Darton, TC; Thanh Tuyen, H; Chung The, H; Newton, PN; Dance, DAB; Phetsouvanh, R; Davong, V; Campbell, JI; Minh Hoang, NV; Thwaites, GE; +3 more... Parry, CM; Pham Thanh, D; Baker, S; (2018) Azithromycin resistance in Shigella spp. in Southeast Asia. Antimicrobial agents and chemotherapy. ISSN 0066-4804 DOI: https://doi.org/10.1128/AAC.01748-17

Downloaded from: http://researchonline.lshtm.ac.uk/464321/

DOI: https://doi.org/10.1128/AAC.01748-17

Usage Guidelines:

Please refer to usage guidelines at https://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2,5/
Azithromycin resistance in *Shigella* spp. in Southeast Asia

Thomas C Darton 1,2, Ha Thanh Tuyen 1, Hao Chung The 1, Paul N Newton 3,4,
David AB Dance 3,4,5, Rattanaphone Phetsouvanh 3, Viengmon Davong 3,
James I Campbell 1, Nguyen Van Minh Hoang 1, Guy E Thwaites 1,4, Christopher M Parry 6,7,
Duy Pham Thanh 1, and Stephen Baker 1,4,*

1 The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University
Clinical Research Unit, Ho Chi Minh City, Vietnam
2 Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield Medical School, Sheffield, United Kingdom
3 Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Vientiane, Laos
4 Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford,
5 Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine,
6 Clinical Sciences, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
7 School of Tropical Medicine and Global Health, Nagasaki University, Japan
8 The Department of Medicine, The University of Cambridge, Cambridge, United Kingdom
* Corresponding author: Professor Stephen Baker, the Hospital for Tropical Diseases, 764 Vo Van Kiet, Quan 5, Ho Chi Minh City, Vietnam. Tel: +84 89241761 Fax: +84 89238904 sbaker@oucru.org

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.
Introduction

Organisms of the bacterial genus *Shigella* are a common cause of moderate to severe diarrhea and dysentery in children attending day-care facilities, those living in resource-limited settings, and travellers to such areas (1-5). In many low to middle-income countries (LMICs), such as Vietnam, endemic shigellosis is now predominantly caused by *Shigella sonnei*. Sustained antimicrobial pressure in LMICs has led to the emergence of resistance to the antimicrobials used for treating shigellosis (6,7). In Southeast Asia, antimicrobial resistance (AMR) in the Shigellae is largely being driven by the expansion of a specific *S. sonnei* lineage, which is known as Global III (8).

AMR within the genus *Shigella* is a problem for clinical management (9,10). The treatment of *Shigella* infections with antimicrobials is recommended by most clinical guidelines, predominantly to reduce the risk of onward transmission and disease complications. The WHO currently recommends ciprofloxacin as first-line treatment, with pivmecillinam, ceftriaxone, and azithromycin as alternative options. However, *Shigella* spp. are adept at acquiring AMR genes and plasmids, and reports of multi-drug resistant (MDR) lineages or isolates with reduced susceptibility to fluoroquinolones and third-generation cephalosporins are increasing globally (11,12).

Some recent recommendations have advocated the oral azalide antimicrobial azithromycin as an alternative treatment for shigellosis, particularly infections caused by MDR organisms or when fluoroquinolones are inappropriate (9,13). Clinical evidence for the efficacy of azithromycin in treating shigellosis is limited (14,15), and there are presently no suitable clinically derived susceptibility breakpoints to facilitate the laboratory identification of *Shigella* spp. exhibiting azithromycin non-susceptibility. Recently updated CLSI guidelines suggest epidemiological cut-off values (ECVs) of Minimum Inhibitory Concentrations (MIC) ≥16mg/L and MIC≥32mg/L to categories non-wild type *S. flexneri* and *S. sonnei*, respectively (16). Data supporting these guidelines are limited, principally originating from reports of an...
international outbreak of *S. flexneri* serotype 3a among men who have sex with men (MSM) (17-19). Here, we aimed to assess the frequency and mechanisms of *Shigella* spp. isolates with decreased susceptibility against azithromycin in Southeast Asia, a setting where fluoroquinolone and third-generation cephalosporin resistance has become common. Additionally, using a large dataset from Vietnam and Laos spanning 18 years, we aimed to calculate suitable breakpoints for assessing *Shigella* susceptibility against azithromycin.

