notificados de CHIK concentraram-se no período de 2014 a 2016, mantendo-se estacionária a partir de 2017. A CHIK é a segunda arbovirose mais prevalente na Região das Américas (33). A Figura 1.5 mostra uma série temporal de arboviroses diagnosticadas nas Américas, no período de 2008 a 2021. Observe que

no ano de 2015 não foram relatados casos de zika, pois os boletins epidemiológicos não foram atualizados; porém o primeiro surto de zika no Brasil ocorreu em 2015.

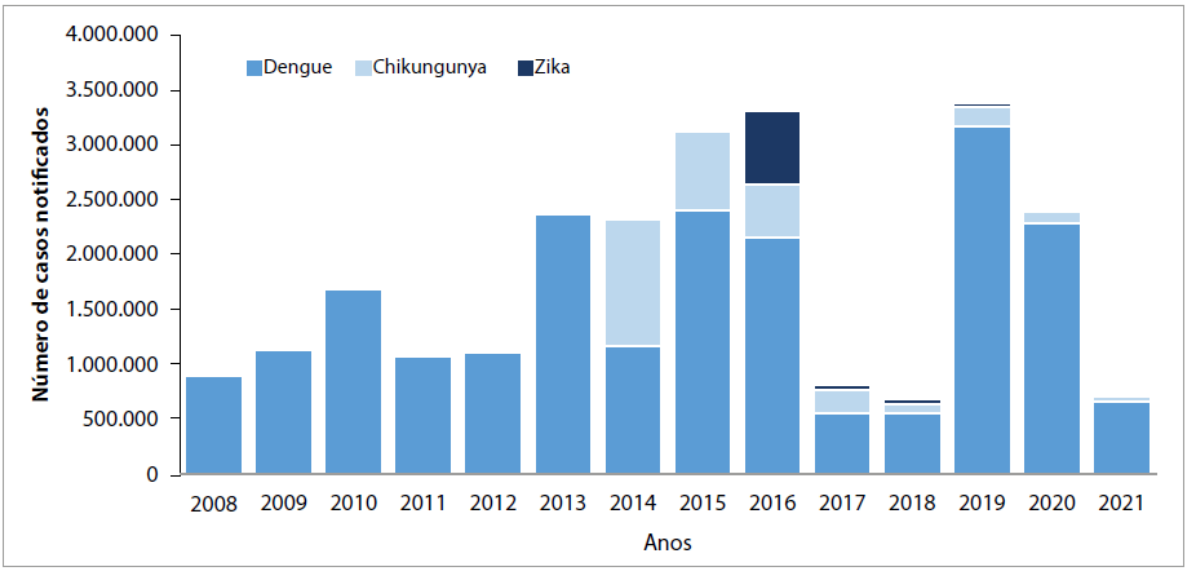

Figura 1.5: Distribuição dos casos notificados de dengue, chikungunya e zika por ano de notificação. Região das Américas, 2008-2021. Fonte: PLISA, OPAS/OMS, 2021 (33).

1.1.4 Etiofisiopatogenia e Manifestações Clínicas da Chikungunya

Após a picada do mosquito, o CHIKV é introduzido na pele humana, misturado com a saliva do vetor, que possui propriedades anti-hemostáticas e imunomoduladoras, facilitando a penetração celular. O vírus penetra em fibroblastos e macrófagos cutâneos, realizando uma primeira replicação. Num segundo momento, o vírus multiplica-se em gânglios linfáticos e depois se dissemina, hematologicamente, para diversos tecidos corporais (34). O CHIKV pode causar danos em diversos órgãos, com tropismo especial para cérebro, fígado, baço, músculos e articulações (34).

A infecção por CHIKV induz resposta da imunidade inata, com produção de marcadores pró-inflamatórios e citocinas, com predomínio de interferon alfa, interleucina 4, Interleucina 10 e interferon-gama (34,35). Chen e colaboradores descreveram que a infecção por CHIKV ativa o inflamassoma NLRP3 em humanos, estando associada ao pico de sintomas inflamatórios. A inflamação está relacionada

a níveis altos de citocinas interleucina-6, quimiocina ligante 2 e fator de necrose tumoral no tecido articular. A inibição deste inflamassoma específico pode reduzir a resposta inflamatória, sendo um caminho para o desenvolvimento de medidas terapêuticas para a CHIK crônica (36). Um importante estudo de meta-análise mostrou que o aumento da interleucina 1β e fator de necrose tumoral α são determinantes nas dores crônicas (37). Na Figura 1.6 há uma esquematização dos principais fenômenos que ocorrem após a introdução do CHIKV no organismo humano.

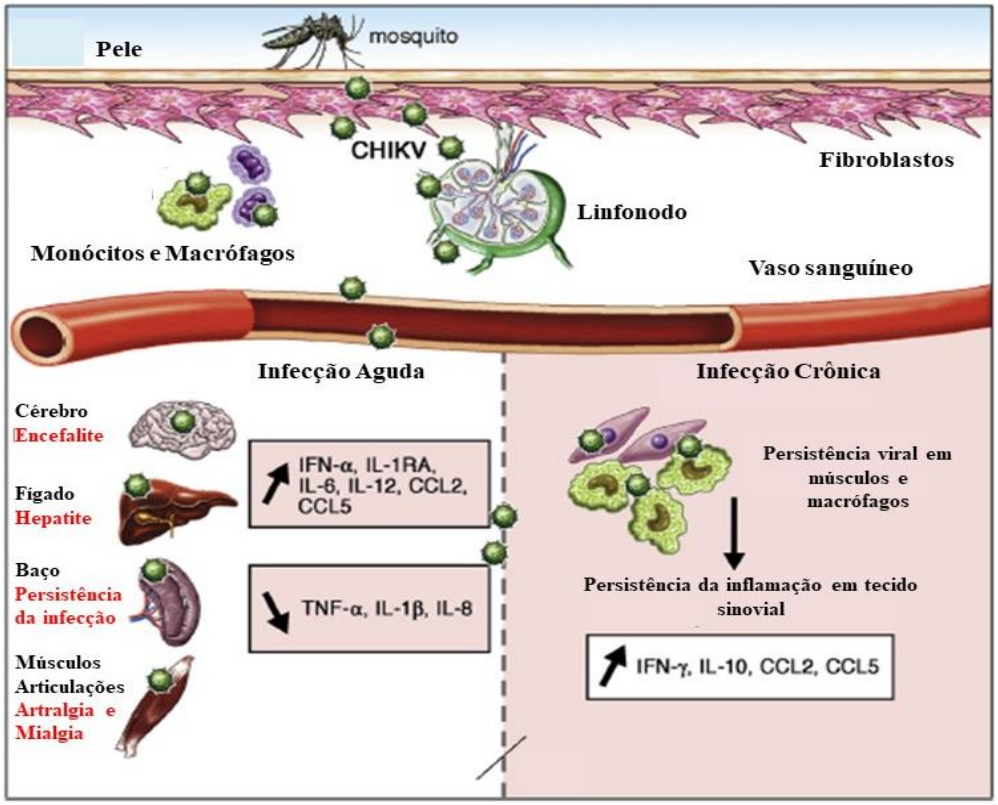

Figura 1.6: Etiofisiopatogenia da chikungunya: após a penetração cutânea, o CHIKV se multiplica em linfonodos regionais e células do tecido conjuntivo; por disseminação hematogênica chega a todos os órgãos. Diferentes citocinas inflamatórias estão presente nas diferentes fases da doença. Adaptado de Petitdemange e cols., 2015 (34).

A infecção pelo CHIKV pode ser assintomática ou produzir um espectro diverso de manifestações clínicas, variando de formas mais brandas a condições graves e incapacitantes (38). Geralmente, a CHIK não está associada à mortalidade

e a taxa de hospitalização é muito baixa na maioria dos estudos em diferentes coortes, onde há predomínio de infecções não graves. A gravidade dos sintomas da doença depende de vários fatores, incluindo: idade, sexo, estado imunológico, predisposição genética e comorbidades (39-43). Apresentações atípicas da doença (0,3 a 1%) e casos graves (0,1%) estão associados às taxas de letalidade, que variam de 10 a 30% (44).

A doença é caracterizada por dor articular intensa de início abrupto, febre alta e erupção cutânea. A infecção é autolimitada e os sintomas agudos geralmente desaparecem após um período de sete a dez dias. Clinicamente, a doença pode se manifestar em três fases: aguda, subaguda e crônica. A poliartralgia é recorrente e debilitante em muitos dos indivíduos infectados, podendo persistir por meses a anos, causando limitação funcional para simples atividades da vida cotidiana. A enfermidade causa incapacitação laborativa persistente, representando um grave problema de saúde pública, por afetar, significativamente, tanto a economia quanto o sistema de saúde (45,46). A Figura 1.7 mostra, de forma esquemática, a relação entre a presença do vírus e a resposta imunológica, para o estabelecimento dos sintomas da doença.

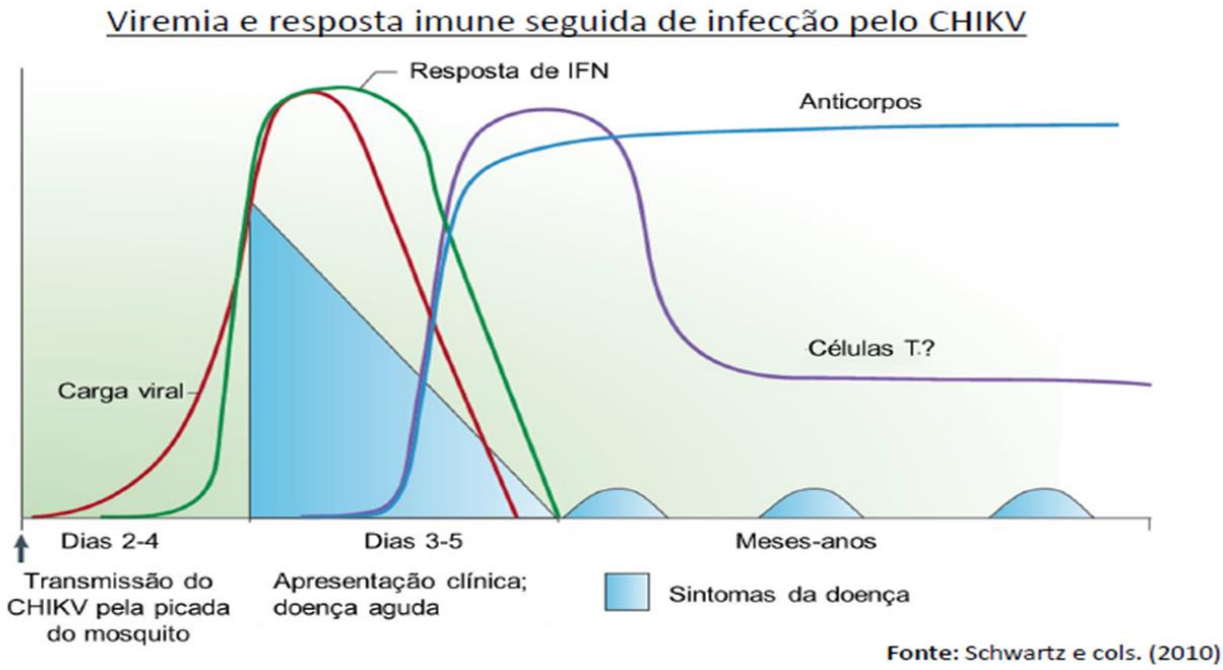

Figura 1.7: Esquematização da resposta imune à infecção do vírus chikungunya, com estabelecimento de relação com as manifestações clínicas da doença. Adaptado de Schwartz et al., 2010 (47).

A fase aguda da doença ocorre nos primeiros 10 dias do início dos sintomas, após um período de incubação que varia de dois a sete dias após a picada do mosquito. Febre alta, artralgia, mialgia, prostração, cefaleia e exantema são os principais sintomas (48). A poliartralgia afeta as articulações dos membros simetricamente e bilateralmente, sendo geralmente acompanhada de edema (43). As articulações dos dedos, tornozelos, punhos, ombros, joelhos, cintura pélvica, pés, cotovelos e coxas são as localizações mais afetadas (42,48). Grave comprometimento dermatológico foi descrito em alguns casos, com máculas purpúricas e lesões vésico-bolhosas na fase aguda da doença (49).

A fase subaguda tem início após o décimo dia de manifestações clínicas e estende-se por três meses. Os pacientes apresentam melhora transitória de sua condição clínica e as recaídas podem ocorrer. Há relato de poliartralgia persistente, com necessidade de medicação analgésica ou anti-inflamatória para aliviar a dor. Essa fase subaguda é representada pelo recrudescimento de manifestações clínicas pré-existentes, ainda que de menor intensidade (38, 42, 48).

A fase crônica é considerada quando a artralgia persiste por mais de três meses, podendo durar alguns meses ou anos. A artralgia e a artrite tendem a ser bilaterais e simétricas, com padrão migratório. A dor assume uma característica intermitente ou constante, podendo ser acompanhada de edema articular ou rigidez articular matinal (42,50). A infecção por CHIKV pode resultar em doença musculoesquelética crônica significativa, que causa sofrimento pessoal, social e econômico, com perda de horas produtivas de trabalho (51). Estudos sugerem que a persistência da dor nas articulações ocorre com maior frequência em mulheres, pessoas com mais de 40 anos e em pessoas com comorbidades preexistentes (52,53). Em pacientes com predisposição para desenvolvimento de doenças reumatológicas pode haver progressão para artropatia destrutiva (45).

Em muitos pacientes, a doença progride para artrite crônica incapacitante. Esta artrite é produto de um processo inflamatório pós-infeccioso com características clínicas e patogênicas que mimetizam a artrite reumatóide (54).

1.1.5 Diagnóstico e Principais Alterações Laboratoriais

A CHIK está intimamente ligada à zika e à dengue, pois são doenças causadas por arbovírus que podem produzir um quadro clínico muito semelhante e

são transmitidas pelo mesmo mosquito, representando um desafio para um diagnóstico clínico adequado. Os sinais e sintomas entre as três doenças são muito semelhantes na fase aguda da doença, principalmente nos primeiros dias de apresentação. Essa semelhança representa um desafio para estabelecimento de um diagnóstico clínico, que pode levar a uma abordagem clínica inadequada, aumentando as chances de complicações (55).

A OMS recomenda três principais testes laboratoriais, preferencialmente em amostras de sangue, para diagnosticar infecções por CHIKV; a saber: isolamento viral, testes sorológicos e reação em cadeia da polimerase da transcriptase reversa em tempo real (rRT-PCR) (56). A escolha dos testes depende do número de dias a partir do início dos sintomas. Isolamento do vírus e rRT-PCR são recomendados para amostras coletadas nos primeiros cinco dias da doença. Os testes de sorologia são recomendados para amostras coletadas a partir de cinco dias após o início da doença. O teste ELISA de Anticorpos Anti-Chikungunya do tipo Imunoglobulina M (Anti-CHIKV-IgM) é o teste sorológico mais recomendado para diagnosticar infecção por CHIKV a partir de sete dias de doença e pode persistir reagente até cerca de 3-6 meses após o início dos sintomas(57). A Figura 1.8 mostra o período ideal (em dias) para a realização dos principais testes laboratoriais no diagnóstico de CHIK.

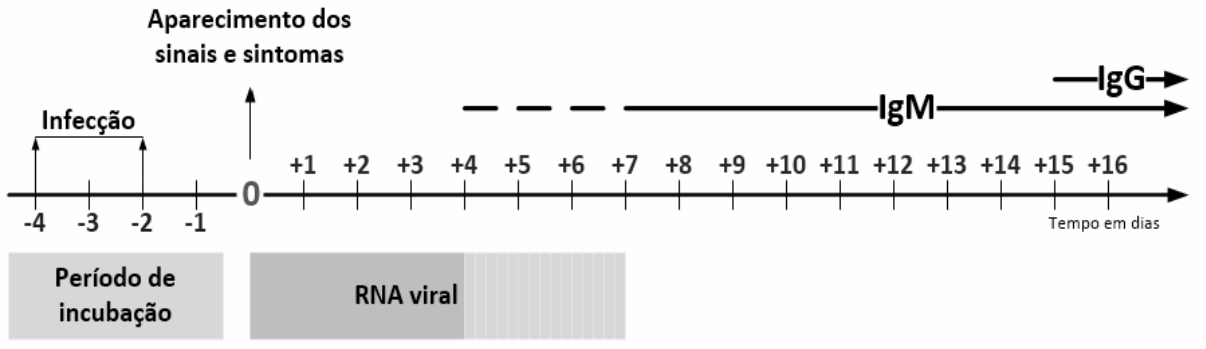

Figura 1.8: Diagrama para o diagnóstico laboratorial de chikungunya, por testes de biologia molecular (rRT-PCR) e testes sorológicos. Adaptado de Sullivan Nicolaides Pathology, 2014 (58)

Os testes sorológicos baseados em antígenos e anticorpos são mais fáceis de realizar e têm baixo custo. Os estudos sobre os testes sorológicos para CHIKV aumentaram nos últimos anos. Porém, a precisão diagnóstica da sorologia é

desconhecida devido aos vários graus de sensibilidades e especificidades dos testes (59).

Andrew e colaboradores (57), em um importante estudo de revisão e meta-análise, concluíram que dependendo do tempo de coleta das amostras, antígenos e testes sorológicos baseados em anticorpos podem diagnosticar com precisão o CHIKV. Os testes de detecção de antígenos são indicados para amostras obtidas durante a fase aguda (1 a 7dias), enquanto os testes de detecção de Anti-CHIKV-IgM e Anticorpos Anti-Chikungunya do tipo Imunoglobulina G (Anti-CHIKV-IgG) podem ser usados para amostras coletadas na fase convalescente (>7 dias após). A combinação dos testes sorológicos IgM e IgG pode diferenciar infecções recentes e passadas (57).

