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Instructions for use

1   *Mastomys natalensis* **is a possible natural rodent reservoir for**

2   **encephalomyocarditis virus.**

3

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28   **Keyword**

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31   **Repositories**

34

35   **Abstract**

36   Encephalomyocarditis virus (EMCV) infects a wide range of hosts and can

37   cause encephalitis, myocarditis, reproductive disorders and diabetes mellitus in selected

38   mammalian species. As for humans, EMCV infection seems to occur by the contact

39   with animals and can cause febrile illnesses in some infected patients. Here we isolated

40   EMCV strain ZM12/14 from a natal multimammate mouse (*Mastomys natalensis*: *M.*

41   *natalensis*) in Zambia. Pairwise sequence similarity of ZM12/14 P1 region consisting of

42   antigenic capsid proteins showed the highest similarity of nucleotide (80.7%) and amino

43   acid (96.2%) sequence with EMCV serotype 1 (EMCV-1). Phylogenetic analysis

44   revealed that ZM12/14 clustered into EMCV-1 at the P1 and P3 regions but segregated

45   from known EMCV strains at the P2 region, suggesting a unique evolutionary history.

46   RT-PCR screening and neutralizing antibody assays for EMCV were performed using

47   collected tissues and serum from various rodents (n=179) captured in different areas in

48   Zambia. We detected the EMCV genome in 19 *M. natalensis* (19/179=10.6%) and

2

49 neutralizing antibody for EMCV in 33 *M. natalensis* (33/179=18.4%). However, we did

50 not detect either the genome or neutralizing antibody in other rodent species. High

51 neutralizing antibody titers ($\geqq$320) were observed in both RT-PCR-negative and -

52 positive animals. Inoculation of ZM12/14 caused asymptomatic persistent infection in

53 BALB/c mice with high antibody titers and high viral loads in some organs, consistent

54 with the above epidemiological results. This study is the first report of the isolation of

55 EMCV in Zambia, suggesting that *M. natalensis* may play a role as a natural reservoir

56 of infection.

57

Introduction

Encephalomyocarditis virus (EMCV) infects a wide range of animal species and causes various conditions ranging from subclinical to lethal disease with myocarditis, encephalitis, neurological disorders, reproductive failure and diabetes mellitus in humans or animals (1). EMCV infection results in different outcomes depending on the host animal species and the virus strains. For example, sudden death caused by EMCV infection has been reported in primates, elephants and various captive animals in zoos (2–11). Dogs show systemic symptoms with encephalitis and myocarditis (12). Importantly, pigs are the most susceptible animal for EMCV, and EMCV infection causes a serious threat to the pig industry with sudden death often associated with myocarditis and reproductive failures including abortion (13–15). As for humans, serological surveys for EMCV have shown seropositivity rates of up to 30%. In addition, higher seropositive rates were observed in populations that have more frequent contact with wild animals such as hunters, indicating that EMCV may be a zoonotic pathogen which could be transmittd from animals to humans (16,17). Subclinical or mild infections are thought to be predominant in humans, but there are some reports showing an association with febrile illness (18,19). While EMCV infection provokes some symptoms in most animal species, rodents such as *Ruttus ruttus* and *Mus musculus* exhibit mainly asymptomatic persistence and disperse viruses for a relatively long period (20–22). A reservoir has been defined as populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to susceptible animals (23). Although there have been no reports of direct transmission from rodents to other animals or humans, these rodent species have been considered to be a potential EMCV reservoir for susceptible animals, such as pigs, wild animals or potentially humans.

