medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY-NC-ND 4.0 International license .

1 **Title Page**

2 **Article summary line:**

3 Viruses in the genus *Gemykibivirus* can be potential causal agents of encephalitis.

4

5 **Running Title:**

6 Gemykibivirus in acute encephalitis patients

7

8

9 **Key words:**

10 acute encephalitis syndrome, Gemykibivirus, metagenomic analysis, emerging infection

11 **Title:**

12 Gemykibivirus detection in acute encephalitis patients from Nepal

13 **Authors:**

14 Eans Tara Tuladhar[1], Smita Shrestha[1], Susan Vernon, Lindsay Droit, Kathie A.

15 Mihindukulasuriya, Mamta Tamang, Lata Karki, Annie Elong Ngono, Bimlesh Jha, Bal Krishna

16 Awal, Bimal Sharma Chalise, Runa Jha, Sujan Shresta, David Wang[2], Krishna Das Manandhar[2]

17 **Author affiliations:**

18 Tribhuvan University Central Department of Biotechnology, Kathmandu, Nepal (E.T. Tuladhar,

19 S. Shrestha, L. Karki, M. Tamang, K.D. Manandhar); National Public Health Laboratory,

20 Kathmandu, Nepal (B. Jha, B.K Awal, R. Jha);Sukraraj Tropical and Infectious Disease

21 Hospital, Kathmandu, Nepal (B.S Chalise); La Jolla Institute for Immunology, California, United

22 States (A. Ngono, S. Shresta); Washington University in St. Louis, Missouri, United States (S.

23 Vernon, L. Droit, K. Mihindukulasuriya, D. Wang)

24 **Footnotes**

25 [1]These authors contributed equally to this article; [2]Corresponding authors

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

26 **Abstract**

27 Acute Encephalitis Syndrome (AES) causes significant morbidity and mortality
28 worldwide. In Nepal, Japanese encephalitis virus (JEV) accounts for ~ 5-20% of AES
29 cases, but ~75% of AES cases are of unknown etiology. We identified a gemykibivirus in
30 CSF collected in 2020 from a male child with AES using metagenomic next-generation
31 sequencing. Gemykibiviruses are single stranded, circular DNA viruses in the family
32 *Genomoviridae*. The complete genome of 2211 nucleotides was sequenced which shared
33 98.69% nucleotide identity to its closest relative, Human associated gemykibivirus 2
34 isolate SAfia-449D. Two real-time PCR assays were designed, and screening of 337 CSF
35 and 164 serum samples from AES patients in Nepal collected in 2020 and 2022 yielded
36 11 CSF and 1 serum sample that were positive in both PCR assays. Complete genomes
37 of 7 of the positives were sequenced. These results identify a candidate etiologic agent of
38 encephalitis in Nepal.

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

39 **Introduction**

40 Encephalitis is a neurological disorder associated with a high mortality rate on a
41 global scale (1). It is the inflammation of the brain parenchyma with clinical features
42 of fever, altered mental state, and/or new onset of seizures. At present, low
43 sociodemographic index regions in Asia and Africa carry the highest burden of
44 encephalitis (2). In the year 2021, a total of 512 cases of AES were reported in Nepal
45 (3) . More than 100 different infectious agents that cause encephalitis are known
46 including bacteria, viruses, fungi, and parasites (4, 5). The major known etiologic
47 agents of encephalitis as reported in Nepal and internationally are Japanese
48 encephalitis virus (JEV), enteroviruses, herpes simplex, and varicella zoster viruses
49 (6, 7). In Nepal, there has been a national surveillance program for JE since 2004
50 wherein CSF and serum samples of suspected viral cases of encephalitis are collected
51 from sentinel sites throughout Nepal with technical support from WHO and analyzed
52 at the National Public Health Laboratory (NPHL) by serology for anti-JEV IgM. In
53 numerous studies in Nepal assessing cases since 2000, ~70-95% of the AES cases per
54 year have no diagnosis (3, 8-11). A significant fraction of encephalitis in other
55 countries similarly lack diagnosis, despite extensive testing (5, 12, 13). In recent
56 years, the application of metagenomic analysis to patients with encephalitis has begun
57 to identify a range of emerging viruses linked to encephalitis (14-19)
58 The *Genomoviridae* family of viruses has single stranded DNA genomes of ~2.1-2.2
59 kb (20) that encode a capsid protein (CP) and a replication associated protein (REP).
60 They have been identified from a wide range of hosts including plants, insects,
61 animals, and humans (20-22). There are ten genera in the *Genomiviridae* family (20).
62 Viruses in the genus *Gemykibivirus* have been identified in multiple human cases and
63 in multiple specimen types including: blood of febrile Tanzanian children (23); the

