

## Antimicrobial Susceptibility to Azithromycin among *Salmonella enterica* Isolates from the United States<sup>∇</sup>

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**Due to emerging resistance to traditional antimicrobial agents, such as ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, azithromycin is increasingly used for the treatment of invasive *Salmonella* infections. In the present study, 696 isolates of non-Typhi *Salmonella* collected from humans, food animals, and retail meats in the United States were investigated for antimicrobial susceptibility to azithromycin. Seventy-two *Salmonella enterica* serotype Typhi isolates from humans were also tested. For each isolate, MICs of azithromycin and 15 other antimicrobial agents were determined by broth microdilution. Among the non-Typhi *Salmonella* isolates, azithromycin MICs among human isolates ranged from 1 to 32  $\mu\text{g/ml}$ , whereas the MICs among the animal and retail meat isolates ranged from 2 to 16  $\mu\text{g/ml}$  and 4 to 16  $\mu\text{g/ml}$ , respectively. Among *Salmonella* serotype Typhi isolates, the azithromycin MICs ranged from 4 to 16  $\mu\text{g/ml}$ . The highest MIC observed in the present study was 32  $\mu\text{g/ml}$ , and it was detected in three human isolates belonging to serotypes Kentucky, Montevideo, and Paratyphi A. Based on our findings, we propose an epidemiological cutoff value (ECOFF) for wild-type *Salmonella* of  $\leq 16 \mu\text{g/ml}$  of azithromycin. The susceptibility data provided could be used in combination with clinical outcome data to determine tentative clinical breakpoints for azithromycin and *Salmonella enterica*.**

Non-Typhi *Salmonella* is the second leading cause of foodborne illness in the United States (34). Each year, approximately 1.0 million people are infected with *Salmonella*, resulting in 19,000 hospitalizations and almost 400 deaths (34). Although antimicrobial treatment is not indicated for uncomplicated infections, antimicrobial agents are potentially life-saving for extraintestinal infections. Likewise, antimicrobial therapy is essential for treating infections of *Salmonella enterica* serotypes Typhi and Paratyphi, the causative agents of enteric fever (31).

Due to widespread resistance to traditional first-line drugs, such as ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, current recommendations suggest using a fluoroquinolone (e.g., ciprofloxacin) or an extended-spectrum cephalosporin (e.g., ceftriaxone) for treating invasive and severe *Salmonella* infections (16, 18). However, *Salmonella* serotype Typhi and paratyphoidal serotypes with decreased susceptibilities to fluoroquinolones, which may be associated with inadequate responses to treatment, have become common, and reports of fluoroquinolone-resistant *Salmonella* strains are increasing (8, 22, 24, 25). Furthermore, extended-spectrum  $\beta$ -lactamase-producing *Salmonella* serotype Typhi and *Salmonella* serotype Paratyphi A have been reported (1, 32). Of even greater concern, some *Salmonella* isolates display concurrent

resistance to quinolones and extended-spectrum cephalosporins, which may require use of an alternative antimicrobial class for management of invasive infections (27, 36).

Azithromycin is an azalide antimicrobial agent that has been demonstrated in clinical trials to be equivalent or superior to chloramphenicol, fluoroquinolones, and extended-spectrum cephalosporins for the management of uncomplicated typhoid fever (3, 4, 13–15, 30). In addition, azithromycin is increasingly considered for the management of bacillary dysentery and invasive nontyphoid *Salmonella* infections (9, 10, 37, 38). Azithromycin shows excellent penetration into most tissues, and it achieves concentrations in macrophages and neutrophils that are >100-fold higher than concentrations in serum (23, 29). These properties, along with azithromycin's long half-life of 2 to 3 days, have increased azithromycin's role in the therapeutic management of infections of the reticuloendothelial system.

Prior to initiation of any antimicrobial therapy, the *in vitro* susceptibility of the disease-causing bacteria to potentially effective antimicrobial agents should be examined. The *in vitro* susceptibilities of bacteria are classified as either "susceptible," "intermediate," or "resistant" and are defined by clinical breakpoints. In the United States, the Food and Drug Administration (FDA) and the Clinical and Laboratory Standards Institute (CLSI) define breakpoints. In Europe, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) is responsible for defining clinical breakpoints for new and existing drugs (20, 21).

