South Asia as a reservoir for the global spread of ciprofloxacin resistant *Shigella sonnei*

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Short title: Ciprofloxacin resistant *Shigella sonnei*

Abstract

Background

Antimicrobial resistance is a major issue in the *Shigellae,* particularly as a specific multidrug resistant (MDR) lineage of *Shigella sonnei* (lineage III) is becoming globally dominant. Ciprofloxacin is a recommended treatment for *Shigella* infections. However, ciprofloxacin resistant *S. sonnei* are being increasingly isolated in Asia, and sporadically reported on other continents.

Methods and Findings

Hypothesising that Asia is the hub for the recent international spread of ciprofloxacin resistant *S. sonnei,* we performed whole genome sequencing on a collection of contemporaneous ciprofloxacin resistant *S. sonnei* isolated in six countries from within and outside of Asia. We reconstructed the recent evolutionary history of these organisms and combined these data with their geographical location of isolation. Placing these sequences into a global phylogeny we found that all ciprofloxacin resistant *S. sonnei* formed a single clade within a Central Asian expansion of Lineage III. Further, our data show that resistance to ciprofloxacin within *S. sonnei* can be globally attributed to a single clonal emergence event, encompassing sequential *gyrA*-S83L, *parC*-S80I and *gyrA*-D87G mutations. Geographical data predict that South Asia is the likely primary source of these organisms, which are being regularly exported across Asia and intercontinentally into Australia, the USA and Europe.

Conclusions

This study shows that a single clone, which is widespread in South Asia, is driving the current intercontinental surge of ciprofloxacin resistant *S. sonnei* and is capable of establishing endemic transmission in new locations. Despite being limited in geographical scope, our work has major implications for understanding the international transfer of antimicrobial resistant *S. sonnei,* and provides a tractable model for studying how antimicrobial resistant Gram-negative community acquired pathogens spread globally.

Introduction

Diarrheal disease is the second most common cause of mortality in children under the age of five years worldwide, equating to approximately 800,000 deaths per year [1]. The recent Global Enteric Multicentre Study (GEMS), a large prospective case-control study focusing on mild and severe paediatric diarrheal illnesses in sub-Saharan Africa and South Asia, found that *Shigella* (a genus of Gram-negative enteric bacteria) were amongst the top four most prevalent diarrhoeal pathogens in these settings [2]. The most recent estimates suggest that *Shigella* infections account for around 125 million cases of diarrhoea annually, with the majority occurring in children in low income countries [3]. There are four *Shigella* species (*dysenteriae*, *boydii*, *flexneri* and *sonnei*), but the overwhelming majority of the current global burden is presently caused by *S. sonnei* and *S. flexneri*. Present-day international epidemiology of the various *Shigella* species is particularly intriguing, as *S. sonnei* is replacing *S. flexneri* as the most common cause of shigellosis worldwide; this pattern is accentuated in regions undergoing rapid economic development [4,5], where *S. flexneri* dominated as recently as a decade ago.

Shigella infections are characterised by the invasion and disruption of the epithelial cells lining the gastrointestinal mucosa, resulting in mucous and/or bloody diarrhoeal discharge. Although shigellosis is typically self-limiting, antimicrobial treatment is used to prevent complications, reduce dysenteric discharge and curb post-symptomatic faecal shedding [6,7]. Consequently, resistance to antimicrobials restricts treatment options, placing vulnerable individuals suffering from shigellosis at increased risk of complications and increasing the likelihood of protracted faecal shedding. One of the current recommended first-line treatments for shigellosis is the fluoroquinolone, ciprofloxacin [8]. The fluoroquinolones target the DNA gyrase, a type II topoisomerase that is essential for bacterial DNA replication and transcription [9].

