Distinct *Salmonella* Enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings

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69

70 **Abstract**

71 An epidemiological paradox surrounds *Salmonella enterica* serovar Enteritidis. In
72 high-income settings, it has been responsible for an epidemic of poultry-associated,
73 self-limiting enterocolitis, whilst in sub-Saharan Africa it is a major cause of invasive
74 nontyphoidal *Salmonella* disease, associated with high case-fatality. Whole-genome
75 sequence analysis of 675 isolates of *S*. Enteritidis from 45 countries reveals the
76 existence of a global epidemic clade and two novel clades of *S*. Enteritidis that are
77 each geographically restricted to distinct regions of Africa. The African isolates
78 display genomic degradation, a novel prophage repertoire and have an expanded,
79 multidrug resistance plasmid. *S*. Enteritidis is a further example of a *Salmonella*
80 serotype that displays niche plasticity, with distinct clades that enable it to become a
81 prominent cause of gastroenteritis in association with the industrial production of
82 eggs, and of multidrug resistant, bloodstream invasive infection in Africa.

83

84 **Introduction**

85

86 *Salmonella enterica* serovar Enteritidis (hereafter referred to as *S*. Enteritidis) has
87 been a global cause of major epidemics of enterocolitis, which have been strongly
88 associated with intensive poultry farming and egg production[1]. The serovar is
89 usually considered to be a generalist in terms of host range and has a low human
90 invasiveness index, typically causing self-limiting enterocolitis[2]. Following a number
91 of interventions in the farming industry involving both improved hygiene and
92 poultry vaccination, epidemic *S*. Enteritidis has been in decline in many countries
93 including the United Kingdom and USA[3,4]. *S.* Enteritidis has also been used
94 extensively since the early 1900s as a rodenticide (named the "Danysz virus"),
95 following development at Institut Pasteur, France. Although by the 1960s,
96 *Salmonella*-based rodenticides had been banned in the US, Germany and the UK, *S*.
97 Enteritidis is still produced as a rodenticide in Cuba, under the name Biorat®[5].

98

99 Serovars of *Salmonella* that cause enterocolitis in industrialised settings are strongly
100 associated with life-threatening invasive nontyphoidal *Salmonella* (iNTS) disease in
101 sub-Saharan Africa (SSA). *S*. Enteritidis and *Salmonella enterica* serovar
102 Typhimurium (*S*. Typhimurium) are the two leading causes of iNTS disease in SSA[6]
103 and both are associated with multidrug resistance (MDR)[7]. The clinical syndrome
104 iNTS disease is associated with immunosuppression in the human host, particularly
105 malnutrition, severe malaria and advanced HIV in young children and advanced HIV
106 in adults[8]. It has been estimated to cause 681,000 deaths per year[9].

107

108 *Salmonella* is a key example of a bacterial genus in which there is a recognizable
109 genomic signature that distinguishes between a gastrointestinal and an extra-
110 intestinal/invasive lifestyle[10], whereby functions required for escalating growth in
111 an inflamed gut are lost when the lineage becomes invasive[11]. In order to investigate
112 whether there were distinct bacterial characteristics explaining the very different
113 epidemiological and clinical profile of epidemic isolates of serotype *S*. Typhimurium

114 from SSA and industrialised settings, whole-genome sequence (WGS) investigations
115 of this serovar were previously undertaken. These revealed a novel pathotype of
116 multilocus sequence type (MLST) ST313 from SSA, which differed from clades that
117 cause enterocolitis in industrialised settings, by showing patterns of genomic
118 degradation potentially associated with more invasive disease and differential host
119 adaptation[12-17].
120
121 In relation to *S.* Enteritidis, there is a growing body of literature on the evolutionary
122 history, phylogeny and utility of WGS for surveillance of *S*. Enteritidis outbreaks[18-20].
123 The broadest study of the phylogeny to date revealed five major lineages, but
124 contained only two African isolates[21]. There have also been limited reports of
125 isolates of *S.* Enteritidis from African patients living in Europe that are MDR and
126 which display a distinct phage type (PT 42)[22,23]. We therefore hypothesized that
127 there are distinct lineages of *S*. Enteritidis circulating in both the industrialised and
128 developing world with different origins, likely distinct routes of spread and that are
129 associated with different patterns of disease, which will display the distinct genomic
130 signatures characteristic of differential adaptation. To investigate this we have
131 collected a highly diverse global collection of *S*. Enteritidis isolates and compared
132 them using whole-genome sequencing, the highest possible resolution typing
133 methodology.
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142 **Results**

143

144 **Isolate collection**

145

146 In total, 675 isolates of *S.* Enteritidis isolated between 1948 and 2013 were
147 sequenced. The collection originated from 45 countries and six continents (Table 1).
148 496/675 isolates were from Africa, with 131 from the Republic of South Africa
149 (RSA), a further 353 from the rest of SSA, and 12 from North Africa (Table 1). There
150 were 343 isolates from normally sterile human sites (invasive), 124 non-invasive
151 human isolates (predominantly stool samples) and 40 from animal, food or
152 environmental sources. The full metadata are described in Supplementary Table 1
153 and have been uploaded to the publically available database Enterobase.

154

155 **Phylogeny**

156

157 675 *S.* Enteritidis genomes and one *Salmonella enterica* serovar Gallinarum were
158 mapped to the *S.* Enteritidis strain P125109 reference sequence, variable regions
159 excluded and the remaining sites were screened for single nucleotide
160 polymorphisms (SNPs). This left an alignment containing a total of 42,373 variable
161 sites, from which a maximum likelihood (ML)-phylogeny was constructed using *S*.
162 Gallinarum, which is a closely related serovar, as an out-group (Figure 1). HierBAPS
163 was run over two rounds, which provided clear distinction between
164 clades/clusters[24]. The phylogeny of *S.* Enteritidis revealed evidence of three clades
165 associated with epidemics, one which we have termed the 'global epidemic clade'
166 and includes the reference PT4 isolate P125109 and two African clades: one
167 predominantly composed of West African isolates (labeled the 'West African clade')
168 and a second composed of isolates predominantly originating in Central and Eastern
169 Africa, called the 'Central/Eastern African clade'). Figure 1 also shows the other
170 clades and clusters predicted by HierBAPS, the largest of which is a paraphyletic

171 cluster from which the global epidemic clade emerged (Outlier Cluster in Figure 1),
172 and a further five smaller clades or clusters predicted by HierBAPS.
173
174 The global epidemic clade contains isolates of multiple phage types, including 4 and
175 1, which have been linked to the global epidemic of poultry associated human
176 enterocolitis[25]. It comprised 250 isolates from 28 countries, including 43 from
177 Malawi and 82 from RSA. They were isolated from across a 63-year period (1948-
178 2013). Antimicrobial susceptibility testing had been performed on 144 isolates and
179 104 were susceptible to all antimicrobials tested, five were multidrug resistant
180 (MDR: resistant to 3 or more antimicrobial classes), one was nalidixic acid resistant
181 and none were extended-spectrum beta-lactamase (ESBL)-producing isolates.
182 Database comparison of the genomes from this clade revealed that 221 (88%) of
183 them contained no predicted antimicrobial resistance (AMR) genes apart from the
184 cryptic resistance gene *aac(6')-Iy*[26].
185
186 The global epidemic clade has emerged from a diverse cluster previously described
187 by Zheng[27], which encompassed 131 isolates (Figure 1: 'Outlier Cluster'). In addition
188 to being paraphyletic, this group was geographically and temporally diverse, and
189 predominantly drug susceptible (59/71 isolates). Whilst the majority of the
190 diversity of phage typed isolates was contained within the global epidemic clade,
191 this cluster alone contained isolates of phage type 14b, which was recently
192 associated with a multi-country outbreak of *S*. Enteritidis enterocolitis in Europe
193 associated with chicken eggs from Germany[28]. There were also 41 isolates from RSA
194 in this clade, where it has been a common cause of bloodstream infection, and 39
195 bloodstream isolates from Malawi. Database comparison of the genomes from this
196 clade revealed that 122 (82%) of these genomes contained no predicted AMR genes
197 apart from the cryptic resistance gene *aac(6')-Iy*.
198
199 There were two related, but phylogenetically and geographically distinct, epidemic
200 clades that largely originated from SSA. The Central/Eastern African clade included