4

82   EMCV is a member of the species of *Cardiovirus A* in the genus *Cardiovirus* in

83   the family *Picornaviridae*, which is the largest group of small non-enveloped positive

84   sense RNA viruses with an icosahedral capsid of 30 nm in diameter. The EMCV genome

85   is approximately 7,800 bp in length and encodes a single open reading frame (ORF),

86   which is translated as a single polyprotein precursor and cleaved by a viral protease to

87   produce mature proteins. The genome organization is as follows: VPg+5′ untranslated

88   region   (UTR)$^{IRES-II}$ [L/1A-1B-1C-1D-2A^{npgp}/2B-2C/3A-3B^{VPg}-3C^{pro}-3D^{pol}]   3′UTR-

89   poly(A). Precursor 1 (P1) composed of four proteins (1A-1D) is the capsid protein. P2

90   composed of 2A-2C and P3 composed of 3A-3D are nonstructural proteins (1,24).

91   Serologically, EMCV is classified as EMCV-1 and EMCV-2, both of which are assigned

92   to the species *Cardiovirus A* by the International Committee on Taxonomy of Viruses

93   (ICTV) (25). Resently, Vyshemirskii et al. have proposed a detailed genetic classification

94   of EMCV based on the nucleotide sequence identity, which contains four members of

95   *Cardiovirus A* (EMCV-1 to 4) and EMCV-1 is subdivided into 7 lineages (A to G) (5).

96   EMCV was firstly discovered in a gibbon ape in 1945 in Florida, USA (26).

97   Thereafter, EMCV was identified in wide range of domestic and wild animals, including

98   pigs, dogs, rodents, primates, elephants, antelopes, lions and birds in all continents except

99   for Antarctica (2,7,8,12,27). In Africa, there were outbreaks in domestic pigs and wild

100   elephants in South Africa (8,10) and primates in Democratic Republic of the Congo (11).

101   In addition, Grobler et al. reported that seropositivity in natal multimammate mice

102   (*Mastomys natalensis*: *M. natalensis*) captured in 1994 in the Kruger National Park, South

103   Africa for EMCV was 37.9% (100/264) (10). However, studies on the serosurveillance

104   of EMCV has not been reported in the subsequent 26 years. Furthermore, there have been

105   no reports on EMCV in either domestic or wild animals in Zambia. In this study, we have

106 isolated infectious EMCV from *M. natalensis* and screened for EMCV infection in

107 Zambian wild rodents using RT-PCR and neutralizing antibody tests. This study revealed

108 a unique molecular evolution of Zambian EMCV and suggests *M. natalensis* is a natural

109 reservoir of EMCV in Zambia. This is the first study of surveillance of EMCV in wildlife

110 in Zambia.

111

112 **<u>Materials and Methods</u>**

113 **Sample collection and ethical statement**

114   A total of 179 wild rodents, including *M. natalensis* and shrews collected in

115 three areas in Zambia from 2012 to 2013 were investigated: 67 rodents and shrews were

116 captured in Mpulungu, 41 in Solwezi and 71 in Mazabuka (Fig. 1). Rodents and shrews

117 were captured using Sherman traps and cage traps and euthanized with diethyl ether,

118 then sera, kidneys, spleens and lungs were collected and kept at -80℃ until use. In

119 collected kidneys, spleens and lungs, we did not observe any macroscopical changes.

120 Captured rodents and shrews were classified into 13 species of rodents and 2 species of

121 shrews by nucleotide sequence analysis of the mitochondrial cytochrome *b* gene, as

122 described previously (28,29). Ethical approval to undertake the present study was

123 provided by the then Zambia Wildlife Authority, which is now the Department of

124 National Parks and Wildlife, Ministry of Tourism and Arts, Zambia.

125

126 **Cells and viruses**

127   Baby hamster kidney 21 (BHK-21 C-13, JCRB Cell Bank, Osaka, Japan) cells

128 were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal

129 bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (PS). Cells

130   were constantly cultured at 37°C with 5% CO_2. For EMCV propagation, BHK-21 cells

131   were infected with EMCV ZM12/14 at a multiplicity of infection (MOI) of 0.1 and

132   maintained for 2 days in static culture with maintenance medium: DMEM with 2% FBS

133   and PS. For virus titration, BHK-21 cells in 96-well plates were infected with EMCV

134   with 10-fold serial dilutions. Appearance of cytopathic effect (CPE) was monitored at 4

135   days post infection (dpi) and the 50% tissue culture infective dose ($TCID_{50}$)/ml was

136   calculated according to the Reed and Muench method.

