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In Vitro Activity of Azithromycin against Nontyphoidal *Salmonella enterica*▽

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The *in vitro* activity of azithromycin against 1,237 nontyphoidal *Salmonella enterica* isolates collected from Finnish patients between 2003 and 2008 was investigated. Only 24 (1.9%) of the isolates tested and 15 (5.1%) of the 294 isolates with reduced fluoroquinolone susceptibility had azithromycin MICs of ≥32 μg/ml. These data show that azithromycin has good *in vitro* activity against nontyphoidal *S. enterica*, and thus, it may be a good candidate for clinical treatment studies of salmonellosis.

Salmonella is one of the most common causes of food-borne illnesses and a major cause of human infections all over the world (23). *Salmonella* infections are usually treated with fluoroquinolones or extended-spectrum cephalosporins. Unfortunately, excessive use of fluoroquinolones both in human and in veterinary medicine has led to increasing numbers of resistant isolates, including nontyphoidal strains of *Salmonella enterica* (18, 19, 28). In addition, the nonclassical quinolone resistance phenotype (the Qnr phenotype), showing reduced susceptibility to ciprofloxacin (MIC of ≥0.125 μg/ml) but susceptibility or only low-level resistance to nalidixic acid (MIC of ≤32 μg/ml), has become more common (6, 14, 19, 20, 22). Extended-spectrum β-lactamase (ESBL) producers have emerged in *Enterobacteriaceae* and in *Salmonella*, and there are reports of the coappearance of ESBL and *qnr* genes in the same transferable genetic elements (5, 10, 21, 27). These resistance problems may jeopardize the treatment of severe *Salmonella* infections. Thus, alternative antibiotics for the treatment of *Salmonella* infections are needed.

Salmonella isolates are intrinsically resistant to erythromycin via active efflux (2) but naturally susceptible to azithromycin (29), which is a 15-membered erythromycin derivative. Resistance to macrolides is usually conferred by mutations in nucleotides A2058 and A2059 of the 23S rRNA, according to the *Escherichia coli* numbering (26). Also, the alteration of the 50S ribosomal subunit proteins L4 (*rlpD*) and L22 (*rlpV*) may lead to macrolide resistance (4).

The purpose of the present study was to determine the *in vitro* activity of azithromycin against nontyphoidal *Salmonella* isolates collected between 2003 and 2008 from Finnish patients. Special attention was paid to isolates with reduced fluoroquinolone susceptibility or showing the Qnr phenotype. In addition, mutations in the 23S rRNA and in the L4 and L22 ribosomal proteins were investigated.

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A total of 1,237 nontyphoidal *Salmonella* isolates (638 domestic and 599 foreign) collected from Finnish patients between 2003 and 2008 were included in this study. Starting in January each year, we collected the first 100 domestic and the first 100 foreign, i.e., collected from Finnish travelers returning from abroad, *Salmonella* isolates. The strains were serotyped at the National Salmonella Reference Centre in Finland.

The MICs of antimicrobial agents for the isolates were determined by the agar dilution method according to the CLSI guidelines (8). Mueller-Hinton II agar (Becton Dickinson, Cockeysville, MD) was used as the culture medium. All 1,237 *Salmonella* isolates were tested for susceptibility to ciprofloxacin, nalidixic acid, and azithromycin (all from Sigma, Steinheim, Germany). We tested 809 selected isolates for erythromycin (Sigma) and 635 for telithromycin (Sanofi Aventis, Paris, France) susceptibility. Control strains for susceptibility testing were as described previously (14). On the basis of earlier publications (1, 16, 17), the MIC breakpoint chosen for reduced ciprofloxacin susceptibility was ≥0.125 μg/ml. For nalidixic acid, CLSI breakpoints were used (9). Based on the EUCAST recommendation for *S. enterica* serovar Typhi (www.eucast.org) and a previous publication (3), the epidemiological cutoff value chosen for azithromycin was ≥32 μg/ml. The susceptibility data were analyzed by using the WHONET 5.4 computer program.

Pyrosequencing was used to detect macrolide resistance causing point mutations in the ribosomal target sites A2058 and A2059 (*E. coli* numbering) of the 23S rRNA in 22 isolates with azithromycin MICs of ≥32 μg/ml, 44 isolates belonging to the Qnr phenotype, and 73 isolates showing erythromycin MICs of ≥32 μg/ml. Pyrosequencing was performed with previously described primers and protocols (15) using a PSQ 96MA pyrosequencer.

