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DISSERTAÇÃO DE MESTRADO

AVALIAÇÃO EPIDEMIOLÓGICA E GENÔMICA DO VÍRUS CHIKUNGUNYA CIRCULANTE NO RIO DE JANEIRO

JOILSON XAVIER DOS SANTOS JUNIOR

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"AVALIAÇÃO EPIDEMIOLÓGICA GENÔMICA DO VÍRUS CHIKUNGUNYA CIRCULANTE NO RIO DE JANEIRO".

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RESUMO

INTRODUÇÃO: A febre Chikungunya é uma doença autolimitada causada pelo vírus chikungunya (CHIKV), que é transmitido pela picada de mosquitos hematófagos infectados do gênero *Aedes*. A maioria dos indivíduos infectados apresentam sintomas dentro de 4 a 7 dias, como febre, exantema maculopapular, poliartralgia e mialgia. Os casos de chikungunya no Brasil são registrados desde que o vírus foi identificado pela primeira vez no país em 2014. Em 2018, o Brasil registrou 87.687 casos prováveis da doença, dos quais, 52.966 casos (60,4%) ocorreram apenas na região Sudeste. O estado do Rio de Janeiro experienciou uma grande epidemia causada pelo CHIKV em 2016, 18.516 casos prováveis. A emergência do CHIKV tem levantado preocupação devido à rápida disseminação do vírus em nova áreas geográficas e às características clínicas associadas à infecção. **OBJETIVO**: Diante do exposto, o objetivo deste estudo foi investigar a epidemiologia genômica do vírus chikungunya circulante no estado do Rio de Janeiro. **MATERIAIS E MÉTODOS**: Nós empregamos o método de sequenciamento por nanoporos, que permite a geração de muitos dados em poucas horas. **RESULTADOS**: Nós geramos com sucesso 11 sequências genômicas do CHIKV a partir de amostras de soro de pacientes sintomáticos residentes em sua maioria (n=6) no município do Rio de Janeiro. As cinco amostras restantes foram de pacientes residentes em quatro municípios vizinhos. As análises filogenéticas, empregando a abordagem do relógio molecular, revelaram que as cepas circulantes no Rio de Janeiro pertenciam ao genótipo africano ECSA e provavelmente este foi introduzido neste estado por volta de julho de 2014. Isto significa que o vírus circulou despercebidamente por 16 meses antes dos primeiros relatos oficias de transmissão autóctone no estado do Rio de Janeiro. **CONCLUSÕES**: Ambos os dados epidemiológicos e filogenéticos sugerem que as cepas da linhagem ECSA foram introduzidas no Rio de Janeiro a partir dos eventos de dispersão do CHIKV da região Nordeste para outras regiões do Brasil.

Palavras-chave: Vírus Chikungunya, Rio de Janeiro, Epidemiologia genômica, MinION, Sequenciamento.

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ABSTRACT

INTRODUCTION: Chikungunya fever is a self-limited disease caused by chikungunya virus (CHIKV), which is transmitted by the bite of infected hematophagous mosquitoes of the genus *Aedes*. Most infected individuals present symptoms within 4 to 7 days, such as fever, maculopapular rash, polyarthralgia and myalgia. Cases of chikungunya in Brazil are recorded since the virus was first identified in the country in 2014. In 2018, Brazil reported 87,687 probable cases of the disease, of which 52,966 cases (60.4%) occurred in the Southeast region only. The state of Rio de Janeiro experienced a major epidemic caused by CHIKV in 2016, reporting 18,516 probable cases. The emergence of CHIKV has raised concern due to the rapid spread of the virus into new geographic areas and the clinical features associated with the infection. **OBJECTIVE**: Thus, the objective of this study was to investigate the genomic epidemiology of chikungunya virus circulating in the state of Rio de Janeiro. **MATERIALS AND METHODS**: We used the nanopore sequencing method that allows the generation of large amounts of data in a few hours. **RESULTS**: We successfully generated 11 CHIKV genomic sequences from serum samples from symptomatic patients residing mostly (n = 6) in the city of Rio de Janeiro. The remaining five samples were from patients residing in four neighbouring municipalities. Phylogenetic analyses, using the molecular clock approach, revealed that the strains circulating in Rio de Janeiro belonged to the ECSA African genotype and this genotype was likely introduced in the state around July 2014. This means that the virus circulated unnoticed for 16 months before the first official reports of autochthonous transmission in the state of Rio de Janeiro. **CONCLUSIONS**: Both epidemiological and phylogenetic data suggest that strains from ECSA lineage were introduced into Rio de Janeiro from CHIKV dispersion events from Northeast region of Brazil to others Brazilian regions.

Key-words: Chikungunya virus, Rio de Janeiro, Genomic epidemiology, MinION, Sequencing.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

26S	RNA subgenômico
49S	RNA genômico
Å	Ångström
CCL2/MCP-1	Proteína quimioatratante de monócitos
CD209	Conjunto de diferenciação 209
CHIKV	vírus chikungunya (*chikungunya virus*)
CLEC4M	Lectina tipo C
CXCL10/IP-10	Proteína 10 indutora de interferon
DCs	Células dendríticas
DENV	vírus da dengue (*dengue virus*)
DNA	Ácido desoxirribonucleico)
E1	Proteína do envelope 1
E2	Proteína do envelope 2
E3	Proteína do envelope 3
ECSA	Genótipo Leste-Central-Sul-Africano
EEEV	vírus encefalite equina do leste (*Eastern equine encephalitis virus*)
GM-CSF	Fator estimulante de colônias de granulócitos e macrófagos
IFN-α	Interferon Alfa
IFN-γ	Interferon
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IL-12	Interleucina 12
IL-1RA	Antagonista do receptor de interleucina 1
IL-6	Interleucina 6
IOL	Linhagem Oceano Índico
Kb	Kilobase
MAYV	vírus mayaro (*Mayaro virus*)
mL	mililitro
NK	Células natural killer
nsP1	Proteína não-estrutural 1
nsP2	Proteína não-estrutural 2

nsP3	Proteína não-estrutural 3
nsP4	Proteína não-estrutural 4
ONNV	vírus O'nyong-Nyong (*o'nyong'nyong virus*)
ORF	Quadro aberto de leitura
PHB	Proibitina
RNA	Ácido ribonucleico
RRV	vírus do rio Ross (*Ross River virus*)
SINV	vírus sindbis (*Sindbis virus*)
T CD4+	Linfócito T com conjunto de diferenciação celular 4
T CD8+	Linfócito T com conjunto de diferenciação celular 8
Th1	Linfócito T auxiliar tipo 1
Th2	Linfócito T auxiliar tipo 2
TNF-α	Fatore de Necrose Tumoral Alfa
VEEV	vírus encefalite equina venezuelana (*Venezuelan equine encephalitis virus)*
WEEV	vírus encefalite equina do oeste (*Western equine encephalitis virus*)
ZIKV	vírus zika (*Zika virus*)

SUMÁRIO

1 INTRODUÇÃO

1.1 A FEBRE CHIKUNGUNYA

A febre Chikungunya é uma doença causada pelo vírus chikungunya (*Chikungunya virus* - CHIKV), que é transmitido pela picada de mosquitos hematófagos infectados, como *Aedes aegypti* e *Aedes albopictus*, os mesmos vetores transmissores do vírus da febre amarela, dengue e zika (REINHOLD *et al.*, 2018).

Os indivíduos infectados pelo CHIKV apresentam sintomas similares aos da dengue, como febre alta de rápido início (>39ºC), exantema maculopapular, dor de cabeça e fadiga (WEAVER; LECUIT, 2015). Estima-se que 87–98% dos pacientes apresentam poliartralgia, sendo este o sintoma com valor preditivo positivo maior que 80% (THIBERVILLE *et al.*, 2013; WEAVER; LECUIT, 2015). Mialgia, predominantemente nos braços e coxas, também tem sido observada em mais de 50% dos casos (THIBERVILLE *et al.*, 2013). Ao contrário da dengue, a maioria dos indivíduos infectados pelo CHIKV são sintomáticos, os quais apresentam os sintomas clínicos característicos dentro de 4-7 dias (THIBERVILLE *et al.*, 2013).

Dois estágios da infecção pelo CHIKV foram descritos. O estágio de infecção aguda caracteriza-se por uma carga viral alta (107 pfu/ml em média), com a duração média da viremia de 6 dias e com resolução em uma a duas semanas dos sintomas da febre, mialgia, artralgia e exantema (BURT *et al.*, 2017; THIBERVILLE *et al.*, 2013). Após o estágio agudo, alguns pacientes apresentam persistente artralgia e dor musculoesquelética (RODRÍGUEZ-MORALES *et al.*, 2016). O estágio crônico, que pode durar até três anos, apresenta significante variação na intensidade e frequência das manifestações clínicas, como dores articulares, fadiga, inchaço e rigidez matinal (BURT *et al.*, 2017; CUNHA; TRINTA, 2017). As dores articulares do estágio crônico geralmente são debilitantes e podem levar à incapacitação persistente que requer tratamentos de longo prazo (WEAVER; LECUIT, 2015).

Embora os casos de morte por chikungunya sejam raros, os pacientes podem estar em maior risco de apresentar complicações graves da doença quando outras comorbidades crônicas estão associadas (CUNHA; TRINTA, 2017). As comorbidades relatadas como mais prevalentes entre pacientes infectados por CHIKV foram hipertensão, seguida por diabetes, doenças cardíacas e asma (BADAWI *et al.*, 2018). Uma proporção 4-5 vezes maior da presença dessas condições crônicas foi observada em pacientes com idade acima de 50 anos quando comparada com pacientes mais jovens (BADAWI *et al.*, 2018).

Até o momento não há tratamento específico ou vacina disponível para prevenir a infecção pelo CHIKV (REZZA; WEAVER, 2019). O tratamento é direcionando primariamente para aliviar os sintomas da doença, com a prescrição de anti-inflamatórios não-esteroides, antipiréticos e analgésicos (WHO, 2019). Métodos preventivos envolvem o controle dos mosquitos vetores da doença, com o uso de inseticidas, repelentes e eliminação dos criadouros com água parada, e a redução do risco de exposição da pele ao mosquito com o uso de telas de proteção, calças e camisas de manga longa (WHO, 2019).

1.2 O VÍRUS CHIKUNGUNYA

A epidemia de uma doença desconhecida, com sinais clínicos semelhantes a dengue, foi registrada entre 1952-1953 na região do Makonde, Tanzânia (antiga Tanganica), África Oriental (ROBINSON, 1955). ROSS (1956) após isolar o agente infeccioso a partir do soro de pacientes na fase aguda, verificou que se tratava de um vírus, nomeado como chikungunya, um termo local que pode ser traduzido como "doença que dobra as articulações", devido à gravidade dos sintomas de dores articulares induzidos pela doença.

O CHIKV é classificado como um membro do gênero *Alphavirus*, da Família *Togaviridae* (STRAUSS; STRAUSS, 1994). A família *Togaviridae* também inclui o gênero *Rubivirus*, unicamente representado pelo vírus rubella, causador da rubéola (CHEN *et al.*, 2018). O gênero *Alphavirus*, por sua vez, inclui arbovírus com distribuição geográfica ampla, presente em todos os continentes, exceto Antártica, e causam doenças em seres humanos e animais (POWERS *et al.*, 2001). Encefalite é causada pelos vírus encefalite equina venezuelana (VEEV), vírus encefalite equina do leste (EEEV) e o vírus encefalite equina do oeste (WEEV), enquanto que artralgia e febre alta são causadas pelos vírus do rio Ross (RRV), vírus O'nyong-Nyong (ONNV), vírus mayaro (MAYV), vírus sindbis (SINV) e o vírus chikungunya (CHIKV). Além disso, doenças neurológicas também têm sido associadas à infecção pelo vírus CHIKV (ACEVEDO *et al.*, 2017; MEHTA *et al.*, 2018; SCHUFFENECKER *et al.*, 2006; VIEIRA *et al.*, 2018).

Análises filogenéticas revelaram que o CHIKV apresenta quatro genótipos (ou linhagens), nomeadas de acordo com sua distribuição geográfica (POWERS *et al.*, 2000) (Figura 1). O genótipo África Ocidental compreende cepas isoladas originalmente na Nigéria e no Senegal (POWERS *et al.*, 2000). O genótipo Asiático inclui originalmente isolados de países asiáticos, tais como Índia, Tailândia e Filipinas (POWERS *et al.*, 2000). O genótipo Leste-Central-Sul-Africano (ECSA) é originalmente enzoótico na África, compreendendo

cepas isoladas inicialmente na antiga República Sul-Africana, Tanzânia, na região Centro-Africana, e Uganda (POWERS *et al.*, 2000). Por último, a linhagem Oceano Índico (IOL), descendente da linhagem ECSA, consiste em cepas que foram isoladas durante uma epidemia registrada em ilhas do Oceano Índico, tais como Comoros e La Reunion, no subcontinente Indiano e no sudeste asiático (POWERS, 2011; SCHUFFENECKER *et al.*, 2006). Apesar da existência de quatro genótipos, o CHIKV possui apenas um sorotipo descrito após verificação da alta similaridade antigênica entre os quatro genótipos (AUERSWALD *et al.*, 2018).

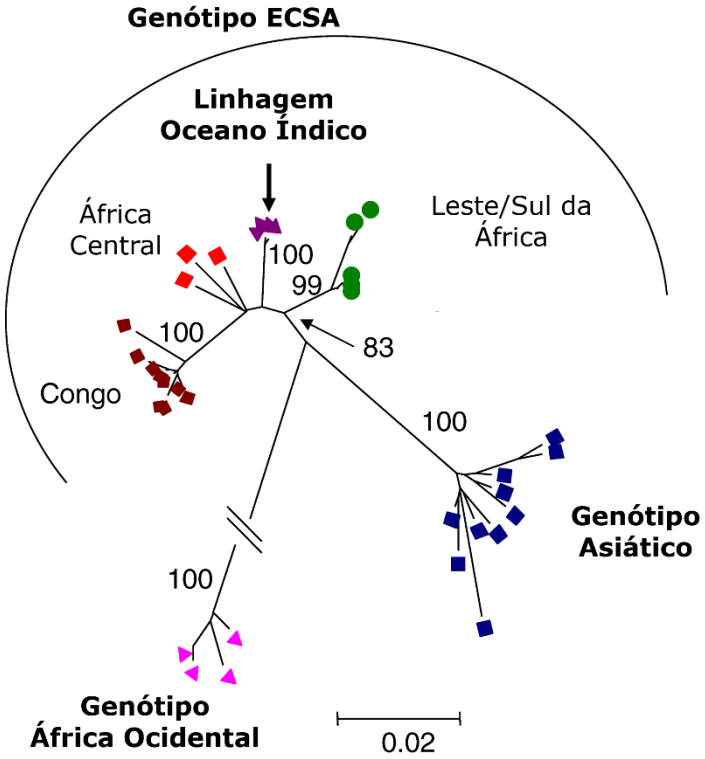

Figura 1. Relações filogenéticas entre isolados representantes dos quatro genótipos do CHIKV. Nessa filogenia fica visível a descendência da linhagem Oceano Índico a partir do genótipo ECSA. Números indicam os valores de reamostragem por bootstrap. Fonte: Adaptado de SCHUFFENECKER *et al.* (2006).

1.2.1 Organização estrutural do genoma do vírus chikungunya

O genoma do CHIKV, assim como em outros alfavírus, consiste em uma molécula de RNA fita simples de polaridade positiva com tamanho de aproximadamente 12 kb (YAP *et al.*, 2017) (Figura 2). O genoma viral possui dois quadros abertos de leitura (ORFs), a região 5′ é "encapada" (do inglês *capped*), enquanto a extremidade 3′ é poliadenilada (SHARMA *et al.*, 2018). A extremidade 5′ do genoma codifica quatro proteínas não estruturais (nsP1, nsP2, nsP3

e nsP4) envolvidas na replicação (YAP *et al.*, 2017). A extremidade 3′ do genoma, por sua vez, codifica cinco proteínas estruturais, a proteína C do capsídeo, a glicoproteína E1, 6K e a poliproteína precursora p62 (ou pE2), que é posteriormente clivada, originando as glicoproteínas E2 e E3 (YAP *et al.*, 2017). As proteínas estruturais estão envolvidas no processo de entrada do vírus na célula hospedeira, montagem dos nucleocapsídeos e brotamento de vírus a partir da membrana da célula hospedeira (SHARMA *et al.*, 2018).

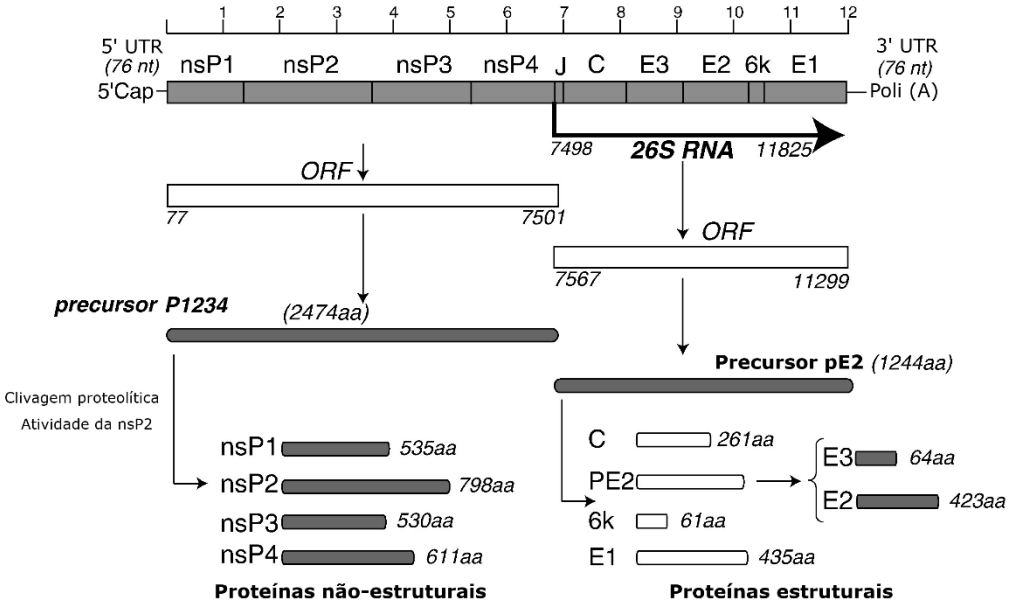

Figura 2. Organização do genoma do CHIKV e produtos gênicos (nsP1–nsP4, proteína do capsídeo, E1, E2, E3, 6K). Duas ORFs estão presentes no genoma do CHIKV. A poliproteína precursora P1234 após sofrer clivagem origina as quatro proteínas não-estruturais (nsP1-nsP4). O precursor pE2, por sua vez, origina as proteínas estruturais (C, E1, E2, E3) e a proteína 6k. Fonte: Adaptado de SOLIGNAT *et al.* (2009).

O vírus possui um núcleo central com as proteínas do capsídeo organizadas icosaedricamente cercando o genoma (LONG *et al.*, 2015) (Figura 3). O cerne do nucleocapsídeo é envelopado por uma membrana lipídica, derivada da membrana da célula hospedeira, na qual as glicoproteínas E1 e E2 estão inseridas (LONG *et al.*, 2015). A partícula viral madura possui um diâmetro de 700 Å e sua superfície externa é composta por 80 peplômeros, estruturas proeminentes expostas na superfície que são formadas por três cópias de um heterodímero de E1 e E2 (LONG *et al.*, 2015; PORTA *et al.*, 2016).

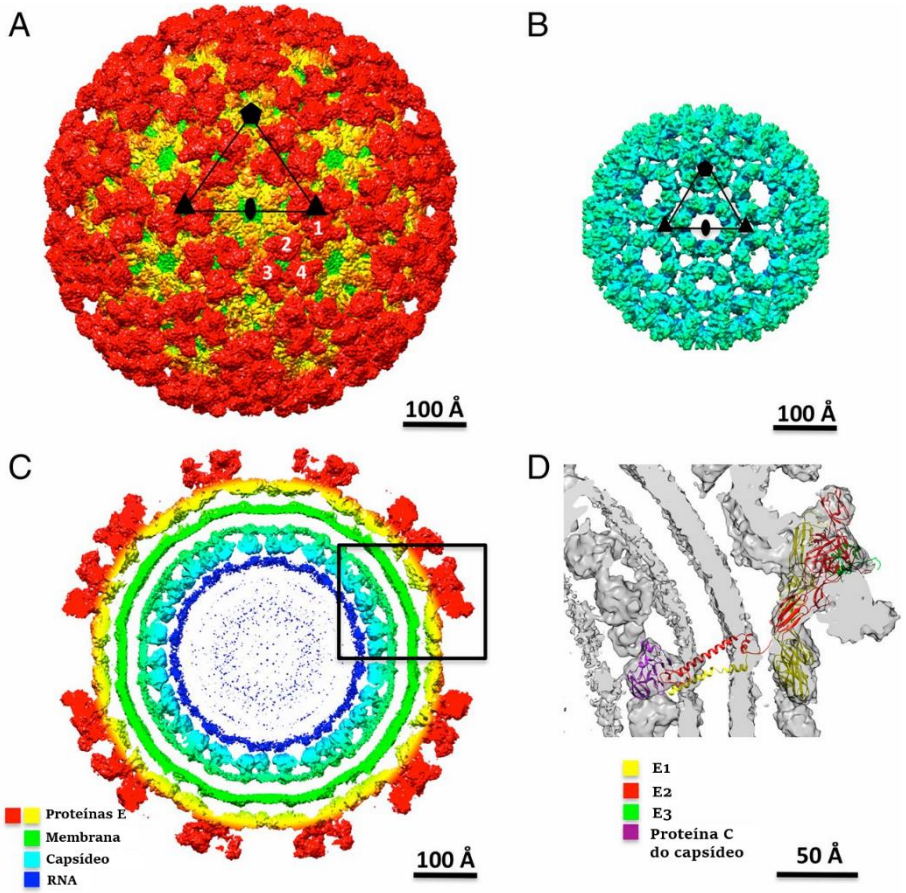

Figura 3. Reconstrução por microscopia crio-eletrônica de partículas semelhantes ao CHIKV imaturo. (A) Mapa tridimensional do CHIKV imaturo. Uma unidade assimétrica icosaédrica é marcada por um triângulo preto. (B) Representação da organização interna da proteína do capsídeo do CHIKV imaturo. (C) Secções transversais centrais do CHIKV imaturo. Componentes do vírus são mostrados em cores diferentes, conforme indicado na legenda na figura. (D) Visão ampliada da região delineada pelo retângulo preto em C. A montagem de uma estrutura E1-p62-C é mostrada no mapa do CHIKV imaturo. Fonte: Adaptado de YAP *et al.* (2017).

1.2.2 Ciclo replicativo do vírus chikungunya

O ciclo de replicação viral do CHIKV é essencialmente semelhante ao ciclo descrito para outros alfavírus (Figura 4) (SOLIGNAT *et al.*, 2009). A replicação ocorre no citoplasma das células de ambos hospedeiros vertebrados e mosquitos (SOLIGNAT *et al.*, 2009). Os vírions entram na célula hospedaria por endocitose mediada por clatrinas, após interações das glicoproteínas E2 com receptores da membrana da célula hospedeira, tais como CD209, proibitina (PHB), glicosaminoglicanos e CLEC4M (ASHBROOK *et al.*, 2014; SCHWARTZ; ALBERT, 2010; VAN DUIJL-RICHTER *et al.*, 2015b). Após endocitose, os vírions são incluídos em endossomos, cujo ambiente interior de ph baixo promove mudanças conformacionais nas glicoproteínas E1/E2 do envelope viral, levando à dissociação da

glicoproteína E1, que por sua vez, induz a fusão da membrana viral com a membrana plasmática do endossomo (VAN DUIJL-RICHTER *et al.*, 2015a; VOSS *et al.*, 2010). Como consequência, o nucleocapsídeo viral é liberado no citoplasma da célula hospedeira (SCHWARTZ; ALBERT, 2010).

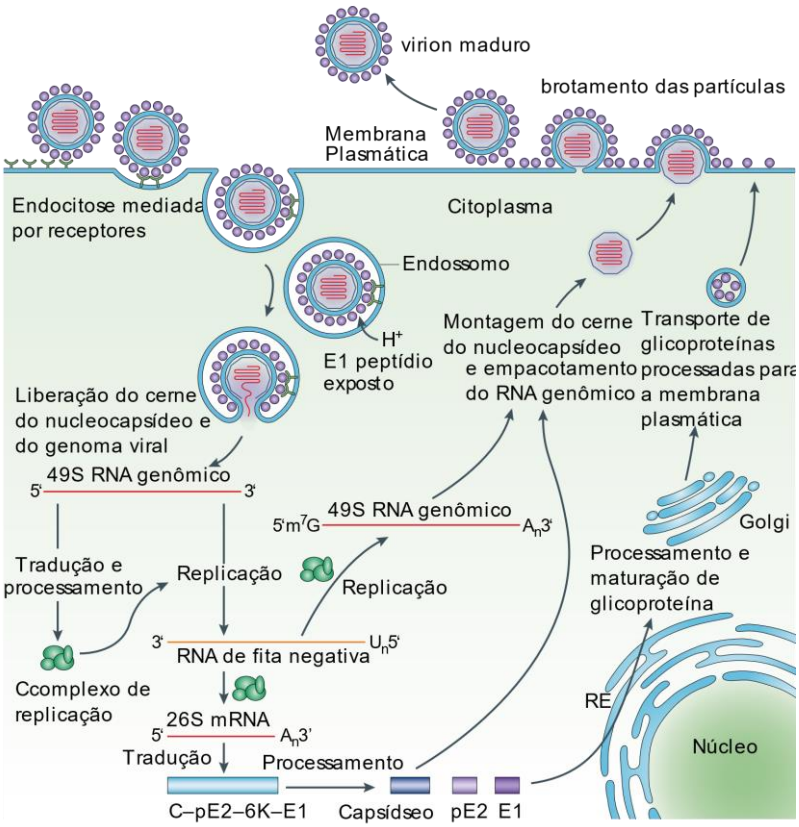

Figura 4. Ciclo de replicação dos alfavírus. Após endocitose da partícula viral, o genoma viral é liberado no citoplasma, onde ocorre a replicação e a síntese das proteínas virais que irão compor os novos vírions, cuja maturação ocorre junto a membrana plasmática. Fonte: Adaptado de SCHWARTZ e ALBERT (2010).

No citoplasma, o nucleocapsídeo se desintegra e libera o RNA viral (SCHWARTZ; ALBERT, 2010). A replicação do RNA do CHIKV é precedida por sua tradução que gera a produção de poliproteínas precursoras que após clivagem originam as proteínas não-estruturais nsP1-nsP4 (BURT *et al.*, 2017). A proteína madura nsP1 atua na síntese da fita negativa do RNA viral, enquanto a nsP1 possui atividade de protease, RNA helicase e RNA trifosfatase (BURT *et al.*, 2017; SCHWARTZ; ALBERT, 2010). nsP3, por sua vez, faz parte da unidade da replicase, e nsP4 consiste na RNA polimerase viral (BURT *et al.*, 2017; SCHWARTZ; ALBERT, 2010).

As proteínas não-estruturais formam o complexo de replicação que sintetiza incialmente a fita negativa do RNA viral, que servirá de fita molde para a síntese da fita positiva de vários RNAs genômicos (49S) e subgenômicos (26S) (RUPP *et al.*, 2015). A tradução RNA subgenômico 26S resulta na síntese da poliproteína precursora das cinco proteínas estruturais (C, E1, E2, E3, 6K) (SCHWARTZ; ALBERT, 2010; SOLIGNAT *et al.*, 2009). A proteína precursora pE2 é exportada para a membrana plasmática onde é clivada originando as proteínas E2 e E3 (SCHWARTZ; ALBERT, 2010). As proteínas estruturais são necessárias para a formação dos vírions maduros que ocorre na membrana plasmática da célula hospedeira (SCHWARTZ; ALBERT, 2010). A montagem dos novos vírions é promovida pela ligação entre o nucleocapsídeo e o RNA viral, e o recrutamento das glicoproteínas do envelope (SCHWARTZ; ALBERT, 2010; SOLIGNAT *et al.*, 2009). Na membrana da célula ocorre o brotamento das partículas virais montadas, com um cerne icosaédrico (SCHWARTZ; ALBERT, 2010).

1.2.3 Aspectos imunopatogênicos da infeção pelo vírus chikungunya

Nos últimos anos, o conhecimento sobre a patogênese da infecção pelo CHIKV tem sido derivado de estudos *in vitro* e *in vivo* com modelos animais com ratos e primatas não-humanos. Cada modelo oferece facetas únicas sobre a patogênese da chikungunya, e também nenhum deles recapitulam completamente a infeção pelo CHIKV em humanos (TANABE *et al.*, 2018b).