**Materials and methods**

**Ethics statement**

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

**Study sites**

The majority of fecal specimens from which *Shigella* spp. were isolated were collected in a series of pediatric studies performed in Vietnam between 1994 and 2012, as previously described (6). Briefly, children presenting with either diarrhea or dysentery were recruited into observational studies (6, 20, 21), or treatment trials (22, 23) performed at the Hospital for Tropical Diseases (HTD), Children’s Hospital 1, or Children’s Hospital 2 in Ho Chi Minh City, Vietnam. Additional microbiology isolates collected for routine diagnostic purposes were also included from Huế Central Hospital in Huế and Khánh Hòa General Hospital in Nha Trang, Vietnam, and Mahosot Hospital in Vientiane, Laos.
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 plNV 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 ermA (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 ermA/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 ermA/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 \( \geq 3 \) 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 defined 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\textsubscript{50} for azithromycin was 4mg/L (MIC\textsubscript{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\( \geq 16\)mg/L was 6/181 (3.3%, 95%CI, 1.4 to 7.4), whereas the proportion of *S. sonnei* isolates with an MIC\( \geq 32\)mg/L was 16/294 (5.4%, 3.2 to 8.9; \( p > 0.05 \)).
Genes conferring decreased susceptibility against azithromycin

Isolates were screened by PCR amplification for the macrolide resistance genes \(\text{erm}A/B/C\), \(\text{mph}A/B\), \(\text{ere}A/B\), and \(\text{msr}A\) and \(\text{mef}A\), which encode antimicrobial efflux mechanisms.

Nucleic acid extractions from 19/475 (4.0%) isolates generated an amplicon for \(\text{mph}A\); 14 \(S.\) sonnei and 5 \(S.\) flexneri (Table 2). The majority of these organisms had azithromycin MICs of \(\geq 32\)mg/L with a corresponding zone of inhibition of \(\leq 14\)mm; three \(S.\) flexneri isolates had azithromycin MICs of 16mg/L and zone sizes of 11 and 12mm (2 isolates) to a 15\(\mu\)g azithromycin disc. A further three organisms produced \(\text{erm}B\) amplicons (3/475, 0.6%). The only \(\text{erm}B\) amplification positive \(S.\) flexneri isolate had a lower MIC (16mg/L) and larger inhibition zone size (12mm) in comparison to the two \(S.\) sonnei isolates (MIC 32mg/L, zone size 9mm). These data suggest that \(S.\) sonnei and \(S.\) flexneri exhibit different distribution of MICs when harboring the \(\text{mph}A\) and/or \(\text{erm}B\) genes.

Determining disc susceptibility breakpoints for azithromycin

The CLSI recently provided ECV for determining azithromycin resistance in \(S.\) flexneri (disc diffusion and MIC) and \(S.\) sonnei (MIC only) (16). While ECVs are not generally recommended for determining clinical susceptibility breakpoints, we used these same criteria in our dataset, given that clinical data on azithromycin usage was not available. We aimed to determine whether the CLSI cut-off values could be used to determine suitable disc diffusion breakpoints for \(S.\) sonnei. Azithromycin disc inhibition zone sizes were available for 181 \(S.\) flexneri and 294 \(S.\) sonnei isolates. A regression analysis for determining the suitability of MIC data to extrapolate disc diffusion breakpoints demonstrated a significant correlation between MIC and disc diffusion zone size for \(S.\) flexneri (rho, -0.845; \(p<0.0001\); Spearman) and to a lesser extent for \(S.\) sonnei (rho, -0.649; \(p<0.001\)).

For \(S.\) flexneri, a breakpoint zone size of \(\leq 15\)mm exhibited good discrimination against a 15\(\mu\)g azithromycin disc to identify non-susceptible isolates. Using an error rate-bounding method, a 3% major error rate was found, and with a \(\leq 15\)mm breakpoint there were no very
major or minor errors when compared an MIC of $\leq 8\text{mg/L}$ (Table 3, Figure 2), thereby fulfilling CLSI recommendations (36). In contrast, while the ECV MIC threshold of $\geq 32\text{mg/L}$ 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 $\leq 11\text{mm}$ resulted in an acceptable discrepancy rate (Table 3), whereas $\leq 12\text{mm}$ 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%...
DNA sequence identity with pEG356 (accession: FN594520.1), which we previously characterized in the Vietnamese S. sonnei isolate, EG356 (38). Similar to plasmid pEG356, p20094 carried a blaCTX-M-24 downstream of an ISecp1. However, this replicon additionally contained an ISCR3 insertion sequence encompassing both the ermB and ermC genes. The IncFII plasmid pEG430-2 (accession LT174531.1) was 68,999 bp and harbored ermB and ermC genes downstream of an IS6 transposase and had a 33,429 bp DNA transfer region comprised of 37 contiguous genes (Figure 4a). Plasmid pEG430-2 shared significant DNA homology to other two other previously sequenced IncFII plasmids, p183660 (KX008967; coverage 86% and identity 98%) and pKSR100 (LN624486, coverage 89%, identity 98%), which were respectively identified in S. sonnei and S. flexneri 3a isolates associated with disease in MSM.

