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1 Poliovirus surveillance in Mayotte, Indian Ocean, reveals encephalomyocarditis virus type 1
2 and a wide diversity of non-polio enteroviruses

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26 **Abstract**

27 In 2023, Mayotte, a French department in the Mozambique channel, experienced a
28 long drought that led to potable water restrictions. Although the French vaccination schedule
29 makes polio vaccination compulsory for children, the large proportion of illegal migrants on
30 the island coupled with the water crisis raised concerns about the establishment of poliovirus
31 transmission chains. Therefore, a surveillance was implemented to detect polioviruses in
32 sewage sampled in the two main wastewater treatment plants. Samples collected from
33 September 2023 through January 2024 were processed following the Global Polio Lab
34 Network's algorithm. Only two polioviruses were detected, both featuring a low number of
35 nucleotide differences compared to vaccine strains, which suggested excretion by recently
36 vaccinated people rather than circulation. The surveillance revealed viruses of the species
37 *Cardiovirus rueckerti* in almost half of the samples. Furthermore, 305 non-polio enteroviruses
38 were detected, of which most belonged to various virus types of species *Enterovirus*
39 *betacoxsackie* (53%) and *E. coxsackiepol* (44%) and species *E. alphacoxsackie* (3%). In
40 conclusion, the poliovirus surveillance did not detect any wild or highly mutated poliovirus
41 strains and did not highlight poliovirus circulation. It gave the opportunity to get the first
42 overview of the non-polio enteroviruses circulating in Mayotte.

43

44 **Accession numbers**

45 GenBank accession numbers PQ009012-PQ009102, PQ566710-PQ566937 and PQ868249-
46 PQ868262.

47

48

49 **Keywords:** encephalomyocarditis virus, enterovirus, environmental surveillance, cardiovirus,
50 Mayotte, poliovirus

51

52 **Introduction**

53 Mayotte is a French overseas department located in the Mozambique Channel. It
54 consists of two main islands, Grande-Terre (363 km²) and Petite-Terre (12 km²), surrounded
55 by islets. It has a tropical climate with two main seasons: the rainy season (November-April)
56 and the dry season (May-October). The population was estimated at 310,000 in January 2024,
57 half of the inhabitants being younger than 18 [1]. The average annual population growth was
58 of 3.8% from 2012 through 2017, fuelled by a relatively high fertility rate (4.5 children per
59 woman vs 1.9 in mainland France) and by sustained immigration: more than half of all adults
60 living in Mayotte were born elsewhere [2]. The median equivalised disposable income for the
61 inhabitants of Mayotte is seven times lower than at national level and 77% of the inhabitants
62 live below the national poverty threshold. About 80% of the potable water is produced from
63 surface water sources made of rivers and hillside reservoirs that are mainly fed during the
64 rainy season [3]. Consequently, any lack of rain can result in a water crisis, as already
65 illustrated in 2017 [4]. Water shortage favours outbreaks of faecal-oral diseases as users turn
66 to the consumption of water resources of insufficient quality [5]. This risk is particularly high
67 in Mayotte where only a small proportion of the population is connected to the sewage
68 system, which contributes to a deterioration of the quality of the environmental water
69 resources, such as surface water or well water. In 2023, a drought in Mayotte led the
70 authorities to introduce water restrictions. In response to the water crisis, the French
71 authorities implemented a strengthened surveillance relying on data from different origins,
72 such as the hospital emergency department, general practitioners, pharmacists and
73 college/high school infirmaries [6]. Wastewater-based surveillance was also implemented
74 against different pathogens [7], including poliovirus (PV), the etiologic agent of poliomyelitis.
75 As a French department, Mayotte follows the French vaccination schedule that relies on the

76 inactivated polio vaccine. Polio vaccination is mandatory for all children born after 1st
77 January 2018 before entering a childcare centre or any collective childhood institutions. In
78 2019, the polio vaccine coverage in Mayotte was estimated at 93.2% among children under 5
79 years, at 45.3% among 7-11 year-old children and at 27.1% among 14-16 year-old children
80 [7]. No routine PV environmental surveillance is conducted in France. The surveillance took
81 place from September 2023 through January 2024 and focused on two wastewater treatments
82 plants (WWTPs) located in Mamoudzou and Dembeni, the two main cities. It was carried out
83 following the Global Polio Laboratory Network (GPLN) algorithm. This surveillance also
84 gave the opportunity to get an overview of the genetic diversity of the non-polio enteroviruses
85 (NPEVs) that circulate in the island and fortuitously revealed numerous encephalomyocarditis
86 virus type 1 (EMCV-1) strains.

87

88 **Material and methods**

89 *Sample collection*

90 Wastewater samples were collected in the two largest WWTPs of the island. One
91 (called “Baobab”) is located in Mamoudzou, the capital city (~72,000 inhabitants) and the
92 other in Dembeni (~16,000 inhabitants). Weekly sampling was scheduled in both WWTPs
93 but, due to logistical issues, no sampling was performed during week #49 while two samples
94 were collected during some weeks. One additional sample came from a pond located in
95 Mamoudzou. The wastewater samples were sent to the Institut Pasteur’s polio laboratory,
96 which is one of the GPLN’s Global Specialized Lab.

