Molecular characterization of pathogenic Clostridium difficile strains

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Applied research

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Comparison of real-time PCR techniques to cytotoxigenic culture methods for diagnosing *Clostridium difficile* infection

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Abstract

In the past decade, incidence of *Clostridium difficile* infections (CDI) with more severe course has increased in Europe and Northern America. Assays that are capable to rapidly diagnose CDI are essential. Two real-time PCRs (LUMC and LvI) targeting *C. difficile* toxin genes (*tcdB*; *tcdA* and *tcdB*, respectively), were compared with the BD GeneOhm PCR (targeting the *tcdB* gene), using cytotoxigenic culture as gold standard. In addition, a real-time PCR targeting the *tcdC* frame shift mutation at position 117 (Δ117 PCR) was evaluated for detecting toxigenic *C. difficile* and the presence of PCR Ribotype (RT) 027 in stool samples. In total, 526 diarrheal samples were prospectively collected and included in the study. Compared with cytotoxigenic culture, sensitivity, specificity, positive predicted value (PPV) and negative predicted value (NPV) were: for PCR LUMC 96.0%, 88.0%, 66.0% and 98.9%, for PCR LvI 100.0%, 89.4%, 69.7% and 100.0%, for PCR Δ117 98.0%, 90.7%, 71.9% and 99.5% and for PCR BD GeneOhm 88.3%, 96.9%, 86.5% and 97.4%. Compared to faeces samples cultured positive for *C. difficile* RT 027, sensitivity, specificity, PPV and NPV values of the Δ117 PCR were: 95.2%, 96.2%, 87.0% and 98.7%. We conclude that all real-time PCRs can be applied as a first screening test in an algorithm for diagnosing CDI. However, the low PPVs hinder the use of the assays as a stand-alone test. Furthermore, the Δ117 PCR may provide valuable information for minimising the spread of the epidemic *C. difficile* RT 027.

Introduction

Clostridium difficile is a major cause of nosocomial diarrhea and pseudomembranous colitis (1). Incidence of *Clostridium difficile* infection (CDI) has increased in the past decade, which is associated with the emergence of the hypervirulent PCR Ribotype (RT) 027 (2,3). The *C. difficile* enterotoxin A (TcdA) and cytotoxin B (TcdB) are considered as major virulence factors, whereas the binary toxin might play a role in virulence through the formation of microtubule-based protrusions thereby increasing the adherence of the bacteria (4-6). CDI can also be caused by strains that produce only TcdB (7), but strains producing TcdA only have not been described. Assays for the rapid diagnosis of CDI are important to prevent the spread of *C. difficile*, in particular for hypervirulent strains like the RT 027. Conventional diagnostic methods for CDI, such as cytotoxigenic culture (CYTGC) are time-consuming and not available at all routine diagnostic laboratories, whereas the performance of rapid enzyme immuno-assays to detect toxins in faeces is insufficient (4,5,11). Previously, we developed a real-time PCR that detects the presence of the *tcdB* gene (8). In this study we have improved the performance of this PCR and compared it with the commercially available BD GeneOhm PCR, and another in-house developed real-time PCR assay that detects the presence of the *tcdA* and *tcdB* gene (9). In addition, we evaluated a real-time PCR that targets the *tcdC* gene frame shift mutation at position 117 which can act as a marker for the RT 027/NAP1 strain (10,11).

Materials and methods

Cytotoxicity assay (CYT) and cytotoxigenic culture (CYTGC)

In total, 526 routine diagnostic diarrheal samples were submitted to the Department of Microbiology at Leeds Teaching Hospitals and tested prospectively by the CYT and CYTGC assays as previously described (12). Briefly, all stool samples (less then 48h old) were stored at 2 to 5°C. Twenty µL of diluted fecal sample (1:5 in PBS) was filtered and added to a monolayer of both *C. sordelli*-antitoxin protected (Prolab Diagnostics, United Kingdom) and unprotected Vero cells. A sample was considered toxin-positive when cell rounding was observed after 24 or 48 hours of incubation. In addition, cultured *C. difficile* isolates from faeces samples that were found negative by the CYT assay, were investigated for toxin production using the CYTGC assay (12). Isolates were inoculated into brain heart infusion broth (BHI). After 48 hours of incubation culture supernatants were added to a monolayer of protected and unprotected Vero cells. Cultured *C. difficile* isolates that were positive for the CYT assay were considered to be positive for the CYTGC assay.

