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Laboratory-developed test for detection of acute *Clostridium difficile* infections with the capacity for quantitative sample normalization

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ABSTRACT

We describe a laboratory-developed test intended for the detection of acute *Clostridium difficile* infections (CDI) with the capacity for quantitative sample normalization. The test is based on the detection of the *tcdB* gene. However, this biomarker is also present among people without symptoms, implying that individuals with diarrhea, not caused by *C. difficile* may nonetheless test positive. Therefore, clinical diagnosis based on this format of testing can be challenging. In order to improve diagnostic assays capability, *tcdB*-based quantification methods were suggested as a potential solution, however they did not increase clinical specificity. We report methodology for a dual biomarker monitoring (total bacterial load and *tcdB* assay), allowing for the calculation of the relative presence of *tcdB* in the total bacterial population in the tested samples. We believe that this approach is clinically relevant to current assays and can improve CDI testing algorithms.

1. Introduction

Clostridium difficile is the major nosocomial pathogen (Loo et al. 2011) that causes virtually all cases of pseudomembranous colitis. There is considerable debate about which test is "the best test" for the detection of *Clostridium difficile* infection (CDI). Using highly sensitive diagnostic tests may reduce transmission but also may lead to unnecessary treatments (Gerding et al. 2016). However, classical ToxB-based enzyme immune assay (EIA), or toxinogenic culture assay (Jensen et al. 2015), can be negative in patients with symptomatic and even life-threatening CDIs (Lashner et al. 1986; Dallal et al. 2002; Sayedy et al. 2010; Fang et al. 2017). With recent "molecular" developments of ultrasensitive EIA (Song et al. 2015; Sandlund et al. 2018), it has become apparent that clinical applicability of the ideal medical device for CDI detection is not going to be improved by novel molecular ultrasensitive technological platforms (Polage et al. 2015; Song et al. 2015) but rather, from our standpoint, by the quantifying and normalizing ToxB content of diarrheic sample and by identifying additional novel disease-specific biomarkers. The examples of ToxB EIA positive asymptomatic cases (Anikst et al. 2016; Fang et al. 2017) were fundamental to the very basic sources of current disagreements among experts in the field. Besides in-adequate calibration of biomarkers performed in the past, the host immune response is not yet considered as a valid biomarker, thus further increasing uncertainty of CDI prediction.

The definition of diarrhea is heterogeneous. Recent reports demonstrated that even among intensive care health professionals (n = 80), almost half defined diarrhea using only the criterion of consistency (liquid or pasty stools), while 48% was also incorporated the criterion of bowel movement frequency (Cláudia Regina Felicetti Lordani 2014). Attempts to quantify diarrheic stool samples were made in the past and they are based on frequency, volume and consistency of samples (Guenter and Sweed 1998). Normal stool water content is ~68%, whereas diarrheic stool is characterized by minimum 85% water content (Bliss et al. 1999). Additionally, the term "severe diarrhea" is characterized by more than 10 loose, watery stools in a single day (William and Blahd 2017). In summary, variable volume/per defecation and variable frequency (during duration of acute phase), together with differential antibiotic uptake (Vincent and Manges 2015) reflects inherent

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variability of microbiota (and other constituents of the stool) and makes absolute quantifications of Tox B (or any other biomarker) unreliable (Fang et al. 2017; Senchyna et al. 2017; Begum et al. 2018; Kamboj et al. 2018; Kim et al. 2018; Madden et al. 2018; Sandlund et al. 2018).

