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Molecular characterization of enteroviruses circulating among pigs and goats in two Central African countries, Cameroon and the Central African Republic.

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1 **Molecular characterization of enteroviruses circulating among pigs and goats in two**

2 **Central African countries, Cameroon and the Central African Republic.**

3

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26 **Abstract**

27 To date, data on animal enteroviruses (EVs) are scarce, especially in Central Africa.
28 The aim of this study was to characterize EVs among pigs and goats in Cameroon and
29 Central African Republic (CAR).

30 A total of 226 pig and goat faecal samples collected in two previous studies carried
31 out in Cameroon and CAR were pooled and were screened with molecular assays targeting
32 EV-Es, EV-Fs and EV-Gs. EV genomes were amplified by RT-PCR and their sequences
33 obtained by Illumina sequencing and *de novo* assembly. Based on the capsid sequences, 27
34 EV-G sequences were identified and assigned to 11 virus types while no EV-E or EV-F was
35 observed. Phylogenetic analysis revealed that the EV-Gs detected in this study were
36 genetically closely related to sequences already reported worldwide. This worldwide
37 circulation of EV-Gs is likely due the massive international trade of live animals. One human
38 EV, EVC-99, which belongs to the species *Enterovirus C*, was detected in pigs. This is the
39 third detection of such an event in a similar context, reinforcing the hypothesis that some EV-
40 Cs could be infecting pigs. Our work provides new data on the genetic diversity of EVs
41 circulating among domestic animals in Central Africa, and particularly in Cameroon with the
42 first study.

43

44 **Keywords:** Enterovirus, Zooanthroponosis, Cameroon, Central African Republic, EV-G,
45 *Enterovirus species G*

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Introduction

Enteroviruses (EVs), members of the *Picornaviridae* family, form a large group of viruses that infect numerous mammal species. The *Enterovirus* genus currently comprises over 300 different virus types, grouped into 15 species: *Enterovirus A-L* and *Rhinovirus A-C* [1]. EV genome contains a long open reading frame (ORF) encoding a polyprotein containing the four structural proteins that form the capsid (VP1 to VP4) and all the non-structural proteins. Among these four proteins that compose the virus capsid, VP1 bears the major neutralizing epitopes. Therefore, the original classification of EVs into serotypes is broadly consistent with the classification based on the VP1-encoding sequences. The VP1-enconding region is thus widely used to determine the virus types of EV field strains through molecular characterization [2, 3].

Members of the species *Enterovirus E, F* and *G* (EV- E, F and G) are closely associated with animal diseases affecting the livestock industry. The species *Enterovirus E* and *Enterovirus F* formed initially a unique species called *Bovine Enterovirus* [4, 5]. EV-Es and EV-Fs have mainly been reported in cattle but they are able to infect a wide range a mammals, including dolphins and primates [5–11]. EV-Es and EV-Fs have been sampled mostly in Asia, with a few cases in the USA, Egypt, Nigeria, Brazil, Europe, Australia, Bangladesh and New Zealand. The species *Enterovirus G* was previously referred to as *Porcine enterovirus* because they were first sampled in suidae. It was subsequently discovered that some members of this species circulate in sheep and goats [5]. EV-Gs have been sampled in Europe, Asia, Americas and Africa. To date, data on EVs that circulate in herd animals are scarce in Central Africa [12–15]. This geographic region seems to harbour a particular ecosystem of EVs circulating in humans, with a relatively high proportion of EV-Cs [12, 16–19] compared to other regions where these viruses are seemingly less abundant. By contrast, in farm animals, the few previous studies carried out in the region revealed EV-

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76 Gs that were genetically close to EVs described on other continents [12, 13, 15].
77 Nevertheless, a recent study has identified a previously unknown EV-G in pigs living in
78 Central African Republic (CAR) [12].