Culture

Culture of isolates was performed as previously described (12). In short, following alcohol shock, samples were cultured on Braziers CCEY agar (Bioconnections, Wetherby, United Kingdom) supplemented with 5 mg/l lysozyme (Sigma, United Kingdom) and without egg yolk supplement. Incubation was done in an anaerobic workstation (Don Whitley, United Kingdom) for at least 48 hours. Grey-brown colonies with the characteristic horse manure odor were identified as *C. difficile*. Whenever the identification of an isolate was questionable the Microgen *C. difficile* latex agglutination kit (Microgen Bioproducts Ltd., Camberley, United Kingdom) was used to confirm the identity.

DNA extraction

Fecal samples were stored at 4°C for 1 week and then frozen at -20°C. Specimen preparation and DNA extraction for the BD GeneOhm Cdiff assay was performed according to the manufacturers' protocol. For the other real-time PCRs, fecal samples were pre-treated with stool transport and recovery (STAR) buffer (Roche, Penzberg, Germany) according to the manufacturers' protocol. For the LUMC real-time PCR, DNA was extracted on the MagNA Pure (Roche) using LC DNA Isolation Kit III (Roche, Penzberg, Germany) according to the manufacturers' protocol. In short, 100 µL supernatant of STAR buffer and chloroform pre-treated fecal sample was added to lysisbuffer (130 µL) and Prot K (20 µL). This mixture was heated at 65°C for 10 min followed by 95°C for 10 min, after which it was centrifuged for 1min at 1000 x g. 200 µL supernatant was used for automated DNA extraction. DNA was eluted in 100 µL elution buffer. The Phocine Herpes Virus (PhHV), which served as internal control, was added to the lysis buffer. For the LvI real-time PCR, DNA was extracted on the NucliSENS easyMAG (Biomérieux, Boxtel, The Netherlands) according to the specific A protocol (Biomérieux, Boxtel, The Netherlands). In short, 150 µL of STAR buffer and chloroform pre-treated faeces suspension was added to 2 *mL* lysis buffer (NucliSENS; Biomérieux, Boxtel, The Netherlands). After incubation for 10 min at room temperature, the total suspension was transferred to the sample vessel including 140 µL magnetic silica beads and used for automated DNA extraction. DNA was eluted in 110 µL elution buffer. The internal control Phocine Herpes Virus (PhHV) was added to the lysis buffer.

Real-time PCR

Amplification of part of the *tcdB* gene by the BD GeneOhm Cdiff PCR was performed on a Smartcycler (Cepheid, United Kingdom) according to the manufacturers' protocol. Primers and probes that were used for the LUMC real-

time PCR and the LvI PCR are described in Table 1. Amplification of the *tcdB* gene by the LUMC real-time PCR was performed on a CFX detection system (Biorad, Veenendaal, The Netherlands) as previously described (8) with some optimizations. The PCR amplification was performed in a 50 µL final volume, containing 25 µL Hotstar mastermix (Qiagen, Venlo, The Netherlands), forward and reverse primers at 80 nM each, 3.5 mM $MgCl_2$, 100 nM *tcdB* probe and 10 µL DNA. The PhHV primers described by Niesters (13) were used with a modified probe. Amplification protocol included an enzyme activation step for 15 min at 95°C, followed by 50 cycles of amplification; 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. The LvI real-time PCR was designed to target both *tcdA* and *tcdB* gene. Amplification of these genes was performed as a multiplex PCR on an AB 7500 PCR system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Table 1: Primers and probes used for in-house developed real-time PCRs.

