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EPIDEMIOLOGY AND SURVEILLANCE

Azithromycin Resistance in Shiga Toxin-Producing *Escherichia coli* in France between 2004 and 2020 and Detection of *mef*(C)-*mph*(G) Genes

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ABSTRACT We described and characterized Shiga-toxin-producing *Escherichia coli* (STEC) strains with high levels of resistance to azithromycin isolated in France between 2004 and 2020. Nine of 1,715 (0.52%) STEC strains were resistant to azithromycin, with an increase since 2017. One isolate carried a plasmid-borne *mef*(C)-*mph*(G) gene combination, described here for the first time for *E. coli*. Azithromycin resistance, although rare, needs consideration, as this treatment may be useful in cases of STEC infection.

KEYWORDS EHEC, azithromycin resistance, fosfomycin, macrolide resistance, macrolides, *mef*(C)-*mph*(G), *mph*(A), *mph*(B), phosphorylases, rifaximin

Shiga-toxin-producing *Escherichia coli* (STEC) strains are major foodborne pathogens responsible for gastrointestinal diseases, ranging from diarrhea to hemolytic uremic syndrome (HUS) (1). Typical STEC isolates carry the intimin (*eae* gene) and the prophage-encoded Shiga-toxin (Stx). Although no clear risk factors for HUS development, except for young age, have been identified, higher levels of Stx production could be an important trigger of HUS occurrence (2). Unlike quinolones or beta-lactams, macrolides and especially azithromycin do not modify or increase Stx production *in vitro* (3, 4) and decrease bacterial shedding after HUS (5).

Azithromycin is one of the most prescribed antibiotics worldwide, among adults (6) and children (7), and could participate in the selection of resistant strains (8). Macrolide resistance is mainly observed in *E. coli* strains with phosphorylases [*mph*(A) to *mph*(O) genes (9, 10)], especially *mph*(A) (11). However, in STEC strains, resistance to azithromycin is poorly studied. Two studies address this subject; one, carried out in France, reported a prevalence of 0.3% (2/508 isolates in 2004 to 2014) (12), and another one, in England in 2017, reported a prevalence of 0.2% (1/430) (13).

Here, we aimed to describe and characterize STEC strains with high-level resistance to azithromycin, isolated in France between 2004 and 2020, and to assess alternative drugs in that case. To this end, we included all STEC strains collected in France between January 2004 and June 2020 by the French National Reference Center for *E. coli* (NRC). For azithromycin-resistant isolates, antimicrobial susceptibility testing was performed either by disk diffusion (rifaximin [40 μg; MAST] and spiramycin [100 μg; Bio-Rad]) or by using Etest (bioMérieux, France) (erythromycin, clindamycin, clarithromycin, fosfomycin, and rifampicin) (Table 1). We used the reference strain *E. coli* ATCC 25922 as a control. Fosfomycin MICs were interpreted using the European Committee

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The authors declare no conflict of interest.

Received 3 October 2021
Returned for modification 27 October 2021
Accepted 26 November 2021

Accepted manuscript posted online 6 December 2021
Published 15 February 2022

TABLE 1 Antibiotic susceptibility of azithromycin-resistant Shiga-toxin producing *E. coli* strains isolated between 2004 and 2020 in France

	MIC (mg/L)						Inhibition zone diam (mm) in disk diffusion	
Isolate	Azithromycin	Erythromycin	Clindamycin	Clarithromycin	Fosfomycin	Rifampicin	Rifaximin	Spiramycin
34396	>256	>256	>256	>256	0.75	16	12	6
36493	>256	>256	>256	>256	0.75	16	16	8
42514	256	>256	>256	>256	0.38	16	13	9
43037	96	>256	>256	>256	1	16	16	9
45195	256	>256	>256	>256	0.5	16	13	6
45381	48	>256	>256	>256	1	16	16	6
45466	64	>256	>256	>256	12	16	17	8
47439	48	>256	>256	>256	0.75	12	15	10
47750	48	>256	>256	>256	0.5	12	17	6
ATCC 25922	4	32	>256	48	1,5	12	12	10

on Antimicrobial Susceptibility Testing (EUCAST; https://mic.eucast.org/) breakpoints (14). The breakpoint of >16 mg/L has been proposed to define resistance to azithromycin in *Salmonella* and *Shigella*, and therefore, it is also often used for intestinal pathogenic *E. coli* (11). No resistance breakpoints are established for the other molecules (rifaximin, erythromycin, clindamycin, clarithromycin, spiramycin, and rifampicin) in *E. coli*.

