

1 **The genetic signatures of *Salmonella* Typhi carriage in the human gallbladder**

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19 **Running title**

20 *Salmonella* Typhi in the human gallbladder

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28 **Abstract**

29 Despite recent gains in typhoid fever control, asymptomatic carriage of *Salmonella* Typhi in the
30 gallbladder remains an enigma. Aiming to understand if *S. Typhi* in the gallbladder are vital for
31 transmission and/or adapted for long-term colonisation we performed whole genome sequencing on a
32 collection of *S. Typhi* isolated from the gallbladders of typhoid carriers. These sequences were
33 compared to contemporaneous sequences from organisms isolated from the blood of acute patients.*S.*
34 *Typhi* carriage was not restricted to any particular genotype or conformation of antimicrobial
35 resistance genes but reflective of the general population. However, gallbladder isolates had a higher
36 genetic variability than acute isolates, with median pairwise SNP distances of 21 and 13 SNPs
37 ($p=2.8 \times 10^{-9}$), respectively. This variation was associated with a higher prevalence of nonsense
38 mutations in the gallbladder isolates in the predominant genotype. Notably, gallbladder isolates
39 displayed a higher frequency of non-synonymous mutations in genes encoding hypothetical proteins,
40 membrane lipoproteins, transport/binding proteins, surface antigens, and carbohydrate degradation.
41 Particularly, we identified several gallbladder-specific non-synonymous mutations involved in LPS
42 synthesis and modification, with some isolates lacking the Vi capsular polysaccharide vaccine target
43 due to a 134Kb deletion. Long-term gallbladder carriage of *S. Typhi* results in atypically long branch
44 lengths that can distinguish between carriage and acute infection. Our data strongly suggests typhoid
45 carriers are unlikely to play a principal role in disease transmission in endemic settings, but that the
46 hostile environment of the human gallbladder may generate new antigenic variants through immune
47 selection.

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49

50 **Keywords**

51 Typhoid fever, *Salmonella* Typhi, gallbladder, *Salmonella* chronic carriage, asymptomatic carrier,
52 H58, antimicrobial resistance, Nepal, epidemiology

53

54 **Background**

55 Typhoid fever, a potentially life-threatening systemic infection caused predominantly by the
56 bacterium *Salmonella enterica* serovar Typhi (*S. Typhi*), remains a significant public health problem
57 in resource-poor settings including parts of Asia and Africa ¹. The disease is contracted via ingestion
58 of contaminated food or water or through contact with individuals excreting the organism ². The
59 majority of typhoid patients fully recover with appropriate treatment; however, some individuals can
60 become asymptomatic carriers and shed infectious bacteria in their faeces for an ill-defined period of
61 time. Asymptomatic carriage of *S. Typhi* has been recognized as a public health threat for more than a
62 century, with infamous typhoid carriers like Mary Mallon, a cook in New York, and Mr N, a milker in
63 England, identified in the early part of the 20th century ^{3,4}.

64
65 Typhoid carriage can be differentiated into three categories depending on the duration of shedding:
66 convalescent (three weeks to three months), temporary (three to twelve months), and chronic (more
67 than one year) ⁵. In endemic regions, an estimated 2-5 percent of acute typhoid patients become
68 chronic carriers, meaning that they continue to intermittently shed the bacteria indefinitely after
69 apparent clinical resolution ^{3,5}. Consequently, chronic carriers are widely believed to be an ecological
70 niche that facilitates the transmission and persistence of typhoid in human populations ⁶. *S. Typhi* is a
71 human-restricted pathogen, meaning that the disease may be theoretically eliminated locally by
72 reducing transmission through targeted treatment, improved sanitation, and mass vaccination.
73 Consequently, understanding the role of chronic carriers in disease transmission, and detecting them
74 prospectively, may accelerate disease elimination.

75
76 Despite substantive gains in understanding the biology of typhoid, we have generated limited new
77 insights into typhoid carriage in recent decades. Data from murine models of *Salmonella* carriage and
78 human clinical investigations have determined that the gallbladder is a key permissive niche for long-
79 term bacterial persistence ⁷⁻¹³. Various epidemiological investigations have shown that gallstones and

80 gallbladder damage may facilitate typhoid carriage^{9,13–17}, and that *Salmonella* preferentially attach to,
81 and form biofilms on, cholesterol-rich gallstones^{7,11,13,18,19}. *S. Typhi* carriage isolates have been
82 previously genetically compared with isolates from acute infection, with the aim of identifying
83 signatures associated with carriage^{20–23}. However, these studies were unable to infer how carriage
84 isolates directly relate to those causing acute disease.

85

86 It is apparent we need a better understanding of the role of the typhoid carrier and associated
87 organisms to generate new approaches to the management of such individuals in endemic locations.
88 Although it is widely accepted that that *S. Typhi* carriage play a key role in the transmission of
89 typhoid in endemic settings it is unknown if carriage organisms are somehow adapted for long-term
90 colonisation. Aiming to address this question, we performed whole genome sequencing and detailed
91 genetic analyses on *S. Typhi* isolated from the gallbladders of typhoid carriers in Kathmandu. We
92 compared these data to the sequences of contemporaneous organisms isolated from the blood of
93 acutely infected patients in the same community over the same time period. Our data provides new
94 insight into the role of typhoid carriage in disease transmission, showing that whilst carriage isolates
95 are reflective of the general *S. Typhi* population circulating in the community, gallbladder carriage
96 subjects organisms to immune pressures, which induces genetic variation and genomic degradation.

97

98 **Results**

99 *The phylogenetic relationships between acute and gallbladder S. Typhi isolates*

100 Between June 2007 and October 2010, we conducted a *Salmonella* carriage study in Kathmandu¹³.
101 Patients undergoing cholecystectomy for acute or chronic cholecystitis were enrolled; bile and stool
102 samples from 1,377 individuals were collected and subjected to microbiological examination.
103 Twenty-four *S. Typhi* were isolated from bile samples taken from these patients and designated as
104 gallbladder isolates. Ninety-six *S. Typhi* isolates recovered from patients with acute typhoid fever
105 living in the same population over the same time period were used for comparison²⁴ (denoted as acute

106 isolates) (Table S1). A phylogenetic analysis of these 120 *S. Typhi* isolates demonstrated that
107 subclade 4.3.1 (H58) was the dominant genotype, constituting 62.5% (15/24) of all gallbladder
108 isolates and 65.6% (63/96) of all acute isolates. The second most common genotype was 3.3.0 (H1),
109 accounting for 12.5% (3/24) and 14.6% (14/96) of all gallbladder and acute isolates, respectively.

