Co-circulation of multidrug-resistant *Shigella* among men who have sex with men, Australia

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Summary: In this study, we combine genomic data with comprehensive epidemiological data on sexual exposure and travel to describe the spread of multidrug-resistant *Shigella* lineages within sexual networks of MSM in Australia, and identify a global outbreak of a multidrug-resistant plasmid.

ABSTRACT

Background

In urban Australia, the burden of shigellosis is either in returning travellers from shigellosis-endemic regions, or in men who have sex with men (MSM). Here, we combine genomic data with comprehensive epidemiological data on sexual exposure and travel to describe the spread of multidrug-resistant *Shigella* lineages.

Methods

A population-level study of all cultured *Shigella* isolates in the state of Victoria, Australia was undertaken from 1 January 2016 through to 31 March 2018. Antimicrobial susceptibility testing, whole genome sequencing (WGS) and bioinformatic analysis of 545 *Shigella* isolates was performed at the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL). Risk factor data on travel and sexual exposure were collected through enhanced surveillance forms or by interview.

Results

Rates of antimicrobial resistance were high, with 17.6% (95/541) and 50.6% (274/541) resistance to ciprofloxacin and azithromycin, respectively. There were strong associations between antimicrobial resistance, phylogeny and epidemiology; specifically, two major MSM-associated lineages were identified, a *S. sonnei* lineage (n=159) and a *S. flexneri* 2a lineage (n=105). Of concern, 147/159 (92.4%) of isolates within the *S. sonnei* MSM-associated lineage harboured mutations associated with reduced susceptibility to recommended oral antimicrobials, namely azithromycin, trimethoprim-sulfamethoxazole and ciprofloxacin. Long-read sequencing demonstrated global dissemination of multidrug-resistant plasmids across *Shigella* species and lineage, but predominantly associated with MSM isolates.

Conclusions

Our contemporary data highlight the ongoing public health threat posed by resistant *Shigella*, both in Australia and globally. Urgent multidisciplinary public health measures are required to interrupt transmission and prevent infection.

Keywords: Shigellosis; epidemiology; genomics; antimicrobial resistance; sexually-transmitted infections.

INTRODUCTION

Shigellosis is estimated to cause 190 million cases of diarrhoea globally per year [1]. In low- and middle-income countries, the burden of shigellosis is concentrated in children, with inadequate sanitation and contaminated food and / or water the most common mode of acquisition. In contrast, shigellosis in high-income countries occurs predominantly in returning travellers, or in men who have sex with men (MSM) [2, 3]. In many high-income countries there are increasing reports of locally-acquired shigellosis in MSM, with endemic shigellosis in males in these countries often considered a sexually-transmitted infection (STI) [2, 4-8].

As in other countries, treatment for shigellosis in Australia is recommended, to reduce both symptoms and asymptomatic bacterial shedding. The recommended first-line oral treatment is ciprofloxacin, and as second-line agents, azithromycin or trimethoprim-sulfamethoxazole (co-trimoxazole) [1]. However, recent studies have highlighted increasing resistance to these agents amongst *Shigella* spp., in low, middle-income and high-income countries [9-13]. Indeed, the World Health Organisation (WHO) and Centers for Disease Control and Prevention (CDC) have declared antimicrobial-resistant (AMR) *Shigella* spp., a major public health threat [14, 15]. Of further concern is a recent advisory from the CDC suggesting that ciprofloxacin may not be suitable for treatment in *Shigella* isolates with minimum inhibitory concentrations (MICs) of 0.12–1.0 μg/mL [16]. Although current interpretive criteria categorise isolates with MICs ≤1.0 μg/mL as susceptible [17], this advisory suggested isolates with MICs of 0.12–1.0 μg/mL may harbour ≥1 mutations associated with fluoroquinolone resistance, which could lead to adverse clinical outcomes and sustained shedding if fluoroquinolones are used for treatment [16]. Further, there are increasing reports of azithromycin resistance in *Shigella* spp., (mainly amongst MSM in urban populations) where azithromycin resistance is largely mediated by the plasmid-encoded *mph(A)* gene [9, 10, 13].

Whole genome sequencing (WGS) has been previously used to describe both the global and regional molecular epidemiology of shigellosis. To date, studies have predominantly assessed either the population structure of individual *Shigella* lineages [18, 19], or have focused on representative subsamples of epidemiologically-suspected epidemics [9, 10, 20]. Unbiased WGS of all cultured *Shigella* isolates in a population could provide valuable insights into possible transmission networks of shigellosis, in addition to providing information on emerging genotypic AMR patterns. Accordingly, we performed WGS of all cultured *Shigella* isolates in the state of Victoria, Australia over a two-year period. We combine genomic data with comprehensive epidemiological data on sexual exposure and travel to demonstrate the spread of highly-resistant *Shigella* lineages within MSM. Further, we contextualised our isolates with data from recent studies of shigellosis in MSM and travellers, and demonstrate global dissemination of highly-related, multi-drug resistant (MDR) plasmids.

METHODS

Setting, bacterial isolates and microbiological testing

In Australia, shigellosis is a notifiable disease under public health legislation, and diagnostic laboratories forward *Shigella* isolates to a reference laboratory. The Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL) is the bacteriology reference laboratory for the state of Victoria, covering a resident population of approximately 6.24 million people. Here, we conducted a retrospective, observational study of all cases of shigellosis associated with a *Shigella* isolate in Victoria between January 1st 2016 and March 31st 2018. Susceptibility testing was performed as detailed in the Supplementary Methods. We classified isolates with a ciprofloxacin MIC of 0.12 – 1 μg/mL as 'high resistance potential' (HRP) isolates [21].