Azithromycin-resistant isolates were subjected to whole-genome sequencing (WGS) using Illumina, and sequences were analyzed as previously described (3, 15).

The genetic support of *mef*(C)-*mph*(G) genes was studied in *E. coli* isolate 45466 using long-read sequencing with Nanopore (Oxford, UK). It was performed with the rapid sequencing kit SQK-RAD and a MinION device with a R9.4 flow cell. We used Unicycler software (v0.4.0) to carry out hybrid assembly of the Illumina and Nanopore sequences (16). Sequences were analyzed with PlasmidFinder (17) and annotated with RAST software (18). Finally, we searched both for homologous plasmids from *Enterobacterales* and for *mef*(C)-*mph*(G) genes with 100% coverage and homology in all *E. coli/Shigella* sequences in the NCBI database.

Between 2004 and 2020, 1,715 STEC strains were isolated by the NRC. Azithromycin MIC_{50} and MIC_{90} were 4 mg/L and 6 mg/L, respectively (range, 0.5 to >256 mg/L; median, 4 mg/L). Nine isolates were resistant to azithromycin (MIC range, 48 to >256 mg/L; median, 64 mg/L), resulting in 0.52% resistance. Of note, only 2/807 (0.25%) azithromycin-resistant isolates were found before 2017 (in 2012 and in 2013 [12]), and the other 7 resistant isolates (7/908; 0.77%) were collected between January 2017 and June 2020 (Tables 1 and 2).

For azithromycin-resistant isolates, MICs of rifampicin and fosfomycin ranged from 12 to 16 mg/L (median, 16 mg/L) and from 0.5 to 12 mg/L (median, 1.25 mg/L), respectively. MICs of other macrolides (erythromycin, clindamycin, and clarithromycin) were >256 mg/L, and spiramycin zone diameter inhibition was 6 to 10 mm (median, 8 mm). Zone diameter inhibition of rifaximin was between 12 and 17 mm (median, 16 mm). Of note, 45466 had the highest fosfomycin MIC (12 mg/L; however, this MIC remains susceptible according to EUCAST criteria) (Table 1).

Resistance to azithromycin was mostly mediated by *mph*(A) ($n = 7$), either alone ($n = 4$) or associated with *mph*(B) ($n = 1$) or *erm*(B) ($n = 2$). One isolate carried the association *mph*(B)/*erm*(B), and one carried the rare couple *mef*(C)/*mph*(G) (strain 45466) (Table 2).

For the latter, long-read sequencing allowed us to identify a 202,201-bp plasmid (p45466-R) harboring the *mef*(C)-*mph*(G) genes with IncHI1A, IncHI1B(R27), and IncFIA(HI1) incompatibility groups (17). These genes are localized in a cassette carrying several other resistance genes conferring resistance to penicillins (bla_{TEM}), sulfamethoxazole/trimethoprim (*dhfr1* and *sul2*), or aminoglycosides [*aph(3)*-Ib and *aph(6)*-I]. The *mef*(C)-*mph*(G) genes are organized in tandem-pair arrangement only 5 nucleotides apart (Fig. 1 and Table 2) and are surrounded by IS*6*-like element IS*26* family transposases and an IS*91* family transposase.