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

64 respiratory tract of an elderly woman with respiratory distress in China (24); feces
65 from diarrhea patients in Brazil (25); blood of healthy blood donors of Brazil (21);
66 blood of HIV positive Cameroonian males (26); and in cervical swab of HIV/HPV
67 infected pregnant females (27). Specific to encephalitis, there are reports of
68 gemykibiviruses in CSF from an encephalitic child from China (15) and CSF from
69 three patients with encephalitis from Sri Lanka (25). Furthermore, analysis of
70 Nepalese sewage yielded the complete genome of a gemykibivirus (25). Here, we
71 used metagenomic next generation sequencing (NGS) to identify the presence of a
72 gemykibivirus in CSF from a patient with encephalitis in Nepal. Further PCR
73 screening identified 12 additional positive cases.
74

75 **Materials and Methods**

76 **Ethical clearance, study population, and collection of biospecimens:**

77 This study was approved by the Nepal Health Research Council of Nepal [approval#
78 274-2020] and the Human Research Protection Office of Washington University in
79 Saint Louis [202004087]. The study population focused on AES patients who were
80 negative for JE IgM. Residual samples from national JE surveillance sentinel sites in
81 Nepal were utilized. The index case CSF sample in the NPHL repository was
82 collected in 2020 from Rupandehi district. For prevalence studies, 122 repository
83 specimens (82 CSF, 40 sera) collected in 2020 and 379 repository specimens (255
84 CSF, 124 sera) from 2022 were tested by PCR for gemykibivirus.

85 **Total nucleic acid extraction**

86 Total nucleic acid extraction was performed using the Invitrogen Pure Link ™ Viral
87 RNA/DNA mini kit [Thermo Fisher Scientific] and eluted in 50 µL volume following
88 the instruction manual of the kit. Samples were stored at -80°C.

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

89 **Metagenomic NGS analysis**

90 Extracted total nucleic acid were randomly amplified as described previously (28)
91 and used for library construction with NEBNext Ultra DNA Library Prep Kit for
92 Illumina (New England Biolabs). The sample library was sequenced on the Illumina
93 MiSeq instrument using the 2 × 250 bp paired-end protocol. NGS data was analyzed
94 for presence of viruses using CZID (29). NGS data is available at ENA:
95 PRJEB72279.

96 **Genome sequencing of index case and additional cases**

97 The NGS contigs were confirmed using PCR, cloning, and sanger sequencing with
98 primers (Appendix Table 1). Using a pair of primers that amplified the entire circular
99 genome, Gemy1xgenomeF
100 (5'TTAATCGATCTAGAGGATCCTTGTTAGATATCCATATGGCGG-3') and
101 Gemy1xgenomeR (5'-TTAGTAATGGGCCCGGATCCACGAGAGGAACACG-3'),
102 three independent PCR reactions were performed, and the resulting fragments were
103 cloned into pCR4.0 and sequenced using the Oxford Nanopore technologies
104 (Plasmidsauraus). Additional positive cases were similarly amplified and whole
105 genomes sequenced to 3X coverage. Complete genomes sequences are available at
106 Genbank (Accession# PP270194-PP270201).

107 **Gemykibivirus qPCR development**

108 Two sets of Taqman real time PCR primers and probes were designed using express
109 software (Applied Biosystems), one targeting the CP gene and the other targeting the
110 REP gene. The primers and the probes were supplied by IDT (Integrated DNA
111 Technologies, USA). The first assay, Gemy_1, targets the CP gene using primers
112 GemykibiTM_8917F (5'-ACCTCTTATCCGGTTTGGCA-3') and
113 GemykibiTM_8917R (5'-AGCGCGAAATTCCTCTTGAC-3') and the probe

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

114 GemykibiTM_8917Probe(5'- [6-
115 FAM]CGGACCTGA[ZEN]CCGGATGCCCGG[3IABkFQ]-3') that uses FAM and
116 the dual quencher Zen and Iowa Black. The second assay, Gemy_2, targets the REP
117 gene with GemykibiTM_9967F (5'-GGTCAGAGCCTAGTGTTGTATG-3')
118 GemykibiTM_9967R (5'-CGACGTTGTCTGTGTCTTCT-3')
119 GemykibiTM_9967Probe (5'-[6-FAM]AAGACACTC[ZEN]TGGGCAAGAAGCC
120 TT[IABkFQ]-3') using the same fluor and quencher.
121 For both assays, standard curves were generated using serial 10-fold dilution ranging
122 from $2x10^8$ to $2x10^1$ copies of positive control plasmid [plasmid PCR4 containing the
123 respective target sequence]. A 20 µL PCR mixture was made comprising 2 µL of
124 extracted nucleic acid sample, 10 µL of 2x TaqMan Fast Advanced Master Mix
125 [Thermo Fisher Scientific], and 5 pmol of each primer and probe. The PCR reactions
126 were performed in 96-well plates on a CFX Opus 96 thermocycler [Bio-Rad] with one
127 negative control nuclease free water in each row and one positive control of $2\ x10^3$
128 copies per plate. The cycling conditions were 50°C for 2 mins, 95°C for 30 secs and
129 40 cycles of 95°C for 5 secs followed by 60°C for 30 secs. The threshold of all plates
130 was set at standard value and data was analyzed using Bio-Rad CFX Maestro 2.3
131 software. Samples were counted as positive if their threshold cycle (Ct) value was less
132 than 33.

