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Identification and Characterization of *Shigella* with Decreased Susceptibility to Azithromycin in the United States, 2005 to 2014

Davina Campbell[1], **Anna Bowen**[1], **Amelia Bhatnagar**[2], **Andre McCullough**[2], **Julian Grass**[1], **Jessica Chen**[2], **Jason P. Folster**[1]

[1]Division of Foodborne, Waterborne and Environmental Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

[2]IHRC, Inc., Atlanta, GA, USA

Abstract

Objectives—To identify *Shigella* isolates in the U.S. with decreased susceptibility to azithromycin (DSA) and characterized the genetic mechanisms responsible for this resistance.

Methods—The National Antimicrobial Resistance Monitoring System (NARMS) at Centers for Disease Control and Prevention (CDC) collects and conducts broth microdilution antimicrobial susceptibility testing on *Shigella* to determine minimum inhibitory concentrations (MIC) for up to 15 drugs, including azithromycin. Isolates with decreased susceptibility to azithromycin were subjected to molecular methods (PCR, whole genome sequencing, and plasmid typing/transformation) to identify the genetic mechanisms of resistance.

Results—A total of 118 isolates with decreased susceptibility to azithromycin were tested; 65 (55%) isolates contained only *mphA*, one (<1%) isolate contained only *ermB*, and 51 (43%) isolates contained both mechanisms. Seven isolates contained IncFII plasmids with *mphA*, *ermB*, or *mphA* and *ermB*, while one isolate contained an IncB/O plasmid with *mphA*. One (<1%) isolate that contained neither *mphA* nor *ermB* contained mutations in *rrlH, rplD*, and *rplV* genes, and an insertion in *rplV*, the function of which are not yet known.

Conclusions—Additional studies are needed to understand the effect on treatment outcomes, epidemiology, and possible additional mechanisms responsible for decreased susceptibility of azithromycin in *Shigella*.

Keywords

Shigella; Antimicrobial Drug Resistance

Shigella, the causative agent of shigellosis, causes approximately 450,000 infections, 6,380 hospitalizations, and 26 deaths annually in the United States (1). Symptoms start within 1-2 days and includes diarrhea, abdominal cramps, nausea, vomiting, and fever. In most individuals, symptoms resolve without treatment within 5 to 7 days, but can last longer

*Corresponding author Jason P. Folster, PhD, National Antimicrobial Resistance Monitoring System, Centers for Disease Control and Prevention, OID/NCEZID/DFWED/EDLB, 1600 Clifton Road, Atlanta GA 30333, gux8@cdc.gov, Ph: (404) 639-4948, Fax. (404) 639-4290.

for immunocompromised persons or young children (2). Shigellosis is transmitted from person to person through the fecal-oral route or via contaminated water or food, and children < 5 years old experience the highest incidence of shigellosis (3). Infections can be self-limited, but antibiotics may be indicated for patients with severe illness or immunocompromising conditions, and to limit the risk of transmission (4). Ampicillin and trimethoprim-sulfamethoxazole were previously recommended for treatment, but high rates of resistance in the United States prompted the use of fluoroquinolones and azithromycin, a macrolide antibiotic, for treatment of shigellosis (5)(2). Decreased susceptibility to azithromycin (DSA) in the United States has been observed in clinical *Shigella* isolates harboring the plasmid-encoded gene, *mphA*, alone or in combination with another plasmid-encoded gene, *ermB* (6). However, it has been difficult to document DSA in clinical settings because of the lack of established clinical breakpoints; therefore, clinical labs are unable to report susceptibility results for azithromycin (7, 8).