Epidemiological investigation

Where possible, all cases of shigellosis in Victoria are interviewed by a public health officer (PHO), or notifying medical practitioners complete an enhanced surveillance questionnaire. Exposure information is collected for the two weeks prior to illness onset. Primary risk factors were considered in one of three main categories: (i) male to male sexual contact within Australia; (ii) international travel-associated, or (iii) other / unknown risk factor. Cases were classified as unknown source only when no risk factors were identified after interview. Travel destinations were categorised using the Standard Australian Classification of Countries, 2nd edition [22]. Information on shigellosis notifications in Victoria was obtained from the National Notifiable Diseases Surveillance System (NNDSS) (http://www9.health.gov.au/cda/source/cda-index.cfm). Data were collected in accordance with the Victorian Public Health and Wellbeing Act 2008 [23] and formal ethical approval was not required as this work was part of enhanced epidemiological surveillance.

Whole genome sequencing and bioinformatic analysis

DNA extraction and WGS of study isolates was performed at MDU PHL (Supplementary Methods). In brief, reads were aligned to a reference genome to identify single nucleotide polymorphisms (SNPs) using Snippy v4.3.5 (https://github.com/tseemann/snippy), with filtering of phage regions identified using Phaster [24] and recombinant regions identified using Gubbins v2.3.4 [25]. The final core SNPs were extracted with SNP-sites [26].

A maximum likelihood phylogeny was produced using IQ-tree (v1.6.5) [27] and population structure investigated using hierarchical Bayesian Analysis of Population Structure (BAPS) [28]. For context, we included data from other WGS-based studies of *Shigella* [10, 19, 29, 30]. We specifically included these previous studies as the epidemiological context (MSM and / or travel) was similar to our study. *De novo* assembly was performed using Unicycler (v.0.4.6) [31], and AMR genes were detected using ABRicate (https://github.com/tseemann/abricate). Point mutations in the quinolone resistance determining region (QRDR) were detected by read-mapping (Supplementary Methods). Four

genomes were selected for PacBio long read sequencing (details in Supplementary Methods). Sequencing data are available at the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) (BioProject PRJNA319594).

Statistical analysis

Comparisons between isolates or clusters were made using a chi-squared test. The Mann-Whitney Rank sum test was used to compare non-normal distributions. All statistical analyses were performed using R (version 3.4.0).

RESULTS

Epidemiological characteristics of cases and antimicrobial susceptibility patterns

In total, 545 *Shigella* isolates (364 *S. sonnei* and 181 *S. flexneri*) isolates underwent WGS, representing 42.9% (545/1,269) of shigellosis notifications in Victoria over the study period. The median age was 35 years (interquartile range, IQR 27-48, range 0-83), with a male to female ratio of 2.4 : 1.0 (Supplementary Figure 1). Over one-third of cases (36.0%; 196/545) were associated with overseas travel as a primary risk factor, and 176 (32.3%) cases identified MSM as a primary risk factor. 541 isolates underwent susceptibility testing (Supplementary Table 1). Decreased susceptibility to azithromycin was identified in 51.4% (93/181) of *S. flexneri* and 50.3% (181/360) of *S. sonnei* (P = 0.81), and resistance to ciprofloxacin in 9.4% (17/181) of *S. flexneri* and 21.7% (78/360) of *S. sonnei* ($P < 0.001$). *S. sonnei* isolates were significantly more likely to be resistant to two or three oral antimicrobials than *S. flexneri* (62.2% vs 40.9%, $P < 0.001$ and 6.9% vs 0.6%, $P < 0.001$, respectively) (Supplementary Table 1).

Correlation of phylogeny with epidemiological risk factors for resistant Shigella spp.

Isolates were interrogated phylogenetically by species, and clustering was assessed using BAPS. Amongst *S. sonnei*, four major BAPS groups were identified (BP1 – BP4; Table 1) (Figure 1 and Supplementary Figures 2-3). An additional cluster (BP5) represented grouping of the most divergent isolates, a known limitation of this clustering approach [28]. As such, we did not consider this a biologically relevant grouping, and this group was excluded from further analyses.

Correlating the phylogeny with epidemiological characteristics revealed clear links with risk factors. Specifically, cases in BP1, BP2 and BP4 were associated with overseas travel (Table 1, Supplementary Figure 2), with strong associations between sub-lineages and region of acquisition. For example, of the 32 cases of *S. sonnei* reporting travel to Southern / Central Asia, 32 cases (100%) were associated with BP1 (Supplementary Figure 4). In keeping with this observation, isolates from BP1 clustered with representative isolates from the Asian ciprofloxacin-resistant *S. sonnei* lineage III, previously described by Chung et al [19]. Further, in the BP1 lineage, isolates were significantly associated with ciprofloxacin resistance (Figure 1 and Table 1); of the 75 ciprofloxacin-resistant isolates in BP1, 74 isolates harboured triple mutations in the QRDR (Figure 1).

