

Effects of ciprofloxacin on the expression and production of exotoxins by *Clostridium difficile*

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Hypervirulent BI/NAP1/027 strains of *Clostridium difficile* have been associated with increased mortality of *C. difficile* infection (CDI). The emergence of highly fluoroquinolone (FLQ)-resistant BI/NAP1/027 strains suggests that FLQ exposure may be a risk factor for CDI development. However, the mechanism for this is not clear. We compared the effects of subinhibitory concentrations of ciprofloxacin on Toxin A and B gene expression and protein production in recent (strain 039) and historical (strain 5325) BI/NAP1/027 clinical isolates with high- and low-level ciprofloxacin resistance, respectively. In the highly ciprofloxacin-resistant isolate (strain 039), ciprofloxacin significantly and dose-dependently increased Toxin A gene expression and shifted its expression to earlier in its growth cycle; TcdB gene expression also increased but was less sensitive to low-dose ciprofloxacin. Maximal Toxin A/B production (4 ng ml⁻¹) was increased twofold and occurred significantly earlier than in the untreated control. In strain 5325, ciprofloxacin at 0.25×MIC markedly increased both *tcdA* and *tcdB* expression but their temporal dynamics were unchanged. Maximal toxin production (250 ng ml⁻¹) was reduced approximately threefold compared with that of the untreated control. These results demonstrate significant differences in ciprofloxacin-induced toxin gene expression and protein production among BI/NAP1/027 isolates, and offer a new paradigm for FLQ-associated CDI caused by recent, highly antibiotic-resistant strains.

Received 13 December 2012

Accepted 14 February 2013

INTRODUCTION

Clostridium difficile is the leading cause of nosocomial diarrhoea in hospitals and long-term healthcare facilities worldwide. The incidence, severity and mortality associated with *C. difficile* infection (CDI) have increased drastically over the past decade due to the emergence of the hypervirulent BI/NAP1/027 (NAP1) strain. *C. difficile* NAP1 strains are characterized by increased cytotoxin [Toxin A (TcdA) and Toxin B (TcdB)] production and by increased resistance to several classes of antibiotics (Carter *et al.*, 2011; Hookman & Barkin, 2009; O'Connor *et al.*, 2009; Bartlett, 1992; Saxton *et al.*, 2009).

CDI is generally associated with recent or current administration of antibiotics, with clindamycin, the penicillins and cephalosporins carrying the greatest risk factors for the disease (O'Connor *et al.*, 2009). Fluoroquinolone (FLQ) antibiotics are among the most commonly prescribed antimicrobials, and several NAP1 outbreaks in hospitals in the United States, Canada and Europe have been attributed to FLQ usage (O'Connor *et al.*, 2009; Saxton *et al.*, 2009;

Deshpande *et al.*, 2008). While FLQs are now considered by some to be a major risk factor for CDI because of hypervirulent, FLQ-resistant strains, several case–control studies have demonstrated no significant relationship between FLQ usage and CDI development (Dhalla *et al.*, 2006; McFarland *et al.*, 2007; Novell & Morreale, 2010).

β-Lactam antibiotic-induced toxin production has been clearly demonstrated for *Staphylococcus aureus* (Stevens *et al.*, 2007) and other Gram-positive pathogens. Vancomycin, metronidazole, linezolid, amoxicillin and clindamycin have also been reported to induce toxin gene expression and increase toxin production by historical strains of *C. difficile* (Pultz & Donskey, 2005; Drummond *et al.*, 2003; Gerber *et al.*, 2008). Although FLQs have been shown to stimulate germination and growth of NAP1 strains (Saxton *et al.*, 2009; Adams *et al.*, 2007), the direct effects of FLQs on toxin gene expression and production by hypervirulent NAP1 strains have not been previously reported. Here, we describe the effects of subinhibitory doses of the FLQ antibiotic ciprofloxacin on *tcdA* and *tcdB* gene expression and soluble TcdA and TcdB production by two strains of *C. difficile* NAP1: the historical 5325 strain and a recent clinical NAP1 isolate, strain 039. These strains exhibit distinct differences in (i) exotoxin production *in vitro*, (ii) resistance to

