The effect of antimicrobials on expression of *Clostridium difficile* toxin B and binary toxin genes *in vitro*

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INTRODUCTION

Toxins A and B are considered the main *C. difficile* virulence factors. Several environmental factors affecting *tcdA* and *tcdB* gene expression have been reported. The presence of glucose and other rapidly metabolizable sugars and certain amino acids in the growth medium have been shown to suppress toxin expression[1,2]. The transition from ambient temperatures to 37˚C or between growth phases has been shown to increase *tcdA* and *tcdB* expression[3,4]. Exposure to sub-MIC levels of various antimicrobials also also increases expression[4]. The commonality between these exposures that induce toxin expression may be the stress exerted on the cells by unfavourable growth conditions.

The *C. difficile* binary toxin is an ADP-ribosyltransferase and its role in pathogenesis is poorly understood. An idea has been put forth that it may enhance virulence by acting in synergy with Toxin A and B and increasing the susceptibility of the host to the effects of these major toxins[5]. If the binary toxin does act in synergy with TcdAB, similar environmental factors may exert similar effects on binary toxin gene expression.

OBJECTIVE

The objective of this research was to characterize the expression of *cdtA*, and its putative regulator, *cdtR* in response to sub-minimum inhibitory concentrations (MIC) of levofloxacin, clindamycin, and enrofloxacin.

MATERIALS AND METHODS

A NAP1/toxinotype III/ribotype 027 and a NAP7/toxinotype V/ribotype 078 strain of *C. difficile* was chosen for study and culture in a growth medium as previously described[6].

To examine the effects of antibiotic exposure, both strains were grown in 1/8 the minimum inhibitory concentration (MIC) for 3 different antibiotics (Table 1); levofloxacin (LEVO), clindamycin (CLIN)and enrofloxacin (ENRO) (all from Sigma-Aldrich, Oakville, ON).

Table 1. The minimum inhibitory concentrations of the antimicrobials assessed and the concentrations used in *C. difficile* ribotypes 027 and 078.

Ribotype	Antimicrobials	MIC (µg/ml)	Concentration used (µg/ml)
027	LEVO	64	8
	CLIN	12	1.5
	ENRO	8	1
078	LEVO	2	0.25
	CLIN	4	0.5
	ENRO	8	1

Ten ml of culture were removed during exponential phase (OD_{600} 0.3-0.4) and early stationary phase (OD_{600} 1.0) for RNA isolation. The cultures were centrifuged and the pellet was resuspended in 5 volumes of RNA*later* reagent (Ambion Inc., Austin, TX) and kept at 4˚C overnight and at -80˚C for long term storage. Isolates were cultured in triplicate.

RNA was isolated using either the The Maxwell® 16 Automated system and the Low Elution Volume Total RNA Isolation kit (Promega, Madison, WI) or the RNeasy mini kit (Qiagen Inc., Mississauga, ON) following the manufacturer's instructions. All RNA samples were DNAse treated with the DNAfree DNAse Kit (Ambion, Austin, TX) according to the manufacturer's instructions and the absence of DNA contamination was confirmed when no amplification of a 16S gene fragment occurred by quantitative real-time PCR (qPCR) as previously described[6] .

MATERIALS AND METHODS

Fifty ng of total RNA were used in reverse transcription reactions using the Omniscript RT PCR kit (Qiagen Inc., Mississauga, ON) and 0.5 mM of each primer as described by the manufacturer. The cDNA was immediately stored at -20˚C until use.

Standard curves for the reference and target gene primers were generated as previously described[6]. The reference genes *gluD*, *tpi* and *gyrA* were used for ribotype 078 and *rpsJ*, *adk* and *rrs* were used for ribotype 027 as previously described[6]. qPCR was performed on all target and reference genes as previously described on 3 biological replicates for exponential and stationary phase for each ribotype[6].

Expression analysis was performed using the geometric mean of the raw reference gene expression values to calculate a normalization factor to normalize expression of the target genes as described by Vandesompele et al[7] Expression levels were compared using the Student's t-test and differences were considered significant if $P < 0.05$.