Antimicrobial resistance is an emerging global issue in *S. sonnei,* with a specific multidrug resistant (MDR) lineage (III) now dominating internationally. Further, organisms belonging to lineage III appear to be highly proficient at acquiring resistance to additional antimicrobials (including third

generation cephalosporins) when they are introduced into new locations [10]. However, given their common usage and broad spectrum of activity, resistance against the fluoroquinolones is the most concerning. Since the first isolation of *S. sonnei* with reduced susceptibility to ciprofloxacin in Japan in 1993 [11], ciprofloxacin resistant *S. sonnei* have been increasingly reported throughout Asia [12–14]. Furthermore, public health laboratories in several non-Asian countries with low incidences of shigellosis have reported the isolation of ciprofloxacin resistant *S. sonnei*, often from individuals reporting recent travel to locations with a high risk of shigellosis [15–17].

Whole genome sequencing has proven to be the gold standard for tracking the international dissemination of clonal bacterial pathogens [18,19], and we have previously exploited this method to study the phylogenetic structure and spread of *S. sonnei* at both national and intercontinental levels [10,20]. Hypothesising that Asia was a hub for the recent international spread of ciprofloxacin resistant *S. sonnei,* we performed whole genome sequencing and phylogenetic characterisation of a collection of ciprofloxacin resistant *S. sonnei* isolated from within and outside Asia, aiming to explore the origins of this growing international epidemic.

Methods

Strain collection

Aiming to investigate the current international upsurge in ciprofloxacin resistant *S. sonnei* in detail, we gathered a collection of 60 contemporary ciprofloxacin resistant *S. sonnei* from six countries for whole genome sequencing. The isolates originated from Asian countries with a high incidence of shigellosis (Vietnam, n=11; Bhutan, n=12; Thailand, n=1; Cambodia, n=1), as well as isolates from countries with a low incidence of shigellosis (Australia, n=19; Ireland, n=16). Twelve additional ciprofloxacin susceptible *S. sonnei* sequences from these settings were also included for phylogenetic context. All strains were isolated independently between 2010 and 2015; details of the isolates used in this study are shown in Table 1.

142 The *S. sonnei* isolates from countries with a high incidence of shigellosis in Asia were collected
143 through diarrhoeal disease surveillance in Bhutan and Thailand and as part of on-going, local IRB
144 approved, hospital based studies in Ho Chi Minh City, Vietnam and Siem Reap, Cambodia [14]. The
145 target patient group for these studies were/are generally hospitalised children aged less than five years
146 residing in close proximity to the study centres. The ciprofloxacin resistant *S. sonnei* from countries
147 with a low incidence of shigellosis (outside Asia) were collected and characterized by the National
148 *Salmonella*, *Shigella* and *Listeria monocytogenes* Reference Laboratory, Galway, Ireland, and the
149 Microbiological Diagnostic Unit Public Health Laboratory, Melbourne, Australia. These isolates were
150 generally, but not exclusively, obtained from patients reporting recent travel to countries with a high
151 incidence of shigellosis in Asia (Table 1). Susceptibility to ciprofloxacin was determined by either
152 disk diffusion, E-test, agar dilution or broth microdilution, depending on the collaborating institution,
153 and susceptibility breakpoints were interpreted according to the European Committee on
154 Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical_breakpoints). Namely, resistance
155 was determined as strains with a zone of inhibition ≤15 mm (5 µg disc) and/or a Minimum Inhibitory
156 Concentration (MIC) >2 µg/ml against ciprofloxacin; the various location specific methods and
157 resulting data are described in Table 1.
158
159 ***Genome sequencing and analysis***
160 All isolated *S. sonnei* were sub-cultured and subjected to DNA extraction prior to whole genome
161 sequencing on various Illumina platforms to produce pair-ended short read sequence; the specific
162 sequencing system and the resulting public database numbers are shown in Table 1. We additionally
163 included 14 *S. sonnei* sequences from organisms isolated in the USA and deposited in the
164 GenomeTrackr Project (NCBI BioProject number PRJNA218110). All sequences were mapped to the
165 *S. sonnei* Ss046 reference sequence (Accession number: NC_007384) using SMALT (version 0.7.4)
166 and SNPs were called against the reference and filtered using SAMtools [21]. To contextualize all
167 ciprofloxacin resistant *S. sonnei* within the global phylogeny, we appended our collection to include
168 133 publicly available sequences from a previous global analysis (accession ERP000182) [20].
169 Previously characterized mobile genetic elements and putative recombination (predicted using