Over-sensitivity of nucleic acid amplification tests (NAAT) among various patient populations can be used for infection control (Dionne et al. 2013; Senchyna et al. 2017; Kamboj et al. 2018; Kim et al. 2018; MacDougall et al. 2018; Madden et al. 2018; Wilmore and Goldenberg 2018). Analysis of Cp values (cycle quantification values in qPCR-based methodology) may offer a means to differentiate active *C. difficile* infection from asymptomatic colonization (Fang et al. 2017; Kamboj et al. 2018; Kim et al. 2018; Madden et al. 2018; Wilmore and Goldenberg 2018). The *tcdB* Cp value, in a same manner as ToxB protein concentration, obtained from ultrasensitive EIA (Pollock 2016; Sandlund et al. 2018), allows valuable feedback for diagnostic stewards. It is evident that validations of clinical qPCR or ultrasensitive EIA require further research, in various clinical settings and contexts, before their clinical use can be widely applied.

In this report, we describe a simple qPCR-based Laboratory Developed Test (LDT), which can be performed directly from the stool swab (but is also compatible with rectal/perirectal swab). It is characterized by 15 min hands-on time, low material cost and has the capacity to overcome/detect PCR inhibition, a typical obstacle for fecal sample material. More importantly, our LDT is amendable to measure total microbial load present in the sample, thus enabling normalization of microbiota dilution-factor within diarrheic stool samples.

2. Material and methods

Sample processing: The 2350 stool swabs (Medicom SafeBasic, Sterile Cotton tipped Applicators, Montreal, QC) included in this analysis were taken from anonymized patient stool material received for *C. difficile* testing. As per our hospital institutional review board (IRB) policies, this project was a laboratory quality improvement program and thus further IRB approval was not required. The stool swabs were transferred into 1 mL of 1 × TE buffer (Tris-EDTA, 100× Solution (Molecular Biology, Fisher BioReagents, Ottawa, Ontario), and mixed by vortexing for 5 min before use. The working procedure was equivalent to the one described in BD GeneOhm™ Cdiff Assay Manual (https://www.bd.com/resource): the 20 μL of sample material was transferred into 100 μL of TE buffer containing a volume of 50 μL of glass beads (0.1 mm, Cell Disruption media, Scientific Industries, Inc., Ocala, FL). After vortexing for 5 min and heating at 95 °C for 5 min, the tube was cooled down to +4 °C, resulting in a crude lysate, of which 3 μL were added directly into the PCR assay. For commercial BD GeneOhm and BD MAX *C. difficile* assays, (BD-Canada, Mississauga, Ontario) and rapid *C. difficile* EIA assay (C. diff Quik Chek Complete, Abbot Diagnostics, Mississauga, Ontario), the protocols were followed as to the manufacturers' specifications.

The *tcdB*_Lab_PCR assay: All oligonucleotides were synthesized by Integrated DNA Technologies (IDT DNA, Iowa, US) and listed on Table 1. *tcdB* gene oligonucleotides: The MTO2F (5′TGCAGCCAAAG TTGTTGAAT3′) and MTO2R (5′GCTCTTTGATTGCTGCACCT3′) primers, together with MTO2P (/56-FAM/TCTGAAGGA/ZEN/TTACCTRTAATT GCAA/3IABkFQ/ are used with QuantiNova Probe PCR kit (Cat No./ID: 208254, Qiagen, Mississauga, Ontario); Inhibition control-relevant oligonucleotides: Test for PCR inhibition was performed in a separate reaction, using the same MTO2F and MTO2R primers, acknowledging that the whole process of PCR inhibition is sequence-dependent. The full sequence of Inhibition control target (INH.CONTR.) was (5′ TGC AGC CAA AGT TGT TGA ATG CAA TGG TCC CAA TGG CTA ACG CGC AGA GCC TTC AGG TCA GAA ATT TTT GCC ATC CGA GAC ATC AGG TGC AGC AAT CAA AGA GC3′), and it is detected by hydrolysis probe with sequence (INH.P) /56-FAM/TTCTGACCT/ZEN/GAAGGCTCT GCGCG/3IABkFQ/.

5′CGGCTAACTMCGTGCCAG3′ as described (Brukner et al. 2015; Glisovic et al. 2018). Amplification of V3 and V4 variable regions of the eubacterial 16S rRNA gene (~460 bp) was performed using 5′ CCTACGGGNGGCWGCAG3′ and 5′GACTACHVGGGTATCTAATCC3′ primers, combined with TaqMan 16S probes (Glisovic et al. 2018). The final concentrations for all primers and probes were 0.5 μM and 0.2 μM, respectively.