97

98 *Sample concentration*

99 Raw wastewater samples were processed as already described (Supplementary Fig.1)
100 [8]. First, 240 mL of raw wastewater were clarified by centrifugation for 10 min at $1,000 \times g$.

101 The supernatant was collected and filtered through a 0.45 µm Nalgene 500 mL Rapid-flow™
102 and concentrated down to a 6-8 mL using Vivaspin 20 centrifugal concentrators with a 10 kDa
103 cutoff. The concentrates were treated with 20% chloroform by vigorous shaking for 1 min.
104 After a centrifugation for 10 min at 1,000 × g, the upper phase was collected and used to
105 inoculate cells.

106

107 *Cell inoculation*

108 Following the GPLN's standard procedures, two cell lines were used for PV detection:
109 RD cells (from human rhabdomyosarcoma tumour tissue) and L20B cells (transgenic murine
110 L cell permanently expressing the poliovirus receptor CD155) [9,10]. HEp-2c cells were also
111 used to isolate NPEVs that do not grow in RD and L20B cells. Cells were grown in Minimum
112 Essential Medium Eagle (Sigma Aldrich) supplemented with 2 mM L-glutamine, 10 mM
113 HEPES and fetal bovine serum (5% for RD and HEp-2c, 10% for L20B) at 37°C in non-
114 ventilated flasks. Growth medium was removed prior to inoculation and replaced by medium
115 with 2% fetal bovine serum. Chloroform-treated concentrates (500 µL per flask) were used
116 for inoculating cells in 25-cm² flasks: five L20B flasks, three RD flasks and one HEp-2c
117 flask. Cells were daily microscopically observed for five consecutive days to observe the
118 appearance of cytopathic effects (CPEs). A blind passage in the same cell line was performed
119 when no CPE was observed. Positive supernatants were clarified and used to inoculate a fresh
120 monolayer of the opposite cell line (RD into L20B and L20B into RD).

121

122 *Poliovirus detection and characterization*

123 Supernatants of positive L20B flasks were screened molecularly for the presence of
124 PV with the ITD 5.2 kit distributed throughout the GPLN [11]. After RNA extraction using

125 the QIAamp viral RNA kit, PV VP1-encoding region was amplified with the Y7R/Q8 primer
126 pair [12] and sequenced by Sanger technique.

127

128 *Amplification of EMCV-1 and NPEV genomes*

129 The EMCV-1 VP1 region was amplified using project-specific primers (EMCV-2563-
130 F and EMCV-3548-R; Table 1). Whole genomes were amplified in two overlapping fragments
131 (Table 1). Reverse transcription was performed with the Maxima H Minus First Strand cDNA
132 Synthesis Kit (Thermo Scientific) and random hexamers. PCR was conducted using Phusion
133 High-Fidelity DNA Polymerase (Thermo Scientific) under the following conditions: 98°C for
134 30 s; 35 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 2 min; and a final extension at 72°C
135 for 10 min.

136 The NPEV genomes were amplified as already described by RT-PCR and semi-nested
137 PCR with primers targeting conserved regions across species *E. alphacoxsackie*, *E.*
138 *betacoxsackie*, *E. coxsackiepol* and *E. deconjuncti* (EV-A, EV-B, EV-C and EV-D,
139 respectively) [13]. These primers generate amplicons spanning a large part of the genome
140 [14], including the VP1-encoding region commonly used for typing [15]. Amplification was
141 performed on RNA extracted from RD and HEp-2c supernatants with CPEs. To overcome
142 cell-culture bias, the same primers were also used directly on RNA extracted from the 28
143 concentrates, regardless of cell culture results (Supplementary Fig.1).

144

145 *Illumina sequencing, bioinformatics and phylogenetic analyses*

146 PCR and/or nested PCR products were purified using NucleoFast plates and
147 sequenced using Illumina technology as described previously [13]. Reads from Illumina
148 sequencing were assembled into contigs through *de novo* assembly using CLC Genomics
149 Workbench using parameters previously optimised [16]. To identify viruses that have the

150 highest similarity with EVs that circulate in Mayotte, a BLAST analysis was performed based
151 on the VP1-encoding region [17]. Multiple sequence alignments were built with CLC Main
152 Workbench and phylogenetic trees were drawn using Mega X [18]. The evolutionary history
153 was inferred by using the Maximum Likelihood method and Tamura-Nei model. Initial trees
154 for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ
155 algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then
156 selecting the topology with superior log likelihood value. The trees were drawn to scale, with
157 branch lengths measured in the number of substitutions per site.

