UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA INTERUNIDADES DE PÓS-GRADUAÇÃO EM BIOINFORMÁTICA

# VIGILÂNCIA GENÔMICA DOS ARBOVIROSES EMERGENTES E REEMERGENTES CIRCULANTES E CO-CIRCULANTES NO ESTADO DE MINAS GERAIS

Felipe Campos de Melo Iani

Belo Horizonte
2021

FELIPE CAMPOS DE MELO IANI

# VIGILÂNCIA GENÔMICA DOS ARBOVIROSES EMERGENTES E REEMERGENTES CIRCULANTES E CO-CIRCULANTES NO ESTADO DE MINAS GERAIS

Tese apresentada ao Programa Interunidades de Pós-Graduação em Bioinformática da Universidade Federal de Minas Gerais, como requisito para obtenção do grau de Doutor em Bioinformática.

Orientador: Dr. Luiz Carlos Júnior Alcantara

Coorientadores: Dra. Marta Giovanetti, Dr. Nuno Rodrigues Faria

Belo Horizonte

2021

043 Iani, Felipe Campos de Melo.
Vigilância genômica dos arboviroses emergentes e reemergentes circulantes e co-circulantes no estado de Minas Gerais [manuscrito] / Felipe Campos de Melo Iani. – 2021.
96 f. : il. ; 29,5 cm.

Orientador: Dr. Luiz Carlos Júnior Alcantara. Coorientadores: Dra. Marta Giovanetti, Dr. Nuno Rodrigues Faria.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa Interunidades de Pós-Graduação em Bioinformática.

1. Biologia Computacional. 2. Zika virus. 3. Febre Amarela. 4. Vírus Chikungunya. 5. Genoma. I. Alcantara, Luiz Carlos Júnior. II. Giovanetti, Marta. III. Faria, Nuno Rodrigues. IV. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. V. Título.

CDU: 573:004

Ficha catalográfica elaborada pela bibliotecária Fabiane C M Reis – CRB 6 - 2680

UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-graduação em Bioinformática

## ATA DA DEFESA DE TESE

### FELIPE CAMPOS DE MELO IANI

Às quatorze horas do dia 04 de agosto de 2021, reuniu-se, através de videoconferência, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho de **Felipe Campos de Melo Iani**, intitulado: **"Vigilância Genômica Dos Arboviroses Emergentes E Reemergentes Circulantes E Co-Circulantes No Estado De Minas Gerais"**, requisito para obtenção do grau de Doutor em **Bioinformática.** Abrindo a sessão, o Presidente da Comissão, Dr. Luiz Carlos Júnior Alcântara, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

| **Prof./Pesq.** | Instituição | Indicação |
|---|---|---|
| Dr. Luiz Carlos Júnior Alcântara - Orientador | Fiocruz/BA | Aprovado |
| Dr. José Lourenço | University of Oxford | Aprovado |
| Dr. Aristóteles Góes Neto | Universidade Federal de Minas Gerais | Aprovado |
| Dra. Érica Azevedo Costa | Universidade Federal de Minas Gerais | Aprovado |
| Dr. Sérgio Caldas | Fundação Ezequiel Dias | Aprovado |

Pelas indicações, o candidato foi considerado: **Aprovado**

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

**Belo Horizonte, 04 de agosto de 2021.**

Documento assinado eletronicamente por **Erica Azevedo Costa, Professora do Magistério Superior**, em 04/08/2021, às 17:20, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por **Sérgio Caldas, Usuário Externo**, em 04/08/2021, às 17:21, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por **Luiz Carlos Junior Alcantara, Usuário Externo**, em 05/08/2021, às 12:18, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por **Jose Miguel Lima de Aguiar Graça Lourenço, Usuário Externo**, em 05/08/2021, às 12:58, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por **Aristoteles Goes Neto, Professor do Magistério Superior**, em 31/08/2021, às 11:37, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

A autenticidade deste documento pode ser conferida no site https://sei.ufmg.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **0871838** e o código CRC **2ADBFAC1**.

## AGRADECIMENTOS

Agradeço primeiramente a minha família que sempre acreditou em mim e me deu todo o suporte necessário para que eu realizasse este trabalho. A minha esposa Helen meu grande amor por toda parceria, incentivo e cumplicidade. Aos meus filhos Laila e Davi que são minha fonte de amor, alegria e diversão. Aos meus pais por todo apoio e pelo exemplo de competência e dedicação aos seus objetivos. Aos meus irmãos Raphael e Gabriella por todo incentivo e parceria.

Ao meu orientador Prof. Dr. Luiz Carlos Junior Alcantara pela confiança em meu trabalho, pelos valiosos ensinamentos e por todas oportunidades que me foram concedidas.

Ao meu coorientador Prof. Dr. Nuno Faria, por ter compartilhado os seus conhecimentos e me proporcionado uma grande experiência e uma excelente vivência científica.

A Funed que me permitiu e me deu todos os subsídios necessários para executar todos estes trabalhos. A Fapemig e a CAPES pelo financiamento de bolsas de estudos que foram essenciais para o alcance destes resultados.

Aos colegas do Serviço de Virologia e Riquestioses por manterem os serviços funcionando com excelência, e aqueles que me ajudaram participando ativamente dos projetos, assinalo todo meu reconhecimento.

A toda equipe do projeto Zibra II por me introduzirem neste mundo da vigilância genômica e me ensinarem todas as etapas dos trabalhos realizados, além de se tornarem grandes parceiros.

E principalmente a Deus por ser minha base e força em toda esta caminhada.

“Basta ser sincero e desejar profundo, você será capaz de sacudir o mundo.”

Raul Seixas

**RESUMO**

Apesar dos avanços da medicina moderna, doenças infeciosas estão entre as principais causas de morbidade e mortalidade no mundo, sendo as arboviroses responsáveis pela maioria dos casos, principalmente os vírus das famílias *Flaviviridae* e *Togaviridae*. Os vírus zika e febre amarela, do gênero *Flavivirus* e família *Flaviviridae*, são uma grande ameaça à saúde pública mundial. Um outro vírus de grande importância médica é o chikungunya, da família *Togaviridae*, responsável por grandes epidemias ao redor do mundo. Com o avanço da tecnologia de sequenciamento genético de nova geração tem sido possível realizar a vigilância genômica para auxiliar as autoridades sanitárias no combate às epidemias causadas por estes vírus emergentes e reemergentes. Nestes trabalhos foram sequenciados 69 genomas do vírus zika, 62 do vírus da febre amarela e 20 genomas do vírus chikungunya, totalizando 151 genomas. Com os resultados destes dados genômicos associados aos dados epidemiológicos foi possível caracterizar melhor as epidemias ocorridas por estes vírus nos últimos anos. A implantação da vigilância genômica no Laboratório de Saúde Pública de Minas Gerais possibilitou gerar dados genômicos que juntamente com os dados epidemiológicos tradicionais e com a utilização de ferramentas de bioinformática possibilitaram estabelecer a história evolutiva e da disseminação geográfica ao longo do tempo destes arbovírus.

Palavras-chaves: zika, febre amarela, sequenciamento, vigilância genômica.

**ABSTRACT**

Despite the advances in modern medicine, infectious diseases are among the main causes of morbidity and mortality in the world, with arboviruses being responsible for most cases, mainly viruses of the Flaviviridae and Togaviridae families. The Zika virus and yellow fever of the genus Flavivirus belonging to the family Flaviviridae are a major threat to public health worldwide. Another virus of great medical importance is the chikungunya of the Togaviridae family responsible for major epidemics around the world. With the advancement of new generation genetic sequencing technology, it has been possible to carry out genomic surveillance to assist health authorities in combating epidemics caused by these emerging and reemerging viruses. In these works, 69 genomes of the Zika virus, 62 of the yellow fever virus and 20 genomes of the Chikungunya virus were sequenced, totaling 151 genomes. With the results of these genomic data associated with epidemiological data, it was possible to better characterize the epidemics that have occurred in recent years. The implementation of genomic surveillance in the public health laboratory of Minas Gerais made it possible to generate genomic data which, together with traditional epidemiological data and with the use of bioinformatics tools, made it possible to establish the evolutionary history and geographical spread over time of these arboviruses.

Key-words: zika, yellow fever, sequencing, genomic surveillance.

**LISTA DE FIGURAS**

## LISTA DE ABREVIAÇÕES

| | |
|---|---|
| C | Proteína do nucleocapsídeo |
| CHIKV | Vírus da chikungunya |
| DENV | Vírus da dengue |
| E | Proteína do envelope, E |
| ECSA | *East-Central-South African* |
| JEV | Vírus da encefalite japonesa |
| Lacen | Laboratório Central de Saúde Pública |
| ICTV | International Committee on Taxonomy of Viruses |
| MAYV | Vírus mayaro |
| nm | Nanometros |
| NS | Proteína não estrutural (*Non structural*) |
| ONNV | vírus O'nyong nyong |
| ORF | Janela de leitura aberta (*open reading frame*) |
| OROV | Vírus oropouche |
| pb | Pares de bases |
| PNH | Primata não humano |
| prM | Proteína pré-membrana |
| PTV | Punta Toro vírus |
| RNA | Ácido ribonucleico |
| RRV | Vírus da febre do rio Ross |
| RT-qPCR | Transcrição Reversa-Reação em cadeira da polimerase em tempo real |
| SLEV | Vírus da encefalite de Saint Louis |
| YFV | Vírus da febre amarela |
| WNV | Vírus do oeste do Nilo |
| ZIKV | Vírus zika |

## SUMÁRIO

## PREFÁCIO

As arboviroses estão entre as principais causas de doenças infeciosas a emergir ou reemergir em todo o mundo, estando dentre as principais causas de morbidade e mortalidade no mundo, apesar dos contínuos avanços da medicina moderna. Nas últimas décadas, com o avanço de plataformas de sequenciamento de nova geração, houve uma redução no custo do sequenciamento tornando-se viável a geração de inúmeros genomas virais completos, o que tem possibilitado o monitoramento das epidemias através da vigilância genômica.

Através dos genomas é possível quantificar a diversidade genética viral, reconstruir as origens da epidemia, estimar as taxas de transmissão, prever a possibilidade de novos surtos e fornecer informações para o desenvolvimento de vacinas e novos medicamentos, bem como melhoramento dos métodos diagnósticos sorológicos e moleculares. Para melhor entendimento das epidemias causadas por arbovírus foi implantado no Laboratório Central de Saúde Pública de Minas Gerais (Lacen-MG) a utilização de dois sequenciadores de nova geração para auxiliar nas atividades de vigilância genômica: i) Ion Torrent PGM (Thermo Fisher Scientific) e ii) MinION (Oxoford Nanopore). Estas novas tecnologias foram utilizadas nesta tese para dar suporte às estratégias de vigilância e monitoramento genômico dos arbovírus (zika, febre amarela e chikungunya) circulantes e co-circulantes no estado de Minas Gerais. Deste modo, a Tese será constituída em sete subdivisões, conforme apresentadas abaixo.

Na primeira seção foi realizada uma breve revisão bibliográfica sobre os temas tratados nesta Tese, abordando itens como histórico, características genômicas e estruturais, manifestações clínicas, tratamentos, diagnósticos, vacinas e epidemiologia dos vírus. O objetivo geral e específicos desse trabalho estão apresentados na segunda seção.

Na terceira seção são apresentados os resultados gerados durante o desenvolvimento desse trabalho, constituído por 4 artigos publicados:

- no primeiro artigo, foi investigada a epidemiologia e a evolução do vírus zika no estado de Minas Gerais. Neste trabalho, combinando dados genômicos e epidemiológicos, reconstruímos o evento de introdução e subsequente transmissão desse patógeno emergente no estado;
- no segundo artigo, realizado em colaboração com a Fiocruz-Amazonas, investigamos a dispersão do vírus zika no estado do Amazonas, explorando os mecanismos de introdução e dispersão nessa região;
- no terceiro artigo, estendemos a utilização da técnica de sequenciamento por nanoporos para entendermos a reemergência do vírus da febre amarela no sudeste do Brasil, no

estado de Minas Gerais, que ocorreu no final de dezembro de 2016. Nesse trabalho, desenvolvemos um protocolo para o sequenciamento do genoma completo do YFV que possibilitou a geração de 62 genomas completos, praticamente dobrando o número de genomas do vírus da febre amarela disponíveis em todo mundo. Além disso, nosso trabalho demonstrou que esta epidemia provavelmente se ateve ao ciclo silvestre da doença;

- no quarto artigo estendemos a utilização dessa tecnologia de sequenciamento inovadora para entendermos a dispersão de um outro arbovírus emergente no Brasil, o vírus chikungunya. Nesse trabalho foi investigada a introdução do genótipo africano (ECSA) pela primeira vez na região norte do país através da geração de 20 genomas completos oriundo do estado de Roraima.

Na quarta seção foi realizada uma discussão integrando todos os trabalhos realizados, demonstrando a importância dos resultados alcançados no auxílio ao combate das epidemias pelas autoridades de saúde pública. Na quinta seção é apresentada a conclusão da Tese, com todos os objetivos que foram alcançados ao longo desses anos.

Por fim, na seção Apêndice, estão elencados um capítulo de livro, outros cinco artigos publicados e mais quatro artigos já submetidos, produzidos durante o desenvolvimento dessa Tese.

# 1 INTRODUÇÃO

## 1.1 Arbovírus

Em comum com todos os organismos, os agentes patogênicos também evoluem. Todos os anos somos surpreendidos com relatos de patógenos humanos, previamente desconhecidos, que ampliaram sua área geográfica de ocorrência, se tornaram menos suscetíveis ao tratamento e à prevenção ou exibiram tendências epidêmicas sem precedentes. Apesar dos avanços da medicina moderna, doenças infeciosas estão entre as principais causas de morbidade e mortalidade no mundo (WHO, 2018b). Nos últimos 60 anos, mais da metade das doenças infecciosas emergentes em humanos têm sido transmitidas por animais (Jones et al., 2008), sendo 72% de origem silvestre. Estes números estão propensos a aumentar à medida em que novos agentes zoonóticos, causadores de doença em humanos, continuam a emergir (Assiri et al., 2013; Reusken et al., 2013; Wang et al., 2013).

No grupo de doenças infecciosas, os arbovírus transmitidos por mosquitos são considerados importantes desafios para a saúde pública. O termo arbovírus surgiu em 1942, advindo da contração da frase em inglês "*Arthropod born virus*" para descrever um grupo de vírus zoonóticos transmitidos por artrópodes, em sua maioria mosquitos e carrapatos, para os vertebrados (on Arboviruses et al., 1967). Este termo não abrange um grupo taxonômico específico, mas engloba um conjunto de vírus com características ecológicas semelhantes (Didier Musso & Gubler, 2016).

Cerca de 545 espécies de arbovírus já foram descritas, das quais mais de 150 estão documentados como causadores de doenças em humanos, sendo a maioria zoonóticas. Os arbovírus são mantidos em um ciclo de transmissão entre artrópodes, que agem como vetores, e animais vertebrados, que atuam como principais hospedeiros e amplificadores. Os seres humanos geralmente são hospedeiros acidentais e não possuem viremia suficiente para infectar os artrópodes, com a exceção de alguns vírus (Figura 1), como dengue (DENV), febre amarela (YFV) e chikungunya (CHIKV) (Didier Musso & Gubler, 2016).

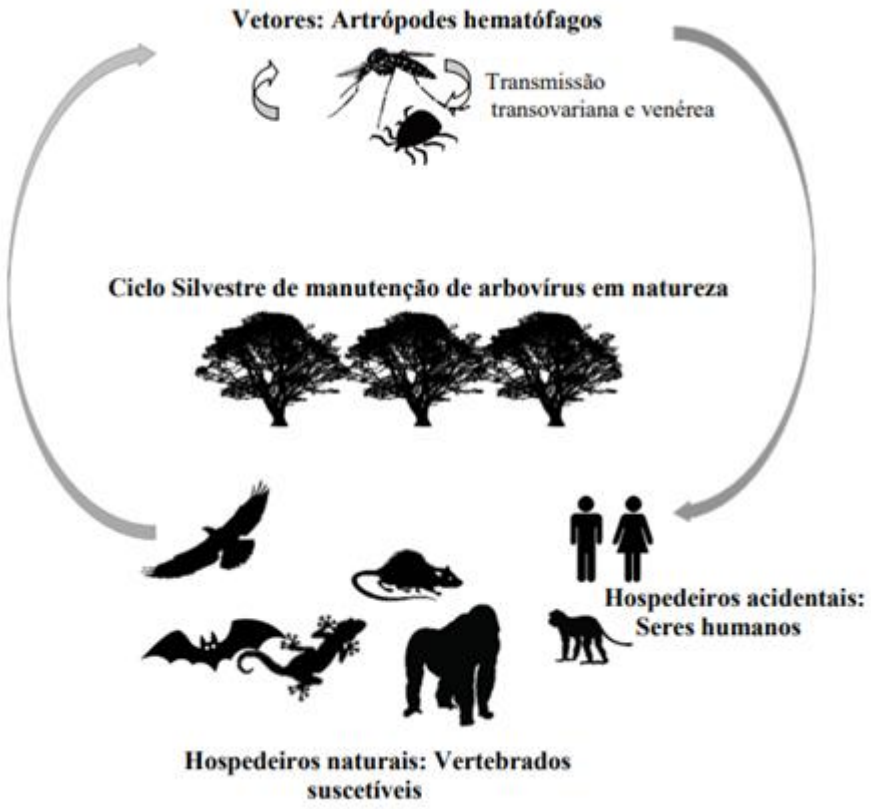



**Figura 1: Manutenção dos arbovírus na natureza.** A manutenção dos arbovírus na natureza envolve artrópodes hematófagos como vetores, os vertebrados como hospedeiros naturais e os seres humanos como hospedeiros acidentais. Fonte: Catenacci, 2017.

Os arbovírus estão entre as principais causas de doenças infeciosas a emergir ou reemergir em todo o mundo. Pertencem a quatro famílias virais (*Bunyaviridae*, *Reoviridae*, *Togaviridae* e *Flaviviridae*) podendo causar doenças humanas graves, como febres hemorrágicas, encefalites e meningites (Wu et al., 2019). Dentre essas famílias, os vírus pertencentes à *Flaviviridae* e *Togaviridae* são os responsáveis pela maioria dos casos de doença em humanos (Brackney, 2017). Apesar da grande preocupação com a dengue, considerada endêmica em quase todo o país, e as recentes epidemias causadas pelos vírus zika (ZIKV), febre amarela e chikungunya (CHIKV), dados epidemiológicos previamente publicados mostram a emergência de outros arbovírus de importância médica no Brasil, como mayaro (MAYV), oropouche (OROV), encefalite de Saint Louis (SLEV), oeste do Nilo (WNV) e punta toro (PTV) (Bastos et al., 2012; Cardoso et al., 2015; Figueiredo & Figueiredo, 2014; Mourão et al., 2009; Perrone et al., 2007; H. B. Vasconcelos et al., 2009).

As arboviroses podem causar uma pluralidade de manifestações clínicas, incluindo indivíduos assintomáticos, quadros de doença febril indiferenciada, moderada ou grave, erupções cutâneas, comprometimento articular, síndrome neurológica e síndrome hemorrágica

(Lopes et al., 2014). As arboviroses estão associadas a grandes epidemias e um consequente aumento dos custos financeiros relacionados ao diagnóstico e ao tratamento, bem como na piora da qualidade de vida dos indivíduos infectados. O diagnóstico é considerado complexo devido à semelhança clínica com outras patologias, a presença de casos assintomáticos ou oligossintomáticos, a dificuldade de acesso aos laboratórios de referência para um diagnóstico molecular e/ou sorológico diferencial e a existência de reações sorológicas cruzadas (Moreli & Costa, 2013).

### 1.1.1 Família *Flaviviridae*

Os Flavivirus são vírus pequenos, esféricos, medindo aproximadamente 50 nm e possuem como material genômico RNA fita simples senso positivo com aproximadamente 11.000 pb codificando uma única janela de abertura (*open reading frame* - ORF) que é sendo flanqueada por duas regiões 5´e 3´ não traduzidas (*untranslated region* - UTR). O genoma é traduzido em uma única poliproteína que posteriormente é clivado em 10 proteínas, sendo três proteínas estruturais (proteína do nucleocapsídeo, C; proteína pré-membrana, prM; e proteína do envelope, E) e sete proteínas não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Pierson & Diamond, 2020) (Figura 2).

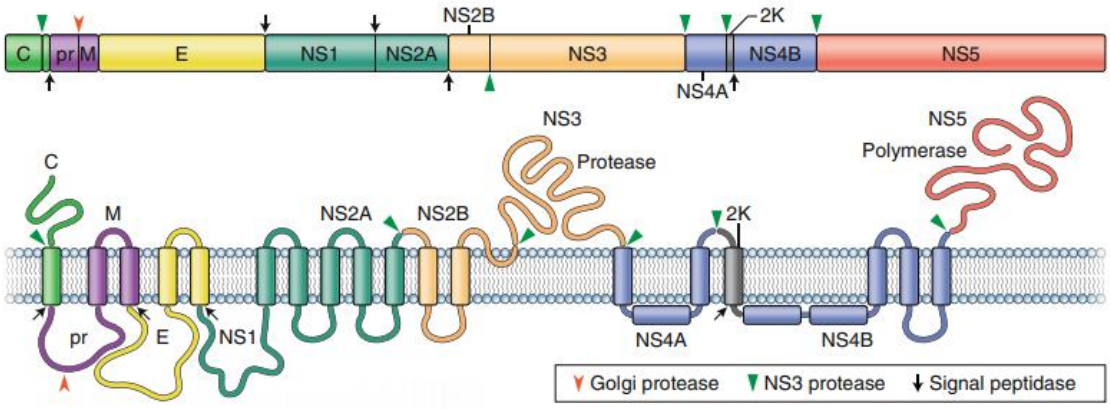



**Figura 2: Estrutura e organização do genoma dos Flavivirus.** Desenho esquemático do genoma dos flavivírus mostrando sua única ORF que codifica uma única poliproteína e seus posteriores pontos de clivagem pelas proteases **(**Adaptado de Pierson et al 2020).

Na estruturação da partícula viral, a proteína C é responsável pela formação do capsídeo auxiliando na proteção do material genético do vírus. Já a proteína prM participa da manutenção conformacional da proteína E e as duas juntas compõem a estrutura da superfície viral

(Lindenbach & Rice, 2007). As proteínas não estruturais têm um papel importante na replicação, montagem, e repressão da resposta inata do hospedeiro à infecção (Chen et al., 2017).

A família *Flaviviridae* engloba quatro gêneros de acordo com o relatório *International Committee on Taxonomy of Viruses* (ICTV), *Flavivirus, Pestivirus, Pegivirus e Hapacivirus*, sendo *Flavivirus* o único que contém arbovírus, como DENV, ZIKV, YFV, WNV, SLEV e encefalite japonesa (JEV) (Cleton et al., 2012; Holbrook, 2017; Simmonds et al., 2017; Stapleton et al., 2011). O gênero *Flavivirus* compreende três grupos distintos de vírus: os transmitidos por mosquitos, os transmitidos por carrapatos e aqueles sem vetores definidos. Os flavivírus estão relacionados a uma gama de doenças em humanos e são uma grande ameaça à saúde pública mundial, principalmente nos países em desenvolvimento (Holbrook, 2017).

Os principais vetores associados à transmissão dos flavivírus são mosquitos do gênero *Aedes* (*Ae. aegypti* e *Ae. Albopictus*) (Muktar et al., 2016), entretanto, mosquitos *Haemagogus* e *Sabethes* também são importantes para a transmissão silvestre de YFV nas Américas (P. F. da Vasconcelos, 2003).

#### 1.1.1.2 Zika vírus

O ZIKV pertence à família *Flaviviridae* e ao gênero *Flavivirus*, sendo classificado em duas linhagens geograficamente e filogeneticamente distintas: o genótipo africano e asiático (Faye et al., 2013). O genótipo asiático do ZIKV surgiu recentemente como uma das mais graves ameaças globais à saúde pública (Baud et al., 2017). Assim como os demais *Flavivirus*, o ZIKV possui um genoma de RNA de senso positivo de cadeia única com 10.794 pb, o genótipo asiático e de 10.617 pb, o genótipo africano, observado nos genomas de referência (Didier Musso & Gubler, 2016).

O ZIKV é transmitido aos humanos principalmente pelo mosquito *Ae. aegypti*, também responsável pela transmissão de DENV e CHIKV (Boyer et al., 2018; Didier Musso & Gubler, 2016), embora existam evidências de transmissão não vetorial por transfusão de sangue (D. Musso et al., 2014), via sexual, principalmente de homem para mulher (Foy et al., 2011; Didier Musso et al., 2015), e perinatal em aproximadamente 26% das mães infectadas (Pomar et al., 2018). Além do *Ae. aegypti* o ZIKV pode ser transmitido por outras espécies do gênero *Aedes*, dentre elas o *Ae. albopictus*, espécie de mosquito com ampla distribuição mundial. Assim como outros arbovírus, como DENV e CHIK, o ZIKV vem se espalhando em territórios onde existe

a infestação destes mosquitos, demonstrando o potencial da emergência deste vírus, principalmente em grandes centros urbanos (Didier Musso & Gubler, 2016).

Estudos demonstraram que os primatas não humanos (PNH) são os prováveis hospedeiros reservatórios que mantém o vírus no ciclo silvestre tanto na África quanto na Ásia, enquanto os mosquitos principalmente do gênero *Aedes* são os vetores. Em localidades em que não existe PNH como na Polinésia Francesa e na Micronésia, o ZIKV provavelmente é mantido em um ciclo humano-mosquito-humano sugerindo a adaptação do vírus ao homem (Didier Musso & Gubler, 2016). No Brasil ainda é incerto como o vírus se mantem. Porém, já foi demonstrado a infecção natural em saguis (*Callithrix sp.*) e macacos-prego (*Sapajus sp*) de vida livre no sudeste brasileiro, além de infecções experimentais em saguis. Estes dados sugerem fortemente o papel relevante dos PNHs no ciclo de transmissão e manutenção do ZIKV em meio urbano e alerta para um possível estabelecimento de um ciclo silvestre ZIKV no Brasil (Terzian et al., 2018).

A infecção por ZIKV causa uma doença autolimitada e até 80% dos casos são assintomáticos (Haby et al., 2018). A maioria dos casos sintomáticos é caracterizada por uma doença febril aguda com febre baixa, dor de cabeça, mialgia, conjuntivite e/ou erupção maculopapular (Ioos et al., 2014). Apesar de ser primariamente uma doença autolimitada, a infecção por ZIKV pode causar doenças neurológicas graves, tais como síndrome de Guillain-Barré e microcefalia em recém-nascidos (Figura 3) . De fato, o vírus já foi encontrado no líquido amniótico, na placenta e no tecido cerebral de fetos e crianças (Baud et al., 2017). Inclusive, o aumento dramático de casos de microcefalia no Brasil associados ao ZIKV, levou a Organização de Saúde (OMS) em fevereiro de 2016 a declarar uma emergência de saúde pública de interesse internacional (WHO, 2016b).

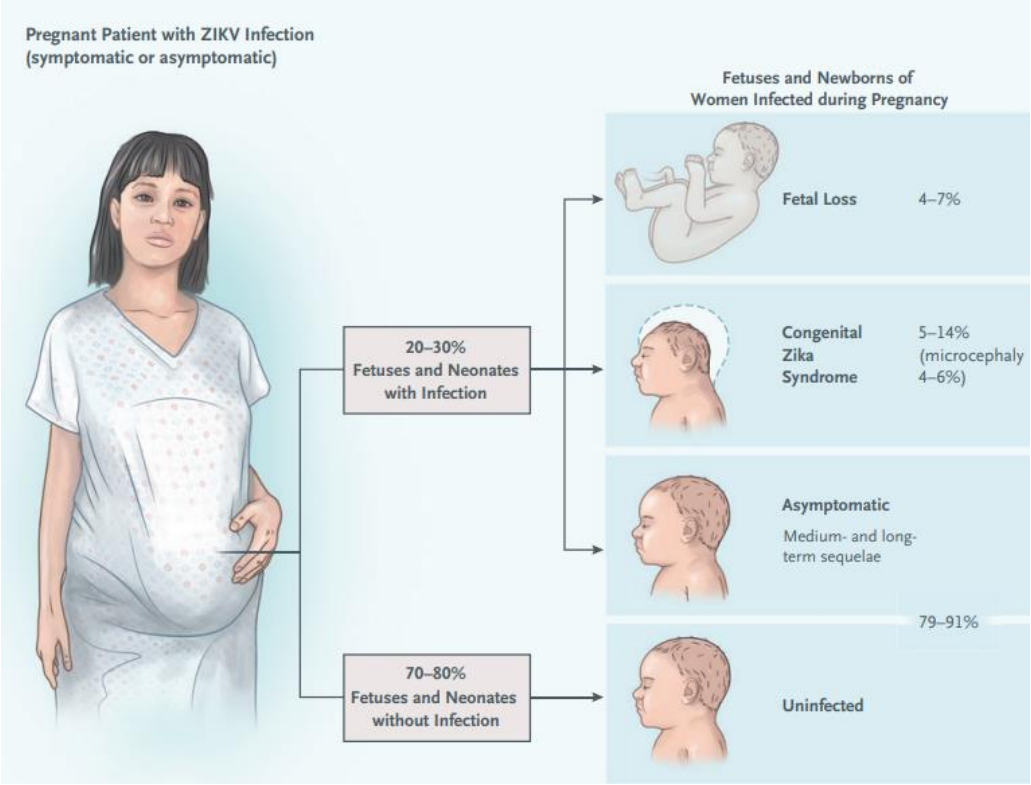



**Figura 3: Ilustração com as estatísticas de casos de infecção congênita pelo ZIKV**. No desenho está demonstrado as porcentagens de transmissão vertical, perda fetal, aquisição da síndrome congênita do Zika e microcefalia associada ao ZIKV entre fetos e bebês de mulheres infectadas pelo vírus (Adaptado de Musso, 2019).

Visto que os sintomas do ZIKV são inespecíficos, o diagnóstico laboratorial se torna fundamental no enfrentamento a doença. As principais formas de diagnosticar o vírus são reação em cadeia da polimerase em tempo real (RT-qPCR) e exames sorológicos (Didier Musso et al., 2019). O resultado positivo para RT-qPCR sinaliza a detecção do RNA viral, confirmando a infecção, porém não significa que aquele paciente ainda possui partículas virais infectantes. Já o resultado negativo por si só não é suficiente para descartar a possibilidade de infecção. O exame sorológico é mais complicado devido a possíveis reações cruzadas com outros flavivírus, consequentemente gerando resultados falso-positivos, o que demanda uma interpretação minuciosa dos casos positivos (Didier Musso et al., 2019).

Apesar de vários estudos demonstrarem componentes com possíveis ações contra o ZIKV, ainda não existe tratamento específico, sendo o suporte terapêutico a única abordagem a ser adotada (Didier Musso et al., 2019). A síndrome de Guillain–Barré associada ao ZIKV é tratada da mesma forma que a síndrome de Guillain–Barré clássica (WHO, 2016a). Bebês com microcefalia necessitam de um cuidado especial com uma equipe multidisciplinar que irá acompanhar seu desenvolvimento (Adebanjo et al., 2006).

A melhor prevenção da população em geral contra o ZIKV ainda é a proteção contra as picadas de mosquitos transmissores. Para a proteção da transmissão via sexual se faz necessário o uso de preservativos ou a abstinência sexual no período posterior a suspeita de infecção (WHO, 2018c). Várias possíveis candidatas a vacinas passaram para a fase clínica 1 e uma passou para fase 2. Porém, devido à baixíssima incidência do ZIKV após a epidemia, se torna muito difícil avaliar a efetividade das candidatas a vacina (Didier Musso et al., 2019).

O ZIKV foi isolado pela primeira vez em 1947, de um macaco sentinela na floresta de Zika, localizada no sudeste da Uganda, na África Oriental (Baud et al., 2017). Os primeiros casos de ZIKV em humanos foram registrados na Nigéria, em 1954 (MacNamara, 1954). O vírus foi isolado pela primeira vez fora do continente africano no ano de 1969, em mosquitos da Malásia, e anos mais tarde, em 1977, os primeiros casos em humanos não africanos foram registrados na Indonésia (Marchette et al., 1969; Olson et al., 1981) (Marchette et al, 1969; Olson et al 1981). O primeiro surto de ZIKV fora da África e Ásia foi registrado na Micronésia, em 2007. A partir daí, epidemias se tornaram comuns, atingindo o Camboja (2010), Polinésia Francesa (2013), Ilhas Cook e Nova Caledônia (2014), Vanuatu, Ilhas Salamão, Samoa e Fiji (2015), e mais recentemente o Brasil (Didier Musso & Gubler, 2016).

Em poucos anos o ZIKV passou de um vírus desconhecido com casos esporádicos na África e Ásia para um vírus quase pandêmico atingindo pelo menos 87 países. Apesar da imunidade populacional adquirida pela infecção natural ter praticamente extinto o ZIKV de várias regiões, durante a epidemia podem ter sido gerados bolsões, como na cidade de São Paulo, que ainda não foram acometidos pelo vírus e se mantém susceptíveis ao vírus, podendo sustentar uma nova epidemia (Didier Musso et al., 2019).

No Brasil, os primeiros casos de transmissão autóctone do ZIKV foram registrados em maio de 2015 nos estados da Bahia e Rio Grande do Norte (Ministério da Saúde, 2017). Desde então, o ZIKV tem ganhado posição de destaque em saúde pública no país. Em 2016, já havia circulação autóctone do vírus registrada em 22 dos 27 estados brasileiros (Ministério da Saúde, 2017). Até agosto de 2020, 294.713 casos de infecção por ZIKV foram confirmados no país (Iani et al., 2021).

Além da recente emergência do ZIKV, outros arbovírus como YFV e CHIKV foram responsáveis por epidemias que impactaram significativamente a saúde pública no Brasil.

#### 1.1.1.3 Febre Amarela

Ao longo da história, a febre amarela causada pelo YFV já possuiu mais de 150 nomes diferentes, sendo que o termo “febre amarela” teve seu uso consagrado em 1750 por Griffin Hughes no livro *“Natural History of Barbadoes*. O vírus teve sua origem na África a aproximadamente 3000 anos e veio para a América provavelmente via navios durante o início do mercado de escravos (Bryant et al., 2007). Na América, o vírus encontrou um ambiente propício para sua propagação com vetores competentes para a eficiente manutenção e transmissão, porém diferenças nos vetores e reservatórios locais obrigaram o vírus a sofrer adaptações (Li & Yang, 2017). A febre amarela impactou a história e a economia da América Latina mais do que da África, além de gerar conflitos entre os próprios cientistas principalmente a respeito de sua origem, modo de transmissão e o agente responsável pela transmissão (Chippaux & Chippaux, 2018).

O YFV foi o primeiro vírus isolado da família *Flaviviridae* e é considerado o protótipo do gênero *Flavivirus* (Monath, 2001). É um vírus icosaédrico, envelopado, com um diâmetro aproximado de 50 nm (Heinz & Allison, 2003) (Figura 4).

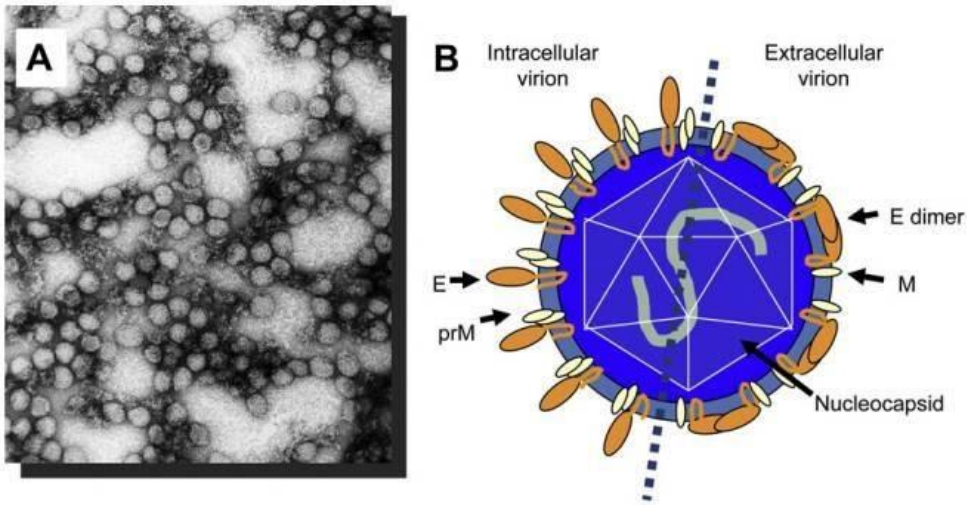



**Figura 4: Vírus da febre amarela**. A) Fotomicrografia mostrando vários vírions da febre amarela (aumento de 234.000 X). B) Estrutura dos vírions imaturo (intracelular) e maduro (extracelular). Fonte: adaptado de Gardner, 2010.

Até o momento, já foram identificados sete genótipos diferentes de YFV, sendo cinco na África (África Ocidental I e II, África Oriental, África Oriental/Central e Angola) e duas nas Américas (América do Sul I e II) os quais foram derivaram do genótipo da África Ocidental (Bryant et al., 2007). No Brasil o genótipo predominante é o América do Sul 1 que possuem 5 linhagens diferentes 1A-1E sendo que as mais antigas 1A, 1B, 1C foram substituídas pelas mais recentes 1D E 1E, esta última responsável pelas recentes epidemias de YFV no Brasil em 2016-2019 (Silva et al., 2020).

O YFV é mantido em dois ciclos de transmissão distintos, classificados como silvestre e urbano (Figura 5). O ciclo silvestre se caracteriza pela transmissão do vírus entre primatas não humanos (PNH) por mosquitos vetores do gênero *Aedes* na África, e pelos mosquitos *Haemagogus* e *Sabethes* nas Américas (Monath, 2001). Neste ciclo, os PNHs atuam como hospedeiros amplificadores, enquanto os mosquitos são considerados transmissores e reservatórios, pois uma vez infectados permanecem ao longo da vida transmitindo também verticalmente para seus descendentes (P. F. da Vasconcelos, 2003).

