

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

2

3 Pham Thanh Duy <sup>1,3</sup>, Nga Tran Vu Thieu <sup>1</sup>, Nguyen Thi Nguyen To <sup>1</sup>, Ho Ngoc Dan Thanh <sup>1</sup>,

4 Sabina Dongol <sup>2</sup>, Abhilasha Karkey <sup>2</sup>, Megan Carey <sup>4</sup>, Buddha Basnyat <sup>2</sup>, Gordon Dougan <sup>4</sup>,

5 Maia A Rabaa <sup>1,3\*</sup>, and Stephen Baker <sup>4\*c</sup>

6

7 <sup>1</sup> The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University

8 Clinical Research Unit, Ho Chi Minh City, Vietnam

9 <sup>2</sup> Oxford University Clinical Research Unit, Patan Academy of Health Sciences, Kathmandu, Nepal

10 <sup>3</sup> Centre for Tropical Medicine, University of Oxford, Oxford, United Kingdom

11 <sup>4</sup> Cambridge Institute of Therapeutic Immunology & Infectious Disease (CITIID) Department of

12 Medicine, University of Cambridge, Cambridge, UK

13

14 \* Joint senior authors

15 <sup>c</sup> Corresponding author: Corresponding author: Prof. Stephen Baker, Cambridge Institute of

16 Therapeutic Immunology & Infectious Disease (CITIID), Cambridge Biomedical Campus, University

17 of Cambridge, Cambridge, United Kingdom CB2 0AW; Email: [sgb47@medschl.cam.ac.uk](mailto:sgb47@medschl.cam.ac.uk)

18

19 **Running title**

20 *Salmonella* Typhi in the human gallbladder

21

22

23

24

25

26

27

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.

48

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 <sup>1</sup>. The disease is contracted via ingestion  
58 of contaminated food or water or through contact with individuals excreting the organism <sup>2</sup>. 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 20<sup>th</sup> century <sup>3,4</sup>.

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) <sup>5</sup>. 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 <sup>3,5</sup>. Consequently, chronic carriers are widely believed to be an ecological  
70 niche that facilitates the transmission and persistence of typhoid in human populations <sup>6</sup>. *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 <sup>7-13</sup>. Various epidemiological investigations have shown that gallstones and

80 gallbladder damage may facilitate typhoid carriage<sup>9,13–17</sup>, and that *Salmonella* preferentially attach to,  
81 and form biofilms on, cholesterol-rich gallstones<sup>7,11,13,18,19</sup>. *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<sup>20–23</sup>. 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 Kathmandu<sup>13</sup>.  
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 comparison<sup>24</sup> (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.

