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**An unusual two-strain cholera outbreak in Lebanon, 2022-2023: a genomic
epidemiology study**

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37

38 **ABSTRACT**

39

40 **Background**

41 Cholera is a bacterial infection caused by the ingestion of contaminated water or food. It
42 principally affects the gastrointestinal system and spreads easily, causing outbreaks. The first
43 case of cholera in this outbreak was detected in Lebanon in October 2022. The outbreak
44 lasted three months, with 8,007 suspected cases (671 laboratory-confirmed) and 23 deaths.
45 We characterised the *Vibrio cholerae* strain responsible for this cholera outbreak.

46 **Methods**

47 In total, 34 *Vibrio cholerae* isolates collected by random sampling of stools, water and plant
48 samples throughout the outbreak and over the affected regions were studied by phenotypic
49 methods and microbial genomics.

50 **Findings**

51 All isolates were *V. cholerae* O1, serotype Ogawa strains from wave 3 of the seventh
52 pandemic El Tor (7PET) lineage. Phylogenomic analysis unexpectedly revealed the presence
53 of two different 7PET strains, a highly unusual finding outside the Bay of Bengal, where
54 several sublineages circulate together. The dominant strain had a narrow antibiotic resistance
55 profile and was phylogenetically related to South Asian *V. cholerae* isolates. The second
56 strain, which was found exclusively in South Lebanon and Beqaa, was resistant to multiple
57 antibiotics, including macrolides, third-generation cephalosporins and cotrimoxazole. It
58 belonged to the AFR13 sublineage and clustered with *V. cholerae* isolates collected in Yemen

59 from 2016 to 2019. This second Lebanese strain also harboured the same multidrug-
60 resistance (MDR) IncC-type plasmid found in Yemeni isolates from 2018.

61 **Interpretation**

62 The 2022-2023 Lebanese cholera outbreak was caused by the simultaneous introduction of two
63 different 7PET strains. The MDR strain was geographically limited, but the spread of this clone
64 or the horizontal transfer of the MDR plasmid to more susceptible clones could affect epidemic
65 cholera case management. Genomic surveillance is crucial to prevent further spread, and to
66 ensure a prompt and effective response to outbreaks.

67

68 **Funding**

69 The study was funded by the Centers for Disease Control (CDC) award number BAA
70 75D301-21-C-12132, a grant awarded to the American University of Beirut, WHO country
71 office Lebanon, the Lebanese University, and Institut Pasteur.

72

73 **Keywords**

74 Two-strain outbreak, cholera, Lebanon, genomic epidemiology, MDR plasmids

75 **RESEARCH IN CONTEXT PANEL**

76

77 **Evidence before this study**

78 Whole-genome sequencing (WGS) has greatly advanced our understanding and the
79 characterisation of *Vibrio cholerae* outbreaks. However, few studies in the Middle East and
80 North Africa (MENA) region have used this powerful technology. We searched PubMed for
81 studies investigating the molecular epidemiology of *V. cholerae* by WGS in the MENA region,
82 including Lebanon, with the terms “cholera*” AND “a country name of MENA countries” with
83 no restrictions on language or date. The very small number of studies identified concerned
84 Yemen and Algeria. All the outbreaks in the MENA region investigated to date and many
85 others worldwide were caused by a single strain introduced once, contrasting with the endemic
86 setting (the Bay of Bengal) in which several lineages circulate together. One manuscript
87 addressing the history of cholera in Africa from a genomic perspective assigned three Lebanese
88 strains from past outbreaks in 1970 and 1993 as O1 Ogawa isolates from waves 1 and 2 of the
89 seventh pandemic lineage (7PET).

90

91 **Added value of the study**

92 We provide the first comprehensive overview of the molecular epidemiology of the *V.*
93 *cholerae* strains responsible for the 2022-2023 Lebanese cholera outbreak. The use of WGS
94 made it possible to distinguish clearly between two phylogenetically distant strains from
95 genomic wave 3 of the 7PET lineage responsible for the Lebanese outbreak and to assign
96 their putative origins to South Asia and Yemen. Based on their different susceptibility
97 patterns (a predominant strain with a narrow resistance profile and a minor strain with an
98 extended resistance profile), WGS excluded the hypothesis of the multidrug-resistant (MDR)
99 minor strain emerging from the susceptible dominant strain through the acquisition of the

100 MDR plasmid, instead clearly demonstrating the seeding of the outbreak by two different
101 introductions.

102

103 **Implications of all available evidence**

104 This study demonstrates the importance of WGS associated with national surveillance for
105 obtaining new insights and perspectives, modifying our perception of *V. cholerae* outbreak.

106 This unexpected occurrence of a two-strain outbreak in a setting considered non-endemic for
107 *V. cholerae* requires tight control by the local health authorities to prevent the sporadic
108 introduction and spread of additional strains. Our findings raise the question of the extent to
109 which the strains identified, particularly those from South Asia, spread in Iraq and Syria,
110 neighbouring countries that declared cholera outbreaks before Lebanon. It is difficult to
111 answer this question due to the lack of strains collected from these countries. Regional
112 surveillance of the causal agent of cholera is therefore essential, to unravel transmission
113 events and monitor the emergence of antimicrobial drug-resistant strains observed in many
114 countries around the world.

115

116

117 INTRODUCTION

118 Cholera is an acute life-threatening diarrhoeal disease caused by two cholera toxin-producing
119 serogroups — O1 and, less frequently, O139 — of a Gram-negative bacterium, *Vibrio*
120 *cholerae*; it occurs following the ingestion of contaminated water and food in endemic and
121 epidemic settings.¹ Cholera continues to be a major public health problem, with 1.3 to 4.0
122 million cases and 21,000 to 143,000 deaths annually according to the World Health
123 Organisation (WHO).² Cholera outbreaks are still raging globally, particularly in countries
124 already bearing the brunt of natural disasters, human turmoil, and weak economic systems.
125 Indeed, the two countries hardest hit by outbreaks in modern history are Yemen (2016-present)
126 and Haiti (2010-2019; September 2022- present)) with record numbers of cases, at 2.5 million
127 (as recorded in April 2021) and 820,000, respectively.^{3,4}

128

129 Lebanon has witnessed several cholera outbreaks throughout its history, the most recent of
130 these past outbreaks occurring between July and December 1993, with a total of 344 cases and
131 29 deaths.⁵ On October 6th, 2022, the Lebanese Ministry of Public Health (MoPH) notified the
132 WHO of two laboratory-confirmed cases of cholera meeting all diagnostic criteria in the
133 absence of an epidemiological link with a confirmed cholera outbreak (i.e. culture, including
134 seroagglutination with *V. cholerae* O1-specific antisera, and confirmation of the presence of
135 the cholera toxin genes by PCR) reported by the North and Akkar governorates in northern
136 Lebanon. The index case, a 51-year-old Syrian man living in an informal settlement in Minieh-
137 Donniyeh district (North governorate), was reported on October 5th, 2022. This patient was
138 admitted to hospital on the October 1st, with rice-water diarrhoea and severe dehydration. The
139 second case occurred in a 47-year-old health worker, possibly through healthcare-associated
140 transmission and corresponding to the first nosocomial infection of this outbreak. Shortly after

141 these two cases were identified, the epidemiological surveillance unit began detecting active
142 cases in the informal settlement inhabited by the index case. In total, about ten additional cases
143 were diagnosed by laboratory testing. *V. cholerae* was also found in sources of drinking water,
144 irrigation, and sewage (October 9, 2022). In parallel, two culture-confirmed cases were
145 identified in Halba (the capital of Akkar Governorate). On the October 10th, an additional four
146 cases were confirmed by culture in Syrian nationals living in an informal settlement in Aarsal,
147 a town in the Baalbek district.