133 **Phylogenetic analysis**

134 Representative protein sequences of the REP gene of prototypes of each genus in the
135 *Genomoviridae* were downloaded from GenBank. Alignments were generated using
136 Clustal Omega (*30*). The alignment converted to fasta via
137 http://sequenceconversion.bugaco.com/converter/biology/sequences/clustal_to_fasta.p
138 hp. Maximum likelihood trees were generated with bootstrapping, using W-IQ-TREE

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

139 (31). Trees were visualized using iTOL (32). All available complete genomes in the
140 species *Gemykibivirus humas2* were downloaded from Genbank along with
141 representative genomes from the species *Gemykibivirus humas 1, 3, 4*, and *5*, and the
142 top 10 additional complete genomes with highest BLASTn scores. Alignments were
143 generated using Clustal Omega (30) Maximum likelihood trees were generated with
144 bootstrapping, using W-IQ-TREE (31).Trees were visualized using iTOL (32) .
145
146 **Results**
147 **Detection of a gemykibivirus by metagenomic NGS**
148 NGS of nucleic acids extracted from the CSF of a male child yielded reads that could
149 be assembled into two contigs that shared 97 and 99% nucleotide similarity with
150 human associated Gemykibivirus2 SAfia-449D (accession# MN765187.1). Using
151 PCR, gaps between the two contigs were spanned to generate a complete circular
152 genome of 2211 nt. To formally assess the taxonomic relationship of this virus to
153 viruses in the family *Genomoviridae*, we generated a maximum likelihood
154 phylogenetic tree of the REP protein with the type species of each genus, in
155 accordance with the ICTV guidelines (20, 22) (Figure 1A). The virus was most
156 similar to the prototype virus from the genus *Gemykibivirus*. To further assess its
157 relationship within the genus *Gemykibivirus*, we generated a maximum likelihood tree
158 using the whole genome sequence (Figure 1B), which demonstrated it is most closely
159 related to Human gemykibivirus 2 SAfia-449D, a virus detected in blood of
160 Tanzanian children (23), with 98.69% nucleotide identity. Based on these criteria, the
161 virus genome from the index case was designated Human gemykibivirus 2
162 Nepal/N0000051/2020. Human gemykibivirus 2 Nepal/N0000051/2020 was also
163 closely related to Gemycircularvirus-SL1 (accession# KP133075), a previously

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

164 reported gemykibivirus detected in CSF from an encephalitis patient in Sri Lanka (25)
165 sharing 97.96% nucleotide identity (33 SNPs across the genome and a 12 bp insertion
166 located in a region of tandem repeat hexamers) and it shared 98.64% identity to
167 another gemykibivirus, Gemycircularvirus NP (accession# KP133080), detected in
168 sewage from Nepal (25).
169 Gene predictions identified three ORFs, characteristic of Gemykibiviruses (Figure 2).
170 The CP ORF is 969 bp, the REP ORF, generated by splicing is 1114 bp, and the
171 unknown ORF3, which overlaps with the REP ORF, is 702 bp. A large intergenic
172 region (LIR) of 127bp is present, and the putative viral origin of replication nona-
173 nucleotide motif 5′-TAAAATTTA-3′ described in Gemycircularvirus NP (accession#
174 KP133080) is conserved. A predicted stem loop in the LIR is observed from
175 nucleotides 23 to 57. The stem loop structure is present in genomoviruses and
176 geminiviruses where it is necessary for rolling circle replication (33, 34).

177 **Identification of additional positive specimens from encephalitis patients**

178 To define the prevalence of this virus, two Taqman real-time PCR assays were
179 designed and validated, one targeting the REP gene and one targeting the CP gene
180 (Appendix Figure 1). A total of 337 CSF samples and 164 serum samples were tested
181 using both assays. Sample with Ct. values <33 for both assays were considered
182 positive. There were three gemykibivirus positive CSF specimens from 2020 and 9
183 positive specimens (8 CSF and 1 serum) from 2022 (Table). The prevalence rate in
184 CSF was 3.3% (11/337) and in serum was 0.6% (1/164). Positive patients ranged
185 from 4 months to 72 years of age. Including the index case, there were 7 male and 6
186 female patients. Geographically, most positive patients were from districts in south-
187 central Nepal (Figure 3). Unfortunately, no additional clinical details are available for
188 the patients in this study besides meeting the acute case definition of encephalitis.