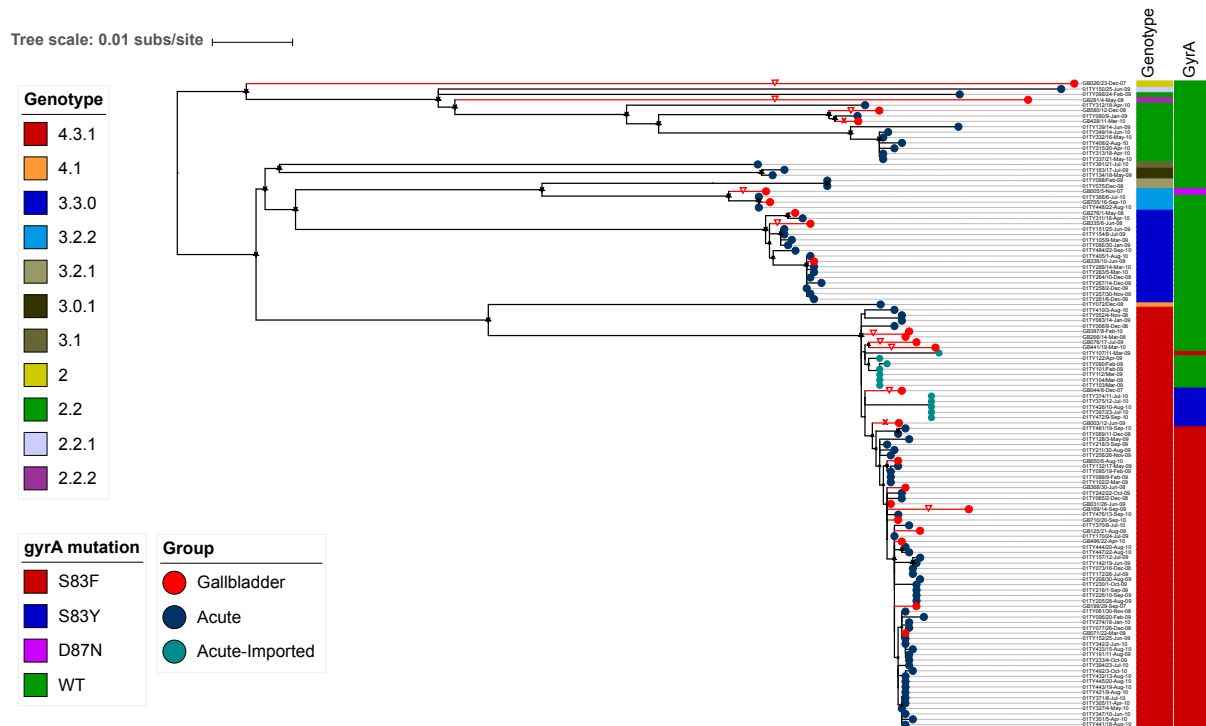
110

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*

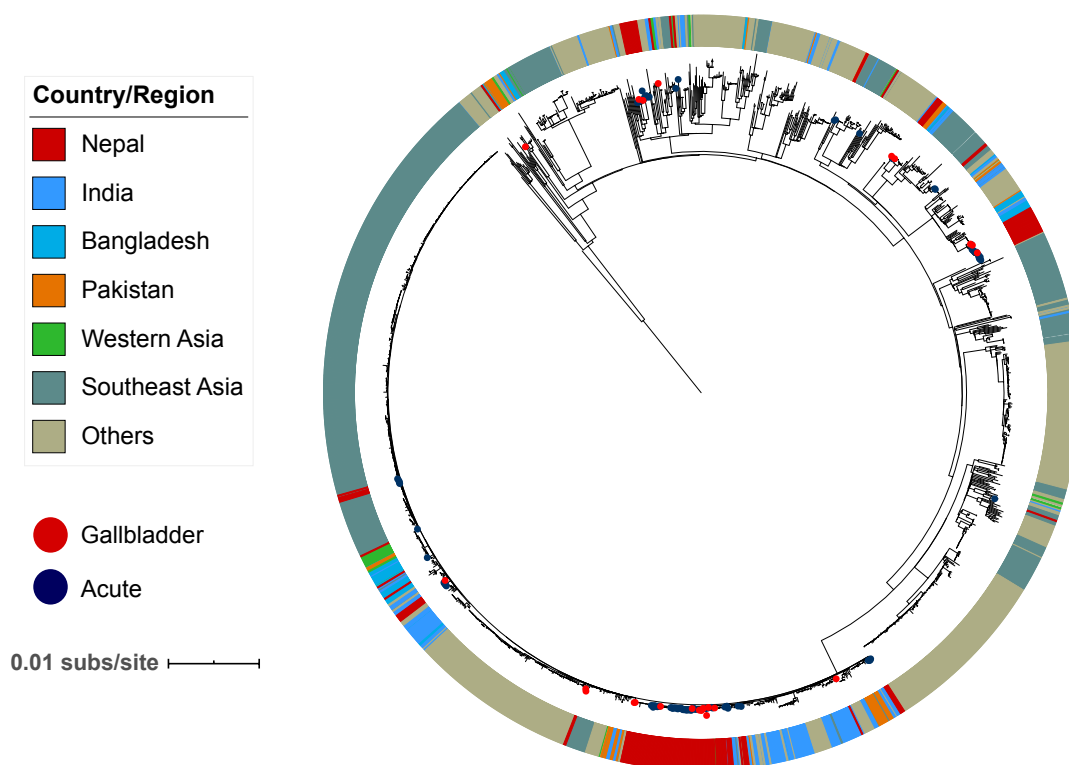
136 Despite the acute and gallbladder *S. Typhi* isolates generally clustering within the same genotypes  
137 across the phylogeny, we observed that a substantial proportion of the gallbladder isolates had higher  
138 genetic variability, which could be distinguished by long terminal branches (Figure 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.