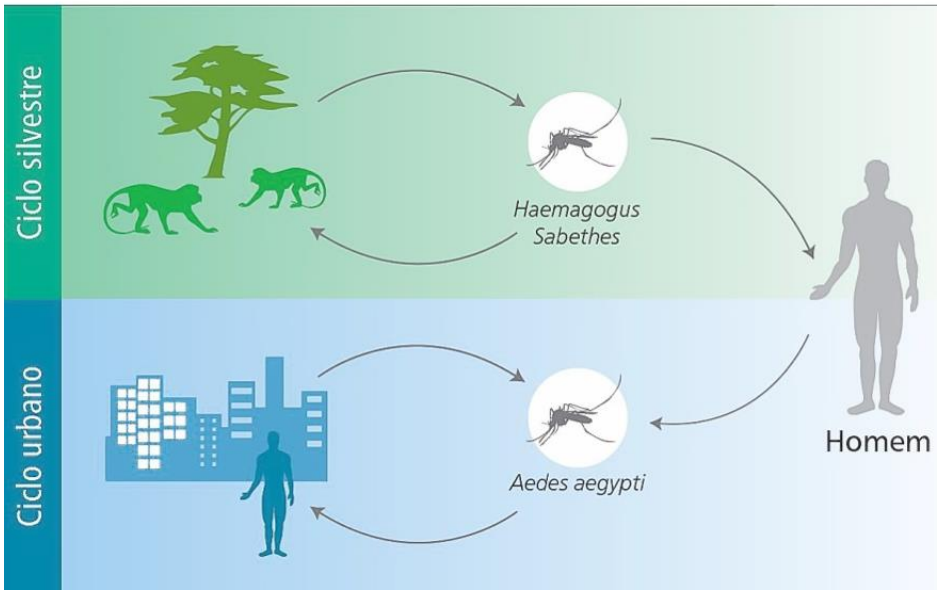



**Figura 5: Ciclos epidemiológicos silvestre e urbano da febre amarela.** O ciclo silvestre envolve a transmissão entre PNH por mosquitos *Haemagogus* e *Sabethes*, enquanto o ciclo urbano envolve a transmissão do vírus aos humanos pelos mosquitos *Aedes aegypti*. Fonte: Ministério da Saúde.

Os PNHs são considerados os hospedeiros primários tanto na África quanto nas Américas. Na África, os PNHs quando infectados costumam não morrer e sim ficarem imunes após o contato com o vírus (Chippaux & Chippaux, 2018). Certos gêneros de primatas, como o *Alouatta*, são mais sensíveis ao YFV e outros parecem ser mais resistentes ao vírus, como o gênero *Cebus*. Já alguns marsupiais e preguiças arboreais parecem desempenhar um papel secundário no ciclo silvestre, principalmente onde não há PNH (P. F. da Vasconcelos, 2003). No ciclo silvestre, o homem é considerado hospedeiro acidental quando penetra neste ciclo durante atividades ocupacionais ou recreativas (Monath, 2001). Como todos os PNH da América do Sul são altamente susceptíveis a infecção pelo YFV e as epizootias costumam ocorrer antes de casos humanos eles podem ser utilizados como sentinelas nos programas de vigilância (Silva et al., 2020).

No ciclo urbano, os humanos atuam como hospedeiros e amplificadores e os principais vetores envolvidos na transmissão são os mosquitos *Ae aegypti*, embora os mosquitos *Ae. albopictus* também possam transmitir (Couto-Lima et al., 2017). Esse ciclo se perpetuará até que se esgotem os humanos susceptíveis ou se elimine os vetores de transmissão (P. F. da Vasconcelos, 2003).

A febre amarela é uma doença infecciosa viral aguda, não contagiosa, de curta duração que apresenta um espectro de sintomas amplo e variável (P. F. da Vasconcelos, 2003). A doença pode variar desde uma infecção leve, com sintomas quase imperceptíveis incluindo febre, dor de cabeça, icterícia, dores musculares, náuseas, vômitos e fadiga, até uma infecção grave, com uma taxa de letalidade entre 20%-50%, causada por sepse viral pansistêmica, com febre, prostração, lesão hepática, renal e miocárdica, hemorragia e choque (Gardner & Ryman, 2010). A última epidemia de febre amarela ocorrida no Brasil em 2016-2017 teve uma taxa de letalidade nos casos graves de 33,6% comparável aos surtos anteriores (Faria et al., 2018).

O diagnóstico de febre amarela pode ser realizado através de duas abordagens principais distintas, a virológica e a sorológica. A virológica inclui: a detecção do genoma viral, do antígeno viral, o isolamento do vírus e a imunohistoquímica. Já a sorológica inclui: o teste de neutralização por redução de placas e o imunoensaio enzimático (WHO, 2018a). O exame de RT-PCR deve ser priorizado por ser mais específico do que os exames sorológicos que podem ocorrer reações cruzadas com outros flavivirus. Entretanto um resultado não detectado no RT-PCR não descarta uma possível infecção sendo necessários exames sorológicos para tentar elucidar o caso (WHO, 2018a). Os exames sorológicos são recomendados a serem realizados após a soroconversão a partir do 6 dia de início dos sintomas. Já o RT- PCR deve ser realizado na fase aguda até o 10 dia do início dos sintomas, porém já foi demonstrado que em alguns casos é possível detectar o RNA viral no soro e na urina até 28 e 47 dias, respectivamente (Silva et al., 2020).

Com a descoberta da manutenção do vírus no ciclo silvestre ficou claro a impossibilidade de eliminar o YFV. Com isso, se reforçou a ideia de que a melhor medida de prevenção seria a vacina. Depois de décadas de estudos e tentativas frustradas, relacionadas a atenuação do vírus, a fabricação em larga escala e a eliminação de contaminantes, foi na década de 1930 que se conseguiu produzir uma primeira geração de vacina atenuada, e relativamente segura nomeada de linhagem 17D. Esta foi posteriormente aperfeiçoada e substituída pela vacina da sublinhagem 17DD utilizada até os dias de hoje (Frierson, 2010). Esta vacina é considerada segura, apesar de observados alguns casos de reações adversas e ainda que apenas

uma dose da vacina ser suficiente para imunizar por toda a vida, este é um tema ainda em debate (Silva et al., 2020). Apesar da existência de uma vacina eficaz, a febre amarela continua sendo um problema de saúde pública, principalmente na África e na América do Sul, com uma incidência anual de aproximadamente 200 mil casos e 30 mil óbitos (P. F. C. Vasconcelos & Monath, 2016).

Ainda não está disponível um tratamento específico para a febre amarela, sendo este considerado apenas suportivo e sintomático (Litvoc et al., 2018). Devido à gravidade da doença e o alto risco de reurbanização, é imprescindível o controle de YFV baseado principalmente no controle dos vetores e na imunização da população em risco (Chippaux & Chippaux, 2018).

Desde o século XV, casos esporádicos e pequenas epidemias forma registradas na África. Na América do Sul, o YFV causou várias epidemias nos séculos principalmente nos séculos 18 e 19, sendo que o primeiro registro no Brasil aconteceu após a epidemia em Pernambuco em 1685 (Chippaux & Chippaux, 2018). A última grande epidemia de febre amarela urbana do Brasil aconteceu em 1929, no Rio de Janeiro, e o último caso de febre amarela urbana registrado foi no Acre em 1942 (P. F. da Vasconcelos, 2003). O ciclo silvestre da febre amarela foi observado no Brasil pela primeira vez em 1889, no estado de São Paulo, sendo confirmado em 1932 no Espírito Santo. Nas décadas seguintes, vários surtos de febre amarela silvestres ocorreram, mas antes restrito à apenas Bacia Amazônica, onde é considerada endêmica, e a partir de 1999 ocorreu uma mudança deste perfil epidemiológico e a maioria dos casos passaram a ocorrer fora da bacia Amazônica (Silva et al., 2020). Já no período de 2016-2018, uma epidemia silvestre ocorreu no sudeste brasileiro, acometendo principalmente os estados de Minas Gerias e Espírito Santo (Faria et al., 2018). Durante essa epidemia, foram confirmados 676 óbitos e 2.043 casos, sendo 777 confirmados por RT-qPCR, distribuídos em 10 estados brasileiros (Faria et al., 2018).

### 1.1.2 Família *Togaviridae*

Os Togavírus são vírus envelopados, esféricos medindo aproximadamente 70 nm de diâmetro. Seu genoma é de RNA fita simples senso positivo variando de 9.700 pb a 11.800 pb de tamanho. A partícula viral consiste em um núcleo capsídeo envolto por uma camada bilipídica embutida com glicoproteínas. Seu genoma possui um *cap* na região 5´ e uma cauda poli-A na região 3´ (Chen et al., 2017). O genoma possui duas ORFs e é traduzido em duas poliproteínas que posteriormente codifica 10 proteínas, sendo seis estruturais (capsídeo, E1, E2, E3, 6K e *Transfame*) e 4 não estruturais (nsP1, nsP2, nsP3 e nsP4). As proteínas estruturais são essenciais na constituição viral. Já as não estruturais formam o complexo da replicação viral, auxiliando na montagem do vírus (Holmes et al., 2020) (Figura 6).

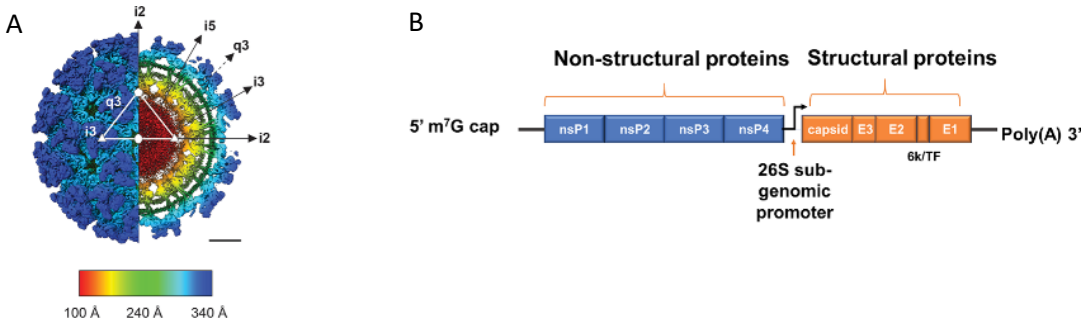



**Figura 6: Representação da partícula viral e do genoma de um Togavirus**. A) Desenho esquemático da partícula viral de um membro da família *Togaviridae*. B) Esquema do genoma de um *Togaviridae* demonstrando as 2 poliproteínas com 6 proteínas estruturais e 4 não estruturais (Adaptado de Holmes, et al 2020).

Até abril de 2019, esta família era composta por dois gêneros o *Alphavirus* e o *Rubivirus* e mais de 30 espécies. Porém o gênero *Rubivirus* foi retirado da família Togaviridae e transferido para a família *Matonaviridae* segundo a classificação do ICTV (Rima et al., 2020).

O gênero *Alphavirus* contém muitos patógenos humanos e veterinários sendo pelo menos nove deles de importância médica: CHIKV, MAYV, vírus da febre do rio Ross (RRV), vírus O'nyong nyong (ONNV), virus Sindbis, vírus da floresta de Barmah, virus da encefalite equina oriental, vírus da encefalite equina do oeste e o vírus da encefalite equina venezuelana (Gubler & Department, 2002).

Diferentemente dos outros Alphavirus o CHIKV não possui a estrutura tipo stem-loop um enhancer na região 5´terminal, portanto ainda não se sabe como a tradução do CHIKV é mantida (Burt et al., 2017).

#### 1.1.2.1 Chikungunya

A febre chikungunya é uma doença viral artrítica debilitante transmitida pela picada de mosquitos *Aedes*. A palavra chikungunya é utilizada tanto para nomear a doença quanto o vírus e significa "aqueles que se dobram" em dialetos africanos Swahili ou Makonde. Ela faz referência à aparência curvada devido a artralgia incapacitante dos pacientes que foram atendidos na primeira epidemia documentada, na Tanzânia, localizada no leste da África, entre 1952 e 1953 (Pialoux et al., 2007).

CHIKV é uma arbovírus membro da família *Togaviridae*, gênero *Alphavirus*, pertencente ao complexo antigênico *Semliki forest*, composto também pelos alfavírus MAYV, RRV, ONNV, Getah, Bebaru e Semlikiforest (Cleton et al., 2012).

Análises filogenéticas demonstraram que o CHIKV apresenta quatro genótipos/linhagens geneticamente distintos, nomeados de acordo com sua distribuição geográfica: (1) Oeste Africano, que é mantido em um ciclo silvático; (2) Asiático, endêmico na Ásia; (3) Leste-Centro-Sul Africano (ECSA), endêmico na África (Powers et al., 2000); e (4) Oceano Índico (IOL), descendente do genótipo ECSA, responsável por várias epidemias (Volk et al., 2010). Durante a epidemia no Oceano Índico foi detectada uma mutação na glicoproteína do envelope que melhorou a adaptação do CHIKV facilitando sua habilidade de infectar e replicar no *Aedes Albopictus* o que deve ter contribuído para a magnitude da epidemia (Burt et al., 2017).

Assim como a febre amarela, a chikungunya tem dois ciclos de transmissão distintos: o silvático e o urbano. Evidências mostram que PNHs são os principais hospedeiros e amplificadores no ciclo silvático. Surtos e epidemias podem ocorrer quando mosquitos mais antropofílicos, como *Ae. aegypti* e/ou *Ae. albopictus*, iniciam um ciclo urbano com o homem sendo o principal hospedeiro (Althouse et al., 2018). Também ocorre a transmissão do CHIKV de mãe para filho causando um número significativo de casos de morbidades e até mortes de neonatal (Contopoulos-Ioannidis et al., 2018).

O período de incubação do CHIKV pode variar entre 2-4 dias e é seguido por uma doença febril abrupta. Os sintomas mais comuns incluem febre alta, calafrio, dor de cabeça, fotofobia, erupção cutânea petequial ou erupção maculopapular e artralgia frequentemente incapacitante. Os casos assintomáticos são observados em aproximadamente 15% (Schwartz & Albert, 2010). A fase aguda da febre chikungunya geralmente é autolimitada durando até duas semanas, mas a artralgia pode permanecer por semanas, meses e até anos. A artralgia

persistente ocorre depois da fase aguda atingindo principalmente as juntas menores, mas também pode afetar juntas maiores (Burt et al., 2017). Apesar de raras, as formas graves da doença podem ocorrer, principalmente, encefalite e encefalopatia, miocardite, hepatite e falência de múltiplos órgãos e culminar em óbito, sendo estes casos menos de 1 em cada 1000, principalmente de idosos ou pessoas com comorbidades preexistentes (Burt et al., 2017; Galán-Huerta et al., 2015).

Não existe tratamento específico para infecção pelo CHIKV. Os tratamentos utilizados são apenas suportivos e para aliviar os sintomas na fase aguda. Se não houver a recuperação natural e a artralgia persistir por longos períodos (poliartralgia difusa pós-CHIKV) se faz necessário um tratamento específico baseado no controle da dor, incluindo terapia inflamatória de longa duração (Galán-Huerta et al., 2015).

O diagnóstico é baseado em critérios clínicos, epidemiológicos e laboratorial. Devido a semelhanças nos sintomas e a possível co-circulação com outros arbovírus, dificultando a diferenciação clínica e epidemiológica, o exame laboratorial de qualidade se faz necessário. Existem 3 principais diagnósticos laboratorial: o isolamento viral, o RT-qPCR e os sorológicos (Vu et al., 2017).

A vacinação é uma ferramenta poderosa no combate a epidemias. As tentativas para se desenvolver uma vacina começaram nos anos 1960 logo após a descoberta do vírus. Mesmo com todo avanço nos métodos bioquímicos e moleculares os cientistas continuam tentando desenvolver uma vacina com as mais variadas estratégias como: vacina inativada, vacina quimérica, vacinas de ácidos nucleicos entre outras, mas sem sucesso. Até hoje não existe nenhuma vacina com autorização de uso (Gao et al., 2019).

Acredita-se que o CHIKV tenha surgido na África e posteriormente se espalhou para a Ásia. Desde sua primeira epidemia em 1952 na Tanzânia, surtos e epidemias tem sido regulamente registrados na África e na Ásia que teve seu primeiro em 1954 nas Filipinas (Burt et al., 2017). Em 2004, foi registrado um surto no Quênia, que se espalhou para Comores e posteriormente para ilhas no Oceano Índico até atingir a Ilha da Reunião, em 2005, sendo o primeiro país ocidental a registrar casos de infecção por CHIKV (Schwartz & Albert, 2010). A epidemia continuou se expandindo até atingir a Índia, Siri Lanka, Tailândia e Malásia, até que, em 2007, o vírus foi identificado na Itália (Galán-Huerta et al., 2015) Em 2011, o CHIKV foi novamente identificado na África, Ásia, Europa e em ilhas do Oceano Índico. Em 2012, ele continuou a se espalhar por estas regiões até ser registrado na Oceania em 2013 (Galán-Huerta et al., 2015). Este aumento de surtos que disseminou o vírus para áreas anteriormente não

endêmicas pode estar relacionado com adaptações genéticas do vírus ao vetor *Aedes Albopictus* (Burt et al., 2017) (Figura 7).

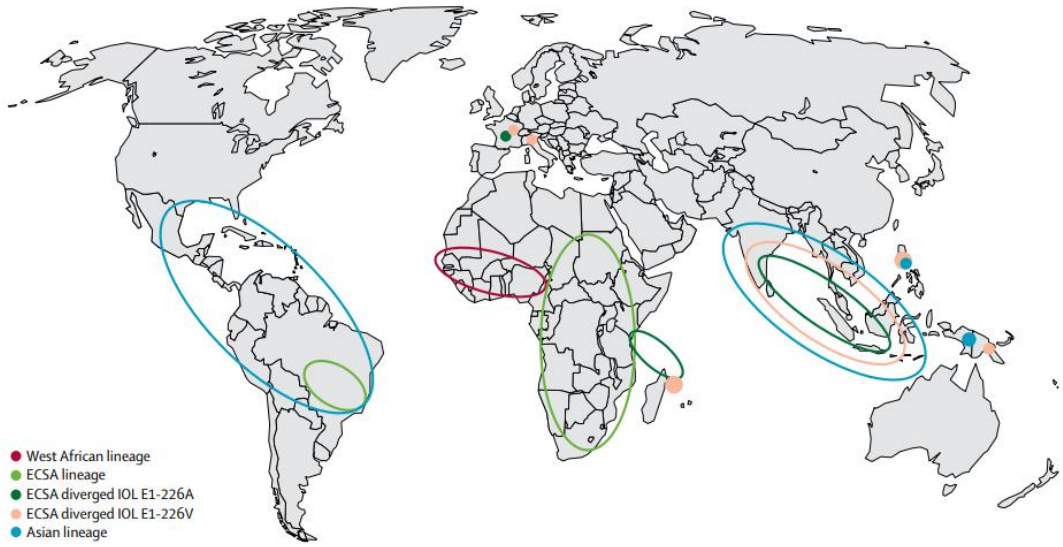



**Figura 7: Distribuição de CHIKV pelo mundo. A figura mostra a distribuição das linhagens do CHIKV nas ilhas do Oceano Índico, África, América e Europa. Fonte: Burt et al, 2017.**

Ainda em 2013, foi estabelecido pela primeira vez um ciclo urbano de CHIKV nas Américas, que teve início na Ilha de São Martinho, no Caribe, e posteriormente se espalhou para as ilhas vizinhas, atingindo a Guiana Francesa em 2014. A partir daí, o vírus se dispersou rapidamente, sendo registrado em 48 regiões das Américas (Burt et al., 2017; Galán-Huerta et al., 2015). No Brasil, o primeiro caso autóctone de infecção por CHIKV foi causado pelo genótipo asiático na cidade de Oiapoque, Amapá, em setembro de 2014. Sete dias depois do primeiro caso confirmado, o genótipo ECSA foi registrado em Feira de Santana, na Bahia (Nunes et al., 2015), espalhando-se rapidamente para outros estados brasileiros (Xavier et al., 2019).

### 1.1.3 Vigilância genômica

O sequenciamento dos genomas virais desempenha um papel importante na luta contra as epidemias emergentes e reemergentes, e foram amplamente utilizadas para compreender a pandemia de gripe H1N1 em 2009 e a propagação do vírus Ebola na África Ocidental em 2013-2016 (Faria et al., 2016). Os genomas analisados permitem caracterizar a diversidade genética viral, reconstruir as origens da epidemia, estimar as taxas de transmissão, sugerir próximos surtos e fornecer informações para o desenvolvimento de vacinas e novos medicamentos, bem

como melhoramento dos métodos diagnósticos sorológicos e moleculares (Faria et al., 2016, 2017).

A vigilância genômica é a combinação de dados genômicos e epidemiológicos aliados a ferramentas de bioinformática que geram informações imprescindíveis para o entendimento do passado e do futuro das viroses humanas circulantes. Um sistema combinado genômica viral, epidemiologia e bioinformática, contínuo e estruturado no nosso país, integrado com dados de vigilância, pode fornecer informações oportunas para dar respostas efetivas contra vários vírus emergentes e reemergentes (WHO, 2020).

Portanto, existe uma necessidade de monitoramento ativo de reservatórios e hospedeiros animais para caracterizar agentes infecciosos e o risco de exposição da comunidade, principalmente em países com históricos de surtos de transmissão zoonótica como o Brasil. Nesse contexto, esta Tese contribuiu de forma substancial para a obtenção de vários genomas completos de vírus circulantes como ZIKV, CHIKV, YFV que proporcionou a avaliação das características virais, variabilidade genética, e evolução utilizando ferramentas de bioinformática, além de gerar dados para o desenvolvimento de testes moleculares e vacinas mais eficazes. Por fim, o uso dos dados gerados nesta Tese associados aos dados epidemiológicos disponibilizados pelo Ministério da Saúde e Secretaria Estadual de Saúde de Minas Gerais (SES-MG), análises de dispersão, curvas epidêmicas e associação com quadros severos da doença puderam ser investigadas de forma mais efetiva.

# 2 OBJETIVOS

## 2.1 Objetivo Geral

- Realizar a vigilância genômica em tempo real das arboviroses emergentes e reemergentes circulantes no estado de Minas Gerais e na Bacia Amazônica.

## 2.2 Objetivos específicos

- Implantar protocolos de sequenciamento dos genomas completos no Lacen-MG para ZIKV, YFV e CHIKV, utilizando o sequenciador portátil MinION;
- Gerar genomas completos de ZIKV, YFV e CHIKV que circularam no estado de Minas Gerais e na Bacia Amazônica durante os surtos recentes;
- Implantar um pipeline, utilizando ferramentas de bioinformática, do sequenciador ao resultado final;
- Demonstrar a situação epidemiológica dos surtos de ZIKV, YFV e CHIKV em Minas Gerais e na Bacia Amazônica.

# 3. RESULTADOS

## 3.1 Artigo 1: *"Epidemiology and evolution of Zika virus in Minas Gerais, Southeast Brazil"*

A transmissão autóctone do ZIKV no Brasil foi identificada pela primeira vez em abril de 2015, com os primeiros casos de microcefalia associados ao ZIKV detectados em outubro de 2015. Apesar dos esforços para compreender a transmissão do ZIKV no Brasil, pouco se sabe sobre a epidemiologia dos vírus e a diversidade genética em Minas Gerais, o segundo estado mais populoso do país. Até janeiro de 2020, 26.817 infecções suspeitas de ZIKV e 86 casos de síndrome congênita foram notificados no estado de MG, sendo o Lacen-MG responsável pelo diagnóstico de 8.552 casos suspeitos de ZIKV e microcefalia. Nesse trabalho, foram gerados dez genomas quase completos de ZIKV diretamente a partir de amostras clínicas. Até 2021, um total de 1.723 casos confirmados foram detectados em MG, com duas ondas epidêmicas principais; a primeira e maior onda epidêmica atingiu o pico em março de 2016 e a segunda onda, menor, atingiu seu pico em março de 2017. A análise utilizando a abordagem do relógio molecular revelou que várias introduções ocorreram em MG entre 2014 e 2015, sugerindo que o vírus circulava despercebido há pelo menos 16 meses antes do primeiro caso laboratorial confirmado, ocorrido retrospectivamente em dezembro de 2015. Nossas descobertas destacaramm a importância das estratégias de vigilância genômica continuada combinada com a epidemiologia tradicional para auxiliar os laboratórios de saúde pública no monitoramento e compreensão da diversidade de arbovírus, que pode ajudar a diminuir o impacto dessas doenças na saúde pública.

Infection, Genetics and Evolution 91 (2021) 104785

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

# Epidemiology and evolution of Zika virus in Minas Gerais, Southeast Brazil

Felipe C.M. Iani [a,b,c], Marta Giovanetti [c,d], Vagner Fonseca [c,e,f], William M. Souza [b,g], Talita E. R. Adelino [a,c], Joilson Xavier [c], Jaqueline G. Jesus [g,h], Maira A. Pereira [a], Marcos V.F. Silva [a], Alana V.B. Costa [a], Erniria C. Silva [i], Márcia C.O. Mendes [i], Ana M.B. Filippis [d], Carlos F. C. Albuquerque [j], André L. Abreu [f], Marluce A.A. Oliveira [a,*], Luiz C.J. Alcantara [c,d,**], Nuno R. Faria [b,h,k,***]

[a] Laboratório Central de Saúde Pública, Fundação Ezequiel Dias, Belo Horizonte, Minas Gerais, Brazil
[b] Department of Zoology, University of Oxford, United Kingdom
[c] Laboratório de Genética Celular e Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[d] Laboratório de Flavivírus, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
[e] KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), College of Health Sciences, University of KwaZuluNatal, Durban 4001, South Africa
[f] Coordenação Geral dos Laboratórios de Saúde Pública/Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília, Distrito Federal, Brazil
[g] Centro de Pesquisa em Virologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
[h] Instituto de Medicina Tropical, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil
[i] Secretaria de Estado de Saúde de Minas Gerais, Belo Horizonte, Brazil
[j] Organização Pan-Americana da Saúde/Organização Mundial da Saúde, Brasília, DF, Brazil
[k] MRC Centre for Global Infectious Disease Analysis, J-IDEA, Imperial College London, London, UK

ARTICLE INFO

*Keywords:*
Zika virus
Epidemiology
Genomic surveillance
Minas Gerais
Brazil

ABSTRACT

Autochthonous Zika virus (ZIKV) transmission in Brazil was first identified in April 2015 in Brazil, with the first ZIKV-associated microcephaly cases detected in October 2015. Despite efforts on understanding ZIKV transmission in Brazil, little is known about the virus epidemiology and genetic diversity in Minas Gerais (MG), the second most populous state in the country. We report molecular and genomic findings from the main public health laboratory in MG. Until January 2020, 26,817 ZIKV suspected infections and 86 congenital syndrome cases were reported in MG state. We tested 8552 ZIKV and microcephaly suspected cases. Ten genomes were generated on-site directly from clinical samples. A total of 1723 confirmed cases were detected in Minas Gerais, with two main epidemic waves; the first and larger epidemic wave peaked in March 2016, with the second smaller wave that peaked in March 2017. Dated molecular clock analysis revealed that multiple introductions occurred in Minas Gerais between 2014 and 2015, suggesting that the virus was circulating unnoticed for at least 16 months before the first confirmed laboratory case that we retrospectively identified in December 2015. Our findings highlight the importance of continued genomic surveillance strategies combined with traditional epidemiology to assist public health laboratories in monitoring and understanding the diversity of circulating arboviruses, which might help attenuate the public health impact of infectious diseases.

## 1. Introduction

Zika virus (ZIKV) is a mosquito-borne virus with a 11 kb positive-sense single-stranded RNA genome. ZIKV belongs to the *Flavivirus* genus (*Flaviviridae* family), the same virus family as dengue virus (DENV), and yellow fever virus (Simmonds et al., 2017). ZIKV was first isolated in 1947 from a sentinel monkey in the Zika forest, located in southeast Uganda, East Africa (Baud et al., 2017). ZIKV infection causes a self-limiting disease and up to 80% of cases are asymptomatic (Haby et al., 2018). Most of the symptomatic cases are characterized by an acute febrile illness with headache, myalgia, conjunctivitis, and/or maculopapular rash (Ioos et al., 2014). However, ZIKV infection can

* Corresponding author.
** Correspondence to: L. C. J. Alcantara, Laboratório de Genética Celular e Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
*** Correspondence to: N. R. Faria, Department of Zoology, University of Oxford, United Kingdom.
*E-mail addresses:* marluce.oliveira@funed.mg.gov.br (M.A.A. Oliveira), luiz.alcantara@ioc.fiocruz.br (L.C.J. Alcantara), n.faria@imperial.ac.uk (N.R. Faria).

https://doi.org/10.1016/j.meegid.2021.104785
Received 1 October 2020; Received in revised form 15 February 2021; Accepted 24 February 2021
Available online 27 February 2021

cause severe neurological disease, such as microcephaly in newborns and Guillain-Barré syndrome in adults (Baud et al., 2017).

ZIKV is mainly transmitted to humans by the *Aedes aegypti* mosquitoes, the same vector of DENV, and Chikungunya virus (CHIKV) (Boyer et al., 2018; Musso and Gubler, 2016). These viruses co-circulate in the same geographic area and cause clinically similar illnesses (Grubaugh et al., 2018). Therefore, the reliable diagnosis of ZIKV infection requires detecting ZIKV RNA through real-time reverse transcription-polymerase chain reaction (RT-qPCR). ZIKV can be classified into two geographically and phylogenetically distinct lineages, the African and Asian genotypes (Faye et al., 2020). The ZIKV Asian genotype has recently emerged as one of the most serious global public health threats (Baud et al., 2017); its first reported epidemic was described in 2007 in Micronesia, followed by outbreaks in 2013–2014 in several Pacific islands and 2015 in Africa (Musso and Gubler, 2016; Faye et al., 2020).

In April 2015, the first autochthonous ZIKV cases were confirmed in northeast Brazil (Ministério da Saúde, 2017). Until August 2020, ZIKV caused 294,713 cases in Brazil (Ministério da Saúde, 2018a, 2020) and 3474 microcephaly cases associated with ZIKV infection until October, 2019 (Ministério da Saúde, 2019). Previous studies investigated ZIKV's transmission in 12 out of 27 states of Brazil, including the federal district (Faria et al., 2016b, 2017; Giovanetti et al., 2020). Moreover, there is no information about the genetic diversity of ZIKV circulating in Minas Gerais, the second most populous Brazilian state, bordering Rio de Janeiro and Sao Paulo states, and located in the southeast region of the country. In this study, we combine epidemiological analysis and portable genome sequencing with data generated at *Fundação Ezequiel Dias* (FUNED), the Public Central Health Laboratory of Minas Gerais, to describe the ZIKV epidemic in Minas Gerais between 2015 and 2020.

## 2. Material and methods

### 2.1. Sample collection

Serum, urine, tissue samples from all patients with ZIKV symptoms attended by public health services in the Minas Gerais States were collected for molecular diagnostics and sent for testing at *Fundação Ezequiel Dias* (FUNED), the Public Central Health Laboratory of Minas Gerais. Sampled patients subjected to molecular diagnostics presented maculopapular rash and at least two symptoms, such as fever, polyarthralgia, peri-articular edema, and conjunctivitis (purulent or hyperemic). All samples were processed under terms of Resolution 510/2016 of national ethical review board (*Comissão Nacional de Ética em Pesquisa*), under the auspices of the ZiBRA2 project (www.zibra2project.org/). The project was approved by the Pan American Health Organization Ethics Review Committee (PAHOERC) no. PAHO-2016-08-0029.

### 2.2. Nucleic acid isolation and RT-qPCR for ZIKV

Viral nucleic acid extraction was performed using the MagNA Pure 96 System (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. Molecular diagnostic was performed by RT-qPCR against the prM target specific to ZIKV (using 5′ FAM as the probe reporter dye) and GoTaq® 1-Step RT-qPCR System (Promega, USA), as previously described (Lanciotti et al., 2008). Cycle threshold (Ct) values were determined for all samples. All procedures were conducted in biological safety cabinets located in physically separated areas. Negative controls were used in all reactions.

### 2.3. ZIKV sequencing and consensus sequences

Genome sequencing was performed in samples with Ct values <35 and availability of epidemiological data, such as date of onset of symptoms, date of sample collection, sex, municipality of residence, and symptoms. A total of 10 ZIKV samples with PCR products yielding sufficient material (DNA concentration after clean-up being >4 ng/μL), were randomly selected for genome sequencing.

Positive samples were submitted to a cDNA synthesis protocol (Quick et al., 2017) using Superscript IV cDNA Synthesis Kit. Then, a multiplex tiling PCR was conducted with Q5 High Fidelity Hot-Start DNA Polymerase (New England Biolabs) using ZIKV sequencing primers scheme designed using Primal Scheme (http://primal.zibraproject.org) (Quick, Grubaugh et al., 2017). The thermocycling conditions involved 40 cycles; reaction conditions were previously reported in (Quick et al., 2017). Sequencing libraries were generated from the barcoded products using the Genomic DNA Sequencing Kit SQK-MAP007 and SQK-LSK208 and library quality was assessed by Qubit quantification after barcoding and adapter ligation steps. DNA was loaded onto R9.4 flow cells (Oxford Nanopore Technologies, United Kingdom). Raw files were basecalled using Guppy (Oxford Nanopore Technologies, United Kingdom), demultiplexed and trimmed using Porechop (https://github.com/rrwick/Porechop) and/or QCAT, and then mapped generating contigs (de novo) and aligned with the reference (GenBank accession number NC_035889.1) using Genome Detective (Vilsker et al., 2019).

### 2.4. Collation and sequence alignment of ZIKV-Asian complete genome datasets

Genotype of the newly generated genomes were identified using Genome Detective (Vilsker et al., 2019). New data was then appended to publicly available data for subsequent analysis. Two ZIKV genome (coverage >50%) data sets were compiled: dataset 1 ($n$ = 474), comprised the data reported in this study ($n$ = 10) plus ($n$ = 464) publicly available ZIKV-Asian genotype genomes available until November 2019 in GenBank and after being filtered by genome coverage. The second one, dataset 2 ($n$ = 196), included only Brazilian strains ($n$ = 177 being 10 from this study) plus the oldest ZIKV Asian sequences (n = 19). Sequence alignment was performed using MAFFT version 7 (Katoh and Standley, 2013) and visually inspected in AliView version 1.26 (Larsson, 2014). Since recombination may impact evolutionary estimates before performing our phylogenetic reconstruction, we employed the 12 recombination detection methods available in RDP version 4 (Martin and Rybicki, 2000) to further search for evidence of recombination in our dataset. Moreover, no evidence of recombination was found.

### 2.5. Maximum likelihood analysis and clock signal estimation

Maximum likelihood (ML) trees were estimated using IQ-TREE 1.6.12 (Nguyen et al., 2014) under an GTR + F + R3 nucleotide substitution model for the dataset 1 and TIM2 + F + R2 for the dataset 2, as indicated by ModelFinder implemented in IQ-TREE (Kalyaanamoorthy et al., 2017). Statistical robustness of tree topology was inspected using 1000 bootstrap replicates; a bootstrap value >80% was considered strong statistical support. To estimate temporal signal in each dataset, sample collection dates were regressed against root-to-tip genetic distances obtained from the ML phylogenies using TempEst 1.5.3 (Rambaut et al., 2016). When precise sampling dates were not available, a precision of 1 month or 1 year in the collection dates was considered. For convenience Brazilian sequences have been grouped into states macro region for which those sequences were generated (Southeast: Minas Gerais, São Paulo, Rio de Janeiro; Northeast: Alagoas, Bahia, Ceará, Maranhão, Paraíba, Pernambuco, Rio Grande do Norte; and North: Amazonas, Pará, Tocantis).

### 2.6. Dated phylogenetics

Time scaled phylogenetic trees were inferred using the BEAST version 1.10.4 statistical framework (Suchard et al., 2018). We used the codon-based SRD06 model (Shapiro et al., 2006) and a non-parametric Bayesian skyline coalescent model (Drummond and Rambaut, 2015) to model changes in effective population size over time and the uncorrelated relaxed molecular clock model (Drummond et al., 2006). Previous

studies have demonstrated this combination to be the best fitting model combination for ZIKV in the Americas (Faria et al., 2017; Grubaugh et al., 2018; Grubaugh et al., 2017). We computed 4 independent runs of 100 million MCMC steps, sampling parameters and trees every 10,000 steps. Convergence of MCMC chains was checked using Tracer v.1.7.1 (Rambaut et al., 2018). Maximum clade trees were summarized using TreeAnnotator version v1.10.4 after discarding 10% as burn-in. Phylogenetics analyses were performed using the Sagarana HPC cluster, CEPAD-ICB-UFMG.

### 2.7. Epidemiological data

We followed the case definition guidelines of the State Health Secretary of Minas Gerais (SES-MG). Specifically, a notified case was defined as a patient that presented maculopapular rash and at least two symptoms, such as fever, polyarthralgia, peri-articular edema, and conjunctivitis (purulent or hyperemic). A probable case was defined as a notified case, excluding the patients with laboratory diagnosis with negative results or diagnosed for other diseases. Confirmed cases were defined as patients with positive laboratory results for ZIKV or clinical-epidemiological criteria. Microcephaly incidence was estimated using the number of confirmed cases divided by the number of live births obtained by DATASUS (www.datasus.saude.gov.br). Incidences were calculated based on the estimated population of Minas Gerais State in 2019, as reported by the Brazilian Institute of Geography and Statistics (www.ibge.gov.br). Association between tested and confirmed cases, population size, incidence, and microcephaly cases in Minas Gerais state was determined by Spearman correlation analysis. Results were plotted using log10 transformed values after correlation analyses. All the statistics and maps were done using RStudio version 1.2.5033 (RStudio Team, 2019).

### 2.8. Data availability

Epidemiological data and phylogenetic trees, XMLs are available on Zibra II Project website repository (https://www.zibra2project.org/epidemiology-and-evolution-of-zika-virus-in-minas-gerais-southeast-brazil/). ZIKV sequences from Minas Gerais State are available on GenBank (accession numbers: MT439638, MT439639, MT439640, MT439641, MT439642, MT439643, MT439644, MT439645, MT439646,

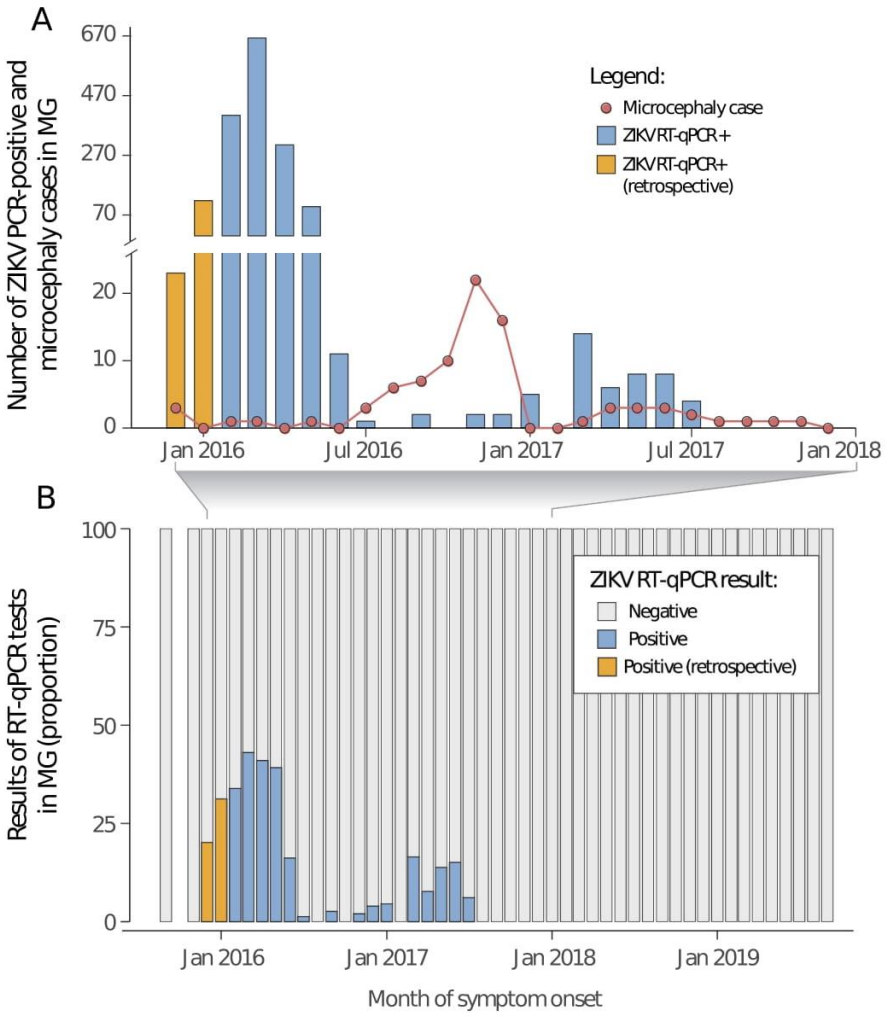



**Fig. 1.** Temporal distribution of zika positive and microcephaly cases in Minas Gerais over 2016–2018. (A) ZIKV RT-qPCR confirmed cases (bars) and microcephaly ZIKV associated cases (red points) per month in Minas Gerais state, southeast Brazil. (B) Comparative bar chart of the number of ZIKV positive and negative RT-qPCR cases per month in Minas Gerais state.