Table 1
tcdB gene Oligonucleotides (MTO2F, MTO2R and MTO2P); qPCR reaction inhibition [MTO2F, MTO2R, INH.P (inhibition probe), INH. CONT. (oligonucleotide-target for estimating PCR inhibition) and MTO2RC (complementary oligonucleotide of MTO2R primer)] and estimation of total bacterial load (16SF, 16SR and 16SP) are presented, together with corresponding Melting Temperatures (Tm), using https://www.idtdna.com/calc/analyzer. The *tcdB* amplicon is 134 bp long; test for PCR inhibitory reaction is producing PCR product of 104 bp and 16S rDNA PCR product is ~460 bp long.

Oligonucleotides used in this work:	
MTO2F	5′TGCAGCCAAAGTTGTTGAAT3′
MTO2R	5′GCTCTTTGATTGCTGCACCT3′
MTO2P	(56-FAM/TCTGAAGGA/ZEN/TTACCTRTAATTGCAA/3IABkFQ/ 5′TGCAGCCAAAGTTGTTGAATGCAATGGTCCCAA
INHIBITION	TGGCTAACGCGCAGAGCCTTCAGGTCAGAAATTTTTGCCATCCGA
CONTR.	GACATCAGGTGCAGCAATCAAAGAGC3′
INH.P	56-FAM/TTCTGACCT/ZEN/GAAGGCTCTGCGCG/3IABkFQ/
16SF	5′CCTACGGGNGGCWGCAG3′
16SR	5′GACTACHVGGGTATCTAATCC3′
16SP	56-FAM/CGGCTAACTMCGTGCCAG31ABkFQ/

PCR cycling: The qPCR was performed on Light Cycler 480 instrument (Roche Diagnostics, Laval, Canada), using LC480 white plates and following PCR program: hold at 94 °C for 2 min and cycling of 45x (94C°, 10 sec, followed by priming, elongation and acquisition of fluorescence at 50 °C for 20 seconds). The RFU (Relative Fluorescence Units) versus time/cycles curve were visually analyzed. Internal PCR positive control was genomic DNA from *C. difficile* ATCC 43255. LC480 software (V 1.5.1.62 SP3) was used to calculate Cp values using second derivate analyses and high sensitivity mode. Decisions of the result were made based on interplay between results of *tcdB* and/or inhibition assays and curve shape, and/or generated Cp value.

Relative normalization: The Cp values of both *tcdB* and 16S rDNA assays, using 10x serially diluted targets, (after nucleic acid isolation), produced "Ct versus $\log_{10}$ (target concentration) curves", or "efficiency curves", with $-3.0 \geq$ slope, covering a linear range of 20–40 and 12–28 Cp units, respectively, indicating a similar level of efficiency. The efficiency of PCR assay from the crude sample (without nucleic acid purification) is compromised by (a) inhibition reaction, which is sample-dependent (due to the high inhibition potential of fecal material) and by (b) the complexity of interactions between the targets (16S rDNA genes of microflora) and broad-range primers. Note that inhibitors are diluted together with the DNA/RNA in efficiency assays. As such, higher dilutions contain lower inhibitor concentrations and thus lack the dominant inhibitory effect, making slope of the efficiency curve lower and efficiency higher. Consequently, the ΔCt values between the concentrated and diluted sample are smaller as predicted, resulting in an amplification efficiency above 100% ($-3.0 \geq$ slope). In general, one must try to work under similar assay efficacies for both *tcdB* and 16S rDNA. Diluting 10-fold sample which is entering 16S rDNA reaction (10-fold, compared to the input of tcdB assay) one can make the efficacy of amplification (*tcdB* and 16S rDNA) similar. Recalculation of 16S rDNA Cp values to represent sample dilution equivalent of *tcdB* assay (i.e. subtract value of ~3.3 Cp units from the measured value) permits calculation of [Cp(*tcdB*)-Cp(16SrDNA)]. We noticed that when DNA was isolated from the same sample volume equivalent (EazyMag, Biomerieux, Montreal), the reaction was rarely inhibited, and additional dilutions were not required. Considering that novel generation of medical devices perform automatic nucleic acid purification, the protocol for calculation of relative *tcdB a*bundance can be straightforward. During the exploratory phase of the assay optimization, both protocols were performed (with and without nucleic acid isolations), producing similar end-results [Cp(*tcdB*)-Cp(16SrDNA)]. Due to the

