1 **Title:**

2 **Frequency of a nucleotide overhang at the 5' end of hemorrhagic fever**
 3 **mammarenavirus genomes in public sequence data**

4

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57

58 **Abstract**

59 Mammarenaviruses, such as Lassa virus and South American hemorrhagic fever
60 (SAHF) virus, cause severe hemorrhagic fevers in humans, and pose major threats to
61 public health. Mammarenaviruses consist of a bi-segmented negative-sense RNA
62 genome in which the 5' and 3' ends form complementary strands that serve as a
63 replication promoter. Some mammarenaviruses have a nucleotide overhang at the 5'
64 genome end. By examining the complementarity of 5' and 3' genome ends using public
65 mammarenavirus genome sequences, we found that the 5' guanine overhang (5'-G
66 overhang) was present more frequently in Lassa and SAHF viruses than in other viruses.
67 The 5'-G overhang in the Lassa and SAHF virus sequences was found to be restricted to
68 the L and S segments, respectively. If the genome end sequence data in the public
69 database are accurate, the 5'-G overhang may be related to the high pathogenicity of
70 mammarenaviruses in humans.

71

72 **Keywords:** Mammarenaviruses, replication promoter, overhang, public sequence data

73

74 Introduction

75 Arenaviruses (genus *Mammarenavirus*, family *Arenaviridae*, order *Bunyavirales*)
 76 are divided into Old World and New World complexes (1). The most significant
 77 pathogen in the Old World complex is Lassa virus, the cause of hemorrhagic Lassa
 78 fever in humans. Lymphocytic choriomeningitis virus (LCMV) is also an Old World
 79 arenavirus that can cause a serious neurological disease called lymphocytic
 80 choriomeningitis, especially in prenatal and immunocompromised humans (2). The
 81 New World arenaviruses are divided into three major clades (A, B, and C), with clade B
 82 containing the Junín, Machupo, Guanarito, and Sabiá viruses, which cause Argentinian,
 83 Bolivian, Venezuelan, and Brazilian hemorrhagic fevers, respectively; collectively,
 84 these are referred to as South America Hemorrhagic fever (SAHF) (3). The
 85 mammarenavirus genome consists of two negative-strand RNA segments encoding four
 86 viral proteins: a large (L) RNA-dependent RNA polymerase (RdRp) and a matrix
 87 protein (Z) within the large RNA segment (L segment), and a nucleocapsid protein (NP)
 88 and a glycoprotein precursor (GPC) within the small RNA segment (S segment). The
 89 genomic RNA is encapsidated by NP and bound at both genome ends by L protein to
 90 form pseudocircularized ribonucleoprotein complexes (RNPs) (4–6). The genomic
 91 RNPs are enveloped by a lipid bilayer decorated with mature viral glycoproteins to form
 92 infectious virions.

93 In mammarenaviruses, the genome ends exhibit terminal complementarity of the 5'
 94 and 3' ends in each of the L and S segments to form a replication promoter. The
 95 interaction of each 5' and 3' end to L protein is essential for the initiation of RNA
 96 synthesis (7,8). The 5' end of some mammarenavirus genomes contains an additional
 97 nontemplated guanine residue (5'-G overhang) (9–12), which has been considered to be
 98 generated by a prime-and-realign mechanism. During mammarenavirus replication, it is
 99 thought that RdRp initiates at position +2 of the promoter to generate a GC dinucleotide
 100 primer that realigns, leading to base pairing between the C and a G at position +1 of the
 101 template (prime-and-realign) (13). Subsequent elongation by RdRp results in the

102 synthesis of antigenomic RNPs with a pseudotemplated G residue at the 5' end (5'-G
103 overhang). The antigenomic RNPs are used as templates for mRNA synthesis, and the
104 GPC and Z mRNAs are transcribed from the S and L segments, respectively. The
105 antigenomic RNPs also serve as templates for GC-primed replication to produce
106 progeny genomic RNPs with 5'-G overhang. The mammarenavirus replication has been
107 also shown to be initiated with CG primer that starts with precise genomic end (+1
108 position) without generating 5'-G overhang (13). It remains unclear whether the
109 initiation of RNA synthesis and the genomic terminal structures are common between
110 all mammarenaviruses, and the biological significance of the 5'-G overhang has not
111 been studied in detail.

