

1 **Real-time genomic surveillance during the 2021 re-emergence of the yellow fever  
2 virus in Rio Grande do Sul State, Brazil**

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23

24 **Abstract**

25 **The yellow fever virus (YFV) re-emergence in Rio Grande do Sul, Brazil, raised big  
26 concerns and led the state to declare a Public Health Emergency of State Importance.  
27 Here, we generated near-complete genomes from the ongoing outbreak in Southern  
28 Brazil, aiming to better understand the phylogenetic aspects and also spatio-temporal  
29 dynamics of the virus. Our findings highlight the path and dispersion in Rio Grande  
30 do Sul and that YFV was reintroduced from São Paulo to the Rio Grande do Sul state  
31 through Paraná and Santa Catarina states, at the end of 2020.**

32 **Keywords:** yellow fever virus, RS outbreak, phylogenetic analysis, NHPs

33

34 **1. INTRODUCTION**

35 Yellow fever (YF) is a viral hemorrhagic fever caused by the Yellow Fever Virus (YFV), the  
36 prototype of the genus *Flavivirus*, family *Flaviviridae* (Monath, 2001). In South America, YFV  
37 is widely spread and maintained in a sylvatic cycle by transmission between non-human  
38 primates (NHP's) and blood-feeding mosquitoes mainly from the genera *Haemagogus*  
39 (Monath and Vasconcelos 2015).

40 In Brazil, the area of YFV occurrence extends from the Amazon region, northern Brazil, to  
41 the state of Rio Grande do Sul (RS), the southernmost region of the country. The latter is  
42 sporadically affected when the virus overflows from the North (endemic area) to the  
43 Southeast and South Regions (Almeida et al., 2012; Delatorre et al., 2019; Figueiredo et al.,  
44 2020; Monath and Vasconcelos, 2015; Possas et al., 2018; Vasconcelos et al. 2004). During  
45 the YFV spreads, NHP's deaths (hereafter called epizootics) precedes human cases,  
46 highlighting the importance of the epizootic surveillance system as a tool for early detection  
47 of YFV circulation. Prompt action in such episodes allows to actively increase vaccination  
48 coverage of human populations in the vicinity of the epizootic event as well as for mapping  
49 virus dispersion (Almeida et al., 2014; Romano et al., 2014).

50 Among South American NHP's genera, *Alouatta* is particularly important for YF surveillance,  
51 because it is the most severely affected by YFV (Almeida et al., 2014; Hill et al., 2020;  
52 Mares-Guia et al., 2020; Moreno et al., 2013). In 2001-2002, following epizootics affecting  
53 *Alouatta* sp., a YF surveillance based on monitoring of living and dead NHP's was  
54 implemented in RS. Unfortunately, between 2008-2009 a new outbreak occurred in RS,  
55 causing thousands of NHP's deaths, and 21 human cases. In the subsequent twelve years  
56 of continued surveillance, no virus circulation was evidenced in the South Region (Almeida  
57 et al., 2012; 2014; 2019). Meanwhile, between 2014 - 2019, a new YFV foci was first  
58 detected in Tocantins state, in the North Region, and reached Southeast and South  
59 Regions, including Brazilian coast states Espírito Santo, Rio de Janeiro and São Paulo,  
60 causing the largest sylvatic outbreak ever recorded (Abreu et al., 2019b; Bonaldo et al.,  
61 2017; Brasil, 2014; Brasil, 2015; Brasil, 2017; Brasil, 2019; Cunha et al., 2019; Silva et al.,  
62 2020). Following these events, from 2019 onwards, YFV continued to spread towards the  
63 South Region, arriving in the southern states of Paraná and Santa Catarina and causing  
64 human cases and hundreds of epizootics (Brasil, 2019; DIVE, 2021; SESA, 2021).

65 Altogether, that YF outbreak affected ten states and the Federal District outside the Amazon  
66 and left behind about two thousand human cases and countless NHP's deaths. That  
67 outbreak was monitored by real-time genomic surveillance (Brasil, 2019), and the

68 phylogenetic analysis revealed the existence of at least two main viral sub-lineages  
69 occurring in Brazil in that period - the "Yellow Fever Virus Minas Gerais/São Paulo"  
70 (hereafter YFV<sub>MG/SP</sub>) and the "Yellow Fever Virus Minas Gerais/Espírito Santo/Rio de  
71 Janeiro" (hereafter YFV<sub>MG/ES/RJ</sub>) (Delatorre et. al., 2019).