"complex" technical resolution of differential PCR inhibitions among 2 assays, the nucleic acid isolation of normalized fecal material is a more reliable procedure for addressing clinical predictive value of CDI qPCR assay.

Amplicon identity of *tcdB* PCR products was confirmed by Sanger sequencing service performed by Genome Quebec, Canada, (http://gqinnovationcenter.com/services/sequencing/ serviceSangerSeq). The 16S rDNA amplicon was obtained by broad range primers, used in the Illumina protocol for 16S Ribosomal RNA Gene Amplicons PCR Preparation for the Illumina MiSeq System. These primers are amplifying the V3 and V4 region of 16S rDNA gene. Multiple sequencing validations showed that these primers approximate metagenomic abundance but do not have low-level taxonomic resolution [29].

Intra and inter-run reproducibility using the fraction of the same sample (where the second run is performed within 4 hours from the time of receiving the sample), were within one Cp units. The stability of our controls among daily runs were preserved (for example, positive control for *tcdB* standard deviations, covering 45 independent runs in March of 2019, was 0.67 Cp units).

3. Results

(1) *Analytical* versus *clinical assay performance*: Over the last 8 years, various commercial assays have been compared to our "in-house" qPCR assay (named "tcdB_Lab_PCR"). These commercial assays included rapid EIA (C. diff Quik Chek Complete) and FDA/Health Canada approved nucleic acid-based assays, like BD GeneOhm Cdiff assay (discontinued) and BD MAX C difficile).

As previously reported by others (Luna et al. 2011; Bandelj et al. 2013; Senchyna et al. 2017; Kamboj et al. 2018; Kim et al. 2018; Wilmore and Goldenberg 2018) and confirmed by us (Table 2), commercial rapid EIA has a low sensitivity [0.72 (95% CI: 0.66–0.78)] threshold, when compared to our in house qPCR test (tcdB_Lab_PCR). GeneOhm Cdiff assay compared with our qPCR (Table 3) has a higher sensitivity [0.77 (95% CI: 0.61–0.89)]. The BD MAX C Difficile assay has a minor sensitivity gains when compared to any other commercial NAAT (Shin et al. 2016). Taken together, re-optimized qPCR assay design, more efficient bacterial lysis and incorporation of re-optimized DNA purification steps, resulted in an increase in analytical sensitivity of *tcdB* gene detection (Seok et al. 2018). However, maximizing analytical sensitivity leads to an increase in the false positive CDI rate and an unnecessary treatment of "carriers" whose symptoms were not related to active *C. difficile* infection (Polage et al. 2016; Fang et al. 2017; Prechter et al. 2017; Kamboj et al. 2018; Kim et al. 2018; Madden et al. 2018). To resolve "assay over-sensitivity issues", the race to establish a quantitative biomarker threshold began (Dionne et al. 2013; Polage et al. 2016; Fang et al. 2017; Senchyna et al. 2017; Kamboj et al. 2018; Kim et al. 2018; Madden et al. 2018; Peng et al. 2018; Sandlund et al. 2018).

