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Azithromycin resistance in *Shigella* spp. in Southeast Asia

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Running title: *Shigella* susceptibility to azithromycin

Abstract

Infection by *Shigella* spp. is a common cause of dysentery in Southeast Asia. Antimicrobials are thought to be beneficial for treatment, however antimicrobial resistance in *Shigella* spp. is becoming widespread. We aimed to assess the frequency and mechanisms associated with decreased susceptibility to azithromycin in Southeast Asian *Shigella* isolates and use these data to assess appropriate susceptibility breakpoints. *Shigella* isolated in Vietnam and Laos were screened for susceptibility against azithromycin (15µg) by disc diffusion and minimum inhibitory concentration (MIC). Phenotypic resistance was confirmed by PCR amplification of macrolide resistance loci. We compared the genetic relationships and plasmid contents of azithromycin resistant *S. sonnei* using whole genome sequences. From 475 available *Shigella* spp. isolated in Vietnam and Laos between 1994 and 2012, 6/181 *S. flexneri* (3.3%, MIC≥16g/L) and 16/294 *S. sonnei* (5.4%, MIC≥32g/L) were phenotypically resistant to azithromycin. PCR amplification confirmed a resistance mechanism in 22/475 (4.6%) isolates (19 *mphA* and 3 *ermB*). Susceptibility data demonstrated the acceptability of *S. flexneri* (MIC≥16g/L, zone≤15mm) and *S. sonnei* (MIC≥32g/L, zone≤11mm) breakpoints with <3% discrepancy. Phylogenetic analysis demonstrated that decreased susceptibility has arisen sporadically in Vietnamese *S. sonnei* on at least seven occasions between 2000 and 2009, but failed to become established. While the proposed susceptibility breakpoints may allow better recognition of resistant isolates, additional studies are required to assess the impact on clinical outcome. The potential emergence of azithromycin resistance highlights the need for alternative management options for *Shigella* infections in endemic countries.

46 **Introduction**

47 Organisms of the bacterial genus *Shigella* are a common cause of moderate to severe diarrhea
48 and dysentery in children attending day-care facilities, those living in resource-limited
49 settings, and travellers to such areas (1-5). In many low to middle-income countries (LMICs),
50 such as Vietnam, endemic shigellosis is now predominantly caused by *Shigella sonnei*.
51 Sustained antimicrobial pressure in LMICs has led to the emergence of resistance to the
52 antimicrobials used for treating shigellosis (6,7). In Southeast Asia, antimicrobial resistance
53 (AMR) in the Shigellae is largely being driven by the expansion of a specific *S. sonnei*
54 lineage, which is known as Global III (8).
55
56 AMR within the genus *Shigella* is a problem for clinical management (9,10). The treatment of
57 *Shigella* infections with antimicrobials is recommended by most clinical guidelines,
58 predominantly to reduce the risk of onward transmission and disease complications. The
59 WHO currently recommends ciprofloxacin as first-line treatment, with pivmecillinam,
60 ceftriaxone, and azithromycin as alternative options. However, *Shigella* spp. are adept at
61 acquiring AMR genes and plasmids, and reports of multi-drug resistant (MDR) lineages or
62 isolates with reduced susceptibility to fluoroquinolones and third-generation cephalosporins
63 are increasing globally (11,12)
64
65 Some recent recommendations have advocated the oral azalide antimicrobial azithromycin as
66 an alternative treatment for shigellosis, particularly infections caused by MDR organisms or
67 when fluoroquinolones are inappropriate (9,13). Clinical evidence for the efficacy of
68 azithromycin in treating shigellosis is limited (14,15), and there are presently no suitable
69 clinically derived susceptibility breakpoints to facilitate the laboratory identification of
70 *Shigella* spp. exhibiting azithromycin non-susceptibility. Recently updated CLSI guidelines
71 suggest epidemiological cut-off values (ECVs) of Minimum Inhibitory Concentrations (MIC)
72 ≥16mg/L and MIC≥32mg/L to categories non-wild type *S. flexneri* and *S. sonnei*, respectively
73 (16). Data supporting these guidelines are limited, principally originating from reports of an