The National Antimicrobial Resistance Monitoring System (NARMS) at Centers for Disease Control and Prevention (CDC) conducts broth microdilution antimicrobial susceptibility testing on *Shigella* to determine minimum inhibitory concentrations (MIC) for up to 15 drugs, and azithromycin has been included since 2011 (4). Fifty-four state and local public health laboratories submit every 20th *Shigella* isolate, and at least 3 representative isolates from outbreaks to NARMS for antimicrobial susceptibility testing (AST). In 2005–2014, CDC received and tested 4248 *Shigella* surveillance isolates and 358 outbreak isolates. For AST, Custom Sensititre™ dry format panels (Thermo Fisher, Cleveland, OH) were used according to manufacturer's instructions. A subset of isolates was tested on Custom Sensititre™ frozen format panels (Thermo Fisher, Cleveland, OH). Quality control (QC) strains recommended by the Clinical and Laboratory Standards Institute (CLSI) were used, including *Staphylococcus aureus* ATCC 29213 for azithromycin, and QC results were interpreted using interpretive criteria from CLSI when available (8). For azithromycin, CLSI has established epidemiological cutoffs (ECVs) for *Shigella* species *sonnei* (MIC ≤ 16 mg/L) and *flexneri* (MIC ≤ 8 mg/L) (8, 9). These cutoffs allow categorization of isolates as either wild-type (WT; no detectable resistance or decreased susceptibility) or non-wild-type (NWT; shows detectable resistance or decreased susceptibility). For this analysis we used our previously defined DSA for *Shigella* as an isolate with an azithromycin MIC >16 μg/mL, regardless of species (11). Isolates of *Shigella* collected through routine surveillance and outbreaks in 2005 to 2014 were evaluated and included in the study if azithromycin MICs showed DSA.

One hundred eighteen isolates (7.3% of all tested) that showed DSA were identified: 82 *Shigella flexneri*, 35 *Shigella sonnei*, and one *Shigella boydii* isolate. PCR-amplification was used to screen these isolates for two macrolide resistance genes, *mphA*, a macrolide-2'-phosphotransferase gene previously described in *Shigella* and *ermB*, a macrolide methyltransferase gene described in several bacteria including *E. coli*, using previously described primers and methods (9, 10). Of the 82 *Shigella flexneri* DSA isolates, 45 were positive for *mphA* only, one for *ermB* only, and 36 were *mphA* and *ermB* positive. Of the 35 *Shigella sonnei* isolates, 19 were positive for *mphA* only and 15 were *mphA* and *ermB* positive. One *Shigella sonnei* isolate contained neither *mphA* nor *ermB*. The *Shigella boydii* isolate was positive for *mphA* only. In total, 65 (55%) isolates contained only *mphA*, one

(<1%) isolate contained only *ermB*, 51 (43%) isolates contained both mechanisms, and one (<1%) isolate contained neither mechanism (Table 1).

To determine the location of these macrolide resistance genes, nine DSA isolates were selected for transformation experiments. Plasmids were purified using Qiagen QiaFilter Midi kit following manufacturer's protocol and electroporated into *E. coli* DH10B Electro MAX competent cells (Invitrogen, Carlsbad, CA) as previously described (11). Transformants were selected by plating on Luria-Bertani (LB) agar plates containing 20 mg/L azithromycin (Sigma-Aldrich, St. Louis, MO). PCR analysis of transformants confirmed the presence or absence of *mphA* and/or *ermB*. Plasmids purified from transformants were typed by plasmid-based replicon typing (12). Seven isolates contained IncFII plasmids with *mphA*, *ermB*, or *mphA* and *ermB*, while one isolate contained IncB/O with *mphA* (Table 2). No transformants were obtained for 2014C-3799, the isolate that contained neither *ermB* nor *mphA*. Whole genome sequencing (WGS) analysis was conducted on isolate 2014C-3799. Genomic DNA was purified with the DNeasy Blood and Tissue kit (Qiagen Inc.) according to the manufacturer's protocol. The whole genome library was constructed using a NexteraXT library kit, and paired-end, base pairreads were generated on a MiSeq (Illumina, San Diego, CA). The raw reads were assembled *de novo* with CLC Genomics Workbench 11 (Qiagen Inc.). The whole genome assembly was analyzed with ResFinder 3.0 (date run 06/29/2018) (http://cge.cbs.dtu.dk/services/) using a threshold of 90% for ID and 60% gene length. The raw reads from the whole genome sequencing were deposited in NCBI's Sequence Read Archive under the accession numbers **SAMN03097542**.

WGS analysis confirmed that 2014C-3799 possessed neither *mphA* nor *ermB*, and it did not contain any other acquired macrolide resistance genes or known 23S rRNA mutations. The azithromycin resistance mechanism could not be transferred with transformation studies, suggesting that the mechanism is likely chromosomal and could be mutational.