Abbreviations: CDI, *Clostridium difficile* infection; FLQ, fluoroquinolone; TcdA, *C. difficile* Toxin A; TcdB, *C. difficile* Toxin B.

ciprofloxacin and (iii) their responses to antibiotic-induced toxin production following ciprofloxacin exposure. Our results demonstrate that recent, highly FLQ-resistant *C. difficile* isolates increase extracellular TcdA/B toxin production in response to ciprofloxacin. These findings offer a new mechanism to explain FLQ-associated CDI caused by NAP1 *C. difficile*.

METHODS

C. difficile strains. Two strains of *C. difficile* were studied. Strain 5325 is a historical NAP1 clinical isolate collected in 1993 (a kind gift from Dr Stewart Johnson, VA Chicago Health Care System, Chicago, IL, USA). *C. difficile* strain 039 was collected in 2005 from a critically ill patient with CDI at Johns Hopkins Medical Center, Baltimore, MD, USA (provided by Dr Karen Carroll). Both organisms were determined as toxinotype III strains by PFGE (data not shown).

Determination of MICs. Ciprofloxacin was purchased from Sigma-Aldrich. The MIC of this antibiotic was determined for *C. difficile* NAP1 strains by using the microbroth dilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for such testing in anaerobes (CLSI, 2004). In brief, 200 µl stationary overnight culture was used to inoculate 20 ml pre-reduced brain heart infusion (BHI) broth. Cultures were grown anaerobically for approximately 2 h at 37 °C until a turbidity equal to the 0.5 McFarland standard (OD₆₃₀ of 0.08–0.1; $\sim 1 \times 10^6$ c.f.u. ml⁻¹) was achieved. The prepared *C. difficile* (50 µl) was then added to duplicate wells of a 96-well plate containing 50 µl of twofold serially diluted (0.5–1024 µg ml⁻¹) ciprofloxacin. Plates were incubated anaerobically at 37 °C for 48 h and growth (turbidity) was assessed by a microplate reader (OD₆₃₀). MICs were defined as the lowest antibiotic concentration that inhibited measurable bacterial growth (i.e. OD₆₃₀ equal to the negative control). MICs were determined five times in triplicate.

Growth curves and RNA isolation. NAP1 isolates were cultured anaerobically overnight in BHI, whereupon bacteria were collected by centrifugation and washed once with fresh BHI. The pellet was resuspended to the original volume (10 ml) in fresh BHI and an aliquot of washed bacteria (4–6 ml) was added to 100 ml fresh, pre-equilibrated BHI to an OD₆₃₀ of 0.08–0.1. The culture was then added to 900 ml fresh, pre-equilibrated BHI and grown anaerobically to an OD₆₃₀ of 0.08–0.1. Aliquots (199 ml) of this stock culture were divided among five individual sterile 500 ml flasks. Ciprofloxacin was prepared in sterile water as a $\times 200$ stock solution with reference to the highest concentration required. Twofold serial dilutions were made from this stock in sterile water and 1 ml of each appropriate stock was added to 199 ml of *C. difficile* culture to give final concentrations of 0.25 \times , 0.125 \times and 0.0625 \times MIC, respectively. Sterile water (1 ml) served as a negative treatment control. At times -3 h (stock culture split), 0 h (antibiotics added) and 6, 12, 24 and 48 h after antibiotic addition, 10 ml samples were removed from each *C. difficile* culture and a small aliquot (20 µl) was used to determine viable c.f.u. The remaining organisms were collected by centrifugation (13 000 g) and used to prepare total RNA as described below for PCR analysis of toxin gene expression. Resultant supernatants were filter-sterilized and frozen at -70 °C for soluble TcdA/B production by ELISA.