148

149 Within three months, the cholera outbreak spread across all eight governorates and 20 of the
150 25 districts in Lebanon (Figure 1). The last positive case was recorded on January 5th, 2023.
151 As of June 2023, the cumulative number of reported cholera cases was 8,007, with 671
152 cumulative culture-confirmed cases and 23 deaths. The outbreak was concentrated in the
153 northern governorates of Akkar and North Lebanon, and in the Beqaa Valley. About 29% of
154 the cases concerned children aged 0-4 years, and 16% of all cases have required hospitalisation.
155 Daily hospitalisation rates peaked one week into the outbreak, with more than 220 patients
156 hospitalised per day. During this period, the case fatality rate (CFR) for cholera reached 11%
157 and the attack rate was highest in the northern districts of Akkar and Minieh-Donniyeh. The
158 intensity of the outbreak necessitated the activation of a multisectoral response to increase
159 cholera preparedness, including greater laboratory capacity for cases of acute watery diarrhoea
160 (AWD) (testing of stools and water) and the training of surveillance and rapid response teams
161 in the early identification of AWD cases. The response to cholera was also strengthened by
162 introducing the oral cholera vaccine (OCV) and ensuring the maintenance of adequate support
163 for intensive care units to prepare for emergencies and the provision of training in the
164 management of cholera and other forms of AWD. The massive cholera vaccination campaign

165 (more than 1 million people) conducted up to January 2023 helped to contain the disease but
166 did not entirely eliminate the risk, particularly as only a single dose of vaccine was
167 administered, rather than the recommended two doses, thus providing protection against
168 cholera for about six months, rather than two years. There is therefore a risk of a resurgence of
169 the disease.

170

171 Two different antimicrobial drug resistance (AMR) profiles differing in prevalence were
172 observed in the *V. cholerae* O1 isolates obtained during the 2022-2023 Lebanese outbreak: a
173 major profile with a narrower resistance spectrum and a minor profile with an extended
174 multidrug-resistant (MDR) profile. Two scenarios could potentially account for this
175 distribution: the first, considered the most likely *a priori*, involves the acquisition of a mobile
176 genetic element, such as an MDR plasmid, by the strain with a narrower resistance profile
177 initially responsible for the outbreak, following some kind of selection pressure and thereby
178 resulting in an MDR profile (as in Yemen).⁶ The second scenario, which is uncommon
179 outside the Bay of Bengal, involves the circulation of two different strains introduced
180 separately into Lebanon. We used whole-genome sequencing (WGS) and genomic
181 epidemiology approaches to determine which of these two scenarios had occurred in
182 Lebanon. We also delved into the genetic basis of antibiotic resistance and virulence in the
183 circulating isolates.

184

185

186 **MATERIALS AND METHODS**

187 **Ethics statement**

188 This research study was based exclusively on bacterial isolates and the corresponding metadata
189 collected for nationwide surveillance of the cholera outbreak by the Lebanese Ministry of
190 Public Health (MoPH) in collaboration with the American University of Beirut (AUB), Rafic
191 Hariri University Hospital, and the “Laboratoire Microbiologie Santé et Environnement”
192 (LMSE). Hence, neither informed consent nor institutional review board (IRB) approval was
193 required.

194

195 ***Vibrio cholerae* isolates**

196 Stools, sewage, water and plant samples were collected by the MoPH and delivered to the
197 bacteriology and molecular microbiology research laboratory at AUB, a WHO collaborating
198 centre for reference and research on bacterial pathogens. In total, 671 clinical isolates of *V.*
199 *cholerae* were identified, with 144 isolates from North Lebanon collected and stored in the “la
200 Collection Microbiologique de l’Université Libanaise (CMUL)” at LMSE at the Lebanese
201 University. We included 18 isolates from AUB and 16 from the LMSE in this study (Appendix
202 1). These isolates were recovered between October and December 2022, for continuous
203 surveillance and prevention, the last positive case being reported on January 5th, 2023.

204

205 **Bacterial culture and identification**

206 The approach to bacterial culture and identification differed between the LMSE and AUB
207 laboratories. At the LMSE, part of each stool sample was plated directly on two different media,

208 a non-selective nutrient-rich agar medium (pH=8.5, 10 g/l NaCl), and a selective agar medium,
209 thiosulphate-citrate-bile salts-sucrose (TCBS) agar (BioMérieux, Marcy-l'Etoile, France).
210 Another portion of each sample was incubated in alkaline peptone water (Bio-Rad, Marnes-la-
211 Coquette, France; 10 g/l NaCl) for 6-8 hours at 35-37°C and was then plated on the same solid
212 media. By contrast, at AUB, the stool sample was incubated in alkaline peptone water for 6
213 hours at 35°C and was then plated on the surface of TCBS agar, MacConkey agar (Bio-Rad),
214 and *Vibrio* Chromagar (CHROMagar, Paris, France). After standard microbiological
215 identification by microscopy (a comma-shaped Gram-negative bacterium) and oxidase tests,
216 *V. cholerae* isolates were identified with API 20E test strips (BioMérieux) and by matrix-
217 assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF), with
218 Vitek MS (BioMérieux) at LMSE or MALDI Biotyper (Bruker Daltonics, Germany) at AUB.
219 Agglutination was performed with specific antisera (Artron Laboratories Inc., British
220 Columbia, Canada for O1 and O139 antisera) and the presence of cholera toxin genes was
221 confirmed with a multiplex PCR method developed by Hoshino and colleagues,⁷ for the first
222 five cases.

223

224 **Antimicrobial drug susceptibility testing**

225 Antimicrobial drug susceptibility testing was performed by the disk diffusion method in
226 accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Tests were
227 performed for 14 antimicrobial agents: piperacillin/tazobactam, cefotaxime, ceftazidime,
228 meropenem, nalidixic acid, ciprofloxacin, levofloxacin, erythromycin, azithromycin,
229 trimethoprim-sulfamethoxazole (cotrimoxazole), tetracycline, doxycycline, nitrofurantoin, and
230 vibriostatic agent O/129. CLSI interpretative criteria for the antibiotic susceptibility testing of
231 *Vibrio spp.* (M45 document) were used when available.⁸ For vibriostatic agent O/129

232 (equivalent to trimethoprim), nitrofurantoin, nalidixic acid, and ciprofloxacin, the
233 interpretative criteria for *Enterobacteriaceae/Salmonella* spp. (M100-S30 document) were
234 used.⁹ The minimum inhibitory concentrations (MICs) of colistin for 19 isolates — 16 from
235 the LMSE and 3 from the AUB — were determined at Institut Pasteur with the Sensititre™
236 system (Thermo Fisher Scientific).

237

238 **Whole-genome sequencing**

239 We studied 34 *V. cholerae* isolates, 16 originating from the LMSE, which were sequenced at
240 the Institut Pasteur in Paris, and 18 isolates originating from AUB, which were sequenced in-
241 house. At Institut Pasteur, genomic DNA was extracted with the Maxwell 16-cell purification
242 kit (Promega, <https://www.promega.com>). The DNA libraries were then prepared at the Institut
243 Pasteur Mutualized Platform for Microbiology (P2M) with the Nextera XT kit (Illumina, San
244 Diego, CA, USA) and sequencing was performed with the NextSeq 500 system (Illumina),
245 generating 150 bp paired-end reads. At AUB, genomic DNA was extracted with the Quick-
246 DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA) and purified with the
247 Genomic DNA Clean & Concentrator™ kit (Zymo Research), according to the manufacturer's
248 protocols. DNA libraries were prepared with the Illumina DNA prep kit (Illumina GmbH,
249 Munich, Germany) and subjected to Illumina MiSeq 2 × 150 bp paired-end sequencing by
250 Illumina MiSeq.

251

252 The resulting reads were filtered with FqCleanER version 21.10
253 (<https://gitlab.pasteur.fr/GIPhy/fqCleanER>) with options -q 28 -l 70 to discard low-quality
254 reads with phred scores <28 and length <70 bp and to remove adaptor sequences.¹⁰

255

256 Two isolates were selected for long-read sequencing, based on geographic origin and
257 representativity of the two circulating strains. Long-read sequencing was performed on isolate
258 CNRVC220127 (alternative name CMUL 009) at Institut Pasteur, with a MinION nanopore
259 sequencer (Oxford Nanopore Technologies), as previously described.⁶ The second isolate
260 sequenced (VIC_202210_72, alternative name VIC11A) was cultured on MacConkey agar.
261 DNA was extracted with the Quick DNA Fungal/Bacterial Miniprep Kit (by ZYMO Research)
262 and cleaned with DNA Clean & Concentrator -5 (by ZYMO Research). The clean DNA was
263 then sequenced with an Oxford Nanopore MinION at AUB. The DNA library was prepared
264 with Rapid Barcoding Kit 96 (SQK-RBK110.96) and sequenced on R9.4.1 flow cells (FLO-
265 MIN106).