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

189 **Whole genome sequencing and phylogenetic analysis of additional positive cases**

190 From the 10 positive specimens with highest Gemykibivirus copy number, we tried to
191 amplify the whole genome using PCR. For 7 of the samples, we obtained amplicons
192 that corresponded to the whole genome. These were cloned and sequenced to 3X
193 coverage to determine their consensus sequences. These 7 genomes varied from the
194 index genome by 4 to 21 SNPs. The intron of the Rep gene contains a region with
195 seven tandemly repeated hexamers, and deletions of one or more of the hexamers are
196 observed in some of the genomes. Phylogenetic analysis of the complete genomes
197 demonstrated that they formed a clade that included known Gemykibiviruses
198 previously detected in human CSF, stool, and blood.

199 **Discussion**

200 We used metagenomic NGS to detect Human gemykibivirus2 Nepal/N0000051/2020
201 in CSF of a male encephalitic child from Rupandehi district (Figure 3) of Nepal which
202 is alongside the border with India. Further screening through qPCR identified an
203 additional 12 positive specimens from patient samples collected in 2020 and 2022.
204 We found that these cases were mostly concentrated in and around the south-central
205 region of Nepal (Figure 3).

206 The identified virus genomes were very closely related (97.96% nt. identity) to a
207 gemykibivirus previously detected in CSF from three encephalitis patients from Sri
208 Lanka (25). In addition, a distinct gemykibivirus (Human gemykibivirus 4) has also
209 been reported in CSF of an encephalitis patient from China (15). Together with our
210 study, these data implicate viruses in the genus *Gemykibivirus* as potential causal
211 agents of encephalitis. There is one report of *Gemykibivirus* in sewage from Nepal
212 from 2012, which is also highly similar to the sequences we detected in Nepalese
213 patients from 2020-2022. This suggests that gemykibivirus has been circulating in

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

214 Nepal for at least the past decade. Furthermore, detection in sewage, raises the
215 possibility that gemykibivirus may be transmitted fecal-orally, similarly to some
216 neurotropic viruses such as polio and enteroviruses. Detection of highly similar
217 viruses in patients in Tanzania (*23*) and Brazil (*21*) suggests that gemykibiviruses are
218 globally widespread.
219 One limitation of this study is that the samples analyzed were residual specimens
220 from a surveillance repository without additional available clinical metadata, thus
221 limiting our knowledge of the precise symptoms, disease severity, and outcomes of
222 these patients. While detection of gemykibiviruses in presumptively sterile CSF
223 supports the hypothesis that they could be causal agents of encephalitis, additional
224 research to culture the virus and establish animal models to fulfill Koch's postulates
225 are needed to definitively establish causality. In addition, more prevalence studies in
226 encephalitis and other diseases are also needed as are serological studies to define the
227 extent of human infection by gemykibiviruses. Finally, additional, unbiased
228 approaches are needed to define the etiologies of encephalitis in Nepal, and
229 worldwide.
230

231 **Acknowledgment.**

232 This project was supported by National Institutes of Health grant U01AI151810.
233

234 **Biographical sketch of first author:** Dr. Tuladhar, MD is currently pursuing a PhD at
235 Tribhuvan University Central Department of Biotechnology, Nepal studying emerging viral
236 infections. [ORCiD:0000-0001-5705-3953]
237
238

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

239 **Corresponding author's mailing address**

240 David Wang

241 ORCiD:0000-0002-0827-196X

242 314-286-1123 (phone)

243 314-286-1232 (FAX)

244 davewang@wustl.edu

245 Washington University in St. Louis School of Medicine

246 660 S. Euclid Ave

247 St. Louis, MO 63110

248 USA

249 Krishna Das Manandhar

250 ORCiD :0000-0002-6798-4935

251 +977 9860 960 577 (Phone)

