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CLINICAL THERAPEUTICS

Antibiotic-Mediated Modulations of Outer Membrane Vesicles in Enterohemorrhagic *Escherichia coli* O104:H4 and O157:H7

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ABSTRACT Ciprofloxacin, meropenem, fosfomycin, and polymyxin B strongly increase production of outer membrane vesicles (OMVs) in *Escherichia coli* O104:H4 and O157:H7. Ciprofloxacin also upregulates OMV-associated Shiga toxin 2a, the major virulence factor of these pathogens, whereas the other antibiotics increase OMV production without the toxin. These two effects might worsen the clinical outcome of infections caused by Shiga toxin-producing *E. coli*. Our data support the existing recommendations to avoid antibiotics for treatment of these infections.

KEYWORDS antibiotics, enterohemorrhagic *E. coli*, hemolytic uremic syndrome, outer membrane vesicles, Shiga toxins

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes most EHEC infections worldwide (1), but the most severe EHEC outbreak, with a high progression rate of infection to life-threatening hemolytic uremic syndrome (HUS), was caused in 2011 by *E. coli* O104:H4 (2), which is a hybrid of EHEC and enteroaggregative *E. coli* (3, 4). Both of these pathogens secrete subsets of their virulence factors, including Shiga toxin (Stx) 2a, the major EHEC virulence molecule, via outer membrane vesicles (OMVs) released during growth (5, 6). Since certain antibiotics increase Stx production (7–9), which may increase the risk of HUS development in patients treated with antibiotics during the initial phase of diarrhea (10–13), antibiotics are not recommended for treatment of EHEC infections (10–15). However, therapeutic or prophylactic administration of antibiotics was necessary in some patients during the *E. coli* O104:H4 outbreak (16–18). In our previous study, we identified several antibiotics potentially useful for treatment of *E. coli* O104:H4-infected patients based on their failure to induce stx_{2a}-harboring bacteriophages and increase total Stx2a production in the outbreak strain (16). However, it is unknown if these antibiotics affect production of OMVs and OMV-associated Stx2a. Here, we determined effects of several antibiotics, including those used during the 2011 outbreak, on OMV production, the amount of OMV-associated Stx2a, and degree of OMV cytotoxicity in EHEC O104:H4 and EHEC O157:H7 (the most common EHEC serotype associated with severe disease) (1, 14).

MICs of the antibiotics used (Table 1) for EHEC O104:H4 outbreak isolate LB226692 (3, 4) and EHEC O157:H7 strain 5791/99 (6) were determined according to the guidelines of the Clinical and Laboratory Standards Institute (19), as described previously (16). To test effects of the antibiotics on the production of OMVs, the amount of OMV-associated Stx2a, and degree of OMV cytotoxicity, the strains were grown until an optical density at 600 nm (OD_{600}) of 0.5 was reached. Aliquots of 150 ml were then supplemented with 1/4 MIC of each antibiotic or with 0.5 μg/ml of mitomycin C (positive control) (20, 21) or left untreated (negative control) and then cultured for 16 h. OMVs were isolated by ultracentrifugation of sterile-filtered supernatants (5, 6) and resuspended in 1 ml of 20 mM Tris-HCl (pH 8.0); numbers of CFU/ml were determined

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TABLE 1 MICs of the antibiotics used for EHEC O104:H4 and O157:H7

Antibiotic	MICs (μg/ml) for:	
	LB226692 (O104:H4)	5791/99 (O157:H7)
Ciprofloxacin[a]	0.25	0.125
Fosfomycin[a]	1.0	2.0
Meropenem[a]	0.016	0.016
Gentamicin[b]	0.125	0.125
Rifaximin[c]	128.0	64.0
Tigecycline[d]	0.25	0.125
Azithromycin[d]	8.0	4.0
Chloramphenicol[b]	8.0	8.0
Polymyxin B[a]	0.5	0.5

[a]From Sigma-Aldrich.
[b]From Appli-Chem.
[c]From Alfa Wassermann.
[d]From Pfizer.

by a standard plate dilution method. OMVs and OMV-associated Stx2a were quantified by immunoblot of OMV preparations (10 μl/lane) with antibodies against the outer membrane protein A (OmpA) (an OMV marker) and Stx2a, respectively (6), and the densitometric signals were normalized to CFU/ml. OMV cytotoxicity titers were determined by Vero cell assay (5). The amounts of OMVs and OMV-associated Stx2a and degree of OMV cytotoxicity produced in the presence of each antibiotic was expressed as a fold increase of that produced by untreated bacteria. Total OMV protein concentrations were determined by Roti-Nanoquant (Carl Roth) and normalized to CFU/ml. The total OMV protein content in the presence of each antibiotic was expressed as a fold increase of that in the absence of antibiotics.