74 international outbreak of *S. flexneri* serotype 3a among men who have sex with men (MSM)
75 (17-19). Here, we aimed to assess the frequency and mechanisms of *Shigella* spp. isolates
76 with decreased susceptibility against azithromycin in Southeast Asia, a setting where
77 fluoroquinolone and third-generation cephalosporin resistance has become common.
78 Additionally, using a large dataset from Vietnam and Laos spanning 18 years, we aimed to
79 calculate suitable breakpoints for assessing *Shigella* susceptibility against azithromycin.
80

81 **Materials and methods**

82 *Ethics statement*

83 Bacterial isolates and data for this investigation originated from clinical studies approved by
84 the scientific and ethical committees of the Hospital for Tropical Diseases in HCMC, all other
85 participating hospitals, and the Oxford Tropical Research Ethics Committee (OXTREC) in
86 the United Kingdom. The study also included the characterization of bacterial isolates
87 submitted for routine diagnostic purposes. Study participants or parents of young participants
88 were required to provide written informed consent for the collection of samples and
89 subsequent analyses, except when samples were collected as part of routine care.
90

91 *Study sites*

92 The majority of fecal specimens from which *Shigella* spp. were isolated were collected in a
93 series of pediatric studies performed in Vietnam between 1994 and 2012, as previously
94 described (6). Briefly, children presenting with either diarrhea or dysentery were recruited
95 into observational studies (6, 20, 21), or treatment trials (22, 23) performed at the Hospital for
96 Tropical Diseases (HTD), Children's Hospital 1, or Children's Hospital 2 in Ho Chi Minh
97 City, Vietnam. Additional microbiology isolates collected for routine diagnostic purposes
98 were also included from Huế Central Hospital in Huế and Khanh Hoa General Hospital in
99 Nha Trang, Vietnam, and Mahosot Hospital in Vientiane, Laos.
100

Microbiology methods

Fecal samples were collected and processed as previously described using standard microbiological methods (6, 24). Briefly, non-lactose fermenting colonies grown on MacConkey and/or Xylose Lysine Desoxycholate (XLD) agar (Oxoid), were identified biochemically (API20E; biomerieux, Vietnam) and by slide agglutination with polyvalent somatic (O) and monovalent serotype-specific grouping antisera (Denka Seiken, Japan in Vietnam & Pro-Lab Diagnostics, UK in Laos). Azithromycin susceptibility testing against was performed at a single laboratory in Vietnam using Kirby-Bauer disc diffusion method (15µg disc) and by MIC antimicrobial gradient diffusion (Etest, AB Biodisk, Sweden on both on Mueller-Hinton agar (Oxoid).

Molecular methods

Genomic DNA was extracted from *S. flexneri* and *S. sonnei* isolates using the Wizard Genomic DNA Extraction Kit (Promega) following the manufacturers' recommendations, with the quality and quantity assessed using the Quant-IT Kit (Invitrogen) prior to sequencing. PCR amplification for the detection of macrolide resistance genes (*mphA/B*, *ermA/B/D*, *ereA/B*, and *mefA/B*) was performed as previously described (25).

In addition, we performed phylogenetic analysis of 247 existing *S. sonnei* genomes (global lineage III) and an additional 68 contemporary genomes of isolates collected during the same period (1995-2011) (6) (accession numbers available in Table S1). Briefly, raw Illumina reads were mapped against an *S. sonnei* reference genome (strain Ss046 chromosome, accession number NC_007382 and pINV B plasmid, accession number NC_00735) using BWA and SNPs were called using SAMtools (26, 27). Phylogenetic reconstruction was performed using multiple alignment of SNPs by maximum-likelihood based phylogenetic inference (RAxML, version 8.2.8) (28) with a GTR+GAMMA substitution model. Bootstrap support for the maximum-likelihood phylogeny was accessed by 1,000 pseudo-replicates. Phylogenetic tree

was displayed and annotated using iTOL (29), highlighting the presence/absence of macrolide resistance genes over the study period among terminal taxa.