201 166 isolates, all but two of which (from RSA) came from this region. Of these,
202 126/155 (82%) were MDR and 148/153 (97%) displayed phenotypic resistance to
203 between one and four antimicrobial classes. All of these genomes contained at least
204 five predicted resistance genes and 128 (77%) contained nine (Table 2 and
205 Supplementary Table 2). 155/165 (94%) of these isolates were cultured from a
206 normally sterile compartment of a human (i.e. blood or cerebrospinal fluid) and
207 were considered to be causing invasive disease (Table 2). The second African
208 epidemic clade was significantly associated with West Africa with 65/66 isolates
209 coming from this region and one isolate from USA. This clade was also associated
210 with drug resistance (62 [94%] resistant to ≥1 antimicrobial class by phenotype and
211 genotype) and human invasive disease (61 [92%]). It also included two isolates that
212 were subtyped as phage type 4.
213
214 The remaining 58 isolates included in this study were extremely diverse,
215 phylogenetically, temporally and geographically. Only two displayed any phenotypic
216 AMR, one of which was MDR. Inspection of the genome revealed that five had
217 predicted AMR genes in addition to *aac(6')-Iy*, four of which were isolated in sub-
218 Saharan Africa. Twenty were associated with invasive human disease, and six were
219 recovered from stool. Three isolates were from stocks of rodenticide and these were
220 phylogenetically remote from both global-epidemic and the two African epidemic
221 clades.
222
223 To add further context to these findings we screened the entire publically available
224 Public Health England (PHE) sequenced *Salmonella* routine surveillance collection,
225 which includes 2,986 *S*. Enteritidis genomes, 265 of which were associated with
226 travel to Africa (Supplementary Figure 1). Within this huge collection, including 61
227 (2.0%) bloodstream isolates and 2670 (89.4%) stool isolates, only 6 isolates (4 from
228 blood culture, 1 from stool) fell within to the West African clade and 1 (from stool)
229 belonged to the Central/Eastern African clade. Notably, these isolates were all either
230 associated with travel to Africa and/or taken from patients of African origin.

231 It is apparent from the location of the archetypal reference isolate and archetypal
232 phage types in the phylogeny (Supplementary Figure 2) that the majority of *S*.
233 Enteritidis studied previously belonged to the global epidemic clade associated with
234 enterocolitis in industrialised countries. Furthermore, its also clear that two
235 additional, previously unrecognized *S*. Enteritidis lineages have emerged, largely
236 restricted to Africa, that are strongly associated with MDR and invasive disease.
237
238 To understand how recently these Africa-associated lineages emerged we used
239 Bayesian Evolutionary Analysis by Sampling Trees (BEAST) to reconstruct the
240 temporal history of the epidemic clades[29]. These data (Supplementary Figure 3)
241 estimate the most recent common ancestor (MRCA) of the Central/Eastern African
242 clade dates to 1945 (95% Credible Interval [CrI]: 1924-1951) and for the West
243 African clade it was 1933 (95% CrI: 1901-1956). We estimate the MRCA of the
244 global epidemic clade originated around 1918 (95% CrI: 1879-1942 –
245 Supplementary Figure 4), with a modern expansion occurring in 1976 (95% CrI:
246 1968-1983), whereas the paraphyletic cluster from which it emerged dates to
247 approximately 1711 (95% CrI: 1420-1868).
248
249 **Contribution of the accessory genome**
250
251 Prophages have the potential to carry non-essential "cargo" genes, which suggests
252 they confer a level of specialization to their host bacterial species, whilst plasmids
253 may confer a diverse array of virulence factors and AMR [30,31]. Therefore it is critical
254 to evaluate the accessory genome in parallel with the core. 622 sequenced genomes
255 were used to determine a pangenome, which yielded a core genome comprising
256 4,076 predicted genes present in ≥90% isolates, including all 12 recognised
257 *Salmonella* Pathogenicity Islands as well as all 13 fimbrial operons found in the
258 P125109 reference[32]. The core gene definition was set to minimize stochastic loss of
259 genes from the core due to errors in individual assemblies across such a large
260 dataset. The accessory genome consisted of 14,015 predicted genes. Of the

261 accessory genes, 324 were highly conserved across the global and two African
262 epidemic clades, as well as the outlier cluster. Almost all were associated with the
263 acquisition or loss of mobile genetic elements (MGEs) such as prophage or plasmids.
264 Prophage regions have been shown to be stable in *Salmonella* genomes and are
265 potential molecular markers, the presence of which has previously been used to
266 distinguish specific clades[13,33].
267
268 The lineage-specific whole gene differences of the major clades are summarized in
269 Figure 2 and plotted against the representatives of the four major clades in
270 Supplementary Figure 5. The lineage specific sequence regions include 57 predicted
271 genes found to be unique to the global epidemic clade (Figure 2), all of which were
272 associated with prophage ϕSE20, a region shown to be essential for invasion of
273 chicken ova and mice in one previous study[34]. There were a further 39 genes
274 conserved in the global epidemic and the paraphyletic outlying cluster, which were
275 absent from both African clades, 26 of which correspond to region of difference
276 (ROD) 21[32]. The Central/Eastern Africa clade contained 77 predicted genes that
277 were absent in the other clades. 33 were associated with the virulence plasmid and
278 a further 40 chromosomal genes were associated with a novel, Fels-2 like prophage
279 region (ϕ*fels-BT*). The West African clade had only 15 distinct predicted genes, 11 of
280 which were plasmid-associated. The two African clades shared a further 102 genes:
281 48, including a leucine-rich repeat region, were associated with a novel prophage
282 region closely related to *Enterobacter* phage P88, 44 were associated with a Gifsy-1
283 prophage found in *S*. Bovismorbificans and eight were associated with a Gifsy-2
284 prophage which has degenerated in the reference P125109.
285
286 The *S.* Enteritidis plasmid is the smallest of the generic *Salmonella* virulence
287 plasmids at 58 kb and is unusual in that it contains an incomplete set of *tra* genes
288 that are responsible for conjugative gene transfer. The phylogeny of the *S*.
289 Enteritidis virulence plasmid backbone was reconstructed using reads that mapped
290 to the *S.* Enteritidis reference virulence plasmid, pSENV. 120/675 (18%) genomes

291 lacked pSENV. The virulence plasmid phylogeny is similar to that of the
292 chromosome, suggesting that they have been stably maintained by each lineage and
293 diversified alongside them (Supplementary Figure 6).
294
295 The virulence plasmids from the African clades were much larger than those held in
296 the other clades at ~90 kb. A representative example was extracted from Malawian
297 isolate D7795, sequenced using long read technology to accurately reconstruct it
298 (PacBio; see methods) and denoted pSEN-BT (Accession number LN879484). pSEN-
299 BT is composed of a backbone of pSENV with additional regions that are highly
300 similar to recently sequenced fragments of an novel *S.* Enteritidis virulence plasmid
301 (pUO-SeVR) isolated from an African patient presenting with MDR invasive *S.*
302 Enteritidis in Spain[22]. Plasmid pSEN-BT harbours nine AMR genes (full list in
303 Supplementary Table 2), plus additional genes associated with virulence and a
304 toxin/antitoxin plasmid addiction system. Of note, plasmids from the West African
305 isolates carry resistance gene chloramphenicol acetyl transferase A1 (*cat*A1),
306 whereas the Central/Eastern African strains carry *cat*A2 and tetracycline resistance
307 gene *tet*(A). Like pSENV, the African virulence plasmid contained an incomplete set
308 of *tra* genes and so is not self-transmissible. This was confirmed by conjugation
309 experiments and is consistent with previous reports[22,23]. These observations
310 suggest that the evolution of the *S.* Enteritidis plasmid mirrors that of the
311 chromosome; it is thus not a 'novel' plasmid, but in different SSA locations has
312 acquired different AMR genes.
313
314 **Multiple signatures of differential host adaptation**
315
316 It has been observed in multiple serovars of *Salmonella* including *S*. Typhi, *S*.
317 Gallinarum and *S*. Typhimurium ST313 that the degradation of genes necessary for
318 the utilization of inflammation-derived nutrients is a marker of that lineage having
319 moved from an intestinal to a more invasive lifestyle[13,14,32,35]. Accordingly, we have
320 looked for similar evidence within a representative example of a MDR, invasive,

