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Subtype-Specific Suppression of Shiga Toxin 2 Released from *Escherichia coli* upon Exposure to Protein Synthesis Inhibitors▽

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Shiga toxins (Stx) are important virulence factors in the pathogenesis of severe disease including hemolytic-uremic syndrome, caused by Stx-producing *Escherichia coli* (STEC). STEC strains increase the release of Stx in vitro following the addition of fluoroquinolones, whereas protein synthesis inhibitors previously have been reported to suppress the release of Stx. The amount of Stx released from wild-type STEC strains incubated with protein synthesis inhibitors was examined by a Vero cell cytotoxicity assay. The amounts released were compared to the Stx type (Stx1 or Stx2) and additionally to the individual subtypes and toxin variants of Stx2. In general, Stx2 release was suppressed significantly upon exposure to protein synthesis inhibitors at MICs, which was not observed in the case of Stx1. Also, the average amount of different Stx2 toxin variants released was suppressed to various levels ranging from 14.0% (Stx2-O157-EDL933) to 94.7% (Stx2d-O8-C466-01B). Clinical studies exploring protein synthesis inhibitors as future candidates for treatment of intestinal infections caused by Stx2-producing STEC should therefore include knowledge of the toxin variant in addition to the subtype.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains cause a broad spectrum of disease ranging from watery or bloody diarrhea to hemolytic-uremic syndrome (HUS), which can be fatal (7, 9). The key virulence trait of STEC is Stx, which can cause microangiopathic alterations of renal endothelial cells that are characteristic of HUS (2, 16).

Two major toxin types, Stx1 and Stx2, are produced by STEC, but Stx2 is more frequently associated with bloody diarrhea and HUS than Stx1 is (3, 4). Stx2 is divided into various subtypes, such as Stx2, Stx2b (previous designation Stx2-O111-PH [15]), Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g, and toxin variants on the basis of phylogenetic sequence (14). Subtypes Stx2 and Stx2c are frequently associated with HUS compared to other subtypes (6, 14). Risk factors for HUS are stx_2, *eae* (intimin-encoding gene), bloody diarrhea, and being a patient younger than 8 years of age, whereas O-serogroup O157 is not independently associated with progression to HUS (5).

Stx is encoded by genes on lysogenic prophages, which can be induced by external factors, such as UV light or antibiotic exposure whereby the production of Stx increases (1, 9, 22). Fluoroquinolones are known to induce the bacteriophage (10, 21, 22), whereas protein synthesis inhibitors have been reported to suppress the release of Stx from STEC (12, 20, 21).

The aim of this study was to investigate the release of Stx from STEC incubated with protein synthesis inhibitors, examining differences among Stx1 and Stx2, including subtypes and toxin variants of Stx2. Another aim of this study was to clarify upon exposure to protein synthesis inhibitors whether toxin release in strains of serogroup O157 is different from toxin release in other O-serogroup strains producing the same toxin variant.

MATERIALS AND METHODS

Bacterial isolates. The STEC strains investigated were isolated from human fecal specimens received at The National Reference Laboratory for Enteropathogenic Bacteria, Division of Microbiology and Diagnostics, Statens Serum Institut, Copenhagen, Denmark.

The following four strains producing Stx1 were tested (serotype shown in parentheses): C126-02 (O157:H$^-$), C1111-02 (O157:H$^-$), C1096-02 (O156:H25), and C118-05 (O146:H21).