Em relação ao tropismo celular do CHIKV nas infeções humanas, tem-se relatado, em experimentos de cultura de tecido, a replicação do CHIKV em células aderentes, tais como células primárias endoteliais e epiteliais, fibroblastos e macrófagos derivados de monócitos (SCHWARTZ; ALBERT, 2010). A replicação do vírus também tem sido observada em células satélites musculares humanas, no entanto, o vírus não consegue infectar, *in vitro*, linfócitos e células dendríticas derivadas de monócitos (SCHWARTZ; ALBERT, 2010; TANABE *et al.*, 2018b). A infecção por CHIKV mostrou-se altamente citopática em cultura de células que rapidamente sofrem morte celular por apoptose após a infecção (SCHWARTZ; ALBERT, 2010). Em cérebros de modelos murinos, o vírus foi capaz de infectar astrócitos e oligodendrócitos, e induzir a produção, por células microgliais, de citocinas e moléculas pro-apoptóticas como IL-12, IFN-α e TNF-α (DAS *et al.*, 2015).

Após a picada por mosquito infectado, o CHIKV replica-se na pele e então dissemina-se, através do sangue, para o fígado e articulações (SCHWARTZ; ALBERT, 2010). Após o período de incubação de 2 a 10 dias surgem os sintomas clínicos da doença (WEAVER *et al.*,

2018). No estágio agudo, linfopenia aguda é frequentemente relatada, a carga viral pode atingir 10^8 partículas virais por mL de sangue, e uma correlação positiva tem sido observada entre o aumento da carga viral e o aumento nos níveis de citocinas e quimiocinas como IFN-α, IL-6, IL-12, IL-1RA, CXCL10/IP-10, e CCL2/MCP-1 (CHOW *et al.*, 2011; SCHWARTZ; ALBERT, 2010).

O estágio agudo da doença é marcado por um aumento significativo dos componentes da resposta imune celular que leva a ativação das células inatas natural killer (NK) (HOARAU *et al.*, 2010). Monócitos e macrófagos derivados de monócitos parecem estar envolvidos com a patologia das dores articulares associadas à doença (TANABE *et al.*, 2018b). Experimentos *in vitro* mostraram que sinoviócitos humanos primários semelhantes a fibroblastos são suscetíveis à infecção por CHIKV e o sobrenadante dessas células infectadas induzem a diferenciação de monócitos/macrófagos em células semelhantes à osteoclastos (PHUKLIA *et al.*, 2013). Estas células levam à produção de altos níveis de mediadores da artrite, tais como IL-6 e TNF-α (PHUKLIA *et al.*, 2013; TANABE *et al.*, 2018b).

As respostas da imunidade inata e adaptativa parecem estar conectadas pela participação das células dendríticas (DCs) na apresentação de antígenos (TANABE *et al.*, 2018b). Em macacos infectados, DCs mostraram-se resistentes à infecção pelo vírus e a mobilização dessas células foi observada após a infecção de astrócitos de ratos neonatos sujeitos a injeção intracerebroventricular do CHIKV (DAS *et al.*, 2015).

Evidência da resposta imune adaptativa aparece aproximadamente uma semana depois que a infecção é resolvida (SCHWARTZ; ALBERT, 2010). Em humanos e em ratos, a produção de anticorpos anti-CHIKV, que tem primariamente como alvo a proteína do envelope viral E2, leva a *clearance* (remoção) do vírus (TANABE *et al.*, 2018b). Tais anticorpos mostraram-se persistente por até 21 meses após o início da doença no plasma de pacientes em fase convalescente (5-14 dias após início da doença) (KAM *et al.*, 2012a). Anti-CHIKV IgG é detectado no início da fase convalescente e a aparência precoce desse anticorpo está associada à proteção contra as complicações crônica da doença (KAM *et al.*, 2012b).

A ativação e proliferação de linfócitos T CD8+ foi observada apresentando pico no estágio inicial agudo da doença em pacientes infectados (WAUQUIER *et al.*, 2011). A resposta imune mediada por linfócitos T CD4+, por sua vez, aumenta na final do estágio agudo doença (WAUQUIER *et al.*, 2011). Além disso, células T CD4+ específicas ao CHIKV têm sido associadas à inflamação independente de IFN-γ nas articulações (TEO *et al.*, 2013).

Durante o estágio crônico da doença, os macrófagos parecem atuar como reservatórios celulares do CHIKV persistente e como reguladores do balanço da resposta imunes dos tipos

Th1/Th2 nos tecidos danificados (TANABE *et al.*, 2018b). A persistência de células infectadas e/ou de antígenos virais desencadeariam repostas pró-inflamatórias prolongadas, com a liberação de IL-6 e GM-CSF (TANABE *et al.*, 2018b; WEAVER *et al.*, 2018). Tem-se argumentado que citocinas, como IL-6 e TNF, promovem atividade osteoclástica e têm sido associadas à osteoclastogênese que pode contribuir para os efeitos da fase crônica da doença (GANESAN *et al.*, 2017).

1.2.4 Epidemiologia

Desde que o CHIKV foi identificado pela primeira vez no leste da África, vários surtos foram registrados no continente africano, incluindo o surto mais recente registrado no Sudão com mais de 13.000 casos até outubro de 2018 (WHO, 2018). A soroprevalência da infecção por CHIKV na África tem sido estimada de 9,7% para IgM e 16,4% para IgG (SIMO *et al.*, 2019). Análises filogenéticas indicam que a dispersão do CHIKV ocorreu a partir do leste Africano para o sul e sudeste da Ásia (WEAVER; FORRESTER, 2015). Desde então, o vírus tem sido identificado em mais de 60 países, e mais de 70 epidemias foram registradas ao redor do mundo entre 1959-2016 (MASCARENHAS *et al.*, 2018; WHO, 2019) (Figura 5).

Desde 1960 surtos de chikungunya têm sido reportados na África e Ásia, com grande atividade nos anos 60 e 80 e então uma diminuição na atividade até 2004 (ZELLER *et al.*, 2016). Ainda, entre 1999 e 2000, a República Democrática do Congo declarou a ressurgência da epidemia de chikungunya após 39 anos, com aproximadamente 50.000 pessoas afetadas (PASTORINO *et al.*, 2004). Durante o período 2004 até 2010 epidemias foram registradas sequencialmente no Quênia, Senegal, Sudão, Camarões e Gabão (THIBERVILLE *et al.*, 2013). Em 2011, uma epidemia de chikungunya foi registrada pela primeira vez na República do Congo (KELVIN, 2011).

Na Ásia, os surtos de chikungunya foram inicialmente associados e confundidos com epidemia de dengue (ZELLER *et al.*, 2016). Grandes surtos foram observados na Tailândia no início dos anos 1960, e na Índia do início dos anos 1960 até a década de 1970 (PETERSEN e POWERS, 2016). Aproximadamente 31% da população de Bangkok, Tailândia, foi infectada durante o surto de 1962 (PETERSEN; POWERS, 2016). O primeiro surto de chikungunya na Malásia foi registrado em 1998, enquanto que na Indonésia, após um intervalo de 15 anos, surtos esporádicos foram identificados em várias regiões entre 2001 e 2002 (LARAS *et al.*, 2005).

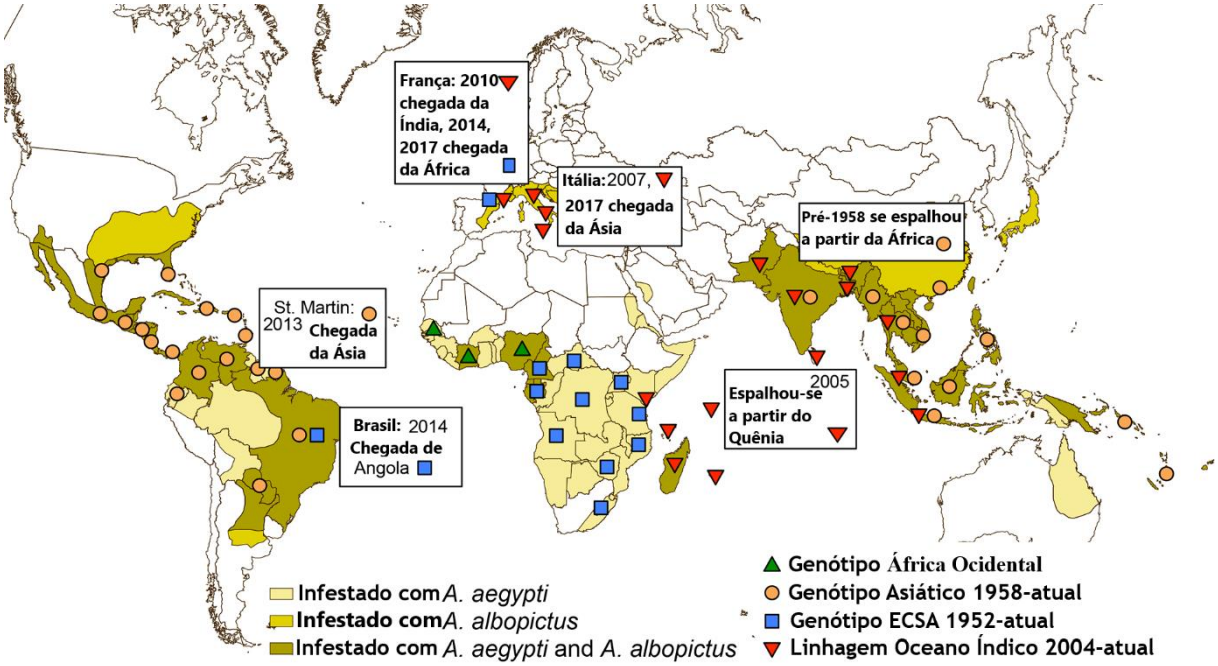

Figura 5. Mapa do mundo com os países onde cadeias autóctones (localmente iniciadas) de transmissão do CHIKV foram identificadas. Além do continente africano, O genótipo ECSA já foi identificado na América e Europa (França). A linhagem do Oceano Índico também foi registrada na França e Itália. Fonte: Adaptado de REZZA e WEAVER (2019).

Cepas do CHIKV identificadas nas regiões central e sul da África foram agrupadas e classificadas como linhagem ECSA (POWERS *et al.*, 2000). Enquanto que cepas isoladas durante surtos na Ásia entre 1958 e 1973 foram agrupadas em um grupo monofilético chamado de linhagem asiática (POWERS *et al.*, 2000). Análises filogenéticas têm estimado que as linhagens ECSA e Asiática divergiram a cerca de 100 anos atrás (VOLK *et al.*, 2010). Ambas as linhagens ECSA e Asiática são responsáveis por epidemias e o fato de que ambas reemergiram quase simultaneamente após anos de relativamente pouca atividade sugere que forças similares podem estar influenciando a reemergência dessas linhagens (PETERSEN; POWERS, 2016).

Em 2004, uma epidemia de chikungunya na costa do Quênia registrou quase meio milhão de casos, que mais tarde atingiu algumas ilhas no sudeste do Oceano Índico (Madagáscar, Seychelles, Maurícia, Mayotte e Réunion) (THIBERVILLE *et al.*, 2013). Análises filogenéticas indicaram que as cepas circulantes durante a epidemia no Oceano Índico eram derivadas do genótipo ECSA e então foram incluídas na linhagem IOL (SCHUFFENECKER *et al.*, 2006). Estudos posteriores com alguns isolados da linhagem IOL mostraram que a epidemia em La Réunion, Oceano Índico, estava associada à uma cepa que

apresentava uma mutação (E1-A226V) no gene da glicoproteína do envelope viral, que conferiu um aumento na eficiência da replicação do CHIKV no mosquito *Aedes albopictus*, levando ao aumento no potencial de transmissão do vírus (SCHUFFENECKER *et al.*, 2006; TSETSARKIN *et al.*, 2007; VAZEILLE *et al.*, 2007).

Durante o pico da epidemia de chikungunya envolvendo cepas da linhagem IOL no Oceano Índico e Ásia, milhares de viajantes infectados promoveram a importação do vírus para outras regiões no mundo (ROSSINI *et al.*, 2016). A cepa IOL dispersou a partir da ilha La Reunion para o subcontinente indiano, causando mais de um milhão de casos da doença entre 2005-2006 (HIGUERA; RAMIREZ, 2019; REZZA; WEAVER, 2019). A partir da Índia a linhagem IOL se espalhou rapidamente para o sudeste da Ásia, Itália (2007) e França (2010), com casos importados alcançando várias regiões no mundo (REZZA *et al.*, 2007; PANNING *et al.*, 2008; CHEN *et al.*, 2016). O surto de chikungunya na Itália, que envolveu aproximadamente 300 pessoas, representou o primeiro surto registrado no clima subtropical numa área que possui apenas o *Aedes albopictus* como espécie vetor (Petersen e Powers, 2016).

Do início de 2006 até 2010 a linhagem ECSA foi a linhagem mais prevalente em alguns países da Ásia, porém isso foi ultrapassado subsequentemente pela linhagem Asiática (HIGUERA; RAMIREZ, 2019). Após várias décadas com poucos relatos de surtos ou casos de chikungunya na Ásia, a linhagem Asiática esteve envolvida na reemergência do CHIKV na Malásia em 2006 e foi o genótipo mais prevalente na Indonésia e Filipinas entre os anos de 2007 e 2014 (ZELLER *et al.*, 2016; WAHID *et al.*, 2017). Em 2011 cepas da linhagem Asiática estiveram circulando em diferentes regiões da Indonésia e causaram um pequeno surto na Nova Caledônia após dois viajantes infectados retornarem da Indonésia, onde o vírus tem circulado por pelo menos uma década (PETERSEN; POWERS, 2016; ZELLER *et al.*, 2016). A linhagem Asiática subsequentemente foi identificada durante surtos na Papua Nova Guiné (2012), em Yap nos Estados Federados da Micronésia (2013), em Tonga, Samoa, Samoa Americana, Tokelau, e polinésia francesa (2014) e na República de Kiribati e nas ilhas Cook (2015) (WEAVER; REISEN, 2010; CHEN *et al.*, 2016)

Os primeiros casos autóctones de chikungunya no hemisfério ocidental ocorreram na ilha de Saint Martin após disseminação da linhagem Asiática, filogeneticamente relacionada com os vírus identificados nos Estados Federados da Micronésia, Filipinas e Indonésia (LEPARC-GOFFART *et al.*, 2014; PETERSEN; POWERS, 2016). A partir de Saint Martin a linhagem Asiática se espalhou para outras 26 ilhas do Caribe e para outros 14 países do continente Americano, resultando em mais de 1 milhão de casos relatados em 2014 nas Américas (PAHO/WHO, 2015).

No Brasil, os primeiros casos autóctones causados pela linhagem Asiática foram registrados em setembro de 2014 no município de Oiapoque, estado do Amapá, na região norte do país (NUNES *et al.*, 2015). Naquele mesmo mês também foi identificado pela primeira vez no Brasil a presença da linhagem ECSA na cidade de Feira de Santana, Bahia, introduzida por um viajante infectado que retornou da Angola. Identificação da transmissão autóctone genótipo ECSA na Bahia sinalizou a emergência e o estabelecimento deste genótipo no país (NUNES *et al.*, 2015). Desde então, o genótipo ECSA tem se espalhado para outros estados brasileiros das regiões Nordeste e Sudeste, exercendo uma ameaça à saúde pública, especialmente devido à cocirculação de outros arbovírus, tais como dengue e zika, que causam manifestações clínicas semelhantes (CHARLYS DA COSTA *et al.*, 2017; CUNHA, M. D. P. *et al.*, 2017; CUNHA, M. S. *et al.*, 2017; LESSA-AQUINO *et al.*, 2018; TANABE *et al.*, 2018a).

A linhagem ESCA foi identificada em 11 cidades do estado de Sergipe num estudo em 2016 (CUNHA, M. D. P. *et al.*, 2017). Tanabe *et al.* (2018a) sequenciaram e caracterizaram o surto de chikungunya no estado de Alagoas em 2016, cujas cepas circulantes estavam relacionadas as cepas da linhagem ECSA da Bahia e de Sergipe. Em Maceió, CHarlys da Costa *et al.* (2017) sequenciaram 23 amostras do CHIKV e verificaram que a linhagem ECSA foi introduzida no estado de Alagoas a partir e um evento único de introdução de uma cepa possivelmente oriunda do estado da Bahia em 2015. Casos autóctones de chikungunya em 2016 também foram relatados no estado do Rio Grande do Sul, onde tem-se registrado também a cocirculação de dengue e zika (GREGIANINI *et al.*, 2017). Além da circulação da linhagem Asiática na região amazônica do Brasil, a introdução e o estabelecimento da linhagem ECSA naquela região foram caracterizados por Naveca *et al.* (2018) que estimaram que esta linhagem foi introduzida em 2016 na cidade de Boa Vista, Roraima, a partir da região Nordeste do Brasil e verificaram a substituição do genótipo Asiático pelo genótipo ECSA naquela região.

O Brasil relatou 277.882 casos prováveis de infecção pelo CHIKV em 2016, e 185.593 casos 2017 (BRASIL, 2018; 2019). Em 2018, o país registrou 87.687 casos prováveis, dos quais 52.966 casos (60,4%) ocorreram apenas na região Sudeste (BRASIL, 2019). Nesta região, o estado do Rio de Janeiro registrou uma epidemia com 18.516 casos prováveis até a semana epidemiológica 52 de 2016 (BRASIL, 2018). A epidemia persistiu, levando o estado a reportar 39.725 casos prováveis em 2018, com os municípios do Rio de Janeiro (10.062 casos) e São Gonçalo (6.261 casos) estando entre os municípios mais afetados pela epidemia (BRASIL, 2019).

1.3 APLICAÇÃO DE MÉTODOS DE BIOINFORMÁTICA NO ESTUDO DA EPIDEMIOLOGIA GENÔMICA DE VÍRUS

Os vírus de RNA são caracterizados por uma alta taxa de mutação que dirige a evolução viral (DUFFY *et al.*, 2008). As altas taxas de mutação, associadas à seleção e à possibilidade de recombinação contribuem para a variabilidade genética nas populações virais (DUFFY *et al.*, 2008; POSADA-CESPEDES *et al.*, 2017). A existência de diferentes cepas virais possui relevância clínica, uma vez que a heterogeneidade genética tem implicações significativas na patogênese, virulência, persistência, resistência à vacinas e drogas antivirais, e provavelmente contribui para o tropismo tecidual (POSADA-CESPEDES *et al.*, 2017). Nesse sentido, a tecnologia de sequenciamento tem sido empregada em muitas aplicações clínicas, para a identificação de novos ou insuspeitos patógenos, assim como para a avaliação da diversidade genética de vírus que causam doenças no ser humano (BARZON *et al.*, 2013; POSADA-CESPEDES *et al.*, 2017).

Novas tecnologias têm permitido a produção de grandes quantidades de dados genômicos em um tempo relativamente curto (POSADA-CESPEDES *et al.*, 2017). Essas tecnologias são coletivamente chamadas de sequenciamento de alto rendimento, e têm permitido a caracterização genética de muitas cepas virais e de mutações de baixa frequência, que têm relevância no contexto de resistência à drogas, pois tais mutações podem facilitar a adaptação viral levando à falha do tratamento (POSADA-CESPEDES *et al.*, 2017; VANDENHENDE *et al.*, 2014).

O advento da tecnologia de sequenciamento por nanoporos permitiu a geração de longas sequências de DNA a partir do sequenciamento de molécula única de DNA em tempo real (GOODWIN *et al.*, 2016). Tal tecnologia é empregada no sequenciador portátil da Oxford Nanopore Technologies, MinION, no qual o sequenciamento acontece quando uma molécula de RNA ou RNA atravessa uma proteína formadora de poro inserida numa membrana (GOODWIN *et al.*, 2016). Uma corrente elétrica é aplicada no poro, e a passagem da molécula de DNA através do poro provoca alterações no padrão da corrente elétrica, que são identificadas por um chip associado (GOODWIN *et al.*, 2016). Uma vez que cada mudança de voltagem é característica para a molécula, os registros dos padrões de alteração na corrente elétrica são analisados por um programa de computador, que por sua vez, faz a identificação da sequência de nucleotídeos presentes na molécula de DNA sequenciada (GOODWIN *et al.*, 2016).

O sequenciador portátil MinION tem sido empregado em situações de campo durante epidemias virais, como por exemplo, durante a epidemia de Ebola em 2014 no Oeste da África,

e durante os trabalhos em um laboratório móvel instalado em um ônibus do projeto Zibra realizado no Nordeste do Brasil em 2016, no contexto da epidemia de Zika (FARIA *et al.*, 2017b; QUICK *et al.*, 2016). Ainda no Brasil, o MinION também foi empregado na caracterização genômica da transmissão selvática do vírus da Febre Amarela durante o surto registrado na região Sudeste entre 2016-2017 (FARIA *et al.*, 2018).

Os dados genômicos podem ser empregados para informar não apenas o diagnóstico de patógenos, mas também a epidemiologia. A epidemiologia genômica utiliza análises genômicas para inferir dinâmicas epidemiológicas de emergência a partir do genoma de vírus amostrados e sequenciados em curtas escalas de tempo epidêmica (GRUBAUGH *et al.*, 2019).

O emprego da abordagem do relógio molecular permite estimar a taxa evolutiva do genoma viral a partir da diversidade genética de uma população viral (DOS REIS *et al.*, 2016). A taxa evolutiva indica quando os isolados amostrados compartilharam um ancestral comum (GRUBAUGH *et al.*, 2019). Tal informação fornece um limite inferior sobre quando um surto iniciou, por exemplo, e por quanto tempo o vírus estava circulando antes da descoberta (GRUBAUGH *et al.*, 2019).

Análises filogenéticas permitem inferir uma árvore que representa as relações filogenéticas entre os genomas virais amostrados e que apresenta ramos medidos em unidade de tempo (GRUBAUGH *et al.*, 2019). Cada ramo indica eventos de transmissão viral de um caso para o próximo, fornecendo informações sobre o surto que se desdobra, que podem ser usadas para identificar as cadeias de transmissão para entender as redes de "quem-infectou-quem" e assim, contribuir com informações que possam ser usadas para interromper a disseminação do vírus e reduzir a magnitude do surto (HALL *et al.*, 2016).

Inferências filogenéticas baseadas em dados genômicos também permitem estimar a taxa na qual o vírus pode estar se espalhando dentro da população e também fornecem informações sobre o papel de uma transmissão zoonótica durante um surto (GRUBAUGH *et al.*, 2019; VOLZ *et al.*, 2009). Além disso, informações genômicas permitem descobrir a identidade e a localização geográfica do hospedeiro reservatório de um vírus (GRUBAUGH *et al.*, 2019). Os métodos de inferência filogeográfica, por sua vez, permitem reconstruir uma história detalhada da dispersão de um vírus a partir da origem do surto (FARIA *et al.*, 2011). Essas análises fornecem estimativas da localização para cada nó ancestral numa árvore filogenética, usando modelos estocásticos, e também podem esclarecer os fatores que impulsionaram a disseminação do vírus, através da integração dos dados genômicos do vírus com conjuntos diversos de metadados (GRUBAUGH *et al.*, 2019).

2 JUSTIFICATIVA

A epidemia de zika e chikungunya no Brasil evidenciou a escassez de dados genômicos sobre as cepas epidêmicas circulantes, como consequência dos reduzidos esforços empregados para a realização da vigilância genômica de vírus emergentes (FARIA *et al.*, 2017b; FARIA *et al.*, 2016). A epidemia de chikungunya no Rio de Janeiro em 2016 registrou uma alta taxa de incidência (111,3 casos/100 mil hab.), no entanto, apesar do elevado número de casos, apenas 18 sequências genômicas do CHIKV isolado no Rio de Janeiro estão disponíveis, e são limitadas as informações existentes sobre a dinâmica de dispersão do vírus durante aquela epidemia.

A capacidade do CHIKV de dispersar geograficamente ao redor do mundo está associada ao aparecimento de mutações espontâneas no genoma viral que podem resultar na adaptação do vírus à diferentes espécies de vetores (CHU; ANG, 2016). Nesse sentido, tem-se argumentado sobre a relevância para a saúde pública da implementação de um sistema de vigilância que monitore a difusão do vírus e o aparecimento de novas variantes genéticas (GARDY; LOMAN, 2017). Tal sistema pode ser alcançado através da implementação de métodos de sequenciamento que proporcionem uma melhora na sensibilidade e especificidade do diagnóstico, além da integração dos dados genômicos e epidemiológicos, com o objetivo de elucidar os padrões de dispersão do vírus durante uma epidemia (GRUBAUGH *et al.*, 2019).

Nesse sentido, neste trabalho foi empregado uma técnica de sequenciamento por nanoporos em tempo real, que permitiu a geração de novas sequências do genoma completo do CHIKV, contribuindo com o aumento da quantidade de dados genômicos disponíveis em bancos de dados públicos sobre o CHIKV circulante no Brasil. Sequências do genoma completo viral permitem a realização de análises filogenética mais robustas e auxiliam na identificação e caracterização de possíveis novas variantes genéticas e mutações que confiram aumento da eficiência na replicação viral (VOLK *et al.*, 2010). As análises filogenéticas realizadas neste estudo fornecem informações sobre os eventos de transmissão viral, assim como a aprovável data do evento de introdução do CHIKV no estado do Rio de Janeiro, contribuindo, dessa forma, para a compreensão do estabelecimento e da dinâmica epidemiológica do CHIKV em áreas urbanas do Rio de Janeiro.

3 HIPÓTESE

A epidemia de chikungunya no estado do Rio de Janeiro em 2016 foi causada por um evento de introdução de uma cepa da linhagem ECSA oriunda da região Nordeste do Brasil.

4 OBJETIVOS

4.2 GERAL

Investigar a epidemiologia genômica do vírus chikungunya circulante no estado do Rio de Janeiro.

4.3 ESPECÍFICOS

a. Sequenciar o genoma do CHIKV a partir de amostras biológicas com diagnóstico molecular positivo;
b. Identificar o genótipo dos isolados sequenciados;
c. Reconstruir a filogenia do CHIKV circulante no Rio de Janeiro;
d. Identificar os eventos de introdução do CHIKV no Rio de Janeiro;
e. Estimar a data de introdução do CHIKV no Rio de Janeiro;

5 RESULTADOS

5.2 MANUSCRITO

Título: Circulation of chikungunya virus East/Central/South African lineage in Rio de Janeiro, Brazil.

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Situação: Submetido

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Objetivos:

a. Sequenciar o genoma do CHIKV a partir de amostras biológicas com diagnóstico molecular positivo para chikungunya;
b. Identificar o genótipo dos isolados sequenciados;
c. Reconstruir a filogenia do CHIKV circulante no Rio de Janeiro;
d. Identificar os eventos de introdução do CHIKV no Rio de Janeiro;
e. Estimar a data de introdução do CHIKV no Rio de Janeiro;

Article

Circulation of chikungunya virus East/Central/South African lineage in Rio de Janeiro, Brazil

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Abstract: The emergence of chikungunya virus (CHIKV) has raised serious concerns due to the virus rapid dissemination into new geographic areas and the clinical features associated with infection. To better understand CHIKV dynamics in Rio de Janeiro, we generated 11 whole genomes by means of real-time portable nanopore sequencing of virus isolates obtained directly from clinical samples. Our phylogenetic reconstructions indicated the circulation of the East-Central-South-African (ECSA) lineage during the 2016 epidemic in Rio de Janeiro. Time-measured phylogenetic analysis combined with CHIKV notified case numbers revealed the ECSA lineage was introduced in Rio de Janeiro around July 2014 (95% Bayesian credible interval: March to late November 2014), indicating the virus was circulating unnoticed in Rio de Janeiro for 16 months before the first confirmed cases. These findings reinforce that continued genomic surveillance strategies are needed to assist in the monitoring

and understanding of arbovirus epidemics, which might help to attenuate public health impact of infectious diseases.

Keywords: Chikungunya virus; Rio de Janeiro; East-Central-South-African; molecular clock; nanopore sequencing; genomic surveillance

1. Introduction

Chikungunya virus (CHIKV) infections have been reported worldwide since the virus was first isolated in Tanzania in 1953 [1]. Over 70 CHIKV epidemics have been reported around the world between 1959 and 2016 [2]. Only in the Americas, more than 1 million cases were notified in 2017, with Brazil reporting 185,593 cases [3,4].

CHIKV is an *Alphavirus* from the *Togaviridae* family that is transmitted via the bite of infected *A. aegypti* and *A. albopictus* mosquitoes [5,6]. CHIKV infection results in a dengue-like febrile disease, whose acute phase is characterized by high fever, macular or maculopapular rash, backache, headache, fatigue, myalgia, and polyarthralgia [7]. Patients may also develop a later chronic condition, with persistent or relapse symptoms of arthropathy [7]. As the majority of CHIKV infected patients are symptomatic with varying degrees of joint or musculoskeletal pains and require long-term treatment, CHIKV represents a serious burden inflected on both public health and economic sector [7-9].