The 50S ribosomal proteins L4 and L22 were amplified, and mutations were screened in 24 isolates having azithromycin MICs of ≥32 μg/ml, of which 6 were of the Qnr phenotype. In addition, 13 isolates having only erythromycin MICs of ≥32

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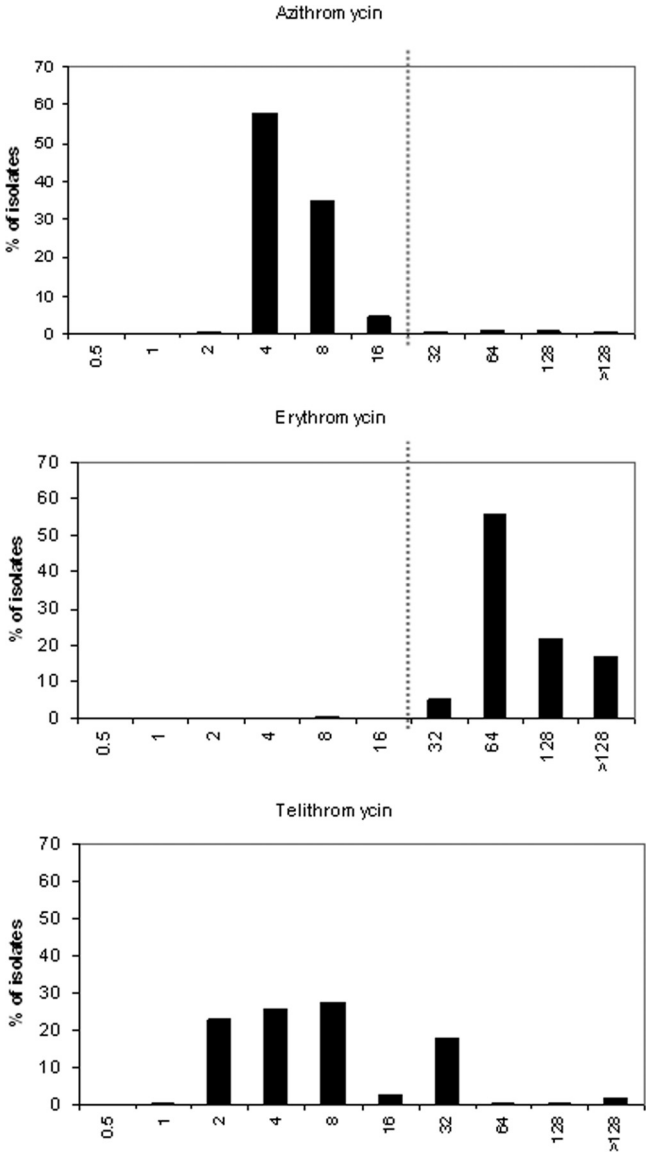

FIG. 1. Histograms of azithromycin (n = 1,237), erythromycin (n = 809), and telithromycin (n = 635) MICs for *S. enterica* isolates collected from Finnish travelers between 2003 and 2008. The vertical line represents the resistance breakpoint.

μg/ml and showing the Qnr phenotype were tested. DNA was prepared as described above. The specific primers used for amplification of the complete L4 and L22 genes *rlpD* and *rlpV* were Salm_L4_f (5′-TGAAGGCGTAAGGGGATAGCA-3′) and Salm_L4_r (5′-TCAGCAGA CGTTCTTCACGAA-3′) and Salm_L22_f (5′-GAAATAAGGTAG GAGGAAGAG-3′) and Salm_L22_r (5′-CCATTGCTAGTCTCCAGAGTC-3′). The PCR conditions were as follows: 94°C for 10 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 33 cycles. Any L4- or L22-positive results were confirmed by direct sequencing of both strands of amplicons using specific PCR primers as previously described (24). Amino acid sequences were then compared with the known *rlpD* and *rlpV* genes by a BLAST search through the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/blast/).

Two different populations of *S. enterica* were detected regarding the azithromycin MIC distribution. The majority of the isolates had azithromycin MICs of 4 to 8 μg/ml, i.e., representing the wild-type population, whereas a minority of the isolates had MICs of 32 to ≥128 μg/ml, i.e., over the epidemiological cutoff value. Between 2003 and 2008, 24 (1.9%) of the 1,237 isolates had azithromycin MICs of ≥32 μg/ml (Fig. 1; Table 1). Nine (1.4%) of the 638 domestic isolates and 15 (2.1%) of the 599 foreign isolates had azithromycin MICs over the epidemiological cutoff value.

Two hundred ninety-four (23.8%) *S. enterica* isolates showed reduced fluoroquinolone susceptibility, and 53 (18.0%) of them showed the Qnr phenotype. Among the isolates with reduced fluoroquinolone susceptibility, 4 (5.2%) domestic and 11 (5.1%) foreign isolates had azithromycin MICs of ≥32 μg/ml. Among the Qnr phenotype isolates, six (11.3%) had azithromycin MICs of ≥32 μg/ml (Table 1). Azithromycin MICs of ≥32 μg/ml were detected among 12 different serovars, *S. enterica* serovar Stanley being the most common one, and all isolates with a Qnr phenotype belonged to *S. enterica* serovar Stanley (Table 1).

Of the *S. enterica* isolates tested, 99.6% (806/809) had erythromycin MICs of ≥32 μg/ml. The erythromycin MICs varied between 8 and ≥128 μg/ml, and the vast majority of the isolates had erythromycin MICs of ≥64 μg/ml (Fig. 1).

