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Original Article

Effects of Ampicillin, Gentamicin, and Cefotaxime on the Release of Shiga Toxins from Shiga Toxin–Producing *Escherichia coli* Isolated During a Diarrhea Episode in Faisalabad, Pakistan

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Abstract

The Shiga toxin–producing *Escherichia coli* (STEC) is an emerging foodborne pathogen. The proportion of cases attributed to STEC in an episode of diarrhea in the Faisalabad region of Pakistan was investigated. In addition, as increase in Shiga toxin (Stx) release after exposure to various antimicrobial agents is widely reported, we also elucidated the *in vitro* effects of three commonly used antibiotics (ampicillin, gentamicin, and cefotaxime) on Stx release. Isolation and detection of STEC was done using enzyme-linked immunosorbent assay and polymerase chain reaction, followed by phenotypic characterization. *In vitro* Stx release from isolated STEC was determined using enzyme-linked immunosorbent assay, and Stx-induced verocytotoxicity was quantified using cytotoxicity detection assay. STEC was detected in 5 (21.7%) of 23 patients. Exposure to minimum inhibitory concentration (MIC) of ampicillin, gentamicin, and cefotaxime resulted in a considerable decrease in toxin release and level of cytotoxicity in most of the STEC isolates when compared with control (without antibiotic exposure). Exposure to sub-MIC of ampicillin resulted in a relative increase in Stx release and cytotoxicity ($p \leq 0.05$) in three of the four isolates tested, whereas a decreasing trend was observed in isolates exposed to sub-MICs of gentamicin and cefotaxime. Sub-MIC of gentamicin resulted in largest decrease in Stx release and a similar trend was observed with cefotaxime to a lesser extent. In conclusion, these *in vitro* observations suggested that sub-MIC of ampicillin may stimulate Stx release and level of cytotoxicity and therefore should be avoided. Gentamicin did not show such effects and therefore may be considered for STEC antimicrobial therapy.

Introduction

Since its first report in 1982, Shiga toxin–producing *Escherichia coli* (STEC) has emerged as a major public health problem. The strains of STEC have been implicated as causative agents of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Thorpe, 2004). STEC is mainly characterized by the presence of two major virulence factors, Shiga toxin 1 (encoded by *stx1*gene) and Shiga toxin 2 (encoded by *stx2* gene) which are cytotoxic to Vero cells. Other major putative virulence factors can also contribute toward pathogenesis, which include intimin (encoded by *eae* gene) and plasmid encoded hemolysin (*hlyA* gene) (Tarr *et al*., 2005).

Beside STEC O157:H7, a number of non-O157 strains have also been reported for STEC infections. Different methods have been used for the identification of STEC. Nonsorbitol fermenting (NSF) STEC O157, the major serotype, can be distinguished as colorless colonies on sorbitol-MacConkey (SMAC) agar because of its inability to ferment sorbitol; however, sorbitol-fermenting (SF) STEC are also emerging as significant pathogens (Johnson *et al*., 2006). Among relatively modern techniques, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have been successfully applied for the identification of SF or NSF, O157, and non-O157 STEC strains (Bettelheim and Beutin, 2003).

The value of antimicrobial agents in treating STEC-mediated infections remains equivocal, because of the risk of increased release of Stx after exposure to different antimicrobial agents, as shown by *in vitro* and *in vivo* studies (Panos *et al*., 2006). Shiga toxin (1 or 2) release has been reported to be augmented by some of the antimicrobial agents, such as cotrimoxazole, cefixime, tetracycline (Walterspiel *et al*., 1992), fosfomycin (Yoh and Honda, 1997a), trimethoprim, azithromycin, gentamicin, ampicillin, penicillin G, streptomycin

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(Grif *et al*., 1998), cefdinir, levofloxacin (Murakami *et al*., 2000), and ciprofloxacin (Zhang *et al*., 2000).