Alterações de exames hematológicos e bioquímicos específicas para CHIK ainda não foram documentadas. Anwar e colaboradores relataram que o nível de hemoglobina pode ser menor em crianças e mulheres e que a contagem de hemácias era significativamente aumentada, além do intervalo de referência nos grupos de meia-idade e idosos. No entanto, eles não documentaram queda significativa nos leucócitos ou nas plaquetas (42). Anfasa e colaboradores mostraram níveis elevados de proteína C reativa (PCR) e ferritina em pacientes na fase aguda da doença, durante uma epidemia na Ilha de Curaçao, no período de 2014 a 2015 (60). Os biomarcadores PCR e velocidade de hemossedimentação (VHS) são testes comumente utilizados na rotina clínica, inclusive no monitoramento da evolução de doenças articulares crônicas. Estudos anteriores falharam em demonstrar uma correlação entre VHS e PCR com a gravidade da doença articular crônica induzida por CHIKV (61,62).

1.1.6 Detecção de CHIKV rRT-PCR em Fluidos Corporais

As técnicas de amplificação de ácido nucleico (NAAT) são as mais amplamente utilizadas nos testes moleculares para diagnóstico virológico, pois permitem a detecção de fragmentos do genoma viral. Várias NAATs foram desenvolvidas para melhorar o desempenho e a especificidade, como a rRT-PCR, na qual os produtos amplificados são detectados e quantificados em cada ciclo da reação (63). Métodos diagnósticos baseados em rRT-PCR têm sido usados para detectar o material genético de agentes infecciosos e são considerados os mais

sensíveis e específicos métodos para sua detecção (64). Especificamente, para o diagnóstico de CHIK, a rRT-PCR continua sendo a técnica mais fidedigna para diagnóstico, uma vez que as reações cruzadas existentes em locais onde circulam muitos arbovírus dificultam o diagnóstico baseado em técnicas sorológicas (65,66).

Os testes diagnósticos para CHIK, rotineiramente, são realizados em amostras de soro ou plasma (67). O uso alternativo de amostras de urina e saliva para o diagnóstico molecular, na fase aguda, de infecções por flavivírus foi descrito em trabalhos com Febre do Oeste do Nilo (68) e em pacientes com dengue (69). Em estudos recentes, envolvendo pacientes infectados pelo vírus zika (ZIKV), as amostras alternativas sugeriram um tempo de excreção maior deste vírus nestes fluidos (70-80).

A detecção do ZIKV no sêmen, com detecção prolongada do RNA viral, foi descrita em alguns estudos (81-90). Estudos envolvendo o isolamento do CHIKV em amostras de saliva e sêmen são poucos e ainda não mostraram resultados conclusivos. Um estudo realizado na Polinésia Francesa, durante um surto de CHIK, conseguiu isolar o vírus em amostras de saliva e urina durante a fase aguda, porém não o encontraram na fase de convalescença da doença (91).

Em estudo realizado por Bandeira e colaboradores foi demonstrada a presença do RNA do CHIKV em amostras de sêmen e urina após 30 dias do início dos sintomas, trazendo perspectivas para novas formas diagnósticas e mecanismos de transmissão da doença (92).

1.1.7 Tratamento

Atualmente, o tratamento para CHIK é direcionado para o alívio dos sintomas, principalmente da dor articular, e ainda não há um consenso definido sobre o tema (93-95). Na infecção aguda, cerca de 95% das pessoas infectadas desenvolvem sintomas, que desaparecem em cerca de duas semanas. Na forma crônica, a artralgia pode durar meses a anos (95,96). No entanto, sem nenhum tratamento padronizado, a criação de planos de tratamento pode ser complexa, pois existe uma vasta quantidade de pesquisas sobre tratamentos naturais e farmacêuticos para os sintomas crônicos da CHIK (93-95,97).

O Ministério da Saúde do Brasil tem um importante guia de abordagem terapêutica para CHIK, no qual fármacos opioides, como tramadol ou codeína, são

indicados para alívio das dores intensas, durante o período agudo da doença. No mesmo protocolo, há indicação de tratamento da dor neuropática com amitriptilina ou gabapentina. Nas fases subagudas e crônicas da doença, o uso de corticoide (prednisona), hidroxicloroquina e metotrexato são indicados em situações específicas (17).

Uma recente revisão sistemática destacou que diretrizes para a abordagem terapêutica da CHIK são controversas e heterogêneas em todo o mundo (95). Embora haja um consenso sobre o tratamento sintomático da doença aguda não grave, há uma falta geral de orientação para os cuidados de suporte. A recomendação do uso e tempo de tratamento dos corticosteroides é o maior problema; onde alguns defendem seu uso na fase aguda, outros desaconselham. Há também variação e conselhos contraditórios sobre o uso de anti-inflamatórios não esteroides na fase aguda da doença. Além disso, a falta de padronização na classificação dos estágios da doença em aguda, subaguda e crônica pode impactar nas recomendações de tratamento dos pacientes (98).

Há muita variação na recomendação do uso de fármacos antirreumáticos, especialmente hidroxicloroquina e metotrexato, no tratamento da CHIK crônica. Os estudos clínicos intervencionistas existentes sobre estes fármacos são limitados, com a falta de metodologias padronizadas. Ainda não há estudos suficientes para a realização de revisões de meta-análises (98).

Um importante trabalho de revisão, realizado por reumatologistas brasileiros, formulou recomendações para o tratamento da CHIK no Brasil, com esquemas terapêuticos baseados nas diferentes fases clínicas da doença. Neste trabalho há indicações mais precisas para o uso de amitriptilina, gabapentina, pregabalina, hidroxicloroquina, metotrexate e drogas bloqueadoras de fator de necrose tumoral (99). A CHIK crônica passou a ser melhor conduzida por médicos reumatologistas; e fluxogramas terapêuticos padronizados consolidaram uma melhor abordagem para diminuir a inflamação crônica e amenizar as possíveis dores neuropáticas que estão associadas aos processos inflamatórios (99).

Há uma necessidade urgente de desenvolvimento de alternativas naturais ou homeopáticas para esta infecção viral emergente, que possibilitem o uso em áreas de poucos recursos, onde esse vírus é mais preocupante (100).

1.2 Hipótese do Estudo

A hipótese principal do estudo foi que o CHIKV estaria presente nos fluidos corporais (sangue, saliva, urina, sêmen e secreção vaginal) desde os primeiros dias da doença e que se manteria detectável durante a fase subaguda desta enfermidade (até três meses).

Uma hipótese secundária foi que a presença deste vírus nos fluidos corporais durante a fase aguda da doença poderia estar relacionada às manifestações clínicas prolongadas da CHIK, principalmente ao que se refere à dor articular (principal sintoma da doença).

1.3 Justificativa

A CHIK representa, na atualidade, um grave problema de saúde pública no território brasileiro. Conhecer a dinâmica viral da doença no organismo humano, nas diferentes fases evolutivas poderá auxiliar grandemente na abordagem médico-laboratorial dos pacientes infectados; estabelecendo uma relação com características sociodemográficas, comorbidades, manifestações clínicas e marcadores de atividade inflamatória.

Há menos de uma década convivemos com esta arbovirose e é gigantesco o impacto econômico negativo, pelas frequentes abstenções laborativas dos indivíduos doentes e pela catastrófica diminuição da qualidade de vida das pessoas que desenvolvem a doença crônica.

A confirmação do período de excreção do CHIKV nos diferentes fluidos corporais poderá contribuir para o desenvolvimento de métodos alternativos de diagnóstico, baseados no período de evolução da doença e para o estabelecimento de políticas públicas de controle da transmissão do CHIKV.

A detecção do CHIKV em diferentes fluidos corporais será de grande valia para estudar diferentes formas de excreção viral e confirmar se há mutações genômicas em diferentes compartimentos corporais, proporcionando substrato para recomendação de amostras alternativas para o diagnóstico laboratorial. Da mesma forma, poderá propor medidas mais eficazes para tratamento e prevenção da doença.

Recentemente, houve um progresso significativo nos estudos científicos de vários aspectos da infecção por CHIKV, mas importantes lacunas remanescentes precisam ser extensivamente abordadas. Em países de clima tropical, como o Brasil, existe uma sobreposição de muitas doenças endêmicas, com manifestações clínicas muito semelhantes. Uma melhor compreensão do comportamento do CHIKV nas diferentes fases da infecção se faz necessária, para o desenvolvimento de intervenções terapêuticas eficazes.

Associada à incapacidade significativa e à redução da qualidade de vida, a artralgia pode persistir por muitos meses após a infecção pelo CHIKV. Compreender a duração esperada da persistência da artralgia é importante para gerenciar as expectativas clínicas em nível individual, também, para estimar os encargos de longo prazo na saúde da população, após uma epidemia de CHIK.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar a presença e duração do CHIKV no sangue, sêmen, secreção vaginal, saliva e urina em indivíduos adultos infectados durante a fase aguda e subaguda da doença e estimar os fatores de risco para a dor articular crônica.

2.2 Objetivos Específicos

- Investigar a presença e duração do CHIKV nos fluidos corporais em pacientes com idade igual ou superior a 18 anos, de ambos os sexos, em um período de três meses;

- Correlacionar fatores sociodemográficos, manifestações clínicas e alterações de exame laboratoriais com a presença de dor crônica.

3. MATERIAL E MÉTODOS

3.1 Desenho de Estudo

Estudo de coorte longitudinal prospectivo, que utilizou a estrutura regular de coleta de dados e de material biológico do projeto “AMOSTRAS ALTERNATIVAS PARA O DIAGNÓSTICO DE CHIKUNGUNYA E PERSISTÊNCIA DO VÍRUS EM FLUIDOS CORPORAIS”, sob responsabilidade de Guilherme Amaral Calvet e em andamento desde abril de 2019.

3.2 Casuística

3.2.1 População e Local de Estudo

O estudo foi realizado a partir da consulta de formulários clínicos e relatórios médico-laboratoriais, produzidos em consultas ambulatoriais, realizadas pela equipe de especialistas do Laboratório de Doenças Febris Agudas (DFA). Os atendimentos por demanda espontânea ou referenciados foram realizados no período de abril de 2019 a maio de 2020. A população atendida é residente da região metropolitana do Rio de Janeiro, ou de qualquer município do estado ou do Brasil, ou de qualquer região tropical do planeta. Referência para viajantes com febre, o Laboratório de DFA é localizado no Instituto Nacional de Infectologia Evandro Chagas (INI), no campus da Fundação Oswaldo Cruz (FIOCRUZ).

3.2.2 Critérios de Inclusão

- Idade igual ou superior a 18 anos de idade.
- Ambos os sexos.
- Pacientes do Ambulatório de DFA do INI, atendidos no período de abril de 2019 a maio de 2020.
- Relato de febre e/ou dor articular de início recente (até sete dias).
- Não ter a intenção de mudar o local de moradia nós próximos três meses.
- Apresentar infecção por CHIKV comprovada por teste de CHIKV rRT-PCR positivo nos espécimes de sangue e/ou urina, coletados na primeira consulta ambulatorial; e/ou sorologia reagente para Anti-CHIK-IgM.

3.2.3 Critérios de Exclusão

- Mulheres grávidas;
- Pacientes com transtornos mentais graves; ou que não permitam a coleta de informações por distúrbios de comunicação; ou falta de fluência na língua portuguesa ou outro idioma compreendido pelo médico assistente;
- Presença de infecção bacteriana evidente (por exemplo, celulite, abscesso, pneumonia).
- Pacientes com outras doenças febris agudas.

3.3 Materiais, Procedimentos e Coleta de Dados

Após a suspeita de infecção por CHIKV foram programadas seis consultas médicas/paciente padronizadas com: anamnese dirigida, exame físico e exames laboratoriais pré-estabelecidos. As primeiras cinco visitas (V1, V2, V3, V4 e V5) tinham intervalos de 15 dias e uma última visita (V6) programada após 90 dias da inclusão no estudo, independentemente dos resultados de rRT-PCR.Cada visita foi constituída por consulta clínica e coleta de material biológico (sangue, urina, saliva, sêmen e secreção vaginal).

Foi utilizado um instrumento de coleta de dados estruturado, preenchido durante uma entrevista com os participantes após assinatura do termo de consentimento livre e esclarecido (TCLE).

Definimos febre como uma temperatura axilar maior ou igual a 37,8 °C.

A captura e o armazenamento de dados foram realizados no programa *Research Electronic Data Capture* (REDCap), utilizados de forma contínua e sistemática. Os dados deste estudo estão contidos na base de dados do Laboratório de Pesquisa Clínica em Doenças Febris Agudas (LapClinDFA).

3.4 Exames Laboratoriais

As amostras de soro e líquidos corporais foram testadas para CHIKV por rRT-PCR. Valores de limiar de ciclo (Ct – “*Cycle threshold*”) menores que 38 e com

curvas sigmoides foram considerados positivos. O soro foi testado para Anti-CHIKV-IgM (Euroimmun®, Luebeck, Alemanha).

A pesquisa de ZIKV e dengue vírus (DENV), por rRT-PCR, também foi realizada em amostras de soro, coletadas na fase aguda da doença, utilizando o kit comercial ZDC, do Instituto de Tecnologia em Imunobiológicos Biomanguinhos. O kit foi aprovado pela Agência Nacional de Vigilância Sanitária - ANVISA (registro nº 80142170032). As amostras de urina coletadas durante a fase aguda também foram testadas para ZIKV por rRT-PCR. Nos casos em que o DENV foi detectado pelo kit ZDC, o protocolo de Lanciotti e colaboradores foi usado para identificar subtipos de DENV (101). Todos os exames do estudo foram realizados no Laboratório Nacional de Referência em Vigilância Epidemiológica de Arbovírus, do Laboratório de Flavivírus, do Instituto Oswaldo Cruz da Fiocruz (Rio de Janeiro, Brasil).

Amostras de sangue adicionais foram coletadas para avaliar os parâmetros hematológicos e bioquímicos de cada paciente. Todos os resultados laboratoriais foram avaliados de acordo com os limites de normalidade de cada exame em relação ao sexo do participante, se aplicável.

3.5 Isolamento Viral – Teste de Infectividade

Amostras de espécimes clínicos com CHIKV detectado por rRT-PCR e valor de Ct correspondente a altas cargas virais foram selecionadas para a realização de isolamento viral em cultura celular.Foi realizada a inoculação de oito amostras de sêmen, inicialmente em cultura celular C6/36 clone de *Aedes albopictus* em tubo de célula, seguido da utilização de placas de cultura celular de VERO (células epiteliais de rim de macaco verde africano). A partícula viral era considerada infecciosa quando capaz de infectar a monocamada celular, elevando assim o título viral.

3.6 Plano de Análise

3.6.1 Desfechos e Variáveis de Interesse

Neste estudo foram utilizadas variáveis sociodemográficas, clínicas, laboratoriais e desfechos de interesse (confirmação do CHIKV por exames

específicos rRT-PCR ou sorologia Anti-CHIK-IgM) e presença de dor após três meses do início dos sintomas.

3.6.2 Análise de Dados

As variáveis sociodemográficas foram descritas por meio de frequências e proporções, para variáveis categóricas, e medianas e intervalos ou intervalos interquartis (IIQ) para variáveis contínuas (102).

Realizamos uma análise exploratória por meio do cálculo de medidas estatísticas resumidas, para avaliar a distribuição do tempo de persistência do CHIKV entre os fluidos. O tempo até a perda de detecção de RNA em cada fluido corporal foi definido como o número de dias entre o início dos sintomas da doença e o primeiro resultado negativo de rRT-PCR. Presumimos que o RNA de CHIKV em todas as amostras era detectável no dia do início dos sintomas. Pacientes que nunca tiveram resultado positivo durante o estudo, mesmo que tivessem apenas uma visita, foram excluídos da análise.

Os participantes com teste positivo para qualquer fluido durante o estudo, sem que um resultado negativo fosse apresentado ao final do acompanhamento, foram censurados. A técnica de Kaplan-Meier (K-M) e os modelos paramétricos de regressão de Weibull foram usados para estimar o tempo até a perda de detecção do RNA de CHIKV entre os fluidos corporais (103,104).

Medianas e intervalo de confiança (IC) de 95% foram usados para produzir os resultados. O modelo de Weibull foi sobreposto na Curva K-M para as amostras do fluido, proporcionando uma análise mais precisa.

Para determinar fatores de risco para dor crônica nas articulações após três meses de infecção por CHIKV, uma análise univariada foi realizada comparando as características iniciais dos dois grupos: "com dor articular" e “sem dor articular" na visita de três meses. Fatores de risco associados à artralgia prolongada, após ajuste para idade, sexo e presença de artrose, foram inseridos em um modelo de Regressão Logística Múltipla (“*Forward Stepwise*”). No modelo final, as variáveis eram retidas se fossem estatisticamente significativos ($p<0,05$) ou se alterassem substancialmente os coeficientes de outras variáveis no modelo. O limite de significância foi definido em 5% (102).

Os dados foram coletados e gerenciados usando o *REDCap*. A análise estatística foi realizada com o software estatístico R, versão 4.1.0 (R Core Team, 2021) e IBM SPSS Statistics 22.0.

3.7 Aspectos Éticos

Trata-se de um projeto intitulado “AMOSTRAS ALTERNATIVAS PARA O DIAGNÓSTICO DE CHIKUNGUNYA E PERSISTÊNCIA DO VÍRUS EM FLUIDOS CORPORAIS”, em andamento desde 2019 no LapClinDFA. O estudo foi aprovado pelo Comitê de Ética em Pesquisa do INI em 26 de fevereiro de 2019, sob registro CAAE: 06779019.0.0000.5262. Os pacientes foram incluídos no estudo após assinatura do TCLE.

