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***Mastomys natalensis* is a possible natural rodent reservoir for
encephalomyocarditis virus.**

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Keyword

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Repositories

The GenBank/EMBL/DDBJ accession numbers for the viral sequences reported in this paper are LC585221-40.

Abstract

Encephalomyocarditis virus (EMCV) infects a wide range of hosts and can cause encephalitis, myocarditis, reproductive disorders and diabetes mellitus in selected mammalian species. As for humans, EMCV infection seems to occur by the contact with animals and can cause febrile illnesses in some infected patients. Here we isolated EMCV strain ZM12/14 from a natal multimammate mouse (*Mastomys natalensis*: *M. natalensis*) in Zambia. Pairwise sequence similarity of ZM12/14 P1 region consisting of antigenic capsid proteins showed the highest similarity of nucleotide (80.7%) and amino acid (96.2%) sequence with EMCV serotype 1 (EMCV-1). Phylogenetic analysis revealed that ZM12/14 clustered into EMCV-1 at the P1 and P3 regions but segregated from known EMCV strains at the P2 region, suggesting a unique evolutionary history. RT-PCR screening and neutralizing antibody assays for EMCV were performed using collected tissues and serum from various rodents (n=179) captured in different areas in Zambia. We detected the EMCV genome in 19 *M. natalensis* (19/179=10.6%) and

neutralizing antibody for EMCV in 33 *M. natalensis* (33/179=18.4%). However, we did not detect either the genome or neutralizing antibody in other rodent species. High neutralizing antibody titers (≥ 320) were observed in both RT-PCR-negative and -positive animals. Inoculation of ZM12/14 caused asymptomatic persistent infection in BALB/c mice with high antibody titers and high viral loads in some organs, consistent with the above epidemiological results. This study is the first report of the isolation of EMCV in Zambia, suggesting that *M. natalensis* may play a role as a natural reservoir of infection.

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 transmitted 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 *Rattus rattus* 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.

EMCV is a member of the species of *Cardiovirus A* in the genus *Cardiovirus* in the family *Picornaviridae*, which is the largest group of small non-enveloped positive sense RNA viruses with an icosahedral capsid of 30 nm in diameter. The EMCV genome is approximately 7,800 bp in length and encodes a single open reading frame (ORF), which is translated as a single polyprotein precursor and cleaved by a viral protease to produce mature proteins. The genome organization is as follows: VPg+5' untranslated region (UTR)^{IRES-II} [L/1A-1B-1C-1D-2A^{np}g^p/2B-2C/3A-3B^{VPg}-3C^{pro}-3D^{pol}] 3'UTR-poly(A). Precursor 1 (P1) composed of four proteins (1A-1D) is the capsid protein. P2 composed of 2A-2C and P3 composed of 3A-3D are nonstructural proteins (1,24). Serologically, EMCV is classified as EMCV-1 and EMCV-2, both of which are assigned to the species *Cardiovirus A* by the International Committee on Taxonomy of Viruses (ICTV) (25). Recently, Vyshemirskii et al. have proposed a detailed genetic classification of EMCV based on the nucleotide sequence identity, which contains four members of *Cardiovirus A* (EMCV-1 to 4) and EMCV-1 is subdivided into 7 lineages (A to G) (5).

EMCV was firstly discovered in a gibbon ape in 1945 in Florida, USA (26). Thereafter, EMCV was identified in wide range of domestic and wild animals, including pigs, dogs, rodents, primates, elephants, antelopes, lions and birds in all continents except for Antarctica (2,7,8,12,27). In Africa, there were outbreaks in domestic pigs and wild elephants in South Africa (8,10) and primates in Democratic Republic of the Congo (11). In addition, Grobler et al. reported that seropositivity in natal multimammate mice (*Mastomys natalensis*: *M. natalensis*) captured in 1994 in the Kruger National Park, South Africa for EMCV was 37.9% (100/264) (10). However, studies on the serosurveillance of EMCV has not been reported in the subsequent 26 years. Furthermore, there have been no reports on EMCV in either domestic or wild animals in Zambia. In this study, we have

isolated infectious EMCV from *M. natalensis* and screened for EMCV infection in
Zambian wild rodents using RT-PCR and neutralizing antibody tests. This study revealed
a unique molecular evolution of Zambian EMCV and suggests *M. natalensis* is a natural
reservoir of EMCV in Zambia. This is the first study of surveillance of EMCV in wildlife
in Zambia.

