

High-Resolution Single Nucleotide Polymorphism Analysis Distinguishes Recrudescence and Reinfection in Recurrent Invasive Nontyphoidal *Salmonella* Typhimurium Disease

Chinyere K. Okoro,¹ Robert A. Kingsley,¹ Michael A. Quail,¹ Anstead M. Kankwatira,² Nicholas A. Feasey,^{2,3} Julian Parkhill,¹ Gordon Dougan,¹ and Melita A. Gordon^{2,3}

¹Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom; ²Malawi-Liverpool-Wellcome Trust Clinical Research Program, Blantyre, Malawi; and ³Wellcome Department of Molecular and Cellular Gastroenterology, Institute of Translational Medicine, University of Liverpool, United Kingdom

Background. Bloodstream infection with invasive nontyphoidal *Salmonella* (iNTS) is common and severe among human immunodeficiency virus (HIV)-infected adults throughout sub-Saharan Africa. The epidemiology of iNTS is poorly understood. Survivors frequently experience multiply recurrent iNTS disease, despite appropriate antimicrobial therapy, but recrudescence and reinfection have previously been difficult to distinguish.

Methods. We used high-resolution single nucleotide polymorphism (SNP) typing and whole-genome phylogenetics to investigate 47 iNTS isolates from 14 patients with multiple recurrences following an index presentation with iNTS disease in Blantyre, Malawi. We isolated nontyphoidal salmonellae organisms from blood (n = 35), bone marrow (n = 8), stool (n = 2), urine (n = 1), and throat (n = 1) samples; these isolates comprised serotypes Typhimurium (n = 43) and Enteritidis (n = 4).

Results. Recrudescence with identical or highly phylogenetically related isolates accounted for 78% of recurrences, and reinfection with phylogenetically distinct isolates accounted for 22% of recurrences. Both recrudescence and reinfection could occur in the same individual, and reinfection could either precede or follow recrudescence. The number of days to recurrence (23–486 d) was not different for recrudescence or reinfection. The number of days to recrudescence was unrelated to the number of SNPs accumulated by recrudescing organisms, suggesting that there was little genetic change during persistence in the host, despite exposure to multiple courses of antibiotics. Of *Salmonella* Typhimurium isolates, 42 of 43 were pathovar ST313.

Conclusions. High-resolution whole-genome phylogenetics successfully discriminated recrudescing iNTS from reinfection, despite a high level of clonality within and among individuals, giving insights into pathogenesis and management. These methods also have adequate resolution to investigate the epidemiology and transmission of this important African pathogen.

Received 28 July 2011; accepted 6 October 2011; electronically published 7 February 2012.

Correspondence: Melita A. Gordon, MA, MD, MRCP, DTM&H, Division of Gastroenterology, Henry Wellcome Laboratories, Nuffield Building, Crown Street, Liverpool University, Liverpool L69 3GE, United Kingdom (magordon@liverpool.ac.uk).

Clinical Infectious Diseases 2012;54(7):955–63

© The Author 2012. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please email: journals.permissions@oup.com. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1093/cid/cir1032

Nontyphoidal salmonellae (NTS) are among the commonest causes of invasive bacterial disease across sub-Saharan Africa [1–4]. Invasive nontyphoidal *Salmonella* (iNTS) disease predominantly affects children with malnutrition, malaria, or human immunodeficiency virus (HIV) [1], and HIV-infected adults, among whom the condition frequently recurs [3]. Case-fatality rates are high in both adults (22%–47%) and children (21%–24%), despite therapeutic intervention [3–5]. The epidemiology and transmission of iNTS are poorly understood.

In HIV-infected adults who are not treated with antiretroviral therapy (ART), iNTS disease is frequently recurrent, with recurrence in 20%–43% of index cases. Of these cases, $\leq 25\%$ have multiple recurrences [3]. Recurrent iNTS disease following a course of appropriate antimicrobials has been attributed to both recrudescence of the same organism and reinfection with a distinctly different strain [3, 6, 7]. Previous analyses of paired isolates from index presentation and recurrence events using serology, antibiotic-resistance profiling, pulsed-field gel electrophoresis, and plasmid profiling identified recrudescence as the most likely driver of recurrent disease in Malawian HIV-infected patients [3]. These subgenomic methods, however, lack the resolution to definitively discriminate between recrudescence and reinfection [6, 8].