There are four CHIKV genotypes (or lineages), which have been named based on their geographical distribution: (i) the West African; (ii) the East/Central/South African (ECSA); (iii) the Asian; and (iv) the Indian Ocean Lineage (IOL), which emerged from the ECSA lineage between 2005 and 2006 [10-12]. Studies have shown that several IOL strains presented a series of mutations including the E1-A226V adaptive mutation that confers an increased replication rate in *Aedes albopictus*, thus enhancing viral transmission potential [11,13,14].

Local transmission of the Asian lineage was detected in Brazil for the first time in September 2014 in Oiapoque municipality, in the northern region [15]. Seven days later, a new cluster of CHIKV infections was notified in the city of Feira de Santana, Bahia state, northeastern Brazil [15]. Sequencing of three isolates revealed those infections in Bahia were caused by the ECSA lineage, signalling its emergence and establishment in the country [15]. Since then, the ECSA genotype has been detected in several other Brazilian states in the northeastern, southeastern and northern regions, exerting a serious threat to public health, especially because the ECSA lineage is associated with higher symptomatic to asymptomatic ratio compared with the Asian lineage circulating in the Caribbean [16-21].

CHIKV infections in Brazil accounted for 277,882 and 185,593 suspected cases in 2016 and 2017, respectively [22]. In 2018, the country registered 87,687 suspected cases, most of which in southeastern Brazil (52,966; 60.4%) [3]. In 2016, Rio de Janeiro state reported 18,516 CHIKV suspected cases until epidemiological week 52 [22]. The epidemic persisted and, in 2018, Rio de Janeiro state reported 39,725 cases, with the municipalities of Rio de Janeiro (10,062 cases) and São Gonçalo (6,261 cases) municipalities being among the most affected by the epidemic [3].

Rio de Janeiro state, located in the southeast of Brazil, is a popular tourist destination, which, only during the Olympic Games in August of 2016, registered the influx of 1,6 million passengers arriving in 12,710 international and domestic flights [23]. The first cases of CHIKV autochthonous transmissions in Rio de Janeiro were registered in late 2015, although there is some serological evidence of imported CHIKV infections acquired abroad in 2010 [24-26]. Limited molecular and evolutionary information are available on the CHIKV epidemic, although sequencing of two partial genomes revealed that the ECSA lineage was the strain circulating in Rio de Janeiro during the 2016 epidemic [27,28].

Rio de Janeiro state, whose capital city is the second biggest of Brazil, is an important hub for busyness, tourism and it is well connected to the main metropolitan regions in the country. Nevertheless, limited information is available about the molecular epidemiology of CHIKV in Rio de

Janeiro, partly because of insufficient genomic surveillance efforts undertaken, despite increasing case numbers over the last years. The lack of complete genome sequences available impairs our understanding of the virus introduction and establishment in the region. We generated 11 complete genomes collected from 2016 to 2018 using portable nanopore sequencing and present a molecular epidemiology that sheds new light on the introduction and establishment of ECSA lineage in Rio de Janeiro.

2. Materials and Methods

2.1. Diagnostic assays

Under the scope of the ZIBRA project, an itinerant virus surveillance project supported by the Brazilian Ministry of Health (zibraproject.org), we analysed 102 samples from patients presenting symptoms compatible with arbovirus infection. Those samples were collected by the Central Laboratory of Public Health that seeked the diagnostic services provided by the National Reference Laboratory for Epidemiological Surveillance of Arbovirus in the Laboratory of Flavivirus at the Oswaldo Cruz Institute (IOC) from the Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, Brazil.

Serum samples were submitted to nucleic acid purification using the Magmax Pathogen RNA / DNA kit in a Thermo 5400630 KingFisher Flex Purification System, in a procedure using magnetic beads in automatic extraction. The RNA detection by RT-qPCR was performed using a Real Time RT-PCR protocol adapted from [29], using Promega GoTaq Probe 1-Step RT-PCR System Kit in Bioer LineGene 9660 equipment. All procedures were conducted in biological safety cabinets located in physically separated areas. Negative controls were used in all reactions.

2.2. cDNA synthesis and multiplex tiling PCR

All 102 positive samples were submitted to a cDNA synthesis protocol [30] using ProtoScript II First Strand cDNA Synthesis Kit. Then, a multiplex tiling PCR was conducted using Q5 High Fidelity Hot-Start DNA Polymerase (New England Biolabs) and CHIKV sequencing primers scheme (divided into two separated pools) designed using Primal Scheme (http://primal.zibraproject.org) [31]. The thermocycling conditions involved 45 cycles, and reaction conditions were previously reported in [31].

2.3. Library preparation and nanopore sequencing

Amplicons were purified using 1x AMPure XP Beads and cleaned-up PCR products concentrations were measured using Qubit™ dsDNA HS Assay Kit on a Qubit 3.0 fluorimeter (ThermoFisher). DNA library preparation was performed on 11 selected samples (selection based on DNA concentration after clean-up being ≥ 4ng) using the Ligation Sequencing Kit (Oxford Nanopore Technologies) and Native Barcoding Expansion 1-12 kit, whose reactions conditions have already been described [31], with the following modifications: both purified PCR products pools were pooled together before barcoding reactions (taking in consideration each amplicon pool DNA concentrations), and one barcode was used per sample in order to maximize the number of samples per flow cell. Sequencing library was generated from the barcoded products using the Genomic DNA Sequencing Kit SQK-MAP007/SQK-LSK208 (Oxford Nanopore Technologies). Sequencing library was loaded onto a R9.4 flow cell, and data was collected for up to 48 hours, but generally less. Acquisition of the final consensus sequences was performed following a previously published protocol [31].

2.4. Phylogenetic and Bayesian analysis

The 11 new genome sequences reported in this study were initially submitted to a genotyping analysis performed by Genome Detective virus tool online (https://www.genomedetective.com/). New sequences were aligned to 242 complete or almost complete CHIKV genome sequences (>10,000 bp), retrieved from NCBI in November 2018 and covering all four existing lineages. We also included 6

sequences from a recent outbreak in Brazilian Amazon region [16], and 16 sequences from Rio de Janeiro recently published [32]. Alignment was performed using MAFFT online program [33]. The complete dataset was assessed for presence of phylogenetic signal by applying the likelihood mapping analysis implemented in the IQ-TREE 1.6.8 software [34]. A maximum likelihood phylogeny was reconstructed from the concatenated dataset (n=242) using IQ-TREE 1.6.8 software under the HKY nucleotide substitution model with 4 gamma categories (HKY+4Γ) which was inferred in jModelTest as the best fitting model [35].

From the ML generated using the concatenated dataset we selected all ECSA taxa from Brazil (ECSA-BR dataset) (n=47). In order to investigate the temporal signal in our CHIKV-ECSA dataset, we regressed root-to-tip genetic distances from this ML tree against sample collection dates using TempEst v 1.5.1 [36]. The ML phylogeny was used as a starting tree for Bayesian time-scaled phylogenetic analysis using BEAST 1.10.2 [37]. In the Bayesian analyses, we used a HKY+4Γ substitution model with a Bayesian skygrid coalescent model with 20 grid points [38]. We computed MCMC duplicate runs of 50 million states each, sampling every 5.000 steps for the ECSA-BR dataset. Convergence of MCMC chains was checked using Tracer v.1.7.1 [39]. Maximum clade trees were summarized from the MCMC samples using TreeAnnotator after discarding 10% as burn-in.

2.5. Epidemiological data assembly

Data of weekly notified CHIKV cases in Brazil were supplied by Brazilian Ministry of Health, and were plotted using the R software version 3.5.1.

2.6. Ethical statement for patient's sample collection

The project was supported by the Pan American World Health Organization (PAHO) and the Brazilian Ministry of Health (MoH) as part of the arboviral genomic surveillance efforts within the terms of Resolution 510/2016 of CONEP (Comissão Nacional de Ética em Pesquisa, Ministério da Saúde; National Ethical Committee for Research, Ministry of Health).

2.7. Data availability

XML files, epidemiological data and new genomic sequences (GenBank under accession numbers MK244632 - MK244642) are available in GitHub repository.

3. Results

To better understand the diversity of CHIKV in some of most affected municipalities from Rio de Janeiro, we generated 11 CHIKV complete genomes (coverage range 62%-83%, mean=73%) from serum samples using a nanopore sequencing approach [31]. Mean threshold cycle value of the generated sequences was 14.86. Most of the isolates are from patients that reside in the municipality of Rio de Janeiro (n=6), and the other five isolates are from four different neighbouring municipalities. Of the sequenced samples, 6 are from female and 5 from male patients. Ten samples are from over 40 years old patients, and one sample is from a 26 years old female. None of the patients had travelled to previous epidemic areas as indicated by the epidemiological data from the local surveillance service. Sequencing statistics and epidemiological details of the sequences generated here are available in Table 1 and 2.

Table 1. Epidemiological data for the sequenced samples.

ID	Sample	Collection date	Sex	Age	Municipality	State
RJ39	Serum	2016-02-19	F	74	Rio de Janeiro	RJ
RJ74	Serum	2016-04-05	M	65	Rio de Janeiro	RJ
RJ83	Serum	2016-04-27	F	88	Rio de Janeiro	RJ
RJ94	Serum	2016-05-02	M	53	Rio de Janeiro	RJ

RJ95	Serum	2016-05-06	F	53	Rio de Janeiro	RJ
RJ96	Serum	2016-05-10	M	54	Rio de Janeiro	RJ
RJ105	Serum	2016-04-19	F	67	São João de Meriti	RJ
RJ111	Serum	2016-04-05	M	57	Mesquita	RJ
RJ125	Serum	2017-03-07	M	70	São Gonçalo	RJ
RJ127	Serum	2017-03-09	F	26	Niteroi	RJ
RJ137	Serum	2018-02-18	F	49	São João De Meriti	RJ

ID=study identifier; Municipality=Municipality of residence; State= RJ-Rio de Janeiro; F=Female; M=Male.

Table 2. Sequencing statistics for the 11 new obtained sequences.

ID	Accession number	Reads	Bases	Coverage (%)	QC	Ct
RJ39	MK244635	58,566	22,587,905	65.7552	1006	14.6
RJ74	MK244632	103,884	39,905,972	62.3518	1006	10.8
RJ83	MK244634	56,876	22,567,764	74.0857	1006	14.1
RJ94	MK244636	64,688	26,009,860	82.5432	1006	14.8
RJ95	MK244633	65,235	25,887,105	74.5852	1006	15.6
RJ96	MK244637	82,622	32,005,376	70.8009	1006	14.7
RJ105	MK244639	51,939	21,750,261	72.5025	1006	15.2
RJ111	MK244638	163,802	63,997,867	76.7863	1006	19.8
RJ125	MK244640	68,806	28,017,200	79.8002	1006	13.3
RJ127	MK244641	73,636	28,692,714	70.1998	1006	15.5
RJ137	MK244642	126,118	48,272,106	75.8805	1006	15.1

ID=study identifier; Accession number=NCBI accession number; QC= Quality control of a flow cell-number of available pores; Ct=RT-qPCR quantification cycle threshold value.

To investigate the phylogenetic relationship of CHIKV in the southeast region of Brazil we estimated Maximum Likelihood (ML) for a dataset comprising 242 international sequences of the four CHIKV lineages. We also investigated the Brazilian ECSA clade in more detail using both ML and Bayesian molecular clock approaches, where we included the recently published ECSA isolates from Brazilian Amazon region [16].

Our ML phylogeny revealed that the newly generated CHIKV isolates from Rio de Janeiro belong to the ECSA lineage, clustering with other Brazilian isolates from the northeastern region that also belong to this lineage (**Figure S1**). These results were confirmed by a genotyping analysis implemented in the Genome Detective virus online tool (https://www.genomedetective.com/).

To investigate the time of introduction of the ECSA lineage in the state of Rio de Janeiro, we performed a sampling time-scaled phylogenetic analysis using a Bayesian Markov Chain Monte Carlo (MCMC) framework [37]. A regression of genetic divergence from root to tip against sampling dates confirmed strong temporal signal (r^2=0.68). Our Bayesian time-scaled phylogenies showed that ten (90.9%) genomes generated in this study formed a well-supported clade (posterior probability support = 1.0) comprising isolates from Rio de Janeiro state, sampled between 2015-2017; while only one isolate from 2018 (isolate RJ137, Accession number: MK244642) was placed in a different cluster with an isolate from Paraíba state (**Figure 1**). Also, an isolate from Sergipe state, northeast Brazil, sampled in 2016, clustered closely to the Rio de Janeiro clade. The observed clustering of the 2016-2017 CHIKV isolates reported here probably reflects geographical proximity of the municipalities where samples were collected in Rio de Janeiro (**Figure 2**). Although the RJ105 and RJ137 isolates are both from the same

municipality of São João de Meriti, they have different sampling collection dates, 2016 and 2018, respectively.

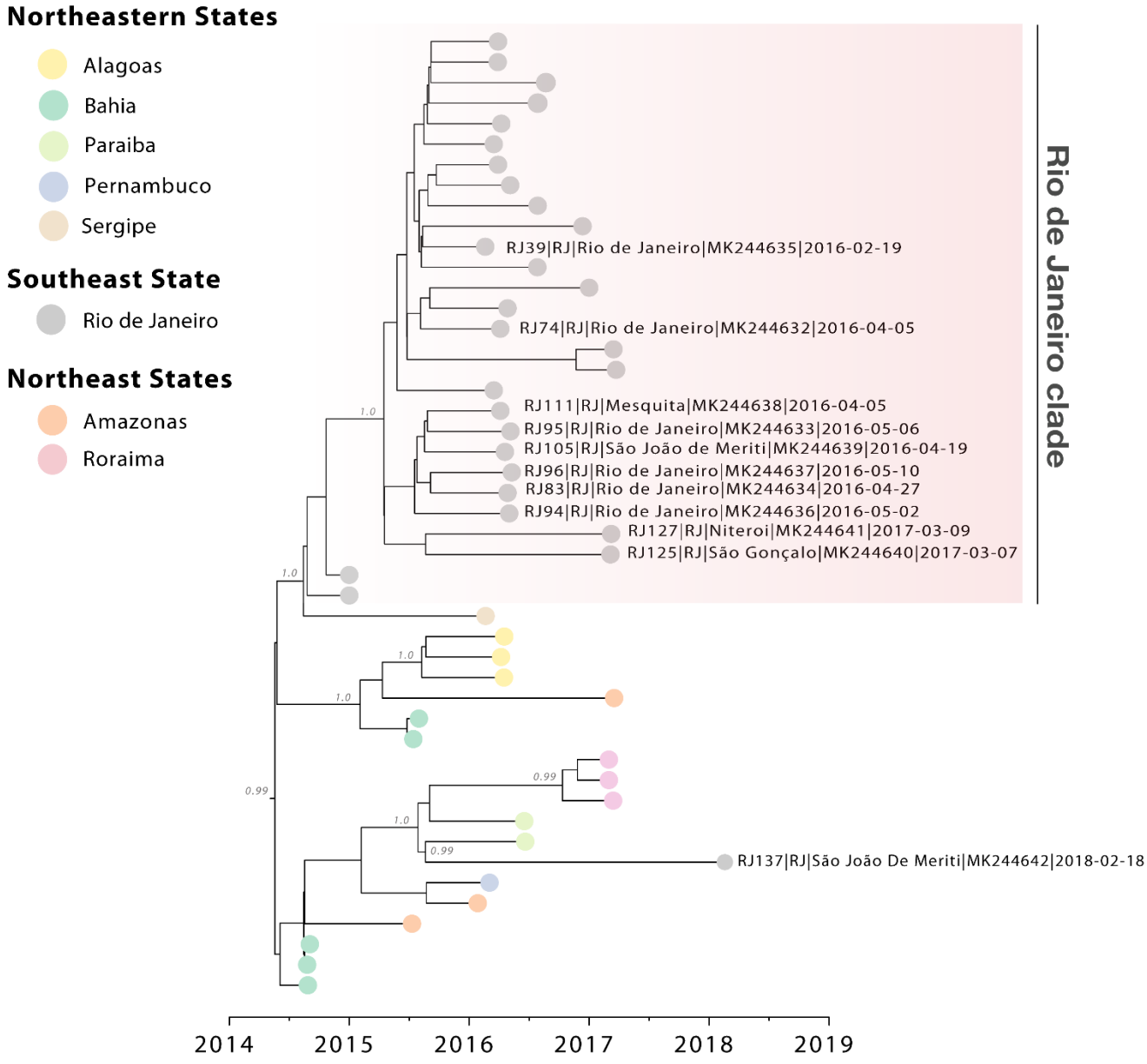

Fig 1. Molecular clock phylogeny of the CHIKV circulating in Rio de Janeiro state. Molecular clock phylogeny obtained using 11 new CHIKV near-complete genome sequences from the 2016-2018 epidemic in Rio de Janeiro, including 36 publicly available Brazilian CHIKV-ECSA lineage sequences. Numbers along branches represent clade posterior probability >0.90. Colours represent different locations.

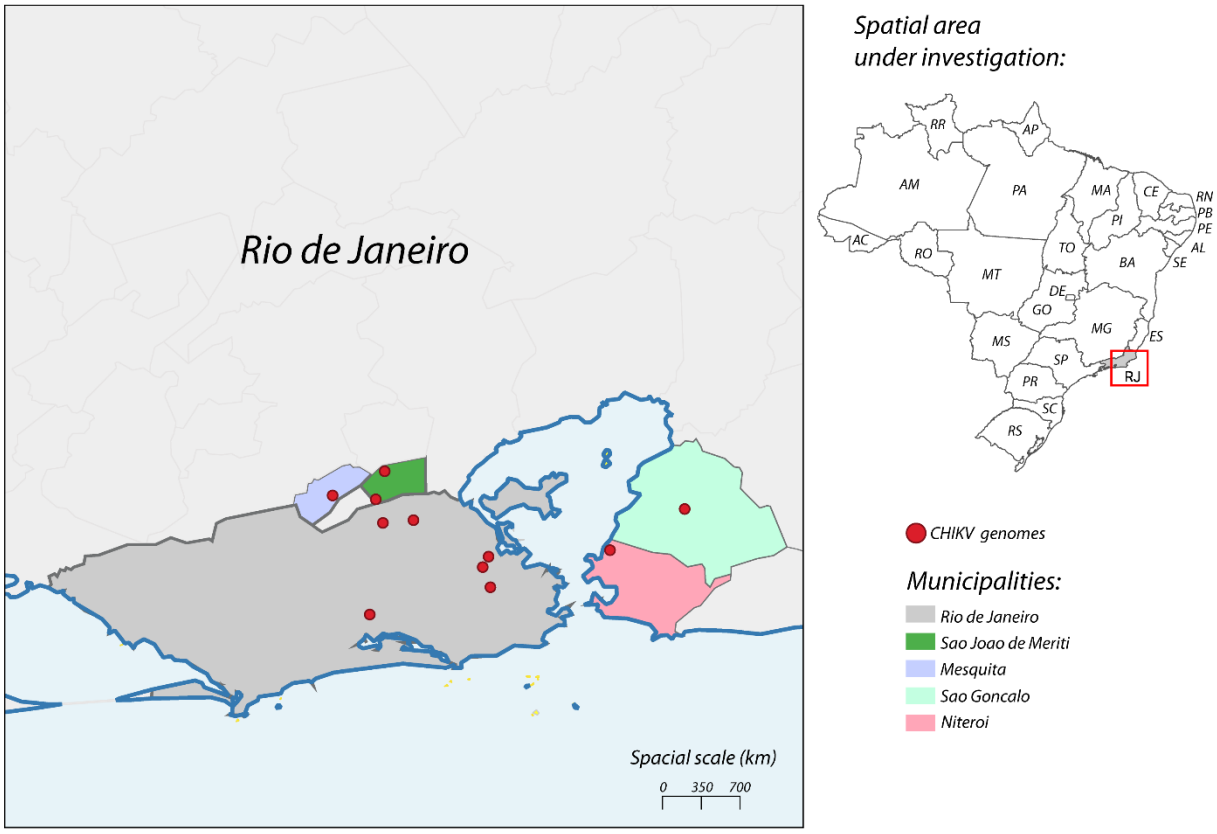

Figure 2. Map of Rio de Janeiro state. Red circles show sampling locations of the isolates generated in this study.

We estimated the date of the most recent common ancestor (tMRCA) of the Rio Janeiro clade to be around July 2014 (95% Bayesian credible interval: March to late November 2014) (**Figure 1**). This estimation suggests the ECSA lineage circulated unnoticed for approximately 16 months before the first reports of CHIKV autochthonous cases in Rio de Janeiro, around November 2015 [24,25]. If we take into account the upper uncertainty interval of our dating estimates, we find that CHIKV-ECSA was introduced in Rio de Janeiro at least one year before its first detection in the state.

Figure 3 shows cumulative case numbers from the northeastern region, southeastern (without computing numbers from Rio de Janeiro state), and from Rio de Janeiro state alone. While the northeastern region presented intense epidemic waves in early 2016 and 2017, reporting 239,714 and 141,312 cases, respectively, the southeastern region reported a lower number of cases in those years, accounting for 28,556 and 32,314 notified cases in 2016 and 2017, respectively. In Rio de Janeiro, after an epidemic peak in April 2016, a small number of cases were reported in 2017. Yet, CHIKV infections increased again in 2018, with an epidemic peak at epidemic week 15 and a total number of 39,208 cumulative cases until epidemic week 44. Rio de Janeiro's epidemic curve also shows that the state alone accounted for 66.8% and 69.7% of the cumulative cases registered in the southeastern region in 2016 and 2018, respectively. While a reduction in the number of cases was observed in the northeastern region in 2018, the southeastern region kept experiencing increased CHIKV transmission (**Figure 3**). In general, CHIKV cases peak in the early months of represented years, consistent with virus dynamics predictions made previously (**Figure 3**) [15,40].

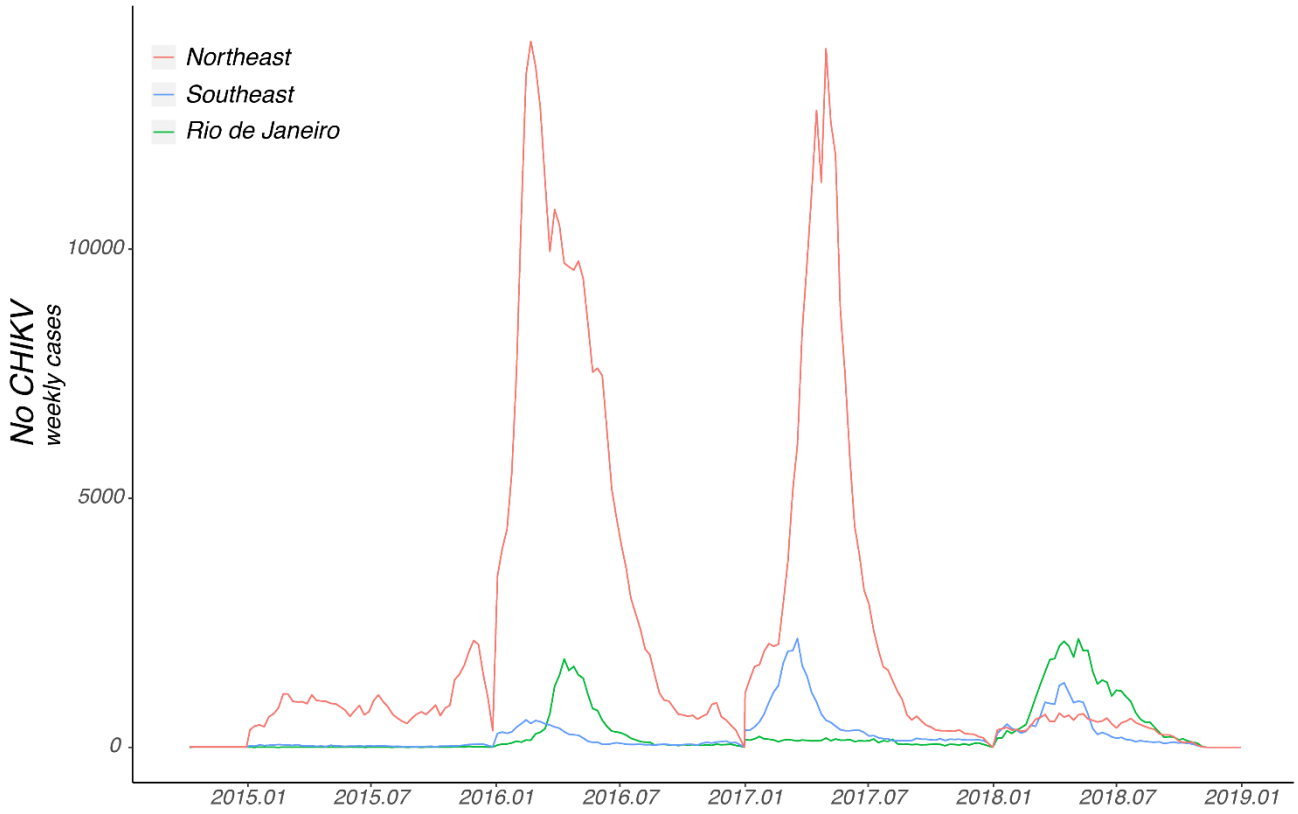

Figure 3. Reported cases of Chikungunya fever by epidemiological week, northeast and southeast Brazil, 2015-2018. Colours represent different locations.

We found that epidemic waves from Rio de Janeiro state and the northeastern region displayed a synchronicity during the period from late 2015 to early months of 2016, and there is a high human mobility between the two regions [23]. From combined epidemiological and genetic data, we found that the northeast Brazil is the most likely source location of the ECSA-lineage strain that was circulating unnoticed during the 2016 epidemic in the state of Rio de Janeiro.

4. Discussion

Rio de Janeiro has had an explosive epidemic of CHIKV from 2016 to 2018. Here, by performing real-time portable nanopore sequencing, we generated 11 near-complete new CHIKV genome sequences from 2016-2018 collected in several municipalities in the Rio de Janeiro state. The new generated genomic data allowed us to estimate the introduction date of the ECSA lineage in Rio de Janeiro around July 2014, suggesting an undetected circulation of the virus for 16 months before the first reports of CHIKV autochthonous transmission in Rio de Janeiro [24,25]. Moreover, our data suggests that the Rio de Janeiro epidemic may have resulted from at least two separate introductions from the northeastern region, where the ECSA lineage was first detected in late 2014 [15].

Genome sequencing has become a powerful tool for tracking virus transmission. Our results confirm the introduction of the CHIVK ECSA in Brazil in the northeast Brazil in mid-2014 [15]. After its introduction, the risk of the ECSA lineage to spread from Bahia to other Brazilian states was estimated to be higher in the northeast and southeast regions due to abundance of competent vectors and climate conditions in those geographical areas [15,40]. CHIKV ECSA lineage spread to other northeastern states, such as Paraiba, Sergipe, Pernambuco and Alagoas states, causing large outbreaks until it arrived in the southeast region, where this lineage caused a total of 25,245 suspected cases in 2016 alone [17,18,21,22,32]. The ECSA lineage also reached the Brazilian Amazon region in 2017 [16].

This pattern of arbovirus cryptic transmission prior to first official reports have been observed before for dengue and zika virus epidemics [41,42]. Genetic and epidemiological analysis indicated the northern of Brazil acted as a source region for dengue, or as stepping-stone spot for the dissemination of arbovirus to other areas of the country, which might have been influenced by the increased human mobility and vector suitability [42]. Similarly, zika virus strains from the 2015-2016 epidemic in Brazil circulated cryptically in the northeastern region and, from there, disseminated to other Brazilian states and countries before its first detection in the Americas [41]. Climatic data suggests that both the northern and northeastern regions are able to sustain year-round transmission of mosquito-borne viruses, making them putative sources of transmission [41].

In our sample we have not found mutations that were previously associated with increased transmission in *Aedes* mosquitos. However, future investigation of the fitness, viral infectivity and evolution of CHIKV in mosquito populations, together with continued genomic surveillance, will determine whether the upsurge in the number of cases in Rio in 2018 was due to the acquisition of specific mutations that increase replication rates in local *Aedes* spp. mosquitoes.

Together, our results indicate CHIKV ECSA lineage might have spread to Rio de Janeiro from the northeastern region of Brazil, and shed light on the epidemiological dynamics of the virus in urban areas of the Rio de Janeiro, where the virus remained undetected by several months before reporting of the first local transmission cases in that state. In conclusion, our study shows that genomic data generated by real time portable sequencing technology can be employed to assist public health laboratories in monitoring and understanding the diversity of circulating mosquito-borne viruses.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Maximum likelihood phylogeny of chikungunya virus.

