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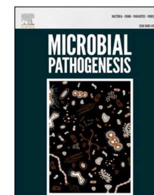

Effects of antimicrobials on Shiga toxin production in high-virulent Shiga toxin-producing *Escherichia coli*

Silje N. Ramstad [a,b,*], Arne M. Taxt [a], Umaer Naseer [c], Yngvild Wasteson [d], Jørgen V. Bjørnholt [a,b], Lin T. Brandal [c]

[a] *Department of Microbiology, Division of Laboratory Medicine, Oslo University Hospital, PB 4956 Nydalen, 0424, Oslo, Norway*
[b] *Institute of Clinical Medicine, University of Oslo, Oslo, Norway*
[c] *Department of Infectious Diseases and Prevention, Norwegian Institute of Public Health, Oslo, Norway*
[d] *Department of Paraclinical Sciences, Norwegian University of Life Sciences, Oslo, Norway*

A R T I C L E I N F O

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A B S T R A C T

Purpose: Antimicrobial treatment of Shiga toxin-producing *Escherichia coli* (STEC) infections is controversial because antimicrobials may stimulate Shiga toxin (Stx) production, and thereby increase the risk of developing haemolytic uremic syndrome (HUS). Previous *in vitro* studies have shown this mainly in infections caused by STEC serotype O157:H7. The aim of this study was to investigate induction of Stx transcription and production in different serotypes of STEC isolated from severely ill patients, following their exposure *in vitro* to six different classes of antimicrobials.

Methods: We investigated Stx transcription and production in 12 high-virulent STEC strains, all carrying the *stx2a* gene, of six different serotypes following their exposure to six classes of antimicrobials. Liquid cultures of the STEC strains were incubated with sub-inhibitory concentrations of the antimicrobials. We used reverse-transcription quantitative PCR to measure the relative expression of Stx2a mRNA and an enzyme-linked immunosorbent assay to quantify Stx production.

Results: In general the antibiotics tested showed only minor effects on transcriptional levels of Stx2a. Ciprofloxacin caused an increase of Stx production in all but two strains, while gentamicin, meropenem and azithromycin did not induce Stx production in any of the STEC strains examined. STEC O104:H4 was the serotype that in greatest extent responded to antimicrobial exposure with an increase of *stx2a* transcription and Stx production.

Conclusion: Gentamicin, meropenem and azithromycin exposure did not result in elevated Stx production. We recommend that this finding is investigated further in the search for candidates for future antimicrobial treatment of STEC.

1. Introduction

Infections caused by Shiga toxin-producing *Escherichia coli* (STEC) can cause a variety of gastrointestinal symptoms, including nausea, abdominal pain and mild to bloody diarrhoea, but may also cause severe complications such as haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic microangiopathy [1–3]. HUS occurs in 5–15% of all STEC infections and mostly affects children [3]. In severe cases supportive therapy is required, but the role of antimicrobial treatment of STEC infections is controversial. Some clinical studies have indicated that antimicrobials can increase the risk of HUS development, and therefore should be avoided [4,5].

The key virulence factors of STEC infection pathogenesis are Shiga toxins (Stx) and the ability to attach to the intestinal epithelium via the attaching and effacing mechanism. Stx are divided into two main types, Stx1 and Stx2, which further are divided into various subtypes [6]. The

Abbreviations: HUS, haemolytic uremic syndrome; NIPH, Norwegian Institute of Public Health; NRL, National Reference Laboratory; NSF, Non sorbitol fermenting; OMV, outer membrane vesicles; RT-PCR, Reverse transcription polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SF, Sorbitol fermenting; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin.

* Corresponding author. Department of Microbiology, Division of Laboratory Medicine, Oslo University Hospital, PB 4956 Nydalen, 0424, Oslo, Norway.
E-mail address: silje.ramstad@fhi.no (S.N. Ramstad).

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different Stx subtypes are associated with different disease severity, with Stx2a being the subtype most frequently associated with HUS development [6–9]. In Norway, STEC isolates with Stx2 encoding genes *stx2a, stx2c* and/or *stx2d* are categorized as high-virulent, and guides subsequent implementation of control measures [10].