We therefore used high-quality, single nucleotide polymorphism (SNP)-based, phylogenetic analyses based on whole-genome sequences to detect phylogenetic differences between index and recurrent isolates at very high resolution. Recently, SNP-based typing methods have been used to demonstrate the genetic variation between very closely related bacterial strains in clinically important settings [9, 10]. We demonstrate here the value of whole-genome phylogenetic methods in resolving variation between African iNTS isolates, to understand pathogenesis and guide effective therapeutic and preventive measures.

MATERIALS AND METHODS

Participants and Isolates

The isolates and clinical events described here arose from a larger study in which consecutive febrile adult (>14 y) patients in Blantyre were recruited. Patients with iNTS were identified at an index event by blood and bone-marrow culture, treated with ciprofloxacin 500 mg twice per day for 10–14 days, and followed up monthly to detect recurrence of invasive disease [11]. Recurrent episodes of invasive iNTS disease were retreated with ciprofloxacin. All iNTS patients were HIV-infected.

At follow-up, we collected blood samples, and we requested bone-marrow sampling, for culture, when there was a febrile episode. Simultaneous mucosal samples (urine, stool, throat swab) were also taken when possible. We used previously described diagnostic microbiological methods [11]. We isolated NTS from blood or bone-marrow samples by automated culture (BacT/Alert 3D, BioMérieux) or pour-plate culture in Columbia agar. Stool, urine, and throat swabs were cultured in selenite broth and subcultured onto xylose–lysine–deoxycholate agar. We used standard biochemical and serological methods to identify NTS, which were then stored at -80°C . Series of all *Salmonella* isolates from 14 individuals who had multiple recurrences of iNTS disease were selected for genomic analysis.

Genomic DNA Preparation

Bacteria were grown on Luria–Bertani (LB) medium then incubated in LB broth overnight at 37°C . We pelleted bacterial cells by centrifugation and extracted DNA using the Wizard Genomic DNA kit (Promega) according to the manufacturer's instructions. We checked DNA quality and quantity by gel electrophoresis and Qubit quantitation platform (Invitrogen). We submitted 20–50 ng/ μL of DNA from each isolate for Illumina sequencing.

Library Preparation and DNA Sequencing

We prepared multiplex libraries with 54 or 76 base-pair (bp) reads and 200 bp insert sizes [9]. We conducted cluster formation, primer hybridization, and sequencing reactions using the Illumina Genome Analyzer System [12] according to standard protocols [9]. An average of 97.3% of the reference was mapped with a mean depth of 53.3-fold in mapped regions across all isolates (Supplementary Table 1). We submitted the sequence data to the European Nucleotide Archive (<http://www.ebi.ac.uk/ena>).

SNP Detection and Phylogenetic Analyses

We mapped the sequence data from each isolate to the non-repetitive genome sequence of the reference SL1344, which was obtained by excluding phage sequences and exact repetitive sequences of ≥ 20 bp identified using the repeat-finding programs nucmer, REPuter, and repeat-match [13–16]. We mapped reads from each isolate onto the reference genome using SSAHA v2.2.1 [17] with a minimum mapping score of 30. We identified SNPs using SSAHA_pileup and filtered them with a minimum mapping quality of 30 and a SNP/mapping quality ratio cutoff of 0.75 [9, 14]. A total of 45 497 chromosomal sites contained a high-quality SNP in at least 1 mapped isolate. We constructed maximum-likelihood phylogenetic trees with RAxML v7.0.4 [18] with alignment of all the concatenated 45 497 variant sites from each of the isolates. We removed from the alignment 4 isolates with $>12\,000$ SNP differences from the reference, as these were unlikely to be *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium), and recalculated the maximum-likelihood tree using 51 isolates with 1463 SNP sites. In both calculations, we used the general time-reversible model with a gamma correction for site variation as the nucleotide substitution model. We calculated likelihood test ratios as previously described [9]. We checked support for nodes on the trees using 100 random bootstrap replicates.

Validation of Phylogenetic Clusters

We used several independent approaches to validate the phylogenetic tree. Concatenated SNP alignments generated for 51 isolates consisting of 1463 chromosomal SNP loci were used to construct additional unrooted phylogenetic trees, via neighbor joining and maximum-parsimony methods with the PHYLIP 3.69 package [19]. We evaluated the support for the nodes of the resultant tree clusters by bootstrap analyses based on 100

Table 1. Sampling and Isolation of Nontyphoidal Salmonellae From Cohort

Site Sampled	By Patient (n = 14)			By Event (n = 71)		
	No. of Patients Sampled	No. of Patients Positive for NTS	Patients Positive for NTS, %	No. of Events Sampled	No. of Events Positive for NTS	Events Positive for NTS, %
Blood	14	13	93	71	32	45
Bone marrow	12	9	75	22	11	50
Stool	7	2	29	15	4	27
Urine	14	3	21	35	3	8