110

111 We identified a significant degree of genetic diversity within this collection of acute and carriage
112 organisms, with multiple less-common genotypes co-circulating, included various clades (4.1, 3.1 and
113 2.2), subclades (3.2.2, 3.0.1, 2.2.2 and 2.2.1), and organisms within primary cluster 2 (Figure 1). The
114 less common genotypes from the gallbladder fell within subclade 3.2.2 (8.3%; 2/24), 2.2.2 (4.2%;
115 1/24), clade 2.2 (8.3%; 2/24) and primary cluster 2 (4.2%; 1/24). Overall, gallbladder isolates were
116 not significantly associated with subclade 4.3.1 in comparison with other genotypes (15/24 versus
117 9/24, $p=0.083$; Chi-squared test). These initial observations indicate that *S. Typhi* carriage was not
118 restricted to any particular *S. Typhi* genotype; instead, the genotype distribution among gallbladder
119 isolates generally reflected a genetic structure similar to that of the acute *S. Typhi* infections
120 circulating in the community.

121

122 *Antimicrobial susceptibility*

123 We speculated resistance to key antimicrobials may facilitate the development of carriage. However,
124 we found that the *S. Typhi* gallbladder isolates did not carry any obviously acquired AMR genes.
125 However, chromosomal mutations associated with reduced susceptibility against fluoroquinolones
126 were common. These fluoroquinolone resistance-associated mutations within the gallbladder
127 organisms were more commonly observed in subclade 4.3.1 than in non-subclade 4.3.1 (73% (11/15)
128 versus 11% (1/9), $p=0.01$; Chi-squared test). In comparing the respective *gyrA* mutation profiles
129 between the acute and gallbladder isolates within subclade 4.3.1, we found that 76.2% (48/63) and
130 60% (9/15) carried the S83F mutation respectively, 7.9% (5/63) and 13.3% (2/15) carried the S83Y
131 mutation, and 15.9% (10/63) and 26.7% (4/15) had no *gyrA* mutation. Consequently, there was no

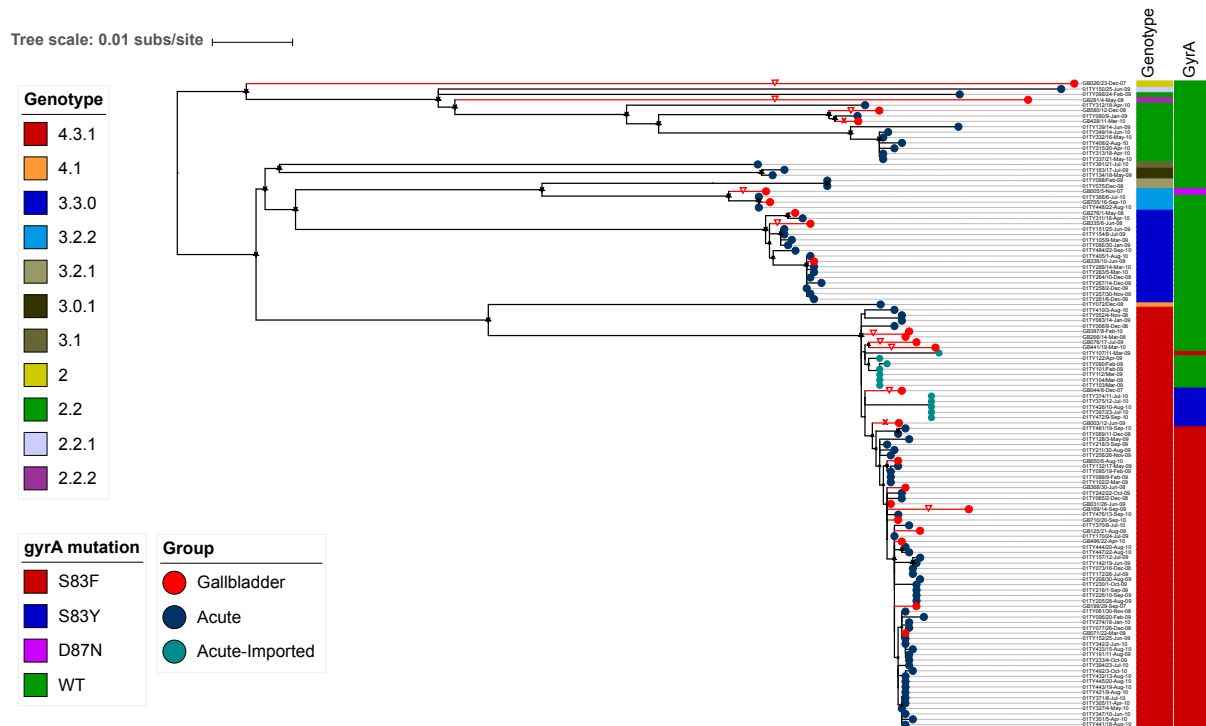


Figure 1. The phylogenetic structure of gallbladder and acute *S. Typhi* isolates collected between 2007 and 2010.

Rooted maximum likelihood tree (*S. Paratyphi A* used as an outgroup to root the tree and pruned for visualization) based on core-genome SNPs of 120 *S. Typhi* isolates with the corresponding metadata: genotype, *gyrA* mutation. Gallbladder and acute isolates are shown as red and dark circles at the terminal nodes, respectively. Acute isolates originating from importation are also highlighted by turquoise circles at the terminal nodes. Terminal branches leading to gallbladder isolates are highlighted in red. Red triangles show gallbladder isolates associated with unusually long terminal branches.

132 significant difference ($p=0.327$; Chi-squared test) in the presence of fluoroquinolone resistance-
133 associated mutations between acute and gallbladder isolates within subclade 4.3.1.

134

135 *Phylogenetic signatures of long-term Salmonella Typhi carriage*

136 Despite the acute and gallbladder *S. Typhi* isolates generally clustering within the same genotypes
137 across the phylogeny, we observed that a substantial proportion of the gallbladder isolates had higher
138 genetic variability, which could be distinguished by long terminal branches (Figure 1). The median
139 pairwise SNP distance of gallbladder isolates within subclade 4.3.1 was 21 SNPs (IQR: 12-24), which
140 was significantly greater than that of the corresponding acute isolates (13 SNPs (IQR: 8-19 SNPs)
141 ($p=2.8 \times 10^{-9}$, Wilcoxon rank-sum test) (Figure S2). Similarly, the median pairwise SNP distance of
142 gallbladder isolates within subclade 3.3.0 (20 SNPs, IQR: 13-22 SNPs) was higher than that of acute
143 isolates (13 SNPs, IQR: 4-15 SNPs) ($p=0.26$, Wilcoxon rank-sum test).