Real-time PCR	Target	Primers and probes	Nucleotide sequence (5'→ 3')
LUMC	TcdB	398CLDs	GAAAGTCCAAGTTTACGCTCAAT
		399CLDas	GCTGCACCTAAACTTACACCA
		551CLD-tq-FAM	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-BHQ1
LvI	TcdA	CD-*tcdA*-F	TTG TAT GGA TAG GTG GAG AAG TCA GT
		CD-*tcdA*-R	AAT ATT ATA TTC TGC ATT AAT ATC AGC CCA T
		CD-*tcdA*-MGB1	FAM-ATA TTG CTC TTG AAT ACA TAA A-NFQ-MGB
		CD-*tcdA*-MGB2	FAM-TAT TGT TCT TGA ATA CAT AAA AC-NFQ-MGB
	TcdB	CD-*tcdB*-F1	GAA ACA GGA TGG ACA CCA GGT T
		CD-*tcdB*-F2	AAG AGG ATG GAC GCC AGG TT
		CD-*tcdB*-R1	ACG GTC TAA CAG TTT TGT GCC A
		CD-*tcdB*-R2	CTG CCC TTC ATA ATG ATC TCT TAT ACG
		CD-*tcdB*-MGB	FAM-AAG AAG CTT AGA AAA TG-NFQ-MGB
Δ117 PCR	TcdC	CD-*tcdC*-F	GCA CAA AGG RTA TTG CTC TAC TGG
		CD-*tcdC*-R1	AGC TGG TGA GGA TAT ATT GCC AA
		CD-*tcdC*-R2	CAA GAT GGT GAG GAT ATA TTG CCA
		CD-*tcdC*wt-MGB	FAM-AAA CAC RCC HAA AAT AA-NFQ-MGB
		CD-*tcdC*mut-MGB	VIC-AAA CAC RCC AAA ATA A-NFQ-MGB
All	PhHV	295PhHVs	GGGCGAATCACAGATTGAATC
		296PhHVas	GCGGTTCCAAACGTACCAA
		531PhHV-tq-CY5	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2* NED-CGC CAC CAT CTG GAT-NFQ-MGB **

BHQ = Black hole quencher; NFQ = Non fluorescent quencher; MGB = Minor groove binder. *used for LUMC PCR; ** used for LVI PCR and Δ117 PCR

Each PCR reaction was performed in a 25 µL final volume, containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), forward and reverse primers at 300 nM each, TaqMan MGB probes at 100 nM each, 2.5 µg bovine serum albumin (Roche), and 5 µL DNA extract. For PhHV, the primers described by Niesters (13) were used, whereas the probe was modified to a MGB-probe. Amplification protocol included 2 min at 50°C, 10 min at 95°C followed by 40 cycles of amplification; 94°C for 15 sec, 60°C for 1 min.

The Δ117 real-time PCR was designed to target the *tcdC* gene frame shift mutation at position 117. This assay utilizes two Taqman MGB probes, a wildtype (WT) probe and a mutant (MUT Δ117) probe respectively, that both can hybridize with part of the *tcdC* gene sequence flanking the 1 bp deletion at position 117. Isolates that do not carry the 1 bp deletion, will give a stronger signal with the WT probe, while 027/NAP1 isolates will do so with the MUT Δ117 probe. Hence, the ΔCt (Ct WT – Ct MUT Δ117) for 027/NAP1 strains will be positive, whereas the ΔCt for other *C. difficile* ribotypes will be negative, which enables discrimination. The primer/probe set was used in the same multiplex setup as the LvI PCR assay described above, with the primers at 300 nM and the MGB probes (5'-FAM and 5'-VIC, 3'-NFQMGB) (Applied Biosystems) at 100 nM. Reactions were run on an ABI 7500 with the same amplification protocol as for the LvI PCR.

PCR ribotyping

PCR ribotyping was performed at the Department of Microbiology at Leeds Teaching Hospitals following the protocol from the *C. difficile* Ribotyping Network for England (CDRNE) laboratory (12).

Data analysis

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined by comparing the real-time PCRs with the CYTGC gold standard; using statistical software PASW 17.0.2. Differences in the sensitivity and specificity between the real-time PCRs were determined by using the McNemar's Test for correlated proportions. Samples with a Ct-value higher then 40 were considered as negative. In addition, samples with an internal control Ct-value that deviated more than 3 Ct-values compared to the internal control Ct-value of the negative control were considered inhibited and discarded from the analyses for the LUMC PCR. For the LVI PCR, samples were considered inhibited and discarded from analysis when the Ct value for the internal control exceeded 34.91 cycles (i.e., the mean Ct value for uninhibited specimens ± 2 standard deviations). The number of inhibited samples for the Δ117 PCR was equally determined as for the LVI PCR. Furthermore, the Δ117 PCR was compared with PCR ribotyping of CYTGC

positive isolates. The BD GeneOhm Cdiff PCR tests were interpreted according to the manufacturers' protocol. The software on the Smart cycler (Cepheid, UK) recorded the results of the PCR assay as positive, negative or unresolved.

