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1 ***Molecular epidemiology of Japanese Encephalitis Virus in pig population of Odisha,***
2 ***Assam and Manipur states of India.***

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21 ## Abstract

22 Japanese encephalitis virus (JEV) comes under the family *Flaviviridae* and genus flavivirus.
23 It predominantly infects the children under the age of 10 years and the case fatality rate can
24 stretch out as high as 30%. Pigs act as reservoir and amplifying intermediate host for JEV.
25 Recent report suggested longer persistence of JEV in tonsil than in circulation of
26 experimentally infected pigs. The current investigation was conducted to understand the
27 prevalence and molecular epidemiology of JEV infection in pigs in three different
28 geographical sites in India (Odisha, Assam and Manipur). Serum samples were tested by
29 ELISA and RT-PCR for detection of JEV, while only RT-PCR was done in case of tonsils
30 tissues collected from pigs slaughtered in abattoir. Prevalence of JEV was highest in Manipur
31 (25.45% in serum and 10.08% in tonsil) but lower in Assam (3.75% in serum and 0% in
32 tonsils) and Odisha (1.49% in serum and 3.7% in tonsils). The percentage of sero-positivity
33 was found to be 3.75% of IgM and 9.9% of IgG in Assam and Odisha respectively. Genotype
34 III (GIII) of JEV was the dominant genotype and sporadic mutations of S83G, H76P, E78Q,
35 C55S, and S64W along with two consistent mutations V46S and V51I were observed in all
36 the GIII strains. Analysis of the E gene sequence revealed a single mutation, S118N in the GI
37 strain. Older pigs (above 7 months) were found to be infected relatively more (8.6%) than
38 younger pigs (age group 3-7 months). In conclusion, the high JE virus infection rate of pig in
39 the current locations suggests the need for continuous surveillance of this virus in pigs which
40 will ultimately help to adopt an effective control strategy to prevent the spread of JE infection
41 to human.

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45 ## Author summary

46 Japanese encephalitis is one of the contributing factors in acute encephalitis syndrome cases
47 reported across India as well as Asia. Primarily young naive human population are affected
48 with JEV. The death rate can be as high as 30% and in about 30%-50% surviving population
49 paralysis, brain damage or other serious permanent sequelae may be observed. The viral load
50 gets amplified in pigs and thus plays a crucial role in transmitting the infection in human
51 communities living in close proximity to pig dwelling. The current study was conducted to
52 demonstrate prevalence of JEV in pig population of three geographical regions of India *viz.*
53 the States of Odisha, Assam and Manipur that have reported JE outbreaks in human
54 population. The current study demonstrates that the rate of infection is 3.28% among pigs in
55 Manipur followed by Assam and Odisha. GIII was found to be the most predominant JEV
56 genotype, while only one GI genotype strain was detected from Odisha region. These
57 findings suggested the need of continuous surveillance of this virus in pigs and proper
58 implementation of human and animal vaccination programme to control the infection.

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Introduction

Flaviviruses are important human and animal pathogens with worldwide distribution. Japanese encephalitis virus (JEV), belonging to this family particularly affects the children below 10 years and the mortality rate can be upto 30% [1] and about 30%-50% of survivors develop permanent neurologic disorder or psychiatric sequelae [2]. JEV is a single stranded RNA virus with a genome length of approximately 11kb. The genome is divided into a structural region containing capsid (C), pre-membrane (prM) and envelope (E) genes and a non-structural region consisting of NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 genes [3]. The NS3 and NS5 genes encode for viral helicase and polymerase enzymes respectively whereas the functions of NS4a and NS4b are not clearly understood [4]. *Culex tritaeniorhyncus* is the dominant transmitting vector [5] whereas other species of mosquitoes namely *Culex modestus* [6], *Culex pipiens* [7], *Culex bitaeniorhyncus* [7] and *Anopheles sinensis* [8] have also been reported. In nature, JEV maintains its life cycle between vectors and reservoir hosts such as pigs, wading birds and bats [2] from where the virus is transmitted to the dead end hosts such as humans and horses which do not develop high viremia to infect mosquito hosts [9, 10]. Similarly, JEV infection to pregnant sows leads to reproductive failures such as abortions and stillbirths [11]. In South East Asia, JEV transmission is mainly associated with the onset of rainy season but it can occur throughout the year in tropical regions [4].

The JEV strains have been characterized genetically depending upon the limited sequencing of C/prM, E and NS5/3′ UTR regions, while based on their evolutionary divergence JEV genotypes are further categorized into GI (GI-a and GI-b), GII, GIII, GIV and GV [12, 13]. A single serotype exists for all the JEV genotypes [14]. The Nakayama and Beijing-1 prototype strains and all other strains isolated from pigs and mosquitoes before 1994 belong to GIII

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91 [15]. However, there are reports indicating recent introduction of GI strain in pigs and
92 mosquitoes [16, 17]. It has also been reported that GI has slowly replaced GIII strain and can
93 become a dominant genotype because of contribution of several viral, environmental and host
94 factors [18]. Moreover, the GI genotype has equal virulence as that of GIII and is responsible
95 for encephalitis in humans in most Asian countries including India [12]. Earlier reports
96 suggested that GIII is the most prevalent genotype in India [19], though there are reports
97 suggesting the presence of GI in Uttar Pradesh [20] and West Bengal [21]. The main cause of
98 viral encephalitis in Asia is JEV. The transmission of JEV is endemic in around 24 countries
99 of South East Asia and Western Pacific regions [22]. More than 3 billion people are at risk of
100 JEV infection with approximately 68,000 clinical cases reported every year [23]. JEV
101 epidemics among human was first reported in Haryana, India in 1990 [24] and a major
102 outbreak was reported from Gorakhpur in Uttar Pradesh, India in 2005 [1]. Subsequent
103 outbreaks also occurred in Malkangiri in 2012 and Manipur in 2016 [4]. Most of the
104 epidemics occurred in the first to third weeks of October in a year [1]. The common clinical
105 manifestations include fever (temperature 38.5°C-40°C), severe headache, convulsions,
106 vomiting and in extreme cases the infections leads to paralysis, coma and death [1].
107 According to National Vector Borne Diseases Control Programme, 216 deaths were reported
108 out of approximately 1731 cases in 2015.

109 JE is mainly reported in the rural areas as most of the Indian population relies on agriculture
110 and the agricultural grounds serve as a potent place for mosquito breeding. As the vector
111 remains in close proximity to its reservoir hosts, this significantly increases the risk of JE
112 transmission to human as well as other hosts such as horses [1]. Pigs are the prime suspects as
113 reservoir for disease transmission [25] and the high viral titer in peripheral blood of pigs
114 ensures transmission of infection to susceptible naive young human population through
115 mosquito bite. Reports also suggest, the transmission of virus from infected to uninfected

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116 pigs occurring through mosquitoes [26]. Recent reports indicates the vector free transmission
117 of JEV through the oronasal secretion of infected pigs to naive pigs [25].

118 There are several reports regarding the mosquito studies in the JE endemic regions of India,
119 however studies on reservoir hosts is limited [19]. As investigating the JEV persistence in the
120 pig population is of utmost importance to take the necessary precautionary measures before
121 the onset of fatal situation, this investigation was conducted to study the molecular
122 epidemiology of JEV in the pig population of JE endemic regions of Odisha, Assam and
123 Manipur in India.