In our study we have isolated and characterized STEC from an episode of diarrhea. Further, *in vitro* Stx release response and verocytotoxicity from isolated STEC strains were elucidated after exposure to minimum inhibitory concentration (MIC) and sub-MIC of ampicillin, gentamicin, and cefotaxime, which are currently among the commonly used antibiotics for the treatment of diarrheal patients in local hospitals.

Materials and Methods

Collection of samples

An episode of diarrhea was reported in 23 children who were from 2 to 10 years of age and admitted to Allied Hospital, Faisalabad, Pakistan, in August 2007. All patients were in hospital for less than 1 week and had four or more stool per 24 h with or without mucus or blood or both. The presenting symptoms ranged from watery to bloody diarrhea, fever, and acute gastroenteritis. Stool samples were initially enriched in 10 mL of tryptic soy broth (Merck, Darmstadt, Germany) and the overnight growth was streaked on MacConkey agar plates (Oxoid, Basingstoke, United Kingdom) and kept at 37°C overnight. No nonlactose-fermenting colonies were observed on these plates. All the colonies had typical morphology of *E. coli*, and thus they were identified as putative *E. coli*. Cultures from each plate were stored in tryptic soy broth (with 20% glycerol) at −20°C. Based on this observation and presenting symptoms, clinical pathologist suspected the STEC involvement. The cultures were transported to our STEC laboratory to determine the involvement of STEC.

Identification of STEC by ELISA

All the putative *E. coli* isolates were enriched in 4 mL of enterohemorrhagic *Escherichia coli* medium (EHEC-Direct Medium; Heipha, Eppelheim, Germany) at 37°C for 24 h with shaking. After incubation, the growth was centrifuged at 12,000 rpm for 5 min and the supernatants were checked for Stx using a commercial enzyme immunoassay kit (Premier EHEC; Meridian, Milano, Italy) as instructed by the manufacturer.

PCR for stx1, stx2, eae, *and* hlyA

All putative *E. coli* isolates were streaked onto SMAC agar (Oxoid) and incubated overnight at 37°C. SF and NSF colonies (five from each plate) were randomly selected from agar plates. For using as template, bacterial suspensions of 10^6 cells were used in the PCR reactions and *stx1* and *stx2* genes were detected using protocols published previously (Friedrich *et al*., 2002). The STEC isolates (*stx1*/*stx2*) were also investigated for the presence of virulence genes *eae* (Zhang *et al*., 2002) and *hlyA* (Schmidt *et al*., 1995) under the conditions described previously. Strain EDL933 was used as positive control, whereas *E. coli* K-12 strain C600 served as negative control. The amplification products of each PCR reaction were separated by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Phenotypic methods

Biochemical identification of *E. coli* was done using an API 20 E system (bioMérieux; Marcy l'Etoile, Lyon, France). Sorbitol fermentation was examined after overnight incubation on SMAC agar (Oxoid). β-Glucornidase activity was tested using Bactident kit (Merck) according to the manufacturer's instructions. The identification of *E. coli* O157:H7 serotype among the isolates was carried out using a latex agglutination test kit (Wellcolex; Remel, Dartford, United Kingdom). Non-O157:H7 serotypes were checked against *E. coli* O-antigens (1–181) and H-antigens (1–56) from boiled cultures using commercially available antisera (Statens Serum Institute, Copenhagen, Denmark). Motility in STEC isolates was also determined by several passages through 0.3% semisolid agar and those which lacked motility were defined as nonmotile.

Determination of MIC

The MICs of ampicillin, gentamicin, and cefotaxime (Oxoid) against various STEC isolates were determined by broth microdilution method (NCCLS, 2000). Briefly, twofold serial dilutions of antibiotics were prepared in Mueller–Hinton broth (Oxoid). Typical colonies of STEC from each SMAC agar plate were touched with a wire loop and transferred to the tube containing 1 mL of toxin-free *Limulus* amebocyte lysate (LAL) reagent water (Cape Cod, MA). An homogenous suspension was made and the turbidity of suspension was adjusted with LAL reagent water to match with the 0.5 McFarland standard so that the final concentration in each tube was $5\times10^{5^\circ}$cfu/mL and incubated at 37°C for 18 h. *E. coli* ATCC 25922 was used as an internal quality control. The MIC was defined as the lowest concentration of antibiotic showing no visible growth after overnight incubation, whereas culture containing 1/2 of the MIC was considered as sub-MIC. In each case, positive control was prepared by inoculating the STEC isolate in the absence of particular antibiotic.