Como objetivo de assegurar a confidencialidade dos dados, todos os arquivos eletrônicos foram mantidos em área restrita ao uso comum. Este projeto foi conduzido de acordo com as normas das Boas Práticas em Pesquisa Clínica.

O estudo seguiu as instruções e as condutas aplicadas às pesquisas com seres humanos, Resolução CNS Nº 466, de 12 de dezembro de 2012, de acordo com a Resolução nº196/96, do Conselho Nacional de Saúde, do Ministério da Saúde. Os participantes foram informados sobre os objetivos, os métodos e o uso de dados da pesquisa. Os princípios de confidencialidade e anonimato foram respeitados. Foi esclarecido que a participação no estudo era voluntária e não remunerada; e que os dados da pesquisa serão apresentados em artigos científicos e em reuniões científicas nacionais ou internacionais.

4. ARTIGOS PUBLICADOS

Os resultados desta tese estão resumidos em três artigos, o primeiro intitulado “Detection of Chikungunya virus in bodily fluids: The INOVACHIK Cohort Study” e publicado 07/03/2022 na revista *Plos Neglected Tropical Diseases*; o segundo intitulado “Chikungunya vírus shedding in semen: a case series” e publicado em 26/08/2022 na revista *Viruses*; e o terceiro intitulado “Predictors of chronic joint after Chikungunya virus infection in the INOVACHIK prospectivecohortstudy” foi submetido em 16/03/2023 na revista *Journal of Clinical Virology*.

4.1 ARTIGO 1 –Detection of Chikungunya virus in bodily fluids: The INOVACHIK Cohort Study.

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Detection of Chikungunya virus in bodily fluids: The INOVACHIK cohort study

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Abstract

Background

Chikungunya is a widely distributed, re-emerging tropical disease caused by the chikungunya virus (CHIKV). Little is known about the duration for which CHIK RNA are detectable in bodily fluids, especially genital secretions, and current evidence is based on small series or case reports. An understanding of viral dynamics across different body compartments can inform diagnostic testing algorithms and public health prevention interventions.

Methodology

A prospective cohort study was conducted to assess the presence and duration of detectable levels of CHIKV RNA in blood, urine, saliva, semen, and vaginal secretions. Men and women ($\geq$ 18 years) with a positive reverse transcriptase-polymerase chain reaction (RT-PCR) test for CHIKV in the acute phase (1–14 days) of the disease were included. After enrollment, clinical data and samples were collected every 15 days over the first 2 months, and a final collection was performed 3 months after recruitment. The Kaplan–Meier interval-censoring method and the parametric Weibull model were fitted to estimate the median time of viral persistence until the lack of CHIKV RNA detection among all body fluids. Punctual estimates of the median time of CHIKV RNA persistence for each fluid were estimated using a 95% confidence interval (CI).

Results

From April to December 2019, 170 participants were screened. Of these, 152 (100 women) were enrolled in the study. The median and interquartile range (IQR) ages for men and women were 39.3 (IQR: 26.9, 50.7) and 43.5 (IQR: 33.8, 53.6) years, respectively. CHIKV RNA was detected in 80.3% (122/152) of serum samples, 23.0% (35/152) of urine samples, 30.3% (46/152) of saliva samples, 14.3% (6/42) of semen samples, and 20.2% (20/99) of vaginal secretion samples. The median time until the loss of CHIKV RNA detection was

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19.6 days (95% CI, 17.5–21.7) in serum, 25.3 days (95% CI, 17.8–32.8) in urine, 23.1 days (95% CI, 17.9–28.4) in saliva, and 25.8 days (95% CI, 20.6–31.1) in vaginal secretion. The number of semen samples available was too small to make statistical estimates, but a last positive sample was obtained from a participant 56 days after the onset of symptoms.

Conclusions

CHIKV RNA could be detected in all bodily fluids studied, including genital secretions during the acute and convalescent phases and additional studies on viral infectivity in semen and vaginal secretions are warranted.

Author summary

This prospective cohort study of adult patients aimed to estimate the presence and duration of detectable levels of chikungunya virus RNA in bodily fluids, including genital secretions, among participants in the acute and convalescent phases of the disease. In addition to the fluids usually used for diagnosis in humans (serum and plasma), we reported the detection of chikungunya virus RNA in all body fluids. Reports have shown that chikungunya virus RNA in serum declines to undetectable levels within 1–2 weeks after symptom onset. The viral persistence in the serum in our study was longer than expected. In addition, we showed that saliva and urine contained detectable viral RNA in both the acute and convalescent phases of the disease. To the best of our knowledge, this is the first cohort study assessing the presence and persistence of CHIKV in genital fluids (vaginal secretions and semen). Knowledge of viral persistence can help inform recommendations for the control, treatment, and prevention of the disease. Additional studies on viral infectivity are warranted.

Introduction

Chikungunya is a widely distributed, re-emerging tropical disease caused by the chikungunya virus (CHIKV) and transmitted by *Aedes aegypti* mosquitoes [1,2]. Prior to 2013, CHIKV cases and some outbreaks were identified in several countries in Africa, Asia, Europe, and the Indian Ocean Islands. In 2013, the first local transmission of the CHIKV in the Americas was identified in the Caribbean countries and territories. The virus then spread throughout most of the Americas in 2014, especially in Brazil [1,2]. To date, three CHIKV genotypes are known: West African, Asian, and East Central South African (ECSA), of which the last two are prevalent in Brazil [3,4].

Clinically, the disease has three main phases: acute, post-acute, and chronic. In the acute phase, symptom onset ranges from 2–12 days following bites by an infected mosquito. This phase is associated with an abrupt onset of fever, headache, arthralgia, myalgia, fatigue, prostration, and rash. Severe joint pain is the most prevalent symptom, described in 90% of cases. The post-acute phase appears after 14 days of illness, following the febrile period. At this stage, joint pain is observed, which may last for up to 3 months. Finally, in the chronic phase, articular manifestations are seen, which are usually debilitating and can persist for many years [5,6].

Routinely, diagnosis is performed using serum or plasma samples [7]. However, the alternative use of urine and saliva samples for molecular diagnosis has been described in the acute

phase of flavivirus infections, such as the West Nile virus [8], dengue virus (DENV) [9], and Zika virus (ZIKV) infections [10]. In addition, several studies have suggested a more extended detection and persistence of ZIKV in selected body fluids, such as saliva, urine, semen, sweat, and rectal samples [11–15].

Gardner et al. showed that oral fluid (saliva) of CHIKV-infected animals and humans might contain infective CHIKV in the acute phase of the disease. Human saliva samples were obtained from 13 CHIKV-positive patients who presented with hemorrhagic manifestations [16]. The prolonged detection of ZIKV RNA in semen has been described in some studies [13,17,18]. Although studies involving the isolation of CHIKV in saliva, urine, and semen samples are scarce, during an outbreak of chikungunya in French Polynesia, the virus was detected in saliva and urine samples in the acute phase of the disease [19]. Bandeira et al. reported CHIKV RNA in semen and urine samples 30 days after symptom onset, bringing new perspectives for alternative diagnostic forms and mechanisms of infection transmission [20], with implications for its prevention and control.

This study aimed to estimate the presence and duration of detectable levels of CHIKV RNA in bodily fluids, namely serum, saliva, urine, semen, and vaginal secretion in the acute and convalescent phases of the disease.

Methods

Ethics statement

INOVACHIK was a prospective cohort study conducted at the Acute Febrile Illness Laboratory, Oswaldo Cruz Foundation outpatient clinic in Rio de Janeiro, Brazil. The institutional review board reviewed and approved the study protocol (CAAE: 06779019.0.0000.5262). Written informed consent wasobtained before participation from all patients.

Study site and cases management

Patients admitted to the hospital or the intensive care unit were not targeted for enrollment to avoid bias towards patients with more severe disease. Thus, the patients enrolled in the study were screened at a general febrile illness outpatient clinic for more generalizable findings. Patients seen at this outpatient clinic are either referred by other health units in Rio de Janeiro or spontaneously seek care.

Men and women aged $\geq$18 years who had developed acute fever or arthralgia (with or without a rash) and no evident focus of bacterial infection within the previous 7 days were enrolled. A standard case report form was used to record information about the epidemiological and clinical features. We defined fever as an axillary temperature $\geq$ 37.5˚C. Patients with symptoms reported for up to 7 days were included in the study. The first visit (with fluid collection) was performed on different days, depending on the patient's arrival at our clinic.

Clinical data and biological samples were collected every 15 days for 2 months, with a final 3-month collection. The first samples for all fluids were collected in the first week of symptoms, and the second samples were collected within 14 days after the onset of symptoms (+/- 3 days as visit window). Data regarding clinical signs and symptoms was collected during the acute phase (1–14 days). All patients were tested over the study duration (3 months), despite undetectable RT-PCR results in all body fluids collected during a visit.

Laboratory tests

Serum, urine, saliva, semen, and vaginal secretion specimens were collected at enrollment, every 15 days for 2 months, and at the 3-months follow-up.

The samples were tested for CHIKV using real-time reverse transcriptase polymerase chain reaction (rRT-PCR). Following the manufacturer's instructions, RNA was extracted using the QIAmp Viral RNA Mini Kit. The general procedures for rRT-PCR for chikungunya, zika, and dengue have been described elsewhere [21,22,23]. The RT-PCR mix was prepared using the GoTaq Probe 1-Step RT-qPCR System and was run using the Applied Biosystems 7500 Real-Time PCR System. Cycle threshold (Ct) values lower than 38 and sigmoid curves were considered positive. In addition, serum was tested for anti-CHIKV-IgM according to the manufacturer's protocol.

As a reference laboratory, all measures to avoid cross-contamination within the samples were adopted. There were different areas designated only for PCR: 1. In two large rooms, RNA extraction (manual or automated) was performed in cabinets with UV light or in a biological safety cabinet, with an adjacent room for adding samples in the PCR mix; 2. One "clean" room was used for mix preparation only; 3. One room with thermocyclers was used for the amplifications.

A non-template control (NTC) was used to check for the absence of sample cross-contamination, contamination of reagents, consumables, and environment. The NTC is a negative "sample" that can be water or negative plasma extracted simultaneously with the clinical samples and included in the amplification process. In all steps, tips with barriers were used, most consumables were disposable, and gloves were changed frequently. In addition, aseptic cleaning was frequently performed in all rooms.

Testing for ZIKV and DENV by rRT-PCR, was also performed for serum samples collected during the acute phase of the disease using a commercial kit (ZDC) from the *Instituto de Tecnologia em Imunobiológicos Biomanguinhos*. The ZDC kit was approved by the *Agência Nacional de Vigilância Sanitária/ANVISA* (registry #80142170032). Urine specimens collected during the acute phase were also tested for ZIKV using rRT-PCR. In cases where the ZDC Kit detected DENV, the protocol by Lanciotti *et al*. was used to identify DENV subtypes [24]. All study tests were performed at the National Reference Laboratory for Epidemiological Surveillance of Arbovirus in the Laboratory of Flavivirus at the Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil.

Statistical analyses

The sociodemographic variables were described using frequencies and proportions for categorical variables and medians and ranges or interquartile ranges (IQRs) for continuous variables [25].

An exploratory analysis was performed by calculating summary statistical measures, and the violin plot was used to assess the distribution of the persistence time of the fluids. The Kaplan–Meier (K–M) curve technique was used to analyze time-to-event outcomes and estimate the probability of survival at various time intervals. Graphs were used to illustrate survival; in this case, the persistence time for each fluid over time [26]. The time until the lack of RNA detection in each bodily fluid was defined as the number of days between the onset of CHIKV symptoms and the first negative RT-PCR result. We assumed that CHIKV RNA in all specimens was detectable on the day of symptom onset. Patients who never tested positive during the study were excluded from the analysis, even if they had only one visit. Parametric Weibull regression models were used to estimate the time until the loss of CHIKV RNA detection in body fluids. Medians and 95% confidence intervals (CIs) were used to report the results. We estimated survival functions and 95% CIs for the Weibull model with median and 95th percentiles [27].

The Weibull curve was used as a smoother curve for the Kaplan–Meier estimator's distribution. The Weibull curve showed the best choice as a smoothing curve to represent the Kaplan–

Meier estimator's distribution for all studied fluids. Statistical analysis was conducted using R, version 3.6 (R Core Team, 2020) and IBM SPSS Statistics 22.0.

Results

Participants characteristics

From April 10th to December 5th, 2019, A total of 170 participants were screened. Of these, 152 patients were enrolled in the study. The reasons for exclusion of the 18 potential participants were as follows: unspecified viral disease (n = 13), bacterial tonsillitis (n = 1), syphilis (n = 1), mononucleosis (n = 1), influenza virus (n = 1), and adverse cutaneous reaction (n = 1), as shown in Fig 1.

The median and interquartile range (IQR) age was 39.3 (IQR; 26.9, 50.7) for men and 43.5 years (IQR; 33.8, 53.6) for women. The majority of the study population was women (n = 100, 65.8%). Most patients were born in the state of Rio de Janeiro (82.6%). Table 1 shows the main sociodemographic characteristics of the study population.

Signs, symptoms, and comorbidities

Arthralgia (99.3%), fever (99.3%), prostration (94.7%), headache (86.8%), taste alteration (81.6%), chills (76.3%), myalgia (71.7%), and retroorbital pain (53.3%) were the most common symptoms reported. Complaints were associated with a rash in 84.9% of these patients. Joint swelling was also a common sign (61.2%), especially in the hands, ankles, and knees. Table 2 shows the signs and symptoms in the acute phase of the disease.

The most frequent comorbidities observed were high blood pressure (19.7%), allergic rhinitis (19.1%), and arthrosis (9.9%). Coinfection with HIV was found in eight patients (5.3%), but their clinical presentations did not differ from those of the rest of the study population.

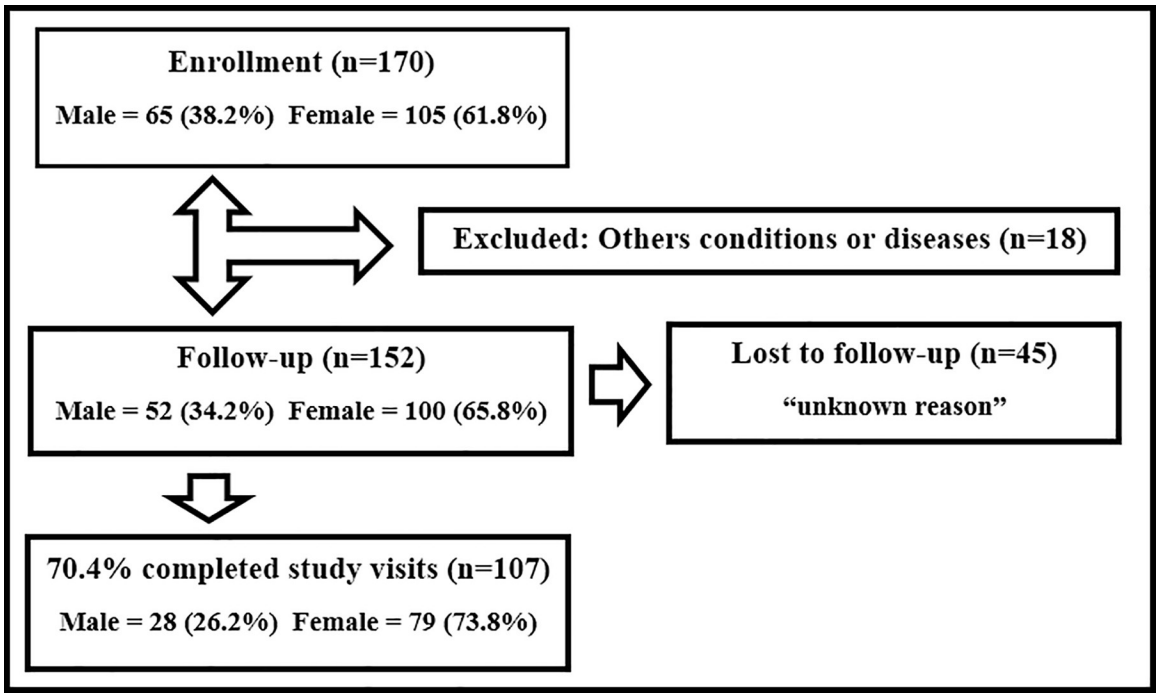

Fig 1. Flow diagram of INOVACHIK Cohort Study.

https://doi.org/10.1371/journal.pntd.0010242.g001

Table 1. Sociodemographic characteristics of the study population, April—December 2019, Rio de Janeiro, Brazil.

Characteristics	n	%
Female	100	65.8%
Male median age (IQR)	39.3 (26.9–50.7)	
Female median age (IQR)	43.5 (33.8–53.6)	
Race		
Black	24	15.8%
White	76	50.0%
Mixed race	51	33.6%
Yellow	1	0.7%
Education level		
Elementary School	44	29.0%
High School	66	43.4%
College	42	27.6%
Marital Status		
Single	70	46.1%
Married / Stable Union	66	43.4%
Divorced / Separated	13	8.6%
Widowed	3	2.0%

IQR: interquartile range.

https://doi.org/10.1371/journal.pntd.0010242.t001

Zika and Dengue virus coinfection

Among the 152 enrolled participants, three (2.0%) had confirmed ZIKV infection, as assessed by rRT-PCR in the serum (n = 2) and urine (n = 1). A positive rRT-PCR result for DENV was found in four participants (2.6%), and all presented with DENV-2 infection.