Abbreviations: NTS, nontyphoidal salmonellae.

replicates. We conducted a Bayesian phylogenetic analysis using MrBayes 3.1 [20, 21]. We used a general time-reversible substitution model with gamma rate variation. The Markov chain Monte Carlo method ran until it reached convergence for 1 000 000 generations and sample frequency of 100. Summaries were based on 15 002 samples from 2 runs. Each run produced 10 001 trees, of which 7501 were included. We used 15 002 final trees for posterior probability distribution calculations; the probability of each partition or clade on resulting tree was given as a credibility value. To find the best clustering of isolates, we used the Bayesian Analysis of Population Structure (BAPS) 5 package [22] to further probe the population structure using Bayesian statistical approaches. We performed a genetic-mixture analysis for each concatenated SNP sequence and the dependencies present between loci within the aligned sequences [23]. We conducted sequential clustering analyses on identified clusters until single-member clusters were obtained [22, 23].

Phylogenetic Relationships of Non-iNTS Typhimurium Isolates

We calculated a separate maximum-likelihood tree including the 4 isolates that had SNP differences of >12 000 from the *Salmonella* Typhimurium SL1344 reference, and 11 additional reference *Salmonella enterica* serovars including *Salmonella enterica* serovar Enteritidis, *Salmonella* Typhi, and *Salmonella* Paratyphi groups A, B, and C (Supplementary Table 2).

Discrimination Between Recrudescence and Reinfection Isolates

We based classification of recrudescence and reinfection isolates primarily on their relationship within the phylogenetic trees by determining whether 1 isolate could have been plausibly derived from another with reference to the structure. We calculated the number of pairwise SNP differences between index and recurrence isolates.

RESULTS

SNP and Phylogenetic Analyses of Recurrent iNTS Disease Isolates Based on Whole-Genome Sequences

We selected 47 index and recurrence NTS isolates from systemic and mucosal sites, from 14 patients who had multiple recurrences

following an index presentation with iNTS. Sampling at different sites is described in Table 1. The isolates used are summarized in Table 2; they comprised 35 blood isolates, 8 bone-marrow isolates, 2 stool isolates, and a single isolate each from an oral-pharyngeal swab and a urine sample. To control the study, we also included 7 previously sequenced Malawian iNTS Typhimurium isolated from blood cultures of febrile pediatric hospital admissions and the gastroenteritis-associated reference SL1344 [24].

We detected high-quality discriminatory SNPs by mapping sequence reads to the *Salmonella* Typhimurium SL1344 reference sequence. Of the isolates, 43 were closely related to the *Salmonella* Typhimurium SL1344 reference, differing by 348–834 SNPs. The other 4 recurrent disease isolates, from participants Q23 and Q255, were found to differ from SL1344 by >12 000 SNPs (Table 3). Serotyping identified Q23A, Q23F1, and Q23F2_BM as *S. Enteritidis*, and this was concordant with their phylogenetic relationships. Isolate Q255F1_U was identified serologically as belonging to *Salmonella* group D and derived phylogenetically from the same branch as *S. Enteritidis* (Figure 1A, Supplementary Figure 1).

The sequences of the 43 iNTS Typhimurium isolates from 14 patients and 8 control iNTS Typhimurium isolates were next subjected to a maximum-likelihood tree analysis to determine the overall population structure in fine detail. We confirmed this tree using several independent approaches, with bootstrap support and clade credibility marked. All the iNTS Typhimurium isolates from patients in the study fell within 3 distinct phylogenetic clusters (Figure 1B). Clusters 1 and 2 included 4 study isolates (9.3%) and 38 study isolates (88.4%), respectively. Both these clusters represented sequence type ST313 *Salmonella* Typhimurium strains. Cluster 3 contained only the index isolate Q303A (2.3%), which is most closely related isolate to the SL1344 reference.