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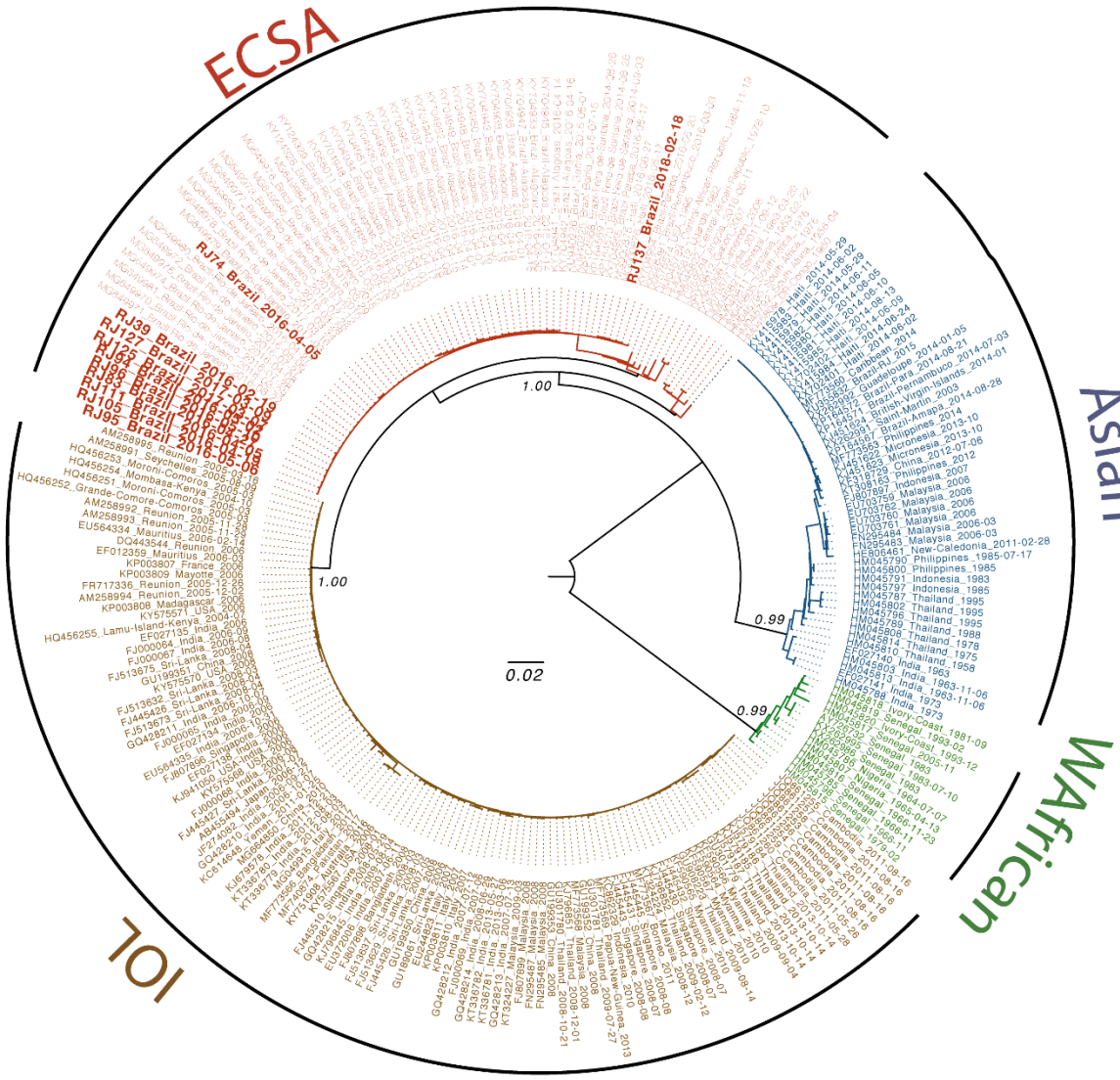

Supplementary Figure 1. Maximum likelihood phylogeny using 11 new CHIKV near-complete genome sequences from Rio de Janeiro generated in this study (in bold) plus 242 CHIKV reference sequences from other genotypes (East-Central South African; Indian Ocean Lineage; Asian; West African). The scale bar is in units of substitutions per site (s/s). Node labels indicate bootstrap support values.

6 DISCUSSÃO

As doenças infecciosas transmitidas por mosquitos têm ultimamente integrado manchetes mundiais desde o surgimento de surtos de arbovírus em grandes áreas urbanas. As mudanças climáticas, indicadas pelas mudanças nos regimes de temperatura e precipitação, têm sido implicadas a influenciar a adequabilidade para a transmissão de doenças transmitidas por vetores, como a dengue (WATTS *et al.*, 2018). No Brasil, a emergência do ZIKV e do CHIKV, em curto espaço de tempo, constitui um ônus preocupante para o sistema público de saúde e para o setor econômico devido à rápida disseminação em novas áreas geográficas, e às características clínicas associada à infecção (ZANOTTO; LEITE, 2018).

Após uma epidemia registrada em 2015 na região Nordeste, os primeiros casos de transmissão autóctone do CHIKV no Rio de Janeiro foram registrados em novembro de 2015 (RIO DE JANEIRO, 2016; BRASIL, 2015). As transmissões continuaram e causaram uma epidemia em 2016 com 18.516 casos reportados no estado do Rio de Janeiro, que sozinho foi responsável por 66.8% dos casos reportados na região Sudeste naquele ano. As primeiras informações sobre as características genéticas das cepas circulantes no Rio de Janeiro durante a epidemia em 2016 revelaram que os isolados analisados pertenciam à linhagem ECSA e estavam agrupados com outros isolados da região Nordeste do país (CUNHA, M. S. *et al.*, 2017; SOUZA *et al.*, 2017). Além disso, essas cepas sequenciadas não apresentavam a mutação A226V, associada à adaptação ao vetor *Aedes albopictus*.

Apesar do elevado números casos reportados, existe limitada informação sobre a epidemiologia genômica do CHIKV da epidemia no Rio de Janeiro em 2016. Os estudos disponíveis apenas determinaram o genótipo da cepa circulante a partir de análises filogenéticas usando sequências parciais do gene E1 (DE SOUZA *et al.*, 2018). No presente estudo, nós geramos 11 novas sequências genômicas do CHIKV circulante no Rio de Janeiro, através do sequenciamento por nanoporos em tempo real. A reconstrução filogenética realizada neste estudo, revelou que estes 11 novos isolados pertencem a linhagem ECSA, corroborando estudos anteriores.

As amostras biológicas utilizadas neste estudo são provenientes, em sua maioria, de pacientes residentes no município do Rio de Janeiro (n=6), enquanto as amostras restantes (n=5) são oriundas de munícios vizinhos. Verificamos que a maioria (n=10) desses pacientes possuíam idade acima de 40 anos. Apesar do baixo número amostral, essa tendência de ocorrência dos casos em pacientes acima de 40 anos tem sido relatada em outros estudos prévios (DE SOUZA *et al.*, 2018; FULLER *et al.*, 2017). Tal tendência pode ser explicada pela possível

existência de um viés no sistema de notificação de casos, uma vez que pacientes de meia-idade ou idosos são mais prováveis a procurar atenção médica, ou porque eles são mais suscetíveis à infecção pelo vírus devido à uma saúde mais comprometida em geral (FULLER *et al.*, 2017).

Estima-se que a linhagem ECSA tenha sido introduzida no Brasil por volta de junho de 2014 em Feira de Santana, Bahia, a partir de um indivíduo que tinha retornado de Angola, como indicado por estreita relação filogenética entre os isolados brasileiros e um isolado de Angola (NUNES *et al.*, 2015). Um ano após a introdução do CHIKV no Brasil, um estudo de soroprevalência em áreas urbanas de duas cidades da Bahia, Feira de Santana e Riachão do Jacuípe, mostrou que mais de 45% da população analisada tinha anticorpos para CHIKV, e a taxa de casos sintomáticos era maior entre pacientes com idades entre 40-59 anos e acima 60 anos (DIAS *et al.*, 2018). Além disso, manifestações clínicas da doença a longo prazo foram observadas em mais de 60% da população sintomática analisada (DIAS *et al.*, 2018).

Após introdução, o risco de propagação da linhagem da ECSA da Bahia para outros estados brasileiros foi estimado sendo mais alto nas regiões Nordeste e Sudeste, devido à abundância de vetores competentes e às condições climáticas naquelas áreas geográficas (FULLER *et al.*, 2017; NUNES *et al.*, 2015). FULLER *et al.* (2017) observaram que em 2016 no Rio de Janeiro a ocorrência de períodos de chuva intensa precedia o surgimento dos casos de infecção pelo CHIKV em 3 semanas, sugerindo que períodos de chuva são preditores de surtos potenciais.

Como previsto, foram então observados eventos de dispersão da linhagem ECSA indicados pelos surtos registrados em outros estados do Nordeste, tais como Paraíba, Sergipe, Pernambuco e Alagoas (TANABE *et al.*, 2018a). Somente na região Nordeste foram registrados 239.714 casos prováveis de infecção por CHIKV em 2016. Ao chegar na região Sudeste, o CHIKV foi responsável por um total de 25.245 casos prováveis somente em 2016. Surtos causados pelo CHIKV também foram relatados na região Norte e Centro-Oeste em 2016 e 2017. Casos importados da doença também foram relatadas no Rio de Janeiro em 2010 e 2012. ALBUQUERQUE *et al.* (2012) relatou um caso de um paciente que, após retornar de uma viagem à Indonésia, buscou atenção médica em 2010 devido à apresentação de febre e dores articulares. A soro conversão para o CHIKV deste paciente foi confirmada por Elisa (IgM) e teste de inibição da hemaglutinação (ALBUQUERQUE *et al.*, 2012). Um segundo relato de caso importado no Rio de Janeiro entre viajantes que retornaram do Caribe entre 2014-2015 (CONTEVILLE *et al.*, 2016). Neste caso, foram obtidas sequências parciais do gene E1, cuja analise filogenética revelou que os quatros isolados pertenciam à linhagem Asiática (CONTEVILLE *et al.*, 2016). A identificação de casos importados de chikungunya no Rio de

Janeiro antes de 2016, destaca o potencial de impacto da mobilidade humana na dinâmica de dispersão de vírus transmitidos por artrópodes (CONTEVILLE *et al.*, 2016). Tal observação foi relatada por NUNES *et al.* (2014) após investigarem a dispersão espaço-temporal da dengue no Brasil. Eles estimaram que o transporte aéreo de humanos e/ou mosquitos determinavam significativamente a dispersão do vírus da dengue no Brasil (NUNES *et al.*, 2014).

Para investigar a data de introdução da linhagem ECSA no Rio de Janeiro, nós realizamos a reconstruções filogenéticas, empregando a abordagem do relógio molecular, usando um *dataset* (conjunto de sequências genômicas do CHIKV) atualizado que, além de conter 30 sequências publicamente disponíveis, continha também as 11 novas sequências genômicas gerada neste estudo e 6 sequências oriundas de um surto recente na região amazônica (NAVECA *et al.*, 2018). As análises filogenéticas estimaram que a linhagem ECSA foi introduzida no estado do Rio de Janeiro por volta de julho de 2014. Isto sugere que cepas da linhagem ECSA circularam de forma despercebida dos serviços de vigilância por 16 meses antes dos primeiros relatos oficiais de transmissão autóctone no Rio de Janeiro. Combinando dados da curva epidêmica do CHIKV da região Nordeste e Sudeste com os resultados das análises filogenéticas, podemos inferir que a região Nordeste é a provável fonte de origem das cepas da linhagem ECSA que circularam despercebidas no Rio de Janeiro até provocar a epidemia em 2016.

Tal dinâmica de dispersão marcada por um período de transmissão críptica do vírus CHIKV tem sido identificada para o CHIKV circulante na região Norte do Brasil, assim como para outros arbovírus emergentes e reemergentes. NAVECA *et al.* (2018) reportaram que cepas da linhagem ECSA, oriundas da região Nordeste, dispersaram em direção à cidade de Boa Vista, Roraima, onde circularam despercebidamente desde julho de 2016 até provocar uma epidemia na cidade em 2017. Além disso, argumenta-se que a linhagem ECSA, após estabelecer-se em Boa Vista em 2016, substituiu com sucesso a linhagem Asiática previamente presente naquela região desde 2014 (NAVECA *et al.*, 2018). Da mesma forma, as cepas do ZIKV da epidemia de 2015-2016 no Brasil circularam de forma críptica na região Nordeste por mais de um ano e, a partir desta região, disseminaram-se para outros estados brasileiros e países antes de sua primeira detecção nas Américas (FARIA *et al.*, 2017b).

Análises genéticas e epidemiológicas, usando isolados do vírus da dengue (DENV) coletados durante a epidemia de dengue no Rio de Janeiro em 2012, indicaram que a região Norte do Brasil atuou como fonte de origem do DENV, ou como *stepping-stones* (‘trampolins’ ou ‘pontos de conectividade’ em português) para a disseminação do arbovírus para outras áreas do país, o que pode ter sido influenciado pelo aumento da mobilidade humana e adequação do

vetor (FARIA *et al.*, 2017a). Dados climáticos sugerem que tanto as regiões Norte e Nordeste são capazes de sustentar durante todo o ano a transmissão de vírus transmitidos por mosquitos, o que as tornam fontes putativas de transmissão de cepas virais para outras regiões do Brasil (FARIA *et al.*, 2017b).

Nas sequências genômicas do CHKV geradas neste estudo, não encontramos as mutações E1-A226V e E2-L210Q, previamente associadas ao aumento da transmissão em mosquitos *Aedes albopictus* (SCHUFFENECKER *et al.*, 2006; TSETSARKIN; WEAVER, 2011). No entanto, investigações futuras sobre a aptidão, infectividade viral e evolução do CHIKV em populações de mosquitos, juntamente com a continuidade da vigilância genômica, esclarecerão se o aumento no número de casos no Rio em 2018 foi devido à aquisição de mutações específicas que aumentam as taxas de replicação em mosquitos *Aedes* spp. locais.

As tecnologias de sequenciamento de genoma completo têm sido empregadas com sucesso no diagnóstico rápido e no rastreamento de doenças infeciosas (GARDY; LOMAN, 2017). O uso de sequências de genoma completo tem permitido a compressão detalhada da dinâmica de transmissão viral durante epidemias (TANG *et al.*, 2017). Neste estudo, nós geramos 11 novas sequências genômicas do CHIKV através do sequenciamento por MinION, que nos permitiram realizar inferências filogenéticas sobre a data do evento de introdução da linhagem ECSA no estado do Rio de Janeiro.

A tecnologia de sequenciamento por nanoporos empregada no sequenciador MinION tem permitido a geração de uma grande quantidade dados genômicas de forma muito rápida. O MinION é um sequenciador que cabe no bolso, e sua a portabilidade associada à geração de grandes quantidades de dados em tempo real torna-o uma ferramenta útil para ser empregada nas respostas rápidas à surtos e epidemias, mesmo em locais difícil acesso ou com recursos limitados (GARDY; LOMAN, 2017). Dessa forma, o MinION pode fornecer dados genômicos sobre os vírus circulantes aos agentes de saúde pública para que estes possam implementar medidas mais eficientes para o controle da epidemia (FARIA *et al.*, 2017b). De fato o MinION já foi empregado em campo nas situações de resposta à epidemias na África e no Brasil. Durante a epidemia de Ebola no Oeste da África em 2015, o sequenciamento por MinION foi empregado para gerar 125 sequências do genoma do vírus ebola que permitiram identificar duas linhagens do vírus, cujas relações filogenéticas indicaram eventos de transmissão através das fronteiras da Guiné e Serra Leoa (QUICK *et al.*, 2016). Já no Brasil, o MinION foi usado para gerar 64 novas sequências do genoma do vírus da febre amarela isolados em Minas Gerais, São Paulo e Rio de Janeiro durante o surto da doença em 2017 (FARIA *et al.*, 2018). As novas sequências do vírus da febre amarela permitiram a realização de análises filogenéticas que revelaram uma

fase inicial de transmissão selvática do vírus e a expansão espacial em direção à áreas previamente livres da doença, indicando que a epidemia era causada por infecções humanas adquiridas durante visitas à áreas florestais (FARIA *et al.*, 2018).

7 CONCLUSÕES

Neste trabalho realizamos o sequenciamento de onze isolados do vírus chikungunya circulante no Rio de Janeiro. As análises filogenéticas indicaram que estes isolados pertencem ao genótipo ECSA e se agrupam em um clado com outros isolados do Rio de Janeiro. As analises filogenéticas também indicaram que o evento de introdução da linhagem ECSA no Rio de Janeiro provavelmente ocorreu em julho de 2014.

Os resultados deste estudo indicam que a linhagem CHIKV-ECSA pode ter sido introduzida no estado do Rio de Janeiro a partir da região Nordeste do Brasil, e lançam luz sobre a dinâmica epidemiológica do vírus em áreas urbanas do Rio de Janeiro, onde o vírus circulou sem ser detectado por vários meses antes dos primeiros relatos de casos de transmissão autóctone no estado. Em conclusão, esse estudo mostra que dados genômicos gerados por tecnologia de sequenciamento portátil em tempo real podem ser empregados para auxiliar os laboratórios de saúde pública no monitoramento e na compreensão da diversidade de vírus circulantes transmitidos por mosquitos.

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APÊNDICE - Artigos publicados não relacionados ao tema da dissertação

Artigo original 1

Emergence of the Zika virus Asian lineage in Angola

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Research In Context

Evidence before this study

We searched PubMed without language restrictions using the keywords 'Zika' and 'Africa' for papers published to October 2018. We also checked available 'Situation Report' publications from WHO for evidence of Zika virus (ZIKV) or congenital Zika disease in Africa. ZIKV African lineage has been detected within Africa since the mid 20th century, yet evidence for spread of the ZIKV Asian lineage within Africa is limited. Two countries in Africa (Cabo Verde and Angola) have reported ZIKV cases that are believed to be caused by a newly introduced Asian lineage virus. Sequence data are critical for confirming and understanding the spread of ZIKV Asian lineage within Africa, but these data are currently limited to a single 193bp fragment of the ZIKV NS1 gene from Angola. In addition, whilst epidemiological data on ZIKV and suspected microcephaly cases have been reported in detail from Cabo Verde, data from Angola are extremely limited.

Added value of this study

We provide a detailed report of detected ZIKV acute cases and suspected microcephaly cases in Angola. We sequence ZIKV genomes from three acutely infected cases. These represent the first three Asian lineage genomes available from Africa, one of which was acquired from a baby with confirmed microcephaly. Analysis of these sequences suggests that ZIKV may have been introduced to Angola between July 2015 and June 2016, after which it likely circulated for at least one year. This provides the first genetic confirmation of autochthonous ZIKV Asian lineage transmission within Africa. We suggest that the virus was more likely introduced to Angola directly from Brazil, rather than from Cabo Verde. Our analyses from Angola, only the second African country to report presence of the Asian virus lineage, therefore improve our understanding of the extent and clinical impact of ZIKV Asian lineage in the continent.

Implications of all the available evidence

The circulation of ZIKV Asian lineage within parts of sub-Saharan Africa is concerning given the potential for continued viral spread across much of the continent. Available evidence suggests that ZIKV has circulated and caused cases of microcephaly in Cabo Verde and in Angola. Detecting additional ZIKV transmission using only clinical data on suspected microcephaly or clusters of mild illness may be challenging in countries where systems for reporting birth defects are limited and infectious disease burden is high. Further spread of the ZIKV Asian lineage would likely not be detected unless molecular surveillance systems for ZIKV are implemented to routinely monitor ZIKV transmission in Africa. Implementation of such a surveillance system is especially important in countries that are linked by high human mobility to areas that have experienced recent or ongoing outbreaks of ZIKV.

Abstract

Background

Zika virus (ZIKV) infections and suspected microcephaly cases have been recently reported in Angola, but no data are available on the origins, epidemiology, and diversity of the virus.

Methods

Serum samples from 54 suspected ZIKV cases, 76 suspected microcephaly cases, and 24 mothers of infants with suspected microcephaly were received by the Angolan Ministry of Health. Computed tomographic brain imaging and serological assays (PRNT) were conducted on one microcephalic infant. All sera were tested for ZIKV by RT-qPCR. 349 samples from HIV+ patients and 336 samples from patients suspected of chikungunya virus or dengue virus infection were also tested. Portable sequencing was used to generate Angolan ZIKV genome sequences, including from a ZIKV+ neonate with microcephaly born in Portugal to an Angolan resident. Genetic and mobility data were analysed to investigate the date of introduction and geographic origin of ZIKV in Angola.

Findings

Four autochthonous cases were ZIKV positive via RT-qPCR, with all positive samples collected between December 2016 and June 2017. Viral genomes were generated for two of these cases, and from the neonate with microcephaly identified in Portugal. Genetic analyses and other data indicate that ZIKV was introduced to Angola from Brazil between July 2015 and June 2016. This introduction likely initiated local ZIKV circulation in Angola that continued until June 2017. The scanned microcephaly case showed brain abnormalities consistent with congenital Zika syndrome and serological evidence for maternal ZIKV infection.

Interpretation

Our analyses confirm the autochthonous transmission of the ZIKV Asian lineage in continental Africa. Conducting ZIKV surveillance throughout Africa is critical in the light of presented evidence for autochthonous ZIKV transmission in Angola, and associated microcephaly cases.

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Background

Zika virus (ZIKV) is an RNA virus of the *Flavivirus* genus that is primarily transmitted by *Aedes sp.* mosquitos. ZIKV is classified into two distinct lineages, the African and the Asian genotypes. Serological studies suggest that ZIKV may be widespread across Africa,[1] yet the interpretation of serological assays is challenging due to extensive cross-reactivity among related flaviviruses.[2] Prior to 2007, ZIKV was identified in only 14 human cases in Africa and Asia[3] and infection was thought to cause only mild symptoms, including fever, headache and rash.[1] However, since 2013, the Asian genotype of ZIKV spread to locations in the Pacific and the Americas, resulting in > 800,000 suspected and confirmed cases of Zika virus disease.[4] The discovery that ZIKV infection during pregnancy can cause severe birth defects and other adverse outcomes,[2] led to a research response that, to date, has been focused largely on the Americas.

Hundreds of millions of people in sub-Saharan Africa live in areas thought to be suitable for ZIKV transmission.[5] Despite evidence of past transmission in Africa of the African genotype of ZIKV,[3] there is a lack of data on recent ZIKV transmission from the continent. Since 2015, three African countries (Guinea-Bissau, Cabo Verde and Angola) have reported suspected human cases of ZIKV and clusters of suspected microcephaly cases.[6–9] Understanding these ZIKV outbreaks is critical for safeguarding public health in Africa and elsewhere.

Obtaining accurate ZIKV surveillance data is challenging because most cases are asymptomatic, symptoms are mild and non-specific, and infections are frequently misclassified.[2] In the absence of complete surveillance data, virus genome sequences have proven important for investigating ZIKV transmission.[10] The current lack of ZIKV genomes from Africa hinders our understanding of the re-emergence of ZIKV in the continent.

Here, we provide the first comprehensive study of the recent ZIKV outbreak in Angola. We report and analyse confirmed ZIKV infected cases and suspected microcephaly cases. Computed tomographic (CT) imaging of one infant confirms the presence of brain abnormalities consistent with congenital Zika syndrome. The mother of this infant was serologically positive for ZIKV infection. We tested available serum samples for ZIKV and used portable sequencing technology to generate three Asian lineage ZIKV genome sequences from individuals infected in Angola, including one individual born with microcephaly.[11] Phylogenetic analyses reconstruct the timings and geographic origin of the Angolan ZIKV outbreak. We corroborate our conclusions using multiple data sources, including the climatic suitability of the *Aedes* mosquito vector, international data on ZIKV incidence and airline passenger numbers. Our study is the first detailed analysis of the introduction to and circulation of ZIKV Asian lineage in Africa.

Materials and Methods

Zika virus surveillance and testing

The Ministry of Health of Angola conducted diagnostic testing of suspected cases of acute ZIKV infection at the Instituto Nacional de Investigação em Saúde (INIS) since late December 2016. Between late December 2016 and November 2018, 54 serum samples were collected from patients following clinical examination. Viral RNA was extracted from these samples using QiaAmp Viral RNA Mini Kits. RT-qPCR testing for the presence of ZIKV, chikungunya virus (CHIKV) and dengue virus (DENV) RNA was conducted using CDC Trioplex kits [12] or Bio-Manguinhos (Fiocruz) ZDC RT-qPCR kits on an Applied Biosystems 7500 Fast machine.

On 1st January 2018, it became mandatory for health providers in Angola to notify the Angolan Direcção Nacional de Saúde Pública (DNSP) of infants with suspected microcephaly (additional **Supplementary Materials and Methods** for case identification details). In total, 76 samples were received from infants between January 2017 and November 2018, with a median time of sample collection of 24 days after birth (mean 65 days, range 0-315 days) (**Figure S1**). Serum samples from 24 mothers who gave birth to infants with suspected microcephaly were also collected. All sera were tested by PCR as described above. Serological testing for toxoplasmosis, rubella and cytomegalovirus (ToRC pathogens) was conducted on eight serum samples from infants with suspected microcephaly using the ViDAS ToRC panel.

An additional 685 serum samples were investigated for ZIKV. Approximately half (n=336) were collected between January and October 2018 from patients with suspected DENV or CHIKV acute infections. These samples were PCR tested for DENV, CHIKV and ZIKV as part of routine diagnosis. The remaining 349 samples were collected between April and November 2017 by the Instituto Nacional de Luta Contra SIDA as part of a separate study investigating antiviral drug resistance among HIV+ patients in Luanda. These samples were retrospectively tested for ZIKV only, to improve the chance of detecting ZIKV cases.

Patients provided written consent forms agreeing that samples could be used for research purposes. Residual samples from Angola were used for viral genomic sequencing with the approval of the National Ethical Committee, Ministry of Health, Angola.

Brain imaging of Angolan infant with microcephaly

CT imaging was conducted on one female child who was born in early August 2017 with suspected microcephaly. The child was born in Angola but diagnosed later at the Microcephaly Reference Centre IPESQ (Campina Grande, Brazil), following travel from Angola to Brazil in early November 2018. Plasma samples were taken from both the mother and child on 30th November 2018 and tested for ZIKV and DENV infection using EuroImmun IgG ELISAs. Plaque reduction neutralisation tests (PRNT) were performed using standard protocols (**Supplementary Materials and Methods**) to quantitate neutralising antibodies

against ZIKV for both the mother and the child. CT scans were conducted to assess whether the child's neurological damage was consistent with congenital Zika syndrome. CT imaging was performed with a 64-section CT scanner (Philips Brilliance).

ZIKV genome sequencing

Sequencing of the ZIKV genome coding regions was attempted using an Oxford Nanopore (ONT) MinION device following previously published protocols.[13] Virus genome sequencing of RT-qPCR+ samples was attempted at INIS (Luanda, Angola). Generation of consensus sequences was undertaken using a previously published and validated bioinformatics pipeline (**Supplementary Materials and Methods**).[13]

Whilst investigations in Angola were underway, ZIKV RNA was detected in the urine of a neonate with microcephaly born in Portugal in November 2017 to a resident of Angola. Details of this case, a short (193 nt) fragment of the ZIKV NS1 gene from the neonate, and ethical permissions are described elsewhere.[11] Virus genome sequencing of that strain was attempted using the methods described above, at the Instituto Nacional de Saúde Doutor Ricardo Jorge, Portugal.

Phylogenetic analyses

The new ZIKV genomic sequences were aligned with 390 other ZIKV genomes publicly available from GenBank using Muscle.[14] Maximum likelihood phylogenies were estimated using RAxML v8.2.11,[15] and molecular clock phylogenies were generated in BEAST v1.10.3 [16] (see **Supplementary Materials and Methods** for further details).

Origins of Angolan ZIKV outbreak

To corroborate the results of our genomic analyses, we investigated countries that could have exported ZIKV to Angola using additional data sources. Specifically, two factors were considered here as contributing to a high risk of ZIKV introduction to Angola: (i) countries with a high local incidence of ZIKV, and (ii) countries with high numbers of air passengers travelling to Angola. The monthly number of passengers to Angola from countries reporting ZIKV was estimated using worldwide ticket sales data from the International Air Transport Association during 2015-2017.[17] The average incidence rate per person per week in each country was estimated from surveillance data on the number of suspected and confirmed cases of ZIKV per epidemiological week.[7,18] See **Supplementary Materials and Methods** for details.

Role of the funding source

The sponsors of the study had no role in study design, data collection, analysis, or writing of the report. The corresponding author had full access to all data used in the study and was responsible for submitting the article for publication.

Results

Confirmed autochthonous ZIKV transmission in Angola

We collated evidence of ZIKV in Angolan residents or in travellers returning from Angola (**Figure 1**). ZIKV infection was first detected by seroneutralisation assay in a traveller returning from Angola in September 2016.[19] In late December 2016, RT-qPCR testing of suspected acute ZIKV infections began at INIS. Between December 2016 and June 2017, four ZIKV cases were confirmed locally by Trioplex RT-qPCR. Three of these cases were identified among 54 symptomatic patients with suspected acute ZIKV infections. The remaining case (from June 2017) was detected through retrospective screening of an unrelated archive of 349 HIV+ samples obtained during April to November 2017. No additional ZIKV cases were detected among 336 samples collected from patients with suspected DENV or CHIKV infections during 2018. None of the ZIKV-positive samples were positive for DENV or CHIKV. Of the 51 ZIKV-negative samples from suspected ZIKV cases, three were positive for DENV-2, and seven were positive for CHIKV.