79 The aim of this study was to provide additional data on the genetic diversity of EVs
80 among domestic animals (pigs and goats) in Cameroon and CAR. Pools of stool samples
81 were molecularly screened with assays targeting EV-Es, -Fs and -Gs. The EV genomes thus
82 detected were sequenced by Illumina techniques and compared to genomes available in
83 public databases.

84

85 **Material and Methods**

86 ***Study Sites and Sample Collection***

87 We performed a retrospective study by analysing animal stool samples previously
88 collected as part of two studies investigating the presence of hepatitis E virus in pigs and
89 goats in Cameroon and CAR [20, 21]. In Cameroon, stools were collected in slaughterhouses
90 in Yaoundé (*Marché huitième*) and Douala (*Marché des chèvres*) after pig slaughter between
91 February 2017 and September 2018. In CAR, anorectal swabs were taken from pigs and goats
92 on farms in 5 districts of Bangui (2nd, 4th, 6th, 7th, 8th) between January and October 2021.
93 These stool samples were stored at the Virology Unit of the *Centre Pasteur du Cameroun* at -
94 80°C and analysed in the present study to detect and characterize animal EVs.

95

96 **RNA extraction and molecular screening**

97 Faecal samples were diluted in phosphate-buffered saline and clarified by
98 centrifugation following the guidelines of the Polio Laboratory Manual [22]. After
99 clarification, individual animal samples were pooled according to their respective animal
100 species and collection sites before molecular screening. RNAs from each pooled suspension

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101 were extracted using the ZYMO_RESEARCH® Kit according to the manufacturer's
102 instructions. EV RNAs were detected by real-time RT-PCR using protocol recently published
103 [12]. Primers and probes were designed to target conserved nucleotide sequences in the 5'
104 UTR of EV-E, EV-F and EV-G. Because of the genetic variability in the 5'UTR, two probes
105 were designed: one targeting the 5'UTRs of EV-E, -F and some EV-Gs (assay A) found in
106 goats and sheep and the second targeting most EV-Gs (Assay B).
107 Detection of EV RNA was performed using SuperScript™ III One-Step RT-PCR
108 System with Platinum Taq (Life Technologies Corporation, USA) in a final volume of 20 μL
109 by mixing 2 μL of extracted RNA with 0.4 μL of SuperScript RT/Platinum III Taq Mix, 10
110 μL of Reaction (2X), 10 pmol of each primer, 5 pmol of each probe, and 4.6 μL of nuclease-
111 free water. The thermocycler profile was 45°C for 15 min, 95°C for 2 min followed by 45
112 cycles of PCR (95°C for 15 s and 60°C for 30s).
113
114 ***EV genome amplification and sequencing***
115 For amplifying the EV genomes prior to sequencing, two overlapping fragments were
116 amplified using generic primers already described [12]. The first half of the genomes was
117 amplified using primers targeting conserved genetic sequence in the 5' untranslated region
118 (5'UTR) and in the cis-replicating element (cre) located within the 2C-encoding region
119 (Table 1). They were designed based on EV-E, -F and -G genetic sequences retrieved from
120 GenBank. To obtain cDNA, reverse transcription (RT) step was perfomed using the Maxima
121 H Minus First Kit Strand cDNA Synthesis Kit (Thermo Scientific, ref K1652) in a volume of
122 20 μL with 4 μL of extracted RNA, 10 nM of each dNTP, 1 μL of HeptaN (0.5 μg.μL^{-1}) and
123 14 μL of nuclease-free water. The mixture was incubated at 65°C for 5 min and then on ice
124 for 2 min; after adding 4 μL of RT buffer (5X) and 1 μL of Maxima H minus enzyme Mix,
125 incubation was performed at 50°C for 30 min and 70°C for 5 min. PCR was performed using