Population Structure of Isolate Groups as a Basis to Define Recrudescence and Reinfection

Overall, >78% of recurrent isolates (25 of 32) represented recrudescence events among the samples we analyzed, and 13 of 14 participants demonstrated recrudescences during the study

Table 2. Isolates Used in the Study^a

Index Isolate	Date of Isolation	Recurrent Isolate 1	Date of Isolation	Recurrent Isolate 2	Date of Isolation	Recurrent Isolate 3	Date of Isolation	Recurrent Isolate 4	Date of Isolation
Q18A	28/03/2002	Q18A_S	28/03/2002	Q18F2	24/05/2002	Q18F3_S	22/06/2002		
Q23A	26/02/2002	Q23F1	25/03/2002	Q23F2_BM	26/04/2002	Q23F5	26/03/2003		
Q134A	30/05/2002	Q134F4	16/10/2002	Q134F8	28/04/2003	Q134F9	06/09/2003		
Q175A	01/07/2002	Q175F4_TH	27/01/2003	Q175F6	07/04/2003				
Q213F1a	02/09/2002	Q213F1b	05/09/2002	Q213F3_BM	21/10/2002				
Q217A	18/03/2002	Q217F3	12/11/2002						
Q255A	17/09/2002	Q255F1_BM	10/10/2002	Q255F2_U	13/11/2002	Q255F4	26/03/2003		
Q258A	21/10/2002	Q258F3_BM	07/01/2003	Q258F4	02/03/2003				
Q285A	22/08/2002	Q285F1_BM	11/11/2002	Q285F5	22/04/2003				
Q303A	26/11/2002	Q303F1	20/12/2002	Q303F3	02/03/2003	Q303F5	22/04/2003		
Q340F1	10/01/2002	Q340F1_BM	10/01/2003	Q340F4	11/05/2003	Q340_F4BM	11/05/2003		
Q363A	03/12/2002	Q363F3	27/03/2003						
Q367A	31/12/2002	Q367F3	17/03/2003						
Q435A	02/03/2003	Q435A2	02/03/2003	Q435F1	07/04/2003	Q435F1_BM	14/04/2003	Q435F2	18/06/2003
Other sequenced African invasive nontyphoidal <i>Salmonella</i> Typhimurium used in the study									
A130	1997								
D22827	2003								
D22889	2003								
D25734	2004								
D25248	2004								
D26275	2004								
D25646	2004								
D23580	2004								

^a Isolates from 14 individual patients (Q) with recurrent invasive nontyphoidal *Salmonella* Typhimurium disease, consisting of index presentation invasive isolates (A) and recurrent isolates at follow-up visits (F1, F2, F3 etc). Isolates are from 5 different tissues: blood (no suffix), bone marrow (_BM), stool (_S), throat (_TH) and urine (_U).

(Table 2). Of recurrent isolates, 22% (7 of 32) represented reinfections, and only 1 patient (Q175) had recurrences that were only a result of reinfection. A further 4 of 14 patients showed a combination of recrudescence and reinfection patterns.

Participants With Only Recrudescence

Isolate clusters from 9 patients—Q18, Q213, Q217, Q340, Q258, Q285, Q363, Q367, and Q435 (Figure 1C and 1D, Table 2)—suggested that all recurrent events for these participants were caused by recrudescence. We considered that these could be defined as recrudescence isolates as they were positioned in discrete clusters on individual phylogenetic branches and were highly genetically related, differing by only 0–6 SNPs (Table 3). Such SNP numbers could easily accumulate as the bacteria persist and replicate within the host. Whereas a single recrudescence isolate group, Q213, was found in cluster 1, the 8 other recrudescence isolate groups fell within cluster 2.

Participants With Only Reinfection

Other isolate pairs or series from the same individual fell on separate branches either within the same cluster or on different

clusters, and we considered that these could be defined as likely reinfections, as later isolates could not be plausibly derived from earlier ones. The numbers of pairwise SNP differences were 5–508 within *Salmonella* Typhimurium serovars, and >12 000 between *Salmonella* Typhimurium and *S. Enteritidis* isolate pairs (Figure 1A, Table 3). For example, patient Q175 experienced only reinfections, at both a mucosal and an invasive site. The index and first recurrence isolates, Q175A and Q175F4_TH respectively, fell on different branches within cluster 2, despite only differing by 5 SNPs (Figure 1D), whereas a second recurrence isolate, Q175F6, was found in cluster 1 (Figure 1C).