At present, no clinical azithromycin breakpoints have been defined for *Enterobacteriaceae*, including *Salmonella*, by either the CLSI or EUCAST (5, 6, 11). By performing antimicrobial

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susceptibility testing on 696 isolates of non-Typhi *Salmonella* isolated from humans, food animals, and retail meats in the United States, we provide data on the range of azithromycin MICs observed among *Salmonella enterica* isolates from the United States. We further present azithromycin MICs for 72 isolates of *Salmonella* serotype Typhi. This information could be combined with clinical outcome data to facilitate establishment of clinical azithromycin breakpoints for *Salmonella*.

#### MATERIALS AND METHODS

In 2008, 54 state and local public health laboratories participating in the National Antimicrobial Resistance Monitoring System (NARMS) forwarded every isolate of *Salmonella* serotype Typhi and every 20th non-Typhi *Salmonella* isolate from human clinical infections to the Centers for Disease Control and Prevention (CDC). Similarly, non-Typhi *Salmonella* isolates from retail meats (chicken breasts, ground turkey, ground beef, and pork chops) were submitted by the states participating in the Foodborne Diseases Active Surveillance Network (FoodNet) for analysis at the U.S. Food and Drug Administration Center for Veterinary Medicine (FDA-CVM). Non-Typhi *Salmonella* isolates from food animals were obtained from carcass rinsates (chicken), carcass swabs (turkey, cattle, and swine), and ground products (chicken, turkey, and beef). Animal samples were collected by the U.S. Department of Agriculture's (USDA) Food Safety Inspection Service (FSIS) from federally inspected slaughter and processing plants throughout the United States and forwarded to the USDA in Athens, GA, for further analysis. In addition, non-Typhi *Salmonella* isolates from animal products were collected through special studies performed at the USDA. At each agency, 232 non-Typhi *Salmonella* isolates were randomly chosen for azithromycin susceptibility determination. Moreover, 60 isolates of *Salmonella* serotype Typhi submitted in 2008 and 12 additional isolates from previous years were tested for azithromycin susceptibility at the CDC. All Typhi isolates were clinical isolates collected from humans.

MICs were determined by broth microdilution using a frozen azithromycin reference panel with concentrations ranging from 0.25 to 256 µg/ml (Sensititre; Trek Diagnostics, Westlake, OH). Antimicrobial susceptibility testing was performed according to the manufacturer's instructions, with *Staphylococcus aureus* ATCC 29213 used as a quality control strain (5). For each isolate, a final inoculum of  $5 \times 10^5$  CFU/ml was targeted. The panels were read after 18 h of incubation at 35°C.

In addition, all isolates were tested for susceptibility to 15 antimicrobial agents included on the NARMS Gram-negative panel (amikacin, ampicillin, amoxicillin-clavulanic acid, ceftiofur, ceftriaxone, cefoxitin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, sulfamethoxazole, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline) and interpreted according to CLSI standards, where available (5). Additional susceptibility testing with azithromycin Etest (bioMérieux, Inc., NC) strips was performed according to the manufacturer's instructions on Mueller-Hinton II agar plates incubated at 37°C for 16 to 20 h.

An MIC histogram was constructed, and the MIC<sub>50</sub> value, representing the MIC at which the growth of 50% of the population is inhibited, was calculated for each of the four sample groups (*Salmonella* serotype Typhi from humans and non-Typhi *Salmonella* from humans, retail meats, and animals). The histograms were further inspected to identify the wild-type MIC distribution.

Genomic DNA was prepared by lysing the bacteria at 95°C and collecting the supernatant following centrifugation. A PCR assay using previously described primers was used to screen for the following macrolide resistance genes: *ereA*, *ereB*, *ermB*, *mefA*, *mphA*, *mphB*, and *mphD* (28). Plasmids were extracted using the Qiagen plasmid midi prep kit (Qiagen, Valencia, CA) and electroporated into electrocompetent *Escherichia coli* DH10B (Invitrogen, Carlsbad, CA).

#### RESULTS

In 2008, 2,379 isolates of non-Typhi *Salmonella* were submitted to the CDC, 1,326 to the USDA, and 495 to the FDA-CVM as part of the NARMS program for enteric bacteria. At each agency, a subset of 232 isolates was randomly chosen to be tested for antimicrobial susceptibility to azithromycin. In addition, 72 isolates of *Salmonella* serotype Typhi were included. Among the *Salmonella* serotype Typhi isolates, 49

(68.1%) were isolated from blood cultures and 16 (22.2%) from stool. The sources of the remaining 7 isolates were not provided by the submitting laboratory.