In contrast to travel-associated lineages, *S. sonnei* cases in BP3 were significantly associated with MSM, with 61% (97/159) of males in this lineage reporting MSM as a primary risk factor (Figure 1 and Table 1). Isolates in BP3 were highly related, with a median pairwise core SNP distance of 4 (IQR 2-6) (Table 1; Supplementary Figure 3A). Further, MSM cases in BP3 were distributed across the study period and not limited to a temporally-restricted outbreak (Supplementary Figure 2). Most isolates in BP3 (139/159; 87%) harboured highly-related plasmids (see below) containing AMR determinants for azithromycin, trimethoprim and sulfonamides (Figure 1). Although isolates in this lineage were not associated with ciprofloxacin resistance at the current CLSI breakpoint of 1 μg/mL, reducing the breakpoint to 0.12 μg/mL resulted in 147/149 (99%) of tested isolates being re-classified

as having HRP for ciprofloxacin. All 149 isolates harboured single mutations in the QRDR (147 *gyrA* S83L, and 2 *gyrA* D87Y) (Figure 1).

Amongst *S. flexneri*, there were four major BAPS groups (BP1 – BP4) (Table 2; Supplementary Figure 3). These groups broadly correlated with serotyping, with the largest group (BP2) corresponding to *S. flexneri* serotype 2a (Supplementary Dataset). An additional cluster (BP5), represented grouping of divergent isolates, was excluded from further analyses. Similar to *S. sonnei*, there were distinct associations between phylogeny, AMR and risk factors. Specifically, MSM status was associated with BP2 (58.4% (52/89) of males in this lineage were MSM), and to a lesser extent, BP1 and BP3 (Table 2), whilst BP4 was more associated with recent travel (Table 2; Figure 2). In contrast to the major BP3 MSM-lineage in *S. sonnei*, the BP2 MSM-lineage in *S. flexneri* was not associated with ciprofloxacin resistance at a lower breakpoint of 0.12 μg/mL, and did not harbour QRDR mutations (Figure 2, Supplementary Dataset). However, 63.8% (67/105) of isolates in the BP2 MSM-lineage of *S. flexneri* harboured azithromycin resistance plasmids similar to those in the *S. sonnei* BP3 MSM- lineage (see below) (Figures 2 & 3).

For context, we undertook combined analyses of 691 *S. sonnei* isolates (364 Australian and 327 international) and 408 *S. flexneri* (171 Australian, 237 international) (Supplementary Figures 4 and 5). For both Australian and the United Kingdom (UK) *S. sonnei* and *S. flexneri*, there was limited clustering of isolates according to region of travel (Supplementary Figures 4-5). Australian *S. sonnei* MSM isolates in BP3 formed a distinct lineage, almost completely comprising Australian isolates (Supplementary Figure 4). In contrast, for *S. flexneri*, international serotype 2a isolates in the previously described MSM clades from Baker et al. [10] clustered together in the phylogeny with Australian isolates in the MSM-associated BP2 lineage (Supplementary Figure 5). Moreover, 31 of these international *S. flexneri* isolates also harboured pKSR100 plasmids, similar to those described

from the UK (see below and Figure 3), further suggesting intercontinental dissemination of the MSM-associated *S. flexneri* 2a lineage.

Global dissemination of MDR plasmids across Shigella species and lineage

To understand the relatedness of multi-resistant plasmids in our study, specifically amongst MSM-associated isolates, long-read sequencing was performed on four genomes, representing *S. flexneri* BP1 and BP2 and *S. sonnei* BP1 and BP3 (Supplementary Dataset). All four plasmids harboured $bla_{\text{TEM-1}}$, *ermB* and *mph(A)*, and three of the plasmids contained an integron with additional AMR genes, namely *sul1*, *dfrA17* and *aadA5* (Figure 3). Comparison with pKSR100 (a conjugative plasmid previously identified in MSM-associated *Shigella* in the UK [7, 10]) revealed a high level of homology between pKSR100 and all four plasmids, (Figure 3). The global relationship of pKSR100 plasmids was further explored by comparing all pKSR100-like plasmids (from our study and other recent work [10, 13]) to pKSR100 (Figure 4). pKSR100-like plasmids were disseminated across *Shigella* species but were predominantly associated with MSM-associated shigellosis in both Australia and overseas (Figure 4).

DISCUSSION

In this study, we demonstrate that local and global dissemination of clinically significant AMR *Shigella* spp., is driven largely by the spread of highly-related, multi-resistant plasmids that are not restricted by *Shigella* species or lineage. By sampling all isolates in a population (rather than representatively sub-sampling known outbreaks), and integrating with epidemiological data, we identified distinct correlations between *Shigella* sublineages and the two major modes of shigellosis acquisition in high-income countries, namely international travel and domestically-acquired MSM-associated shigellosis, each associated with approximately one-third of all cases in this study.