158

159 **Results**

160 *Detection of two Sabin-like polioviruses*

161 A total of 28 wastewater samples were tested. All were positive on RD cells, and 17
162 (61%) were L20B positive (Fig. 1). Of these, only two contained PVs, identified by VP1
163 sequencing as serotypes 2 and 3, both derived from Sabin strains. Sabin-derived PVs are
164 classified as ‘Sabin-like’ or ‘vaccine-derived’ based on VP1 nt differences. Both PVs had 2 nt
165 differences and were thus classified as Sabin-like. Whole-genome sequencing showed that the
166 type 2 isolate derived from the novel oral polio vaccine strain (nOPV2) used since 2021 [19].

167

168 *Many samples contained encephalomyocarditis viruses*

169 Fifteen samples showed CPEs on L20B cells but contained neither PVs nor NPEVs
170 and were classified as non-EV (NEV). To confirm the absence of EV, RT-PCR targeting
171 conserved EV regions was performed, yielding no expected bands, but the product was
172 nonetheless submitted to Illumina sequencing. *De novo* assembly and BLAST analysis
173 identified short contigs matching EMCV-1, species *Cardiovirus rueckerti* (formerly

174 *Cardiovirus A*), genus *Cardiovirus*, family *Picornaviridae*. No other virus was identified
175 among the contigs.

176 Primers were designed against conserved nt sequences located on both sides of the
177 EMCV-1 VP1-encoding region and used to screen the 17 NEV supernatants. Bands at the
178 expected size were obtained for 13 samples, thus revealing the presence of EMCV-1. As some
179 samples contained multiple EMCV-1s, 20 distinct sequences were obtained. Based on the
180 capsid-encoding region, different genetic lineages of EMCV-1 have been previously defined
181 [20–23]. The viruses detected in Mayotte did not belong to any of them and fell into three
182 separate groups, each of which gathers sequences with >85% nt similarity (Fig. 2). All
183 sequences from Dembeni formed a single group (DEM in Fig.2) while the sequences from
184 Mamoudzou split into two groups (MMZ1 and MMZ2). Whole genome amplification was
185 performed on six viruses representative of the three distinct lineages. In the 3D-encoding
186 region, which constitutes the 3' terminal part of the open reading frame, the clustering of these
187 viruses into three lineages was congruent with that observed in the VP1-encoding region
188 (Supplementary Fig.2).

189

190 *Samples contained numerous non-polio enteroviruses*

191 Illumina sequencing and *de novo* assembly yielded 350 contigs covering the capsid
192 region, with some found in both concentrates and supernatants from the same sample. After
193 removing duplicates, 305 distinct capsid sequences remained. Per sample, the number of
194 distinct contigs ranged from 3 to 26 (Fig. 3A), with a higher average in Dembeni WWTP
195 samples (15.2) than in Baobab WWTP samples (9.9) ; the pond sample contained 4 distinct
196 VP1 sequences (Fig. 3A).

197 The 305 NPEVs thus detected belonged to three species, *E. alphacoxsackie*, *E.*
198 *betacoxsackie* and *E. coxsackiepol*. EV-Bs were majority (n=162), accounting for 53.1%

199 (162/305) of the total NPEVs. They were the only EVs detected in RD supernatants and were
200 majority in HEp-2c supernatants (28/46), which also contained EV-Cs (Fig.3B). By contrast,
201 EV-Cs accounted for 85.9% (116/135) of the EVs detected in concentrates (Fig.3B). Only 9
202 EV-As were detected, all in concentrates (Fig.3B).

203 The few EV-As belonged to 6 different virus types and EV-Bs fell into 7 virus types.
204 Only one coxsackievirus (CV) B5 was detected while between 20 to 35 specimens were
205 detected for each of the other EV-B types, namely CVB3, echovirus (E) 6, E7, E11, E19 and
206 E24 (Fig.3C). The EV-Cs belonged to 8 virus types (Fig.3C), two of which (CVA13 and EV-
207 C99) accounted each for about one third of the total EV-Cs (52/134 and 43/134, respectively).
208 Among the other EV-Cs, 36 belonged to virus types that are regularly reported worldwide
209 (CVA11, CVA20, CVA24 and EV-C96) while 3 belonged to two virus types (CVA1 and EV-
210 C116) that were underreported for a long time because they are refractory to cultivation in
211 standard cell lines [24].