The following 25 strains producing Stx2 were applied (serotype and subtype/toxin variant in parentheses): C832-02 (O26:H11; Stx2-O157-EDL933), C218-03 (O121:H19; Stx2-O157-EDL933), C388-02 (O157:H7; Stx2-O157-EDL933), C528-03 (O157:H7; Stx2-O157-EDL933), C532-03 (O157:H7; Stx2-O157-EDL933), C699-03 (O101:H$^-$; Stx2-O157-EDL933), C597-03 (O145:H$^-$; Stx2-O48-94C), C269-03 (O26:H$^-$; Stx2-O48-94C), C349-03 (O145:H$^-$; Stx2-O48-94C), C770-02 (O157:H$^-$; Stx2-O48-94C), C546-03 (O146:H28; Stx2b-O111-PH), C305-02B (O146:H28; Stx2b-O111-PH), C61-03 (O75:H$^-$; Stx2b-O111-PH), C1354-02 (O88:H8; Stx2b-O111-PH), C1112-02A (O145:H$^-$; Stx2c-O157-FLY16), C1211-02 (O157:H$^-$; Stx2c-O157-FLY16), C1386-02 (O145:H$^-$; Stx2c-O157-FLY16), C396-03 (O157:H7; Stx2c-O157-FLY16), C618-03 (O157:H7; Stx2c-O157-FLY16), C306-02 (O145:H$^-$; Stx2c-O157-FLY16), C86-97 (O113:K$^-$:H4; Stx2d3-O157-7278), C572-03 (O113:H4; Stx2d3-O157-7278), C466-01B (O8:H19; Stx2d-O8-C466-01B), C472-01 (O51:H49; Stx2d-O8-C466-01B), and C165-02 (O73:H16; Stx2d-O73-C165-02). Stx2 toxin was divided into various subtypes and toxin variants on the basis of partial sequencing of the most variable part of the $stxAB_2$ operon as described by Persson et al. (14).

The control strains, enteropathogenic *Escherichia coli* serotype O157:H16 strain C135-03 and nonpathogenic *Escherichia coli* K-12 strain D2103, which do not have the ability to produce Stx, were used to verify that no bacterial factors other than Stx caused Vero cell cytotoxicity.

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Cytotoxicity assay. Cytotoxicity (as a percentage) was measured using CytoTox 96 nonradioactive cytotoxicity assay (Promega). Standardized suspensions (10^7 CFU/ml) of STEC strains in Evan's medium (SSI Diagnostica, Hillerød, Denmark) were incubated for 24 h at 37°C with or without antimicrobial drugs. The bacteria were removed by centrifugation (18,000 × *g*, 15 s) and membrane filtration (0.22 μm) of the supernatants. The filtrates were added in triplicate to a subconfluent monolayer of Vero cells (CCL-81; American Type Culture Collection) prepared in 96-well plates (7.5×10^3 cells per well). The cytotoxic effect of the filtrates was determined after 24 h by measuring the amount of lactate dehydrogenase (LDH) released to the culture medium upon Vero cell lysis. By a reaction catalyzed by LDH, a red colored product (formazan) was formed proportionally to the concentration of LDH and was measured spectrophotometrically (490 nm) by using a standard enzyme-linked immunosorbent assay microplate reader.

Antimicrobials. The protein synthesis inhibitors used were gentamicin (Garamycin; Schering-Plough, Belgium), azithromycin (Zitromax; Pfizer, Denmark), and telithromycin (Aventis Pharma Deutschland GmbH, Germany). Moreover, the fluoroquinolone ciprofloxacin (Ciproxin; Bayer, Germany) was used in assessment of Stx prophage induction. The bacteria were incubated with the protein synthesis inhibitors at MICs (3.0 μg/ml for gentamicin, 0.25 μg/ml for azithromycin, and 8.0 μg/ml for telithromycin) and at 0.25× MIC for gentamicin and azithromycin. Incubation of selected strains with ciprofloxacin was performed at the ciprofloxacin MIC (0.016 μg/ml). The levels of MIC were determined after 24 h of incubation using viable counting to detect growth. The MIC determinations were performed on a panel of strains producing all the subtypes/toxin variants, and the highest MIC detected for each antimicrobial was subsequently used.

Statistics. The mean of triple responses for each strain incubated with an antimicrobial drug and the mean response without application of antimicrobials were compared by one-way analysis of variance, followed by Dunnet's test (a P value of <0.05 was considered significant).

The relative average decrease in the release of each toxin variant upon the addition of protein synthesis inhibitors was calculated. The decreases of toxin variants were thereby compared by one-way analysis of variance and then by Student-Newman-Keuls test (a P of <0.05 was considered significant).