Of specific concern was the high prevalence of resistance to oral agents currently recommended for the treatment of shigellosis. Approximately 50% of all isolates displayed resistance to azithromycin, representing to date, the highest reported azithromycin resistance rate in *Shigella* spp. globally [13, 32, 33]. Notably, azithromycin resistance was significantly more common in MSM-associated *Shigella* (93% in BP3 *S. sonnei* and 71% in BP2 *S. flexneri*), signalling the demise of azithromycin as a suitable treatment option for domestically-acquired shigellosis in MSM in our population. The reasons for this high rate of azithromycin resistance are multifactorial, and likely related to both host and pathogen factors. First, the recommended use of azithromycin as an empiric agent for the syndromic treatment of urethritis is likely to have exerted selection pressure for the emergence of azithromycin-resistance in MSM [34], a hypothesis corroborated by the contemporaneous emergence of azithromycin resistance in other sexually-transmitted pathogens such as *Neisseria gonorrhoeae*, *Treponema pallidum* and *Mycoplasma genitalium* [35-38]. Second, the finding that *mph(A)* was harboured on genetically similar pKSR100-like plasmids regardless of species or lineage highlights the apparent ease of horizontal transmission of these particular plasmids, an observation supported by the identification of similar plasmids from multiple geographic locations and epidemiological contexts [7, 9, 10, 13]. Collectively, our data support the existence of a global outbreak of a highly successful azithromycin resistance plasmid, predominantly in MSM populations.

Compared to azithromycin resistance, ciprofloxacin resistance was largely associated with international travel to areas of high endemicity (particularly South East and Central Asia), rather than local populations, in keeping with the fact that ciprofloxacin is a restricted antibiotic in Australia [39]. However, when an MIC threshold of 0.12 μg/mL was applied, approximately 57% of all tested isolates in our study were classified as HRP, compared to only 18% at the current resistance breakpoint of >1.0 μg/mL. The difference was largely driven by the MSM-associated *S. sonnei* BP3 lineage, in which all HRP isolates harboured single mutations (mainly *gyrA* S83L) in the QRDR. This finding is of specific concern given: (i) the sequential development of resistance in the QRDR, in which the *gyrA* S83L mutation is a first step [12], and (ii) the concurrent high prevalence of

azithromycin resistance in MSM-associated shigellosis, which may increase therapeutic use of ciprofloxacin, leading to a detrimental 'Catch 22' situation of ciprofloxacin use further driving resistance in this population. Future work should closely monitor clinical outcomes associated with such cases, and indeed, recent guidelines suggest that antimicrobial treatment should now only be reserved for 'high-risk' populations (e.g. immunocompromised patients; the very young or elderly, and individuals at risk of causing outbreaks, such as food handlers or childcare workers) [21].

Given our finding of several travel-associated cases within major MSM-associated *S. sonnei* and *S. flexneri* lineages, it is plausible that MSM lineages were imported into Australia, with subsequent onwards transmission in the context of a successful epidemiological triad of host (dense MSM networks with high-risk sexual behaviours), pathogen (highly infectious bacterial species with transmissible resistance determinants) and environment (selection pressure from azithromycin use). Indeed, many isolates in the Australian *S. flexneri* 2a MSM-associated lineage were highly related at a core genome level to isolates from a previous UK study [10], further highlighting the importance of global travel in propagating shigellosis outbreaks. Moreover, the hypothesis of importation and domestic spread of pathogens in MSM is also supported by recent outbreaks of other pathogens, such as hepatitis A virus [40] and azithromycin-resistant *Neisseria gonorrhoeae* [41], demonstrating the need for improved understanding of the factors that may promote such outbreaks, such as HIV co-infection, pre-exposure prophylaxis, asymptomatic carriage, and circumstantial features such as social networking applications and recreational drug use.

In addition to MSM-associated shigellosis, the other major burden of disease in our study was amongst returning travellers. Previous work from the UK has demonstrated travel-associated *Shigella* sub-lineages, with distinct phylogeographic associations [9]. Here, we broaden this genomic framework extensively to include isolates from returned travellers in Australia. Like the UK study, we observed similar patterns of triple QRDR mutations in *Shigella* isolates from Southern Central Asia

(mainly India, Pakistan and Nepal), and South-East Asia (mainly Vietnam and Cambodia) further highlighting these regions as reservoirs of resistant enteric pathogens [12], a situation that has parallels with the global emergence of fluoroquinolone-resistant *Salmonella* Typhi [42]. Our study further demonstrates the utility of genomic surveillance in detecting emerging genotypic AMR patterns, using isolates from returned travellers as a proxy for assessing AMR in other regions.

Key strengths of our study include our contemporary sampling frame (i.e. 2016 – 2018); comprehensive coverage of cultured cases of shigellosis, and integration of detailed epidemiological data. Further, previous studies have demonstrated high interconnectivity of MSM populations in urban Australia [43], meaning that our findings are likely to have applicability across major cities in Australia. Moreover, the inclusion of epidemiologically relevant international isolates provides additional geographic context [9, 10, 13].

Although we received all cultured isolates in the state during the study, this represented only 43% of shigellosis notifications over the study period. This limitation applies to all WGS-based studies of shigellosis, whereby the increasing use of molecular testing for enteric pathogens reduces the availability of isolates for additional analyses [44]. This situation has marked parallels with the use of molecular testing for *N. gonorrhoeae*, where reduced bacterial culture is compromising the ability to detect multi-resistant isolates [45]. In an era of increasing AMR in STIs, it is critical that concerted efforts are made to ensure continuation of culture-based surveillance.

In conclusion, we present to our knowledge, the first population-based genomic surveillance of shigellosis in Australia. We demonstrate the global dissemination of a multi-resistant plasmid, present across multiple continents, and highly associated with MSM. This represents a significant and immediate health threat not only in Australia, but also globally. Urgent multidisciplinary public health

measures are required, including enhanced contact tracing of multi-resistant cases of shigellosis (which could be informed by WGS data), improved antimicrobial stewardship, improved information on clinical outcomes of resistant shigellosis, and heightened awareness of shigellosis as an STI.

