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INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming, obligately anaerobic rod-shaped bacterium. After its first isolation in 1935 (Hall & O'Toole, 1935), the role of C. difficile as an important cause of antibiotic-associated diarrhoea remained undiscovered for a long time. Fortythree years later, in 1978, a correlation between C. difficileassociated diarrhoea (CDAD), pseudomembranous colitis (PMC) and antibiotics was established (Bartlett et al., 1978; Larson et al., 1978). The clinical appearance of C. difficile infection ranges from asymptomatic to mild self-limiting diarrhoea to severe diarrhoea with possible complications such as perforation, ileus and toxic megacolon. Nearly all

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Abbreviations: CDAD, Clostridium difficile-associated diarrhoea; CLI, clindamycin; LZD, linezolid; MTR, metronidazole; PMC, pseudomembranous colitis; VAN, vancomycin.

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Effect of sub-MIC concentrations of metronidazole, vancomycin, clindamycin and linezolid on toxin gene transcription and production in Clostridium difficile

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Clostridium difficile is the major cause of hospital-acquired infectious diarrhoea. Several antimicrobials are known to induce and promote C. difficile-associated diarrhoea (CDAD). The impact of metronidazole (MTR), vancomycin (VAN), clindamycin (CLI) and linezolid (LZD) on growth, toxin gene transcription and toxin production in C. difficile was investigated. Four C. difficile strains were grown with and without sub-MIC concentrations of MTR, VAN, CLI and LZD (0.5× MIC) and growth was measured by colony counts. Toxin production was detected using ELISA (for toxin A) and a cytotoxicity assay (for toxin B) in culture supernatants and also in sonicated cells. Real-time PCR was used to measure transcription of the toxin A and B genes. The aim of this work was to combine analysis of toxin A and B production by ELISA or cell culture assay with transcriptomic analysis. The four strains showed similar growth and different levels of toxin production in the absence of antibiotics. An antibiotic-free control showed toxin production at a late stage when the plateau phase of bacterial growth was reached, whereas antibioticexposed strains showed earlier toxin production. All of the antibiotics used except CLI increased the transcription rate of toxin genes. The findings of this study show that sub-MIC concentrations of antibiotics can cause changes in gene transcription of the major virulence factors of C. difficile. This study describes a new method for transcriptomic analysis of toxin genes in C. difficile.

> antibiotics are thought to be able to initiate C. difficile infection, but broad-spectrum agents such as cephalosporins, clindamycin and, as described recently, fluoroquinolones are the agents most frequently reported to be associated with CDAD and PMC (Dodson & Borriello, 1996; Jones et al., 1997; Kelly et al., 1994; Pepin et al., 2005). The pathogenesis is known to start with the colonization of the gut with C. difficile after the resident bacterial microbiota has been eliminated by antibiotics. The overgrowth of virulent strains of C. difficile results in the production of their major virulence factors: toxin A and toxin B (Farrell & LaMont, 2000). With molecular masses of 308 and 207 kDa, respectively, these toxins are the largest bacterial toxins known and express potent cytotoxicity and enterotoxicity (Dove et al., 1990). Toxin production is affected by a large number of factors, such as temperature, biotin limitation, amino acid concentration, glucose concentration and alteration of the oxidationreduction potential (Barc et al., 1992; Dupuy & Sonenshein, 1998; Honda et al., 1983; Ikeda et al., 1998; Karlsson et al., 1999; Onderdonk et al., 1979). An interesting effect on the production of toxin was described

by Onderdonk et al. (1979), whereby subinhibitory concentrations of vancomycin (VAN) and penicillin increased toxin production during continuous culture. It was supposed that the marked elevation of toxin titres in supernatant fluids was caused by a net efflux from the intracellular to the extracellular compartments induced by cell-wall-active antibiotics, although the mechanism of toxin increase remained unclear. Several years later, a toxin-inducing effect in C. difficile was recognized for clindamycin (CLI) and cephalosporins (Honda et al., 1983). Recently, many studies have provided information about the physiological effects of sub-MIC antibiotic concentrations in different species (Davies et al., 2006). The described changes induced by different antibiotics are highly diverse. Several studies on pharmacodynamic effects in C. difficile including sub-MIC concentrations have been published (Drummond et al., 2003; Odenholt et al., 2007). However, none has investigated the association of antimicrobials and toxin production with mRNA levels.