212 It is noteworthy that the percentage of nt similarity between the Mayotte EV-Cs and
213 their respective closest matches found in GenBank was relatively low (80.8-91.2%) compared
214 to the percentage of similarity observed for EV-As (90.8-99.0%) and EV-Bs (82.8-98.7%)
215 (Supplementary Fig.3). CVA13s and EV-C99s are the most abundant virus types in our
216 dataset. The strains belonging to these two virus types have been previously classified into
217 different groups [25,26]. The Mayotte's CVA13s fell into two clusters (Fig.4A and
218 Supplementary Fig.4): cluster B, which contains numerous specimens sampled in Madagascar
219 in the 2000s and 2010s and one specimen from Chad, and cluster A, which contains viruses
220 sampled in different continents. Interestingly, within these two clusters, Mayotte's CVA13s
221 clearly formed separate branches (bootstrap values of 100% and 98%) compared to viruses
222 sampled elsewhere. Most EV-C99s from Mayotte fell into genotype D, which contained
223 exclusively viruses from Madagascar before this study (Fig.4B and Supplementary Fig.5) but,

224 unlike CVA13s, they split into different groups within this genotype. One Mayotte's EV-C99
225 was linked to a strain sampled in Senegal in 2014 (genotype F) [27] while six others grouped
226 together into a new genotype (Fig.4B and supplementary Fig.5). Considering the virus types
227 besides CVA13 and EV-C99, Mayotte EVs showed genetic links to strains from various
228 continents, without peculiar association with a specific geographic region (Supplementary
229 Table 1).

230

231 **Discussion**

232 This study was conducted at the request of health authorities amid drought-related
233 waterborne outbreak risks in Mayotte. Since no form of oral polio vaccine is used in France,
234 all PVs found in Mayotte are imported strains. The low number of PVs over 14 weeks
235 surveillance suggests no active PV circulation. Even though the temperature of the wastewater
236 samples was not monitored during the transportation, the isolation of NPEVs from all of them
237 indicates good sample quality. Nonetheless, the surveillance was limited to two WWTPs,
238 which, despite being the largest, cover only a small portion of the population. In Mayotte,
239 only 20% of households are connected to collective WWTPs while 35% use septic tanks and
240 45% are not connected to any water treatment system [28].

241 Nearly half of sewage samples in this study were classified as NEVs under the GPLN
242 algorithm, an unusually high rate. Previous studies in Haiti, India, Nigeria reported NEV rates
243 below 7% in wastewater [29–31]. Nonetheless, a similar observation was made in Taiwan
244 where 94 mammalian orthoreoviruses (family *Seoriviridae*, genus *Orthoreovirus*) were
245 isolated in L20B cells from 300 sewage samples [32]. The isolation of EMCV-1 in L20B cells
246 is not a surprise, since this virus grows in L cells [33], the precursors of L20B cells. The high
247 detection rate and genetic diversity across sites suggest active circulation in Mayotte. As
248 EMCV-1 can infect humans, it is possible that EMCV-1 circulates among humans in Mayotte

249 but the genetic clustering of Dembele and Mamoudzou isolates into separate groups does not
250 support this hypothesis. Swine constitutes an effective reservoir for this virus but they are
251 almost non-existent in Mayotte [34]. Therefore, it is likely that the EMCV-1 isolates came
252 from rodents that live within the sewer system, especially black rats (*Rattus rattus*), which are
253 present in Mayotte [35] and constitute a reservoir for this virus [36]. Nonetheless, as EMCV-1
254 infects a wide range of mammals and birds [37], it is impossible to ascertain the source of the
255 viruses detected in this study.

256 Because of its geographic and socio-demographic characteristics, Mayotte likely
257 constitutes an original biotope for human viruses: it is in the Indian Ocean but, as a French
258 department, it has close links with mainland France; furthermore, many people enter in the
259 island from the Union of the Comoros and from continental Africa. Before this study, data
260 about EVs in Mayotte were very scarce since they relied on two reports only: one relates an
261 outbreak of acute haemorrhagic conjunctivitis that took place in 2012 and was probably due
262 to a CVA24 variant [38]; the second one reports the detection of few EV-Bs in patients with
263 meningitis in 2019 [39]. The high number of EVs (>300) found in a limited set of sewage
264 samples highlights the interest of wastewater to get an overview of the EVs that circulate
265 among humans. Capturing full EV diversity landscape is nonetheless challenging: virus
266 isolation facilitates genomic amplification but misses non-cultivable virus types; by contrast,
267 direct amplification from concentrates may fail due to inhibitors and overlook minority EVs.
268 These biases are obvious in our study, since the RD arm only revealed EV-Bs while EV-Cs
269 dominated in the sequences generated directly from the concentrates. The two approaches
270 complement each other, and their combination increases the number of EVs detected. The
271 pattern of our dataset, with a high proportion of EV-Cs, particularly CVA13, aligns with
272 previous observations in Madagascar and sub-Saharan Africa where CVA13 abundancy was
273 consistently observed regardless of EV detection and genome amplification methods [26,40–

274 46]. Genetic close relationships between certain EVs in Mayotte and those from other
275 continents suggest regular importations into the island. Conversely, the greater genetic
276 divergence between EV-Cs from Mayotte and from other regions may stem from less frequent
277 EV-C importations. Nonetheless, non-polio EV-Cs are likely undersampled due to their
278 exclusion from clinical surveillance, as they are not linked to severe clinical manifestations,
279 unlike EV-As, EV-Bs and EV-D68 [47].