Acknowledgements: We thank staff of the Enteric Reference Laboratory in the Microbiological Diagnostic Unit Public Health Laboratory, and Kaye Surge and Andrea Sant'ana from the Victorian Department of Health and Human Services.

Funding: DAW and JCK are supported by an Early Career Fellowships from the National Health and Medical Research Council (NHMRC) of Australia (GNT1123854 and GNT1142613). BPH is supported by a Practitioner Fellowship from the NHMRC (GNT1105905). Work in this study was supported by a Project Grant from the NHMRC (GNT1147735). Salary support for DJI was supported by an NHMRC Project Grant (APP1129770) and the European Union's Horizon 2020 research and innovation programme under grant agreement 643476. MDK is supported by a Career Development Fellowship from the NHMRC (GNT1145997).

Conflicts: EC reports grants from Merck and personal fees from Gilead Sciences and Seqirus Australia. All other authors declare no conflicts.

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FIGURE LEGENDS

Figure 1. Population structure of 364 Australian *Shigella sonnei* isolates included in this study. The mid-point rooted phylogenetic tree of *Shigella sonnei* is plotted on the left. A total of 16 additional representative genomes are included to provide reference to established population structure [29]. The

tips are colored by primary risk factor. BAPs groups are highlighted. Phenotypic resistance profiles are shown next to corresponding genotypic mutations for ciprofloxacin (number of QRDR mutations; see text), azithromycin (*mphA*), trimethoprim (any *dfr* mutation) and sulfonamides (any *sul* mutation). In addition, the effect of reducing the ciprofloxacin breakpoint to 0.12 μg/mL is displayed ('high resistance potential'; HRP).

Figure 2. Population structure of 171 Australian *Shigella flexneri* isolates and 20 additional representative genomes are included to provide reference to established population structure [28]. The mid-point rooted phylogenetic tree of *Shigella flexneri* is plotted on the left. The tips are colored by primary risk factor. BAPs groups are highlighted. Phenotypic resistance profiles are shown next to corresponding genotypic mutations for ciprofloxacin (number of QRDR mutations; see text), azithromycin (*mphA*), trimethoprim (any *dfr* mutation) and sulfonamides (any *sul* mutation). In addition, the effect of reducing the ciprofloxacin breakpoint to 0.12 μg/mL is displayed ('high resistance potential'; HRP).

Figure 3. Comparison of four Australian pKSR100-like plasmids with pKSR100 (SF7955).

The structure of the pKSR100 plasmid is shown at the bottom of each panel with the comparison plasmid at the top. A) *S. flexneri* 1c strain AUSMDU00008355 (BP1 lineage); B) *S. flexneri* 2a strain AUSMDU00008332 (BP2 lineage); C) *S. sonnei* biotype g strain AUSMDU00008333 (BP1 lineage) and D) *S. sonnei* biotype g strain AUSMDU00008361 (BP3 lineage). Plasmid genes are shown in orange with genes mediating resistance to different drug classes highlighted in: green ($bla_{\text{TEM-1}}$), purple (*mphA* and *ermB*) and blue for (*dfrA17*, *sul1* and *aadA*). The shaded grey indicates regions with nucleotide homology of ≥90 percent of higher.

Figure 4. Dissemination of multi-drug resistant plasmid within and between *Shigella* spp.

A. Comparisons of plasmids detected in four Australian *Shigella* isolates. Plasmid genes are shown in orange with genes mediating resistance to different drug classes highlighted in: (i) green for $_{bla\text{TEM-1}}$, (ii) purple for *mphA* and *ermB* and (iii) blue for *dfrA17*, *sul1* and *aadA*. The shaded grey indicates sequence homology regions of ≥90 percent of higher. B) The inferred phylogeny of pKSR100-like plasmids within *S. flexneri* and *S. sonnei* of isolates that had ≥90% of nucleotide coverage to the pKSR100 reference. The primary risk factor, the species membership and serotype for the isolates are shown to the right of the phylogeny. The *Shigella* species of an isolate is plotted to the right of the phylogeny. A binary heatmap illustrates the presence/absence of six genes known to be mobilized on the pKSR100-like plasmids.

Table 1. Characteristics of *Shigella sonnei* isolates included in this study and associations with phylogenetic groupings.