Despite the erratic distribution of the mphA gene in the 2000 and 2010 S. sonnei isolates, sequence analysis demonstrated that these isolates likely carried mphA on a similar IncI plasmid backbone of a comparable size. A de novo assembly of S. sonnei DE105 effectively produced an entire plasmid sequence of 113,548 bp, designated as pDE105 (accession number: MG569891) (Figure 4b). Plasmid pDE105 was analogous in size and structure to a previously described IncI plasmid pHV292 from an E. coli identified in the poultry production system in Switzerland (accession: KM377239.1). The mphA gene was located downstream of an IS3/IS911 transposase (orfA-orfB) and several additional AMR genes associated with a mpA transposon and conferring resistance against sulphamides (folP), streptomycin (strePAB), β-lactams (bla-TEM-1), and tetracycline (tetA-tetR). Plasmid pDE105 also contained a type IV secretion system with tral/traJ genes responsible for conjugal transfer and an operon for pilus biosynthesis (pilI, pilQ, pilM, pilN, pilO, and pilP).

We lastly performed plasmid isolation and sequencing on an additional S. sonnei isolate (DE891), which was distantly related to DE105. A de novo plasmid assembly produced seven contiguous sequences of 115 kb 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-resistant plasmids appear not to have been maintained within the population, which may be
associated with a lack of antimicrobial selection pressure, heterogeneity in the populations sampled, or simply due to instability of the described resistance plasmids. There was only one example in the S. sonnei population in which an mphA-harboring plasmid sub-clade was maintained for at least two years (2000-2001).

Given the limited antimicrobial treatment options available for Shigella-associated dysentery and the now widespread use of azithromycin, it is critical that laboratories can identify clinical isolates non-susceptible to azithromycin. We assessed the suitability of recently published ECVs for use as clinical susceptibility breakpoints. The MIC and disc zone sizes for S. flexneri in this study were consistent with the ECV guidance proposed by CLSI for MIC and disc diffusion measurements to identify non-wild type S. flexneri isolates, based on the detection of a resistance mechanism (16). In contrast, the distribution of MICs for azithromycin in S. sonnei were not concordant with the CLSI ECV guidance with a skew to the right. Our data support a higher ECV and susceptibility breakpoint for S. sonnei of ≥32mg/L, and that a tentative zone size of ≤11mm around a 15µg azithromycin disc can identify non-wild type isolates. These thresholds are supported by confirmatory PCR amplifications and genome sequencing which corroborated the presence of azithromycin resistance gene in these 22 non-wild type isolates, and demonstrated an acceptably small proportion of discrepancies according to CLSI criteria (36).

Limitations to our interpretations include the retrospective nature of the data analysis from the associated collection of organisms and a lack of clinical outcome data. The clinical impact of reductions in azithromycin susceptibility is uncertain, as azithromycin achieves a high concentration in intracellular compartments, such as within macrophages and colonic epithelial cells. The pathogenesis of Shigella spp. requires colonic epithelial cells for invasion, intracellular survival, and replication (8). Consequently a positive clinical outcome may be achieved even in the context of reduced in vitro susceptibility. Additionally, broth or 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.

Acknowledgements

We are grateful to all of the study participants and patients who have taken part in these studies. We also gratefully acknowledge the support of participant’s parents and additional clinical and laboratory staff for their assistance in collection and processing of samples and
bacterial isolates respectively. In Lao, we are very grateful to Assoc. Prof. Bounthaphany Bounxouei the Director of Mahosot Hospital, the staff of Mahosot Hospital, Assoc. Prof. Chanphomna Vongsamphan, the Director of Department of Health Care, Ministry of Health, and Assoc. Prof. Boukong Syhavong, Minister of Health, Lao PDR for their very kind help and support.