112 In this study, we constructed a list of mammarenavirus promoters by using public
113 sequence data to characterize the promoter structures of various species of
114 mammarenaviruses. We examined the conservation of sequences of promoters among
115 mammarenaviruses, and analyzed the genomic sequences for the presence of 5'- and
116 3'-overhangs.

117

118 **Methods**

119 **List of mammarenavirus promoters**

120 Forty-six mammarenavirus species were registered on the International
121 Committee on Taxonomy of Viruses (ICTV) list (<https://talk.ictvonline.org/>) on
122 December 7th, 2021. The complete sequences of S and L segments of
123 mammarenaviruses available on the National Center for Biotechnology Information
124 (NCBI) associated with Genbank accession numbers as listed in Supplementary Table 1
125 were used for the analysis. The sequences of the extreme 40 nucleotides (nts) of each of
126 the 5' and 3' ends of mammarenaviruses were collected, and the complementarity
127 between the 5' and 3' ends of the sequences was examined. The counts of G:C and A:U
128 complementarity and each of the nts (A, U, G, and C) in the promoter region are shown

129 in Supplementary Table 1. The conservation of the nts in the promoter was analyzed by
130 using a sequence logo generator, Weblogo (<https://weblogo.berkeley.edu/logo.cgi>).

131 **Analysis of the 5'- overhang in the sequences of the *Arenaviridae* family**

132 Sequences annotated as *Arenaviridae* were downloaded from the NCBI refseq
133 database on January 7th, 2022. There were 5,274 *Arenaviridae* sequences in the
134 database. Sequences shorter than 1,000 nt (n = 2,343) were omitted from this analysis.
135 We first checked for the presence of the promoter sequence, CGCA, in the 8-nt ends of
136 the sequences. The promoter sequence was present in both ends of 502 sequences. Then,
137 the overhangs were detected from the 502 sequences by a custom Python script. The
138 codes used for this analysis is available on GitHub
139 (https://github.com/shohei-kojima/Arenaviridae_overhang_analysis_2022).

140

141 **Results and Discussion**

142 **Construction of a list of promoters in mammarenaviruses**

143 To examine the conservation of the promoter sequence among virus species, we
144 compared the both extreme 40 nts of the mammarenavirus genome sequences registered
145 on the ICTV list. Some mammarenavirus sequences available on the NCBI database did
146 not have complete genomic terminal information (14,15). Sequences that showed
147 complementarity between +1 and +4 within 0 to 2 nt shifting of the 5' or 3' ends for
148 both L and S segments were selected (Figure 1A). We obtained 23 mammarenavirus
149 promoter sequences of both the L and S segments, as listed in Supplementary Table 1
150 (shown as the positive-strand antigenomic forms). The conservation of the extreme 38
151 nts (without the overhang) of each of the 5' and 3' ends of the L and S segments among
152 the 23 virus species was analyzed by using a sequence logo generator, Weblogo (Figure
153 1B). The first 5 nts of the 5' and 3' ends were completely conserved in both the L and S
154 segments in the 23 mammarenaviruses. Although the +6 nt in the 3' end varied in the L
155 segment, that in the S segment was U in almost all samples. This difference may result
156 in differences in the promoter strength of the L and S segments to create these segments