The aim of this study was to determine the effect of sub-MIC concentrations of antibiotics on the growth and toxin production of four strains of C. difficile. Four antibiotics were investigated, comprising agents that are used for the treatment of CDAD [VAN and metronidazole (MTR)], predispose for it (CLI) or have recently been discussed as a treatment possibility [linezolid (LZD)]. The study included experiments to differentiate between toxin levels in supernatant and sonicated cells. The increased toxin levels noted could reflect either a release of preformed toxin from the bacterial cell or synthesis of additional toxin. Previous studies that also aimed to differentiate between toxin levels in supernatants and sonicated cells have discussed enhanced production or release of toxin (Nakamura et al., 1982; Onderdonk et al., 1979). Here, a new method of quantitative real-time PCR using LightCycler technology was developed to determine the levels of gene transcription of the toxin A and B genes, tcdA and tcdB, in C. difficile.

METHODS

Strains of C. *difficile*. Four strains of C. *difficile* were used in this study: VPI 10463 (ATCC 43255), strain 2932 (a local endemic strain, toxinotype VIII, that was isolated at University Hospital Leipzig, Germany, which has a deletion in the pathogenicity locus and is unable to produce toxin A), strain 47 and strain 317 (both local endemic strains that were isolated at Bonn University Hospital, Germany). Strains VPI 10463, 47 and 317 all belong to toxinotype 0. Strains 2932 and 47 were positive for the *ermB* gene and showed MLS_B resistance. All strains were tested four times for their susceptibility to the four antibiotics investigated in this study using the Etest on Columbia agar. PCR of the *tcdA* and *tcdB* sequences was positive for all strains, but strain 2932 produced a shortened 1.3 kb A1 fragment due to the deletion in the pathogenicity locus. All strains were stored at -80 °C.

Antimicrobials. Antibiotic powders used in this study were VAN, MTR, CLI (all from Sigma) and LZD (Pfizer). Antibiotics were stored according to the manufacturer's instructions and prepared fresh daily

in sterile distilled water as a $10\,\times\,$ solution with reference to the highest concentration required.

Growth curves. Strains were grown on pre-reduced cycloserine/ cefoxitin/fructose agar (Oxoid) in an anaerobic chamber (Heraeus Instruments) for 48 h (George *et al.*, 1979). Anaerobic conditions provided by the anaerobic chamber were 80 % N₂, 15 % CO₂ and 5 % H₂ at 37 °C. After incubation and a purity check on pre-reduced Columbia agar (Oxoid) for another 48 h, one colony was inoculated in 5 ml pre-reduced brain heart infusion (BHI) broth (Oxoid) for 24 h. Ten microlitres was then added to another 5 ml pre-reduced BHI broth and incubated for 12 h.

Bacterial density was related to McFarland standards (McFarland $1=3 \times 10^8$ c.f.u. ml⁻¹; bioMérieux). By measuring OD₅₅₀, an inoculum of 3×10^6 cells ml⁻¹ was incubated in 250 ml BHI containing the appropriate antibiotic concentration (0.5 × MIC). An antibiotic-free control was used for each strain. All actions were performed under anaerobic conditions in an anaerobic chamber. All growth media were pre-reduced.

Bacterial growth was measured by counting plated cells at 0, 4, 8, 12, 16, 20 and 24 h. At each time point, serial 10-fold dilutions of the culture were plated on pre-reduced Columbia agar and incubated for 48 h. For each time point, samples were taken as follows: (i) 1 ml for subsequent toxin A ELISA; (ii) 1 ml for subsequent detection of toxin B in cell culture; and (iii) 25 ml for RNA extraction. Samples (i) and (ii) were centrifuged for 5 min at 6000 g and the supernatants were filtered (0.22 $\mu m;$ Sartorius) and transferred to new 1.5 ml tubes. The remaining pellets were sonicated three times (Vibra-Cell; Sonics & Materials) for 2 min, centrifuged for 4 min at 5300 g, filtered and transferred to new 1.5 ml tubes. Both supernatants and sonicated pellets were stored at -80 °C until toxin analysis. Sample (iii) was centrifuged for 5 min at 5300 g. The supernatant was discarded and the approximately 200 µl pellet was retrieved. The RNA was stabilized by the addition of 400 µl RNAprotect Bacteria Reagent (Qiagen) and stored at -80 °C until extraction. Samples were not stored longer than 1 week. All growth curves were performed in triplicate.