Table 2
Diagnostic Tests and inter-rater agreement (EIA vs tcdB_Lab_PCR); (DAG) Stat (https://www.biostats.com.au/DAG_Stat/Paper.html) was used to calculate statistics for assessment of diagnostic tests and inter-rater agreements using referenced material (Mackinnon 2000). The 1667 stool samples were tested by EIA and tcdB_Lab_PCR (gold standard) in parallel (see Material and Methods section) and the relevant statistical parameters are presented.

		EIA		
		Positive	Negative	
tcdB_Lab_PCR	Positive	144	55	199
	Negative	33	1435	1468
		177	1490	1667

Sensitivity = 0.72 (95% CI: 0.66–0.78); Specificity = 0.98 (95% CI: 0.97–0.98); Efficiency (Correct classification rate) = 0.95 (95% CI: 0.94–0.96); Predictive value of positive test = 0.81 (95% CI: 0.75–0.87); Predictive value of negative test = 0.96 (95% CI: 0.95–0.97).

Table 3
Diagnostic Tests and inter-rater agreement (BD GeneOhm vs tcdB_Lab_PCR); DAG Stat (https://www.biostats.com.au/DAG_Stat/Paper.html) was used to calculate statistics for assessment of diagnostic tests and inter-rater agreements using referenced material [25]. The 454 stool samples were tested by BD GeneOhm Cdiff (gold standard) and tcdB_Lab_PCR in parallel (see Material and Methods section) and the relevant statistical parameters are presented.

		tcdB_Lab_PCR		
		Positive	Negative	
GeneOhm C diff	Positive	30	9	39
	Negative	0	415	415
		30	424	454

Sensitivity = 0.77 (95% CI: 0.61–0.89), Specificity 1.0; Efficiency (Correct classification rate) = 0.98 (95% CI: 0.96–0.99); Predictive value of negative test = 0.98 (95% CI: 0.96–0.99); Predictive value of positive test 1.00 (95% CI: 88.43- MAX).

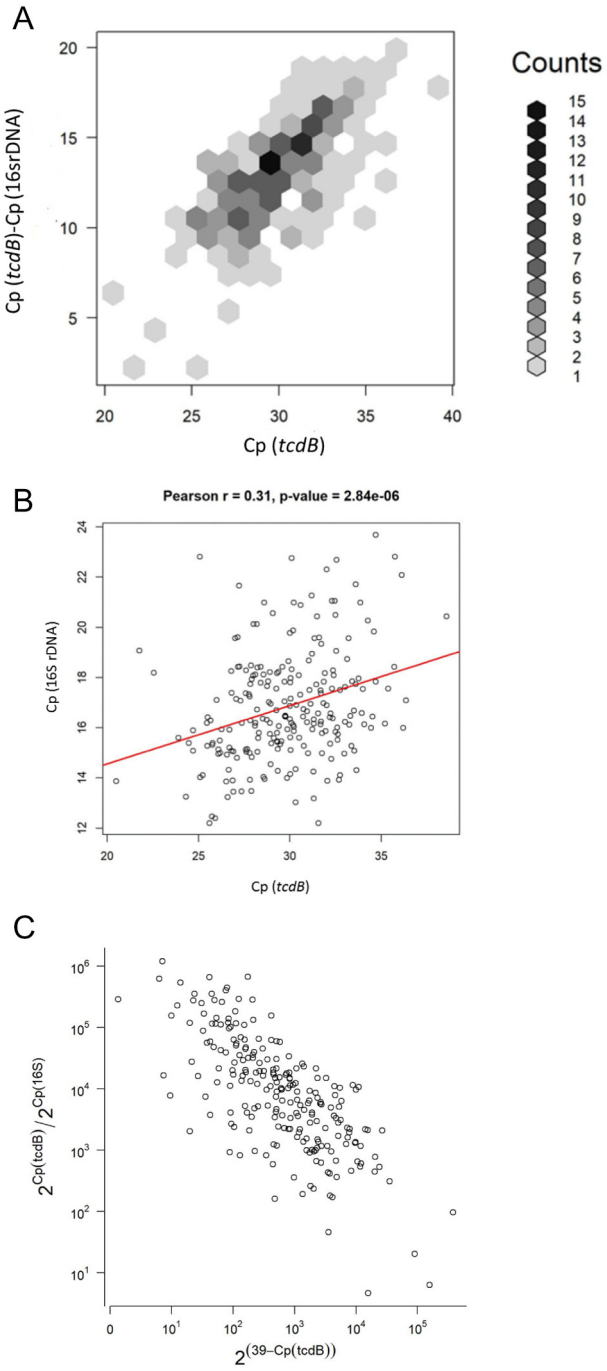
(2) *Sample normalization*: Bacterial biomass is a major component (25–54%) of the organic fraction of the feces (Rose et al. 2015). Stool swab sample processing involves technical variability, which can be improved by homogenizing the stool prior to subsampling and by using standard volume input prior to sample processing (Gorzelak et al. 2015). However, in the real-life situation, the frequency and volume of diarrheal stools of critical patients is not extensively and systematically monitored/recorded. In these cases, mass/volume normalization of the input feces material will not provide intended sample normalization. The only alternative is to perform relative normalization, using additional biomarker within the fecal sample. For example, the data presented on Fig. 1A shows that for the same absolute values of *tcdB* gene copy numbers (related to Cp values of *tcdB*) relative frequency of toxinogenic *C. difficile* in the total bacterial flora (Cp (16S)-Cp (*tcdB*)) varies greatly. The strategy of relative normalization of biomarkers is described in gene expression studies (Bustin 2017), and includes a second biomarker which responds linearly on the further dilutions of the sample material. Our clinical data shows weak linear trend among Cp values (Fig. 1B), between 16S rDNA and *tcdB* copy numbers, despite that individual biological variability is superimposed over this trend.