321 Central/Eastern African clade isolate, D7795, that was isolated from the blood of a
322 Malawian child in 2000. The draft genome sequence of D7795 closely resembles
323 that of P125109, however, in addition to the novel prophage repertoire and plasmid
324 genes described above, it harbours a number of predicted pseudogenes or
325 hypothetically disrupted genes (HDGs)[11].
326
327 In total, there were 42 putative HDGs in D7795, many of which are found in genes
328 involved in gut colonisation and fecal shedding as well as various metabolic
329 processes such as cobalamine biosynthesis which is a cofactor for anaerobic
330 catabolism of inflammation-derived nutrients, such as ethanolamine, following
331 infection[36]. Curation of the SNPs and insertions or deletions (indels) predicted to be
332 responsible for pseudogenisation across the Central/Eastern African clade and West
333 African clade revealed 37/42 predicted HDGs were fixed in other representatives of
334 the Central/East African clade, with 27 of them being present in over 90% of
335 isolates from that clade. Relatively fewer HDGs in D7795 (19/42) were present in
336 representatives of the West African clade, although 13 were present in ≥90% of
337 isolates (Supplementary Table 3).
338
339 In addition to this evidence of reductive evolution in D7795, there were 363 genes
340 containing non-synonymous (NS)-SNPs, which change the amino acid sequence and
341 so may have functional consequences[37]. The two African clades were screened for
342 the presence of these NS-SNPs and 131 were found to be present and completely
343 conserved across both clades, including NS-SNPs in 43 genes encoding predicted
344 membrane proteins, 36 metabolic genes and 23 conserved hypothetical genes
345 (Supplementary Table 4). Furthermore many of these NS-SNPs fall in genes within
346 the same metabolic pathways as the HDGs (see Supplementary Note for detailed
347 description). Supplementary Table 5 provides a list of some of the common traits
348 identified amongst the functions of genes lost independently by D7795, *S.* Typhi and
349 *S*. Gallinarum. The disproportionate clustering of mutations in membrane structures

350 observed in the African clades is yet another sign of differential host adaptation
351 analogous to that reported in both *S*. Typhi[35] and *S*. Gallinarum[32].
352
353 **Biolog™ growth substrate platform profiling**
354
355 The Biolog™ platform was utilized to generate a substrate growth utilisation profile
356 for selected *S*. Enteritidis isolates. Corresponding signal values of replicate pairs of a
357 Central/Eastern African isolate (D7795) and a global epidemic isolate (A1636) were
358 compared using principal component analysis and found to be highly consistent. In
359 total, 80 metabolites showed evidence of differential metabolic activity (Figure 3).
360 Evaluation of data from the Central/Eastern African isolate using Pathway Tools
361 software revealed that 14/27 (52%) of pathways with evidence of decreased
362 metabolic activity at 28˚C had a corresponding component of genomic degradation.
363 This was also true for 12/30 (40%) of pathways with evidence of decreased
364 metabolic activity at 37°C.
365
366 Instances of reduced metabolic activity in a Central/Eastern African strain (D7795)
367 compared to a global epidemic strain (A1636) included dulcitol and glycolic acid in
368 the glycerol degradation pathway, propionic acid in the propanediol pathway and
369 ethylamine and ethanolamine. These are all vitamin B12 (cobalamin) dependent
370 reactions, for which there was a corresponding signature of genomic degradation.
371 Also there was reduced activity in response to three forms of butyric acid, alloxan
372 and allantoic acid metabolism. Allantoin can be found in the serum of birds, but not
373 humans and is utilised as a carbon source during *S.* Enteritidis infection of
374 chickens[38], and HDGs relating to allantoin have been noted in *S.* Typhimurium
375 ST313[13]. The full list of differences is detailed in Supplementary Table 6 and 7. This
376 is a further sign of decreased metabolism of the Central/Eastern African isolate in
377 the anaerobic environment of the gut.
378
379 **Chick infection model suggests divide in host range**

380

381 Given the phenotypic differences observed in the genotypically distinct global and
382 African clades, we hypothesized that these lineages could have differing infection
383 phenotypes in an *in vivo* challenge model. We compared the infection profile of a
384 member of the Central/Eastern African clade (D7795) to the reference global
385 epidemic strain P125109 in an avian host. The chicken group infected with P125109
386 showed mild hepatosplenomegaly consistent with infection by this *Salmonella*
387 serovar and cecal colonization (Figure 4A-C). In contrast, the Central/Eastern
388 African strain displayed significantly reduced invasion at 7 dpi of both liver
389 (p=0.027) and spleen (p=0.007), however cecal colonization was not significantly
390 reduced (p=0.160). This is in marked contrast to the behavior of *S*. Typhimurium
391 ST313, which is more invasive in a chick infection model[12].
392
393
394 **Discussion**
395
396 *S.* Enteritidis is an example of a successful *Salmonella* lineage with the apparent
397 ability to adapt to different hosts and transmission niches as and when
398 opportunities for specialization have presented. Langridge *et al* recently evaluated
399 the Enteritidis/Gallinarum/Dublin lineage of *Salmonella*, revealing components of
400 the nature and order of events associated with host-range and restriction[39]. In the
401 present study, we have highlighted the plasticity of *S*. Enteritidis, providing evidence
402 of three distinct epidemics of human disease. In addition we show multiple
403 additional clades and clusters that demonstrate the huge reservoir of diversity
404 amongst *S.* Enteritidis from which future epidemics might emerge.
405
406 An important question posed by this study is why have distinct clades of *Salmonella*
407 emerged to become prominent causes of iNTS disease in Africa, from a serotype
408 normally considered to be weakly invasive? The presence of a highly
409 immunosuppressed population due to the HIV pandemic is clearly a key host factor

410 that facilitates the clinical syndrome iNTS disease[40,41]. In addition to human host
411 factors, there are two distinct African epidemic lineages that have emerged in the
412 last 90 years. Both lineages are significantly associated with a novel prophage
413 repertoire, an expanded, MDR-augmented virulence plasmid, and patterns of
414 genomic degradation with similarity to other host-restricted invasive *Salmonella*
415 serotypes including *S*. Typhi and *S*. Gallinarum and to clades of *S.* Typhimurium
416 associated with invasive disease in Africa[13,32,35]. This pattern of genomic
417 degradation is concentrated in pathways specifically associated with an enteric
418 lifestyle, however it is noteworthy that in the chick infection model, the African *S*.
419 Enteritidis invaded the chick liver and spleen less well than the global pandemic
420 clade. This raises the possibility that the two clades occupy different ecological
421 niches outside the human host or that they behave differently within the human
422 host and screening of the huge *S.* Enteritidis collection from routine *Salmonella*
423 surveillance by PHE supports the assertion that these lineages are geographically
424 restricted to Africa. This study therefore indicates a need to understand what these
425 ecological niches might be, and then to define the transmission pathways of African
426 clades of *S*. Enteritidis, in order to facilitate public health interventions to prevent
427 iNTS disease.
428
429 The evolution of the *S*. Enteritidis virulence plasmid is intriguing; pSENV is the
430 smallest of the known *Salmonella* virulence-associated plasmids, but in SSA, the
431 plasmid has nearly doubled in size partly through the acquisition of AMR genes. The
432 absence of *tra* genes necessary for conjugal transfer either indicates that MDR status
433 has evolved through acquisition of MGEs multiple times or through clonal expansion
434 and vertical transmission of the plasmid to progeny. The available data suggest that
435 the former scenario has happened twice, once in West Africa, and once in
436 Central/Eastern Africa.
437
438 Despite *S*. Enteritidis being reported as a common cause of bloodstream infection
439 (BSI) in Africa[6,7] the Global Enteric Multicenter Study (GEMS) found that *Salmonella*

440 serotypes were an uncommon cause of moderate to severe diarrhoea in African
441 children less than 5-years of age[42]. Our data associating the African lineages with
442 invasive disease is also consistent with data presented in a recent Kenyan study
443 comparing a limited number and diversity of *S*. Enteritidis isolates from blood and
444 stool. Applying the lineages defined in this study to the genome data reported from
445 Kenya showed that 20.4% of isolates from that study belonging to the global clade
446 were associated with invasive disease, whereas 63.2% of the isolates in that study
447 belonging to our Central/Eastern African clade were associated with invasive
448 disease[43]. The remaining isolates were associated with cases of enterocolitis or
449 asymptomatic carriage, confirming that the Central/Eastern African clade can also
450 cause enterocolitis. The association of *S*. Enteritidis clades circulating in sub-
451 Saharan Africa with iNTS disease may reflect the fact that their geographical
452 distribution permits them to behave as opportunistic invasive pathogens in a setting
453 where advanced immunosuppressive disease is highly prevalent in human
454 populations.
455
456 In summary, two clades of *S.* Enteritidis have emerged in Africa, which have
457 different phenotypes and genotypes to the strains of *S*. Enteritidis circulating in the
458 industrial world. These strains display evidence of changing host adaptation,
459 different virulence determinants and multi-drug resistance, a parallel situation to
460 the evolutionary history of *S*. Typhimurium ST313. They may have different
461 ecologies and/or host ranges to global strains and have caused epidemics of BSI in
462 at least three countries in SSA, yet are rarely responsible for disease in South Africa.
463 An investigation into the environmental reservoirs and transmission of these
464 pathogens is warranted and urgently required.
465
466
467
468 **URLs:**
469

470 Enterobase: https://enterobase.warwick.ac.uk/

471

472 **Data access:** Accession numbers for sequencing data including both raw sequencing

473 reads and assembled sequences are available in Supplementary Table 1.