Toxin A ELISA. Levels of toxin A were measured by ELISA in supernatants as well as in sonicated cells using the ProSpecT 2 kit (Alexon-Trend) according to the manufacturer's instructions. Cells of each sample were sonicated three times for 2 min each using a Vibra-Cell. Two hundred microlitres of each sample was used in the assay. Reading of the microtitre plates was performed at a wavelength of 450 nm. An A_{450} reading above 0.1–3 was considered positive. No further dilutions of pellet and supernatant were made when A_{450} was above 3.

Toxin B cell culture. Toxin B titres from culture supernatants and from sonicated cells were measured using a cytotoxicity assay. An African green monkey kidney (Vero) cell line was used and suspended in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal calf serum (FCS; Seromed-Biochrom KG) and 2 µl gentamicin (Ratiopharm) ml⁻¹. The FCS was free of C. difficile toxin B antibodies. A negative control of gentamicin-free DMEM showed no cytopathic effect. Cell density was measured microscopically in a counting chamber (Feinoptik) and 2×10^4 cells were added to each well of a 96-well plate (Greiner). The cells were incubated for 24 h at 37 °C to achieve a homogeneous monolayer. One hundred microlitres of supernatant or sonicated cells diluted in DMEM was added to the Vero cell monolayers at dilutions ranging from 1:3 to 1:6561. After 48 h, the cytotoxic effect was documented using an inverted microscope equipped with a camera. The last dilution resulting in cytopathic effect was reported as the titre. All cytotoxicity tests were performed in duplicate.

Extraction of RNA. Two volumes of RNAprotect Bacteria Reagent (Qiagen) were added to the bacterial pellet to stabilize the bacterial mRNA. Because there is no stable expression of housekeeping genes in *C. difficile*, an internal standard was used to control RNA loss during the extraction process and to enable an accurate quantification of mRNA levels. The standard was of a similar length to the target sequence and contained the same primer binding sequence. Binding of hybridization probes by the RNA standard was different due to specifically introduced sequences. Thus measuring both standard and original target sequences in one tube after exposure to RNA extraction, reverse transcription and amplification was possible. Bacterial RNA was prepared using peqGOLD RNA Pure (peqLab) according to the manufacturer's instructions. Three samples were extracted for each condition. RNA concentration and purity were determined by measuring $A_{260/280}$.

After extraction, a DNase I (RQ1 RNase-Free DNase; Promega) treatment step was performed using 1 U DNase (μ g RNA)⁻¹ at 37 °C for 30 min. RQ1 DNase Stop Solution (Promega) was used to terminate the reaction and the DNase was inactivated by incubation at 65 °C for 10 min.

Reverse transcription. LightCycler RT-PCR was performed using a Qiagen OneStep RT-PCR kit. Specific primers and two different reverse transcriptases (Omniscript and Sensiscript) were used. Each transcriptase is highly specific for a certain length of template mRNA.

For cDNA synthesis, 4 μ l RNA was added to 20 μ l RT master mix, which contained each specific primer at a concentration of 1.2 μ M, 400 μ M dNTPs, 0.8 μ l Qiagen OneStep RT-PCR enzyme mix and 8 U RNasin (Promega) per reaction. The incubation time was 30 min at 50 °C. The initial PCR activation and also inactivation of the reverse transcriptases were performed at 95 °C for 15 min.

LightCycler RT-PCR. A primer pair was used to amplify a segment within the C. difficile tcdA1 gene (411 bp) and a second primer pair was chosen to amplify a segment within the C. difficile tcdB2 gene (403 bp) (Table 1). Specific hybridization probes (Table 2) labelled with LightCycler Red (LC Red) and fluorescein were designed. Synthesis of probes was carried out at Tib-MolBiol (Berlin, Germany). The master mix used for amplification and hybridization of probe based on detection of the tcdA1- and tcdB2-specific amplicons was as follows: 375 nM each primer, 150 nM each hybridization probe, 250 µM each dNTP, 1 U Platinum Taq DNA polymerase (Invitrogen), 10 ng BSA (Sigma) μ l⁻¹, 2 μ l corresponding template RNA, and 5 mM MgCl₂ for the tcdA1 PCR and 3.75 mM MgCl₂ for the *tcdB2* PCR. Negative and positive controls were included in all experiments. The negative control sample was prepared by replacing the DNA template with PCR-grade water. The positive control sample was prepared by adding 2 µl C. difficile RNA standard to the master mix. The following PCR profile was used for amplification and hybridization probe-based detection of the tcdA1- and tcdB2-specific amplicons: initial denaturation for 2 min at 95 °C; amplification for 50 cycles of 10 s at 95 °C, 10 s at 50 °C and 20 s at 72 °C (temperature transition rate: 20 °C s⁻¹; acquisition mode: single); followed by cooling for 2 min at 40 °C. Readout of LC Red 640 values was performed in channel F2 and readout of LC Red 705 values in channel F3.