MT439647).

## 3. Results

The first ZIKV RT-qPCR confirmed case in Minas Gerais state was reported on February 19, 2016 by the Ezequiel Dias Foundation (available on the *Gerenciador de Ambiente Laboratorial* - an electronic system maintained by the Ministry of Health), although if the official bulletin from the MG's Secretary of Health was only published in March 7, 2016 (Secretaria de Estado da Saúde de Minas Gerais, 2016). In an attempt to obtain evidence of ZIKV transmission before its first detection, we retrospectively assessed 513 samples collected from September 2015 to January 2016 from patients with acute febrile illness. Of these, we found 141 ZIKV RT-qPCR-positive samples (8.2% of all RT-qPCR confirmed cases) collected between December 6, 2015 and January 31, 2016, revealing that ZIKV was likely circulating unnoticed for more than 2 months in Minas Gerais before its first detection. (Fig. 1A).

Time-series of the ZIKV RT-qPCR positive cases show two epidemic waves: the 'first ZIKV wave with 94% of all RT-qPCR confirmed cases (1623 cases) between December 2015 to May 2016 with a peak of cases registered on March 2016 (with $n = 663$, 41% of all RT-qPCR confirmed cases of the first ZIKV wave) (Fig. 1A) and a second, smaller epidemic wave with 51 RT-qPCR confirmed cases from March to July 2017 (3% of all RT-qPCR confirmed cases detected during the study period) (Fig. 1B).

We next evaluated ZIKV incidence across Minas Gerais regions.The *Norte de Minas* (north region) was the mesoregion with the highest incidence of RT-qPCR confirmed cases during the first ZIKV wave (Fig. 2A). The Ipatinga municipality accounted for the highest number of ZIKV cases confirmed by clinical-epidemiological criteria ($n = 2546$) and with RT-qPCR ($n = 79$), which reported an incidence of 29.99 per 100,000 inhabitants in 2016. Besides, the highest number of RT-qPCR confirmed cases was detected in Belo Horizonte city (state capital city) ($n = 486$) (Supplementary Fig. 1A), that recorded an incidence of 19.34 per 100,000 inhabitants (Supplementary Fig. 1B). Interestingly, the last ZIKV RT-qPCR confirmed cases in the Minas Gerais state were detected on July 31, 2017. ZIKV RT-qPCR confirmed cases were predominantly in adults (18 to 40 years old) (78.81%, $n = 1358/1723$). The median age was 30.28 years (interquartile range: 23–35), ranging from 1 day to 78 years. Samples were characterized by mean Ct of 29.3 (range 21.21 to 39.99) (Fig. 2B). We found that municipality population size was moderately associated with ZIKV confirmed cases (Spearman's rank coefficient of 0.38, $p = 6e\text{-}08$). Moreover, we found a strong correlation between the number of total cases tested per municipality versus ZIKV confirmed (Spearman's rank coefficient of 0.8, $p < 2.2e\text{-}16$ (Supplementary Fig. 2A and B).

Most RT-qPCRconfirmed cases were in females (91.6%, $n = 1578$) (Fig. 2C), most likely due to guidelines in testing. Noteworthy, we report one case in embryonic fragment of an abortion of an eight-week RT-qPCR positive pregnant woman (accession number GenBank: MT439640) and 43 RT-qPCR confirmed cases in children (0 to 12 years

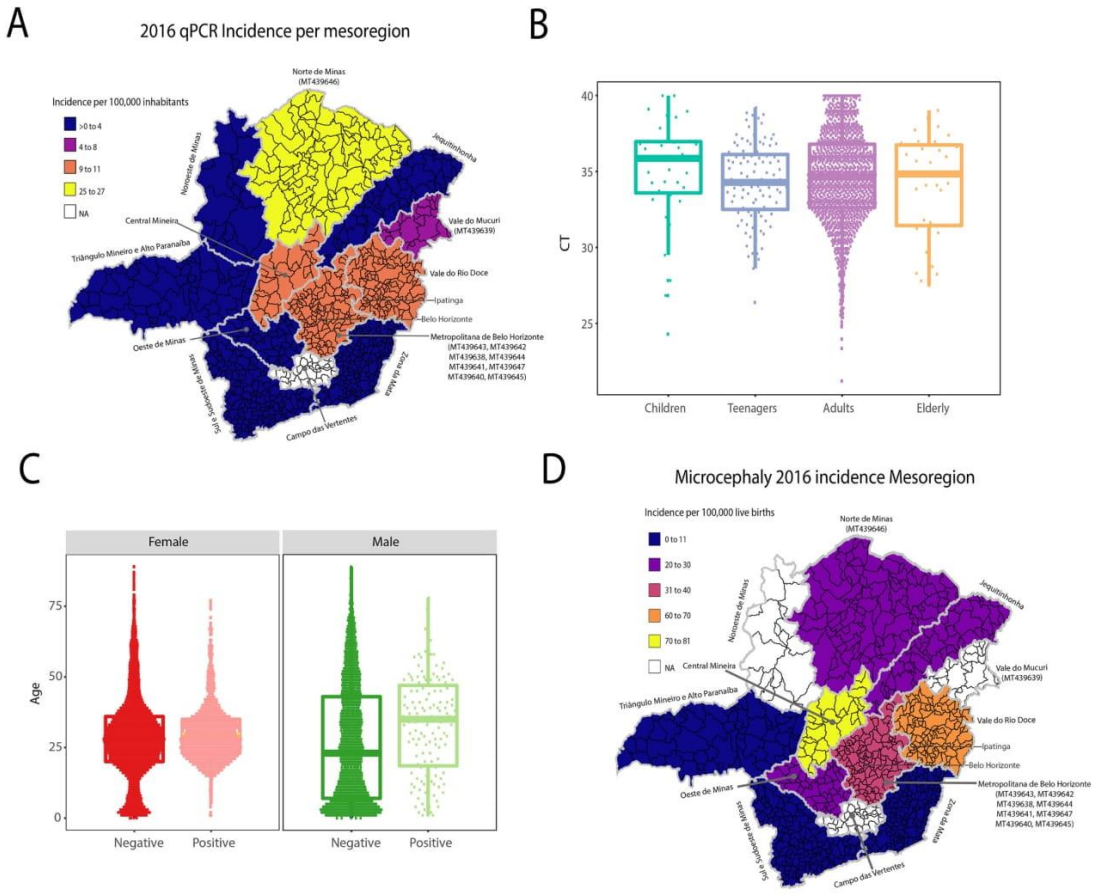



**Fig. 2.** (A) Minas Gerais mesoregion map showing 2016 Zika virus incidence per 100,000 inhabitants and the origin location of novel sequences; (B) RT-qPCR confirmed cases by age groups and RT-qPCR cycle threshold; (C) RT-qPCR test results by sex and age; (D) 2016 microcephaly associated with Zika virus infection incidence per live birth Minas Gerais mesoregion map and the origin location of novel sequences.

old). The ZIKV outbreak (2016–2017) in Minas Gerais caused 83 microcephaly cases confirmed by ZIKV infection in 43 municipalities, 16.9% (14/83) were reported in Belo Horizonte city (Ministério da Saúde, 2018b). The *MG Central mesoregion* had the highest 2016 microcephaly incidence 80.77 per 100,000 newborns (Fig. 2D). In the state, most microcephaly cases were reported in November 2016 ($n$ = 22), approximately eight months after the peak of the first ZIKV wave (Fig. 1a). The Spearman correlation analysis showed an association between microcephaly and the number of newborns ($r = 0.67$, $p$ = 1e-06). A correlation was also observed between microcephaly and confirmed cases per municipality ($r = 0.58$, $p = 0.0012$) (Supplementary Fig. 2C and D).

Using handheld nanopore technologies, we obtained 10 ZIKV genome sequences, (coverage range 60.5%–91.5%, mean = 76.56%), five from Belo Horizonte, and other five from municipalities of Betim, Matozinhos, Montes Claros, Santa Luzia, and Teofilo Otoni. All samples sequenced in this study were from the first ZIKV wave in Minas Gerais state and they had mean Ct value of 29.28 (range 23.87–33.45). Mean threshold cycle value of the generated sequences, sequencing statistics and epidemiological data are detailed in Table 1.

To better understand the transmission dynamics of ZIKV in MG state we estimate an initial maximum likelihood (ML) phylogenetic tree (Supplementary Fig. 3) analysis on the 10 new sequences combined with another 464 publicly available ZIKV-Asian genotype genomes. Our estimated phylogeny identified that the newly ZIKV genomes obtained in this study fell within the ZIKV American clade (bootstrap score = 100) (Supplementary Fig. 3).

To assess the evolution of ZIKV in MG state, we performed Bayesian time-measured phylogenetic analysis using a molecular clock model on a dataset 2 ($n$ = 196) comprising the 10 new sequences from MG plus other Brazilian strains ($n$ = 167) plus the oldest ZIKV Asian sequences (n = 19) sequences. A regression of genetic divergence from root to tip against sampling dates confirmed sufficient temporal signal (r2 = 0.734) (Supplementary Fig. 4). Our maximum clade credibility (MCC) tree identified that at least three independent introductions of ZIKV occurred in MG state between end-October 2014 and early-April 2015 (see clades MG1 MG2 and MG3 in Fig. 3B). We observed that MG2 clade includes isolate MT439646 which was also closely placed to isolates MT439644 and MT439643 in the ML tree, with a bootstrap value of 0.91. MG2 internal nodes present low bootstrap values which might reflect sequence low quality and coverage. Furthermore, formation of clades MG1 and MG3 were also observed in the ML tree, which is consistent with our time-measured Bayesian phylogeny. We estimated the most recent common ancestor (TRMCA) for each clade with a 95% of high posterior density to be early-April 2015 (November 2014 to September 2015) for the MG1, end-December 2014 (August 2014 to May 2015) for the MG2, and end-October 2014 (June 2014 to February 2015) for the MG3. We also identified three isolates from 2016 outside those three main clades. Isolate MT439641, sampled in April 2016, clusters with sequences from Northeast Brazil. Isolate MT439647, sampled in April 2016, clusters with sequences from Northeast and Southeast Brazil, and isolate MT439645 sampled in March 2016 falls basally to a clade containing sequences from Southeast Brazil. Taken together these data suggest that multiple independent introduction events have occurred into MG state mainly from Southeastern (MG1 and MG2), and Northeastern (MG3) Brazilian states (Fig. 3A, B).

## 4. Discussion

In this study, combining genomics and epidemiological data we investigate the ZIKV outbreak in Minas Gerais State between 2015 and 2017. Epidemiological data reveal that two main waves were responsible for the ZIKV epidemic in MG state, the first and largest one registered in 2016, followed by a second smaller one in 2017, which peaked around March, during the rainy season, a period with climatic suitability for arbovirus transmissions, such as DENV in the state (Aguiar et al., 2014). Interestingly, the MG state as well as other bordering states such as São Paulo, Goias and Espirito Santo, were not characterized by a third epidemic wave between 2018 and 2019 as previously reported for the Amazon region (Giovanetti et al., 2020; Secretaria de Estado da Saúde de Minas Gerais, 2020).

The generated genomic data allowed us to estimate the introduction date of the ZIKV-Asian lineage in MG to have occurred between end-October 2014 and early-April 2015, suggesting an undetected circulation of the virus for 16 months before the first reports of ZIKV transmission in the state (Secretaria de Estado da Saúde de Minas Gerais, 2016) following a pattern of cryptic transmission that have been already observed before for zika as well as for other mosquito-borne viruses such as dengue and chikungunya virus epidemics (Faria et al., 2017; Xavier et al., 2019; Faye et al., 2020). According to the Minas Gerais State Health Secretary epidemiological bulletin, ZIKV autochthony in MG was reported on 8 of March (Secretaria de Estado da Saúde de Minas Gerais, 2016). Prior to that, only imported cases had been registered.

Moreover, our data suggest that the circulation of the ZIKV-Asian

**Table 1**
Sequencing and epidemiological statistics.

| Accession number | Lab_id | Coverage (%) | Reads | Depth of Coverage | NT (Identity (%)) | AA (Identity (%)) | I/D/M/F | Ct value | Sample Type | Municipality | Collection Date | Onset date | Age | Sex |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MT439639 | 2032/16 M | 91.5 | 68,415 | 4130.4 | 99.1 | 98.7 | 0/12 | 33.45 | Serum | Teofilo Otoni | 11/04/16 | 11/04/16 | 28 | Female |
| MT439643 | BH 06 | 87.5 | 4285 | 217.7 | 99.5 | 99.5 | 0/5 | 26.96 | Serum | Belo Horizonte | 12/04/16 | NA | 28 | Female |
| MT439642 | BH 05 | 84.2 | 5035 | 268.4 | 99.5 | 99.5 | 0/5 | 26.05 | Serum | Belo Horizonte | 06/04/16 | 05/04/16 | 17 | Female |
| MT439638 | 1295/16 M | 80.4 | 54,258 | 3826.4 | 98.9 | 98.3 | 0/15 | 33.14 | Serum | Belo Horizonte | 24/03/16 | 21/03/2016 | 27 | Female |
| MT439644 | BH 07 | 79.8 | 3524 | 214.2 | 99.5 | 99.2 | 0/3 | 27.5 | Serum | Santa Luzia | 20/04/16 | 18/04/16 | 25 | Female |
| MT439641 | BH 02 | 73.4 | 2053 | 126.1 | 99.5 | 99.5 | 0/1 | 23.87 | Serum | Matozinhos | 05/04/16 | 01/04/16 | 37 | Female |
| MT439647 | BH 21 | 72.1 | 4383 | 391.7 | 99.5 | 99.2 | 0/7 | 30.04 | Serum | Belo Horizonte | 14/04/16 | 13/03/16 | 18 | Female |
| MT439640 | 1377/16 M | 69.1 | 21,624 | 2277.7 | 98.4 | 97.8 | 15/4 | 32.09 | Tissue | Belo Horizonte | 24/03/16 | Abortion | NA | NA |
| MT439645 | BH 16 | 67.1 | 2745 | 208.5 | 99.6 | 99.4 | 0/3 | 29.8 | Serum | Betim | 18/03/16 | 16/03/16 | 30 | Female |
| MT439646 | BH 19 | 60.5 | 4173 | 455.1 | 99.4 | 98.8 | 1/2 | 29.95 | Serum | Montes Claros | 06/04/16 | 04/04/16 | 23 | Female |

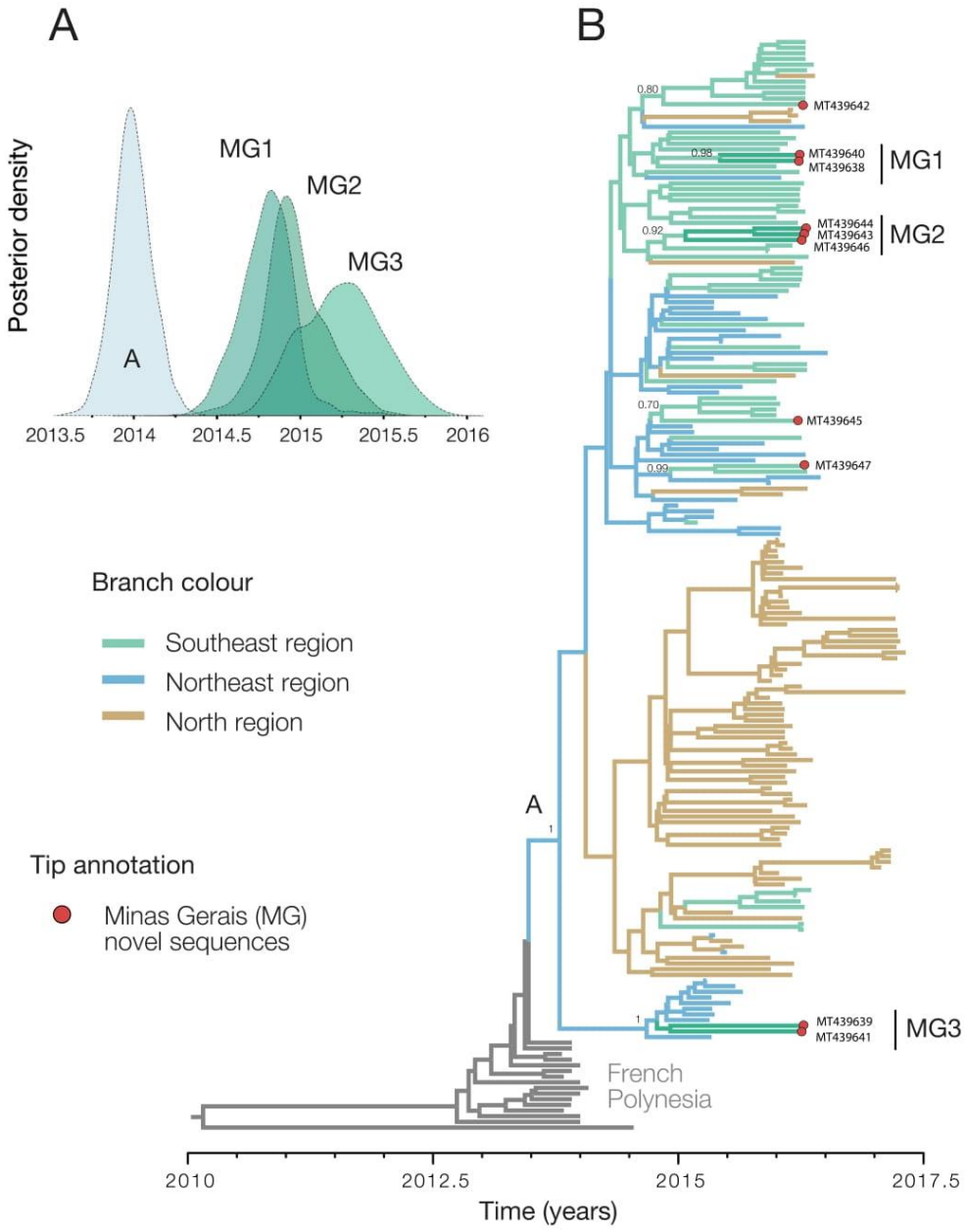



**Fig. 3.** Phylogenetic analysis of the introduction of Zika virus to Minas Gerais state. A) Dynamics of ZIKV introduction events in Minas Gerais state. Dates of introduction events were estimated from sequence data using a phylogenetic approach. B) Maximum clade credibility phylogeny estimated from Zika virus genomes with a molecular clock phylogenetic approach. Sequences are coloured according to sampling location. Clade A contains the MG Zika virus (red) and other closely related sequences from Brazil (green, blue and yellow indicate sampling location in Brazil). Clade posterior probabilities are shown at well-supported nodes.

genotype in MG may resulted from at least three independent introduction events over 2014 and 2015 (Fig. 3), which we infer to have occurred, during a period characterized by high climatic suitability for arbovirus transmission in the region (Aguiar et al., 2014). Further, we found evidence that two (MG1 and MG2) clades had originated from the southeast region whereas a third one (MG3 clade) appears to have originated directly from Northeast Brazil, that have played a significant role in the establishment and dissemination of ZIKV in the Americas (Faria et al., 2016).

Based on retrospective investigations reported by Ministry of Health, it was confirmed that ZIKV was the etiological agent of six microcephaly cases in December 2015 (Ministério da Saúde, 2018b), two months before the first ZIKV cases reported in Minas Gerais state on February 2016 by FUNEDThese findings also corroborate with our estimates reinforcing the idea of a likely ZIKV cryptic transmission before its first detection in the state.

Together, our results shed light on the epidemiological dynamics of the ZIKV-Asian genotype into MG state, showing that genomic data generated by portable sequencing technology can be employed to assist public health laboratories in monitoring and understanding the diversity of circulating mosquito-borne viruses.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104785.

## Funding

This work was supported by Decit/SCTIE/BrMoH/CNPq (440685/2016-8; 421598/2018-2), by CAPES (88887130716/2016-00) and by the European Union's Horizon 2020 Research and Innovation Programme under ZIKAlliance Grant Agreement no. 734548. F.C.M.I and J. X. are supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. F.C.M.I is also supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). M.G. is supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPER J. N.R.F. is supported by a Wellcome Trust and Royal Society Sir Henry Dale Fellowship (204311/Z/16/

Z). N.R.F is also supported by a Medical Research Council-São Paulo Research Foundation (FAPESP) CADDE partnership award (MR/S0195/1 and FAPESP 18/14389-0) (http://caddecentre.org/). V.F. is supported by the South African Medical Research Council (MRC-RFA-UFSP-01-2013/UKZN HIVEPI) and the NIH H3Abi-oNet network, which is an initiative of the Human Health and Heredity in Africa Consortium (H3Africa). W.M.S. is supported by the São Paulo Research Foundation, Brazil (2017/13981-0 and 2019/24251-9). J.G.J. s supported by FAPESP (2018/17176-8 and 2019/12000-1, 18/14389-0).

## Declaration of Competing Interest

This work does not have any relationships with business related issues, and no conflict of interest exits in the submission of this manuscript.

## Acknowledgements

We acknowledge The Brazilian Ministry of Health for the microcephaly cases data disponibilization in special *Wanderson* Oliveira and *Sulamita* Brandão Barbiratto and Darlan Candido for the initial preparation of sequence dataset. We also thanks Minas Gerais State Scretariat for Health8 for providing the epidemiological data and the Ezequiel Dias Foundation for the facilities availability.

## References

Aguiar, M., Rocha, F., Pessanha, J.E.M., Mateus, L., Stollenwerk, N., 2014. Carnival or football, is there a real risk for acquiring dengue fever in Brazil during holidays seasons? Sci. Rep. 5, 1–12. https://doi.org/10.1038/srep08462.

Baud, D., Gubler, D.J., Schaub, B., Lanteri, M.C., Musso, D., 2017. An update on Zika virus infection. Lancet 390, 2099–2109. https://doi.org/10.1016/S0140-6736(17)31450-2.

Boyer, S., Calvez, E., Chouin-Carneiro, T., Diallo, D., Failloux, A., 2018. An overview of mosquito vectors of Zika virus. Microbes Infect. 20, 646–660. https://doi.org/10.1016/j.micinf.2018.01.006.

Drummond, A.J., Rambaut, A., 2015. Bayesian evolutionary analysis by sampling trees. Bayesian Evol Anal with BEAST 8, 79–96. https://doi.org/10.1017/CBO9781139095112.007.

Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, 699–710. https://doi.org/10.1371/journal.pbio.0040088.

Faria, N.R., Sabino, E.C., Nunes, M.R.T., Alcantara, L.C.J., Loman, N.J., Pybus, O.G., 2016. Mobile real-time surveillance of Zika virus in Brazil. Genome Med 8, 97. https://doi.org/10.1186/s13073-016-0356-2.

Faria, N.R., Do Socorro Da Silva Azevedo, R., MUG, Kraemer, Souza, R., Cunha, M.S., Hill, S.C., et al., 2016b. Zika virus in the Americas: Early epidemiological and genetic findings. Science 352, 345–349. https://doi.org/10.1126/science.aaf5036.

Faria, N.R., Quick, J., Claro, I.M., Thézé, J., De Jesus, J.G., Giovanetti, M., et al., 2017. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. Nature 546, 406–410. https://doi.org/10.1038/nature22401.

Faye, O., de Lourdes, Monteiro M., Vrancken, B., Prot, M., Lequime, S., Diarra, M., et al., 2020. Genomic epidemiology of 2015–2016 Zika virus outbreak in Cape Verde. Emerg. Infect. Dis. 26 (6), 1084–1090. https://doi.org/10.3201/eid2606.190928.

Giovanetti, M., Faria, N.R., Lourenço, J., Goes de Jesus, J., Xavier, J., Claro, I.M., et al., 2020. Genomic and epidemiological surveillance of zika virus in the amazon region. Cell Rep 30, 2275–2283 e7. https://doi.org/10.1016/j.celrep.2020.01.085.

Grubaugh, N.D., Ladner, J.T., Kraemer, M.U.G., Dudas, G., Tan, A.L., Gangavarapu, K., et al., 2017. Genomic epidemiology reveals multiple introductions of Zika virus into the United States. Nature 546, 401–405. https://doi.org/10.1038/nature22400.

Grubaugh, N.D., Faria, N.R., Andersen, K.G., Pybus, O.G., 2018. Genomic insights into Zika virus emergence and spread. Cell 172, 1160–1162. https://doi.org/10.1016/j.cell.2018.02.027.

Haby, M.M., Pinart, M., Elias, V., Reveiz, L., 2018. Prevalence of asymptomatic Zika virus infection: a systematic review. Bull. World Health Organ. 96, 402–413D. https://doi.org/10.2471/BLT.17.201541.

Ioos, S., Mallet, H.P., Leparc Goffart, I., Gauthier, V., Cardoso, T., Herida, M., 2014. Current Zika virus epidemiology and recent epidemics. Med. Mal. Infect. 44, 302–307. https://doi.org/10.1016/j.medmal.2014.04.008.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., Von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. https://doi.org/10.1038/nmeth.4285.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010.

Lanciotti, R.S., Kosoy, O.L., Laven, J.J., Velez, J.O., Lambert, A.J., Johnson, A.J., et al., 2008. Genetic and serologic properties of Zika virus associated with an epidemic, yap state, Micronesia, 2007. Emerg. Infect. Dis. 14, 1232–1239. https://doi.org/10.3201/eid1408.080287.

Larsson, A., 2014. AliView: a fast and lightweight alignment viewer and editor for large datasets. Bioinformatics 30, 3276–3278. https://doi.org/10.1093/bioinformatics/btu531.

Martin, D., Rybicki, E., 2000 Jun. RDP: detection of recombination amongst aligned sequences. Bioinformatics. 16 (6), 562–563. https://doi.org/10.1093/bioinformatics/16.6.562. 10980155.

Ministério da Saúde, 2017. Boletim Epidemiológico Secretaria de Vigilância em Saúde – Ministério da Saúde - até a Semana Epidemiológica 52, 2016, 48.

Ministério da Saúde, 2018a. Boletim Epidemiológico 47: Situação epidemiológica da infecção pelo vírus Zika no Brasil, de 2015 a 2017. Bol Epidemiológico S 49, 1–12.

Ministério da Saúde, 2018b. Registro de Eventos em Saúde Pública (RESP-Microcefalia). http://www.resp.saude.gov.br/microcefalia#/painel (accessed November 6, 2018).

Ministério da Saúde, 2019. Síndrome congênita associada à infeccção pelo vírus Zika, 50.

Ministério da Saúde, 2020. Monitoramento dos casos de arboviroses urbanas transmitidas pelo Aedes. Bol Epidemiológico Arboviroses 51, 1–13.

Musso, D., Gubler, D.J., 2016. Zika virus. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.00072-15.

Nguyen, L., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2014. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies, 32, pp. 268–274. https://doi.org/10.1093/molbev/msu300.

Quick, J., Grubaugh, N.D., Pullan, S.T., Claro, I.M., Smith, A.D., Gangavarapu, K., et al., 2017. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat. Protoc. 12, 1261–1266. https://doi.org/10.1038/nprot.2017.066.

Rambaut, A., Lam, T.T., Max Carvalho, L., Pybus, O.G., 2016. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol 2, vew007. https://doi.org/10.1093/ve/vew007.

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior summarization in Bayesian phylogenetics using tracer 1.7. Syst. Biol. 67, 901–904. https://doi.org/10.1093/sysbio/syy032.

RStudio Team, 2019. RStudio: Integrated Development Environment for R.

Secretaria de Estado da Saúde de Minas Gerais, 2016. Boletim epidemiológico de monitoramento dos casos de Dengue, Febre Chikungunya e Febre Zika. Nº 10. Semana Epidemiológica 11, 15/03/2016.

Secretaria de Estado da Saúde de Minas Gerais, 2020. Boletim Epidemiologico.

Shapiro, B., Rambaut, A., Drummond, A.J., 2006. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol. Biol. Evol. 23, 7–9. https://doi.org/10.1093/molbev/msj021.

Simmonds, P., Becher, P., Bukh, J., Gould, E.A., Meyers, G., Monath, T., et al., 2017. ICTV virus taxonomy profile: Flaviviridae. J Gen Virol 98, 2–3. https://doi.org/10.1099/jgv.0.000672.

Suchard, M.A., Lemey, P., Baele, G., Ayres, D.L., Drummond, A.J., Rambaut, A., 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol 4, 1–5. https://doi.org/10.1093/ve/vey016.

Vilsker, M., Moosa, Y., Nooij, S., Fonseca, V., Ghysens, Y., Dumon, K., et al., 2019. Genome detective: an automated system for virus identification from high-throughput sequencing data. Bioinformatics 35, 871–873. https://doi.org/10.1093/bioinformatics/bty695.

Xavier, J., Giovanetti, M., Fonseca, V., Thézé, J., Gräf, T., Fabri, A., et al., 2019. Circulation of chikungunya virus east/central/ south African lineage in Rio de Janeiro, Brazil. PLoS One 14, 1–14. https://doi.org/10.1371/journal.pone.0217871.

## 3.2 Artigo 2: *"Genomic and Epidemiological Surveillance of Zika Virus in the Amazon Region".*

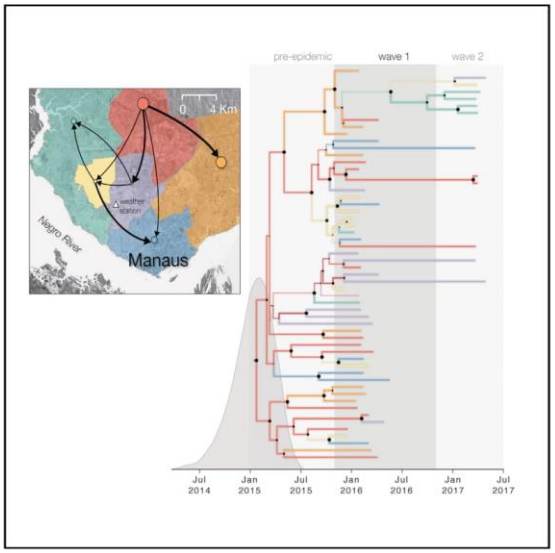
O ZIKV causou uma epidemia explosiva associada a resultados clínicos graves nas Américas. Em junho de 2018, 4.929 suspeitas de infecções por ZIKV e 46 casos de síndrome congênita foram relatados em Manaus, Amazonas. Embora Manaus seja o principal pólo demográfico da região amazônica, pouco é conhecido sobre a epidemia de ZIKV nessa região, em termos de transmissão e diversidade genética viral. Utilizando o sequenciador portátil, geramos 59 genomas de ZIKV em Manaus. Análises filogenéticas indicaram múltiplas introduções de ZIKV do região nordeste brasileira para Manaus. Análises genômicas espaciais do movimento do vírus entre seis áreas em Manaus sugeriram que bairros populosos do norte atuaram como fontes de transmissão do vírus para outros bairros. Este estudo revelou como a epidemia de ZIKV foi iniciada e mantida dentro da maior metrópole urbana do Amazonas. Esses resultados podem contribuir para melhorar a resposta da saúde pública aos surtos no Brasil.

Article

Cell Reports

# Genomic and Epidemiological Surveillance of Zika Virus in the Amazon Region

## Graphical Abstract




## Authors

Marta Giovanetti, Nuno Rodrigues Faria, José Lourenço, ..., Felipe Gomes Naveca, Oliver G. Pybus, Luiz Carlos Alcantara

## Correspondence

nuno.faria@zoo.ox.ac.uk (N.R.F.), oliver.pybus@zoo.ox.ac.uk (O.G.P.), luiz.alcantara@ioc.fiocruz.br (L.C.A.)

## In Brief

Zika virus has caused an explosive epidemic linked to severe clinical outcomes in the Americas, but little is known about the epidemic in the Brazilian state of Amazonas. To gain insights into the routes of ZIKV introduction, Giovanetti et al. tracked the virus by sequencing genomes from infected patients from this region.

## Highlights

- Epidemiological data reveal three ZIKV epidemic waves (2016, 2017, and 2018) in Manaus
- Our results suggest multiple introductions of ZIKV from northeastern Brazil to Manaus
- ZIKV cases in Manaus resulted from a single introduction event (January 2015)
- Spatial analysis suggested that northern neighborhoods acted as sources for transmission

Giovanetti et al., 2020, Cell Reports *30*, 2275–2283
February 18, 2020 
https://doi.org/10.1016/j.celrep.2020.01.085

CellPress

Cell Reports
Article

OPEN ACCESS
CellPress

# Genomic and Epidemiological Surveillance of Zika Virus in the Amazon Region

Marta Giovanetti,[1,2,22] Nuno Rodrigues Faria,[2,3,22,*] José Lourenço,[3] Jaqueline Goes de Jesus,[1] Joilson Xavier,[2] Ingra Morales Claro,[4] Moritz U.G. Kraemer,[3] Vagner Fonseca,[2,5] Simon Dellicour,[6,7] Julien Thézé,[3] Flavia da Silva Salles,[4] Tiago Gräf,[8] Paola Paz Silveira,[8] Valdinete Alves do Nascimento,[9] Victor Costa de Souza,[9] Felipe Campos de Melo Iani,[2,10] Emerson Augusto Castilho-Martins,[11] Laura Nogueira Cruz,[12] Gabriel Wallau,[13] Allison Fabri,[1] Flávia Levy,[1] Joshua Quick,[14] Vasco de Azevedo,[2] Renato Santana Aguiar,[8] Tulio de Oliveira,[5] Camila Bôtto de Menezes,[15] Marcia da Costa Castilho,[16] Tirza Matos Terra,[17] Marineide Souza da Silva,[17] Ana Maria Bispo de Filippis,[1] André Luiz de Abreu,[12] Wanderson Kleber Oliveira,[18] Julio Croda,[19] Carlos F. Campelo de Albuquerque,[20] Marcio R.T. Nunes,[21] Ester Cerdeira Sabino,[4] Nicholas Loman,[14] Felipe Gomes Naveca,[9] Oliver G. Pybus,[3,*] and Luiz Carlos Alcantara[1,2,23,*]

[1]Laboratório de Flavivírus, Instituto Oswaldo Cruz Fiocruz, Rio de Janeiro, Brazil
[2]Laboratório de Genética Celular e Molecular, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[3]Department of Zoology, University of Oxford, Oxford OX1 3PS, UK
[4]Instituto de Medicina Tropical, Universidade de São Paulo, São Paulo, Brazil
[5]KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), College of Health Sciences, University of KwaZuluNatal, Durban 4001, South Africa
[6]Spatial Epidemiology Lab, Université Libre de Bruxelles, Bruxelles, Belgium
[7]KU Leuven, Department of Microbiology and Immunology, Rega Institute, Laboratory for Clinical and Epidemiological Virology, Leuven, Belgium
[8]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil
[9]Laboratório de Ecologia de Doenças Transmissíveis na Amazônia, Instituto Leônidas e Maria Deane, Fiocruz, Manaus, Brazil
[10]Laboratório Central de Saúde Pública, Fundação Ezequiel Dias, Belo Horizonte, Brazil
[11]Faculdade de Medicina, Universidade Federal do Amapá, Macapá, Brazil
[12]Coordenação Geral dos Laboratórios de Saúde Pública/Secretaria de Vigilância em Saúde, Ministério da Saúde (CGLAB/SVS-MS), Brasília, Distrito Federal, Brazil
[13]Fundação Oswaldo Cruz - Instituto Aggeu Magalhães, Recife, Pernambuco, Brazil
[14]Institute of Microbiology and Infection, University of Birmingham, Birmingham, UK
[15]Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil
[16]Departamento de Virologia, Fundação de Medicina Tropical Doutor Heitor Vieira Dourado, Manaus, Amazonas, Brazil
[17]Laboratório Central de Saúde Pública do Amazonas, Manaus, Amazonas, Brazil
[18]Secretaria de Vigilância em Saúde, Ministério da Saúde (SVS-MS), Brasília, Distrito Federal, Brazil
[19]Departamento de Vigilância de Doenças Transmissíveis/Secretaria de Vigilância em Saúde, Ministério da Saúde (DEVIT/SVS-MS), Brasília, Distrito Federal, Brazil
[20]Organização Pan-Americana da Saúde/Organização Mundial da Saúde (OPAS/OMS), Brasília-DF, Brazil
[21]Center for Technological Innovations, Evandro Chagas Institute, Ministry of Health, Ananindeua, Pará, Brazil
[22]These authors contributed equally
[23]Lead Contact
*Correspondence: nuno.faria@zoo.ox.ac.uk (N.R.F.), oliver.pybus@zoo.ox.ac.uk (O.G.P.), luiz.alcantara@ioc.fiocruz.br (L.C.A.)
https://doi.org/10.1016/j.celrep.2020.01.085

## SUMMARY

Zika virus (ZIKV) has caused an explosive epidemic linked to severe clinical outcomes in the Americas. As of June 2018, 4,929 ZIKV suspected infections and 46 congenital syndrome cases had been reported in Manaus, Amazonas, Brazil. Although Manaus is a key demographic hub in the Amazon region, little is known about the ZIKV epidemic there, in terms of both transmission and viral genetic diversity. Using portable virus genome sequencing, we generated 59 ZIKV genomes in Manaus. Phylogenetic analyses indicated multiple introductions of ZIKV from northeastern Brazil to Manaus. Spatial genomic analysis of virus movement among six areas in Manaus suggested that populous northern neighborhoods acted as sources of virus transmission to other neighborhoods. Our study revealed how the ZIKV epidemic was ignited and maintained within the largest urban metropolis in the Amazon. These results might contribute to improving the public health response to outbreaks in Brazil.