280

281 **Conclusions**

282 Environmental surveillance conducted in Mayotte during the water crisis did not
283 detect any circulating virulent polioviruses. However, EMCV-1 was incidentally identified in
284 nearly half of the collected samples. This was the first study to detect many non-polio
285 enteroviruses present in Mayotte.

286

287 **Authors' contributions**

288 SW and MB conceived the study design. AT, and MLJ conducted the experiments and
289 generated the virus genomic sequences from Illumina raw data. All authors contributed to
290 interpretation of data. MB wrote the draft and all authors reviewed and approved the final
291 manuscript.

292

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302

303 **Competing interests**

304 The authors have no conflicts of interest to declare.

305

306 **Ethical approval**

307 The study used wastewater samples whose analysis does not require ethical approval.

308

309 **Data availability**

310 Virus genetic sequences were submitted to GenBank (accession numbers PQ009012-
311 PQ009102, PQ566710-PQ566937 and PQ868249-PQ868262).

312

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468 **Captions**

469

470 Fig.1. Timeline of surveillance. Each square represents a wastewater sample.

471

472 Fig.2. Phylogenetic relationships between EMCV-1 strains detected in Mayotte and strains
473 available in public database, based on the VP1-encoding region. Circles indicate the strains
474 identified in our study, of which those whose genome was fully sequenced are underlined. The
475 other strains are named by using their respective GenBank accession number, country (ISO
476 3166-1 alpha-3 codes) and year of sampling. A strain belonging to the species *Cardiovirus*
477 *theileri* was used as outgroup to root the tree. Bootstrap values of the main nodes are shown.