The individual variability of CDI burden is illustrated in Fig. 1C, where the relative abundance of toxinogenic *C. difficile* in total bacterial micro-flora is related to the copy number of *tcdB* genes in the same fecal sample. The greater the toxinogenic *C. difficile* load the patient has, the smaller is the abundance of residual bacterial microflora. Our first attempt to use 16S rDNA qPCR assay (as a sample normalization assay) failed due to the frequent presence of sample-induced PCR inhibition. This PCR reaction inhibition was characterized by the fact that even at 10x serial dilution of the typical sample fecal material (used for *tcdB* detection), similar Cp values were present as for the undiluted samples. However, at greater than 10x serial dilutions, we began to observe the typical Cp shift of ~Cp = 3.3 units, with each subsequent 10x dilution (data not shown). This indicated that stool sample is sufficiently diluted to produce quantitative values of total bacterial load which are out of the inhibition range and that relative normalization of *tcdB* reflects relative frequency of *C. difficile* in the total bacterial fecal load. This number is independent of sampling variability.

(3) *Further increased analytical sensitivity can be achieved by introducing novel amplicons, under the same fluorophores:* During our validation study we used qPCR amplicons previously described in literature (van den Berg et al. 2007; Matsuda et al. 2012) in parallel with our "in-house" monoplex reaction and as an equimolar combination of primer and probes in a duplex reaction, under the same 6-FAM fluorophore. We noticed that discrepant data among BD GeneOhm and our "in house" assay could be "resolved" by using a combination of these 2 different qPCRs under the same fluorophore, thus showing capacity to improve limits of detection (LoD), if required.

In summary, the tcdB_Lab_PCR assay comprises of non-proprietary PCR materials and reagents, is cost-effective, workflow and hands-on

time efficient and presents an example of a Laboratory Developed Test which allows developmental flexibility and improved predictive power. The described protocol does not require DNA isolation, but mechanical lysis of bacterial cells, which is performed with a simple glass beads protocol. Inhibition control (INH.CONTR.), is spiked target oligonucleotide which generates a PCR product with the same primers as the *tcdB* PCR assay, thus enabling adjustment of analytical sensitivity to the intended sensitivity threshold. Our tcdB_Lab_PCR assay can be used to detect presence of *tcdB* with the high negative predictive value (NPV) for CDI. Moreover, the assay is unique in its capacity to perform relative normalization of variable liquid stool material, which we believe is paramount for improving the clinical impact of *tcdB* quantifications in CDI testing algorithms.