Plasmid isolation and sequencing

Bacterial conjugation was performed as described previously by combining representative isolates carrying *ermB* (EG430), *mphA* (DE891) and *E. coli* J53 (sodium azide resistant) (30). *E. coli* transconjugants were selected on media containing sodium azide (100mg/L) and azithromycin (24mg/L). *ErmB/mphA*-containing plasmids were extracted using plasmid Midi kit (Qiagen) and sequenced using the MiSeq Illumina platform with 2x250bp pair-end reads. *De novo* assembly was performed using SPADES v3.6.2 and annotated using Prokka (v1.11) (31,32). ABACAS was used to map all the assembled contigs against a concatenated reference sequence containing *S. sonnei* Ss046 chromosome (NC_007382), virulence plasmid pSs046 (NC_007385.1) and three small plasmids commonly found in *S. sonnei* belonging to Global lineage III: spA (NC_009345.1) spB (NC_009346.1), spC (NC_009347.1) (33). The unmapped assembled sequences were presumed to contain *ermB*/*mphA*-encoding plasmids and Incompatibility (Inc) groups were then determined using *in silico* PCR by mapping the primers described previously to these unmapped sequences using an in-house script at the Sanger Institute (34). The presence of the *ermB/mphA* plasmid was confirmed by BLASTN searching the plasmid sequences to the previously sequenced plasmids in Genbank and comparative analysis was performed and visualized using ACT (35).

Statistical analysis

Statistical analysis of *Shigella* spp. isolates was limited to *S. flexneri* and *S. sonnei* only, as insufficient numbers of other species were available (Table 1). For comparisons of proportions of non-susceptible isolates, intermediate and resistant isolates were grouped together and compared with the proportion of susceptible isolates using Fisher's exact test. Comparison of MIC measurements from different time periods was performed by ANOVA and subsequent Dunn's test with Bonferroni correction for multiple testing, with a threshold

of $p<0.05$ considered significant. To determine appropriate azithromycin breakpoints, MIC histograms were constructed and disc zone diameter breakpoints were selected using the modified error rate-bounding method of Metzler and De Haan, according to CLSI recommendations (36).

Accession no(s). The sequence for plasmid pDE105 has been deposited in GenBank under accession no. MG569891.

Results

Decreased susceptibility to azithromycin in Shigella spp. in Southeast Asia

Data from a total of 517 *Shigella* (198 *S. flexneri*, 308 *S. sonnei*, and 11 others) isolated between 1994 and 2012 in Vietnam (6 studies, 472 isolates) and Laos (45 isolates) (Table 1) were available for antimicrobial susceptibility analysis. In this collection of organisms, 180/198 (91%) *S. flexneri* were defined as being MDR (resistant to ≥3 classes of antimicrobials), 3/196 (2%) were resistant to ceftriaxone, and 78/196 (40%) were resistant to nalidixic acid. In contrast, significantly fewer *S. sonnei* isolates were MDR (181/308, 59%; $p<0.0001$), while a greater proportion exhibited resistance to ceftriaxone (92/307, 30%; $p<0.0001$), and nalidixic acid (174/307, 69%; $p=0.0003$) (20).

From the 517 *Shigella* isolates collected over the defied period, 479 were recovered and available for azithromycin susceptibility testing; 181/479 (37.8%) *S. flexneri*, 294/479 (61.4%) *S. sonnei*, and 4/479 (0.8%) isolates belonging to other *Shigella* species (not considered further). The distributions of the azithromycin MICs against azithromycin of the 475 *Shigella* isolates collected over the sampling period are shown in Figure 1. The combined MIC_{50} for azithromycin was 4mg/L (MIC_{90}, 8mg/L); the *S. sonnei* isolates exhibited a higher range of MIC values (IQR, 4 to 8 mg/L) in comparison with the *S. flexneri* isolates (IQR, 2 to 4mg/L). The proportion of *S. flexneri* isolates with an MIC≥16mg/L was 6/181 (3.3%, 95%CI, 1.4 to 7.4), whereas the proportion of *S. sonnei* isolates with an MIC≥32mg/L was 16/294 (5.4%, 3.2 to 8.9; $p>0.05$).