474

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500 LLL, GL, SRH, AEM, MF, MA. Isolate acquisition and processing and clinical data
501 collection: NAF, KHK, JJ, XD, CMe, SK, CMl, RSO, FXW, SLH AMS, MM, PD, CMP, JC, NF,
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504

505 References

506 1. Ward, L.R., Threlfall, J., Smith, H.R. & O'Brien, S.J. Salmonella enteritidis
507 epidemic. *Science* **287**, 1753-4; author reply 1755-6 (2000).

508 2. Jones, T.F. *et al.* Salmonellosis outcomes differ substantially by serotype. *J*
509 *Infect Dis* **198**, 109-14 (2008).

510 3. O'Brien, S.J. The "decline and fall" of nontyphoidal salmonella in the United
511 kingdom. *Clin Infect Dis* **56**, 705-10 (2013).

512 4. Braden, C.R. Salmonella enterica serotype Enteritidis and eggs: a national
513 epidemic in the United States. *Clin Infect Dis* **43**, 512-7 (2006).

514 5. Friedman, C.R., Malcolm, G., Rigau-Perez, J.G., Arambulo, P., 3rd & Tauxe,
515 R.V. Public health risk from Salmonella-based rodenticides. *Lancet* **347**, 1705-6
516 (1996).

517 6. Reddy, E.A., Shaw, A.V. & Crump, J.A. Community-acquired bloodstream
518 infections in Africa: a systematic review and meta-analysis. *Lancet Infect Dis* **10**,
519 417-32 (2010).

520 7. Feasey, N.A. *et al.* Three Epidemics of Invasive Multidrug-Resistant Salmonella
521 Bloodstream Infection in Blantyre, Malawi, 1998-2014. *Clin Infect Dis* **61 Suppl 4**,
522 S363-71 (2015).

523 8. Feasey, N.A., Dougan, G., Kingsley, R.A., Heyderman, R.S. & Gordon, M.A.
524 Invasive non-typhoidal salmonella disease: an emerging and neglected tropical
525 disease in Africa. *Lancet* **379**, 2489-99 (2012).

526 9. Ao, T.T. *et al.* Global burden of invasive nontyphoidal salmonella disease,
527 2010(1). *Emerg Infect Dis* **21**(2015).

528 10. Nuccio, S.P. & Baumler, A.J. Reconstructing pathogen evolution from the ruins.
529 *Proc Natl Acad Sci U S A* **112**, 647-8 (2015).

530 11. Nuccio, S.P. & Baumler, A.J. Comparative analysis of Salmonella genomes
531 identifies a metabolic network for escalating growth in the inflamed gut. *MBio* **5**,
532 e00929-14 (2014).

533 12. Parsons, B.N. *et al.* Invasive non-typhoidal Salmonella typhimurium ST313 are
534 not host-restricted and have an invasive phenotype in experimentally infected
535 chickens. *PLoS Negl Trop Dis* **7**, e2487 (2013).

536 13. Kingsley, R.A. *et al.* Epidemic multiple drug resistant Salmonella Typhimurium
537 causing invasive disease in sub-Saharan Africa have a distinct genotype.
538 *Genome Res* **19**, 2279-87 (2009).

539 14. Okoro, C.K. *et al.* Signatures of adaptation in human invasive Salmonella
540 Typhimurium ST313 populations from sub-Saharan Africa. *PLoS Negl Trop Dis*
541 **9**, e0003611 (2015).

542 15. Herrero-Fresno, A. *et al.* The role of the st313-td gene in virulence of Salmonella
543 Typhimurium ST313. *PLoS One* **9**, e84566 (2014).

544 16. Ramachandran, G., Perkins, D.J., Schmidlein, P.J., Tulapurkar, M.E. & Tennant,
545 S.M. Invasive Salmonella Typhimurium ST313 with naturally attenuated flagellin
546 elicits reduced inflammation and replicates within macrophages. *PLoS Negl Trop*
547 *Dis* **9**, e3394 (2015).

548 17. Okoro, C.K. *et al.* Intracontinental spread of human invasive Salmonella
549 Typhimurium pathovariants in sub-Saharan Africa. *Nat Genet* **44**, 1215-21
550 (2012).

551 18. Allard, M.W. *et al.* On the evolutionary history, population genetics and diversity
552 among isolates of Salmonella Enteritidis PFGE pattern JEGX01.0004. *PLoS One*
553 **8**, e55254 (2013).

554 19. den Bakker, H.C. *et al.* Rapid whole-genome sequencing for surveillance of
555 Salmonella enterica serovar enteritidis. *Emerg Infect Dis* **20**, 1306-14 (2014).

556 20. Taylor, A.J. *et al.* Characterization of Foodborne Outbreaks of Salmonella
557 enterica Serovar Enteritidis with Whole-Genome Sequencing Single Nucleotide
558 Polymorphism-Based Analysis for Surveillance and Outbreak Detection. *J Clin*
559 *Microbiol* **53**, 3334-40 (2015).

560 21. Deng, X. *et al.* Genomic epidemiology of Salmonella enterica serotype Enteritidis
561 based on population structure of prevalent lineages. *Emerg Infect Dis* **20**, 1481-9
562 (2014).

563 22. Rodriguez, I., Guerra, B., Mendoza, M.C. & Rodicio, M.R. pUO-SeVR1 is an
564 emergent virulence-resistance complex plasmid of Salmonella enterica serovar
565 Enteritidis. *J Antimicrob Chemother* **66**, 218-20 (2011).

566 23. Rodriguez, I., Rodicio, M.R., Guerra, B. & Hopkins, K.L. Potential international
567 spread of multidrug-resistant invasive Salmonella enterica serovar enteritidis.
568 *Emerg Infect Dis* **18**, 1173-6 (2012).

569 24. Cheng, L., Connor, T.R., Siren, J., Aanensen, D.M. & Corander, J. Hierarchical
570 and spatially explicit clustering of DNA sequences with BAPS software. *Mol Biol*
571 *Evol* **30**, 1224-8 (2013).

572 25. Rodrigue, D.C., Tauxe, R.V. & Rowe, B. International increase in Salmonella
573 enteritidis: a new pandemic? *Epidemiol Infect* **105**, 21-7 (1990).

574 26. Magnet, S., Courvalin, P. & Lambert, T. Activation of the cryptic aac(6')-Iy
575 aminoglycoside resistance gene of Salmonella by a chromosomal deletion
576 generating a transcriptional fusion. *J Bacteriol* **181**, 6650-5 (1999).

577 27. Zheng, J. *et al.* Genetic diversity and evolution of Salmonella enterica serovar
578 Enteritidis strains with different phage types. *J Clin Microbiol* **52**, 1490-500
579 (2014).

580 28. Inns, T. *et al.* A multi-country Salmonella Enteritidis phage type 14b outbreak
581 associated with eggs from a German producer: 'near real-time' application of
582 whole genome sequencing and food chain investigations, United Kingdom, May
583 to September 2014. *Euro Surveill* **20**(2015).

584 29. Drummond, A.J. & Rambaut, A. BEAST: Bayesian evolutionary analysis by
585 sampling trees. *BMC Evol Biol* **7**, 214 (2007).

586 30. Thomson, N. *et al.* The role of prophage-like elements in the diversity of
587 Salmonella enterica serovars. *J Mol Biol* **339**, 279-300 (2004).

588 31. Rotger, R. & Casadesus, J. The virulence plasmids of Salmonella. *Int Microbiol*
589 **2**, 177-84 (1999).

590 32. Thomson, N.R. *et al.* Comparative genome analysis of Salmonella Enteritidis
591 PT4 and Salmonella Gallinarum 287/91 provides insights into evolutionary and
592 host adaptation pathways. *Genome Res* **18**, 1624-37 (2008).

593 33. Cooke, F.J. *et al.* Prophage sequences defining hot spots of genome variation in
594 Salmonella enterica serovar Typhimurium can be used to discriminate between
595 field isolates. *J Clin Microbiol* **45**, 2590-8 (2007).

596 34. Shah, D.H., Zhou, X., Kim, H.Y., Call, D.R. & Guard, J. Transposon mutagenesis
597 of Salmonella enterica serovar Enteritidis identifies genes that contribute to
598 invasiveness in human and chicken cells and survival in egg albumen. *Infect*
599 *Immun* **80**, 4203-15 (2012).