Differences in mean suppression of toxin release between O-serogroup O157 and non-O157 strains were analyzed by *t* test for strains producing the same toxin variant upon incubation with protein synthesis inhibitors at MICs and 0.25× MIC (a $P < 0.05$ was considered to be significant).

RESULTS

A total of 29 STEC wild-type strains producing toxin type Stx1 and 7 different Stx2 toxin variants were exposed to protein synthesis inhibitors, and the level of released Stx was measured by a Vero cell cytotoxicity assay. The relative decreases in toxin release, which are shown in Table 1, were determined as the ratio of the difference in toxin release between strains incubated with and without protein synthesis inhibitors and the response without antimicrobial drugs. The effects of protein synthesis inhibitors on each Stx2 variant appeared to be similar, regardless of drug class, and therefore, the average toxin release across the antimicrobials was used for statistical analysis in order to obtain the necessary statistical power. This is further justified by the fact that the three antimicrobials used have the same mechanism of action.

Protein synthesis inhibitors at MICs. The average toxin release from strains producing Stx1 increased by 4.6% when these strains were exposed to protein synthesis inhibitors at MICs. In contrast, the average release of toxin was decreased for all strains producing Stx2 toxin variants: toxin release decreased by 14.0% for the least decreased variant (Stx2-O157-EDL933) and up to 94.7% (Stx2d-O8-C466-01B).

One strain (C532-03) producing toxin variant Stx2-O157-EDL933 exposed to gentamicin or azithromycin at MICs displayed significant increases in toxin release, whereas an insignificant increase was observed following the addition of telithromycin. Another strain producing Stx2-O157-EDL933 (C528-03) displayed a significant increase in toxin release when incubated with telithromycin and an insignificant increase and decrease when subjected to gentamicin and azithromycin, respectively.

Despite these increases in toxin release, the average decrease of 14.0% for strains producing toxin variant Stx2-O157-EDL933 that were exposed to protein synthesis inhibitors at MICs proved to be a statistically significant decrease ($P < 0.0005$ determined by *t* test). Except for the two strains producing Stx2-O157-EDL933, no strains producing Stx2 exhibited an increase in toxin release upon the addition of protein synthesis inhibitors at MICs.

The average responses of strains producing Stx1 and the seven toxin variants of Stx2 incubated with protein synthesis inhibitors at MICs were all found to be significantly unique, except for the average release from the strain producing toxin variant Stx2d-O73-C165-02 (76.5% decrease), which was not found significantly dissimilar to the 73.7% decrease of the strain producing Stx2c-O157-FLY16 and the 85.1% decrease of the strain producing Stx2b-O111-PH.

Ciprofloxacin at MIC. Toxin release in the majority of the strains producing Stx2 toxin variants increased or increased only moderately during incubation with ciprofloxacin. The average amounts of toxin released were increased by 18.8%, 19.3%, 21.6%, and 67.9% from strains producing Stx2d3-O157-7278, Stx2c-O157-FLY16, Stx1, and Stx2d-O73-C165-02, respectively. However, average decreased toxin releases of 1.2%, 2.9%, and 22.9% were obtained from strains producing Stx2-O157-EDL933, Stx2-O48-94C, and Stx2b-O111-PH, respectively. Only the strain producing toxin variant Stx2d-O8-C466-01B responded much differently, as ciprofloxacin decreased the toxin release by 92.2%.

Protein synthesis inhibitors at sub-MICs. Gentamicin and azithromycin were applied at 0.25× MIC and prompted highly variable responses from the STEC strains. In short, strains producing Stx1 and strains producing three Stx2 toxin variants (Stx2-O157-EDL933, Stx2b-O111-PH, and Stx2c-O157-FLY16) displayed significant increases and decreases in toxin release compared to the response during incubation without protein synthesis inhibitors. Strain C165-02 producing toxin variant Stx2d-O73-C165-02 was observed to significantly increase toxin release when exposed to gentamicin and azithromycin. Significant decreases in toxin release were detected only from strains producing toxin variants Stx2-O48-94C and Stx2d-O8-C466-01B, whereas strains producing Stx2d3-O157-7278 displayed no significant alterations of Stx2 release (data not shown).