Specific IgM antibodies against CHIKV

Specific IgM antibodies against CHIKV were detected in 146 participants (96.1%). Six participants had a single visit with negative IgM antibodies against CHIKV; therefore, it was not possible to document the occurrence of seroconversion. The lack of IgM class antibody production in these participants was probably because sample collection was performed 2 days after symptom onset in five participants and 3 days after symptom onset in another participant.

CHIKV detection in bodily fluids

Among the enrolled participants, 122 had detectable CHIKV RNA (80.3%) in serum in at least one specimen (Table 3), and eight (5.3%) had CHIKV RNA detection in more than one serum sample. CHIKV RNA was detected in urine samples only once in 35 (23.0%) of 152 participants (Table 3). CHIKV RNA was detected in urine samples from 30/100 (30.0%) female participants and only 5/52 (9.6%) male participants. Among the 152 enrolled participants, 46 (30.3%) had CHIKV RNA detected in at least one saliva specimen (Table 3), four (2.6%) of whom had positive results more than once. Of the 52 male participants, 42 provided at least one semen sample. Only six participants (14.3%) had detectable CHIKV RNA in semen, of which two had a second detection. All but one female participant provided at least one vaginal secretion specimen for RT-PCR analysis. CHIKV RNA was present in 20 participants (20.2%), with a single detection performed in 19 participants and twice in one participant. Of note, six

Table 2. Signs or Symptoms at Acute Phase of the Disease (1–14 days).

Sign/Symptoms	(n)	Percentage
Fever	151	99.3%
Arthralgia	151	99.3%
Prostration	144	94.7%
Headache	132	86.8%
Rash	129	84.9%
Taste alteration	124	81.6%
Chills	116	76.3%
Pruritis	115	75.7%
Anorexia	114	75.0%
Myalgia	109	71.7%
Backache	100	65.8%
Nausea	95	62.5%
Edema	93	61.2%
Eye pain	81	53.3%
Photophobia	70	46.1%
Sweating	50	32.9%
Diarrhea	47	30.9%
Abdominal Pain	46	30.3%
Lymphadenopathy	36	17.1%
Eye Congestion	30	19.7%
Dyspnea	28	18.4%
Vomiting	26	17.1%
Light Bleeding	26	17.1%
Odynophagy	25	16.4%
Runny nose	23	15.1%
Nasal congestion	21	13.8%
Otalgia	18	11.8%
Cough	16	10.5%
Hoarseness	14	9.2%
Dysuria	9	5.9%

https://doi.org/10.1371/journal.pntd.0010242.t002

participants had their first CHIKV RNA detection at or after the third study visit (≥1 month after symptom onset).

Of the 152 enrolled participants, 114 were included in the persistence analysis. The reasons for exclusion were as follows: only reactive anti-Chikungunya IgM during the whole study

Table 3. Detection of CHIKV RNA in Body Fluids, According to Gender for 152 enrolled participants*.

Body Fluid	Total Patients	Positive Patients	Detection Percentage	Male n (%)	Female n (%)
Serum	152	122	80.3%	45 (86.5)	77 (77.0)
Urine	152	35	23.0%	5 (9.6)	30 (30.0)
Saliva	152	46	30.0%	14 (26.9)	32 (32.0)
Semen	42	6	14.3%	6 (14.3)	Not Applicable
Vaginal Secretions	99	20	20.2%	Not Applicable	20 (20.2)

* Data were derived from a combination of all study visits. The first samples (for all fluids) were collected within the first week after symptom onset. Samples were collected every 15 days for 2 months and at 3 months of follow-up. Each participant collected a maximum of six samples for each body fluid.

https://doi.org/10.1371/journal.pntd.0010242.t003

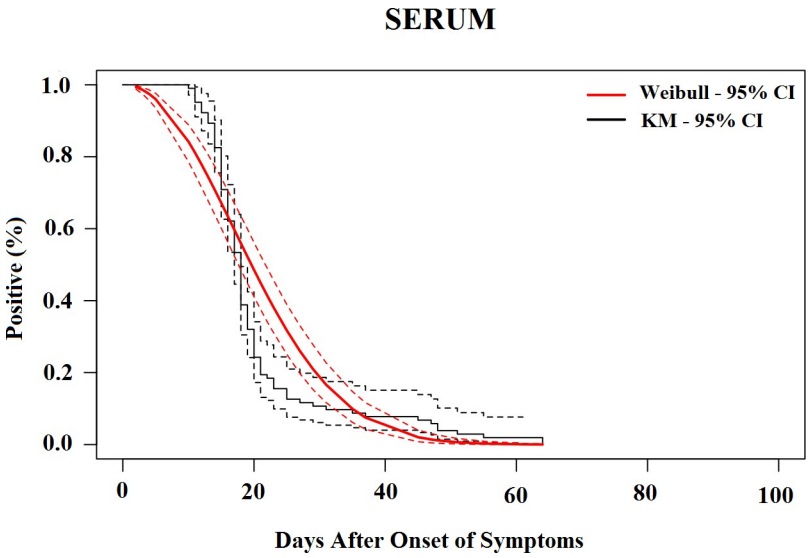

Fig 2. Survival curves (Kaplan–Meier analysis) of CHIKV RNA persistence in Serum, with Weibull fit.

https://doi.org/10.1371/journal.pntd.0010242.g002

(n = 25) without a positive RT-PCR result, the first detection of CHIKV RNA in any bodily fluids occurred during or after the third study visit (n = 6), coinfection with DENV-2 (n = 4), and ZIKV (n = 3). A total of 34 (29.8%) participants were lost to follow-up for unknown reasons, and 80 (70.2%) completed the study for persistence analysis.

The median time for the loss of CHIKV RNA detection was 19.6 days (95% CI, 17.5–21.7) in the serum (Fig 2), 25.3 days (95% CI, 17.8–32.8) in urine (Fig 3), 23.1 days (95% CI, 17.9–28.4) in saliva (Fig 4) and 25.8 days (95% CI, 20.6–31.1) in vaginal secretions (Fig 5). The number of semen samples available was too small for statistical estimation. Nevertheless, the maximum detection of CHIKV RNA was observed 56 days after the onset of symptoms in a study participant.

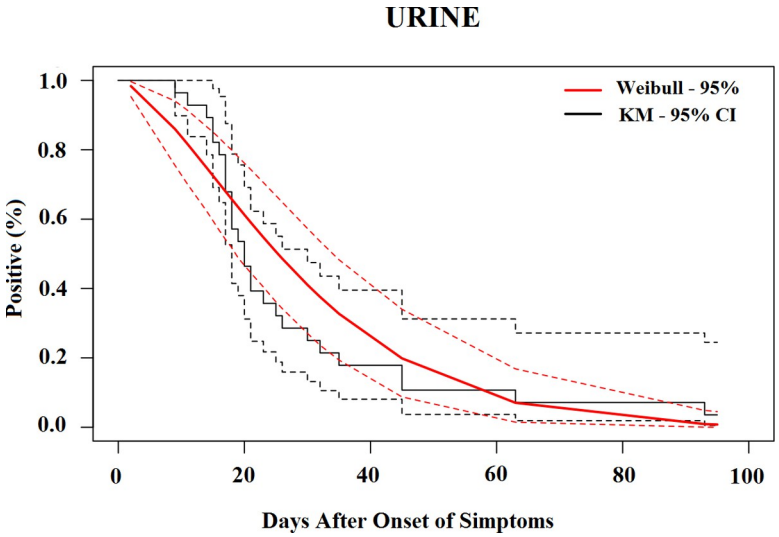

Fig 3. Survival curves (Kaplan–Meier analysis) of CHIKV RNA persistence in Urine, with Weibull fit.

https://doi.org/10.1371/journal.pntd.0010242.g003

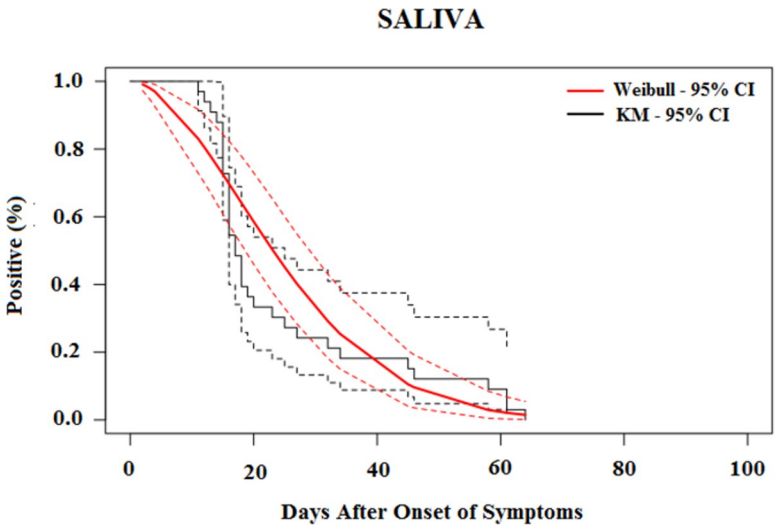

Fig 4. Survival curves (Kaplan–Meier analysis) of CHIKV RNA persistence in Saliva, with Weibull fit.

https://doi.org/10.1371/journal.pntd.0010242.g004

Table 4 shows the percentiles from the Weibull models and their 95% CIs until the loss of CHIKV RNA in selected body fluids. The 95th percentile of time was 39.7 days (95% CI, 35.7 to 43.7) in serum, 68.4 days (95% CI, 49.2 to 87.7) in saliva, 52.9 days (95% CI, 41.8 to 63.9) in urine, and 48.8 days (95% CI, 37.7 to 59.8) in vaginal secretions based on the Weibull model.

Figs 6–9 shows RT-PCR Ct values for CHIKV detection for each fluid studied, allowing the understanding of the strength of the signal in the different body fluids over time. Samples with a Ct < 38 (dashed line) were considered positive. Each circle indicates a positive result. As expected, Ct values were lower for all body fluids mainly in serum samples in the acute phase suggesting higher viral loads.

Real-time reverse transcription PCR cycle threshold values for Chikungunya virus. Samples with Ct <38 (dashed line) were considered to be positive. Each circle indicates a positive result.

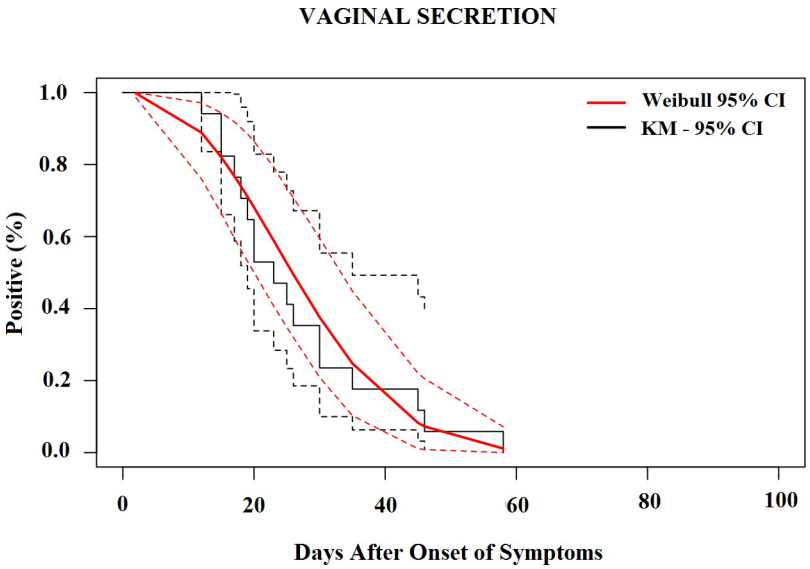

Fig 5. Survival curves (Kaplan–Meier analysis) of CHIKV RNA persistence in Vaginal Secretion, with Weibull fit.

https://doi.org/10.1371/journal.pntd.0010242.g005

Table 4. Percentiles from the Weibull models until loss of CHIKV RNA in body fluids (in days).

Body Fluid	Percentile	0.95 LCL	0.95 UCL
	25th		
Serum	12.83	10.98	14.68
Saliva	13.93	8.37	19.49
Urine	14.09	9.78	18.39
Vaginal Secretions	17.65	13.34	21.96
	Median		
Serum	19.60	17.47	21.73
Saliva	25.31	17.83	32.80
Urine	23.14	17.91	28.37
Vaginal Secretions	25.84	20.61	31.07
	75th		
Serum	27.38	24.79	29.96
Saliva	40.53	30.11	50.96
Urine	34.22	27.58	40.86
Vaginal Secretions	34.90	28.26	41.54
	95th		
Serum	39.69	35.73	43.65
Saliva	68.42	49.18	87.66
Urine	52.87	41.83	63.91
Vaginal Secretions	48.75	37.71	59.78

LCL: Lower Confidence Limit, UCL: Upper Confidence Limit

https://doi.org/10.1371/journal.pntd.0010242.t004

Discussion

This longitudinal study reported CHIKV detection by RT-PCR in several bodily fluids, including genital secretions, during the acute and convalescent phases of the disease (up to 3

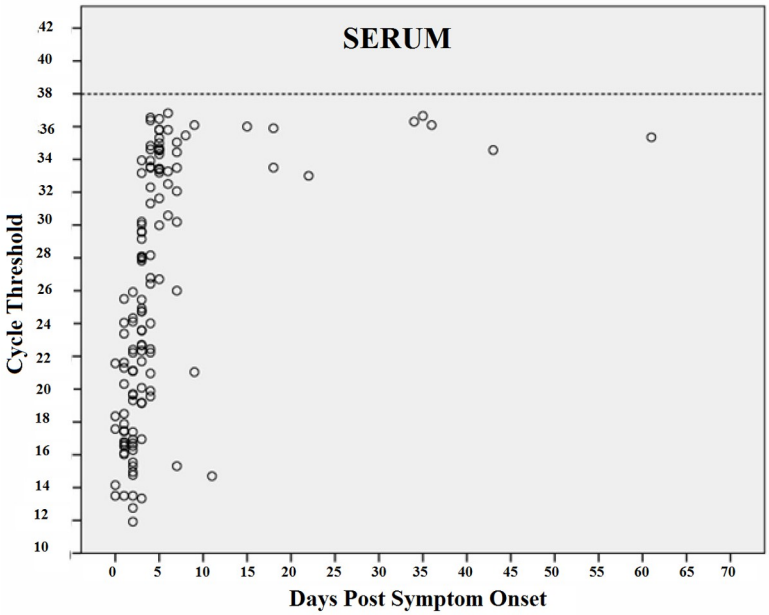

Fig 6. CHIKV Cycle Threshold (Ct) by days after the onset of symptoms in Serum.

https://doi.org/10.1371/journal.pntd.0010242.g006

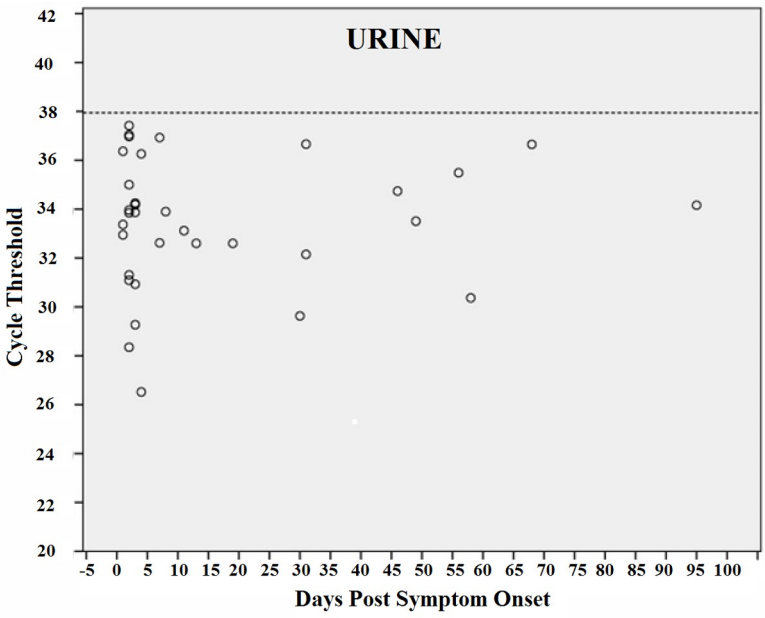

Fig 7. CHIKV Cycle Threshold (Ct) by days after the onset of symptoms in Urine.

https://doi.org/10.1371/journal.pntd.0010242.g007

months). We demonstrated that CHIKV RNA was detected more than 30 days in all the fluids studied. In addition, serum, urine, and saliva had detectable virus levels and persistence for more than 60 days, while urine had them for more than 90 days. To the best of our knowledge,