600 35. Parkhill, J. *et al.* Complete genome sequence of a multiple drug resistant
601 Salmonella enterica serovar Typhi CT18. *Nature* **413**, 848-52 (2001).

602 36. Thiennimitr, P. *et al.* Intestinal inflammation allows Salmonella to use
603 ethanolamine to compete with the microbiota. *Proc Natl Acad Sci U S A* **108**,
604 17480-5 (2011).

605 37. Kingsley, R.A. *et al.* Genome and transcriptome adaptation accompanying
606 emergence of the definitive type 2 host-restricted Salmonella enterica serovar
607 Typhimurium pathovar. *MBio* **4**, e00565-13 (2013).

608 38. Dhawi, A.A. *et al.* Adaptation to the chicken intestine in Salmonella Enteritidis
609 PT4 studied by transcriptional analysis. *Vet Microbiol* **153**, 198-204 (2011).

610 39. Langridge, G.C. *et al.* Patterns of genome evolution that have accompanied host
611 adaptation in Salmonella. *Proc Natl Acad Sci U S A* **112**, 863-8 (2015).

612 40. Feasey, N.A. *et al.* Modelling the Contributions of Malaria, HIV, Malnutrition and
613 Rainfall to the Decline in Paediatric Invasive Non-typhoidal Salmonella Disease
614 in Malawi. *PLoS Negl Trop Dis* **9**, e0003979 (2015).

615 41. Gordon, M.A. *et al.* Non-typhoidal salmonella bacteraemia among HIV-infected
616 Malawian adults: high mortality and frequent recrudescence. *AIDS* **16**, 1633-41
617 (2002).

618 42. Kotloff, K.L. *et al.* Burden and aetiology of diarrhoeal disease in infants and
619 young children in developing countries (the Global Enteric Multicenter Study,
620 GEMS): a prospective, case-control study. *Lancet* **382**, 209-22 (2013).

621 43. Kariuki, S. & Onsare, R.S. Epidemiology and Genomics of Invasive Nontyphoidal
622 Salmonella Infections in Kenya. *Clin Infect Dis* **61 Suppl 4**, S317-24 (2015).

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633 **Figures**

634

635 Figure 1: Maximum likelihood phylogeny of *S*. Enteritidis based on 675 isolates
636 rooted to *S*. Gallinarum. There are 3 epidemic clades; 2 African epidemic clades and
637 a global epidemic clade. Scale bar indicates nucleotide substitutions per site.

638

639 Figure 2: Differences in accessory genomes of 4 major clades. Approximate position
640 of prophages in chromosome is depicted, although prophages are not drawn to scale

641

642 Figure 3: Heat map revealing changes in metabolic activity of Central/Eastern
643 African clade isolate D7795 when compared to global epidemic isolate A1636 at 28
644 and 37˚C. The figure also displays whether there are corresponding mutations in
645 genes related to the affected metabolic pathway. (NSSNP=Non-synonymous single
646 nucleotide polymorphism, HDG = Hypothetically disrupted gene)

647

648 Figure 4: *Salmonella* isolation from a chick infection model demonstrates failure of
649 Central/Eastern African clade isolate to invade chicken spleen (4A) and liver (4B) or
650 to colonize chicken caeca (4C) at 7 days post infection (dpi) (n=24 at this time point)
651 compared to the global epidemic clade. Numbers are expressed as colony forming
652 units (CFU) per gram of tissue

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660 **Tables**

661 Table 1: Summary of metadata (n) by region in numbers

662

Region	Total	Site of isolation			Antimicrobial resistance phenotype				
		Human Invasive	Human non-invasive	Food/Animal/ Environment	Drug susceptible	Resistant to 1-2 1st line	MDR*	Fluoroquinolone	ESBL†
Asia	11	5	5	1	0	0	0	0	0
Europe	61	0	16	24	2	0	0	0	0
South America	27‡	3	6	7	8	0	0	0	0
North Africa	12	9	1	1	9	0	0	2	0
Sub-Saharan Africa	353	269	22	7	99	64	149	0	3
Republic of South Africa	131	57	74	0	83	44	4	0	0

663

664 *Multidrug resistant: resistant to ≥3 antimicrobials

665 †Extended spectrum beta lactamase producing

666 ‡Uruguay strains previously characterised by Betancor [44]

667

668 Table 2: Metadata summarised by clade

Major Clade/cluster	Site of isolation N (%)				Number (%) of antimicrobial resistance genes*		
	Human Invasive	Human non-invasive	Food/Animal/ Environment	Unknown	1-3	4-6	7-9
West African	61 (92)	1 (2)	0 (0)	4 (6)	22 (33)	9 (14)	35 (66)
Central/Eastern African	155 (93)	7 (4)	0 (0)	5 (3)	0 (0)	11 (7)	156 (93)
Global epidemic	94 (38)	95 (38)	31 (12)	30 (12)	243 (97)	7 (3)	0 (0)
Outlier cluster	51 (38)	36 (27)	27 (20)	20 (15)	128 (96)	3 (2)	3 (2)

669

670 *All isolates contained cryptic aminoglycoside acetyltransferase gene *aac(6')-ly*[26]

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677 **Online Methods**

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679 Bacterial Isolates

680

681 *S*. Enteritidis isolates were selected on the basis of six factors; date of original
682 isolation, antimicrobial susceptibility pattern, geographic site of original isolation,
683 source (human [invasive vs stool], animal or environmental), phage type (where
684 available), and multilocus variable number tandem repeat (MLVA) type (where
685 available). *S.* Enteritidis P125109 (EMBL accession no. AM933172) isolated from a
686 poultry farm from the UK was used as a reference[32]. The full metadata are in
687 Supplementary Table 1. Isolates have been attributed to region according to United
688 Nations statistical divisions.

689

690 Sequencing, SNP-calling, construction of phylogeny and comparative genomics

691

692 PCR libraries were prepared from 500 ng of DNA as previously described[45]. Isolates
693 were sequenced using Illumina GA II, HiSeq 2000 and MiSeq machines (Illumina,
694 San Diego, CA, USA) and 150 bp paired-end reads were generated. The strains were
695 aligned to *Salmonella* Enteritidis reference genome P125109 using a pipeline
696 developed in-house at the Wellcome Trust Sanger Institute (WTSI). For each isolate
697 sequenced, the raw sequence read pairs were split to reduce the overall memory
698 usage and allow reads to be aligned using more than one CPU. The reads were then
699 aligned using SMALT, a hashing based sequence aligner. The aligned and unmapped
700 reads were combined into a single BAM file. Picard was used to identify and flag
701 optical duplicates generated during the making of a standard Illumina library, which
702 reduces possible effects of PCR bias. All of the alignments were created in a
703 standardized manner, with the commands and parameters stored in the header of
704 each BAM file, allowing for the results to be easily reproduced.

705

706 The combined BAM file for each isolate was used as input data in the SAMtools

707 mpileup program to call SNPs and small indels, producing a BCF file describing all of
708 the variant base positions[46]. A pseudo-genome was constructed by substituting the
709 base call at each variant or non-variant site, defined in the BCF file, in the reference
710 genome. Only base calls with a depth of coverage >4 or quality >50 were considered
711 in this analysis. Base calls in the BCF file failing this quality control filter were
712 replaced with the "N" character in the pseudo-genome sequence.
713
714 All of the software developed is freely available for download from GitHub under an
715 open source license, GNU GPL 3.
716
717 Phylogenetic modelling was based on the assumption of a single common ancestor,
718 therefore variable regions where horizontal genetic transfer occurs were
719 excluded[47,48]. A maximum likelihood (ML) phylogenetic tree was then built from the
720 alignments of the isolates using RAxML (version 7.0.4) using a GTR+I+G model[49].
721 The maximum-likelihood phylogeny was supported by 100 bootstrap pseudo-
722 replicate analyses of the alignment data. Clades were predicted using Hierarchical
723 Bayesian Analysis of Population Structure (HierBAPS)[24]. This process was repeated
724 to construct the plasmid phylogeny, using reads that aligned to pSENV.
725 To ascertain the presence of the clusters defined by HierBAPs in the Public Health
726 England (PHE) routine *Salmonella* surveillance collection, seventeen isolates
727 representing the diversity of the collection were compared against 2986 *S*.
728 Enteritidis PHE genomes. Single linkage SNP clustering was performed as
729 previously described[50]. A maximum-likelihood phylogeny showing the integration
730 of the seventeen isolates with 50-SNP cluster representatives of the PHE *S*.
731 Enteritidis collection was constructed as above. FASTQ reads from all PHE
732 sequences in this study can be found at the PHE Pathogens BioProject at the
733 National Center for Biotechnology Information (Accession PRJNA248792).
734
735 Temporal reconstruction was performed using Bayesian Evolutionary Analysis
736 Sampling Trees (BEAST version 1.8.2)[51]. A relaxed lognormal clock model was