252 krishna.manandhar@gmail.com

253 Central Department of Biotechnology, Tribhuvan University

254 TU Road, Kirtipur, Nepal

255

256

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .
257 **References**

258 1. Why encephalitis matters? Report of the virtual meeting, 28-29 June 2022. Geneva:
259 World Health Organization; 2023.
260 2. Wang H, Zhao S, Wang S, Zheng Y, Wang S, Chen H, et al. Global magnitude of
261 encephalitis burden and its evolving pattern over the past 30 years. J Infect. 2022
262 Jun;84(6):777-87.
263 3. Department of Health Services Annual Report 2078/79. Kathmandu: Government of
264 Nepal Ministry of Health and Population; 2021/2022.
265 4. Granerod J, Cunningham R, Zuckerman M, Mutton K, Davies NW, Walsh AL, et al.
266 Causality in acute encephalitis: defining aetiologies. Epidemiol Infect. 2010 Jun;138(6):783-
267 800.
268 5. Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, et al.
269 Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis.
270 2006 Dec 15;43(12):1565-77.
271 6. Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, et al.
272 Causes of encephalitis and differences in their clinical presentations in England: a
273 multicentre, population-based prospective study. Lancet Infect Dis. 2010 Dec;10(12):835-44.
274 7. Rayamajhi P, Nepal G, Ojha R, Rajbhandari R, Gajurel BP, Karn R. Evaluating
275 cognitive outcomes in adult patients with acute encephalitis syndrome: a prospective study
276 from a tertiary care center in Nepal. Encephalitis. 2022 Apr;2(2):36-44.
277 8. Khanal B, Singh RR, Bhattacharya SK. Japanese encephalitis in eastern Nepal: access
278 to the laboratory diagnosis. 15th ICID Abstracts / International Journal of Infectious
279 Diseases. 2012:e88.
280 9. Dhital B, Bhat N. Clinico-epidemiological presentation of acute encephalitis
281 syndrome in patients visiting a tertiary hospital in Kathmandu, Nepal: A descriptive cross-
282 sectional study. Journal of Gandaki Medical College-Nepal. 2022;15(2):168-72.
283 10. Rayamajhi A, Ansari I, Ledger E, Bista KP, Impoinvil DE, Nightingale S, et al.
284 Clinical and prognostic features among children with acute encephalitis syndrome in Nepal; a
285 retrospective study. BMC Infect Dis. 2011 Oct 28;11:294.
286 11. Kumar Pant D, Tenzin T, Chand R, Kumar Sharma B, Raj Bist P. Spatio-temporal
287 epidemiology of Japanese encephalitis in Nepal, 2007-2015. PLoS One.
288 2017;12(7):e0180591.
289 12. Huppatz C, Durrheim DN, Levi C, Dalton C, Williams D, Clements MS, et al.
290 Etiology of encephalitis in Australia, 1990-2007. Emerg Infect Dis. 2009 Sep;15(9):1359-65.
291 13. Mailles A, Stahl JP, Steering C, Investigators G. Infectious encephalitis in france in
292 2007: a national prospective study. Clin Infect Dis. 2009 Dec 15;49(12):1838-47.
293 14. Edridge AWD, Deijs M, Namazzi R, Cristella C, Jebbink MF, Maurer I, et al. Novel
294 Orthobunyavirus Identified in the Cerebrospinal Fluid of a Ugandan Child With Severe
295 Encephalopathy. Clin Infect Dis. 2019 Jan 1;68(1):139-42.
296 15. Zhou C, Zhang S, Gong Q, Hao A. A novel gemycircularvirus in an unexplained case
297 of child encephalitis. Virol J. 2015 Nov 24;12:197.
298 16. Smits SL, Zijlstra EE, van Hellemond JJ, Schapendonk CM, Bodewes R, Schurch
299 AC, et al. Novel cyclovirus in human cerebrospinal fluid, Malawi, 2010-2011. Emerg Infect
300 Dis. 2013;19(9):1511-3.
301 17. Liu Q, Wang X, Xie C, Ding S, Yang H, Guo S, et al. A Novel Human Acute
302 Encephalitis Caused by Pseudorabies Virus Variant Strain. Clin Infect Dis. 2021 Dec
303 6;73(11):e3690-e700.