TABLE 2 Whole-genome sequencing results of azithromycin-resistant Shiga toxin-producing *E. coli* strains isolated between 2004 and 2020 in France

Isolate	Collection yr	Patient age (yr)	Clinical disease[a]	Quality sequencing data			Serotype	Sequence type	Virulence factor(s)	Macrolide resistance gene(s)
				N_{50} (bp)	Contig no.	Mean genome size (bp)				
34396	2012	62	HUS	278,669	279	5,300,106	O17/O44:H18	69	Stx2d	*mph*(A), *erm*(B)
36493	2013	<1	HUS	94,290	408	5,562,733	O26:H11	29	Stx2d, Eaeβ1	*mph*(A)
42514	2017	28	BD	29,905	469	5,171,176	O117:H7	6880	Stx1a	*mph*(A)
43037	2017	5	HUS	48,855	301	5,125,621	O146:H8	8356	Stx1c	*mph*(A)
45195	2018	24	BD	39,047	332	5,095,835	ND:H7	5292	Stx1a	*mph*(A), *erm*(B)
45381	2019	29	BD	52,202	318	5,272,679	O146:H8	8356	Stx1c	*mph*(A), *mph*(B)
45466	2019	1	BD	64,220	472	5,942,917	O26:H11	21	Stx1a, Eaeβ1, EhxA	*mph*(G), *mef*(C)
47439	2020	1	HUS	79,390	382	5,411,518	O111:H8	16	Stx1a, Stx2a, Eaeγ2, EhxA	*mph*(A)
47750	2020	44	BD	70,538	333	5,642,051	O157:H7	11	Stx1a, Stx2c, Eaeγ1, EhxA	*mph*(B), *erm*(B)

[a]HUS, hemolytic uremic syndrome; BD, bloody diarrhea.

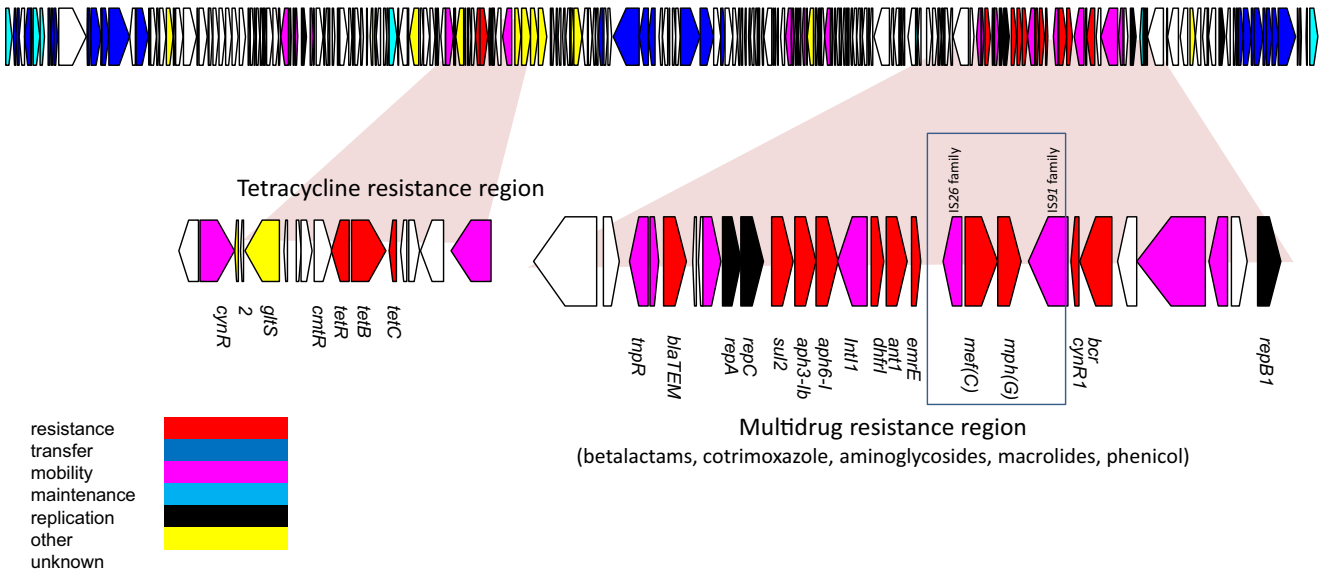

FIG 1 Annotation of the 202-kb plasmid p45466 from *E. coli* strain 45466, harboring the *mef*(C)-*mph*(G) gene pair involved in macrolide resistance.

After subjecting this plasmid to a BLAST search in the NCBI database, we found that it was close to several plasmids described in *Enterobacterales*, with the closest being p14ODMR, described in *E. coli* 14OD0056 with a 99.98% identity and a coverage of 94% (accession number MG904992.1). Interestingly, none of these plasmids carry the *mef*(C)-*mph*(G) genes.