## INTRODUCTION

Zika virus (ZIKV) is a flavivirus with an 11 kb positive-sense RNA genome that has caused an explosive epidemic in the Americas linked to severe congenital syndromes, including microcephaly (Petersen et al., 2016). ZIKV transmission occurs via the bite of infected *Aedes aegypti* mosquitoes, although sexual and vertical transmission, as well as transmission through blood transfusion, have been also reported (Petersen et al., 2016). Since the first detection of ZIKA in northeastern Brazil in May 2015 (Zanluca

OPEN ACCESS
CellPress

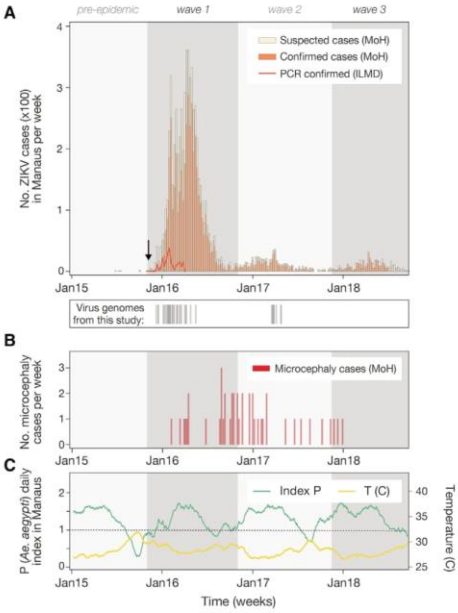



**Figure 1. Zika Virus Transmission in Manaus**

(A) ZIKV confirmed (dark orange bars) and suspected (light orange bars) cases per week in Manaus municipality notified from the Brazilian Ministry of Health (MoH), number of weekly RT-PCR-positive cases (red line) tested at Instituto Leonidas & Maria Deane (ILMD) FIOCRUZ, Amazonas, Brazil. Below, the dates of sample collection of the virus genomes generated in this study are shown using gray bars with transparency, such that darker shading reflects more dense sampling.

(B) Number of microcephaly cases per week in Manaus municipality notified to the Brazilian Ministry of Health.

(C) Daily transmission potential of *Aedes aegyti* in Manaus inferred using MVSE R-package (Obolski et al., 2019) from Manaus' climatic data. Relative humidity and temperature (degrees Celsius) were collected by an INMET weather station (for exact location, see Figure 7B).

et al., 2015; Campos et al., 2015), the country has reported nearly 1 million confirmed and suspected ZIKV infections, the greatest number among the 52 territories in the Americas that have reported ZIKV transmission (Pan American Health Organization, 2017; Zhang et al., 2017; Perkins et al., 2016). In recent years Brazil has been gripped by a wave of severe and overlapping epidemics of mosquito-borne viruses, which together have challenged passive syndromic surveillance and led to increased morbidity and disability (de Oliveira et al., 2017; Cao-Lormeau et al., 2016; Grubaugh et al., 2017). The northeastern and southeastern regions of Brazil have been severely hit by the ZIKV epidemic and account for 75% of reported ZIKV cases in Brazil between 2016 and 2018 (Secretaria de Vigilância em Saúde, Ministério da Saúde, 2018a; 2018b).

Although a smaller number of ZIKV infections have been reported in the northern region of Brazil encompassing the Amazon, several studies suggest that the region may be a location of entry for *Aedes*-borne viruses to Brazil, and increased epidemiological surveillance in the region is needed. For example, Brazilian dengue virus (DENV) and chikungunya virus (CHIKV) seem to have emerged first in the Amazon region before spreading to other, more densely populated locations (Nunes et al., 2012, 2014, 2015). The Amazon region is also home to a high diversity of mosquito-borne viruses (Vasconcelos et al., 1992), including Mayaro (Azevedo et al., 2009) and Oropouche (Azevedo et al., 2007), and an ecosystem that, under inadequate management, may facilitate the emergence and re-emergence of mosquito-borne virus epidemics (Faria et al., 2018; Vasconcelos, 2001). Moreover, climatic data suggest the possibility of year-round endemic transmission of arboviruses in the Amazon, which stands in contrast to seasonal epidemics in the southeastern, southern, and central western regions of Brazil (Messina et al., 2016; Obolski et al., 2019).

Between January 2015 and September 2018, Amazonas, the largest federal unit in Brazil, reported 4,929 Zika cases, including 46 cases of microcephaly in newborns. Most of these cases were reported in Manaus, the largest urban metropolis in the Amazon region, and cases were reported across several epidemic seasons (Secretaria de Vigilância em Saúde, Ministério da Saúde, 2019). However, the epidemic transmission and genomics of ZIKV in the Amazon region remain poorly understood. It is also unclear how and where Zika may have persisted in the Amazon region across epidemic seasons. Following our previous experience of using a mobile laboratory to investigate the genomic epidemiology of ZIKV in Brazil (Faria et al., 2016a), we used portable genome sequencing to locally generate ZIKV genomes from infected patients residing in Manaus. Samples were collected from febrile cases between December 2015 and April 2017. We use molecular epidemiology analysis to uncover the diversity and persistence of ZIKV in Manaus and its transmission in the Amazon region.

## RESULTS

### Expansion of the ZIKV Epidemic in Manaus

Up to September 2018, 6,987 ZIKV cases were reported by the main public health laboratory in Amazonas. The first PCR-confirmed case was identified in early November 2015 (Figure 1A, vertical arrow), and the first epidemic wave ("wave 1") peaked in mid-April 2016 (n = 288 cases/week) (Figure 1A). A second, smaller epidemic wave ("wave 2") peaked in early April 2017 (n = 32 cases/week) and was followed by a third epidemic wave ("wave 3") around mid-April 2018 (n = 30 cases/week). The estimated basic reproductive number of ZIKV from the first epidemic wave is $R_0 \sim 2.69$ (95% credible interval 2.32–3.11), in line with previous estimates (Faria et al., 2016b; Caminade et al., 2017).

Figure 1B shows the temporal distribution of microcephaly cases (n = 46) reported in Manaus between 2015 and 2018. To investigate the temporal association between confirmed cases of Zika and microcephaly cases in Manaus, we use a Poisson regression model that accounts for cross-correlation. We find evidence of a possible temporal association between the ZIKV

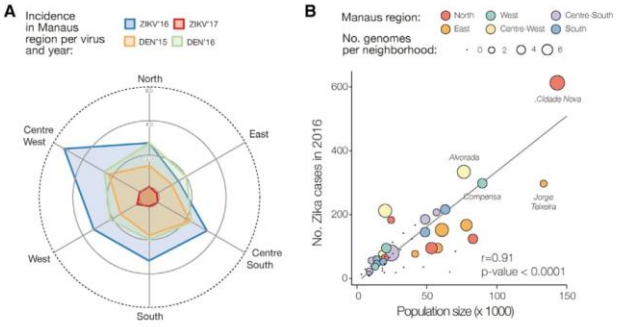



**Figure 2. Spatial Incidence of Zika Virus in Manaus**

(A) Circular plot shows ZIKV incidence (blue, 2016; red, 2017) and DENV incidence (orange, 2015; green, 2016) in the different areas of Manaus. The units for the incidence are cases per 1,000 inhabitants per year.

(B) Number of yearly ZIKV cases plotted against number of inhabitants per Manaus area (detailed data on ZIKV cases per neighborhood can be found in Table S3). The names of the four neighborhoods with the largest numbers of cases are shown. Circle sizes are proportional to the number of sequenced genomes per neighborhood, which are colored by region of Manaus.

See Table S2 for more details.

and microcephaly time series ($p < 0.001$, cross-correlation coefficient = 0.43, for December 19, 2015, to January 20, 2018). This model estimates that the microcephaly time series lags the ZIKV cases by 29 weeks in Manaus (Table S1).

To better understand the epidemic transmission of ZIKV in Manaus, we compiled climatic data from a weather station in Manaus city center and evaluated ZIKV transmission potential using the estimated suitability index *P* (Lourenço et al., 2017; Obolski et al., 2019). The estimated *P* index consistently reveals high suitability ($P > 1$) during the epidemic waves. According to the estimated *P* index, mosquitoes are able to contribute to transmission of ZIKV in Manaus throughout most of the year; that is, each year includes one or more long periods of time during which $P > 1$ (Figure 1C). The association between *P* and ZIKV cases was high (cross-correlation coefficient = 0.815, coefficient $p = 0.037$). Moreover, we estimate that ZIKV cases lag the *P* index time series by 4.7 weeks on average (Table S1).

## Highest ZIKV Incidence in the Center-West Region of Manaus

We next explored the spatial distribution of ZIKV cases within individual neighborhoods of Manaus. Neighborhood-level yearly notified cases for ZIKV (2016 and 2017) and DENV (2015 and 2016) were made available from the Brazilian Ministry of Health, and case counts were grouped into six areas of Manaus city: north, west, east, center-west, center-south, and south (Figure 2). In 2016, ZIKV incidence in Manaus was highest in the center-west area (5.3 cases per 1,000 inhabitants); within this city area, the Dom Pedro neighborhood had the highest incidence (10.5 cases per 1,000 inhabitants; Table S2). The lowest incidence was recorded in the east area of Manaus (1.5 cases per 1,000 inhabitants). Incidence in all Manaus neighborhoods in 2017 was negligible (Figure 2A; Tables S2 and S3). As expected, ZIKV case numbers and neighborhood population size were strongly positively associated ($\rho = 0.91$, $p < 0.0001$; summarized in Figure 2B and detailed in Table S2), with 24% of ZIKV cases in Manaus being reported in the most populous north area of the city. We found a moderate association between ZIKV and DENV incidence per area in 2016 ($\rho = 0.47$, $p = 0.0002$), although DENV incidence was on average 1.4-fold lower in 2016 compared with that of ZIKV, possibly because of previous circulation of DENV and therefore accumulation of herd immunity to DENV (Figure 3).

## Molecular Diagnostics and Genome Sequencing from Clinical Samples

A total of 525 samples from patients (68% woman [359 of 525]) visiting either local clinics or the main hospital in Manaus municipality between February 2014 and April 2017 were screened previously at Instituto Leonidas & Maria Deane (ILMD/FIOCRUZ) of Amazonas, the Central Laboratory of Public Health of Amazonas (LACEN-Amazonas), and the Flavivirus Laboratory at FIOCRUZ Rio de Janeiro (LABFLA/FIOCRUZ) using an in-house quantitative real time PCR assay targeting the ZIKV envelope gene region (Naveca et al., 2017). Of the tested samples, 218 (42%) tested positive for ZIKV, of which 158 (72.5%) were from female patients. For positive samples, PCR cycle threshold (Ct) values were on average 34.18 (range 15.19–41.01). We selected samples with Ct values of 38 or less for genome sequencing, resulting in 106 samples with an average Ct of 31.38 (range 15.19–38.00) (Table S4). These selected qRT-PCR-positive samples were obtained on average 3 days (range 0–14 days) after the onset of symptoms (Table S4) and were obtained from patients who resided in 40 different neighborhoods in Manaus. We used a MinION handheld nanopore sequencer to generate virus genome sequences from positive samples using our previously validated approach (Quick et al., 2017; Faria et al., 2017b). We successfully generated 59 complete and near complete genome sequences (average coverage 73%; see Figure 4; Table S5).

## Genomic History of ZIKV in the Capital City of Amazonas State

To better understand the establishment and transmission of ZIKV in Manaus, we added our newly generated consensus genome sequences to a global dataset of 423 ZIKV genomes, including recently released ZIKV genomes from Angola and Cuba (Hill et al., 2019; Grubaugh et al., 2019), and we estimated an initial maximum likelihood (ML) phylogenetic tree (Figure 5). We find that 93% (55 of 59) of the novel Manaus isolates fall within a single large well-supported monophyletic clade (bootstrap score

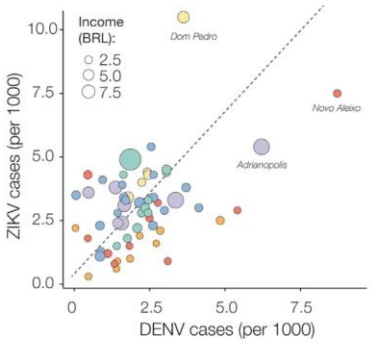



**Figure 3. Number of Yearly ZIKV Cases Plotted against Number of DENV Cases per 1,000 Inhabitants per Year per Manaus Area**

The names of the neighborhoods with largest numbers of cases are shown. Circle sizes are proportional to the number of sequenced genomes per neighborhood, which are colored by region of Manaus. Detailed data on ZIKV cases per neighborhood can be found in Table S3. See Table S2 for more details.

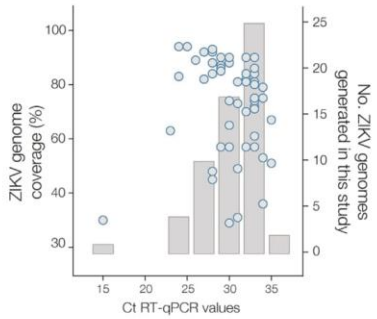



**Figure 4. Zika Virus Sequencing Statistics**

The percentage of ZIKV genome sequenced plotted against qRT-PCR Ct value for each sample (n = 59). Each circle represents a sequence recovered from an infected individual in Manaus.

[BS] = 94%) within the ZIKV American clade. This suggests that the ZIKV epidemic in Manaus was caused primarily by a single introduction, resulting in a large epidemic clade, named hereafter the Manaus clade.

We also identified four isolates from 2016 outside the main clade. Isolate AMA14, sampled in April 2016, falls basally to a clade containing six sequences from Chinese travelers infected in February 2016 in Venezuela (Sun et al., 2017) and a single sequence from the Dominican Republic. Isolate AMA59, sampled in January 2016, clusters with sequences from southeastern Brazil. A small clade containing isolates AMA53 and AMA20, sampled in January and April 2016, respectively, is closely related to a sequence from northeastern Brazil, and this resulting cluster is a sister clade to other sequences from the midwestern, southeastern, and northeastern regions of Brazil and also to three isolates from Angola (which likely derived from northeastern Brazil; Hill et al., 2019). Taken together these data suggest at least four independent introductions into Manaus. Although one isolate from Venezuela clusters together within the Manaus clade, we cannot make speculations about the transmission route between the two countries, because we do not have enough information about the epidemiological data of the sample, as well as a larger number of samples from Venezuela.

## Spatiotemporal Evolution of ZIKV in Manaus

We estimated a timescale for the evolution of the Manaus clade using the best-fitting molecular clock model. A regression of genetic divergence from root to tip against sampling dates confirmed sufficient temporal signal ($r^2$ = 0.62) (Figure 6). The evolutionary rate of the Manaus clade was calculated to be $1.09 \times 10^{-3}$ substitutions/site/year (s/s/y; 95% Bayesian credible interval [BCI] $7.7 \times 10^{-4}$ to $1.43 \times 10^{-3}$ s/s/y). This is in line with previous analyses of other ZIKV datasets from the Americas (Faria et al., 2016b, 2017b). We estimate the date of the most recent common ancestor (MRCA) of the ZIKV Manaus clade to be around January 2015 (95% BCI August 2014 to May 2015) (Figure 7A). Although this date represents a lower bound on the age of the Manaus clade, the estimated time of the MRCA of the Manaus clade coincides with a period of high ZIKV transmission potential in the city (Figure 1C).

In the Manaus clade, most of the sequences sampled from different city regions are interspersed, suggesting a highly interconnected dispersion pattern. We thus investigated the movement of ZIKV among geographic areas in Manaus using a discrete trait phylogenetic model. We find strong statistical support that the Manaus clade originated in the north area of the city (location posterior support = 0.92; Figure 7A). Our analysis identifies the north and east areas as probable source locations of ZIKV transmission in Manaus, seeding most of the virus lineage movement events within the city. The north and east are the most populated and least economically developed areas of Manaus, which suggests a possible link between ZIKV transmission and socioeconomic factors at a within-city level. ZIKV genome sequences from the center-south area were not phylogenetically clustered, indicating a lack of local virus transmission there. In contrast, six of ten strains from the west area of Manaus form a single monophyletic clade that resulted from an introduction during the peak of the epidemic in 2016 (Figures 1 and 7). These strains were isolated in April 2017, so this lineage may have circulated unnoticed for 10 months before detection.

We also estimated the contributions of different geographic areas of Manaus to the persistence of ZIKV in the city by estimating the waiting times between virus lineage movements (Markov rewards) across the phylogeny of the Manaus clade. Our results support the hypothesis that the north area of Manaus acts as the main source location (42% of the total branch duration in the time-scaled phylogeny is inferred to be located in the north area). Finally, we used our spatial analysis to infer the location in Manaus of ZIKV lineages that persisted across epidemic waves (Figure 1; see also Figure 7A). Our results suggest that ZIKV was

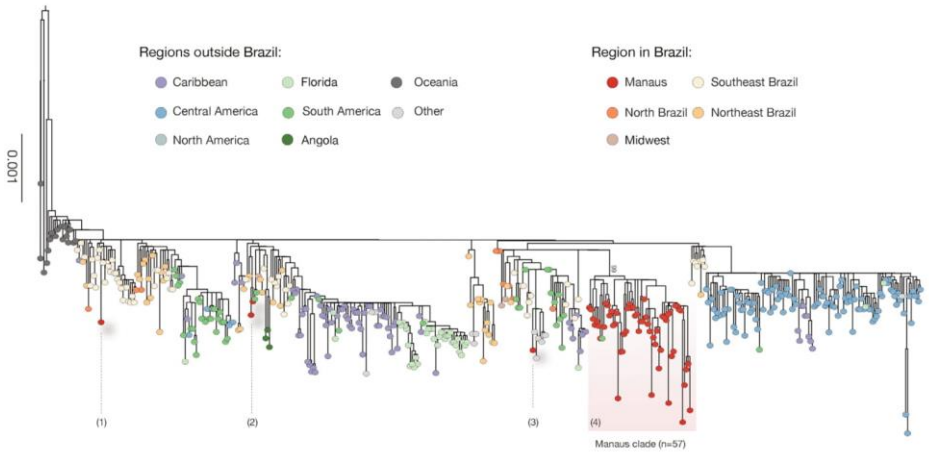



**Figure 5. Maximum Likelihood Phylogeny of Zika Virus in the Americas**

Maximum likelihood phylogeny was estimated with 482 complete or near complete genome sequences from Oceania and from the Americas. Sequences or clades from Manaus are numbered from 1 to 4, with the Manaus clade (4) being supported by a 94% bootstrap score. Colors represent different locations. Scale bar represents expected substitutions per nucleotide site.

able to persist locally across the 2015 and 2017 epidemic seasons in the north, east, south, and west areas of Manaus, which are also the four most populated areas of the city (Table S3).

## DISCUSSION

In this study we characterized disease transmission in the large ZIKV outbreak in Manaus, Amazonas, in northern Brazil, using a combination of portable genome sequencing and epidemiological analysis. We find that the ZIKV epidemic in Manaus, the largest metropolis in the Amazon region, was ignited by an introduction of a single virus lineage, most likely from northeastern Brazil, which we infer was introduced around January 2015. This was a time of high climatic suitability for arbovirus transmission. We further show that the virus persisted locally until at least April 2017. Spatial genetic analysis indicates that the virus was introduced first to the northern neighborhoods of Manaus, from which the virus lineages seeded other nearby areas.

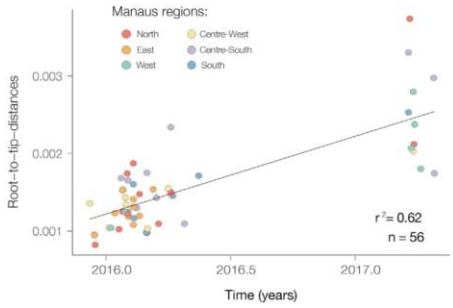



**Figure 6. Root-to-Tip Plot**

Regression of sequence sampling dates against root-to-tip genetic distances in a maximum likelihood phylogeny of the Manaus clade. Sequences are colored according to the six areas of Manaus (north, west, east, center-west, center-south, and south).

Analysis of the 59 ZIKV complete and partial genome sequences from 30 different neighborhoods in Manaus generated here provides a high-resolution contribution to our understanding of the introduction and progression of ZIKV in Brazil and to the transmission of ZIKV in tropical urban regions. Our analysis indicates that ZIKV was introduced to Manaus from the northeastern region of Brazil on at least four occasions. This agrees with our previous work that has found that northeastern Brazil played a significant role in the establishment and dissemination of ZIKV in the Americas (Faria et al., 2016b, 2017b).

Although evidence of cross-border transmissions among locations that share a tropical climate is frequent and has been observed previously in the region, for example, for DENV serotype 4 (Nunes, 2012) and CHIKV (Naveca et al., 2019), and although our results show that one isolate from Venezuela, a country with a high suitability for *Ae. aegypti* that has direct river connections to Manaus, clusters within the Manaus clade (Figure 5), we cannot exclude that some of the ZIKV introductions to Manaus were from Venezuela, therefore we cannot make speculations about the direction of the transmissions between

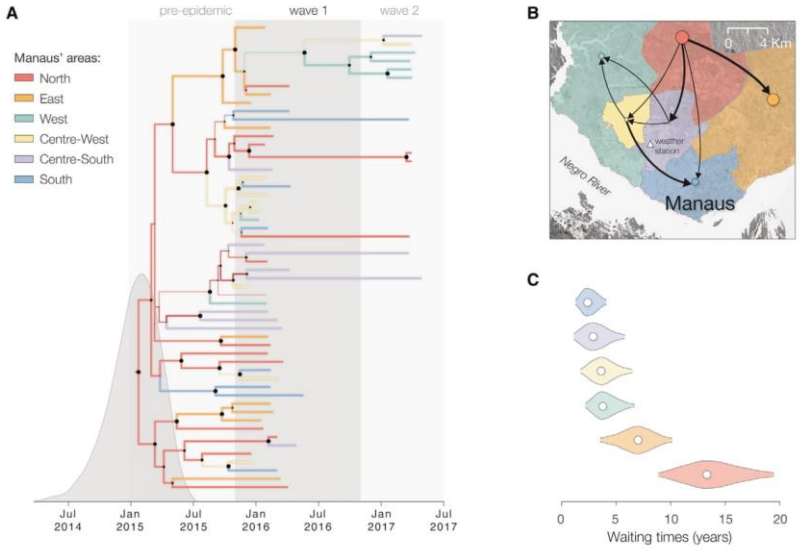



**Figure 7. Phylogeography of ZIKV within Manaus**

(A) Maximum clade credibility phylogeographic tree of the Manaus outbreak clade (n = 56). Branch colors represent most probable inferred locations. The black circles at internal nodes are sized in proportion to clade posterior probabilities. The branch thicknesses are sized in proportion to the most probable inferred locations.

(B) Map showing the inferred patterns of Zika virus transmission within areas of Manaus. Circles are proportional to the population size of each area of the city. The arrows are sized in proportion to the diffusion dispersal rate.

(C) Violin plot showing the posterior distribution of the total duration of phylogeny branches that are inferred to be located in each region of Manaus (Markov rewards). Colors represent different areas in Manaus as indicated in (A). The posterior distribution was calculated from 9,000 sampled trees.

the two countries, because of the lack of epidemiological data linked to this sample as well as a larger samples number from Venezuela.

Our within-city phylogeographic reconstruction is consistent with a gravity-like model of ZIKV dissemination, with virus transmission being driven by the most populated areas, which act as source locations, as shown previously for other infectious diseases (e.g., Kraemer et al., 2017, 2019a). The north area of Manaus has had the highest rate of population growth in recent years and has the second lowest income of all areas in the city. This suggests that demographic and socioeconomic factors have likely determined the incidence and persistence of the virus across Manaus neighborhoods (Lindoso and Lindoso, 2009; Hagan et al., 2016; Wilder-Smith et al., 2017). Our within-city phylogeographic reconstruction (Figure 7) further indicates that ZIKV transmission persisted through multiple epidemic waves in several neighborhoods.

It is important to note that phylogeographic analyses can be affected by sampling bias. In this study we compiled an updated dataset of ZIKV genome sequences dataset; comparatively few sequences from Brazil from 2017 and 2018 are available. This matches the small number of reported ZIKV cases in the country during this period, but undersampling may affect our conclusions concerning clustering with Manaus lineages after 2016. Regarding the within-city reconstructions, our sampling effort was successful in capturing ZIKV diversity in all main regions; the variation in sampling sizes obtained is approximately proportional to the number of ZIKV cases reported for each region in 2016 and 2017 (Table S3).

Epidemiological analysis of suspected ZIKV infections indicates a dominant epidemic wave of transmission in Manaus that peaked around mid-April 2016, followed by a second smaller wave in 2017. A third small epidemic peak in suspected cases can be noted around April 2018. We also find evidence that local microcephaly cases are correlated with local ZIKV cases and lag the latter by ~29 weeks. The introduction and spread of ZIKV over two or three consecutive waves in a given location has been observed previously and explained by the temporal accumulation of herd immunity (Lourenço et al., 2017; Ferguson et al., 2016). It has been reported also that the vast majority of Zika infections go unnoticed, and it is possible that the high similarity of case definitions for DENV, CHIKV, and ZIKV, which co-circulate in the Amazon region (Nunes et al., 2012, 2015; Vasconcelos et al., 1992; Naveca et al.,

2019; da Costa et al., 2018) could have resulted in a significant number of ZIKV infections being classified as either dengue or chikungunya at the beginning of the epidemic.

We find that local among-season ZIKV transmission in the Brazilian Amazon is consistent with sustained local year-round ecological suitability for *Aedes* spp., as previously predicted from climatic data alone (Bogoch et al., 2016), and also with the indication of a possible ZIKV persistence through natural vertical transmission in *Ae. aegypti* populations in Manaus (da Costa et al., 2018; Izquierdo-Suzán et al., 2019; Chaves et al., 2019), although these cases require more caution because the non-specific methodology used. Genetic and epidemiological analysis have indicated the northern region of Brazil has acted as a source region for DENV or as stepping-stone for the dissemination of arboviruses to other areas of the country (Faria et al., 2018) these trends may have been influenced by increases in human mobility and vector suitability (Kraemer et al., 2019b). Taken together, these results emphasize the ecological suitability of Manaus for the establishment of *Aedes*-borne viruses and highlight the need for continued arbovirus surveillance in Amazon urban areas.

In summary, we provide evidence for sustained local transmission of ZIKV in Manaus, Amazonas, between 2015 and 2017, and we reveal the epidemiological connections between Manaus and other locations in South America.

The spread of ZIKV in Manaus was mediated by climatic, socioeconomic, and demographic conditions, as well as by herd immunity (Lourenço et al., 2017), and our results shed light on the epidemiological dynamics of the virus urban tropical locations. Our work also provides an example of the relevance of integrating genetic and epidemiological surveillance when investigating arbovirus transmission (Kraemer et al., 2018). Ultimately such integration should aim for earlier detection of transmission of novel pathogens and for more real-time prediction of disease spread. The generation of genomic data by portable sequencing technology in local public health laboratories, as demonstrated here, can contribute substantially to these goals. Given the biodiversity of the Amazon basin, improving disease surveillance the region is crucial, both to improve public health responses and to increase our understanding of the diversity of known and unknown mosquito-borne viruses that co-circulate in the region.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Sample collection
  - Ethical statement
- METHOD DETAILS
  - Nucleic acid isolation and RT-qPCR
  - cDNA synthesis and whole genome nanopore sequencing
  - Generation of consensus sequences
  - Collation of ZIKV complete genome datasets
  - Maximum likelihood analysis and clock signal estimation
  - Dated phylogenetics
  - Phylogeographic analysis
  - Epidemiological analysis
  - Temporal association between Zika virus and microcephaly cases
  - Daily Aedes-ZIKV transmission potential (P index)
- QUANTIFICATION AND STATISTICAL ANAYLSIS
  - Maximum Likelihood Phylogenetic Analysis
  - Dated phylogenetics and Phylogeographic analysis
  - Epidemiological analysis and Temporal association between Zika virus and microcephaly cases
  - Daily Aedes-ZIKV transmission potential (P index)
- DATA AND CODE AVAILABILITY
  - Data availability

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.085.

### ACKNOWLEDGMENTS

We thank Oxford Nanopore Technologies for technical support and QIAGEN for support of the ZIBRA-2 (Zika in Brazil Real Time Analyses-Second Round) project with reagents and equipment. This work was supported by Decit/SCTIE/MoH and CNPq (440685/2016-8 and 440856/2016-7); by CAPES (88887.130716/2016-00, 88881.130825/2016-00, and 88887.130823/2016-00); by the European Union (EU) Horizon 2020 Programme through ZIKAlliance (PRES-005-FEX-17-4-2-33); by the Medical Research Council and FAPESP CADDE partnership award (MR/S0195/1); and by the Oxford Martin School. N.R.F. is supported by a Sir Henry Dale Fellowship (204311/Z/16/Z). M.U.G.K. is supported by The Branco Weiss Fellowship - Society in Science, administered by ETH Zurich, and acknowledges funding from a Google Faculty Award and a Training Grant from the National Institute of Child Health and Human Development (T32HD040128). S.D. is supported by Fonds National de la Recherche Scientifique (FNRS; Belgium). The activities of the A.M.B.F. laboratory were supported by the Faperj under the grant no. E-26/2002.930/2016, by the International Development Research Centre (IDRC) Canada under the grant 108411-001) and the EU Horizon 2020 Programme through ZikaPlan and ZikAction (grants 734584 and 734857). M.G. is supported by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

### AUTHOR CONTRIBUTIONS

Conception and Design, M.G., N.R.F., N.L., O.G.P., and L.C.A. ; Investigations, M.G., N.R.F., F.G.N., J.G.J., J.X., I.M.C., F.S.S., P.P.S., V.A.N., V.C.S., F.C.M.I., G.W., E.A.C.-M., A.F., and F.L.; Data Curation, M.G., N.R.F., J.L., M.U.G.K., V.F., S.D., J.T., O.G.P., and L.C.J.A.; Formal Analysis, M.G., N.R.F., J.L., M.U.G.K., S.D., and L.P.; Writing – Original Draft Preparation, M.G., N.R.F., J.L., M.U.G.K., F.G.N., O.G.P., and L.C.J.A.; Revision, M.G., N.R.F., J.L., M.U.G.K., F.G.N., O.G.P., T.G., M.R.T.N., T.O., and L.C.J.A.; Resources, L.N.C., M.C.C., F.G.N., T.M.T., M.S.S., A.M.B.F., A.L.A., W.K.O., J.C., C.F.C.A., and L.C.J.A.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 3, 2019
Revised: December 16, 2019
Accepted: January 24, 2020
Published: February 18, 2020

OPEN ACCESS
CellPress

## REFERENCES


Azevedo, R.S.S., Nunes, M.R.T., Chiang, J.O., Bensabath, G., Vasconcelos, H.B., Pinto, A.Y., Martins, L.C., Monteiro, H.A., Rodrigues, S.G., and Vasconcelos, P.F. (2007). Reemergence of Oropouche fever, northern Brazil. Emerg. Infect. Dis. *13*, 912–915.

Azevedo, R.S.S., Silva, E.V., Carvalho, V.L., Rodrigues, S.G., Nunes-Neto, J.P., Monteiro, H., Peixoto, V.S., Chiang, J.O., Nunes, M.R., and Vasconcelos, P.F. (2009). Mayaro fever virus, Brazilian Amazon. Emerg. Infect. Dis. *15*, 1830–1832.

Bogoch, I.I., Brady, O.J., Kraemer, M.U.G., German, M., Creatore, M.I., Kulkarni, M.A., Brownstein, J.S., Mekaru, S.R., Hay, S.I., Groot, E., et al. (2016). Anticipating the international spread of Zika virus from Brazil. Lancet *387*, 335–336.

Caminade, C., Turner, J., Metelmann, S., Hesson, J.C., Blagrove, M.S., Solomon, T., Morse, A.P., and Baylis, M. (2017). Global risk model for vector-borne transmission of Zika virus reveals the role of El Niño 2015. Proc. Natl. Acad. Sci. U S A *114*, 119–124.

Campos, G.S., Bandeira, A.C., and Sardi, S.I. (2015). Zika virus outbreak, Bahia, Brazil. Emerg. Infect. Dis. *21*, 1885–1886.

Cao-Lormeau, V.M., Blake, A., Mons, S., Lastère, S., Roche, C., Vanhomwegen, J., Dub, T., Baudouin, L., Teissier, A., Larre, P., et al. (2016). Guillain-Barré syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet *387*, 1531–1539.

Chaves, B.A., Junior, A.B.V., Silveira, K.R.D., Paz, A.D.C., Vaz, E.B.D.C., Araujo, R.G.P., Rodrigues, N.B., Campolina, T.B., Orfano, A.D.S., Nacif-Pimenta, R., et al. (2019). Vertical transmission of Zika virus (Flaviviridae, Flavivirus) in Amazonian Aedes aegypti (Diptera: Culicidae) delays egg hatching and larval development of progeny. J. Med. Entomol. *56*, 1739–1744.

da Costa, C.F., da Silva, A.V., do Nascimento, V.A., de Souza, V.C., Monteiro, D.C.D.S., Terrazas, W.C.M., Dos Passos, R.A., Nascimento, S., Lima, J.B.P., and Naveca, F.G. (2018). Evidence of vertical transmission of Zika virus in field-collected eggs of Aedes aegypti in the Brazilian Amazon. PLoS Negl. Trop. Dis. *12*, e0006594.

Darriba, D., Taboada, G.L., Doallo, R., and Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods *9*, 772–778.

de Oliveira, W.K., de França, G.V.A., Carmo, E.H., Duncan, B.B., de Souza Kuchenbecker, R., and Schmidt, M.I. (2017). Infection-related microcephaly after the 2015 and 2016 Zika virus outbreaks in Brazil: a surveillance-based analysis. Lancet *390*, 861–870.

Drummond, A.J., Ho, S.Y., Phillips, M.J., and Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. PLoS Biol. *4*, e88.

Faria, N.R., Sabino, E.C., Nunes, M.R.T., Alcantara, L.C.J., Loman, N.J., and Pybus, O.G. (2016a). Mobile real-time surveillance of Zika virus in Brazil. Genome Med. *8*, 97.

Faria, N.R., Azevedo, R.D.S.D.S., Kraemer, M.U.G., Souza, R., Cunha, M.S., Hill, S.C., Thézé, J., Bonsall, M.B., Bowden, T.A., Rissanen, I., et al. (2016b). Zika virus in the Americas: early epidemiological and genetic findings. Science *352*, 345–349.

Faria, N.R., da Costa, A.C., Lourenço, J., Loureiro, P., Lopes, M.E., Ribeiro, R., Alencar, C.S., Kraemer, M.U.G., Villabona-Arenas, C.J., Wu, C.H., et al.; NHLBI Recipient Epidemiology and Donor Evaluation Study-III (REDS-III) (2017a). Genomic and epidemiological characterisation of a dengue virus outbreak among blood donors in Brazil. Sci. Rep. *7*, 15216.

Faria, N.R., Quick, J., Claro, I.M., Thézé, J., de Jesus, J.G., Giovanetti, M., Kraemer, M.U.G., Hill, S.C., Black, A., da Costa, A.C., et al. (2017b). Establishment and cryptic transmission of Zika virus in Brazil and the Americas. Nature *546*, 406–410.

Faria, N.R., Kraemer, M.U.G., Hill, S.C., Goes de Jesus, J., Aguiar, R.S., Iani, F.C.M., Xavier, J., Quick, J., du Plessis, L., Dellicour, S., et al. (2018). Genomic and epidemiological monitoring of yellow fever virus transmission potential. Science *361*, 894–899.

Ferguson, N.M., Rodríguez-Barraquer, I., Dorigatti, I., Mier-Y-Teran-Romero, L., Laydon, D.J., and Cummings, D.A. (2016). Benefits and risks of the Sanofi-Pasteur dengue vaccine: modeling optimal deployment. Science *353*, 1033–1036.

Ferreira, M.A.R., and Suchard, M.A. (2008). Bayesian analysis of elapsed times in continuous-time Markov chains. Can. J. Stat. *78*, 355–368.

Fonseca, V., Libin, P.J.K., Theys, K., Faria, N.R., Nunes, M.R.T., Restovic, M.I., Freire, M., Giovanetti, M., Cuypers, L., Nowé, A., et al. (2019). A computational method for the identification of Dengue, Zika and Chikungunya virus species and genotypes. PLoS Negl. Trop. Dis. *213*, 72–131.

Gill, M.S., Lemey, P., Faria, N.R., Rambaut, A., Shapiro, B., and Suchard, M.A. (2013). Improving Bayesian population dynamics inference: a coalescent-based model for multiple loci. Mol. Biol. Evol. *30*, 713–724.

Grubaugh, N.D., Ladner, J.T., Kraemer, M.U.G., Dudas, G., Tan, A.L., Gangavarapu, K., Wiley, M.R., White, S., Thézé, J., Magnani, D.M., et al. (2017). Genomic epidemiology reveals multiple introductions of Zika virus into the United States. Nature *546*, 401–405.

Grubaugh, N.D., Saraf, S., Gangavarapu, K., Watts, A., Tan, A.L., Oidtman, R.J., Ladner, J.T., Oliveira, G., Matteson, N.L., Kraemer, M.U.G., et al.; GeoSentinel Surveillance Network (2019). Travel surveillance and genomics uncover a hidden Zika outbreak during the waning epidemic. Cell *178*, 1057–1071.e11.

Hagan, J.E., Moraga, P., Costa, F., Capian, N., Ribeiro, G.S., Wunder, E.A., Jr., Felzemburgh, R.D., Reis, R.B., Nery, N., Santana, F.S., et al. (2016). Spatiotemporal determinants of urban Leptospirosis Transmission: Four-Year prospective cohort study of slum residents in Brazil. PLoS Negl. Trop. Dis. *10*, e0004275.

Hasegawa, M., Kishino, H., and Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. *22*, 160–174.

Hill, S.C., Vasconcelos, J., Neto, Z., Jandondo, D., Zé-Zé, L., Aguiar, R.S., Xavier, J., Thézé, J., Mirandela, M., Micolo Cândido, A.L., et al. (2019). Emergence of the Asian lineage of Zika virus in Angola: an outbreak investigation. Lancet Infect. Dis. *19*, 1138–1147.

Izquierdo-Suzán, M., Zárate, S., Torres-Flores, J., Correa-Morales, F., González-Acosta, C., Sevilla-Reyes, E.E., Lira, R., Alcaraz-Estrada, S.L., and Yocupicio-Monroy, M. (2019). Natural vertical transmission of Zika virus in larval Aedes aegypti populations, Morelos, Mexico. Emerg. Infect. Dis. *25*, 1477–1484.

Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution *30*, 772–780.