Previously, serotypes have been used to predict and classify the virulence potential of STEC, based on their association with outbreaks and severity of disease in humans [8]. Therefore, non-sorbitol fermenting (NSF) STEC O157:H7 was initially the focus of STEC surveillance and research, since this serotype has been the aetiological agent of most STEC outbreaks and HUS cases worldwide [11–13]. However, the large German STEC outbreak in 2011 deviated from the ordinary pattern. The outbreak was caused by an *E. coli* O104:H4 strain originally classified as an enteroaggregative *E. coli*, which had acquired the Stx2a encoding gene. The disease outcome of that outbreak was also different from the usual pattern, as 22% of the cases developed HUS, most of whom were adults [14]. In recent years there has been more focus on non-O157 STEC strains as important contributors to infections and outbreaks [15]. In Norway, STEC O145:H25, O26:H11, O103:H25, and sorbitol fermenting (SF) as well as NSF O157:H7 are the most common serotypes causing HUS [7,9].

The *stx* genes are carried by lambdoid prophages, which insert their genomes into the bacterial chromosome. The phage-genes responsible for the switch between lysogenic and lytic states are co-regulated by the bacterial SOS-response system [16]. The triggering of this system, with the subsequent induction of the lytic cycle of the phage, production of Stx, cell lysis and release of toxins and new Stx prophages, is the main reason why antimicrobial treatment of HC and/or HUS is contraindicated [17]. Another theory is that antibiotics may lead to changes in the normal gut flora and this may make room for the STEC and Stx to reach the intestinal wall, or that it could lead to bacterial death and subsequent release and spreading of the intra-cellular Stx [18].

On the other hand, studies on STEC O104:H4 infections have found that treatment with antimicrobials do not increase the risk of HUS and may have positive effects on the clinical outcome [19,20]. Most previous studies aiming at explaining these conflicting observations have focused on a limited number of STEC serotypes, mostly NSF O157:H7 and O104:H4, but have not provided any conclusive recommendations [21–23]. It is suggested that individual STEC strain characteristics, as well as the class and dosing of the antimicrobials used, are important factors influencing the effects of antimicrobials on Stx production and thereby the likelihood of HUS development.

To investigate this in further detail, we designed a study to examine the effects of sub-inhibitory concentrations of six different classes of antimicrobials on Stx production in high-virulent STEC strains, all carrying *stx2a* gene, of various serotypes isolated from cases of severe STEC infections in Norway.

2. Materials and methods

2.1. STEC strains

In total, twelve high-virulent STEC strains were investigated. Eleven high-virulent STEC strains, all *stx2a* positive, isolated from severely ill patients in Norway between 2000 and 2013 were selected from the National Reference Laboratory (NRL) for Enteropathogenic Bacteria at the Norwegian Institute of Public Health (NIPH), where they had been previously characterized (serotypes and virulence genes) (Table 1) [7, 9]. The collection of strains to be studied consisted of two strains of each of the four serotypes most frequently associated with HUS in Norway, two strains of serotype O104:H4, one associated with the German HUS outbreak in 2011 (strain 3) [25], and two strains of serotype O103:H25, one causing a severe foodborne outbreak in Norway in 2006 (strain 7) [26] were analysed. The reference strain EDL933 (ATCC® 43895™) was included as one of two NSF O157:H7 strains.

Table 1
Characteristics of STEC strains examined.

			Virulence genes		
Strain number	Serotype	Year of isolation	*stx*-subtype	*eae*	*aggR*
1	O145:H25	2013	*stx2a*	*eae*	–
2	O145:H25	2009	*stx2a*	*eae*	–
3	O104:H4	2011	*stx2a*	–	*aggR*
4	O104:H4	2006	*stx2a*	–	*aggR*
5	SF O157:H7	2009	*stx2a*	*eae*	–
6	SF O157:H7	2008	*stx2a*	*eae*	–
7	O103:H25	2006	*stx2a*	*eae*	–
8	O103:H25	2005	*stx2a*	*eae*	–
9	O26:H11	2012	*stx2a*	*eae*	–
10	O26:H11	2010	*stx2a*	*eae*	–
11	NSF O157:H7	1999	*stx2a*	*eae*	–
12[a]	NSF O157:H7	1982	*stx2a* + *stx1*	*eae*	–

SF= Sorbitol fermenting, NSF = non-sorbitol fermenting, a EDL 933 STEC reference strain.