Analysis of toxin gene expression and production. RNA was isolated from collected bacterial pellets using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's recommendations. Contaminating genomic DNA was removed by two rounds of DNase treatment (DNA-free kit; Ambion), and the final RNA yield and

quality were assessed by UV absorbance and agarose gel electrophoresis, respectively. Changes in *tcdA* and *tcdB* gene expression were assessed by real-time RT-PCR. For cDNA synthesis, 1 µg total RNA was converted to cDNA using M-MuLV reverse transcriptase (10 U ml⁻¹; New England BioLabs), 1 mM random hexamer primers (Invitrogen) and 0.5 mM dNTPs (Invitrogen); all final concentrations. Reverse transcriptase reactions were performed at 37 °C for 2 h and were terminated by heating to 95 °C for 5 min. cDNA samples were diluted 1:4 and 1:40 for strains 039 and 5325, respectively, in sterile water and used for subsequent PCR. In brief, real-time RT-PCR was performed using a 7500 Fast Real-time PCR System (Applied Biosystems) by using the RT² real-time SYBR green/Rox PCR master mix (SuperArray) and the following cycles: 10 min at 95 °C and then 40 cycles each at 95 °C for 15 s and 55 °C for 60 s. Primer sequences used for RT-PCR are listed in Table 1. Relative gene expression was determined using the 2^{- $\Delta\Delta$ Ct} method (Livak & Schmittgen, 2001). Sample mean Ct of 16S rRNA (internal control gene) was subtracted from the sample mean Ct of the *tcdA* and *tcdB* genes (Δ Ct). Delta-Ct (Δ Ct) of the no-treatment control for each time point was subtracted from the mean Δ Ct of each experimental sample ($\Delta\Delta$ Ct). This 2^{- $\Delta\Delta$ Ct} method yields fold change in gene expression of the gene of interest normalized to the expression of the 16S rRNA internal control and relative to the no-treatment control.

Soluble TcdA and TcdB levels were measured (in combination) in collected culture supernatant samples using the Wampole Tox A/B II kit (TechLabs) as described by Merrigan *et al.* (2010). TcdB purified from a NAP1 isolate (a kind gift from Dr Jimmy Ballard, University of Oklahoma Health Sciences Center; stock concentration 300 µg ml⁻¹) was used to construct a standard curve. Samples were diluted when necessary to obtain readings within the linear range of the standard curve. All samples were tested in triplicate.

RESULTS

MICs of historical and recent NAP1 strains

The MIC of ciprofloxacin was determined for both the recent 039 and historical 5325 *C. difficile* NAP1 strains. According to the CLSI and NCCLS interpretative categories for resistance, sensitive organisms have an MIC <8.0 µg ml⁻¹ (CLSI, 2004). In the recent 039 NAP1 strain, a strong resistance to ciprofloxacin was observed (MIC=256 µg ml⁻¹), while the historical 5325 strain displayed a lower resistance to the antibiotic (MIC=8 µg ml⁻¹).

Growth of recent and historical NAP1 strains in the presence of ciprofloxacin

The addition of subinhibitory concentrations of ciprofloxacin during the early exponential phase had little effect on growth of either strain 039 (Fig. 1) or strain 5325 (Fig. 2), although the absolute amounts of drug used differed by more than tenfold between strains. Some minor differences in growth dynamics were noted between strains, irrespective of the presence of antibiotic. Growth of both strains peaked at ~6 h; however, the recent 039 strain gradually declined thereafter, whereas the viability of the historical 5325 strain dropped by more than 1 log between 6 and 10 h and then maintained or slightly increased this level thereafter.