Kraemer, M.U.G., Faria, N.R., Reiner, R.C., Jr., Golding, N., Nikolay, B., Stasse, S., Johansson, M.A., Salje, H., Faye, O., Wint, G.R.W., et al. (2017). Spread of yellow fever virus outbreak in Angola and the Democratic Republic of the Congo 2015-16: a modelling study. Lancet Infect. Dis. *17*, 330–338.

Kraemer, M.U.G., Cummings, D.A.T., Funk, S., Reiner, R.C., Faria, N.R., Pybus, O.G., and Cauchemez, S. (2018). Reconstruction and prediction of viral disease epidemics. Epidemiol. Infect. *4*, 1–7.

Kraemer, M.U.G., Golding, N., Bisanzio, D., Bhatt, S., Pigott, D.M., Ray, S.E., Brady, O.J., Brownstein, J.S., Faria, N.R., Cummings, D.A.T., et al. (2019a). Utilizing general human movement models to predict the spread of emerging infectious diseases in resource poor settings. Sci. Rep. *9*, 5151.

Kraemer, M.U.G., Reiner, R.C., Brady, O.J., Messina, J.P., Gilbert, M., Pigott, D.M., Yi, D., Johnson, K., Earl, L., and Marczak, L.B. (2019b). Past and future spread of the arbovirus vectors Aedes aegypti and Aedes albopictus. Nat. Microbiol. *4*, 854–863.

Lanciotti, R.S., Kosoy, O.L., Laven, J.J., Velez, J.O., Lambert, A.J., Johnson, A.J., Stanfield, S.M., and Duffy, M.R. (2008). Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg. Infect. Dis. *14*, 1232–1239.

Lemey, P., Rambaut, A., Drummond, A.J., and Suchard, M.A. (2009). Bayesian phylogeography finds its roots. PLoS Comput. Biol. *5*, e1000520.

Lindoso, J.A., and Lindoso, A.A. (2009). Neglected tropical diseases in Brazil. Rev. Inst. Med. Trop. Sao Paulo *51*, 247–253.

Loman, N.J., and Quinlan, A.R. (2014). Poretools: a toolkit for analyzing nanopore sequence data. Bioinformatics *30*, 3399–3401.

Lourenço, J., Maia de Lima, M., Faria, N.R., Walker, A., Kraemer, M.U., Villabona-Arenas, C.J., Lambert, B., Marques de Cerqueira, E., Pybus, O.G., Alcantara, L.C., and Recker, M. (2017). Epidemiological and ecological determinants of Zika virus transmission in an urban setting. eLife *6*, 290–82.

Messina, J.P., Kraemer, M.U., Brady, O.J., Pigott, D.M., Shearer, F.M., Weiss, D.J., Golding, N., Ruktanonchai, C.W., Gething, P.W., Cohn, E., et al. (2016). Mapping global environmental suitability for Zika virus. eLife *5*, 5–18.

Naveca, F.G., Nascimento, V.A.D., Souza, V.C., Nunes, B.T.D., Rodrigues, D.S.G., and Vasconcelos, P.F.D.C. (2017). Multiplexed reverse transcription real-time polymerase chain reaction for simultaneous detection of Mayaro, Oropouche, and Oropouche-like viruses. Mem. Inst. Oswaldo Cruz *112*, 510–513.

Naveca, F.G., Claro, I., Giovanetti, M., de Jesus, J.G., Xavier, J., Iani, F.C.M., do Nascimento, V.A., de Souza, V.C., Silveira, P.P., Lourenço, J., et al. (2019). Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon. PLoS Negl. Trop. Dis. *13*, e0007065.

Nunes, M.R., Faria, N.R., Vasconcelos, H.B., Medeiros, D.B., Silva de Lima, C.P., Carvalho, V.L., Pinto da Silva, E.V., Cardoso, J.F., Sousa, E.C., Jr., Nunes, K.N., et al. (2012). Phylogeography of dengue virus serotype 4, Brazil, 2010-2011. Emerg. Infect. Dis. *18*, 1858–1864.

Nunes, M.R., Palacios, G., Faria, N.R., Sousa, E.C., Jr., Pantoja, J.A., Rodrigues, S.G., Carvalho, V.L., Medeiros, D.B., Savji, N., Baele, G., et al. (2014). Air travel is associated with intracontinental spread of dengue virus serotypes 1-3 in Brazil. PLoS Negl. Trop. Dis. *8*, e2769.

Nunes, M., et al. (2012). Phylogeography of dengue virus serotype 4, Brazil, 2010-2011. Emerg. Infect. Dis. *18*, 1858–1864.

Nunes, M.R.T., Faria, N.R., de Vasconcelos, J.M., Golding, N., Kraemer, M.U., de Oliveira, L.F., Azevedo, Rdo.S., da Silva, D.E., da Silva, E.V., da Silva, S.P., et al. (2015). Emergence and potential for spread of chikungunya virus in Brazil. BMC Med. *13*, 102.

Obolski, U., Perez, P.N., Villabona-Arenas, C.J., Thézé, J., Faria, N.R., and Lourenço, J. (2019). MVSE: an R-package that estimates a climate-driven mosquito-borne viral suitability index. Methods Ecol. Evol. *10*, 1357–1370.

Pan American Health Organization (2017).www.paho.org.

Perez-Guzman, P.N., Carlos Junior Alcantara, L., Obolski, U., de Lima, M.M., Ashley, E.A., Smithuis, F., Horby, P., Maude, R.J., Lin, Z., Kyaw, A.M.M., and Lourenço, J. (2018). Measuring mosquito-borne viral suitability in Myanmar and implications for local Zika virus transmission. PLoS Curr. *10*, 16–37.

Perkins, T.A., Siraj, A.S., Ruktanonchai, C.W., Kraemer, M.U., and Tatem, A.J. (2016). Model-based projections of Zika virus infections in childbearing women in the Americas. Nat. Microbiol. *1*, 16126.

Petersen, L.R., Jamieson, D.J., and Honein, M.A. (2016). Zika Virus. N. Engl. J. Med. *375*, 294–295.

Quick, J., Grubaugh, N.D., Pullan, S.T., Claro, I.M., Smith, A.D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T.F., Beutler, N.A., et al. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat. Protoc. *12*, 1261–1276.

Rambaut, A., Lam, T.T., Max Carvalho, L., and Pybus, O.G. (2016). Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol. *2*, vew007.

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., and Suchard, M.A. (2018). Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. Syst. Biol. *67*, 901–904.

Secretaria de Vigilância em Saúde, Ministério da Saúde (2018a). Monitoramento dos casos de dengue, febre de chikungunya e doença aguda pelo vírus Zika até a semana epidemiológica 45 de 2018. Boletim Epidemiológico *49* (*53*). http://portalarquivos2.saude.gov.br/images/pdf/2018/novembro/29/BE-2018-58-SE-45.pdf.

Secretaria de Vigilância em Saúde, Ministério da Saúde (2018b). Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a semana epidemiológica 52, 2017. Boletim Epidemiológico *49* (*2*). http://portalarquivos2.saude.gov.br/images/pdf/2018/janeiro/23/Boletim-2018-001-Dengue.pdf.

Secretaria de Vigilância em Saúde, Ministério da Saúde (2019). Síndrome congênita associada à infecção pelo vírus Zika: situação epidemiológica, ações desenvolvidas e desafios de 2015 a 2019. Boletim Epidemiológico. http://portalarquivos2.saude.gov.br/images/pdf/2019/dezembro/05/be-sindrome-congenita-vfinal.pdf.

Shapiro, B., Rambaut, A., and Drummond, A.J. (2006). Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol. Biol. Evol. *23*, 7–9.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics *30*, 1312–1313.

Suchard, M.A., Lemey, P., Baele, G., Ayres, D.L., Drummond, A.J., and Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol. *4*, vey016.

Sun, J., Wu, D., Zhong, H., Guan, D., Zhang, H., Tan, Q., Zhou, H., Zhang, M., Ning, D., Zhang, B., et al. (2017). Returning ex-patriot Chinese to Guangdong, China, increase the risk for local transmission of Zika virus. J. Infect. *75*, 356–367.

Thézé, J., Li, T., du Plessis, L., Bouquet, J., Kraemer, M.U.G., Somasekar, S., Yu, G., de Cesare, M., Balmaseda, A., Kuan, G., et al. (2018). Genomic epidemiology reconstructs the introduction and spread of Zika virus in Central America and Mexico. Cell Host Microbe *23*, 855–864.e7.

Vasconcelos, L. (2001). New forums out of sustainability—recent trends at local level. Proceedings of the First World Planning Congress – ACSP-AESOP-APSA-ANZAPS *32*, 11–15.

Vasconcelos, P.F.C., Travassos Da Rosa, A.P.A., Dégallier, N., Travassos Da Rosa, J.F.S., and Pinheiro, F.P. (1992). Clinical and ecoepidemiological situation of human arboviruses in Brazilian Amazonia. J. Brazil. Assoc. Adv. Sc. *44*, 117–124.

Wickham, H. (2016). In: Elegant Graphics for Data Analysis (New York: Springer-Verlag).

Wilder-Smith, A., Gubler, D.J., Weaver, S.C., Monath, T.P., Heymann, D.L., and Scott, T.W. (2017). Epidemic arboviral diseases: priorities for research and public health. Lancet Infect. Dis. *17*, e101–e106.

Yu, G., Tsan-Yuk Lam, T., Zhu, H., and Guan, Y. (2018). Two Methods for Mapping and Visualizing Associated Data on Phylogeny Using Ggtree. Biology and Evolution *35*, 3041–3043.

Zanluca, C., Melo, V.C., Mosimann, A.L., Santos, G.I., Santos, C.N., and Luz, K. (2015). First report of autochthonous transmission of Zika virus in Brazil. Mem. Inst. Oswaldo Cruz *110*, 569–572.

Zhang, Q., Sun, K., Chinazzi, M., Pastore Y Piontti, A., Dean, N.E., Rojas, D.P., Merler, S., Mistry, D., Poletti, P., Rossi, L., et al. (2017). Spread of Zika virus in the Americas. Proc. Natl. Acad. Sci. U S A *114*, E4334–E4343.

OPEN ACCESS CellPress

## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Virus strains | | |
| Zika Virus strains from Amazon | This Study | N/A |
| Biological Samples | | |
| Serum, urine, cerebrospinal fluid samples from patients visiting either local clinics or the main hospital in Manaus municipality of Amazonas state | Amazonas State<br>- Instituto Leonidas & Maria Deane (ILMD/FIOCRUZ) of Amazonas<br>- The Central Laboratory of Public Health of Amazonas (LACEN-Amazonas)<br>- The Flavivirus Laboratory at FIOCRUZ Rio de Janeiro (LABFLA/FIOCRUZ) | N/A |
| Critical Commercial Assays | | |
| QIAamp Viral RNA Mini Kit | QIAGEN | Cat # 204443 |
| TaqMan Fast Virus 1-Step Master Mix | Thermo-Fisher Scientific | Cat # 4444436 |
| ProtoScript® II First Strand cDNA Synthesis Kit | New England Biolabs | Cat # E6560L |
| Q5 High-Fidelity DNA polymerase | New England Biolabs | Cat # M0491L |
| Agencourt AMPure XP | Beckman Coulter | Cat # A63880 |
| Qubit dsDNA HS Assay Kit | QIAGEN | Cat # Q32851 |
| CDC monoplex assay | Lanciotti et al., 2008 | N/A |
| Native Barcoding Expansion 1-12 (PCR-free) | Oxford Nanopore Technologies | Cat # EXP-NBD104 |
| DNA Sequencing Kit SQK-MAP007/ SQK-LSK108 | Oxford Nanopore Technologies | Cat # SQK-LSK108 |
| R9.4 flowcell | Oxford Nanopore Technologies | Cat # FLO-MIN106 |
| Deposited Data | | |
| 59 Zika virus sequences 59 from Manaus, Amazonas State, Brazil have been deposited in the National Center for Biotechnology Information (NCBI) GenBank | This Study | National Center for Biotechnology Information (NCBI) GenBank: MK216687-MK216688; MK216690-MK216738; MK216740-MK216745; MK216747- MK216748. |
| 423 publicly available Zika virus sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank | N/A | National Center for Biotechnology Information (NCBI) GenBank: MK829154, MK829153, MK829152, MH544701, MH513600, MH513599, MH513598, MH157213, MH157208, MH157202, MH063265, MH063264, MH063263, MH063262, MH063261, MH063260, MH063259, MF783073, MF783072, MF073359, MF073358, MF073357, MG595216, MG494697, MF988743, KY441403, KY441402, KY441401, MF438286, MF384325, MF167360, MF159531, MF801426, MF801425, MF801424, KY606273, MF801423, MF801422, MF801421, MF801420, MF801419, MF801418, MF801417, KY606274, MF801416, MF801415, MF801414, MF801413, MF801412, MF801411, MF801410, |

(Continued on next page)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| | | MF801409, MF801408, MF801407, MF801406, MF801405, MF801404, |
| | | MF801403, MF801402, MF801401, MF801400, MF801399, MF801398, |
| | | MF801397, MF801396, KY606272, MF801395, MF801394, KY606271, |
| | | MF801393, MF801392, MF801391, MF801390, MF801389, MF801388, |
| | | MF801387, MF801386, MF801385, MF801384, MF801383, MF801382, |
| | | MF801381, MF801380, MF801379, MF801378, MF801377, MF434522, |
| | | MF434521, MF434520, MF434519, MF434518, MF434517, MF434516, |
| | | KX446950, KX446951, KX856011, KU870645, KY785416, KY014319, |
| | | KY785471, KY014310, KY014311, KY014312, KY785418, KY785414, |
| | | KY785444, KY785461, KY785442, KY014315, KY785452, KY014327, |
| | | KY014306, KY785448, KY785458, KY785431, KY785421, KX421195, |
| | | MF098770, MF098771, KY785454, KU501216, KU501217, KX262887, |
| | | KX766029, KY120349, KY325465, KY328289, KY631493, KY693676, |
| | | KY693677, KY765317, KY765318, KY765323, KY765324, KY765325, |
| | | KY927808, KX520666, KX830930, KY785437, KY785436, KY014317, |
| | | KY785446, KY785451, KY014320, KY014308, KY785410, KY014307, |
| | | KY014309, KY785411, KY785427, KY785479, KY785480, KY785467, |
| | | KY785425, KY785426, KY785409, KY014305, KY785469, KY014313, |
| | | KY785423, KY785449, KY785463, KY785455, KY785417, KY785475, |
| | | KY785465, KY785483, KY785433, KY785439, KY014303, KY014321, |
| | | KY785415, KY014297, KY785450, KY014301, KY785429, KY785456, |
| | | KY014296, KY014304, KY785420, KY785476, KY014300, KY014302, |
| | | KY014318, KY785466, KY785485, KY785477, KY785441, KY785419, |
| | | KY785428, KY014314, KY014295, KY785445, KY785412, KY785443, |
| | | KY785457, KY014325, KY785440, KY785474, KY014298, KY014326, |
| | | KY014316, KY014324, KY014322, KY014323, KX247646, KY014299, |

(*Continued on next page*)

OPEN ACCESS CellPress

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| | | KY785422, KY785472, KY785468, KY272991, KX156775, KX156774, |
| | | KX156776, KU926309, MF098764, MF098765, MF098766, MF098769, |
| | | MF098768, KX702400, KU926310, MF098767, KY785453, KY785478, |
| | | KY785484, KY785473, KY785470, KY785460, KY785435, KY785434, |
| | | KY785447, KY785413, KY785482, KY785424, KY785438, KY785430, |
| | | KY785432, KY785481, KY785464, KY785462, KY785459, KX101065, |
| | | KX101067, KX101064, KU940224, KU940227, KU940228, KX101063, |
| | | KX101062, KX101061, KX101060, KX101066, KU312312, KU312313, |
| | | KU312314, KU312315, KU501215, KU509998, KU646827, KU646828, |
| | | KU647676, KU527068, KU707826, KU497555, KU729218, KU729217, |
| | | KX197192, KU365780, KU365779, KU365778, KU365777, KX280026, |
| | | KU321639, KU740184, KU758868, KU758869, KU758870, KU758871, |
| | | KU758872, KU758873, KU758874, KU758875, KU758876, KU758877, |
| | | KU761564, KU820897, KU820898, KU853012, KU922960, KU937936, |
| | | KU955590, KU991811, KX051563, KX056898, KX087101, KX087102, |
| | | KX197205, KX198135, KX212103, KX269878, KX377337, KX548902, |
| | | KX601168, KX673530, KX766028, KX811222, KX879603, KX879604, |
| | | KY003153, KY003154, KY003155, KY003156, KY003157, KY014328, |
| | | KY014329, KY317936, KY317937, KY317938, KY317939, KY317940, |
| | | KY348640, KY558989, KY558990, KY558991, KY558992, KY558993, |
| | | KY558994, KY558995, KY558996, KY558997, KY558998, KY558999, |
| | | KY559000, KY559001, KY559002, KY559003, KY559004, KY559005, |
| | | KY559006, KY559007, KY559008, KY559009, KY559010, KY559011, |
| | | KY559012, KY559013, KY559014, KY559015, KY559016, KY559017, |
| | | KY559018, KY559019, KY559020, KY559021, KY559022, KY559023, |
| | | KY559024, KY559025, KY559026, KY559027, KY559028, KY559029, |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| | | KY559030, KY559031, KY559032, KY631492, KY693678, KY693679, |
| | | KY693680, KY817930, KY989971, KX447521, KX447520, KX447519, |
| | | KX447518, KX447517, KX447516, KX447515, KX447514, KX447513, |
| | | KX447512, KX447511, KX447510, KX447509, KX369547, KJ776791, |
| | | KU681081, KU681082, JN860885, EU545988, KY075932, KX922706, |
| | | KX922707, KX832731, KX838904, KX838905, KX838906, KX922708, |
| | | KY075937, KY075938, KY075939, KY075933, KY317936, KY317937, |
| | | KY317939, KY317940, KY317938, MF159531, MH063260, MH063261, |
| | | MH063262, MH063263, KX842449, MH063264, MH063265, FL257|/H, |
| | | KY075934, KX922703, KY075935, KX922704, KX922705, KY075936. |
| Oligonucleotides | | |
| ZIKV 1086 5′-CCGCTGCCCAACACAAG-3′ | Lanciotti et al., 2008 | N/A |
| ZIKV 1162c 5′-CCACTAACGTTCTTTTGCAGACAT-3′ | Lanciotti et al., 2008 | N/A |
| ZIKV 1107-FAM 5′-AGCCTACCTTGACAAGCAGTCA GACACTCAA /3IABkFQ −3′ | Lanciotti et al., 2008 | N/A |
| Software and Algorithms | | |
| BEAST | Suchard et al., 2018 | http://beast.community |
| jModelTest2 | Darriba et al., 2012 | https://github.com/ddarriba/jmodeltest2 |
| MAFFT | Katoh and Standley, 2013 | https://mafft.cbrc.jp/alignment/server/ |
| RAxML v8 | Stamatakis, 2014 | https://github.com/stamatak/standard-RAxML |
| Zika Virus Typing tool | Fonseca et al., 2019 | http://www.krisp.org.za/tools.php |
| PrimalScheme | Quick et al., 2017 | http://primal.zibraproject.org |
| QGIS | QGIS Development Team | https://qgis.org/en/site/ |
| R Statistical Computing Software | The R Foundation | https://www.r-project.org/ |
| R-package bdskytools | N/A | https://github.com/laduplessis/bdskytools |
| R-package ggplot2 | Wickham, 2016 | https://ggplot2.tidyverse.org |
| R-package ggtree | Yu et al., 2018 | https://github.com/YuLab-SMU/ggtree |
| TempEst | Rambaut et al., 2016 | http://beast.community/tempest |
| Tracer | Rambaut et al., 2018 | http://beast.community/tracer |
| Albacore | Loman and Quinlan, 2014 | https://github.com/nanoporetech |
| Nanopolish | Loman and Quinlan, 2014 | https://github.com/jts/nanopolish |
| Porechop | Loman and Quinlan, 2014 | https://github.com/rrwick/Porechop |
| Other | | |
| Alignment used in phylogenetic analyses, including 196 publicly available Zika virus sequences and 59 Zika virus sequences generated in this study | This Study | N/A |

## LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for laboratory resources and reagents should be directed to and will be fulfilled by the corresponding author, Luiz Carlos Junior Alcantara (luiz.alcantara@ioc.fiocruz.br). Requests for computational resources and files should be

OPEN ACCESS
CellPress

directed to and will be fulfilled by the corresponding authors, Oliver G. Pybus (oliver.pybus@zoo.ox.ac.uk) and Nuno Rodrigues Faria (nuno.faria@zoo.ox.ac.uk). This study did not generate new unique reagents.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Sample collection

Samples (serum, urine, cerebrospinal fluid) from patients visiting either local clinics or the main hospital in Manaus municipality of Amazonas state were collected for molecular diagnostics and sent for testing at IMLD/FIOCRUZ, LACEN-Amazonas and LABFLA/FIOCRUZ. Sampled individuals that were subjected to molecular diagnostics presented exanthema accompanied by two or more of the following symptoms: fever, headache, conjunctivitis, arthralgia, myalgia, and edema. The majority of samples were linked to a digital record that collated epidemiological and clinical data such as date of sample collection, municipality of residence, neighborhood of residence, demographic characteristics (age and sex) and date of onset of clinical symptoms (Tables S4 and S5).

### Ethical statement

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). The diagnostic of ZIKV infection at ILMD was approved by the Ethics Committee of the State University of Amazonas (CAAE: 56.745.116.6.0000.5016).

## METHOD DETAILS

### Nucleic acid isolation and RT-qPCR

Most of the Zika-suspected clinical samples were screened for ZIKV RNA from serum (86%), urine (3.5%) and cerebrospinal fluid (CSF) (11%). Samples were obtained from 0 to 31 days after the onset of symptoms. Viral RNA was isolated from 140 μl samples using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. An internal positive control, the *Escherichia coli* bacteriophage MS2 (ATCC 15597-B1), was used during the RNA extraction as previously describe (Naveca et al., 2017). Cycle threshold (Ct) values were determined for all samples by probe-based reverse transcription quantitative real-time PCR (RT-qPCR) against the envelope (ENV) gene target for ZIKV detection (using 5′ FAM as the probe reporter dye) (Lanciotti et al., 2008) using the following primers 5′-CCGCTGCCCAACACAAG-3′ (forward) 5′-CCACTAACGTTCTTTTGCAGACAT. 3′ (reverse) and probe 5′- 6-FAM-AGCCTACCT/ZEN/TGACAAGCAGTCAGACACTCAA /3IABkFQ. RT-qPCR assays were performed with TaqMan Fast Virus 1-Step Master Mix in a reaction of 10 μL using a final concentration of 0.3 μM for primers and 0.1 μM for probe in a StepOnePlus Real-Time PCR System (Applied Biosystems) installed at the Real-Time PCR Platform of ILMD-FIOCRUZ.

### cDNA synthesis and whole genome nanopore sequencing

DNA amplification and sequencing were attempted on the 106 selected RT-PCR positive samples that exhibited Ct-values < 38, in order to increase the genome coverage of clinical samples by nanopore sequencing (Quick et al., 2017). Extracted RNA was converted to cDNA using the Protoscript II First Strand cDNA synthesis Kit (New England Biolabs, Hitchin, UK) and random hexamer priming. Whole-genome amplification by multiplex PCR was attempted using the previously published Zika Asian primer scheme and 45 cycles of PCR using Q5 High-Fidelity DNA polymerase (NEB) as previously described (Quick et al., 2017). PCR products were cleaned-up using AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and quantified using fluorimetry with the Qubit dsDNA High Sensitivity assay on the Qubit 3.0 instrument (Life Technologies). PCR products for samples yielding sufficient material (more than 4ng/μL as determined using Qubit) were barcoded and pooled in an equimolar fashion using the Native Barcoding Kit (Oxford Nanopore Technologies, Oxford, UK). Sequencing libraries were generated from the barcoded products using the Genomic DNA Sequencing Kit SQK-MAP007/SQK-LSK208 (Oxford Nanopore Technologies) and were loaded onto a R9.4 flowcell. All sequencing was performed at ILMD-FIOCRUZ.

### Generation of consensus sequences

Consensus sequences for each barcoded sample were generated following a previously published approach (Quick et al., 2017). Briefly, raw files were basecalled using Albacore (Loman and Quinlan, 2014), demultiplexed and trimmed using Porechop. Nanopolish variant calling was applied to the assembly to detect single nucleotide variants to the reference ZIKV genome (KJ776791). Only positions with ≥ 20x genome coverage were used to produce consensus alleles. Regions with lower coverage, and those in primer-binding regions were masked with N characters.

### Collation of ZIKV complete genome datasets

Two complete or near-complete ZIKV genome datasets were generated. Dataset 1 (n = 482) comprised the data reported in this study (n = 59) plus a larger and updated dataset including recently released data from the ZIKV epidemic in Angola and Cuba (Hill et al., 2019; Grubaugh et al., 2019). Subsequently, to investigate the dynamic of the ZIKV infection within Manaus, genetic analyses

were conducted on a smaller dataset including only sequences pertaining to the largest clade of virus strains circulating in Manaus (n = 56).

### Maximum likelihood analysis and clock signal estimation

Maximum likelihood (ML) trees were estimated using RAxML v8 (Stamatakis, 2014) under an HKY nucleotide substitution model (Hasegawa et al., 1985), with a gamma distribution of among site rate variation (HKY + G + I) as selected by jModeltest.v.2 (Darriba et al., 2012). Statistical robustness of tree topology was inspected using 1000 bootstrap replicates; a bootstrap value > 80% was considered notable. To estimate temporal signal in each dataset, sample collection dates were regressed against root-to-tip genetic distances obtained from the ML phylogenies using TempEst (Rambaut et al., 2016). When precise sampling dates were not available, a precision of 1 month or 1 year in the collection dates was considered.

### Dated phylogenetics

To estimate time-calibrated phylogenies dated from time-stamped genome data, we conducted phylogenetic analysis using the Bayesian software package BEASTv.1.10.2 (Suchard et al., 2018). As previously (Thézé et al., 2018), we used the HKY nucleotide substitution model with codon partitions (Shapiro et al., 2006) and Bayesian Skygrid tree prior (Gill et al., 2013) with an uncorrelated relaxed clock with a lognormal distribution (Drummond et al., 2006). Analyses were run in duplicate in BEASTv.1.10.2 (Suchard et al., 2018) for 50 million MCMC steps, sampling parameters and trees every 5000$^{th}$ step. A non-informative continuous time Markov chain reference prior on the molecular clock rate was used (Ferreira and Suchard, 2008). Convergence of MCMC chains was checked using Tracer v.1.7.1 (Rambaut et al., 2018). Maximum clade credibility trees were summarized using TreeAnnotator after discarding 10% as burn-in.

### Phylogeographic analysis

We investigated the dynamics of ZIKV infection and virus lineage movements in Manaus using a sampled set of time-scaled phylogenies and the sampling location (area in Manaus) of each geo-referenced ZIKV sequence, as shown in Table S6. We discretised sequence sampling locations by considering 6 distinct geographic areas of the Manaus city: north (n = 13), east (n = 9), south (n = 8), west (n = 6), central-west (n = 10), and center-south (n = 10), as shown in Figure 7. Phylogeographic reconstructions were conducted using the asymmetric discrete trait model implemented in BEASTv1.10.2 (Lemey et al., 2009). As part of the flexible discrete trait phylogeographic approach implemented in BEASTv1.10.2, we also estimated posterior expectations both the number of transitions among areas (Markov jumps) and the waiting times between transitions (Markov rewards) (Gill et al., 2013). Maximum clade credibility trees were summarized using TreeAnnotator after discarding 10% as burn-in. While the sampling is relatively homogeneous among sampled locations, the phylogeographic reconstruction will remain sampling dependent. For example, sampling effort could impact on the estimated transition frequencies among locations. However, with careful interpretation, phylogeographic analysis can provide valuable information about dispersal dynamics, including information about linkages that would not be evident without genomic data.

### Epidemiological analysis

Number of weekly Zika virus cases in the municipality of Manaus were obtained from the Brazilian Ministry of Health. Cases were defined as suspected ZIKV infection when patients presented maculopapular rash and at least two of the following symptoms: fever, conjunctivitis, polyarthralgia or periarticular edema. Details and limitations of Zika virus surveillance approach based on notified or suspected cases have been described in more detail elsewhere (Faria et al., 2017b). The epidemic basic reproductive number, $R_0$, was estimated as previously described (Faria et al., 2017a). In brief, we fit a simple exponential growth rate model to weekly case counts from the first epidemic wave in Manaus. The period of exponential growth was selected, and a linear model was fitted to estimate the weekly exponential growth rate ($r$). We then derived reproductive number $R_0$ from $r$ and a probability density distribution of the epidemic generation time. We assume a gamma-distribution function for the generation time with a mean of 20 days and a standard deviation of 7.4 days. We also explored other scenarios with generation time of 10 days.

### Temporal association between Zika virus and microcephaly cases

The number of weekly microcephaly cases in the municipality of Manaus were obtained from the Brazilian Ministry of Health and are available. Zika virus and microcephaly case counts (n = 46) were compared using a Poisson regression model with Akaike Information Criteria to find the best-fitting time-lagged model. In this case, p value is the explanatory power of the Zika confirmed for microcephaly case counts to indicate the evidence for their association. Coefficients, cross-correlations and time-lags (in epidemiological weeks) for each comparison can be found in Table S1.

### Daily Aedes-ZIKV transmission potential (P index)

Estimation of mosquito-borne virus suitability (P index) was calculated using a climate-driven method as previously described in (Obolski et al., 2019). The index P measures the reproductive (transmission) potential of an adult female mosquito for a given point in time. Manaus' average daily temperature and relative humidity (%) between 01/01/2014 to 21/01/2019 were obtained from the Instituto Nacional de Metereologia (INMET) weather station number 82331 (latitude: −3.11, longitude: −59.95). Climate data was

OPEN ACCESS
CellPress

downloaded from INMET's website (http://www.inmet.gov.br/portal/). Moreover, for a comparison between the suitability index P and Zika confirmed cases, we considered Zika non-negative counts as continuous and applied a log(x+1) transformation. We focus on the epidemic season 14th November 2015 to 10th August 2016. An autoregressive integrated moving average (ARIMA) model was used to account for any residual autocorrelation (P). In this case, the p value reflects the explanatory power of suitability for Zika virus confirmed cases. We note that by using the index P as a proxy for transmission potential using climate data from a single weather station in Manaus, we do not take into account the possible effects of microclimate across the city. Although having been demonstrated to be highly correlated with mosquito-borne incidence in other cities of Brazil and elsewhere (Obolski et al., 2019; Perez-Guzman et al., 2018), the index P is not informed by factors that may play a role in transmission potential in Manaus, such as abundance of vegetation and human density or mobility.

## QUANTIFICATION AND STATISTICAL ANAYLSIS

### Maximum Likelihood Phylogenetic Analysis

To assess the suitability of substitution models for our ZIKV alignment we performed a statistical model selection procedure based on the Akaike information criterion, using jModelTest2 (Darriba et al., 2012). This identified the best fitting substitution model (HKY + G + I) for ML phylogenetic analysis. A phylogenetic bootstrap analysis with 100 replicates using RAxML v8 (Stamatakis, 2014) was conducted to evaluate the statistical support for nodes of the ML phylogeny.

### Dated phylogenetics and Phylogeographic analysis

To assess whether our data was suitable for a molecular clock phylogenetic analysis, we evaluated the temporal evolutionary signal in our ZIKV alignment using the statistical approaches in TempEst (Rambaut et al., 2016). A linear regression between sample collection dates and root-to-tip genetic distances obtained from the ML phylogeny indicated that the feasibility of a molecular clock approach. A Bayesian MCMC approach implemented in BEAST v1.10.2 (Suchard et al., 2018) was used to infer molecular clock.

### Epidemiological analysis and Temporal association between Zika virus and microcephaly cases

A linear regression model developed and described in Faria et al., (2017a), was used to assess the correlation between Zika virus and microcephaly cases, for each each distinct Manaus's neighborhoods.

### Daily Aedes-ZIKV transmission potential (P index)

Estimation of mosquito-borne virus suitability (P index) was calculated using a climate-driven method as previously described in (Obolski et al., 2019). An autoregressive integrated moving average (ARIMA) model was used to account for any residual autocorrelation (P). This model measures the reproductive (transmission) potential of an adult female mosquito for a given point in time and explains the variation in the ZIKV transmission potential.

## DATA AND CODE AVAILABILITY

### Data availability

New sequences have been deposited in GenBank under accession numbers MK216687-MK216688; MK216690-MK216738; MK216740-MK216745; MK216747- MK216748.

## 3.3 Artigo 3: “Genomic and epidemiological monitoring of yellow fever virus transmission potential”

A epidemia de YFV no Brasil no período de 2016 a 2018 foi a maior em décadas. A recente descoberta de YFV em mosquitos *Aedes* destaca a necessidade de monitorar o risco de reestabelecimento da transmissão urbana desse vírus nas Américas. Utilizando um conjunto de abordagens epidemiológicas, espaciais e genômicas para caracterizar a transmissão de YFV, nós demonstramos que a distribuição de idade e sexo dos casos humanos é característica de transmissão silvestre. A análise de casos combinados com genomas gerados localmente revelou uma fase inicial da transmissão silvestre e expansão espacial em direção a áreas anteriormente livre do vírus, seguido por um aumento no espalhamento viral para humanos no final de 2016. Nossos resultados estabeleceram uma estrutura para monitorar a transmissão de YFV em tempo real, que certamente contribuirá para o controle de futuras epidemias.

RESEARCH ARTICLE

YELLOW FEVER

# Genomic and epidemiological monitoring of yellow fever virus transmission potential

N. R. Faria[1*†], M. U. G. Kraemer[1,2,3*], S. C. Hill[1*], J. Goes de Jesus[4*], R. S. Aguiar[5*], F. C. M. Iani[6,7*], J. Xavier[4], J. Quick[8], L. du Plessis[1], S. Dellicour[9], J. Thézé[1], R. D. O. Carvalho[7], G. Baele[9], C.-H. Wu[10], P. P. Silveira[5], M. B. Arruda[5], M. A. Pereira[6], G. C. Pereira[6], J. Lourenço[1], U. Obolski[1], L. Abade[1,11], T. I. Vasylyeva[1], M. Giovanetti[4,7], D. Yi[12], D. J. Weiss[13], G. R. W. Wint[1], F. M. Shearer[13], S. Funk[14], B. Nikolay[15,16], V. Fonseca[7,17], T. E. R. Adelino[6], M. A. A. Oliveira[6], M. V. F. Silva[6], L. Sacchetto[7], P. O. Figueiredo[7], I. M. Rezende[7], E. M. Mello[7], R. F. C. Said[18], D. A. Santos[18], M. L. Ferraz[18], M. G. Brito[18], L. F. Santana[18], M. T. Menezes[5], R. M. Brindeiro[5], A. Tanuri[5], F. C. P. dos Santos[19], M. S. Cunha[19], J. S. Nogueira[19], I. M. Rocco[19], A. C. da Costa[20], S. C. V. Komninakis[21,22], V. Azevedo[7], A. O. Chieppe[23], E. S. M. Araujo[4], M. C. L. Mendonça[4], C. C. dos Santos[4], C. D. dos Santos[4], A. M. Mares-Guia[4], R. M. R. Nogueira[4], P. C. Sequeira[4], R. G. Abreu[24], M. H. O. Garcia[24], A. L. Abreu[25], O. Okumoto[25], E. G. Kroon[7], C. F. C. de Albuquerque[26], K. Lewandowski[27], S. T. Pullan[27], M. Carroll[28], T. de Oliveira[4,17,29], E. C. Sabino[20], R. P. Souza[19], M. A. Suchard[30,31], P. Lemey[9], G. S. Trindade[7], B. P. Drumond[7], A. M. B. Filippis[4], N. J. Loman[8], S. Cauchemez[15,16*], L. C. J. Alcantara[4,7*†], O. G. Pybus[1*†]

**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

[1]Department of Zoology, University of Oxford, Oxford, UK. [2]Computational Epidemiology Lab, Boston Children's Hospital, Boston, MA, USA. [3]Department of Pediatrics, Harvard Medical School, Boston, MA, USA. [4]Laboratório de Flavivírus, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil. [5]Laboratório de Virologia Molecular, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. [6]Laboratório Central de Saúde Pública, Instituto Octávio Magalhães, FUNED, Belo Horizonte, Minas Gerais, Brazil. [7]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [8]Institute of Microbiology and Infection, University of Birmingham, Birmingham, UK. [9]Department of Microbiology and Immunology, Rega Institute, KU Leuven, Leuven, Belgium. [10]Department of Statistics, University of Oxford, Oxford, UK. [11]The Global Health Network, University of Oxford, Oxford, UK. [12]Department of Statistics, Harvard University, Cambridge, MA, USA. [13]Malaria Atlas Project, Big Data Institute, Nuffield Department of Medicine, University of Oxford, Oxford, UK. [14]Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK. [15]Mathematical Modelling of Infectious Diseases and Center of Bioinformatics, Institut Pasteur, Paris, France. [16]CNRS UMR2000: Génomique Évolutive, Modélisation et Santé, Institut Pasteur, Paris, France. [17]KwaZulu-Natal Research, Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa. [18]Secretaria de Estado de Saúde de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [19]Núcleo de Doenças de Transmissão Vetorial, Instituto Adolfo Lutz, São Paulo, Brazil. [20]Instituto de Medicina Tropical e Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil. [21]Retrovirology Laboratory, Federal University of São Paulo, São Paulo, Brazil. [22]School of Medicine of ABC (FMABC), Clinical Immunology Laboratory, Santo André, São Paulo, Brazil. [23]Coordenação de Vigilância Epidemiológica do Estado do Rio de Janeiro, Rio de Janeiro, Brazil. [24]Departamento de Vigilância das Doenças Transmissíveis da Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília-DF, Brazil. [25]Secretaria de Vigilância em Saúde, Coordenação Geral de Laboratórios de Saúde Pública, Ministério da Saúde, Brasília-DF, Brazil. [26]Organização Pan - Americana da Saúde/Organização Mundial da Saúde - (OPAS/OMS), Brasília-DF, Brazil. [27]Public Health England, National Infections Service, Porton Down, Salisbury, UK. [28]NIHR HPRU in Emerging and Zoonotic Infections, Public Health England, London, UK. [29]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa. [30]Department of Biostatistics, UCLA Fielding School of Public Health, University of California, Los Angeles, CA, USA. [31]Department of Biomathematics and Human Genetics, David Geffen School of Medicine at UCLA, University of California, Los Angeles, CA, USA.
*These authors contributed equally to this work.
†Corresponding author. Email: nuno.faria@zoo.ox.ac.uk (N.R.F.); luiz.alcantara@ioc.fiocruz.br (L.C.J.A.); oliver.pybus@zoo.ox.ac.uk (O.G.P.)