2.2. Antimicrobials and antimicrobial susceptibility testing

We decided to expose each strain to half of their minimum inhibitory concentrations (½ MIC) of each antimicrobial. The MIC values were determined for each antimicrobial using the broth microdilution method according to the ISO 20776-1 standard. The following antimicrobials, belonging to different classes or showing promising effects on Stx-production in previous studies [21,22], were selected for inclusion: azithromycin, doxycycline, gentamicin, rifampicin, meropenem, and ciprofloxacin. Azithromycin, doxycycline, gentamicin, and rifampicin are protein-synthesis inhibitors, meropenem is an inhibitor of cell-wall synthesis, and ciprofloxacin is a DNA-synthesis inhibitor. The antimicrobials were purchased from Merck (Darmstadt, Germany). Reference strain *E. coli* ATCC® 25922™ was included as quality control.

2.3. Antimicrobial exposure and isolation of RNA and protein

For each STEC strain, two parallel, independent experiments were performed. In brief, an overnight culture was grown in 5 ml Luria Bertani (LB) broth at 37 °C. A starter culture was made in 90 ml LB broth inoculated with 300 μl of the overnight culture and incubated with shaking in a water bath until the optical density (OD600) reached 0.2–0.4. The starter culture was divided into eight 15 ml centrifuge tubes, with 9.9 ml culture in each. Six of the tubes were inoculated with 100 μl of one of the different dissolved and diluted antimicrobials, such that the final concentration of each antimicrobial in the centrifuge tube was at ½ MIC of the respective STEC strain. To the seventh tube, 100 μl sterile water was added (non-exposed sample), and to the last tube, 100 μL of 50 μg/ml mitomycin C (Merck, Darmstadt, Germany) was added as a positive control sample for the induction experiments. The cultures were incubated overnight in the 15 ml centrifuge tubes (with the caps tightly closed) at 37 °C with 750 rpm shaking. OD was measured (Biochrom Ultrospec 10, Cambridge, United Kingdom) every hour after adding the antimicrobials for 5 h, then once again after overnight incubation.

Based on the growth curves, RNA isolation was performed after 2 h, at the end of exponential growth. After 2 h incubation with antimicrobials 200 μl of each culture was withdrawn, RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) was added as described by the manufacturer, and the samples were stored frozen at −80 °C until use. Stx production was quantified after 23–25 h; the induction experiments were terminated by centrifuging (6000 rcf, for 10 min at room temperature) the cultures followed by filtration of the supernatants (0.22 μm, Sartorius, Gottingen, Germany). Supernatants containing the Stx

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protein fraction were stored at −80 °C until use.

2.4. Relative quantification of Stx2a mRNA

Samples for RNA isolation were thawed, and the RNA isolated by using the RNeasy Kit (Qiagen, Hilden, Germany) following the instructions from the manufacturer. Quantitative real-time PCR (qPCR) and the ΔΔCt method were used to compare the relative quantities of Stx2a mRNA transcribed in the cultures that had been exposed to antimicrobials with the non-exposed cultures for each STEC strain, as previously described [27]. Briefly, the RNA concentration was measured (NanoDrop 1000 Spectrophotometer; Thermo Scientific, Wilmington, USA) and controlled for degradation by gel electrophoresis (Lonza, Basel, Switzerland). The samples were diluted to RNA concentrations of either 80 ng/μl or 100 ng/μl in each sample, with all samples from each culture experiment diluted to the same concentration, before mixing them with the Qiagen Reverse Transcription Kit (Hilden, Germany). Every sample was run in triplicate on qPCR as previously described [27]. Negative controls for the RT-reaction, the DNase reaction, and the qPCR were included in each run. A fold change ≥2 was considered significant based on previous reports of the fact that natural variation in the transcription of the endogenous control (*gapA*) is less than two-fold [27].