137

138   **Virus isolation**

139   Mixed tissue homogenates of kidney, spleen and lung of each rodent and shrew

140   were prepared using BioMasher II (Nippi, Tokyo, Japan). After centrifugation at

141    $3,000 \times g$ for 5 min, supernatants were inoculated to BHK-21 cells with 2 ml isolation

142   medium [DMEM supplemented with 10% FBS, PS, 25 μg/ml gentamycin, 1%

143   antibiotic-antimycotic solution (Wako, Osaka, Japan) and 25 mM 4-(2-hydroxyethyl)-1-

144   piperazineethanesulfonic acid (HEPES)] in 15 ml tissue culture tubes. Cells were

145   cultured for 7 days in rolling condition of 0.3 rpm/min and inoculated cells were

146   subsequently blind passaged twice in BHK-21 cells.

147

148   **Viral genome sequencing**

149   Viral RNA was extracted from the supernatant of the infected BHK-21 cells

150   using TRIzol LS reagent (Invitrogen, Carlsbad, CA). Double-strand cDNA was

151   constructed by PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio, Shiga,

152   Japan) and subjected to sequence library construction using Nextera XT DNA Library

153   Preparation Kit (Illumina, San Diego, CA). The 300 bp paired-end sequencing was

154 performed on an illumina MiSeq sequencer (Illumina). Sequence reads were trimmed

155 and assembled into contigs by *de novo* assembly using CLC Genomics Workbench 20.1

156 (Qiagen, Hilden, Germany). The obtained contigs were analyzed by blastn program

157 (National Center for Biotechnology Information, Bethesda, MD).

158

159 **qRT-PCR**

160   Total RNA was extracted from culture supernatants or 10% tissue homogenates

161 using TRIzol LS reagent and subjected to qRT-PCR using THUNDERBIRD Probe

162 One-step qRT-PCR Kit (TOYOBO, Osaka, Japan). The primer and probe sequences for

163 EMCV ZM12/14 were as follows: forward primer 5′-

164 TCTTCTTGTGGCGACGAATTA-3′; reverse primer 5′-

165 GTCTTGTTAGCGGGTGTTATCT-3′; probe 5′-

166 /FAM/TCCTGTCTT/ZEN/TGCCAGATTTGTTCTCACC/IABkFQ/-3′ (Integrated

167 DNA Technologies, Coralville, IA). Serially diluted RNA from the culture supernatants

168 containing EMCV were used to generate a standard curve for the conversion of Ct

169 values to $TCID_{50}$.

170

171 **RT-PCR and sequencing**

172   Total RNA was extracted from kidneys of rodents and shrews from Mpulungu

173 and spleens from Solwezi and Mazabuka using TRIzol (Invitrogen). To detect multiple

174 EMCV strains with a high degree of nucleotide sequence diversity, a universal

175 degenerate primer set was designed based on the consensus amino acid sequence of

176 EMCV 3D gene from 50 strains previously registered to GenBank: forward primer 5′-

177 RARYCTVGCAAAGACAGG-3′; reverse primer 5′-CKGTACTCCACASTYTC-3′.

8

178 RT-PCR assay was performed using SuperScript IV One-Step RT-PCR System

179 (Invitrogen) with the following thermal cycling conditions: 50°C for 10 min, 98°C for 2

180 min, 40 cycle of 98°C for 10 sec, 50°C for 10 sec and 72°C 30 sec, followed by 72°C

181 for 5min. PCR amplicons (312 bp in length) were sequenced by direct sequencing

182 methods.