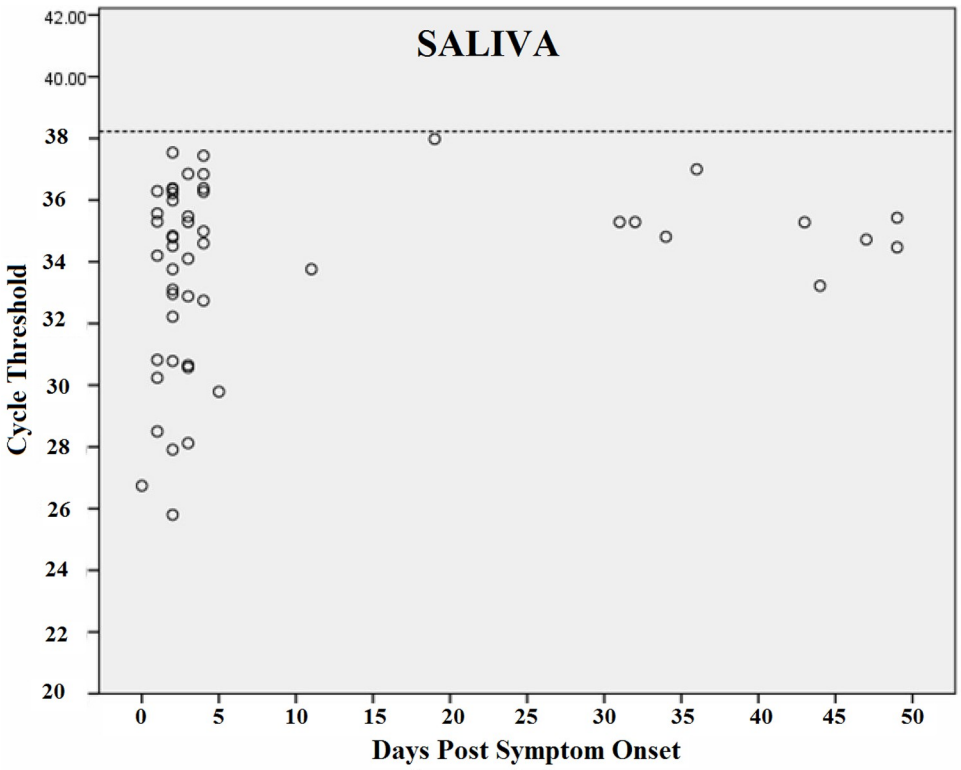

Fig 8. CHIKV Cycle Threshold (Ct) by days after the onset of symptoms in Saliva.

https://doi.org/10.1371/journal.pntd.0010242.g008

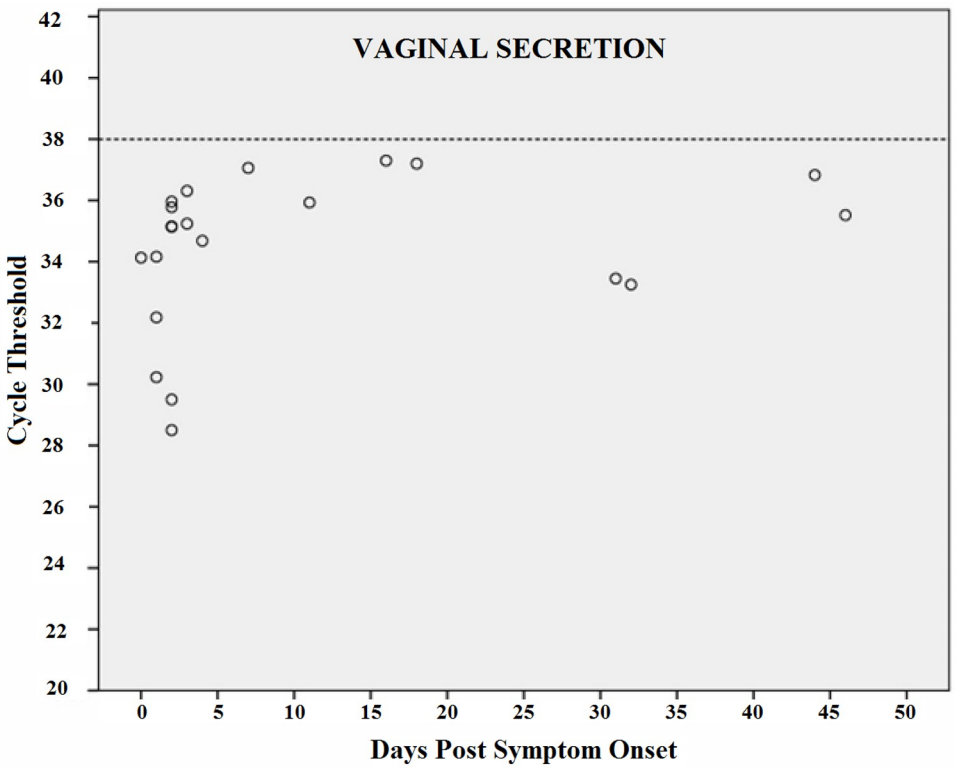

Fig 9. CHIKV Cycle Threshold (Ct) by days after the onset of symptoms in Vaginal Secretion.

https://doi.org/10.1371/journal.pntd.0010242.g009

this was the first cohort study to assess the persistence of CHIKV RNA in genital fluids (vaginal secretions and semen).

Females outnumbered male participants in the diagnosis of chikungunya. Similar results have been described in other studies [28–30]. A combination of fever, arthralgia, and prostration was the most prevalent presentation in our cohort, which is consistent with the results described by Anwar et al. [29].

Since 2014, the presence of co-circulating arboviruses (dengue, zika, and chikungunya) has increased the chance of coinfection. Epidemiological findings from a surveillance study for acute febrile illnesses including 948 participants, showed that 247 (26.1%) had evidence of an acute arboviral infection, of which 224 (23.6%) were single infections and 23 (2.4%) were coinfections [31]. Specifically, 13 (1.4%) patients tested positive for DENV/CHIKV coinfection and nine (0.9%) for CHIKV/flavivirus coinfection [28]. In another study, Dos Santos *et al.* reported five (9.6%) patients with coinfection with DENV-2 among 52 participants diagnosed with chikungunya [32]. Our cohort had similar results where coinfection of ZIKV and CHIKV was reported in three (2.0%) participants and 14 patients (9.2%) had reactive acute-phase anti-DENV IgM. DENV-2 was detected in only four participants (2.6%). We did not observe differences in symptom severity in patients with these coinfections.

We observed that the detection rate of CHIKV RNA was significantly higher in blood, saliva, and urine during the first week of symptom onset, which is consistent with other studies reporting viral presence during the acute phase of the disease [19,20]. In addition, saliva and urine did not increase the detection rate of CHIKV RNA in the acute phase of the disease, and, in concordance with Musso et al., blood was the sample of choice for chikungunya diagnosis [19]. CHIKV RNA persistence in the serum in our study was longer than expected. Most

literature reports showed that CHIKV RNA in serum declines to undetectable levels within 1–2 weeks after symptom onset [33–35].

We also detected CHIKV RNA in urine 95 days after symptom onset. A similar study by Bandeira *et al*. reported the maximum viral persistence in urine after 30 days [20]. Interestingly, in our cohort, CHIKV RNA was detected in 30% of urine samples from female participants and in only 9.6% of male participants. We did not find reports evaluating RT-PCR RNA detection rates in urine samples by sex, but contamination by menstrual blood can be a reasonable explanation, although all the guidelines for urine collection were given to the study participants. Additionally, the collection was not performed during the menstrual period.

To the best of our knowledge, this is the first prospective study to monitor and detect CHIKV RNA in vaginal secretions. We detected CHIK RNA up to 46 days after the acute onset of symptoms in vaginal secretion samples. We did not perform statistical estimates for semen as the number of samples was small, but the maximum detection of CHIKV RNA in semen was 56 days after the onset of symptoms in a study participant.

Although we detected CHIKV in semen and vaginal secretions, it was impossible to assess its potential for sexual transmission as viral isolation was not attempted. In addition, this study did have an appropriate study design to establish sexual transmission due to the endemic nature of the infection, making it difficult to ascertain the actual route of transmission, sexual or vectorial, but additional studies on viral infectivity are warranted. Therefore, it was out of the scope of this study to assess the sexual transmission of CHIKV.

Chikungunya diagnosis in humans is mainly based on RNA detection in serum or plasma samples. However, we have demonstrated that saliva and urine could be considered as potential alternative samples for diagnosis in the acute and convalescent phases of the disease. Diagnostic algorithms using urine or saliva as alternative samples have the advantage of being quick, easy-to-perform, and being less invasive than blood collection. The demonstration of longer persistence of CHIKV in bodily fluids may help diagnosis in later stages of the disease.

This study has some limitations. 1) As the visits and sample collections were scheduled every 15 days, we may have underestimated the exact viral persistence time in the different body fluids; 2) the median duration of CHIKV in semen was evaluated in a small number of patients, because of difficulties in sample collection, mainly due to joint pain in the acute phase of the disease; and 3) the follow-up time was limited to 90 days, making it impossible to assess the maximum persistence of CHIKV in all bodily fluids.

Knowledge of chikungunya viral persistence, infectivity and epidemiology can inform recommendations for control, treatment, and prevention of the disease, and contribute to public health programs.

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4.2 ARTIGO 2– Chikungunya virus shedding in semen: a case series.

viruses

MDPI

Communication

Chikungunya Virus Shedding in Semen: A Case Series

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Abstract: Background: Chikungunya is a viral disease that is transmitted by mosquitoes. It is characterized by an acute onset of fever and severe arthralgia. Methods: We describe six cases of acute and post-acute chikungunya in which viral RNA was detected in semen. Conclusions: The most prolonged detection period was 56 days after illness onset. We attempted to cultivate positive semen samples, but virus isolation was unsuccessful in all cases.

Keywords: chikungunya; semen; detection; genitals; shedding; persistence

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1. Introduction

Chikungunya is a neglected tropical disease caused by chikungunya virus (CHIKV), an RNA arbovirus belonging to the Togaviridae family (genus, *Alphavirus*). CHIKV is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes [1]. Prior to 2013, CHIKV outbreaks were identified in Africa, Asia, Europe, and the Indian and Pacific Oceans. After 2013, the virus spread throughout most Americas, and arrived in Brazil in 2014 [2–4]. Chikungunya is characterized by intense joint pain with an abrupt onset, a high fever, and rash. The post-acute phase can involve recurrent joint pain and debilitating arthritis that may last for months or even years [5].

The detection and prolonged persistence of Zika virus (ZIKV) RNA in semen have been described in some studies [6,7]. In addition, prolonged persistence of Ebola and Marburg viruses in semen has been reported [8]. More recently, yellow fever virus and Chapare virus have been isolated from semen during the convalescent period of infection [9,10]. Although studies involving the isolation of CHIKV in semen samples are scarce, Bandeira et al. [11] reported CHIKV RNA in semen and urine samples 30 days after symptom onset.

The knowledge of viral persistence in genital fluids is imperative to elucidate different forms of infection and possible reactional activity in response to the continuity of the virus in the body, which has relevance for the diagnosis and pathophysiology of viral diseases. Confirmation of the detection and persistence of the virus in semen is useful in health programs, especially for viruses with high rates of mortality or morbidity [12].

The present study aimed to describe the detection and duration of CHIKV RNA detected in semen samples obtained from six symptomatic men.

2. Materials and Methods

From 16 April 2019 to 10 October 2019, six male patients infected by CHIKV, and with the virus detectable in semen samples using real-time reverse transcriptase polymerase chain reaction (rRT-PCR), were followed for 3 months at the Acute Febrile Illnesses outpatient clinic of Oswaldo Cruz Foundation in Rio de Janeiro, Brazil. The patients were enrolled in a cohort study to evaluate the presence and duration of CHIKV infection in

bodily fluid samples obtained from adult patients. The local ethics committee reviewed and approved the study (CAAE:06779019.0.0000.5262). Clinical data and samples (blood, urine, saliva, and semen) were collected at enrollment and every 15 days for 2 months, with a final collection at 3 months, for a total of six visits [13].

The samples were tested for CHIKV, ZIKV, and dengue virus (DENV) using rRT-PCR. RNA was extracted using the QIAmp Viral RNA Mini Kit. The general procedures for rRT-PCR for chikungunya, Zika, and dengue have been described elsewhere [14–16]. The rRT-PCRs mix was prepared using the GoTaq Probe 1-Step RT-qPCR System and was run using the Applied Biosystems 7500 Real-Time PCR System. Cycle threshold (Ct) values <38 and sigmoid curves were considered positive. Additional blood samples were collected to assess each patient's hematological and biochemical parameters at each scheduled visit.

2.1. Testing the Conditions for Virus Isolation

To establish the conditions for viral isolation in cell culture, three semen samples with Ct 32, and one with Ct 34, with sufficient sample volume for testing and subsequent extractions, were initially diluted (1:4) in culture medium 199 without fetal bovine serum (FBS). Then, 100 µL was inoculated in a monolayer of VERO cells (kidney epithelial cells of an African green monkey) and grown in 12.5 cm^2 flasks under four conditions: 1. The diluted inoculum was filtered with 0.22 µm syringe filter and removed from washing with 199 medium without FBS after adsorption; 2. The diluted inoculum was filtered with a 0.22 µm syringe filter and maintained after adsorption; 3. The diluted unfiltered inoculum was removed by washing with 199 medium without FBS after adsorption; 4. The diluted unfiltered inoculum was maintained after adsorption. All inoculums were adsorbed to the cell monolayer for one hour at 37 °C/5% CO_2.

After incubation, 1.5 mL of culture medium 199 containing 2.5% FBS was added. Flasks containing only the cell monolayer without manipulating the medium exchange were used as negative controls during the inoculation process. All flasks were incubated at 37 °C/5% CO_2 for up to 14 days and monitored daily for cytopathic effects using an inverted microscope. Aliquots of 140 µL of supernatants from all flasks were taken at 3, 7, and 14 days of incubation, and were subjected to viral RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen, Inc., Hilden, Germany. https://www.qiagen.com/us/ accessed on 10 July 2020), according to the protocol described by the manufacturer, and stored at −70 °C. RNAs was tested for the detection of CHIKV by rRT-PCR, as described previously [14].

2.2. Virus Isolation

After testing the viral isolation conditions, all semen samples that were previously positive by rRT-PCR were inoculated in cell culture. The eight samples were diluted in culture medium 199 without FBS, six at a ratio of 1:2 (50 µL of semen + 50 µL of culture medium), and two samples at 1:10 (10 µL of semen + 90 µL of culture medium), owing to the scarcity of the original sample. A volume of 100 µL was inoculated into a monolayer of VERO cells grown in 12.5 cm^2 flasks. The inoculums were not filtered and were maintained after adsorption. All inoculums were adsorbed to the cell monolayer for one hour at 37 °C/5% CO_2, and, after incubation, 1.5 mL of culture medium 199 containing 2.5% FBS was added. Flasks were incubated at 37 °C/5% CO_2 for up to 14 days and monitored daily for cytopathic effects using an inverted microscope. Aliquots of 140 µL of supernatant from all flasks were taken at 3, 7, and 14 days of incubation and were subjected to viral RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen, Inc., https://www.qiagen.com/us/ accessed on 10 July 2020), according to the protocol described by the manufacturer, and stored at −70 °C. RNA was tested for the detection of CHIKV by rRT-PCR, as described previously [14].

3. Results

All patients presented with symptomatic disease and moderate clinical manifestations. Their median age was 43.5 years (ranging, 33–56 years). There were no significant changes in the hematological or biochemical parameters at any of the study visits. All patients

had chikungunya confirmed by detectable rRT-PCR in the serum during enrollment or seroconversion of anti-CHIKV-IgM.

Patient 1: A 56-year-old male with hypertension and asthma complained of high fever (>40 °C), myalgia, asthenia, taste alteration, and moderate polyarthralgia for 4 days. He also described episodes of vomiting and nausea for 2 days. He developed a cutaneous rash 5 days after symptom onset. After the acute phase, mild symmetrical arthralgia in the shoulders, knees, and ankles resolved within six weeks without anti-inflammatory drug use.

Patient 2: A 48-year-old male previously healthy, with a 3-day history of fever, chills, headache, severe arthralgia, and prostration. On the seventh day, he developed a diffuse rash on the trunk, abdomen, and limbs. Joint pain was symmetrical (mainly in the shoulders, wrists, knees, and ankles) and lasted for more than six weeks. The patient was treated with prednisone (20 mg/day for 7 days) with a favorable outcome. Coinfection with DENV type 2 was detected.

Patient 3: A 51-year-old male with hypertension presented with a 4-day history of acute fever, malaise, generalized rash, and pain in multiple joints. The patient presented with edema in the hands and knees. The polyarthralgia was severe, lasted throughout the study period (three months), and was localized mainly in the knees. He was administered prednisone (20 mg/day for 14 days) and gabapentin (300 mg/day for 30 days) with partial improvement of his arthralgia.

Patient 4: A 33-year-old previously healthy man developed an abrupt onset of fever, chills, prostration, and moderate polyarthralgia affecting the shoulders, wrists, proximal interphalangeal joints, knees, ankles, and metatarsophalangeal joints. He had a 3-day history of a disseminated maculopapular rash. The fever lasted for 3 days, and mild joint pain lasted 8 weeks, without any pharmacological intervention.

Patient 5: A 39-year-old man with three days of fever, chills, and sweating associated with severe pain in the shoulders, wrists, knees, ankles, and feet. Exanthema presented for 3 days. Polyarthralgia was severe and persisted throughout the follow-up period. Prednisone (20 mg/day for 20 days) was prescribed for partial pain relief.

Patient 6: A 35-year-old man with a 5-day history of moderate fever, headache, prostration, myalgia, joint pain, and exanthema. The polyarthralgia was severe and lasted for more than 8 weeks. The patient received prednisone (20 mg/day for 21 days) with partial clinical relief of his symptoms.

Twenty-three semen samples were collected from these six patients, and eight were CHIKV-rRT-PCR-positive (Table 1). The maximum detection of CHIKV RNA in semen was 5, 18, 18, 56, 28, and 36 days after the onset of symptoms in patients 1 to 6, respectively (Figure 1).

Table 1. Detection of CHIKV RNA in bodily fluids.