Toxin assay at MIC and sub-MIC levels

The cultures containing MIC and sub-MIC of the antibiotic were centrifuged at 10,000 rpm for 10 min and the supernatants were used for the quantification of toxin amount using a commercial ELISA kit (Premier EHEC; Meridian) that uses 96-well microtiter plate coated with monoclonal antibodies specific for *E. coli* Shiga toxin 1 and 2. Each test was performed in duplicate according to the manufacturer's guidelines and the results were expressed as mean ± standard deviation.

The toxin release in the supernatant of control group (without antibiotic) was also measured. The amount of toxin released in the culture supernatant of isolate exposed to antibiotic was defined relative to the amount of toxin released in the culture supernatant of control group.

Assay for verocytotoxicity

Culture supernatants containing MIC and sub-MIC were filtered through 0.22 μm pore-size membrane filters (Roth, Karlsruhe, Germany). These sterile filtrates were tested (in triplicate) for verocytotoxicity in 96-well microtiter tissue culture plates as described previously (Grif *et al*., 1998). High and low controls were also included in each experiment. The high control referred to the maximum lactate dehydrogenase (LDH) release from control wells containing Vero cells, after exposure to Triton X-100 which causes maximum cell death. The minimum LDH release (low control) was determined by adding minimal essential media into the wells.

The microtiter plates were kept at 37°C for 48 h in 5% CO_2 incubator. For the quantification of Vero cell death or cytotoxicity, LDH release from damaged cells was measured using a cytotoxicity LDH kit (Roche Diagnostics, Mannheim, Germany). The cytotoxicity level of supernatant from pure culture (without antibiotic) was also determined.

Statistical analysis

The percentage amount of relative release of toxin was determined as follows: (optical density of culture supernatant pre-exposed to antibiotics/optical density of control group)×100. In the cytotoxicity experiment the percentage cytotoxicity was calculated as follows: (exposure value − low control)/(high control − low control)×100. One-way analysis of variance was performed to test the effect of antibiotics on cytotoxicity as induced by STEC isolates. The relative increase or decrease (%) was determined as the differences in cytotoxicity level between cultures that were exposed to antibiotics and those that were not.

Results

STEC detection by ELISA and PCR

Of the 23 putative *E. coli* isolates, five (21.7%) were ELISA positive for Stx production (Table 1). PCR analysis confirmed the presence of one or both Shiga toxin genes (*stx1* and *stx2*) in these isolates. Isolates M31 and M72 were found positive for *stx1*, M24 and M108 for *stx2*, and M91 for both genes (Table 1). PCR targeting virulence factors intimin (*eae*) and enterohemolysin (*hlyA*) showed positive amplification in all STEC isolates (Table 1).

Phenotypic characteristics

ELISA and PCR positive isolates were confirmed as *E. coli* by API 20 E. Sorbitol fermentation, β-glucornidase activity, and motility profile of STEC isolates are summarized in Table 1. Serotyping showed that of the five STEC, two were found to be O157:H7 (M24 and M108), one nonmotile O157 (M91), one O103:H2 (M72), and one nontypeable (M31) (Table 1).

Effect of antibiotics on toxin release

Broth microdilution ranges and MICs of ampicillin, gentamicin, and cefotaxime against STEC isolates are listed in Table 2. Although the isolate M31 was positive for STEC by ELISA and PCR, it was not included in this experiment as it yielded low toxin amount with ELISA and was nontypeable for O antigen. MIC level of all three antibiotics resulted in considerable decrease (data not shown) in Stx release from all four STEC isolates relative to control (without antibiotic); however, exposure to sub-MIC caused a variable response. Sub-MIC of ampicillin displayed a relative increase in toxin release by 90%, 20%, and 15.8% from isolates M72, M108, and M24, respectively, whereas no notable change was observed from isolate M91. Exposure to sub-MIC of gentamicin resulted in largest decrease in toxin release ranging from 38.5% to 88.2%, whereas the other antibiotic tested cefotaxime induced a relative decrease which ranged from 20% to 69.7% (Fig. 1).