157 in a different ratio in infected cells as proposed in other segmented RNA viruses (16,17).
158 To further characterize the promoter structure, the counts of G:C and A:U
159 complementarity and A, U, G, and C in the first 40 nts (for 38 to 39 nts in the opposite
160 strand of 2-nt and 1-nt overhangs, respectively) of the L and S segments were
161 determined (Figure 1C and Supplementary Table 1). G:C complementarity was
162 significantly higher than A:U complementarity in both the L and S segments; this result
163 differed from that of tri-segmented viruses belonging to the order *Bunyavirales*, which
164 showed higher A:U complementarity in the promoters (18). The L and S segments
165 contained significantly less A than U, G, and C in the 5' ends, which results in an nt
166 bias between the genomic and antigenomic promoters. This may function to regulate the
167 replication balance between genome and antigenome to produce different ratios of
168 various viral RNA species as observed in infected cells (19). The 5'-CGCA-UGCG-3'
169 sequence was the most conserved genome end sequence in the 23 mammarenaviruses
170 (Figure 1D, non-overhang). The 5'-GCGCA-UGCG-3' sequence showed the presence
171 of an unpaired 5' guanine nt (Figure 1D, 5'-G overhang). Among the 23 viruses, the
172 5'-G overhang was found in the Lassa, Guaranito, Junín, Machupo, Sabiá, and Oliveros
173 viruses (Figure 1D), and the 2-nt 3'-overhang was found only in the Lunk virus S
174 segment (Supplementary Table 1) in the public database. All viruses, except for
175 Oliveros virus (20,21), with the 5'-G overhang are associated with human hemorrhagic
176 fever, and are categorized as biosafety level 4 pathogens.

177 **Analysis for the 5'-G overhang in the *Arenaviridae* family using public sequence** 178 **data**

179 To expand the study of viruses having the 5'-G overhang, we next analyzed
180 sequences with 5'-CGCA-UGCG-3' (non-overhang), 5'-GCGCA-UGCG-3' (5'-G
181 overhang), or 5'-(N)NCGCA-UGCGN(N)-3' (1- or 2-nt overhang [not the 5'-G
182 overhang]) in sequences of the *Arenaviridae* family (Figure 2A). Among the virus
183 sequences, artificially generated sequences (e.g., generated by reverse genetics virus
184 engineering) were excluded. We found that 345 sequences did not have the overhang

185 (5'-CGCA-UGCG-3'), whereas 58 sequences had the 5'-G overhang
186 (5'-GCGCA-UGCG-3'; Supplementary Table 2). The sample numbers of over 5
187 sequences per virus species are shown in Figure 2B. We found that virus genomes with
188 the 5'-G overhang were restricted to the Lassa, LCMV, Junín, Machupo, Guanarito,
189 Sabiá, and Oliveros viruses (Figure 2B and C). All LCMVs with the 5'-G overhang
190 found in this study were clones obtained from virus-inoculated laboratory mice (22,23).
191 We next determine which segments (L or S) contained the 5'-G overhang in the Lassa,
192 Junín, Machupo, Guanarito, Sabiá, and Oliveros viruses. Guanarito virus sequences had
193 the 5'-G overhang only in the L segment, Lassa and Oliveros virus sequences had the
194 5'-G overhang only in the S segment, and the other sequences had it in both segments
195 (Figure 2D and E). L segments with the 5'-G overhang were found only in the New
196 World viruses causing SAHF. In general, the Old World viruses are geographically
197 confined to the African continent, with the exception of LCMV that circulates globally.
198 The New World viruses are found on the South and North American continents.
199 Regardless of the region where the viruses are distributed, the 5'-G overhang was found
200 specifically in hemorrhagic fever virus sequences on both continents. Although Oliveros
201 virus is a New World virus, it had the 5'-G overhang in the L segment, unlike other
202 SAHF viruses. This slight difference in the Oliveros virus from other SAHF viruses
203 might be the reason for its non-pathogenic characteristic.

204 **Minor genome end sequences in the database**

205 Among the 447 sequences described in Figure 2A, we found 35 sequences that did
206 not have 5'-CGCA-UGCG-3' (non-overhang) or 5'-GCGCA-UGCG-3' (5'-G
207 overhang). Two minor non-overhang sequences, i.e., 5'-GCGCA-UGCGC-3' and
208 5'-UCGCA-UGCGA-3', were found in some viruses, including biosafety level 4
209 pathogens (5'-GCGCA-UGCGC-3' in Lassa, Lujo, and Junín viruses, and
210 5'-UCGCA-UGCGA-3' in Chapare virus; Figure 3A). These sequences may have
211 evolved from the 5' overhang with an additional nt on the opposite end of the overhang.
212 We also found some variations in the 5' overhang (not the 5'-G overhang; Figure 3B).