RESULTS

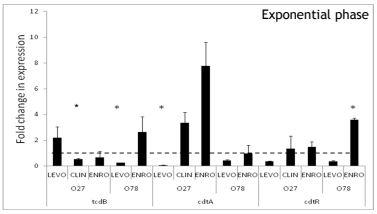

Figure 1. Fold change in gene expression of *C. difficile* ribotypes 027 and 078 in response to sub-MIC antimicrobial treatments (levo : levofloxacin, clin: clindamycin, enro: enrofloxacin) during exponential phase growth. Stars indicate fold changes in expression that are statistically significant ($P < 0.05$). The dashed line indicates a ratio of 1 which is no change in expression. Below 1 indicates repression of expression and above 1 indicates and increase in expression.

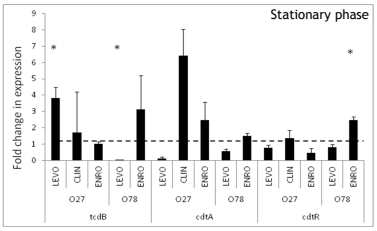

Figure 2. Fold change in gene expression of ribotypes 027 and 078 in response to sub-MIC antimicrobial treatments during stationary phase growth. Stars indicate fold changes in expression that are statistically significant ($P < 0.05$). The dashed line indicates a ratio of 1 which is no change in expression. Below 1 indicates repression of expression and above 1 indicates and increase in expression.

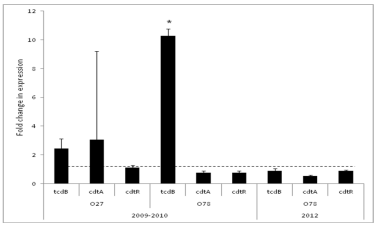

Figure 3. Fold change in gene expression of ribotypes 027 and 078 comparing growth phases between data collected in 2009-2010 and 2012. Stars indicate fold changes in expression that are statistically significant ($P < 0.05$). The dashed line indicates a ratio of 1 which is no change in expression. Below 1 indicates repression of expression and above 1 indicates and increase in expression.

RESULTS

Standard curves were performed for all primer pairs, in order to use the slope of the curve to calculate PCR efficiencies. The efficiencies range between 1.69 - 1.97.

Figures 1 and 2 display the fold change in expression of each of the target genes for both ribotypes in response to sub-MIC exposure to antimicrobials for exponential and stationary phase growth, respectively. Figure 3 displays the fold change expression between data collected in 2009-2010 and 2012.

DISCUSSION

Clindamycin administration is a well-recognized risk factor for CDI, its effect on gene expression in ribotype 027 was insignificant with the exception of the exponential phase *tcdB* repression which has been previously reported[4]. Possibly, toxin repression allows colonization of the intestinal cells to progress unhindered by the immune response which would be triggered by toxin production. Toxin gene expression could then resume upon completion of the course of clindamycin.

The effect of clindamycin in ribotype 078 was not examined because of its high susceptibility, since there was concern about studying the effect of the antimicrobial far below a biologically relevant concentration.

Levofloxacin exposure resulted in increased expression of *tcdB* in ribotype 027 but decreaesd expression of other genes. Although this effect has not been previously reported, this result is consistent with a study that showed higher yields of toxin B in feces of *C. difficile* infected fluoroquinolone-exposed mice compared with controls[8]. In contrast, *tcdB* was repressed in ribotype 078. It is unclear why such a disparity occurred. The stimulatory effect of levofloxacin on *tcdB* expression could play an important role in virulence of this strain.

Enrofloxacin lacks any anaerobic spectrum[9] and was chosen to determine if the effects on gene expression were unrelated to their mechanisms of action. It had no significant effect except for *cdtR* in ribotype 078. An increase in expression was observed in both phases in this strain. There was no increase in *cdtA* expression supporting the notion that regulatory mechanisms other than *cdtR* exist and *cdtA* expression is not completely dependent on or correlated with *cdtR* expression.

The inconsistent results between growth phases of untreated ribotype 078 between 2009-2010 and 2012 experiments are concerning. There was no statistical significance between the *cdtA* and *cdtR* expression levels between the experiments but the difference between *tcdB* expression levels were statistically significant (*P*= 0.011).

Additional studies will determine if the changes in expression are a result of a specific interaction between the antimicrobial and bacterial cell or are part of a more generalized stress response. and what role the altered expression of these genes play in survival, adaptation, colonization and/or the pathogenesis of infection.

The impact of antimicrobials (and other drugs) on gene expression and subsequent bacterial survival, adaptation, colonization and virulence requires further study.

REFERENCES

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