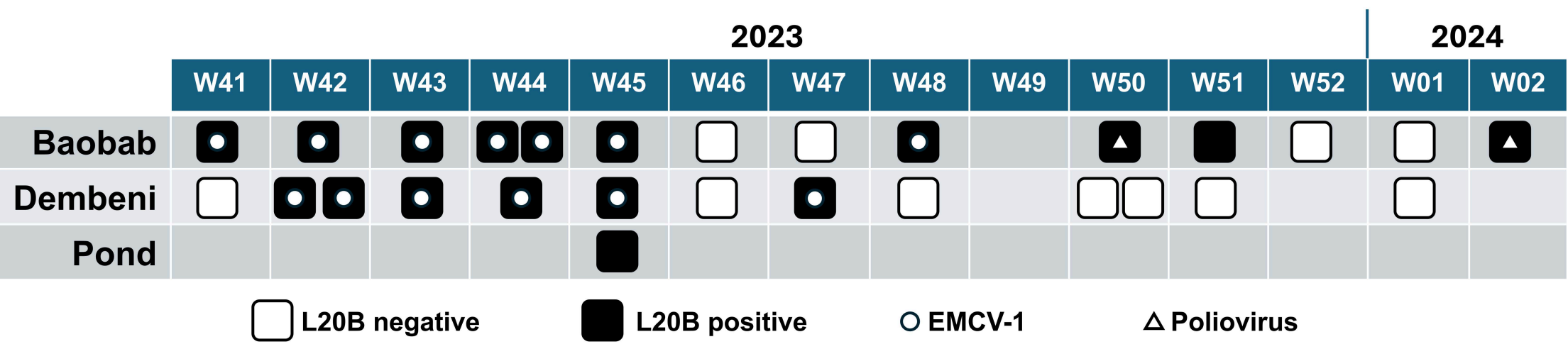
478

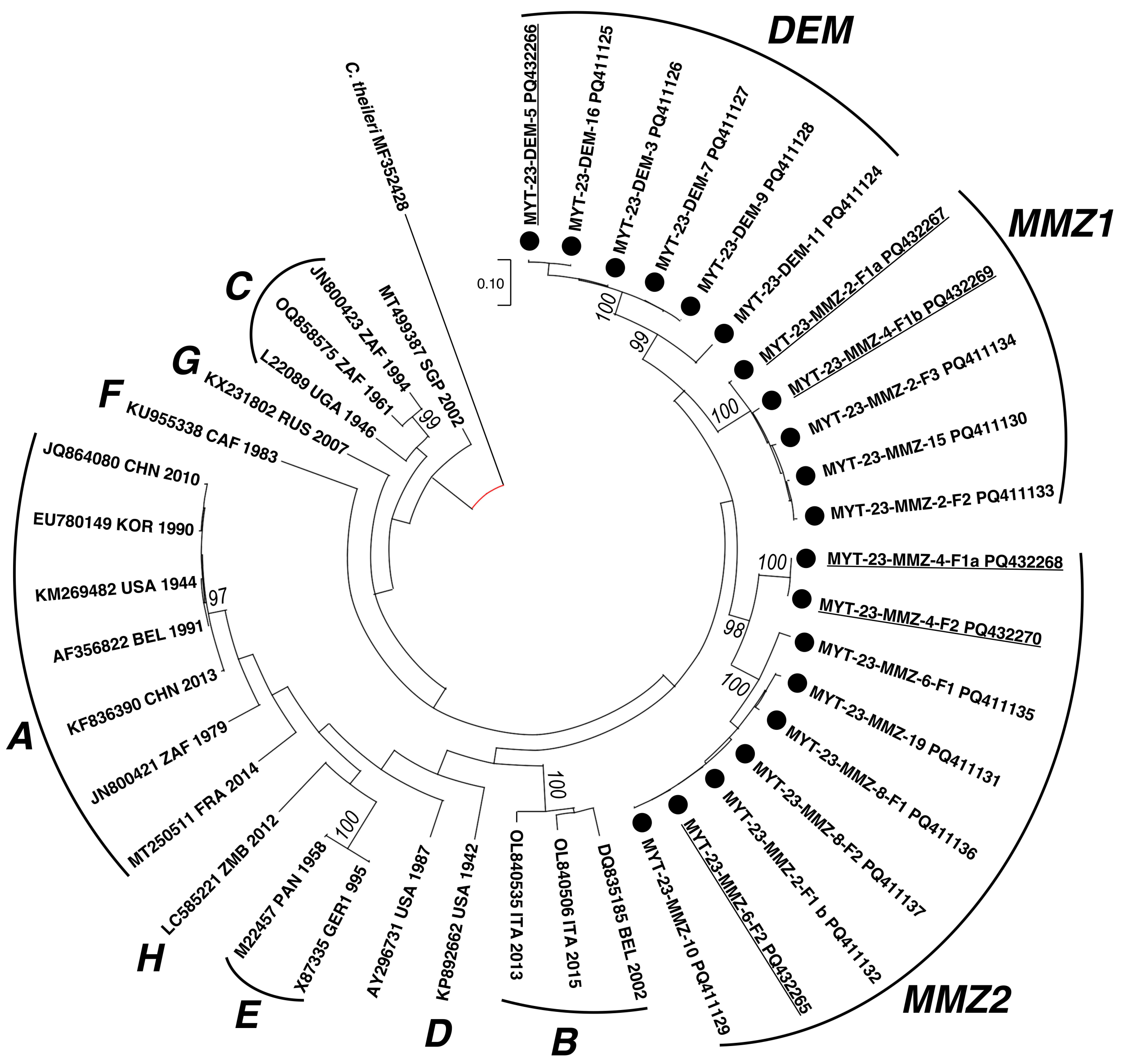
479 Fig.3. A. Number of VP1-encoding sequences per sample. The short horizontal bar indicates
480 the average for each wastewater treatment plant. B. Total number of VP1-encoding sequences
481 detected in RD and HEp-2c cells supernatants and directly from concentrates. C. Distribution
482 of the EVs into species and virus types.