**Funding**

This work was supported by the National Institutes for Health Research (Academic Clinical Lectureship grant number 3557 to TCD); the Academy of Medical Sciences and Wellcome Trust (Clinical Lecturer Starter Grant number SGCL015/1005 to TCD); the Wellcome Trust (grant number 098051 to HCT, grant number 089276/2/09/2 to JIC and GT, grant number 106698/Z/14/Z to DABD, PNN, RP and VD); the Oak Foundation (leadership fellow grant number B9R00910 to PTD); and the Wellcome Trust and Royal Society (Sir Henry Dale Fellowship grant number 10008/Z/12/Z to SB).

**Transparency**

The authors declare no competing interests.

**References**


21. Thompson CN, Phan VT, Le TP, Pham TN, Hoang LP, Ha V, Nguyen VM, Pham VM, Nguyen TV, Cao TT, Tran TT, Nguyen TT, Dao MT, Campbell JI, Nguyen TC, Tang CT, Ha MT, Farrar J, Baker S. 2013. Epidemiological features and risk factors...
of Salmonella gastroenteritis in children resident in Ho Chi Minh City, Vietnam.


27. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with


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

<table>
<thead>
<tr>
<th>Country/Study code</th>
<th>Period</th>
<th>Shigella species</th>
<th>Antimicrobial resistance markers n/N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S. flexneri</td>
<td>S. sonnei</td>
</tr>
<tr>
<td>Vietnam/MS</td>
<td>1994-1998</td>
<td>58</td>
<td>22</td>
</tr>
<tr>
<td>Vietnam/DE</td>
<td>2000-2002</td>
<td>42</td>
<td>62</td>
</tr>
<tr>
<td>Laos</td>
<td>2006-2012</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>Vietnam/EG</td>
<td>2007-2008</td>
<td>30</td>
<td>78</td>
</tr>
<tr>
<td>Vietnam/Huế</td>
<td>2008-2010</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td>Vietnam/AV</td>
<td>2009-2010</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td>Vietnam/KH</td>
<td>2009-2010</td>
<td>8</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>198</td>
<td>308</td>
</tr>
</tbody>
</table>

**Notes:**
- DSA, decreased sensitivity to azithromycin (S. flexneri MIC ≥ 16 mg/L; S. sonnei MIC ≥ 32 mg/L)
- NAL, nalidixic acid (zone < 19 mm); CRO, ceftriaxone resistant organism (zone < 23 mm)
- MDR, multidrug resistant: intermediate or resistant to ≥3 classes of antimicrobials: penicillins (ampicillin), cephems (ceftriaxone), folate inhibitors (trimethoprim), phenicols (chloramphenicol), tetracyclines (tetracycline), quinolones (specifically nalidixic acid resistance), aminoglycosides (gentamicin)
- Study code, as per description in Reference 6.
- NA, not available; ° 1 S. boydii; 1 S. dysenteriae, 6 NA; ² 1 S. boydii; ² 2 S. boydii.
Table 2. Source, microbiological and genotypic characteristics of Shigella spp. isolates with decreased susceptibility to azithromycin