124 Materials and methods

125 Sample collection

126 Blood and tonsil tissue from slaughtered pigs were collected from the endemic regions of
127 Odisha, Assam and Manipur from August 2017 - March 2019 (Fig 1). The animals used in
128 the study were being processed as part of the normal work in the abattoir. The tissue and
129 blood samples were collected by qualified practicing veterinarians from animals slaughtered
130 in the abattoir as a part of the routine process after obtaining necessary consent for the same.

131 The serum was separated from the blood sample by centrifugation at 5000 rpm for 5 mins at
132 4°C and aliquoted in properly labeled vials. Tonsil sample of around 1gram was collected
133 from slaughtered pig and stored in RNA later (Ambion, Thermo Fischer Scientific, USA)
134 [16]. Both the serum and the tonsil tissues were stored at -80°C until further processing.

135 Fig 1. Outline Map of India showing three different States of Odisha, Assam and Manipur.
136 The colored areas represent the districts of different States to focus the exact location from
137 where samples were collected (https://commons.wikimedia.org/wiki/Atlas_of_the_world).

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139 **Viral RNA extraction from serum and RT-PCR**

140 The viral RNA was extracted from serum samples as referred by Desingu *et al*, 2016 [19].
141 For this, 140µl of swine serum was used and extraction was done, using viral RNA isolation
142 kit (QIAmp Viral RNA Mini Kit Qiagen, Germany) according to the manufacturer's protocol.
143 The viral RNA was quantified and cDNA was prepared with random hexamers using 1µg of
144 viral RNA by Superscript III first strand cDNA synthesis kit (Invitrogen, USA). This was
145 followed by a PCR reaction using Taq DNA polymerase (Thermoscientific, USA) with initial
146 denaturation at 95°C for 5 mins followed by 35 cycles of 95°C for 45 s, 45°C for 45 s and
147 72°C for 30 s with the final extension of 10 mins, amplifying 400bp of envelope (E) gene
148 using specific primers [27]. The amplified products were electrophoresed on 2% agarose gel
149 and viewed under UV transilluminator.

150 **Total RNA extraction from tonsil tissue and RT-PCR**

151 The total RNA was extracted from tonsil tissue as per the method described by Mendez *et al*,
152 2011 [28]. The tonsil tissue was homogenized in 500µl of TRIzol (Invitrogen, USA) by
153 sterile homogenizer and total RNA was extracted using manual trizol extraction method and
154 quantified. Approximately 1µg of total RNA was used to prepare cDNA with random
155 hexamers using Superscript III first strand cDNA synthesis kit (Invitrogen, USA). The 400bp
156 partial region of E gene was amplified as mentioned earlier.

157 **Serology**

158 The serum samples were also subjected to indirect ELISA to detect the presence of IgG
159 antibodies against JEV using Porcine JE IgG ELISA Kit (Glory Science Co., Ltd, USA) in
160 accordance with the manufacturer's instructions.

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161 The IgM antibody detection against JEV in pig serum was also carried out by using Porcine
162 Japanese Encephalitis IgM antibody (JE-IgM AB) ELISA kit (Genexbio Health Science Pvt.
163 Ltd.) as per manufacturer's protocol.

164 **Sequencing and phylogenetic analysis using E gene sequence**

165 The positive serum and tissue samples were subjected to bi-directional sequencing with
166 overlapping primers using an automated DNA sequencer (Applied biosystem®3500 series
167 genetic analyser), based on the Sanger's dye terminating method. The sequence obtained was
168 analyzed for its correct identity using the BLAST tool of NCBI and confirmed.

169 The obtained sequences were aligned by the ClustalW tool [29] and the phylogenetic analysis
170 was performed using the MEGA6.06 software [30]. The phylogenetic tree was constructed
171 using the Neighbor-Joining method [31] and the evolutionary distances were compared using
172 the maximum composite likelihood method [32]. The statistical significance of the
173 phylogenetic tree was tested with a bootstrap value of 1000 pseudo-replicate datasets [33].

174 **Mutational analysis**

175 The nucleotide sequences of the JEV E gene of the isolated strains were translated to protein
176 sequences using the ExPASy translate tool (https://web.expasy.org/translate/). The sequences
177 were then aligned with their respective prototype strain using the Clustal Omega tool
178 (https://www.ebi.ac.uk/Tools/msa/clustalo/). The variations in the protein sequences were
179 highlighted.

180 **Accession numbers**

181 The E gene sequences (n=36) were submitted to GenBank database with accession numbers
182 MK421340, MK940903, MK940904, MK940905, MN115386, MN115387, MK491507,
183 MH376692, MK682387, MK692888, MK692889, MK692890, MK692891, MK692892,

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184 MK692893, MK692894, MK518053, MK952776, MK952775, MK962309, MK962310,
185 MK975821, MK975822, MK975823, MK975824, MN029019, MN010526, MN010527,
186 MN010528, MN010529, MN029013, MN029014, MN029015, MN029016, MN029017,
187 MN029018.

188 Results

189 In the present study, the total number of serum samples screened from Odisha, Assam and
190 Manipur were 402, 400 and 55 respectively; whereas the corresponding numbers for tonsil
191 samples were 27, 55 and 248. The number of JEV positive serum samples detected by means
192 of RT-PCR was 6 out of 402 (1.49%), 15 out of 400 (3.75%) and 14 out of 55 (25.45%) and
193 for tissue samples 1 out of 27 (3.7%), 0 out of 55 (0%) and 25 out of 248 (10.08%) in the
194 respective States (Fig 2A and 2B). On the other hand, 15 out of 400 (3.75%) of IgM and 40
195 out of 402 (9.9%) of IgG positive cases were reported from Assam and Odisha respectively.

196 Fig 2 Detection of JEV in samples of pig collected between August 2017-March 2019 by RT-
197 PCR.

198 (A) Number of positive cases from the serum samples collected from pigs. (B) Positive
199 number of cases from the tonsils tissues collected from the slaughtered pigs.

200 District-wise distribution of JE positive cases

201 As per the district-wise distribution for all the three states, the overall prevalence of JEV for
202 serum samples was found to be 1.49% in Malkangiri, 25.45% in Imphal West, whereas
203 3.33% in Jorhat, 5.71% in Lakhimpur, 4.00% in Dhemaji and 1.67% in Kamrup (S1A Table).
204 Besides this, percentage of positivity for tonsils tissues by RT-PCR were found to be 3.7% in
205 Malkangiri, 8.23% in Imphal West, 35.89% in Imphal East, 3.4% in Kakching and 100% in
206 Bishnupur (S1B Table). However, district wise distribution was not available for Assam

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207 tonsil samples. Further, there was not a single positive case for tonsil sample by RT-PCR in
208 Assam (S1B Table).