Effect on verocytotoxicity

Sterile supernatants pretreated with MIC of ampicillin caused a significant decrease in the level of cytotoxicity (relative to control) in case of STEC isolates M24, M108, and M91, whereas a statistically nonsignificant increase was seen in case of isolate M72. However, sterile filtrates of isolates M24, M108, and M72 pre-exposed to sub-MIC exhibited a significant increase ($p \leq 0.05$) in cytotoxicity but no effect was observed in case of isolate M91 (Table 3). In all four isolates, the MIC of gentamicin resulted in a statistically significant decrease in cytotoxicity when compared with control (Table 3), whereas at sub-MIC level a nonsignificant decrease was detected. MIC level of cefotaxime inflicted a significant relative decrease in the level of cytotoxicity from three of the four isolates, whereas its sub-MIC did not show any significant effect on cell death (Table 3).

Discussion

STEC are important enteric pathogens which cause high morbidity and mortality. The incidence of STEC in developing countries, particularly because of absence of diagnostic facilities, is probably underestimated and overlooked (Tarr *et al.*, 2005).

STEC O157:H7 is the predominant serotype; however, there are more than 250 non-O157 STEC serotypes implicated in human infections. It is imperative to make precise and comprehensive diagnosis for both O157 and non-O157 (SF or NSF) STEC strains. For detection of STEC, many laboratories use a commercial immunoassay that detect Shiga toxin in fecal cultures (Fach *et al.*, 2003). On the other hand, PCR can detect *stx* genes from pure bacterial colonies, crude DNA

Table 1. Biochemical and Molecular Characterization of Shiga Toxin–Producing *Escherichia coli* Isolates

STEC isolates	*ELISA*	*PCR*				*Sorbitol fermentation*	*β-Glucornidase*	*Serotypes*	*Motility*
		stx1	stx2	eae	hlyA				
M24	+	−	+	+	+	−	−	O157:H7	+
M31	+	+	−	+	+	+	+	ONT[a]:H19	+
M108	+	−	+	+	+	−	−	O157:H7	+
M91	+	+	+	+	+	−	−	O157:H$^-$	−
M72	+	+	−	+	+	+	+	O103:H2	+

[a]ONT, O nontypeable.
STEC, Shiga toxin-producing *Escherichia coli*; PCR, polymerase chain reaction; +, positive; −, negative.

Table 2. Broth Microdilution Ranges and Minimum Inhibitory Concentration (μg/ml) of Different Antibiotics Against Shiga Toxin–Producing *Escherichia coli* Isolates

	Ampicillin		*Gentamicin*		*Cefotaxime*	
STEC isolates	*MIC (μg/mL)*	*Dilution range (μg/mL)*	*MIC (μg/mL)*	*Dilution range (μg/mL)*	*MIC (μg/mL)*	*Dilution range (μg/mL)*
M24	7.812	125–0.97	0.97	62.5–0.48	0.78	50–0.39
M108	15.62	125–0.97	1.95	62.5–0.48	0.78	12.5–0.09
M91	15.62	125–0.97	1.95	62.5–0.48	0.39	12.5–0.09
M72	7.812	125–0.97	0.97	62.5–0.48	0.58	37.5–0.29

MIC, minimum inhibitory concentration.

extracts, and mix culture growth, with more sensitivity and specificity (Ramotar *et al*., 1995).

In our study an episode of diarrhea at a local hospital was investigated for its STEC proportion. Both ELISA and PCR were used to detect Stx toxins and Stx genes.