Gubbins) were removed [20], resulting in a gap-free alignment of 211 non-duplicate pseudo-whole genome sequences of 4,738 SNPs. A whole genome phylogeny was inferred from this alignment using RAxML v8.1.3 under the GTRGAMMA substitution model, and sufficient bootstrap replicates were determined automatically using the extended majority rule (MRE) bootstrap convergence criterion. In order to obtain a refined phylogenetic structure of the Central Asia clade, we applied the aforementioned approach to a set of 97 *S. sonnei* sequences (86 novel sequences and 11 historical sequences) belonging to this clade. This resulted in an alignment of 1,121 SNPs, which was used for phylogenetic inference.

Results

Fluoroquinolone resistant Shigella sonnei in a global context

We constructed a whole genome phylogeny of *S. sonnei*, incorporating sequences from 133 globally representative isolates and 86 novel isolates from Vietnam, Cambodia, Thailand, Bhutan, Australia, Ireland and the USA. The novel sequences included 60 from ciprofloxacin resistant (MIC >2 μg/ml) organism and 26 from ciprofloxacin susceptible organisms (or of unknown ciprofloxacin susceptibility isolated in the USA). The overall tree topology reflected the previously described global phylogenetic structure [20], confirming the presence of four distinct lineages (I, II, III and IV); lineage III was the most commonly represented and the most widely geographically distributed (Figure 1A). All ciprofloxacin resistant *S. sonnei* formed a single well-supported monophyletic clade within the Central Asian expansion of Lineage III (Central Asia III); an MDR group that is closely related but distinct from the Global III clade (Figure 1A and 1B).

The emergence of a fluoroquinolone resistant Shigella sonnei clone

We next performed a more detailed phylogenetic reconstruction of the Central Asia III clade, incorporating sequence data from the 60 phenotypically ciprofloxacin resistant isolates and 26 others (ciprofloxacin susceptible or of unknown ciprofloxacin susceptibility), along with 11 historical Central Asia III sequences sourced from our previous global study (Figure 1B) [20]. The majority of the Central Asia III isolates carried more than three antimicrobial resistance genes, encoding

198 resistance to a wide range of first-line drugs including tetracycline, streptomycin and co-trimoxazole.
199 We additionally examined the genome sequence data for mutations in the Quinolone Resistance
200 Determining Region (QRDR) within the DNA gyrase gene (*gyrA*) and the topoisomerase IV gene
201 (*parC*), the regions encoding the target residues for fluoroquinolone activity. Overlaying these
202 mutations on the phylogenetic tree indicated that the *gyrA*-S83L mutation, the first sequential
203 mutation which confers reduced susceptibility against fluoroquinolones, has arisen independently
204 within the Central Asia III clade on at least four separate occasions (Figure 1B). Amongst the isolates
205 examined here for the first time, extensive resistance to ciprofloxacin can be attributed to a single
206 clonal emergence event, via the sequential accumulation of *gyrA*-S83L followed by *parC*-S80I and
207 *gyrA*-D87G, except for a single outlier that was isolated in Australia (Figure 1B). These three QRDR
208 mutations were also shared by ten phenotypically uncharacterized *S. sonnei* from the USA, thus
209 providing genotypic evidence for ciprofloxacin resistance. The single outlier isolate shares the *gyrA*-
210 S83L and *parC*-S80I QRDR mutations of the other ciprofloxacin resistant isolates, but harbours *gyrA*-
211 D87N rather than a *gyrA*-D87G, and is within a closely related out group of the major ciprofloxacin
212 resistant clone (Figure 1B).
213
214 ***South Asia as a hub of fluoroquinolone resistant Shigella sonnei***
215 We additionally mapped the country of isolation and patient travel history onto the Central Asia III
216 phylogeny to investigate the geographical structure of the clade (Figure 1B). For the ciprofloxacin
217 resistant *S. sonnei* isolated from countries with a low incidence of shigellosis (Ireland, Australia and
218 USA) and for which data on recent travel history was confirmed (27/45; 60%), India was the most
219 commonly reported travel destination (21/27; 78%). The majority of the isolates associated with travel
220 to India clustered closely with strains isolated in neighbouring Bhutan. These data suggest that South
221 Asia is the primary source of ciprofloxacin resistant *S. sonnei* that have increasingly been isolated
222 both inside and outside of Asia in recent years. Further, greater genetic diversity was observed within
223 the South Asian *S. sonnei* than within the other sampled countries (Figure 1B), suggesting that this
224 region acts as the most likely geographical source population.
225