2.5. Relative quantification of Stx

For Stx quantification, a commercial ELISA kit (RIDASCREEN Verotoxin kit R-biopharm, Darmstadt, Germany) was used according to the manufacturer's instructions. A standard curve was made with the non-exposed supernatant of each culture experiment, to ensure that all measurements were within the linear range. All samples were diluted to reach an absorbance within the linear range. For each experiment, all samples were run in triplicate in the same ELISA plate. Triplicates of the negative control of the ELISA kit ("blank") were added to every plate. To calculate the fold change relative to the non-exposed sample, the mean of the OD for each sample was calculated, the mean of the blanks subtracted, and then each mean was multiplied by the respective dilution factor and divided by that of its respective non-exposed sample.

3. Results

3.1. Antimicrobial induction

The control strain, ATCC25922, was within the accepted MIC range according to the EUCAST quality control guidelines (v9.0) for ciprofloxacin, gentamicin, and meropenem, and in line with previously published MICs for azithromycin, doxycycline, and rifampicin [28–30]. The MIC values for the six antimicrobials are shown in Table S1 (supplementary). All strains were categorized as susceptible to ciprofloxacin, gentamicin and meropenem according to the EUCAST breakpoint tables (v9.0). Azithromycin, doxycycline and rifampicin do not have established breakpoints by EUCAST.

Growth curves for each STEC strain, exposed and not exposed to antimicrobials, from two independent experiments are shown in Fig. S1 (supplementary). All cultures were in late exponential growth phase 2 h after antimicrobial exposure (with the exception of mitomycin C exposed cultures), with OD600 values varying from 1.05 to 2.15. We therefore chose to collect samples for transcriptional analyses at this time-point as the culture conditions were stable and the RNA-levels were high.

3.2. Sub-inhibitory levels of antimicrobials and effects on stx2a transcription

Levels of *stx2a* transcription in exposed state (relative to non-exposed state) for all STEC strains examined are shown in Fig. 1. The positive control mitomycin C increased *stx2a* transcription from 12 to