144

145 We mapped the contemporary acute and gallbladder *S. Typhi* sequences onto the global *S. Typhi*
146 phylogeny, which indicated that the majority of these Nepalese acute and gallbladder *S. Typhi* isolates
147 fell within known domestic genotypes, with limited evidence of importation from other countries
148 (Figure S1). This observation suggests that the long terminal branches associated with gallbladder
149 isolates were unlikely to be driven by the importation of these organisms from alternative countries.

150

151 We next estimated and plotted the nearest phylogenetic distances (NPDs) between each taxon and its
152 nearest neighbour on the subclade 4.3.1 tree versus the year of isolation, the age of the individual
153 from whom the organism was isolated, and the *gyrA* mutation profile. We hypothesized that the
154 annual distribution of NPDs of *S. Typhi* acute isolates would represent the phylogenetic diversity
155 (mutation accumulation) occurring annually via acute disease transmission and would be comparable
156 over multiple years. Alternatively, we considered that *S. Typhi* in the gallbladder may develop
157 characteristic adaptive mutations facilitating long-term persistence, causing them to gradually become

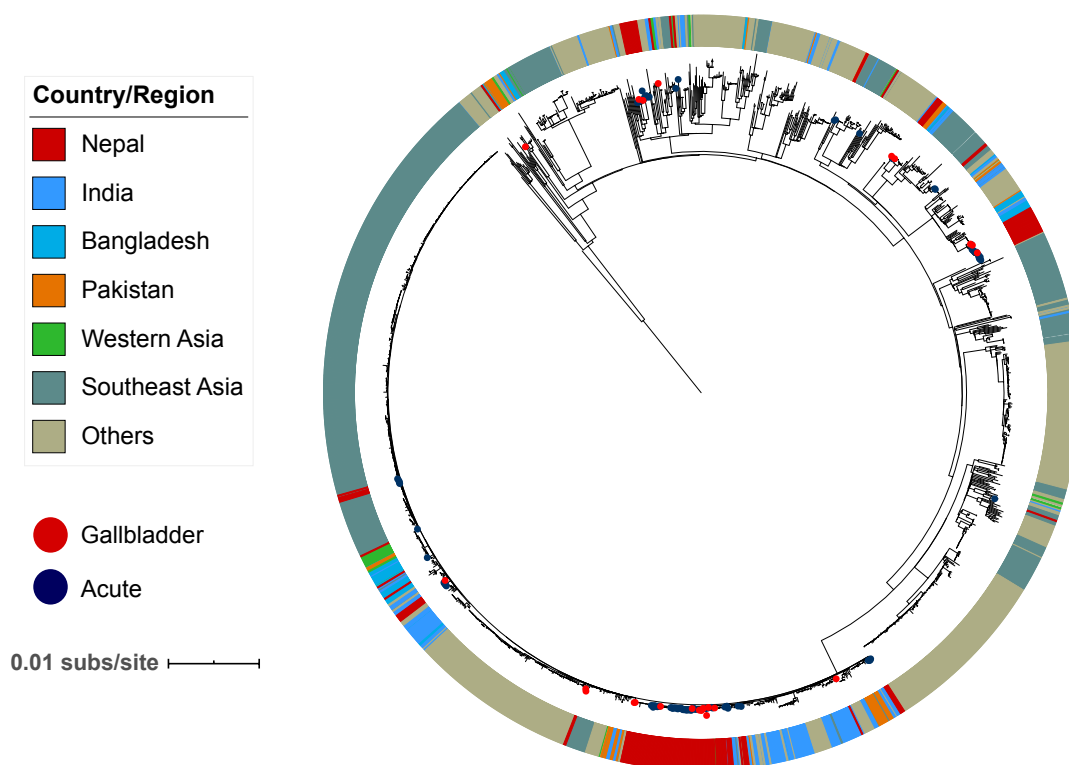


Figure S1. Phylogenetic structure of acute and gallbladder *Salmonella* Typhi isolates from Nepal in the global context

Acute and gallbladder *S. Typhi* isolates from this study are highlighted in blue and red circles; respectively, at the terminal nodes. The outer ring exhibits the location of the isolates from Nepal and its neighbouring countries as well as other regions in the world.

158 increasingly distinct from contemporaneous acute isolates, leading to greater phylogenetic distances
159 relative to their neighbours. In addition, given that all acute subclade 4.3.1 isolates here exhibited a
160 *gyrA* mutation, the gallbladder subclade 4.3.1 isolates without a *gyrA* mutation were more likely to
161 have colonized the gallbladder prior to nalidixic acid resistance becoming commonplace.

162

163 Our analyses showed that the average (\pm SD) NPD per year of acute subclade 4.3.1 isolates was
164 comparable; specifically, 0.00163 (\pm 0.00202) substitutions/site (\sim 3.6 (\pm 4.4) SNPs) in 2008; 0.00110
165 (\pm 0.00229) substitutions/site (\sim 2.4 (\pm 5) SNPs) in 2009, and 0.00144 (\pm 0.00238) substitutions/site
166 (\sim 3.2 (\pm 5.2) SNPs) in 2010. The majority of the subclade 4.3.1 gallbladder isolates (8/10) for which
167 NPDs fell within the annual NPD distribution of acute subclade 4.3.1 isolates were associated with
168 comparable terminal branch lengths and had a *gyrA* S83F mutation. Based on these findings, we
169 surmised that gallbladder colonization with these isolates was likely to have occurred relatively
170 recently in these individuals. Notable exceptions were two gallbladder isolates (GB266 and GB387)
171 that did not possess a *gyrA* mutation and were associated with long terminal branches but had low
172 NPDs as they clustered closely within the main phylogeny (Figures 1 and 2). Further, our data showed
173 that all subclade 4.3.1 gallbladder isolates exhibiting abnormally high NPDs were associated with
174 long terminal branches, indicative of chronic carriage (Figure 2). In particular, two subclade 4.3.1
175 gallbladder isolates (GB76 and GB441) lacked *gyrA* mutations, two others (GB003 and GB044) had
176 *gyrA* S83Y mutations, and the remaining one (GB169) exhibited *gyrA* mutation S83F. With respect to
177 the age distribution, typhoid carriers were significantly older (median age 36 years, range: 20-67) than
178 patients with acute illness (median age 16 years, range: 0-31) ($p=3.8 \times 10^{-8}$, Wilcoxon rank-sum test).
179 The gallbladder isolates thought to have originated from chronic carriers (based on above data) were
180 obtained from individuals between aged between 27 and 40 years, which was older than the majority
181 of the sampled acute typhoid patients; however, there was no significant difference in age distribution
182 between those estimated to be recent and chronic carriers (Figure 2).