737 initially employed. The results of this model indicated that a constant clock model
738 was not appropriate, as the posterior of the standard deviation of the clock rate did
739 not include zero. A range of biologically plausible population models (constant,
740 exponential and skyline) was investigated. Skyline models can be biased by non-
741 uniform sampling and we observed a strong similarity between reconstructed
742 skyline population and the histogram of sampling dates and so this model was
743 excluded. The exponential models consistently failed to converge and were
744 excluded. Thus, for all datasets, lognormal clock and constant population size
745 models were used. The computational expense required for this analysis precluded
746 running estimators for model selection. However, we note that Deng et al used the
747 same models in their analysis of 125 *S*. Enteritidis isolates. Default priors were used
748 except for ucld.mean, Gamma(0.001,1000), initial: 0.0001; exponential.popSize,
749 LogNormal(10,1.5), initial: 1[21].
750
751 Three chains of 100 million states were run in parallel for each clade of the four
752 major HierBAPS clades, as well as a fourth chain without genomic data to examine
753 the influence of the prior, which in all cases was uninformative. The final results, as
754 used here, all had effective sample sizes (ESS) of over 200 and had convergence
755 between all three runs. For the Global and Global Outlier lineages, the datasets were
756 not computationally feasible to analyse. We thus created 3 further random subsets
757 of the data by drawing n isolates from each sampled year where n was sampled
758 from a Poisson distribution where $\lambda=2$. The posteriors of all subsets were extremely
759 similar and runs were combined to produce the final most recent common ancestor
760 (MRCA) estimates.
761
762 In order to gain a detailed insight into genomic differences, a single high quality
763 sequence from Malawian *S*. Enteritidis isolate D7795 was aligned against the
764 P125109 using ABACAS and annotated[52]. Differences were manually curated against
765 the reference using the Artemis Comparison Tool (ACT)[53]. Sections of contigs which
766 were incorporated into the alignment, but which did not align with P125109 were

767 manually inspected and compared to the public databases using BLASTn. When
768 these regions appeared to be novel prophages, they were annotated using the phage
769 search tool PHAST and manually curated[54]. In order to investigate whether the SNPs
770 and/or indels that were predicted to be responsible for pseudogene formation in
771 D7795 were distinct to that isolate or conserved across both African epidemic
772 clades, all isolates were aligned to P12509 and the relevant SNPs/indels
773 investigated using *in-silico* PCR of the aligned sequences. Manual curation was
774 performed to confirm the nature of all pseudogene-associated SNPs/indels. NS-SNPs
775 identified in D7795 were sorted throughout the African clades by extracting and
776 aligning the appropriate gene sequences from P125109 and D7795. The coordinates
777 of the NS-SNPs were then used to identify the relevant sequence and determine the
778 nature of the base.
779
780 Accessory genome
781 The pangenome for the dataset was predicted using ROARY [55]. Genes were
782 considered to be core to *S*. Enteritidis if present in ≥90% of isolates. A relaxed
783 definition of core genome was used as assemblies were used to generate it and the
784 more assemblies one uses, the more likely it is that a core gene will be missed in one
785 sample due to an assembly error. The remaining genes were considered to be core
786 to the clades/clusters predicted by HierBAPS if present in ≥75% if isolates from
787 within each clade/cluster. These genes were then curated manually using ACT to
788 search for their presence and position in P125109 or the improved draft assembly
789 of representative isolates of each of the other clades if not present in P125109. Any
790 large accessory regions identified were blasted against the assembled genomes of
791 the entire collection to confirm they were grossly intact.
792
793 Plasmid identification
794 Plasmid DNA was extracted from isolate D7795 using the Kado & Liu method and
795 separated by gel-electrophoresis alongside plasmids of known size, to estimate the
796 number and size of plasmids present[56]. Plasmid conjugation was attempted by

797 mixing 100 µL of overnight culture of donor and recipient strains (rifampicin
798 resistant *Escherichia coli* C600) on Luria-Bertani agar plates and incubating
799 overnight at 26°C and 37°C. The plasmid was sequenced using the PacBio platform
800 to gain long reads and a single improved draft assembly, which was aligned against
801 P125109 plasmid pSENV (Accession Number HG970000). For novel regions of the
802 plasmid from isolate D7795, genes were predicted using GLIMMER and manual
803 annotations applied based on homology searches against the public databases, using
804 both BLASTn and FASTA. The plasmid phylogeny was reconstructed using the same
805 methodology as the chromosome; a maximum likelihood (ML) phylogenetic tree
806 was built from the alignments of the isolates using RAxML (version 7.0.4) using a
807 GTR+I+G model
808
809 Identification of AMR genes
810
811 A manually curated version of the Resfinder database was used to investigate the
812 isolates for the presence of AMR genes[57]. To reduce redundancy, the database was
813 clustered using CD-HIT-EST[58], with the alignment length of the shorter sequence
814 required to be 90% the length of the longer sequence. All other options were left as
815 the defaults. The representative gene of each cluster was then mapped with SMALT
816 to the assemblies of each isolate to identify and matches with an identity of 90% or
817 greater were considered significant, in line with the default clustering parameters of
818 CD-HIT-EST. Where partial matches were identified at the ends of contigs, having an
819 identity of 90% or greater to the matched region of the gene, potential AMR gene
820 presence was recorded. To confirm presence of these partial matches, raw
821 sequencing reads of the pertinent isolates were mapped using SMALT to these genes
822 to check for 90% identity across the entire gene.
823
824 Biolog™ growth substrate platform profiling
825

826 The Biolog™ platform enables the simultaneous quantitative measurement of a
827 number of cellular phenotypes, and therefore the creation of a phenotypic profile of
828 a variety of assay conditions[59]. Incubation and recording of phenotypic data were
829 performed using an OmniLog® plate reader.
830
831 In these experiments, two replicates of D7795 were compared to two replicates of a
832 PT4 like strain at 28˚C and 37˚C to represent environmental and human
833 temperatures. Biolog™ plates PM1-4 and 9 (Carbon source [PM1,PM2], nitrogen
834 source [PM3] and phosphor and sulphur source [PM4] metabolism and osmotic
835 pressure [PM9]) were used. Each well was inoculated as described below, thereby
836 testing 475 conditions at once (each plate has one negative control well).
837
838 The isolates were cultured overnight on LB-agar at 37°C in air to exclude
839 contamination. Colonies were scraped off plates and dispensed into IF-0a solution
840 (Biolog) to a cell density corresponding to 81% transmittance. For each plate used,
841 880 µL of this cell suspension was added to 10 mL IF-10b GP/GP solution (Biolog)
842 and 120 µL dye mix G (Biolog). This was then supplemented with a 1 mL solution of
843 7.5 mM D-ribose (Sigma), 2 mM magnesium chloride, 1 mM calcium chloride, 2 mM
844 sodium pyrophosphate (Sigma), 25 µM L-arginine (Sigma), 25 µM L-methionine
845 (Sigma), 25 µM hypoxanthine (Sigma), 10 µM lipoamide (Sigma), 5 µM nicotine
846 adenine dinucleotide (Sigma), 0.25 µM riboflavin (Sigma), 0.005% by mass yeast
847 extract (Fluka) and 0.005% by mass Tween 80 (Sigma). 100µl of this mixture was
848 dispensed into each well on the assay plate. Plates were then allowed to equilibrate
849 in air for 5 min prior to being sealed in airtight bags and loaded into the Omnilog
850 machine (Biolog). Plates were scanned every 15 min for 48 hours while incubated at
851 28°C and 37°C in air. Culture under anaerobic conditions was unavailable. Two
852 paired replicates were performed for each of the two isolates.