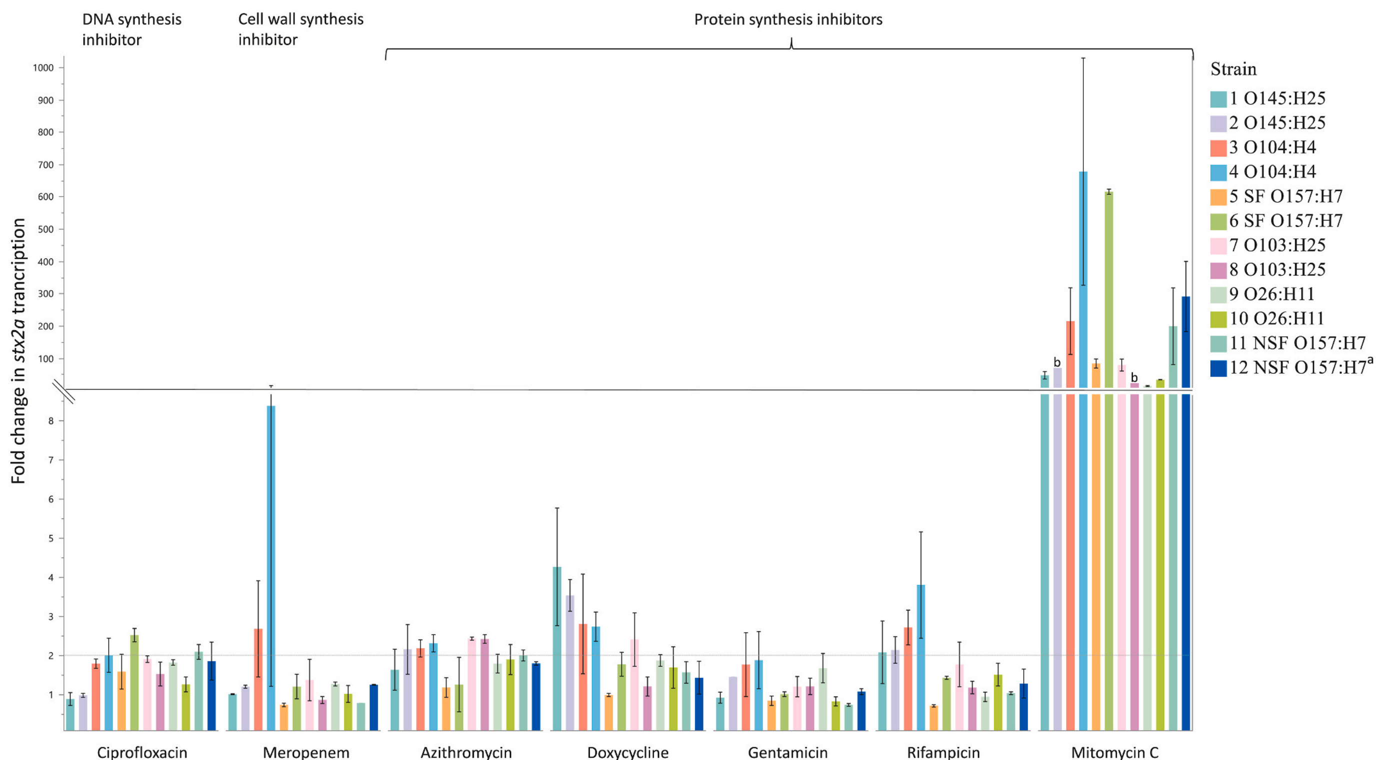

Fig. 1. *Stx2a* transcription in cultures exposed to ½ MIC of antimicrobials displayed as fold change relative to non-exposed cultures for each STEC strain examined. Samples were drawn 2 h after exposure start. Shown is mean of two independent experiments (error bars indicate min and max value). Mitomycin C was included as a positive control. The horizontal grey line indicates a fold change of 2, a fold change of ≥2 was considered significant [27]. SF = Sorbitol fermenting, NSF = non-sorbitol fermenting. [a] EDL 933 STEC reference strain, [b] the bars are based on only one replicate. Figure was made using JMP pro 15 (SAS Institute Inc.).

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1030 fold, thereby demonstrating the applicability of the experimental set-up. For the other antimicrobials, there were some variations between the different combinations of serotypes and antimicrobials, although no consistent pattern was observed. However, serotype O104:H4 often deviated from the other serotypes, with higher induction of Stx2a mRNA for some of the antimicrobials. Overall the antimicrobials had little effect on *stx2a* transcription after 2 h of exposure. Ciprofloxacin did not induce *stx2a* transcription in six (50%) of the STEC strains examined. Meropenem did not induce *stx2a* transcription in ten strains and for the remaining two strains (strains 3 and 4, both O104:H4) transcription was above two-fold in only one of the replicate experiments. However, it is noteworthy that there was apparently 15-fold induction of transcription in strain 4 O104:H4 in one replicate, the highest seen across all strains and antimicrobials in this experiment. Azithromycin did not induce *stx2a* transcription in three strains, and only three strains (strain 4, O104:H4, and strains 7 and 8, both O103:H25) were induced in both experiments. Doxycycline did not influence *stx2a* transcription in four strains, five strains were induced in only one of the culture experiments, although reaching above four-fold transcription for two strains in one replicate. Gentamicin did not influence *stx2a* transcription in nine strains and in three strains (strains 3 and 4, both O104:H4 and strain 9, O26:H11) it only induced *stx2a* transcription in one of the culture experiments. Rifampicin did not affect the *stx2a* transcription in seven of the strains and only two strains (strains 3 and 4, both O104:H4) were induced in both culture experiments, but with strain 4 being induced to over 5-fold transcription in one of the replicates.

3.3. Sub-inhibitory levels of antimicrobials and effects on Stx production

The effects of exposure to antimicrobials on Stx production after 23–25 h, as analysed by ELISA, are shown in Fig. 2. Mitomycin C had a pronounced effect on Stx production in all strains, inducing a >10 fold increase of Stx-toxin in all but two strains. Exposure to ciprofloxacin also generally led to an increase of Stx production, as observed in ten of the 12 strains included in the current study. The notable exceptions were the two strains of serotype O145:H25, which were the same two strains which also displayed the least increase of toxin production when exposed the mitomycin C. Noteworthy, the three antimicrobials meropenem, azithromycin and gentamicin did not induce Stx production in any of the 12 high-virulent STEC strains examined.