184 *Genes conferring decreased susceptibility against azithromycin*

185 Isolates were screened by PCR amplification for the macrolide resistance genes *ermA/B/C*,
186 *mphA/B*, *ereA/B*, and *msrA* and *mefA*, which encode antimicrobial efflux mechanisms.
187 Nucleic acid extractions from 19/475 (4.0%) isolates generated an amplicon for *mphA*; 14 *S.*
188 *sonnei* and 5 *S. flexneri* (Table 2). The majority of these organisms had azithromycin MICs of
189 ≥32mg/L with a corresponding zone of inhibition of ≤14mm; three *S. flexneri* isolates had
190 azithromycin MICs of 16mg/L and zone sizes of 11 and 12mm (2 isolates) to a 15µg
191 azithromycin disc. A further three organisms produced *ermB* amplicons (3/475, 0.6%). The
192 only *ermB* amplification positive *S. flexneri* isolate had a lower MIC (16mg/L) and larger
193 inhibition zone size (12mm) in comparison to the two *S. sonnei* isolates (MIC 32mg/L, zone
194 size 9mm). These data suggest that *S. sonnei* and *S. flexneri* exhibit different distribution of
195 MICs when harboring the *mphA* and/or *ermB* genes.
196
197 ***Determining disc susceptibility breakpoints for azithromycin***
198 The CLSI recently provided ECV for determining azithromycin resistance in *S. flexneri* (disc
199 diffusion and MIC) and *S. sonnei* (MIC only) (16). While ECVs are not generally
200 recommended for determining clinical susceptibility breakpoints, we used these same criteria
201 in our dataset, given that clinical data on azithromycin usage was not available. We aimed to
202 determine whether the CLSI cut-off values could be used to determine suitable disc diffusion
203 breakpoints for *S. sonnei*. Azithromycin disc inhibition zone sizes were available for 181 *S.*
204 *flexneri* and 294 *S. sonnei* isolates. A regression analysis for determining the suitability of
205 MIC data to extrapolate disc diffusion breakpoints demonstrated a significant correlation
206 between MIC and disc diffusion zone size for *S. flexneri* (rho, -0.845; $p<0.0001$; Spearman)
207 and to a lesser extent for *S. sonnei* (rho, -0.649; $p<0.001$).
208
209 For *S. flexneri*, a breakpoint zone size of ≤15mm exhibited good discrimination against a
210 15µg azithromycin disc to identify non-susceptible isolates. Using an error rate-bounding
211 method, a 3% major error rate was found, and with a ≤15mm breakpoint there were no very

major or minor errors when compared an MIC of ≤8mg/L (Table 3, Figure 2), thereby fulfilling CLSI recommendations (36). In contrast, while the ECV MIC threshold of ≥32mg/L appeared to define non-susceptible *S. sonnei*, no clear demarcation in disc diffusion zone size measurements was observed (Figure 2). The largest azithromycin zone of inhibition in the *S. sonnei* isolates with a known azithromycin resistance mechanism was 9mm. We aimed to identify the largest zone size concordant with a permissible CLSI error rate. We determined that a cut-off of ≤11mm resulted in an acceptable discrepancy rate (Table 3), whereas ≤12mm resulted in a 6.5% major error rate.

Plasmid structures and phylogenetic context of azithromycin resistant Shigella sonnei

As observed previously, phylogenetic analyses confirmed that all genome-sequenced Vietnamese *S. sonnei* isolates belonged to the same clade of the Global III lineage (37). Investigation of the accessory genome confirmed that resistance to azithromycin within these *S. sonnei* isolates was mediated by either *ermB* or *mphA* in 16 of the sequenced isolates (Figure 3). Two of the 16 azithromycin-resistant isolates carried an *ermB* gene; the remaining 14 carried an *mphA* gene. Notably, unlike the phenotypes of reduced susceptibility to fluoroquinolones and resistance against third generation cephalosporins (38), these azithromycin resistance genes were not restricted to individual sub-lineages or clonal expansions. Indeed, we estimated that between 2001 and 2008 *ermB* was acquired independently on at least two separate occasions, whilst *mphA* was acquired on at least five separate occasions, forming a small sub-clade of azithromycin-resistant organisms on two instances (Figure 3). However, these azithromycin resistance genes were transient and appeared not to be maintained within the population.