209 **Age, month and sex-wise distribution of positive cases of JE in pigs detected by RT-PCR**

210 The number of JEV positive cases detected in pig serum in the above mentioned states was
211 found to be 5.21%, 1.67% and 77.7% respectively for the age group 3-7 months, whereas
212 above 7 months, it was found to be 0%, 6.88% and 15.2%, respectively in Odisha, Assam and
213 Manipur (Fig 3A and 3B). However, for tonsil samples from Manipur 11.12% (3 out of 27)
214 was found to be positive under age group 3-7 months, whereas 9.95% (22 out of 221) was
215 found positive in the age group above 7 months. Details regarding age-wise distribution of
216 positive cases in respect of tonsil samples were not available for Odisha and Assam. In the
217 current study, in Odisha more number of positive cases was found during the pre-monsoon
218 season whereas in Assam, number of positive cases was more during monsoon and post-
219 monsoon periods. The sex-wise distribution of JEV serum positive cases in the three states
220 were 1.85%, 2.56 % and 40.0% in males, and 7.93%, 4.88%, 8.0% in females (Fig 4A and
221 4B). Similar details in respect of tonsil samples were not known for all the three states.

222 Fig 3. Age-wise distribution of RT-PCR positive JEV cases detected from pig serum.

223 (A) Positive number of cases under age group 3-7 months. (B) Number of positive cases
224 above the age of 7 months

225 Fig 4 Sex-wise distribution of RT-PCR positive JEV in pig serum.

226 (A)Number of RT-PCR positive male cases. (B) Number of RT-PCR positive females cases.

227 **Sequencing and phylogenetic analysis**

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228 In the present study, a total of 61 samples including serum and tonsil were found to be
229 positive by RT-PCR. The PCR products which were found positive were confirmed by
230 sequencing. Out of 61 positive samples 36 sequences were submitted to the GenBank. All the
231 strains bearing GenBank accession numbers are listed in Table S2.

232 The genotype I and III strains were found to be circulating among the pigs of the three states
233 under study, of which, genotype III was found to be the predominant one. The genotype III
234 strains (n=60) were found in all the three States whereas a single case of genotype I (n=1)
235 was reported from Malkangiri district of Odisha.

236 The phylogenetic tree was constructed with reference JEV strains of genotype I (n=19),
237 genotype II (n=2), genotype III (n=6), genotype IV (n=2) and genotype V (n=2) including the
238 strains isolated in the present study. All of the isolated strains in the present study were
239 clustered into genotype I and genotype III. The isolated strain with accession number
240 MK421340 clustered with the other GI strains reported from Vietnam, India, Korea, China,
241 Taiwan and Japan (Fig 5). However, the rest of the strains clustered in genotype III including
242 the prototype Nakayama strain.

243 Fig 5. The phylogenetic analysis of the E gene of JEV strains from pigs. The phylogenetic
244 tree was constructed using neighbor-joining method with 1000 bootstrap value by the
245 MEGA6.06 software. The reference strains of GI, GII, GIII, GIV and GV were obtained from
246 the GenBank database. Viruses are depicted by accession number/country/year of isolation.
247 The close circle, close triangle and open square represents the Odisha, Assam and Manipur
248 isolates respectively. The bootstrap values are indicated at major branch points and scale bar
249 indicates nucleotide substitution per sites.

250 **Presence of mutations in the isolated strains**

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251 The amino acid sequence of the translated protein coded by the E gene of the detected GI
252 strain was aligned with that of the respective prototype strain as well as to the strain isolated
253 from human in 2005 from Gorakhpur, India using the Clustal Omega tool. It was noticed that
254 the JEV GI strain isolated from Odisha was approximately 99% identical with a single
255 mutation (S118N) observed within the amino acid region of 1-156 of the E gene (S3A Table
256 and S1A Fig). Similarly, after the alignment of GIII E amino acid sequences with the
257 prototype strain, it was observed that they were approximately 96-98% identical. The
258 sequence analysis showed the presence of two consistent mutations V46S and V51I in all the
259 GIII strains of the present study. Further, S83G mutation was observed in five Assam strains,
260 two mutations S64W and H76P were observed in strain with accession number MH376692,
261 whereas a single mutation E78Q was observed in Odisha strain (MK491507) and C55S
262 mutation was found in Manipur strain (MK518053) (S3B Table and S1B Fig).

263 **Discussion**

264 Pigs act as an amplifier host of JEV, thus can be a key suspect of spreading infection to
265 human living in close proximity [4]. The detection of JEV in pigs was reported earlier from
266 Cambodia, Singapore, Vietnam, Australia, Japan, China, Thailand, Laos, Indonesia, India etc
267 (Fig 6) [22, 34-42], however the studies on pigs for the presence of JE virus and its genotypes
268 in circulation among pigs in India are limited [19]. Hence, the current investigation was
269 mainly focused on the JE virus detection in pig serum and tonsil in three different JE endemic
270 states of India namely Odisha, Assam and Manipur.

271 Fig 6.The World map showing reported cases of JEV detection in pig population
272 (https://commons.wikimedia.org/wiki/Atlas_of_the_world).

273 In the current investigation, a large number of JE positive cases were detected among pigs in
274 Manipur State of India, as compared to the other two states. Moreover, it was noticed that the

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275 genotype III strain was predominant in all the three states with a single case of genotype I in
276 Odisha.

277 In the present study, high sero-positivity was observed in pigs from the districts of Odisha,
278 Assam and Manipur. There are reports that *Culex tritaeniorhynchus* acts as a transmitting
279 vector and majorly found around the pig farming areas throughout the year. There are reports
280 which suggest that the pigs are the potential feeding hosts for *Culex tritaeniorhynchus* [43,
281 44]. The presence of virus in tissue sample suggests that the virus can also invade tonsil
282 tissue along with CNS as was reported earlier [25]. According to previous reports, in
283 experimental JEV infected pigs, the viral load was noticed to be highest in tonsils as
284 compared to other secondary lymphoid organs as well as CNS. In tonsils, even after 11 days
285 of post infection no significant reduction was observed in viral RNA. Further, live virus was
286 also detected in tonsil tissues [25].

287 The higher percentage of positive cases detected in Manipur (Imphal West district) and
288 Assam (Lakhimpur district) might be due to larger pig population of the north eastern regions
289 of India [45]. The male pigs were found to be infected more with JE virus compared to
290 females. Moreover, the rate of infection was higher in the pigs above 7 months of age
291 compared to 3-7 months. Pigs over 11 months of age were also found positive for JEV in
292 Cambodia [46]. It might be due to pig to pig transmission through oronasal route in pig
293 rearing farms [25]. The JE positivity was observed throughout the year in the current study
294 with slight increase during the monsoon and post-monsoon periods. Earlier reports also
295 suggested that the JE detection goes up during the monsoon and the virus can be detected in
296 pig throughout the year in tropical regions [4].

297 Here, the genotype III strain was found to be circulating predominantly among pig population
298 of the three states. This was reported earlier that genotype III is the most prevalent strain in

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299 pig population of India as well as other parts of Asia such as China [19, 47]. Interestingly, a
300 single strain of genotype I was detected in pig population of Odisha. There are reports which
301 indicate the recent introduction of genotype I in human in India [20], however GI JEV strain
302 has never been detected previously from pig. The presence of genotype I was also reported
303 from other parts of Asia, suggesting it as an emerging strain [15]. The highest nucleotide
304 identity for the GenBank submitted sequence of genotype I (GenBank Accession number
305 MK421340) was found to be 99.74% with Yunan isolate YNTC07290. Whereas, for
306 genotype III the submitted sequences of GenBank showed highest nucleotide identity of
307 approximately 99% with Indian isolate JEV/SW/IVRI/395A/2014**.** As reported earlier also,
308 genetic diversity is low for the E gene at nucleotide and amino acid level [23]. The
309 mutational analysis showed a single mutation in GI strain, whereas sporadic mutations along
310 with two consistent mutations were observed for all the GIII strains.