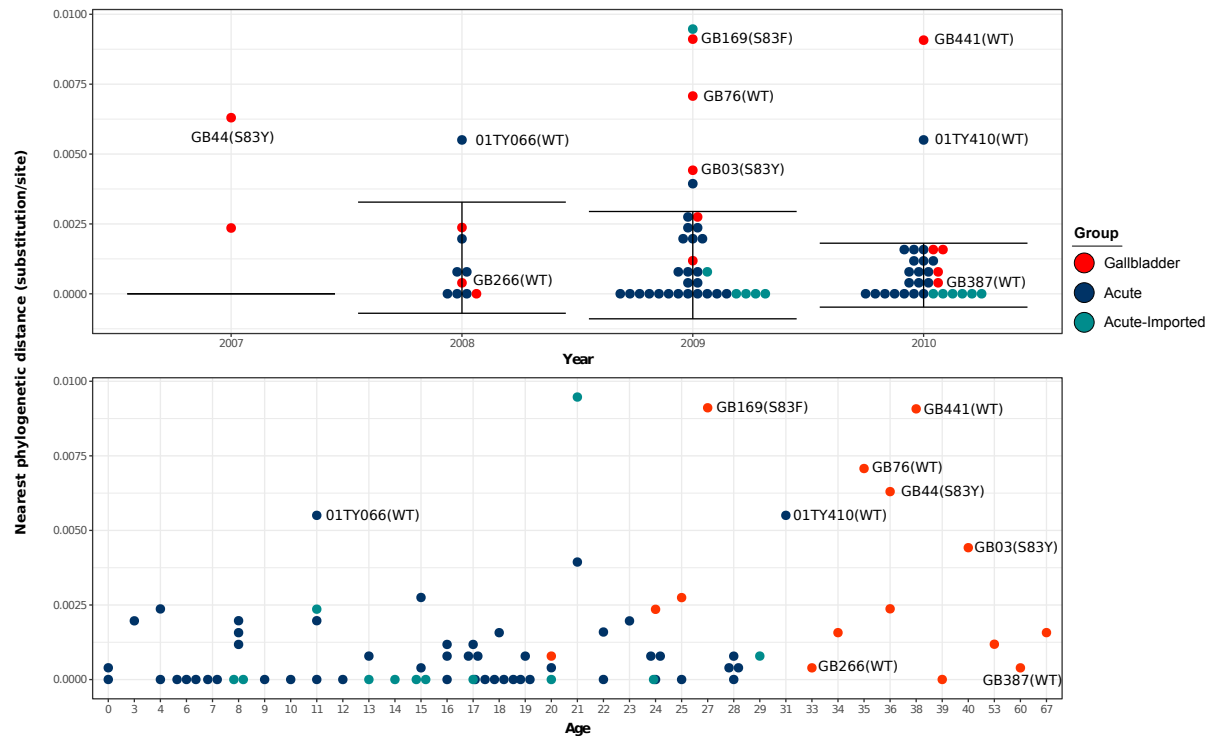


158 increasingly distinct from contemporaneous acute isolates, leading to greater phylogenetic distances  
159 relative to their neighbours. In addition, given that all acute subclade 4.3.1 isolates here exhibited a  
160 *gyrA* mutation, the gallbladder subclade 4.3.1 isolates without a *gyrA* mutation 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