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .
304 18. Manso CF, Bibby DF, Mohamed H, Brown DWG, Zuckerman M, Mbisa JL.
305 Enhanced Detection of DNA Viruses in the Cerebrospinal Fluid of Encephalitis Patients
306 Using Metagenomic Next-Generation Sequencing. Front Microbiol. 2020;11:1879.
307 19. Quan PL, Wagner TA, Briese T, Torgerson TR, Hornig M, Tashmukhamedova A, et
308 al. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerg Infect Dis.
309 2010 Jun;16(6):918-25.
310 20. Varsani A, Krupovic M. Family Genomoviridae: 2021 taxonomy update. Arch Virol.
311 2021 Oct;166(10):2911-26.
312 21. Zucherato VS, Giovanetti M, Costa LOA, Krause LMF, Alves DCC, Moreira RMA,
313 et al. Molecular identification of the emerging Human Gemykibivirus-2 (HuGkV-2) among
314 Brazilian blood donors. Transfus Apher Sci. 2023 Feb;62(1):103516.
315 22. International Committee on Taxonomy of Viruses (ICTV):
316 https://ictv.global/taxonomy/. [cited 2023; Available from: https://ictv.global/taxonomy/
317 23. Cordey S, Laubscher F, Hartley MA, Junier T, Keitel K, Docquier M, et al. Blood
318 virosphere in febrile Tanzanian children. Emerg Microbes Infect. 2021 Dec;10(1):982-93.
319 24. Wang J, Li Y, He X, Ma J, Hong W, Hu F, et al. Gemykibivirus Genome in Lower
320 Respiratory Tract of Elderly Woman With Unexplained Acute Respiratory Distress
321 Syndrome. Clin Infect Dis. 2019 Aug 16;69(5):861-4.
322 25. Phan TG, Mori D, Deng X, Rajindrajith S, Ranawaka U, Fan Ng TF, et al. Small
323 circular single stranded DNA viral genomes in unexplained cases of human encephalitis,
324 diarrhea, and in untreated sewage. Virology. 2015 Aug;482:98-104.
325 26. Orf GS, Olivo A, Harris B, Weiss SL, Achari A, Yu G, et al. Metagenomic Detection
326 of Divergent Insect- and Bat-Associated Viruses in Plasma from Two African Individuals
327 Enrolled in Blood-Borne Surveillance. Viruses. 2023 Apr 21;15(4).
328 27. Siqueira JD, Curty G, Xutao D, Hofer CB, Machado ES, Seuanez HN, et al.
329 Composite Analysis of the Virome and Bacteriome of HIV/HPV Co-Infected Women
330 Reveals Proxies for Immunodeficiency. Viruses. 2019 May 7;11(5).
331 28. Lim ES, Zhou Y, Zhao G, Bauer IK, Droit L, Ndao IM, et al. Early life dynamics of
332 the human gut virome and bacterial microbiome in infants. Nat Med. 2015 Oct;21(10):1228-
333 34.
334 29. Kalantar KL, Carvalho T, de Bourcy CFA, Dimitrov B, Dingle G, Egger R, et al.
335 IDseq-An open source cloud-based pipeline and analysis service for metagenomic pathogen
336 detection and monitoring. Gigascience. 2020 Oct 15;9(10).
337 30. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable
338 generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol
339 Syst Biol. 2011 Oct 11;7:539.
340 31. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. W-IQ-TREE: a fast online
341 phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 2016 Jul
342 8;44(W1):W232-5.
343 32. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic
344 tree display and annotation. Nucleic Acids Res. 2021 Jul 2;49(W1):W293-W6.
345 33. Yu X, Li B, Fu Y, Jiang D, Ghabrial SA, Li G, et al. A geminivirus-related DNA
346 mycovirus that confers hypovirulence to a plant pathogenic fungus. Proc Natl Acad Sci U S
347 A. 2010 May 4;107(18):8387-92.
348 34. Heyraud F, Matzeit V, Schaefer S, Schell J, Gronenborn B. The conserved
349 nonanucleotide motif of the geminivirus stem-loop sequence promotes replicational release of
350 virus molecules from redundant copies. Biochimie. 1993;75(7):605-15.
351

352

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

353 Table: Patient demographics and qPCR Ct. values of gemykibivirus positive cases

Sample ID[†]	Specimen	Age group [yrs]	Gender	Ct. Gemy_1[‡]	Ct. Gemy_2[§]	Specimen collection date	Districts[¶]	Province
N0000218	CSF	11-15	M	32.42	30.68	2020 Feb	Kaski	Gandaki
N0000256	CSF	0-5	F	29.41	29.34	2020 July	Myagdi	Gandaki
N0000300	CSF	6-10	F	32.95	31.46	2020 Sep	Palpa	Lumbini
N0000358	Serum	0-5	M	29.27	29.94	2022 Feb	Sarlahi	Madhesh
N0000359	CSF	0-5	F	27.4	27.08	2022 Feb	Kapilvastu	Lumbini
N0000395	CSF	0-5	M	30.74	30.73	2022 April	Kaski	Gandaki
N0000434	CSF	56-60	M	29.02	30.06	2022 June	Lalitpur	Bagmati
N0000468	CSF	30-35	F	30.84	31.47	2022 July	Chitwan	Bagmati
N0000490	CSF	unknown	M	29.86	32.08	2022 July	Syangja	Gandaki
N0000545	CSF	70-75	F	26.84	25.35	2022 Aug	Kathmandu	Bagmati
N0000546	CSF	20-25	M	24.65	23.53	2022 Aug	Kathmandu	Bagmati
N0000722	CSF	20-25	F	28.74	29.72	2022 Nov	Chitwan	Bagmati

354 [*]Ct., cycle threshold; yrs, years.

355 [†]The sample IDs are coded and are not known outside the research group.

356 [‡]Gemy_1: qPCR targeting capsid protein gene.

357 [§]Gemy_2: qPCR targeting replication associated protein (REP) gene.

358 [¶]Districts involved are highlighted in Figure 3 as shade.