Characteristic	BAPS group [a]				*P* [b]
	BP1	BP2	BP3	BP4	
Median pairwise SNP difference (IQR)	50 (41-75)	168 (144-189)	4 (2-6)	106 (64-120)	
Phenotypic resistance	Number resistant / isolates tested (% resistant)				
Ampicillin	37/98 (37.8)	10/40 (25)	150/159 (94.3)	11/57 (19.3)	<0.001
Ciprofloxacin (CIP)	75/98 (76.5)	0/40	2/159 (1.3)	1/57 (1.8)	<0.001
Azithromycin (AZT)	27/98 (27.6)	0/40	148/159 (93.1)	6/57 (10.5)	<0.001
Ceftriaxone	19/98 (19.4)	0/40	11/159 (6.9)	5/57 (8.8)	0.002
Trimethoprim (TMP)	97/98 (99.0)	26/40 (65.0)	159/159 (100)	57/57 (100)	<0.001
Sulfathiozole (SUL)	86/98 (87.8)	27/40 (67.5)	157/159 (98.7)	44/57 (77.2)	<0.001
Gentamicin	3/97 (3.1)	0/40	0/159	1/57 (1.8)	0.13
Meropenem	2/98 (2.0)	1/40 (2.5)	1/159 (0.6)	0/57	NA
Resistant to 2 oral antimicrobials	71/98 (72.4)	0/40 (0)	147/159 (92.4)	6/57 (10.5)	<0.001
Resistant to 3 oral antimicrobials	22/98 (22.4)	0/40 (0)	2/159 (1.3)	1/57 (1.8)	<0.001
'High resistance potential' for ciprofloxacin	82/88 (93.2)	0/39 (0)	147/149 (98.6)	12/52 (23.1)	<0.001
Epidemiological characteristic					
Males	54/102 (52.0)	17/40 (42.5)	144/159 (90.6)	25/57 (43.8)	<0.001
Median age (IQR)	28.5 (10.5-43)	36.5 (25.25-44.75)	38 (30-48)	34 (24-55)	<0.001
Overseas travel or contact with traveller	62/102 (60.7)	25/40 (62.5)	12/159 (7.6)	39/57 (68.4)	<0.001
Male to male sexual contact	8/102 (7.8)	3/40 (7.6)	97/159 (61.0)	2/57 (3.5)	<0.001
Other / unknown	32/102 (31.3)	12/40 (30.0)	50/159 (31.4)	16/57 (28.1)	0.49

Abbreviations: SNP, single nucleotide polymorphism; IQR, interquartile range; BAPS, Bayesian Analysis of Population Structure; BP, BAPS group; NA, not applicable

[a] Four isolates that belonged to a divergent group (BP5) were excluded from this analysis (see text). Two Australian isolates initially identified as BP4 were also excluded from the BP4 analysis.

[b] 2 x 4 χ^2 test

Table 2. Characteristics of *Shigella flexneri* isolates included in this study and associations with phylogenetic groupings.

Characteristic	BAPS group [a]	*P* [a]

	BP1	BP2	BP3	BP4	
Median pairwise SNP difference (IQR)	162 (125-181)	139 (6-283)	91 (70.25-615)	259 (118.25-325.75)	
Phenotypic resistance	Number resistant / isolates tested (% resistant)				
Ampicillin	26/32 (81.3)	102/105 (97.1)	15/16 (93.8)	6/10 (60.0)	<0.001
Ciprofloxacin	2/32 (6.3)	12/105 (11.4)	0/16 (23.5)	2/10 (20.0)	0.30
Azithromycin	12/32 (37.5)	74/105 (70.5)	6/16 (37.5)	0/10 (0)	<0.001
Ceftriaxone	0/32 (0)	1/105 (1.0)	0/16 (0)	0/10 (0)	0.86
Trimethoprim	24/32 (75.0)	92/105 (87.6)	2/16 (12.5)	7/10 (70.0)	<0.001
Sulfathiozole	20/32 (62.5)	67/105 (63.8)	3/16 (18.6)	6/10 (60.0)	0.008
Gentamicin	1/32 (3.1)	0/105 (0)	0/16 (0)	0/10 (0)	0.25
Meropenem	0/32 (0)	0/105 (0)	0/16 (0)	0/10 (0)	NA
Resistant to 2 oral antimicrobials	13/32 (40.6)	58/105 (55.2)	0/16 (0)	2/10 (20.0)	<0.001
Resistant to 3 oral antimicrobials	0/32 (0)	1/105 (1.0)	0/16 (0)	0/10 (0)	0.91
'High resistance potential' for ciprofloxacin	18/29 (62.1)	14/97 (14.4)	0/15 (0)	6/10 (60.0)	<0.001
Epidemiological characteristic					
Males	25/32 (78.1)	89/105 (84.8)	13/16 (81.3)	5/10 (50.0)	0.001
Median age (IQR)	39 (30.25-47.75)	37 (28-50)	35.5 (30.25-49.25)	30 (7.25-38)	0.19
Overseas travel or contact with traveller	11/32 (34.4)	19/105 (18.0)	6/16 (37.5)	6/10 (60.0)	0.008
Male to male sexual contact	7/32 (21.9)	52/105 (49.5)	6/16 (37.5)	1/10 (10.0)	0.003
Other / unknown	14/32 (43.8)	34/105 (32.4)	4/16 (25.0)	3/10 (30.0)	0.05

Abbreviations: SNP, single nucleotide polymorphism; IQR, interquartile range; BAPS, Bayesian Analysis of Population Structure; BP, BAPS group; NA, not applicable

[a] Seven isolates that belonged to divergent group (BP5) were excluded from this analysis (see text). Ten isolates that belonged to distant serotype 6 were also excluded.