Additional *in silico* analysis of the azithromycin resistance plasmids demonstrated that *ermB* was associated with two differing plasmid structures; *S. sonnei* 20094 harbored an IncFI plasmid (p20094) and *S. sonnei* EG430 carried an IncFII plasmid (pEG430-2). The IncFI plasmid (p20094) was assembled and found to be approximately 82kb in size, sharing 99%

240 DNA sequence identity with pEG356 (accession: FN594520.1), which we previously
241 characterized in the Vietnamese *S. sonnei* isolate, EG356 (38). Similar to plasmid pEG356,
242 p20094 carried a $bla_{\text{CTX-M-24}}$ downstream of an IS*Ecp1*. However, this replicon additionally
243 contained an ISCR3 insertion sequence encompassing both the *ermB* and *ermC* genes. The
244 IncFII plasmid pEG430-2 (accession LT174531.1) was 68,999bp and harbored *ermB* and
245 *ermC* genes downstream of an IS6 transposase and had a 33,429bp DNA transfer region
246 comprised of 37 contiguous genes (Figure 4a). Plasmid pEG430-2 shared significant DNA
247 homology to other two other previously sequenced IncFII plasmids, p183660 (KX008967;
248 coverage 86% and identity 98%) and pKSR100 (LN624486, coverage 89%, identity 98%),
249 which were respectively identified in *S. sonnei* and *S. flexneri* 3a isolates associated with
250 disease in MSM.
251
252 Despite the erratic distribution of the *mphA* gene in the 2000 and 2010 *S. sonnei* isolates,
253 sequence analysis demonstrated that these isolates likely carried *mphA* on a similar IncI
254 plasmid backbone of a comparable size. A *de novo* assembly of *S. sonnei* DE105 effectively
255 produced an entire plasmid sequence of 113,548bp, designated as pDE105 (accession
256 number: MG569891) (Figure 4b). Plasmid pDE105 was analogous in size and structure to a
257 previously described IncI plasmid pHV292 from an *E. coli* identified in the poultry
258 production system in Switzerland (accession: KM377239.1). The *mphA* gene was located
259 downstream of an IS3/IS911 transposase (*orfA*-*orfB*) and several additional AMR genes
260 associated with a *tnpA* transposon and conferring resistance against sulphanomides (*folP*),
261 streptomycin (*strepAB*), ß-lactams (*bla*-TEM-1), and tetracycline (*tetA*-*tetR*). Plasmid
262 pDE105 also contained a type IV secretion system with *traI*/*traJ* genes responsible for
263 conjugal transfer and an operon for pilus biosynthesis (*pilI, pilQ, pilM, pilN, pilO*, and *pilP*).
264
265 We lastly performed plasmid isolation and sequencing on an additional *S. sonnei* isolate
266 (DE891), which was distantly related to DE105. A *de novo* plasmid assembly produced seven
267 contiguous sequences of 115kb spanning 99.6% of pDE105 and had 99% DNA sequence

identity. These data confirmed a common IncI plasmid backbone within the *mphA* positive Vietnamese *S. sonnei*. Mapping the remaining *mphA* plasmid sequences against pDE105, we found that they all shared a common genetic synteny (~90kb), which contained the same resistance gene cassettes.

Discussion

Azithromycin is a commonly though to be last resort drug for dysentery, but an increasing number of reports of decreased susceptibility against azithromycin in *Shigella* isolates is concerning. This problem has been observed in disparate populations including among MSM in affluent areas and children with dysentery in LMICs. Antimicrobial options for treating MDR and/or ciprofloxacin-resistant *Shigella* spp. are limited, especially for children or when an oral antimicrobial is required. In this large set of clinical *Shigella* spp. isolates collected over 18 years in Vietnam and Laos, both countries in which *Shigella*-associated dysentery in endemic, we found a low proportion (~5%) of *Shigella* isolates with decreased susceptibility to azithromycin. This low rate of non-susceptibility may be associated with the initial low rates of nalidixic acid and ciprofloxacin resistance and thus limited azithromycin usage. To our knowledge, this is the largest collection of *Shigella* spp. exhibiting decreased susceptibility against azithromycin reported from this region. Plasmid-mediated acquisition of *mphA* and *ermB* were identified as the principal mechanisms for azithromycin resistance.