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126 cDNA as template and the Phusion High-Fidelity DNA polymerase kits (Thermo scientific)
127 as previously described. The reaction mixture was as follows: 2 μL of cDNA, 5 μL of
128 Phusion HF Buffer (5X), 10 nM μL of each dNTP, 10 pmol of each primer (EV-EFG-271-F/
129 EV-EFG-4441-R), and 0.5 μL of enzyme. The thermocycler profile was: 98°C for 30 s
130 followed by 35 cycles of amplification (98°C for 10 s, 50°C for 30 s and 72°C for 2 min) and
131 a final extension step at 72°C for 10 min. If no band appeared on the agarose gel at the
132 expected size, two nested PCRs were performed in parallel with the primer pairs EV-EF-381-
133 F/ EV-EF-4357-R and EV-G-491-F/ EV-G-4294-R by using 2 μL of the product of the first
134 PCR as template under the same thermal conditions.

135 The second half of the EV-G genomes was amplified according to the same protocol
136 using a sense primer (EV-G-4263-F) that targets a conserved sequence within the 2C-
137 encoding region and a reverse primer (J-polyT) that targets the polyA tail (Table 1).

138 After electrophoresis on an agarose gel, the products of the PCR or nested PCR with
139 bands of the expected size were purified on a NucleoFast®_96_PCR_Plate-DNA prior to
140 sequencing by Illumina technology [23]. Sequencing was performed using the Nextera XT
141 DNA Library Preparation kit according to the manufacturer's recommendations. Libraries
142 were built using 0.1 ng of DNA with the Nextera XT DNA Library Preparation kit in a
143 PCRmax Alpha Cycler 1 Thermal Cycler (Cole-Parmer). After purification on AMPure beads
144 (Beckman), the libraries were controlled using the High Sensitivity D1000 assay (Agilent) on
145 a TapeStation 4200 (Agilent). Sizing was achieved by electrophoresis on a PippinPrep
146 System with the PippinPrep kit CDF1510 (Ozyme).

147

148 ***Sequence analysis and molecular typing of EVs isolates***

149 The Illumina raw sequences were analysed using CLC Genomics software (version
150 22). Contigs were built *de novo* and the contigs that correspond to EV genomic sequences

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151 were flagged through a BLAST analysis. Molecular typing of EV strains was based on the

152 capsid-encoding genomic region, especially the VP1-encoding sequence. 5 The complete VP1

153 nucleotide sequences were aligned using the ClustalW program and phylogenetic trees were

154 reconstructed using the MEGA X program [24]. The Neighbour-joining algorithm was used

155 to generate the initial tree. 12 The percentage of replicate trees in which the associated taxa

156 clustered together in the bootstrap test was calculated from 1,000 replicates.

157 The nucleotide sequences of 27 EV-Gs and 1 EV-C 4 isolated in this study have been

158 deposited in GenBank with the accession numbers PQ043000-PQ043027.

159

160

161 **Results**

162 ***Origin of the samples***

163 A total of 226 animal stools were analysed in this retrospective study, including 136

164 38 collected from pigs in slaughterhouses in Cameroon (Yaounde n=58 and Douala n=78); and

165 90 collected from pigs (n=60) and goats (n=30) on farms in Bangui, CAR. Individual animal

166 samples were pooled according to their respective animal species and collection site, and we

167 obtained 20 pools for pigs (6 pools in Yaounde, 8 pools in Douala and 6 pools in Bangui) and

168 3 pools for goats (only in Bangui).

169

170 ***Detection of EV-Gs and one EV-C in stools and sequencing***

171 After pooling and RNA extraction, EVs were detected by reel time RT-PCR using a

172 primer pair and two probes targeting the 5'UTR. The probe A (targeting the 5'UTRs of EV-

173 E, -F and some EV-Gs) gave no positive result while the probe B (targeting most EV-Gs) was

174 positive for twelve pools from pigs including 6 in Cameroon and 6 in CAR (Table 2).