In this study, most isolates analyzed contained *mphA* with or without *ermB*, and these genes were located on mobile genetic elements, mainly IncFII plasmids. Potential spread of these mechanisms by virtue of their location on plasmids is concerning, and further work is needed to determine the extent to which this occurs. We did identify one DSA *Shigella sonnei* isolate (2014C-3799) that contained neither *mphA* nor *ermB*, and it's mechanism responsible for DSA warranted further investigation. In other bacterial species, mutations in the usually multi-copy *rrlH* gene encoding the 23S ribosomal protein commonly confer macrolide resistance. However, *Shigella* have seven copies of this gene, leading to the hypothesis that 23S rRNA mutations in all seven copies may be difficult to acquire and maintain, possibly explaining why this mechanism has not been observed in clinical isolates. A limited number of studies have identified low-level azithromycin resistance in lab-derived *Shigella* strains due to mutations in the *rplD* and *rplV* genes, which encode the L4 and L22 ribosomal proteins (13). In *E. coli*, erythromycin resistance, mostly in lab-derived strains, has been associated with 23S rRNA mutations at nucleotide positions 2058, 2059, and 2062 of the *rrlH* gene (14, 15). Sequencing analysis of the *rrlH* genes of 2014C-3799 did not identify any known macrolide resistance mutations but did identify novel *rrlH* mutations at nucleotide positions 2203 and 2211; the role of these mutations in

the DSA phenotype remains unknown. Additionally, sequencing analysis of *rplD* identified a His165Gln mutation and sequencing analysis of *rplV* identified a Leu46Gln mutation. An insertion of 30 nucleotides at position 221 encoding an 11-amino-acid sequence, LFVDEGPSMK, in the *rplV* gene was also identified. Both the mutations and the insertion were previously reported in DSA *Shigella* and *Escherichia coli* and may contribute to decreased susceptibility to azithromycin (13). The high copy number of *rrlH* makes it extremely difficult to perform allelic exchange and our attempts to overexpress our *rrlH* variant in a wildtype *rrlH* background failed (data not shown).

Decreased susceptibility to azithromycin is concerning in light of the high prevalence of *Shigella* resistant to ampicillin and trimethoprim-sulfamethoxazole, increasing resistance to quinolones, and a rise in use of azithromycin for empiric treatment of diarrhea (6, 16, 17). Clinical outcome data are needed to help set clinical breakpoints for azithromycin. Continued surveillance is critical to understand trends of DSA, and to identify new or changing mechanisms responsible for DSA.

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Table 1.

Mechanisms associated with decreased susceptibility to azithromycin in *Shigella* isolates collected through the National Antimicrobial Resistance Monitoring System (NARMS), 2005 – 2014

Organism	mphA	ermB	mphA and ermB	Neither mphA or ermB	Total Tested
Shigella boydii	1	0	0	0	1
Shigella flexneri	45	1	36	0	82
Shigella sonnei	19	0	15	1	35
Total Positive for Mechanism (n)	65	1	51	1	118
Percent positive for mechanism	55%	0.9%	43%	0.9%	100%

Table 2.

Azithromycin susceptibility, plasmids, and resistance genes for *Shigella* isolated in the United States, 2005 – 2014

Isolate	Genus	Species	Azithromycin MIC (mg/L) with dried panel	Azithromycin MIC (mg/L) with frozen panel	Year	Plasmid Inc Type	DSA Mechanism
AM23591	*Shigella*	*sonnei*	>16	256	2005	IncB/O	*mphA*
2013C-3849	*Shigella*	*flexneri*	>16	64	2007	IncFII	*mphA*
2012C-3670	*Shigella*	*sonnei*	>16	256	2012	IncFII	*mphA*
2014C-4197	*Shigella*	*sonnei*	>16	-	2012	IncFII	*mphA/ermB*
2013C-3575	*Shigella*	*flexneri*	>16	64	2010	IncFII	*ermB*
2012C-3948	*Shigella*	*sonnei*	>16	>256	2012	IncFII	*mphA/ermB*
2012AM-0331	*Shigella*	*sonnei*	>16	-	2012	IncFII	*mphA/ermB*
2013AM-2113	*Shigella*	*sonnei*	>16	64	2013	IncFII	*mphA*
2014C-3799	*Shigella*	*sonnei*	>16	-	2014	none	none

Dashes (-) represent not tested

MIC, minimum inhibitory concentration; *Inc*, Incompatability/Replicon; DSA, decreased susceptibility to azithromycin