As human-restricted pathogens, *Shigella* spp. likely acquire resistance from the colonizing microbiota by plasmid transfer. This phenomenon has previously been demonstrated with *E. coli* donating *mphA* to *S. sonnei* (25). All of the identified *mphA*-associated plasmids have previously been described in *E. coli*, supporting their role as a reservoir from which AMR *Shigella* spp. may emerge. We demonstrate that the mechanism of azithromycin resistance to *Shigella* spp. arose sporadically during this period through at least seven plasmid acquisition events at different time points (from 2000 to 2009). *Shigella* spp. harboring azithromycin-resistance plasmids appear not to have been maintained within the population, which may be

296 associated with a lack of antimicrobial selection pressure, heterogeneity in the populations
297 sampled, or simply due to instability of the described resistance plasmids. There was only one
298 example in the *S. sonnei* population in which an *mphA*-harboring plasmid sub-clade was
299 maintained for at least two years (2000-2001).
300
301 Given the limited antimicrobial treatment options available for *Shigella*-associated dysentery
302 and the now widespread use of azithromycin, it is critical that laboratories can identify
303 clinical isolates non-susceptible to azithromycin. We assessed the suitability of recently
304 published ECVs for use as clinical susceptibility breakpoints. The MIC and disc zone sizes
305 for *S. flexneri* in this study were consistent with the ECV guidance proposed by CLSI for
306 MIC and disc diffusion measurements to identify non-wild type *S. flexneri* isolates, based on
307 the detection of a resistance mechanism (16). In contrast, the distribution of MICs for
308 azithromycin in *S. sonnei* were not concordant with the CLSI ECV guidance with a skew to
309 the right. Our data support a higher ECV and susceptibility breakpoint for *S. sonnei* of
310 ≥32mg/L, and that a tentative zone size of ≤11mm around a 15µg azithromycin disc can
311 identify non-wild type isolates. These thresholds are supported by confirmatory PCR
312 amplifications and genome sequencing which corroborated the presence of azithromycin
313 resistance gene in these 22 non-wild type isolates, and demonstrated an acceptably small
314 proportion of discrepancies according to CLSI criteria (36).
315
316 Limitations to our interpretations include the retrospective nature of the data analysis from the
317 associated collection of organisms and a lack of clinical outcome data. The clinical impact of
318 reductions in azithromycin susceptibility is uncertain, as azithromycin achieves a high
319 concentration in intracellular compartments, such as within macrophages and colonic
320 epithelial cells. The pathogenesis of *Shigella* spp. requires colonic epithelial cells for
321 invasion, intracellular survival, and replication (8). Consequently a positive clinical outcome
322 may be achieved even in the context of reduced *in vitro* susceptibility. Additionally, broth or
323 agar dilution methods are the recognized standard method for MIC determination, and a

previous study has demonstrated potential issues with measuring disc diffusion and Etests to determine azithromycin susceptibility (39). In a small study, Jain *et al.* demonstrated a double zone phenomenon for both methods and reported that broth dilution MICs corresponded with values intermediate to inner and outer zones. While zone size interpretation may be a limitation, we additionally performed genotypic screening for associated resistance genes on all isolates, confirming our phenotypic testing results. Despite these limitations, the major strengths of our analyses include the large dataset of clinical isolates, the wide range of azithromycin MICs and the repeat testing of all isolates at a single center, thus limiting inter-laboratory technical and interpretation errors.