266

267 The sequences of the two isolates were assembled from both long and short reads, by two
268 different methods. For CNRVC220127, a hybrid approach was used in UniCycler v.0.4.8.¹⁰
269 For VIC_202210_72, a combination of Raven¹¹ v.1.6.0 (<https://github.com/lbcb-sci/raven>),
270 Medaka v.1.4.4 (<https://github.com/nanoporetech/medaka>) and Polypolish¹² v.0.5.0
271 (<https://github.com/rrwick/Polypolish/>) was used. The large plasmid of VIC_202210_72 was
272 then annotated with Bakta¹³ v.1.5.0, corrected manually and visualised with BRIG v.0.95
273 (<http://sourceforge.net/projects/brig>).¹⁰

274

275 **Genomic analysis**

276 For construction of a globally representative set of isolates, we downloaded and included in
277 this study sequences available either in raw-read format or as assembled genomes in the

278 European Nucleotide Archive (ENA) (<https://www.ebi.ac.uk/ena>) and GenBank
279 (<https://www.ncbi.nlm.nih.gov/genbank/>) databases (Appendix 2).

280

281 The phylogenomic analysis was performed as previously described.¹⁴ Briefly, the paired-end
282 reads were mapped onto the reference genome of *V. cholerae* O1 El Tor strain N16961, also
283 known as A19 (GenBank accession numbers LT907989 and LT907990) with Snippy. The
284 single-nucleotide variants (SNVs) were then called with Snippy v.4.6.0/Freebayes v.1.3.2
285 (<https://github.com/tseemann/snippy>), using the following parameters: a minimum read
286 coverage of 4, a minimum base quality of 13, a mapping quality of 60, and a 75% read
287 concordance at a locus for a variant to be reported. Finally, core-genome SNVs were then
288 aligned in Snippy for phylogenetic inference.

289

290 Repetitive sequences (insertion sequences and the TLC-RS1-CTX region) and recombinogenic
291 regions (VSP-II) were masked.¹⁵ Putative recombinogenic regions were identified and masked
292 with Gubbins v.3.2.0.¹⁰ A maximum likelihood (ML) phylogenetic tree was constructed from
293 an alignment of 10,632 chromosomal SNVs, with RAxML v. 8.2.12, under the GTR model,
294 with 200 repetitions for bootstrapping.¹⁶ This global tree was rooted on the A6 genome and
295 visualised with iTOL v.6 (<https://itol.embl.de/>).¹⁷

296

297 Short reads from Illumina were assembled *de novo* with SPAdes v.3.15.2.¹⁰ The presence of
298 various genetic markers (O1 *rfb* gene, whole locus of VSP-II, *ctxB*, and *wbeT*) was investigated
299 with BLAST v.2.2.26 against reference sequences, as previously described.^{14,15} The presence
300 and type of acquired antibiotic resistance genes (ARGs) or ARG genetic structures were

301 investigated with ResFinder v.4.0.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>),
302 PlasmidFinder v.2.1.1 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>), and BLAST analysis
303 against GI-15, Tn7, and SXT/R391 integrative and conjugative elements (ICE).¹⁵ The
304 sequences assembled *de novo* were examined with BLAST to look for mutations of genes
305 encoding resistance to nitrofurans (*VC_0715* and *VC_A0637*), resistance to quinolones (*gyrA*
306 and *parC*) or restoring susceptibility to polymyxin B (*vprA*), as previously described.^{15,18}

307

308 **Data availability**

309 Short reads were submitted to the ENA under study project PRJEB65303 (Appendix 2).
310 Assemblies resulting from long-read sequencing were submitted to GenBank under project
311 PRJNA1013428 (CP134060-CP134061 for CNRVC220127/CMUL009 and CP134057-
312 CP134059 for VIC11-A).

313

314

315 RESULTS

316 Antimicrobial drug susceptibility testing results

317 Antimicrobial drug susceptibility testing in the Lebanese laboratories (LMSE and AUB)
318 identified two different AMR profiles (Table 1 and Appendix 1) in the 671 *Vibrio cholerae* O1
319 isolates recovered during the outbreak. One of these profiles predominated, accounting for
320 94.7% (636/671) of the isolates collected across Lebanon, including the 19 isolates tested at
321 Institut Pasteur. It displayed resistance to nitrofurantoin, the vibriostatic agent O/129, and
322 nalidixic acid only, and decreased susceptibility to ciprofloxacin. The second profile was a
323 minor profile accounting for only 5.6% (35/671) of the isolates. It was characterised by
324 resistance to third-generation cephalosporins (cefotaxime and ceftazidime), macrolides
325 (erythromycin and azithromycin), sulphonamides, the vibriostatic agent O/129, cotrimoxazole,
326 nitrofurantoin and nalidixic acid, and decreased susceptibility to ciprofloxacin. This second
327 AMR profile was found exclusively in isolates originating from the South of Lebanon (Tyr)
328 and Beqaa.

329

330 Phylogenetic and genomic features of *V. cholerae* O1 isolates

331 We selected 34 *V. cholerae* O1 isolates for further analysis on the basis of their AMR profiles
332 (Figure 1a); 31 presented the predominant AMR profile (limited resistance) and three presented
333 the minor AMR profile (extended resistance) (Table 1). Genome sequencing confirmed that all
334 34 isolates belonged to serogroup O1, serotype Ogawa and biotype El Tor (sequence type
335 ST69). All the *V. cholerae* O1 isolates (Table 1) displayed the following genomic features: (i)
336 the *ctxB7* variant of the cholera toxin subunit B gene, (ii) the toxin-coregulated pilus gene

337 subunit A gene variant *tcpA*^{CIRS101}, and (iii) a deletion (Δ VC0495–0512) in the *Vibrio* seventh
338 pandemic island II (VSP-II) (Table 1).

339

340 All 34 *V. cholerae* O1 isolates had (i) a deletion of about 10 kb in the chromosomal
341 ICE*Vch*Ind5 integrative and conjugative element, resulting in the loss of four genes encoding
342 resistance to streptomycin (*strA* and *strB*), sulphonamides (*sul2*), and chloramphenicol (*floR*),
343 but not the fifth gene encoding resistance to the vibriostatic agent O/129 (*dfrA1*), (ii)
344 mutations of the chromosomal *VC0715* (resulting in the R169C substitution) and *VCA0637*
345 (resulting in a premature codon stop at Q5) nitroreductase genes, leading to nitrofurantoin
346 resistance, (ii) a mutation of the chromosomal *VC1320* (*vprA*) (D89N) gene re-establishing
347 susceptibility to polymyxin B, and (iv) mutations of the chromosomal DNA gyrase *gyrA*
348 (S83I) and topoisomerase IV *parC* (S85L) genes, leading to nalidixic acid resistance and
349 decreased susceptibility to ciprofloxacin. The three isolates with the second AMR profile also
350 carried an IncC of about 139 kb (formerly IncA/C₂). This plasmid displayed 100% nucleotide
351 sequence identity to the MDR plasmid, pCNRVC190243 (GenBank accession number
352 OW443149.1),⁶ found in *Vibrio cholerae* O1 isolates from Yemen in 2018-2019 (Figure 2).⁶
353 The backbone of pCNRVC190243 harboured a 20 kb pseudo-compound transposon,
354 Yem*Vch*MDRI, flanked by IS26 insertion sequences and encompassing genes encoding
355 aminoglycoside resistance (*aadA2*), a quaternary ammonium compound efflux pump (*qac*),
356 sulphonamide resistance (two copies of the *sul1* gene), an extended-spectrum beta-lactamase
357 (ESBL; *bla*_{PER-7}), and macrolide resistance (*mph(A)*, *mph(E)*, and *msr(E)*). The resistance to
358 cotrimoxazole of isolates with the second AMR profile probably resulted from the
359 simultaneous presence of the plasmid-borne *sul1* gene and the chromosomal *dfrA1* gene. The
360 pCNRVC190243 plasmid had a nucleotide sequence 99.98% identical to that of
361 pYA00120881 (GenBank accession number MT151380) identified in Zimbabwean *Vibrio*

362 *cholerae* O1 isolates collected in 2015 and 2018, but it carried a different multidrug-
363 resistance region, containing, in particular, the ESBL gene *bla*_{CTX-M-15}.¹⁹

364

365 One isolate (VIC_202211_60) also harboured a putative small Col3M colicin plasmid
366 encoding a plasmid-mediated quinolone resistance protein, QnrD1, a member of the Qnr family
367 protecting DNA-gyrase and topoisomerase IV against quinolones. However, this had no effect
368 on the quinolone resistance profile of this isolate.