RESULTS AND DISCUSSION

The MICs of the four antibiotics for each strain are shown in Table 3. Bacterial growth was assessed by counting colonies to determine the number of c.f.u. Fig. 1 shows growth curves for all four strains with and without the antibiotics. Variations in growth patterns among the four strains in the untreated control were small. After 4 h, each strain reached the exponential phase and after 12 h they reached the stationary phase. Under the influence of antibiotics, the stationary phase was reached later, confirming the results of an earlier study (Drummond et al., 2003). In antibiotic-free medium, toxin production in all four strains started at the same time. After 16 h, toxin B was found in the supernatant, whereas toxin A was detectable after 20 h. Generally, toxin B was detectable 4 h earlier than toxin A, and toxins were detectable 4 h earlier in sonicated cells than in supernatant. The levels of toxins measured by ELISA and cytotoxicity assay in supernatant and pellet for strain VPI 10463 are shown in Table 4. However, the amount of toxin produced was different among the strains. Strain VPI 10463 and 317 (titre of 1:6561 after 24 h) produced more toxin B than strains 2932 and 47 (titre of 1:2187 after 24 h) in supernatant and pellet. After 24 h, all strains except strain 2932 produced toxin A that exceeded the measurable level of the assay $(A_{450} \text{ of } 3.0).$

Under the influence of subinhibitory concentrations of antibiotics, variations in growth, toxin production and transcriptomic activity among the strains were observed. Subinhibitory concentrations of antibiotics seemed to delay bacterial growth and increased the time until the stationary phase was reached. Comparing the antibiotic-treated strains with the untreated control, toxin detection was possible up to 12 h earlier (Table 4).

During bacterial growth (4-16 h), toxin A and B production is supposed to be down-regulated due to the expression of *tcdC* or, as recently described, *codY* (Dineen *et al.*, 2007; Hundsberger *et al.*, 1997). The results of this study showed increased toxin A and B detection during the exponential phase for those strains exposed to sub-MIC VAN, MTR and LZD (Table 4). This effect was not noticed for CLI. In the stationary growth phase (20–24 h), toxin production reached and stayed at the same level as in the

Table 1. Primer sequences for amplification of C. difficile tcdA1 and tcdB2 sequences

Gene	Primer	Sequence (5′→3′)	Position (nt)	Length
tcdA1	A1F	GAGAAGTCAGTGATATTGCTCTTGA	12616-12640	411 bp
	A1R	CCCTGATATCTATCCCATGATTACT	13026-13002	
tcdB2	YT100	GTGCAGCAATCAAAGAGCTAAGTG	6980-7003	404 bp
	YT101	TGAACCACCTTCCATTCTCCAG	7383–7362	

Probe	Target gene	Sequence (5′→3′)*
HP A1-3	A1	AGGCTATAGTTGAATCTTCTACCACT-FL
HP A1-4		LC Red 640-AAGCATTACAGCTACTAGAGGAAGAGATT-PH
HP B2-3	B2	AAGGAGTATTTACTTTATTAGATGATAA-FL
HP B2-4		LC Red 640-ATAATGATGCCACAAGATGATTTAG-PH
HP-BpaF	RNA standard	GTTCTACCAAAGACCTGCCTGGGC-FL
НР Вра		LC Red 705-AGACAAGCCTGGAACCACTGGTAC-PH

Table 2.	Hybridization	probe	sequences
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*LC Red, LightCycler Red; FL, fluorescein; PH, phosphate.

controls without antibiotics. The results of toxin mRNA quantification were similar (Fig. 2).

To compare mRNA levels under different growth conditions, the number of mRNA copies was divided by the number of c.f.u. ml^{-1} (i.e. mRNA per cell). During the exponential phase of bacterial growth, the transcription ratio was increased up to 10^6 -fold under the influence of VAN, MTR and LZD, whereas the level of mRNA per cell in the untreated control remained below the level in the antibiotic-treated strains (Fig. 2). In the stationary phase, the transcription activity of antibiotic-treated strains was reduced. However, the antibiotic-free control showed high numbers of mRNA per cell. Again, results for CLI were different (no increase in mRNA per cell) from those for the other antibiotics used.