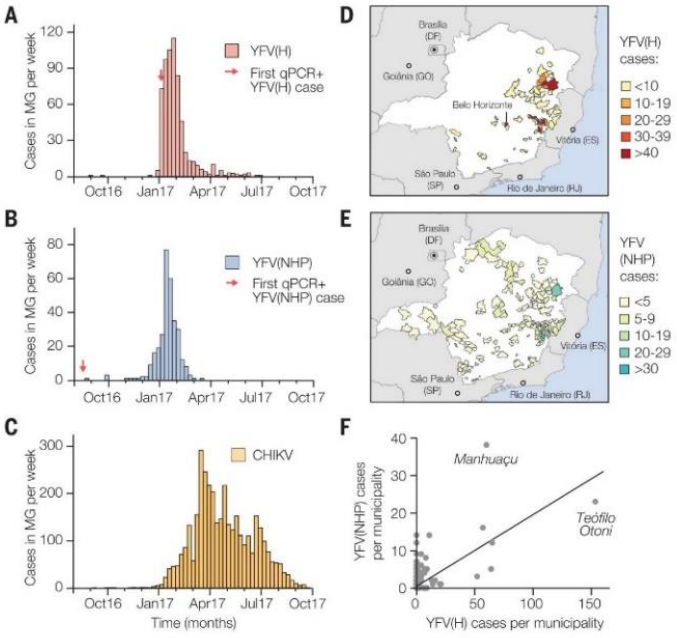



**Fig. 1. Spatial and temporal epidemiology of YFV and CHIKV in Minas Gerais (MG).** (**A**) Time series of human (H) YFV cases in MG (676 cases across 61 municipalities)—confirmed by serology, reverse transcription quantitative PCR (RT-qPCR), or virus isolation—during the first YFV epidemic wave (August 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

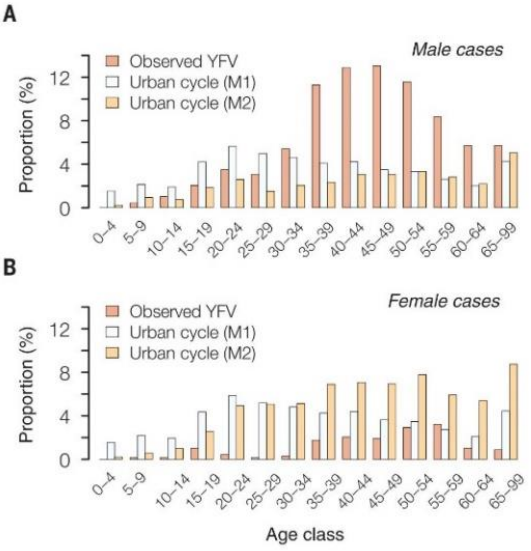



**Fig. 2. Age and sex distribution of YFV cases in MG, 2016–2017.** Red bars show the proportion of observed YFV cases in MG that occur in each age class, in (**A**) males and (**B**) females. These empirical distributions are different from those predicted under two models (M1, pale blue bars; M2, orange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

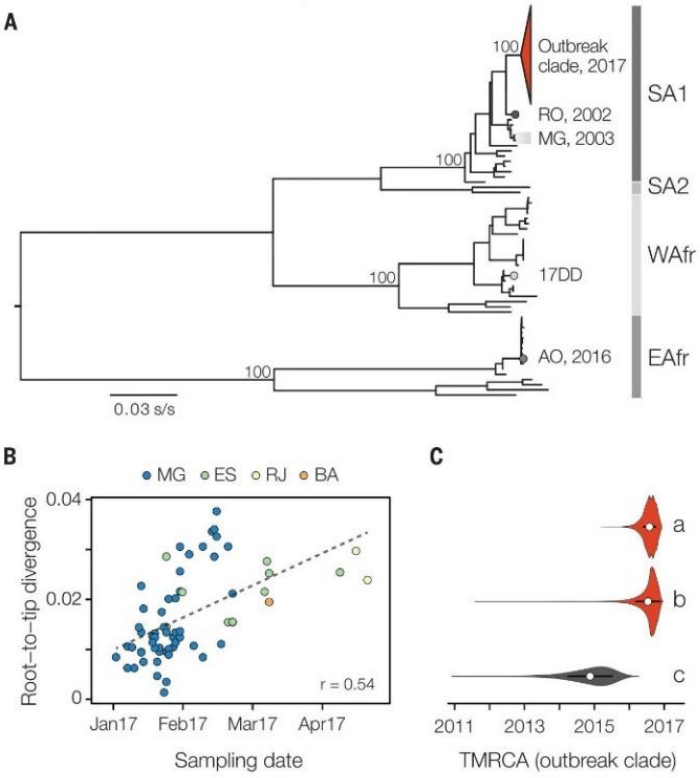



**Fig. 3. Molecular phylogenetics of the Brazilian YFV epidemic.** (**A**) Maximum likelihood phylogeny of complete YFV genomes showing the outbreak clade (red triangle) within the South American I (SA1) genotype (Fig. 4 and fig. S6). SA2, WAfr, and EAfr indicate the South America II, West Africa, and 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

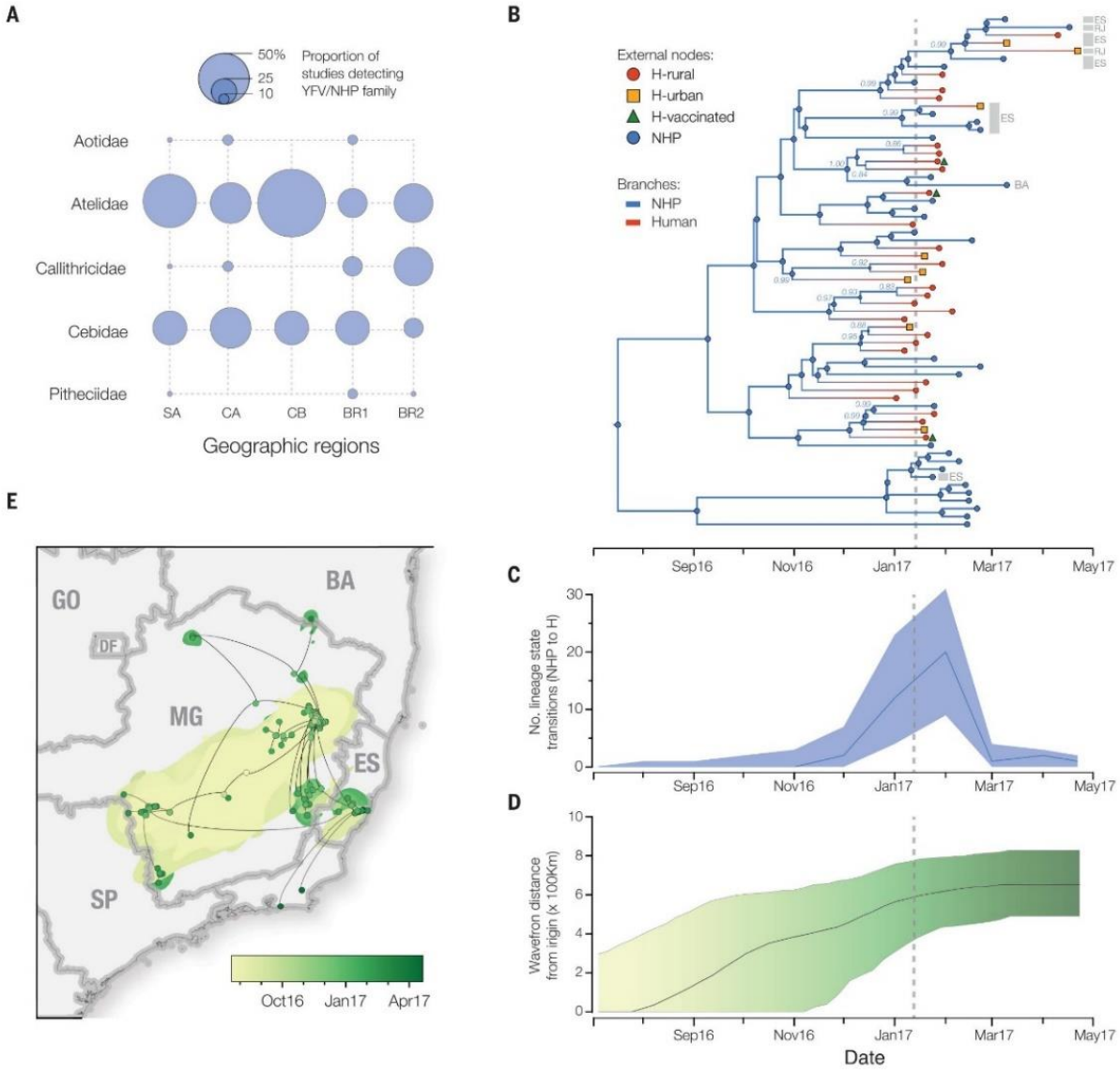



**Fig. 4. Spatial and evolutionary dynamics of YFV outbreak.** (**A**) Frequency of detection of YFV in NHPs in the Americas (*50*). Circle sizes represent the proportion of published studies ($n$ = 15) that have detected YFV in each primate family and region. SA, South America (except Brazil); CA, Central 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.

### REFERENCES AND NOTES


1. T. Garske *et al.*, *PLOS Med.* **11**, e1001638 (2014).
2. C. I. Paules, A. S. Fauci, *N. Engl. J. Med.* **376**, 1397–1399 (2017).
3. M. Theiler, H. H. Smith, *J. Exp. Med.* **65**, 767–786 (1937).
4. F. M. Shearer *et al.*, *Lancet Infect. Dis.* **17**, 1209–1217 (2017).
5. M. R. Nunes *et al.*, *J. Virol.* **86**, 13263–13271 (2012).
6. J. P. Mutebi, H. Wang, L. Li, J. E. Bryant, A. D. T. Barrett, *J. Virol.* **75**, 6999–7008 (2001).
7. J. J. von Lindern *et al.*, *J. Gen. Virol.* **87**, 895–907 (2006).
8. E. Wang *et al.*, *Virology* **225**, 274–281 (1996).
9. G. J. Chang, B. C. Cropp, R. M. Kinney, D. W. Trent, D. J. Gubler, *J. Virol.* **69**, 5773–5780 (1995).
10. M. N. O. Segura, F. C. C. Castro, *Atlas de Culicídeos na Amazônia Brasileira* (Instituto Evandro Chagas Press, 2007), vol. 167.
11. J. da C. Cardoso *et al.*, *Emerg. Infect. Dis.* **16**, 1918–1924 (2010).
12. A. A. Grobbelaar *et al.*, *Emerg. Infect. Dis.* **22**, 1854–1855 (2016).
13. M. U. G. Kraemer *et al.*, *Lancet Infect. Dis.* **17**, 330–338 (2017).
14. O. Franco, *História da Febre-Amarela no Brasil* (Ministério da Saúde, DNERU, 1969).

15. R. A. G. B. Consoli, R. Lourenço de Oliveira, *Principais Mosquitos de Importância Sanitária no Brasil* (FioCruz, 1994).
16. J. Vainio, "Yellow fever" (World Health Organization, 1998).
17. M. U. Kraemer *et al.*, *eLife* **4**, e08347 (2015).
18. Secretaria de Vigilância em Saúde, Ministério da Saúde, "Emergência epidemiológica de febre amarela no Brasil, no período de Dezembro de 2016 a Julho de 2017" (Boletim Epidemiológico vol. 48, 2017); http://portalarquivos2.saude.gov.br/images/pdf/2017/setembro/06/2017_027.pdf.
19. P. F. Vasconcelos *et al.*, *J. Med. Virol.* **65**, 598–604 (2001).
20. Pan American Health Organization/World Health Organization, "Epidemiological update yellow fever 16 Feb 2018" (PAHO/WHO, 2018); www.paho.org/hq/index.php?option=com_topics&view=readall&cid=2194&Itemid=40784&lang=en.
21. F. M. Shearer *et al.*, *Lancet Glob. Health* **6**, e270–e278 (2018).
22. Instituto Evandro Chagas. www.iec.gov.br/portal/descoberta/ (2018).
23. R. Lourenço-de-Oliveira, M. Vazeille, A. M. B. de Filippis, A. B. Failloux, *Trans. R. Soc. Trop. Med. Hyg.* **98**, 43–54 (2004).
24. D. Couto-Lima *et al.*, *Sci. Rep.* **7**, 4848 (2017).
25. N. R. Faria *et al.*, *PLOS Currents Outbreaks*, 10.1371/currents.outbreaks.c97507e3e48efb946401755d468c28b2 (2016).
26. N. R. Faria *et al.*, *Sci. Rep.* **7**, 15216 (2017).
27. S. H. Tuboi, Z. G. A. Costa, P. F. da Costa Vasconcelos, D. Hatch, *Trans. R. Soc. Trop. Med. Hyg.* **101**, 169–175 (2007).
28. PAHO. "Outbreak of Yellow Fever in Paraguay" (PAHO, 2009).
29. A. Nasidi *et al.*, *Trans. R. Soc. Trop. Med. Hyg.* **83**, 401–406 (1989).
30. S. D. Thiberville *et al.*, *PLOS Negl. Trop. Dis.* **7**, e2004 (2013).
31. M. Ribeiro, C. M. F. Antunes, *Rev. Soc. Bras. Med. Trop.* **42**, 523–531 (2009).
32. J. Quick *et al.*, *Nat. Protoc.* **12**, 1261–1276 (2017).
33. M. C. Bonaldo *et al.*, *Mem. Inst. Oswaldo Cruz* **112**, 447–451 (2017).
34. A. Moreira-Soto *et al.*, *Clin. Microbiol. Infect.* 10.1016/j.cmi.2018.01.026 (2018).
35. Secretaria de Vigilância em Saúde, Ministério da Saúde, "Reemergência da Febre Amarela Silvestre no Brasil, 2014/2015: Situação epidemiológica e a importância da vacinação preventiva e da vigilância intensificada no período sazonal" (Boletim Epidemiologico vol. 46, 2015); http://portalarquivos2.saude.gov.br/images/pdf/2015/outubro/19/2015-032–FA-ok.pdf.
36. P. F. C. Vasconcelos *et al.*, *Emerg. Infect. Dis.* **10**, 1578–1584 (2004).
37. F. P. Câmara, A. L. B. B. Gomes, L. M. F. Carvalho, L. G. V. Castello, *Rev. Soc. Bras. Med. Trop.* **44**, 297–299 (2011).
38. P. F. Vasconcelos *et al.*, *Cad. Saude Publica* **17** (suppl.), 155–164 (2001).
39. G. Dudas *et al.*, *Nature* **544**, 309–315 (2017).
40. M. S. Gill, P. Lemey, S. N. Bennett, R. Biek, M. A. Suchard, *Syst. Biol.* **65**, 1041–1056 (2016).
41. T. G. Vaughan, D. Kühnert, A. Popinga, D. Welch, A. J. Drummond, *Bioinformatics* **30**, 2272–2279 (2014).
42. G. Dudas, L. M. Carvalho, A. Rambaut, T. Bedford, *eLife* **7**, e31257 (2018).
43. O. G. Pybus *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 15066–15071 (2012).
44. P. Lemey, A. Rambaut, J. J. Welch, M. A. Suchard, *Mol. Biol. Evol.* **27**, 1877–1885 (2010).
45. L. Jung, I. Mourthe, C. E. V. Grelle, K. B. Strier, J. P. Boubli, *PLOS ONE* **10**, e0129789 (2015).
46. E. Flacio, L. Engeler, M. Tonolla, P. Müller, *Parasit. Vectors* **9**, 304 (2016).
47. A. Estrada *et al.*, *Sci. Adv.* **3**, e1600946 (2017).
48. R. A. Nascimento, R. A. M. Montano, *Neotrop. Biol. Conserv.* **8**, 79–87 (2013).
49. A. J. Auguste *et al.*, *Emerg. Infect. Dis.* **21**, 99–102 (2015).
50. P. R. Stephens *et al.*, *Ecology* **98**, 1476 (2017).


## ACKNOWLEDGMENTS

We thank FUNED-MG and the Brazilian YFV surveillance network for their contributions. N.R.F. thanks J. F. Drexler for sharing data. N. Trovão for discussions, F. Campos for proof imaging, and P. Fonseca for proofreading. We thank Oxford Nanopore Technologies for technical support. L.C.J.A. thanks QIAGEN for reagents and equipment. A.C.d.C. and E.C.S. thank Illumina, Zymo Research, Sage Science, and Promega for donation of reagents. **Funding:** This work was supported in part by CNPq 400354/2016-0 and FAPESP 2016/01735-2. N.R.F. is supported by a Sir Henry Dale Fellowship (204311/Z/16/Z), internal GCRF grant 005073, and John Fell Research Fund grant 005166. This research received funding from the ERC (grant agreement 614725-PATHPHYLODYN) and from the Oxford Martin School. M.U.G.K. acknowledges funding from a Branco Weiss Fellowship, administered by ETH Zurich, a Training Grant from the National Institute of Child Health and Human Development (T32HD040128), and the National Library of Medicine of the National Institutes of Health (R01LM010812 and R01LM011965). S.D. is funded by the Fonds Wetenschappelijk Onderzoek (FWO, Flanders, Belgium). G.B. acknowledges support from the Interne Fondsen KU Leuven/Internal Funds KU Leuven. A.C.d.C. is funded by FAPESP 2017/00021-9. B.B.N. and S.C. are supported by the EU's Horizon 2020 Programme through ZIKAlliance (grant 734548), the Investissement d'Avenir program, the Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases program (grant ANR-10-LABX-62-IBEID), the Models of Infectious Disease Agent Study of the National Institute of General Medical Sciences, the AXA Research Fund, and the Association Robert Debré. P.L. and M.A.S. acknowledge funding from the European Research Council (grant agreement 725422-ReservoirDOCS) and from the Wellcome Trust Collaborative Award 206298/Z/17/Z. P.L. acknowledges support from the Research Foundation, Flanders (Fonds voor Wetenschappelijk Onderzoek, Vlaanderen, G066215N, G0D5117N, and G0B9317N). **Author contributions:** N.R.F., L.C.J.A., S.C.H., A.M.B.F., M.U.G.K., S.C., and O.G.P. designed the study. S.C.H., J.J.G., R.S.d.A., F.C.M.I., J.X., R.D.O.C., J.T., M.G., L.C.J.A., and N.R.F. undertook fieldwork. S.C.H., J.Q., J.J.G., A.C.d.C., S.C.V.K., V.F., and T.d.O. undertook experiments. N.R.F., L.d.P., J.T., S.D., G.B., O.G.P., C.-H.W., T.I.V., and P.L. performed genetic analyses. M.U.G.K., S.C., S.F., J.L., U.O., L.A., D.Y., and N.R.F. performed epidemiological and cartographic analyses. B.N., F.M.S., and N.R.F. performed historical YFV review. N.R.F., M.U.G.K., L.C.J.A., S.C., and O.G.P. wrote the manuscript. E.C.S., J.T., L.d.P., R.P.S., P.L., C.F.C.d.A., R.S.d.A., and A.M.B.F. edited the manuscript. All other authors were involved in collection, processing, sequencing, and bioinformatics of samples and geographic data. All authors read and approved the contents of the manuscript. **Competing interests:** N.J.L. and L.C.J.A. received free-of-charge reagents in support of the project from Oxford Nanopore Technologies. A.C.d.C. and E.C.S. received reagents at no cost from Illumina, Zymo Research, Sage Science, and Promega. **Data and materials availability:** Raw data, code, and analysis files are available via the GitHub repository (https://github.com/arbospread/YFV-monitoring). See https://github.com/zibraproject/zika-pipeline/tree/master/schemes for MinION sequencing protocols. Genome sequences generated here are available under GenBank accession numbers MH018064 to MH018115 and MH484423 to MH484434.

## SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/361/6405/894/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S10
Tables S1 to S9
References (*51*–*107*)
Movie S1

31 March 2018; accepted 20 July 2018
Published online 23 August 2018
10.1126/science.aat7115

### 3.4 Artigo 4: *"Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon"*.

Até o final de 2017, o Brasil notificou o maior número de infecções causadas por CHIKV nas Américas. Nós investigamos um grande surto de CHIKV em Boa Vista, município na região amazônica brasileira. O sequenciamento rápido do genoma de 20 novos isolados e subsequente análise genética revelaram que a linhagem ECSA foi introduzida em Roraima a partir da região nordeste do Brasil por volta de julho de 2016. Análises epidemiológicas sugeriram um número reprodutivo básico (R0) de 1,66, o que sugere que aproximadamente 39% da população de Roraima estava infectada com CHIKV-ECSA. Dado o domínio de CHIKV genótipo asiático nas Américas, nossos dados destacaram a rápida disseminação do genótipo ECSA, que é menos compreendido e menos caracterizado no Brasil. Investigações sobre potenciais associações entre os casos de CHIKV e a diversidade genética das linhagens circulantes são fundamentais para melhor avaliar o impacto desse vírus no Brasil e no mundo.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

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

**Felipe Gomes Naveca[1], Ingra Claro[2], Marta Giovanetti[3,4], Jaqueline Goes de Jesus[3,5], Joilson Xavier[3,5], Felipe Campos de Melo Iani[4,6], Valdinete Alves do Nascimento[1], Victor Costa de Souza[1], Paola Paz Silveira[7], José Lourenço[8], Mauricio Santillana[9,10], Moritz U. G. Kraemer[8,11], Josh Quick[12], Sarah C. Hill[8], Julien Thézé[8], Rodrigo Dias de Oliveira Carvalho[4], Vasco Azevedo[4], Flavia Cristina da Silva Salles[2], Márcio Roberto Teixeira Nunes[13], Poliana da Silva Lemos[13], Darlan da Silva Candido[8], Glauco de Carvalho Pereira[4], Marluce Aparecida Assunção Oliveira[4], Cátia Alexandra Ribeiro Meneses[14], Rodrigo Melo Maito[14], Claudeth Rocha Santa Brígida Cunha[14], Daniela Palha de Sousa Campos[15], Marcia da Costa Castilho[16], Thalita Caroline da Silva Siqueira[15], Tiza Matos Terra[17], Carlos F. Campelo de Albuquerque[18], Laura Nogueira da Cruz[19], André Luis de Abreu[19], Divino Valerio Martins[19], Daniele Silva de Moraes Vanlume Simoes[19], Renato Santana de Aguiar[7], Sérgio Luiz Bessa Luz[1], Nicholas Loman[12], Oliver G. Pybus[8], Ester C. Sabino[2], Osnei Okumoto[19], Luiz Carlos Junior Alcantara[3,4]*, Nuno Rodrigues Faria[8]***

**1** Laboratório de Ecologia de Doenças Transmissíveis na Amazônia, Instituto Leônidas e Maria Deane, FIOCRUZ, Manaus, Brazil, **2** Instituto de Medicina Tropical e Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil, **3** Laboratório de Flavivírus, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil, **4** Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **5** Laboratório de Patologia Experimental, Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil, **6** Laboratório Central de Saúde Pública, Instituto Octávio Magalhães, FUNED, Belo Horizonte, Minas Gerais, Brazil, **7** Laboratório de Virologia Molecular, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, **8** Department of Zoology, University of Oxford, South Parks Road, Oxford, United Kingdom, **9** Harvard Medical School, Department of Pediatrics, Boston, MA, United States of America, **10** Computational Health Informatics Program, Boston Children's Hospital, Boston, MA, United States of America, **11** Computational Epidemiology Lab, Boston Children's Hospital, Boston, MA, United States of America, **12** Institute of Microbiology and Infection, University of Birmingham, Birmingham, United Kingdom, **13** Evandro Chagas Institute, Brazilian Ministry of Health, Ananindeua, Brazil, **14** Laboratório Central de Saúde Pública, Boa Vista, Roraima, Brazil, **15** Superintendência de Vigilância em Saúde, Secretaria Municipal de Saúde de Boa Vista, Roraima, Brazil, **16** Departamento de Virologia, Fundação de Medicina Tropical Doutor Heitor Vieira Dourado, Manaus, Amazonas, Brazil, **17** Laboratório Central de Saúde Pública do Amazonas, Manaus, Amazonas, Brazil, **18** Organização Pan—Americana da Saúde/Organização Mundial da Saúde—(OPAS/OMS), Brasília-DF, Brazil, **19** Secretaria de Vigilância em Saúde, Ministério da Saúde (SVS/MS), Brasília-DF, Brazil

* luiz.alcantara@ioc.fiocruz.br (LCJA); nuno.faria@zoo.ox.ac.uk (NRF)



OPEN ACCESS

**Citation:** Naveca FG, Claro I, Giovanetti M, de Jesus JG, Xavier J, Iani FCdM, et al. (2019) Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon. PLoS Negl Trop Dis 13(3): e0007065. https://doi.org/10.1371/journal.pntd.0007065

**Editor:** Rebecca C. Christofferson, Louisiana State University, UNITED STATES

**Received:** December 6, 2018

**Accepted:** February 1, 2019

**Published:** March 7, 2019



**Data Availability Statement:** XML files and datasets analysed in this study are available in the GitHub respository (https://github.com/arbospread/chik-amazon). New sequences have been deposited in GenBank under accession numbers MK121891-MK121908 (CHIKV-ECSA) and MK134712-MK134713 (CHIKV-Asian).

**Funding:** The ZIBRA-2 project is funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant 440685/2016-8) and Coordenação de Aperfeiçoamento de Pessoal de

## 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-

PLOS | NEGLECTED TROPICAL DISEASES



Nível Superior (grant 88887.130716/2016-00). FGN is funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (http://www.cnpq.br, grant 440856/2016–7) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (http://www.capes.gov.br, grants 88881.130825/2016–00 and 88887.130823/2016–00). L.C.J.A. is supported by the EU's Horizon 2020 Programme through ZIKAlliance (grant PRES-005-FEX-17-4-2-33). N.R.F. is funded by a Royal Society and Wellcome Trust Sir Henry Dale Fellowship (204311/Z/16/Z), internal GCRF grant 005073, and John Fell Research Fund grant 005166. This research received funding from the ERC (grant agreement 614725-PATHPHYLODYN). MS was partially supported by the National Institute of General Medical Sciences of the NIH (USA) under award number R01GM130668. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

**Competing interests:** The authors declare no competing interests.

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.

## Introduction

In August 2014, local transmission of chikungunya virus (CHIKV) was detected in Brazil for the first time, with cases being reported nearly simultaneously in Oiapoque (Amapá state, north Brazil) and Feira de Santana (Bahia state, northeast Brazil), two municipalities separated

by >2000 km distance. Genetic analysis confirmed the co-circulation of distinct virus lineages in Brazil: the Asian genotype (CHIKV-Asian) was introduced to Oiapoque possibly from neighbouring French Guiana, while the East-Central-South-African genotype (CHIKV-ECSA) was introduced to Feira de Santana from a traveller returning from Angola [1].

Since 2014 and until the end of September 2018, a total of 697,564 CHIKV cases have been notified in Brazil (including 94,672 laboratory-confirmed cases). This is the largest number recorded in any of the 51 countries or territories reporting local CHIKV transmission in the Americas [2]. The virus has been circulating in the Americas since 2013 where approximately 260 million people live in areas at-risk of transmission [2–4]. Similar to the recent Zika virus epidemic [5], the rapid spread of CHIKV in the Americas, including in Brazil, results from several factors, including the establishment and abundance of competent *Aedes* spp. vectors, lack of population immunity, poor housing quality, and increased mobility of vectors and humans between regions reporting current presence of the virus [6,7].

Chikungunya virus is an enveloped, non-segmented, single-stranded positive polarity RNA alphavirus that is a member of the *Togaviridae* family and is transmitted predominately by the *Aedes aegypti* and *Aedes albopictus* vectors, which are widespread in Brazil [8]. There are four main genotypes: (i) the West African genotype is maintained in an enzootic cycle in Africa, (ii) the Asian genotype, which is endemic in Asia, (iii) the East-Central-South-African genotype, endemic to Africa, and (iv) the Indian Ocean Lineage (IOL) genotype, an epidemic lineage that emerged from the ECSA genotype around 2004 and swept through the Indian Ocean region causing a series of explosive outbreaks [9].

The first symptoms of CHIKV infection are a rapid increase in temperature (>38.9˚C), followed by severe, often debilitating polyarthralgia. Serological data from La Reunion, Philippines and the Indian Ocean island of Mayotte suggest that 75–97% of persons infected with CHIKV develop symptomatic infections [10]. 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 [11].

Throughout Asia and the Americas, chikungunya virus outbreaks have been associated with unique clinical features [12], including long-lasting symptoms [13], and high mortality resulting from complications associated with CHIKV infection [14, 15]. 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 [13, 16]. 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 [17], mostly due to the co-circulation and co-infection with viruses that cause overlapping symptoms, such as Zika and dengue viruses [18–20]. 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 [21, 22]. 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 [23].

Until the end of 2016, 83.3% of the cases in Brazil were reported in northeast region of the country [24]. 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 [25]. Within Brazil's northern states, Roraima has been implicated as a stepping-stone to virus introductions from other Latin American regions, such as dengue [26],

and yellow fever virus in the past [27]. 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 [28].

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

## Methods

### Connectivity in study area

Roraima is the northernmost of Brazil's 27 federal units (Fig 1A) and has an estimated population of 450,479, of whom 284,313 live in the capital city of Boa Vista (ibge.gov.br/). Despite being Brazil's least populated federal unit, Roraima is one of the best-connected Brazilian states in the Amazon basin [29]. Within Brazil, Roraima is connected to Amazonas state in the south via the road BR-174. This road also connects Roraima's capital city, Boa Vista, to the states of Bolivar and Amazonas in Venezuela in the north. Further, the road BR-401 links Boa Vista to Guyana in the east. There are four daily flights connecting Boa Vista with Brasília, capital of Brazil, as well as six weekly flights to Manaus, the capital city of Amazonas state and the biggest city in the north of the country, with connecting daily nonstop flights to all other Brazilian states/regions and international destinations, including important international airport hubs in Panamá City and Miami, USA. There are also less-commonly used seasonal fluvial networks that connect Boa Vista and Manaus via the Amazonas river.

### Chikungunya virus case count time series

The Roraima State Central Laboratory (LACEN-RR) is responsible for the differential diagnosis of suspected arbovirus cases presenting to Roraima's public health units. Between Jan 2014

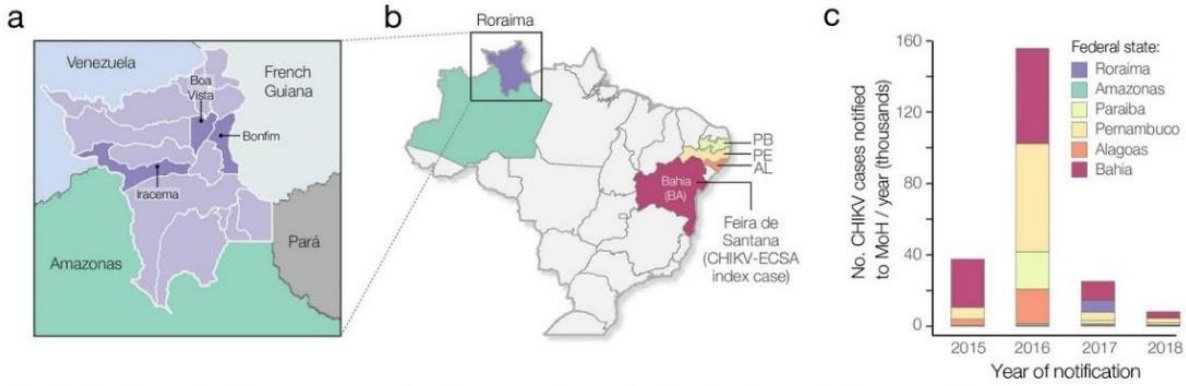



**Fig 1. Context of this study.** A. Map showing municipalities of Roraima state, including Boa Vista, bordering countries (Venezuela and French Guiana) and bordering Brazilian federal states (Amazonas and Pará). B. Map of Brazilian states, showing the states from which CHIKV sequence data in this study was analysed (Bahia, Alagoas, Pernambuco, Paraíba, Amazonas and Roraima). C. Barplot showing the annual number of notified CHIKV cases in selected states of Brazil (data obtained from the Brazilian Ministry of Health). Map was made with Natural Earth. Free vector and raster map data at naturalearthdata.com.

https://doi.org/10.1371/journal.pntd.0007065.g001

and September 2018, LACEN-RR notified 5,928 CHIKV cases in Boa Vista alone, 3,795 of these laboratory-confirmed, to the National Reportable Disease Information System (SINAN). Case count time series are available from Github (https://github.com/arbospread/chik-amazon). We follow the Brazilian Ministry of Health's guidelines and define a notified CHIKV case as a suspected case characterized by (i) acute onset of fever >38.5˚C, (ii) severe arthralgia and/or arthritis not explained by other medical conditions, and (iii) residing or having visited epidemic areas within 15 days before onset of symptoms. A laboratory-confirmed case is a suspected case confirmed by laboratory methods such as (i) virus isolation in cell culture, (ii) detection of viral RNA, (iii) detection of virus-specific IgM antibodies in a single serum sample collected in the acute or convalescent stage of infection; or (iv) a four-fold rise of IgG titres in samples collected during the acute 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) n° 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 [30]. All qRT-PCR results were corroborated using a second protocol [31]; 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 [32]), 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 [33]. Sequencing statistics can be found in S1 Table. In brief, we employed a protocol with cDNA synthesis using random primers followed by strain-specific multiplex PCR [33]. 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 genome amplification by multiplex PCR was attempted using the CHIKAsia-nECSA primer scheme and 35 cycles of PCR using Q5 High-Fidelity DNA polymerase (NEB) as described in [33]. PCR products were cleaned up using AmpureXP purification beads (Beckman Coulter, High Wycombe, UK) and quantified using fluorimetry with the Qubit dsDNA High Sensitivity assay on the Qubit 3.0 instrument (Life Technologies). PCR products for samples yielding sufficient material were barcoded and pooled in an equimolar fashion

**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 S1 Table. 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 |

https://doi.org/10.1371/journal.pntd.0007065.t001

using the Native Barcoding Kit (Oxford Nanopore Technologies, Oxford, UK). Sequencing libraries were generated from the barcoded products using the Genomic DNA Sequencing Kit SQK-MAP007/SQK-LSK108 (Oxford Nanopore Technologies). Libraries were loaded onto a R9/R9.4 flow cell and sequencing data were collected for up to 48hr. Consensus genome sequences were produced by alignment of two-direction reads to a CHIKV virus reference genome (GenBank Accession number: N11602) as previously described in [33]. Positions with≥20× genome coverage were used to produce consensus alleles, while regions with lower coverage, and those in primer-binding regions were masked with N characters. Validation of the sequencing protocol was previously performed in [33].

## Collation of CHIKV-ECSA complete genome datasets

Genotyping was first conducted using the phylogenetic arbovirus subtyping tool available at http://www.krisp.org.za/tools.php. Complete and near complete sequences were retrieved from GenBank on June 2017 [34]. Two complete or near-complete CHIKV genome datasets were generated. Dataset 1 included ECSA-PreAm (ECSA sampled outside the Americas) and ECSA-Br (ECSA sequences sampled in the Americas) sequences. This dataset contained 36 complete genomes from the ECSA genotype, including 7 from East and Central Africa (HM045823 from Angola 1962; HM045784 from Central African Republic 1984; HM045812 from Uganda 1982; KY038947 from Central African Republic 1983; HM045793 from Central African Republic 1986; HM045822 from Central African Republic 1978; and KY038946 from Central African Republic 1975). Dataset 1 also included 29 sequences from Brazil, including the new 18 genomes reported here from the ECSA lineage and 3 genomes from the outbreak caused by the ECSA lineage in June 2016 in Maceió, Alagoas states, northeast Brazil (Fig 1A)

[35]. Dataset 2 (ECSA-Br) included only the 29 Brazilian genome sequences. Using a robust nonparametric test [36], 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 [37]. 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 [38]. 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 [9], 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 [39]. 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) [40], b) the Bayesian skyride plot [41], and c) the Bayesian skygrid model [42], 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 [42]. 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) [43]. 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 [43].

## Epidemiological analysis

The epidemic basic reproductive number ($R_0$) was estimated from monthly confirmed cases, as previously described [32, 44]. 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 [45]. 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 [46], and (b) in unexpected and emerging mosquito-borne disease outbreaks such as the 2015–2016 Latin American Zika outbreak [47]. 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 [46, 47] 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 comparison, GT search activity for the term "Chikungunya" was collected for the same time period for Manaus municipality (Amazonas state). The synchronicity of GT time series and notified and confirmed case counts from Boa Vista and Manaus was assessed using the Spearman's rank correlation test in the R software [48].

## Results

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

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

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

ML and Bayesian phylogenetic analyses reveal that the ECSA sequences from Brazil form a single well-supported clade (bootstrap support = 100), hereafter named as ECSA-Br clade; which contains strong temporal signal ($r^2 = 0.84$) as measured by a regression of genetic divergence against sampling dates (**Figs 2B and 3**). Thus we estimated the evolutionary time-scale of the ECSA-Br lineage using several well-established molecular clock coalescent methods. Our substitution rate estimates indicate that the ECSA-Br lineage is evolving at $7.15 \times 10^{-4}$ substitutions per site per 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

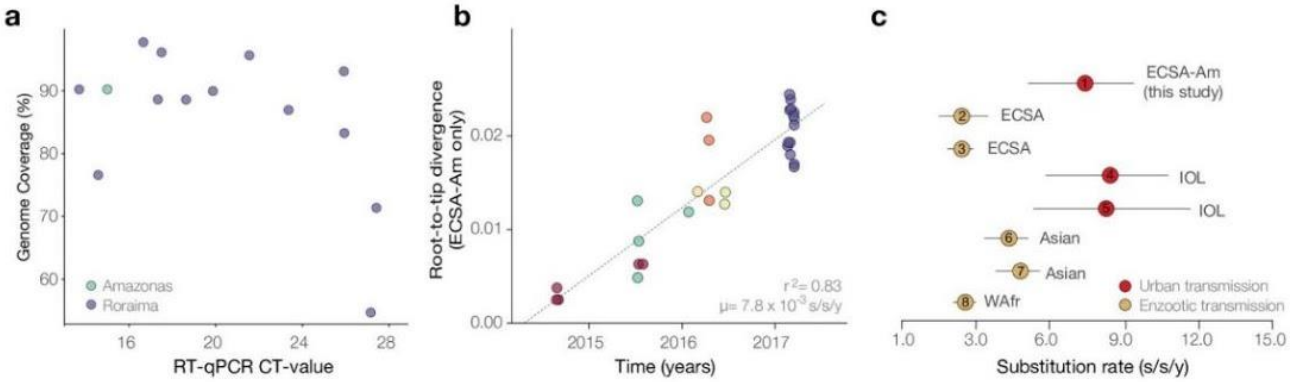



**Fig 2. Sequencing statistics, temporal signal and evolutionary rates of the CHIKV-ECSA lineage.** A. Genome coverage plotted against RT-qPCR CT-values for the newly generated sequence data. B. Genetic divergence regressed against dates of sample collection for dataset 2 (CHIKV-ECSA-Br lineage). C. Evolutionary rate estimates for the CHIKV-ECSA-Br lineage obtained by this study (circle number 1) compared to published evolutionary rates obtained for other lineages. Circles numbered 2 to 8 represent point estimates reported in [1, 9, 80]. Horizontal bars represent 95% highest posterior density credible intervals for evolutionary rates.

https://doi.org/10.1371/journal.pntd.0007065.g002

evolutionary rates estimated for the epidemic lineage circulating in the Indian Ocean region (Fig 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 [49].