Our data also show evidence of regional diversification of ciprofloxacin resistant *S. sonnei* within Asia. The phylogenetic structure is highly suggestive of a clonal expansion of ciprofloxacin resistant *S. sonnei* in Southeast Asia, specifically within Vietnam, as indicated by a long branch with 100% bootstrap support (Figure 1B). We additionally noted that *S. sonnei* nested within this clonal expansion were also isolated from travellers returning from countries including Cambodia and Thailand, indicating that isolates from this lineage have spread widely across Southeast Asia, as well as having been introduced into Australia on at least five separate occasions. An additional subpopulation of ciprofloxacin resistant *S. sonnei*, isolated in Ireland (five individuals with no recent history of travel and one individual returning from Germany) and the USA, are also likely representative of an expansion of this clone within Europe and the USA (Figure 1B). Whilst it was not possible to identify the geographical source definitively, the isolates most closely related to this European/USA subpopulation originated in India and Bhutan, again suggesting South Asia was the most likely origin. These two examples of subpopulation clonal expansions in Southeast Asia and Europe/USA indicate that this clone of ciprofloxacin resistant *S. sonnei* is also capable of sustained circulation upon introduction into new locations.

Discussion

Here we provide direct evidence for the on-going global expansion of *S. sonnei* exhibiting new and clinically relevant antimicrobial resistance profiles. What is more, this study has significant implications for understanding the international trafficking of antimicrobial resistant bacterial pathogens. We suggest that as a single-serotype, human-adapted pathogen with a clonal population structure, *S. sonnei* serves as a tractable model for understanding how Gram-negative antimicrobial resistant pathogens are being regularly mobilised around the globe.

This is the first study that has used whole genome sequencing to examine the emergence and global spread of ciprofloxacin resistant *S. sonnei*. Our data show that all sequenced extant ciprofloxacin resistant *S. sonnei*, though sourced from disparate geographical locations, belonged to a single clonal expansion of Lineage III, with South Asia being the most likely hub for its origin and spread. Our

findings support previous hypotheses suggesting that ciprofloxacin resistant *S. sonnei* in industrialised countries is being imported from South Asia [15,16]. A recent estimation of worldwide antimicrobial usage reported that India was the largest consumer of antimicrobials in 2010 [22]. Additionally, the fluoroquinolones are ranked as the most common antimicrobial prescribed for acute enteric diseases in India and Bangladesh [23,24]. The intensive use of fluoroquinolones in a region where there are foci of high population density and inconsistent access to sanitation is likely to have contributed to emergence of ciprofloxacin resistant enteric bacteria, such as *S. sonnei* and *Salmonella* Typhi, on the Indian subcontinent [19]. Global dissemination of these organisms is likely facilitated by the volume of travel between these regions and other areas of the world.

