Towards understanding Clostridioides difficile colonization

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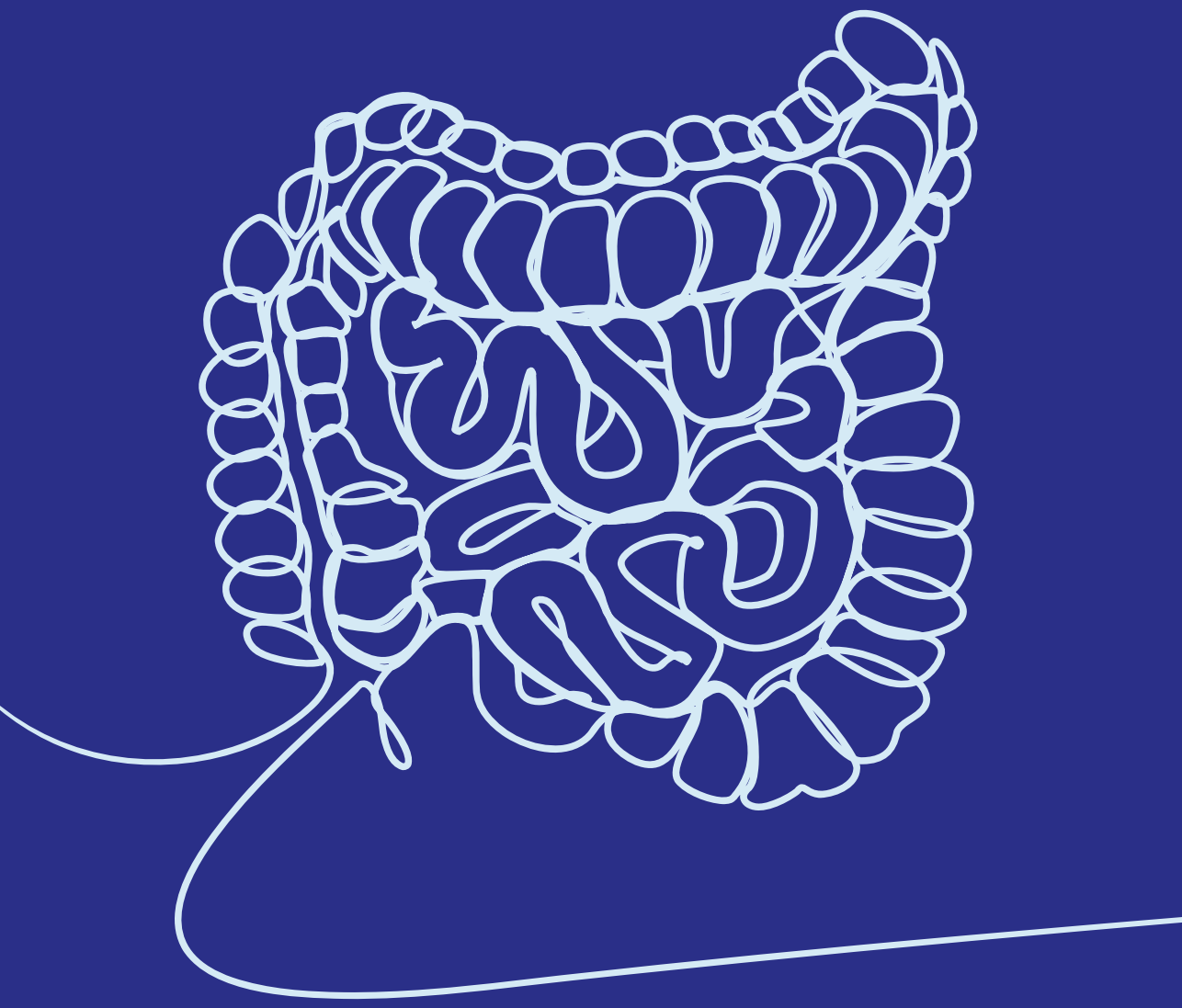
CHAPTER 4

Nucleic acid amplification test quantitation as predictor of toxin presence in *Clostridium difficile* infection