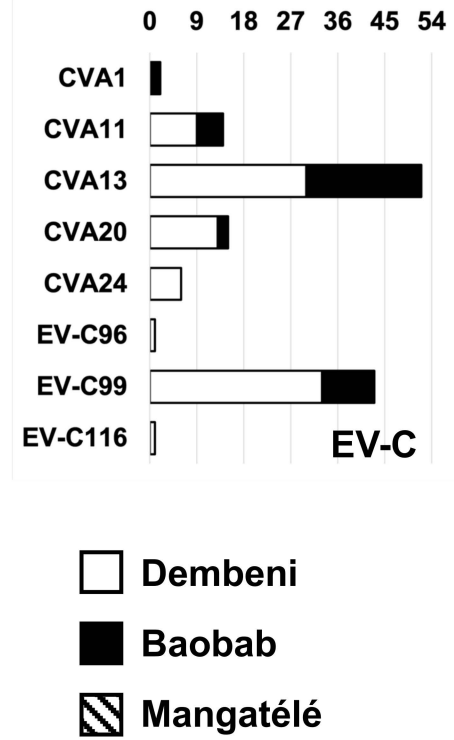
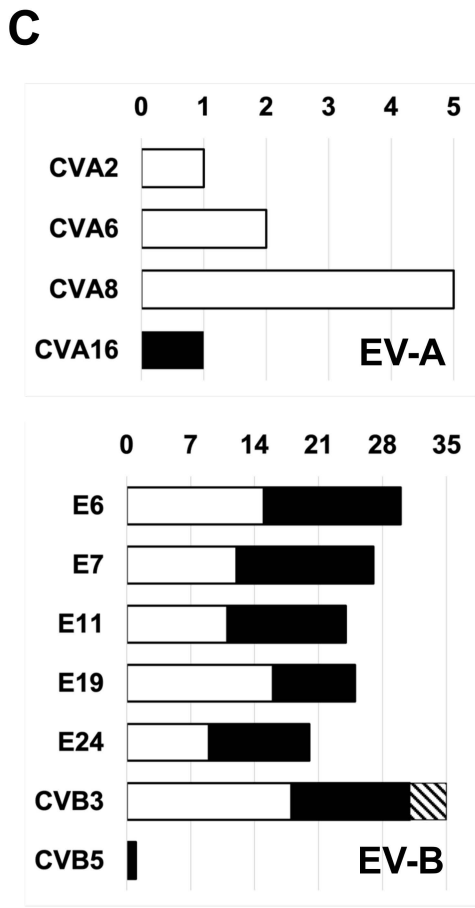
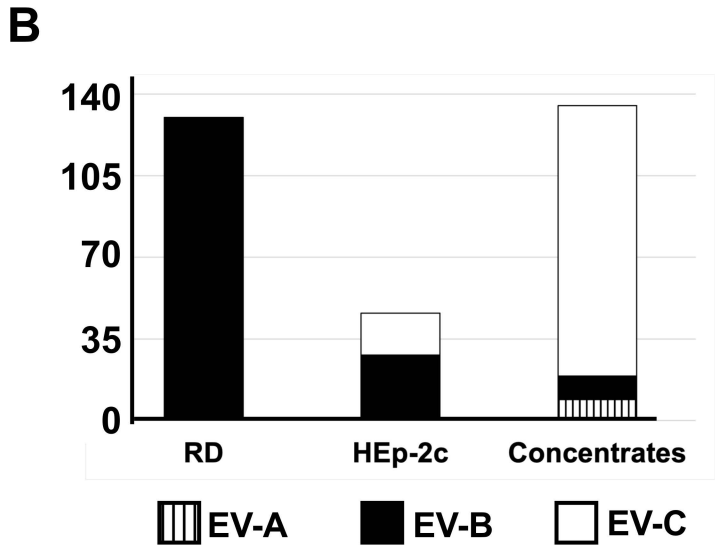
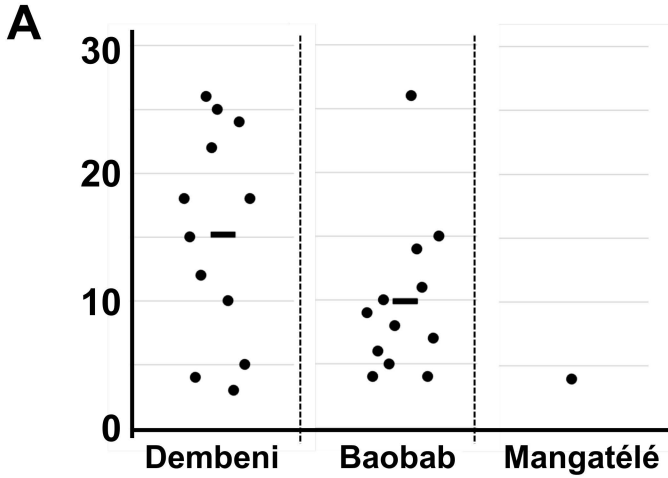
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484 Fig.4. Phylogenetic relationships between CVA13 (A) and EV-C99 (B) strains, based on the
485 VP1-encoding region. Circles indicate the strains identified in Mayotte in our study (full
486 circles) and previously in Madagascar (open circles). Triangles indicate the prototype strains.
487 Bootstrap values of the main nodes are shown. For a better readability, some subtrees have
488 been collapsed. Trees showing sequence names and accession numbers are provided as
489 Supplementary Figure 4 and Supplementary Figure 5).