853 After completion of the run, the signal data were compiled and analysed using the
854 limma package in 'R' described previously[60]. A log-fold change of 0.5 controlling for

855 a 5% false discovery rate was used as a cut-off for investigating a specific metabolite
856 further using Pathway Tools[61] and whether the metabolic change was related to
857 pseudogenes and non synonymous(NS)-SNPs in genes in the respective genomes.
858
859 *In vivo* Infection Model
860
861 Two isolates were used in the animal models: *S*. Enteritidis P125109 and D7795.
862 Unvaccinated commercial female egg-layer Lohmann Brown chicks (Domestic Fowl
863 [*Gallus gallus*]) were obtained from a commercial hatchery and housed in secure
864 floor pens at a temperature of 25°C. Eight chicks per strain per time point were
865 inoculated by gavage at 10 days (d) of age and received a dose of ~10^8 *Salmonella*
866 colony forming units (CFU) in a volume of 0.2 mL. Subsequently, four to five birds
867 from each group were humanely killed at 3, 7 or 21 d post-infection (p.i.). At post
868 mortem, the liver, spleen, and caecal contents were removed aseptically,
869 homogenised, serially diluted and dispensed onto Brilliant Green agar (Oxoid) to
870 quantify colony forming units (CFU) as described previously[62]. Statistical analysis
871 was performed using SPSS, version 20 (IBM). Kruskal-Wallis was used to compare
872 bacterial loads between infected groups.
873
874 All work was conducted in accordance with the UK legislation governing
875 experimental animals, Animals (Scientific Procedures) Act 1986, under project
876 licence 40/3652 and was approved by the University of Liverpool ethical review
877 process prior to the award of the project license. The licensing procedure requires
878 power calculations to determine minimal group sizes for each procedure to ensure
879 results are significant. For these experiments a group size of 8 birds per time point
880 was chosen, based on a variation in 1.0 $\log_{10}$ in bacterial count between groups as
881 being significant along with prior experience of *Salmonella* infection studies. Groups
882 were randomly selected on receipt from the hatchery and investigators conducting
883 animal experiments were not blinded, as the current UK code of practice requires all
884 cages or pens to be fully labeled with experimental details. No animals were

885 excluded from the analysis. All animals were checked a minimum of twice daily to
886 ensure their health and welfare.
887
888 Code availability & URLs
889
890 Software is referenced and URLS are provided below. All software is open source.
891
892 BEAST: http://beast.bio.ed.ac.uk/
893 Biolog$^{TM:}$ http://www.biolog.com
894 BLASTn: http://blast.ncbi.nlm.nih.gov
895 limma package: www.bioconductor.org
896 PacBio platform: http://www.pacificbiosciences.com/
897 Picard: https://broadinstitute.github.io/picard
898 'R': www.R-project.org
899 SMALT: www.sanger.ac.uk/science/tools/smalt-0
900 United Nations statistical divisions:
901 www.unstats.un.org/unsd/methods/m49/m49regin.htm

902 Supplementary References

903 44. Betancor, L. *et al.* Genomic and phenotypic variation in epidemic-spanning
904 Salmonella enterica serovar Enteritidis isolates. *BMC Microbiol* **9**, 237 (2009).

905 45. Harris, S.R. *et al.* Whole-genome sequencing for analysis of an outbreak of
906 meticillin-resistant Staphylococcus aureus: a descriptive study. *Lancet Infect Dis*
907 **13**, 130-6 (2013).

908 46. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics*
909 **25**, 2078-9 (2009).

910 47. Holt, K.E. *et al.* High-throughput sequencing provides insights into genome
911 variation and evolution in Salmonella Typhi. *Nat Genet* **40**, 987-93 (2008).

912 48. Croucher, N.J. *et al.* Rapid pneumococcal evolution in response to clinical
913 interventions. *Science* **331**, 430-4 (2011).

914 49. Stamatakis, A. RAxML-VI-HPC: maximum likelihood-based phylogenetic
915 analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-90
916 (2006).

917 50. Philip Ashton, S.N., Tansy Peters, Rediat Tewolde, Martin Day, Michel
918 Doumith, Jonathan Green, Claire Jenkins, Anthony Underwood, Catherine
919 Arnold, Elizabeth de Pinna, Tim Dallman, Kathie Grant. Revolutionising
920 Public Health Reference Microbiology using Whole Genome Sequencing:
921 Salmonella as an exemplar. *bioRxiv* (2015).

922 51. Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A. Bayesian phylogenetics
923 with BEAUti and the BEAST 1.7. *Mol Biol Evol* **29**, 1969-73 (2012).

924 52. Assefa, S., Keane, T.M., Otto, T.D., Newbold, C. & Berriman, M. ABACAS:
925 algorithm-based automatic contiguation of assembled sequences. *Bioinformatics*
926 **25**, 1968-9 (2009).

927 53. Carver, T.J. *et al.* ACT: the Artemis Comparison Tool. *Bioinformatics* **21**, 3422-3
928 (2005).

929 54. Zhou, Y., Liang, Y., Lynch, K.H., Dennis, J.J. & Wishart, D.S. PHAST: a fast
930 phage search tool. *Nucleic Acids Res* **39**, W347-52 (2011).

931 55. Page, A.J. *et al.* Roary: Rapid large-scale prokaryote pan genome analysis.
932 *Bioinformatics* (2015).

933 56. Kado, C.I. & Liu, S.T. Rapid procedure for detection and isolation of large and
934 small plasmids. *J Bacteriol* **145**, 1365-73 (1981).

935 57. Zankari, E. *et al.* Identification of acquired antimicrobial resistance genes. *J*
936 *Antimicrob Chemother* **67**, 2640-4 (2012).

937 58. Li, W. & Godzik, A. Cd-hit: a fast program for clustering and comparing large sets
938 of protein or nucleotide sequences. *Bioinformatics* **22**, 1658-9 (2006).

939 59. Tracy, B.S., Edwards, K.K. & Eisenstark, A. Carbon and nitrogen substrate
940 utilization by archival Salmonella typhimurium LT2 cells. *BMC Evol Biol* **2**, 14
941 (2002).

942 60. Croucher, N.J. *et al.* Dominant role of nucleotide substitution in the diversification
943 of serotype 3 pneumococci over decades and during a single infection. *PLoS*
944 *Genet* **9**, e1003868 (2013).

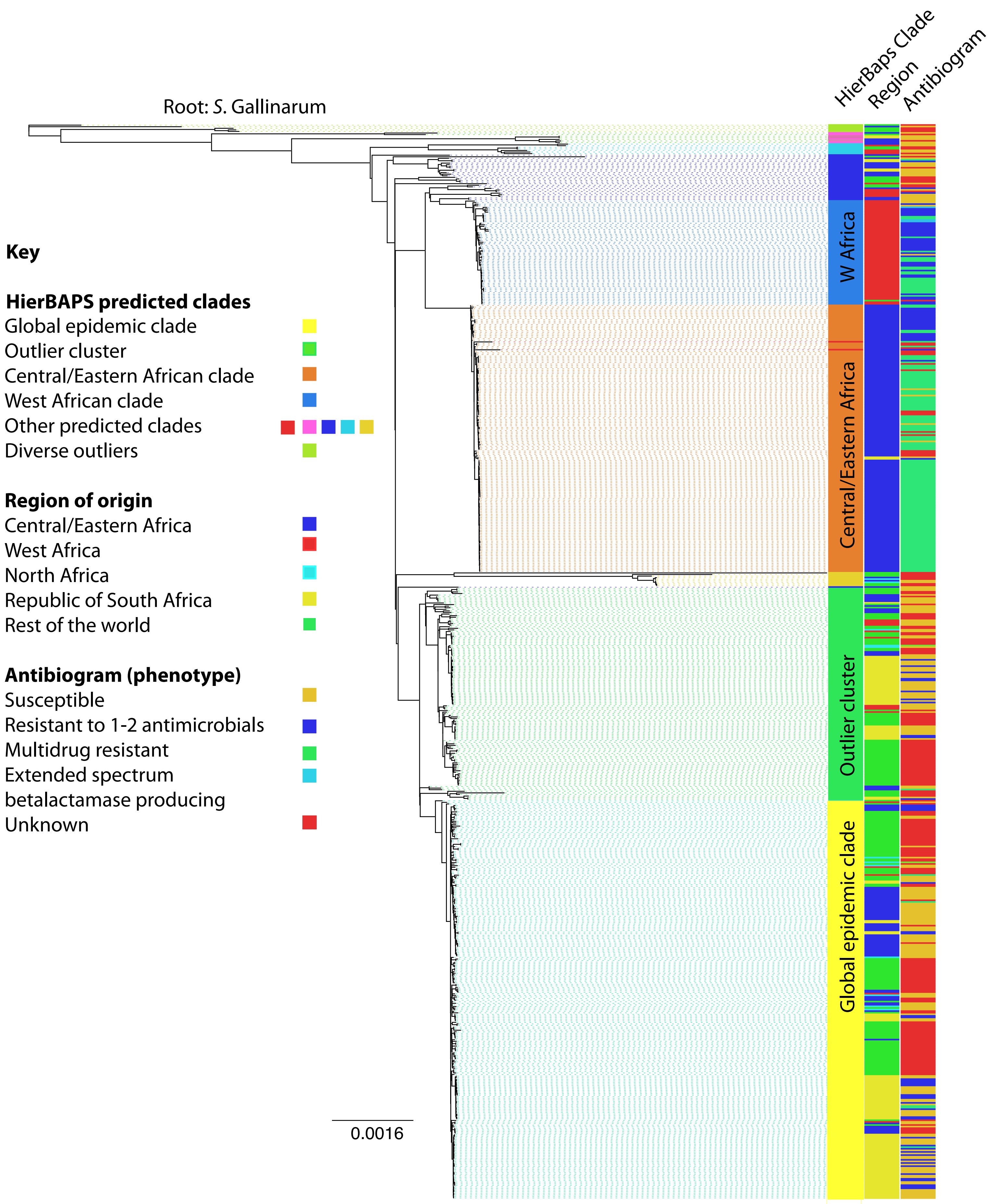
945 61. Karp, P.D. *et al.* Pathway Tools version 13.0: integrated software for
946 pathway/genome informatics and systems biology. *Brief Bioinform* **11**, 40-79
947 (2010).