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175 After screening by Taqman reactions, two pairs of generic primers were used to
176 perform RT-PCR and, when necessary, a subsequent nested-PCR. Both primer pairs target
177 conserved sequences in both sides of the capsid-encoding region: the sense primers target the
178 5'UTR and the antisense primers targeted the cis-replicating element (cre) located within the
179 2C-encoding region. The corresponding amplicons were ~3,800 nt-long and encompassed the
180 whole capsid and a large fraction of the 5'UTR and P2 genomic regions. Of the 12 pools that
181 gave positive results through molecular screening, 9 pools were positives after RT-PCR with
182 amplicons at the expected size for each of the 9 pools (Table 2).

183 After amplification, Illumina sequencing and data analyses, nucleotide sequences
184 corresponding to the complete VP1 capsid gene for every EV isolate were considered for
185 typing of EVs. The number of VP1 sequences per pool of animal stools ranged from 1 to 7.
186 Overall, 28 VP1 sequences were identified, 27 of which belonged to the species EV-G and 1
187 to the species EV-C. No EV-E or -F was characterized in the pig and goat samples analysed
188 in our study.

189 The EV-C came from a pool of pig samples collected in CAR and was an EV-C99.
190 EV-Gs have been classified into 28 virus types (EV-G1 to EV-G28) based on VP1
191 relationships higher than 75% nt identity [13]. Our 27 EVG VP1 sequences were assigned to
192 11 virus types already described worldwide with 2 types (EVG-1 and 4) found in both
193 Cameroon and CAR, 3 types (EV-G8, G14 and G17) and 6 types (EV-G2, G3, G6, G9, G13
194 and G15) found only in CAR and Cameroon, respectively (Figure 1).

195 To obtain complete genomes of animal EVs, the second half of the EV-G genomes
196 was amplified using primers that targets a conserved sequence within the 2C-encoding region
197 and a reverse primer (J-polyT) that targets the polyA tail (Table 1). Of the 9 pools that gave a
198 positive result through 5'half genome amplification, 2 pools were positives for 3'half genome
199 amplification. Thus, we obtained two complete genomes of EV-G (Table 2).

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201 ***EV-Gs found in Central Africa are genetically close to EV-Gs sampled in other regions***

202 5 In order to investigate the genetic relationships between the EVs detected in our study
203 and previously reported EVs, we used two genomic regions independently (the 5'UTR and
204 2A-2B regions), which surround the capsid coding region. Genetic sequences with high
205 similarity were found and phylogenetic trees drawn 26 using the Basic Local Alignment Search
206 Tool (BLAST). The phylogenetic analyses of our sequences based on both regions have
207 shown that sequences are not cluster by virus types, indicating that intertypic recombination
208 takes place between EV-Gs in both sides of the capsid-encoding region (Figure 2).
209 Interestingly, Cameroon and 2 CAR EV-Gs identified here were closely related to EV-Gs
210 previously sampled in different continents and do not form topotypes specific to Central
211 Africa, which suggests a global circulation of EV-Gs.

212

213 **Discussion**

214 The aim of our study was to identify EVs circulating among domestic animals (pigs
215 and goats) in two Central African countries, Cameroon and the Central African Republic. To
216 30 our knowledge, this is the first study reporting genetic diversity of EVs in domestic animals
217 in Cameroon, and the second in CAR after the one recently published [12]. Globally, we
218 found EV-Gs in the animal samples but neither 14 EV-E nor EV-F. EV-Es and EV-Fs are mostly
219 sampled in cattle but some isolates were previously reported from different other mammal
220 species, including goats [10]. Previous studies in CAR and Gabon [12, 15] also reported the
221 absence of EV-Es and EV-Fs in stools of herd animals, including cattle, which suggest that
222 EV-Es and EV-Fs were not abundant in herd animals in this region when these different
223 studies were carried out (from Feb 2017 through Aug 2022).