Comparison of average Stx2 release of toxin variants revealed that release of Stx2d-O73-C165-02 was significantly higher than release of all other toxin variants (all $P < 0.001$). Furthermore, the average toxin release of Stx2d-O8-C466-01B (all $P \leq 0.05$) and Stx2b-O111-PH (all $P \leq 0.017$) was significantly decreased compared to the other toxin variants (data not shown).

O-serogroup O157 versus non-O157. Coherence in O serogroup and toxin release was investigated by comparing the average responses of O157 strains and non-O157 strains for the release of Stx1 and three toxin variants of Stx2 (Table 2). Incubation of strains producing toxin variant Stx2-O157-EDL933 with protein synthesis inhibitors at MICs caused a significantly greater

TABLE 1. Relative decreases in toxin release from STEC strains upon exposure to ciprofloxacin or protein synthesis inhibitors at MICs[a]

Toxin type or subtype	Toxin variant	Strain	O serotype	*eae*[b]	Relative % decrease in toxin release[c] in STEC strains exposed to:				Avg[d]
					Ciprofloxacin (0.016 g/ml)	Gentamicin (3 μg/ml)	Azithromycin (0.25 μg/ml)	Telithromycin (8 g/ml)	
Stx1		C126-02	O157:H$^-$	+	−52.5*	−14.6	−14.4	−7.8	
		C1111-02	O157:H$^-$	+	3.3	−8.7	−11.2*	−3.9	
		C1096-02	O156:H25	+	ND	−0.8	1.3	15.1*	
		C118-05	O146:H21	−	−15.5	−10.1	−10.4	10.1	−4.6 ± 1.8
Stx2	Stx2-O157-EDL933	C832-02	O26:H11	+	24.4*	49.9*	29.8*	43.8*	
		C218-03	O121:H19	+	−8.6	20.0*	19.8*	29.4*	
		C388-02	O157:H7	+	ND	22.7*	10.8*	21.6*	
		C528-03	O157:H7	+	−61.5*	0.6	−5.3	22.2*	
		C532-03	O157:H7	+	47.2*	−21.1*	−19.8*	−13.9	
		C699-03	O101:H$^-$	+	4.5	11.6*	11.0*	19.3*	14.0 ± 2.7
	Stx2-O48-94C	C597-03	O145:H$^-$	+	−8.5	52.2*	44.5*	83.4*	
		C269-03	O26:H$^-$	+	30.0*	32.6*	8.8	55.1*	
		C349-03	O145:H$^-$	+	−29.4	65.2*	51.0*	50.9*	
		C770-02	O157:H$^-$	+	19.6	52.9*	26.2*	59.5*	48.5 ± 3.2
Stx2b	Stx2b-O111-PH	C546-03	O146:H28	−	22.3*	92.6*	86.3*	92.1*	
		C305-02B	O146:H28	−	ND	60.8*	63.9*	78.2*	
		C61-03	O75:H$^-$	−	ND	90.4*	85.1*	90.5*	
		C1354-02	O88:H8	−	23.5*	94.7*	92.6*	94.4*	85.1 ± 1.9
Stx2c	Stx2c-O157-FLY16	C1112-02A	O145:H$^-$	+	ND	82.1*[e]	89.9*	91.9*	
		C1211-02	O157:H$^-$	+	−14.6	80.5*	78.0*	83.7*	
		C1386-02	O145:H$^-$	+	ND	69.7*	74.4*	85.2*	
		C396-03	O157:H7	+	9.3	48.9*	50.3*	63.1*	
		C618-03	O157:H7	+	−20.7*	86.7*	68.6*	98.5*	
		C306-02	O145:H$^-$	+	−51.2*	48.5*	69.8*	58.8*	73.7 ± 2.2
Stx2d	Stx2d3-O157-7278	C86-97	O113:K$^-$:H4	−	−76.6*	6.5	19.2*	29.6*	
		C572-03	O113:H4	−	39.0	48.1*	42.0*	57.9*	33.9 ± 4.3
	Stx2d-O8-C466-01B	C466-01B	O8:H19	−	90.9*	98.6*	98.5*	101.0*	
		C472-01	O51:H49	+	93.6*	96.3*	82.8*	91.1*	94.7 ± 1.7
	Stx2d-O73-C165-02	C165-02	O73:H16	−	−67.9	78.7*	69.4*	81.4*	76.5 ± 2.2