It is obvious that there is a strong association between use of antibiotics, *C. difficile* infection, CDAD and PMC. A prerequisite for *C. difficile* infection, the depletion of the normal protective bowel flora, is caused by broad-spectrum antibiotics. Once the balance of the gut is disturbed, opportunistic micro-organisms can cause an infection via the now-available binding sites and the excess of nutrients. The use of antibiotics leads to suppression of colonization resistance and promotes the development and progress of *C. difficile* infection, CDAD and PMC (Farrell & LaMont, 2000). Consequently, *C. difficile* is the major cause of hospital-acquired infectious diarrhoea.

In the last several years, studies have been published on antibiotics inducing CDAD not only by alteration of commensal gut flora but also by influencing pathogenicity via physiological effects (Drummond *et al.*, 2003). Over 20 years ago, the first reports suggested that certain antibiotics increased the production of the two *C. difficile* toxins

Table	3.	MICs	of the	four	antibiotics	(ma	$ ^{-1})$
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MIC	VPI 10463	Strain 2932	Strain 47	Strain 317
VAN	0.5	1	2	1
MTR	0.0175	0.06	0.75	0.25
LZD	0.75	16	1.5	1.5
CLI	4	>256	>256	4

(Honda *et al.*, 1983; Onderdonk *et al.*, 1979). As with *C. difficile*, antibiotics are considered to have an effect on the expression of virulence factors in other species, including *Escherichia coli*, *Vibrio cholerae*, meticillin-sensitive *Staphylococcus aureus* and meticillin-resistant *S. aureus* (Levner *et al.*, 1977; Ohlsen *et al.*, 1998; Yoh *et al.*, 1983). It is evident that concentrations of antibiotic lower than those required for inhibition induce multiple effects in almost all bacterial species (Davies *et al.*, 2006).

As shown in Table 4 and Fig. 2, toxin production was initiated earlier with subinhibitory concentrations of antibiotics than in antibiotic-free medium. Even at subinhibitory concentrations, antibiotics seem to cause stress to bacteria. The stress response includes activation of several genes that are thought to affect toxin promoters. A recent study showed that UviA, a UV-inducible regulator from Clostridium botulinum, was induced by treatment with UV light or mitomycin C after the cell experienced DNA damage. UviA is related to a special class of σ factors found in Clostridium species, responsible for activating transcription of toxin genes (Dupuy et al., 2005). Temperature also seems to be a factor influencing toxin production (Karlsson et al., 2003). Another study showed that antibiotics increase the levels of GroEL, a protein that works as a surface adhesin and helps C. difficile colonize recently vacated binding sites left by depleted normal bacterial flora (Hennequin et al., 2003). These findings suggest that, under adverse conditions such as the presence of antibiotics, increased expression levels of certain genes may help C. difficile to survive.

Four different antibiotics with distinct modes of action were investigated. VAN as a glycopeptide inhibits a late stage in bacterial cell wall peptidoglycan synthesis; MTR inhibits nucleic acid synthesis and leads to cell death. The oxazolidinone LZD inhibits protein synthesis by binding at the P site of the ribosomal 50S subunit. No studies have investigated the sub-MIC effects of LZD in *C. difficile* before. As a member of the lincosamide group, CLI inhibits bacterial protein synthesis and acts specifically on the 50S subunit of the bacterial ribosome. Application of usual doses results in bacteriostatic activity. Higher concentrations, as reached *in vitro*, may even be bactericidal (Spizek & Rezanka, 2004). CLI affects protein synthesis by blocking the ribosome.



Fig. 1. Growth of the four strains tested following exposure to 0.5× MICs of the described antibiotics. Strains: VPI 10463 (a), 2932 (b), 47 (c) and 314 (d).

Table 4. Toxin levels in supernatant and sonicated cell pellet

Results are shown for strain VPI 10463 only. Toxin A was measured by ELISA and is given as A_{450} values; toxin B was measured using a cytotoxicity assay and the results are given as titres. Positive results for either assay are shown in bold.