The antibiotics differentially modulated OMV and OMV-Stx2a production and OMV cytotoxicity in strains LB226692 and 5791/99 (Fig. 1). Ciprofloxacin strongly increased production of OMVs (250-fold and 183-fold) and OMV-associated Stx2a (143-fold and 123-fold) and increased OMV cytotoxicity (1024-fold and 512-fold) in the respective strains. Similar effects were elicited by mitomycin C, which increased OMV production (568-fold and 470-fold, respectively), OMV-associated Stx2a (332-fold and 275-fold, respectively), and OMV cytotoxicity (1,024-fold). Fosfomycin also increased OMV production in each strain (77-fold and 24-fold, respectively), as did meropenem (27-fold and 14-fold, respectively) and polymyxin B (9-fold and 7-fold, respectively). However, none of the last three antibiotics affected the amount of OMV-associated Stx2a and OMV cytotoxicity in any of the strains. Gentamicin, rifaximin, tigecycline, and azithromycin did not influence OMV production in any strain but differentially modulated OMV-associated Stx2a and, thus, OMV cytotoxicity. Specifically, in strain LB226692, rifaximin, tigecycline, and azithromycin significantly reduced OMV-associated Stx2a and OMV cytotoxicity, whereas these antibiotics had no effects on these characteristics in strain 5791/99. No changes in OMV production, but significant decreases of OMV-associated Stx2a and OMV cytotoxicity, were elicited in both strains by chloramphenicol (Fig. 1). The upregulations of OMV production by ciprofloxacin, fosfomycin, meropenem, polymyxin B, and mitomycin C determined by immunoblot with anti-OmpA antibody (Fig. 1) were confirmed in each strain by quantifying the total OMV protein contents (Table 2).

We demonstrate for the first time that particular antibiotics significantly upregulate OMV production in EHEC O104:H4 and O157:H7. These effects on vesiculation occur together with or without modulation of OMV-associated Stx2a. This plausibly reflects different mechanisms involved in OMV and OMV-Stx2a production and different abilities of the particular antibiotics to interfere with these mechanisms. Specifically, the upregulation of EHEC vesiculation by the SOS response inducers ciprofloxacin and mitomycin C (21, 22) is consistent with the involvement of the SOS response in ciprofloxacin-mediated increase of OMV production in *Pseudomonas aeruginosa* (22) and in mitomycin C-mediated increase of vesiculation in *Shigella dysenteriae* type 1 (20).

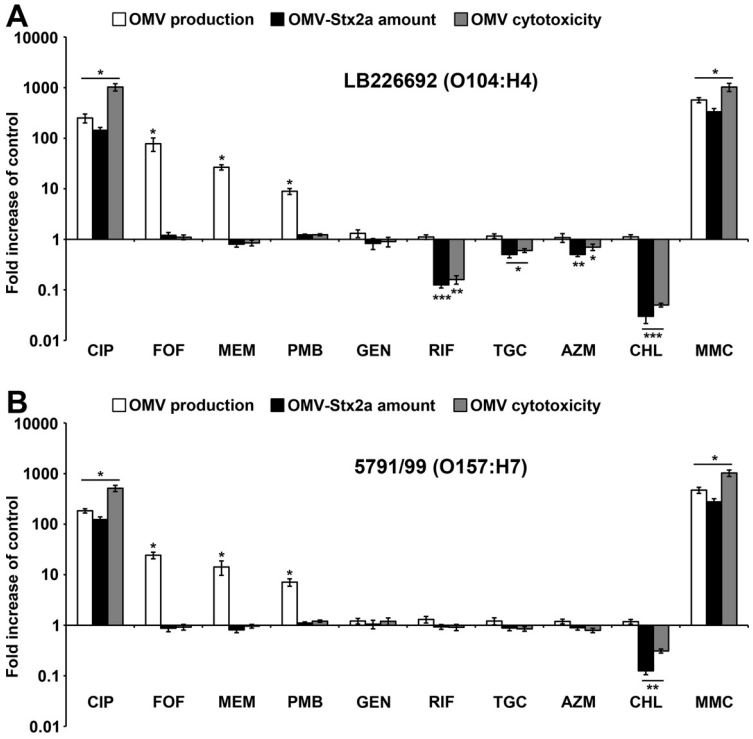

FIG 1 Effects of antibiotics on the production of OMVs, OMV-associated Stx2a, and OMV cytotoxicity in EHEC O104:H4 and O157:H7. Strains LB226692 (O104:H4) (A) and 5791/99 (O157:H7) (B) were grown for 16 h in the presence of 1/4 MIC of ciprofloxacin (CIP), fosfomycin (FOF), meropenem (MEM), polymyxin B (PMB), gentamicin (GEN), rifaximin (RIF), tigecycline (TGC), azithromycin (AZM), or chloramphenicol (CHL), 0.5 μg/ml of mitomycin C (MMC) (positive control), or without antibiotics (negative control). OMVs and OMV-associated Stx2a were quantified by immunoblot with anti-OmpA and anti-Stx2a antibodies, respectively, and normalized to CFU/ml. OMV cytotoxicity was determined by Vero cell assay. The data show a fold increase in each respective characteristic in the presence of the indicated antibiotic compared to untreated culture (set up as 1.0), and are expressed as means ± standard deviations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (one-sample *t* test with *P* value adjustments for multiple comparisons by Benjamini–Hochberg method).