311 In the present investigation, paired samples of serum and tonsil could not be collected due to
312 operational issues in the abattoir, which could have offered better insights into the merit of
313 testing tonsil tissue (if any) rather than serum samples for detection/quantification of JEV by
314 RT-PCR. Further, a well-designed survey for JEV infection/disease prevalence in human and
315 mosquito population during the study period would have allowed possibilities to correlate
316 JEV infection in humans and pigs in the same geographical region.

317 The current study limited to three geographical sites has indicated that JEV prevalence in pigs
318 can be very high in areas where human JEV outbreak has been reported. We suggest that JEV
319 outbreaks in human population might be controlled by vaccinating pigs in areas of high
320 prevalence of the viral load in the animal reservoir – a strategy that has not been widely
321 adopted.

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463 ## Supporting information captions

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464 S1A Table. District-wise distribution of the positive JEV cases by RT-PCR from the pig
465 serum samples collected during the study period. (DOC)

466 S1B Table. District-wise distribution of the positive JEV cases by RT-PCR from the pig
467 tonsil samples collected during the study period. (DOC)

468 S2 Table. Accession numbers for the positive serum and tonsil samples from three different
469 States of India. (DOC)

470 S3A Table. Amino acid substitutions in the envelope (E) gene of the studied JEV genotype I
471 (GI) strain in comparison to the prototype strain. (DOC)
472 S3B Table. Amino acid substitutions in the envelope (E) gene of the studied JEV genotype III
473 (GIII) strains in comparison to the prototype Nakayama strain. (DOC)
474 S1A Fig. Alignments of JEV E amino acid sequences of GI strain detected in the study in
475 comparison to strains isolated from mosquito and human from China and India respectively.
476 Conserved amino acids are kept as star marked. The substitution mutations are represented in
477 colour with highlight (DOC).

478 S1B Fig. Alignments of JEV E amino acid sequences of GIII strain detected in the study in
479 comparison to prototype Nakayama strain. Conserved amino acids are kept as star marked.
480 The substitution mutations are represented in colour with highlight. (DOC)
481
482