72 Since the virus entered the RS neighboring state (Santa Catarina) in 2019 (Brasil, 2019;  
73 DIVE, 2020), following its progression to the south, health authorities of RS focused its  
74 surveillance efforts at border municipalities. Aimed for this, a surveillance team from the  
75 State Health Department was working to promote vaccination and raise awareness of people  
76 who live in that region to report NHP's deaths (CEVS, 2021). Noteworthy, at the end of 2020  
77 and beginning 2021, the virus continued its route southwards causing the first cases of  
78 NHP's (*Alouatta guariba clamitans*) deaths on RS.

79 Although the virus has crossed Paraná and Santa Catarina territories, to date, no YFV  
80 genomes of viruses circulating in these states were publicly available and little is known  
81 about the dynamics of spatial spread of the YFV in southern Brazil. In this way, aiming to fill  
82 this information gap, here we sequence the first YFV genomes from the edge of the current  
83 YFV expansion wave in the South Region of Brazil, recovered from NHP's samples during  
84 YF epizootics surveillance in RS.

85

86 **2. METHODS**

87 **2.1 - Ethics statement**

88 This study comprised analysis of routinely collected surveillance data performed by the state  
89 and municipalities health departments and followed guidelines of the Ministry of Health of  
90 Brazil and Brazilian National Committee for Ethics in Research. All samples were obtained  
91 from dead NHPs. This study followed the guide to epizootic surveillance in non-human  
92 primates and entomology applied to yellow fever surveillance (Brasil, 2017) and Institutional  
93 Animal Care and Use Committees (IACUCs) review the Use of Nonhuman Primates (Tardif  
94 et al., 2013). This study was conducted in accordance with Brazilian legislation under the  
95 SISBIO/ICMBio/MMA authorizations for activities with scientific purpose 75734-1 and  
96 SISGEN license AF40BCA.

97 **2.2 - Sample collection**

98 All NHP's samples were collected in municipalities in the Northeast Region of the state of  
99 Rio Grande do Sul, Brazil. Samples from liver, kidney, lung, spleen and heart were collected  
100 in the field from dead animals and were kept refrigerated (4°C) or frozen in dry ice and  
101 dispatched to the central office of the State Health Department, where they were stored at -  
102 80°C. All collections followed biosafety protocols and were in accordance with the state YF  
103 surveillance strategy carried out by the Environmental Health Surveillance Division, State  
104 Center of Health Surveillance, State Health Department of RS, and the Ministry of Health of  
105 Brazil. Data concerning the geo-located origin of the animals, date of sampling and post-  
106 mortem findings were registered.

107 **2.3 - RT-qPCR**

108 YFV RNAs were extracted from NHP's tissue (liver and kidney) samples spotted on FTA®  
109 classic filter paper (Whatman). A hole punch was used to excise a single dried 6 mm  
110 diameter circle. To avoid carryover contamination, the instrument was disinfected with  
111 bleach, water and ethanol 100% (Bonne et al., 2008). The material was then lysed using  
112 proteinase K and lysis buffer containing carrier RNA at 56 °C, for 15 min, according to  
113 PureLink Viral Mini kit protocol (Invitrogen). The lysates samples were finally extracted using  
114 automated extraction (Loccus, Extracta Kit FAST). Viral RNA was detected using two  
115 previously published RT-qPCR protocols (Domingo et al., 2012). Samples which were  
116 positive at RT-qPCR with cycle thresholds (CT) below 25 were sent to Baculovirus  
117 Laboratory, in University of Brasilia, for sequencing.