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Abstract

Multi-step algorithmic testing in which a sensitive nucleic acid amplification test (NAAT) is followed by a specific toxin A and B Enzyme Immunoassay (Tox A/B EIA) is among the most accurate methods for *Clostridium difficile* infection (CDI) diagnosis. The obvious shortcoming of this approach is that multiple tests must be performed to establish a CDI diagnosis, which may delay treatment. As such, we sought to determine whether a preliminary diagnosis could be made on the basis of the quantitative result of the first test in algorithmic testing, which provides a measure of organism burden. To do so, we retrospectively analyzed two large collections of samples (n=2669 and n=1718, respectively) that were submitted to the laboratories of two Dutch hospitals for CDI testing. Both hospitals apply a two-step testing algorithm in which a NAAT is followed by a Tox A/B EIA. From all samples, 208 and 113 samples tested positive by NAAT, respectively. Within these NAAT-positive samples, significantly lower mean *Cq* values were found in patients whose stool eventually tested positive for toxin than in patients who tested negative for toxin (24.4 vs 30.4 and 26.8 vs 32.2, $p<0.001$ for both cohorts). Receiver operating characteristics (ROC) curve analysis was performed to investigate the ability of *Cq* values to predict toxin status and yielded areas under the curve (AUCs) of 0.826 and 0.854. Using the optimal *Cq* cut-off values, prediction of the eventual Tox A/B EIA results was accurate in 78.9% and 80.5% of samples, respectively. In conclusion, *Cq* values can serve as predictors of toxin status, but due to the suboptimal correlation between the two tests, additional toxin testing is still needed.

Introduction

Clostridium difficile (recently reclassified as *Clostridioides difficile* based on phenotypic, chemotaxonomic and phylogenetic analyses (1) – for simplicity and consistency with previous literature *C. difficile* will be used in this paper) is an anaerobic, spore-producing bacterium that is responsible for *C. difficile* infection (CDI), the leading cause of nosocomial infectious diarrhea (2). Symptoms range from mild self-limiting diarrhea to potentially life-threatening fulminant colitis (3, 4). CDI occurs when alterations in the gut microbiome, particularly antibiotic-induced disruptions, create conditions favorable for *C. difficile* proliferation (5). Proliferation is followed by production of one or two enterotoxins, known as toxins A and B (Tox A/B), and in some strains a binary toxin, *C. difficile* transferase (CDT), whose inflammatory and necrotic effects on colonic tissue mediate the clinical symptoms of CDI (2).

CDI diagnostic methods continue to present problematic shortcomings. Establishing a CDI diagnosis is dependent on demonstrating the presence of toxin or toxigenic organism in stool samples (6). The two reference methods for doing so, cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC), are lengthy, laborious techniques whose clinical implementation is unrealistic. Therefore, rapid tests with the same aims in mind have been developed. Enzyme immunoassays (EIA) can be used to either detect toxin (Tox A/B EIA) or glutamate dehydrogenase (GDH EIA), an abundant enzyme whose presence is indicative of *C. difficile* (both toxigenic and non-toxigenic strains). Similarly, nucleic acid amplification tests (NAAT) can detect the presence of toxin-producing genes. While these rapid tests are easily carried out in a clinical setting, they too suffer from drawbacks. Tox A/B EIA use was once widespread, given the etiologic relationship between toxin and clinical symptoms, but recognition of its low sensitivity (6) has changed this paradigm. Increasingly, NAATs have gained popularity, given their ease of use and high sensitivity. However, there is considerable debate about whether the presence of toxigenic organism alone warrants a diagnosis of CDI or should instead be considered *C. difficile* colonization (7-10). This has prompted the creation of multi-step algorithms, where a first sensitive test, a NAAT or GDH EIA, is used to screen for the organism, which in the event of a positive result reflexes to a highly specific second test for toxin detection, the Tox A/B EIA (6).

The algorithmic approach is currently recommended by common guidelines, such as those published by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (6). And while algorithms do well to minimize false positive and negative results,

their obvious shortcoming is that in the event of a positive first test, a second test must be performed to establish a diagnosis, potentially delaying treatment and isolation of true CDI patients, or leading to premature treatment of non-CDI patients. In light of this shortcoming, we sought to determine whether the quantitative result of the first test, a Tox A/B or Tox B NAAT, in a two-step algorithm could predict the eventual outcome of the second test, a Tox A/B EIA.

Methods

Study design and population

This study was performed using CDI testing data from two Dutch hospitals, the Leiden University Medical Center (LUMC) (a tertiary care university-affiliated hospital) and Amphia Hospital (a large general hospital). In both hospitals CDI diagnoses are established using a two-step algorithm recommended by ESCMID, in which a NAAT for the toxin A producing gene (*tcdA*) and/or toxin B producing gene (*tcdB*) is, in the event of a positive result, followed by a Tox A/B EIA. All consecutive stool samples (both from inpatients and outpatients) that underwent CDI testing by this algorithm were considered. Samples from infants were only included if a specific request for CDI testing was made. For the LUMC samples were included from January 2016 – March 2017, and for Amphia Hospital samples were included from January 2016 – January 2017. Additionally, LUMC data from adult asymptomatic patients who, upon admission for non-CDI-related reasons agreed to have their stool tested for *C. difficile* and were found positive by culture, were included as a control. In the LUMC only, culture and ribotyping was performed on NAAT positive samples.