Participants With Both Recrudescence and Reinfection

Isolate groups from 4 patients, Q23, Q134, Q255, and Q303, provided examples of disease recurrence resulting from combinations of recrudescence and reinfection within 1 participant (Figure 1D, Table 2). The index and recurrence isolates Q23A, Q23F1, and Q23F2_BM were identified as iNTS Enteritidis isolates, based on serology and phylogeny, and these F1 and F2 isolates were most likely early recrudescence episodes. A later

Table 3. Single Nucleotide Polymorphism Differences for Recrudescence and Reinfection Isolates

Index Isolate	Recurrent Isolate 1	SNPs in Recurrent Isolate 1	Recurrent Isolate 2	SNPs in Recurrent Isolate 2	Recurrent Isolate 3	SNPs in Recurrent Isolate 3	Recurrent Isolate 4	SNPs in Recurrent Isolate 4
Recrudescence								
Q18A	Q18A_S	0	Q18F2	0	Q18F3_S	0		
Q213F1a	Q213F1b	2	Q213F3_BM	6				
Q217A	Q217F3	0						
Q258A	Q258F3_BM	1	Q258F4	1				
Q285A	Q285F1_BM	2	Q285F5	1				
Q340F1	Q340F1_BM	1	Q340F4	0	Q340_F4BM	0		
Q363A	Q363F3	0						
Q367A	Q367F3	1						
Q435A	Q435FA2	2	Q435F1	2	Q435F1_BM	1	Q435F2	1
Reinfection								
Index Isolate	Recurrent Isolate 1	SNPs in Recurrent Isolate 1	Recurrent Isolate 2	SNPs in Recurrent Isolate 2				
Q175A	^a Q175F4_TH	5	^a Q175F6	470				
Recrudescence and Reinfection								
Index Isolate	Recurrent Isolate 1	SNPs in Recurrent Isolate 1	Recurrent Isolate 2	SNPs in Recurrent Isolate 2	Recurrent Isolate 3	SNPs in Recurrent Isolate 3		
Q23A	Q23F1	0	Q23F2_BM	0	^a Q23F5	38569		
Q134A	Q134F4	1	^a Q134F8	11	Q134F9	11		
Q255A	Q255F1_BM	0	^a Q255F2_U	36974	^a Q255F4	9		
Q303A	^a Q303F1	505	Q303F3	508	Q303F5	507		

Abbreviation: SNP, single nucleotide polymorphism.

^a Isolate that represents a reinfection at a mucosal or invasive site, with a nontyphoidal *Salmonella* pathovar that is phylogenetically distinct from the chronologically previous isolate. Isolates are labeled according individual patients (Q) with recurrent invasive nontyphoidal *Salmonella* Typhimurium disease, consisting of index presentation invasive isolates (A) and recurrent isolates at follow-up visits (F1, F2, F3 etc). Isolates are from 5 different tissues: blood (no suffix), bone marrow (_BM), stool (_S), throat (_TH) and urine (_U).

invasive reinfection with an *Salmonella* Typhimurium isolate, Q23F5, was identified, which differed from the index *S. Enteritidis* cluster by >38 000 SNPs.

For participant Q134, the index and recurrence isolates, Q134A and Q134F4, formed a recrudescence pair differing by a single SNP but fell on a different phylogenetic branch within cluster 2 (Figure 1D) from a later reinfection isolate, Q134F8. Q134F9 thereafter represented a new recrudescence pair with Q134F8. The index and first recurrence isolates Q255A and Q255F1_BM formed an identical recrudescence pair; whereas a recurrence isolate, Q255F4, fell on a separate branch within cluster 2 (Figure 1D), which was indicative of a later invasive reinfection. Interestingly, the urine sample Q255F1_U had 36 974 SNP loci different from the index isolate and represented a mucosal reinfection with another NTS serovar, which was closely related to *S. Enteritidis* (Table 2, Supplementary Figure 1).

For participant Q303, only a single disease episode resulted from reinfection whereas all later isolates represented recrudescence. The index isolate Q303A, found in cluster 3 (Figure 1B), differed from the reinfection isolate Q303F1

found on a single phylogenetic branch in cluster 2, by >500 SNPs (Figure 1D, Table 2). Q303F1 subsequently showed recrudescence on 2 occasions (Q303F3 and Q303F5), when only 2–3 additional SNP differences were observed.

The time to recurrence ranged from 23 to 486 days between recrudescence pairs, and 24 to 464 days between reinfection pairs. The time to recurrence showed no correlation with the number of SNP differences between index and recrudescence isolates (Figure 2).