118 **2.4 - Genome sequencing**

119 All samples that met the previous criteria were submitted to cDNA synthesis protocol using  
120 LunaScript™ RT SuperMix Kit (NEB) following the manufacturer's instructions. Then, a  
121 multiplex tiling PCR was attempted using the previously published YFV primers (Faria et al.,  
122 2018) and 40 cycles (denaturation: 95°C/15 s and annealing/extension: 65°C/5 min) of PCR  
123 using Q5 high-fidelity DNA polymerase (NEB). Amplicons were purified using 1x AMPure XP  
124 beads (Beckman Coulter), and cleaned-up PCR product concentrations were measured  
125 using a QuantiFluor® dsDNA System assay kit on a Quantus™ Fluorometer (Promega).  
126 DNA library preparation was performed using the Ligation sequencing kit SQK-LSK309  
127 (Oxford Nanopore Technologies) and the Native barcoding kit (EXP-NBD104 and EXP-  
128 NBD114; Oxford Nanopore Technologies, Oxford, UK). The sequencing library (23 samples  
129 and a negative control per run) was loaded onto a R9.4 flow cell (Oxford Nanopore  
130 Technologies) and sequenced between 6 to 18 hours using MiNKOW software. The  
131 RAMPART (Version 1.2.0, ARTIC Network) package was used to monitor coverage depth  
132 and genome completion. The resulting Fast5 files were basecalled and demultiplexed using  
133 Guppy (Version 4.4.2, Oxford Nanopore Technologies). Variant calling and consensus  
134 genome assembly was carried out with Medaka (Version 1.0.3, Oxford Nanopore  
135 Technologies) using the sequence JF912190 as the reference genome.

136 **2.5 - Phylogenetic analysis**

137 To perform phylogenetic analysis, we selected from NCBI all near-complete YFV sequences  
138 (YFV-set-1, n=359, sequences > 8 kb excluding sequences from vaccine and patents).  
139 Then, we make a subset of sequences belonging to recent (2015 to 2021) extra-Amazonian  
140 region waves, including clades YFV<sub>MG/ES/RJ</sub> and YFV<sub>MG/SP</sub> (YFV-subset, n=264). Metadata as  
141 samples collection date and geographic coordinates were retrieved from GenBank files or  
142 from genome associated publications (manual curation). Genomes generated here (n = 23)  
143 combined with YFV-set-1 were aligned with MAFFT v.7.480 (Katoh and Standley, 2013).  
144 The Maximum-likelihood tree was inferred using IQTREE, with the GTR+F+I+Γ<sub>4</sub> model. YFV-  
145 subsets combined with the newly determined genomes were used to construct a time-scaled  
146 tree in Nextstrain (<https://nextstrain.org/ncov>). The new genome sequences will be sent to  
147 the NCBI GenBank database to obtain accession numbers.

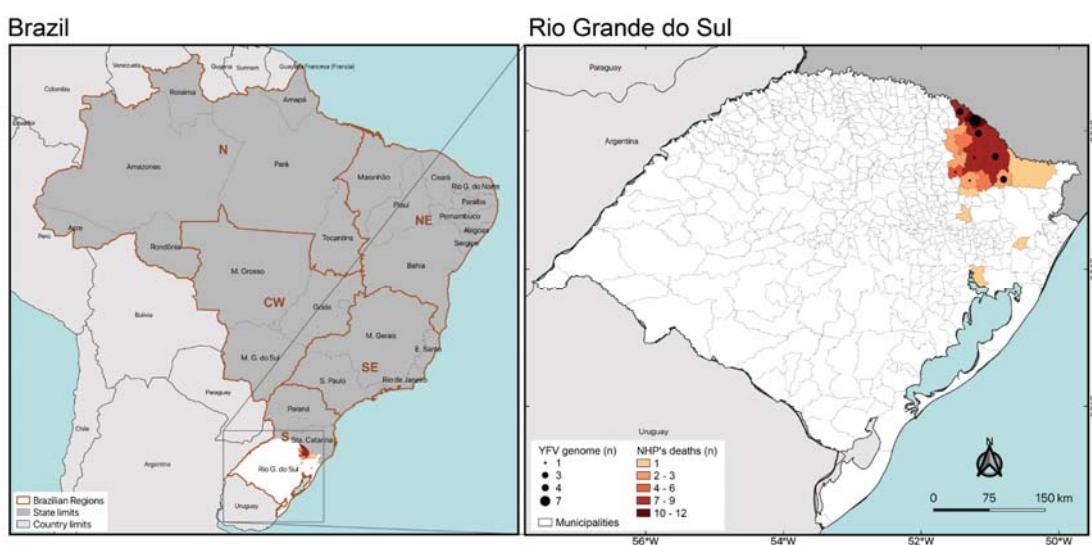
148 **2.6 - Epidemiological and geographic information**

149 Epidemiological data about human cases (from 2016 to March 8<sup>th</sup>, 2021) and epizootics  
150 (from 2014 to March 8<sup>th</sup>, 2021) related to YF in Brazil, was provided by the Ministry of Health  
151 of Brazil by the law of access to information. Numbers of Epizootics in RS were provided by  
152 Division of Environmental Health Surveillance from Rio Grande do Sul State Health  
153 Department. Maps presenting the results were generated through free software QGIS  
154 version 2.18.