Diagnostic tests

Both hospitals use an in-house NAAT targeting *tcdB* only (LUMC) or both *tcdB* and *tcdA* (Amphia). For both sites, we used the *tcdB Cq* value for all calculations. LUMC's NAAT was performed as previously described (11). For the in-house NAAT in the Amphia hospital, DNA extraction was performed using the Nuclisens EasyMag system (bioMerieux, Marcy-l'Etoile, France). This in-house assay has been been validated internally and complies with the quality criteria described in the requirements of the International Organization for Standardization (ISO 15189:2012). In short, feces of approximately the size of a pinhead was suspended in 1ml of stool transport and recovery (STAR) buffer (Roche Diagnostics, Almere, The Netherlands) and frozen before further processing. After thawing, samples

were first homogenized in the Magnalyser (Roche Diagnostics, Almere, The Netherlands) (30 seconds, speed 6000xg) and thereafter centrifuged (1 minute, speed 14.000xg). A total of 100 microliters supernatant was used for automated nucleic acid extraction using the EasyMag system. Amplification of the *tcdA* and *tcdB* genes was performed on an ABI Taqman 7500 real time PCR system (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Primers and probes that were used for the Amphia NAAT have been described before (12). Taqman Universal PCR Master Mix (Applied biosystems, Nieuwekerk a/d IJssel, the Netherlands) and PCR plates were prepared using the PIRO pipetting robot (Dornier, Lindau, Germany) and contained 20microliters of mastermix and 5 microliters of extracted DNA. The amplification protocol included 5 min at 50C, 10 min at 95C followed by 45 cycles of amplification; and 95C for 10s, 60C for 32 seconds. Both laboratories used phocine herpes virus as internal control to test for PCR inhibition. For both hospitals, NAAT results were quantitated by measuring quantification cycle (*Cq*); the cycle value at which fluorescence from amplification exceeds the background fluorescence, serving as an indirect measure of how many copies of DNA were present in the sample tested. At the LUMC a VIDAS® *C. difficile* Toxin A & B (bioMerieux, Marcy-l'Etoile, France) was performed; values greater than 0.37 were considered positive according to the manufacturer's instructions. Amphia used an Immuno*Card* Toxins A&B (Meridian Bioscience, Cincinnati, OH, USA); results were not quantitative, instead presented as positive/negative. Both assays were performed according to manufacturer's instructions.

On working days, NAATs were performed on day of receipt. During weekend days and holidays, NAAT was performed on the following working day. In case of a positive NAAT, Tox A/B EIA was performed on the same or following day. Samples were stored at 4°C until tested. Culture and ribotyping of NAAT positive samples from the LUMC cohort was performed as previously described (13).

Statistical analysis

Average *Cq* values were compared by t-test and ANOVA. The ability of *Cq* values to predict toxin presence was assessed by receiver operating characteristic (ROC) curves. Positive predictive values and negative predictive values were calculated for different *Cq* cut-off points. Results were considered significant below the 0.05 level. Analyses of data were performed using SPSS version 23.0 statistical software (IBM, Armonk, NY, USA) and STATA SE version 12.1 statistical software (StataCorp, Texas, USA).

Results

LUMC

In total, 2669 unformed stool samples from patients suspected of CDI were tested by an in-house NAAT. Of these, 2424 had a negative result and 17 showed inhibition on NAAT and were excluded from further analysis. Of the remaining 228 NAAT positive samples 20 were excluded from further analysis as the Tox A/B EIA was not performed (either because there was insufficient feces to perform the assay or because the assay was stopped for any other reason). The remaining samples underwent testing by Tox A/B EIA, yielding a final sample size of 208 (Figure 1a). Quantification cycle (*Cq*) values in patients with positive (n=78) and negative (n=130) Tox A/B EIA results and in asymptomatic individuals who were found to be asymptomatically colonized by *C. difficile* upon hospital admission via culture are shown in Figure 2a. Comparable mean *Cq* values were observed in symptomatic patients negative for toxin (30.4, 95% CI 29.5-31.3) and asymptomatic carriers (29.2, 95% CI 27.3-31.2), while symptomatic patients with a positive toxin result had significantly lower mean *Cq* values per ANOVA (24.4, 95% CI 23.5-25.3, $p<0.001$). Seventeen outliers that were positive by Tox A/B EIA with high *Cq* values were retested by *tcdB* NAAT. The mean *Cq* value in these samples did not decrease after retesting. Samples were evaluated for PCR inhibition or irregular amplification curves, but neither was found to be a cause for these anomalies. Clinical data showed that only one of these samples was submitted during metronidazole treatment for CDI, 14 samples were submitted while no CDI antibiotics were used and for 2 samples antibiotic use was not clear. All but one of these 17 samples were positive in culture, yielding 11 different ribotypes. The only culture negative sample was from a patient with a clinical suspicion of a CDI recurrence 4 months after a previous episode. After the positive CDI test result, the patient was treated with oral metronidazole.