Among the 232 non-Typhi *Salmonella* isolates randomly selected from human isolate submissions, *Salmonella enterica* serotypes Enteritidis (19.0%) and Typhimurium (13.4%) were most common. Of these, 84.1% were isolated from stool cultures and 9.1% from blood cultures. The remaining 16 isolates were isolated from urine, other sources, or nonspecified sources. Among the isolates randomly selected for analysis from food animal submissions, *Salmonella enterica* serotypes Kentucky (16.4%), Heidelberg (9.5%), and Montevideo (7.3%) were most common, and among the isolates randomly selected from retail meats, *Salmonella enterica* serotypes Heidelberg (19.6%), Hadar (16.8%), and Typhimurium variant O:5– (10.8%) predominated. Among the animal isolates, 227 were obtained from the following sample sources: chicken (38.8%), cattle (37.9%), turkey (15.4%), and swine (7.9%). The remaining five isolates from the USDA were special study isolates obtained from ready-to-eat products and eggs. Among the retail meat isolates, 116 (50.0%) originated from ground turkey, 95 (40.9%) from chicken breast, 12 (5.2%) from pork chops, and 9 (3.9%) from ground beef.

Among the non-Typhi *Salmonella* isolates from all sources, 64 (9.2%) displayed resistance to ceftriaxone (MIC  $\geq 4$  µg/ml) and 15 (2.2%) showed decreased susceptibility to ciprofloxacin (MIC  $\geq 0.125$  µg/ml). The azithromycin MICs among the isolates collected from humans ranged from 1 to 32 µg/ml, whereas the MICs among the animal and retail meat isolates ranged from 2 to 16 µg/ml and 4 to 16 µg/ml, respectively (Fig. 1). All three distributions peaked at 8 µg/ml, and the MIC<sub>50</sub> value for each distribution was 8 µg/ml. Three isolates collected from humans displayed a MIC of 32 µg/ml. These isolates comprised *Salmonella* serotypes Kentucky, Montevideo, and Paratyphi A. The serotype Paratyphi A isolate was obtained from blood, whereas the serotype Kentucky and Montevideo isolates were obtained from stool. By PCR, these three isolates were negative for genes associated with macrolide resistance (*ereA*, *ereB*, *ermB*, *mefA*, *mphA*, *mphB*, and *mphD*) (28). Moreover, plasmid extractions and electroporation of plasmids into *E. coli* DH10B did not yield any transformants. Retesting of these isolates by Etest yielded MICs of 16 µg/ml.

Among the 72 isolates of *Salmonella* serotype Typhi, 5 (7.0%) showed resistance to ampicillin (MIC  $\geq 32$  µg/ml), 3 (4.2%) to chloramphenicol (MIC  $\geq 32$  µg/ml), and 7 (9.7%) to trimethoprim-sulfamethoxazole (MIC  $\geq 4$  µg/ml). Fifty-eight (81.0%) isolates showed decreased susceptibility to ciprofloxacin (MIC  $\geq 0.125$  µg/ml). Of these, 12 (16.7%) showed clinical resistance (ciprofloxacin MIC  $\geq 4$  µg/ml), according to current CLSI guidelines (6). The azithromycin MICs among the *Salmonella* serotype Typhi isolates ranged from 4 to 16 µg/ml (Fig. 2).

#### DISCUSSION

Currently, azithromycin is recommended for the treatment of both shigellosis and invasive salmonellosis by the World Health Organization and the American Academy of Pediatrics (2, 37, 38) and is increasingly used for the management of