Table 1. Primer sequences for amplification of *C. difficile* *tcdA* and *tcdB* gene sequences

Gene	Primer	Sequence	Accession number
<i>tcdA</i>	tcdA-F	CAACACCTTAACCCAGCCATA	AM180355
	tcdA-R	AGAGTTTTCTGCGGTAGCTGA	AM180355
<i>tcdB</i>	tcdB-F	ATCTGGAGAATGGAAGGTGGT	AM180355
	tcdB-R	TGATGGTGCTGAAAAGAAGTG	AM180355
16S rRNA	16S-F	AGCGGTGAAATGCGTAGATAT	AM180355
	16S-R	CAGCGTCAGTTACAGTCCAGA	AM180355

Ciprofloxacin induces *tcdA* and *tcdB* gene expression and toxin production

Ciprofloxacin upregulated expression of both *tcdA* and *tcdB* in the recent NAP1 039 strain, although the dynamics of expression were subtly different between the two genes

(Fig. 1). Specifically, ciprofloxacin significantly and dose-dependently induced *tcdA* expression by 6 h (Fig. 1, solid line), which returned to baseline by 10 h. Ciprofloxacin-induced *tcdB* expression demonstrated a biphasic increase, peaking first at 6 h and then again at 24 h (Fig. 1; broken