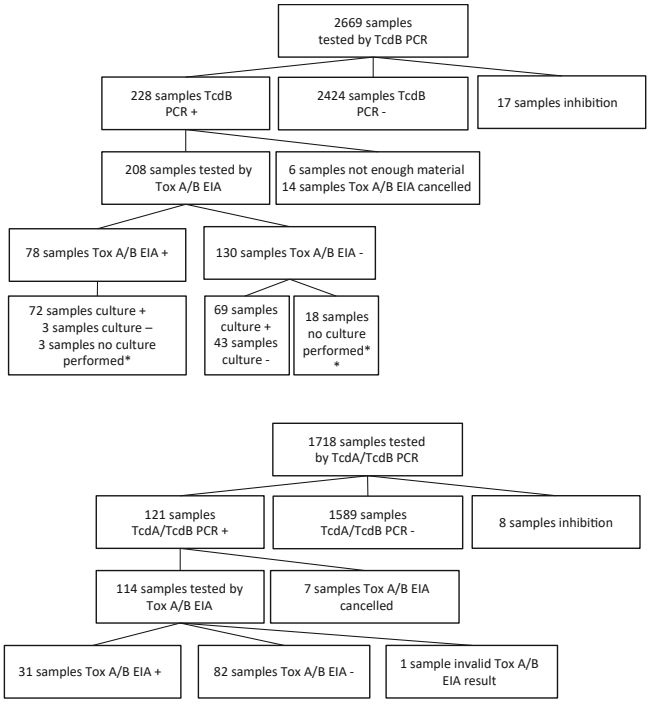

Figure 1. Flowchart of included samples. 1a. LUMC cohort. 1b. Amphia cohort.
* no culture performed because a culture with positive result was performed within the previous week.
** no culture performed because a culture with positive result was performed within the previous week (n=1) or because the positive *tcdB* result was obtained retrospectively during implementation phase of the *tcdB* NAAT, when samples were routinely tested by Tox A/B EIA only.

Based on the significantly lower *Cq* values observed in toxin positive samples, a receiver operating characteristic (ROC) curve was generated to calculate *Cq* values' ability to predict Tox A/B EIA outcome (Fig 3a). The area under the curve (AUC) was found to be 0.826 ($p<0.001$), with an ideal cut-off value of 25.3 cycles (the value best able to discriminate between outcomes – 78.9% of samples would be correctly classified as Tox A/B EIA positive or negative using this *Cq* cut-off value). Measures of accuracy of the ideal cut-off value and others are shown in Table 1.

As LUMC data included PCR ribotypes, we investigated whether ribotype had an effect on our findings. Ribotype distribution was comparable between Tox A/B EIA positive and negative patients by chi-squared test ($p=0.26$) and we did not find any differences in the mean *Cq* values between different ribotype categories ($p=0.55$ for toxin negative samples and $p=0.11$ for toxin positive samples, respectively) (Table 2).