369

370 We then placed these 34 *V. cholerae* O1 isolates from the Lebanon 2022-2023 outbreak in a
371 global context by constructing a maximum-likelihood phylogeny of 1,465 7PET genomic
372 sequences using 10,632 SNVs evenly distributed over the non-repetitive, non-recombinant core
373 genome. All 34 isolates clustered in the genomic wave 3 clade of the 7PET lineage, and more
374 particularly in the subclade containing isolates with the *ctxB7* allele (Figure 3). These 34
375 isolates also differed from other isolates previously recovered in Lebanon, including those
376 isolated in 1970 and 1993, which clustered within genomic waves 1 and 2 of the 7PET lineage,
377 respectively (Figure 3).

378

379 Our phylogenomic analysis revealed that the 34 *V. cholerae* O1 isolates were distributed
380 between two different clusters according to their AMR profiles (Figure 3). Indeed, the 31
381 isolates with the predominant profile clustered together (median pairwise distance of 1.5 [range
382 0–9] core-genome SNVs) and with many other isolates originating from the Pakistan 2022
383 outbreak, from India (2019-2022), and one isolate from the Iraq 2022 outbreak. The three

384 remaining isolates with the extended AMR profile clustered together (no SNV between them)
385 and with Yemeni isolates recovered between 2016 and 2019.

386

387 **DISCUSSION**

388 After approximately three decades without cholera, Lebanon recently suffered an outbreak
389 extending from October 2022 to January 2023 (Figure 1).²⁰ The two different AMR profiles
390 observed in the Lebanese isolates initially suggested the possibility of a mother strain relatively
391 susceptible to antibiotics acquiring an MDR plasmid early in the outbreak, or of two different
392 strains circulating simultaneously, this second possibility being considered less likely.
393 However, the high discrimination power of WGS made it possible to distinguish two different
394 strains of *V. cholerae* O1 serotype Ogawa harbouring the *ctxB7* allele from genomic wave 3 of
395 the 7PET lineage, and, thus, to conclude that the 2022-2023 cholera outbreak in Lebanon was
396 caused by two phylogenetically distant strains rather than a single strain that subsequently
397 acquired an MDR plasmid. The two-strain outbreak scenario was initially considered unlikely
398 because outbreaks in countries non-endemic for cholera generally occur following a single
399 introduction of a single strain, contrasting with the situation in the Bay of Bengal, where many
400 lineages circulate simultaneously.^{21,22} This two-strain outbreak in Lebanon is thus unusual, as
401 it stems from two different introductions outside the endemic setting. The two strains
402 concerned had different AMR profiles and different patterns of circulation in Lebanon. The
403 strain with the narrower AMR profile predominated in all affected regions of Lebanon,
404 including North Lebanon in particular, whereas the strain with the broader AMR profile was
405 found only in South Lebanon and Beqaa.

406

407 Globally, the 7PET lineage has been responsible for the repeated spread of the seventh
408 pandemic from the Bay of Bengal in South Asia to the rest of the world through three epidemic
409 waves.^{15,23} The wave 3 clade carrying the *ctxB7* allele first emerged in Kolkata (India) in
410 2006,²⁴ subsequently spreading to other parts of the world, including Haiti and Yemen, and
411 across Africa.^{6,19,25,26}

412

413 In the global phylogenetic tree, the predominant Lebanese strain clustered with isolates from
414 South Asia, including isolates from the 2022 Pakistani outbreak collected locally (GenBank
415 bioproject PRJNA916827, <https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA916827>)
416 or in travellers from the US and Australia with a history of travel to Pakistan.¹⁴ These isolates
417 were considered the direct ancestors of the Lebanese strain (Figure 3). Two very recently
418 published studies revealed that the strain circulating in the 2022 Pakistani outbreak was also
419 the ancestor of a strain circulating in South Africa and Malawi considered to belong to the
420 AFR15 sublineage.^{14,27} Indeed, although the Lebanese strains were not identified and analysed
421 in the studies performed in Malawi, their phylogenetic tree incorporated the same genomes
422 from Pakistan and showed the grouping of these genomes with the AFR15 sublineage, thereby
423 revealing similarity between the predominant Lebanese strain and the AFR15 sublineage. A
424 sublineage closely related to AFR15 may therefore have been imported into the Middle East
425 region directly from South Asia or indirectly via Africa. Interestingly, one isolate
426 (PNUSAV00294, SRR20325463)¹⁴ collected in the US in June 2022 from a traveller with a
427 history of recent travel to Iraq clustered with the Lebanese isolates, suggesting that the
428 predominant strain in this Lebanese outbreak was the same strain that swept the region (Iraq
429 and Syria) shortly before the Lebanese outbreak, causing outbreaks beginning on June 20th,
430 2022 in Iraq,²⁸ and September 10th, 2022 in Syria.²⁹ One month after the declaration of the
431 Syrian outbreak, Lebanon declared its first index case in a Syrian refugee residing in North

432 Lebanon, providing additional support for the theory that the predominant strain in Lebanon
433 was imported from Syria. According to the United Nations High Commission for Refugees,
434 Lebanon has the largest refugee population per capita in the world, with an estimated 1.5
435 million Syrian refugees living on its soil. Nevertheless, our ability to infer precise transmission
436 routes through genomic analysis is hampered by the lack of availability of isolates from the
437 various affected countries in the region, including Syria and Iraq.

438

439 The minor strain displaying MDR grouped with isolates from the 2019 outbreak in Yemen,
440 suggesting the probable direct introduction of this strain from Yemen into South Lebanon and
441 Beqaa, the areas from which it was exclusively isolated. Like the Yemeni isolates, this strain
442 carried an IncC plasmid (pCNRVC190243) bearing determinants of resistance to
443 cotrimoxazole, macrolides, third-generation cephalosporins and aminoglycosides. The Yemeni
444 epidemic comprised several waves but was seeded by a single introduction linked to the 7PET
445 sublineage AFR13, which was recently transmitted from South Asia into East Africa and from
446 there to Yemen.^{6,18} Before 2018-2019, AFR13 isolates had features resembling those of the
447 predominant Lebanese strain, including a narrow resistance profile, due partly to an
448 ICE*Vch*Ind5 deletion and the subsequent loss of four of five AMR genes. However, a plasmid-
449 carrying AFR13 clone, the pCNRVC190243-carrying AFR13 clone, began to emerge in late
450 2018 and displayed resistance to many therapeutically relevant drugs. The spread of this MDR
451 clone was driven by the therapeutic overuse of macrolides.⁶ Our multidrug-resistant minor
452 strain is, therefore, a Yemeni AFR13 clone carrying a self-transmissible MDR plasmid. The
453 spread of this strain would greatly decrease treatment options and jeopardise cholera case
454 management. This bleak scenario might occur if the multidrug-resistant Yemeni clone manages
455 to expand beyond its current geographic location and spread throughout Lebanon or if it passes
456 its plasmid to the predominant strain (sublineage closely related to AFR15).