Results

Comparing the real-time PCRs with the gold standard CYTGC

In total, we evaluated 526 diarrheal samples, of which 101 samples (19.2%) were positive in the CYTGC assay. Of 101 positive samples, 13 were derived from CYT-assay negative samples. For the evaluation of the LUMC PCR 10 samples (1.9%) were excluded from the analysis due to inhibition during the amplification step, whereas 16 samples (3.1%) and 15 samples (2.9%) were inhibited and excluded from the analysis of the LvI PCR and Δ117 PCR, respectively. Five samples (1%) were recorded as unresolved by the BD GeneOhm PCR assay and excluded from the analysis. Sensitivity, specificity, PPV and NPV for all PCR methods against CYTGC are shown in Table 2. All stool samples that tested positive for *C. difficile* by the CYTGC assay were also detected by the LvI PCR. Comparable sensitivity was achieved by the LUMC PCR (96%) and Δ117 PCR (98%), while the sensitivity of the commercial BD GeneOhm PCR was lower (88.3%). The difference in sensitivity between the BD GeneOhm PCR and the three in-house developed PCRs was significant with *p-values* of 0.00, 0.01 and 0.04 for the LvI PCR, the Δ117 PCR and the LUMC PCR, respectively. In contrast, the BD GeneOhm PCR showed higher specificity (96.9%) compared to the LvI PCR (89.4%), the Δ117 PCR(90.7%) and the LUMC PCR (88.0%). The difference in specificity between the BD GeneOhm PCR and all three in house developed PCRs was significant with a p-value off 0.00. Compared to CYTGC, all PCRs had similar NPV ranging from 97.4 to 100%. The BD GeneOhm PCR had the highest PPV (86.5%) compared to the LvI PCR (69.7%), the Δ117 PCR (71.9%) and the LUMC PCR (66.0%).

Discrepancy analysis

Analysis of false positive results showed an overlap of the numbers of false positives detected by the different PCR methods (Figure 1A). In total, 13 false positive results (14% of total amount of positives) were found by the BD GeneOhm PCR compared to CYTGC. Of these false positives, 54% (n = 7) was also detected as such by all other PCR methods. Compared to CYTGC, the most false positives (34%) were detected by the LUMC PCR, whereas the LvI PCR and the Δ117 PCR had30% and 28% false positive samples, respectively.

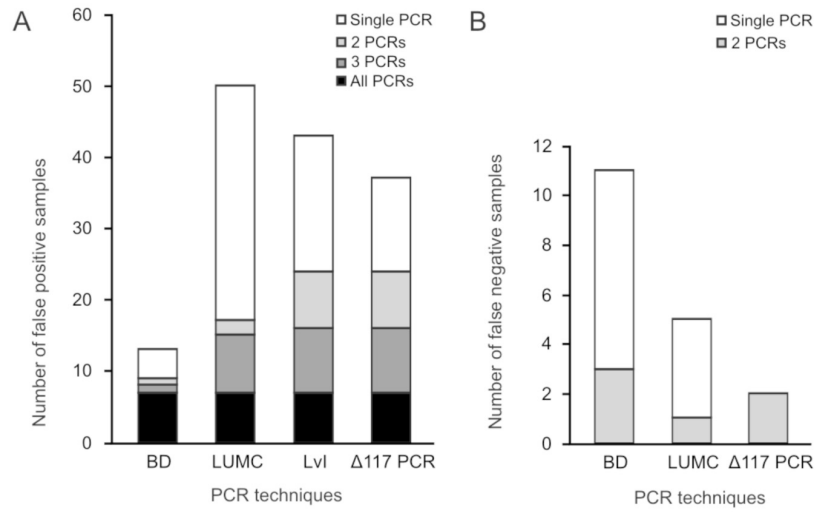

Figure 1: (A) False positive results and **(B)** false negative results detected by real-time PCRs with overlapping samples**.** All false positive and false negative results from each PCR method compared to the CYTGC assay were analyzed for resemblances. Resemblances in false positive and false negative results were ordered by PCR method. No false negative results were found by the LvI real-time PCR.

Table 2. Comparison of four real-time PCR methods with gold standard CYTGC.