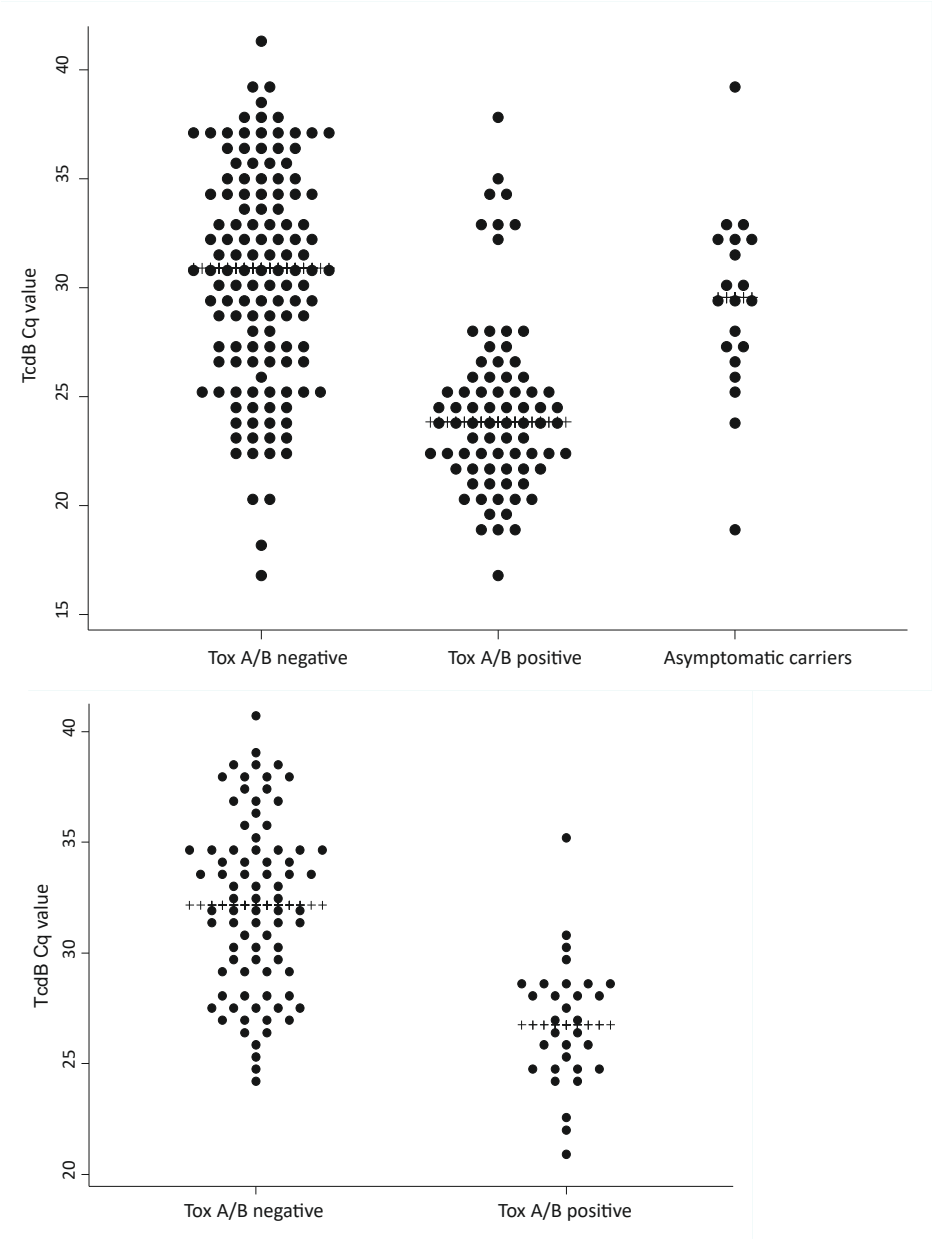

Figure 2. Dotplots of observed *Cq* values. 2a. LUMC cohort and asymptomatic carriers. 2b. Amphia cohort.

Table 1. Accuracy of the ability of NAAT *Cq* cutoff values to predict Tox A/B EIA outcomes.

Cq cut-off value	sensitivity (%) (95% CI)	specificity (%) (95% CI)	accuracy (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
LUMC cohort					
22.0	24.36 (15.35-35.40)	96.92 (92.31-99.16)	69.71 (62.98-75.87)	82.61 (62.65-93.08)	68.11 (65.23-70.86)
23.0	41.03 (30.01-52.75)	93.85 (88.23-97.31)	74.04 (67.52-79.86)	80.0 (66.02-89.17)	72.62 (68.68-76.24)
24.0	51.28 (39.69-62.77)	88.46 (81.68-93.4)	74.52 (68.03-80.29)	72.73 (61.25-81.81)	75.16 (70.50-79.30)
25.3	71.79 (60.47-81.41)	83.08 (75.51-89.08)	78.85 (72.66-84.19)	71.79 (62.92-79.25)	83.08 (77.36-87.58)
26.0	75.64 (64.6-84.65)	77.69 (69.56-84.52)	76.92 (70.59-82.47)	67.05 (59.04-74.17)	84.17 (78.06-88.82)
27.0	82.05 (71.72-89.83)	73.08 (64.60-80.48)	76.44 (70.08-82.03)	64.65 (57.49-71.20)	87.16 (80.67-91.69)
28.0	87.18 (77.68-93.68)	68.46 (59.73-76.33)	75.48 (69.05-81.17)	62.39 (55.94-68.42)	89.90 (83.14-94.14)
29.0	89.74 (80.79-95.47)	64.62 (55.75-72.80)	74.04 (67.52-79.86)	60.34 (54.38-66.02)	91.30 (84.33-95.35)
Amphia Hospital cohort					
24.0	9.68 (2.04-25.75)	100 (95.60-100)	75.22 (66.22-82.86)	100	74.55 (72.3-76.67)
25.0	29.03 (14.22-48.04)	97.56 (91.47-99.7)	78.76 (70.07-85.89	81.82 (50.72-95.16)	78.43 (74.33-82.04)
26.0	38.71 (21.85-57.81)	95.12 (87.98-98.66)	79.65 (71.04-86.64)	75.0 (51.13-89.59)	80.41 (75.55-84.51)
27.0	51.61 (33.06-69.85)	91.46 (83.2-96.5)	80.53 (72.02-87.38)	69.57 (51.01-83.38)	83.33 (77.55-87.86)
28.0	64.52 (45.37-80.77)	78.05 (67.54-86.44)	74.34 (65.26-82.09)	52.63 (40.63-64.33)	85.33 (78.12-90.46)
29.0	87.1 (70.17-96.37)	74.39 (63.56-83.4)	77.88 (69.10-85.14)	56.25 (46.46-65.57)	93.85 (85.83-97.46)
30.0	90.32 (74.25-97.96)	68.29 (57.08-78.13)	74.34 (65.26-82.09)	51.85 (43.44-60.16)	94.92 (86.31-98.22)
31.0	96.77 (83.30-99.92)	64.63 (53.30-74.88)	73.45 (64.32-81.32)	50.85 (43.40-58.26)	98.15 (88.45-99.73)
32.0	96.77 (83.30-99.92)	56.10 (44.7-67.04)	67.26 (57.79-75.79)	45.45 (39.29-51.77)	97.87 (86.89-99.69)