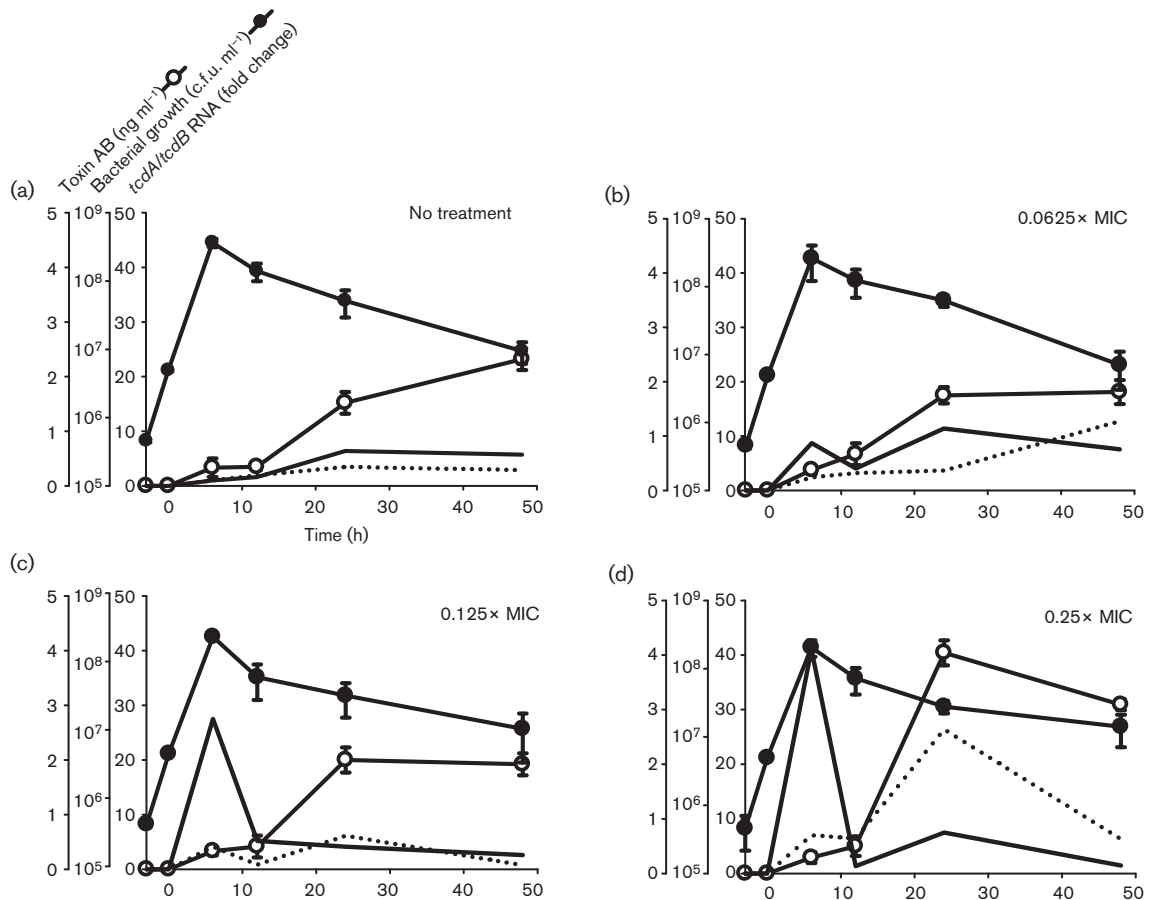


Fig. 1. Effects of ciprofloxacin on the growth, toxin gene expression and soluble toxin production by the *C. difficile* NAP1 039 strain. (a) Antibiotic-free cultures, (b) 0.0625xMIC (16 µg ml⁻¹), (c) 0.125xMIC (32 µg ml⁻¹) and (d) 0.25xMIC (64 µg ml⁻¹). Ciprofloxacin was added, at the final concentrations indicated, during early exponential phase growth (designated time 0). Samples were collected in duplicate over 48 h for quantification of viable *C. difficile*, and for measurement of toxin gene expression by real-time RT-PCR, and TcdA/B production by ELISA. Fold change in mRNA expression for each strain is made relative to that in its respective 'No treatment' control at 6 h (which was virtually zero in both strains). Filled line, *tcdA* mRNA; dotted line, *tcdB* mRNA.