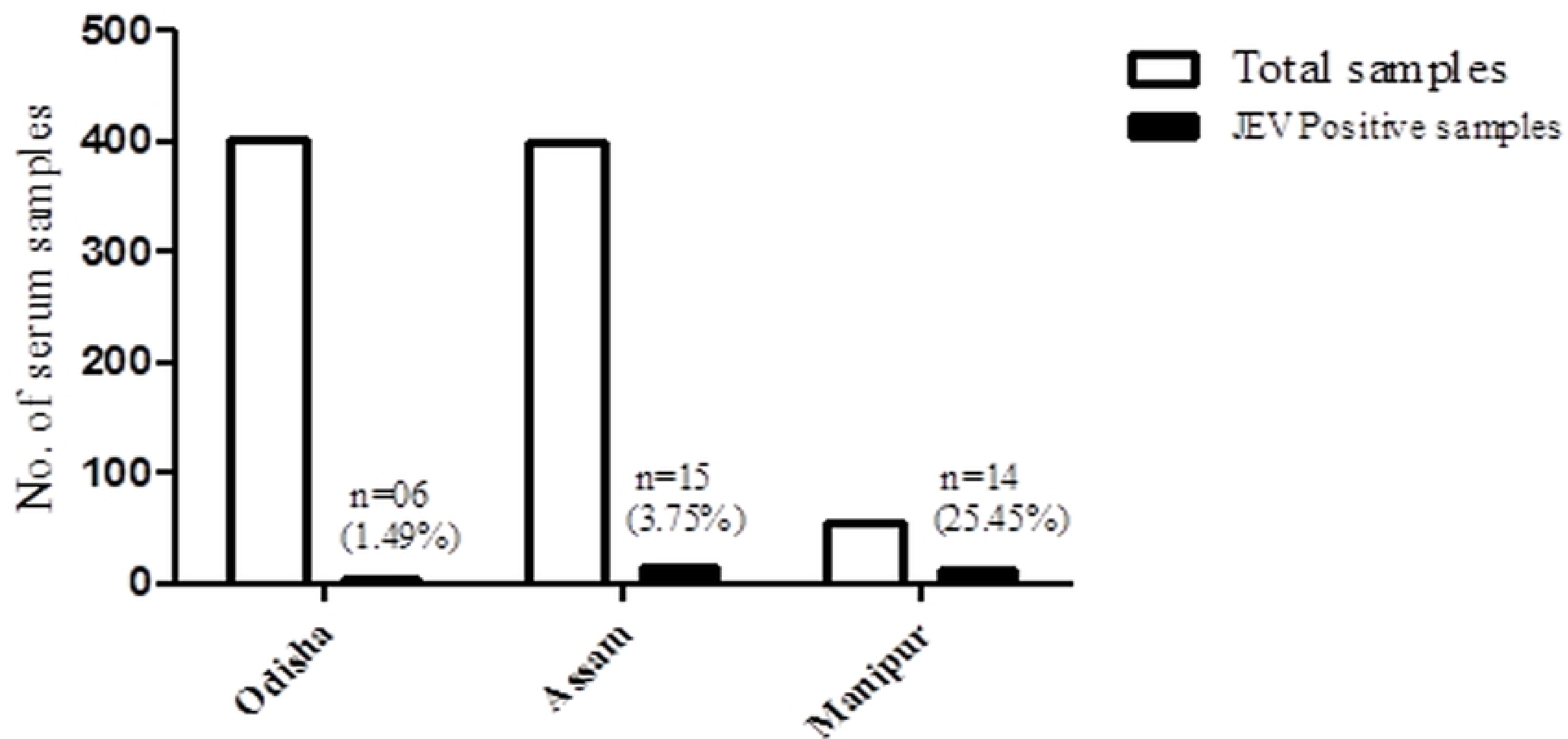


Figure 2A

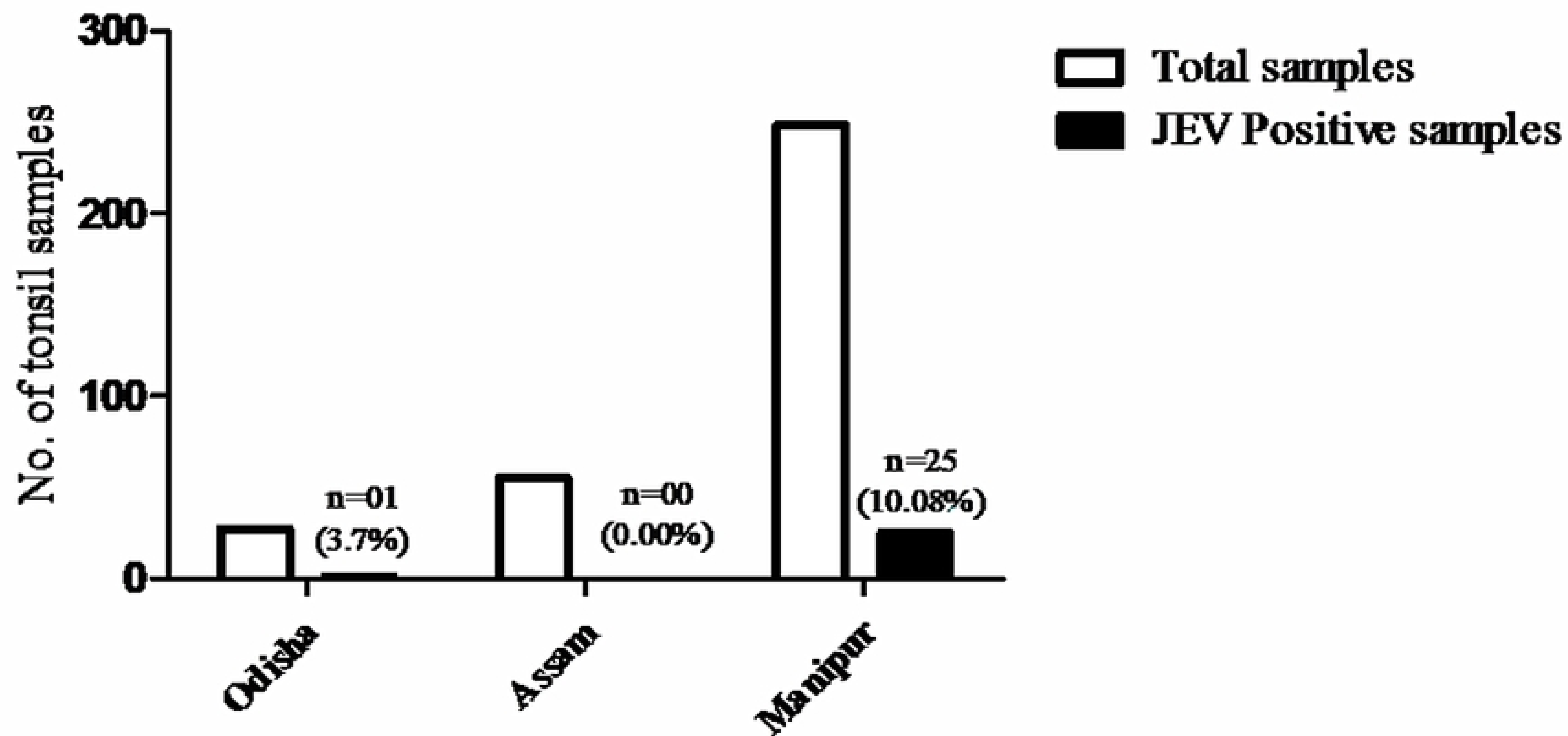


