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Research note

Evidence for multiple sylvatic transmission cycles during the 2016–2017 yellow fever virus outbreak, Brazil

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ABSTRACT

Objectives: Since December 2016, Brazil has experienced an unusually large outbreak of yellow fever (YF). Whether urban transmission may contribute to the extent of the outbreak is unclear. The objective of this study was to characterize YF virus (YFV) genomes and to identify spatial patterns to determine the distribution and origin of YF cases in Minas Gerais, Espírito Santo and Rio de Janeiro, the most affected Brazilian states during the current YFV outbreak.
Methods: We characterized near-complete YFV genomes from 14 human cases and two nonhuman primates (NHP), sampled from February to April 2017, retrieved epidemiologic data of cases and used a geographic information system to investigate the geospatial spread of YFV.
Results: All YFV strains were closely related. On the basis of signature mutations, we identified two cocirculating YFV clusters. One was restricted to the hinterland of Espírito Santo state, and another formed a coastal cluster encompassing several hundred kilometers. Both clusters comprised strains from humans living in rural areas and NHP. Another NHP lineage clustered in a basal relationship. No signs of adaptation of YFV strains to human hosts were detected.
Conclusions: Our data suggest sylvatic transmission during the current outbreak. Additionally, cocirculation of two distinct YFV clades occurring in humans and NHP suggests the existence of multiple sylvatic transmission cycles. Increased detection of YFV might be facilitated by raised awareness for arbovirus-mediated disease after Zika and chikungunya virus outbreaks. Further surveillance is required, as reemergence of YFV from NHPs might continue and facilitate the appearance of urban transmission cycles.
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Introduction

Since December 2016, Brazil has been affected by an unusually large and expanding yellow fever (YF) outbreak, with over 3500 suspected cases reported and several hundred deaths [1]. Descriptive epidemiologic evidence suggests that the outbreak so far shows a sylvatic transmission pattern, with human infections being acquired from nonhuman primates (NHP) via forest-associated mosquito species [1]. However, recent research has identified urban mosquito vectors to be competent for transmission of YF virus (YFV),

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suggesting a risk of reemergence of urban YF in Brazil [2]. In the context of the present outbreak, only two complete NHP YFV genomes from a single geographic location have been sequenced so far [3]. The lack of genome data is of concern for public health, as it hinders the distinction between sylvatic YF that is endemic in Brazil [4] and urban YF that would require high-priority public health interventions. To elucidate transmission cycles, we aimed to phylogenetically characterize YFV strains from three of the most affected Brazilian states.

Methods

Human specimens were sent for diagnostics by attending physicians. NHP samples were sent within flavivirus surveillance programs to the Flavivirus Reference Laboratory of the Oswaldo Cruz Institute. Genomic sequencing of YFV strains is routinely done to differentiate between wild-type YFV infection and vaccine-associated adverse effects [5]. The study was approved by the institutional research ethics board under protocol IOC 274/05. Samples originated in the states of Minas Gerais (MG), Espírito Santo (ES) and Rio de Janeiro (RJ), areas massively affected by YF since 2016 [6]. YFV infection was confirmed by real-time RT-PCR [7]. YFV genomes were amplified by overlapping nested RT-PCR assays and were Sanger sequenced (Supplementary Table S1).

Results

The median age of human patients was 43 years (range, 1.5–71 years). A total of 71.4% of the affected subjects lived in rural areas (Table 1). Adult age and rural origin of the cases in this study were consistent with data from Brazilian authorities identifying rural workers aged 20 to 43 years as the most exposed group during the current YF outbreak [1,8]. Only four subjects lived in urban areas, all with a history of travel to rural areas. Only one of the patients reported previous YF vaccination, but the subject reported YF symptoms at the time of vaccination.