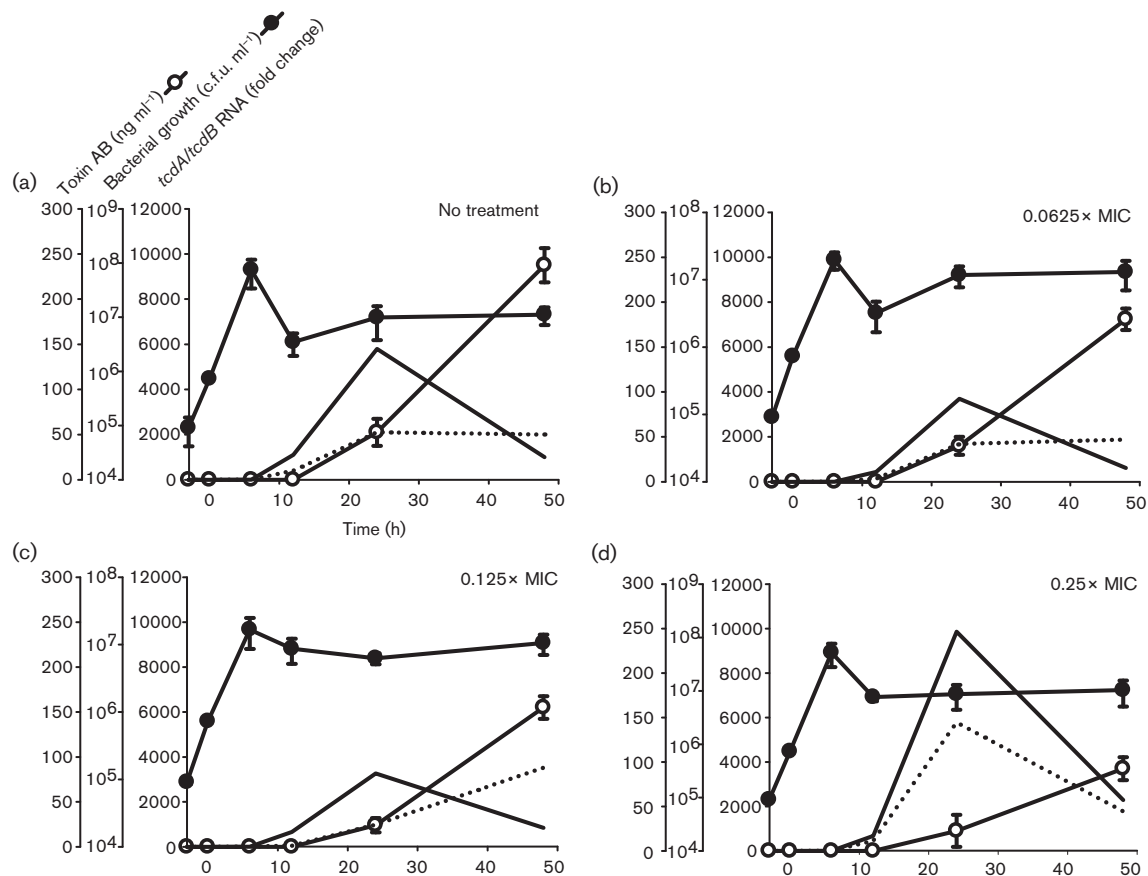


Fig. 2. Effects of ciprofloxacin on the growth, toxin gene expression and soluble toxin production by the *C. difficile* NAP1 5325 strain. (a) Antibiotic-free cultures, (b) 0.0625×MIC (0.5 µg ml⁻¹), (c) 0.125×MIC (1 µg ml⁻¹) and (d) 0.25×MIC (2 µg ml⁻¹). Ciprofloxacin was added, at the final concentrations indicated, during early exponential phase growth (designated time 0). Samples were collected in duplicate over 48 h for quantification of viable *C. difficile*, and for measurement of toxin gene expression by real-time RT-PCR, and TcdA/B production by ELISA. Fold change in mRNA expression for each strain is made relative to that in its respective 'No treatment' control at 6 h (which was virtually zero in both strains). Filled line, *tcdA* mRNA; dotted line, *tcdB* mRNA.

line). For both genes, ciprofloxacin-induced upregulation of *tcdA* and *tcdB* was maximal in the 0.25 × MIC (2 µg ml⁻¹)-treated culture, being 40-fold and fourfold greater, respectively, than antibiotic-free controls (Fig. 1a, d).

The early and significant ciprofloxacin-induced increase in toxin gene expression in strain 039 resulted in an approximately twofold increase in TcdA/B production and shifted the time to peak toxin level from 48 h in untreated control cultures (Fig. 1a; peak 2.3 ng ml⁻¹) to 24 h in ciprofloxacin-treated cultures (Fig. 1d; peak 4.0 ng ml⁻¹). At 48 h, TcdA/B levels declined slightly but remained higher than untreated cultures (Fig. 3).

In contrast to the highly ciprofloxacin-resistant recent 039 NAP1 isolate, subinhibitory concentrations of ciprofloxacin minimally stimulated *tcdA* and *tcdB* expression by the historical 5325 NAP1 strain (Fig. 2), and did so during the stationary phase of growth. Specifically, 1.7- and 2.7-fold increases in *tcdA* and *tcdB* transcripts, respectively, were

observed in cultures containing ciprofloxacin at 0.25 × MIC (2 µg ml⁻¹) at 24 h (Fig. 2d). Toxin expression levels were comparable with those of antibiotic-free cultures for all other ciprofloxacin treatments tested between 6 and 48 h. Consistent with the findings described for toxin gene expression, extracellular TcdA/B production by strain 5325 NAP1 was not increased by ciprofloxacin. In fact, 0.5–2.0 µg ciprofloxacin ml⁻¹ (0.0625–0.25 × MIC) dose-dependently decreased soluble TcdA/B levels in stationary phase supernatants when compared with no-treatment control cultures (Fig. 4).

DISCUSSION

The incidence, severity and mortality associated with *C. difficile*-associated disease (CDI) have worsened during the last decade, largely due to the emergence of hypervirulent NAP1 strains with their increased toxin production and