[b] 2 x 4 χ^2 test

Figure 1

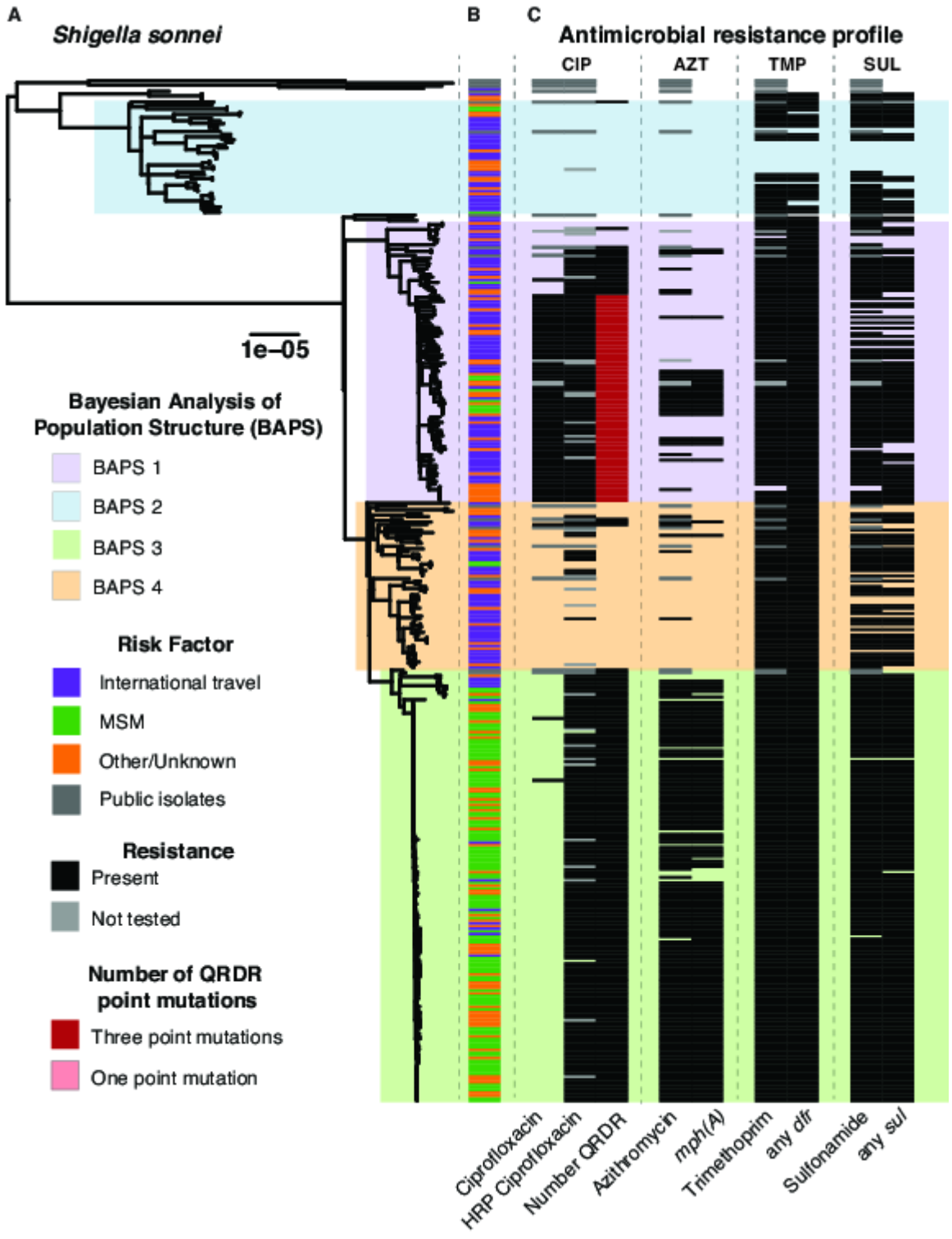

Figure 2