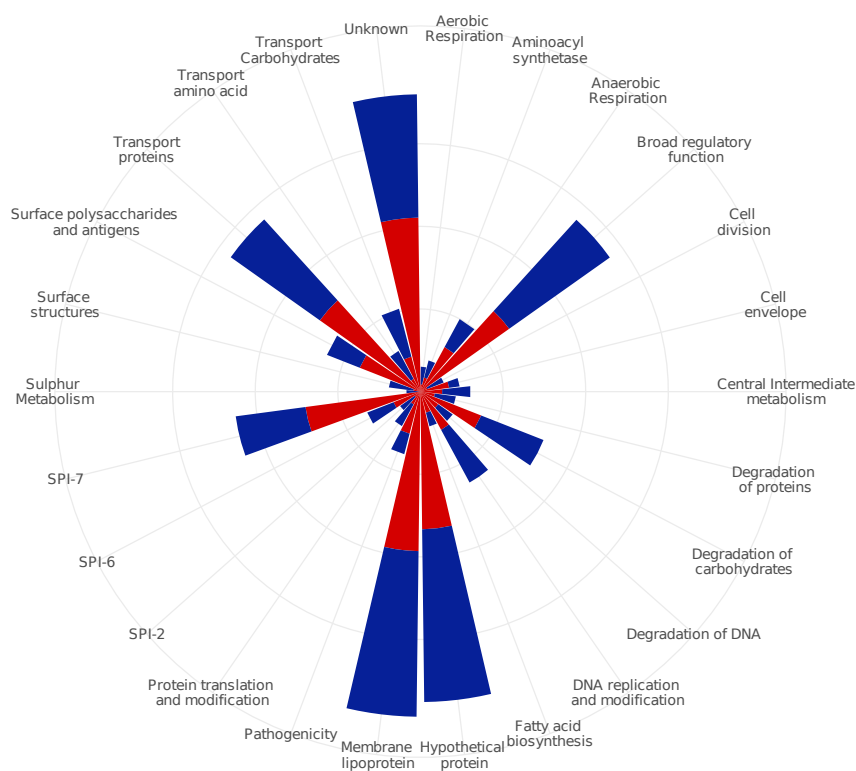


210 proteins were higher in gallbladder than acute isolates (Figure 3). Notably, the data showed that the  
211 proportion of NSs in the *viaB* operon (encoding the Vi antigen, target of the typhoid conjugate  
212 vaccine (TCV)) was significantly higher in gallbladder isolates compared to the acute 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 capsule<sup>25</sup>.  
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<sup>26</sup>. 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 suggestions<sup>27</sup>, 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 patients<sup>28-31</sup>, 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*<sup>32,33</sup>.

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<sup>34,35</sup>. 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<sup>36,37</sup>.  
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 carriage<sup>19</sup>. 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 plasma<sup>38</sup> 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 Vi<sup>39-41</sup>. 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<sup>42,43</sup>. LPS is also a  
339 key structural component of the biofilm extracellular matrix forming on human gallstones<sup>19</sup>. The

340 disruption of genes involved in LPS biosynthesis of *S. Typhimurium* may have a negative influence  
341 on biofilm production and attachment<sup>44</sup>. 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 typhoid<sup>13</sup>.

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 Kathmandu<sup>13</sup>. 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 comparison<sup>24</sup> (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 <sup>45</sup>. Etests<sup>®</sup> 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 ( $\leq 0.06 \mu\text{g/mL}$ ), intermediate ( $0.12\text{-}0.5 \mu\text{g/mL}$ ) and  
400 resistant ( $\geq 1 \mu\text{g/mL}$ ) following CLSI guidelines <sup>45</sup>.

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 $\mu\text{l}$  of single colony suspensions with 50 $\mu\text{l}$  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 methods<sup>46</sup>. 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  $\leq 20$ , read  
424 depth  $\leq 5$  or heterozygous base calls. SNPs in phage regions, repetitive sequences or recombinant  
425 regions were excluded,<sup>47,48</sup> 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 framework<sup>49</sup>.

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](http://www.sanger.ac.uk)) using the genome annotation of *S. Typhi* CT18<sup>50</sup>.

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)<sup>51</sup>.  
439 Resfinder<sup>52</sup> and Plasmidfinder<sup>53</sup> 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+ $\Gamma$ ). 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 previously<sup>54</sup>. 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

#### 483 *Funding*

484 This work was supported by a Wellcome senior research fellowship to Stephen Baker to  
485 (215515/Z/19/Z). DTP is funded as leadership fellow through the Oak Foundation. The funders had  
486 no role in the design and conduct of the study; collection, management, analysis, and interpretation of  
487 the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for  
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

#### 500 *Acknowledgments*

501 We wish to acknowledge all members of the enteric infections group at Oxford University Clinical

502 Research Unit (OUCRU) in Vietnam and Nepal and the study team at Patan Hospital.

503

#### 504 **References**

505 1 Crump JA, Crump JA, Luby SP, Luby SP, Mintz ED, Mintz ED. The global burden of typhoid  
506 fever. *Bull World Health Organ* 2004; **82**: 346–53.