[a] The cytotoxicities of the control strains, enteropathogenic *E. coli* strain C135-03 and *E. coli* K-12 strain D2103, were 3.0% and 0.3%, respectively.
[b] The presence (+) or absence (−) of the *eae* gene, which encodes the adhesion molecule intimin.
[c] The relative decreases in toxin release were determined as the ratio of the difference in toxin release between strains incubated with and without protein synthesis inhibitors and the response without antimicrobial drugs. The values of relative decrease in toxin release are averages of triplicate cultures unless indicated otherwise. Negative decreases are increases in toxin release compared to toxin release in strains that were not exposed to antimicrobial agents. An asterisk indicates that the toxin release value is significantly different from the value in a strain that was not exposed to antimicrobial agents (5% level of significance). ND, not determined.
[d] The averages of percent decreases in toxin release for strains producing toxin type Stx1 or the indicated toxin variants were all significantly different from each other ($P \leq 0.001$), except that the value for toxin variant Stx2d-O73-C165-02 was not significantly different from the value for Stx2c-O157-FLY16 or Stx2b-O111-PH, and the values for Stx2d-O73-C165-02 and Stx2d-O8-C466-01B, Stx2d-O8-C466-01B and Stx2b-O111-PH, and Stx2b-O111-PH and Stx2c-O157-FLY16 differed only with significance of $0.001 < P < 0.05$. Sample statistics are mean ± standard error of the mean.
[e] Average of duplicate cultures.

decrease in toxin release from non-O157 strains (26.1%) than from O157 strains (2.0%). The two strains producing Stx2-O157-EDL933 (C532-03 and C528-03) that displayed increased toxin release following exposure to protein synthesis inhibitors at MICs were both O-serogroup O157. Stx1-producing strains displayed an average increase in toxin release from O157 strains but an average decrease from non-O157 strains at both MICs and sub-MICs. This tendency was also seen at the sub-MIC for the strain producing toxin variant Stx2-O157-EDL933.

DISCUSSION

The hypothesis that antimicrobial therapy of STEC infections will increase the risk of developing HUS has been widely discussed (11). Since the production of Stx seems to be regulated by an induction of the integrated bacteriophage that encodes the toxins, it is suggested that the regulation of Stx-encoding phages play a direct role in STEC pathogenesis (19).

The majority of strains investigated carried the gene encoding intimin (*eae*). One strain producing Stx1 (C118-05) did not have *eae*; however, its response was similar to the responses of the other strains carrying stx_1, indicating that this virulence gene did not contribute to cytotoxicity. Furthermore, the control strains, enteropathogenic *E. coli* strain C135-03 and *E. coli* K-12 strain D2103, were included in the study to demonstrate that no other bacterial factors affected cytotoxicity. These observations confirmed that the cytotoxicity measured is a function of the amount of Stx released.

Interestingly, relative average decreases in toxin release were seen for all strains producing seven Stx2 toxin variants incubated with protein synthesis inhibitors at MICs. The 25

TABLE 2. Relative decreases in toxin release from O-serogroup O157 strains and non-O157 strains exposed to protein synthesis inhibitors