While azithromycin resistance among *Shigella* spp. causing dysentery and diarrhea was not common in the 18-year period between 1994 and 2012 in the sampled locations, the increasing proportion of MDR, fluoroquinolone and third generation cephalosporin resistant isolates will inevitably lead to the increasing use of azithromycin. During the sampling period, *Shigella* spp. with decreased susceptibility to azithromycin emerged on several separate occasions, but failed to become established in the population. Azithromycin is being increasingly used for the treatment of suspected and confirmed *Shigella* infections in LMICs, despite limited evidence. In this study we have developed tentative susceptibility breakpoints that we suggest should be evaluated in other locations. Correlation with proposed breakpoints and clinical outcomes in azithromycin-treated patients is a further priority. MIC and disc susceptibility breakpoints are urgently needed for the active global surveillance for azithromycin resistant strains of *Shigella* spp. Assessment of new alternative treatments are also required to stay ahead of this potential public health problem.

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Transparency

The authors declare no competing interests.

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522 **Table 1**. Origin of *Shigella* isolates and frequency of selected resistance azithromycin markers

Country/Study code	Period	*Shigella* species				Antimicrobial resistance markers n/N (%)			
		S. flexneri	*S. sonnei*	Other	Total	DSA	NAL	CRO	MDR
Vietnam/MS	1994-1998	58	22	0	80	3/70 (4.3)	1/80 (1.3)	0/80 (0)	57/80 (72.5)
Vietnam/DE	2000-2002	42	62	8[A]	112	10/93 (10.8)	32/111 (28.8)	1/111 (0.9)	80/112 (71.4)
Laos	2006-2012	35	9	1[B]	45	0/45 (0)	14/45 (31.1)	0/45 (0)	34/45 (75.6)
Vietnam/EG	2007-2008	30	78	2[C]	110	4/104 (3.8)	75/108 (69.4)	22/108 (20.3)	96/110 (87.3)
Vietnam/Huế	2008-2010	21	37	0	58	1/56 (1.8)	27/58 (46.6)	7/58 (12.0)	24/58 (41.4)
Vietnam/AV	2009-2010	4	58	0	62	3/61 (4.9)	58/62 (93.5)	47/62 (75.8)	52/62 (83.9)
Vietnam/KH	2009-2010	8	42	0	50	1/50 (2.0)	47/50 (94.0)	18/50 (36.0)	25/50 (50)
Total		198	308	11	517	22/479 (4.8)	254/514 (49.4)	95/514 (18.5)	368/517 (71.2)

523
524 DSA, decreased sensitivity to azithromycin (*S. flexneri* MIC≥16mg/L; *S. sonnei* MIC≥32mg/L)
525 NAL, nalidixic acid (zone<19mm); CRO, ceftriaxone resistant organism (zone<23mm)
526 MDR, multidrug resistant: intermediate or resistant to ≥3 classes of antimicrobials: penicillins (ampicillin), cephems (ceftriaxone), folate inhibitors
527 (trimethoprim), phenicols (chloramphenicol), tetracyclines (tetracycline), quinolones (specifically nalidixic acid resistance), aminoglycosides (gentamicin)
528 Study code, as per description in Reference 6.
529 NA, not available; [A] 1 *S. boydii*, 1 *S. dysenteriae*, 6 NA; [B] 1 *S. boydii*; [C] 2 *S. boydii*.

530 **Table 2.** Source, microbiological and genotypic characteristics of *Shigella* spp. isolates with decreased susceptibility to azithromycin