183

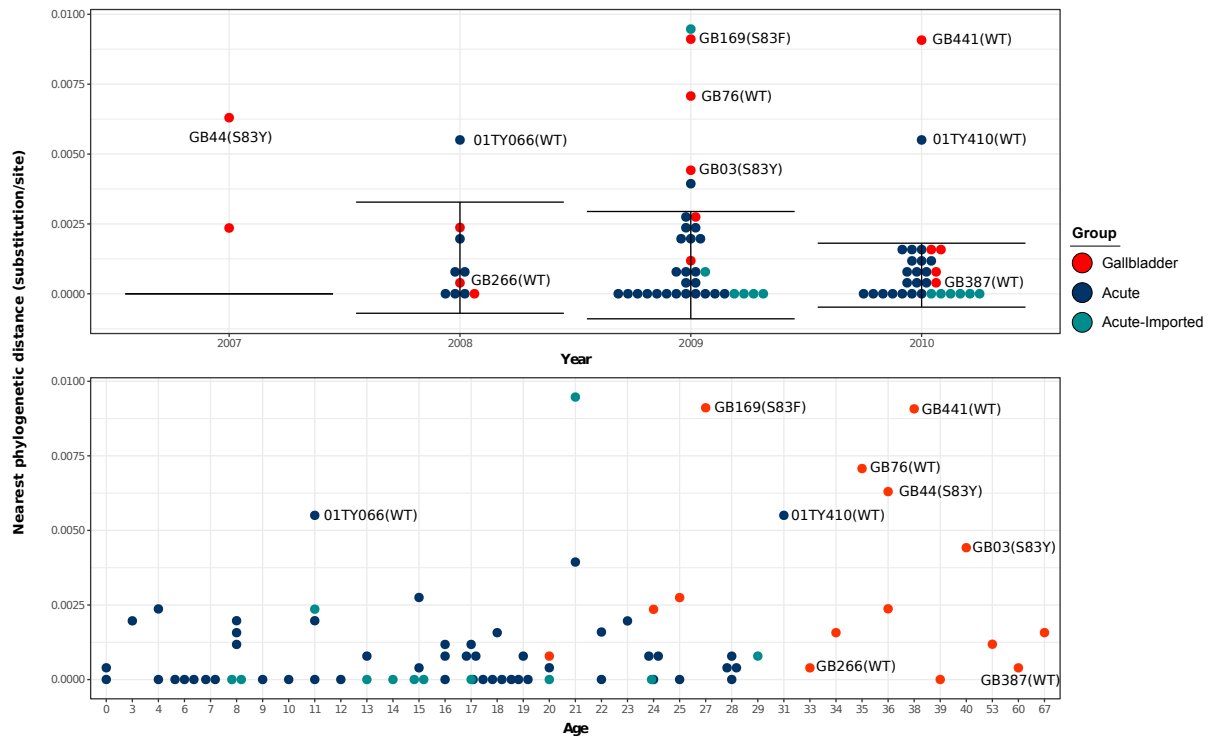


Figure 2. The Distribution of nearest phylogenetic distances of gallbladder and acute H58 isolates over the study period

Each circle represents the phylogenetic distance from each isolate to its nearest neighbour on the phylogenetic tree. The error bar represents the average phylogenetic distance to the nearest neighbour (\pm standard deviation) for acute H58 isolates. Gallbladder and acute isolates estimated to have originated from chronic carriers are labelled with their corresponding strain names.

184 *The genetic traits of Salmonella Typhi gallbladder isolates*

185 To identify potentially adaptive mutations associated with typhoid carriage, all nonsynonymous SNPs
186 (NSs) occurring exclusively within the *S. Typhi* gallbladder genome sequences were identified and
187 grouped by their predicted or known function. A corresponding analysis was performed for all NSs in
188 the acute *S. Typhi* isolates. A total of 228 gallbladder-specific NSs (212 missense and 16 nonsense
189 mutations) and 469 acute-specific NSs (437 missense and 32 nonsense mutations) were identified. In
190 general, there was no significant difference ($p=0.924$; Chi-square test) in the proportion of nonsense
191 mutations out of total specific NSs in the gallbladder versus the acute isolates across all genotypes.
192 However, among subclade 4.3.1 isolates, the proportion of nonsense mutations out of total specific
193 NSs was significantly higher for gallbladder isolates than for acute isolates (10/60 compared to 2/67,
194 Fisher exact test, $p=0.01$). These data suggest that gene degradation resulting from nonsense
195 mutations was more common in the subclade 4.3.1 gallbladder isolates compared to the subclade 4.3.1
196 acute isolates.

197
198 Inactivated genes in the gallbladder isolates included genes involved in the synthesis of peptidoglycan
199 (*pbpC*), vitamin B12 receptor (*btuB*), general stress response regulator (*rpoS*), a laterally acquired
200 protein in SPI-7 (STY4562), membrane transport protein (STY3932), central metabolism (STY0230,
201 *ggt*), hypothetical proteins (STY0929 and STY4178), and osmotically inducible lipoprotein E
202 precursor (*osmE*) (Table 1).

203
204 Overall, the gallbladder- and acute-specific NSs across all genotypes could be grouped into 78
205 functional categories. The highest prevalence of these NSs was found in genes encoding hypothetical
206 proteins, membrane lipoproteins, unknown functions, transport/binding proteins, SPI-7, general
207 regulatory functions, surface polysaccharides and antigens, carbohydrate degradation, and DNA
208 replication/modification. The proportions of NSs in SPI-7, surface polysaccharides and antigens,
209 pathogenicity, cell envelope, anaerobic respiration, fatty acid biosynthesis and transport/binding

210 proteins were higher in gallbladder than acute isolates (Figure 3). Notably, the data showed that the
211 proportion of NSs in the *viaB* operon (encoding the Vi antigen, target of the typhoid conjugate
212 vaccine (TCV)) was significantly higher in gallbladder isolates compared to the acute isolates across
213 all genotypes (9/228 compared to 7/469, Chi-squared test, $p=0.04$). Similar results were obtained
214 when considering only *S. Typhi* isolates belonging to subclade 4.3.1, with gallbladder isolates having
215 more specific NSs in the *viaB* operon than the acute isolates (5/60 compared to 1/67, Fisher's exact
216 test, $p=0.09$). Additionally, we identified two gallbladder isolates (GB428 and GB003) that had lost
217 the Vi capsular polysaccharide due to the deletion of the entire SPI-7 region (134kb).