Toxin type or toxin variant	Protein synthesis inhibitor concn	Relative % decrease[a] in toxin release from:		P^b
		O157 strains	Non-O157 strains	
Stx1	MIC	−10.1 ± 1.6	0.9 ± 2.6	0.0009
	0.25× MIC	−24.1 ± 15.2	15.8 ± 5.4	0.03
Stx2-O157-EDL933	MIC	2.0 ± 3.5	26.1 ± 2.6	<0.0001
	0.25× MIC	−18.8 ± 6.8	12.4 ± 2.9	0.0003
Stx2-O48-94C	MIC	46.2 ± 3.7	49.3 ± 4.8	NS
	0.25× MIC	13.9 ± 8.9	4.6 ± 3.9	NS
Stx2c-O157-FLY16	MIC	73.1 ± 3.2	74.4 ± 3.0	NS
	0.25× MIC	1.7 ± 7.8	−0.1 ± 6.4	NS

[a] The relative decreases in toxin release were determined as the ratio of the difference in toxin release between strains incubated with and without protein synthesis inhibitors and the response without antimicrobial drugs. Negative decrease values correspond to increases in toxin release. Sample statistics are mean ± standard error of the mean.

[b] The relative decreases in toxin release for O157 strains and non-O157 strains were compared, and the *P* values for the differences are shown. NS, not significant on a 5% level of significance.

strains producing seven Stx2 variants exhibited relative average decreases ranging from 94.7% (Stx2d-O8-C466-01B) to 14.0% (Stx2-O157-EDL933). In contrast, the strain producing Stx1 generated an average increase (4.6%) in toxin release, which may occur due to induction of the s*tx*-carrying bacteriophage. The s*tx* genes are located directly downstream of the late phage promoter ($p_{R'}$), and transcription from $p_{R'}$ due to phage induction is crucial for the production of Stx2 but is unnecessary for high-level Stx1 production (18, 19). These findings may confirm that protein synthesis inhibitors are able to suppress Stx2 production but not Stx1 production. The $p_{R'}$ promoter is the primary regulatory element in Stx2 production (19). Different decreases in the amounts of Stx2 released from the seven strains studied that produced Stx2 variants may therefore be due to variations in the $p_{R'}$ regions present in the individual strains.

The decreases in the amounts of Stx2 released upon exposure to protein synthesis inhibitors found in the present in vitro study are of clinical interest, since the most severe clinical complications of STEC infections are associated with strains producing subtype Stx2 and subtype Stx2c (6, 8). In contrast, the use of protein synthesis inhibitors is not obvious in drug therapy of infections due to Stx1-producing strains.

Significant differences in average suppression of toxin release were identified for toxin variants of the same Stx2 subtype. The toxin variants of subtype Stx2 (Stx2-O157-EDL933 and Stx2-O48-94C) showed average suppressions of 14.0% and 48.5%, respectively; furthermore, the toxin variants of subtype Stx2d (Stx2d3-O157-7278, Stx2d-O73-C165-02, and Stx2d-O8-C466-01B) were suppressed 33.9%, 76.5%, and 94.7%. These variations within subtypes indicate that the toxin variant can be a marker of the toxin release following application of protein synthesis inhibitors.

STEC strains belonging to O-serogroup O157 have traditionally been considered to cause more severe clinical outcome than strains of other O serogroups (13, 16). However, epidemiological studies indicate that the development of HUS is attributed to other risk factors, such as the presence of the stx_2 and *eae* genes but is not independently associated with the O-serogroup O157 of the infecting STEC (5, 6). Nevertheless, the present data show that it is significantly more difficult to suppress toxin release of Stx1 and toxin variant Stx2-O157-EDL933 from O157 strains compared to non-O157 strains when the strains were exposed to protein synthesis inhibitors. The promoter strength of $p_{R'}$ might be stronger for the strain producing Stx2-O157-EDL933 than for strains producing other Stx2 variants, and in association with a presently unknown factor that covaries with the O157 strains producing Stx2-O157-EDL933, this could explain the lower decrease in the relative toxin release for this O serogroup.

To avoid introducing bias when comparing toxin variants due to a higher ratio of strains representing O-serogroup O157, strains included in this study were selected to achieve an equal number of O157 strains and non-O157 strains producing each toxin variant. This was attained for Stx1, Stx2-O157-EDL933, and Stx2c-O157-FLY16. Toxin variant Stx2-O48-94C is represented by only one strain of O-serogroup O157 out of four. However, Stx2-O48-94C was still found to be significantly different from Stx2-O157-EDL933 when all O157 strains producing the two toxin variants were omitted from the statistical test or if all non-O157 strains were excluded ($P < 0.001$ in both cases). Furthermore, the observed difference between the strains producing toxin variants of Stx2d cannot be due to bias that arose from the ratio of O157 strains, as no strains of this subtype are O157.