No mutations in A2058 or A2059 of the 23S rRNA gene were detected among any of the isolates tested. Sequencing of the 50S ribosomal proteins L4 and L22 revealed G235A and C379T mutations in the *rlpD* gene and G25A in the *rlp*V gene,

TABLE 1. Twenty-four *S. enterica* isolates showing azithromycin MICs of ≥32 μg/ml

Isolate no.	Yr	Strain	Origin	*S. enterica* serovar	AZM[a] MIC (μg/ml)	*qnr* gene[b]
1	2003	s2099	Thailand	Newport	32	−
2	2003	s2018	Thailand	Stanley	64	+
3	2003	s2021	Thailand	Rissen	>128	−
4	2003	s2085	Thailand	Stanley	64	+
5	2003	s2086	Thailand	Stanley	64	+
6	2003	s2181	Finland	Poona	32	−
7	2003	s2137	Finland	Typhimurium	128	−
8	2003	s2195	Finland	Virchow	>128	−
9	2004	s2236	Thailand	Stanley	32	+
10	2004	s2265	Thailand	Stanley	128	+
11	2004	s2280	Thailand	Stanley	64	+
12	2004	s2389	Finland	Typhimurium	128	−
13	2004	s2391	Finland	Typhimurium	128	−
14	2005	s2439	Egypt	Blockley	64	−
15	2005	s2477	Egypt	Bredeney	>128	−
16	2005	s2608	Finland	Blockley	64	−
17	2006	s2635	Thailand	Emek	64	−
18	2006	s2768	Finland	Blockley	128	−
19	2006	s2829	Finland	Saintpaul	64	−
20	2007	s2868	Malaysia	Stanley	128	−
21	2007	s2948	Finland	Hvittingfoss	128	−
22	2008	s3141	Thailand	Rissen	64	−
23	2008	s3082	Thailand	Typhimurium	128	−
24	2008	s3139	Thailand	Enteritidis	128	−

[a] AZM, azithromycin.
[b] +, present; −, absent.

TABLE 2. Mutations found in the 50S ribosomal proteins L4 and L22 and corresponding MICs

S. enterica serovar	Origin	Yr	Mutation in L4 and L22		MIC[a]				
			rlpD	*rlpV*	AZM	ERY	TEL	CIP	NAL
Blockley	Egypt	2005	G235A		64	>128	>128	0.5	>512
Blockley	Finland	2005	G235A		64	>128	>128	0.25	>512
Blockley	Finland	2006	G235A		128	>128	>128	0.25	>512
Saintpaul	Finland	2006	G235A		64	>128	>128	0.03	16
Typhimurium	Thailand	2008	G235A		128	>128	>128	0.06	16
Typhimurium	Finland	2004	G235A		128	64	ND[b]	0.25	512
Montevideo	Thailand	2004	C379T	G25A	4	64	32	0.5	32
Montevideo	Thailand	2007	C379T	G25A	8	>128	32	0.5	16
Montevideo	Thailand	2008	C379T	G25A	8	>128	4	0.5	16

[a] AZM, azithromycin; ERY, erythromycin; TEL, telithromycin; CIP, ciprofloxacin; NAL, nalidixic acid.
[b] ND, not determined.

respectively. A Glu79-Lys substitution in the *rlpD* gene was found in six isolates belonging to three different serovars (Table 2). An Arg127-Trp substitution in the *rlpD* gene was found in three *S. enterica* serovar Montevideo isolates, which also had an Asp9-Asn substitution outside the coding region (Table 2).

Salmonella isolates are intrinsically resistant to erythromycin via the AcrAB efflux pump (2) but naturally susceptible to azithromycin (29). Azithromycin has shown good efficacy in the treatment of patients suffering from typhoid fever (7, 12, 13, 25, 30), and there has been speculation that azithromycin could be used for empirical therapy of traveler's diarrhea (11, 31). The present study was performed to determine the *in vitro* activity of azithromycin toward nontyphoidal *Salmonella* isolates collected from Finnish patients between 2003 and 2008. Our results show that while nearly all (99.6%) of our *S. enterica* isolates had erythromycin MICs of ≥32 μg/ml, only 24 isolates had azithromycin MICs of ≥32 μg/ml. Azithromycin showed good *in vitro* efficacy also against *S. enterica* isolates with reduced fluoroquinolone susceptibility, although 11.3% of the Qnr phenotype isolates showed azithromycin MICs of ≥32 μg/ml.

These data show that azithromycin has good *in vitro* activity against nontyphoidal *S. enterica* isolates. Although highly azithromycin-resistant isolates did occur, azithromycin was effective even against the isolates with reduced fluoroquinolone susceptibility, including those showing the Qnr phenotype. Based on these results, azithromycin is a good candidate for clinical treatment studies of salmonellosis.

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