Materials and Methods

Sample collection and ethical statement

A total of 179 wild rodents, including *M. natalensis* and shrews collected in
three areas in Zambia from 2012 to 2013 were investigated: 67 rodents and shrews were
captured in Mpulungu, 41 in Solwezi and 71 in Mazabuka (Fig. 1). Rodents and shrews
were captured using Sherman traps and cage traps and euthanized with diethyl ether,
then sera, kidneys, spleens and lungs were collected and kept at -80°C until use. In
collected kidneys, spleens and lungs, we did not observe any macroscopical changes.
Captured rodents and shrews were classified into 13 species of rodents and 2 species of
shrews by nucleotide sequence analysis of the mitochondrial cytochrome *b* gene, as
described previously (28,29). Ethical approval to undertake the present study was
provided by the then Zambia Wildlife Authority, which is now the Department of
National Parks and Wildlife, Ministry of Tourism and Arts, Zambia.

Cells and viruses

Baby hamster kidney 21 (BHK-21 C-13, JCRB Cell Bank, Osaka, Japan) cells
were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal
bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (PS). Cells

were constantly cultured at 37°C with 5% CO₂. For EMCV propagation, BHK-21 cells were infected with EMCV ZM12/14 at a multiplicity of infection (MOI) of 0.1 and maintained for 2 days in static culture with maintenance medium: DMEM with 2% FBS and PS. For virus titration, BHK-21 cells in 96-well plates were infected with EMCV with 10-fold serial dilutions. Appearance of cytopathic effect (CPE) was monitored at 4 days post infection (dpi) and the 50% tissue culture infective dose (TCID₅₀)/ml was calculated according to the Reed and Muench method.

Virus isolation

Mixed tissue homogenates of kidney, spleen and lung of each rodent and shrew were prepared using BioMasher II (Nippi, Tokyo, Japan). After centrifugation at 3,000×g for 5 min, supernatants were inoculated to BHK-21 cells with 2 ml isolation medium [DMEM supplemented with 10% FBS, PS, 25 µg/ml gentamycin, 1% antibiotic-antimycotic solution (Wako, Osaka, Japan) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)] in 15 ml tissue culture tubes. Cells were cultured for 7 days in rolling condition of 0.3 rpm/min and inoculated cells were subsequently blind passaged twice in BHK-21 cells.

Viral genome sequencing

Viral RNA was extracted from the supernatant of the infected BHK-21 cells using TRIzol LS reagent (Invitrogen, Carlsbad, CA). Double-strand cDNA was constructed by PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) and subjected to sequence library construction using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). The 300 bp paired-end sequencing was

performed on an illumina MiSeq sequencer (Illumina). Sequence reads were trimmed and assembled into contigs by *de novo* assembly using CLC Genomics Workbench 20.1 (Qiagen, Hilden, Germany). The obtained contigs were analyzed by blastn program (National Center for Biotechnology Information, Bethesda, MD).

qRT-PCR

Total RNA was extracted from culture supernatants or 10% tissue homogenates using TRIzol LS reagent and subjected to qRT-PCR using THUNDERBIRD Probe One-step qRT-PCR Kit (TOYOBO, Osaka, Japan). The primer and probe sequences for EMCV ZM12/14 were as follows: forward primer 5'-TCTTCTTGTGGCGACGAATTA-3'; reverse primer 5'-GTCTTGTTAGCGGGTGTATCT-3'; probe 5'-/FAM/TCCTGTCTT/ZEN/TGCCAGATTTGTTCTCACC/IABkFQ/-3' (Integrated DNA Technologies, Coralville, IA). Serially diluted RNA from the culture supernatants containing EMCV were used to generate a standard curve for the conversion of Ct values to TCID₅₀.

RT-PCR and sequencing

Total RNA was extracted from kidneys of rodents and shrews from Mpulungu and spleens from Solwezi and Mazabuka using TRIzol (Invitrogen). To detect multiple EMCV strains with a high degree of nucleotide sequence diversity, a universal degenerate primer set was designed based on the consensus amino acid sequence of EMCV 3D gene from 50 strains previously registered to GenBank: forward primer 5'-RARYCTVGCAAAGACAGG-3'; reverse primer 5'-CKGTACTCCACASTYTC-3'.