359

360

361

362

363

364

365

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

366 Appendices

367 Appendix Table: Primers used for PCR

Primer name	Sequence 5' to 3'	Binding Sites
Gemy8917-9967F	TCAACGACCTCTGATACATACC	429 <- 451
Gemy8917-9967R	TAGATGAGTTCCACCATCAGC	2034 -> 2055
Gemi1625confirmR	GGCATTGCAATTATGGCTTATGGT	88 -> 113
Gemi1625confirmF	GGCAAGAAACTCGTCCACTGGG	1194 <- 1215
Gemy9967-8917R	TCATAATCTGCTCCGTGTTCCT	1133 -> 1154
GemykibiTM_9967F	GGTCAGAGCCTAGTGTTGTATG	1419 <- 1440
Gemy1xgenomeF	TTAATCGATCTAGAGGATCCTTGTTAGATATCCATATGGCGG	1160 -> 1187
Gemy1xgenomeR	TTAGTAATGGGCCCGGATCCACGAGAGGAACACG	1146 <- 1165

368

369 **Figure Legends.**

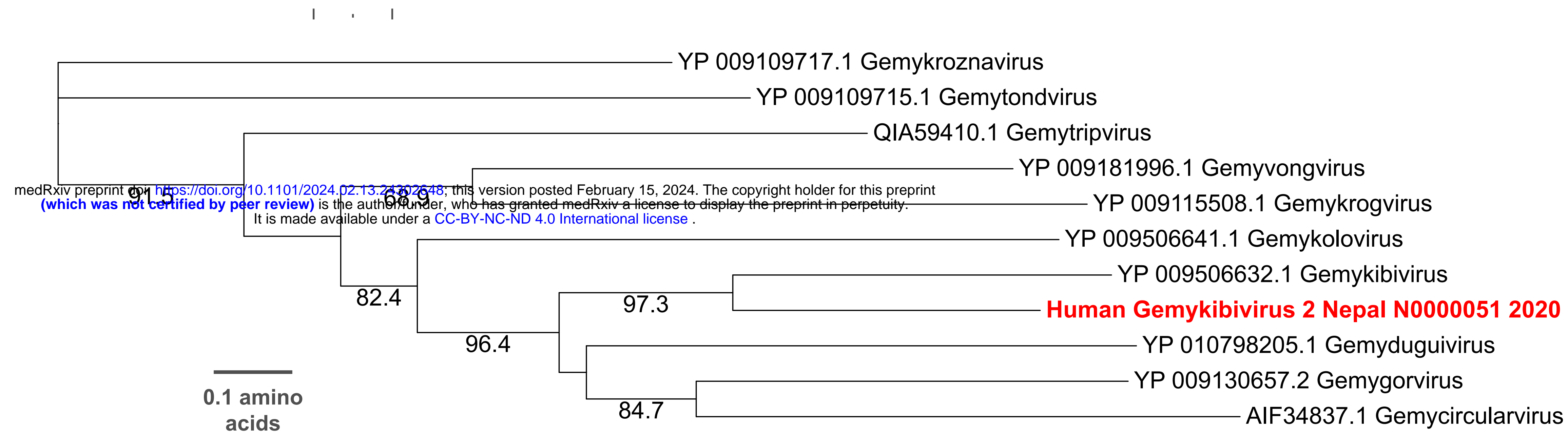
370

371 **Figure 1.** Phylogenetic trees of (A) amino acid sequences of the index case REP protein and
372 representatives of each genus in the family *Genomoviridae;* (B) whole genome nucleotide
373 sequences of the positive samples from Nepal compared to other genomes within the genus
374 *Gemykibivirus.*