DISCUSSION

Recurrence of iNTS Typhimurium disease may be either a result of recrudescence, where the index isolate and the recurrent isolate are either genetically indistinguishable or plausibly phylogenetically derived one from the other, or a result of reinfection, where the subsequent recurrent isolate is phylogenetically distinct from the index isolate [6]. To explain the nature of recurrent iNTS disease, we analyzed isolate series from 14 patients with multiple recurrences of iNTS. The high-resolution SNP-typing and

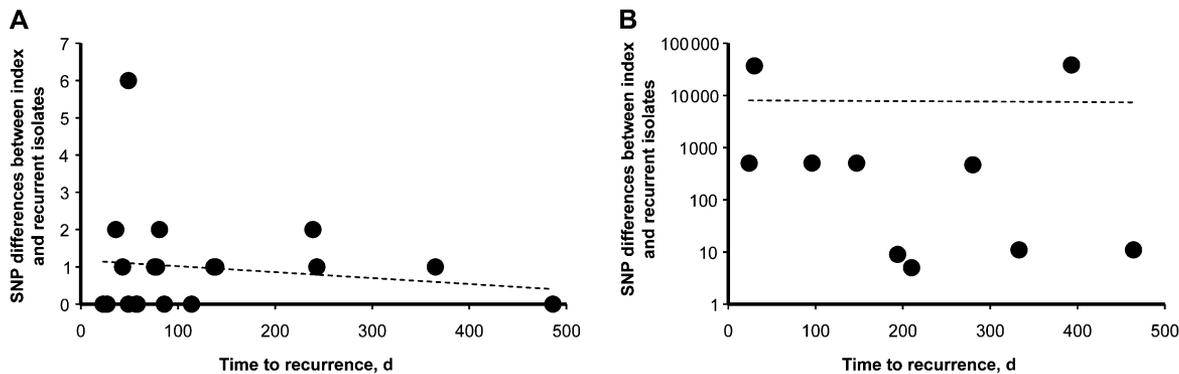


Figure 2. Linear regression plots of single nucleotide polymorphism differences between recrudescence pairs (A) and reinfection pairs (B) against time to recurrence. R^2 values for A = 0.03; B = 0.0002. Abbreviation: SNP, single nucleotide polymorphism.

The high recurrence rate we observed is a well-known feature of NTS infections associated with HIV infection [25–28]. The NTS might reemerge from a suppurative focus of infection (eg, endothelium, biliary or urinary tract, or musculoskeletal sites) [28–31] to cause recurrence, although we have not found this to be a frequent occurrence in our setting [3]. The larger study, from which the participants and isolates for this investigation were drawn [11], showed that there is an intracellular element of iNTS in HIV infection, that there is persistence and replication of NTS in the bone-marrow, and that recurrence is associated with an apparent failure of immunological control, all of which suggests that recrudescence from an intracellular sanctuary site might be the most likely explanation for recurrent iNTS disease in these patients.

Environmental factors such as contaminated food and water supplies, exposure to asymptomatic human carriers [31], or hospital-acquired infection [31, 32] could also potentially contribute to recurrent iNTS Typhimurium disease as a result of re-exposure and reinfection, especially in immunocompromised patients [33]. Clearly, repeated re-exposure might result in infection with a very closely related or identical isolate, or else with a new, unrelated isolate. Our study has not addressed these important possibilities, but there is an urgent need for comprehensive epidemiological investigation to understand the environmental reservoirs and transmission of NTS in sub-Saharan Africa. The methods we describe here would provide the resolution that would be critical for such investigations.

It is possible that there is heterogeneity in the population structure of *Salmonellae* within an individual, which could be underappreciated by sampling a single colony from each primary culture. We found, however, that 78% of recurrent isolates were near-identical to their preceding isolate, even from different sites and even over time. The data therefore point to the strain population within these individuals being homogeneous.

At the time of sampling (2002–2003), multidrug resistant (MDR) iNTS Typhimurium isolates (resistant to ampicillin, chloramphenicol, and cotrimoxazole) were epidemic in Blantyre, Malawi, accounting for >75% of iNTS isolates [4, 34]. Repeated treatment of index and recurrent disease with ciprofloxacin did not, however, prevent multiple recurrences. All but 1 of the *Salmonella* Typhimurium isolates identified in our study were of multilocus sequence typing sequence type 313 (ST313), a novel *Salmonella* Typhimurium pathovar that has been associated exclusively with invasive disease in Africa and which shows genome degradation suggesting host adaptation to humans [35]. During 2001, an MDR ST313 clade represented by D23580 (cluster 2) clonally replaced a previously dominant ST313 clade represented by A130 (cluster 1), in both Malawi and Kenya. It is therefore unsurprising that most of our study isolates were closely related to D23580, forming a highly clonal cluster with <450 chromosomal variable sites. Both clades have several drug resistance markers borne on a virulence plasmid, but both remain susceptible to fluoroquinolones [35].