Assay	Samples included	Samples inhibited	Result	CYTGC Assay (N)		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%)	NPV (%)
				+	-				
LUMC PCR	526	10	+	97	50	96.0	88.0	66.0	98.9
			-	4	365	(90.3-98.5)	(84.5-90.7)		
LvI PCR	522[b]	16	+	99	43	100.0	89.4	69.7	100.0
			-	0	364	(96.3-100)	(86.1-92.1)		
Δ117 PCR[a]	522[b]	15	+	97	38	98.0	90.7	71.9	99.5
			-	2	370	(92.9-99.4)	(87.5-93.1)		
BD GeneOhm	512[c]	N/A	+	83	13	88.3	96.9	86.5	97.4
			-	11	405	(80.3-93.3)	(94.8-98.2)		

For each PCR method the number of samples included and inhibited is shown. Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of the PCR methods are given as percentages, and the corresponding 95% confidence interval (95% CI) is shown in parentheses.[a]. The lowest Ct value belonging to either the *tcdC* wildtype or mutant probe was used for the evaluation of the Δ117 PCR as a screening assay. [b] 4 samples were not present in the collection. [c] data for 14 samples were not available.

Fourteen percent of the false positives detected by the LUMC PCR were also detected as such by all other PCR methods, whereas for the LvI PCR and the Δ117 PCR 16% and 19% of the false positives had a similar test outcome by all other PCR methods. Figure 1B shows the number of false negative results detected by the real-time PCRs. No false negative results were found by the LvI real-time PCR with the CYTGC as standard. Compared to CYTGC, 11 false negative results (2.6% of the total amount of negatives) were detected by the BD GeneOhm PCR, whereas the LUMC PCR and the Δ117 PCR had 1.1% and 0.5% false negative samples, respectively. None of the false negative samples were detected as such by all three PCR methods; only overlapping results between two PCR methods were found.

Comparing the Δ117 PCR with PCR ribotyped CYTGC positive samples

Of the 99 CYTGC positive samples, a total of 21 samples were typed as RT 027 by PCR-Ribotyping (Table 3). The Δ117 PCR was able to confirm 20 of these samples (95%) by detection of the 1 bp deletion at position 117 in the *tcdC* gene, with a ΔCt (Ct WT – Ct MUT Δ117) = + 2.9 cycles difference on average. Compared with CYTGC positive, RT 027 samples, sensitivity, specificity, PPV and NPV values were for this assay: 95.2%, 96.2%, 87.0% and 98.7%. The Δ117 PCR detected 3 samples carrying the Δ117 mutation, which were ribotyped as RT 005, RT 106 and an unknown RT, not RT 027.

Table 3: Comparison of the LvI Δ117 PCR with PCR ribotyping.

Assay	Samples included[a]	Result	Ribotyping of cultured isolates		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
			Ribotype 027	Ribotype non-027				
Δ117 PCR	99	+	20	3[b]	95.2	96.2	87.0	98.7
		-	1	73				

All CYTGC positive samples (N=99) were analyzed by the Δ117 PCR. Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of the PCR method are given as percentages.[a] Only CYTGC positive samples were included.[b] Three CYTGC positive samples were typed as PCR Ribotype 005, Ribotype 106, and rare Ribotype (not 027).

Discussion

Rapid and accurate diagnosis of CDI is essential for patient management and prevention of nosocomial transmission. A main issue in diagnosing CDI is that most conventional tests do not have sufficient performance for applying it as a standalone test. Molecular tests are increasingly applied for diagnosing CDI and are also mentioned in a recent published guideline as potentially rapid assays with better performances (4). This study was performed to compare the diagnostic value of three in-house developed real-time PCRs and a commercially available BD GeneOhm Cdiff assay, using the appropriate gold standard on 526 prospectively collected stool samples. The sensitivity of the in-house developed PCRs was better than the BD GeneOhm test, in contrast to the specificity. Subsequently, NPVs were similar whereas the PPV was the highest for the BD GeneOhm test (86.5%). Peterson *et al.* (14) evaluated a real-time PCR that targeted the *tcdB* gene of *C. difficile* and reported a sensitivity of 93.3% and a specificity of 97.4% This is in line with what has been reported by Sloan *et al.* (15) on the performance of a real-time PCR, which was directed against the *tcdC* gene. They reported a sensitivity of 86% and specificity of 97%. The sensitivity and specificity reported by both studies are comparable to what has been found in this study for the BD GeneOhm Cdiff PCR.