Used at MICs, the protein synthesis inhibitors significantly suppressed the toxin release from all strains producing Stx2, except from two strains producing toxin variant Stx2-O157-EDL933 (C532-03 and C528-03). Thus, the use of protein synthesis inhibitors for treatment of infections with most STEC strains carrying stx_2 appears promising. However, treating infections caused by Stx2-O157-EDL933-producing strains of O-serogroup O157 is not feasible, since both strains that caused an increase in toxin release were of serotype O157:H7. Consequently, these data suggest that it should be mandatory to determine the O serogroup of Stx2-O157-EDL933-producing strains prior to antimicrobial treatment with protein synthesis inhibitors.

In a Danish STEC cohort (255 strains), isolated in the period from January 1997 to October 2003, the Stx2 subtype/variant distribution was determined and associated with clinical data, including HUS. All 20 HUS cases were associated with subtype Stx2 and/or Stx2c. Nineteen HUS cases involved toxin variant Stx2-O157-EDL933 or Stx2-O48-94C either alone (11 cases) or in combination with variant Stx2c-O157-FLY16 (8 cases). Finally, one strain causing HUS produced the Stx2c-O157-FLY16 variant (15). Of the 11 strains producing Stx2-O157-EDL933 and the 8 strains producing Stx2-O48-94C, 8 strains and 1 strain, respectively, belonged to O-serogroup O157. In this cohort, 12 HUS cases might have benefitted from antimicrobial treatment with protein synthesis inhibitors prohibiting fulminant STEC pathogenesis, including HUS.

Ciprofloxacin applied at MIC caused an increase in toxin release from approximately half of the strains tested, whereas only two strains producing Stx2-O157-EDL933 increased the

amount of toxin released as a result of incubation with protein synthesis inhibitors. Ciprofloxacin was observed to suppress the toxin release from a strain producing Stx2d-O8-C466-01B by 92.2%, which could indicate that this toxin variant is independent of phage induction, which was observed for an activatable Stx2d strain (O91:H21, B2F1) by Teel et al. (17).

The subinhibitory concentration is clinically relevant only until the concentration of antimicrobial drug reaches the therapeutic level of the target, because the administered doses are recommended to be well above the MIC. Hence, it will be of importance that the antimicrobial reaches the inhibitory concentration quickly when therapy is initiated and maintained well above the MIC until the infection is cured in order to avoid increase of toxin release of the STEC. Increase in toxin release is expected to enlarge the risk of systemic damage to the renal endothelial cells (22) and is therefore to be avoided. In this study, incubation with protein synthesis inhibitors at sub-MICs displayed highly variable responses, including increases in toxin release.

The level of decrease in toxin release for the different toxin variants shows much variation. In a clinical perspective, it might be more beneficial to treat infections due to STEC producing toxin variants that are more easily suppressed in vitro. Therefore, it seems important to determine the toxin variant of the infecting STEC before antimicrobial therapy is initiated. The Stx2 subtype and variant determination should be referred to national reference laboratories of clinical microbiology if not feasible in local clinical laboratories.

In conclusion, the significant difference in response to protein synthesis inhibitors between Stx2 toxin variants presented in this study emphasizes the relevance of determining not only the Stx2 subtype but also the toxin variant before antimicrobial treatment is considered. Further clinical studies are required to confirm the possible beneficial effect of protein synthesis inhibitors in management of STEC infections. In general, antimicrobial treatment of STEC increases the risk of HUS because of toxin liberation from lysed bacterial cells. Therefore, clinical studies of antimicrobial treatment of STEC raise serious ethical considerations. Our in vitro study suggests that introductory clinical studies should focus on protein synthesis inhibitors.

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