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Absolute versus relative presentation of tcdB Clostridium difficile biomarker

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Abstract

Clostridium difficile infection is the major etiologic agent of antibiotic-associated pseudomembranous colitis, a disease that can be fatal if unrecognized, or untreated. On average, there are 15000 deaths and 500000 new cases per year, in the USA. Diagnostic biomarkers currently used are the tcdB gene, or its gene product (toxin B). Clinical interpretation of the assay is particularly challenging: (1) biomarker detection is possible without manifestation of symptoms and (2) missing biomarker due to assay sensitivity limitations can be fatal. To resolve clinical uncertainty, quantitative analysis has been considered. Despite multiple efforts, a quantitative tcdB/toxB threshold with a meaningful clinical impact has yet to be established. Herein we shed light as to why mass/volume-based normalisations were fruitless in the past. Specifically, measuring total bacterial flora (using "universal bacterial" 16S qPCR rDNA assay) to calculate relative abundance of *C difficile*, we demonstrate a strong and significant negative correlation between tcdB biomarker of *C difficile* (R^2=0.73, N=227, P=10^{-39}) and the rest of gut microorganisms. The new parameter (Cq (toxB)-Cq (16SrDNA)) is calculated from two biomarkers, is independent of sampling variability and inherently incorporates the destructive character of *C difficile* on the rest of micro-flora. By incorporating relative abundance of tcdB (in context of the total bacterial flora), and correcting for "biomass wash-out/dilution effects of acute diarrhea , these biomarkers could collectively enhance the predictive value of CDI testing.

Introduction

Bacterial biomass is the major component (25-54% of dry solids) of the organic fraction of the feces [1]. Stool, stool swabs, or rectal swabs are types of samples used in molecular diagnostics of diarrhea. Sample processing involves various technical variabilities. For instance, sampling of heterogeneous gut microbiome content of the stool can be improved by homogenizing the stool prior to subsampling and by using standard volume input (use of 10 microliter microbiology loop) prior to sample processing [2]. However, despite all pre-cautions, intestinal bowel material is "differentially diluted" among different patients, due to case dependent differential "biomass wash-outs", during presentation of acute diarrheal symptoms, the total bacterial load could vary up to few log values [3]. Whenever pre-analytical sampling variability is large [1,2,4-8] and quantification is intended to establish the clinical status of the patient, normalization based on sample mass, or sample volume is not effective.

Recent reports demonstrate that cycle threshold values (Cq) of tcdB assay overlap between symptomatic and asymptomatic patients. Median Cq value of the tcdB PCR in toxigenic *C. difficile*-positive healthy individuals is significantly higher than for symptomatic patients, indicating that lower tcdB gene load is present in the stool of persons with asymptomatic colonization. However, a numerical consensus is lacking, and future diagnostic strategy is not conclusive [9-16]. Despite the evidence that both qPCR and novel "ultra-sensitive" enzyme immunoassay technologies possess single molecule resolution for CDI detection, the pre-analytical variability of sampling, may at least in part explain the current inability to establish sharp quantitative thresholds with meaningful clinical value [12,17-20]. Since normalization of diarrhea based on mass or volume, does not correct for the past wash-outs of intestinal content during acute CDI phase, symptomatic and asymptomatic toxB/tcdB positive samples will provide partially overlapping absolute quantitative values. We applied relative normalization of Cq values, a strategy well described and promoted by "typical" gene expression studies and summarized in MIQE guidelines [21,22]. Herein, we report that "the rest of bacterial microorganisms" shows negative correlation with tcdB biomarker, thus presenting valuable biomarker capable of clearly identifying samples with extremely high tcdB burden, independent from the absolute concentration of tcdB per volume and/or mass of fecal material.