The presence of ZIKV in Angola during 2016 and 2017 was also indicated by the detection of ZIKV in two travellers; one to Portugal [11] and another returning to Taiwan [20] (**Figure 1**). Whilst suspected cases were reported across Angola (**Figure 2**), all confirmed cases were residents of (or had travel history involving) Luanda or the neighbouring province of Bengo (**Figures 1 & 2**).

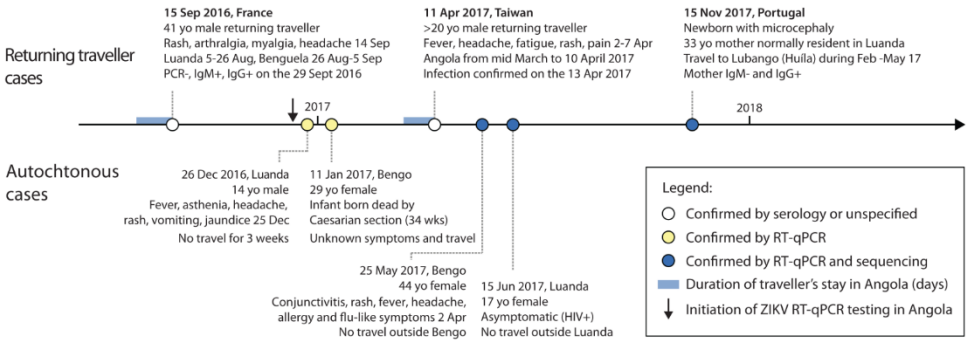

Figure 1. Confirmed Angola-associated ZIKV cases. Patient data and symptoms are reported where known. Travellers returning from Angola are shown above the line and local cases are shown below.

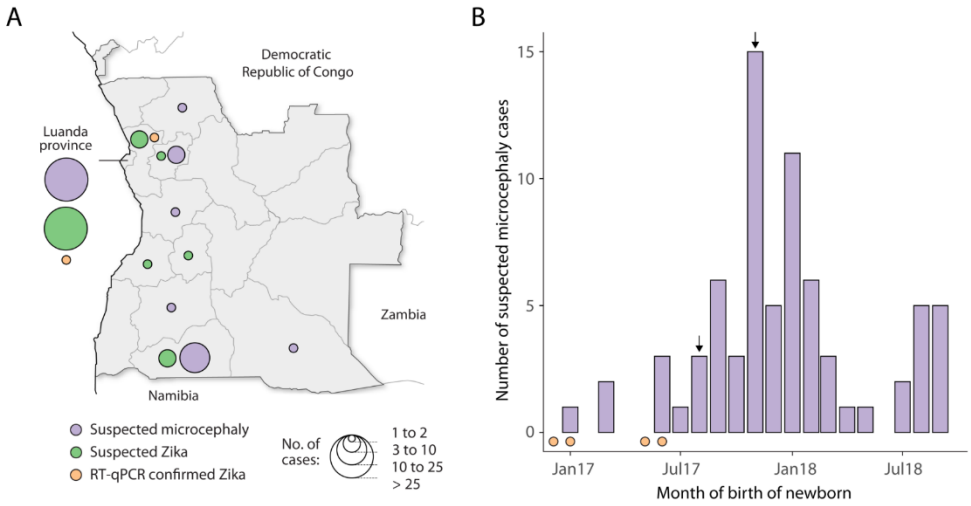

Figure 2. Suspected acute ZIKV cases and suspected microcephaly cases identified in Angola. A) Spatial distributions of suspected ZIKV cases and suspected microcephaly cases. Circle sizes indicate the number of cases per province. B) Time series of suspected microcephaly cases in Angola. Dates of birth, rather than report dates, are shown, so cases are included only if date of birth was recorded (96% of cases). Arrows mark the month of birth of two microcephaly cases that were independently identified and confirmed in Brazil (August 2017, confirmed here), and Portugal (November 2017 [11]). Red dots on the horizontal axis indicate the sampling times of four PCR-confirmed ZIKV infections, identified in non-microcephalic patients in Angola.

Microcephaly in Angola

Suspected microcephaly cases identified in Angola before November 2018 are shown in **Figure 2**. The number of neonates identified with suspected microcephaly peaks around November 2017, and subsequently declines (**Figure 2B**). The peak occurs several months after several PCR-confirmed ZIKV acute cases were detected in Angola (**Figure 2B**, red dots). However, the temporal and geographical distributions of suspected microcephaly and ZIKV cases are difficult to interpret with confidence because the total number of reported cases is low, the consistency of case detection and reporting are likely highly variable, and the intensity of surveillance efforts has changed over time (**Supplementary Materials and Methods**).

Of the 76 samples from infants with suspected microcephaly, none were positive for ZIKV by RT-qPCR. No maternal samples (n=24) tested PCR positive for ZIKV. This is not surprising, given that most infant samples were collected long after ZIKV is expected to be cleared from blood fluids [21] (median time between birth and sample collection was 24 days, **Figure S1**), and because ZIKV infection may have occurred months prior to birth. Only 8 samples from infants with suspected

microcephaly could be tested for infection with ToRC pathogens; none showed evidence of recent ToRC infection.

In addition to the suspected microcephaly cases reported to INIS, one Angolan microcephaly case was identified in Brazil. The 21 year-old mother of this child was a long-term resident of Moxico province in Angola, but travelled to Luanda during the second and third months of pregnancy and reports a rash during this visit (week 10 of pregnancy; January 2017). Confirmed ZIKV cases were identified in Luanda and Bengo province at this time (**Figure 1**). The neonate was delivered in early August 2017 in Angola, coincident with the rise in suspected microcephaly cases (**Figure 2**). CT scans of the child's brain conducted at 15 months confirmed microcephaly through reduced cerebral volume, and showed abnormalities consistent with congenital Zika syndrome observed in Brazil and elsewhere [22] (**Figure 3**). When tested serologically, the child was IgG negative for both DENV and ZIKV by ELISA, and weakly positive for neutralising antibody response by PRNT (PRNT90 titre = 40). This result does not exclude intrauterine ZIKV exposure, as the child was 15 months -old at testing (see **Materials and Methods**) and maternally acquired IgG antibodies are present for only 6-12 months after birth.[23] The mother was strongly IgG positive for ZIKV by ELISA, with a weaker DENV IgG response. PRNT assays confirmed the strong neutralising antibody response in the mother, with a PRNT90 titre of 1280. This result strongly suggests that the mother had been previously infected with ZIKV.

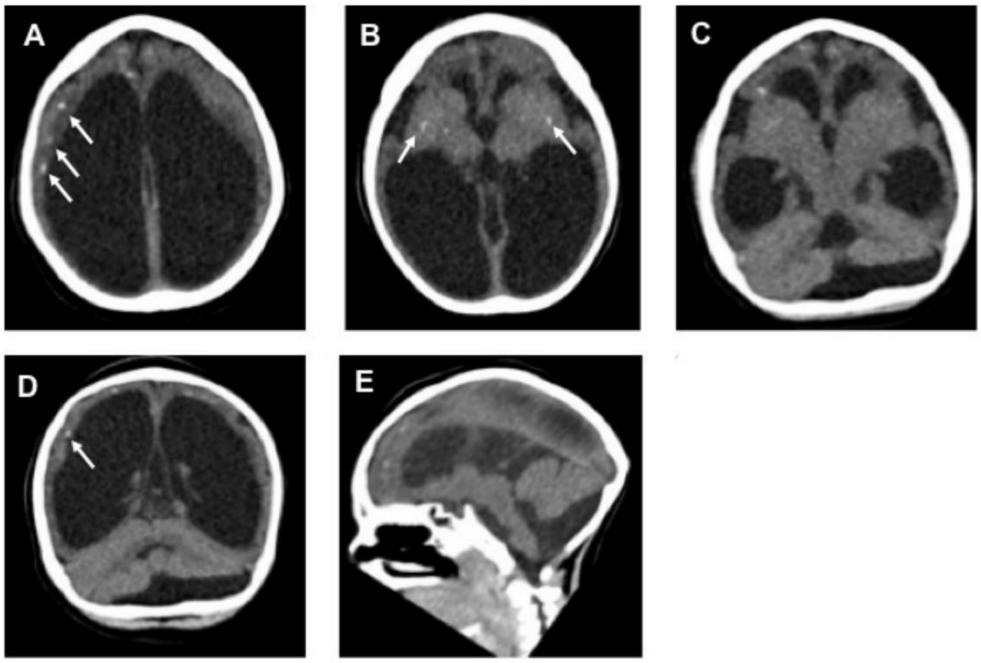

Figure 3. Brain CT image of an Angolan child with microcephaly. (A) Compensatory ventriculomegaly and calcification areas in the subcortical region (arrows). (B) Calcification in the basal ganglia (arrows). (C) Pachygyria. (D) Dysgenesis of the cerebellum and calcification in subcortical region (arrows). (E) Brainstem hypoplasia.

Sequencing and genetic analysis of ZIKV

We sequenced three virus genomes from patients infected with ZIKV in Angola. **Table S1** provides sequencing statistics and GenBank accession numbers. The three Angolan ZIKV sequences differ from each other at 34 nucleotide sites. Three of these variable sites cause amino acid changes: Y135H (in sample Z3), Y3038H and D3344E (both in sample PoHuZV_634939, isolated from a microcephaly patient [11]). Neither of the two amino acid changes observed in strain PoHuZV_634939 are seen in either of the two most closely related ZIKV genomes obtained from infants born with microcephaly in Brazil (GenBank accession numbers KU729217, KU527068).

Maximum likelihood (ML) phylogenies indicate that all three ZIKV strains from Angola form a single, well-supported monophyletic clade within the ZIKV Asian genotype lineage currently circulating in the Americas (**Figure S3;** bootstrap score = 0·96). Thus the ZIKV outbreak in Angola likely resulted from a single successful introduction of the virus, followed by autochthonous transmission within Angola.

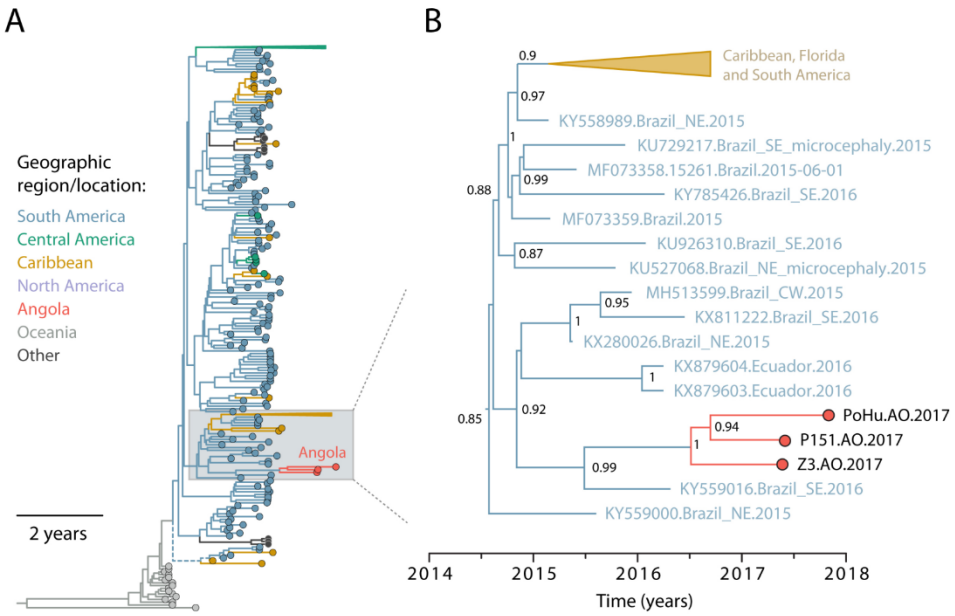

Figure 4. Phylogenetic analysis of the introduction of ZIKV to Angola. A) Maximum clade credibility phylogeny, estimated from complete and near-complete ZIKV genomes using a molecular clock phylogeographic approach. Branch colours indicate the most probable locations of ancestral lineages. Triangular clades represent larger groups of sequences that have been collapsed for visual clarity. B) Expansion of the clade in panel A containing the Angolan ZIKV (green) and closely related sequences from the Americas (red). Clade posterior probabilities are shown at well-supported nodes.

We used a Bayesian phylogenetic framework to investigate when and from where ZIKV was introduced to Angola. Our analyses indicate that ZIKV was likely introduced to Angola from Brazil (**Figure 4**), possibly from the southeast of Brazil, where the most closely related virus to the Angola ZIKV clade was sampled (accession number KY559016; posterior probability = 0·99). The estimated date of the most recent common ancestor of the ZIKV Angolan sequences is June 2016 (Bayesian 95% credible interval, January 2016-October 2016) (**Figure 4**). The date of divergence of the Angolan ZIKV cluster from the most closely related Brazilian ZIKV sequence is July 2015 (February 2015-February 2016) (**Figure 4**). These estimates therefore indicate that ZIKV was introduced to Angola from Brazil between July 2015 and June 2016. Thus ZIKV may have circulated in Angola for 3 to 14 months before being identified for the first time, via a traveller returning from Angola.[19]

Evaluation of the outbreak's geographic origin

A ZIKV outbreak was reported in Cabo Verde during 2015-2016, from which no genetic sequence data are currently available.[7] The absence of ZIKV genetic data from key locations, such as Cabo Verde (**Figure 5**), means that we cannot unambiguously infer the geographic origin of the Angolan ZIKV outbreak using phylogenetic analysis alone. We therefore analysed global ZIKV incidence and human mobility data to predict the likely source of ZIKV in Angola.

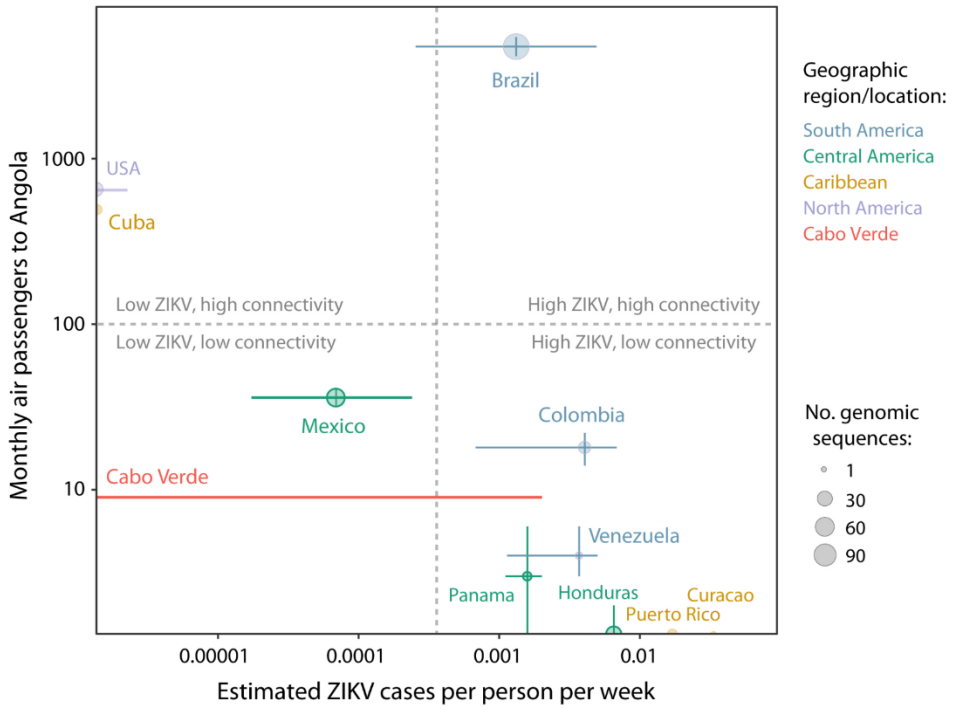

Figure 5. Factors affecting the likelihood of introduction of Asian lineage ZIKV into Angola. The vertical axis shows the median number of air passengers travelling to Angola per month during the most likely time for the most recent common ancestor of the Angolan ZIKV cluster (January 2016 – October 2016).

Vertical bars show the 25% and 75% quartiles of monthly passenger numbers. The horizontal axis shows the median weekly number of suspected or confirmed ZIKV cases per person during the same period. Horizontal bars show the quartiles of ZIKV case numbers. Point diameters are proportional to the total number of genomic sequences currently available from each country. The 11 countries shown include all those with the with the seven highest median passenger numbers and the seven highest number of ZIKV cases per person. Colour indicate geographic region (red: Africa, green: Central America, purple: South America, blue: USA, orange: Caribbean).

We found that 80% of all air passengers who travelled to Angola from countries reporting ZIKV began their journey in Brazil, whilst only 0·15% originated from Cabo Verde. The number of passengers that travelled to Angola per month was therefore over 500 times higher from Brazil than from Cabo Verde (**Figure 5)**. Crucially, Brazil is the only country that exhibits both high connectivity with Angola via air travel and a high average rate of ZIKV cases per person (**Figure 5**, top right). Further, the climatic suitability for *Aedes*-borne viral transmission was more closely synchronised between Angola and Brazil than Angola and Cabo Verde (**Figure S4**). Introduction of ZIKV to a new location is most likely to be successful when environmental suitability for the mosquito vector is high in both the source and destination locations. Thus, multiple lines of evidence (ZIKV incidence, mobility data, and virus phylogenetic analysis) support the hypothesis that ZIKV was introduced directly to Angola from Brazil.

Discussion

Here, we characterise the first known outbreak of Asian lineage ZIKV in continental Africa. We tested 54 suspected cases of acute ZIKV infection and 76 suspected microcephaly cases sampled across Angola by RT-qPCR. Three suspected cases of acute ZIKV infection, all notified between December 2016 and May 2017, were ZIKV positive. A further ZIKV+ case was detected in a cohort of 349 HIV+ patients sampled in Luanda in June 2017. To understand the origins of the Angolan outbreak, we sequenced ZIKV genomes from two autochthonous cases and a confirmed microcephalic neonate, born in Portugal to a resident of Luanda. These represent the first ZIKV Asian lineage genomes reported from Africa, and the first genome from a ZIKV associated microcephaly case in Africa.

We analysed suspected microcephaly cases reported to INIS during 2017-2018. The fact that we found no ZIKV-positive cases among 76 suspected microcephaly cases does not indicate the virus was absent from this group, because the samples were collected long after birth, at which point ZIKV viremia is expected to have declined to undetectable levels in blood fluids.[21] To maximise ZIKV detection sensitivity in suspected microcephaly cases, sera and urine samples for RT-qPCR should be collected as soon as possible after birth, and ideally within the first few days.[24] Systems for the detection and reporting of birth defects are limited in sub-Saharan Africa, so the spatiotemporal distribution of suspected cases must be interpreted cautiously. In addition to the 76 suspected

microcephaly cases identified in Angola, two cases of microcephaly acquired in Angola have been confirmed in other countries. Both cases (one of which is clinically reported here) have strong serological evidence for maternal ZIKV exposure and brain abnormalities consistent with congenital Zika syndrome.[11,22] Further, both confirmed microcephaly cases were born a few months after the date of sampling of PCR-confirmed ZIKV infections in Angola. A time lag of ~5 months between ZIKV incidence and occurrence of microcephaly cases has been observed elsewhere.[25] We show here that ZIKV detected in one these confirmed microcephaly cases [11] belongs to a virus lineage originating from the Americas, and which likely circulated in Angola for at least 12 months. Continued identification of confirmed cases of acute ZIKV infection and ZIKV-associated microcephaly in Angola remains a priority to understand the clinical consequences of ZIKV in Africa.

Phylogenetic analysis shows that the three Angolan ZIKV genomes form a single clade whose common ancestor dates to June 2016 or earlier. Successful introductions of ZIKV to new regions often occur during seasons of peak climatic suitability for *Aedes-borne* transmission.[10,26] Under this scenario, ZIKV would most likely have entered Angola between November 2015 and April 2016 (**Figure S4**). Detection of a single ZIKV outbreak clade in Angola is compatible with two scenarios, (i) a single successful introduction that initiated local ZIKV transmission in Angola , continuing until at least June 2017 or (ii) recurrent but later introduction to Angola of viruses belonging to a specific ZIKV lineage present in Brazil. The latter is much less likely, as it is improbable that three or more independent introductions of ZIKV to Angola belonged to just one of the many different lineages of ZIKV that co-circulated in Brazil.[10] If the former scenario is correct, then ZIKV may have circulated undetected in Angola for at least 3 months before a case was first detected in a returning traveller,[19] and at least 6 months before a local case was detected (**Figure 1**). Similar or longer periods of cryptic ZIKV transmission have been reported in the Americas [10] and attributed in part to the difficulties in identifying clinical cases when infections are asymptomatic or mildly symptomatic.[27] Retrospective screening of stored samples and identification of young children with suspected microcephaly, will help to determine the magnitude and duration of undetected ZIKV transmission in Angola since 2015.

We conclude that ZIKV was introduced to Angola from Brazil. This is consistent with previous predictions that, given the presence of ZIKV in Brazil, Angola is the African country most at-risk of importing the virus.[28] Transmission of mosquito-borne viruses between these two countries was demonstrated previously by the spread of CHIKV East-Central-South African genotype (ECSA) from Angola to Brazil in June 2014.[29] The introduction of ZIKV to Angola underscores the need to coordinate viral surveillance strategies across countries that share high human interconnectivity and similar vector-borne transmission potential, regardless of the transnational distance involved. Detecting ZIKV transmission using only clinical data on clusters of mild illness or suspected microcephaly would be extremely difficult in this setting. Molecular surveillance systems are therefore needed to monitor further ZIKV spread in Africa, including those countries potentially at risk of ZIKV introduction from Angola.[30]

The epidemiology and clinical significance of Asian and African ZIKV lineages in Africa is unclear. Widespread screening of pregnant women for STORCH pathogen infections, sensitive ZIKV antibody-based assays, and follow-up of children born with abnormalities would improve our understanding of the extent and causes of birth defects in Africa. Additional genomic surveillance of other mosquito-borne viruses, such as dengue and Japanese encephalitis virus, is required to inform effective intervention strategies to ameliorate disease burden in Angola. Previously, genomes of emerging viruses have been typically generated in a small number of highly resourced genomic centres, located in wealthy nations. The introduction of low cost, portable sequencing technology to laboratories in Africa heralds an era in which virus genome sequencing is widespread, real-time, and sustainable, with the potential to directly inform public health responses to future emerging outbreaks.

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Contributions

SCH performed laboratory work and analyses, and wrote the manuscript. JV, ZN, DJ, JX, MM, ALMC, FV, CSS, CC, SPS, DQ, CD, BP and IMC performed laboratory work on samples identified in Angola and epidemiological surveillance. LZZ and MJA identified and helped with sequencing of the Portuguese case. RSA assisted laboratory work in Angola, and performed investigations on the microcephalic infant identified in Brazil along with AM, BLFSR, AT, LMH and GSA. CHW helped with analyses. AGW, KK, MK and DSC assisted with analysis of global mobility and incidence data. JT and NRF conducted bioinformatics and assisted phylogenetic analyses. LCJA and ECS oversaw parts of the laboratory work. EL and OGP contributed to the overall design, provided interpretation of the results

and commented on manuscript drafts. NRF and JA led the design and execution of the study, and oversaw all analyses and interpretation. All authors have seen and approved of the final text.

Declaration of interests

KK is the founder of BlueDot, a social enterprise that develops digital technologies for public health. AW and KK received employment or consulting income from BlueDot during this research. No other authors have conflicts of interest to declare.

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Data sharing

Sequence alignments, XMLs and tree files are available at GitHub (XXX to be completed prior to acceptance XXX). ZIKV genome sequences are available on GenBank with accession numbers XXX-XXX (pending).

Artigo original 2

PLOS Neglected Tropical Diseases

Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon

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Abstract:	Background Since its first detection in the Caribbean in late 2013, chikungunya virus (CHIKV) has affected 51 countries in the Americas. The CHIKV epidemic in the Americas was caused by the CHIKV-Asian genotype. In August 2014, local transmission of the CHIKV-Asian genotype was detected in the Brazilian Amazon region. However, a distinct lineage, the CHIKV-East-Central-South-America (ECSA)-genotype, was detected nearly simultaneously in Feira de Santana, Bahia state, northeast Brazil. The genomic diversity and the dynamics of CHIKV in the Brazilian Amazon region remains poorly understood despite its importance to better understand the epidemiological spread and public health impact of CHIKV in the country. Methodology/Principal Findings We report a large CHIKV outbreak (5,928 notified cases between August 2014 and August 2018) in Boa vista municipality, capital city of Roraima's state, located in the Brazilian Amazon region. In just 48 hours, we generated 20 novel CHIKV-ECSA genomes from the Brazilian Amazon region using MinION portable genome sequencing. Phylogenetic analyses revealed that despite an early introduction of the Asian genotype in 2015 in Roraima, the large CHIKV outbreak in 2017 in Boa Vista was caused by an ECSA-lineage most likely introduced from northeastern Brazil. Epidemiological analyses suggest a basic reproductive number of R0 of 1.66, which translates in an estimated 39 (95% CI: 36 to 45) % of Roraima's population infected with CHIKV-ECSA. Finally, we find a strong association between Google search activity and the local laboratory-confirmed CHIKV cases in Roraima.

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Describe where the data may be found in full sentences. If you are copying our sample text, replace any instances of XXX with the appropriate details. • If the data are **held or will be held in a public repository**, include URLs, accession numbers or DOIs. If this information will only be available after acceptance, indicate this by ticking the box below. For example: *All XXX files are available from the XXX database (accession number(s) XXX, XXX.*). • If the data are all contained **within the manuscript and/or Supporting Information files**, enter the following: *All relevant data are within the manuscript and its Supporting Information files.* • If neither of these applies but you are able to provide **details of access elsewhere**, with or without limitations, please do so. For example: *Data cannot be shared publicly because of [XXX]. Data are available from the XXX Institutional Data Access / Ethics Committee (contact via XXX) for researchers who meet the criteria for*	XML files and datasets analysed in this study are available in the GitHub repository (http://github.com/arbospread/chikv-amazon). New sequences have been deposited in GenBank under accession numbers MK121891-MK121908 (CHIKV-ECSA) and MK134712-MK134713 (CHIKV-Asian).

Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon

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Abstract

Background

Since its first detection in the Caribbean in late 2013, chikungunya virus (CHIKV) has affected 51 countries in the Americas. The CHIKV epidemic in the Americas was caused by the CHIKV-Asian genotype. In August 2014, local transmission of the CHIKV-Asian genotype was detected in the Brazilian Amazon region. However, a distinct lineage, the CHIKV-East-Central-South-America (ECSA)-genotype, was detected nearly simultaneously in Feira de Santana, Bahia state, northeast Brazil. The genomic diversity and the dynamics of CHIKV in the Brazilian Amazon region remains poorly understood despite its importance to better understand the epidemiological spread and public health impact of CHIKV in the country.

Methodology/Principal Findings

We report a large CHIKV outbreak (5,928 notified cases between August 2014 and August 2018) in Boa vista municipality, capital city of Roraima's state, located in the Brazilian Amazon region. We generated 20 novel CHIKV-ECSA genomes from the Brazilian Amazon region using MinION portable genome sequencing. Phylogenetic analyses revealed that despite an early introduction of the Asian genotype in 2015 in Roraima, the large CHIKV outbreak in 2017 in Boa Vista was caused by an ECSA-lineage most likely introduced from northeastern Brazil. Epidemiological analyses suggest a basic reproductive number of R_0 of 1.66, which translates in an estimated 39 (95% CI: 36 to 45) % of Roraima's population infected with CHIKV-ECSA. Finally, we find a strong association between Google search activity and the local laboratory-confirmed CHIKV cases in Roraima.

Conclusions/Significance

This study highlights the potential of combining traditional surveillance with portable genome sequencing technologies and digital epidemiology to inform public health surveillance in the Amazon region. Our data reveal a large CHIKV-ECSA outbreak in Boa Vista, limited potential for future CHIKV outbreaks, and indicate a replacement of the Asian genotype by the ECSA genotype in the Amazon region.

Author Summary

Until the end of 2017, Brazil notified the highest number of infections caused by chikungunya virus (CHIKV) in the Americas. We investigated a large CHIKV outbreak in Boa vista municipality in the Brazilian Amazon region. Rapid portable genome sequencing of 20 novel isolates and subsequent genetic analysis revealed that ECSA lineage was introduced from northeastern Brazil to Roraima around July 2016. Epidemiological analyses suggest a basic reproductive number of R_0 of 1.66, which suggests that approximately 39% of Roraima's population was infected with CHIKV-ECSA. Given the dominance of the CHIKV-Asian genotype in the Americas, our data highlights the rapid spread of a less understood and poorly characterized CHIKV-ECSA genotype in Brazil. Investigations on potential associations between public health impact of CHIKV and genetic diversity of circulating strains are warranted to better evaluate its impact in Brazil and beyond.