507 2 Schwartz E. Typhoid and Paratyphoid Fever. *Trop Dis Travel* 2010; **366**: 144–53.

508 3 Ledingham JCG. Mr N the milker , and Dr Koch ' s concept of the healthy carrier. *Lancet*  
509 1999; **353**: 1354–6.

510 4 Carrier of typhoid fever-1912. ; i.

511 5 Parry CM, Hien TT, Dougan G, *et al.* Typhoid fever. *N Engl J Med* 2002; **347**: 1770–82.

512 6 Gonzalez-Escobedo G, Marshall JM, Gunn JS. Chronic and acute infection of the gall bladder  
513 by *Salmonella* Typhi: understanding the carrier state. DOI:10.1038/nrmicro2490.

514 7 Prouty AM, Schwesinger WH, Gunn JS. Biofilm formation and interaction with the surfaces of  
515 gallstones by *Salmonella* spp. *Infect Immun* 2002; **70**: 2640–9.

516 8 Basnyat B, Baker S. Typhoid carriage in the gallbladder. *Lancet*. 2015; **386**: 1074.

517 9 Schiøler H, Christiansen ED, Høybye G, Rasmussen SN, Greibe J. Biliary calculi in chronic  
518 *Salmonella* carriers and healthy controls: a controlled study. *Scand J Infect Dis* 1983; **15**: 17–  
519 9.

520 10 Marshall JM, Flechtner AD, La Perle KM, Gunn JS. Visualization of extracellular matrix

- 521 components within sectioned Salmonella biofilms on the surface of human gallstones. *PLoS*  
522 *One* 2014; **9**. DOI:10.1371/journal.pone.0089243.
- 523 11 Crawford RW, Rosales-Reyes R, Ramirez-Aguilar M d. I. L, Chapa-Azuela O, Alpuche-  
524 Aranda C, Gunn JS. Gallstones play a significant role in Salmonella spp. gallbladder  
525 colonization and carriage. *Proc Natl Acad Sci* 2010; **107**: 4353–8.
- 526 12 Gonzalez-Escobedo G, Gunn JS. Gallbladder epithelium as a niche for chronic salmonella  
527 carriage. *Infect Immun* 2013; **81**: 2920–30.
- 528 13 Dongol S, Thompson CN, Clare S, *et al.* The Microbiological and Clinical Characteristics of  
529 Invasive Salmonella in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal.  
530 *PLoS One* 2012; **7**. DOI:10.1371/journal.pone.0047342.
- 531 14 Levine MM, Black RE, Lanata C. Precise estimation of the numbers of chronic carriers of  
532 Salmonella typhi in Santiago, Chile, an endemic area. *J Infect Dis* 1982; **146**: 724–6.
- 533 15 Mian MF, Pek EA, Chenoweth MJ, Coombes BK, Ashkar AA. Humanized mice for  
534 Salmonella typhi infection: new tools for an old problem. *Virulence* 2011; **2**: 248–52.
- 535 16 Mateen MA, Saleem S, Rao PC, Reddy PS, Reddy DN. Ultrasound in the diagnosis of  
536 Typhoid fever. *Indian J Pediatr* 2006; **73**: 681–5.
- 537 17 Mathur R, Oh H, Zhang D, *et al.* A mouse model of salmonella typhi infection. *Cell* 2012;  
538 **151**: 590–602.
- 539 18 Crawford RW, Gibson DL, Kay WW, Gunn JS. Identification of a bile-induced  
540 exopolysaccharide required for salmonella biofilm formation on gallstone surfaces. *Infect*  
541 *Immun* 2008; **76**: 5341–9.
- 542 19 Marshall JM, Flechtner AD, La Perle KM, Gunn JS, Heuser J. Visualization of Extracellular  
543 Matrix Components within Sectioned Salmonella Biofilms on the Surface of Human  
544 Gallstones. *PLoS One* 2014; **9**: e89243.
- 545 20 Yap KP, Gan HM, Teh CSJ, *et al.* Genome sequence and comparative pathogenomics analysis  
546 of a salmonella enterica serovar typhi strain associated with a typhoid carrier in Malaysia. *J.*