155 **3. RESULTS**

156 Between January and March 08<sup>th</sup> (date of collection of our last sequenced sample), 78  
157 epizootics were notified to RS State Health Department and Ministry of Health of Brazil  
158 surveillance, of which, in 42 (54%) sample collection could be performed. From these, 34  
159 (81%) tested positive for YFV by RT-qPCR assay. All confirmed YFV-positive NHP's were  
160 *Alouatta guariba clamitans* and it became evident that, at the date of collection of our last  
161 sequenced sample, the virus remained in circulation in the state for at least 10 weeks, with  
162 epizootics occurring mainly in the Northeast Region of RS near to the border with Santa  
163 Catarina state (**Figure 1**).

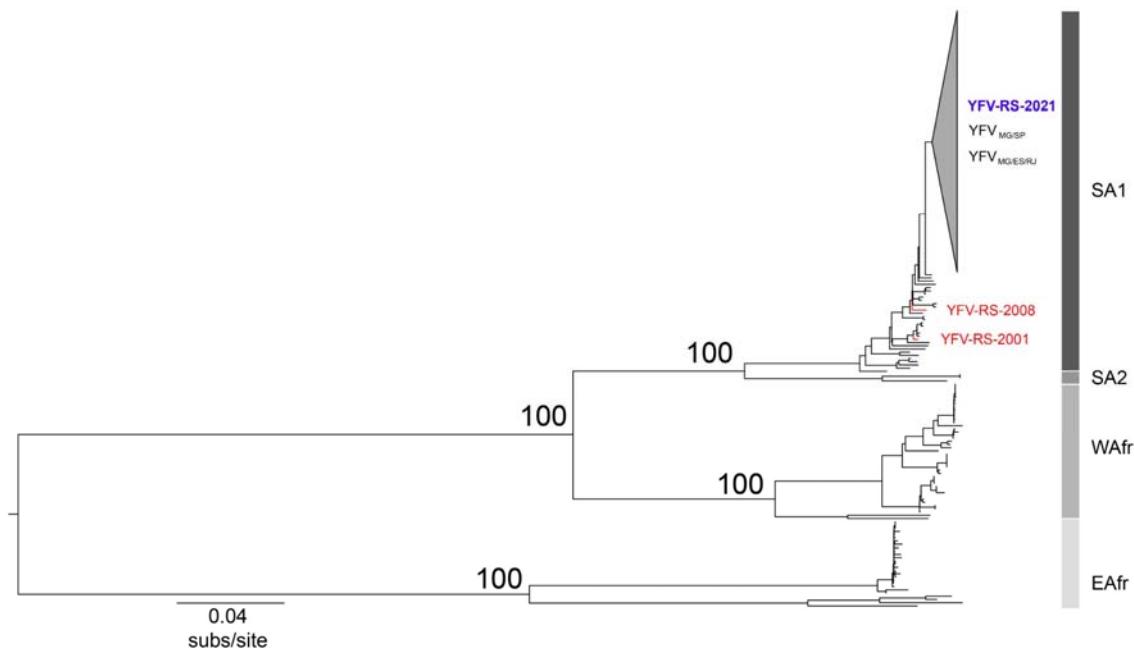
164



176 **Table 1.** List of genomes generated in this study showing date of collection, sample name,  
177 latitude, longitude, cycle threshold (Ct) and coverage.