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224 The detection of one EV-C99 in pig stools is of interest. EV-C99s were initially not
225 distinguished from members of another virus type named coxsackievirus (CV) A24. They
226 were assigned to a new virus type, EV-C99, based on pairwise identities, similarity plots and
227 phylogenetic analyses [25]. EV-C99s have been sampled worldwide and are particularly
228 abundant in children in sub-Saharan Africa [12, 26–28]. This virus type has already been
229 described in dogs in Gabon [15] and in chimpanzees in Democratic Republic of Congo [29]
230 but, to our knowledge, our study constitutes the first report of a human EV-C99 in pig.
231 However, this is the second time that human EV-Cs are reported in pigs, as two human EV-
232 Cs (CVA17 and CVA24) were recently identified in pigs in CAR [12]. To our knowledge,
233 the ability of EV-Cs to infect animals has never been demonstrated. The hypothesis of a
234 passive carriage occurring through consumption of contaminated water, faeces or food cannot
235 be ruled out. Nonetheless, previous studies had demonstrated sustained transmission in pigs
236 of one lineage of CV-B4 and one lineage of CV-B5, following the crossing of the species
237 barrier by these two human members of the species *Enterovirus B* [5, 30, 31]. The low
238 number of EV-Cs detected in pig samples in this study and in previous ones does not suggest
239 an active transmission of EV-Cs from pig to pig, but further investigations are needed to
240 determine whether pigs could constitute efficient reservoirs for these viruses.

241 Our study identified 11 EV-G types genetically very close to EV-Gs sampled in
242 various regions of the world, suggesting a global circulation of EV-Gs. These results concur
243 with previous observations made in CAR [12] and can be explained by to the high number of
244 live pigs imported every year by sub-Saharan countries including Cameroon and CAR. The
245 livestock trade contributes to the global dissemination of viruses and can lead to the
246 transboundary spread of diseases that have a high economical and/or ecological impact, such
247 as African swine fever that spread out from Africa and reached Europe and Asia [32].

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248 In conclusion, this study conducted in Cameroon and CAR reported a diversity of animal
249 EV-Gs that are genetically linked to strains sampled in other continents, while no EV-E or -F
250 was detected. The presence of EV-C99 in pigs reinforces the hypothesis that domestic pigs
251 could be infected by human EVs, but their role as an effective reservoir remains to be
252 determined.

253

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256 Pasteur International Bioresources network, *Plateforme de microbiologie mutualisée*) for
257 processing their samples.

258

259 **Data Summary**

260 Genetic sequences were submitted to GenBank (accession numbers PQ043000-
261 PQ043027)

262

263 **Ethics**

264 This study was approved by the Cameroonian Ministry of Livestock, Fisheries and Animal
265 Industries (N°000050/L/MINEPIA/SG/DREPIA/CE).

266

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271

272 **Conflict of Interest**

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273 The authors do not report any conflict of interest.

274

275 **Author contributions**

276 Conceptualized the study: AFM, MCEZ, SSM, RN, MB. Collected the samples:

277 AFM, MPMNY. Performed the experiments: AFM, MLJ. Performed the analysis: AFM,

278 MLJ, MB. Wrote the manuscript: AFM, MCEZ, MB.

279

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385 **Figure 1**. Phylogenetic relationships between Cameroon EV-Gs, CAR EV-Gs and other

386 specimens available in public databases, based on the VP1-encoding region. Sequences from

387 Cameroon are indicated by green circles, while those from Central Africa Republic are

388 indicated by red circles. The other sequences are named by using their respective genotype,

389 country (ISO 3166-1 alpha-3 codes), year of sampling and GenBank accession number.