To our knowledge there is a paucity of information on STEC infections in Pakistan, and to date, there are only two reports. Mufti *et al.* (1999) found only two STEC isolates in a prospective study of 99 diarrhea patients. The true incidence of STEC might be overlooked because of the lack of utilizing sensitive and specific techniques such as ELISA and PCR. In our previous work, we detected 11 (%) STEC from 200 stool samples using multiplex PCR on crude DNA from mix culture; however, STEC were not fully characterized (Mohsin *et al*., 2007). In our study we have used comprehensive methods based on EHEC-ELISA, culturing, and PCR to detect STEC. Biochemical characterization and complete serotyping were also conducted. Using ELISA and PCR strategies, STEC was found in 5 (21.7%) of 23 putative *E. coli* cultures. The 18 other *E. coli* isolates were non-STEC and hence not further studied. Although the sample size was small, the STEC serotype O157:H7/H$^-$ was found in three isolates, one was serotype O103:H2 and one was nontypeable (Table 1). Our finding is significant because it points toward the possibility of large outbreaks due to STEC and therefore stresses the need of incorporation of STEC detection in the routine diagnosis of enteric pathogens.

In our study we addressed another point of great contention, that is, role of commonly used antimicrobial agents (ampicillin, cefotaxime, and gentamicin) for treatment of diarrhea/STEC infections in local hospitals. Previous studies have shown that antimicrobial therapy for STEC-related infections is controversial. In a multivariate analysis, a strong association between antibiotic use and progression to HUS was observed (Wong *et al*., 2000). In another retrospective study, antibiotic treatment of patients with STEC enteritis showed higher risk of HUS (Dundas *et al*., 2001). More recently, Iijima *et al*. (2008) emphasized that antibiotics should not be administrated to patients with possible enteric STEC infections. Contrary to this, a meta-analysis suggested that antibiotic administration for STEC O157:H7 infection is not harmful and has no effect on developing HUS (Safdar *et al*., 2002). However, to date there is no recommended antibiotic that can ameliorate this illness (Iijima *et al*., 2008).

Ampicillin belongs to the penicillin group of betalactam antibiotics, which has several advantages in children (Diniz-Santos *et al*., 2006). Ito *et al*. (1997) reported that ampicillin caused an increase in Stx (1 or 2) release in culture supernatants of STEC, but another study found a decrease in Stx release with ampicillin (Yoh *et al*., 1997b). In our study, exposure

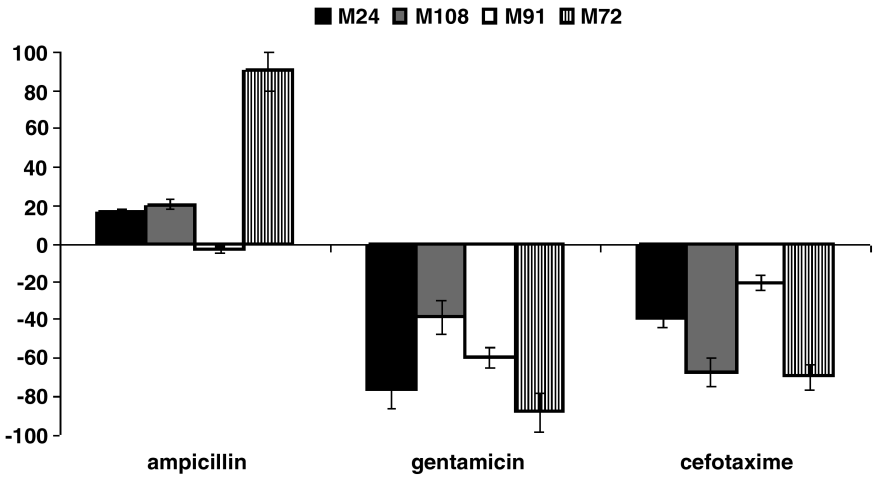

FIG. 1. Relative Shiga toxin release (%) at subminimum inhibitory concentration of antibiotics. The difference in the mean values of relative amount of toxin release (%) induced by ampicillin, gentamicin, and cefotaxime at subminimum inhibitory concentration level from Shiga toxin–producing *Escherichia coli* isolates. Bars extending upward from zero line of control showed a relative increase, whereas bars toward downward exhibited a relative decrease.