218

219 *Positive selection associated with typhoid carriage*

220 Finally, we investigated signatures of positive selection by identifying analogous genetic variation
221 detected in different gallbladder isolates. Among the gallbladder specific NSs, a number of different
222 mutations were present in the same gene or the same biological pathway in at least two
223 phylogenetically unlinked gallbladder isolates. For example, within the *viaB* operon, there were two
224 NSs at codon 137 and 462 in the *tviE* gene (isolates GB580 and GB026) and six NSs in codons 166,
225 504, 506, 508, 665 and 752 in the *tviD* gene (isolates GB005, GB026, GB076, GB125 and GB281,
226 respectively). Both genes facilitate the polymerization and translocation of the Vi capsule²⁵.
227 Convergent NSs were also observed in the *rpoS* gene (sigma factor sigma-38) of isolates GB125
228 (nonsense mutation at codon 247) and GB705 (NSs at codon 94 and 250), which may impact general
229 stress response and nutrient starvation. A further example was NSs at codon 59 and 230 in the *degS*
230 gene (serine protease) (isolates GB005 and GB169). *DegS* is a component of the DegS-DegU two-
231 component regulation system involved in expression of several degradative enzymes for salt stress
232 responses and growth-limiting conditions. Additionally, three isolates (GB005, GB026 and GB705)
233 each possessed an NS (codons 335, 406 and 946, respectively) in STY1242 (*ptsG* - glucose-specific
234 PTS system IIBC component). *PtsG* enzyme is a component of the glucose-specific
235 phosphotransferase system and plays a role in phosphorylation and translocation of glucose across the

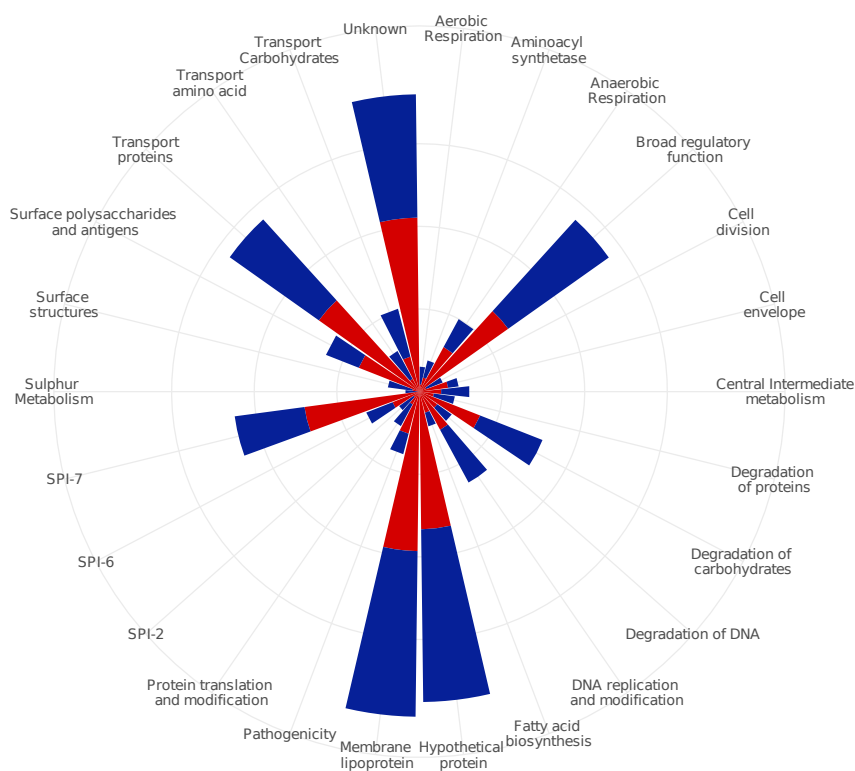


Figure 3. Functional classes of *Salmonella* Typhi genes associated with the highest prevalence of gallbladder-specific nonsynonymous SNPs versus acute-specific nonsynonymous SNPs

Functional classes are shown on the outermost circle. Four circles from the middle represent 5 – 10 – 15 – 20 percent of the cumulative percentage of functional classes. Red and blue blocks are representatives of gallbladder and acute isolates, respectively.

236 bacterial membrane, and is induced in carbon-limited conditions ²⁶. NSs in several other genes were
237 observed in >2 carriage isolates, including STY0429 (*SbcC* - exonuclease), STY0661 (*dmsC* -
238 molybdopterin containing oxidoreductase membrane anchor subunit), STY1447 (putative ribulose-5-
239 phosphate 3-epimerase) and STY2760 (*ratA* - putative exported protein) (Table 2).

240

241 *Evidence of selective pressure on lipopolysaccharide*

242 With respect to convergent mutations within the same biological pathways, there were a number of
243 gallbladder-specific NSs involved in LPS O-antigen synthesis and modification; for example, an NS
244 in the *rfc* gene (regulator of O-antigen polymerization) in isolate GB441, an NS in STY2629 (LPS
245 modification acyltransferase) in isolate GB335, two NSs in *rfbE* (CDP-tyvelose-2-epimerase) and
246 *rfaG* genes (LPS core biosynthesis protein) in isolate GB281, and three NSs in the *rfbK*
247 (phosphomannomutase), *manB* (phosphomannomutase), and *rfaD* genes (ADP-L-Glycero-D-
248 mannoheptose-6-epimease) in isolate GB026. *RfbK* and *manB* are both related to GDP-mannose
249 synthesis for the LPS, and *rfaD* is an enzyme that catalyzes the conversion of ADP-D-glycerol-D-
250 mannoheptose to ADP-L-glycerol-D-mannoheptose, a precursor for the synthesis of inner-core LPS.