213 The 5'-CGCGCA-UGCGC-3' sequence found in the Lassa and Junín viruses seemed to
 214 be a 1-nt addition to the 5' end of the minor non-overhang shown in Figure 3A. The
 215 3'-C overhang (5'-CGCA-UGCGC-3') was found in the Lassa, Oliveros, and other
 216 virus sequences (Figure 3B). When viral replication occurs using the genome with a
 217 5'-G overhang as a template, the 3' end of the newly replicated RNA terminates with a
 218 C complementary to the overhanging G. It has been presumed that mammarenaviruses
 219 remove this last C to retain genome integrity (13). The 3'-C overhang may represent
 220 this C that was not removed during the replication process. The 3' overhang
 221 (5'-GCGCA-UGCGCG-3') found in Lassa virus (Figure 3C) was also considered to
 222 have arisen from a minor non-overhang. There were a few virus sequences possessing a
 223 2-nt overhang both in the 5' and 3' ends, i.e., an unidentified *arenavirus* sp. and Lunk
 224 virus (Figure 3B and C). The mechanism of their generation and their biological
 225 properties remain unknown.

226 **Dependence on the accuracy of the sequence database**

227 All of the results of this study depend on the accuracy of the public genome
 228 sequences. If the genome end sequences in the NCBI database were correct and
 229 accurately registered, hemorrhagic fever viruses appear to have a higher frequency than
 230 other non-pathogenic arenaviruses of containing the 5'-G overhang in their genomes.
 231 However, some of the sequences in the database were obtained from full-genome
 232 amplification via reverse transcription polymerase chain reaction, and in those cases, the
 233 extreme sequences are derived from oligonucleotide primers. Further experiments are
 234 needed to validate whether the 5'-G overhang correlates with pathology. Even if a
 235 mutant arenavirus that has an artificially attached or removed 5'-G overhang is
 236 produced using reverse genetics, it would be difficult to evaluate whether changes in its
 237 pathogenicity are due to the presence or absence of the 5'-G overhang, because the virus
 238 can automatically synthesize the overhang by the prime-and-realign mechanism of the L
 239 protein. Identification of the functional region in the L protein that is involved in the

240 prime-and-realign mechanism and that confers a 5'-G overhang is essential to prove a
241 correlation between the 5'-G overhang and viral pathogenicity.

242 **The advantage of the 5'-G overhang for arenavirus growth and pathogenesis**

243 The high frequency of the 5'-G overhang in hemorrhagic fever virus sequences in
244 the public database suggests that hemorrhagic fever viruses have a higher ability than
245 other viruses to produce the overhang via prime-and-realign. It was hypothesized that
246 the 5'-G overhang might increase the pathogenicity of these viruses in humans. It has
247 been proposed that the 5'-G overhang in the promoter protects this portion of
248 double-stranded RNA from being recognized by retinoic acid-inducible gene I (24). The
249 presence of the 5'-G overhang was shown to be important for stimulating the RNA
250 synthesis of purified Machupo and Lassa virus L protein (25,26). These facts indicate
251 that the presence of the 5'-G overhang may be an important factor associated with viral
252 pathogenicity via the enhancement of host immune evasion and polymerase activity.