Finally, we identified six isolates carrying the combination *mef*(C)-*mph*(G) with 100% identity and coverage that were *E. coli*/*Shigella* in the NCBI database. Of note, 2 of these also carried *stx* genes (Table 3).

While exploring antibiotic resistance among STEC strains in France, we observed low but increasing rates of high-level resistance to azithromycin. Isolates identified in our study were from various serotypes and sequence types, showing that the diffusion is not due to the emergence of a particular clone.

Interestingly, we identified an azithromycin-resistant *E. coli* strain (strain 45466) carrying the gene combination *mef*(C)-*mph*(G), encoding the Mph(G) phosphotransferase associated with the Mef(C) efflux pump. To our knowledge, this is the first description of an azithromycin-resistant *E. coli* strain carrying this combination. The *mef*(C)-*mph*(G) gene association was first described in *Photobacterium damselae*, an indigenous marine bacterium known as a zoonotic pathogen (19, 20). The combination of both genes is synergic and is associated with a high azithromycin MIC (19).

The *mef*(C)-*mph*(G) genes are located on a plasmid of 202 kb whose main structure was found in several other *Enterobacterales*. The presence of the *mef*(C)-*mph*(G) genes in the whole-genome sequences of six *E. coli*/*Shigella* in the NCBI database supports the idea that the diffusion of these genes is not an isolated event. However, as there are only genomic data, it is not possible to determine the azithromycin MICs for these isolates and thus be sure of the expression of these genes.

TABLE 3 Whole-genome sequence data (from public databases) of *E.coli*/*Shigella* isolates carrying *mef*(C)-*mph*(G) genes

Strain	Biosample ID	Country	Source	Origin	Serotype	Sequence type	Virulence factor(s)
IHIT32077	SAMN14279035	Spain		Environmental	O128:H26	2197	
978891	SAMN15933666	United Kingdom	Human	Clinical	O166:H28	1819	Stx1c, EhxA
As Lw Down3-2	SAMN11124839	Germany	River sediment	Environmental	O93:H28	4038	
B P Zu-1	SAMN11125139	Germany	Raw sewage	Environmental	O8:H19	201	Stx2e
S18-17	SAMN11125178	Germany	River sediment	Environmental	O149:H1	5748	
Win2012_WWKa_NEU_19	SAMN06641869	Germany	Wastewater inflow	Environmental	O143:H4	117	

The triggering role of antibiotics in HUS is still debated (21). Some bactericidal antibiotics (co-trimoxazole and fluoroquinolones, for example) increase Stx production *in vitro* and therefore could increase the risk of progression to HUS (22, 23). Inversely, azithromycin is associated with a decrease in the STEC inoculum and in Stx release and therefore probably decreases the risk of developing HUS (3, 22). The use of azithromycin is currently being assessed in a clinical trial concerning patients with HUS (NCT02336516). Therapeutic alternatives to azithromycin were proposed in some countries, such as rifamycins (especially rifaximin) in the United States and salts of fosfomycin in Japan (24). Both are associated with a favorable clinical outcome when administered early after the first symptoms of bloody diarrhea (24–26). Although no critical inhibition zone diameters exist for these two antibiotics, all isolates had inhibition zone diameters or MICs similar to those of wild-type *E. coli* ATCC 25922, suggesting susceptibility, except for the 45466 isolates, which had a higher fosfomycin MIC than other isolates.

Together, these results show that resistance to azithromycin in STEC strains remains rare but tends to increase in France; hence the need to test sensitivity before therapeutic use. Here, we describe a new combination of genes, *mef*(C)-*mph*(G), in *E. coli* that was initially described in a marine bacterium. *In vitro* data suggest that rifaximin or fosfomycin could be interesting therapeutic alternatives in STEC infections with azithromycin-resistant *E. coli*.

Data availability. Raw reads have been deposited in GenBank under BioProject ID PRJNA735027.

ACKNOWLEDGMENTS

This study was supported by internal funding.

We have no conflicts of interest to declare.