- 547 Bacteriol. 2012; **194**: 5970–1.
- 548 21 Baddam R, Kumar N, Shaik S, Lankapalli AK, Ahmed N. Genome dynamics and evolution of  
549 Salmonella Typhi strains from the typhoid-endemic zones. *Sci Rep* 2014; **4**.  
550 DOI:10.1038/srep07457.
- 551 22 Ong SY, Pratap CB, Wan X, *et al.* The Genomic Blueprint of Salmonella enterica subspecies  
552 enterica serovar Typhi P-stx-12. *Stand Genomic Sci* 2013; **7**: 483–96.
- 553 23 Baddam R, Kumar N, Shaik S, *et al.* Genome sequencing and analysis of Salmonella enterica  
554 serovar Typhi strain CR0063 representing a carrier individual during an outbreak of typhoid  
555 fever in Kelantan, Malaysia. *Gut Pathog* 2012; **4**: 20.
- 556 24 Koirala S, Basnyat B, Arjyal A, *et al.* Gatifloxacin versus ofloxacin for the treatment of  
557 uncomplicated enteric fever in Nepal: an open-label, randomized, controlled trial. *PLoS Negl*  
558 *Trop Dis* 2013; **7**: e2523.
- 559 25 Virlogeux I, Waxin H, Ecobichon C, Popoff MY. Role of the *viaB* locus in synthesis, transport  
560 and expression of Salmonella typhi Vi antigen. *Microbiology* 1995; **141**: 3039–47.
- 561 26 Steinsiek S, Bettenbrock K. Glucose transport in Escherichia coli mutant strains with defects  
562 in sugar transport systems. *J Bacteriol* 2012; **194**: 5897–908.
- 563 27 Hatta M, Pastoor R, Scheelbeek PFD, *et al.* Multi-locus variable-number tandem repeat  
564 profiling of Salmonella enterica serovar Typhi isolates from blood cultures and gallbladder  
565 specimens from Makassar, South-Sulawesi, Indonesia. *PLoS One* 2011; **6**.  
566 DOI:10.1371/journal.pone.0024983.
- 567 28 Baker S, Holt K, Van De Vosse E, *et al.* High-throughput genotyping of Salmonella enterica  
568 serovar Typhi allowing geographical assignment of haplotypes and pathotypes within an urban  
569 district of Jakarta, Indonesia. *J Clin Microbiol* 2008; **46**: 1741–6.
- 570 29 Holt KE, Baker S, Dongol S, *et al.* High-throughput bacterial SNP typing identifies distinct  
571 clusters of Salmonella Typhi causing typhoid in Nepalese children. *BMC Infect Dis* 2010; **10**:  
572 144.

- 573 30 Baker S, Holt KE, Clements ACA, *et al.* Combined high-resolution genotyping and geospatial  
574 analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol* 2011; **1**:  
575 110008.
- 576 31 Holt KE, Dolecek C, Chau TT, *et al.* Temporal Fluctuation of Multidrug Resistant Salmonella  
577 Typhi Haplotypes in the Mekong River Delta Region of Vietnam. *PLoS Negl Trop Dis* 2011;  
578 **5**: e929.
- 579 32 Holt KE, Thomson NR, Wain J, *et al.* Pseudogene accumulation in the evolutionary histories  
580 of Salmonella enterica serovars Paratyphi A and Typhi. *BMC Genomics* 2009; **10**: 36.
- 581 33 McClelland M, Sanderson KE, Clifton SW, *et al.* Comparison of genome degradation in  
582 Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid.  
583 *Nat Genet* 2004; **36**: 1268–74.
- 584 34 Felix A. Detection of chronic typhoid carriers by agglutination tests. *Lancet* 1938; **232**: 738–  
585 41.
- 586 35 Nolan CM, White PC, Feeley JC, Brown SL, Hambie EA, Wong KH. Vi serology in the  
587 detection of typhoid carrierS. *Lancet* 1981; **317**: 583–5.
- 588 36 House D, Ho VA, Diep TS, *et al.* Antibodies to the Vi capsule of Salmonella Typhi in the  
589 serum of typhoid patients and healthy control subjects from a typhoid endemic region. *J Infect*  
590 *Dev Ctries* 2008; **2**: 308–12.
- 591 37 Gupta A, My Thanh NT, Olsen SJ, *et al.* Evaluation of community-based serologic screening  
592 for identification of chronic Salmonella Typhi carriers in Vietnam. *Int J Infect Dis* 2006; **10**:  
593 309–14.
- 594 38 Lanata CF, Ristori C, Jimenez L, *et al.* Vi serology in detection of chronic salmonella typhi  
595 carriers in an endemic area. *Lancet* 1983; **322**: 441–3.
- 596 39 Coynault C, Robbe-Saule V, Norel F. Virulence and vaccine potential of Salmonella  
597 typhimurium mutants deficient in the expression of the RpoS ( $\sigma(S)$ ) regulon. *Mol Microbiol*  
598 1996; **22**: 149–60.