Figure 2B

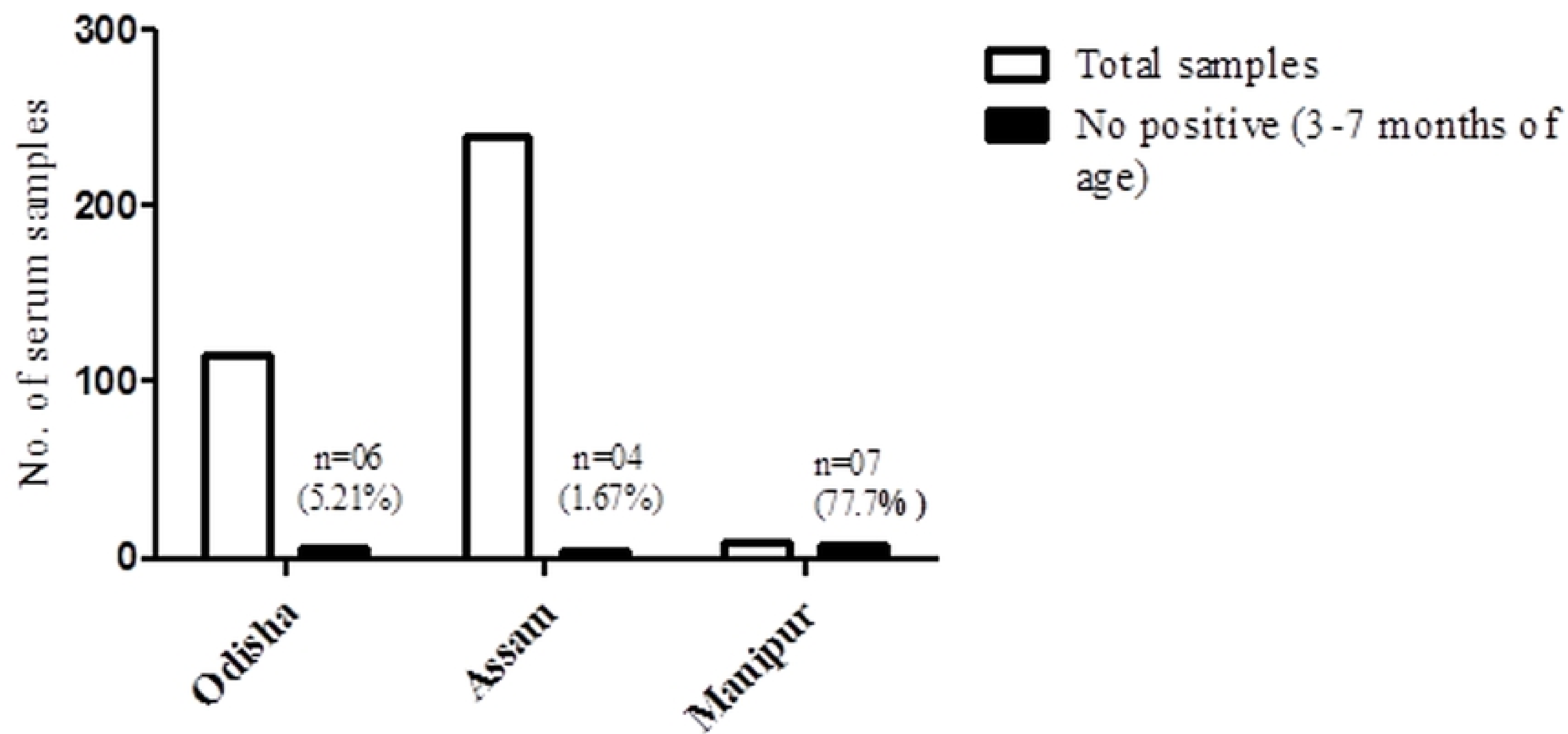
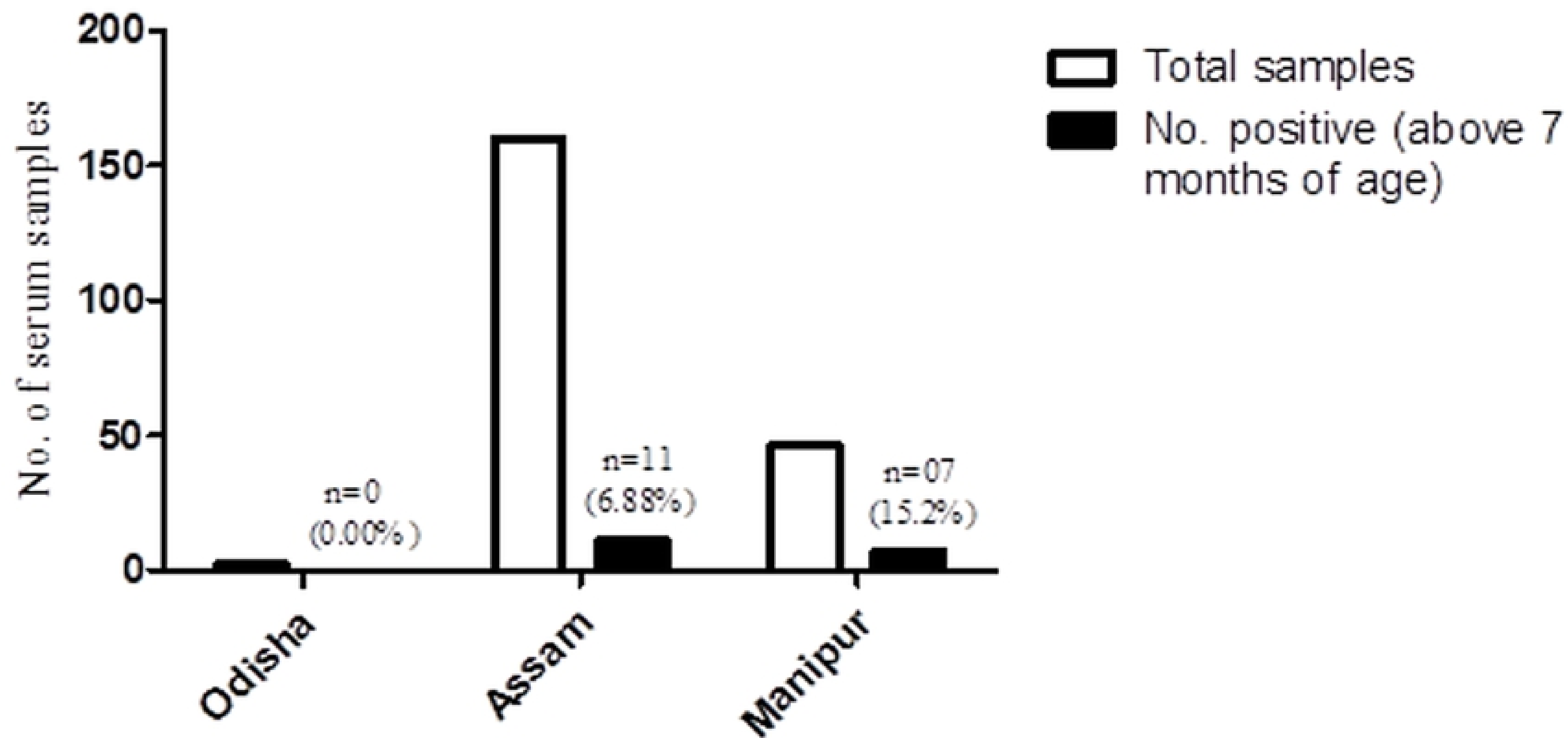