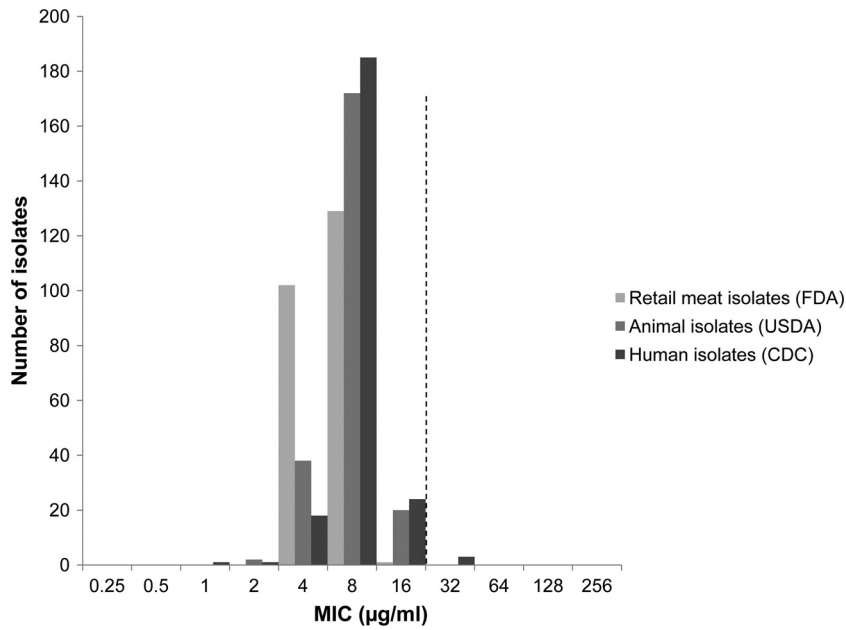


FIG. 1. Azithromycin MIC distributions for 696 isolates of non-Typhi *Salmonella enterica* collected from humans, food animals and retail meats through the National Antimicrobial Resistance Monitoring System (NARMS), 2008. The dashed line denotes a proposed epidemiological cutoff value (ECOFF) for WT isolates of  $\leq 16$   $\mu\text{g/ml}$ .

uncomplicated enteric fever (3, 4, 13–15, 30). However, clinical breakpoints for azithromycin and *Salmonella* have yet to be defined. Clinical breakpoints are necessary to detect emerging and changing patterns of resistance and to guide clinicians in the selection of effective antimicrobial therapy. The first step toward defining clinical breakpoints is to collect relevant data, including (i) pharmacodynamic data of the drug, (ii) pharmacological properties of the drug, (iii) clinical outcome data, and (iv) microbiological data, i.e., MIC data for the specific patho-

gen in question (7, 35). In this paper, we provide MIC data for 696 isolates of non-Typhi *Salmonella* and 72 isolates of *Salmonella* serotype Typhi that could contribute to establishing susceptibility breakpoints for *Salmonella* and azithromycin.

The 696 non-Typhi *Salmonella* isolates included in the present study were collected from various sources, including humans, animals, and retail meats in the United States. The MIC distributions for the different sample sources were similar, and the majority of the isolates in each distribution displayed MIC

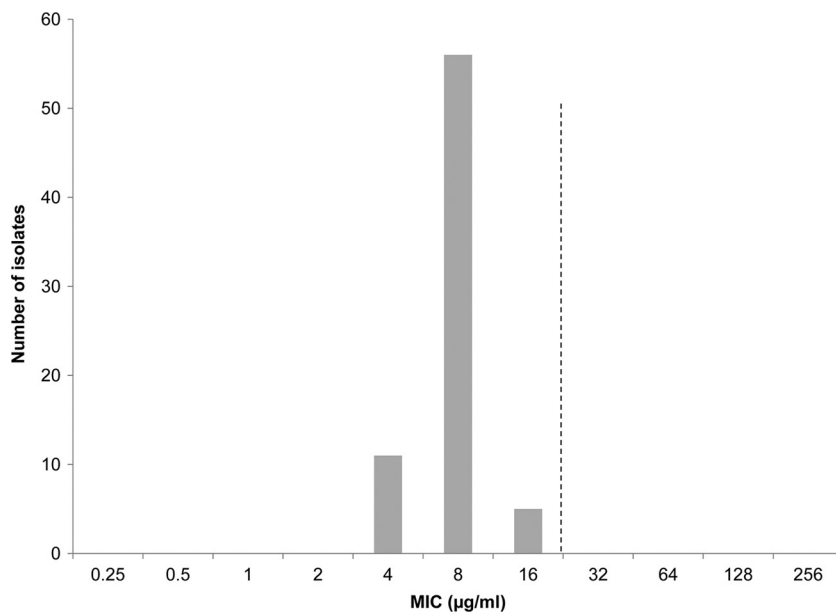


FIG. 2. Azithromycin MIC distribution for 72 isolates of *S. enterica* serotype Typhi collected by the National Antimicrobial Resistance Monitoring System (NARMS). The dashed line denotes a proposed epidemiological cutoff value (ECOFF) for WT isolates of  $\leq 16$   $\mu\text{g/ml}$ .

values of 4 to 8  $\mu\text{g/ml}$ . Thus, overall, azithromycin showed similar activities against non-Typhi *Salmonella* isolates obtained from various sources, including isolates displaying resistance to ceftriaxone and decreased susceptibility to ciprofloxacin. Our findings are consistent with a study reporting azithromycin susceptibility among non-Typhi *Salmonella* isolates collected in Finland (17) and are also consistent with MIC distribution data presented for *Salmonella* serotype Typhi and *Shigella* (15, 19).

