Reference gene evaluation and toxin gene expression in *Clostridium difficile*

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Introduction

Quantitative real-time PCR (qPCR) is a sensitive and accurate technique for quantification of gene expression. Stable reference genes are required for normalization of expression data to correct for intrinsic and technical variations introduced throughout the experimental process [1]. To date, no thorough evaluation of *C. difficile* housekeeping genes has been performed to identify an ideal, stable candidate reference gene. The objective of this study was to evaluate 8 different possible reference genes to identify optimal genes or gene combination for use in gene expression studies and to use these genes to assess expression of 2 toxin genes (*cdtA*, and *tcdB*) and the putative binary toxin regulator (*cdtR*) in response to growth phase and an antibiotic treatment.

Materials and Methods

Bacterial strains. Three *C. difficile* isolates were chosen for study: NAP2/toxinotype 0/ribotype 001, NAP1/toxinotype III/ribotype 027 and a NAP7/toxinotype V/ribotype 078. Isolates were grown in a *C. difficile* growth medium (2) and incubated anaerobically at 37°C. Ten ml of culture were removed at the exponential (OD_{600} 0.3-0.4) and stationary phases (OD_{600} 1.0) of growth, centrifuged, and the pellets were resuspended in 2 volumes of RNAlater (Ambion, Austin, TX) reagent and stored at -80°C immediately until use.

Candidate Reference Genes. A total of 8 candidate reference genes were assessed for stability in the *C. difficile* isolates during exponential and stationary phases of growth (Table1). All putative reference genes fragments were amplified, purified, and cloned into a pDrive cloning vector (Qiagen) in order to generate a standard curve to determine PCR efficiency. In triplicate, a $\log_{10}$ dilution series of plasmids harbouring the reference gene fragments (confirmed by sequencing) were assayed and PCR efficiencies were determined using the slope of the curve and the formula: 10E (-1/slope).

Reference Gene Validation. Total RNA was isolated from the 2 phases of the 3 *C. difficile* isolates using a Maxwell®16, a robotic, magnetic bead-based system (Promega) and samples were treated with the Turbo DNA-free DNase system (Ambion). Fifty ng was used in a reverse transcription reaction using the Omniscript Reverse Transcription Kit (Qiagen). In triplicate, cDNA from both phases of growth from the three strains were subjected to qPCR for each candidate reference gene. The stability of the 8 reference genes was examined using the visual basic application for excel program geNorm (3). Relative expression levels were entered into the geNorm program and stability values (designated M) were calculated based on the mean of pairwise variation between each gene versus all other candidate genes tested. A stepwise elimination of genes based on highest M-values (indicating the lowest stability), allows for each gene to be ranked based on its expression stability.

***cdtA*, *cdtR*, and *tcdB* qPCR.** The 3 genes identified by geNorm to exhibit the most overall stability for each strain were used to normalize expression of 3 target genes in response to growth phase and a sub-MIC (1/4) concentration of levofloxacin (Levo). Expression levels were compared using the Student's t-test and differences were considered significant if $p<0.05$.

Results

Table 1. Features of the 8 candidate *C. difficile* reference genes assessed for stability in 3 *C. difficile* ribotypes and between exponential and stationary phases of growth.

Candidate reference genes	Slope of the standard curve	PCR efficiency	Melting Temperature (°C)
rpoA	-4.0	1.78	77.97
tpi	-4.4	1.69	77.59
rho	-4.2	1.73	77.59
gyrA	-4.1	1.75	80.97
gluD	-3.5	1.93	79.70
rrs	-3.4	1.97	85.68
rpsJ	-3.6	1.89	78.67
adk	-3.7	1.86	77.35

•All primers used have PCR efficiency values that ranged between 1.69 and 1.98 (Table 1) and all reactions produced single PCR products of the expected size when visualized on an agarose gel.

•The most stable reference genes overall, as indicated by the lowest M-values, were *rrs*, *adk*, and *rpsJ* while the genes *rho* and *rpoA* gave the highest M-value (indicating the lowest stability).