Figure 3A



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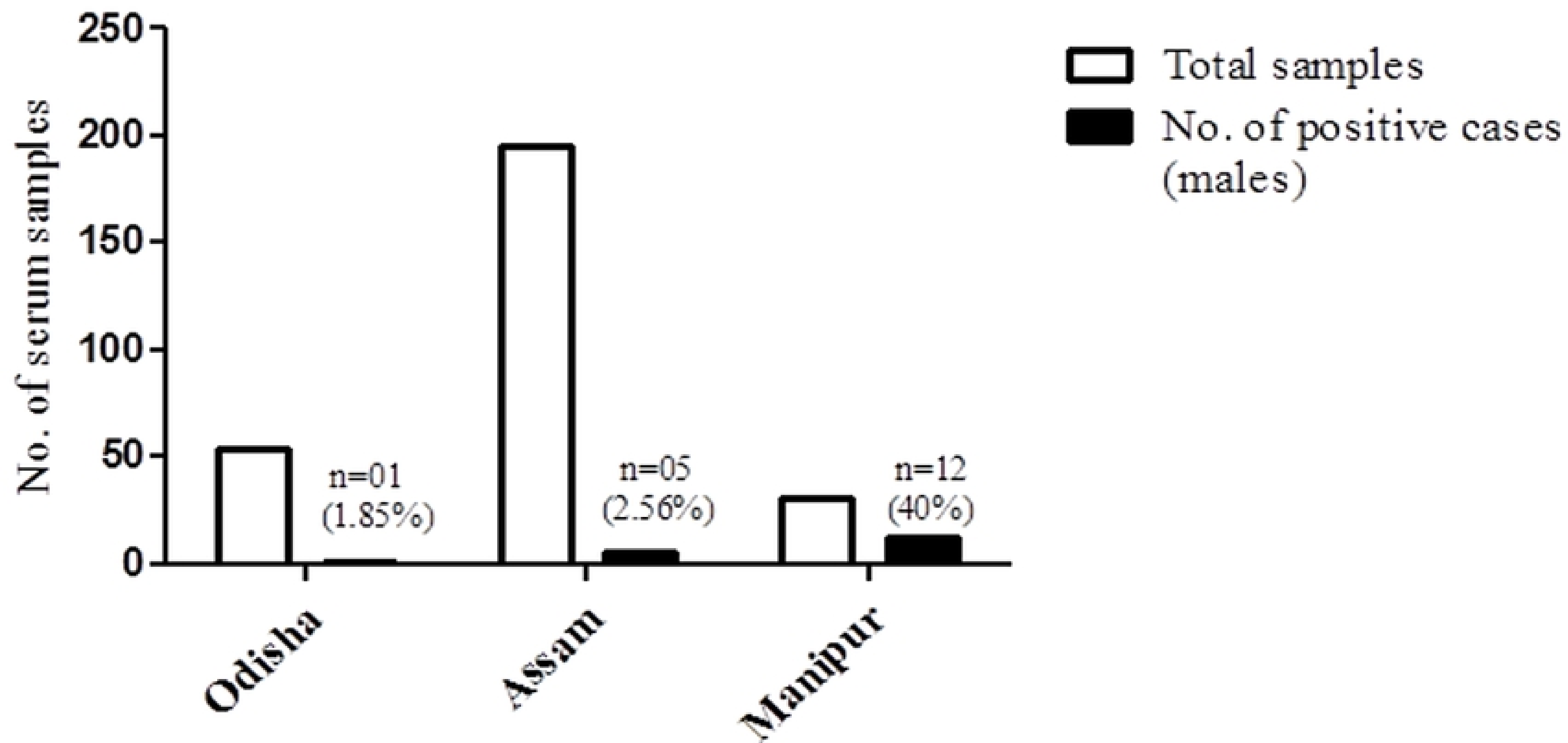