390 Bootstrap values are indicated if >70%.

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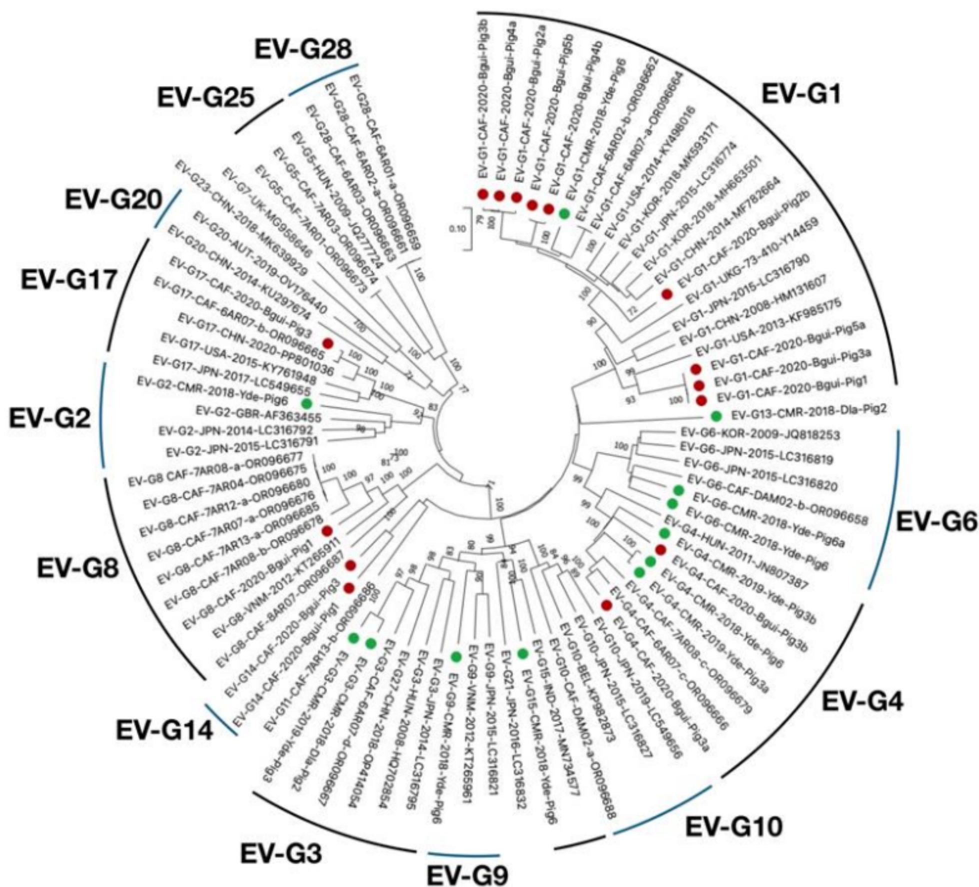

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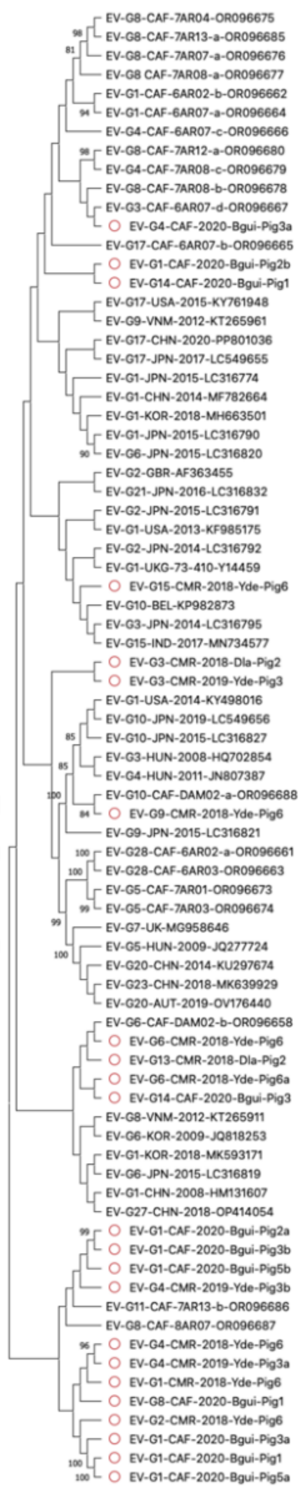
395 **Figure 2.** Phylogenetic relationships between EV-Gs found in our study and other