Values were calculated as follows: sensitivity= samples with a *Cq* value beneath the cut-off/all toxin positive samples; specificity= samples with a *Cq* value above the cut-off/all toxin negative samples; accuracy= all correctly classified specimens; positive predictive value = chance of positive Tox A/B EIA result among samples with a *Cq* value beneath the cut-off; negative predictive value= chance of negative Tox A/B EIA result among samples with a *Cq* value above the cut-off

Amphia Hospital

A total of 1,718 unformed stool samples suspected of CDI were tested by an in-house NAAT (different from the LUMC NAAT). Of these, 1,589 had a negative result and 8 showed inhibition and were excluded from further analysis. Seven of the 121 NAAT positive samples were not tested by Tox A/B EIA (2 were repeat samples from the same patient on the same day, 1 sample was a gut biopsy and 4 other samples were not tested otherwise). The remaining 114 samples underwent Tox A/B EIA testing. One sample had an invalid result on the second test (no detectable color in the reaction port) and was also excluded from further analysis, yielding a final sample size of n=113 (Figure 1b). *Cq* values in toxin positive (n=31) and negative (n=82) samples are shown in Figure 2b. Significantl lower mean *Cq* values were found in toxin positive patients compared to toxin negative patients (26.8, 95% CI 25.8-27.9 vs 32.2, 95% CI 31.3-33.0, $p<0.001$). Evaluation of the one outlier positive by Tox A/B EIA with a high *Cq* value revealed a normal shape of the amplification curve but no diarrhea anymore at time of results (without treatment).

Like the other cohort, an ROC curve was generated for determining *Cq* values' ability to predict the outcome of Tox A/B EIA testing (Fig 3b). AUC was 0.854 ($p<0.001$), with an ideal cut-off value of 27.0 (80.5% of samples correctly classified). Measures of accuracy of the ideal cut-off value and others are shown in Table 1

Discussion

This study sought to determine whether quantitation of NAAT results could predict the presence or absence of toxin in subsequent testing. Significantly lower *Cq* values were found in stool samples that tested positive for toxin in two large cohorts from different hospitals. Concomitant ROC curves in both cohorts showed that, using the optimal *Cq* cut-off value, the toxin result could be predicted in at least 78% of the samples. With the recent emergence of NAATs as stand-alone tests or as first step in an algorithm, there has been increasing interest in the use or non-use of quantitation of NAAT results. There is a growing body of work showing an association between *Cq* values and toxin presence: toxin-positive samples are associated with lower *Cq* values or higher bacterial load (14-21). Toxin presence is generally thought to be associated with CDI severity and outcome (7, 8). Some studies indeed found *Cq* values to be predictors of clinical severity or outcome, probably mediated through the presence or absence of toxins (21-23), although this was not confirmed in all studies (24, 25). A very recent paper was the first to describe the

Table 2. Ribotype distribution and mean *Cq* values for toxin positive and toxin negative samples (LUMC cohort).