253

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259 Y.N., S.K., M.K., A.S., T.I., T.W., Y.A., H.S., K.N., T.K. and Y.M. conceived
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262

263 **Competing interests**

264 The authors declare no competing interests.

265

266

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Figure 1. Comprehensive analysis of the genomic promoters in mammarenaviruses
 (A) Schematic diagram of mammarenavirus promoter sequences and the complementarity of the 5' and 3' ends. (B) The sequence conservation of the 38 nts in the 5' and 3' genome ends of the L and S segments (non-overhang region) among 23 mammarenavirus species was analyzed by a sequence logo generator, Weblogo. (C) Counts of G:C and A:U complementarity and each nt in the first 40 nts. Bars represent the means and statistical deviations. Inferential statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey's test. * $p < 0.01$, ** $A > U$, G , and C in 5' end ($p < 0.05$), *** $A > U$, G , and C in 3' end ($p < 0.05$). (D) Schematics of the first 4 to 5 nts in the non-overhang and 5'-G overhang genome ends. Virus species possessing the 5'-G overhang are shown. All sequences are shown as the positive-strand antigenomic form.

Figure 2. Analysis of the overhang at the genome end in the *Arenaviridae* family
 (A) Schematic diagram of the analysis of genome end sequences in the *Arenaviridae* family. Viral antigenome sequences starting with 5'-CGCA and ending with UGCG-3' were recognized as not having the overhang (non-overhang), and those starting with 5'-GCGCA and ending with UGCG-3' were recognized as having the 5'-G overhang. (B) The number of sequences per virus species recognized as not having (non-overhang) or having the 5'-G overhang shown in panel A. (C) The percentage of sequences possessing the 5'-G overhang among the total sequences. HFV: hemorrhagic fever virus. The full virus names are shown in Supplementary Table 2. Reptarena: unidentified reptarena virus strains. (D) The number of sequences possessing the 5'-G overhang in the L and S segments. (E) The percentage of sequences possessing the 5'-G overhang in the L and S segments ($L + S = 100\%$).

Figure 3. Minor genome end sequences in the *Arenaviridae* family

370 (A) Minor sequences without the overhang (non-overhang). (B) Minor sequences with
371 the 5' overhang. (C) Sequences with the 3' overhang. Additional nts outside of the
372 5'-CGCA-UGCG-3' sequence are shown in green. HFVs are shown in red.

A

Sequences of 46 mammarenaviruses registered on the ICTV list

- 5'- and 3'-complementarity (C) analysis of 40 nts
- 5'- and 3'-overhang analysis (underline) available under a [CC-BY 4.0 International license](#).

5' - CGCACCGGGGAUCCUAGGCAUUUUUGGUUGCGCAAUU
 ***** * ***** *
 3' - GCGUGUACCUAGGAUCCGAUAACCUAACGCGAAACGAA
 +01234

Data selection for analysis

1. Complement +1 to +4 nts
2. Complement +1 to +4 nts within 2 nt overhang

Complete list of promoters (Supplementary Table 1)

D

Non-overhang

5' - CGCA
 3' - GCGU

5'-G overhang

5' - GCGCA
 3' - GCGU

Lassa virus
 Guanarito virus
 Junín virus
 Machupo virus
 Sabiá virus
 Oliveros virus

B

C

Figure 1

Figure 2

A Non-overhang

5'-**G**CGCA **Lassa (4)**
 3'-**C**GCGU **Lujo (3)**
 Junín (1)
 Keijut (1)
 Luna (1)
 S5-like (1)
 Univ of Giessen (1)

5'-**U**CGCA **Chapare (2)**
 3'-**A**GCGU

B 5' overhang

5'-**U**CGCA **LCMV (2)**
 3'- GCGU

5'-**C**GCGCA **Lassa (4)**
 3'- C GCGU **Junín (1)**

5'-**A**UCGCA **Guanarito (1)**
 3'- C GCGU

5'-**UU**CGCA **Wehzhou (1)**
 3'- A GCGU

5'-**UG**CGCA **Arena sp. (1)**
 3'- GCGU

C 3' overhang

5'- CGCA **Lassa (4)**
 3'-**C**GCGU **Luna (1)**
 Oliveros (1)
 Reptarena (1)

5'- UCGCA **LCMV (1)**
 3'-**AA**GCGU

5'- CGCA **Lunk (1)**
 3'-**G**GCGU

5'- GCGCA **Lassa (1)**
 3'-**G**GCGU

5'- CGCA **Arena sp. (1)**
 3'-**AC**GCGU

Figure 3