Isolate ID	Organism	Year	Age (years)	Azithromycin susceptibility MIC (mg/L)	Azithromycin susceptibility Zone (mm)	Resistance gene	ESBL	MDR
MS025	*S. flexneri* 2a	1994-1998	0.75	32	11	*mphA*	-	+
MS052	*S. flexneri*	1994-1998	0.83	16	14	*mphA*	-	+
MS055	*S. flexneri* 6	1994-1998	0.92	512	6	*mphA*	-	+
DE0088	*S. sonnei*	2000	4.00	512	6	*mphA*	-	+
DE0105	*S. sonnei*	2000	1.50	512	6	*mphA*	-	+
DE0108	*S. sonnei*	2000	1.50	512	6	*mphA*	-	+
DE0185	*S. sonnei*	2000	0.67	512	6	*mphA*	-	+
DE0199	*S. sonnei*	2000	2.42	512	6	*mphA*	-	+
DE0490	*S. sonnei*	2000	1.67	512	6	*mphA*	-	+
DE0579	*S. sonnei*	2001	4.00	512	6	*mphA*	-	+
DE0885	*S. sonnei*	2001	3.00	512	6	*mphA*	-	+
DE0891	*S. sonnei*	2001	1.50	128	6	*mphA*	-	+
DE1336	*S. sonnei*	2002	1.92	512	6	*mphA*	-	+
EG0094	*S. sonnei*	2007	2.58	256	6	*mphA*	-	+
EG0352	*S. sonnei*	2007	2.50	256	6	*mphA*	-	+
EG0419	*S. flexneri* 2a	2007	1.92	16	12	*ermB*	-	+
EG0430	*S. sonnei*	2008	3.00	32	9	*ermB*	+	+
Huế 49	*S. flexneri*	2009	4.00	128	6	*mphA*	-	+
KH 39	*S. flexneri*	2009	0.75	16	12	*mphA*	-	+
20094	*S. sonnei*	2010	1.42	32	9	*ermB*	+	+
20343	*S. sonnei*	2010	1.58	512	6	*mphA*	+	+
30295	*S. sonnei*	2010	1.75	512	6	*mphA*	+	+

Table 3. Discrepancy rates of false-susceptible and false-resistant isolates detected using proposed breakpoint criteria using an error rate-bounding method

Organism (breakpoint, g/L)	MIC range	Number	Discrepancies N(%)	
			Very major	Major
S. flexneri (≤8)	≥R + 1	3	0	NA
	R + S	4	0	1 (25)
	≤S + 1	191	NA	5 (2.6)
	Total	198	0	6 (3.0)
S. sonnei (≤16)	≥R + 1	14	0	NA
	R + S	2	0	0
	≤S + 1	292	NA	3 (1.0)
	Total	308	0	3 (1.0)

R, non-susceptible MIC; S, susceptible MIC; NA, not applicable

535 **Figure 1.** The distribution of azithromycin MICs for *S. flexneri* and *S. sonnei* in Southeast
536 Asia
537 Histograms showing the number of *S. sonnei* (green) and *S. flexneri* (blue) collected in 7
538 studies performed in Southeast Asia between 1994 and 2012 exhibiting different MICs
539 against azithromycin (mg/L).
540
541 **Figure 2.** The relationship between azithromycin MIC and inhibition zone size in Southeast
542 Asian *Shigella* spp.
543 Plots showing the relationship between inhibition zone size (mm, x-axis) and MIC (mg/L, y-
544 axis) for azithromycin in *S. flexneri* (blue, left) and *S. sonnei* (green, right). The squares are
545 colored with respect to the number of isolates in each group, the number of isolate in each
546 group is additionally provided.
547
548 **Figure 3.** Phylogenetic tree of *S. sonnei* in Southeast Asia
549 Phylogenetic tree of 261 *S. sonnei* genomes (global lineage III) and an additional 54 genomes
550 of isolates collected during the same period (1995-2011). Tree constructed through 2,812
551 chromosomal SNPs. Phylogenetic reconstruction was performed using multiple alignments of
552 SNPs by maximum-likelihood based phylogenetic inference and displayed and annotated
553 using iTOL. The year/period of isolation is highlighted in the outer ring and the organisms
554 with reduced susceptibility against azithromycin; *mphA* positive isolates are highlighted in red
555 and *ermB* positive isolates are highlighted in blue.
556
557 **Figure 4.** Maps of azithromycin *S. sonnei* azithromycin resistant plasmids pDE105 and
558 pEG403_2
559 Maps of A) pDE105 and B) pEG403_2 azithromycin resistance plasmids isolated from
560 Vietnamese *S. sonnei.* The coding sequences of are number consecutively and notable
561 genes/regions are highlighted, which include DNA transfer regions, replication, antimicrobial

562 resistance, and the azithromycin resistance genes (*ermB* and *mphA*, respectively). The size

563 (bp) of each plasmid are shown in the center.

564