183

184 **Virus neutralization tests**

185   Sera from rodents and shrews were heat-inactivated at 56°C for 30 min and

186 two-fold serially diluted from 1:10 to 1:640 at the reaction steps with maintenance

187 medium. Then the diluted serum (12.5 μl) was mixed with an equal volume of

188 maintenance medium, containing 100 $TCID_{50}$ of EMCV. The mixture was incubated at

189 37°C for 2 h. After incubation, the serum-EMCV mixture was added to the suspension

190 of BHK-21 cells (2×10^4 cells/175 μl) and cultured for 4 days in 96-well plates. Virus

191 back-titration was included in each test to validate input amounts of the virus. The

192 highest serum dilution which completely inhibited CPE development was adopted to the

193 neutralizing antibody titer, and a neutralizing antibody titer greater than 1:30 was

194 considered seropositive according to a previous report (30).

195

196 **Phylogenetic analysis**

197   The genome sequence of EMCV ZM12/14 was aligned with reference EMCV

198 sequences from GenBank using ClustalW algorithm with default parameters and

199 applied to pairwise sequence identity comparison in CLC Genomics Workbench 20.1.

200 Phylogenetic trees were constructed by the Maximum-Likelihood (ML) method using

201 models of GTR+G+I for full-length of P1, P2, and P3 and K2+G for PCR amplicons, as

9

202 the best fit models, with bootstrap values of 1,000 replicates in the MEGA10 software

203 (31). Possible recombination events were searched using the RDP4 software with

204 default settings (32).

205

206 **Experimental infection of isolated EMCV in laboratory mice**

207 Five-week-old male BALB/c mice were inoculated intraperitoneally (i.p.) with

208 10^6 TCID$_{50}$ of EMCV. After the inoculation, clinical signs and body weight changes

209 were monitored for 14 dpi. At 14 dpi, heart, brain, spleen, testis, serum and feces were

210 collected from the mice. The neutralizing antibody titers of the serum were determined,

211 and the viral load of the organs and feces were estimated by qRT-PCR as described

212 above.

213

214 **Results**

215 **Virus isolation and genome sequencing**

216 Obvious CPE with cell rounding and detachment was observed in BHK-21

217 cells inoculated with tissue homogenates from one *M. natalensis* captured in Mpulungu,

218 which showed no macroscopic signs of serious infection. We tentatively named the

219 isolated virus as ZM12/14. Titration assays revealed the infectious titer of ZM12/14 in

220 the culture supernatant reached up to 2×10^9 TCID$_{50}$/ml. High-throughput sequencing

221 and *de novo* assembly with an average contig coverage of 14,172.4 allowed the

222 determination of nearly the complete genome sequence of ZM12/14 consisting of a

223 single ORF (6,879 nucleotides) encoding a polyprotein (2,292 amino acids), incomplete

224 5′-UTR (576 nt) and a complete 3′-UTR (120 nt) with poly A tail. The determined

225 sequence of ZM12/14 was deposited in GenBank (Accession No. LC585221). BLASTn

226 search revealed the genome sequence of ZM12/14 is the closest to that of EMCV strain

227 M (accession no. M37588). Overall the EMCV strain ZM12/14 was successfully

228 isolated from a *M. natalensis* in Mpulungu, Zambia.

229

230 **Sequence comparison and phylogenetic analysis**

231 To investigate the degree of sequence similarity between ZM12/14 virus and

232 EMCV reference strains, pairwise sequence identity was determined based on

233 nucleotide and amino acid sequences of P1, P2, P3 and ORF, as well as 1D, 2C and 3D

234 (Table 1). The results revealed that the ZM12/14 isolate shared the highest sequence

235 similarity with EMCV-1 strains in any examined regions and specifically P1 and 1D

236 region, which contain the main antigenic determinants located on the capsid protein,

237 ZM12/14 shared 80.7-77.5% nucleotide and 96.2-85.1% amino acid sequence identity

238 in P1, and 82.4-75.5% nucleotide and 92.8-89.8% amino acid sequence identity in 1D

239 with EMCV-1 strains. The highest P1 nucleotide sequence identity (80.7%) to that of

240 ZM12/14 was found in strains JZ1203 and YM13, which were isolated from mice and

241 pigs in China, respectively (Supplemental table 1).