RT-PCR assay was performed using SuperScript IV One-Step RT-PCR System (Invitrogen) with the following thermal cycling conditions: 50°C for 10 min, 98°C for 2 min, 40 cycle of 98°C for 10 sec, 50°C for 10 sec and 72°C 30 sec, followed by 72°C for 5min. PCR amplicons (312 bp in length) were sequenced by direct sequencing methods.

Virus neutralization tests

Sera from rodents and shrews were heat-inactivated at 56°C for 30 min and two-fold serially diluted from 1:10 to 1:640 at the reaction steps with maintenance medium. Then the diluted serum (12.5 µl) was mixed with an equal volume of maintenance medium, containing 100 TCID₅₀ of EMCV. The mixture was incubated at 37°C for 2 h. After incubation, the serum-EMCV mixture was added to the suspension of BHK-21 cells (2×10⁴ cells/175 µl) and cultured for 4 days in 96-well plates. Virus back-titration was included in each test to validate input amounts of the virus. The highest serum dilution which completely inhibited CPE development was adopted to the neutralizing antibody titer, and a neutralizing antibody titer greater than 1:30 was considered seropositive according to a previous report (30).

Phylogenetic analysis

The genome sequence of EMCV ZM12/14 was aligned with reference EMCV sequences from GenBank using ClustalW algorithm with default parameters and applied to pairwise sequence identity comparison in CLC Genomics Workbench 20.1. Phylogenetic trees were constructed by the Maximum-Likelihood (ML) method using models of GTR+G+I for full-length of P1, P2, and P3 and K2+G for PCR amplicons, as

the best fit models, with bootstrap values of 1,000 replicates in the MEGA10 software (31). Possible recombination events were searched using the RDP4 software with default settings (32).

Experimental infection of isolated EMCV in laboratory mice

Five-week-old male BALB/c mice were inoculated intraperitoneally (i.p.) with 10^6 TCID₅₀ of EMCV. After the inoculation, clinical signs and body weight changes were monitored for 14 dpi. At 14 dpi, heart, brain, spleen, testis, serum and feces were collected from the mice. The neutralizing antibody titers of the serum were determined, and the viral load of the organs and feces were estimated by qRT-PCR as described above.

Results

Virus isolation and genome sequencing

Obvious CPE with cell rounding and detachment was observed in BHK-21 cells inoculated with tissue homogenates from one *M. natalensis* captured in Mpulungu, which showed no macroscopic signs of serious infection. We tentatively named the isolated virus as ZM12/14. Titration assays revealed the infectious titer of ZM12/14 in the culture supernatant reached up to 2×10^9 TCID₅₀/ml. High-throughput sequencing and *de novo* assembly with an average contig coverage of 14,172.4 allowed the determination of nearly the complete genome sequence of ZM12/14 consisting of a single ORF (6,879 nucleotides) encoding a polyprotein (2,292 amino acids), incomplete 5'-UTR (576 nt) and a complete 3'-UTR (120 nt) with poly A tail. The determined sequence of ZM12/14 was deposited in GenBank (Accession No. LC585221). BLASTn

search revealed the genome sequence of ZM12/14 is the closest to that of EMCV strain M (accession no. M37588). Overall the EMCV strain ZM12/14 was successfully isolated from a *M. natalensis* in Mpulungu, Zambia.

Sequence comparison and phylogenetic analysis

To investigate the degree of sequence similarity between ZM12/14 virus and EMCV reference strains, pairwise sequence identity was determined based on nucleotide and amino acid sequences of P1, P2, P3 and ORF, as well as 1D, 2C and 3D (Table 1). The results revealed that the ZM12/14 isolate shared the highest sequence similarity with EMCV-1 strains in any examined regions and specifically P1 and 1D region, which contain the main antigenic determinants located on the capsid protein, ZM12/14 shared 80.7-77.5% nucleotide and 96.2-85.1% amino acid sequence identity in P1, and 82.4-75.5% nucleotide and 92.8-89.8% amino acid sequence identity in 1D with EMCV-1 strains. The highest P1 nucleotide sequence identity (80.7%) to that of ZM12/14 was found in strains JZ1203 and YM13, which were isolated from mice and pigs in China, respectively (Supplemental table 1).