Our new data highlight the limitations of current typing protocols for tracking *S. sonnei*. It had been previously observed that some of the ciprofloxacin resistant *S. sonnei* isolates in this study (originating from Bhutan and Ireland) shared a similar *Xba*I Pulse Field Gel Electrophoresis (PFGE) pattern [14,15]. This pulsotype has been observed previously in India and Bangladesh [12,13,25–27], as well as in Canada [28], Belgium [29] and Japan [30], where the association with ciprofloxacin resistance was inconsistent. However, PFGE in this context did not offer sufficient granularity to link all of the isolates or provide sufficient resolution into the regional evolution of *S. sonnei*. Our phylogenetic analyses show that this pulsotype is associated with a phylogenetic lineage, supporting the notion that this pulsotype actually represents a widespread and pervasive subclade of Central Asia III.

This work has limitations. First, the lack of historical organisms from South Asia restricts our inference to only the contemporary situation. Further, additional present organisms from other settings would have improved our understanding of the current geographical spread of this clonal group. Notwithstanding these limitations, whole genome sequencing of these geographically disparate organisms has provided data at the highest resolution for deciphering the emergence and international spread of ciprofloxacin resistant *S. sonnei*. Future studies interrogating extensive spatial and temporal collections of ciprofloxacin resistant *S. sonnei,* as well as the *S. sonnei* diversity specific to South Asia

prior to and during the emergence of antimicrobial resistance, are essential to further elucidate the origins and epidemiological dynamics of these populations. These supplementary investigations will greatly aid our efforts in controlling the spread of the current ciprofloxacin resistant clone and to prevent future emergent antimicrobial resistant bacterial populations.

In conclusion, the international surge of ciprofloxacin resistant *S. sonnei* clone poses a substantial global health challenge, and our data show this threat is not only manifested in sporadic cases from returning travellers but also the establishment of endemic transmission in new settings. The latter is already evident in high shigellosis incidence areas such as Southeast Asia. Therefore, integrative efforts from both the research community and public health authorities should be prioritised to track, monitor and prevent the international spread of this key enteric pathogen.

Declaration of interests

We declare no competing interests.

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Table 1. The origins of the *Shigella sonnei* isolates and sequences used in this study

Country	Isolates in Central Asia clade (N)	Ciprofloxacin resistant isolates (N)	Study or institute origin	Patient group	Region of recent travel history (N)	Sequencing platform/Public database	Ciprofloxacin susceptibility
Bhutan	12	12	Diarrhoeal disease surveillance in JDWNRH[a], Thimphu, Bhutan (AFRIMS)	Hospitalised children <5 years old	NA	Illumina HiSeq 2000	Disk diffusion/E-te
Vietnam	11	11	Diarrhoeal disease surveillance in Ho Chi Minh City, Vietnam (OUCRU)	Hospitalised children < 5 years old	NA	Illumina MiSeq	Disk diffusion/E-te
Thailand	8	1	Diarrhoeal disease surveillance in Thailand (AFRIMS)	Hospitalised children <5 years old	NA	Illumina HiSeq 2000	Disk diffusion
Cambodia	1	1	Diarrhoeal disease surveillance in Siem Reap, Cambodia (COMRU)	Hospitalised children <5 years old	NA	Illumina HiSeq 2000	Disk diffusion
Ireland	20	16	National *Salmonella, Shigella,* and *L. monocytogenes* Reference Laboratory, Galway, Ireland	Primarily patients with recent travel history	India (9), Germany (1), Morocco (1), No travel (5), Unknown (4)	Illumina HiSeq 2000	Broth microdilutio
Australia	20	19	Microbiological Diagnostic Unit Public Health Laboratory in Melbourne, Australia	Patients with recent travel history	India (15), Cambodia (3), Thailand (1), Southeast Asia (1).	Illumina NextSeq	Agar dilution
USA	14	10	Genome Trackr; Centre for Disease Control and Prevention, USA	Unknown	Unknown	Illumina MiSeq (BioProject PRJNA218110)	*in silico* assessment QRDR mutations