Date	Sample name	Lat	Long	Ct	Coverage
25 January	Pinhal da Serra 01	-27.8757	-51.2260	20	194
03 February	Pinhal da Serra 02	-27.8757	-51.2260	11	156
03 February	Pinhal da Serra 03	-27.8757	-51.2260	11	135
08 February	Monte Alegre dos Campos 01	-28.5355	-51.5023	10	30
08 February	Pinhal da Serra 05	-27.8346	-51.1995	10	284
11 February	Pinhal da Serra 07	-27.8843	-51.1632	13	53
19 February	Esmeralda 01	-28.0930	-51.1124	12	360
19 February	Pinhal da Serra 08	-27.8933	-51.1116	9	303
19 February	Pinhal da Serra 09	-27.8403	-51.2505	10	1572
20 February	Muitos Capões 01	-28.2196	-51.2135	8	309
22 February	Barracão 02	-27.7315	-51.3688	11	247
22 February	Barracão 03	-27.7315	-51.3688	9	153
22 February	Barracão 04	-27.7315	-51.3688	15	666
22 February	Vacaria 01	-28.2900	-50.8116	9	568
22 February	Vacaria 02	-28.2900	-50.8116	10	416
24 February	Esmeralda 02	-28.1698	-50.9228	15	207
24 February	Vacaria 04	-27.9216	-51.2187	11	533
25 February	Esmeralda 03	-27.9754	-51.0557	9	153
25 February	Esmeralda 04	-27.9754	-51.0557	11	294
26 February	Monte Alegre dos Campos 02	-28.7591	-51.2527	11	84
03 March	Monte Alegre dos Campos 03	-28.6724	-50.7997	14	722
04 March	André da Rocha 02	-28.5850	-51.5757	20	1008
08 March	Ipê 01	-28.7591	-51.2527	12	2024