457

458 Similar IncC plasmids were previously observed in other *V. cholerae* strains, including the
459 pYAM00120881 plasmid identified in Zimbabwe in 2018, which has a backbone almost
460 identical to that of pCNRVC190243.¹⁹ Intriguingly, the pYAM00120881-carrying AFR13
461 clone caused a six-month-long outbreak in Zimbabwe, with over 10,000 suspected cases,¹⁹
462 demonstrating a certain degree of plasmid stability in a 7PET *V. cholerae* strain sustaining an
463 outbreak and even providing evidence against the presumed plasmid instability in this species.⁶
464 It has been suggested that the 10 kb deletion in SXT/R391 ICE (*ICEVchInd5*) seen in our
465 isolates might render these isolates fit to host MDR IncC plasmids stably, with deleterious
466 consequences for antibiotic susceptibility in the future.⁶

467

468 Our genomic analysis revealed general consistency with the phenotypic AMR profile. All the
469 isolates also harboured the *catB9* gene, but this gene does not confer chloramphenicol
470 resistance,³⁰ consistent with the chloramphenicol susceptibility of the isolates (data not shown).
471 Resistance to polymyxin B has been used as a marker of *V. cholerae* O1 biotype El Tor since
472 the start of the seventh cholera pandemic, as a means of differentiating this biotype from the
473 classical biotype, which was susceptible to polymyxin B. The susceptibility to polymyxins of
474 our isolates may be due to the VprA (VC1320) D89N substitution, as previously reported in
475 the AFR13 and AFR14 sublineages.^{18,26}

476

477 Lebanon has been struggling with an unprecedented, multifaceted crisis since 2019, including
478 severe economic collapse, the COVID-19 pandemic, the explosion in the Port of Beirut in
479 August 2020 and a high burden of refugees. This major crisis and the fragile infrastructure of

480 Lebanon favoured this outbreak. The economic crisis, with all its implications, affected all
481 aspects of the outbreak. Access to safe water was hampered by the lack of electricity and the
482 inadequacy of the sewage system. The country was already suffering from a shortage of
483 medical and diagnostic supplies in addition to the global shortage of oral cholera vaccines, and
484 laboratory supplies for cholera diagnosis. Nevertheless, tremendous collaborative efforts were
485 initiated, under the auspices of the Ministry of Public Health in Lebanon and in collaboration
486 with several national and international organisations including, but not limited to the WHO,
487 UNICEF, UNHCR, and ICRC, making it possible to halt the spread of the disease within three
488 months of the declaration of the index case. However, we are well aware of the possibility of
489 disease resurgence and of another outbreak, particularly as the neighbouring countries have not
490 yet brought their own outbreaks under control.

491

492 In conclusion, the outbreak in Lebanon was caused by two different strains: one with a narrower
493 AMR profile related to South Asian isolates and the other with an extended AMR profile
494 similar to the Yemeni AFR13 *V. cholerae* strain. However, as isolates from the neighbouring
495 countries are missing from the phylogenetic analysis, it may be difficult to establish a
496 comprehensive history for this outbreak. Regional surveillance of the causal agent of cholera
497 by microbial genomics methods is, thus, paramount for the reliable inference of transmission
498 routes and for tracking and monitoring the emergence of any AMR, particularly after the
499 worrying switch of several AFR13 strains from a limited to an extended MDR phenotype
500 following the acquisition of IncC-type plasmids. ⁶

501

502

503 **CONFLICTS OF INTEREST**

504 The authors declare that there are no conflicts of interest.

505

506 **ACKNOWLEDGMENT**

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508 study, including the American University of Beirut and the American University of Beirut
509 Medical Center, the LMSE, Rafik Hariri University Hospital, the Ministry of Public Health
510 Lebanon, The Lebanese Red Cross, Institut Pasteur, the WHO, and the CDC.

511

512 **Figure 1. Geographic location at which the *V. cholerae* O1 El Tor isolates sequenced were**
513 **obtained and number of reported cholera cases**

514 a, Cumulative number of suspected cholera cases per day vs. the confirmed number of cases
515 per day in Lebanon until January 3, 2023. The dates on which the isolates sequenced in this
516 study were obtained are shown under the epidemic curve. b, Geographic locations at which the
517 34 sequenced *V. cholerae* O1 El Tor isolates were obtained in Lebanon.

518

519 **Figure 2. Circular map and comparative analysis of the IncC2 plasmid found in some *V.***
520 ***cholerae* O1 isolates from Lebanon in 2022**

521 Circles from innermost to outermost indicate (1) the nucleotide position of the plasmid of the
522 VIC_202210_72 isolate (alternative name pVIC-11A), (2) the alignment throughout the
523 plasmids between pVIC-11A (Lebanon, 2022) in dark green, pCNRVC190243 (Yemen, 2019)
524 (GenBank accession number OW443149.1) in medium green, and pYA00120881 (Zimbabwe,

525 2018) (GenBank accession number MT151380) in light green, (3) the G+C content map of
526 pVIC-11A, and (4) the coding sequences (CDS) map of pVIC-11A, in which green arrows
527 indicate antimicrobial drug resistance CDS, dark blue arrows transposase and integrase CDS,
528 red arrows the CDS involved in conjugative transfer, yellow arrows those involved in the
529 structure and cellular processes, and light blue CDS with other functions. The names of
530 resistance genes within the YemVchMDRI are indicated above the corresponding CDS.

531

532 **Figure 3. Maximum-likelihood phylogeny of *Vibrio cholerae* O1 El Tor isolates collected**
533 **in Lebanon in 2022, compared with 1,465 reference seventh pandemic *V. cholerae* El Tor**
534 **genomic sequences**

535 A6 was used as the outgroup. Blue arrows represent the three genomic waves and the black
536 arrow indicates the acquisition of the *ctxB7* allele. The colour coding in the first column shows
537 the geographic origins of the isolates, and African sublineages (AFR1, AFR3–AFR14) are
538 shown on the left. The red colour in the second column indicates the Lebanese origin of the
539 isolates. A magnification of the clades containing the two strains from Lebanon (red square
540 corresponding to the predominant strain and blue square corresponding to the multidrug-
541 resistant minor strain) is shown on the left with red text indicating the Lebanese isolates. For
542 each genome, its name (or accession number), the country in which contamination occurred
543 and the year of sample collection are indicated at the tip of the branch. Scale bars indicate the
544 number of nucleotide substitutions per variable site. Blue dots correspond to bootstrap values
545 $\geq 90\%$.

546

547 **Table 1.** Characteristics of the two epidemic strains of *Vibrio cholerae* O1 involved in the
548 cholera outbreak in Lebanon in 2022-2023*

Category	Predominant strain (<i>n</i> = 31)	Minor strain (<i>n</i> = 3)
Serogroup and serotype,	O1, Ogawa	O1, Ogawa
Sequence type	ST69	ST69
Lineage	7PET	7PET
Genomic wave	3	3
Genetic markers	<i>ctxB7</i> , <i>tcpA</i> ^{CIRS101} , VSP-IIΔ	<i>ctxB7</i> , <i>tcpA</i> ^{CIRS101} , VSP-IIΔ
AMR profile, selected antimicrobial drugs		
Cefotaxime	Susceptible	Resistant
Meropenem	Susceptible	Susceptible
Erythromycin	Susceptible	Resistant
Azithromycin	Susceptible	Resistant
Nalidixic acid	Resistant	Resistant
Ciprofloxacin	Intermediate	Intermediate
Tetracycline	Susceptible	Susceptible
O/129	Resistant	Resistant
Trimethoprim/ sulfamethoxazole	Susceptible	Resistant
Nitrofurantoin	Resistant	Resistant
Colistin	Susceptible	Susceptible
Horizontally acquired AMR elements	ICE <i>Vch</i> Ind5Δ [†]	ICE <i>Vch</i> Ind5Δ, pCNRVC190243 [‡]
Horizontally acquired AMR genes	<i>dfrA1</i> [†]	<i>dfrA1</i> , <i>aadA2</i> , <i>sul1</i> , <i>bla</i> _{PER-7} , <i>mph(A)</i> , <i>msr(E)</i> , <i>mph(E)</i>

Chromosomal gene mutations	AMR phenotype	AMR phenotype
<i>gyrA</i> _S83I and <i>parC</i> _S85L	Resistance to nalidixic acid; decreased susceptibility to ciprofloxacin	Resistance to nalidixic acid; decreased susceptibility to ciprofloxacin
<i>VC0715</i> _R169C and <i>VCA0637</i> _Q5Stop	Resistance to nitrofurantoin	Resistance to nitrofurantoin
<i>vprA</i> _D89N	Susceptibility to colistin	Susceptibility to colistin

549 AMR, antimicrobial resistance; 7PET, seventh pandemic *V. cholerae* biotype El Tor lineage;
550 VSP-II Δ , deletion encompassing VC0495–VC0512 (according to GenBank accession no.
551 AE003852) in *Vibrio* seventh pandemic island II (VSP-II); ICE*Vch*Ind5 Δ , deletion
552 encompassing ICE*Vch*Ind50011–ICE*Vch*InD50019 (according to GenBank accession no.
553 GQ463142) in ICE*Vch*Ind5, an integrative conjugative element (ICE) of the SXT/R391
554 family; †one isolate (VIC_202211_60) also had a Col3M plasmid carrying the *qnrD1* gene,
555 #GenBank accession no. OW443149.1.