Table 3. Relative Cytotoxicity (%) upon Exposure to Antibiotics

	Relative cytotoxicity (%) upon exposure to MIC and sub-MIC of antibiotic					
	Ampicillin		*Gentamicin*		*Cefotaxime*	
STEC isolates	*MIC*	*sub-MIC*	*MIC*	*sub-MIC*	*MIC*	*sub-MIC*
M24	−30.70[a]	15[a]	−40[b]	−6.5	−47[b]	−2.5
M108	−19.12[a]	11[a]	−48.62[b]	−14.12	−21.62[b]	−8.7
M91	−36[b]	1	−65.3[b]	1.7	−25[a]	−12
M72	5	11[a]	−39.2[a]	−0.6	−20.55	7.95

Relative cytotoxicity in percentage was calculated as described in the Materials and Methods section. Negative values showed decrease in cytotoxicity relative to that caused by control group (without particular antibiotic).
[a] $p \leq 0.05$ considered significant.
[b] $p \leq 0.01$ considered highly significant.

to MIC of ampicillin resulted in a decrease in toxin release and cytotoxicity, but exposure to its sub-MIC augmented toxin release and had significant increases ($p < 0.05$) in the level of cytotoxicity in three of the four isolates. It has been documented that the increase in toxin amount could be due to lysis of cell wall followed by release of cell-bounded toxin or perhaps stimulated expression of toxin genes under instant bacterial response (Kimmitt *et al*., 2000; Panos *et al*., 2006).

Findings of some *in vitro* studies have shown that some cephalosporins such as cefixime and cefdinir stimulate the release of Stx in *E. coli* O157:H7 (Ito *et al*., 1997; Grif *et al*., 1998; Murakami *et al*., 2000). However, our results showed that MIC of cefotaxime caused a considerable decrease in Stx release and cytotoxicity in all STEC isolates, whereas sub-MIC exposure did not induce significant effect on cytotoxicity.

Aminoglycosides are clinically important group of antibiotics that inhibit protein synthesis and have a broad spectrum of activity. Gentamicin has been the most popular aminoglycoside in recent times because of trust built over years and relatively low cost. There are conflicting reports regarding its effect on release of both Stx1 and Stx2. One study reported its increasing effect on toxin release from STEC (Grif *et al*., 1998); however, the recent studies demonstrated suppression in toxin release from STEC carrying *stx*2 upon exposure to MIC or sub-MIC of gentamicin (Pedersen *et al*., 2008). We found a considerable decrease in Stx release at MIC and sub-MIC of gentamicin. Sterile filtrates containing MIC of gentamicin showed significant decrease in cytotoxicity from all STEC isolates, whereas decrease caused by sub-MIC was found statistically nonsignificant. Although the exact mechanism of toxin suppression remains to be revealed, it has been proposed that the decrease in toxin amount could be due to inhibition of protein synthesis, as some antibiotic molecules attach to the membrane-bounded ribosome involved in protein synthesis (Shibl, 1984). The suppression of late gene promoter of *stx* gene in case of protein synthesis inhibitor (gentamicin) is also reported, promising use of these agents in STEC infections (Pedersen *et al*., 2008).

Conclusions

STEC were found to be important pathogens causing diarrhea in local scenario. Our *in vitro* experimental data suggest that cefotaxime and gentamicin are safe at MIC level. However, further studies including more replicates and number of isolates are necessary to corroborate the hypothesis drawn from our preliminary study. Moreover, *in vivo*, factors such as plasma concentration of drug, antibiotic resistance, and host immune response may also influence the response to an antibiotic. The increase in toxin release and cytotoxicity at sub-MIC of ampicillin in some cases has highlighted that it is better to avoid its use in STEC-related illness, and if proper diagnosis is not available, in all diarrhea cases. This is especially relevant to developing countries where, because of financial constrains, inadequate, low-dose self-treatment for insufficient period is common and usually the treatment is stopped as soon as the severity of symptoms subsides.

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Disclosure Statement

No competing financial interests exist.

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