Figure 4A

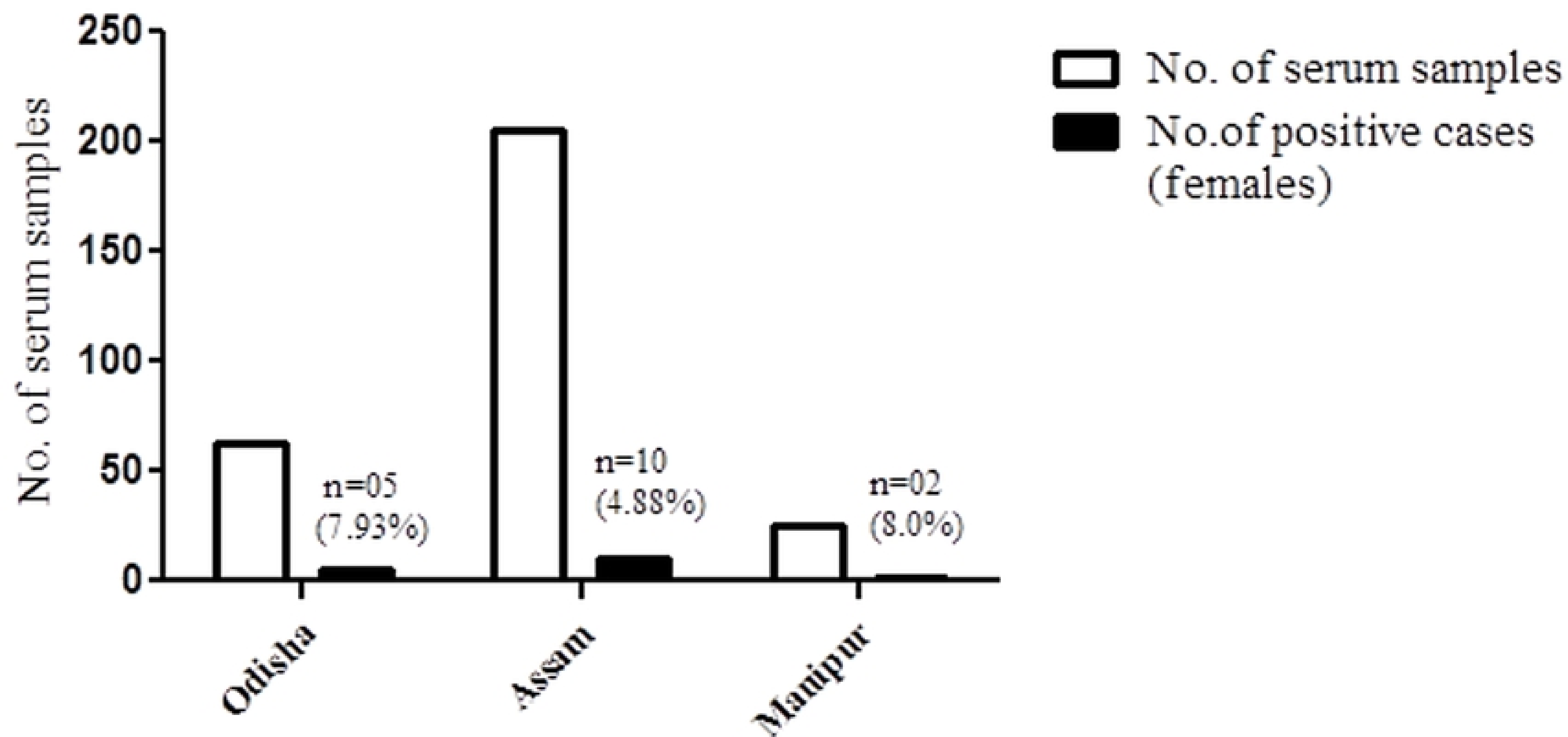

Figure 4B

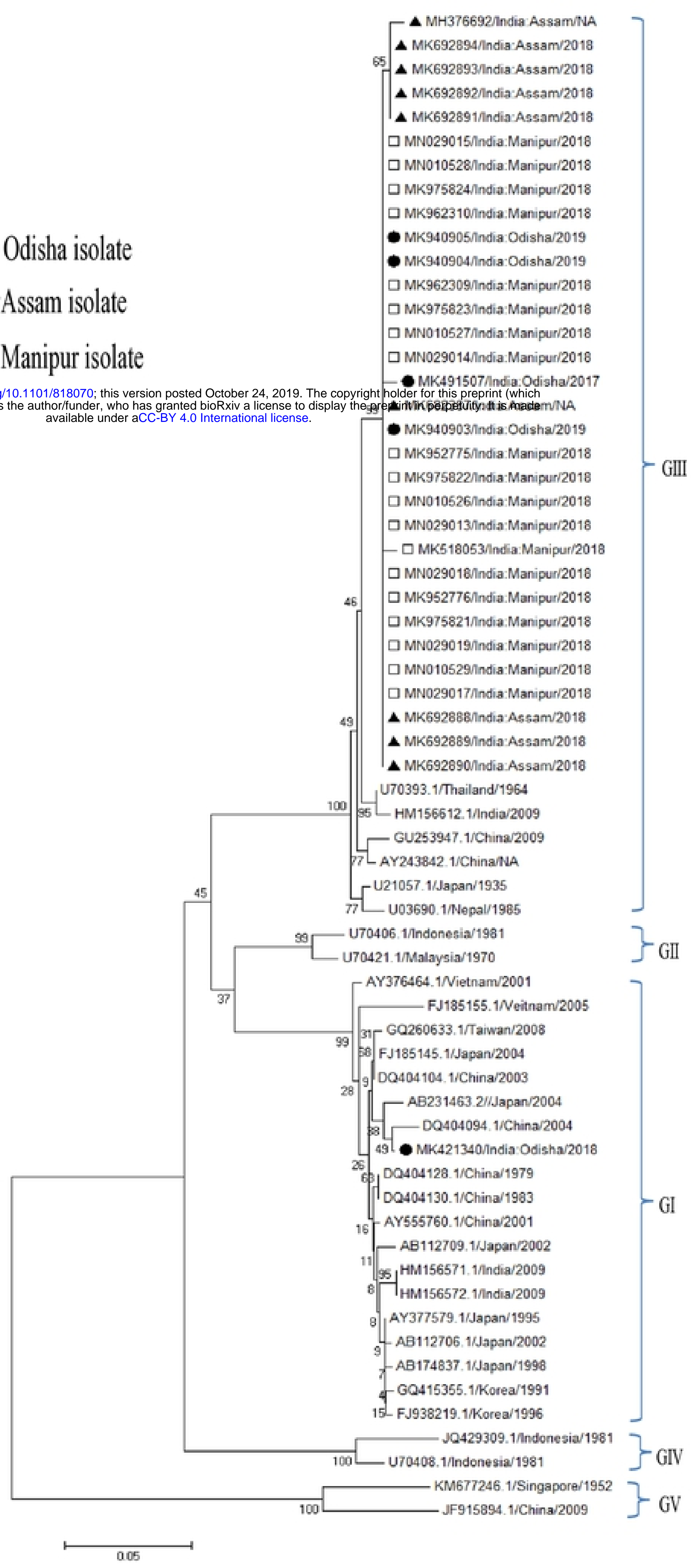

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Figure 5

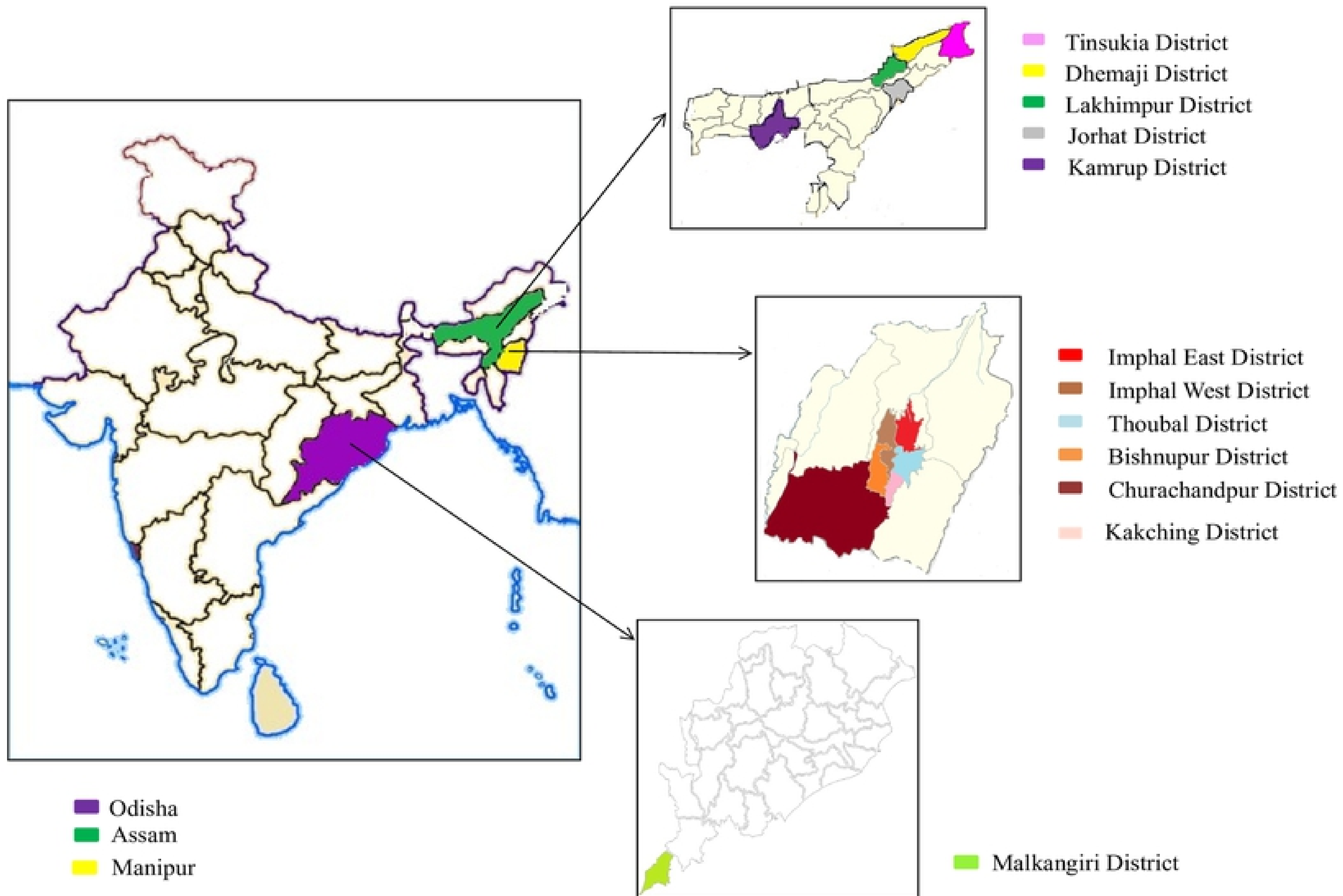

Figure 1

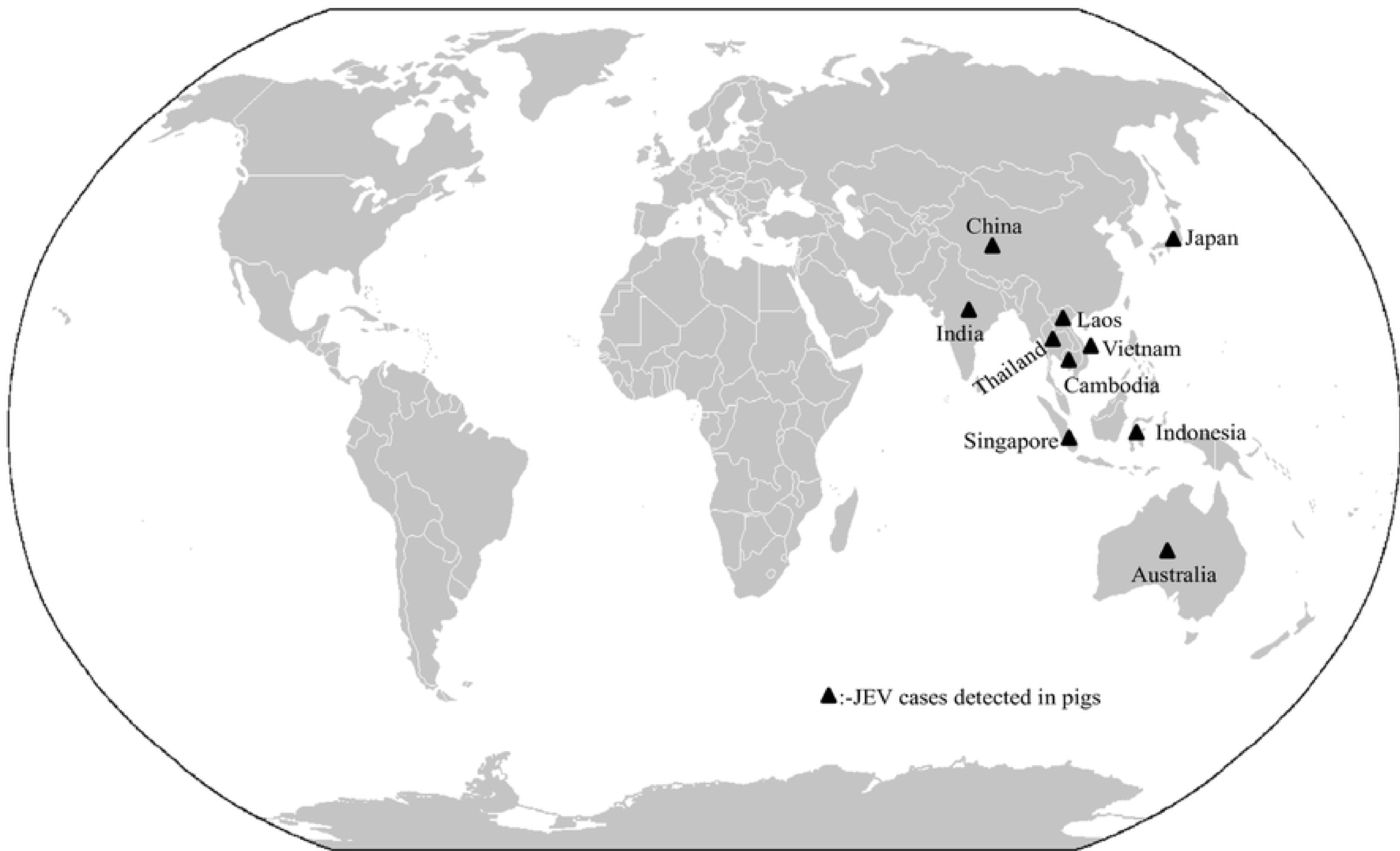

Figure 6