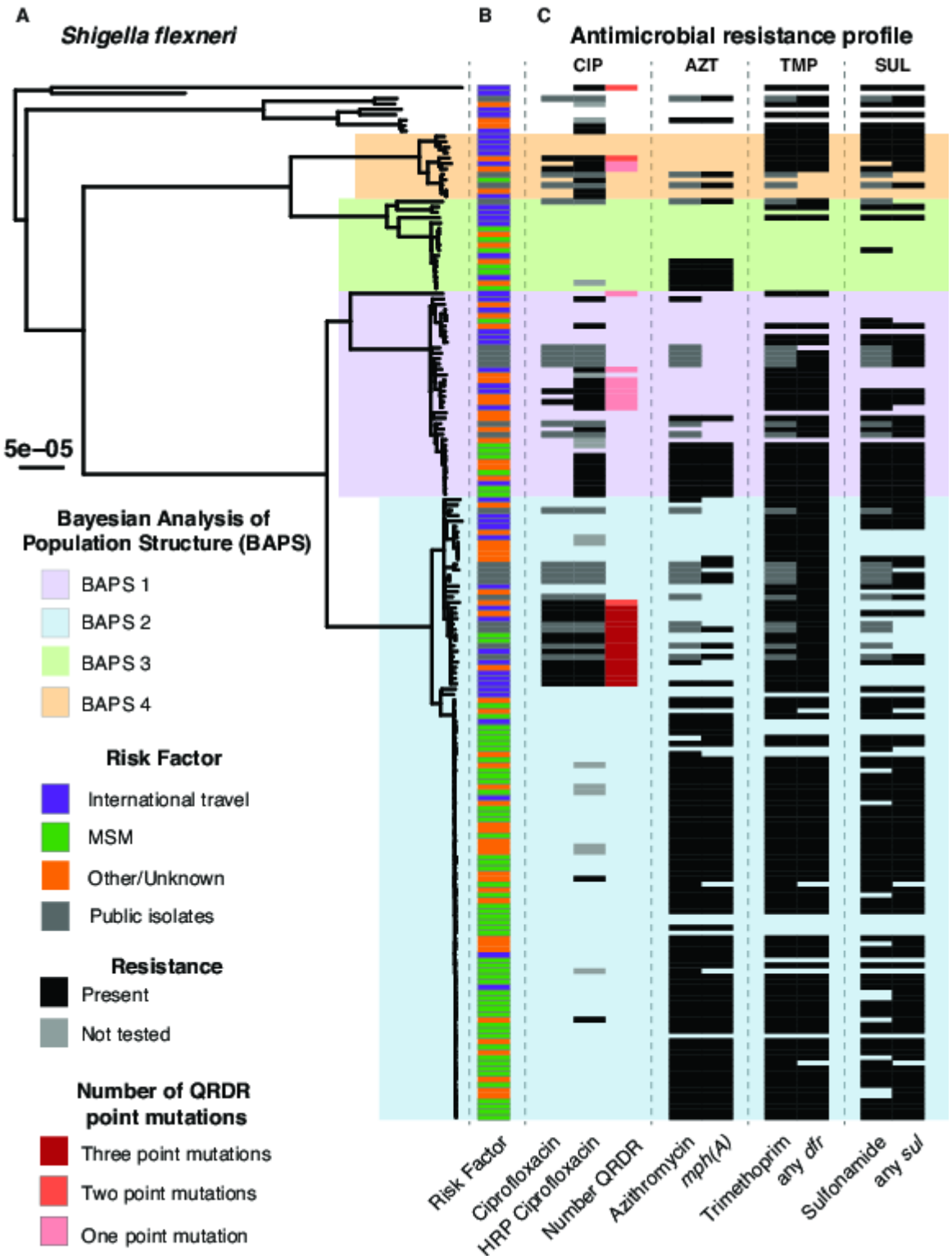

Figure 3

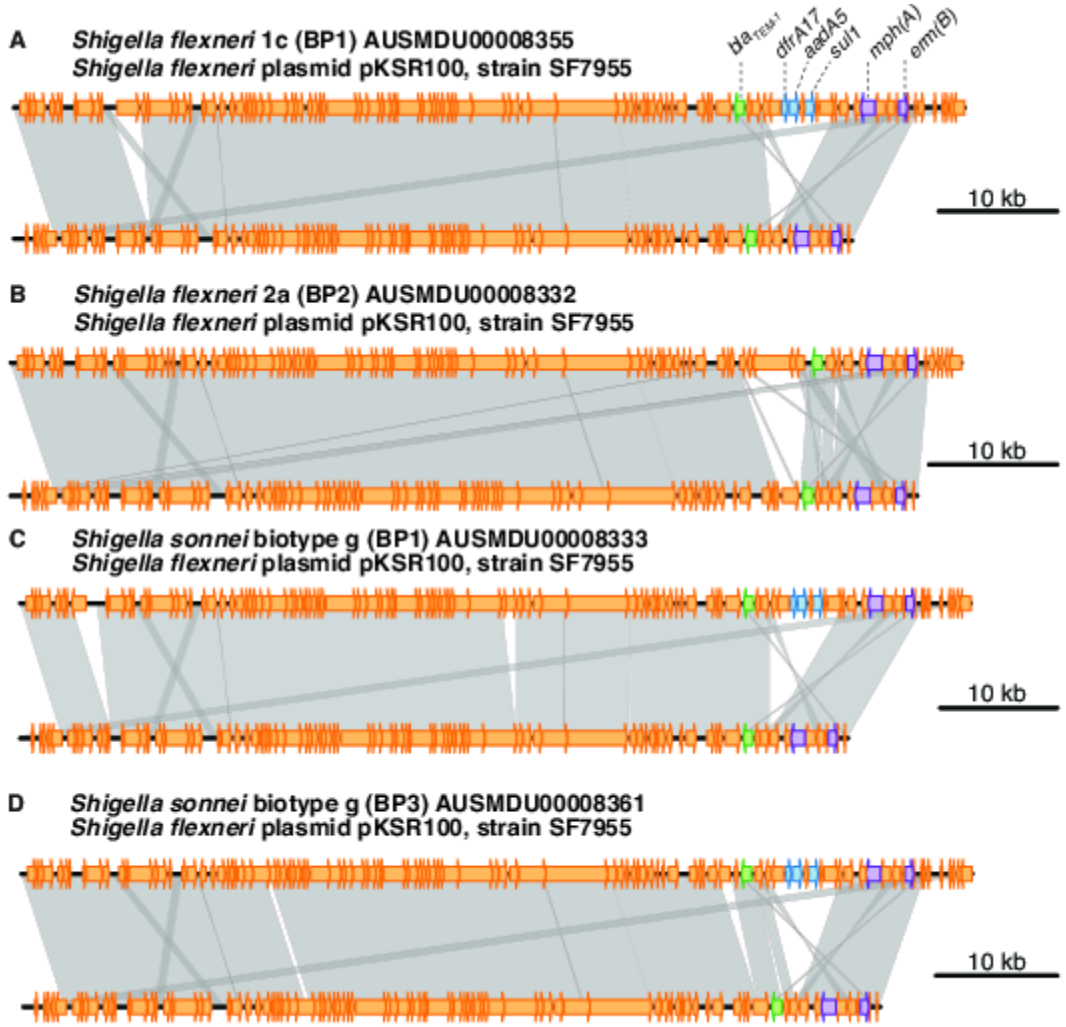

Figure 4