Our study had a prevalence of toxigenic *C. difficile* positive samples of approximately 20%, repeated samples from positive patients were excluded. The prevalence was high, due to a selection of faeces samples with high suspicion of CDI. The PPV is dependent on the prevalence of the disease in the tested population. Several studies report that in the hospital 5%-10% of the antibiotic-associated diarrhea samples contain *C. difficile* (16-18). In the community the prevalence of CDI is close to 2% (19,20). Therefore, we calculated PPVs for all PCR methods at prevalence's of 2%, 5% and 10%. The calculated PPVs at 10% and 5% prevalence decreased to 76% and 60% for the BD GeneOhm PCR, 51% and 33% for the LvI PCR, 54% and 36% for the Δ117 PCR and 47% and 30% for the LUMC PCR. At 2% prevalence PPVs are 37% (BD GeneOhm), 16% (LvI), 18% (Δ117) and 14% (LUMC).

The performance of the LUMC real-time PCR reported in this evaluation was better than reported previously by van den Berg *et al.* (8). This difference can be explained by the difference in prevalence of CDI positive samples used in this study (20%) and the previous study (6%). Furthermore, we optimized PCR conditions (PCR-mix, modified probe), and reached a detection limit of 10^3 CFU/gram stool samples (data not shown) which is improved compared to 10^5 CFU/gram as reported by van den Berg *et al.* (14).

When the false positive PCR results were analyzed, 54% of the false positives detected by the BD GeneOhm PCR were also detected as such by the other PCRs. This suggests that 54% of the false positives contained *C. difficile* specific *tcdB* DNA, since the samples were detected by three PCRs targeting the *tcdB* gene using different primer sets. In addition, these samples were also found positive by the Δ117 PCR using the *tcdC* gene as a target. It can not be excluded that the cultures were false negative due to previous antibiotical treatment but discrepancies with CYTGC assay still remain present. We consider this finding as an important lack of the currently available gold standard and think that future clinical studies are necessary to interpret the findings more precisely.

In comparison to CYTGC positive samples with *C. difficile* RT 027, the Δ117 PCR targeting the *tcdC* gene Δ117 1 bp deletion has a high concordance of 95.2%. This high concordance makes the utility of the Δ117 PCR for direct detection of the epidemic strain promising, although further research is needed to determine if other *C. difficile* RTs contain the *tcdC* point mutation at position 117 and, consequently, are detected by this PCR. Furthermore, the performance of this PCR for detection of toxigenic *C. difficile* indicates that this assay has the potential to diagnose CDI, without pre-screening for the toxin genes A and B. The *tcdC* gene has been recognized as a putative negative regulator of *tcdA* and *tcdB* and thereby is indicative of the presence of the pathogenicity locus (21).

A difference between the three in-house developed real-time PCRs was the percentage of inhibited samples. In total, 3.1% (n = 16), and 2.9% (n=15) of all samples (n = 522) tested by the LvI PCR and Δ117 PCR were inhibited respectively, whereas 1.9% (n = 10) of all samples (526) tested by the LUMC PCR were inhibited. The PCRs used different DNA extraction methods and different platforms which might contribute to the observed differences.

Most rapid diagnostic tests do not have sufficient performance for applying it as a standalone test. Recently, Planche *et al.* (17) defined that a test is applicable as a standalone test when a sensitivity of at least 90% and a specificity of at least 97% is reached. The three in-house developed PCRs lack specificity, whereas the BD GeneOhm PCR lacks sensitivity resulting in too low PPVs of all PCRs ranging from 66% to 86.5% at 20% CDI-prevalence. These PPVs decrease substantially when calculating PPVs for CDI-prevalence's that are more common for a clinical setting (10%) or observed in the community (2%). None of our evaluated real-time PCR methods fulfilled the criteria defined by Planche *et al.* (17). Therefore, it was concluded that they cannot be applied as a standalone test. This finding is in line with what has been found for toxin detection assays and other molecular based assays by other studies (12,16,17). However, due to their high NPVs all four evaluated PCR methods can be applied as a first negative screening test for CDI

in a two-step algorithm. In this algorithm the PCR assay is followed by a second confirmation step to confirm the first positive test result.

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