Since the SOS responses triggered by ciprofloxacin and mitomycin C also lead to induction of stx_{2a}-harboring bacteriophages (9, 16, 21), the OMV upregulation is accompanied by upregulation of OMV-associated Stx2a and, thus, OMV cytotoxicity after exposure to these agents. In contrast, fosfomycin, meropenem, and polymyxin B

TABLE 2 Modulation of OMV production in EHEC O104:H4 and O157:H7 by antibiotics and mitomycin C determined by quantification of total OMV protein contents

	LB226692 (O104:H4)		5791/99 (O157:H7)	
Antibiotic	**Fold increase[a]**	***P*[b]**	**Fold increase[a]**	***P*[b]**
None	1.0 ± 0.13	1.00	1.0 ± 0.16	1.00
Ciprofloxacin	298.22 ± 51.18	<0.05	217.28 ± 42.53	<0.05
Fosfomycin	83.13 ± 21.97	<0.05	31.13 ± 6.04	<0.05
Meropenem	32.25 ± 5.43	<0.05	19.32 ± 5.12	<0.05
Polymyxin B	12.17 ± 3.65	<0.05	9.03 ± 1.88	<0.05
Gentamicin	1.25 ± 0.31	0.40	1.24 ± 0.36	0.59
Rifaximin	1.16 ± 0.27	0.46	1.29 ± 0.58	0.60
Tigecycline	1.24 ± 0.32	0.40	1.23 ± 0.39	0.59
Azithromycin	1.23 ± 0.26	0.40	1.19 ± 0.52	0.63
Chloramphenicol	1.03 ± 0.12	0.71	1.12 ± 0.38	0.64
Mitomycin C	616.96 ± 86.27	<0.05	536.89 ± 79.99	<0.05

[a]Fold increase of total OMV protein concentration in the presence of the indicated antibiotic compared to that in the absence of antibiotic. Means of data from three experiments ± standard deviations are shown.
[b]*P* values for the differences between the total OMV protein concentrations in the presence of the indicated antibiotics compared to that in the absence of antibiotics (one-sample *t* test with *P* value adjustments for multiple comparisons by the Benjamini–Hochberg method); a *P* value of <0.05 was considered significant.

plausibly increase OMV production by acting as envelope stressors (9, 23, 24). The increase of EHEC OMV production by these antibiotics is in agreement with similar effects reported for meropenem in *P. aeruginosa* (25), for imipenem (another carbapenem) in *Stenotrophomonas maltophilia* (24), and for polymyxin B in non-EHEC *E. coli* (23) and *P. aeruginosa* (26). However, the failures of fosfomycin, meropenem, and polymyxin B to induce stx_{2a}-harboring phages and increase Stx production (8, 9, 16) result in an exclusive increase of OMVs without OMV-associated Stx2a. Altogether, our observations of OMV upregulation by antibiotics which induce the SOS response or act as envelope stressors are in agreement with the identification of OMV production as a novel type of bacterial stress response (22, 23, 26, 27). In general, our data support the recommendations to avoid antibiotics for treatment of EHEC infections (10–15). Specifically, the observation that ciprofloxacin strongly upregulates the OMV-associated Stx2a, which is toxic to human glomerular and brain microvascular endothelial cells (6), the major targets during HUS (14, 28), confirms that administration of ciprofloxacin may enhance the risk of HUS development. Furthermore, EHEC O104:H4 OMVs induce, via their non-Stx2a components (lipopolysaccharide and flagellin), secretion of interleukin 8 (5), one of the proinflammatory cytokines involved in the pathogenesis of HUS (14, 28). Thus, even increased OMV production itself without Stx2a upregulation, as observed for fosfomycin, meropenem, and polymyxin B, might worsen the clinical outcome of an EHEC infection. Although administration of fosfomycin was not associated with an increased risk of HUS development (29), the upregulation of OMV production by this antibiotic shown for the first time in our study warrants caution in its use for treatment of EHEC-infected patients. In contrast to the other antibiotics, inhibitors of protein synthesis did not upregulate OMVs or OMV-associated Stx2a in EHEC O104:H4 and O157:H7. Thus, these agents, particularly rifaximin and azithromycin, which were used during the 2011 EHEC O104:H4 outbreak (17, 18), might be considered options if antibiotic therapy of EHEC-infected patients is inevitable.

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M.B., A.B., and A.M. designed the study; A.B., L.K., and M.B. performed experiments; M.B and A.B analyzed data; H.K. and A.M. supervised the study and raised funding; M.B. wrote the manuscript; and all authors edited and approved the final version.

The authors declare no conflict of interest.

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