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 (S1 Fig), 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) (Fig 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) (Fig 3); similar dating estimates under different coalescent models (S2 Fig). In contrast to the Roraima strains, sequences from Manaus were found to be interspersed with isolates from Bahia and Pernambuco (Fig 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, 50, 51]. Our demographic reconstructions indicate that the outbreak in Roraima 2017 probably represents the third epidemic wave spreading across Brazil (S3 Fig).

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 [44]. We find that $R_0 \approx 1.66$ (95% CI: 1.51–1.83), in line with previous reports from other settings [52–54]. A

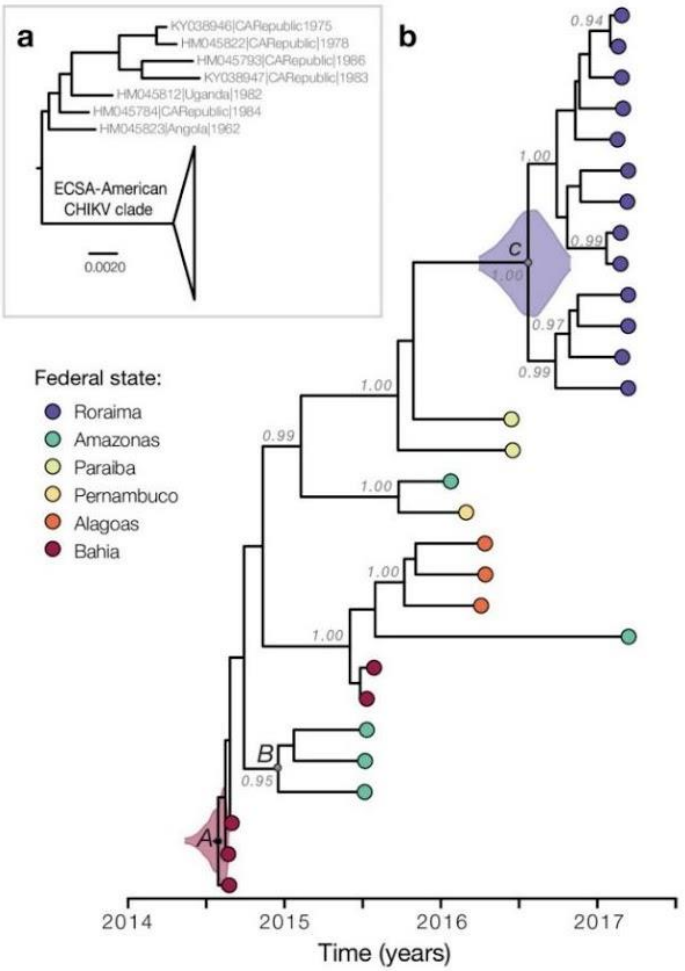



**Fig 3. Genetic analysis of the CHIKV-ECSA genotype.** A. Maximum likelihood phylogeny depicting the monophyletic clade containing all the Brazilian ECSA isolates (ECSA-Br lineage). B. Time-calibrated phylogeny of all available CHIKV-ECSA whole genome sequences from Brazil, including 18 novel genomes from Roraima and Amazonas states. Colours correspond to state of sample collection. Violin plots show 95% Bayesian credible intervals for associated node heights [39].

https://doi.org/10.1371/journal.pntd.0007065.g003

sensitivity analysis considering different exponential growth phase periods resulted in a lower bound for $R_0$ of around 1.23 (S4 Fig). 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 [13, 16]. 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 [55], 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 [11, 56, 57], then the chances of a reported a symptomatic CHIKV case decrease to 5–7% [10]. Case reports suggest that the beginning of the exponential phase of the outbreak was in December 2016 (S4 Fig), 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 (Fig 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: $r = 0.92$, Fig 4D–4E). Additionally, the timing of the peak of Google searches corresponds to that of notified and confirmed cases with a peak in July 2017 (Fig 4A and 4C, Fig 4B and 4F). 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 publicly available. This fact highlights the potential utility of monitoring disease-related searches during the outbreak. Interestingly, we find some web-search activity in Roraima before June 2016, particularly in September 2014, March 2015 and March 2016 (Fig 4F). These patterns are distinct to those in the Amazonas neighbouring state (notified cases: $r = 0.65$; confirmed cases: $r = 0.15$), which shows an early peak in November 2014, soon after the estimated age of *node B* (Fig 3B), followed by a peak in February 2016 and another in March 2017 (Fig 4C). These multiple peaks in internet search queries are consistent with the timing of at least 3 introductions detected in our phylogenetic analyses (Fig 3B), each possibly resulting in small epidemic waves of CHIKV in Manaus and 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 [11], and slightly lower than the 45.7–57.1% observed in two serosurveys conducted in the same state [13], where the ECSA lineage also seems to predominate. 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-

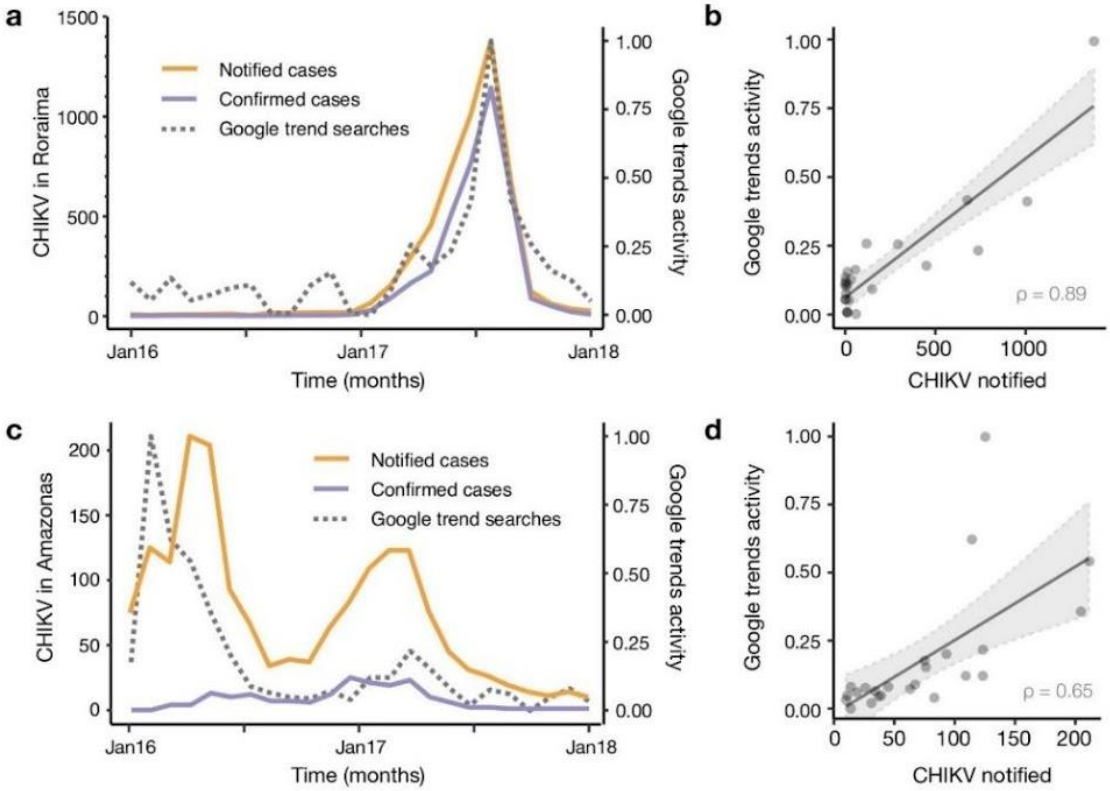



**Fig 4. Digital surveillance of chikungunya in the Brazilian Brazil.** Notified (orange) and confirmed (purple) cases in the central public health laboratories in Roraima state (a) and the Amazonas state (c) from January 2016 to January 2018. Dashed grey lines in (a) and (c) represent Google Trends activity for the term "chikungunya" in Roraima and Amazonas, respectively. Panels b and d show the correlation between Google Trends activity and cases notified in Roraima and Amazonas, respectively. Strength of the association was measured using the Spearman's rank correlation coefficient (in panel b: p-value < 0.001; panel d: p-value = 5.183e-09).

https://doi.org/10.1371/journal.pntd.0007065.g004

related alphavirus that may provide some level of cross-reactivity [58, 59] and is associated with *Haemagogus* spp. vectors [60], but has also been identified in *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes [66]. MAYV has been detected in the north [61–65] and centre-west [22, 66–70] regions of Brazil. Moderate to high prevalence of MAYV IgM have been found in urban northern areas [61], 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 [56], future comparisons of epidemiological parameters across different regions from where no genotype data is available should be taken with caution. Given the rapid spread of different CHIKV lineages, novel diagnostic tools may be needed to evaluate the proportion of individuals infected by each genotype.

Different CHIKV circulating lineages may have remarkably different public health consequences. Lineage-specific clinical presentations have been recently highlighted by a recent index cluster study which showed that 82% of CHIKV infections caused by the ECSA lineage are symptomatic, in comparison to only 52% of symptomatic infections caused by the Asian genotype [56]. While the Asian lineage seems to have circulated cryptically for 9 months before its first detection in the Caribbean [3], the faster detection of the ECSA lineage in Brazil could

at least in part be a consequence of a higher rate of symptomatic to asymptomatic infections of the ECSA lineage circulating in Brazil. The time lag between the phylogenetic estimate of the date of introduction of a virus lineage and the date of the first confirmed case in a given region, enables us to identify surveillance gaps between the arrival and discovery of a virus in that region [71].

We used genomic data collected over a 3-year period to estimate the genetic history of the CHIKV-ECSA-Br lineage. We estimate that the CHIKV-ECSA-Br lineage arrived in Roraima around July 2016, whilst the first confirmed CHIKV cases in Roraima occurred earlier, in August 2014. That the discovery date anticipates the estimated date of introduction can be explained by initial introduction(s) of the Asian linage (from the north of Brazil or from other south American regions) resulting in only limited onwards transmission, followed by the replacement of the Asian lineages by an epidemiological successful ECSA lineage. Transmission of the Asian genotype during this period is in line with an increase in notified and confirmed cases, as well internet search query data between August 2014 and June 2016. It is also possible that ecological conditions may have dampened the transmission of the Asian genotype between August 2014 (detection of autochthonous transmission of the Asian genotype in the north region of Brazil) and July 2016 (estimated arrival of the ECSA in Roraima). In the future, fine-scaled, high-resolution measures of transmission potential that take into account daily changes in humidity and temperature will help addressing the impact of climatic changes in the arbovirus epidemiology in the Brazilian Amazon. Nationwide molecular and seroprevalence studies combined with epidemiological modelling [72] 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 [9]. 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 [73]. In line with this, CHIKV-ECSA was recently detected in *Aedes aegypti* from Maranhão [74] and Rio de Janeiro states [75].

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 [76]. 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 [32]. 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 [26, 77], and northeast Brazil and Haiti [32, 78]. 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

[28]. 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 [27]. In the near future, the increasing rapidity and decreasing cost of genome sequencing in poorly sampled areas, combined with emerging theoretical approaches [79], 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.

## Supporting information

**S1 Fig. 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.
(TIF)

**S2 Fig. Dating estimates obtained under different coalescent models.** Estimates for node A (time of the most recent common ancestor, in dark red, see Fig 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.
(TIF)

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

**S4 Fig. 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.
(TIF)

**S5 Fig. Monthly suitability for the A*edes aegypti* mosquito.**
(TIF)

**S1 Table. Minion sequencing statistics.**
(DOCX)

## Acknowledgments

We are thankful to all personnel from Secretaria de Vigilância em Saúde SVS/MS, PAHO/Brazil, Roraima and Boa Vista Health Surveillance System that coordinated surveillance and helped with data collection and assembly. We thank Oxford Nanopore Technologies for the support to the ZIBRA-2 (Zika in Brazil Real time Analyses-second round) project with

additional flowcells and corresponding reagents, and also thank QIAGEN for donation of consumables.

## Author Contributions

**Conceptualization:** Sarah C. Hill, Vasco Azevedo, Márcio Roberto Teixeira Nunes, Poliana da Silva Lemos, Carlos F. Campelo de Albuquerque, Oliver G. Pybus, Ester C. Sabino, Osnei Okumoto, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Data curation:** Felipe Gomes Naveca, Ingra Claro, Marta Giovanetti, Jaqueline Goes de Jesus, Victor Costa de Souza, Julien Thézé, Flavia Cristina da Silva Salles, Cátia Alexandra Ribeiro Meneses, Rodrigo Melo Maito, Claudeth Rocha Santa Brígida Cunha, Daniela Palha de Sousa Campos, Marcia da Costa Castilho, Laura Nogueira da Cruz, André Luis de Abreu, Divino Valerio Martins, Daniele Silva de Moraes Vanlume Simoes, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Formal analysis:** Ingra Claro, Marta Giovanetti, Jaqueline Goes de Jesus, Joilson Xavier, Felipe Campos de Melo Iani, Paola Paz Silveira, José Lourenço, Mauricio Santillana, Julien Thézé, Rodrigo Dias de Oliveira Carvalho, Darlan da Silva Candido, Nuno Rodrigues Faria.

**Funding acquisition:** Felipe Gomes Naveca, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Investigation:** Felipe Gomes Naveca, Ingra Claro, Marta Giovanetti, Joilson Xavier, Felipe Campos de Melo Iani, Valdinete Alves do Nascimento, Victor Costa de Souza, Paola Paz Silveira, José Lourenço, Moritz U. G. Kraemer, Josh Quick, Rodrigo Dias de Oliveira Carvalho, Márcio Roberto Teixeira Nunes, Poliana da Silva Lemos, Darlan da Silva Candido, Cátia Alexandra Ribeiro Meneses, Rodrigo Melo Maito, Claudeth Rocha Santa Brígida Cunha, Daniela Palha de Sousa Campos, Marcia da Costa Castilho, Thalita Caroline da Silva Siqueira, Tiza Matos Terra, Carlos F. Campelo de Albuquerque, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Methodology:** Jaqueline Goes de Jesus, Joilson Xavier, Felipe Campos de Melo Iani, Victor Costa de Souza, Paola Paz Silveira, Mauricio Santillana, Josh Quick, Sarah C. Hill, Julien Thézé, Flavia Cristina da Silva Salles, Renato Santana de Aguiar, Nicholas Loman, Nuno Rodrigues Faria.

**Project administration:** Felipe Gomes Naveca, Valdinete Alves do Nascimento, Sarah C. Hill, Sérgio Luiz Bessa Luz, Luiz Carlos Junior Alcantara.

**Resources:** Valdinete Alves do Nascimento, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Software:** Nuno Rodrigues Faria.

**Supervision:** Felipe Gomes Naveca, Ingra Claro, Marta Giovanetti, Jaqueline Goes de Jesus, Glauco de Carvalho Pereira, Marluce Aparecida Assunção Oliveira, Oliver G. Pybus, Ester C. Sabino, Osnei Okumoto, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Validation:** Valdinete Alves do Nascimento, Victor Costa de Souza, Mauricio Santillana, Moritz U. G. Kraemer, Julien Thézé, Flavia Cristina da Silva Salles, Nuno Rodrigues Faria.

**Visualization:** Nuno Rodrigues Faria.

**Writing – original draft:** Felipe Gomes Naveca, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

**Writing – review & editing:** Felipe Gomes Naveca, Ingra Claro, José Lourenço, Mauricio Santillana, Moritz U. G. Kraemer, Sarah C. Hill, Vasco Azevedo, Márcio Roberto Teixeira Nunes, Poliana da Silva Lemos, Darlan da Silva Candido, Glauco de Carvalho Pereira, Cátia Alexandra Ribeiro Meneses, Rodrigo Melo Maito, Claudeth Rocha Santa Brígida Cunha, Daniela Palha de Sousa Campos, Marcia da Costa Castilho, Thalita Caroline da Silva Siqueira, Tiza Matos Terra, Carlos F. Campelo de Albuquerque, Laura Nogueira da Cruz, André Luis de Abreu, Divino Valerio Martins, Daniele Silva de Moraes Vanlume Simoes, Renato Santana de Aguiar, Sérgio Luiz Bessa Luz, Nicholas Loman, Oliver G. Pybus, Ester C. Sabino, Osnei Okumoto, Luiz Carlos Junior Alcantara, Nuno Rodrigues Faria.

## References

1. Nunes MR, Faria NR, de Vasconcelos JM, Golding N, Kraemer MU, de Oliveira LF, et al. Emergence and potential for spread of Chikungunya virus in Brazil. BMC medicine. 2015; 13:102. Epub 2015/05/16. https://doi.org/10.1186/s12916-015-0348-x PMID: 25976325; PubMed Central PMCID: PMC4433093.
2. Organization PAWH. Number of Reported Cases of Chikungunya Fever in the Americas, by Country or Territory. Washington: 2017.
3. Sahadeo NSD, Allicock OM, De Salazar PM, Auguste AJ, Widen S, Olowokure B, et al. Understanding the evolution and spread of chikungunya virus in the Americas using complete genome sequences. Virus Evol. 2017; 3(1):vex010. Epub 2017/05/10. https://doi.org/10.1093/ve/vex010 PMID: 28480053; PubMed Central PMCID: PMCPMC5413804.
4. Nsoesie EO, Kraemer MU, Golding N, Pigott DM, Brady OJ, Moyes CL, et al. Global distribution and environmental suitability for chikungunya virus, 1952 to 2015. Euro surveillance. 2016; 21(20). Epub 2016/05/31. https://doi.org/10.2807/1560-7917.ES.2016.21.20.30234 PMID: 27239817; PubMed Central PMCID: PMCPMC4902126.
5. Faria NR, Lourenco J, Cerqueira EM, Lima MM, Pybus OG, Alcantara LCJ. Epidemiology of Chikungunya Virus in Bahia, Brazil, 2014–2015. PLoS Currents Outbreaks. 2016; 1. https://doi.org/10.1371/currents.outbreaks.c97507e3e48efb946401755d468c28b2 PMID: 27330849
6. Johansson MA. Chikungunya on the move. Trends in Parasitology. 2015; 31(2):43–5. Epub 2015/02/05. https://doi.org/10.1016/j.pt.2014.12.008 PMID: 25649340; PubMed Central PMCID: PMCPMC4583061.
7. van Benthem B, Lambin E, Somboon P, Panart K, Oskam L, Vanwambeke S, et al. Spatial patterns of and risk factors for seropositivity for dengue infection. The American Journal of Tropical Medicine and Hygiene. 2005; 72(2): 201–8. PMID: 15741558
8. Kraemer MUG, Sinka ME, Duda KA, Mylne A, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife. 2015;10.7554(eLife.08347).
9. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, et al. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. Journal of Virology. 2010; 84(13):6497–504. Epub 2010/04/23. https://doi.org/10.1128/JVI.01603-09 PMID: 20410280; PubMed Central PMCID: PMC2903258.
10. Staples JE, Breiman RF, Powers AM. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clinical Infectious Diseases. 2009; 49(6):942–8. Epub 2009/08/12. https://doi.org/10.1086/605496 PMID: 19663604.
11. Cunha RV, Trinta KS, Montalbano CA, Sucupira MV, de Lima MM, Marques E, et al. Seroprevalence of Chikungunya Virus in a Rural Community in Brazil. PLoS Negl Trop Dis. 2017; 11(1):e0005319. Epub 2017/01/21. https://doi.org/10.1371/journal.pntd.0005319 PMID: 28107342; PubMed Central PMCID: PMCPMC5287455.
12. Figueiredo LTM. Large outbreaks of Chikungunya virus in Brazil reveal uncommon clinical features and fatalities. Revista da Sociedade Brasileira de Medicina Tropical. 2017; 50(5):583–4. Epub 2017/11/22. https://doi.org/10.1590/0037-8682-0397-2017 PMID: 29160502.
13. Dias JP, Costa M, Campos GS, Paixao ES, Natividade MS, Barreto FR, et al. Seroprevalence of Chikungunya Virus after Its Emergence in Brazil. Emerging Infectious Diseases. 2018; 24(4):617–24. Epub 2018/03/20. https://doi.org/10.3201/eid2404.171370 PMID: 29553317; PubMed Central PMCID: PMCPMC5875253.
14. Brito CAA, Teixeira MG. Increased number of deaths during a chikungunya epidemic in Pernambuco, Brazil. Memorias do Instituto Oswaldo Cruz. 2017; 112(9):650–1. Epub 2017/09/14. https://doi.org/10.1590/0074-02760170124 PMID: 28902292; PubMed Central PMCID: PMCPMC5572452.

15. Freitas ARR, Cavalcanti L, Von Zuben AP, Donalisio MR. Excess Mortality Related to Chikungunya Epidemics in the Context of Co-circulation of Other Arboviruses in Brazil. PLoS Currents. 2017; 9. Epub 2017/12/22. https://doi.org/10.1371/currents.outbreaks.14608e586cd321d8d5088652d7a0d884 PMID: 29263941; PubMed Central PMCID: PMCPMC5731794.

16. Cunha MDP, Santos CAD, Neto DFL, Schanoski AS, Pour SZ, Passos SD, et al. Outbreak of chikungunya virus in a vulnerable population of Sergipe, Brazil-A molecular and serological survey. Journal of Clinical Virology. 2017; 97:44–9. Epub 2017/11/04. https://doi.org/10.1016/j.jcv.2017.10.015 PMID: 29100064.

17. Silva MM, Rodrigues MS, Paploski IA, Kikuti M, Kasper AM, Cruz JS, et al. Accuracy of Dengue Reporting by National Surveillance System, Brazil. Emerging Infectious Diseases. 2016; 22(2):336–9. Epub 2016/01/27. https://doi.org/10.3201/eid2202.150495 PMID: 26812472; PubMed Central PMCID: PMCPMC4734515.

18. Pessoa R, Patriota JV, Lourdes de Souza M, Felix AC, Mamede N, Sanabani SS. Investigation Into an Outbreak of Dengue-like Illness in Pernambuco, Brazil, Revealed a Cocirculation of Zika, Chikungunya, and Dengue Virus Type 1. Medicine. 2016; 95(12):e3201. Epub 2016/03/26. https://doi.org/10.1097/MD.0000000000003201 PMID: 27015222; PubMed Central PMCID: PMCPMC4998417.

19. Sardi SI, Somasekar S, Naccache SN, Bandeira AC, Tauro LB, Campos GS, et al. Coinfections of Zika and Chikungunya Viruses in Bahia, Brazil, Identified by Metagenomic Next-Generation Sequencing. Journal of Clinical Microbiology. 2016; 54(9):2348–53. https://doi.org/10.1128/JCM.00877-16 PMID: 27413190; PubMed Central PMCID: PMCPMC5005514.

20. Cardoso CW, Paploski IA, Kikuti M, Rodrigues MS, Silva MM, Campos GS, et al. Outbreak of Exanthematous Illness Associated with Zika, Chikungunya, and Dengue Viruses, Salvador, Brazil. Emerging Infectious Diseases. 2015; 21(12):2274–6. Epub 2015/11/20. https://doi.org/10.3201/eid2112.151167 PMID: 26584464; PubMed Central PMCID: PMCPMC4672408.

21. Mavian C, Rife BD, Dollar JJ, Cella E, Ciccozzi M, Prosperi MCF, et al. Emergence of recombinant Mayaro virus strains from the Amazon basin. Scientific Reports. 2017; 7(1):8718. Epub 2017/08/20. https://doi.org/10.1038/s41598-017-07152-5 PMID: 28821712; PubMed Central PMCID: PMCPMC5562835.

22. Vieira CJ, Silva DJ, Barreto ES, Siqueira CE, Colombo TE, Ozanic K, et al. Detection of Mayaro virus infections during a dengue outbreak in Mato Grosso, Brazil. Acta Trop. 2015; 147:12–6. Epub 2015/03/31. https://doi.org/10.1016/j.actatropica.2015.03.020 PMID: 25817238.

23. Del Valle SY, McMahon BH, Asher J, Hatchett R, Lega JC, Brown HE, et al. Summary results of the 2014–2015 DARPA Chikungunya challenge. BMC Infectious Diseases. 2018; 18(1):245. Epub 2018/05/31. https://doi.org/10.1186/s12879-018-3124-7 PMID: 29843621; PubMed Central PMCID: PMCPMC5975673.

24. Silva NMD, Teixeira RAG, Cardoso CG, Siqueira Junior JB, Coelho GE, Oliveira ESF. Chikungunya surveillance in Brazil: challenges in the context of Public Health. Epidemiol Serv Saude. 2018; 27(3):e2017127. Epub 2018/09/06. https://doi.org/10.5123/S1679-49742018000300003 PMID: 30183867.

25. Bogoch II, Brady OJ, Kraemer MUG, German M, Creatore MI, Kulkarni MA, et al. Anticipating the international spread of Zika virus from Brazil. Lancet. 2016; 387(10016):335–6. Epub 2016/01/19. https://doi.org/10.1016/S0140-6736(16)00080-5 PMID: 26777915; PubMed Central PMCID: PMCPMC4873159.

26. Nunes MR, Faria NR, Vasconcelos HB, Medeiros DB, Silva de Lima CP, Carvalho VL, et al. Phylogeography of dengue virus serotype 4, Brazil, 2010–2011. Emerging Infectious Diseases. 2012; 18(11):1858–64. https://doi.org/10.3201/eid1811.120217 PMID: 23092706; PubMed Central PMCID: PMC3559147.

27. Faria NR, Kraemer MUG, Hill SC, Goes de Jesus J, Aguiar RS, Iani FCM, et al. Genomic and epidemiological monitoring of yellow fever virus transmission potential. Science. 2018; 361(6405):894–9. Epub 2018/08/25. https://doi.org/10.1126/science.aat7115 PMID: 30139911.

28. Olival KJ, Hosseini PR, Zambrana-Torrelio C, Ross N, Bogich TL, Daszak P. Host and viral traits predict zoonotic spillover from mammals. Nature. 2017; 546(7660):646–50. Epub 2017/06/22. https://doi.org/10.1038/nature22975 PMID: 28636590; PubMed Central PMCID: PMCPMC5570460.

29. Weiss DJ, Nelson A, Gibson HS, Temperley W, Peedell S, Lieber A, et al. A global map of travel time to cities to assess inequalities in accessibility in 2015. Nature. 2018; 553(7688):333–6. Epub 2018/01/11. https://doi.org/10.1038/nature25181 PMID: 29320477.

30. Naveca FG, Nascimento VAD, Souza VC, Nunes BTD, Rodrigues DSG, Vasconcelos P. Multiplexed reverse transcription real-time polymerase chain reaction for simultaneous detection of Mayaro, Oropouche, and Oropouche-like viruses. Memorias do Instituto Oswaldo Cruz. 2017; 112(7):510–3. Epub 2017/06/08. https://doi.org/10.1590/0074-02760160062 PMID: 28591313; PubMed Central PMCID: PMCPMC5452489.

31. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. Chikungunya virus in US travelers returning from India, 2006. Emerging Infectious Diseases. 2007; 13(5):764–7. https://doi.org/10.3201/eid1305.070015 PMID: 17553261; PubMed Central PMCID: PMCPMC2738459.

32. Faria NR, Quick J, Claro IM, Theze J, de Jesus JG, Giovanetti M, et al. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. Nature. 2017; 546(7658):406–10. Epub 2017/05/26. https://doi.org/10.1038/nature22401 PMID: 28538727; PubMed Central PMCID: PMCPMC5722632.

33. Quick J. G, N. D., Pullan S. T., Claro I. M., Smith A. D., Gangavarapu k., Oliveira G., Robles-Sikisaka R., Rogers T. F., Beutler N. A., Burton D. R., Lewis-Ximenez L. L., de Jesus J. G., Giovanetti M., Hill S., Black A., Bedford T., Carroll M. W., Nunes M., Alcantara L. C., Sabino E. C., Baylis S. A., Faria N. R., Loose M., Simpson J. T., Pybus O. G, Andersen K. G., Loman N. J. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nature Protocols. 2017; 12:1261–1276. https://doi.org/10.1038/nprot.2017.066 PMID: 28538739

34. Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Research. 2016; 44(D1):D67–72. https://doi.org/10.1093/nar/gkv1276 PMID: 26590407; PubMed Central PMCID: PMCPMC4702903.

35. da Costa AC, Thézé J., Komninakis S. C. V., Sanz-Duro R. L., Felinto, M. R. L., Moura L. C. C., Barroso I. M. O., Santos L. E. C., Nunes M. A., L., Moura A. A., Lourenco J., Deng X., Delwart E. L., Guimaraes M. R. A. S., Pybys O. G., Sabino E. C., Faria N. R. Spread of Chikungunya Virus East/Central/South African Genotype in Northeast Brazil. Emerging Infectious Diseases. 2017; 23(10).

36. Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. Genetics. 2006; 172(4):2665–81. https://doi.org/10.1534/genetics.105.048975 PMID: 16489234; PubMed Central PMCID: PMC1456386.

37. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014; 30(9):1312–3. https://doi.org/10.1093/bioinformatics/btu033 PMID: 24451623; PubMed Central PMCID: PMCPMC3998144.

38. Rambaut A, Lam TT, Fagundes de Carvalho L, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evolution. 2016; 2(vew007).

39. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol. 2018; 4(1):vey016. Epub 2018/06/27. https://doi.org/10.1093/ve/vey016 PMID: 29942656; PubMed Central PMCID: PMCPMC6007674.

40. Drummond AJ, Rambaut A, Shapiro B, Pybus OG. Bayesian coalescent inference of past population dynamics from molecular sequences. Molecular Biology and Evolution. 2005; 22(5):1185–92. https://doi.org/10.1093/molbev/msi103 PMID: 15703244.

41. Minin VN, Bloomquist EW, Suchard MA. Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. Molecular Biology and Evolution. 2008; 25(7):1459–71. Epub 2008/04/15. https://doi.org/10.1093/molbev/msn090 PMID: 18408232; PubMed Central PMCID: PMC3302198.

42. Gill MS, Lemey P, Faria NR, Rambaut A, Shapiro B, Suchard MA. Improving Bayesian population dynamics inference: a coalescent-based model for multiple loci. Molecular Biology and Evolution. 2013; 30(3):713–24. https://doi.org/10.1093/molbev/mss265 PMID: 23180580; PubMed Central PMCID: PMC3563973.

43. Drummond AJ, Ho SY, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. PLoS Biology. 2006; 4(5):e88. https://doi.org/10.1371/journal.pbio.0040088 PMID: 16683862; PubMed Central PMCID: PMC1395354.

44. Lourenço J, Monteiro M. L., Valdez T., Rodrigues J. M., Pybus O. G., Faria N. R. Epidemiology of the Zika virus outbreak in the Cabo Verde Islands, West Africa. PLoS Currents. 2018. https://doi.org/10.1371/currents.outbreaks.19433b1e4d007451c691f138e1e67e8c PMID: 29637009

45. Salje H, Lessler J, Paul KK, Azman AS, Rahman MW, Rahman M, et al. How social structures, space, and behaviors shape the spread of infectious diseases using chikungunya as a case study. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113(47):13420–5. Epub 2016/11/09. https://doi.org/10.1073/pnas.1611391113 PMID: 27821727; PubMed Central PMCID: PMCPMC5127331.

46. Chan EH, Sahai V, Conrad C, Brownstein JS. Using web search query data to monitor dengue epidemics: a new model for neglected tropical disease surveillance. PLoS Negl Trop Dis. 2011; 5(5):e1206. Epub 2011/06/08. https://doi.org/10.1371/journal.pntd.0001206 PMID: 21647308; PubMed Central PMCID: PMCPMC3104029.

47. Teng Y, Bi D, Xie G, Jin Y, Huang Y, Lin B, An X, Feng D, Tong Y, Dynamic Forecasting of Zika Epidemics Using Google Trends. PLoS One 2017; 12(1)e0165085. https://doi.org/10.1371/journal.pone.0165085 PMID: 28060809

48. Team RC. R: A Language and Environment for Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.

49. Tsetsarkin KA, McGee CE, Volk SM, Vanlandingham DL, Weaver SC, Higgs S. Epistatic roles of E2 glycoprotein mutations in adaption of chikungunya virus to Aedes albopictus and Ae. aegypti mosquitoes. PloS one. 2009; 4(8):e6835. Epub 2009/09/01. https://doi.org/10.1371/journal.pone.0006835 PMID: 19718263; PubMed Central PMCID: PMCPMC2729410.

50. Tanabe ELL, Tanabe ISB, Santos ECD, Marques J, Borges AA, Lima MC, et al. Report of East-Central South African Chikungunya virus genotype during the 2016 outbreak in the Alagoas State, Brazil. Revista do Instituto de Medicina Tropical de Sao Paulo. 2018; 60:e19. Epub 2018/04/26. https://doi.org/10.1590/s1678-9946201860019 PMID: 29694603; PubMed Central PMCID: PMCPMC5956549.

51. Charlys da Costa A, Theze J, Komninakis SCV, Sanz-Duro RL, Felinto MRL, Moura LCC, et al. Spread of Chikungunya Virus East/Central/South African Genotype in Northeast Brazil. Emerging infectious diseases. 2017; 23(10):1742–4. Epub 2017/09/21. https://doi.org/10.3201/eid2310.170307 PMID: 28930031; PubMed Central PMCID: PMCPMC5621546.

52. Robinson M, Conan A, Duong V, Ly S, Ngan C, Buchy P, et al. A model for a chikungunya outbreak in a rural Cambodian setting: implications for disease control in uninfected areas. PLoS Negl Trop Dis. 2014; 8(9):e3120. Epub 2014/09/12. https://doi.org/10.1371/journal.pntd.0003120 PMID: 25210729; PubMed Central PMCID: PMC4161325.

53. Perkins TA, Metcalf CJ, Grenfell BT, Tatem AJ. Estimating drivers of autochthonous transmission of chikungunya virus in its invasion of the americas. PLoS currents. 2015; 7. Epub 2015/03/05. https://doi.org/10.1371/currents.outbreaks.a4c7b6ac10e0420b1788c9767946d1fc PMID: 25737803; PubMed Central PMCID: PMC4339250.

54. Salje H, Cauchemez S, Alera MT, Rodriguez-Barraquer I, Thaisomboonsuk B, Srikiatkhachorn A, et al. Reconstruction of 60 Years of Chikungunya Epidemiology in the Philippines Demonstrates Episodic and Focal Transmission. The Journal of infectious diseases. 2016; 213(4):604–10. Epub 2015/09/28. https://doi.org/10.1093/infdis/jiv470 PMID: 26410592; PubMed Central PMCID: PMCPMC4721913.

55. Dias JP, Costa MCN, Campos GS, Paixao ES, Natividadde MS, Barreto FR, et al. Seroprevalence of Chikungunya Virus after Its Emergence in Brazil. Emerging infectious diseases. 2018; 24(4):617–24. https://doi.org/10.3201/eid2404.171370 PMID: 29553317

56. Bustos Carrillo F, Collado D, Sanchez N, Ojeda S, Lopez Mercado B, Burger-Calderon R, et al. Epidemiological Evidence for Lineage-specific Differences in the Risk of Inapparent Chikungunya Virus Infection. Journal of virology. 2018. Epub 2018/11/23. https://doi.org/10.1128/JVI.01622-18 PMID: 30463967.

57. Sissoko D, Moendandze A, Malvy D, Giry C, Ezzedine K, Solet JL, et al. Seroprevalence and risk factors of chikungunya virus infection in Mayotte, Indian Ocean, 2005–2006: a population-based survey. PloS one. 2008; 3(8):e3066. Epub 2008/08/30. https://doi.org/10.1371/journal.pone.0003066 PMID: 18725980; PubMed Central PMCID: PMCPMC2518850.

58. Hassing RJ, Leparc-Goffart I, Tolou H, van Doornum G, van Genderen PJ. Cross-reactivity of antibodies to viruses belonging to the Semliki forest serocomplex. Euro Surveillance. 2010; 15(23). Epub 2010/06/16. PMID: 20546691.

59. Powers AM, Brault AC, Shirako Y, Strauss EG, Kang W, Strauss JH, et al. Evolutionary relationships and systematics of the alphaviruses. Journal of Virology. 2001; 75(21):10118–31. Epub 2001/10/03. https://doi.org/10.1128/JVI.75.21.10118-10131.2001 PMID: 11581380; PubMed Central PMCID: PMC114586.

60. Vasconcelos PFC, Travassos da Rosa A. P. A., Degallier N., Travassos da Rosa J. F. S., Pinheiro F. R. Clinical and ecoepidemiological situation of human arboviruses in Brazilian Amazonia. Journal of the Brazilian Association for the Advancement of Science. 1992; 43(2):117–24.

61. Mourao MP, Bastos Mde S, de Figueiredo RP, Gimaque JB, Galusso Edos S, Kramer VM, et al. Mayaro fever in the city of Manaus, Brazil, 2007–2008. Vector Borne and Zoonotic Diseases. 2012; 12(1):42–6. Epub 2011/09/20. https://doi.org/10.1089/vbz.2011.0669 PMID: 21923266; PubMed Central PMCID: PMCPMC3249893.

62. Receveur MC, Grandadam M, Pistone T, Malvy D. Infection with Mayaro virus in a French traveller returning from the Amazon region, Brazil, January, 2010. Euro Surveillance. 2010; 15(18). Epub 2010/05/13. PMID: 20460093.

63. Azevedo RS, Silva EV, Carvalho VL, Rodrigues SG, Nunes-Neto JP, Monteiro H, et al. Mayaro fever virus, Brazilian Amazon. Emerging Infectious Diseases. 2009; 15(11):1830–2. Epub 2009/11/07. https://doi.org/10.3201/eid1511.090461 PMID: 19891877; PubMed Central PMCID: PMCPMC2857233.

64. LeDuc JW, Pinheiro FP, Travassos da Rosa AP. An outbreak of Mayaro virus disease in Belterra, Brazil. II. Epidemiology. The American Journal of Tropical Medicine and Hygiene. 1981; 30(3):682–8. Epub 1981/05/01. PMID: 6266264.

65. Causey OR, Maroja OM. Mayaro virus: a new human disease agent. III. Investigation of an epidemic of acute febrile illness on the river Guama in Para, Brazil, and isolation of Mayaro virus as causative agent. The American journal of tropical medicine and hygiene. 1957; 6(6):1017–23. Epub 1957/11/01. PMID: 13487974.

66. Serra OP, Cardoso BF, Ribeiro AL, Santos FA, Slhessarenko RD. Mayaro virus and dengue virus 1 and 4 natural infection in culicids from Cuiaba, state of Mato Grosso, Brazil. Memorias do Instituto Oswaldo Cruz. 2016; 111(1):20–9. Epub 2016/01/20. https://doi.org/10.1590/0074-02760150270 PMID: 26784852; PubMed Central PMCID: PMCPMC4727432.