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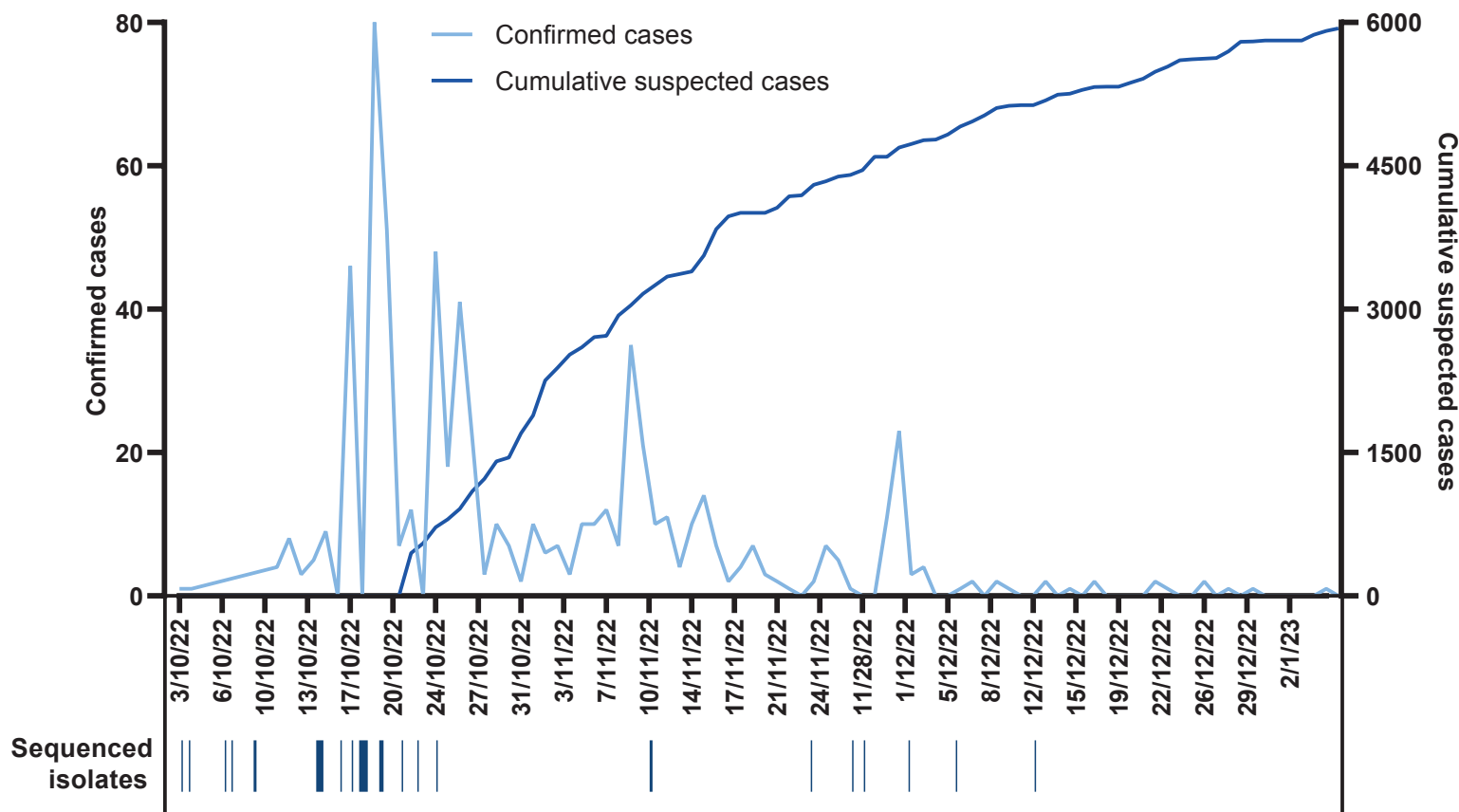
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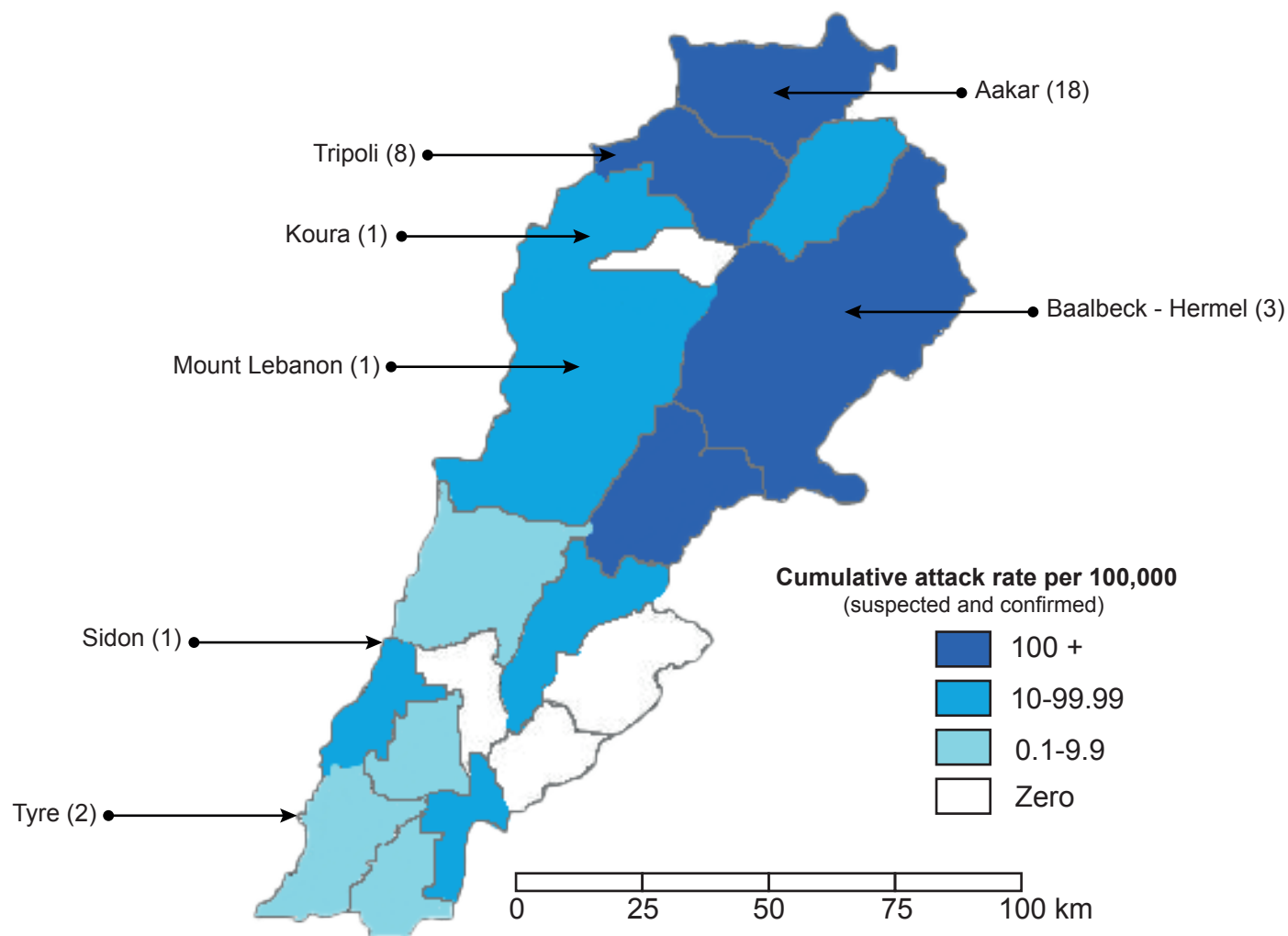
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627