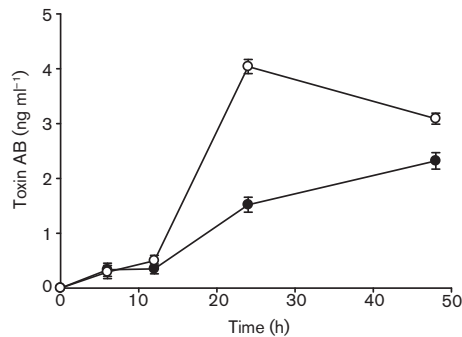


Fig. 3. Effects of ciprofloxacin on Toxin A and B production by the *C. difficile* NAP1 039 strain. Soluble Toxin A and B measured by ELISA in the 64 $\mu\text{g ml}^{-1}$ -treated (0.25 \times MIC) culture. Ciprofloxacin was added at the final concentration during early exponential phase growth, designated time 0. Culture supernatant samples were collected over 48 h for detection of TcdA/TcdB by ELISA. Data shown are the means \pm SD of three replicates. ●, No treatment; ○, 64 mg ml^{-1} .

broad antibiotic resistance profile. FLQ resistance in NAP1 strains ranges from mild (8 $\mu\text{g ml}^{-1}$) to strong (>512 $\mu\text{g ml}^{-1}$), and more recent isolates are trending towards the high end of the resistance spectrum (Saxton *et al.*, 2009). As a result, FLQ use has now become a major risk factor for CDI.

The traditional paradigm for the pathogenesis of antibiotic-associated CDI emphasizes antibiotic-induced alterations of the normal gut micro-organisms such that colonization and proliferation of toxinogenic *C. difficile* are favoured. However, a newer concept is emerging that antibiotic exposure induces CDI by not only changing

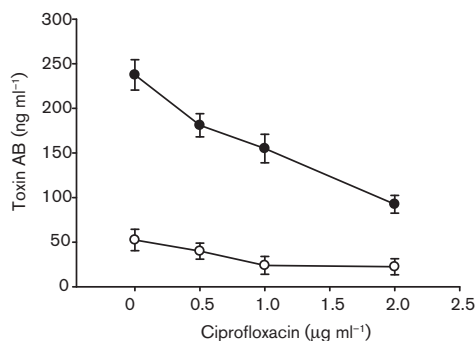


Fig. 4. Dose-dependent decreases in Toxin A and B production in *C. difficile* NAP1 5325 strain following ciprofloxacin exposure. Culture supernatant samples were collected at 24 and 48 h following the addition of ciprofloxacin at final concentrations of 0, 0.5, 1.0 and 2.0 $\mu\text{g ml}^{-1}$ (antibiotic free, 0.0625, 0.125 and 0.25 \times MIC, respectively) for detection of TcdA/TcdB by ELISA. Data shown are the means \pm SD of three replicates. ○, 24 h; ●, 48 h.

colonic micro-organisms but also by affecting the virulence properties of the organism. Honda *et al.* (1983) and Onderdonk *et al.* (1979) each demonstrated enhanced *C. difficile* enterotoxin production in response to sublethal amounts of antibiotics having different mechanisms of action. Similarly, Gerber *et al.* (2008) and Drummond *et al.* (2003) each showed that sub-MICs of drugs that either promote CDI (i.e. amoxicillin, clindamycin, cephalosporins) or those typically used to treat the infection (i.e. metronidazole, vancomycin, linezolid) upregulated toxin production by traditional (non-NAP1) strains of *C. difficile*. Both studies concluded that antibiotic-induced increases in *C. difficile* toxin production were part of a stress-induced response. Also, Gerber *et al.* (2008) have shown that antibiotic-induced toxin production in both laboratory strains and in traditional *C. difficile* clinical isolates is associated with a shift in toxin production to earlier stages of growth and that this is correlated with upregulation of *tcdA* and *tcdB* gene expression. Our findings extend these observations to include the toxigenic effects of FLQs on the more recent, hypervirulent NAP1 strains.

We further demonstrate that different NAP1 isolates, even within the same toxinotype, respond differently to subinhibitory antibiotic pressure based on their level of FLQ resistance, with increased toxin production being greatest in the highly FLQ-resistant strain. It is worth noting that the historical NAP1 5325 strain naturally produces nearly 250 times greater amounts of *tcdA* and *tcdB* transcripts and 100 times greater amounts of TcdA and TcdB than the recent NAP1 039 strain. Thus, the high background levels of toxin production in the no-treatment control may have masked any detectable antibiotic-induced increase in exotoxin production in this strain.