	Fluid	Visit 1 Status (Ct)	Visit 2 Status (Ct)	Visit 3 Status (Ct)	Visit 4 Status (Ct)	Visit 5 Status (Ct)	Visit 6 Status (Ct)
Patient 1	**Semen**	**Positive (34.12)**	NC	Negative	Negative	Negative	NC
	Serum	**Positive (22.23)**	Negative	Negative	Negative	Negative	Negative
	Urine	Negative	Negative	Negative	Negative	Negative	Negative
	Saliva	**Positive (32.74)**	Negative	Negative	Negative	Negative	Negative

Table 1. *Cont.*

	Fluid	Visit 1 Status (Ct)	Visit 2 Status (Ct)	Visit 3 Status (Ct)	Visit 4 Status (Ct)	Visit 5 Status (Ct)	Visit 6 Status (Ct)
Patient 2	**Semen**	NC	**Positive (32.29)**	Missed	Negative	Missed	Missed
	Serum	**Positive (13.50)**	Negative	Missed	Negative	Missed	Missed
	Urine	Negative	Negative	Missed	Negative	Missed	Missed
	Saliva	**Positive (26.74)**	Negative	Missed	Negative	Missed	Missed
Patient 3	**Semen**	**Positive (28.06)**	**Positive (34.60)**	Negative	NC	NC	Missed
	Serum	**Positive (25.45)**	Negative	Negative	Negative	Negative	Missed
	Urine	Negative	Negative	Negative	Negative	Negative	Missed
	Saliva	**Positive (32.88)**	Negative	Negative	Negative	Negative	Missed
Patient 4	**Semen**	NC	Negative	Negative	**Positive (32.73)**	Negative	Negative
	Serum	Negative	Negative	Negative	Negative	Negative	Negative
	Urine	Negative	Negative	Negative	**Positive (35.49)**	Negative	Negative
	Saliva	Negative	Negative	Negative	Negative	Negative	Negative
Patient 5	**Semen**	NC	**Positive (34.70)**	**Positive (32.35)**	Negative	Negative	Missed
	Serum	**Positive (27.96)**	Negative	Negative	Negative	Negative	Missed
	Urine	Negative	Negative	Negative	Negative	Negative	Missed
	Saliva	Negative	Negative	Negative	Negative	Negative	Missed
Patient 6	**Semen**	Negative	Negative	**Positive (34.51)**	Negative	Negative	Missed
	Serum	**Positive (33.36)**	Negative	**Positive (36.65)**	Negative	Negative	Missed
	Urine	Negative	Negative	Negative	Negative	Negative	Missed
	Saliva	Negative	Negative	Negative	Negative	Negative	Missed

Ct: Cycle Threshold; NC: Not Collected; Missed: missed visit

Virus Isolation

From the initial experiment to test the best conditions for viral inoculation, it was possible to recognize that there was no need for the filtering step of the semen samples to minimize possible bacterial contamination, since the cell cultures did not present typical turbidity, maintaining the transparent and translucent supernatant throughout the incubation period. After 3 days of inoculation, one of the four samples showed the beginning of morphological changes in the cell monolayer, resembling the expected cytopathic effect, progressing gradually over the next few days, with cell detachment after 7 days of inoculation. Such evolution initially occurred in cultures with diluted inoculums with and without filtration, and was maintained after adsorption. The same was observed later in the flasks that received the diluted inoculum of the same sample, with and without filtration, which was washed after adsorption. However, the rRT-PCR results of the supernatants from all samples collected at three points after incubation were negative. rRT-PCR of the original sample corresponding to the inoculations that would seem to progress in cytopathic effect was performed, but the result was negative.

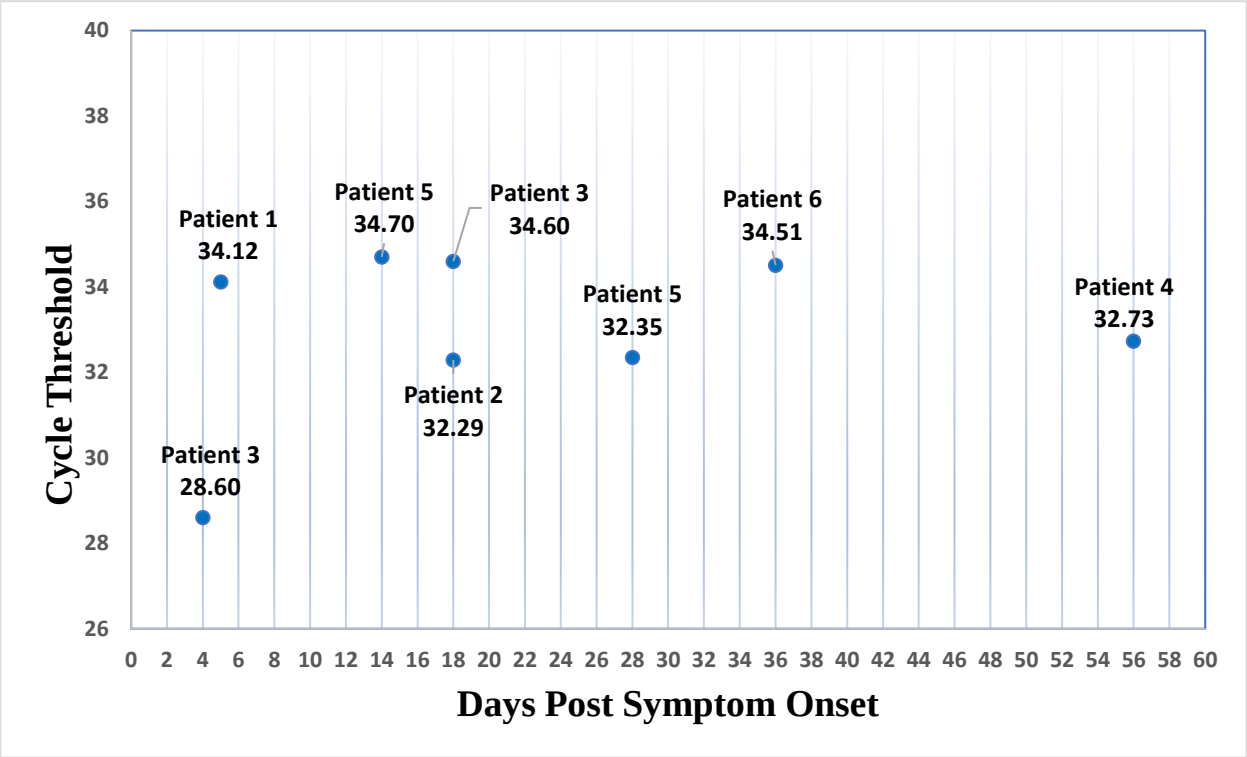

Figure 1. CHIKV cycle threshold by days after the onset of symptoms in semen.

A new viral inoculation was carried out with eight semen samples available under the conditions described in the methodology section. Three days after inoculation, two flasks showed complete detachment of the cell monolayer, one of which was inoculated with the sample diluted 1:10. Other cell monolayers began to show morphological changes, with cell detachment beginning 7 days after inoculation, and total detachment after 14 days. rRT-PCR of supernatants collected throughout the incubation period showed amplification with Ct 35 at the three collection points from a single sample initially diagnosed with Ct 28, which was considered as negative isolation, since the highest Ct maintained throughout the incubation period possibly refers to the inoculum remnant. All supernatants from the other samples tested negative.

4. Discussion

In this case series, CHIKV could be detected in semen samples from patients with either acute or convalescent disease. The longest CHIKV RNA detection period was 56 days after symptom onset in a 33-year-old, previously healthy man. All virus isolation attempts were unsuccessful for all samples.

Efforts to demonstrate virus detection in genital secretions of individuals infected with viruses that had previously been unknown to be sexually transmitted are necessary to investigate additional forms of transmission that could be of public health importance. Sexual transmission of Ebola virus was confirmed in Liberia in 2015 [17]. Sexual transmission of ZIKV has been reported for symptomatic male partners [18], and some studies have documented the prolonged detection of ZIKV RNA in semen [6,19]. Lalle et al. [20] described the presence of DENV RNA in semen up to 37 days post-symptom onset, when viremia and viruria were undetectable [20].

Bandeira et al. [11] described CHIKV RNA in semen and urine samples 30 days after symptom onset, and argued that prednisolone may have contributed to prolonged viral shedding. In our study, four patients were treated with prednisone; however, in this series, the patient with the highest viral detection in the semen (56 days) had not taken anti-inflammatory drugs.

Although CHIKV RNA was detected in the semen in our case series, the virus was not detected by rRT-PCR in the cultured samples. One explanation is that the cycle threshold values found in our samples were high, ranging from 28 to 34, which may correspond to a low viral load in semen. Viral cultures perform better with low Ct values in inoculated samples [18]. For example, low Ct values (20 to 28), viable infectious viruses, and prolonged shedding in semen have been reported in ZIKV studies and are related to sexual transmission [21,22]. Difficulty in viral isolation from the semen of patients, mainly with long-term viral shedding, has also been reported in studies of patients with ZIKV [23].

Factors intrinsic to the nature of the biological sample can influence the degradation of nucleic acid viruses by the presence of nucleases and the integrity of the viral particle, which can suppress viral infection and produce cytotoxic substances for cell culture [24,25]. In addition, storage of fresh samples without preservatives, as well as freezing and thawing processes, can be unfavorable for viral conservation, as demonstrated by the loss of detection of the viral genome in the original samples after re-extraction and repetition of rRT-PCR [26,27].

Additional limitations of our study were the small sample size and the short follow-up period. Infectivity is a prerequisite for pathogen transmission, and depends on factors such as the infectious dose and exposure route. Therefore, virus isolation remains the only direct and definitive approach for proving infectivity [28]. Twenty-seven different viruses have shown varying persistence in human semen [12]. The presence of viruses in semen may be more common than is currently understood, and viruses known as "non-sexually transmitted" should not be considered to be absent from genital secretions. All followed-up patients were informed about the possibility of being a carrier of CHIKV for a longer period, although we did not have confirmation of the risk of infectivity.

Studies on viral detection and semen persistence benefit clinical practice and public health, especially for viruses that can cause high chronic morbidity, such as CHIKV. Further studies are needed to evaluate the potential infectivity of semen and the sexual transmission of chikungunya.

Author Contributions: Conceptualization, E.B.M., G.A.C., F.d.B.-N. and A.M.B.F.; patient enrollment, E.B.M., G.A.C., M.F.B.S., I.C.V.M. and A.P.-C.; laboratory tests, F.d.B.-N., C.D.S.R., C.C.S., S.A.S., A.A.F., V.G.-C., M.A.M.M.-G., N.R.C.F., A.S.S. and M.A.S.P.; formal analysis, E.B.M., G.A.C. and F.d.B.-N.; writing—original draft preparation, E.B.M., G.A.C., P.B., F.d.B.-N. and A.M.B.F.; writing—review and editing, E.B.M., G.A.C., P.B. and A.M.B.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was reviewed and approved by Oswaldo Cruz Foundation Ethics Committee (CAAE: 06779019.0.0000.5262).

Informed Consent Statement: Informed consent was obtained from all the subjects involved in the study.

Data Availability Statement: The data presented in this study are not publicly available but are available on request from the corresponding author.

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4.3 ARTIGO 3 – Predictors of chronic joint pain after Chikungunya virus infection in the INOVACHIK prospective cohort study.

Title: **Predictors of chronic joint pain after Chikungunya virus infection in the INOVACHIK prospective cohort study**

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ABSTRACT

Background: Chikungunya can cause persistent chronic joint pain. Knowledge of risk factors for disease progression is important to preventing and controlling complications. This study aimed to identify the factors associated with chronic joint pain.

Methods: A prospective cohort study was established in a reference center, Rio de Janeiro. Men and women (≥ 18 years) in the acute phase of chikungunya were included. Clinical data and samples were collected during three months. Risk factors were evaluated by multivariate analysis and they were analyzed using logistic regression models.

Results: 107 patients were followed up. The incidence of joint tenderness was 61.7%.Diarrhea (adjusted odds ratio [AOR] 5.94, 95% confidence interval [CI]: 1.67–21.13), severe joint pain in the wrists (AOR 15.22, 95% CI:2.35–98.65), and CHIKV real-time reverse transcription polymerase chain reaction positivity up to 5 days post onset of symptoms in urine (AOR 5.87, 95% CI: 1.01–34.10) and saliva (AOR 4.02, 95% CI:1.10–14.63) as predictors of persistent chronic pain.

Conclusions: Musculoskeletal symptoms are not the only determinants of chronic pain and careful evaluation of CHIKV detection in alternative body fluids (saliva and urine) during the early phase of the disease is warranted.

Keywords: Chikungunya fever; arthralgia; chronic pain; risk factors; cohort study.

INTRODUCTION

Chikungunya (CHIK) is transmitted to humans through the bite of insects of the genus *Aedes* spp. Chikungunya virus (CHIKV) has caused millions of human infections worldwide [1,2].

Clinically, the disease has three main phases: acute (lasting 7–14 days), subacute (lasting up to 3 months), and chronic (more than 3months) [4,5]. The acute phase features high fever, arthralgia, myalgia, prostration, headache, and rash [6,7]. In the subacute phase, patients exhibit only transitory improvements in their clinical condition with frequent relapses. Persistent polyarthralgia has been reported [3,7]. The chronic phase may last for a few months or years, with arthralgia assuming an intermittent or constant characteristic [3]. Some studies suggest that the persistence of joint pain occurs more frequently in females, in people over 40 years of age, and in those with preexisting comorbidities [7,8].

Some studies have described prolonged detection of CHIKV RNA in bodily fluids. In French Polynesia, CHIKV was detected in saliva and urine samples in the acute phase of the disease [9]. Bandeira et al. reported CHIKV in semen and urine samples 30 days after symptom onset [10]. Martins et al. demonstrated that CHIKV was detected in serum, urine, and saliva for more than 60 days, especially in urine with RNA detection for more than 90 days [6].

Chronic CHIK causes personal and economic distress [11]. Therefore, the identification of predictors to chronic diseases would be helpful in therapeutic patient management. This study aimed to identify the factors associated with chronic joint pain.

METHODS

Data collection and patient recruitment

A prospective cohort study was conducted in Rio de Janeiro, Brazil. The Institutional Review Board reviewed and approved the study protocol (CAAE:06779019.0.0000.5262). Informed consent was obtained for all participants. Patients were screened from a general febrile illness outpatient clinic.

Men and women aged ≥18 years who had developed acute fever or arthralgia were enrolled. Detailed procedures of the INOVACHIK cohort study have been previously presented[6]. Follow-up visits occurred for 3 months, including every15 days for 2 months, with a final 3-month visit.

Serum, urine, and saliva specimens were collected at all study visits. The samples were tested for CHIKV using real-time reverse transcriptase polymerase chain reaction (rRT-PCR). The general procedures for rRT-PCR of Chikungunya have been described elsewhere [12]. Cycle threshold (Ct) values < 38 and sigmoid curves were considered positive. In addition, the serum was tested for anti-CHIKV-IgM.

Additional blood samples were collected to assess hematological and biochemical parameters.

Definitions

Assessment of disease activity by the physician or by the patient was determined in all clinical visits, considering tenderness, pain, and swelling of the joints. A numerical rating scale was used, ranging from 0 (“no pain”) to 10 (“pain as bad as it could be”). The absolute values of pain were grouped into three categories:1–4 (mild pain), 5 or 6 (moderate pain), and 7–10 (severe pain). On 3-month visit, the patients were assigned to two groups, "patients without joint tenderness" and "patients with joint

tenderness," based on the assessment of disease activity by the physician, which included evaluation of the pain, irrespective of the severity.

For the statistical analysis, we considered the presence of any joint pain in the right or left side of the body as "yes," if at least one joint in the body side was painful. In addition, metacarpophalangeal (MCP) and proximal and distal interphalangeal joints were grouped as "hands". A confirmed case of Chikungunya was defined as either detected rRT-PCR or reactive IgM for CHIKV.

Statistical analysis

The sociodemographic, clinical, and laboratory variables were described using frequencies and proportions for categorical variables and medians and ranges or interquartile ranges (IQRs) for continuous variables [13]. The chi-square test or Fisher's exact test was used to test the association between categorical variables. Univariate and multivariate logistic regression analyses were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between joint tenderness at the 3months visit and the variables under consideration, and collinearity was assessed using correlation matrices or association tests. Considering a statistical significance level of 10% for variable selection, the variables that showed significance in the univariate analysis and those that were clinically important (age, sex, and presence of arthrosis) were included in the multivariate analysis. Backward elimination (at the same level of significance) was performed for these variables. Only the variables that were statistically significant at the 5% level were included in the final model. The importance of each variable in its contribution to the final model was verified using Wald and likelihood ratio tests. The model fit was evaluated using a goodness-of-fit test.

Statistical analyses were conducted using Statistical Package for the Social Sciences Statistics 22.0.

RESULTS

A total of 170 participants were screened from April to December 2019. Of these, 152 were enrolled in the study. Eighteen patients were excluded because of the diagnosis of Chikungunya was not confirmed. Among patients with confirmed CHIKV infection, 107 (70.4%) completed the follow-up. The incidence of joint tenderness 3 months post onset of symptoms was 61.7% (66/107), as shown in Figure 1.