67. da Costa VG, de Rezende Feres VC, Saivish MV, de Lima Gimaque JB, Moreli ML. Silent emergence of Mayaro and Oropouche viruses in humans in Central Brazil. International journal of infectious diseases. 2017; 62:84–5. Epub 2017/07/26. https://doi.org/10.1016/j.ijid.2017.07.016 PMID: 28739422.

68. Brunini S, Franca DDS, Silva JB, Silva LN, Silva FPA, Spadoni M, et al. High Frequency of Mayaro Virus IgM among Febrile Patients, Central Brazil. Emerging infectious diseases. 2017; 23(6):1025–6. Epub 2017/05/19. https://doi.org/10.3201/eid2306.160929 PMID: 28518022; PubMed Central PMCID: PMCPMC5443426.

69. Pauvolid-Correa A, Juliano RS, Campos Z, Velez J, Nogueira RM, Komar N. Neutralising antibodies for Mayaro virus in Pantanal, Brazil. Memorias do Instituto Oswaldo Cruz. 2015; 110(1):125–33. Epub 2015/03/06. https://doi.org/10.1590/0074-02760140383 PMID: 25742272; PubMed Central PMCID: PMCPMC4371226.

70. Zuchi N, Heinen LB, Santos MA, Pereira FC, Slhessarenko RD. Molecular detection of Mayaro virus during a dengue outbreak in the state of Mato Grosso, Central-West Brazil. Memorias do Instituto Oswaldo Cruz. 2014; 109(6):820–3. Epub 2014/08/21. https://doi.org/10.1590/0074-0276140108 PMID: 25141284; PubMed Central PMCID: PMCPMC4238776.

71. Grubaugh ND, Faria NR, Andersen KG, Pybus OG. Genomic Insights into Zika Virus Emergence and Spread. Cell. 2018; 172(6):1160–2. Epub 2018/03/10. https://doi.org/10.1016/j.cell.2018.02.027 PMID: 29522736.

72. Lourenco J, Maia de Lima M, Faria NR, Walker A, Kraemer MU, Villabona-Arenas CJ, et al. Epidemiological and ecological determinants of Zika virus transmission in an urban setting. eLife. 2017; 6. Epub 2017/09/10. https://doi.org/10.7554/eLife.29820 PMID: 28887877; PubMed Central PMCID: PMCPMC5638629.

73. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife. 2015; 4:e08347. Epub 2015/07/01. https://doi.org/10.7554/eLife.08347 PMID: 26126267; PubMed Central PMCID: PMC4493616.

74. Aragao CF, Cruz ACR, Nunes Neto JP, Monteiro HAO, da Silva EVP, da Silva SP, et al. Circulation of Chikungunya virus in Aedes aegypti in Maranhao, Northeast Brazil. Acta Trop. 2018; 186:1–4. Epub 2018/06/23. https://doi.org/10.1016/j.actatropica.2018.06.022 PMID: 29932932.

75. Souza TM, Azeredo EL, Badolato-Correa J, Damasco PV, Santos C, Petitinga-Paiva F, et al. First Report of the East-Central South African Genotype of Chikungunya Virus in Rio de Janeiro, Brazil. PLoS Currents. 2017; 9. Epub 2017/03/14. https://doi.org/10.1371/currents.outbreaks.4200119978d62ccaa454599cd2735727 PMID: 28286701; PubMed Central PMCID: PMCPMC5325710.

76. Faria NR, da Costa AC, Lourenco J, Loureiro P, Lopes ME, Ribeiro R, et al. Genomic and epidemiological characterisation of a dengue virus outbreak among blood donors in Brazil. Scientific Reports. 2017; 7(1):15216. Epub 2017/11/11. https://doi.org/10.1038/s41598-017-15152-8 PMID: 29123142; PubMed Central PMCID: PMCPMC5680240.

77. Nunes MR, Palacios G, Faria NR, Sousa EC Jr., Pantoja JA, Rodrigues SG, et al. Air travel is associated with intracontinental spread of dengue virus serotypes 1–3 in Brazil. PLoS Negl Trop Dis. 2014; 8(4):e2769. https://doi.org/10.1371/journal.pntd.0002769 PMID: 24743730; PubMed Central PMCID: PMCPMC3990485.

78. White SK, Mavian C, Salemi M, Morris JG Jr., Elbadry MA, Okech BA, et al. A new "American" subgroup of African-lineage Chikungunya virus detected in and isolated from mosquitoes collected in Haiti, 2016. PloS One. 2018; 13(5):e0196857. Epub 2018/05/11. https://doi.org/10.1371/journal.pone.0196857 PMID: 29746539; PubMed Central PMCID: PMCPMC5944945.

79. Obolski U, Perez P. N., Villabobna-Arenas C. J., Faria, N. R., Lourenco J. MVSE: an R-package that estimates a climate-driven mosquito-borne viral suitability index. bioRxiv. 2018. https://doi.org/10.1101/360701.

80. Cherian SS, Walimbe AM, Jadhav SM, Gandhe SS, Hundekar SL, Mishra AC, et al. Evolutionary rates and timescale comparison of Chikungunya viruses inferred from the whole genome/E1 gene with special reference to the 2005–07 outbreak in the Indian subcontinent. Infection, genetics and evolution. 2009; 9(1):16–23. Epub 2008/10/23. https://doi.org/10.1016/j.meegid.2008.09.004 PMID: 18940268.

## 4 DISCUSSÃO GERAL

A emergência e reemergência de alguns arbovírus no Brasil nos últimos anos, principalmente ZIKV, CHIKV e YFV, demonstrou novamente a importância médica e epidemiológica deste grupo de vírus. Apesar da maioria dos casos não apresentarem gravidade, sendo muitos deles até assintomáticos, cada espécie destes vírus pode apresentar sérios problemas para o indivíduo e para a saúde coletiva (Faria et al., 2018; Iani et al., 2021; Naveca et al., 2019).

A epidemia de ZIKV demonstrou ser uma preocupação mundial, principalmente devido a infecções congênitas que ocasionaram um aumento significativo nos casos de microcefalia (Faria et al., 2017). A partir dos dados genômicos e epidemiológicos, foi possível identificar a linhagem asiática circulando em Minas Gerais e no Amazonas. Em ambos os estados foi possível perceber uma circulação silenciosa do vírus por até mais de um ano até ser detectado pelo sistema de vigilância. Também foi possível perceber que a maioria dos casos de microcefalia ocorreram aproximadamente 8 meses após o pico da epidemia em 2016, corroborando outras evidências de que o vírus pode estar relacionado aos casos de microcefalia, principalmente em gestantes no primeiro trimestre, apesar do ZIKV ser transmitido durante toda a gestação (Baud et al., 2017; Giovanetti et al., 2020; Iani et al., 2021).

Já a epidemia de febre amarela, mesmo que restrita ao ciclo silvestre, demonstrou uma alta taxa de letalidade (33,6 %), semelhante ao encontrado em epidemias anteriores (Faria et al., 2018), e o alto risco que a possível urbanização desta doença pode ocasionar. O sequenciamento de YFV também demonstrou utilidade para descartar possíveis infecções relacionadas à reativação do vírus após vacinação, uma vez que os indivíduos vacinados apresentaram infecção pelo genótipo circulante (América do Sul I) e não pelo vacinal (Oeste Africano) (Faria et al., 2018).

A limitação de indivíduos por longos períodos, até mesmo anos, causada pela febre chikungunya explicitou o potencial de uma epidemia de CHIKV na população (Dias et al., 2018). Através da vigilância genômica foi identificado a substituição da linhagem asiática, a primeira a ser introduzida no norte do Brasil, pela linhagem africana (ECSA). Foi estimado que esta linhagem possivelmente infectou 39% da população de Roraima na epidemia de 2017 (Naveca et al., 2019). As diferentes linhagens desencadeiam diferentes manifestações clínicas e o conhecimento da linhagem circulante pode ajudar nas intervenções da vigilância epidemiológica (Naveca et al., 2019).

As informações obtidas nos genomas virais são uma fonte de informação de extrema importância no entendimento da dinâmica das epidemias. A redução dos custos do sequenciamento genético de nova geração facilitou o acesso de laboratórios públicos a esta tecnologia, permitindo a implantação da vigilância genômica no Lacen-MG. A combinação da vigilância genômica com a vigilância tradicional e ferramentas de bioinformática demostraram ser bastante úteis para auxiliar no enfrentamento de surtos e epidemias em Minas Gerais e no Brasil.

## 5. CONCLUSÃO

O relativo fácil acesso à tecnologia de NGS permitiu ao Lacen-MG implantar as metodologias de sequenciamento necessárias para gerar vários novos genomas de alguns principais arbovírus que circulam no estado de Minas Gerais e no Brasil, como o ZIKV, YFV e CHIKV. Além das metodologias de laboratório úmido, também foi possível implantar todas as etapas de análises de bioinformática, o que deu uma grande autonomia para o Lacen-MG, por não depender mais de parceiros para a realização destas análises, melhorando e acelerando o alcance dos resultados. Com os dados genômicos avaliados de forma conjunta com a epidemiologia tradicional, foi possível estabelecer a história evolutiva e da disseminação geográfica ao longo do tempo destes arbovírus.

Estes resultados rápidos e de qualidade dão um valioso suporte para as autoridades da saúde pública tomarem decisões relacionadas ao enfrentamento de surtos e epidemias. Após a implantação destes protocolos, além dos trabalhos já publicados e submetidos, o Lacen-MG também auxiliou a vigilância estadual na resolução de problemas como infecções vacinais *versus* selvagens para o YFV e vírus do sarampo, além do sequenciamento do genoma completo dos vírus influenza, para auxiliar na atualização vacinal realizada anualmente pelo Ministério da Saúde e Organização Mundial de Saúde.

A geração de dados genômicos virais em tempo real por um Lacen, como demonstrado aqui, contribui de forma substancial para respostas mais efetivas e assertivas pela vigilância em saúde públicas de Minas Gerais e do Brasil.

# 6. Apêndice

## 6.1 Artigos publicados

**I.** Field and Classroom Initiatives For Portable Sequence-Based Monitoring Of Dengue Virus In Brazil. Nature Communications, V. 12, P. 2296, 2021.

**II.** The ongoing COVID-19 epidemic in Minas Gerais, Brazil: insights from epidemiological data and SARS-CoV-2 whole genome sequencing. Emerging Microbes & Infections, v. 9, p. 1824-1834, 2020.

**III.** Dengue diagnostics: serious inaccuracies are likely to occur if pre-analytical conditions are not strictly followed. Memórias do Instituto Oswaldo Cruz, v. 115, p. 1/e200287-7, 2020.

**IV.** Pluripotency of Wolbachia against Arboviruses: the case of yellow fever. Gates Open Research, v. 3, p. 161, 2019.

**V.** Persistence of Yellow fever virus outside the Amazon Basin, causing epidemics in Southeast Brazil, from 2016 to 2018. PLoS Neglected Tropical Diseases, v. 12, p. e0006538, 2018.

**VI.** West Nile Virus in Brazil. PATHOGENS, v. 10, p. 896, 2021.

**VII.** Genomic evidence of SARS-CoV-2 reinfection case with the emerging B.1.2 variant in Brazil. JOURNAL OF INFECTION JCR, v. 83, p. 237-279, 2021.

**VIII.** Promoting Responsible Research and Innovation (RRI) During Brazilian Activities of Genomic and Epidemiological Surveillance of Arboviruses. FRONTIERS IN PUBLIC HEALTH JCR, v. 9, p. 11, 2021.

**IX.** Field and classroom initiatives for portable sequence-based monitoring of dengue virus in Brazil. Nature Communications JCR, v. 12, p. 2296, 2021.

**X.** CCL3, CCL5, IL-15, IL-1Ra and VEGF compose a reliable algorithm to discriminate classes of adverse events following 17DD-YF primary vaccination according to cause-specific definitions. Vaccine. 2021 Jul 13;39(31):4359-4372.

## 6.2 Capitulo de livro

**I.** Pan-genomics of virus and its applications. Pan-genomics: Applications, Challenges, and Future Prospects. 1ed.: Elsevier, 2020, v. , p. 237-250

## 6.3 Artigos submetidos

**I.** Spotted Fever in the morphoclimatic domains of an endemic area in Brazil. Submitted to "Journal of Venomous Animals and Toxins including Tropical Diseases" in 18 March 2021

## 7. REFERÊNCIAS BIBLIOGRÁFICAS


Adebanjo, T., Godfred-Cato, S., Viens, L., Fischer, M., Erin Staples, J., Kuhnert-Tallman, W., Walke, H., Oduyebo, T., Polen, K., Peacock, G., Meaney-Delman, D., Honein, M. A., Rasmussen, S. A., & Moore, C. A. (2006). *Virgin Islands, 2015 1105 Tdap Vaccination Coverage During Pregnancy-Selected Sites*. *66*(41). https://www.cdc.gov/mmwr/cme/conted_info.html#weekly.

Althouse, B. M., Guerbois, M., Cummings, D. A. T., Diop, O. M., Faye, O., Faye, A., Diallo, D., Sadio, B. D., Sow, A., Faye, O., Sall, A. A., Diallo, M., Benefit, B., Simons, E., Watts, D. M., Weaver, S. C., & Hanley, K. A. (2018). Role of monkeys in the sylvatic cycle of chikungunya virus in Senegal. *Nature Communications*, *9*(1), 1–10. https://doi.org/10.1038/s41467-018-03332-7

Assiri, A., Al-Tawfiq, J. A., Al-Rabeeah, A. A., Al-Rabiah, F. A., Al-Hajjar, S., Al-Barrak, A., Flemban, H., Al-Nassir, W. N., Balkhy, H. H., Al-Hakeem, R. F., Makhdoom, H. Q., Zumla, A. I., & Memish, Z. A. (2013). Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: A descriptive study. *The Lancet Infectious Diseases*, *13*(9), 752–761. https://doi.org/10.1016/S1473-3099(13)70204-4

Bastos, M. D. S., Figueiredo, L. T. M., Naveca, F. G., Monte, R. L., Lessa, N., De Figueiredo, R. M. P., Gimaque, J. B. D. L., João, G. P., Ramasawmy, R., & Mourão, M. P. G. (2012). Short report: Identification of oropouche Orthobunyavirus in the cerebrospinal fluid of three patients in the Amazonas, Brazil. *American Journal of Tropical Medicine and Hygiene*, *86*(4), 732–735. https://doi.org/10.4269/ajtmh.2012.11-0485

Baud, D., Gubler, D. J., Schaub, B., Lanteri, M. C., & Musso, D. (2017). An update on Zika virus infection. *The Lancet*, *390*(10107), 2099–2109. https://doi.org/10.1016/S0140-6736(17)31450-2

Boyer, S., Calvez, E., Chouin-Carneiro, T., Diallo, D., & Failloux, A. B. (2018). An overview of mosquito vectors of Zika virus. *Microbes and Infection*, *20*(11–12), 646–660. https://doi.org/10.1016/j.micinf.2018.01.006

Brackney, D. E. (2017). Implications of autophagy on arbovirus infection of mosquitoes. *Current Opinion in Insect Science*, *22*, 1–6. https://doi.org/10.1016/j.cois.2017.05.001

Bryant, J. E., Holmes, E. C., & Barrett, A. D. T. (2007). Out of Africa: A molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS Pathogens*, *3*(5), 0668–0673. https://doi.org/10.1371/journal.ppat.0030075

Burt, F. J., Chen, W., Miner, J. J., Lenschow, D. J., Merits, A., Schnettler, E., Kohl, A., Rudd, P. A., Taylor, A., Herrero, L. J., Zaid, A., Ng, L. F. P., & Mahalingam, S. (2017). Chikungunya virus: an update on the biology and pathogenesis of this emerging pathogen. *The Lancet Infectious Diseases*, *17*(4), e107–e117. https://doi.org/10.1016/S1473-3099(16)30385-1

Cardoso, B. F., Serra, O. P., Da Silva Heinen, L. B., Zuchi, N., De Souza, V. C., Naveca, F. G., Dos Santos, M. A. M., & Slhessarenko, R. D. (2015). Detection of oropouche virus segment s in patients and in culex quinquefasciatus in the state of mato grosso, Brazil. *Memorias Do Instituto Oswaldo Cruz*, *110*(6), 745–754. https://doi.org/10.1590/0074-02760150123

Chen, S., Wu, Z., Wang, M., & Cheng, A. (2017). Innate immune evasion mediated by flaviviridae non-structural proteins. *Viruses*, *9*(10), 1–19. https://doi.org/10.3390/v9100291

Chippaux, J., & Chippaux, A. (2018). Yellow fever in Africa and the Americas: a historical and epidemiological perspective. *Journal of Venomous Animals and Toxins Including Tropical*

*Diseases*, *24*(1), 20. https://doi.org/10.1186/s40409-018-0162-y

Cleton, N., Koopmans, M., Reimerink, J., Godeke, G. J., & Reusken, C. (2012). Come fly with me: Review of clinically important arboviruses for global travelers. *Journal of Clinical Virology*, *55*(3), 191–203. https://doi.org/10.1016/j.jcv.2012.07.004

Contopoulos-Ioannidis, D., Newman-Lindsay, S., Chow, C., & LaBeaud, A. D. (2018). Mother-to-child transmission of Chikungunya virus: A systematic review and meta-analysis. *PLoS Neglected Tropical Diseases*, *12*(6), 1–20. https://doi.org/10.1371/journal.pntd.0006510

Couto-Lima, Di., Madec, Y., Bersot, M. I., Campos, S. S., Motta, M. D. A., Dos Santos, F. B., Vazeille, M., Da Costa Vasconcelos, P. F., Lourenço-De-Oliveira, R., & Failloux, A. B. (2017). Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent Aedes populations. *Scientific Reports*, *7*(1), 1–12. https://doi.org/10.1038/s41598-017-05186-3

Dias, J. P., Costa, M. da C. N., Campos, G. S., Paixão, E. S., Natividade, M. S., Barreto, F. R., Itaparica, M. S. C., Goes, C. de S. B., Oliveira, F. L. S., Santana, E. B., Silva, N. S. J., Brito, C. A. A., Rodrigues, L. C., Sardi, S. I., Saavedra, R. C., & Teixeira, M. G. (2018). Seroprevalence of Chikungunya virus in 2 urban areas of Brazil 1 year after emergence. *Emerging Infectious Diseases*, *24*(4), 617–624. https://doi.org/10.3201/eid2404.171370

Faria, N. R., Do Socorro Da Silva Azevedo, R., Kraemer, M. U. G., Souza, R., Cunha, M. S., Hill, S. C., Thézé, J., Bonsall, M. B., Bowden, T. A., Rissanen, I., Rocco, I. M., Nogueira, J. S., Maeda, A. Y., Da Silva Vasami, F. G., De Lima Macedo, F. L., Suzuki, A., Rodrigues, S. G., Cruz, A. C. R., Nunes, B. T., … Vasconcelos, P. F. C. (2016). Zika virus in the Americas: Early epidemiological and genetic findings. *Science*, *352*(6283), 345–349. https://doi.org/10.1126/science.aaf5036

Faria, N. R., Kraemer, M. U. G. G., Hill, S. C., Jesus, J. G. de, Aguiar, R. S., Iani, F. C. M. M., Xavier, J., Quick, J., Plessis, L. du, Dellicour, S., Thézé, J., Carvalho, R. D. O. O., Baele, G., Wu, C.-H. H., Silveira, P. P., Arruda, M. B., Pereira, M. A., Pereira, G. C., Lourenço, J., … Pybus, O. G. (2018). Genomic and epidemiological monitoring of yellow fever virus transmission potential. *Science*. https://doi.org/10.1126/science.aat7115

Faria, N. R., Quick, J., Claro, I. M., Thézé, J., De Jesus, J. G., Giovanetti, M., Kraemer, M. U. G., Hill, S. C., Black, A., Da Costa, A. C., Franco, L. C., Silva, S. P., Wu, C. H., Raghwani, J., Cauchemez, S., Du Plessis, L., Verotti, M. P., De Oliveira, W. K., Carmo, E. H., … Pybus, O. G. (2017). Establishment and cryptic transmission of Zika virus in Brazil and the Americas. *Nature*, *546*(7658), 406–410. https://doi.org/10.1038/nature22401

Faye, O., Faye, O., Diallo, D., Diallo, M., Weidmann, M., & Sall, A. A. (2013). Quantitative real-time PCR detection of Zika virus and evaluation with field-caught Mosquitoes. *Virology Journal*, *10*(1), 1. https://doi.org/10.1186/1743-422X-10-311

Figueiredo, M. L. G. de, & Figueiredo, L. T. M. (2014). Review on Infections of the Central Nervous System by St. Louis Encephalitis, Rocio and West Nile Flaviviruses in Brazil, 2004-2014. *Advances in Microbiology*, *04*(13), 955–961. https://doi.org/10.4236/aim.2014.413106

Foy, B. D., Kobylinski, K. C., Foy, J. L. C., Blitvich, B. J., da Rosa, A. T., Haddow, A. D., Lanciotti, R. S., & Tesh, R. B. (2011). Probable Non-Vector-borne Transmission of Zika Virus, Colorado, USA. *Emerging Infectious Diseases*, *17*(5), 880–882. https://doi.org/10.3201/eid1705.101939

Frierson, J. G. (2010). The yellow fever vaccine: A history. *Yale Journal of Biology and Medicine*, *83*(2), 77–85.

Galán-Huerta, K. A., Rivas-Estilla, A. M., Fernández-Salas, I., Farfan-Ale, J. A., & Ramos-Jiménez, J. (2015). Chikungunya virus: A general overview. *Medicina Universitaria*, *17*(68), 175–183. https://doi.org/10.1016/j.rmu.2015.06.001

Gao, S., Song, S., & Zhang, L. (2019). Recent Progress in Vaccine Development Against

Chikungunya Virus. *Frontiers in Microbiology*, *10*(December). https://doi.org/10.3389/fmicb.2019.02881

Gardner, C. L., & Ryman, K. D. (2010). Yellow fever: a reemerging threat. (Special Issue: Emerging pathogens.). *Clinics in Laboratory Medicine*, *30*(1), 237–260. https://doi.org/10.1016/j.cll.2010.01.001.Yellow

Giovanetti, M., Faria, N. R., Lourenço, J., Goes de Jesus, J., Xavier, J., Claro, I. M., Kraemer, M. U. G., Fonseca, V., Dellicour, S., Thézé, J., da Silva Salles, F., Gräf, T., Silveira, P. P., do Nascimento, V. A., Costa de Souza, V., de Melo Iani, F. C., Castilho-Martins, E. A., Cruz, L. N., Wallau, G., … Alcantara, L. C. (2020). Genomic and Epidemiological Surveillance of Zika Virus in the Amazon Region. *Cell Reports*, *30*(7), 2275-2283.e7. https://doi.org/10.1016/j.celrep.2020.01.085

Gubler, D. J., & Department. (2002). The Global Emergence/Resurgence of Arboviral Diseases As Public Health Problems Duane. *Control of Communicable Diseases Manual*, *33*, 330–342. https://doi.org/10.2105/ccdm.2745.022

Haby, M. M., Pinart, M., Elias, V., & Reveiz, L. (2018). Prevalence of asymptomatic Zika virus infection: A systematic review. *Bulletin of the World Health Organization*, *96*(6), 402-413D. https://doi.org/10.2471/BLT.17.201541

Heinz, F. X., & Allison, S. L. (2003). Flavivirus Structure and Membrane Fusion. *Advances in Virus Research*, *59*, 63–97. https://doi.org/10.1016/S0065-3527(03)59003-0

Holbrook, M. R. (2017). Historical perspectives on flavivirus research. *Viruses*, *9*(5), 1–19. https://doi.org/10.3390/v9050097

Holmes, A. C., Basore, K., Fremont, D. H., & Diamond, M. S. (2020). A molecular understanding of alphavirus entry. *PLoS Pathogens*, *16*(10), 1–20. https://doi.org/10.1371/journal.ppat.1008876

Iani, F. C. M., Giovanetti, M., Fonseca, V., Souza, W. M., Adelino, T. E. R., Xavier, J., Jesus, J. G., Pereira, M. A., Silva, M. V. F., Costa, A. V. B., Silva, E. C., Mendes, M. C. O., Filippis, A. M. B., Albuquerque, C. F. C., Abreu, A. L., Oliveira, M. A. A., Alcantara, L. C. J., & Faria, N. R. (2021). Epidemiology and evolution of Zika virus in Minas Gerais, Southeast Brazil. *Infection, Genetics and Evolution*, *91*(October 2020), 1–7. https://doi.org/10.1016/j.meegid.2021.104785

Ioos, S., Mallet, H. P., Leparc Goffart, I., Gauthier, V., Cardoso, T., & Herida, M. (2014). Current Zika virus epidemiology and recent epidemics. *Medecine et Maladies Infectieuses*, *44*(7), 302–307. https://doi.org/10.1016/j.medmal.2014.04.008

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990–993. https://doi.org/10.1038/nature06536

Li, Y., & Yang, Z. (2017). Adaptive diversification between yellow fever virus West African and South American Lineages: A genome-wide study. *American Journal of Tropical Medicine and Hygiene*, *96*(3), 727–734. https://doi.org/10.4269/ajtmh.16-0698

Lindenbach, B. D., & Rice, C. M. (2007). Flaviviridae: The Viruses and Their Replication. *Fields Virology*, 1101–1151.

Litvoc, M. N., Novaes, C. T. G., & Lopes, M. I. B. F. (2018). Yellow fever. *Revista Da Associação Médica Brasileira*, *64*(2), 106–113. https://doi.org/10.1590/1806-9282.64.02.106

Lopes, N., Nozawa, C., & Linhares, R. E. C. (2014). Características gerais e epidemiologia dos arbovírus emergentes no Brasil. *Revista Pan-Amazônica de Saúde*, *5*(3), 55–64. https://doi.org/10.5123/s2176-62232014000300007

MacNamara, F. N. (1954). Zika virus: A report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *48*(2), 139–145. https://doi.org/10.1016/0035-9203(54)90006-1

Marchette, N. J., Garcia, R., & Rudnick, A. (1969). Isolation of Zika Virus from Aedes Aegypti Mosquitoes in Malaysia. *The American Journal of Tropical Medicine and Hygiene*, *18*(3), 411–415. https://doi.org/10.4269/ajtmh.1969.18.411

Ministério da Saúde. (2017). *Boletim Epidemiológico Secretaria de Vigilância em Saúde − Ministério da Saúde - até a Semana Epidemiológica 52, 2016* (Vol. 48, Issue Tabela 2). http://portalarquivos2.saude.gov.br/images/pdf/2017/abril/06/2017-002-Monitoramento-dos-casos-de-dengue--febre-de-chikungunya-e-febre-pelo-v--rus-Zika-ate-a-Semana-Epidemiologica-52--2016.pdf

Monath, T. P. (2001). Yellow fever: An update. *Lancet Infectious Diseases*, *1*(1), 11–20. https://doi.org/10.1016/S1473-3099(01)00016-0

Moreli, M. L., & Costa, V. G. da. (2013). A systematic review of molecular diagnostic methods for the detection of arboviruses in clinical specimens in Brazil and the importance of a differential diagnosis. *Virology Discovery*, *1*(1), 1. https://doi.org/10.7243/2052-6202-1-1

Mourão, M. P. G., Bastos, M. S., Gimaque, J. B. L., Mota, B. R., Souza, G. S., Grimmer, G. H. N., Galusso, E. S., Arruda, E., & Figueiredo, L. T. M. (2009). Oropouche fever outbreak, Manaus, Brazil, 2007-2008. *Emerging Infectious Diseases*, *15*(12), 2063–2064. https://doi.org/10.3201/eid1512.090917

Muktar, Y., Tamerat, N., & Shewafera, A. (2016). Aedes aegypti as a Vector of Flavivirus. *Journal of Tropical Diseases*, *04*(05). https://doi.org/10.4172/2329-891x.1000223

Musso, D., Nhan, T., Robin, E., Roche, C., Bierlaire, D., Zisou, K., Shan Yan, A., Cao-Lormeau, V. M., & Broult, J. (2014). Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. *Eurosurveillance*, *19*(14), 20761. https://doi.org/10.2807/1560-7917.ES2014.19.14.20761

Musso, Didier, & Gubler, D. J. (2016). Zika virus. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00072-15

Musso, Didier, Ko, A. I., & Baud, D. (2019). Zika Virus Infection — After the Pandemic. *New England Journal of Medicine*, *381*(15), 1444–1457. https://doi.org/10.1056/nejmra1808246

Musso, Didier, Roche, C., Robin, E., Nhan, T., Teissier, A., & Cao-Lormeau, V. M. (2015). Potential sexual transmission of zika virus. *Emerging Infectious Diseases*, *21*(2), 359–361. https://doi.org/10.3201/eid2102.141363

Naveca, F. G., Christofferson, R. C., Claro, I., Giovanetti, M., Jesus, J. G. de, Xavier, J., Iani, F. C. de M., Nascimento, V. A. do, Souza, V. C. de, Silveira, P. P., Lourenço, J., Santillana, M., Kraemer, M. U. G., Quick, J., Hill, S. C., Thézé, J., Carvalho, R. D. de O., Azevedo, V., Salles, F. C. da S., … Faria, N. R. (2019). Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon. *PLOS Neglected Tropical Diseases*. https://doi.org/10.1371/journal.pntd.0007065

Nunes, M. R. T., Faria, N. R., de Vasconcelos, J. M., Golding, N., Kraemer, M. U. G., de Oliveira, L. F., da Silva Azevedo, R. do S., da Silva, D. E. A., da Silva, E. V. P., da Silva, S. P., Carvalho, V. L., Coelho, G. E., Cruz, A. C. R., Rodrigues, S. G., da Silva Gonçalves Vianez, J. L., Nunes, B. T. D., Cardoso, J. F., Tesh, R. B., Hay, S. I., … da Costa Vasconcelos, P. F. (2015). Emergence and potential for spread of Chikungunya virus in Brazil. *BMC Medicine*, *13*(1). https://doi.org/10.1186/s12916-015-0348-x

Olson, J. G., Ksiazek, T. G., Suhandiman, G., & Triwibowo, V. (1981). Zika virus, a cause of fever in central java, indonesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *75*(3), 389–393. https://doi.org/10.1016/0035-9203(81)90100-0

on Arboviruses, W. H. O. S. G., Disease, H., & Organization, W. H. (1967). *Arboviruses and human disease : report of a WHO scientific group [meeting held in Geneva from 26 September to 1 October 1966]* (p. Russian version of nos. 366-380 bound together (ba).

World Health Organization.

Perrone, L. A., Narayanan, K., Worthy, M., & Peters, C. J. (2007). The S Segment of Punta Toro Virus (Bunyaviridae, Phlebovirus) Is a Major Determinant of Lethality in the Syrian Hamster and Codes for a Type I Interferon Antagonist. *Journal of Virology*, *81*(2), 884–892. https://doi.org/10.1128/JVI.01074-06

Pialoux, G., Gaüzère, B. A., Jauréguiberry, S., & Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. In *Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(07)70107-X

Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nature Microbiology*, *5*(6), 796–812. https://doi.org/10.1038/s41564-020-0714-0

Pomar, L., Vouga, M., Lambert, V., Pomar, C., Hcini, N., Jolivet, A., Benoist, G., Rousset, D., Matheus, S., Malinger, G., Panchaud, A., Carles, G., & Baud, D. (2018). Maternal-fetal transmission and adverse perinatal outcomes in pregnant women infected with Zika virus: Prospective cohort study in French Guiana. *BMJ (Online)*, *363*. https://doi.org/10.1136/bmj.k4431

Powers, A. M., Brault, A. C., Tesh, R. B., & Weaver, S. C. (2000). Re-emergence of chikungunya and o'nyong-nyong viruses: Evidence for distinct geographical lineages and distant evolutionary relationships. *Journal of General Virology*, *81*(2), 471–479. https://doi.org/10.1099/0022-1317-81-2-471

Reusken, C. B. E. M., Haagmans, B. L., Müller, M. A., Gutierrez, C., Godeke, G. J., Meyer, B., Muth, D., Raj, V. S., Vries, L. S. De, Corman, V. M., Drexler, J. F., Smits, S. L., El Tahir, Y. E., De Sousa, R., van Beek, J., Nowotny, N., van Maanen, K., Hidalgo-Hermoso, E., Bosch, B. J., … Koopmans, M. P. G. (2013). Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: A comparative serological study. *The Lancet Infectious Diseases*, *13*(10), 859–866. https://doi.org/10.1016/S1473-3099(13)70164-6

Rima, B., Balkema-buschmann, A., Dundon, W. G., Duprex, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R., Lee, B., Rota, P., & Wang, L. (2020). *Virus Taxonomy The ICTV Report on Virus Classification and Taxon Nomenclature Paramyxoviridae Chapter Paramyxoviridae Virion Genome organization and replication*. 1–36.

Schwartz, O., & Albert, M. L. (2010). Biology and pathogenesis of chikungunya virus. *Nature Reviews Microbiology*, *8*(7), 491–500. https://doi.org/10.1038/nrmicro2368

Silva, N. I. O., Sacchetto, L., De Rezende, I. M., Trindade, G. D. S., Labeaud, A. D., De Thoisy, B., & Drumond, B. P. (2020). Recent sylvatic yellow fever virus transmission in Brazil: The news from an old disease. *Virology Journal*, *17*(1), 1–12. https://doi.org/10.1186/s12985-019-1277-7

Simmonds, P., Becher, P., Bukh, J., Gould, E. A., Meyers, G., Monath, T., Muerhoff, S., Pletnev, A., Rico-Hesse, R., Smith, D. B., & Stapleton, J. T. (2017). ICTV virus taxonomy profile: Flaviviridae. *Journal of General Virology*, *98*(1), 2–3. https://doi.org/10.1099/jgv.0.000672

Stapleton, J. T., Foung, S., Muerhoff, A. S., Bukh, J., & Simmonds, P. (2011). The GB viruses: A review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family Flaviviridae. *Journal of General Virology*, *92*(2), 233–246. https://doi.org/10.1099/vir.0.027490-0

Terzian, A. C. B., Zini, N., Sacchetto, L., Rocha, R. F., Parra, M. C. P., Del Sarto, J. L., Dias, A. C. F., Coutinho, F., Rayra, J., da Silva, R. A., Costa, V. V., Fernandes, N. C. C. D. A., Réssio, R., Díaz-Delgado, J., Guerra, J., Cunha, M. S., Catão-Dias, J. L., Bittar, C., Reis, A. F. N., … Nogueira, M. L. (2018). Evidence of natural Zika virus infection in neotropical non-human primates in Brazil. *Scientific Reports*, *8*(1), 1–15. https://doi.org/10.1038/s41598-018-34423-6

Vasconcelos, P. F. da. (2003). Febre amarela. *Revista Da Sociedade Brasileira de Medicina Tropical*, *36*(2), 275–293.

Vasconcelos, H. B., Azevedo, R. S. S., Casseb, S. M., Nunes-Neto, J. P., Chiang, J. O., Cantuária, P. C., Segura, M. N. O., Martins, L. C., Monteiro, H. A. O., Rodrigues, S. G., Nunes, M. R. T., & Vasconcelos, P. F. C. (2009). Oropouche fever epidemic in Northern Brazil: Epidemiology and molecular characterization of isolates. *Journal of Clinical Virology*, *44*(2), 129–133. https://doi.org/10.1016/j.jcv.2008.11.006

Vasconcelos, P. F. C., & Monath, T. P. (2016). Yellow Fever Remains a Potential Threat to Public Health. *Vector-Borne and Zoonotic Diseases*, *16*(8), 566–567. https://doi.org/10.1089/vbz.2016.2031

Volk, S. M., Chen, R., Tsetsarkin, K. A., Adams, A. P., Garcia, T. I., Sall, A. A., Nasar, F., Schuh, A. J., Holmes, E. C., Higgs, S., Maharaj, P. D., Brault, A. C., & Weaver, S. C. (2010). Genome-Scale Phylogenetic Analyses of Chikungunya Virus Reveal Independent Emergences of Recent Epidemics and Various Evolutionary Rates. *Journal of Virology*, *84*(13), 6497–6504. https://doi.org/10.1128/jvi.01603-09

Vu, D. M., Jungkind, D., & LaBeaud, A. D. (2017). Chikungunya Virus. *Clinics in Laboratory Medicine*, *37*(2), 371–382. https://doi.org/10.1016/j.cll.2017.01.008

Wang, D., Hu, W., Chen, J., Jie, Z., Zhu, W., Gao, Z., Xiang, N., Shen, Y., Yu, H., Li, D., Gao, G. F., Ph, D., Wu, G., Wang, Y., Yuan, Z., Ph, D., Shu, Y., & Ph, D. (2013). *Human Infection with a Novel Avian-Origin Influenza A (H7N9) Virus*. https://doi.org/10.1056/NEJMoa1304459

WHO. (2016a). *Assesssment and management of Guillain-Barré syndrome in the context of Zika virus infection: interim guidance update*. World Health Organization.

WHO. (2016b). *WHO announces a Public Health Emergency of International Concern*. https://www3.paho.org/hq/index.php?option=com_content&view=article&id=11640:2016-who-statement-on-1st-meeting-ihr-2005-emergency-committee-on-zika-virus&Itemid=135&lang=en

WHO. (2018a). *Epidemiological Update: Yellow Fever - 7 december 2018*. *January 2017*, 1–7. https://www.paho.org/hq/index.php?option=com_docman&view=download&slug=7-december-2018-yellow-fever-epidemiological-update&Itemid=270&lang=en

WHO. (2018b). *Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2016*. https://www.who.int/healthinfo/global_burden_disease/estimates/en/index1.html

WHO. (2018c). *Zika virus*. https://www.who.int/news-room/fact-sheets/detail/zika-virus

WHO. (2020). *Nota Técnica : Caracterização genômica de SARS-CoV-2 e variantes circulantes na região das Américas Caracterização genômica do SARS-CoV-2* (Vol. 2, Issue 6).

Wu, P., Yu, X., Wang, P., & Cheng, G. (2019). Arbovirus lifecycle in mosquito: Acquisition, propagation and transmission. *Expert Reviews in Molecular Medicine*, *21*. https://doi.org/10.1017/erm.2018.6

Xavier, J., Giovanetti, M., Fonseca, V., Thézé, J., Gräf, T., Fabri, A., de Jesus, J. G., de Mendonça, M. C. L., dos Santos Rodrigues, C. D., Mares-Guia, M. A., dos Santos, C. C., de Oliveira Tosta, S. F., Candido, D., Nogueira, R. M. R., de Abreu, A. L., Oliveira, W. K., Campelo de Albuquerque, C. F., Chieppe, A., de Oliveira, T., … Alcantara, L. C. J. (2019). Circulation of chikungunya virus East/Central/ South African lineage in Rio de Janeiro, Brazil. *PLoS ONE*, *14*(6), 1–14. https://doi.org/10.1371/journal.pone.0217871