We also performed ML phylogenetic analysis based on nucleotide sequences of P1, P2 and P3 region separately (Fig. 2). Virus names and lineages were annotated to the trees according to the previous study (5). EMCV-4 was not included in the phylogenetic tree, because only a small part of P1 sequence was available. In the ML trees of P1 and P3, ZM12/14 fell into a cluster of EMCV-1. Meanwhile, topology of P2 indicated that ZM12/14 were segregated from all EMCVs, including EMCV-1, EMCV-2 and EMCV-3. The phylogenetic incongruence led us to conduct an exploratory recombination analysis using RDP4 program based on the alignment of nucleotide

sequence of ZM12/14 and other EMCV strains; however, this analysis detected no evidence of recombination in the genome of ZM12/14.

Prevalence of EMCV among wild rodents and shrews in Zambia

We performed RT-PCR and virus neutralization test to investigate EMCV prevalence among wild rodents and shrews in Mpulungu, Solwezi and Mazabuka. Of the 179 serum samples of wild rodents and shrews, 33 samples (18.4%) were seropositive for EMCV, and 19 of these were positive in both RT-PCR and neutralization tests (Table 2). EMCV genome was detected in samples from Mpulungu and Solwezi, whereas EMCV-seropositive individuals were confirmed in all three areas. Notably, all of the animals that were positive for EMCV genome and/or neutralizing antibodies for EMCV are *M. natalensis*. Most of serum samples that were positive in EMCV neutralization test had high neutralizing antibody titers (≥ 320 in Fig. 3). In addition, these high neutralizing antibody titers were observed in not only RT-PCR-negative samples (n=14) but also RT-PCR-positive samples (n=19) (Table 2). All amplicons were subsequently sequenced (Accession No. LC585222-40) and the partial 3D sequences were subjected to pairwise sequence comparison and construction of phylogenetic tree (Fig. 4). EMCV strains from Mpulungu and Solwezi shared 86.6-86.3% nucleotide sequence identity and independently formed clusters in EMCV-1, inferring geographic range evolution of EMCV in Zambian *M. natalensis* (Fig.1).

Experimental infection of isolated EMCV in laboratory mice

It has been reported that EMCV strains isolated from symptomatic pigs and dogs cause various symptoms in laboratory rodents (12,27,33,34). To investigate the

pathogenicity of EMCV isolated from *M. natalensis*, three laboratory mice were experimentally inoculated with ZM12/14. All the inoculated mice did not develop clinical symptoms or significant weight loss during the observation period of 14 days. After euthanizing at 14 dpi, serum samples were subjected to a neutralization test, and a neutralizing antibody titer of ≥ 260 was observed in all mice. The ZM12/14 genome was detected by qRT-PCR in hearts, brains, spleens and feces with the wide titer range from 1.1×10^2 to 6.9×10^4 TCID₅₀/whole organ (Fig. 5). These results suggested that ZM12/14 causes asymptomatic persistent infection in rodents, which is consistent with the screening results in *Zambian M. natalensis*.

Discussion

Wild rodents are considered to be the natural reservoirs of EMCV. In previous studies, EMCV were isolated from a wide range of wild rodents; including rats (*Rattus* spp.) (35–38), mice (*Mus* spp.) (39,40), squirrels (*Sciurus* spp.) (41), dormice (*Myoxus glis*) (42), water-rats (*Hydromys chrysogaster*) (43), cotton rats (*Sigmodon hispidus*) (44), spiny rats (*Proechimys guyannensis*) (45). In South Africa, serological survey of wild rodents in the Kruger National Park revealed that *M. natalensis* showed high seropositivity rates (37.9%); however, further integrated studies of genetic and serological analysis are necessary to understand the distribution and evolution of EMCV (10). In this study, an EMCV strain named as ZM12/14 was isolated from a wild *M. natalensis*. Thereafter *Zambian* wild rodents and shrews were screened for EMCV infection by RT-PCR and virus neutralization tests. Because available samples of the wild rodents kept at -80°C were limited, we extracted RNAs from kidneys of Mpulungu rodents and spleens of rodents in Solwezi and Mazabuka for RT-PCR screening, which

were examined in a survey of poxviruses, paramyxoviruses and parvoviruses (29,46,47). As a result, a high prevalence of EMCV in *M. natalensis* was observed, consistent with the previous report from South Africa (10). Interestingly, there were certain number of *M. natalensis* which had both high neutralizing antibody titer and detectable viral RNA. Wild rodents are considered to be natural reservoir of EMCV (10-12) and our results provide evidence that *M. natalensis* is a possible reservoir of EMCV in the African continent, including Zambia.