	Mean Ct value	
Ribotype	*for Tox A/B EIA – samples(n)*	*for Tox A/B EIA + samples (n)*
001	35.55 (2)	26.38 (4)
002	32.23 (4)	21.15 (2)
003		22.35 (2)
005	30.93 (3)	22.83 (3)
012	26.6 (2)	22.35 (2)
013		22.75 (2)
014/020	28.46 (14)	22.56 (15)
015		22.96 (5)
017	24.7 (1)	
019		32.0 (1)
023		30.55 (2)
026		22.2 (1)
031	23.9 (1)	
034	30.3 (1)	
037	32.65 (2)	
050	34.4 (1)	21.8 (1)
053	26.3 (1)	
057	33.4 (1)	27.3 (1)
070	30.9 (1)	21.9 (2)
076		27.7 (1)
078/126	28.3 (15)	25.79 (15)
081	24.2 (2)	
104		32.7 (1)
123	30.9 (1)	
127		24.1 (1)
154		23.9 (1)
156		18.6 (1)
168	30.2 (1)	
198		23.6 (1)
258		22.8 (1)
262	25.3 (1)	
265	31.0 (3)	29.55 (2)
293	23.95 (2)	
328	27.78 (4)	
356	23.4 (1)	
unknown	26.92 (5)	25.06 (5)

performance characteristics of NAAT *Cq* cut-offs for discriminating toxin-positive and toxin-negative stool samples (26). Our study adds to the literature by confirming that *Cq* values can indeed be used to predict toxin status. In our cohorts, the optimal *Cq* cutoff detected toxin-positive samples with a positive and negative predictive value of 71.8% and 83.1% and 69.6% and 83.3%, respectively. Our study also indicates that local assessment of NAAT performance is warranted to determine a cut-off value that can be used for clinical use, as *Cq* values are semi-quantitative, and depend on many factors concerning sample material, used materials and assay.

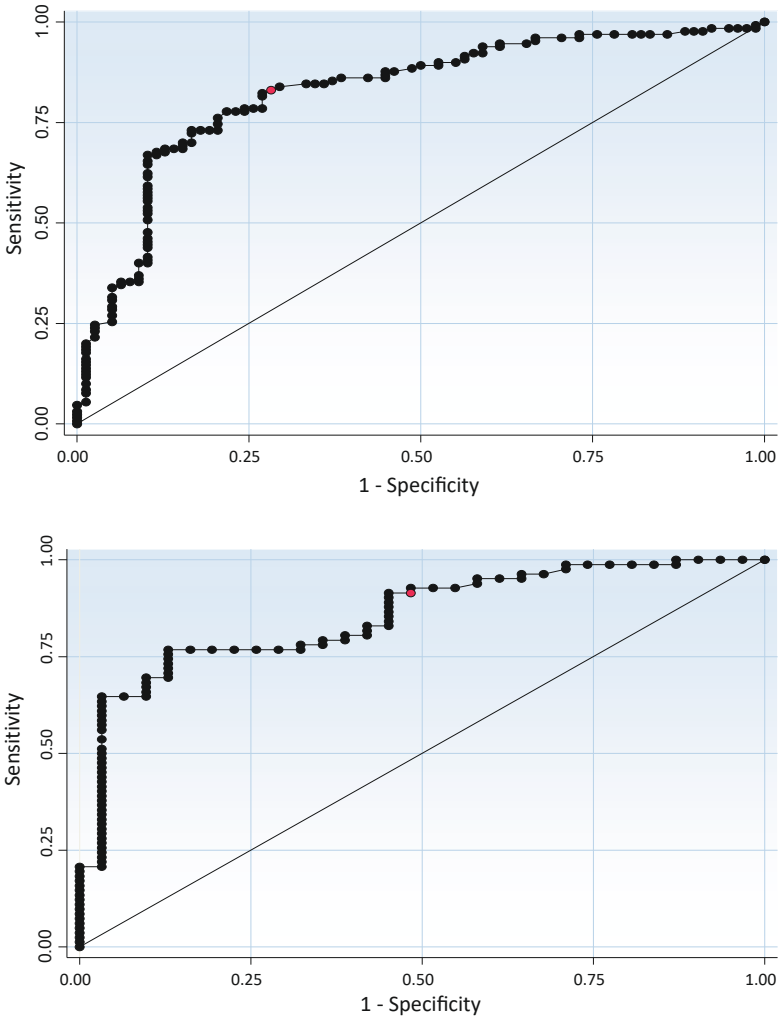

Figure 3. ROC curves assessing the ability of *Cq* values to predict presence of toxin. Optimal cut-off point shown in red. (Top) LUMC cohort. (Bottom) Amphia Hospital cohort.