251

252 **Discussion**

253 As stakeholders consider introduction of a new TCV into their national immunization programmes,
254 research into the role of chronic carriers in bacterial persistence and disease transmission in endemic
255 settings is needed to forecast the longer-term impact of vaccination on the transmission dynamics of
256 typhoid and inform appropriate public health measures. However, epidemiological investigations of
257 typhoid carriage are challenging, given that this population is problematic to identify and follow.
258 Currently, the environmental factors driving the evolution of *S. Typhi* within the gallbladder are
259 poorly understood and little is known about the adaptive mechanisms that may promote long-term
260 survival. Our study is the largest genomic investigation of *S. Typhi* gallbladder carriage in a typhoid
261 endemic setting, which allowed us to provide unprecedented insight into the genetic and phylogenetic

Table 2. Nonsynonymous mutations associated with positive selection in gallbladder isolates

Position in CT18	S/NS	Gene	Product	Functional class	GB005	GB026	GB044	GB076	GB125	GB169	GB199	GB266	GB281	GB335	GB368	GB387	GB441	GB580	GB705
complement (437771..440875)	NS	STY0429	exonuclease SbcC	Degradation of DNA				R394H					L646P						
661366..662133	NS	STY0661	molybdopterin-containing oxidoreductase	Unknown		E5K	V9M												
1196033..1197466	NS	STY1242	PTS, glucose-specific IIBC component	Transport carbohydrates	A112T	H136R													G316S
complement (1399142..1399774)	NS	STY1447	ribulose-5-phosphate 3 epimerase	Unknown		E164K							I101V						
complement (2220042..2221727)	NS	STY2389	two-component system sensor kinase	Broad regulatory function									P11L	V49A					
complement (2331373..2334009)	NS	STY2499	DNA gyrase subunit A	DNA - replication and modification	D87N								L824F						
complement (2915077..2916069)	NS	rpoS	RNA polymerase sigma subunit (sigma-38)	Broad regulatory function					W247*										T94P, E250V
3370486..3371556	NS	degS	serine protease	Degradation of proteins	E230K					V59A									
complement (4516537..4518273)	NS	tviE	Glycosyl transferases	SPI-7		A462T													H137Y
complement (4519050..4521545)	NS	tviD	Vi polysaccharide biosynthesis protein	SPI-7	F166L	P752Q, V508I		G506C	R665H				Q504L						

S1 Table. Gallbladder and acute *Salmonella* Typhi isolates and their associated metadata

262 signatures associated with typhoid carriage, but also to utilize these data to infer the potential role of
263 typhoid carriage in disease transmission.

264

265 Our data demonstrated, contrary to previous suggestions²⁷, that carriage of typhoid in the gallbladder
266 was not restricted to any particular genotype and was associated a diverse range of bacterial
267 genotypes, which largely mirrored the genetic structure of the bacterial population causing acute
268 disease in Nepal. Further, typhoid carriage was not confined to specific AMR phenotypes, signifying
269 that carriage is not associated with treatment failure with specific antimicrobials interacting with
270 corresponding AMR profiles. However, by comparing the pairwise SNP distances between
271 gallbladder and acute isolates within the same genotype, we found that gallbladder isolates displayed
272 significantly greater genetic diversity compared to acute isolates, which suggests that long-term
273 exposure to the gallbladder environment results in different accumulated adaptive mutations over time
274 than would be generated in acute isolates. Our phylogenetic reconstruction of *S. Typhi* revealed that a
275 number of gallbladder isolates had atypically long terminal branches, signifying that chronic carriage
276 isolates may have a distinct phylogenetic signature which could be potentially utilized for the
277 identification of organisms arising from chronic carriers. Further investigating this phenomenon, we
278 found that the annual distribution of NPDs of acute isolates, which likely reflects mutation
279 accumulation in the natural environment, was highly comparable across years and could be exploited
280 to disaggregate recent carriers from longer-term carriers. If carriers are relevant, then we would
281 predict they would be proportionally more important in causing acute disease in immunised
282 populations with reduced environmental transmission. Therefore, we can use the annual NPD
283 distribution to assess the impact of typhoid vaccination on disease transmission dynamics in endemic
284 areas.

285

286 The role of chronic carriage in disease transmission represents one of the most long-standing
287 questions in typhoid fever. Though typhoid carriers have been widely considered as an important

288 source of infection, their exact contribution to transmission in endemic areas is not well understood.
289 Previous molecular epidemiological studies in endemic regions highlighted an abundance of long-
290 cycle environmental transmission in these settings, with a wide diversity of co-circulating bacterial
291 genotypes isolated from acute typhoid patients²⁸⁻³¹, suggesting that person-to-person transmission
292 makes a minimal contribution to new typhoid cases in an endemic area. Here, few gallbladder isolates
293 clustered in close proximity or were directly linked with acute isolates and had long terminal
294 branches. These observations suggest that these organisms play a negligible role in causing onward
295 acute infections. Notably, none of the pre-surgical stool cultures from these patients undergoing
296 cholecystectomy were positive for *S. Typhi*. However, the infectivity and transmission fitness of
297 gallbladder isolates must be investigated further, as we cannot rule out the possibility that gallbladder
298 isolates can become a more important source of infection when environmental transmission is
299 successfully reduced. Further, the fact that gallbladder isolates display greater genetic variation than
300 acute isolates implies that the gallbladder may act as an important ecological niche for generating
301 novel genotypes.

302
303 By identifying NS mutations occurring specifically in gallbladder isolates and classifying them into
304 predicted functional classes for comparison with those of acute isolates, we found that gene
305 degradation by nonsense mutation was significantly higher in gallbladder compared to acute isolates
306 within subclade 4.3.1. The effects of gene inactivation on phenotype, fitness and adaptation of
307 carriage isolates inside the gallbladder are currently unknown. Further investigation of this
308 phenomenon is necessary, as gene inactivation has been shown to be an important molecular
309 mechanism in human adaptation in the evolutionary history of *S. Typhi*^{32,33}.