Serotype O104:H4, which appeared prone to induction by antimicrobials at the transcriptional level, also stood out in the toxin-analyses, displaying a pronounced increase of toxin production in response to doxycycline and rifampicin. Rifampicin did not induce Stx production in the other 11 strains, whereas the induced strain (strain 4, O104:H4) produced almost five and ten times more Stx than the respective non-exposed sample in each experiment. Doxycycline did not affect Stx production in nine strains, whereas three of the strains (strain 4, O104:H4, 9 and 10, O26:H11) were affected by doxycycline with an average fold change between almost two fold to almost four fold compared to the respective non-exposed culture. In concordance with the *stx2a* transcription results strain 4, (O104:H4), stood out as an easily induced strain. The other O104:H4 (strain 3), associated with the German 2011 outbreak, was all over less induced than its fellow serotype in both experiments.

4. Discussion

To address the dilemma of whether or not to administer antimicrobial treatment in severe STEC infections we investigated the effects of six different antimicrobials on *stx2a* transcription and Stx production in 12 high-virulent STEC of various serotypes. We found that sub-inhibitory concentrations of meropenem, azithromycin and gentamicin, did not increase Stx production in any of the STEC strains examined.

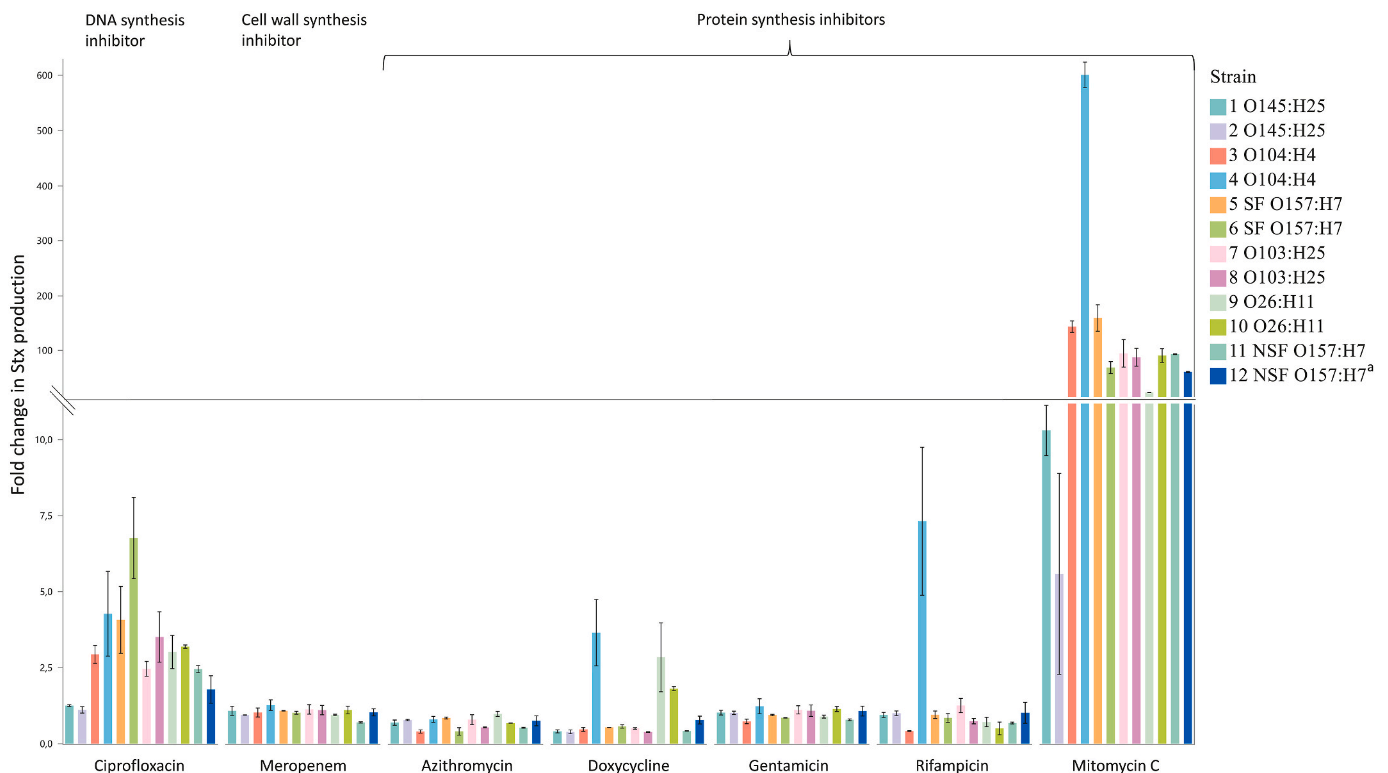

Fig. 2. Stx production in cultures exposed to ½ MIC of antimicrobials displayed as fold change relative to non-exposed cultures for each STEC strain examined. Samples were drawn 24 ± 1 h after exposure start. Shown is mean of two independent experiments (error bars indicate min and max value). Mitomycin C was included as a positive control. SF = Sorbitol fermenting, NSF = non-sorbitol fermenting. [a] EDL 933 STEC reference strain. Figure was made using JMP pro 15 (SAS Institute Inc.).

All experimental strains produced more Stx when exposed to ciprofloxacin compared to the corresponding non-exposed samples, although only a few strains showed more than a two-fold increase in expression of Stx2a mRNA. These results were expected since ciprofloxacin inhibits DNA-replication and thereby trigger the bacterial SOS-response and thus gives rise to increased Stx production.

Meropenem, an inhibitor of cell-wall synthesis, do not directly lead to induction of the SOS-response and stands out as one of the most promising antimicrobial candidates in our study. It did not induce Stx production in any of the strains examined, nor did it induce *stx2a* transcription, with the notable exception of serotype O104:H4. Therefore, meropenem should be included in future *in vitro* investigations. Other *in vitro* studies have shown that meropenem did not increase the Stx production or *stx2a* transcription significantly in the O104:H4 German outbreak strain [21,22]. Further, a clinical study found that patients treated with a combination of meropenem and ciprofloxacin (and sometimes rifaximin) eradicated STEC O104:H4 much more rapidly than non-treated patients [19].