EMCV can infect a wide range of animal species and impact especially on pig production. EMCV causes an acute myocarditis (usually causing sudden death) in young pigs and/or reproductive failure in sows, resulting in economic loss to pig farmers (13–15). It has been reported that rodents contribute to outbreaks of EMCV in pig farms as transmitters. (35,48–50). Although EMCV infection has not been reported in any other animals in Zambia, our study demonstrated the high EMCV prevalence in Zambian *M. natalensis*, highlighting the possible risk of EMCV infection in other animals, such as pigs. In addition to pigs, EMCV infection can also cause fatal diseases in a wide range of non-livestock species (2–4), including many kinds of non-human primates (5–7), African elephants (*Loxodonta africana*) (8,9), considered endangered species listed in the International Union for Conservation of Nature and Natural Resources (IUCN) red list. Africa is the only continent in which outbreaks of EMCV have been reported from a population of free-ranging wild animals (10,11), whereas most of EMCV outbreak among exotic animals in other areas occurred in zoos. From the perspective of species diversity conservation, EMCV transmission in wild rodents would be considered. Further studies of prevalence of EMCV in pig farms and wild

animals should be directed to estimate the risk of EMCV outbreak and the need for rodent control programs in Zambia.

EMCV was initially assumed to consist of a single genotype; however, increasing numbers of EMCV sequence data have revealed high genetic diversity. Recently, EMCV was serologically divided in two groups, (EMCV-1 and 2) (25), that is also accepted by ICTV. It has been proposed that classification of EMCV based on nucleotide sequence should be divided into EMCV-1, 2, 3 and 4, and EMCV-1 subdivided into 7 lineages (5). The group of EMCV-1 to 4 was defined by criteria extrapolating from the genus *Enterovirus* (51,52); the same virus types share $\geq 75\%$ nt ($> 85\%$ aa) identity in 1D region and $\geq 90\%$ aa identity in P1 region. In addition, different lineages of EMCV-1 share $< 83\%$ nucleotide sequence identity in 1D and $< 85\%$ in P1. In accordance with these criteria, ZM12/14 can be assigned to a new lineage H of EMCV-1. Phylogenetic trees of P1 and P3 region also indicated that ZM12/14 can be classified in EMCV-1, which is consistent with pairwise sequence comparison result; however, the phylogenetic tree of the P2 region showed that ZM12/14 separates from the clade of EMCV-1 and even EMCV-2 and 3 without any recombination evidence (Fig. 2). These results suggest that EMCV in Zambia has a unique evolutionary history.

Pathogenicity and tissue tropism of EMCV seemed to vary depending on virus strain and host species; however, detailed information is still unclear. The pathogenicity of EMCV to laboratory mice and rats has been reported to vary from asymptomatic to fatal accompanying encephalitis, myocarditis or diabetes mellitus (53). Previous studies demonstrated that EMCV strains G424/90 and B279/95 isolated from pigs showing clinical signs caused mainly asymptomatic infection in Wistar rats and BALB/c mice

(21,22). In contrast, strains NJ08 and BD2 were fatal for laboratory BALB/c mice (27,33,34). Experimental infection of ZM12/14 to BALB/c mice showed no clinical signs, despite the high neutralizing antibody titer and viral RNA detection in some organs suggesting the establishment of systemic infection (Fig. 5). The pathogenicity of ZM12/14 in pigs and other animals will require further study.

In conclusion, the EMCV strain ZM12/14 isolated from *M. natalensis* in Zambia, had unique phylogenetic features. Given the high detection rate of the EMCV-genome and neutralizing antibody for EMCV in *M. natalensis*, this rodent species may be one of the reservoirs in African countries. Consequently, our study updates the knowledge of the current situation of EMCV in wild rodents in the African continent and highlights the potential risk of EMCV infection in domestic and wild animals and potentially humans in Zambia.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval

Ethical approval to undertake the present study was provided by the then Zambia Wildlife Authority, which is now the Department of National Parks and Wildlife, Ministry of Tourism and Arts, Zambia. All animal experiments were performed at the Animal BSL-2 facility of the Research Center for Zoonosis Control of Hokkaido University, which has been certified by The Association for Assessment and Accreditation of Laboratory Animal Care International, and followed the basic guidelines for animal experiments of the Ministry of Education, Culture, Sports,

Science, and Technology (MEXT) of Japan. All animal experiments were approved by the President of Hokkaido University after review by the Animal Care and Use Committee of Hokkaido University (No. 19-0019).