4. Discussion

Standard enzyme immunoassays (EIAs), although rapid and specific, lack sensitivity for CDI detection (Bogaty et al. 2017). Conversely, negative NAAT results allow for the safe exclusion of CDI (Gerding et al. 2016; Shin et al. 2016), however if the patient has CDI-like symptoms, NAAT positives results will "trigger" antibiotic treatment, even if *C. difficile* is not the real cause of the symptoms (Prechter et al. 2017). To overcome these limitations many laboratories have now implemented complex testing algorithms for CDI diagnosis which combine the results of NAATs and EIAs in multiple steps (Polage et al. 2016; Peng et al. 2018).

The efforts to establish quantitative, or semi-quantitative thresholds, with valuable clinical relevance for detection of CDI were significant (Folkers et al. 2010; Luna et al. 2011; Bandelj et al. 2013; Fang et al. 2017; Kamboj et al. 2018; Kim et al. 2018; MacDougall et al. 2018; Madden et al. 2018; Takahashi et al. 2018; Wilmore and Goldenberg 2018). Recent reports demonstrate that Cp values of *tcdB* assay overlap between symptomatic and asymptomatic patients (Fang et al. 2017; Senchyna et al. 2017; Kamboj et al. 2018; Kim et al. 2018; Madden et al. 2018; Wilmore and Goldenberg 2018). Notably, median Cp 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 having asymptomatic colonization. These reports are supported by numerous clinical data (Dionne et al. 2013; Senchyna et al. 2017; Begum et al. 2018; Kamboj et al. 2018; Wilmore and Goldenberg 2018). However, there is no consensus about a threshold value. Despite the evidence that both PCR and novel ultra-sensitive EIA technologies possess single molecule resolution for CDI detection, the pre-analytical variability of sampling, might partially explain our inability to establish quantitative thresholds with meaningful clinical sensitivity. How does one "standardize" stool samples submitted for diagnosis? Total volume and frequency of liquid stool per time period of acute diarrhea are not considered as reliable parameters for sample normalization. Nevertheless, these parameters significantly contribute to the measurements of absolute concentration of biomarker (per volume, or per mass). Normalization based on mass/

Fig. 1. A: Frequency distribution of Cp values among 227 *tcdB* positive samples: the 3-dimensional distribution of frequency counts of *tcdB* positive clinical samples (presented by gray-scale) as a function of absolute Cp values of *tcdB* gene (X-axis) and *relative* values (vs. total bacterial load), Cp (*tcdB*)-Cp (16 s), presented on Y-axis. One dot is an average bin/count of two neighboring Cp units on XY plane. Note that the same absolute values of *tcdB* load can assume very different relative values.B: Paired Cp values among 2 assays are reflecting *C difficile* and total bacterial load for each sample: paired Cp (*tcdB*) and Cp (16S rDNA) values from *tcdB* positive samples (227) are plotted, where X-axis is Cp (*tcdB*) versus Y axis: Cp (16S rDNA), for the same sample. The line is showing the weak linear trend and corresponding P-value is reported. Differential biomass wash-outs during acute phase of diarrhea can explain "linear" trend among (paired) values of these 2 assays. Deviations from linear trend are reflecting inherent biological variability, i.e. differential CDI burden.C: Relative abundance of 227 *tcdB* positive toxinogenic *C. difficile* in total bacterial micro-flora: To calculate relative frequency of *C. difficile*, i.e. to estimate total bacterial load versus toxinogenic *C. difficile* load ((Y axis), we used data from 16S rDNA and *tcdB* qPCR assays, using {2^[Cp(*tcdB*)-Cp (16S)]}. The Cp values are in inverse relation to the number of bacteria in the stool (Brukner et al. 2015). Serial dilutions of *C. difficile* bacteria were used to estimate absolute values of *C. difficile* bacterial load present in the sample (X-axis; scaling was generated using standard titration curve, using relation between number of *C. difficile* colony forming units and Cp values of *tcdB* qPCR assay). The relative number of *C. difficile* genome equivalents, comparing to the total bacterial genome equivalents is presented as relative abundance (Y-axis). Note that 16 s rDNA assay is un-accurate for high dilutions of stool/fecal (bacterial) biomass, [bellow 1000 bacteria (Cq > 32)], due to the sample-specific reduced complexity and 'non-universality' of the broad-spectrum primers. We are working with much higher number of total bacterial genome equivalent (GE) and diversity within such sample is much higher. For those samples, 10x serial dilutions are linearly responding to the increase in Cp values by ~3.3 Cp units, (since $2^{3.3} \sim 10$), until the threshold of Cp ~32, after which the accuracy drops. Since stool swabs produce Cp values of 16S qPCR in the range $12 < Cp < 24$, the uncertainty of ~1000 genome equivalents do not compromise observed trend. One can conclude that *C. difficile* burden is in significant negative correlation with the rest of microflora ($R^2 = 0.73$, $P = 10^{-39}$): this additional information can help us in removing effect of biomass dilution and quantify CDI burden toward total flora.