Clinical implementation of these findings may be beneficial. Algorithmic testing requires more time to establish a CDI diagnosis than standalone tests, which has been shown to negatively impact patient care (27). One way of addressing this problem would be to use *Cq* values to establish a preliminary diagnosis. This can be either done by using the optimal *Cq* cut-off to consider samples likely toxin positive or negative. Using this approach, as many samples as possible will be classified correctly. One might however also argue, that toxin positive samples should not be missed, as delayed treatment or delayed isolation measures may negatively impact patient care and *C. difficile* transmission. In that case, a *Cq* cut-off with a high negative predictive value should be chosen to classify samples with *Cq* values above this cut-off as probable toxin negative. As an example, *Cq* cut off values of 29.0 and 32.0 for the LUMC and Amphia cohort, would correctly classify 91.3% and 97.9% of samples with a *Cq* value above this cut-off as negative, respectively. A preliminary diagnosis based on one of these two approaches might for instance be used when a clinician considers CDI treatment of a patient before results of toxin testing are available. However, we do recognize that the correlation between *Cq* values and toxin positivity and the positive and negative predictive values of the diverse cut-off values are far from perfect. We therefore think that *Cq* values may be helpful in doubtful cases, but NAAT quantitation should not be seen as a surrogate for free toxin testing or clinical judgment. It would be interesting to investigate if the incorporation of *Cq* values in an algorithm improves patient outcomes, compared to testing where a diagnosis, and consequent treatment, is exclusively dependent on demonstrating presence of toxin.

Our study had some limitations. First, we used an EIA to detect toxin, tests that are known to suffer from low sensitivities. Automated Tox A/B EIA such as the VIDAS® *C. difficile* Toxin A & B used by LUMC have reported sensitivities ranging from 53 to 98% – 0.98 compared to CCNA; membrane-type Tox A/B EIA such as the Immuno*Card* Toxins A&B have sensitivities ranging from 85 to 96% compared to CCNA (6). It is possible that some of the outliers we observed, with low *Cq* values but no toxin present, were actually false negatives in the Tox A/B EIA. Ideally CCNA, the gold standard of toxin detection, should have been used instead, but as these analyses were conducted retrospectively using clinical data where toxin testing is done by Tox A/B EIA, this was not possible. In the study by Senchyna and colleagues a membrane-type EIA detecting both GDH and Tox A/B, CCNA and a well-type Tox A/B EIA were combined to detect toxin positive samples (26). Using these combined tests as the reference standard, the optimal CT cutoff detected toxin-positive samples with a bit higher positive predictive value of 81.7% than in our cohorts, which may thus be explained by the more sensitive reference standard they used. A second limitation of our

study is that we analysed all samples that were tested for CDI and did not exclude samples from the same patient, samples from children, samples during the same diarrheal episode or samples submitted during or after treatment. The heterogeneity of the cohorts may therefore have obscured some associations, like higher *Cq* values for certain ribotypes, as was previously reported for ribotype 014 (20), or an aberrant association between *Cq* values and toxin positivity in children. However, the inclusion of all submitted samples led to a cohort that is representative for the actual situation. Information on repeat samples and CDI treatment is often lacking and the eventual ribotype (if CDI is confirmed) is not available yet at the moment the samples arrive at the laboratory. We therefore think that this study demonstrated the usefulness of NAAT quantitation in two unbiased cohorts which were highly representative for samples that are submitted for CDI testing, both in a universal and a general hospital.

Besides the representative cohorts that were used, there were some other strengths in our study. First of all, samples from the LUMC cohort underwent culture and PCR ribotyping and we were therefore able to evaluate any differences in *Cq* levels between different ribotype categories. Culture and ribotyping results were also used to evaluate the outliers. As 16/17 outlier samples had positive cultures and the one remaining sample had a clear clinical suspicion of CDI, false positive Tox A/B EIA results were considered less likely. A laboratory and clinical evaluation including retesting by *tcdB* NAAT was performed, but no clear explanation for the outliers with high *Cq* values but positive Tox A/B EIA was found. Another strength of our study is the unique comparison to a third group of asymptomatic carriers, which clearly demonstrated that *Cq* levels in asymptomatic carriers and symptomatic patients testing negative for toxins are comparable, suggesting that the latter group indeed represents CDI carriers with diarrhea not due to CDI.

In conclusion, we found *Cq* values to be predictors of toxin status in two large representative cohorts, although the suboptimal accuracy underscores the need of additional Tox A/B EIA testing. Additional studies are needed to determine if the inclusion of *Cq* values in algorithmic testing may aid clinicians in a faster but still accurate preliminary CDI diagnosis while awaiting the results of free toxin testing.

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Potential conflicts of interest

None to declare

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