Keywords

Chikungunya, East-Central-South-African, surveillance, Amazon region, MinION genome sequencing, traditional epidemiology, genomic epidemiology, digital epidemiology, phylodynamics.

104 **Introduction**

105 In August 2014, local transmission of chikungunya virus (CHIKV) was detected in
106 Brazil for the first time, with cases being reported nearly simultaneously in Oiapoque
107 (Amapá state, north Brazil) and Feira de Santana (Bahia state, northeast Brazil), two
108 municipalities separated by >2000 km distance. Genetic analysis confirmed the co-
109 circulation of distinct virus lineages in Brazil: the Asian genotype (CHIKV-Asian)
110 was introduced to Oiapoque possibly from neighbouring French Guiana, while the
111 East-Central-South-African genotype (CHIKV-ECSA) was introduced to Feira de
112 Santana from a traveller returning from Angola [1].

113 Since 2014 and until the end of September 2018, a total of 697,564 CHIKV
114 cases have been notified in Brazil (including 94,672 laboratory-confirmed cases). This
115 is the largest number recorded in any of the 51 countries or territories reporting local
116 CHIKV transmission in the Americas [2]. The virus has been circulating in the
117 Americas since 2013 where approximately 260 million people live in areas at-risk of
118 transmission [2-4]. Similar to the recent Zika virus epidemic [5], the rapid spread of
119 CHIKV in the Americas, including in Brazil, results from several factors, including
120 the establishment and abundance of competent *Aedes* spp. vectors, lack of population
121 immunity, poor housing quality, and increased mobility of vectors and humans
122 between regions reporting current presence of the virus {Johansson, 2015 #4746;Van
123 Benthem, 2005 #4745}.

124 Chikungunya virus is an enveloped, non-segmented, single-stranded positive
125 polarity RNA alphavirus that is a member of the *Togaviridae* family and is
126 transmitted predominately by the *Aedes aegypti* and *Aedes albopictus* vectors, which
127 are widespread in Brazil [7]. There are four main genotypes: (i) the West African
128 genotype is maintained in an enzootic cycle in Africa, (ii) the Asian genotype, which
129 is endemic in Asia, (iii) the East-Central-South-African genotype, endemic to Africa,
130 and (iv) the Indian Ocean Lineage (IOL) genotype, an epidemic lineage that emerged
131 from the ECSA genotype around 2004 and swept through the Indian Ocean region
132 causing a series of explosive outbreaks [8].

133 The first symptoms of CHIKV infection are a rapid increase in temperature
134 (>38.9°C), followed by severe, often debilitating polyarthralgia. Serological data
135 from La Reunion, Philippines and the Indian Ocean island of Mayotte suggest that
136 75-97% of persons infected with CHIKV develop symptomatic infections [9].
137 Seroprevalence data from Brazil suggests that 45.7 to 57.1% Riachão do Jacuípe

and of Feira de Santana, both located in Bahia state, were exposed to CHIKV in 2015, with a total of 32.7% to 41.2% of the population reporting symptoms [10].

Throughout Asia and the Americas, chikungunya virus outbreaks have been associated with unique clinical features [11], including long-lasting symptoms [12], and high mortality resulting from complications associated with CHIKV infection [13, 14]. In Brazil, a striking proportion of 68.1 to 75% of the population with positive serological results reporting symptoms contracted a chronic form of the disease [12, 15]. However, the epidemiological features, genomic diversity, and transmission dynamics of recent CHIKV outbreaks in this country remain poorly understood. Inferences that are based only on clinical-epidemiological notifications are complicated by underreporting of cases by the national reporting system [16], mostly due to the co-circulation and co-infection with viruses that cause overlapping symptoms, such as Zika and dengue viruses [17-19]. Moreover, CHIKV serological tests may cross-react with other alphaviruses, such as Mayaro virus, that circulate in the north and centre-west regions of Brazil [20, 21]. In this context, it is challenging to use only clinical-epidemiological and serological data to evaluate the true extent of the disease. Moreover, accurate incidence data is critical to forecast and provide prediction of the course of epidemics [22].

Until the end of 2016, 83.3% of the cases in Brazil were reported in northeast region of the country [23]. However, in 2017, Roraima state, located in the Amazon basin in the north of Brazil, reported its first large CHIKV outbreak. Roraima is the northernmost state of Brazil, lies in the Amazon basin, borders Venezuela and French Guiana to the north, and Amazonas and Pará states to the south, and its equatorial climate favours year round transmission of mosquito-borne viruses [24]. Within Brazil's northern states, Roraima has been implicated as a stepping-stone to virus introductions from other Latin American regions, such as dengue [25], and yellow fever virus in the past [26]. Moreover, the Amazon region has recently been highlighted as a region with high transmission potential of vector-borne diseases [4] and, more generally, a region with high potential for virus zoonoses and emergence [27].

Due to its connectivity and potential impact on global epidemiology of vector-borne and zoonotic virus from the Amazon basin, it is important to improve genomic pathogen surveillance in Roraima. By August 2018, the public health laboratory of Boa Vista (capital city of Roraima state) had reported 5,928 CHIKV cases, 3,795 of

172 which were laboratory-confirmed. Here we a use combination of on-site portable
173 virus genome sequencing, and epidemiological analysis of case count and web search
174 data to describe the circulation, genetic diversity, epidemic potential and attack rates
175 of a large CHIKV outbreak in Boa Vista.
176

177 # Methods

178 **Connectivity in study area**

179 Roraima is the northernmost of Brazil's 27 federal units (**Figure 1a**) and has an
180 estimated population of 450,479, of whom 284,313 live in the capital city of Boa
181 Vista (ibge.gov.br/). Despite being Brazil's least populated federal unit, Roraima is
182 one of the best-connected Brazilian states in the Amazon basin [28]. Within Brazil,
183 Roraima is connected to Amazonas state in the south via the road BR-174. This
184 road also connects Roraima's capital city, Boa Vista, to the states of Bolivar and
185 Amazonas in Venezuela in the north. Further, the road BR-401 links Boa Vista to
186 Guyana in the east. There are four daily flights connecting Boa Vista with Brasília,
187 capital of Brazil, as well as six weekly flights to Manaus, the capital city of
188 Amazonas state and the biggest city in the north of the country, with connecting
189 daily nonstop flights to all other Brazilian states/regions and international
190 destinations, including important international airport hubs in Panamá City and
191 Miami, USA. There are also less-commonly used seasonal fluvial networks that
192 connect Boa Vista and Manaus via the Amazonas river.
193
194 **Chikungunya virus case count time series**
195 The Roraima State Central Laboratory (LACEN-RR) is responsible for the differential
196 diagnosis of suspected arbovirus cases presenting to Roraima's public health units.
197 Between Jan 2014 and September 2018, LACEN-RR notified 5,928 CHIKV cases in
198 Boa Vista alone, 3,795 of these laboratory-confirmed, to the National Reportable
199 Disease Information System (SINAN). Case count time series are available from
200 Github (https://github.com/arbospread/chik-amazon). We follow the Brazilian
201 Ministry of Health's guidelines and define a notified CHIKV case as a suspected case
202 characterized by (i) acute onset of fever >38.5°C, (ii) severe arthralgia and/or arthritis
203 not explained by other medical conditions, and (iii) residing or having visited
204 epidemic areas within 15 days before onset of symptoms. A laboratory-confirmed
205 case is a suspected case confirmed by laboratory methods such as (i) virus isolation in
206 cell culture, (ii) detection of viral RNA, (iii) detection of virus-specific IgM
207 antibodies in a single serum sample collected in the acute or convalescent stage of
208 infection; or (iv) a four-fold rise of IgG titres in samples collected during the acute
209 phase, in comparison with a sample collected in the convalescent period.

Ethics statement

Residual anonymized clinical samples were processed in accordance with the terms of Resolution 510/2016 of CONEP (National Ethical Committee for Research, Brazilian Ministry of Health), under the auspices of the ZiBRA project (http://www.zibraproject.org/). The project was approved by the Pan American Health Organization Ethics Review Committee (PAHOERC) nº PAHO-2016-08-0029.

Nucleic acid isolation and RT-qPCR

Residual anonymized clinical diagnostic samples were sent to Instituto Leônidas e Maria Deane, FIOCRUZ Manaus, Amazonas, Brazil, for molecular diagnostics as part of the ZiBRA-2 project. Total RNA extraction was performed with QIAmp Viral RNA Mini kit (Qiagen), following manufacturer's recommendations. Samples were first tested using a multiplexed qRT-PCR protocol against CHIKV, dengue virus (DENV1-4), yellow fever virus, Zika virus, Oropouche virus and Mayaro virus [29]. All qRT-PCR results were corroborated using a second protocol [30]; comparable Ct values were obtained with the two protocols. CHIKV positive samples tested negative for all other arboviruses tested. Samples were selected for sequencing based on Ct-value <30 (to maximize genome coverage of clinical samples by nanopore sequencing [31]), and based on the availability of epidemiological metadata, such as date of onset of symptoms, date of sample collection, gender, municipality of residence, and symptoms (**Table 1**). We included a total of 13 samples from Roraima state plus 5 additional samples from patients visiting the LACEN-Amazonas in Manaus.

Complete genome MinION nanopore sequencing

Sequencing was attempted on samples with Ct-value ≤30 at Instituto Leônidas e Maria Deane, FIOCRUZ Manaus. We used an Oxford Nanopore MinION device with protocol chemistry R9.4, as previously described [32]. Sequencing statistics can be found in **Table S1 (Julien)**. In brief, we employed a protocol with cDNA synthesis using random primers followed by strain-specific multiplex PCR [32]. Extracted RNA was converted to cDNA using the Protoscript II First Strand cDNA synthesis Kit (New England Biolabs, Hitchin, UK) and random hexamer priming. CHIKV

243 genome amplification by multiplex PCR was attempted using the
244 CHIKAsianECSA primer scheme and 35 cycles of PCR using Q5 High-Fidelity
245 DNA polymerase (NEB) as described in [32]. PCR products were cleaned up using
246 AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and
247 quantified using fluorimetry with the Qubit dsDNA High Sensitivity assay on the
248 Qubit 3.0 instrument (Life Technologies). PCR products for samples yielding
249 sufficient material were barcoded and pooled in an equimolar fashion using the
250 Native Barcoding Kit (Oxford Nanopore Technologies, Oxford, UK). Sequencing
251 libraries were generated from the barcoded products using the Genomic DNA
252 Sequencing Kit SQK-MAP007/SQK-LSK108 (Oxford Nanopore Technologies).
253 Libraries were loaded onto a R9/R9.4 flow cell and sequencing data were collected
254 for up to 48 hr. Consensus genome sequences were produced by alignment of two-
255 direction reads to a CHIKV virus reference genome (GenBank Accession number:
256 N11602) as previously described in [32]. Positions with ≥ 20× genome coverage
257 were used to produce consensus alleles, while regions with lower coverage, and
258 those in primer-binding regions were masked with N characters. Validation of the
259 sequencing protocol was previously performed in [32].
260
261 **Collation of CHIKV-ECSA complete genome datasets**
262 Genotyping was first conducted using the phylogenetic arbovirus subtyping tool
263 available at http://www.krisp.org.za/tools.php. Complete and near complete
264 sequences were retrieved from GenBank on June 2017 [33]. Two complete or near-
265 complete CHIKV genome datasets were generated. Dataset 1 included ECSA-
266 PreAm (ECSA sampled outside the Americas) and ECSA-Br (ECSA sequences
267 sampled in the Americas) sequences. This dataset contained 36 complete genomes
268 from the ECSA genotype, including 7 from East and Central Africa (HM045823
269 from Angola 1962; HM045784 from Central African Republic 1984; HM045812
270 from Uganda 1982; KY038947 from Central African Republic 1983; HM045793
271 from Central African Republic 1986; HM045822 from Central African Republic
272 1978; and KY038946 from Central African Republic 1975). Dataset 1 also included
273 29 sequences from Brazil, including the new 18 genomes reported here from the
274 ECSA lineage and 3 genomes from the outbreak caused by the ECSA lineage in
275 June 2016 in Maceió, Alagoas states, northeast Brazil (**Figure 1a**) [34]. Dataset 2

(ECSA-Br) included only the 29 Brazilian genome sequences. Using a robust nonparametric test [35], no evidence of recombination was found in both datasets.

Maximum likelihood analysis and temporal signal estimation

Maximum likelihood (ML) phylogenetic analyses were performed for each dataset using RAxML v8 [36]. We used a GTR nucleotide substitution model with 4 gamma categories (GTR+4Γ). In order to investigate the evolutionary temporal signal in each dataset, we regressed root-to-tip genetic distances against sample collection dates using TempEst [37]. For both datasets we obtained a strong linear correlation (dataset 1: r^2=0.93; dataset 2: r^2=0.84) suggesting these alignments contain sufficient temporal information to justify a molecular clock approach. However, for dataset 1, the Angola/M2022/1962 strain was positioned substantially above the regression line. Previous investigations have suggested this strain may have been the result of contamination or high passage in cell culture [8], so this sequence was removed from subsequent analyses.

Molecular clock phylogenetic analysis

To estimate time-calibrated phylogenies we used the BEAST v.1.10.1 software package [38]. To infer historical trends in effective population size from the genealogy we used several different coalescent models. Because preliminary analysis indicated oscillations in epidemic size through time (as also expected from national case report data), we used three flexible, non-parametric models: a) the standard Bayesian skyline plot (BSP; 10 groups) [39], b) the Bayesian skyride plot [40], and c) the Bayesian skygrid model [41], with 45 grid points equally spaced between the estimated TMRCA of the CHIKV-ECSA genotype in Brazil and the date of the earliest available isolate, collected in 18 March 2017 [41]. For comparison, we also used a constant population size coalescent model. We tested two molecular clock models: a) the strict molecular clock model, which assumes a single rate across all phylogeny branches, and b) the more flexible uncorrelated relaxed molecular clock model with a lognormal rate distribution (UCLN) [42]. Because the marginal posterior distribution of the coefficient of variation of the UCLN model did not exclude zero (most likely due to the small alignment size), we used a strict molecular model in all analyses. For each coalescent model, Markov

Chain Monte Carlo analyses were run in duplicate for 10 million steps using a ML starting tree, and the GTR+4Γ codon partition (CP)1+2,3 model [42].

Epidemiological analysis

The epidemic basic reproductive number (R_0) was estimated from monthly confirmed cases, as previously described [31, 43]. Because (*i*) the Asian genotype was circulating in the north region of Brazil since 2014 [1], and (*ii*) we observed a relatively small number of cases both in the notified and confirmed time series, we assume cases from June 2014 and December 2016 did not represent autochthonous transmission of CHIVK-ECSA. We assume a mean generation time of 14 days, as previously reported elsewhere for an outbreak caused by an Indian Ocean lineage (IOL), a subclade of the ECSA genotype [44]. We report R_0 estimates for different values of the generation time (g) parameter, along with corresponding estimates of the epidemic exponential growth rate, per month (r).

Web search query data

Available in near-real time, disease-related Internet search activity has been shown to track disease activity (a) in seasonal mosquito-borne disease outbreaks, such as those caused by dengue [42, 82], and (b) in unexpected and emerging mosquito-borne disease outbreaks such as the 2015-2016 Latin American Zika outbreak [45]. Here, we investigated whether we could find a meaningful relationship between Internet search activity and the local chikungunya outbreak in Roraima. Indeed, novel Internet-based data sources have the potential to complement traditional surveillance by capturing early increases in disease-related search activity that may signal an increase in the public's perception of a given public health threat and may additionally capture underlying increases in disease activity. Internet searches may be particularly important and indicative of changes in disease transmission early during an outbreak, when ongoing information on the virus transmission is obfuscated by a lack of medical surveillance. In addition, Internet search trends may also help track disease activity in populations that may not seek formal medical care. We used the Google Trends (GT) tool [45] to compile the monthly fraction of online searches for the term "Chikungunya", that originated from Boa Vista municipality (Roraima state), between January 2014 and July 2018. For

342 comparison, GT search activity for the term “Chikungunya” was collected for the
343 same time period for Manaus municipality (Amazonas state). The synchronicity of
344 GT time series and notified and confirmed case counts from Boa Vista and Manaus
345 was assessed using the Spearman’s rank correlation test in the R software [46].
346
347 **Data availability**
348 XML files and datasets analysed in this study are available in the GitHub
349 repository (https://github.com/arbospread/chik-amazon). New sequences have been
350 deposited in GenBank under accession numbers MK121891-MK121908 (CHIKV-
351 ECSA) and MK134712-MK134713 (CHIKV-Asian).

352 **Results**

353 Although most CHIKV notified cases in Brazil were reported in 2016 (**Figure 1**),
354 in Roraima, the majority of notified and confirmed cases in Roraima state were
355 reported in 2017 (5,027 notified cases and 3,720 laboratory-confirmed infections).
356 The number of cases in Roraima started increasing exponentially in January 2017,
357 and the outbreak peaked in July 2017.

358 We selected 15 RT-qPCR+ virus isolates from autochthonous cases in
359 Roraima state (11 from Boa Vista, 1 from Bonfim, and 1 from Iracema
360 municipalities) (**Table 1**) with a cycle threshold (Ct) ≤30 (mean 20.3, range 13.7 –
361 27.41). We included two isolates from two infected travellers returning to Roraima in
362 December 2014, and an additional five isolates from Amazonas state (all from
363 Manaus municipality), sampled between July 2015 and March 2017. In less than 48
364 hours genome sequence data was obtained for all selected isolates and in less than
365 72 hours preliminary results were shared with local public health officials and the
366 Brazilian Ministry of Health. A mean genome coverage of 86% (20x) per base pair
367 was obtained for the sequenced data; mean coverage increased to 90% when
368 focusing on samples with Ct<26 (**Figure 2a**). Coverage of individual sequences and
369 epidemiological information for each sequenced isolate can be found in **Table 1**.

370 Identification of virus genotypes was conducted using phylogenetic analysis of
371 full-length genome datasets (manual classification) and using an online phylogenetic
372 analysis tool (automated classification). Both approaches identified the ECSA
373 genotype as the dominant genotype circulating in both Roraima and Manaus between
374 2015 and 2017. However, two cases from late 2014 returning from Venezuela to
375 Roraima (AMA294 and AMA295) were classified as Asian genotype, the dominant
376 lineage circulating in Latin America.

377 ML and Bayesian phylogenetic analyses reveal that the ECSA sequences from
378 Brazil form a single well-supported clade (bootstrap support = 100), hereafter named
379 as ECSA-Br clade; which contains strong temporal signal ($r^2 = 0.84$) as measured by a
380 regression of genetic divergence against sampling dates (**Figs, 2b and 3**). Thus we
381 estimated the evolutionary time-scale of the ECSA-Br lineage using several well-
382 established molecular clock coalescent methods. Our substitution rate estimates
383 indicate that the ECSA-Br lineage is evolving at 7.15×10^{-4} substitutions per site per
384 year (s/s/y; 95% Bayesian credible interval: $5.04 – 9.55 \times 10^{-4}$). This estimated rate is

higher than that estimated for endemic lineages, and is similar to the evolutionary rates estimated for the epidemic lineage circulating in the Indian Ocean region (**Figure 2c**). A closer inspection of amino acid mutations indicate that the ECSA-Br strains lack both the A226V (E1 protein) and the L210Q (E2 protein) mutations that has been reported to increase virus transmissibility and persistence in *Ae. albopictus* populations in the Indian Ocean [47].

This is consistent with the establishment of the ECSA genotype in Brazil following the introduction of a single strain to the Americas [1]. The two isolates collected in late 2014 in Roraima cluster together and fall as expected within the diversity of other Asian genotype sequences from the Americas. Our phylogenetic reconstruction suggests at least five separate introductions of the Asian genotype strain Brazil (**Figure S1**), in contrast to a single introduction of the ECSA genotype followed by onward transmission. Moreover, all 13 ECSA isolates sampled in Roraima (*node C*) cluster together with maximum phylogenetic support (bootstrap support = 100; posterior probability = 1.00) (**Figure 3**). We consistently estimate the date of the most recent common ancestor of ECSA-Br Roraima clade to be mid-July 2016 (95% BCI: late March to late October 2016) (**Figure 3**); similar dating estimates under different coalescent models (**Figure S2**). In contrast to the Roraima strains, sequences from Manaus were found to be interspersed with isolates from Bahia and Pernambuco (**Figure 3**), indicating separate introductions of the CHIKV-ECSA lineage, some in early 2015 (*node B*), possibly from the northeast region of Brazil. Interestingly, according to travel history reports, the first autochthonous transmission of CHIKV in Manaus was linked to an index patient who reported spending holidays in Feira de Santana (Bahia state) in early 2015, during a period when this city was experiencing a large CHIKV outbreak [5]. The date of *node A* was estimated to be around mid-July 2014 (95% BCI: early Jul – late Aug 2014), shortly after the arrival of the presumed index case in Feira de Santana, Bahia [5]. This is in line with a single introduction to Bahia (*node A*), followed by subsequent waves of transmission across the northeast and southeast regions of Brazil [5, 48, 49]. Our demographic reconstructions indicate that the outbreak in Roraima 2017 probably represents the third epidemic wave spreading across Brazil (**Figure S3**).

Next, we used notified case counts to estimate the basic reproductive number, R_0, of the epidemic. R_0 is the average number of secondary cases caused by an infected individual and can be estimated from epidemic growth rates during its

early exponential phase [43]. We find that $R_0 \approx 1.66$ (95% CI: 1.51 – 1.83), in line with previous reports from other settings [50-52]. A sensitivity analysis considering different exponential growth phase periods resulted in a lower bound for R_0 of around 1.23 (**Figure S4**). To gain insights into the possible magnitude of the outbreak and local surveillance capacity we used the equilibrium end state of a simple susceptible-infected-recovered (SIR) model: $N = S + I + R$, $S \sim 1/R_0$, $I \sim 0$, with N being the total population size of Roraima. Using this simple mathematical approach, we obtain an attack rate (R) of 0.39 (95% CI: 0.36 – 0.45), slightly lower than elsewhere in Brazil [12, 15]. This corresponds to an estimated 110,882 (95% CI: 102,352 – 127,940) infected individuals, and a case detection rate of 5.34% (95% CI: 4.63 – 5.79). This implies that approximately 1 case was notified for every 19 infections. If we assume 32.7 – 41.2% of the estimated infections are symptomatic, as previously reported in Bahia and Sergipe [53], then we estimate that the local observation success of symptomatic cases was between 12.8 – 16.1%. However, if we assume that 75 – 97% of people infected with CHIKV will develop symptomatic infections, as reported for the Indian Ocean lineage [10, 54, 55], then the chances of a reported a symptomatic CHIKV case decrease to 5 – 7% [9]. Case reports suggest that the beginning of the exponential phase of the outbreak was in December 2016 (**Figure S4**), while genetic data suggests that the outbreak clade emerged around July 2016. However, between August 2014 and June 2016, 612 CHIKV notified cases and 40 confirmed cases were reported by the LACEN-RR. It is therefore likely that prior to Jan 2017, low but non-neglectable transmission of the Asian genotype occurred in Roraima.

We investigated the public's awareness of the chikungunya outbreak by retrospectively monitoring Google searches of the search term "chikungunya" in Roraima state from January 2014 to July 2018 (**Figure 4**). As a comparison, we performed a similar search focusing on the neighbouring state of Amazonas. We found that web search activity and CHIKV cases counts in Roraima are highly correlated (notified cases: $r = 0.89$; confirmed cases: $r = 0.92$, **Figure 4d – e**). Additionally, the timing of the peak of Google searches corresponds to that of notified and confirmed cases with a peak in July 2017 (**Figure 4a and c, Figure 4b and f**). It is important to note that web search activity was available weeks or months before the final number of confirmed (and suspected) cases were made

452 publicly available. This fact highlights the potential utility of monitoring disease-
453 related searches during the outbreak. Interestingly, we find some web-search
454 activity in Roraima before June 2016, particularly in September 2014, March 2015
455 and March 2016 (**Figure 4f**). These patterns are distinct to those in the Amazonas
456 neighbouring state (notified cases: $r = 0.65$; confirmed cases: $r = 0.15$), which
457 shows an early peak in November 2014, soon after the estimated age of *node B*
458 (**Figure 3b**), followed by a peak in February 2016 and another in March 2017
459 (**Figure 4c**). These multiple peaks in internet search queries are consistent with the
460 timing of at least 3 introductions detected in our phylogenetic analyses (**Figure**
461 **3b**), each possibly resulting in small epidemic waves of CHIKV in Manaus and
462 Amazonas states.

Discussion

In this study we characterized an outbreak caused by CHIKV in Boa Vista city, Roraima state, northern Brazil, using a combination of genetic, laboratory-confirmed and -suspected, and digital search data. Our findings show that an ECSA lineage was introduced in Roraima around July 2016, six months before the beginning of the exponential increase in case numbers. Using simple epidemiological models, we show that on average 1 in 17 (95% CI: 14 – 20) symptomatic CHIKV cases, a fraction of the 110,882 (95% CI: 102,352 – 127,940) estimated number of infections, sought medical care during the outbreak of CHIK ECSA in Roraima. Incidence of CHIKV notified cases was strongly associated with fluctuation in Google search activity in Roraima. Moreover, this study represent the first effort to generate on-site complete CHIKV genome sequences. Our results deliver a genomic and epidemiological description of the largest outbreak ever reported in north Brazil, revealing the circulation of the ECSA lineage in the Amazon region.

We estimate that 39% (95% CI: 36 – 45%) of Roraima's population was infected with CHIKV-ECSA-Br during the outbreak in 2017. Our estimates are higher than the 20% seropositive observed in a rural community in Bahia [10], and slightly lower than the 45.7 – 57.1% observed in two serosurveys conducted in the same state [12], where the ECSA lineage also seems to predominate [50]. The observed differences in terms of the proportion of the population exposed to CHIKV in Roraima compared to previous estimates from the northeast region could result from partial protection resulting from low-level transmission of the CHIKV-Asian genotype during 2014 – 2016 in the north region. Alternatively, some level of cross-protection could have been conferred by previous exposure to Mayaro virus (MAYV); Mayaro is an antigenically-related alphavirus that may provide some level of cross-reactivity [56, 57] and is associated with *Haemagogus* spp. vectors [58], but has also been identified in *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes [59]. MAYV has been detected in the north [60-64] and centre-west [21, 59, 65-68] regions of Brazil. Moderate to high prevalence of MAYV IgM have been found in urban northern areas [60], which could explain the limited spread of CHIKV in Manaus compared to Roraima. Finally, because CHIKV notified cases will be influenced by the apparent rate of infection associated to the genotype causing an outbreak {Bustos Carrillo, 2018 #4712}, future comparisons of epidemiological parameters across different regions from where no genotype data is available should

497 be taken with caution. Given the rapid spread of different CHIKV lineages, novel
498 diagnostic tools may be needed to evaluate the proportion of individuals infected by
499 each genotype.
500 Different CHIKV circulating lineages may have remarkably different public
501 health consequences. Lineage-specific clinical presentations have been recently
502 highlighted by a recent index cluster study which showed that 82% of CHIKV
503 infections caused by the ECSA lineage are symptomatic, in comparison to only 52%
504 of symptomatic infections caused by the Asian genotype [54]. While the Asian
505 lineage seems to have circulated cryptically for 9 months before its first detection in
506 the Caribbean [3], the faster detection of the ECSA lineage in Brazil could at least in
507 part be a consequence of a higher rate of symptomatic to asymptomatic infections of
508 the ECSA lineage circulating in Brazil. The time lag between the phylogenetic
509 estimate of the date of introduction of a virus lineage and the date of the first
510 confirmed case in a given region, enables us to identify surveillance gaps between the
511 arrival and discovery of a virus in that region [69].
512 We used genomic data collected over a 3-year period to estimate the genetic
513 history of the CHIKV-ECSA-Br lineage. We estimate that the CHIKV-ECSA-Br
514 lineage arrived in Roraima around July 2016, whilst the first confirmed CHIKV cases
515 in Roraima occurred earlier, in August 2014. That the discovery date anticipates the
516 estimated date of introduction can be explained by initial introduction(s) of the Asian
517 linage (from the north of Brazil or from other south American regions) resulting in
518 only limited onwards transmission, followed by the replacement of the Asian lineages
519 by an epidemiological successful ECSA lineage. Transmission of the Asian genotype
520 during this period is in line with an increase in notified and confirmed cases, as well
521 internet search query data between August 2014 and June 2016. It is also possible that
522 ecological conditions may have dampened the transmission of the Asian genotype
523 between August 2014 (detection of autochthonous transmission of the Asian genotype
524 in the north region of Brazil) and July 2016 (estimated arrival of the ECSA in
525 Roraima). In the future, fine-scaled, high-resolution measures of transmission
526 potential that take into account daily changes in humidity and temperature will help
527 addressing the impact of climatic changes in the arbovirus epidemiology in the
528 Brazilian Amazon. Nationwide molecular and seroprevalence studies combined with
529 epidemiological modelling [70] will help to determine the proportion of cases caused

by the ECSA compared to the Asian lineage in different geographic settings, and to identify which populations are still at risk of infection in Brazil.