Recrudescence was a commoner event than reinfection in our study: 78% of recurrent isolates represented recrudescence events, and 13 of 14 participants demonstrated recrudescences during the study. The number of days (23–486 d) between isolation of index and recurrent isolates had no obvious impact on the numbers of SNP differences accumulated, suggesting that these isolate groups comprise single clonal haplotypes with virtually no genetic change over time, despite exposure to multiple courses of ciprofloxacin. In contrast, 22% of recurrent isolates represented reinfections, and only 1 patient (Q175) had recurrences that were only a result of reinfection. A further 4 of 14 patients showed a combination of recrudescence and reinfection patterns. From our results, it was clear that recrudescences and reinfections could occur in either order, could occur over varying time scales, could occur multiply or singly, and could occur in either main cluster group.

We studied 4 incidences of infection with another iNTS serovar, Enteritidis, which accounted overall for 21% of iNTS events in Blantyre between 1998 and 2004 [4]. We have observed 1 incidence (Q255) of urinary reinfection with *S. Enteritidis* and 1 case (Q23) with an index series of recrudescence, invasive *S. Enteritidis* infections. These data are sufficient to demonstrate that, as with *Salmonella* Typhimurium, both reinfection with and recrudescence of *S. Enteritidis* may occur in HIV-associated iNTS disease.

Although sampling from mucosal sites was not as comprehensive as from invasive sites, the overall yield from mucosal sites was low, suggesting that prolonged mucosal carriage may be a relatively rare event. The nasopharyngeal and urine isolates (Q175F4_TH and Q255F2_U) represented incidences of later reinfection of the mucosa with a genetically different isolate, which was unrelated to any invasive isolates from the same individual. This is in contrast to the 2 stool samples (Q18A_S and Q18F3_S), which were longitudinally identical to the invasive isolates from the same participant, consistent with a hypothesis that the gut is the main site of invasion of iNTS.

Our results highlight the efficacy and applicability of whole-genome phylogenetics in understanding the pathogenesis of iNTS. The discriminatory power of this approach is likely to be equally necessary in a large-scale epidemiological investigation of this emerging pathogen.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/cid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the Wellcome Trust, United Kingdom (grant number 076964) and the Wellcome Trust Training Fellowship in Clinical Tropical Medicine to MAG; the Wellcome Trust Sanger Institute (grant number 098051) PhD fellowship to CKO.

Potential conflict of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Ethical approval statement. All participants gave written informed consent, and the study was approved by Liverpool School of Tropical Medicine Research Ethics committee and by the University of Malawi, College of Medicine Research Ethics Committee.

References

- Graham SM. Nontyphoidal salmonellosis in Africa. *Curr Opin Infect Dis* 2010; 23:409–14.
- Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: A systematic review and meta-analysis. *Lancet Infect Dis* 2010; 10:417–32.
- Gordon MA, Banda HT, Gondwe M, et al. Non-typhoidal *Salmonella* bacteraemia among HIV-infected Malawian adults: High mortality and frequent recrudescence. *AIDS* 2002; 16:1633–41.
- Gordon MA, Graham SM, Walsh AL, et al. Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis* 2008; 46:963–9.
- Gordon MA. *Salmonella* infections in immunocompromised adults. *J Infect* 2008; 56:413–22.
- Wain J, Hien TT, Connerton P, et al. Molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serovar Typhi from Vietnam: Application to acute and relapse cases of typhoid fever. *J Clin Microbiol* 1999; 37:2466–72.
- Rubino S, Spanu L, Mannazzu M, et al. Molecular typing of non-typhoid *Salmonella* strains isolated from HIV-infected patients with recurrent salmonellosis. *AIDS* 1999; 13:137–9.
- Islam A, Butler T, Ward LR. Reinfection with a different Vi-phage type of *Salmonella typhi* in an endemic area. *J Infect Dis* 1987; 155: 155–6.
- Harris SR, Feil EJ, Holden MT, et al. Evolution of MRSA during hospital transmission and intercontinental spread. *Science* 2010; 327: 469–74.
- Croucher NJ, Harris SR, Fraser C, et al. Rapid pneumococcal evolution in response to clinical interventions. *Science* 2011; 331:430–4.
- Gordon MA, Kankwatira AM, Mwafulirwa G, et al. Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: An emerging disease pathogenesis. *Clin Infect Dis* 2010; 50:953–62.
- Bentley DR, Balasubramanian S, Swerdlow HP, et al. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* 2008; 456:53–9.
- Holt KE, Parkhill J, Mazzoni CJ, et al. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. *Nat Genet* 2008; 40:987–93.
- He M, Sebahia M, Lawley TD, et al. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc Natl Acad Sci U S A* 2010; 107:7527–32.
- Kurtz S, Choudhuri JV, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R. REPuter: The manifold applications of repeat analysis on a genomic scale. *Nucleic Acid Res* 2001; 29:4633–42.
- Kurtz S, Phillippy A, Delcher AL, et al. Versatile and open software for comparing large genomes. *Genome Biol* 2004; 5:R12.
- Ning Z, Cox AJ, Mullikin JC. SSAHA: A fast search method for large DNA databases. *Genome Res* 2001; 11:1725–9.
- Stamatakis A. RAXML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 2006; 22:2688–90.
- Felsenstein J. PHYLIP (phylogeny inference package) [computer program]. Version 3.69. Seattle, Washington: Department of Genome Sciences, University of Washington. Distributed by author, 2005.
- Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003; 19:1572–4.
- Altekar G, Dwarkadas S, Huelsenbeck JP, Ronquist F. Parallel Metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. *Bioinformatics* 2004; 20:407–15.
- Corander J, Tang J. Bayesian analysis of population structure based on linked molecular information. *Math Biosci* 2007; 205: 19–31.
- Corander J, Marttinen P, Siren J, Tang J. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics* 2008; 9:539.
- Hoiseh SK, Stocker BA. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 1981; 291:238–9.
- Jacobs JL, Gold JW, Murray HW, Roberts RB, Armstrong D. *Salmonella* infections in patients with the acquired immunodeficiency syndrome. *Ann Intern Med* 1985; 102:186–8.