948 62. Salisbury, A.M., Bronowski, C. & Wigley, P. Salmonella Virchow isolates from
949 human and avian origins in England--molecular characterization and infection of
950 epithelial cells and poultry. *J Appl Microbiol* **111**, 1505-14 (2011).

951

952 Competing financial interests:

953 The authors declare no competing financial interests.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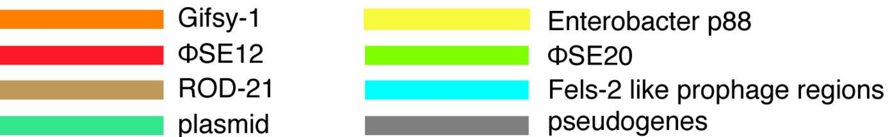
HierBaps Clade
Region
Antibiogram
Key
HierBAPS predicted clades
Global epidemic clade
Outlier cluster
Central/Eastern African clade
West African clade
Other predicted clades
Diverse outliers
Region of origin
Central/Eastern Africa
West Africa
North Africa
Republic of South Africa
Rest of the world
Antibiogram (phenotype)
Susceptible
Resistant to 1-2 antimicrobials
Multidrug resistant
Extended spectrum
betalactamase producing
Unknown
W Africa
Central/Eastern Africa
Outlier cluster
Global epidemic clade
0.0016

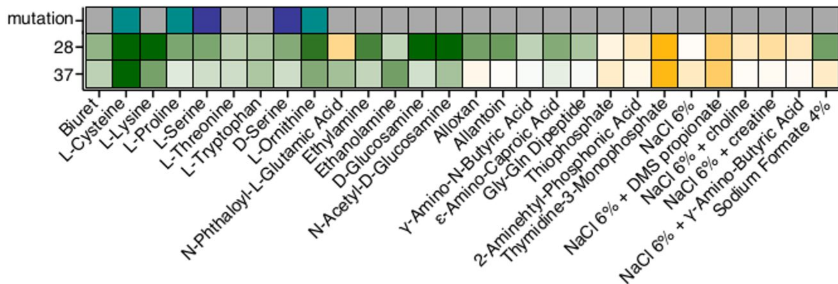
W. Africa
11
ΦSE12 (12)
P88 (48)
Fels2-BT (40)
Gifsy1(44)
E. Africa
2
40
Global
ΦSE12 (4)
ΦSE20(57)
ROD21(26)
Fels2-like (40)
2
Global Outliers
30

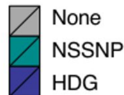


Carbon Sources

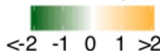
mutation
28
37

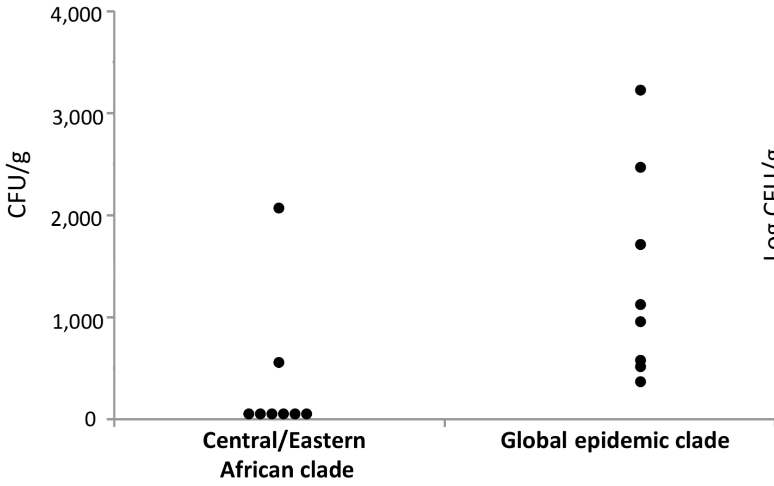
L-Arabinose
N-Acetyl-D-Glucosamine
D-Saccharic Acid
D-Galactose
D-Alanine
D-Mannose
Dulcitol
L-Fucose
Formic Acid
D-Mannitol
Tween 20
L-Rhamnose
D-Fructose
α-D-Glucose
D-Melibiose
Tween 40
α-Keto-Butyric Acid
α-Methyl-D-Galactoside
α-D-Lactose
Lactulose
Sucrose
D-Glucose-1-Phosphate
Tween 80
α-Hydroxy-Butyric Acid
β-Methyl-D-Glucoside
Glycyl-L-Aspartic Acid
Propionic Acid
Mucic Acid
Glycolic Acid
L-Threonine
N-Acetyl-β-D-Mannosamine
Malic Acid
Melibionic Acid
L-Tartaric Acid
Dihydroxyacetone





Log Fold-change in Metabolic Activity

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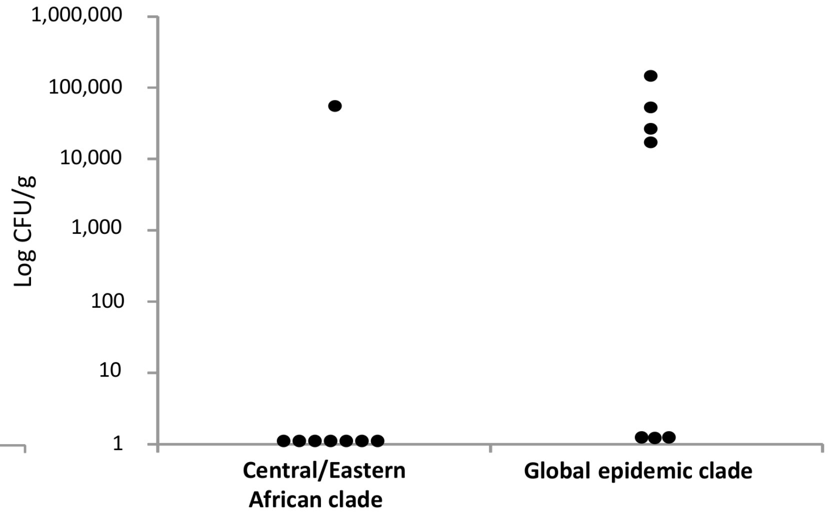
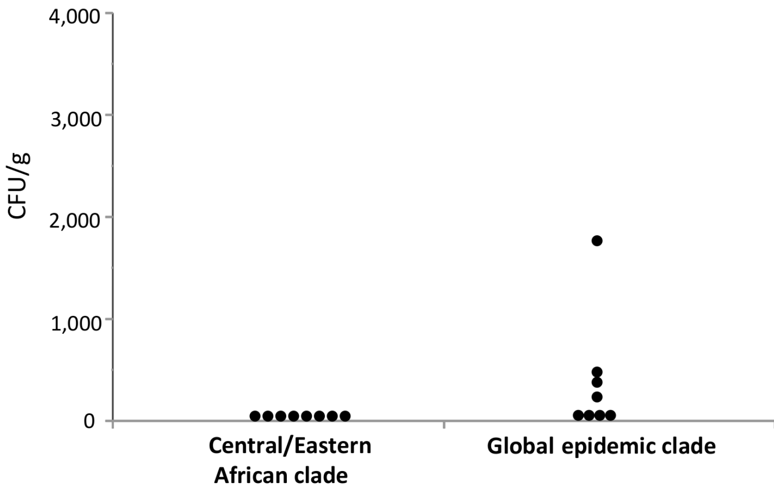
CFU/g
4,000
3,000
2,000
1,000
0
Central/Eastern African clade
Global epidemic clade


4C: Caeca 7 dpi
Log CFU/g
1,000,000
100,000
10,000
1,000
100
10
1
Central/Eastern African clade
Global epidemic clade


4B: Liver 7 dpi
CFU/g
4,000
3,000
2,000
1,000
0
Central/Eastern African clade
Global epidemic clade