We estimated high rates of nucleotide substitution for this lineage, which equates to around 8 (95% BCI: 6 – 11) nucleotide substitutions per year across the virus genome. Such rates are similar to the evolutionary rates estimated for the IOL lineage; these are typical of urban and epidemic transmission cycles in locations with an abundance of suitable hosts and lack of herd immunity [8]. None of the mutations associated previously with increased transmissibility of the IOL lineage in *Ae. albopictus* mosquitos in the Indian Ocean region were identified in this study. However, it is currently unclear whether we should expect the same mutations to be linked with increased transmission in *Aedes* spp. populations both from Brazil and from Southeast Asia. Further, it is possible that CHIKV in Brazil is transmitted mainly by the *Ae. aegypti* vector that is abundant throughout Brazil [71]. In line with this, CHIKV-ECSA was recently detected in *Aedes aegypti* from Maranhão [72] and Rio de Janeiro states [73].

The past dengue serotype 4 genotype II outbreak in Brazil ignited in the north of the country, and is inferred to have been introduced from Venezuela to Roraima, before spreading to the northeast and southeast region of Brazil [74]. Our genetic analysis reveals at least four instances of ECSA-Br virus lineage migration in the opposite direction, i.e., from northeastern to northern Brazil. Such a pattern may not be surprising due to the year-round persistence of *Aedes aegypti* mosquitos in the northeast and the north areas [31]. Within-country transmission will be dictated by human mobility, climatic synchrony, and levels of population immunity. Moreover, international spread of the ECSA-Br linage is expected to regions linked to Brazil. Previous analyses of dengue virus serotypes has identified a strong connectivity between north Brazil and Venezuela [25, 75], and northeast Brazil and Haiti [31, 76]. In addition, Angola and Brazil are linked by human mobility and synchronous climates that have facilitated the migration of CHIKV-ECSA [1] and Zika virus (http://virological.org/t/circulation-of-the-asian-lineage-zika-virus-in-angola/248).

Improving surveillance in the Amazon region may help anticipate transmission of vector-borne diseases and also spillover from wild mammals of zoonotic viruses of particular concern [27]. Genomic portable sequencing of vector-borne viral infections in the Amazon may is particularly important in the context of early identification of circulation of strains newly (re)-introduced from wildlife. For

example, yellow fever strains collected in Roraima seem to be at the source of the 2016-2018 yellow fever virus outbreak in southeast Brazil, which has affected large urban centres in Minas Gerais, São Paulo and Rio de Janeiro [26]. In the near future, the increasing rapidity and decreasing cost of genome sequencing in poorly sampled areas, combined with emerging theoretical approaches [77], will facilitate the investigation of possible associations between arbovirus lineage diversity, mosquito vectors, reservoir species, and transmission potential.

Finally, the reported synchronicities between notified chikungunya case counts in Roraima and the chikungunya-related Internet searches originated in the region highlight the potential complementarity that Internet search activity may offer in future disease outbreaks. Specifically, given that disease-related search activity can be monitored in near-real time, early signals of increases in disease activity may be spotted weeks or months before lab-confirmed case counts may be available in an unfolding outbreak.

Acknowledgments

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587 **Figure Legends**

588 **Figure 1. Context of this study.** A. Map showing municipalities of Roraima state,
589 including Boa Vista, bordering countries (Venezuela and French Guiana) and
590 bordering Brazilian federal states (Amazonas and Pará). B. Map of Brazilian states,
591 showing the states from which CHIKV sequence data in this study was analysed
592 (Bahia, Alagoas, Pernambuco, Paraíba, Amazonas and Roraima). C. Barplot showing
593 the annual number of notified CHIKV cases in selected states of Brazil (data obtained
594 from the Brazilian Ministry of Health). Map was made with Natural Earth. Free
595 vector and raster map data at naturalearthdata.com.
596
597 **Fig. 2. Sequencing statistics, temporal signal and evolutionary rates of the**
598 **CHIKV-ECSA lineage.** A. Genome coverage plotted against RT-qPCR CT-values
599 for the newly generated sequence data. B. Genetic divergence regressed against dates
600 of sample collection for dataset 2 (CHIKV-ECSA-Br lineage). C. Evolutionary rate
601 estimates for the CHIKV-ECSA-Br lineage obtained by this study (circle number 1)
602 compared to published evolutionary rates obtained for other lineages. Circles
603 numbered 2 to 8 represent point estimates reported in [1, 8, 78]. Horizontal bars
604 represent 95% highest posterior density credible intervals for evolutionary rates.
605
606 **Figure 3. Genetic analysis of the CHIKV-ECSA genotype.** A. Maximum likelihood
607 phylogeny depicting the monophyletic clade containing all the Brazilian ECSA
608 isolates (ECSA-Br lineage). B. Time-calibrated phylogeny of all available CHIKV-
609 ECSA whole genome sequences from Brazil, including 18 novel genomes from
610 Roraima and Amazonas states. Colours correspond to state of sample collection.
611 Violin plots show 95% Bayesian credible intervals for associated node heights [38].
612
613 **Figure 4. Digital surveillance of chikungunya in the Brazilian Brazil.** Notified
614 (orange) and confirmed (purple) cases in the central public health laboratories in
615 Roraima state (a) and the Amazonas state (c) from January 2016 to January 2018.
616 Dashed grey lines in (a) and (c) represent Google Trends activity for the term
617 “chikungunya” in Roraima and Amazonas, respectively. Panels b and d show the
618 correlation between Google Trends activity and cases notified in Roraima and
619 Amazonas, respectively. Strength of the association was measured using the
620 Spearman’s rank correlation coefficient (in panel b: p-value < 0.001; panel d: p-value
621 = 5.183e-09).

Table 1. Epidemiological data for virus isolates from Roraima (RR) and Amazonas (AM). CT=cycle threshold, *d*=days from onset of symptoms to sample collection. Corresponding sequencing statistics are available in **Table S1**. Isolates were collected around 2.3 (range: 0 – 5) days after onset of symptoms. Acc. Number = GenBank accession number.

Isolate	State, Municipality	Acc. Number	Ct RT-qPCR	Coverage (%)	Age, Sex	Collection date	*d*
AMA290	AM, Manaus	MK121891	NA	90.2	76, F	15/07/2015	5
AMA291	AM, Manaus	MK121892	NA	80.7	48, F	15/07/2015	4
AMA292	AM, Manaus	MK121893	NA	90.2	50, M	15/07/2015	0
AMA293	AM, Manaus	MK121894	NA	84.4	42, M	31/01/2016	4
AMA294	RR, Boa Vista	MK134712	NA	90.2	45, F	01/12/2014	2
AMA295	RR, Unknown	MK134713	NA	90.2	9, F	11/11/2014	1
AMA74	AM, Manaus	MK121895	15	90.2	32, F	20/03/2017	2
AMA346	RR, Boa Vista	MK121896	13.7	90.2	30, F	03/03/2017	1
AMA350	RR, Bonfim	MK121897	27.15	54.7	32, F	20/02/2017	1
AMA352	RR, Boa Vista	MK121898	17.33	88.6	3, F	22/02/2017	1
AMA354	RR, Boa Vista	MK121899	23.36	86.9	19, F	17/03/2017	1
AMA362	RR, Iracema	MK121900	18.63	88.6	31, F	17/03/2017	1
AMA364	RR, Boa Vista	MK121901	25.93	83.3	19, F	17/03/2017	2
AMA366	RR, Boa Vista	MK121902	19.87	90.0	36, F	17/03/2017	2
AMA368	RR, Boa Vista	MK121903	25.91	93.1	26, F	15/03/2017	2
AMA369	RR, Boa Vista	MK121904	21.55	95.6	52, M	02/03/2017	3
AMA374	RR, Boa Vista	MK121905	27.41	71.4	64, F	02/03/2017	4
AMA379	RR, Boa Vista	MK121906	17.5	96.1	38, F	27/02/2017	4
AMA381	RR, Boa Vista	MK121907	16.66	97.7	31, F	27/02/2017	4
AMA382	RR, Boa Vista	MK121908	14.58	76.6	30, F	05/03/2017	1

Supplementary Figure Legends

Figure S1. Maximum likelihood phylogenetic tree of the CHIKV Asian genotype. Includes isolates from Southeast Asia, Americas and Brazil. Isolates represented by blue tips were sampled in Roraima, while isolates shown in red represent other strains sampled in Brazil.

Figure S2. Dating estimates obtained under different coalescent models. Estimates for node A (time of the most recent common ancestor, in dark red, see Figure 3b), node B (main Amazonas clade, in green), and node C (Roraima clade, in purple) are shown for different non-parametric models (Bayesian skygrid, skyride, skyline) and for a simple constant population size model.

Figure S3. Demographic dynamics of CHIKV ECSA-Br lineage in Brazil. Fluctuation of effective population size over time as inferred through a Bayesian skygrid coalescent model.

Figure S4. Exponential Period of the CHIKV epidemic in Boa Vista municipality, Roraima state. Log number of notified cases per month are plotted against number of months since January 2015.

671 **Supplementary Table**

672 **Table S1. Minion sequencing statistics**

673

Isolate	Mapped reads	Average depth coverage	Bases covered >10x	Bases covered > 25x	Reference covered (%)
AMA290	13624	767	10276	10258	90.2
AMA291	60261	2047	9489	9280	80.7
AMA292	68090	2746	10402	10223	90.2
AMA293	64953	2096	9745	9701	84.4
AMA294	21361	701	10252	10022	90.2
AMA295	16370	531	10188	10077	90.2
AMA74	42276	1951	10396	10195	90.2
AMA346	31210	1225	10243	10208	90.2
AMA350	63672	1673	7522	7168	54.7
AMA352	13530	536	10219	10184	88.6
AMA354	22214	752	10082	9985	86.9
AMA362	9938	398	10237	10128	88.6
AMA364	28494	1079	9813	9577	83.3
AMA366	38228	1441	10264	10224	90.0
AMA368	12968	503	11122	10825	93.1
AMA369	7280	311	11225	11149	95.6
AMA374	7030	305	10225	8805	71.4
AMA379	7970	348	11226	11092	96.1
AMA381	7522	327	11214	11208	97.7
AMA382	14040	411	9915	9424	76.6

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Figure 1

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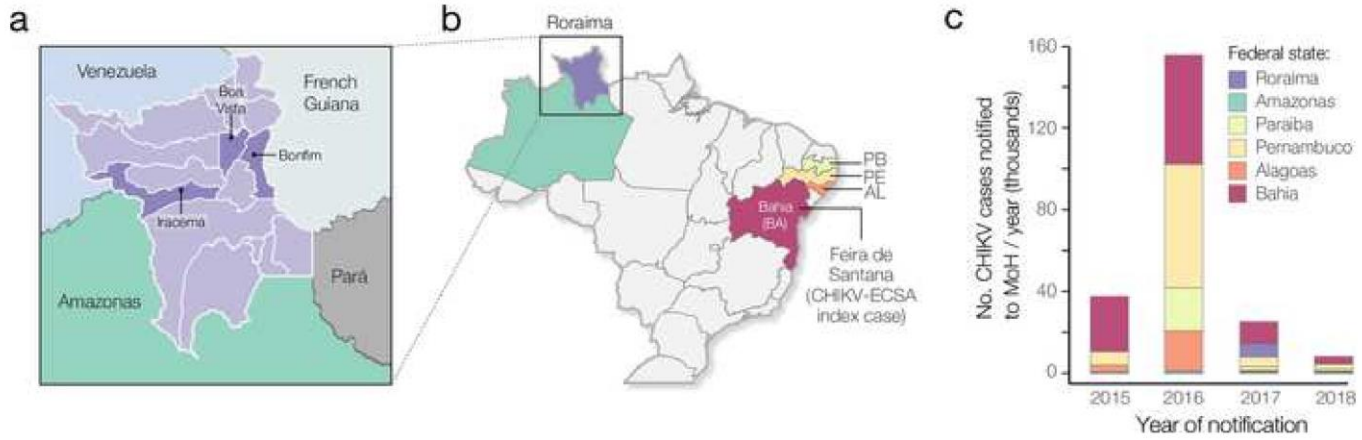

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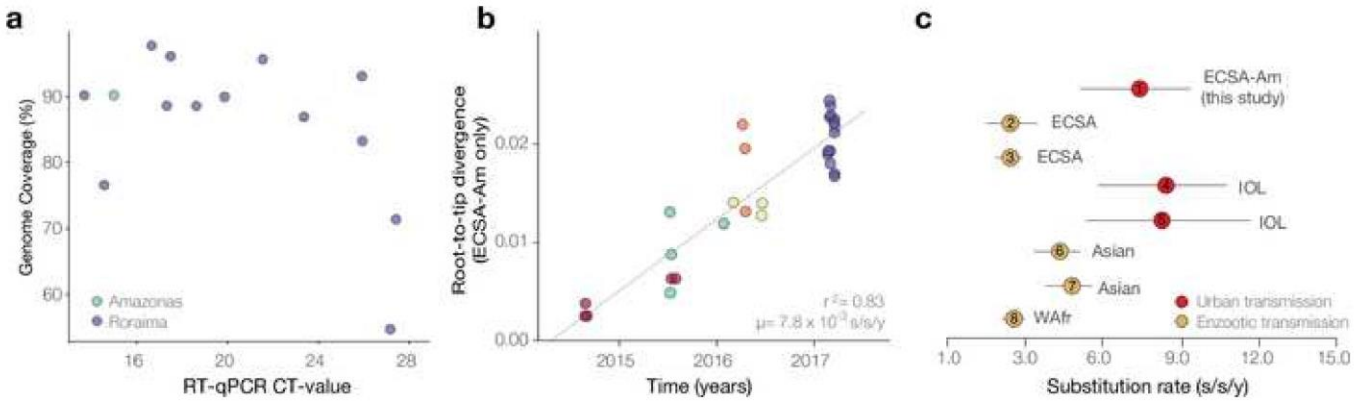

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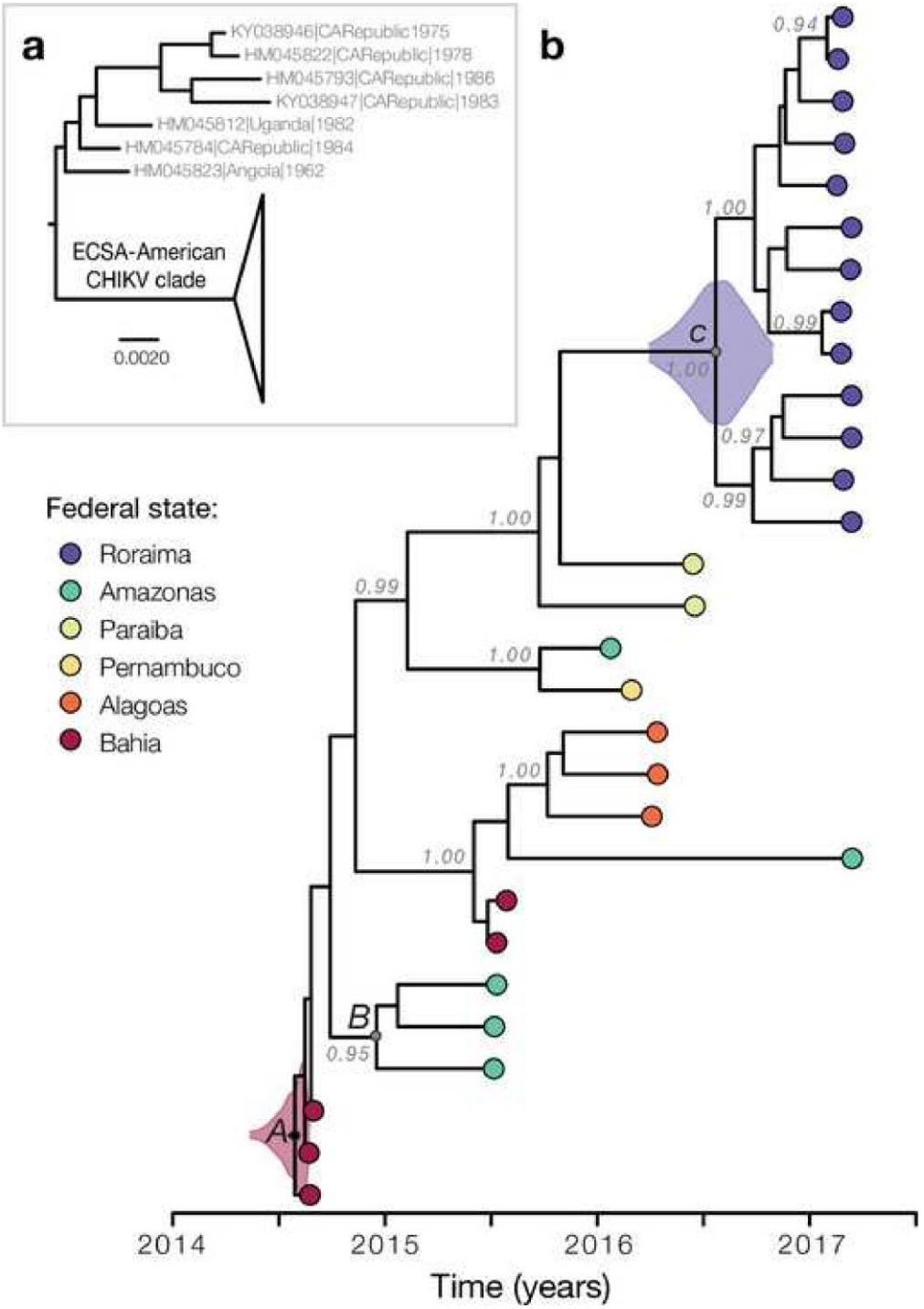

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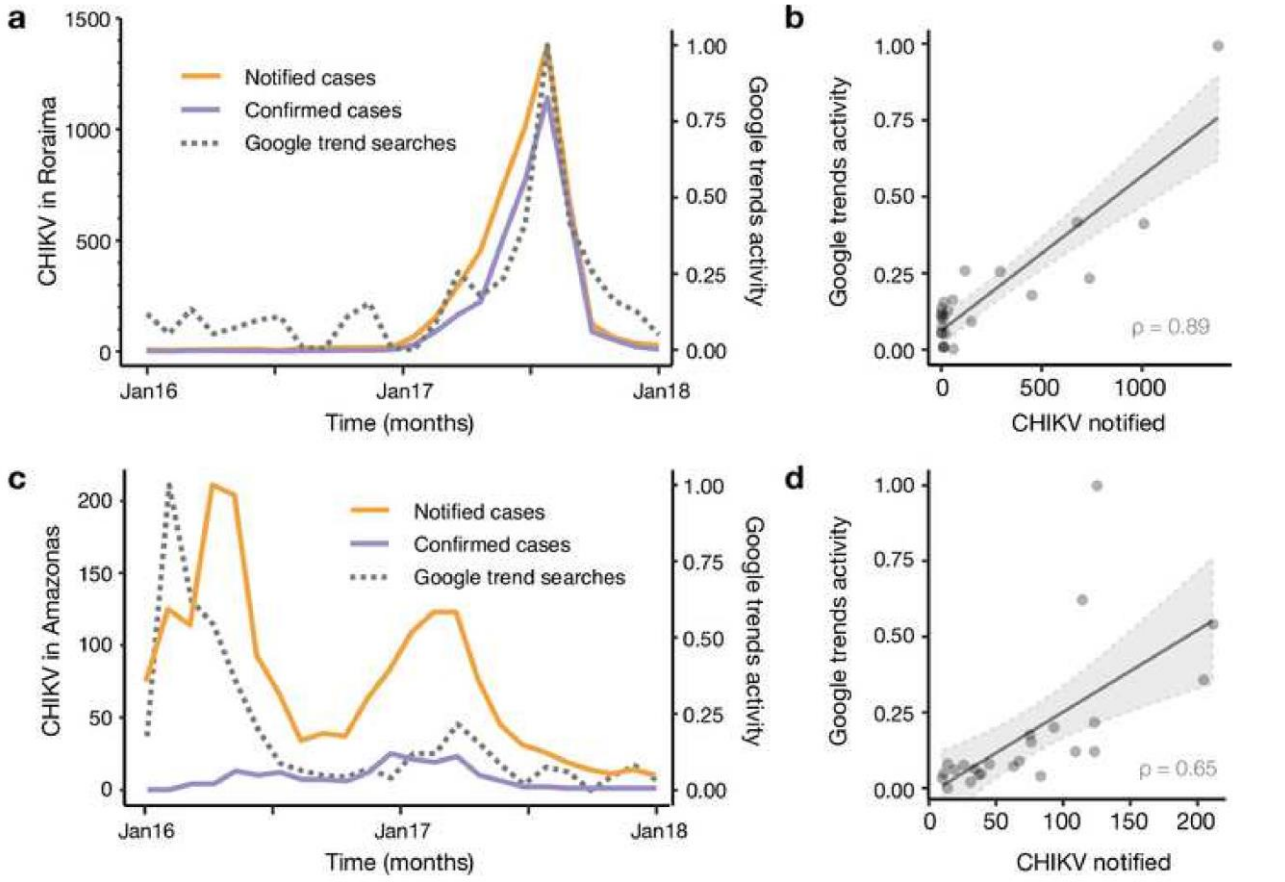

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RESEARCH ARTICLE

YELLOW FEVER

Genomic and epidemiological monitoring of yellow fever virus transmission potential

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The yellow fever virus (YFV) epidemic in Brazil is the largest in decades. The recent discovery of YFV in Brazilian *Aedes* species mosquitos highlights a need to monitor the risk of reestablishment of urban YFV transmission in the Americas. We use a suite of epidemiological, spatial, and genomic approaches to characterize YFV transmission. We show that the age and sex distribution of human cases is characteristic of sylvatic transmission. Analysis of YFV cases combined with genomes generated locally reveals an early phase of sylvatic YFV transmission and spatial expansion toward previously YFV-free areas, followed by a rise in viral spillover to humans in late 2016. Our results establish a framework for monitoring YFV transmission in real time that will contribute to a global strategy to eliminate future YFV epidemics.

Yellow fever (YF) is responsible for 29,000 to 60,000 deaths annually in South America and Africa (*1*) and is the most severe mosquito-borne infection in the tropics (*2*). Despite the existence of an effective YF vaccine since 1937 (*3*), an estimated >400 million unvaccinated people live in areas at risk of infection (*4*). Yellow fever virus (YFV) is a member of the *Flaviviridae* family and is classified into four genotypes: East African, West African, South American I, and South American II (*5*–*9*). In the Americas, YFV transmission occurs mainly via the sylvatic cycle, in which nonhuman primates (NHPs) are infected by tree-dwelling mosquito vectors such as *Haemagogus* spp. and *Sabethes* spp. (*10*, *11*). YFV transmission can also occur via an urban cycle, in which humans are infected by *Aedes* spp. mosquitoes that feed mostly on humans (*12*, *13*).

Brazil has recently experienced its largest-recorded YF outbreak in decades, with 2043 confirmed cases and 676 deaths since December 2016 (supplementary text and fig. S1) (*14*). The last YF cases in Brazil attributed to an urban cycle were in Sena Madureira, in the northern state of Acre, in 1942 (*15*). An intensive eradication campaign eliminated *Aedes aegypti* and YF from Brazil in the 1950s (*16*), but the vector became reestablished in the 1970s and *Aedes* spp. mosquitoes are now abundant across most of Brazil (*17*). The consequences of a reignition of urban cycle transmission in Brazil would be serious, as an estimated 35 million people in areas at risk for YFV transmission in Brazil remain unvaccinated (*4*). New surveillance and analytical approaches are therefore needed to monitor this risk in real time.

Yellow fever virus outbreak in Brazil, 2016–2017

Between December 2016 and the end of June 2017, there were 777 polymerase chain reaction (PCR)–confirmed human cases of YF across 10 Brazilian states—mostly in Minas Gerais (MG) (60% of cases), followed by Espírito Santo (32%), Rio de Janeiro (3%), and São Paulo (3%) (*18*). The fatality ratio of severe YF cases was estimated at 33.6%, comparable to previous outbreaks (*19*, *20*). Despite the exceptional magnitude and rapid expansion of the outbreak, little is known about its genomic epidemiology. Further, it is uncertain how the virus is spreading through space, as well as between humans and NHPs, and analytical insights into the contribution of the urban cycle to ongoing transmission are lacking.

To characterize the 2017 YFV outbreak in Brazil, we first compared time series of confirmed cases in humans (n = 683) and NHPs (n = 313) reported until October 2017 by public health institutes in MG, the epicenter of the outbreak (Fig. 1, A and B, and fig. S2). The time series are strongly associated (cross-correlation coefficient = 0.97; $P < 0.001$). Both peak in late January 2017, and we estimate that human cases lag behind those in NHPs by 4 days (table S1). NHP cases are geographically more dispersed

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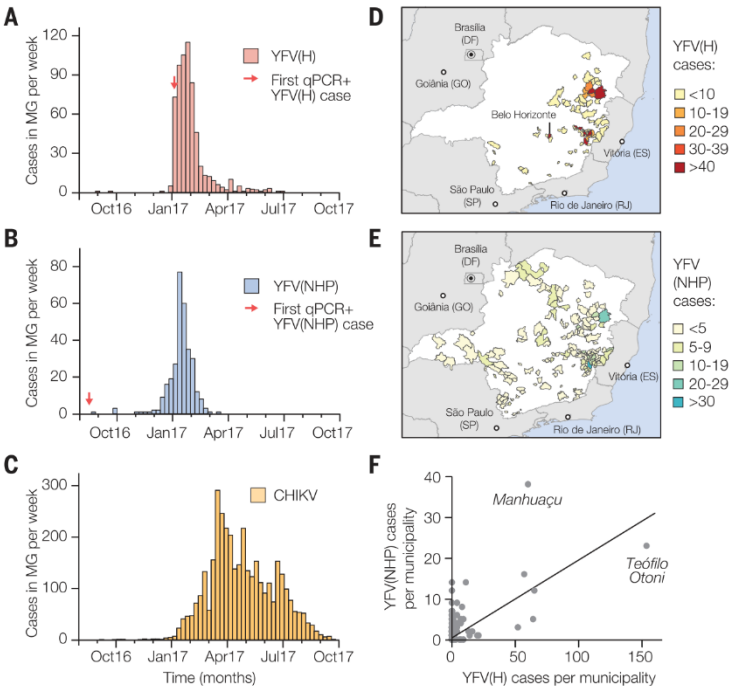

Fig. 1. Spatial and temporal epidemiology of YFV and CHIKV in Minas Gerais (MG). (**A**) Time series of human (H) YFV cases in MG (676 cases across 61 municipalities)—confirmed by serology, reverse transcription quantitative PCR (RT-qPCR), or virus isolation—during the first YFV epidemic wave (August 2016 to October 2017). (**B**) Same as in (A) but showing NHP YFV cases (313 cases across 90 municipalities) confirmed by RT-qPCR. (**C**) Same as in (A) but showing human CHIKV cases (3668 cases across 129 municipalities). (**D**) Geographic distribution of human YFV cases in MG. (**E**) Geographic distribution of NHP YFV cases in MG. Figure S3 shows the corresponding geographic distribution of CHIKV cases. (**F**) Association between the number of human and NHP cases in each municipality of MG (Pearson's $r = 0.62$; $P < 0.0001$; nonparametric Spearman's rank $\rho = 0.32$; $P < 0.05$).

in MG than human cases, which are more concentrated in the Teófilo Otóni and Manhuaçu municipalities (Fig. 1, D and E). Despite this, the numbers of human and NHP cases per municipality are positively correlated (Fig. 1F).

To establish whether human cases are acquired in proximity to potential sources of sylvatic infection, we estimated the distance between the municipality of residence of each human case and the nearest habitat of potential transmission, determined by using the enhanced vegetation index (EVI) (*21*) (supplementary materials). The average minimum distance between areas with EVI > 0.4 and the residence of confirmed human cases is only 5.3 km. In contrast, a randomly chosen resident of MG lives, on average, ≥51 km away from areas with EVI > 0.4. Similarly, human YFV cases reside, on average, 1.7 km from the nearest NHP case, whereas the mean minimum distance of a randomly chosen MG resident to the nearest NHP case is 39.1 km. This is consistent with YF infection risk being greatest for people who reside or work in forested areas where sylvatic transmission occurs. We find that most human cases (98.5%) were reported in municipalities with estimated YFV vaccination coverage above the 80% threshold recommended by the World Health Organization (WHO). On average, human cases would need to travel 65 km from their place of residence to reach an area where vaccination coverage is <80% (*4*).