310
311 We additionally found evidence for the enrichment of NSs in genes encoding the Vi polysaccharide
312 capsule in gallbladder isolates. The Vi antigen is immunogenic and anti-Vi antibody gradually wanes
313 in acute typhoid patients after recovery, but can be detected in plasma from chronic carriers^{34,35}. Data

314 from sero-surveillance studies for chronic carriage have commonly reported elevated anti-Vi
315 antibodies in healthy individuals, which could be associated with carriage or repeated infections^{36,37}.
316 Immunofluorescent staining of biofilms produced by *S. Typhi* on the surface of human gallstones
317 demonstrated an abundance of Vi capsule on the surface of the colonising bacteria, suggesting that *S.*
318 *Typhi* constitutively expresses Vi during carriage¹⁹. The increased frequency of nonsynonymous
319 mutations in the *viaB* operon (*tviB*, *tviD* and *tviE*) of gallbladder isolates, combined with high anti-Vi
320 antibody titres in plasma³⁸ suggest that *S. Typhi* residing in the gallbladder are under sustained
321 immune pressure. The observation that two gallbladder isolates lacked genes encoding proteins for Vi
322 capsule biosynthesis again suggests that these were subject to selective pressure and that the loss of Vi
323 may be an adaptive mechanism for long-term survival. The generation of Vi-negative *S. Typhi* may
324 also question the possibility of their proliferation following mass immunization with TCV.
325
326 Identifying genes under selection among gallbladder isolates is crucial for understanding the
327 evolutionary forces and bacterial adaptation to the gallbladder environment during carriage.
328 Signatures of positive selection were detected in a number of genes containing differing gallbladder-
329 specific NS mutations in at least two phylogenetically unlinked gallbladder isolates. Many of these
330 genes are associated with gene regulation under stress and virulence gene expression. For example,
331 the global regulatory gene *rpoS* is responsible for general stress responses and nutrient starvation, and
332 regulates biofilm formation, colonization of Peyer's patches, persistence in the spleen and the
333 synthesis of Vi³⁹⁻⁴¹. The *degS* gene is involved in salt stress responses and growth-limiting
334 conditions; STY1242 (*ptsG* - glucose-specific PTS system IIBC component) is activated during
335 carbon starvation. These observations suggest that *S. Typhi* is exposed to a range of differing stressors
336 within the gallbladder. Furthermore, the genes responsible for LPS biosynthesis had additionally
337 accumulated NS mutations. LPS is the major component of the outer membrane of Gram-negative
338 bacteria and represents one of the main factors contributing to bile salt resistance^{42,43}. LPS is also a
339 key structural component of the biofilm extracellular matrix forming on human gallstones¹⁹. The

340 disruption of genes involved in LPS biosynthesis of *S. Typhimurium* may have a negative influence
341 on biofilm production and attachment⁴⁴. The enrichment of NS mutations in genes involved in LPS
342 biosynthesis and modification will lead to structural changes in LPS, which we predict will enhance
343 bile resistance and biofilm formation.

344

345 This study has its limitations. The number of gallbladder and acute isolates was relatively small and
346 thus might affect the interpretation of the phylogenetic distances between some of the gallbladder
347 isolates and their nearest neighbour. Specifically, our ability to infer associations with uncommon
348 genotypes was limited. Additionally, the identified phylogenetic signature inferred to be associated
349 with carriage was not observed for all gallbladder isolates, due to an underrepresentation in the
350 acutely infected population. Additionally, it was impossible to determine the duration of carriage to
351 confirm our findings, as most typhoid carriers from our study do not recall a history of typhoid¹³.

352 However, our data suggest that the potential duration of carriage within our gallbladder isolates was
353 variable, which led to variable terminal branch lengths. Despite these limitations, our study is unique
354 and opens up new possibilities for evaluating associations between gallbladder-specific genetic
355 variation and phenotypic differences to better understand the biology of this infectious disease
356 paradox.

357

358 **Conclusions**

359 We conclude that typhoid carriage is not associated with any specific genotype nor driven by AMR
360 phenotypes. However, we show that long-term gallbladder carriage results in atypically long
361 phylogenetic branch lengths that can be used to distinguish between carriage and acute infection.
362 Additionally, we found evidence that typhoid carriers are unlikely to play a major role in disease
363 transmission in endemic settings such as Kathmandu, and long-cycle transmission is the primary
364 driver of disease transmission in highly endemic settings. Public health efforts should continue to
365 focus on providing people with safe water and promoting safe food handling and the introduction of

366 TCV to interrupt environmental transmission in endemic settings. It remains important to further
367 investigate the epidemiology, genomics, biology and public health impacts of carriage in parallel to
368 the deployment of these public health measures. The role of carriers may become increasingly
369 important as we move toward eradication, especially as immune selection appears to play a critical
370 role in gallbladder colonisation.

371

372 **Methods**

373 *Sampling*

374 Between June 2007 and October 2010, we conducted a *Salmonella* carriage study at Patan Hospital in
375 Kathmandu¹³. In brief, patients undergoing cholecystectomy for acute or chronic cholecystitis were
376 enrolled; bile and stool samples from these patients were subjected to microbiological examination. *S.*
377 Typhi were isolated from bile samples taken from these patients (referred to as gallbladder isolates).
378 Additionally, *S.* Typhi isolates recovered from patients with acute typhoid fever living in the same
379 population recruited into a randomized controlled trial were used for a comparison²⁴ (referred to as
380 acute isolates) (Table S1).

381

382 *Bacterial isolation and antimicrobial susceptibility testing*

383 Bile and stool were collected from all cholecystectomy patients for culture. Bile was inoculated into
384 equal volumes of Selenite F broth and Peptone broth and incubated at 37°C overnight. Broth was
385 subcultured onto MacConkey agar and Xylene Lysine Deoxycholate (XLD) agar. After overnight
386 incubation at 37°C, the plates were examined for the growth of Gram-negative bacteria and colonies
387 were identified by API20E (bioMerieux, France). *S.* Typhi were confirmed by slide agglutination
388 using specific antisera (Murex Biotech, Biotech, England).

389

390 For the acute isolates, 5-10 ml of blood was taken from all patients with a clinical suspicion of
391 typhoid fever and inoculated into media containing tryptone soya broth and sodium polyanethol

392 sulphonate (up to 25mL). Blood culture bottles were incubated for up to seven days, with blind sub-
393 cultures at 24 hours, 48 hours, and 7 days, or when the broth became cloudy on sheep blood,
394 chocolate, and MacConkey agar. Presumptive *Salmonella* colonies were identified as above.

395
396 Antimicrobial susceptibility testing was performed by the modified Bauer-Kirby disc diffusion
397 method with zone size interpretation based on CLSI guidelines ⁴⁵. Etests[®] were used to determine
398 MICs following the manufacturer's recommendations (bioMérieux, France). Ciprofloxacin MICs were
399 used to categorise *S. Typhi* isolates as susceptible (≤ 0.06 $\mu\text{g/mL}$), intermediate (0.12-0.5 $\mu\text{g/mL}$) and
400 resistant (≥ 1 $\mu\text{g/mL}$) following CLSI guidelines ⁴⁵.