REFERENCES

1. Williams DM, Sreedhar SS, Mickell JJ, Chan JCM. 2002. Acute kidney failure: a pediatric experience over 20 years. Arch Pediatr Adolesc Med 156:893–900. https://doi.org/10.1001/archpedi.156.9.893.
2. Joseph A, Cointe A, Mariani Kurkdjian P, Rafat C, Hertig A. 2020. Shiga toxin-associated hemolytic uremic syndrome: a narrative review. Toxins 12:67. https://doi.org/10.3390/toxins12020067.
3. Cointe A, Birgy A, Bridier-Nahmias A, Mariani-Kurkdjian P, Walewski V, Lévy C, Cohen R, Fach P, Delannoy S, Bidet P, Bonacorsi S. 2020. *Escherichia coli* O80 hybrid pathotype strains producing Shiga toxin and ESBL: molecular characterization and potential therapeutic options. J Antimicrob Chemother 75:537–542. https://doi.org/10.1093/jac/dkz484.
4. Ohara T, Kojio S, Taneike I, Nakagawa S, Gondaira F, Tamura Y, Gejyo F, Zhang H-M, Yamamoto T. 2002. Effects of azithromycin on shiga toxin production by *Escherichia coli* and subsequent host inflammatory response. Antimicrob Agents Chemother 46:3478–3483. https://doi.org/10.1128/AAC.46.11.3478-3483.2002.
5. Nitschke M, Sayk F, Härtel C, Roseland RT, Hauswaldt S, Steinhoff J, Fellermann K, Derad I, Wellhöner P, Büning J, Tiemer B, Katalinic A, Rupp J, Lehnert H, Solbach W, Knobloch JK-M. 2012. Association between azithromycin therapy and duration of bacterial shedding among patients with Shiga toxin-producing enteroaggregative *Escherichia coli* O104:H4. JAMA 307:1046–1052. https://doi.org/10.1001/jama.2012.264.
6. Hicks LA, Taylor TH, Hunkler RJ. 2013. U.S. outpatient antibiotic prescribing, 2010. N Engl J Med 368:1461–1462. https://doi.org/10.1056/NEJMc1212055.
7. Fleming-Dutra KE, Demirjian A, Bartoces M, Roberts RM, Taylor TH, Hicks LA. 2018. Variations in antibiotic and azithromycin prescribing for children by geography and specialty—United States, 2013. Pediatr Infect Dis J 37:52–58. https://doi.org/10.1097/INF.0000000000001708.
8. Seidman JC, Coles CL, Silbergeld EK, Levens J, Mkocha H, Johnson LB, Muñoz B, West SK. 2014. Increased carriage of macrolide-resistant fecal *E. coli* following mass distribution of azithromycin for trachoma control. Int J Epidemiol 43:1105–1113. https://doi.org/10.1093/ije/dyu062.
9. Gomes C, Martínez-Puchol S, Palma N, Horna G, Ruiz-Roldán L, Pons MJ, Ruiz J. 2017. Macrolide resistance mechanisms in Enterobacteriaceae: focus on azithromycin. Crit Rev Microbiol 43:1–30. https://doi.org/10.3109/1040841X.2015.1136261.
10. Pawlowski AC, Stogios PJ, Koteva K, Skarina T, Evdokimova E, Savchenko A, Wright GD. 2018. The evolution of substrate discrimination in macrolide antibiotic resistance enzymes. Nat Commun 9:112. https://doi.org/10.1038/s41467-017-02680-0.
11. Gomes C, Ruiz-Roldán L, Mateu J, Ochoa TJ, Ruiz J. 2019. Azithromycin resistance levels and mechanisms in *Escherichia coli*. Sci Rep 9:6089. https://doi.org/10.1038/s41598-019-42423-3.
12. Jost C, Bidet P, Carrère T, Mariani-Kurkdjian P, Bonacorsi S. 2016. Susceptibility of enterohaemorrhagic *Escherichia coli* to azithromycin in France and analysis of resistance mechanisms. J Antimicrob Chemother 71:1183–1187. https://doi.org/10.1093/jac/dkv477.
13. Day M, Doumith M, Jenkins C, Dallman TJ, Hopkins KL, Elson R, Godbole G, Woodford N. 2017. Antimicrobial resistance in Shiga toxin-producing *Escherichia coli* serogroups O157 and O26 isolated from human cases of diarrhoeal disease in England, 2015. J Antimicrob Chemother 72:145–152. https://doi.org/10.1093/jac/dkw371.
14. Leclercq R, Cantón R, Brown DFJ, Giske CG, Heisig P, MacGowan AP, Mouton JW, Nordmann P, Rodloff AC, Rossolini GM, Soussy C-J, Steinbakk M, Winstanley TG, Kahlmeter G. 2013. EUCAST expert rules in antimicrobial susceptibility testing. Clin Microbiol Infect 19:141–160. https://doi.org/10.1111/j.1469-0691.2011.03703.x.
15. Jones G, Pardos de la Gandara M, Herrera-Leon L, Herrera-Leon S, Varela Martinez C, Hureaux-Roy R, Abdallah Y, Nisavanh A, Fabre L, Renaudat C, Mossong J, Mattheus W, Huard C, Le Borgne C, de Valk H, Weill F-X, Jourdan-Da Silva N. 2019. Outbreak of *Salmonella enterica* serotype Poona in infants linked to persistent Salmonella contamination in an infant formula manufacturing facility, France, August 2018 to February. Euro Surveill 24:2019. https://doi.org/10.2807/1560-7917.ES.2019.24.13.1900161.
16. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595.
17. Carattoli A, Hasman H. 2020. PlasmidFinder and in silico pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). Methods Mol Biol 2075:285–294. https://doi.org/10.1007/978-1-4939-9877-7_20.

18. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9:75. https://doi.org/10.1186/1471-2164-9-75.
19. Nonaka L, Maruyama F, Miyamoto M, Miyakoshi M, Kurokawa K, Masuda M. 2012. Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobacterium damselae* subsp. damselae isolated from marine aquaculture environment. Microbes Environ 27:263–272. https://doi.org/10.1264/jsme2.me11338.
20. Sugimoto Y, Suzuki S, Nonaka L, Boonla C, Sukpanyatham N, Chou H-Y, Wu J-H. 2017. The novel mef(C)–mph(G) macrolide resistance genes are conveyed in the environment on various vectors. J Glob Antimicrob Resist 10:47–53. https://doi.org/10.1016/j.jgar.2017.03.015.
21. Safdar N, Said A, Gangnon RE, Maki DG. 2002. Risk of hemolytic uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 enteritis: a meta-analysis. JAMA 288:996–1001. https://doi.org/10.1001/jama.288.8.996.
22. Bielaszewska M, Idelevich EA, Zhang W, Bauwens A, Schaumburg F, Mellmann A, Peters G, Karch H. 2012. Effects of antibiotics on Shiga toxin 2 production and bacteriophage induction by epidemic *Escherichia coli* O104:H4 strain. Antimicrob Agents Chemother 56:3277–3282. https://doi.org/10.1128/AAC.06315-11.
23. Nassar FJ, Rahal EA, Sabra A, Matar GM. 2013. Effects of subinhibitory concentrations of antimicrobial agents on *Escherichia coli* O157:H7 Shiga toxin release and role of the SOS response. Foodborne Pathog Dis 10:805–812. https://doi.org/10.1089/fpd.2013.1510.
24. Kakoullis L, Papachristodoulou E, Chra P, Panos G. 2019. Shiga toxin-induced haemolytic uraemic syndrome and the role of antibiotics: a global overview. J Infect 79:75–94. https://doi.org/10.1016/j.jinf.2019.05.018.
25. Ochoa TJ, Chen J, Walker CM, Gonzales E, Cleary TG. 2007. Rifaximin does not induce toxin production or phage-mediated lysis of Shiga toxin-producing *Escherichia coli*. Antimicrob Agents Chemother 51:2837–2841. https://doi.org/10.1128/AAC.01397-06.
26. Jiang ZD, Ke S, Palazzini E, Riopel L, Dupont H. 2000. In vitro activity and fecal concentration of rifaximin after oral administration. Antimicrob Agents Chemother 44:2205–2206. https://doi.org/10.1128/AAC.44.8.2205-2206.2000.