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Organism</th>
<th>Year</th>
<th>Age (years)</th>
<th>Azithromycin susceptibility MIC (mg/L)</th>
<th>Zone (mm)</th>
<th>Resistance gene</th>
<th>ESBL</th>
<th>MDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS025</td>
<td>S. flexneri 2a</td>
<td>1994-1998</td>
<td>0.75</td>
<td>0.75</td>
<td>32</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MS052</td>
<td>S. flexneri 2a</td>
<td>1994-1998</td>
<td>0.83</td>
<td>16</td>
<td>14</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MS055</td>
<td>S. flexneri 6</td>
<td>1994-1998</td>
<td>0.92</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0088</td>
<td>S. sonnei</td>
<td>2000</td>
<td>4.00</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0105</td>
<td>S. sonnei</td>
<td>2000</td>
<td>1.50</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0108</td>
<td>S. sonnei</td>
<td>2000</td>
<td>1.50</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0185</td>
<td>S. sonnei</td>
<td>2000</td>
<td>0.67</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0199</td>
<td>S. sonnei</td>
<td>2000</td>
<td>2.42</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0490</td>
<td>S. sonnei</td>
<td>2000</td>
<td>1.67</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0579</td>
<td>S. sonnei</td>
<td>2001</td>
<td>4.00</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0855</td>
<td>S. sonnei</td>
<td>2001</td>
<td>3.00</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE0891</td>
<td>S. sonnei</td>
<td>2001</td>
<td>1.50</td>
<td>128</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DE1336</td>
<td>S. sonnei</td>
<td>2002</td>
<td>1.92</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EG0094</td>
<td>S. sonnei</td>
<td>2007</td>
<td>2.58</td>
<td>256</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EG0352</td>
<td>S. sonnei</td>
<td>2007</td>
<td>2.50</td>
<td>256</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EG0419</td>
<td>S. flexneri 2a</td>
<td>2007</td>
<td>1.92</td>
<td>16</td>
<td>12</td>
<td>ermA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EG0430</td>
<td>S. sonnei</td>
<td>2008</td>
<td>3.00</td>
<td>32</td>
<td>9</td>
<td>ermA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hul 49</td>
<td>S. flexneri</td>
<td>2009</td>
<td>4.00</td>
<td>128</td>
<td>6</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>KH 39</td>
<td>S. flexneri</td>
<td>2009</td>
<td>0.75</td>
<td>16</td>
<td>12</td>
<td>mphA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>20094</td>
<td>S. sonnei</td>
<td>2010</td>
<td>1.42</td>
<td>32</td>
<td>9</td>
<td>ermA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20343</td>
<td>S. sonnei</td>
<td>2010</td>
<td>1.58</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30295</td>
<td>S. sonnei</td>
<td>2010</td>
<td>1.75</td>
<td>512</td>
<td>6</td>
<td>mphA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3. Discrepancy rates of false-susceptible and false-resistant isolates detected using proposed breakpoint criteria using an error rate-bounding method

<table>
<thead>
<tr>
<th>Organism (breakpoint, g/L)</th>
<th>MIC range</th>
<th>Number</th>
<th>Very major</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em> (≤8)</td>
<td>≥R + 1</td>
<td>3</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>R + S</td>
<td>4</td>
<td>0</td>
<td>1 (25)</td>
</tr>
<tr>
<td></td>
<td>≤S + 1</td>
<td>191</td>
<td>NA</td>
<td>5 (2.6)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>198</td>
<td>0</td>
<td>6 (3.0)</td>
</tr>
<tr>
<td><em>S. sonnei</em> (≤16)</td>
<td>≥R + 1</td>
<td>14</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>R + S</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≤S + 1</td>
<td>292</td>
<td>NA</td>
<td>3 (1.0)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>308</td>
<td>0</td>
<td>3 (1.0)</td>
</tr>
</tbody>
</table>

R, non-susceptible MIC; S, susceptible MIC; NA, not applicable
Figure 1. The distribution of azithromycin MICs for *S. flexneri* and *S. sonnei* in Southeast Asia. Histograms showing the number of *S. sonnei* (green) and *S. flexneri* (blue) collected in 7 studies performed in Southeast Asia between 1994 and 2012 exhibiting different MICs against azithromycin (mg/L).

Figure 2. The relationship between azithromycin MIC and inhibition zone size in Southeast Asian *Shigella* spp. Plots showing the relationship between inhibition zone size (mm, x-axis) and MIC (mg/L, y-axis) for azithromycin in *S. flexneri* (blue, left) and *S. sonnei* (green, right). The squares are colored with respect to the number of isolates in each group, the number of isolate in each group is additionally provided.

Figure 3. Phylogenetic tree of *S. sonnei* in Southeast Asia. Phylogenetic tree of 261 *S. sonnei* genomes (global lineage III) and an additional 54 genomes of isolates collected during the same period (1995-2011). Tree constructed through 2,812 chromosomal SNPs. Phylogenetic reconstruction was performed using multiple alignments of SNPs by maximum-likelihood based phylogenetic inference and displayed and annotated using iTOL. The year/period of isolation is highlighted in the outer ring and the organisms with reduced susceptibility against azithromycin; *mphA* positive isolates are highlighted in red and *ermB* positive isolates are highlighted in blue.

Figure 4. Maps of azithromycin *S. sonnei* azithromycin resistant plasmids pDE105 and pEG403_2. Maps of A) pDE105 and B) pEG403_2 azithromycin resistance plasmids isolated from Vietnamese *S. sonnei*. The coding sequences of are number consecutively and notable genes/regions are highlighted, which include DNA transfer regions, replication, antimicrobial
resistance, and the azithromycin resistance genes (*ermB* and *mphA*, respectively). The size (bp) of each plasmid are shown in the center.