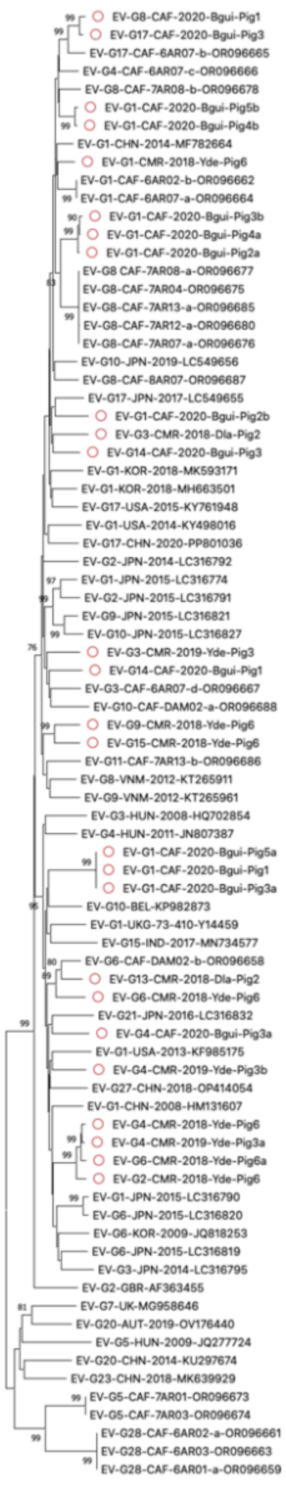
396 specimens available in public databases, based on the 5'UTR (left) and 2A-2B (right)

397 regions. The sequences found in our study are indicated by red circles. The other sequences

398 are named by using their respective genotype, country (ISO 3166-1 alpha-3 codes), year of

399 sampling and GenBank accession number. Bootstrap values are indicated if ≥70%.





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401 **Table 1**. Primers used in our study

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	Name	5'-3' sequence
Molecular screening		
Sense primer	EV-EFG-546-F	CTAATCCCAACCTCSGAGC
Antisense primer	EV-EFG-632-R	ACCSAAAGTAGTCTGTTCC
Probe A	EV-EF-probe	FAM-CCAGTGTTGCTACGTCGTAA-TAMRA [a]
Probe B	EV-G-probe	Cy5-GGYGTCGTAACGGGYAACTCTGTG-IBRQ [b]
Amplification of the 5' half of the genome		
1 st PCR		
Sense primer	EV-EFG-271-F	GGTCAAGCACTTCTGTYTC
Antisense primer	EV-EFG-4441-R	CGDTKCTTGBTCTTGAACTG
Nested PCR		
Sense primer	EV-EF-381-F	CRGYGGTAGCTCTGRRDRATG
Antisense primer	EV-EF-4357-R	GCRTTYTTYCTRCARTGRTG
Nested PCR		
Sense primer	EV-G-491-F	GAATGCKGCTAATCCTAACC
Antisense primer	EV-G-4294-R	CCAAANADGGTYTCYTGYTG
Amplification of the 3' half of the genome		
Sense primer	EV-G-4263-F	CCAACYACRGAGCAGCARG
Antisense primer	J-polyT	CAGGAAACAGCTATGACTTTTTTTTTTTTTTTTTTTTTTVN

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[a] FAM: 6-carboxyfluorescein; TAMRA: 6-carboxy-tetramethyl-rhodamine

[b] Cy5: Cyanine 5; IBRQ: 3' Iowa Black® RQ

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413 **Table 2:** Molecular screening and RT-PCR results

Host	Number of pools	Number of positive pools by screening		Number of pools from which an amplicon was obtained by RT-PCR	
		Probe A	Probe B	5'half amplification	3'half amplification
Cameroon					
Pigs	14	0	6	3	1
Central African republic					
Pigs	6	0	6	6	1
Goats	3	0	0	0	0

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