Risk of YFV urban transmission

YFV was detected in *Aedes albopictus* mosquitoes caught in MG in January 2017 (*22*). Further, experiments suggest that *Aedes* spp. mosquitoes from southeast Brazil can transmit Brazilian YFV, although perhaps less effectively than vectors from elsewhere in the country (*23*, *24*). It is therefore important to investigate whether YFV cases in MG occur where and when *Aedes* spp. vectors are active. To do so, we analyzed confirmed chikungunya virus (CHIKV) cases from MG (Fig. 1C).

CHIKV is transmitted by the urban mosquitoes *Ae. aegypti* and *Ae. albopictus* (*25*). There were 3755 confirmed CHIKV cases in MG during January 2015 to October 2017. The CHIKV epidemic in MG in 2017 began later and lasted longer than the YFV outbreak (Fig. 1C), consistent with the hypothesis that YFV and CHIKV in the region are transmitted by different vector species. However, 29 municipalities with human YFV cases also reported CHIKV cases (Fig. 1D and fig. S3), indicating that YFV is indeed present in municipalities with *Aedes* mosquitoes. The mean

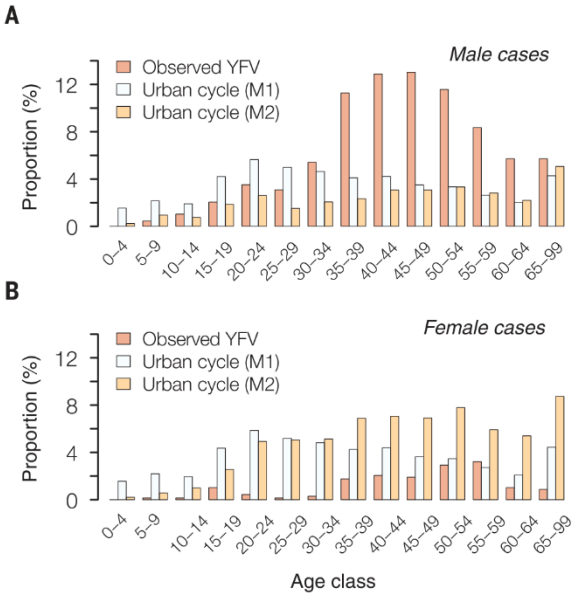

Fig. 2. Age and sex distribution of YFV cases in MG, 2016–2017. Red bars show the proportion of observed YFV cases in MG that occur in each age class, in (**A**) males and (**B**) females. These empirical distributions are different from those predicted under two models (M1, pale blue bars; M2, orange bars) of urban cycle transmission (see text for details).

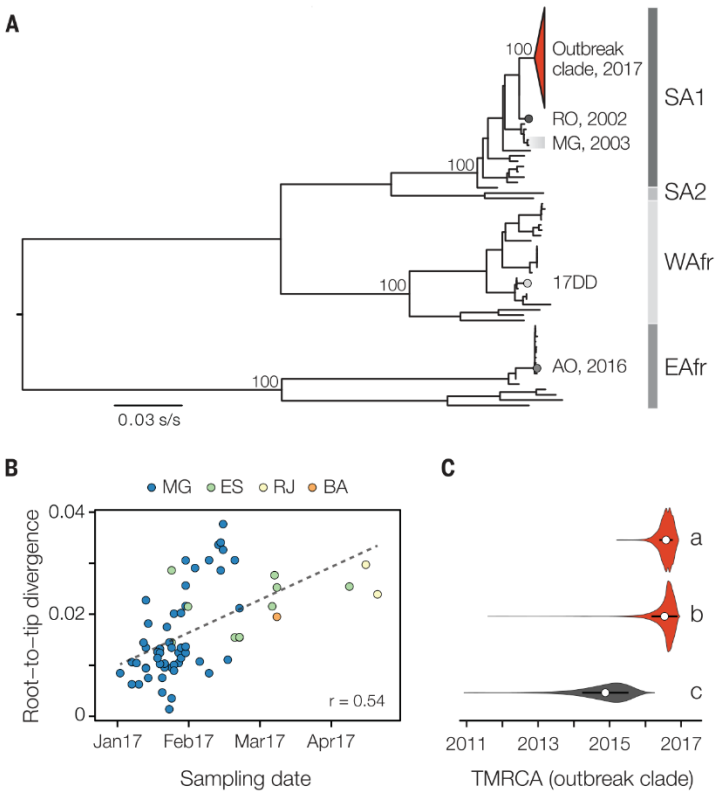

Fig. 3. Molecular phylogenetics of the Brazilian YFV epidemic. (**A**) Maximum likelihood phylogeny of complete YFV genomes showing the outbreak clade (red triangle) within the South American I (SA1) genotype (Fig. 4 and fig. S6). SA2, WAfr, and EAfr indicate the South America II, West Africa, and East Africa genotypes, respectively. For clarity, five YFV strains introduced to Venezuela from Brazil (*49*) are not shown. The scale bar is in units of substitutions per site (s/s). Node labels indicate bootstrap support values. RO 2002, strain BeH655417 from Roraima; MG 2003, two strains from the previous YF outbreak in MG in 2003; 17DD, the vaccine strain used in Brazil; AO 2016, YFV outbreak Angola in 2015–2016 (*13*). (**B**) Root-to-tip regression of sequence sampling date against genetic divergence from the root of the outbreak clade (fig. S6). Sequences are colored according to sampling location (MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; BA, Bahia). (**C**) Violin plots showing estimated posterior distributions (white circles denote means) of the time of the most recent common ancestor (TMRCA) of the outbreak clade. Estimates were obtained using two different datasets (gray, SA1 genotype; red, outbreak clade) and under different evolutionary models: a, uncorrelated lognormal relaxed clock (UCLN) model with a skygrid tree prior with covariates specifically, the time series data shown in Fig. 1, A to C; also see fig. S7); b, UCLN model with a skygrid tree prior without covariates; c, fixed local clock model (see supplementary materials).

YFV vaccination rate in districts with both YFV and CHIKV cases is 72.6% (range = 61 to 78%) (*4*). Thus, relatively high vaccination rates in the locations in MG where YF spillover to humans occurs, and potentially lower vector competence (*23*, *24*), may ameliorate the risk of establishment of an urban YFV cycle in the state. However, adjacent urban regions (including São Paulo and Rio de Janeiro) have lower vaccination rates (*4*), receive tens of millions of visitors per year (*26*), and have recently experienced many human YFV cases (*20*). Thus, the possibility of sustained urban YFV transmission in southern Brazil and beyond necessitates continual virological and epidemiological monitoring.

We sought to establish a framework to evaluate routes of YFV transmission during an outbreak from the characteristics of infected individuals. Specifically, we assessed whether an outbreak is driven by sylvatic or urban transmission by comparing the age and sex distributions of observed YFV cases with those expected under an urban cycle in MG. For example, an individual's risk of acquiring YFV via the sylvatic cycle depends on their likelihood to travel to forested areas, an occurence that is typically highest among male adults (*27*). In contrast, under an urban cycle, we expect more uniform exposure across age and sex classes, similar to that observed for urban cases in Paraguay (*28*) and Nigeria (*29*).

The male-to-female sex ratio of reported YFV cases in MG is 5.7 (85% of cases are male), and incidence is highest among males aged 40 to 49 (Fig. 2). We compared this distribution to that expected under two models of urban cycle transmission (supplementary materials). In model M1, age and sex classes vary in vaccination status but are equally exposed to YFV, a scenario that is typical of arboviral transmission (*30*). Under model M1, predicted cases are characterized by a sex ratio ~1, and incidence peaks among individuals aged 20 to 25 (Fig. 2). In model M2, we assume that the pattern of YFV exposure among age and sex classes follows that observed for CHIKV. The sex ratio of reported CHIKV cases in MG is 0.49 (33% of cases are male) (fig. S4). Under model M2, predicted incidence is highest in females aged >30. The discrepancy between the observed distribution and that predicted under the two urban cycle models indicates that the YF epidemic in MG is dominated by sylvatic transmission. This method shows that age- and sex-structured epidemiological data can be used to qualitatively evaluate the mode of YFV transmission during an outbreak.

Genomic surveillance of the Brazilian YFV outbreak

During a YF outbreak, it is important to undertake virological surveillance to (i) track epidemic origins and transmission hotspots, (ii) characterize genetic diversity to aid molecular diagnostics, (iii) detect viral mutations associated with disease severity, and (iv) exclude the possibility that human cases are caused by vaccine reversion. We generated 62 complete YF genomes from infected humans (n = 33) and NHPs (n = 29) from the most affected Brazilian states, including MG (n = 51), Espírito Santo (n = 8), Rio de Janeiro (n = 2), and Bahia (n = 1) (Fig. 3 and table S3). We also report two genomes from samples collected in 2003 during a previous YFV outbreak in MG from 2002 to 2003 (*31*). Genomes were generated in Brazil using a combination of methods (tables S5 to S7); half were generated in MG using a MinION portable YFV sequencing protocol adapted from (*32*) (tables S4 and S5). This protocol was made publicly available in May 2017 after the completion of pilot sequencing experiments using a cultured vaccine strain (supplementary materials). Median genome coverages were similar for samples obtained from NHPs [99%; median cycle threshold value (Ct) = 11] and from human cases (99%; median Ct = 16) (tables S5 to S7).

To put the newly sequenced YFV genomes in a global context, we added our genomes to a pool of 61 publicly available genomes (*33*, *34*). We developed and applied an automated online phylogenetic tool to identify and classify YFV gene sequences (also publicly available, see supplementary materials). Phylogenies estimated by

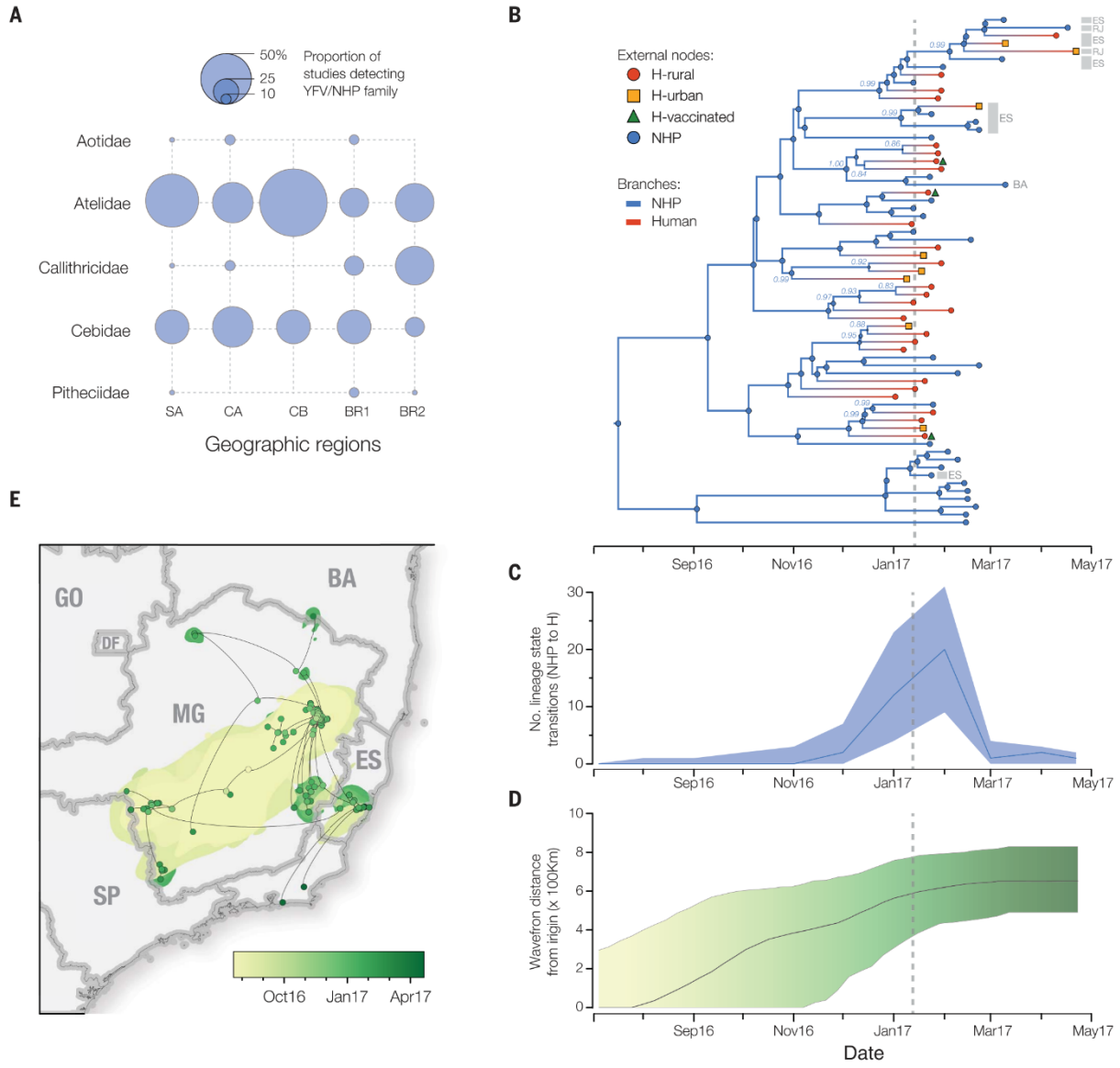

Fig. 4. Spatial and evolutionary dynamics of YFV outbreak. (**A**) Frequency of detection of YFV in NHPs in the Americas (*50*). Circle sizes represent the proportion of published studies (n = 15) that have detected YFV in each primate family and region. SA, South America (except Brazil); CA, Central America; CB, Caribbean; BR1, Brazil (before 2017); BR2, Brazil (this study). (**B**) Maximum clade credibility phylogeny inferred under a two-state (human and NHP) structured coalescent model. External node symbols denote sample type. Gray bars and labels indicate sample location (RJ, Rio de Janeiro; ES, Espírito Santo; BA, Bahia; others were sampled in MG). Internal nodes whose posterior state probabilities are >0.8 are annotated by circles. Node labels indicate posterior state probabilities for selected nodes. Internal branches are blue for NHPs and red for humans. Figure S8 shows a fully annotated tree. (**C**) Average number of YFV phylogenetic state transitions (from NHPs to humans) per month. Solid line, median estimate; shaded area, 95% BCI. (**D**) Expansion of the YFV epidemic wavefront estimated using a continuous phylogeographic approach (*35*). At each time point the plot shows the maximum spatial distance between phylogeny branches and the inferred location of outbreak origin. Solid line, median estimate; shaded area, 95% BCI. The dashed lines in (B) to (D) indicate when YF was declared a public health emergency in MG (13 January 2017). (**E**) Reconstructed spatiotemporal diffusion of the YFV outbreak. Phylogeny branches are arranged in space according the locations of phylogeny nodes (circles). Locations of external nodes are known, whereas those of internal nodes are inferred (*44*). DF, Distrito Federal; GO, Goiás; SP, São Paulo. Shaded regions represent 95% credible regions of internal nodes. Nodes and uncertainty regions are colored according to time.

this tool, along with maximum likelihood and Bayesian methods, consistently place the Brazilian outbreak strains in a single clade within the South America I (SA1) genotype with maximum statistical support (bootstrap = 100%; posterior probability > 0.99) (Fig. 3A and fig. S5).

The outgroup to the outbreak clade is strain BeH655417, a human case sampled in Alto Alegre, Roraima, north Brazil, in 2002. In contrast, isolates sampled during the previous outbreak in MG in 2003 are more distantly related to the outbreak clade within the SA1 genotype (Fig. 3A). Thus, the 2017 outbreak was more likely caused by a YFV strain introduced from an endemic area, possibly northern or center-west Brazil (*35*), than by the reemergence of a lineage that had persisted in MG. Although low sampling densities mean that this conclusion is provisional, similar scenarios have been suggested for previous Brazilian epizootics (*36*). The 14-year gap between the current outbreak and the date of the most closely related nonoutbreak strain agrees with the reported periodicity of YF outbreaks in northern Brazil (*37*), thought to be dictated by vector abundance and the accumulation of susceptible NHP hosts (*19*, *38*).

At least seven humans from MG with PCR-confirmed YFV received a YF vaccine before the onset of symptoms. To test that these occurrences were caused by natural infection, and not by vaccine reactivation, we sequenced the YFV genomes from three of these cases (Fig. 3A and table S3). Our phylogenetic analysis clearly shows that these represent natural infections caused by the ongoing outbreak and are conclusively not derived from the 17DD vaccine strain (which belongs to the West African YFV genotype) (Fig. 3A and fig. S6).

Unifying YFV epidemiology and molecular evolution

Virus genomes are a valuable source of information about epidemic dynamics (*39*) but are rarely used to investigate specific YFV outbreaks in detail. Here we show how a suite of three analytical approaches, which combine genetic, epidemiological, and spatial data, can provide insights into YFV transmission.

First, we used a Bayesian method (*40*) to explore potential covariates of fluctuations in the effective population size of the YFV outbreak in 2017. After finding that genetic divergence in the outbreak clade accumulates over the time scale of sampling (Fig. 3B and fig. S6), albeit weakly, we sought to determine which epidemiological time series best describe trends in inferred YFV effective population size. We found that effective population size fluctuations of the YFV outbreak are well explained by the dynamics of both human and NHP YFV cases (inclusion probability: 0.37 for human cases and 0.63 for NHP cases) (table S8). These two YFV time series explain the genetic diversity dynamics of the ongoing outbreak 10^3 times more effectively than CHIKV incidence (inclusion probability <0.001), which represents transmission by *Aedes* spp. vectors. One benefit of this approach is that epidemiological data contribute to estimation of the outbreak time scale. By incorporating YFV incidence data into evolutionary inference, we estimate the time of the most recent common ancestor (TMRCA) of the outbreak clade to be late July 2016 [95% Bayesian credible interval (BCI): March to November 2016] (Fig. 3C and fig. S7), consistent with the date of the first PCR-confirmed case of YFV in a NHP in MG (Jul 2016). The uncertainty around the TMRCA estimate is reduced by 30% when epidemiological and genomic data are combined, compared with genetic data alone (Fig. 3C).

Second, to better understand YFV transmission between humans and NHPs, we measured the movement of YFV lineages between the NHP reservoir and humans, using a phylogenetic structured coalescent model (*41*). Although previous studies have confirmed that YFV is circulating in five neotropical NHP families (Aotidae, Atelidae, Callitrichidae, Pitheciidae, and Cebidae) (Fig. 4A), thus far NHP YFV genomes during the 2017 outbreak have been recovered only from *Alouatta* spp. (family Cebidae) (*33*). In this analysis, we used the TMRCA estimate obtained above (Fig. 3C) to inform the phylogenetic time scale (Fig. 4B). All internal nodes in the outbreak phylogeny whose host state is well supported (posterior probability >0.8) are inferred to belong to the NHP population, consistent with an absence of urban transmission and in agreement with the large number of NHP cases reported in southeast Brazil (*20*). Despite this, we caution that hypotheses of human-to-human transmission linkage should not be tested directly using phylogenetic data alone, owing to the large undersampling of NHP infections. Notably, the structured coalescent approach reveals substantial changes in the frequency of NHP-to-human host transitions through time, rising from zero around November 2016 and peaking in February 2017 (Fig. 4C). This phylogenetic trend matches the time series of confirmed YFV cases in MG (Fig. 1, A and B), demonstrating that viral genomes, when analyzed using appropriate models, can be used to quantitatively track the dynamics of zoonosis during the course of an outbreak (*42*).

Third, we used a phylogenetic relaxed random walk approach to measure the outbreak's spatial spread (*43*) (supplementary materials and methods and table S9). When projected through space and time (Fig. 4, D and E, and movie S1), the phylogeny shows a southerly dissemination of virus lineages from their inferred origin in MG toward densely populated areas, including Rio de Janeiro and São Paulo (where YF vaccination was not recommended until July 2017 and January 2018, respectively). We estimate that virus lineages move, on average, 4.25 km/day (95% BCI: 2.64 to 10.76 km/day) (*44*). This velocity is similar when human YFV terminal branches are removed (5.3 km/day) and therefore most likely reflects YFV lineage movement within the sylvatic cycle and not the movement of asymptomatic infected humans. These rates are higher than expected given the distances typically travelled by NHPs in the region (*45*) and suggest the possibility that YFV lineage movement may have been aided by human activity—e.g., transport of infected mosquitoes in vehicles (*46*) or hunting or illegal trade of NHPs in the Atlantic forest (*47*, *48*). The epidemic wavefront (maximum distance of phylogeny branches from the inferred epidemic origin) expanded steadily between August 2016 and February 2017 at an estimated rate of ~3.3 km/day. Therefore, by the time YF was declared a public health emergency in MG (13 January 2017; dashed lines in Fig. 4, B to D), the epidemic had already expanded ~600 km (Fig. 4D) and caused >100 reported cases in both humans and NHPs (Fig. 1). Notably, the first detection in humans in December 2016 was concomitant with the outbreak's spatial expansion phase (Fig. 4D) and the rise in estimated NHP-to-human zoonoses (Fig. 4C); both were likely driven by an increase in the abundance of sylvatic vectors. Thus, the outbreak lineage appeared to circulate among NHPs in a widening geographic area for several months before human cases were detected.

Conclusion

Epidemiological and genomic surveillance of human and animal populations at risk is crucial for early detection and rapid containment of YFV transmission. The YFV epidemic in Brazil continues to unfold with an increase in cases since December 2017. Longitudinal studies of NHPs are needed to understand how YFV lineages disseminate across South America between outbreaks and how epizootics are determined by the dynamics of susceptible animals in the reservoir. To achieve the WHO's goal to eliminate YF epidemics by 2026, YF surveillance necessitates a global, coordinated strategy. Our results and analyses show that rapid genomic surveillance of YFV, when integrated with epidemiological and spatial data, could help anticipate the risk of human YFV exposure through space and time and monitor the likelihood of sylvatic versus urban transmission. We hope that the toolkit introduced here will prove useful in guiding YF control in a resource-efficient manner.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S9
References (*51*–*107*)
Movie S1

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Letter to the Editor

Genetic evidence of Zika virus in mother's breast milk and body fluids of a newborn with severe congenital defects

On the basis of the upsurge in the number of newborns with neurologic disorders in the northeast, in November 2015 the Brazilian Ministry of Health declared a public health emergency of national concern [1]. On the basis of evidence for a potential association between microcephaly and other neurologic disorders and Zika virus (ZIKV) infection, the World Health Organization declared a public health emergency of international concern on 1 February 2016 [1]. Here we report genetic evidence of ZIKV RNA in the mother's breast milk and in the serum and urine of a newborn with severe congenital defects.

The institutional review boards at the Instituto Gonçalo Moniz (Fiocruz-Bahia) approved the present study, and the subject provided written and informed consent before her participation.

A 32-year-old pregnant woman from the municipality of Feira de Santana (Bahia, Brazil) reported diffuse pruritic cutaneous rash and joint pain during the ninth gestational week. Serologic tests for toxoplasmosis, herpesvirus 1 and 2, dengue and chikungunya viruses were negative. Immunoglobulin G ELISA was positive and immunoglobulin M ELISA was negative for rubella and cytomegalovirus.

During her 22nd gestational week, morphologic ultrasound revealed alterations in the fetus' left hand, confirmed at week 23 via ultrasound and supported after birth by X-ray (Fig. 1(A) and (B)).

At week 30, foetal biometry appeared to be consistent with gestational age, with the exception of a reduced cephalic circumference, indicating microcephaly. Delivery was performed by C-

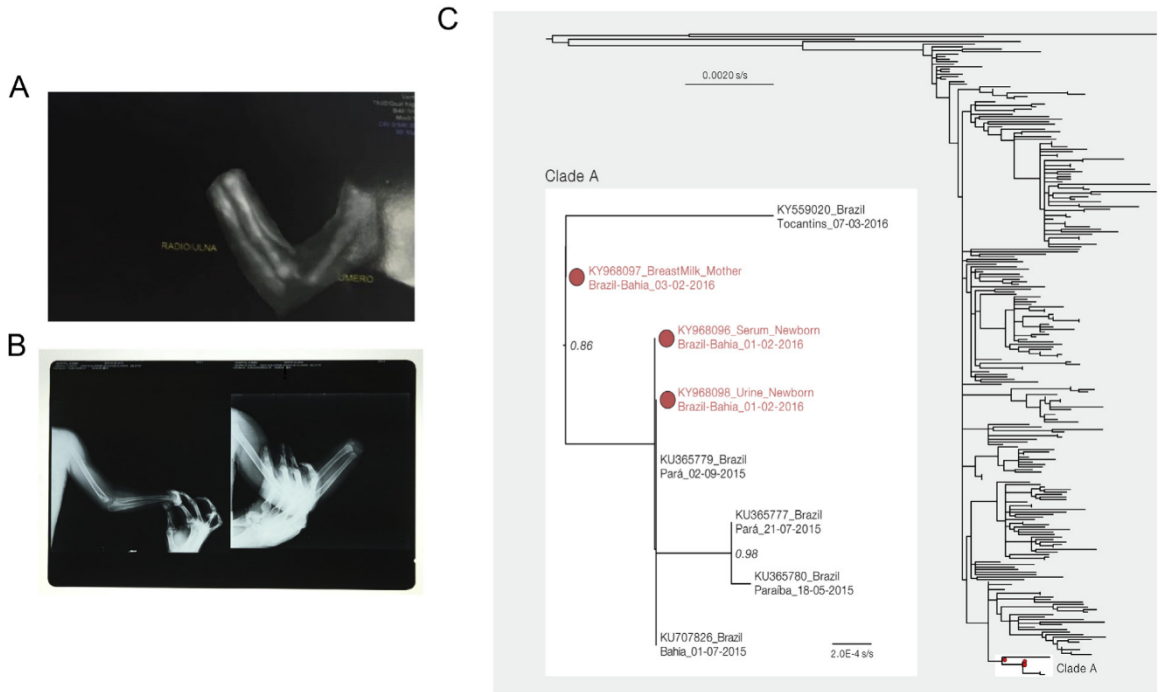

Fig. 1. (A, B) Clinical evaluation of congenital abnormalities. (A) Foetal ultrasound performed during 23rd gestational week revealed morphologic alteration in left hand of fetus. (B) X-ray evaluation performed after second year of birth confirmed defect of left upper limb. (C) Phylogenetic analysis of a newborn with congenital defects. Maximum likelihood phylogeny of NS5 sequences. Phylogeny was estimated using PhyML. Data set used contained new sequences recovered from mother's breast milk and from serum and urine of newborn, in addition to 254 publicly available complete genome sequences of Zika virus Asian genotype sampled in South America, Asia, Europe, Caribbean, North America and Pacific Islands. Sequences obtained from present study subjects are highlighted in red. Scale bar is in units of nucleotide substitutions per site. Inset shows close-up view of clade A containing newly isolated strains from Feira de Santana, Bahia (February 2016).

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section in the 38th gestational week, at which time the mother's blood and a fragment of placenta were collected. A male neonate was born with a weight of 2502 g and a head circumference of 29 cm, classified as severe microcephaly by Intergrowth-21st standard (*Z* score −3.4) [2]. A craniofacial disproportion, abnormal skull morphology with a sloping forehead and a defect of the left upper limb with ring constriction and residual nubbins were observed, indicating the presence of a possible amniotic band syndrome lesion (Fig. 1(A) and (B)).

Samples of the newborn's blood and urine were collected in the first 24 hours of birth, and breast milk was collected 2 days after delivery. Virus RNA was extracted from clinical samples and submitted to real-time quantitative PCR as a reference [3]. Although the placenta sample was positive with a threshold cycle of 33.0, the low virus loads in other samples prevented virus detection by real-time quantitative PCR. A specific set of primers corresponding to the NS5 gene were then designed for conventional nested PCR (Supplementary Material), obtaining ZIKV-specific reverse transcriptase PCR amplification products from newborn serum and urine and the mother's breast milk, while the mother's serum yielded no detectable products. NS5 gene fragments (426 bp) were then obtained using Sanger sequencing. The ZIKV sequences generated from the mother's breast milk and newborn urine and serum were deposited in GenBank under accession numbers KY968096, KY968098 and KY968097, respectively. Although genetic data alone cannot provide definitive evidence of direct vertical transmission, phylogenetic analysis indicated that the sequences generated from the mother's and newborn's body fluids clustered together with strong support (bootstrap support = 0.86, Fig. 1(C)). As expected, maximum likelihood analysis of the present and the other reference ZIKV genome sequences revealed that all belong to the Asian genotype.

Other authors have already described ZIKV in the breast milk of mothers of neonates born without microcephaly [4,5]. Here we report the presence of ZIKV both in the mother's breast milk and in the serum and urine of a newborn with congenital defects.

Transparency declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2018.06.008.

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