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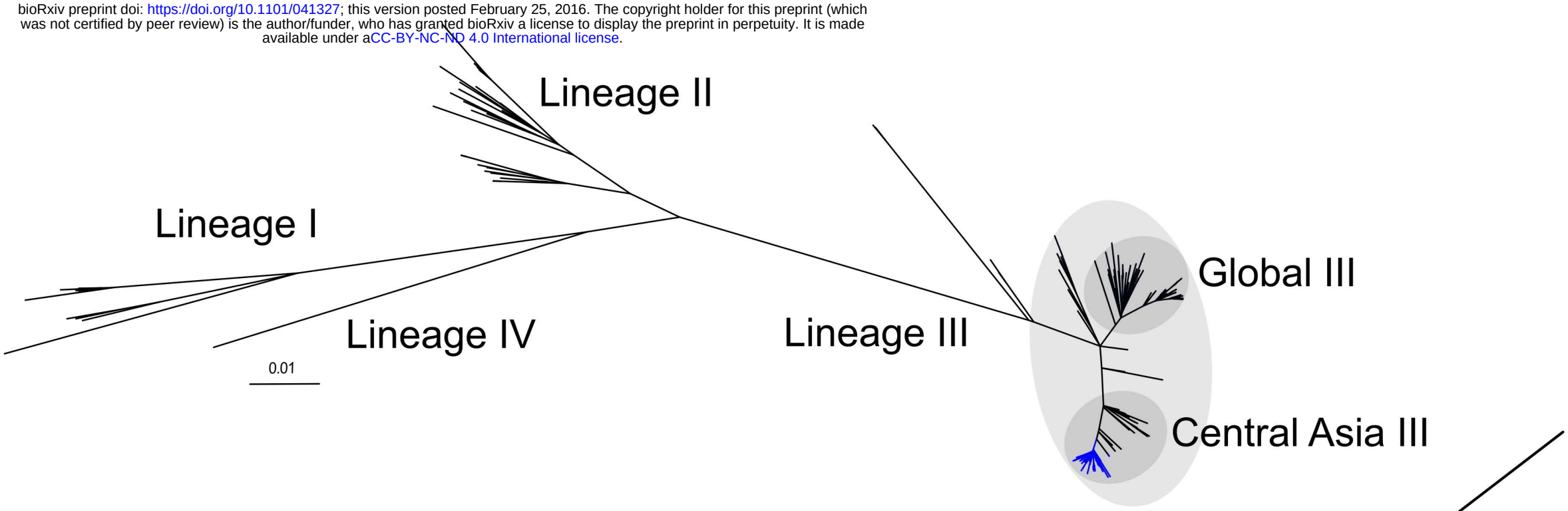
NA, Not applicable

Figure 1. The phylogenetic structure of ciprofloxacin resistant *Shigella sonnei* in a international context

A) Unrooted maximum likelihood phylogeny of 211 globally representative *S. sonnei*, sequences from ciprofloxacin resistant isolates (highlighted by the blue branches). Maj indicated by numbers (I, II, III and IV) as defined in Holt et al. 2012, with clades Glob Central Asia III within lineage III highlighted. Horizontal bar indicates the number of s per site per year. B) Unrooted maximum likelihood phylogeny of Central Asia III, com *sonnei* sequences. Branch colours indicate region of isolation (where no travel history i region of recent travel (where travel history confirmed) according to the keys. For isola confirmed recent travel, a coloured circle at the tip indicates the region where the isola (multiple coloured circles are indicative of multiple isolates). Labelled arrows indicate where the mutations *gyrA*-S83L, *gyrA*-D87N, *gyrA*-D87G and *parC*-S80I have arisen. background shading denotes isolates exhibiting ciprofloxacin resistance conferred by t (*gyrA*-S83L, *parC*-S80I and *gyrA*-D87G (or *gyrA*-D87N)). Subpopulations A and B, a in the darker blue shaded areas, denoting clonal expansions in Southeast Asia and Euro respectively. Numbers above major branches indicate bootstrap support values, and ho denotes the number of substitutions per site per year

A



B