volume does not correct for the past wash-outs of intestinal contents. Consequently, symptomatic and asymptomatic ToxB/*tcdB* positive samples will get partially overlapping absolute values. The 500 copies of *tcdB* gene (or 500 copies of ToxB protein) can be detected only by a sensitive method, but what is unknown, is how much microbiota and/or macromolecular content of the sample is "washed out" during the acute diarrhea time period? Is the laboratory detecting 500 copies per PCR tube from samples having 100 mL of liquid stool per 24 h since initial onset of diarrhea (situation 1), or is this sampling the 5th defecation of the same day, each having volume of 300 mL (situation 2)? In the second situation, a fecal biomass is 15× "washed-out and/or diluted" compared to the first case, despite having the same absolute values of *tcdB* molecules.

Considering the number of copies of *tcdB* gene, we observed that the overlap of Cp values for published data (Fang et al. 2017, Senchyna et al. 2017, Kamboj et al. 2018, Kim et al. 2018, Madden et al. 2018, Wilmore and Goldenberg 2018) does not extend more than 7 Cp units ($2^7 = 128$). As seen on Fig. 1B, *tcdB* load is correlated directly with the total bacteria load, reflecting a sampling "dilution" problem. Higher *tcdB* load is typically associated with a higher total bacterial load, i.e. lower Cp (16S rDNA) values and vice versa. Yet, different stool samples can possess the same absolute Cp value of *tcdB* (for example, see Cp = 30 on X-axis), but *tcdB* relative copy numbers can vary from 0.1% to 10% of total bacterial flora. In general, the relative copy number of *tcdB* gene covers from 10 to 10^6 -fold range (Y axis, Fig. 1C). A recent high resolution profiling of the gut microbiome suggested the extent of *Clostridium difficile* burden among CDI patients to be ~1.78% (average), while healthy controls have values close to 0.008% (Daquigan et al. 2017). Therefore, measuring relative frequency of *tcdB* against total bacterial flora, not only corrects for a "biomass wash-out/dilution" effects of the fecal sample during acute diarrhea, but also captures the individual's ratio of *C. difficile* with respect to the total microbiota, making the combination of these two biomarkers more disease-specific. Collectively, considering inherent variability of liquid stool samples, we suggest that the relative normalization described herein presents a valid option for future clinical studies intended on implementing Cp toxin gene reporting and measuring its impact on patient care and antibiotic stewardship [5–9]. This report offers methodology to perform relative normalization of *tcdB*values and facilitates the generation of more (clinically) reliable analytical thresholds.

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