All viruses detected clustered in a distinct sister clade to YFV strains obtained from humans and NHP in Brazil and Venezuela between 2000 and 2010 (Fig. 1(A)). From one of the human cases and one NHP in our study, the YFV polyprotein gene could only be characterized at about 80% completeness. To maximize the representation of NHP in our data set, we conducted a separate phylogenetic analysis including these two partially characterized polyprotein genes (asterisks in Fig. 1(B)). In both analyses, a YFV strain obtained from a NHP clustered in phylogenetically basal position to the other YFV outbreak strains. Additionally, two apically clustering viruses from NHP were more closely related to strains from humans than to other strains derived from NHP. This tree topology pointed at a higher YFV genetic diversity in NHP than in humans and was consistent with the evolution of the outbreak strains in NHP reservoirs.

Within the data set including all YFV strains from this study and the two previously published strains [3], two to 28 mutual nucleotide differences translating to up to eight amino acid differences were observed. In the description of the two previously characterized YFV genomes from this outbreak, eight unique amino acid residues located in the C, NS3 and NS5 domains were found compared to other YFV strains [3]. Indeed, all these eight residues were conserved in our larger genomic data set. However, the previously described strains were genetically closely related [3]. In our larger data set, 55 synonymous (*dS*) and 27 nonsynonymous (*dN*) exchanges were observed between YFV outbreak strains (Fig. 1(C)). Whether any of the mutations in the outbreak strains or a combination thereof may alter the virus phenotype will therefore require careful investigation.

To further elucidate transmission cycles, we analysed the polymorphic sites within all YFV strains from this study as well as the two previously published strains [3]. As shown in Fig. 1(D), six clades could be differentiated by unique mutations. Three YFV strains from human cases (termed 2–4 in Fig. 1(D)) showed unique mutations that were not shared by any other virus. This pattern might be expected upon human-to-human transmission. However, these three lineages showed relatively high mutual sequence distances of nine to 16 nucleotide exchanges located in different parts of the virus genome. This spoke against their evolution during human-to-human transmission and instead suggested a common source of all viruses in NHP. Two larger viral clades (termed 5 and 6 in Fig. 1(D)) contained signature mutations in the nonstructural domains NS2b, NS3, NS4b and NS5, irrespective of strain origin from humans or NHP. No evidence for site-specific diversifying selection was observed in human YFV strains [9]. The mean *dN*/*dS* ratio in the complete data set was low, at 0.14 (95% confidence interval, 0.08–0.23), which was similar to a previous analysis showing strong purifying selection in YFV [10]. Clade 5 appeared to

Table 1
Subject characteristics

ID	Vaccination history	Age (years)	Municipality	State	Residence zone	Collection date	Host	Polyprotein gene completion (GenBank accession no.)
YF5	NA	24	Alfredo Chaves	ES	Rural	23/03/2017	Human	Complete (MF170980)
YF3	NA	54	Brejetuba	ES	Rural	03/03/2017	Human	Complete (MF170975)
YF6	NA	54	Cariacica	ES	Urban	29/03/2017	Human	Complete (MF170974)
YF9	No	30	Cariacica	ES	Urban	31/03/2017	Human	Complete (MF170972)
YF18	No	65	Casimiro de Abreu	RJ	Urban	18/03/2017	Human	Complete (MF170969)
YF2	No	71	Conceição do Castelo	ES	Rural	10/02/2017	Human	Complete (MF170977)
YF12	No	45	Domingos Martins	ES	Rural	10/04/2017	Human	Complete (MF170973)
YF17	No	41	Domingos Martins	ES	Urban	21/03/2017	Human	Complete (MF170970)
YF1	Yes (January 2017)	50	Laranja da Terra	ES	Rural	25/01/2017	Human	Complete (MF170976)
YF13	No	46	Marechal Floriano	ES	Rural	13/04/2017	Human	Complete (MF170968)
YF4	NA	1.5	Porciuncula	RJ	Rural	26/02/2017	Human	C-NS4b (MF185661) 6995 nucleotides
YF11	No	32	Santa Leopoldina	ES	Rural	05/04/2017	Human	Complete (MF170981)
YF7	NA	2	Santa Maria de Jetibá	ES	Rural	28/03/2017	Human	Complete (MF170978)
YF10	No	64	Santa Maria de Jetibá	ES	Rural	06/04/2017	Human	Complete (MF170979)
YF14	NA	NA	São Roque de Minas	MG	NA	30/01/2017	NHP	Complete (MF170971)
YF16	NA	NA	São Sebastião do Alto	RJ	NA	27/03/2017	NHP	C-NS4b (MF185660) 6995 nucleotides

C, core; ES, Espírito Santo; MG, Minas Gerais; NA, not available; NHP, nonhuman primate; NS4b, nonstructural domain 4b; RJ, Rio de Janeiro.