242 We also performed ML phylogenetic analysis based on nucleotide sequences of

243 P1, P2 and P3 region separately (Fig. 2). Virus names and lineages were annotated to

244 the trees according to the previous study (5). EMCV-4 was not included in the

245 phylogenetic tree, because only a small part of P1 sequence was available. In the ML

246 trees of P1 and P3, ZM12/14 fell into a cluster of EMCV-1. Meanwhile, topology of P2

247 indicated that ZM12/14 were segregated from all EMCVs, including EMCV-1, EMCV-

248 2 and EMCV-3. The phylogenetic incongruence led us to conduct an exploratory

249 recombination analysis using RDP4 program based on the alignment of nucleotide

11

250   sequence of ZM12/14 and other EMCV strains; however, this analysis detected no

251   evidence of recombination in the genome of ZM12/14.

252

253   **Prevalence of EMCV among wild rodents and shrews in Zambia**

254   We performed RT-PCR and virus neutralization test to investigate EMCV

255   prevalence among wild rodents and shrews in Mpulungu, Solwezi and Mazabuka. Of

256   the 179 serum samples of wild rodents and shrews, 33 samples (18.4%) were

257   seropositive for EMCV, and 19 of these were positive in both RT-PCR and

258   neutralization tests (Table 2). EMCV genome was detected in samples from Mpulungu

259   and Solwezi, whereas EMCV-seropositive individuals were confirmed in all three areas.

260   Notably, all of the animals that were positive for EMCV genome and/or neutralizing

261   antibodies for EMCV are *M. natalensis*. Most of serum samples that were positive in

262   EMCV neutralization test had high neutralizing antibody titers ($\geqq$320 in Fig. 3). In

263   addition, these high neutralizing antibody titers were observed in not only RT-PCR-

264   negative samples (n=14) but also RT-PCR-positive samples (n=19) (Table 2). All

265   amplicons were subsequently sequenced (Accession No. LC585222-40) and the partial

266   3D sequences were subjected to pairwise sequence comparison and construction of

267   phylogenetic tree (Fig. 4). EMCV strains from Mpulungu and Solwezi shared 86.6-

268   86.3% nucleotide sequence identity and independently formed clusters in EMCV-1,

269   inferring geographic range evolution of EMCV in Zambian *M. natalensis* (Fig.1).

270

271   **Experimental infection of isolated EMCV in laboratory mice**

272   It has been reported that EMCV strains isolated from symptomatic pigs and

273   dogs cause various symptoms in laboratory rodents (12,27,33,34). To investigate the

274   pathogenicity of EMCV isolated from *M. natalensis*, three laboratory mice were

275   experimentally inoculated with ZM12/14. All the inoculated mice did not develop

276   clinical symptoms or significant weight loss during the observation period of 14 days.

277   After euthanizing at 14 dpi, serum samples were subjected to a neutralization test, and a

278   neutralizing antibody titer of $\geqq 260$ was observed in all mice. The ZM12/14 genome was

279   detected by qRT-PCR in hearts, brains, spleens and feces with the wide titer range from

280   1.1×10^2 to 6.9×10^4 TCID$_{50}$/whole organ (Fig. 5). These results suggested that ZM12/14

281   causes asymptomatic persistent infection in rodents, which is consistent with the

282   screening results in Zambian *M. natalensis*.

283

284   **Discussion**

285   Wild rodents are considered to be the natural reservoirs of EMCV. In previous

286   studies, EMCV were isolated from a wide range of wild rodents; including rats (*Rattus*

287   spp.) (35–38), mice (*Mus* spp.) (39,40), squirrels (*Sciurus* spp.) (41), dormice (*Myoxus*

288   *glis*) (42), water-rats (*Hydromys chrysogaster*) (43), cotton rats (*Sigmodon hispidus*)

289   (44), spiny rats (*Proechimys guyannensis*) (45). In South Africa, serological survey of

290   wild rodents in the Kruger National Park revealed that *M. natalensis* showed high