26. Nadelman RB, Mathur-Wagh U, Yancovitz SR, Mildvan D. *Salmonella* bacteremia associated with the acquired immunodeficiency syndrome (AIDS). *Arch Intern Med* **1985**; 145:1968–71.
27. Smith PD, Macher AM, Bookman MA, et al. *Salmonella typhimurium* enteritis and bacteremia in the acquired immunodeficiency syndrome. *Ann Intern Med* **1985**; 102:207–9.
28. Levine WC, Buehler JW, Bean NH, Tauxe RV. Epidemiology of nontyphoidal *Salmonella* bacteremia during the human immunodeficiency virus epidemic. *J Infect Dis* **1991**; 164:81–7.
29. Fernández Guerrero ML, Ramos JM, Núñez A, de Górgolas M. Focal infections due to non-typhi *Salmonella* in patients with AIDS: Report of 10 cases and review. *Clin Infect Dis* **1997**; 25:690–7.
30. Hohmann EL. Nontyphoidal salmonellosis. *Clin Infect Dis* **2001**; 32: 263–9.
31. Kariuki S, Revathi G, Kariuki N, et al. Invasive multidrug-resistant non-typhoidal *Salmonella* infections in Africa: Zoonotic or anthroponotic transmission? *J Med Microbiol* **2006**; 55:585–91.
32. Keddy KH, Dwarika S, Crowther P, et al. Genotypic and demographic characterization of invasive isolates of *Salmonella* Typhimurium in HIV co-infected patients in South Africa. *J Infect Dev Ctries* **2009**; 3:585–92.
33. Morpeth SC, Ramadhani HO, Crump JA. Invasive non-Typhi *Salmonella* disease in Africa. *Clin Infect Dis* **2009**; 49:606–11.
34. Gordon MA, Graham SM. Invasive salmonellosis in Malawi. *J Infect Dev Ctries* **2008**; 2:438–42.
35. Kingsley RA, Msefula CL, Thomson NR, et al. Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res* **2009**; 19: 2279–87.



Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Okoro, CK;Kingsley, RA;Quail, MA;Kankwatira, AM;Feasey, NA;Parkhill, J;Dougan, G;Gordon, MA

Title:

High-Resolution Single Nucleotide Polymorphism Analysis Distinguishes Recrudescence and Reinfection in Recurrent Invasive Nontyphoidal *Salmonella Typhimurium* Disease

Date:

2012-04-01

Citation:

Okoro, C. K., Kingsley, R. A., Quail, M. A., Kankwatira, A. M., Feasey, N. A., Parkhill, J., Dougan, G. & Gordon, M. A. (2012). High-Resolution Single Nucleotide Polymorphism Analysis Distinguishes Recrudescence and Reinfection in Recurrent Invasive Nontyphoidal *Salmonella Typhimurium* Disease. CLINICAL INFECTIOUS DISEASES, 54 (7), pp.955-963. <https://doi.org/10.1093/cid/cir1032>.

Persistent Link:

<http://hdl.handle.net/11343/260635>

License:

CC BY-NC