The highest MIC value observed in the present study was 32  $\mu\text{g/ml}$ , and it was detected in three human isolates belonging to serotypes Kentucky, Montevideo, and Paratyphi A. These isolates were all negative for *ereA*, *ereB*, *ermB*, *mefA*, *mphA*, *mphB*, and *mphD*, genes associated with macrolide resistance (28). In addition, attempts to transfer the resistance failed in all three isolates, indicating a lack of plasmid-mediated mechanisms. Other possible mechanisms include mutations in the 23S rRNA genes or the *rlpD* and *rlpV* genes, the last two of which encode ribosomal proteins L4 and L22, respectively (33). Further investigations will be necessary to determine whether these isolates acquired a resistance mechanism or whether they belong to the wild-type distribution and their slightly elevated MICs are due to normal variation in the testing methodologies. The latter possibility is supported by the fact that these three isolates displayed MICs of 16  $\mu\text{g/ml}$  upon retesting with Etest.

Studies investigating azithromycin resistance mechanisms in *Salmonella* are scarce. Gunell and colleagues reported an *rlpD* mutation among isolates with azithromycin MICs of 64 to 128  $\mu\text{g/ml}$  (17). Moreover, an isolate of *Salmonella enterica* serotype Stanley collected through NARMS in the United States in 1999 displayed an azithromycin MIC of 128  $\mu\text{g/ml}$  and harbored the *mphA* gene (12; J. P. Folster et al., unpublished data).

Although clinical breakpoints are essential for defining clinical resistance, they might not always be the most sensitive tool for detecting isolates with an acquired resistance mechanism. The detection of resistance mechanisms is becoming increasingly important as surveillance programs recognize their role in the global control of antimicrobial resistance. In this context, the use of microbiological breakpoints or epidemiological cut-off values (ECOFFs) is useful. The concept of ECOFFs was introduced by EUCAST as a way of distinguishing bacteria without resistance (wild type [WT]) from those with acquired resistance (21). The WT ECOFF is expressed as  $\leq x$   $\mu\text{g/ml}$  and serves to divide the bacterial population into two groups: those that are wild type (WT) and those that acquired resistance and are non-wild type (NWT) (21). Based on this study and the work of EUCAST and Gunell and coauthors, we propose an ECOFF for WT *Salmonella* isolates of  $\leq 16$   $\mu\text{g/ml}$  of azithromycin (11, 17). This means that isolates with an MIC of  $\geq 32$   $\mu\text{g/ml}$  could be considered NWT for surveillance purposes. Additional studies would be required to confirm the accuracy of the proposed ECOFF and determine whether isolates displaying an MIC value of 32  $\mu\text{g/ml}$  belong to the wild-type distribution.

Whether NWT isolates should be classified as clinically resistant remains to be determined following the accumulation of clinical endpoint data. Recently, the first case of azithromycin treatment failure in a patient with invasive *Salmonella* infec-

tion was reported (26). The *Salmonella* serotype Paratyphi A isolate that initially displayed an azithromycin MIC of 64  $\mu\text{g/ml}$  later displayed an MIC of 256  $\mu\text{g/ml}$  when it was isolated from a second blood culture (26).

In summary, the azithromycin MIC distributions for *Salmonella* serotype Typhi and non-Typhi *Salmonella* isolates were concordant, and the majority of the isolates showed MICs of 4 to 8  $\mu\text{g/ml}$ . Based on data from this study and the work of others (11, 17), we suggest an ECOFF for WT *Salmonella* of  $\leq 16$   $\mu\text{g/ml}$  of azithromycin. Moreover, as azithromycin use increases for the management of invasive salmonellosis, we encourage national and international antimicrobial susceptibility testing consensus groups to consider the MIC distribution data presented here to contribute to the establishment of clinical breakpoints for azithromycin and *Salmonella enterica*.

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