In our studies, we observed an \sim 18 h delay between the peaks of toxin gene expression and soluble toxin production in the recent 039 *C. difficile* isolate. Two mechanisms could explain this finding. First, Stevens *et al.* (2007) demonstrated that subinhibitory concentrations of nafcillin induced and prolonged mRNA transcripts for several toxins in *S. aureus*. Thus, it is plausible that sublethal doses of ciprofloxacin trigger a similar mechanism in *C. difficile*, inducing early *tcdA* and *tcdB* transcript production and increasing their half-lives until their translation in the stationary phase. Second, it is also possible that, following translation, TcdA and TcdB proteins remain intracellular during exponential phase growth and are released once organisms have entered the stationary phase. This latter scenario seems likely, as the holin-like protein, TcdE, is principally expressed in the stationary phase of growth where it is thought to facilitate exotoxin (TcdA and TcdB) release through permeabilization of the *C. difficile* cell wall (Tan *et al.*, 2001). In addition, normal cell death and lysis of *C. difficile* organisms during the stationary phase probably contribute to TcdA and TcdB release into the external milieu. Both of these potential mechanisms are currently being investigated in our laboratory.

Marked ciprofloxacin-induced upregulation of toxin expression and production by the recent NAP1 039 strain, which is 32 times more resistant to ciprofloxacin than the historical 5325 isolate, suggests that the mechanisms governing high-level antibiotic resistance are linked to pathways controlling virulence factor production. It is not known whether this phenomenon can be generalized to all hyper-drug-resistant, toxin-producing human pathogens, such as *Pseudomonas aeruginosa* or methicillin-resistant *S. aureus*. Such a phenomenon occurring *in vivo* could severely complicate management and adversely affect outcomes for patients with life-threatening infections due to toxin-producing organisms. It would also suggest that a conserved mechanism exists which could be exploited as a therapeutic target for treatment of a broad spectrum of infections caused by hyper-resistant 'superbugs'.

As the NAP1 039 and 5325 strains displayed diverse responses following challenge with ciprofloxacin, it is possible that these heterogeneous effects reflect strain-dependent responses to ciprofloxacin, given that only one FLQ antibiotic and two strains of *C. difficile* were examined. While our observations indicated that FLQ stimulated toxin production in some NAP1 strains, specific mechanisms of FLQ resistance have been identified in *C. difficile* that also contribute to FLQ-related complications of CDI (Ruiz, 2003).

In conclusion, exposure to several classes of antibiotics triggers CDI. Historically, most believed this was due to a suppression of normal bowel micro-organisms followed by colonization and proliferation of toxinogenic strains of *C. difficile*. However, our studies show that ciprofloxacin upregulates toxin gene expression and protein production in a hypervirulent NAP1 strain of *C. difficile* that is highly resistant to this antibiotic. Thus, our findings have important clinical implications when highly FLQ-resistant strains are prevalent in the community or hospital environments. In these instances, FLQ use could increase the incidence and severity of *C. difficile* colitis when patients are colonized with highly resistant organisms by increasing toxin production. Unfortunately, most clinical laboratories do not routinely culture *C. difficile* or determine susceptibility patterns of isolates. Thus, with the recent emergence of highly drug-resistant NAP1 strains, performance of routine antibiograms may gain renewed importance in the clinical microbiology laboratory and antibiotic stewardship committees may consider recommending the limited use of certain antibiotics when hyper-resistant strains are present.

ACKNOWLEDGEMENTS

This material is based upon work supported in part by the US Department of Veterans Affairs, Office of Research and Development Biomedical Laboratory Research Program (M. J. A., A. E. B., D. L. S.) and by the National Institutes of Health (grant P20 RR016454/GM103408 from the Idaho INBRE Program of the National Center for Research Resources).

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