•If strain specific analysis is performed the three most stable reference genes for the ribotype 027, 078 and 001 strains are: *rpsJ*, *adk*, and *rrs*; *gluD*, *tpi*, and *gyrA*; *rrs*, *gluD*, and *rho*, respectively (Table 2).

Table 2. Ranking of the 8 *C. difficile* candidate reference genes.

Rank	Overall (M values)	Ribotype 027 (M values)	Ribotype 078 (M values)	Ribotype 001 (M values)
1(best)	*rpsJ* (0.66)	*rpsJ* (0.38)	*gluD* (0.37)	*rrs* (0.53)
1	*adk* (0.66)	*adk* (0.38)	*tpi* (0.37)	*gluD* (0.53)
3	*rrs*(0.88)	*rrs* (0.54)	*gyrA* (0.58)	*rho* (0.75)
4	*gluD* (0.95)	*gluD* (0.63)	*rrs* (0.80)	*tpi* (0.85)
5	*gyrA* (1.03)	*rho* (0.71)	*rpsJ* (0.91)	*rpsJ* (0.94)
6	*tpi* (1.11)	*rpoA* (0.91)	*adk* (1.03)	*adk* (1.01)
7	*rpoA* (1.29)	*gyrA* (1.14)	*rho* (1.11)	*gyrA* (1.09)
8 (worst)	*rho* (1.44)	*tpi* (1.33)	*rpoA*(*1.29*)	*rpoA* (1.24)

•Levo treatment suppressed expression of *cdtA* and *cdtR* in the 027 and 078 strains but an increase in expression was observed in *tcdB* the 027 strain.

•Levo treatment also resulted in 2 significant increases in expression between exponential and stationary phase (*cdtR* and *tcdB*) in 027 but no significant effect on expression in 001.

•Repression of expression was observed in 078, the effect most pronounced for *tcdB*. This 078 strain differs from the 027 and 001 strain in that it is levo susceptible while the other 2 isolates are resistant.

Discussion

•Increased *tcdA* and *tcdB* expression has previously been reported in response to stationary phase growth versus exponential and to antibiotic treatment (4,5).

•Overall, a similar trend was observed for *cdtA* expression between growth phases compared to *tcdB* (results reported here and in the literature (4)). This suggests regulation of these toxins may occur by similar mechanisms.

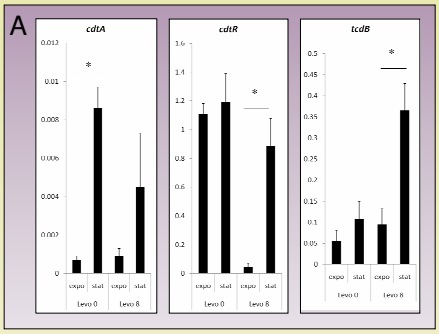

Figure 1. Relative gene expression of *cdtA*, *cdtR*, and *tcdB* in (A) a ribotype 027, (B) a ribotype 078, and *tcdB* in (C) a ribotype 001 strain using qPCR and the 3 most stable reference genes for each ribotype as determined by geNorm. The stars indicate a statistically significant difference between stationary and exponential phase using the Student's t-test ($p<0.05$).

•The variability of expression between isolates likely reflects the heterogeneity of *C. difficile*, particularly in isolates belonging to different ribotypes. This heterogeniety is also likely why there is strain dependent reference gene stability. Based on these results, we would recommend assessing the stability of multiple genes within each strain being studied.

•In order to get a more thorough understanding of the effects of antibiotics treatment on expression, additional antibiotics such as clindamycin or other fluoroquinolones will be investigated as well as expression in other 078 isolates that are levofloxacin resistant.

References: (1) Huggett et al. 2005. Genes and Immun. 6:279:284. (2) Metcalf et al. 2010.Anaerobe.16:439-443. (3) Vandesompele et al. 2002. Genome Biol.3:7. (4) Merrigan et al. 2010. J. Bacteriol. Epub ahead of print. (5) Gerber et al. 2008. J. Med. Microbiol. 57:776-783.