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Figure legends

Fig. 1. Map of Zambia showing the locations of rodent sampling. Rodent collections were carried out in Mpulungu (Northern Province), Solwezi (North-Western Province) and Mazabuka (Southern Province). EMCV strain ZM12/14 was isolated from *M. natalensis* collected in Mpulungu area.

Fig. 2. Phylogenetic analysis of EMCV isolates based on the nucleotide sequence of P1, P2 and P3 regions. The species of *Cardiovirus B* were included as the outer group. Taxon of EMCV strain ZM12/14 was highlighted in the black square. In addition to serotype EMCV-1 and EMCV-2, EMCV-3 and lineages A-G proposed by Vyshemirskii *et al.* are shown as indicated (5). Phylogenetic trees were constructed by the Maximum-Likelihood method using Models of GTR+G+I with bootstrap values of 1,000 replicates.

Fig. 3. Distribution of neutralizing antibody titer of *M. natalensis*. Black or white bars indicate RT-PCR-positive or -negative samples, respectively. The titer greater than 1:30 was considered as seropositive. The neutralizing test was performed twice.

Fig. 4. Phylogenetic analysis of Zambian EMCVs and EMCV genomes deposited in GenBank, based on nucleotide sequences of partial 3D region (277 bp in length). In addition to serotype EMCV-1 and EMCV-2, EMCV-3 and lineages A-G proposed by Vyshemirskii *et al.* are shown (5). Phylogenetic trees were constructed by the Maximum-Likelihood method using Models of K2+G with bootstrap values of 1,000 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 TCID₅₀ based on the standard curve. The values in the graphs were expressed as
578 mean $\pm$ 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 RT-PCR	No. of samples ^a				Total
	+	+	-	-	
	+	-	+	-	
Mpulungu					
<i>Mastomys natalensis</i>	5	3	0	19	27
<i>Crocidura hirta</i>	0	0	0	19	19
<i>Crocidura luna</i>	0	0	0	1	1
<i>Rattus rattus</i>	0	0	0	3	3
<i>Aethomys chrysophilus</i>	0	0	0	6	6
<i>Cricetomys gambianus</i>	0	0	0	3	3
<i>Saccostomus sp.</i>	0	0	0	3	3
<i>Squirrel</i>	0	0	0	2	2
<i>Grammomys sp.</i>	0	0	0	1	1
<i>Steatomys sp.</i>	0	0	0	1	1
<i>Gerbilliscus leucogaster</i>	0	0	0	1	1
Subtotal	5	3	0	59	67
Solwezi					
<i>Mastomys natalensis</i>	14	4	0	12	30
<i>Crocidura luna</i>	0	0	0	7	7
<i>Rattus rattus</i>	0	0	0	1	1
<i>Arvicanthis niloticus</i>	0	0	0	1	1
<i>Saccostomys campestris</i>	0	0	0	1	1
<i>Mus minutoides</i>	0	0	0	1	1
Subtotal	14	4	0	23	41
Mazabuka					
<i>Mastomys natalensis</i>	0	7	0	46	53
<i>Crocidura hirta</i>	0	0	0	4	4
<i>Rattus rattus</i>	0	0	0	1	1
<i>Aethomys chrysophilus</i>	0	0	0	5	5
<i>Saccostomus campestris</i>	0	0	0	2	2
<i>Steatomys sp.</i>	0	0	0	2	2
<i>Graphiurus sp.</i>	0	0	0	4	4
Subtotal	0	7	0	64	71
Total	19	14	0	146	179

^a+, positive, -, negative

DEMOCRATIC
REPUBLIC OF THE
CONGO

Mpulungu

Solwezi

ZAMBIA

Mazabuka

0 150 km

Fig. 1

P3

Fig. 4

Fig. 5