All four protein-synthesis inhibitors showed promising results regarding reduced Stx production, except in serotypes O104:H4 and O26:H11. At the *stx2a* transcription level, the results were variable, but were most promising for gentamicin. As gentamicin is not absorbed across the intestinal wall, high intestinal concentrations of gentamicin could be achieved if administered orally, and that may be a possible approach for future treatment of STEC infection. Although per oral administration of aminoglycosides is not an established therapeutic approach today, this has been studied for the eradication of colonizing carbapenemase-producing Gram-negative bacteria with favourable results and could be explored further [31]. There are few clinical studies that include gentamicin, but as gentamicin targets the 30S ribosomal subunit it should not trigger the SOS-response system and therefore in theory be a safer choice for treatment of STEC infections.

In our study azithromycin resulted in the lowest toxin production overall in the 12 high-virulent STEC strains. The *stx2a* transcription results were not as encouraging as the Stx quantification, although only three strains (strain 4, O104:H4, and strains 7 and 8, both O103:H25) were induced more than two-fold in both experiments. These results are consistent with two studies that reported that sub-MIC levels of azithromycin had no *in vitro* effect on toxin production and that azithromycin did not induce *stx2a* transcription in STEC O104:H4 or EDL933 [21,42]. This is also in support of the proposition by Agger et al. who in a review suggest that STEC infections could be treated orally with a protein-synthesis inhibitor for three days, followed by an inhibitor of cell-wall synthesis for seven days [32]. Azithromycin targets the 50S ribosomal subunit and as gentamicin, should not trigger the SOS-response system, but will inhibit the RNA-dependent protein synthesis and thereby the production of Stx.

Variable results for both *stx2a* mRNA and total Stx production were observed between and within serotypes. Such variation is in line with other comparable *in vitro* studies indicating that not only the antimicrobial used, but also the characteristics of the examined STEC are of importance when it comes to expression of *stx2a* mRNA and production of Stx after antimicrobial exposure [22,33,34]. Differences within serotypes were observed, reflected in the differing results for the two O104:H4 strains, where strain 4 was more easily induced than the German outbreak strain (strain 3). This underlines the importance of the strain specific traits. Differing properties of the *stx* phage itself has been proposed as an important factor which may influence the level of Stx production, and it has been shown that same serotypes of STEC may harbour different *stx* phages [35,36]. Genetic variation exists even within the subgroup of *stx2a* phages, and it is currently not known whether these differences may have clinical implications.