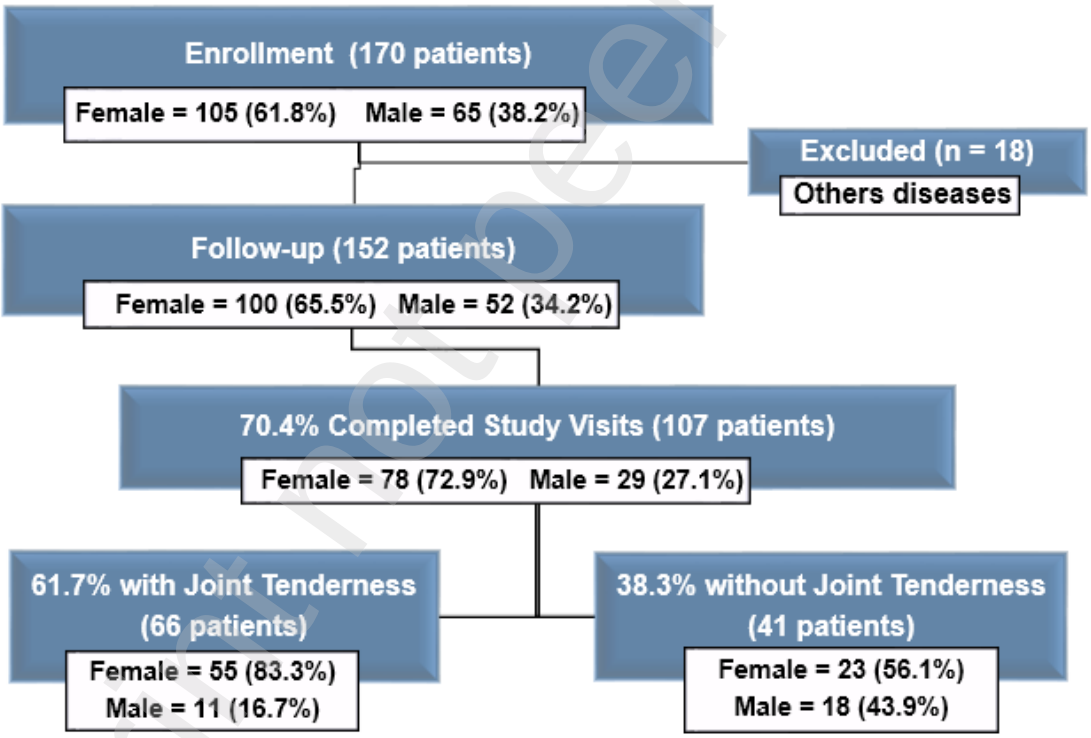

Figure 1: Flow diagram of the INOVACHIK cohort study.

Among the 107 patients, 78 (72.9%) were female. Participants over 45 years of age represented more than half of the study population, and the median age was 45.3 (IQR: 33.2–54.8) years. Hypertension and allergic rhinitis were the most frequently

reported comorbidities in this study. In the acute phase of the disease, besides fever and arthralgia, the inclusion criteria were prostration, headache, exanthem, taste alteration, itching, anorexia, myalgia, nausea/vomiting, and retro-orbital pain, reported by more than 50% of the participants. Table 1 shows the sociodemographic and clinical characteristics of the participants.

Table 1 here

In the acute phase of the disease, CRP levels were elevated in almost all the patients (98/107; 91.6%). Leukopenia was present in 45 patients (42.1%), while anemia and thrombocytopenia were noted in 12 (11.2%) and 13 (12.1%), respectively. Liver function tests for aspartate aminotransferase and alanine aminotransferase were abnormal in 37.4% and 21.5%, respectively. rRT-PCR results for CHIKV were detected within the first 5 days of illness in serum (69.2%), saliva (28.0%), and urine (15.0%). The median Ct for serum, urine, and saliva was23.0 (IQR:17.8–29.9), 33.9 (IQR:31.7–35.9), and34.8 (IQR:32.9–36.2), respectively. Table 2 shows the main abnormal laboratory findings during the acute phase of the disease and the rRT-PCR results for the serum, saliva, and urine.

Table 2 here

Among 107 patients, 92.1% had symmetrical joint involvement. Joint tenderness was frequent in the ankles (87/107, 81%), MCP joints (83/107, 77.6%), knees (81/107, 75.7%), wrists (79/107, 73.8%), shoulders (77/107, 72.0%), and MTP joints (70/107, 65.4%). Associated articular edema was less observed during the acute phase, mainly in the ankles (53/107, 49.5%), knees (33/107, 30.8%), MCP joints (31/107,29.0%), and MTP joints (30/107,28.0%). The median score for the assessment of disease activity by the physician was 7 (IQR:5–8), with severe intensity observed in 62 participants

(57.9%).Stiffness occurred in the morning in 84 patients (78.5%). Table 3 shows the main characteristics of osteoarticular involvement in the study cohort during the acute phase.

Table 3 here

Variables associated with chronic joint pain were examined by univariate analysis; women were at a higher risk of joint tenderness than men (OR 3.91, 95% CI:1.60–9.57).The risk also increased with age (OR 1.05 for each 1-year increase, 95% CI:1.02–1.03), previous diagnosis of osteoarthrosis (OR 9.81,95% CI:1.23–78.14), reporting of itching (OR 4.18, 95% CI:1.58–11.09), and diarrhea (OR 2.86,95% CI:1.14–7.13). Among the laboratory results, CHIKV rRT-PCR positivity in urine (OR 6.79,95% CI: 1.43–32.24) and saliva (OR 2.86, 95% CI:1.08–7.60)up to 5 days post onset of symptoms was statistically associated with chronic joint tenderness. Significant differences were also observed for reported morning stiffness of more than 30 minutes (OR 2.60,95% CI:1.01–6.66), intensity of joint tenderness during the physical examination in wrists (moderate pain: OR 2.67[95% CI:1.01–7.05] and severe pain: OR 5.78 [95% CI:1.34–24.92]), severe pain in the knees(OR 5.45,95% CI:1.24–24.09) and ankles (mild pain: OR 3.63 [95% CI:1.02–12.94], moderate pain: OR 4.67 [95% CI:1.43–15.20], and severe pain: OR 8.56 [95% CI:2.30–31.87]), and severe pain in the MTP joints (OR 4.31,95% CI: 1.42–13.09). Peri-articular edema observed during the physical examination was also significant for the ankles (OR 2.35,95% CI:1.06–5.24) and MTP joints (OR 2.60,95% CI:1.00–6.77) (**Supplemental Table**).

Diarrhea (adjusted OR [AOR]5.94, 95% CI:1.67–1.13), CHIKV rRT-PCR positivity in urine up to 5days post onset of symptoms (AOR 5.87, 95% CI:1.01–34.10), CHIKV rRT-PCR positivity in saliva up to 5 days post onset of symptoms (AOR 4.02,

95% CI:1.10–14.63), and severe joint pain in the wrists (AOR 15.22, 95% CI:2.35–98.65) were the factors associated with chronic joint pain in the multivariate analysis (Table 4).

Table 4 here

DISCUSSION

In this well-established cohort of confirmed patients with CHIKV, we identified predictors of chronic pain among several factors present in the acute phase of the disease. Our analysis found that diarrhea, detection of CHIKV rRT-PCR in urine and saliva up to 5 days after illness onset, and severe pain in the wrist were risk factors for the development of chronic pain.

Our study demonstrated that the incidence of chronic joint tenderness was high 3 months after disease onset, with 61.7% of the participants having varying intensities of arthralgia. This incidence was similar to that in some cohort studies [14,15], in contrast with other studies showing that the percentage of patients with chronic joint pain after CHIKV infection was lower and varied from 40.2 to 45.2% [16-18]. One explanation is thatin those studies, arthralgia was evaluated through telephone interviews [16-18], with a high risk of memory bias. In our cohort, pain assessment was performed prospectively by medical consultation with clinical examination during all visits. This may have contributed to a more reliable assessment of the pain. Although pain is considered a subjective condition, which may reflect a difference in the pain threshold in the patient's report or the physician's reference, in our study, the assessment of disease activity, including joint pain, was similar between participants and the physicians.

Most (83.3%) of the patients who developed chronic joint pain in our cohort were women, who presented a higher risk of developing chronic pain after CHIKV

198 infection than men, according to the world literature [14-16,18-23]. Whether women are
199 more susceptible to chronic inflammatory processes due to the production of pro-
200 inflammatory cytokines by monocytes during the menstrual cycle and ovulation [24,25]
201 or whether estradiol increases the production of antibodies, protecting younger women
202 [26,27] is unknown. The risk also increased with age in univariate analysis, possibly
203 because of a more compromised immune system over the years, as suggested by some
204 authors [18,23].

205 The presence of previous joint diseases, such as osteoarthritis, is strongly
206 associated with chronic progression [28]. However, recent studies have not established a
207 significant association between arthrosis and chronic post-Chikungunya pain [14,19]. In
208 our study, 14 patients reported a history of osteoarthrosis, and all but one had persistent
209 joint pain after 3 months of illness onset.

210 In our analysis, diarrhea was a predictor of the persistence of joint tenderness,
211 and patients with diarrhea were more likely to become dehydrated. According to
212 Bertolotti et al., the aggravating role of dehydration during the acute phase of CHIKV
213 infection isan acute phase factor significantly associated with chronicity [19].
214 Dehydration can also result in varying degrees of cartilage damage, which can
215 predispose patients to joint disease for an extended period [29,30].

216 Our study showed that detecting CHIKV rRT-PCR in urine and saliva samples
217 up to the fifth day of illness was significantly associated with the persistence of joint
218 pain, possibly due to the presence of a high viral load during the early acute phase of the
219 disease in these fluids, thus enabling the possibility of detecting the virus, in addition to
220 the blood. A cohort study on Reunion Island suggested that a higher viral load in the
221 blood in the acute phase of the illness is associated with chronic arthralgia lasting 12
222 months [31]. In our study, the mean Ct values, considered a proxy for viral load, were

higher in the urine and saliva than in the blood. rRT-PCR in the blood up to 5 days after illness onset was not associated with chronic joint pain in our cohort. Consequently, this hypothesis of a high viral load in fluids in the acute phase of the disease and the persistence of pain after 3 months of illness does not apply to our study.

In a recently published cohort study, joint pain, edema, and multiple articular involvements, including those of the hands and feet, were identified as risk factors for chronicity beyond 3 months of illness onset [32]. Morales et al. also used a scoring system that included the presence of edema, among other variables (SHERA - Sex, Hypertension, Edema, Retroocular Pain, Age), to select patients with a high risk of developing chronic arthralgia in the acute CHIKV infection [14]. In our study, although the presence of articular edema in some joints was associated with persistent pain in the univariate analysis of our cohort, these findings were not retained in the final multivariate model.

Many studies have shown that the highest prevalence and intensity of pain occurs in the distal joints of the limbs [12, 17-19,23]. In addition, severe wrist pain during acute illness was highly associated with the risk of persistent arthralgia in our study, suggesting that inflammation during this period increases the likelihood of persistent symptoms.

This study has some limitations. First, all patients were from the outpatient clinic; thus, the results may not be generalizable to more severely ill patients who required hospitalization or remained in the emergency units. Second, despite the study's adequate sample size, the rate of loss to follow-up was relatively high (29.6%), which may have affected the statistical analysis, especially in the group without chronic joint tenderness because we assumed that people without persistent pain were more likely to drop out of the study than the those in the symptomatic group. Finally, the time of

follow-up was limited to 3 months when the results of Chikungunya serology (IgM) could not be distinguished between those who had a chronic evolution and those who did not.

Chronic arthralgia limits daily and professional activities, causing psychological disturbances that affect the family economy. In this study, we found significant risk factors associated with the persistence of chronic pain after 3 months of CHIKV infection. Hydration procedures are encouraged with more intensity for patients with diarrhea in the acute phase of the disease. Furthermore, adequate treatment of the inflammatory process during the acute phase could reduce the incidence of chronic CHIKV disease. In addition, we found that musculoskeletal symptoms were not the only determinants of chronic pain in Chikungunya disease, and careful evaluation of Chikungunya virus detection in alternative body fluids (saliva and urine) in the early phase of the disease is warranted.

Knowledge of these factors can help develop new studies with earlier therapeutic interventions for people at a greater risk of developing chronic pain. Patients at a high risk of chronicity should be closely monitored.

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AUTHORS' CONTRIBUTION: EBM and GAC - Conceived and designed the experiments; EBM, MFBS, ICVM, APC and GAC – data collection; EBM, MSBQ and GAC - data analysis; FBN, CDSR, CCS, SAS, AAF, VCG, NRCF and AMBF – performed molecular and serological experiments; EBM and GAC - wrote the paper; GAC, AMBF and PB critically reviewed the manuscript; All authors read and approved the final manuscript.

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Table 1: Baseline characteristics and clinical symptoms of participants with confirmed Chikungunya infection (N = 107), Rio de Janeiro, April 2019– January, 2020.

Characteristics	Subcategory	n	%
Sex	Male	29	27
	Female	78	73
Age	≤ 45 years	52	49
	> 45 years	55	51
Race	White	52	49
	Non-White	55	51
Highest educational attainment	University or higher	47	44
	Upper secondary school or lower	60	56
Marital Status	Married or living with a partner	48	45
	Single, separated, divorced, or widowed	59	55
Main Comorbidity	Hypertension	25	23
	Allergic rhinitis	23	22
	Osteoarthrosis	14	13
	Hypothyroidism	11	10
	Diabetes Mellitus	9	8.4
	Asthma	8	7.5
	HIV Infection	5	4
Body Mass Index (BMI)	Underweight/Normal	23	22
	Overweight/obesity	84	79
Smoking	Current	8	7.5
Alcohol consumption	Yes	42	39

Main symptoms (Acute phase)	Fever	107	100
	Arthralgia	107	100
	Prostration	102	95
	Headache	93	87
	Exanthem	88	82
	Taste alteration	87	81
	Itching	84	79
	Anorexia	81	76
	Myalgia	78	73
	Nausea/vomiting	70	65
	Retro-orbital pain	55	51
	Photophobia	49	46
	Diarrhea	35	33
	Abdominal pain	32	30
	Lymphonode enlargement	27	25
	Nonpurulent conjunctivitis	19	18
	Dyspnea	18	17
	Oropharyngeal pain	18	17
	Nasal congestion	17	16
	Coryza	16	15
	Earache	15	14
	Cough	11	10

***IQR: Interquartile range**

Table 2. Acute laboratory results of participants with confirmed Chikungunya infection (N = 107), Rio de Janeiro, April 2019– January, 2020

Laboratory abnormalities	n	%
Elevated C-reactive protein	98	91,6
Elevated gamma-glutamyltransferase	59	55.1
Elevated erythrocyte sedimentation rate	57	53.3
Elevated lactate dehydrogenase	55	51.4
Leukopenia	45	42.1
Elevated aspartate aminotransferase	40	37.4
Lymphopenia, absolute	27	25.2
Elevated alanine aminotransferase	23	21.5
Thrombocytopenia	13	12.1
Anemia	12	11.2
Elevated creatinine	12	11.2
Elevated urea	10	9.3
Elevated alkaline phosphatase	10	9.3
Decreased albumin	10	9.3
CHIKV IgM (at 3 months follow-up)		
Positive	94	87.9
Negative	13	12.1
CHIKV rRT-PCR (≤ 5 days post onset of symptoms)		
Serum		
Positive	74	69.2
Missing	20	18.7
Urine		
Positive	16	15
Missing	24	22.4
Saliva		
Positive	30	28
Missing	26	24.3

Table 3: Assessment of disease activity, joint tenderness, and joint swelling of participants with confirmed Chikungunya infection (N = 107), Rio de Janeiro, April 2019– January, 2020

Characteristics	Subcategory	n	%
Assessment of disease activity by the physician, numerical rating scale*	Mild (1-4 points)	23	21.5
	Moderate (5-6 points)	22	20.6
	Severe (7-10 points)	62	57.9
Morning joint stiffness		84	78.5
Joint tenderness	Ankles	87	81.3
	Metacarpophalangeal joints	83	77.6
	Knees	81	75.7
	Wrists	79	73.8
	Shoulders	77	72.0
	Metatarsophalangeal joints	70	65.4
	Elbows	57	53.3
	Proximal interphalangeal joints	53	49.5
	Hips	44	41.1
Joint swelling	Ankles	53	49.5
	Knees	33	30.8
	Metacarpophalangeal joints	31	29.0
	Metatarsophalangeal joints	30	28.0
	Wrists	24	22.4
	Proximal interphalangeal joints	13	12.1
	Shoulders	7	6.5
	Elbows	7	6.5
	Hips	3	2.8

* Assessment of disease activity by the physician or by the patient: Considering the tenderness, pain, and swelling of joints, how active was the disease on the medical consultation day

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Table 4. Factor associated with joint tenderness over three months post onset of symptoms (univariate and multivariate logistic regression analysis among participants with confirmed CHIKV infection (n=107), Rio de Janeiro, April 2019– January, 2020

Factors		Patients without joint tenderness		Patients with joint tenderness								
Variable	**Subcategory**	**n**	**%**	**n**	**%**	**Crude OR**	**95% CI**		**p-value**	**Ajdusted OR****	**95% CI**		**p-value**
Sex													
	Male	18	43.9	11	16.7	1.00				1.00			
	Female	23	56.1	55	83.3	3.91	1.60	9.57	.003	3.17	0.94	10.67	.063
Age (per year increase)*													
Median (IQR)		38.0 (27.3-50.5)				46.5 (38.6-58.1)	1.05	1.02	1.08	.003	1.03	0.99	1.08	.088
Race													
Osteoarthrosis													
	No	40	97.6	53	80.3	1.00				1.00			
	Yes	1	2.4	13	19.7	9.81	1.23	78.14	.031	5.76	0.60	55.17	.129
Allergicrhinitis													
	No	36	87.8	48	72.7	1.00							
	Yes	5	12.2	18	27.3	2.70	0.92	7.96	.072	-	-	-	-
Itching													
	No	15	36.6	8	12.1	1.00							
	Yes	26	63.4	58	87.9	4.18	1.58	11.09	.004	-	-	-	-
Diarrhea													
	No	33	80.5	39	59.1	1.00				1.00			
	Yes	8	19.5	27	40.9	2.86	1.14	7.13	.025	5.94	1.67	21.13	.006
Lymphopenia, absolute													
	No	27	65,9	53	80,3	1,00							
	Yes	14	34,1	13	19,7	0,47	0,20	1,15	.098	-	-	-	-
CHIKV RT-PCR positive in urine (≤ 5 days post onset of symptoms)													