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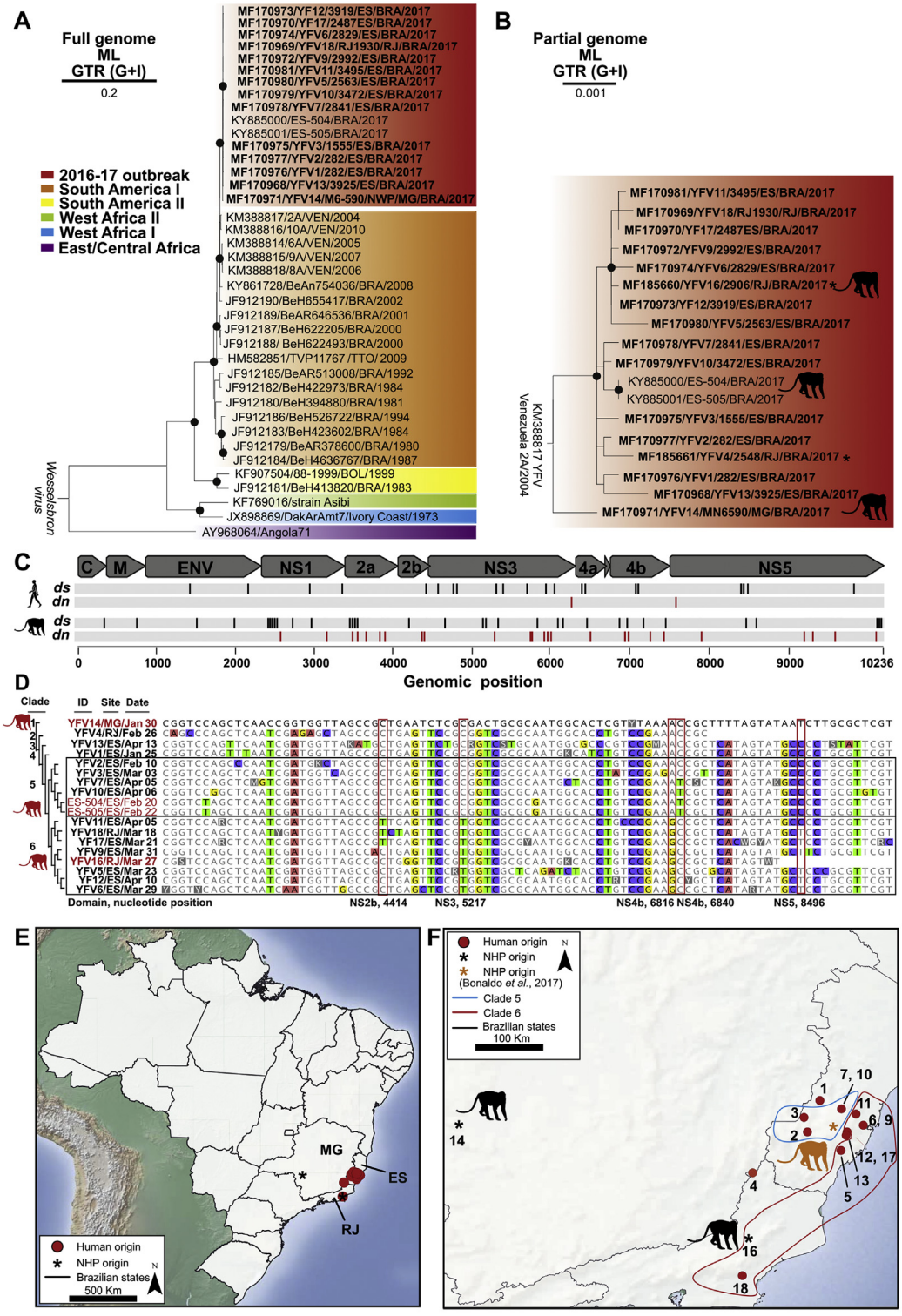