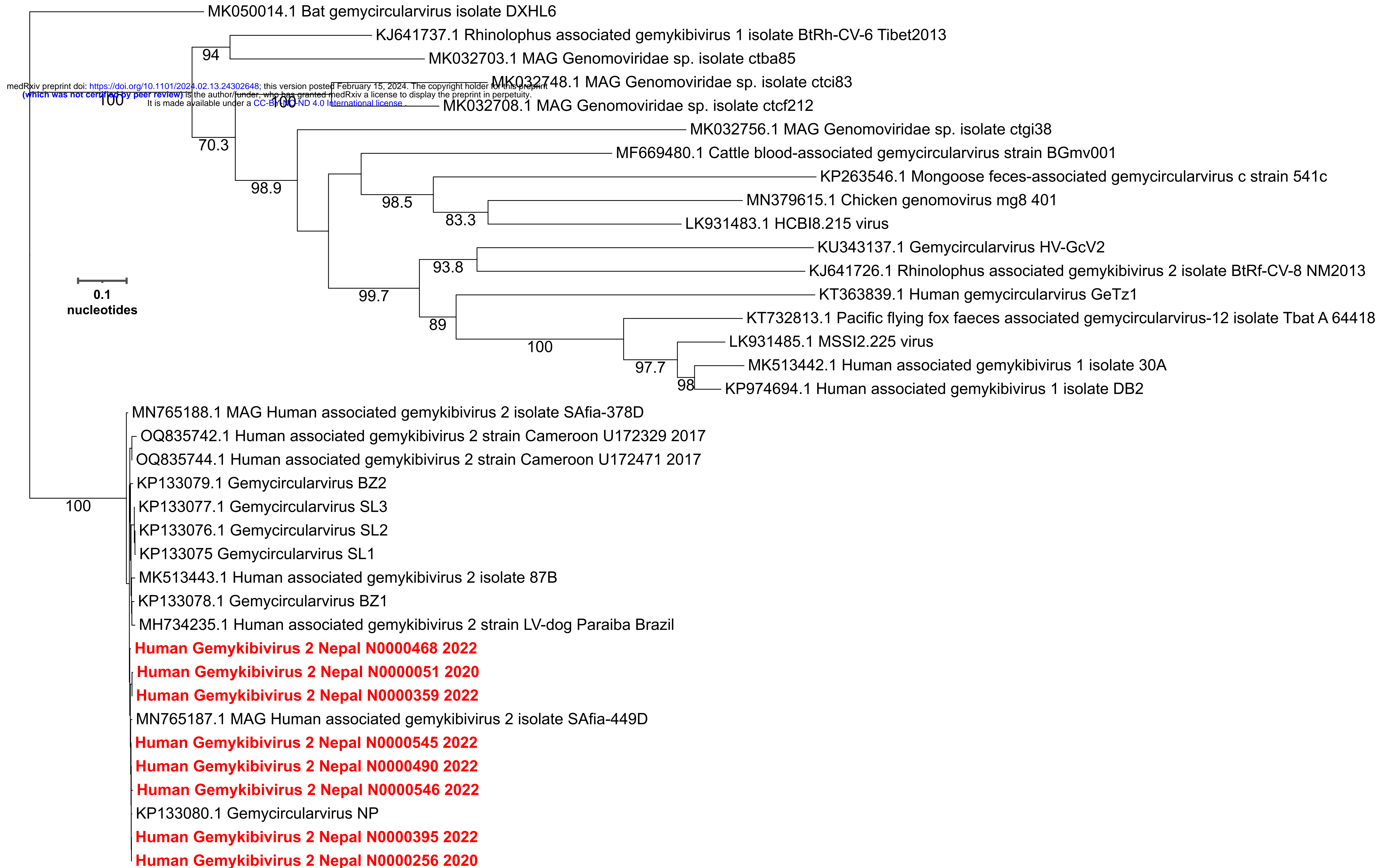
375 **Figure 2.** Schematic of the genome of Human gemykibivirus 2 Nepal/N0000051/2020.

376 **Figure 3.** Map of Nepal and locations of the gemykibivirus positive cases [shaded].

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint
(which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .



medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .



medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .

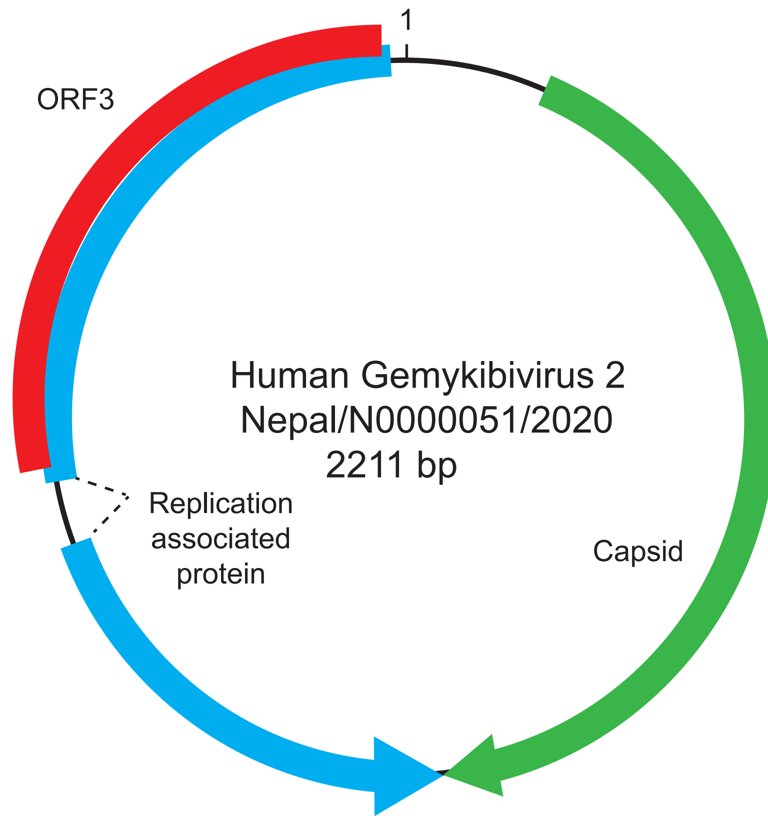


Figure3. Map of Nepal and locations of the gemykibivirus positive cases [shaded].

medRxiv preprint doi: https://doi.org/10.1101/2024.02.13.24302648; this version posted February 15, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted medRxiv a license to display the preprint in perpetuity.
It is made available under a CC-BY-NC-ND 4.0 International license .