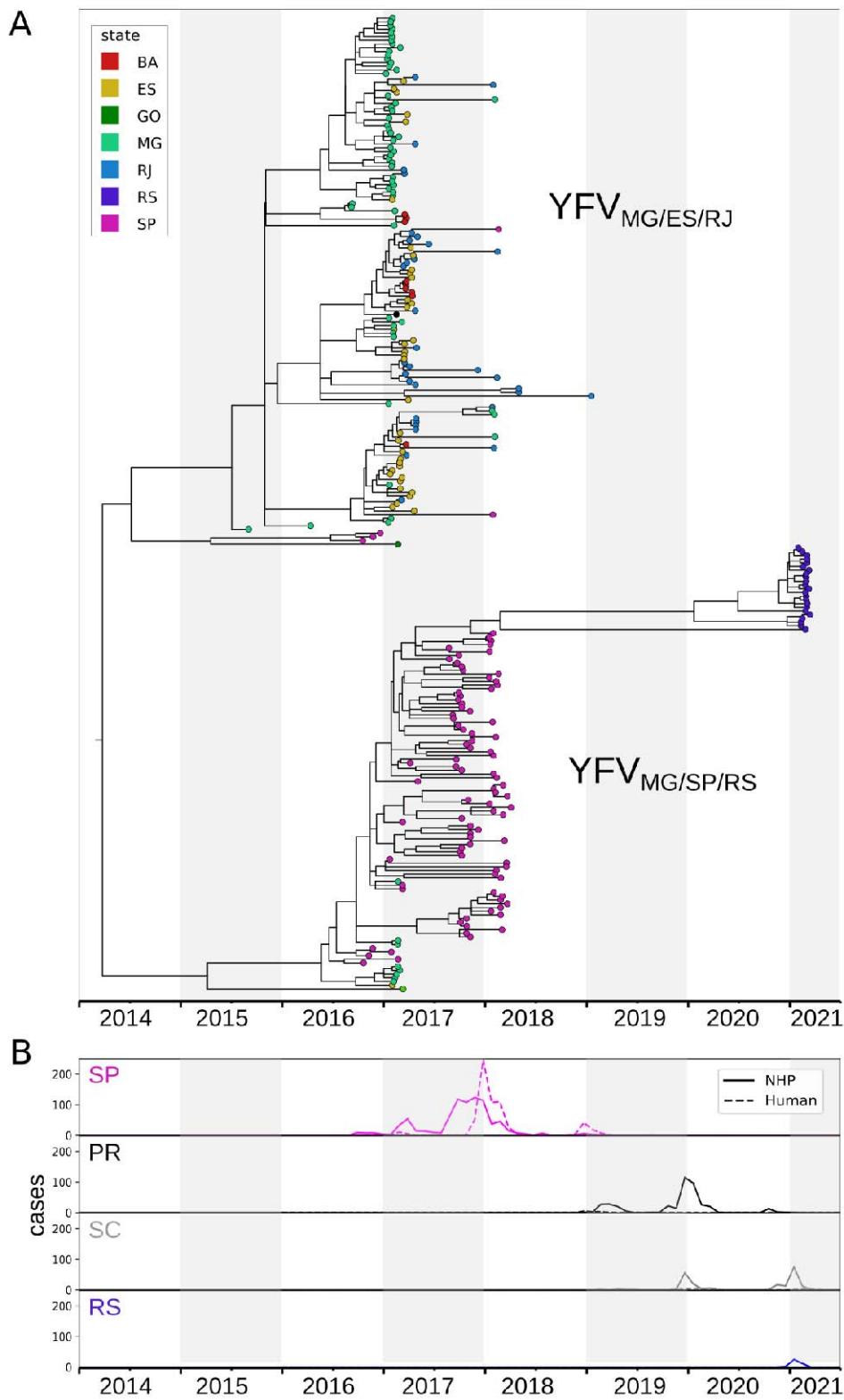
178



179

180 **Figure 2.** Phylogenetic tree of YFV based on 381 near-complete genomes. The gray collapsed group  
181 includes  $YFV_{MG/ES/RJ}$  and  $YFV_{MG/SP}$  clades and all genomes from RS sequenced in this study. South  
182 America 1, South America 2, West Africa and East Africa genotypes are indicated. Previous genomes  
183 from RS, JF912189 (2001) and KY861728 (2008), are highlighted in red.

184 To understand the spatial and evolutionary dynamics of recent YFV dispersion from endemic  
185 areas to southern regions of Brazil we made an analysis using a subset of sequences  
186 belonging to the South America 1 genotype on Nextstrain. The time-scaled phylogenetic tree  
187 (**Figure 3A**) shows that YFV from RS sequenced here are clustered with the YFV<sub>MG/SP</sub> sub-  
188 lineage, named Yellow Fever Virus Minas Gerais/São Paulo/Rio Grande do Sul (hereafter  
189 YFV<sub>MG/SP/RS</sub>), revealing that the origin of this isolates is São Paulo. Furthermore, the time-  
190 scaled phylogenetic tree suggests more than one entry in RS. Despite the lack of genomic  
191 data from Paraná and Santa Catarina, epidemiological data of epizootics and human cases  
192 due to YF from São Paulo and South Region (Paraná, Santa Catarina and RS), as shown in  
193 **Figure 3B**, suggest that the recent YFV dispersion wave achieve RS passing through  
194 Paraná (2018/2020) and Santa Catarina (2019/2021).



195

196 **Figure 3.** Spatio-temporal YFV spread. A) Time-scaled phylogenetic tree of YFV<sub>MG/ES/RJ</sub> and  
197 YFV<sub>MG/SP/RS</sub> sub-lineages. B) Epizootics and human cases due to YF, reported in São Paulo (SP),  
198 Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS) by month (Source: Ministry of Health  
199 of Brazil).

200 **4. DISCUSSION**

201 In this study we applied real-time genomic surveillance to obtain sequences of YFV from  
202 NHP's found dead by the YF surveillance system in RS state, southern Brazil, at the  
203 beginning of the re-emergence of the virus in 2021. The new introduction of YFV in RS has  
204 raised big concerns and led the state to declare a Public Health Emergency of State  
205 Importance. To better understand YFV genetic distribution during the recent outbreak in  
206 Southern Brazil, we generated 23 complete and nearly complete genomes from the first  
207 peak of the epidemic curve from NHP's cases in the northeast of RS state.

208 In Brazil, over the past five years, previous genetic analyses have indicated the circulation of  
209 at least two distinct sub-lineages, in two different transmission routes. The first one was YFV  
210 MG/ES/RJ, which circulated in the east of Minas Gerais in 2016, entering the west of Espírito  
211 Santo and following to the north of Rio de Janeiro. From there, in 2017, the virus spread  
212 southwards, arriving at the border with São Paulo in 2018 (Bonaldo et al., 2017; Delatorre et  
213 al., 2019; Faria et al., 2018; Goméz et al, 2018). About march 2017, NHP's samples infected  
214 by this sub-lineage were also found in Bahia, a Northeast Region state in Brazil (Goes de  
215 Jesus et al., 2020). Meanwhile, the second sub-lineage, named YFV<sub>MG/SP</sub>, circulated in the  
216 west of Minas Gerais and the northwest of São Paulo in 2016-2017, from where it advanced  
217 towards the south and east of that state, reaching the most densely populated region of the  
218 country between 2017-2018 (Hill et al., 2020; Rezende et. al., 2018). From 2018 to 2021  
219 YFV was detected in NHP and humans in Paraná and/or Santa Catarina (Brasil, 2019; DIVE,  
220 2021; SESA, 2021). Although the lack of genomic information available from Paraná and  
221 Santa Catarina, the spatiotemporal distribution of epizootic and human cases in South  
222 Region combined with phylogenetic analysis presented here suggest that YFV wave toward  
223 South Region of Brazil is a continuity of sub-lineage YFV<sub>MG/SP</sub> (now called YFV<sub>MG/SP/RS</sub>)  
224 expansion.