Fig. 1. Sampling sites and phylogenetic analyses. (A) Full polyprotein gene maximum likelihood (ML) tree constructed using GTR+G+I substitution model and complete deletion option, showing relationships between viruses characterized in this study (bold) (GenBank accession no. MF185660-MF185661 and MF170969-MF170980) and representative YFV genotypes. A total of 8034 nucleotide positions were included in the final data set. Wesselsbron virus (GenBank accession no. NC_012735) was used as an outgroup. (B) Partial polyprotein gene ML tree constructed as above, showing relationships between all strains known from the current outbreak. There were a total of 6916 positions in the final data set. YFV strain 2A/Venezuela (GenBank accession no. KM_388817) was used as an outgroup. Solid circles at nodes in (A) and (B) denote support of grouping of above 80% from 500 bootstrap replicates. Asterisks denote partial sequences. Evolutionary analyses were conducted in MEGA 6.0. (C) YFV genome organization showing synonymous substitutions (*dS*) in black and nonsynonymous substitutions (*dN*) in red of all variable sites across 14 YFV polyprotein genes of human origin, and four YFV polyprotein genes of NHP origin available from the current outbreak. The 2K domain located between NS4a and 4b is not detailed in the genomic representation for graphical reasons. *dN*/*dS* estimations were performed using FUBAR (http://www.datamonkey.org/fubar/). (D) Alignment showing all polymorphic sites across YFV outbreak strains. Strains from NHP are shown in red. Red boxes denote mutations that separate lineages identified by numbers to the left. Grey boxes denote clades 5 and 6. (E) Map of Brazil showing origin of samples in states Minas Gerais (MG), Espírito Santo (ES) and Rio de Janeiro (RJ). (F) Sample origin and host species per Brazilian municipality. Maps were created by using QGIS2.14.3 (https://www.qgis.org/en/site/) and freely available data (http://www.naturalearthdata.com/).

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be restricted to a region in the hinterland of the ES state (Fig. 1(E) and (F)). In contrast, clade 6 contained viruses sampled across distances exceeding 200 km along the Brazilian coast (Fig. 1(E) and (F)).

Discussion

A striking attribute of the current YF outbreak is its geographic extension, with cases having been reported across 2000 km [1]. This vast extent would be most compatible with human movement and transmission, as suggested by phylogeographic analyses indicating that the currently circulating YFV strain may have originated from an ancestor imported to Brazil from Trinidad and Tobago [11]. Although limitations of our study include the restricted geographical coverage that precludes geographically representative conclusions, the coastal distribution of one of the YFV clusters we observed may be consistent with virus transfer aided by human movement.

However, our molecular and epidemiologic data were not supportive of predominantly human-to-human transmission. Instead, the rural origin of the human cases in our study, together with the lack of signal for molecular adaptation of YFV strains to human hosts, suggested predominantly *in situ* sylvatic transmission and maintenance. This has been observed in shorter time spans during epidemics, and it is believed that *in situ* maintenance shapes the evolutionary dynamics of YFV genotype I [11]. Additionally, cocirculation of two distinct YFV clades occurring in humans and NHP at the same time suggests parallel existence of multiple sylvatic transmission cycles. Geographically representative genomic data sets will be needed from human cases, NHP and potential mosquito vectors involved in sylvatic or urban cycles [2,12,13] to permit assessments of other YF foci that were not covered in our study, confirm the factors responsible for the expansion of YFV in Brazil and discover if human-to-human transmission plays a role in YFV dissemination.

Finally, we cannot exclude a reporting bias, potentially causing unrelated sylvatic foci to be confused with a large contiguous outbreak. Hypothetically, such a reporting bias may be caused by raised awareness for arbovirus infections due to the Zika and chikungunya virus outbreaks affecting Brazil during the last years [14]. Brazil is responding to the outbreak by large vaccination programmes, which will be required to prevent further expansion and potential reoccurrence of urban cycles [15].

Transparency declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2018.01.026.

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