INTRODUCTION
Toxins A and B are considered the main C. difficile virulence factors. Several environmental factors affecting toxigenic C. difficile gene expression have been reported. The presence of glucose and other rapid 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 increases expression\(^5\). The commonalities 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 TcdB and increasing the susceptibility of the host to the effects of these major toxins\(^6\). If the binary toxin does act in synergy with TcdB, 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\(^7\).

To examine the effects of antibiotic exposure, both strains were grown in 1/8 the minimum inhibitory concentration (MIC) for 3 biological replicates for ribotype 027 and 078.

Ten ml of culture were removed during exponential phase \((\text{OD}\_600 \text{nm} 0.3-0.4)\) and early stationary phase \((\text{OD}\_600 1.0)\) for RNA isolation. The cultures were centrifuged and the pellet was resuspended in 5 volumes of RNAlater reagent (Ambion Inc., Austin, TX) and kept at 4°C overnight and at -80°C for long term storage. Isolates were cultured in triplicate.

The effect of antimicrobials on expression of C. difficile toxin B and binary toxin genes \textit{in vitro}

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RESULTS
Fifty ng of total RNA were used in reverse transcription reactions using the Omniscript RT PCR kit (Qiagen Inc., Mississauga, ON) and 0.5 ml 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\(^8\). The reference genes g3pdh, gpd and gyrA were used for ribotype 078 and rplA, adk and rrs were used for ribotype 027 as previously described\(^9\). qPCR was performed using targeted reference genes as previously described on 3 biological replicates for exponential and stationary phase for each ribotype\(^6\).

Expression analysis was performed using the comparative threshold cycle \((\Delta \Delta Ct)\) method to calculate a normalization factor to normalize expression of the target genes as described by Vandesompele et al\(^6\). Expression levels were compared using the Student’s t-test and differences were considered significant if \(\text{P} \leq 0.05\).

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\(^10\). 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 rrsB in ribotype 027 but decreased 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 control\(^11\). 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 anerobic spectrum\(^12\) 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 cdtB expression levels between the experiments, but the difference between rrsB expression levels were statistically significant \((\text{P} \geq 0.011)\).

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