291   seropositivity rates (37.9%); however, further integrated studies of genetic and

292   serological analysis are necessary to understand the distribution and evolution of EMCV

293   (10). In this study, an EMCV strain named as ZM12/14 was isolated from a wild *M.*

294   *natalensis*. Thereafter Zambian wild rodents and shrews were screened for EMCV

295   infection by RT-PCR and virus neutralization tests. Because available samples of the

296   wild rodents kept at -80°C were limited, we extracted RNAs from kidneys of Mpulungu

297   rodents and spleens of rodents in Solwezi and Mazabuka for RT-PCR screening, which

298 were examined in a survey of poxviruses, paramyxoviruses and parvoviruses

299 (29,46,47). As a result, a high prevalence of EMCV in *M. natalensis* was observed,

300 consistent with the previous report from South Africa (10). Interestingly, there were

301 certain number of *M. natalensis* which had both high neutralizing antibody titer and

302 detectable viral RNA. Wild rodents are considered to be natural reservoir of EMCV

303 (10-12) and our results provide evidence that *M. natalensis* is a possible reservoir of

304 EMCV in the African continent, including Zambia.

305   EMCV can infect a wide range of animal species and impact especially on pig

306 production. EMCV causes an acute myocarditis (usually causing sudden death) in

307 young pigs and/or reproductive failure in sows, resulting in economic loss to pig

308 farmers (13–15). It has been reported that rodents contribute to outbreaks of EMCV in

309 pig farms as transmitters. (35,48–50). Although EMCV infection has not been reported

310 in any other animals in Zambia, our study demonstrated the high EMCV prevalence in

311 Zambian *M. natalensis*, highlighting the possible risk of EMCV infection in other

312 animals, such as pigs. In addition to pigs, EMCV infection can also cause fatal diseases

313 in a wide range of non-livestock species (2–4), including many kinds of non-human

314 primates (5–7), African elephants (*Loxodonta africana*) (8,9), considered endangered

315 species listed in the International Union for Conservation of Nature and Natural

316 Resources (IUCN) red list. Africa is the only continent in which outbreaks of EMCV

317 have been reported from a population of free-ranging wild animals (10,11), whereas

318 most of EMCV outbreak among exotic animals in other areas occurred in zoos. From

319 the perspective of species diversity conservation, EMCV transmission in wild rodents

320 would be considered. Further studies of prevalence of EMCV in pig farms and wild

321 animals should be directed to estimate the risk of EMCV outbreak and the need for

322 rodent control programs in Zambia.

323   EMCV was initially assumed to consist of a single genotype; however,

324 increasing numbers of EMCV sequence data have revealed high genetic diversity.

325 Recently, EMCV was serologically divided in two groups, (EMCV-1 and 2) (25), that is

326 also accepted by ICTV. It has been proposed that classification of EMCV based on

327 nucleotide sequence should be divided into EMCV-1, 2, 3 and 4, and EMCV-1

328 subdivided into 7 lineages (5). The group of EMCV-1 to 4 was defined by criteria

329 extrapolating from the genus *Enterovirus* (51,52); the same virus types share $\geqq$ 75% nt

330 (> 85% aa) identity in 1D region and $\geqq$ 90% aa identity in P1 region. In addition,

331 different lineages of EMCV-1 share < 83% nucleotide sequence identity in 1D and <

332 85% in P1. In accordance with these criteria, ZM12/14 can be assigned to a new lineage

333 H of EMCV-1. Phylogenetic trees of P1 and P3 region also indicated that ZM12/14 can

334 be classified in EMCV-1, which in consistent with pairwise sequence comparison

335 result; however, the phylogenetic tree of the P2 region showed that ZM12/14 separates

336 from the clade of EMCV-1 and even EMCV-2 and 3 without any recombination

337 evidence (Fig. 2). These results suggest that EMCV in Zambia has a unique evolutional

338 history.