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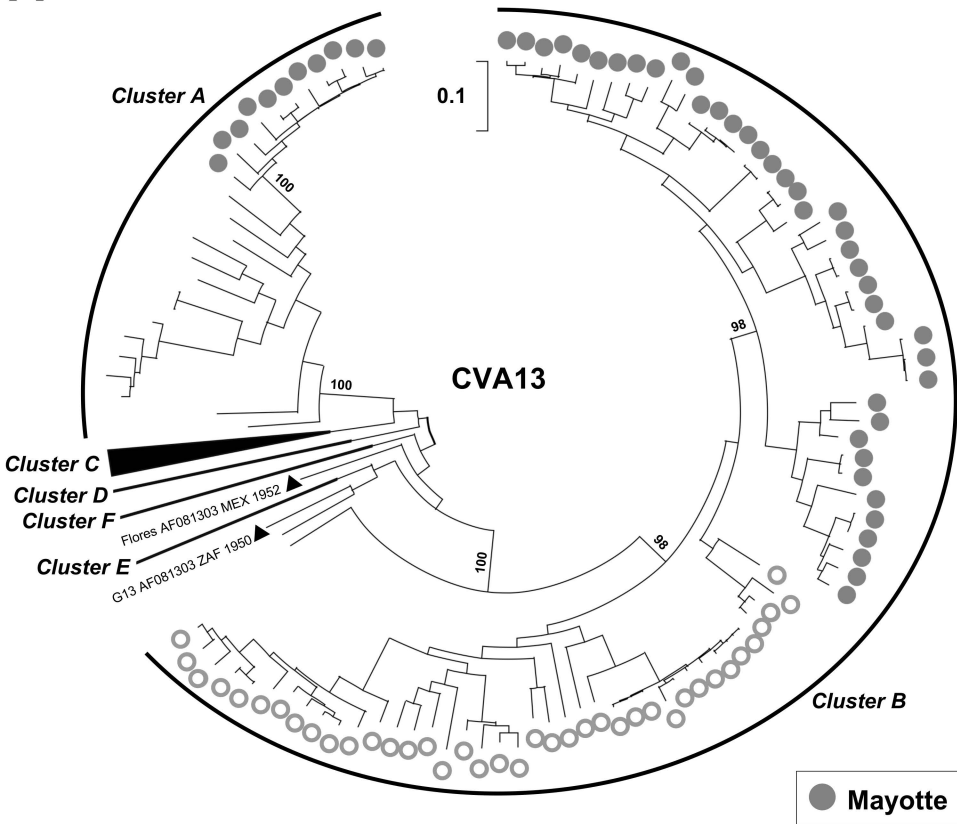
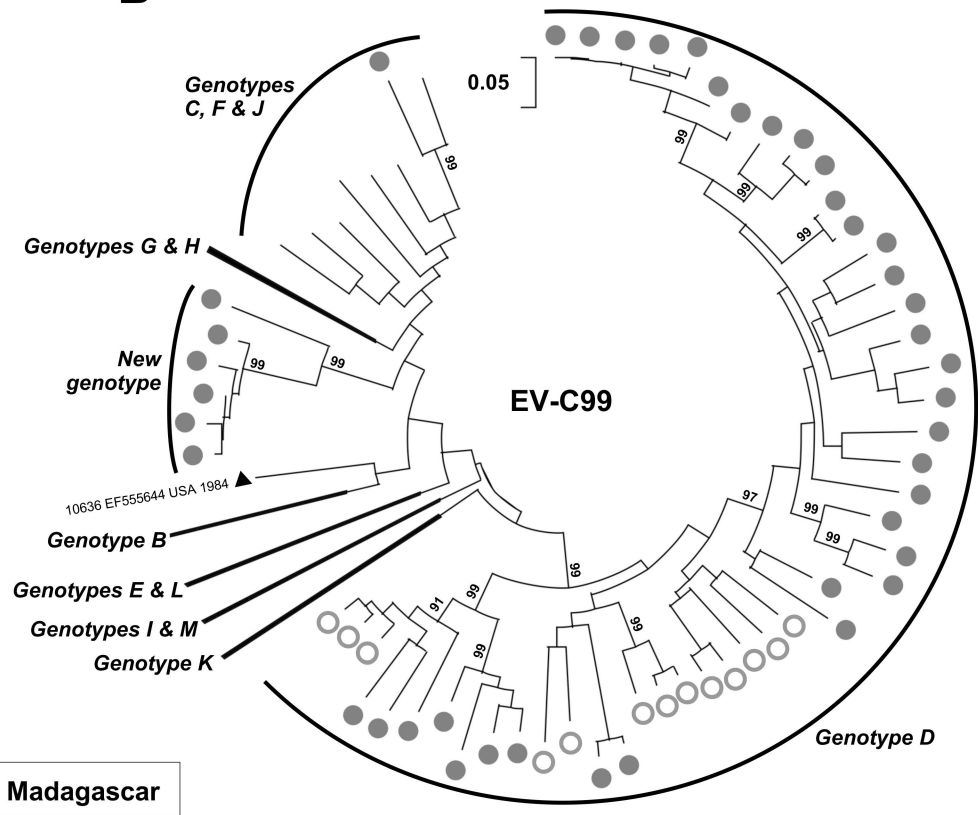
A**B**

Table 1. Primers used in this study.

Step	Primer name	5'→3' sequence	Reference
POLIOVIRUSES			
<i>One-step RT-PCR</i>	Y7R	GGTTTTGTGTCAGCITGYAAYGA	a
	Q8	AAGAGGTCTCTRTTCCACAT	a
EMCV-1			
	EMCV-2563-F	CAATGGTCAGTGCGGGTAAG	This study
	EMCV-3548-R	CACCCTAATCTCAAGTCCTC	This study
	EMCV-310-F	CTGGCCCTGTCTTCTTGACG	This study
	EMCV-4188-R	GCATCACTGCTATYGTCATC	This study
	EMCV-4015-F	CTTGCTCGATGCCAACGAG	This study
	EMCV-7745-R	CTACTCTAGTCTATCTTGyraag	This study
NON-POLIO ENTEROVIRUSES			
<i>Reverse transcription</i>	Oligo-dT(18)	TTTTTTTTTTTTTTTTTTT	
<i>PCR</i>	UG52	CAAGCACTTCTGTTTCCCCGG	b
	EV-CRE-R	CGGBRTTGSWCTTGAAC TG	c
<i>Semi-nested PCR</i>	UG53	TGGCTGCTTATGGTGACAAT	b
	EV-CRE-R	CGGBRTTGSWCTTGAAC TG	c

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