	No	33	80.5	34	51.5	1.00				1.00		
	Yes	2	4.9	14	21.2	6.79	1.43	32.24	.016	5.87	1.01	34.10	.049
	Without information	6	14,6	18	27.3	2.91	1.03	8.24	.044	1.54	0.22	10.76	.661
CHIKV RT-PCR positive in saliva (≤ 5 days post onset of symptoms)													
	No	26	63.4	25	37.9	1.00				1,00			
	Yes	8	19.5	22	33.3	2.86	1.08	7.60	.035	4.02	1.10	14.63	.035
	withoutinformation	7	17.1	19	28.8	2.82	1.01	7.87	.047	2.20	0.29	16.43	.443
Morning joint stiffness													
	No	13	31.7	10	15.2	1.00							
	Yes	28	68.3	56	84.8	2.60	1.01	6.66	.047	-	-	-	-
Jointtendernessintensity													
Wrists													
	None	16	39	12	18.2	1.00				1.00			
	Mild	7	17.1	11	16.7	2.10	0.63	7.01	.230	3.37	0.67	16.87	.139
	Moderate	15	36.6	30	45.5	2.67	1.01	7.05	.048	3.73	0.94	14.76	.061
	Severe	3	7.3	13	19.7	5.78	1.34	24.92	.019	1.,22	2.35	98.65	.004
Hands													
	None	10	24.4	10	15.2	1.00							
	Mild	14	34.1	14	21.2	1.00	0.32	3.15	1.000	-	-	-	-
	Moderate	14	34.1	29	43.9	2.07	0.70	6.12	.188	-	-	-	-
	Severe	3	7.3	13	19.7	4.33	0.94	20.03	.061	-	-	-	-
Knees													
	None	15	36.6	11	16.7	1.00							
	Mild	9	22	18	27.3	2.73	0.89	8.33	.078	-	-	-	-
	Moderate	14	34.1	25	37.9	2.44	0.88	6.73	.086	-	-	-	-
	Severe	3	7.3	12	18.2	5.45	1.24	24.09	.025	-	-	-	-
Ankles													
	None	14	34.1	6	9.1	1.00							

	Mild	9	22	14	21.2	3.63	1.02	12.94	.047	-	-	-	-
	Moderate	12	29.3	24	36.4	4.67	1.43	15.20	.011	-	-	-	-
	Severe	6	14.6	22	33.3	8.56	2.30	31.87	.001	-	-	-	-
Metatarsophalangealjoints													
	None	20	48.8	17	25.8	1.00							
	Mild	4	9.8	8	12.1	2.35	0.60	9.20	.219	-	-	-	-
	Moderate	11	26.8	19	28.8	2.03	0.76	5.44	.158	-	-	-	-
	Severe	6	14.6	22	33.3	4.31	1.42	13.09	.010	-	-	-	-
Joint swelling													
Wrists													
	No	36	87.8	47	71.2	1.00							
	Yes	5	12.2	19	28.8	2.91	0.99	8.54	.052	-	-	-	-
Hands													
	No	33	80.5	43	65.2	1.00							
	Yes	8	19.5	23	34.8	2.21	0.88	5.56	.093	-	-	-	-
Ankles													
	No	26	63.4	28	42.4	1.00							
	Yes	15	36.6	38	57.6	2.35	1.06	5.24	.036	-	-	-	-
Metatarsophalangealjoints													
	No	34	82.9	43	65.2	1.00							
	Yes	7	17.1	23	34.8	2.60	1.00	6.77	.051	-	-	-	-

* indcates continuous variables; IQR: Interquartile range

**Adjusted for gender, age, osteoarthrosis, allergic rhinitis, itching, diarrhea, lymphopenia, CHIKV RT-PCR positive in urine, CHIKV RT-PCR positive in saliva, morning joint stiffness, joint tenderness intensity (wrists, hands, knees, ankles, metatarsophalangeal), and joint swelling (wrists, hands, ankles, metatarsophalangeal)

407

5. DISCUSSÃO

Este estudo confirmou a detecção de CHIKV por rRT-PCR em vários fluidos corporais, incluindo secreções genitais, durante as fases aguda e subaguda da doença (até 3 meses). O RNA do CHIKV foi detectado por mais de 30 dias em todos os fluidos estudados. Além disso, soro, urina e saliva apresentaram níveis de vírus detectáveis e persistência por mais de 60 dias, enquanto a urina os apresentou por mais de 90 dias. Até onde sabemos, este foi o primeiro estudo de coorte a avaliar a persistência do CHIKV em fluidos genitais (secreções vaginais e sêmen).

Nesta coorte bem estabelecida de pacientes infectados com CHIKV, identificamos os preditores de dor crônica entre vários fatores apresentados na fase aguda da doença. Nossa análise constatou que diarreia, detecção de CHIKV por rRT-PCR na urina e saliva até cinco dias após o início da doença e dor intensa no punho foram os fatores de risco para o desenvolvimento de dor crônica.

As mulheres superaram em número os participantes homens no diagnóstico de CHIK. Resultados semelhantes foram descritos em outros estudos (42,105,106). Uma combinação de febre, artralgia e prostração foi a apresentação mais prevalente em nossa coorte, o que está em conformidade com os resultados descritos por Anwar e colaboradores em um representativo estudo de coorte transversal com 187 participantes (42).

A taxa de detecção do RNA do CHIKV foi significativamente maior no sangue, saliva e urina durante a primeira semana do início dos sintomas, em concordância com outros estudos que relataram a presença viral durante a fase aguda da doença (91,92). Além disso, a saliva e a urina não aumentaram a taxa de detecção do RNA do CHIKV na fase aguda da doença e, de acordo com Musso e colaboradores, o sangue deve ser a amostra de primeira escolha para o diagnóstico de CHIK (91). A persistência do RNA do CHIKV no soro em nosso estudo foi mais longa do que o esperado. A maioria dos artigos publicados confirma que o RNA do CHIKV no sangue diminui para níveis indetectáveis ao final do período agudo da doença (48,53,107).

Detectamos o RNA do CHIKV na urina até 95 dias após o início dos sintomas. Estudo semelhante, realizado no Brasil, mostrou a persistência viral máxima na urina por até 30 dias (92). Curiosamente, em nossa coorte, o RNA do CHIKV foi detectado em 30% das amostras de urina de participantes do sexo feminino e em apenas 9,6%

dos participantes do sexo masculino. Não encontramos relatos avaliando as taxas de detecção de CHIKV por rRT-PCR em amostras de urina por sexo, mas a contaminação por sangue menstrual pode ser uma explicação razoável, embora todas as orientações para coleta de urina tenham sido dadas aos participantes do estudo. Além disso, não era permitida a coleta de urina para mulheres durante o período menstrual.

Até onde sabemos, este é o primeiro estudo prospectivo a monitorar e detectar o RNA do CHIKV em secreções genitais. Detectamos o RNA do CHIKV até 46 dias após o início dos sintomas em amostras de secreção vaginal. Não realizamos estimativas estatísticas para o sêmen porque o número de amostras era pequeno, mas a detecção máxima do CHIKV por rRT-PCR no sêmen foi de 56 dias após o início dos sintomas em um participante do estudo.

Esforços para confirmar a presença de vírus em secreções genitais para doenças cuja transmissão sexual não é conhecida são necessários para investigar outras formas de transmissão, podendo ser de relevante importância para a saúde pública. A transmissão sexual do vírus Ebola foi confirmada na Libéria em 2015 (108). A transmissão sexual do ZIKV foi relatada para parceiros masculinos sintomáticos (109), e alguns estudos documentaram a detecção prolongada do RNA do ZIKV no sêmen (110,111). Lalle e colaboradores descreveram a presença de RNA do DENV no sêmen até 37 dias após o início dos sintomas, quando a viremia e a virúria eram indetectáveis (112).

Embora o RNA do CHIKV tenha sido detectado no sêmen em nossa série de casos, o vírus não foi detectado por rRT-PCR nas amostras cultivadas. Uma explicação é que os valores de Ct encontrados em nossas amostras foram altos, variando de 28 a 34, o que pode corresponder a uma baixa carga viral no sêmen. Culturas virais funcionam melhor com valores baixos de Ct em amostras inoculadas (108). Por exemplo, vírus infecciosos viáveis (com Ct de 20 a 28) e liberação prolongada de sêmen foram relatados em estudos de ZIKV e estão relacionados à transmissão sexual (89,113). Dificuldade no isolamento viral do sêmen de pacientes, principalmente com excreção viral de longo prazo, também foi relatada em estudos de pacientes com ZIKV (109).

O isolamento do vírus continua sendo a única abordagem direta e definitiva para provar a infectividade por via sexual (114). Vinte e sete vírus diferentes

mostraram persistência variável no sêmen humano (115). A presença de vírus no sêmen pode ser mais comum do que se sabe atualmente, e os vírus conhecidos como “não sexualmente transmissíveis” não devem ser considerados ausentes das secreções genitais. Todos os pacientes acompanhados em nossa coorte foram informados sobre a possibilidade de serem portadores do CHIKV por mais tempo, embora não tenhamos a confirmação do risco de infectividade. Estudos sobre detecção viral e persistência em secreções genitais beneficiam a prática clínica e a saúde pública, principalmente para vírus que podem causar alta morbidade crônica, como o CHIKV.

Nosso estudo mostrou uma alta prevalência de dor articular crônica após três meses do início da doença, em que 61,7% dos participantes referiam artralgia, com intensidades variadas. Este resultado foi coerente com alguns estudos de coorte (116,117), mas em contraste com outros estudos que mostram porcentagens menores de artralgia crônica após infecção por CHIKV,variando em torno de 40,2 a 45,2% (118-120). Uma recente revisão de estudos de coorte mostrou uma prevalência esperada de 72,2% para artralgia crônica após infecção por CHIKV (121). Uma explicação é que em alguns estudos a artralgia foi avaliada por meio de entrevistas telefônicas (118-120), com alto risco de viés de metodologia. Em nossa coorte, a avaliação da dor foi realizada, prospectivamente, por consulta médica presencial, com exame clínico durante todas as consultas. Isso pode ter contribuído para uma avaliação mais confiável da dor. A dor é considerada uma condição subjetiva, podendo apresentar uma diferença no limiar de dor no relato do paciente ou na referência do médico. Em nosso estudo, a avaliação da atividade da doença, incluindo dor nas articulações, foi semelhante entre os relatos dos participantes e dos médicos.

A maioria (83,3%) dos participantes que desenvolveram dor articular crônica em nossa coorte era do sexo feminino, confirmando maior risco em relação aos homens, em conformidade com a literatura mundial (116-118, 120-129). Em um estudo de coorte realizado na Colômbia, com 410 participantes, para avaliar a dor crônica até três meses após a infecção por CHIKV, as mulheres representaram 89,2% (130). Há hipótese de que as mulheres são mais susceptíveis a processos inflamatórios crônicos devido à produção de citocinas pró-inflamatórias, pelos monócitos durante o ciclo menstrual e a ovulação (131,132).Também é sugerido que

o estradiol aumenta a produção de anticorpos, protegendo mulheres mais jovens (133,134).

O risco de desenvolver dor crônica pós-CHIK também aumentou com a idade na análise univariada de nossa amostra, possivelmente por esse grupo de pessoas apresentarem um sistema imunológico mais comprometido ao longo dos anos, conforme sugerido por alguns autores (120-129).

A presença de doenças articulares prévias, como osteoartrite, é fortemente associada à dor articular crônica pós-CHIK (135). No entanto, estudos recentes não estabeleceram associação significativa entre artrose e dor crônica após infecção por CHIKV (116,122). Em nosso estudo, 14 pacientes relataram história de osteoartrose e todos, exceto um, apresentavam dor articular persistente após três meses do início da doença.

Em nossa análise, a diarreia foi um preditor da persistência da dor articular, e os pacientes com este sintoma têm maior probabilidade de apresentar desidratação. De acordo com Bertolotti e colaboradores, o papel agravante da desidratação durante a fase aguda da infecção pelo CHIKV é um fator de risco significativamente associado à cronicidade da doença (122). A desidratação também pode resultar em vários graus de danos à cartilagem articular, podendo predispor às doenças articulares por um período prolongado (136,137).

Este estudo mostrou que a detecção do CHIKV por rRT-PCR em amostras de urina e saliva até o quinto dia da doença foi significativamente associada à persistência da dor nas articulações. Um estudo de coorte conduzido na Ilha da Reunião sugeriu que uma maior carga viral no sangue, na fase aguda da doença, estava associada à artralgia crônica com duração de até 12 meses (138). Em nosso estudo, os valores médios de Ct, foram maiores na urina e na saliva do que no sangue. Detecção de CHIKV por rRT-PCR no sangue até cinco dias após o início da doença não foi associado à dor articular crônica em nossa coorte. Consequentemente, esta hipótese de alta carga viral em fluidos na fase aguda da doença e persistência da dor após três meses de doença não foi demonstrada em nosso estudo.

Em um recente estudo de coorte, dor nas articulações, edema e múltiplos acometimentos articulares, incluindo as articulações das mãos e pés, foram identificados como fatores de risco para cronicidade após três meses do início da

doença (139). Morales e colaboradores também utilizaram um sistema de pontuação que incluiu a presença de edema, entre outras variáveis (SHERA - Sexo, Hipertensão, Edema, Dor retro-orbitária e Idade), para selecionar pacientes com alto risco de desenvolver artralgia crônica na infecção aguda pelo CHIKV (116). Em nosso estudo, embora a presença de edema articular em algumas articulações tenha sido associada à dor persistente na análise univariada, esses achados não foram mantidos no modelo multivariado final.

Muitos estudos têm mostrado que a maior prevalência e a intensidade da artralgia ocorrem nas articulações distais dos membros (48,50,51,119,120,122,129). A dor intensa no punho durante a doença aguda foi altamente associada ao risco de artralgia persistente em nosso estudo, sugerindo que a inflamação durante esse período aumenta a probabilidade de sintomas persistentes até três meses.

Este estudo teve algumas limitações: 1) como as visitas e coletas de amostras foram agendadas a cada 15 dias, podemos ter subestimado o tempo exato de persistência viral nos diferentes fluidos corporais; 2) a duração mediana do CHIKV no sêmen foi avaliada em um pequeno número de pacientes, por dificuldades na coleta de amostras, principalmente devido a dores articulares na fase aguda da doença; 3) o tempo de seguimento foi limitado a 90 dias, impossibilitando a avaliação da persistência máxima do CHIKV em todos os fluidos corporais; 4) todos os participantes eram ambulatoriais, portanto os resultados podem não ser generalizáveis para pacientes mais graves, que necessitaram de internação ou permaneceram nas unidades de emergência; 5) apesar do tamanho amostral adequado, a taxa de perda de acompanhamento foi alta (29,6%), podendo ter afetado a análise estatística, especialmente no grupo sem dor articular crônica, porque supomos que pacientes sem dor são mais propensos a abandonar o estudo, em relação àqueles do grupo sintomático.

O diagnóstico de CHIK em humanos é baseado principalmente na detecção de RNA em amostras de soro ou plasma. No entanto, demonstramos que a saliva e a urina podem ser consideradas como possíveis amostras alternativas para diagnóstico nas fases aguda e subaguda da doença. Algoritmos de diagnóstico usando urina ou saliva como amostras alternativas têm a vantagem de serem rápidos, fáceis de executar e menos invasivos do que a coleta de sangue. A

demonstração de maior persistência do CHIKV em fluidos corporais poderá auxiliar no diagnóstico em fases mais avançadas da doença.

Estudos sobre detecção viral e persistência dos fluidos genitais beneficiam a prática clínica e as estratégias de saúde pública, principalmente para vírus que podem causar alta morbidade crônica, como o CHIKV. Novos estudos são necessários para avaliar o potencial de infectividade do sêmen e a transmissão sexual do CHIKV.

A artralgia crônica limita as atividades diárias e profissionais, causando transtornos psicológicos e afetando a economia familiar. Neste estudo, encontramos fatores de risco significativos associados à persistência da dor crônica após três meses de infecção pelo CHIKV. Procedimentos de hidratação devem ser incentivados com mais intensidade para pacientes com diarreia na fase aguda da doença. Além disso, o tratamento adequado do processo inflamatório durante a fase aguda poderia reduzir a incidência de doença crônica por CHIKV. Além disso, identificamos que os sintomas musculoesqueléticos não são os únicos determinantes da dor crônica na CHIK, e uma avaliação cuidadosa da detecção do CHIKV em fluidos corporais alternativos (saliva e urina) na fase inicial da doença deve ser investigada. O conhecimento desses fatores pode ajudar a desenvolver novos estudos com intervenções terapêuticas mais precoces para pessoas com maior risco de desenvolver dor crônica.

6. CONCLUSÕES

i. O RNA de CHIKV foi detectado em todos os fluidos corporais estudados, incluindo secreções genitais, durante as fases aguda e subaguda da doença, até três meses do início dos sintomas.

ii. O RNA do CHIKV foi detectado por mais de 30 dias em todos os fluidos estudados. Soro, urina e saliva apresentaram níveis de vírus detectáveis e persistência por mais de 60 dias, enquanto a urina apresentou por mais de 90 dias.

iii. A detecção máxima do RNA de CHIKV foi de 95, 61, 56, 49 e 46 dias na urina, soro, sêmen, saliva e secreções vaginais, respectivamente.

iv. A taxa de detecção do RNA de CHIKV foi significativamente maior no sangue, saliva e urina durante a primeira semana de evolução da doença, com diminuição progressiva nas semanas subseqüentes.

v. Foram identificados quatro fatores de risco para o desenvolvimento de artralgia crônica após três meses da infecção pelo CHIKV: diarreia, detecção de CHIKV por rRT-PCR na urina até 5 dias após a doença, detecção de CHIKV por rRT-PCR na saliva até 5 dias após a doença e dor intensa no punho.

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