Antibiotic	Toxin	Time (h)						
		0	4	8	12	16	20	24
Control	Toxin A/supernatant	0	0	0	0.003	0.035	0.157	1.085
	Toxin A/pellet	0	0	0	0.003	1.392	3.0	3.0
	Toxin B/supernatant	0	0	0	0	27	81	243
	Toxin B/pellet	0	0	0	9	729	6561	6561
VAN	Toxin A/supernatant	0.005	0.321	0.596	0.567	0.564	0.650	0.560
	Toxin A/pellet	0.018	0.125	1.470	3.0	3.0	3.0	3.0
	Toxin B/supernatant	0	81	243	243	243	243	243
	Toxin B/pellet	3	81	243	729	729	729	729
MTR	Toxin A/supernatant	0.007	0.057	0.350	1.154	3.0	3.0	3.0
	Toxin A/pellet	0.038	0.456	3.0	3.0	3.0	3.0	3.0
	Toxin B/supernatant	0	81	243	729	6561	6561	6561
	Toxin B/pellet	27	243	2187	6561	6561	6561	6561
LZD	Toxin A/supernatant	0.009	0.003	0.005	0.029	0.199	3	3
	Toxin A/pellet	0.057	0.052	1.652	3	3	3	3
	Toxin B/supernatant	0	0	3	27	81	2187	6561
	Toxin B/pellet	0	27	243	2187	2187	6561	6561
CLI	Toxin A/supernatant	0.023	0.018	0.022	0.040	0.066	0.156	1.983
	Toxin A/pellet	0.047	0.037	0.054	0.441	2.264	2.096	2.181
	Toxin B/supernatant	0	0	0	3	27	81	2187
	Toxin B/pellet	3	3	9	243	2187	6561	6561





It seems that many antibiotics at low concentrations have the ability to influence gene transcription in a way that is distinct from their inhibitory effect (Goh *et al.*, 2002; Yim *et al.*, 2006). An example of the unexpected effects of antibiotics was found by Rothstein *et al.* (2005). They showed that β -lactam antibiotics can stimulate the glutamate transporter GLT1 in cell culture and in animal models (Rothstein *et al.*, 2005).

Further studies of the interactions of sub-MIC concentrations of antibiotics with bacteria and the host could establish effects as described in this study as pharmacological parameters. Pharmacodynamics are important to determine optimal dosing schedules of antibiotics. The relevance of sub-MIC effects may be enhanced when it comes to resistant strains. Here, not only is the MIC higher than in susceptible strains, but the sub-MIC range is also extended. It is possible that a therapeutically applied supraMIC instantly becomes a sub-MIC in a patient with resistant strains. In this case, it seems possible that the antibiotic does not cause a reduction in *C. difficile* levels, but an induction of virulence factors. Although there is only one report on resistance against MTR and VAN, which was not confirmed with further studies (Pelaez *et al.*, 2002), the possibility of resistance occurring in the future cannot be excluded.

The present study showed that subinhibitory concentrations of various antibiotics modulated transcription of the *tcdA* and *tcdB* genes encoding the *C. difficile* toxins A and B. The main findings were that VAN, MTR and LZD strongly induced *tcdA* and *tcdB* transcription and toxin production in the exponential phase of bacterial growth. This study also indicated that, under subinhibitory concentrations of antibiotics, the increase and earlier release of toxins is a specific process and cannot be explained simply by stress phenomena resulting from destabilization of the bacterial cell.

It is difficult to interpret the effects of antibiotics at sub-MICs with reference to their clinical use. Observations from *in vitro* experiments cannot be transferred to *in vivo* conditions. However, it can be assumed that exposure to antibiotics above the MIC will always be followed by sub-MICs. At this stage of research, advice on clinical therapy based on the results of this study cannot be given. The gut model used by Baines *et al.* (2005) could be a promising approach for a closer *in vivo*-like investigation of antimicrobial agents and toxin production in *C. difficile.*

Recent reports of new epidemic strains of ribotype 027 indicate that there is a correlation between disease severity and different toxin strain variants (Warny *et al.*, 2005). LZD was found to be effective against *C. difficile* (Ackermann *et al.*, 2003), and VAN and MTR are first-line therapy drugs in the treatment of *C. difficile* infection. The results presented here show that, *in vitro*, these antibiotics can induce the production of toxins A and B in *C. difficile*.

To our knowledge, this is the first report that describes a mRNA quantification method based on LightCycler technology in *C. difficile*. As most CDADs are multifactorial, further studies, including animal models and clinical trials, are necessary to elucidate more clearly the effects of antibiotics on the pathogenesis of severe *C. difficile* infection. As a major nosocomial pathogen, *C. difficile* continues to be the subject of intense research, providing a greater understanding of the complex pathogenesis by which CDAD develops. In this regard, sensitive gene-based hybridization techniques, such as that described in this report, are valuable tools that may help gain further insights into the pathogenesis and physiology of *C. difficile*.

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