401

402 *Vi* agglutination assay

403 Two gallbladder isolates of *S. Typhi* (GB003 and GB428) that lacked the *Vi* polysaccharide
404 biosynthesis (*viaB*) operon were grown on LB agar plates supplemented with increasing
405 concentrations (1mM, 85mM and 170mM) of NaCl. *Vi* agglutinations were performed on microscope
406 slides by mixing 10 μl of single colony suspensions with 50 μl of *Vi* antisera (Murex Biotech, Biotech,
407 England). Agglutination was recorded after gently agitating the slide for 1 minute. Two gallbladder
408 isolates of *S. Typhi* (GB125 and GB169) containing the *viaB* operon were used as controls.

409

410 *Whole genome sequencing and SNP analyses*

411 Total genomic DNA from acute and gallbladder *S. Typhi* isolates was extracted using the Wizard
412 Genomic DNA Extraction Kit (Promega, Wisconsin, USA) (Table S1). 50ng of genomic DNA was
413 subjected to library preparation using the Nextera DNA library prep kit; whole genome sequencing
414 (WGS) was performed on an Illumina MiSeq platform following the manufacturer's
415 recommendations to generate 250bp paired end reads.

416

417 Single nucleotide polymorphisms (SNPs) were called using previously described methods⁴⁶. Briefly,
418 all reads were mapped to the reference sequence of *S. Typhi* strain CT18 (Accession no: AL513382),
419 plasmid pHCM1 (AL513383) and pHCM2 (AL513384) using SMALT (version 0.7.4). Candidate
420 SNPs were called against the reference sequence using SAMtools and filtered with a minimal
421 mapping quality of 30 and a quality ratio cut-off of 0.75. The allele at each locus in each isolate was
422 determined by reference to the consensus base in that genome. This process was performed using
423 *samtools mpileup* and by removing low confidence alleles with consensus base quality ≤ 20 , read
424 depth ≤ 5 or heterozygous base calls. SNPs in phage regions, repetitive sequences or recombinant
425 regions were excluded,^{47,48} which resulted in a final set of 2,186 chromosomal SNPs. SNPs were
426 subsequently annotated using the parseSNPTable.py script in the RedDog pipeline (<https://github.com/katholt/RedDog>). From the identified SNPs in *S. Typhi* genomes, a subset of 68 were used to
427 assign *S. Typhi* isolates to previously defined lineages according to the existing extended *S. Typhi*
428 genotyping framework⁴⁹.

430

431 To identify the potential function of genes containing key SNPs, we investigated the known or
432 predicted functions of the identified genes. We identified SNPs occurring exclusively in either acute
433 or gallbladder isolates and genes containing these SNPs were grouped by their predicted or known
434 function based on the *S. Typhi* functional classification scheme developed by the Sanger Institute
435 (www.sanger.ac.uk) using the genome annotation of *S. Typhi* CT18⁵⁰.

436

437 The antimicrobial resistance (AMR) gene and plasmid contents of *S. Typhi* isolates were determined
438 using a local assembly approach with ARIBA (Antimicrobial Resistance Identifier by Assembly)⁵¹.
439 Resfinder⁵² and Plasmidfinder⁵³ were used as reference databases of antimicrobial resistance genes
440 and plasmid replicons, respectively.

441

442

443 *Phylogenetic analyses and pairwise SNP distance*

444 A maximum likelihood phylogenetic tree was reconstructed from the SNP alignment of 120 *S. Typhi*
445 isolates (an *S. Paratyphi A* isolate was included as an outgroup) using RAxML (version 8.2.8) with
446 the generalized time-reversible model and a Gamma distribution to model the site-specific rate
447 variation (GTR+ Γ). Support for the maximum likelihood (ML) tree was assessed via bootstrap
448 analysis with 1,000 pseudoreplicates. Pairwise phylogenetic distances depicting the phylogenetic
449 branch length separating each pair of taxa within subclade 4.3.1 (H58) were estimated using the
450 function *cophenetic* in the ape package (v4.1) in R (v3.3.2). Phylogenetic distances between each
451 taxon and its nearest neighbour on the phylogenetic tree of subclade 4.3.1 were plotted using ggplot2.
452 To investigate the phylogenetic structure of acute and gallbladder *S. Typhi* isolates from Nepal in the
453 global context, a second maximum likelihood tree was inferred from a separate alignment of 23438
454 SNPs identified from 120 Nepali *S. Typhi* along with 1820 globally representative *S. Typhi* described
455 previously⁵⁴. A *S. Paratyphi A* isolate was included as an outgroup to root the tree. Support for this
456 ML tree was assessed via 100 bootstrap replicates.

457

458 Pairwise genetic distances (the difference in the number of SNPs) within and between acute and
459 gallbladder *S. Typhi* isolates were estimated from the SNP alignment using the ape (v4.1) and
460 adegenet (v2.0.1) packages in R (v3.3.2). Pairwise SNP distances were extracted and plotted using the
461 function *pairDistPlot* in the adegenet package. The Wilcoxon rank-sum test was used for testing the
462 difference in the average pairwise SNP distances between groups.

463

464 **Declarations**

465 *Ethics approval and consent to participate*

466 This study was conducted according to the principles expressed in the Declaration of Helsinki and
467 was approved by the institutional ethical review boards of Patan Hospital, The Nepal Health Research
468 Council and The Oxford University Tropical Research Ethics Committee (OXTREC, Reference

469 number: 2108). All enrollees were required to provide written informed consent for the collection and
470 storage of all samples and subsequent data analysis. In the case of those under 18 years of age, a
471 parent or guardian was asked to provide written informed consent.

472

473 *Consent for publication*

474 Consent for publication was incorporated as a component of entrance into the study.

475

476 *Availability of data and materials*

477 The raw sequence data generated from this study are available in the European Nucleotide Archive
478 (ENA) under the accession numbers described in Table S1.

479

480 *Competing interests*

481 The authors declare no competing interests.

482

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488 publication.

489

490 *Authors' contributions*

491 Conceptualization: SB

492 Formal analysis: PTD, NTVT, NTNT, MAR

493 Provided samples: SD, AK, BB

494 Methodology: PTD, NTVT, NTNT, HNDD, SD, AK, MC, MAR

495 Writing original draft: DPT, MAR, SB

496 Review and editing: DPT, MC, GD, MAR, SB

497 Read and approved final version of manuscript: PDT, NTVT, NTNT, HNDD, SD, AK, MC, BB, GD,

498 MAR, SB

499

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503

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