- 599 40 Nickerson CA, Curtiss R. Role of sigma factor RpoS in initial stages of Salmonella  
600 typhimurium infection. *Infect Immun* 1997; **65**: 1814–23.
- 601 41 Santander J, Wanda S-Y, Nickerson CA, Curtiss R, III. Role of RpoS in fine-tuning the  
602 synthesis of Vi capsular polysaccharide in Salmonella enterica serotype Typhi. *Infect Immun*  
603 2007; **75**: 1382–92.
- 604 42 Prouty AM, Van Velkinburgh JC, Gunn JS. Salmonella enterica serovar typhimurium  
605 resistance to bile: Identification and characterization of the tolQRA cluster. *J Bacteriol* 2002;  
606 **184**: 1270–6.
- 607 43 Gunn JS. Mechanisms of bacterial resistance and response to bile. *Microbes Infect* 2000; **2**:  
608 907–13.
- 609 44 Kim SH, Wei CI. Molecular characterization of biofilm formation and attachment of  
610 Salmonella enterica serovar typhimurium DT104 on food contact surfaces. *J Food Prot* 2009;  
611 **72**: 1841–7.
- 612 45 CLSI. Performance Standards for Antimicrobial Susceptibility Testing. Informational  
613 Supplement. 2014 DOI:10.1186/1476-0711-9-23.
- 614 46 Thanh DP, Karkey A, Dongol S, *et al.* A novel ciprofloxacin-resistant subclade of h58.  
615 Salmonella typhi is associated with fluoroquinolone treatment failure. *Elife* 2016; **5**.  
616 DOI:10.7554/eLife.14003.
- 617 47 Roumagnac P, Weill F-X, Dolecek C, *et al.* Evolutionary history of Salmonella typhi. *Science*  
618 2006; **314**: 1301–4.
- 619 48 Holt KE, Parkhill J, Mazzoni CJ, *et al.* High-throughput sequencing provides insights into  
620 genome variation and evolution in Salmonella Typhi. *Nat Genet* 2008; **40**: 987–93.
- 621 49 Wong VK, Baker S, Connor TR, *et al.* An extended genotyping framework for Salmonella  
622 enterica serovar Typhi, the cause of human typhoid. *Nat Commun* 2016; **7**: 12827.
- 623 50 Parkhill J, Dougan G, James KD, *et al.* Complete genome sequence of a multiple drug resistant  
624 Salmonella enterica serovar Typhi CT18. *Nature* 2001; **413**: 848–52.

- 625 51 Hunt M, Mather AE, Sánchez-Busó L, *et al.* ARIBA: rapid antimicrobial resistance  
626 genotyping directly from sequencing reads. *bioRxiv* 2017; : 1–21.
- 627 52 Zankari E, Hasman H, Cosentino S, *et al.* Identification of acquired antimicrobial resistance  
628 genes. *J Antimicrob Chemother* 2012; **67**: 2640–4.
- 629 53 Carattoli A, Zankari E, García-Fernández A, *et al.* In Silico detection and typing of plasmids  
630 using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother*  
631 2014; **58**: 3895–903.
- 632 54 Wong VK, Baker S, Pickard DJ, *et al.* Phylogeographical analysis of the dominant multidrug-  
633 resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission  
634 events. *Nat Genet* 2015; **47**: 632–9.