Our laboratory experiments were designed to mimic several of the characteristics of an *in vivo*-situation as closely as possible. The bacterial strains were grown and exposed to the antimicrobials in small, closed tubes which have limited oxygen access and could easily transform to anaerobic conditions over time. We chose to conduct the induction experiments under oxygen-limited conditions to provide a similar environment to that of the gastrointestinal tract, where there is an O_2 gradient from the anaerobic lumen towards the epithelium [37]. Previous studies have shown that Stx2 is less induced under anaerobic or semi-anaerobic conditions [38,39] and this could partly explain the relatively low levels of induction in our study as compared to similar studies [21,22].

Our experiments were conducted using an antimicrobial concentration of ½ MIC, which is in line with previously published studies in the field [21,22]. The sub-MIC was chosen because this is hypothesized to induce the *stx* phage without killing the experimental culture. A sub-MIC is also relevant in a clinical setting after administration of antimicrobials, both before and after the antimicrobial reaches therapeutic level *in vivo*. A higher concentration of antimicrobials might have given higher induction level and should also be tested along with therapeutic concentrations.

RNA isolation was conducted after 2 h, when the cultures were at the end of exponential growth and the bacteria presumably were under anaerobic conditions in some parts of the tubes. This time-point was chosen because the cultures were still in exponential phase where the cultures are most stable and have little influence from external stress-factors as nutrient depletion. The metabolic activity is high and therefore the RNA levels are high. This time-point was also chosen by Corogeanu et al., who isolated RNA after 2 h of antimicrobial exposure [22]. The cultures were vortexed before sampling of mRNA, which means that the different atmospheric conditions within the tube (semi-anaerobic at the top and more anaerobic towards the bottom) were mixed. Unpredictable oxygen availability may give unstable induction of *stx2a* transcription, adding to the complex picture of an STEC-infection, as there is not necessary a continuous provision of oxygen in the host environment.

The differences observed between the RT-qPCR and the ELISA results presumably reflect that the mRNA samples show a snapshot of transcriptional levels at the point of sampling, while the ELISA analyses show the total accumulated amount of Stx produced during the total 23–25 h of exposure. The mRNA data should be interpreted with caution, as transcription of *stx2a* at a certain time-point may be very sensitive to subtle experimental conditions which are difficult to control. Nevertheless, the high amounts of stx2a mRNA obtained after induction by mitomycin C demonstrate that transcription do occur under the experimental circumstances. STEC have recently been shown to harbour a mechanism for Stx release without the concurrent lysis of the bacteria through the discharge of small outer membrane vesicles (OMVs) containing Stx [40]. The ELISA method used for Stx quantification in our study only measures free Stx in the supernatant, and does not measure Stx inside OMVs. Both microaerobic conditions and some types of antimicrobials have been reported to increase the release of OMV associated Stx, however, of the antimicrobials tested here, only ciprofloxacin has been shown to have this effect [24,41].

In conclusion, meropenem, gentamicin and azithromycin were the most promising antimicrobial agents that did not increase production of Stx of high-virulent STEC. Further studies are needed to examine how different concentrations and combinations of these antimicrobials affect STEC at different time-points. We confirm previous observations that exposure to ciprofloxacin generally leads to an increase of Stx for most serotypes of high-virulent STEC. Various serotypes may respond differently to antimicrobial exposure and strain specific traits seem to influence how a particular strain of STEC responds to antibiotic exposure and these traits should be investigated further.

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Ethics approval

Our study only included anonymized data. Consequently, ethical approval and informed consent from the patients were not required.

Author statement

Silje N Ramstad: Methodology, Writing - original draft and review & editing, data curation, Arne M Taxt: Methodology; Project administration; Funding acquisition; Supervision; Writing - review & editing. Umaer Naseer: Methodology; Writing - review & editing. Yngvild Wasteson: Methodology; Funding acquisition; Supervision; Writing - review & editing. Jørgen V Bjørnholt: Methodology; Supervision; Writing - review & editing. Lin T Brandal: Conceptualization; Methodology; Funding acquisition; Supervision; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104636.

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