225 Previous records of occurrence of YF in RS reveal that the border between Argentina and  
226 Brazil (northwest of RS) is historically the first affected area, as happened in 1947, 1966,  
227 2001, 2002 and 2008/2009 (Almeida et al. 2012, 2014; Bejarano, 1974; Cardoso et al.,  
228 2010; Franco, 1969; Vasconcelos et al., 2003). In the epizootic here reported, the virus  
229 followed a different route reaching northeast of RS, a route which matches with recent  
230 dispersion of YFV in Santa Catarina and Paraná, coming from the southeast of São Paulo  
231 state. Furthermore, our data lead us to believe that samples from Santa Catarina and  
232 Paraná, if sequenced, might be grouped with sub-lineage YFV<sub>MG/SP/RS</sub>. Sequencing samples  
233 from these states are essential to better understand the dispersion pattern of YFV while  
234 circulating in the South Brazil Region.

235 Interestingly, the sequences obtained here, represent virus samples of the farthest place  
236 reached by YFV dispersion in Brazil, since it spread from the endemic area, about 2014, in  
237 this expansion wave. Rio Grande do Sul is the south limit for NHP's distribution in the  
238 Americas (Printes and Liesenfeld, 2001) and the farthest state from YF endemic area, which  
239 is the source of YFV in all extra-Amazon circulations (Monath and Vasconcelos, 2015).  
240 Consequently, it is expected to be the south limit for YFV spread in the Americas too.  
241 Possibly, the 2021 YFV lineage from RS presents accumulated genetic changes over seven  
242 years of circulation, since the first detection of an extra endemic area, in 2014, in the State of  
243 Tocantins (Brazil, 2019).

244 Epidemic outbreaks in the sylvatic cycle of YFV demands a high density of competent  
245 vectors and susceptible NHP's presence, acting as an amplifier for the virus (Abreu et al.,  
246 2019b; Cardoso et al., 2010; Mares-Guia et al., 2020; Pinheiro et al., 2019). Importantly, the  
247 YFV expansion wave (2014-2021) revealed the suitability of climate and ecological  
248 conditions for the occurrence of YFV outbreaks in several Brazilian regions, even though in  
249 the three southernmost states of Brazil (Paraná, Santa Catarina and RS), which have a  
250 subtropical temperate climate and great loss of forest areas, through which viral circulation  
251 can occur (Abreu et al., 2019a; Delatorre et al., 2019; Sacchetto et al., 2020a; Sacchetto et  
252 al., 2020b; Rosa et al., 2021). Therefore, it is important to strengthen surveillance. Our  
253 findings confirm the need for continued surveillance for early detection of outbreaks and the  
254 use of genetic tools to determine the origin and understanding of YFV entry and dispersion  
255 in RS.

256 Ultimately, we present here the first sequences of the front of the current YFV wave heading  
257 south and show that these sequences are related with the lineage identified in SP during  
258 YFV circulation in that state in 2017-2018. However, further studies of the virus dispersion  
259 including ecological, climatic, and anthropogenic factors associated with the disease cases  
260 are needed to improve predictive power, allowing rapid decisions into surveillance and  
261 prevention efforts. Furthermore, we are performing continuous real-time genomic  
262 surveillance at RS, once there is an ongoing outbreak over NHP's, and new genome  
263 sequencing and ecological analysis are in progress. Lastly, our work shows the importance  
264 of capacity building for inter-institutional exchange of data and human resources, to  
265 strengthen the epidemic surveillance and outbreak management during pandemics.

266

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281

282 **Competing interests**

283 The authors have declared that no competing interests exist.

284

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