339   Pathogenicity and tissue tropism of EMCV seemed to vary depending on virus

340 strain and host species; however, detailed information is still unclear. The pathogenicity

341 of EMCV to laboratory mice and rats has been reported to vary from asymptomatic to

342 fatal accompanying encephalitis, myocarditis or diabetes mellitus (53). Previous studies

343 demonstrated that EMCV strains G424/90 and B279/95 isolated from pigs showing

344 clinical signs caused mainly asymptomatic infection in Wistar rats and BALB/c mice

345 (21,22). In contrast, strains NJ08 and BD2 were fatal for laboratory BALB/c mice

346 (27,33,34). Experimental infection of ZM12/14 to BALB/c mice showed no clinical

347 signs, despite the high neutralizing antibody titer and viral RNA detection in some

348 organs suggesting the establishment of systemic infection (Fig. 5). The pathogenicity of

349 ZM12/14 in pigs and other animals will require further study.

350   In conclusion, the EMCV strain ZM12/14 isolated from *M. natalensis* in

351 Zambia, had unique phylogenetic features. Given the high detection rate of the EMCV-

352 genome and neutralizing antibody for EMCV in *M. natalensis*, this rodent species may

353 be one of the reservoirs in African countries. Consequently, our study updates the

354 knowledge of the current situation of EMCV in wild rodents in the African continent

355 and highlights the potential risk of EMCV infection in domestic and wild animals and

356 potentially humans in Zambia.

357

358 **Conflicts of interest**

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

360

361 **Ethical approval**

362 Ethical approval to undertake the present study was provided by the then Zambia

363 Wildlife Authority, which is now the Department of National Parks and Wildlife,

364 Ministry of Tourism and Arts, Zambia. All animal experiments were performed at the

365 Animal BSL-2 facility of the Research Center for Zoonosis Control of Hokkaido

366 University, which has been certified by The Association for Assessment and

367 Accreditation of Laboratory Animal Care International, and followed the basic

368 guidelines for animal experiments of the Ministry of Education, Culture, Sports,

369 Science, and Technology (MEXT) of Japan. All animal experiments were approved by

370 the President of Hokkaido University after review by the Animal Care and Use

371 Committee of Hokkaido University (No. 19-0019).

372

382

383

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548

549

Figure legends

551 Fig. 1. Map of Zambia showing the locations of rodent sampling. Rodent collections

552 were carried out in Mpulungu (Northern Province), Solwezi (North-Western Province)

553 and Mazabuka (Southern Province). EMCV strain ZM12/14 was isolated from *M.*

554 *natalensis* collected in Mpulungu area.

555

556 Fig. 2. Phylogenetic analysis of EMCV isolates based on the nucleotide sequence of P1,

557 P2 and P3 regions. The species of *Cardiovirus B* were included as the outer group.

558 Taxon of EMCV strain ZM12/14 was highlighted in the black square. In addition to

559 serotype EMCV-1 and EMCV-2, EMCV-3 and lineages A-G proposed by Vyshemirskii

560 *et al*. are shown as indicated (5). Phylogenetic trees were constructed by the Maximum-

561 Likelihood method using Models of GTR+G+I with bootstrap values of 1,000

562 replicates.

563

564 Fig. 3. Distribution of neutralizing antibody titer of *M. natalensis*. Black or white bars

565 indicate RT-PCR-positive or -negative samples, respectively. The titer greater than 1:30

566 was considered as seropositive. The neutralizing test was performed twice.

567

568 Fig. 4. Phylogenetic analysis of Zambian EMCVs and EMCV genomes deposited in

569 GenBank, based on nucleotide sequences of partial 3D region (277 bp in length). In

570 addition to serotype EMCV-1 and EMCV-2, EMCV-3 and lineages A-G proposed by

571 Vyshemirskii *et al*. are shown (5). Phylogenetic trees were constructed by the

572 Maximum-Likelihood method using Models of K2+G with bootstrap values of 1,000

573 replicates.