a

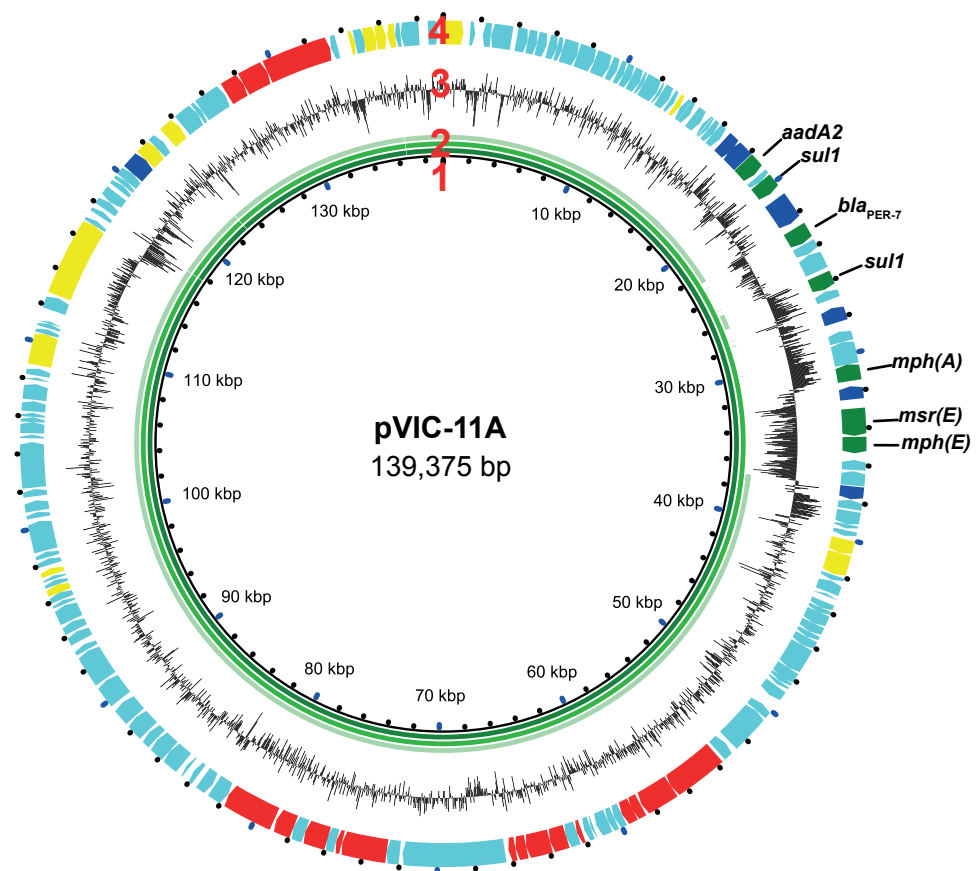


b



Circle 4

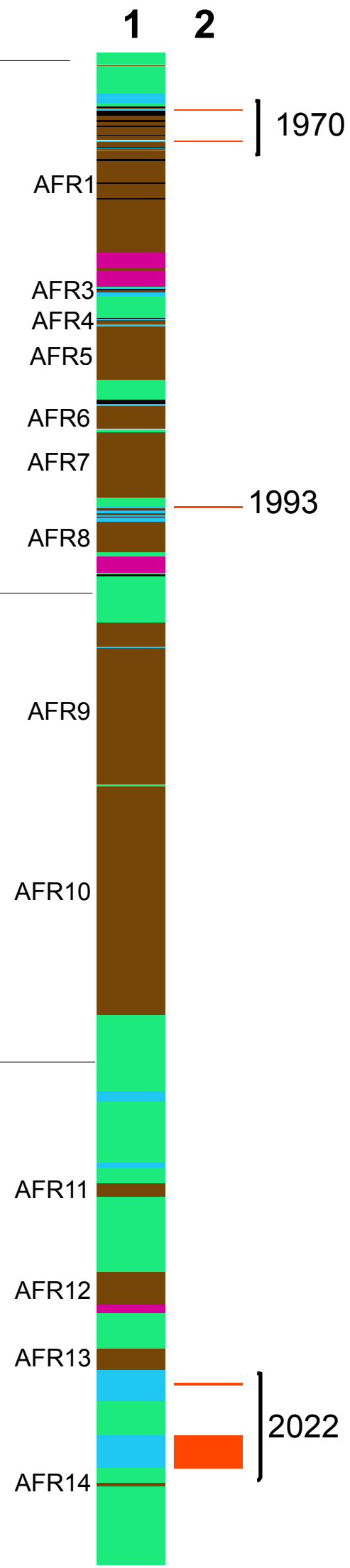
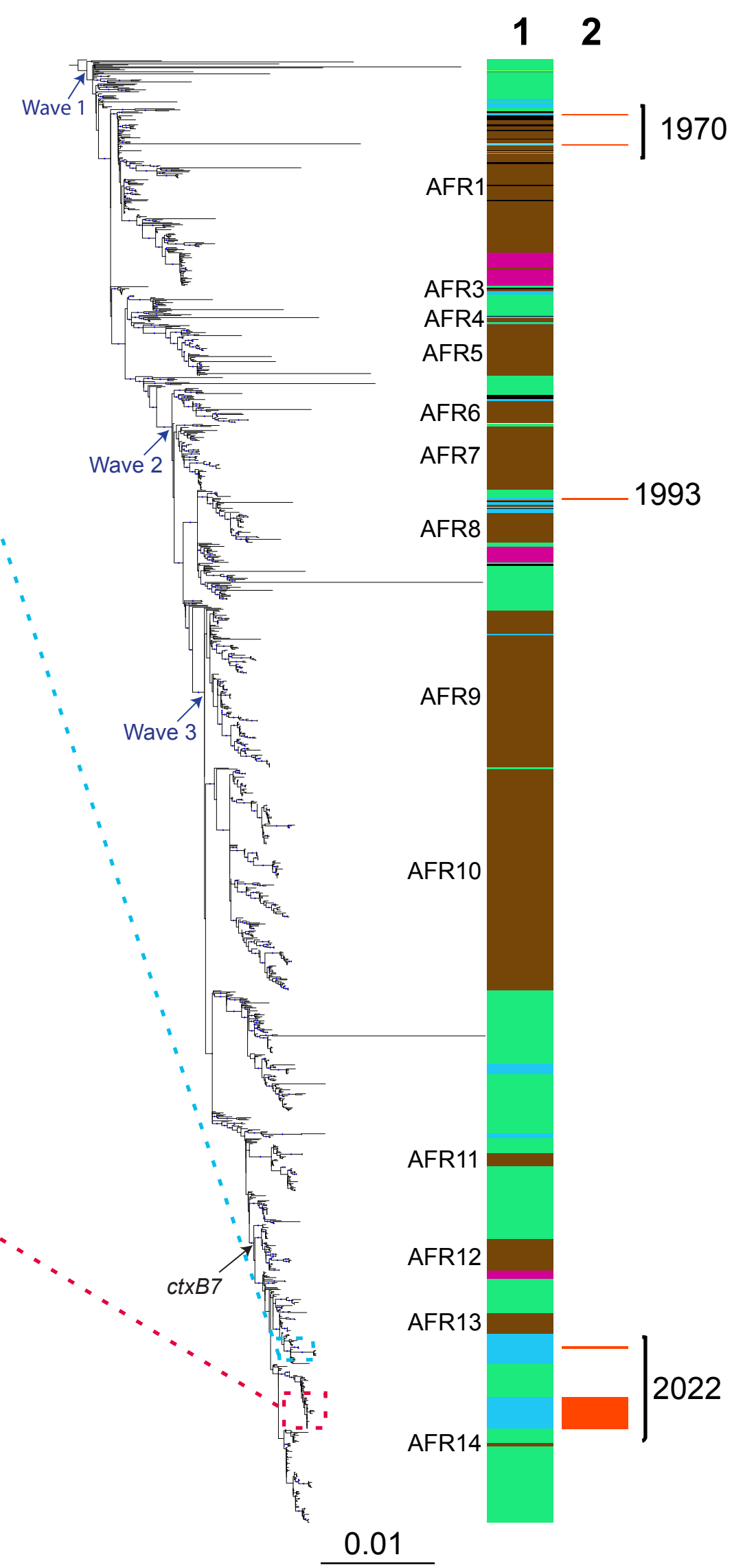
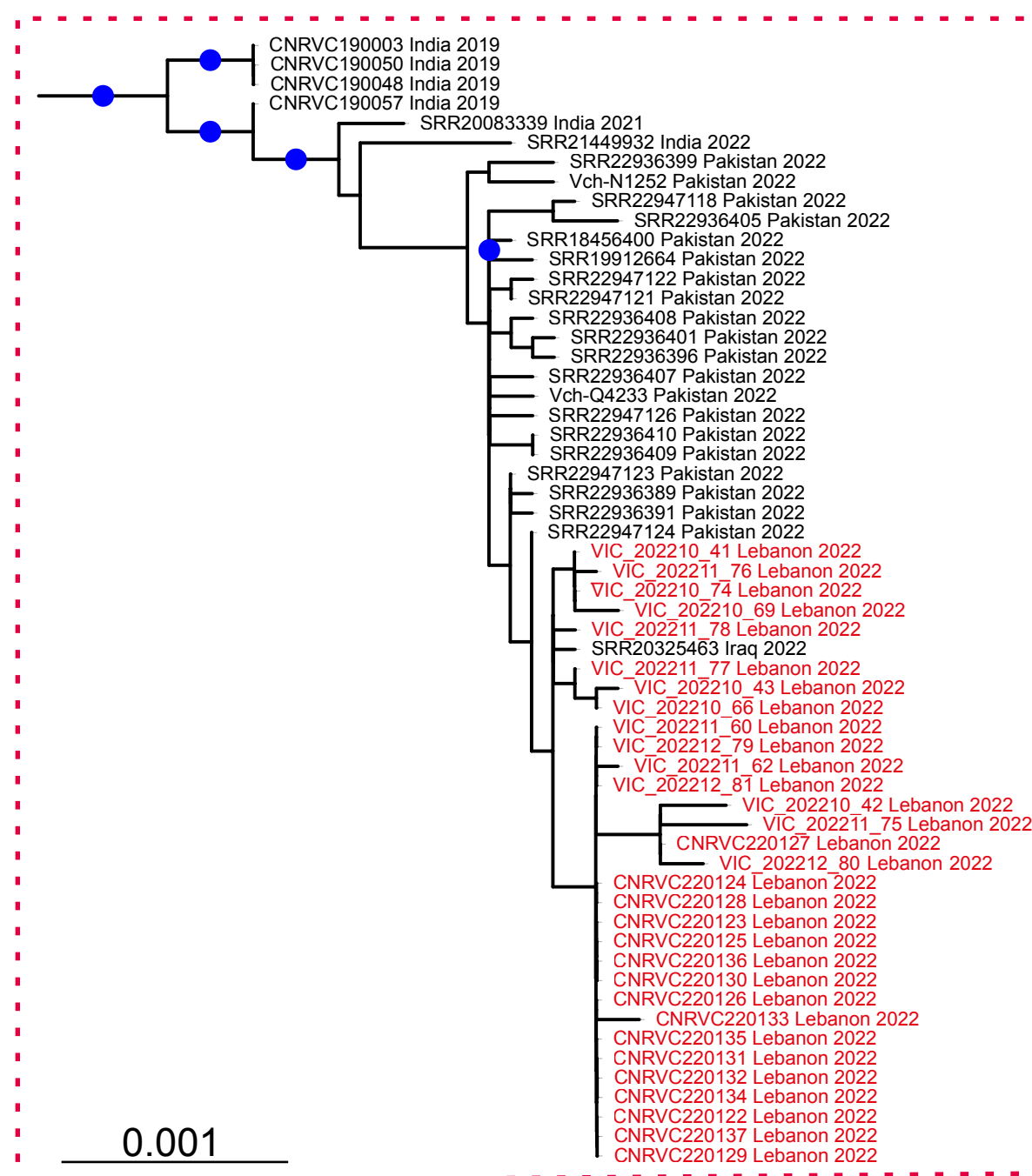
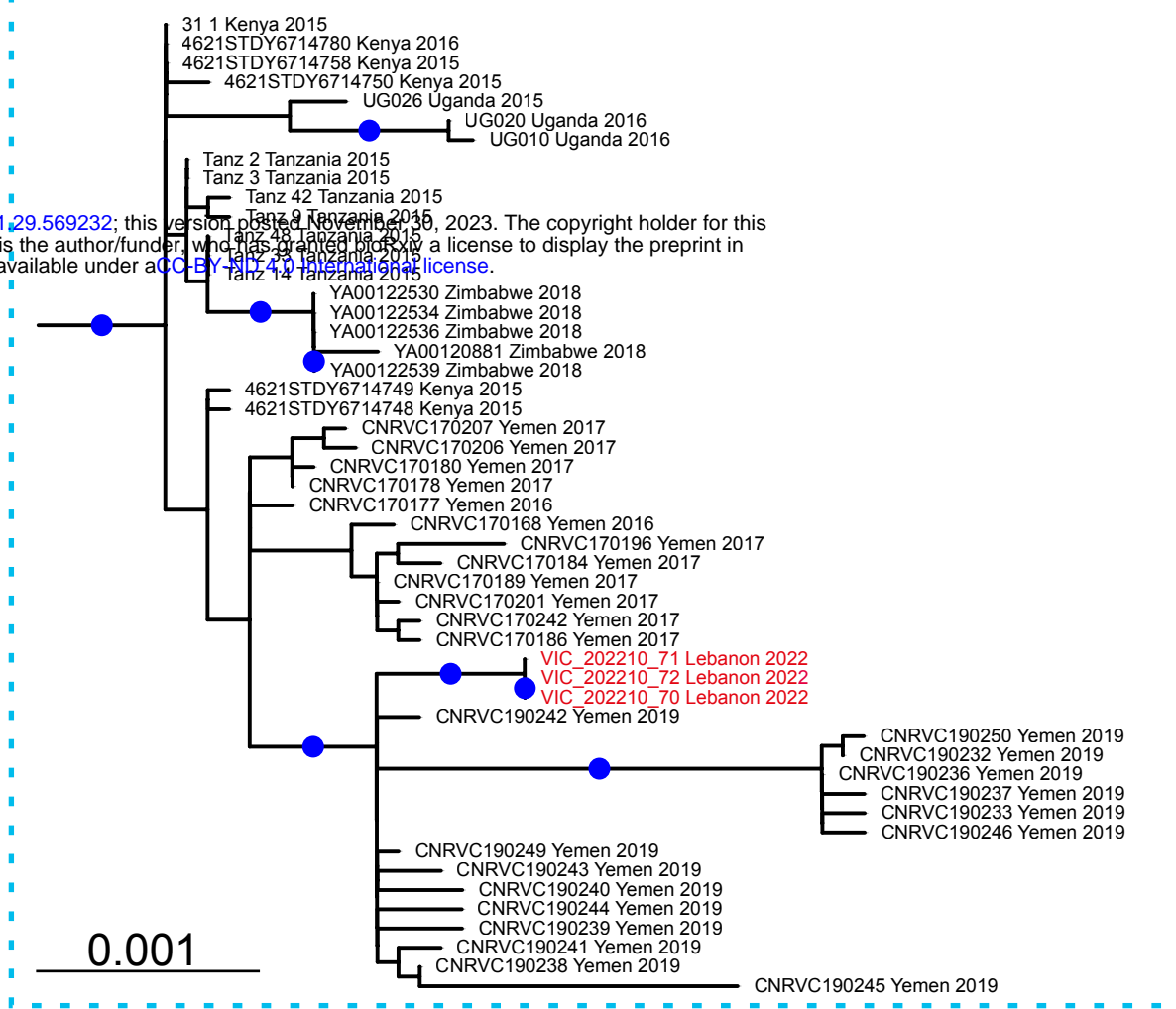
- Antimicrobial drug resistance
- Transposase and integrase
- Conjugative transfer
- Structure and cellular processes
- Other



Circle 2

- V. cholerae* plasmid pVIC-11A (Lebanon, 2022)
- V. cholerae* plasmid pCNRVC190243 (Yemen, 2019)
- V. cholerae* plasmid pYA00120881 (Zimbabwe, 2018)

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1. Geographic region

- Africa
- Asia
- America
- Europe
- Middle East
- Oceania
- Unknown

2. Country

- Lebanon
- Other