Material and methods

Sample processing was equivalent to the one described in BD GeneOhm TM C diff Assay Manual (https://www.bd.com/resource). All oligonucleotides were synthesized by Integrated DNA Technologies (IDT) (Iowa, US): tcdB primers MTO2F (5'TGCAGCCAAAGTTGTTGAAT3') and MTO2R (5'GCTCTTTGATTGCTGCACCT3'), and probe MTO2P (/56-FAM/TCTGAAGGA/ZEN/TTACCTRTAATT GCAA/3IABkFQ/; QuantiNova Probe PCR kit (Cat No./ID: 208254, Qiagen, Canada);

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The full sequence of Inhibition Control Target was (5'TGCAGCCAA AGTTGTTGAATGCAATGGTCCCAATGGCTAACGCGCAGAG CCTTCAGGTCAGAAATTTTTGCCATC CGAGACATCAGGTG CAGCAATCAAAGAGC3'), detected by hydrolysis probe /56-FAM/ TTCTGACCT/ZEN/GAAGGCTCTGCGCG/3IABkFQ/. Described tcdB qPCR assay was validated with multiple commercial assays in the past (C difficile Quick Check Complete, Alere; BD Max, BD and BD GeneOhm, BD) and used over last 10 years in our institution to detect symptomatic patients which are positive for tcdB biomarker. The relative normalization of samples was obtained using universal 16S rDNA amplicons, as a measure of total bacterial load, as described [23]. The qPCR was performed on Roche Light Cycler 480 instrument, following PCR program: hold at 94°C for 2 minutes and cycling of 45x (94°C, 10sec, and following by priming, elongation and acquisition of fluorescence at 50°C for 20 seconds). The RFU (Relative Fluorescence Units) versus cycles curves were visually analyzed. Internal PCR positive control was genomic DNA from *C. difficile* ATCC 43255, adjusted to produce positive and stable Cq values. LC480 software (V 1.5.1.62 SP3) was used to calculate Cq values using second derivate analyses and high sensitivity mode. Decisions of the result was made by operator (positive/negative/ inhibition), based on interplay between results of tcdB and/or inhibition assays and curve shape, and/or generated Cq value. Total bacterial load quantifications were performed on tenfold-diluted sample (comparing to equivalent tcdB assay) to assure that inhibition of 16S rDNA reaction was not a dominant factor in defining Cq value. The difference in Cq values among 16S and tcdB reactions (each done in separate wells) was calculated, after correction for sample dilution.

Results and discussion

The 227 clinical stool samples, declared as positive for tcdB by laboratory developed test (tcdB qPCR), were collected in 2015 in the Clinical Microbiology Laboratory, Jewish General Hospital. Total bacterial load (Cq qPCR values of 16s rDNA) was measured in these samples as second biomarker. The average value for Cq of tcdB in this group of patients was 28.8, covering a minimum-maximum range of 20.5 to 38.6. The standard deviation among repetitive measurements for any of these assays was lower than 2 Cq units. The second biomarker was chosen with the intention of normalizing pre-analytical sample variability, i.e. to calculate relative abundance of *C. difficile* in total stool flora. The Cq values of total bacterial load among collected clinical samples (16S rDNA qPCR) showed a distribution with an average of Cq=16.6 and minimum-maximum range of 12.2 to 23.7. Using paired numerical data (tcdB and 16S rDNA) for each sample, a relative abundance of *C. difficile* was calculated, thereby correcting for sampling variations which are beyond control (e.g. stool mass, stool volume and frequency of defecation). When absolute Cq values of tcdB and relative values were plotted against each other, a strong correlation (R^2=0.73, N=227, P=10^{-39}) was obtained. The number Cq (tcdB) minus Cq (16S rDNA) reflects the relative abundance of tcdB in total flora. The average value for this number was 12.9, with a minimum-maximum range between 2.2 to 20.2. The bigger this number is, the smaller the relative abundance of *C difficile* is. If this number was kept constant and independent from the concentration of tcdB biomarker, we would assume that *C. difficile* is in commensal relation with the rest of bacterial microorganisms. However, data suggests that overgrowth of *C. difficile* strongly suppresses the rest of gut flora (by competition), suggesting aggressive overgrowth of gut eco-system by the pathogenic species. The relative abundance of toxicogenic *C. difficile* in asymptomatic cases usually represents a minor fraction [24]. For example, a recent high-resolution profiling of the gut microbiome reveals the extent of *C. difficile* burden, suggesting that abundance of *C. difficile* among CDI patients is 1.78% or higher, which is quite different from healthy controls (0.008%) [25]. In summary, by measuring relative abundance of tcdB against total bacterial flora, the method corrected for a "biomass wash-out/dilution" effects of the fecal samples during acute diarrhea and captured the individual's ratio of *C. difficile* vs. the rest of the microbiota. The combination of these two biomarkers could enhance the predictive value of test for CDI detection.

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