574

575   Fig. 5. Viral loads of ZM12/14 in the tissues and feces of challenged BALB/c mice

576   (n=3) by using qRT-PCR. The Ct values of viral genome in each sample were converted

577   to TCID$_{50}$ based on the standard curve. The values in the graphs were expressed as

578   mean ± SD of three technical replicates.

579

Table. 1 Pairwise sequence identity (%) with ZR12/14 virus

Virus	P1		1D		P2		2C		P3		3D		ORF	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
EMCV-1	80.7-77.5	96.2-85.1	82.4-75.5	92.8-89.8	78.3-76.2	87.4-84.3	77.7-75.7	92.0-90.5	79.0-74.9	88.9-84.0	81.3-77.0	90.3-89.1	79.5-76.3	91.1-86.9
EMCV-2	67.8-66.7	75.7-75.6	60.4-59.7	64.3-63.8	72.3-71.6	79.2-77.6	75.6-74.5	89.0-87.4	73.6-72.4	81.7-80.6	75.9-75.8	86.7-86.4	71.2-70.5	79.1-78.3
EMCV-3	74.7	91.1-90.9	71.8-71.5	86.8-86.4	71.3	78.0	75.4-75.3	88.4	72.4	80.9	75.6	86.0	73.3	84.1-84.0
EMCV-4	-	-	65.3-64.8	71.5-71.1	-	-	-	-	-	-	-	-	-	-

EMCV-1: NC_001479, AF356822, AF525466, AJ617356, AJ617357, AJ617358, AJ617359, AJ617360, AJ617361, AJ617362, AY296731, DQ288856, DQ464062, DQ464063, DQ517424, DQ835184, DQ835185, EU371993, EU780148, EU780149, EU979545, EU979548, FJ604852, FJ604853, FJ897755, HM641897, JN800421, JN800422, JN800423, DQ294633, JQ864080, KC110082, KC110083, KC110084, KC762214, KF293299, KF598860, KF598861, KF598862, KF598863, KF598864, KF709977, KF771002, KF836386, KF836387, KF836388, KF836389, KF836390, KJ524643, KM269482, KP892662, KU664327, KU955338, KX231802, L22089, L40427, M20167, M22457, M22458, M37588, M54935, M88547, MH191297, X00463, X67502, X74312, X87335, Y15445, Y15448

EMCV-2: JX257003, MN547968

EMCV-3: KC310737, KC310738

EMCV-4: KT944132, KT944133

Table. 2 EMCV prevalence in wild rodents in Zambia

Neutralization test	+	+	-	-	Total
RT-PCR	+	-	+	-	
Mpulungu					
Mastomys natalensis	5	3	0	19	27
Crocidura hirta	0	0	0	19	19
Crocidura luna	0	0	0	1	1
Rattus rattus	0	0	0	3	3
Aethomys chrysophilus	0	0	0	6	6
Cricetomys gambianus	0	0	0	3	3
Saccostomus sp.	0	0	0	3	3
Squirrel	0	0	0	2	2
Grammomys sp.	0	0	0	1	1
Steatomys sp.	0	0	0	1	1
Gerbilliscus leucogaster	0	0	0	1	1
Subtotal	5	3	0	59	67
Solwezi					
Mastomys natalensis	14	4	0	12	30
Crocidura luna	0	0	0	7	7
Rattus rattus	0	0	0	1	1
Arvicanthis niloticus	0	0	0	1	1
Saccostomys campestris	0	0	0	1	1
Mus minutoides	0	0	0	1	1
Subtotal	14	4	0	23	41
Mazabuka					
Mastomys natalensis	0	7	0	46	53
Crocidura hirta	0	0	0	4	4
Rattus rattus	0	0	0	1	1
Aethomys chrysophilus	0	0	0	5	5
Saccostomus campestris	0	0	0	2	2
Steatomys sp.	0	0	0	2	2
Graphiurus sp.	0	0	0	4	4
Subtotal	0	7	0	64	71
Total	19	14	0	146	179

[a]+; positive, -; negative

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5