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ARTICLE

Diagnosis of *Clostridium difficile*: real-time PCR detection of toxin genes in faecal samples is more sensitive compared to toxigenic culture

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Abstract The diagnosis of *Clostridium difficile* infection (CDI) requires the detection of toxigenic *C. difficile* or its toxins and a clinical assessment. We evaluated the performance of four nucleic acid amplification tests (NAATs) detecting toxigenic *C. difficile* directly from faeces compared to routine toxigenic culture. In total, 300 faecal samples from Danish hospitalised patients with diarrhoea were included consecutively. Culture was performed in duplicate (routine and 'expanded toxigenic culture': prolonged and/or re-culture) and genotypic toxin profiling by polymerase chain reaction (PCR), PCR ribotyping and toxinotyping (TT) were performed on culture-positive samples. In parallel, the samples were analysed by four NAATs; two targeting *tcdA* or *tcdB* (illumigene® *C. difficile* and PCRFast® *C. difficile A/B*) and two multi-target real-time (RT) PCR assays also targeting *cdt* and *tcdC* alleles characteristic of epidemic and potentially more virulent PCR ribotypes 027, 066 and 078 (GeneXpert® *C. difficile*/Epi and an 'in-house RT PCR' two-step algorithm). The multi-target assays were significantly more sensitive compared to routine toxigenic culture ($p<0.05$) and significantly more robust to inhibition compared to PCRFast ($p<0.001$). Duplicate 'expanded toxigenic culture' increased the culture-positive rate by 29 % compared to routine culture. The ability of the GeneXpert and in-house assays to correctly classify PCR ribotype 027 was high (>95 %), and in-house PCR displayed 100 % correct identification of PCR ribotypes 066 and 078. Furthermore, the presence of the PCR enhancer bovine serum albumin (BSA) was found to be related to high sensitivity and low inhibition rate. Rapid laboratory diagnosis of toxigenic *C. difficile* by RT PCR was accurate.

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Introduction

Over the past decade, the prevalence and severity of *Clostridium difficile* infection (CDI) has increased, due to outbreaks of epidemic strains, notably BI/NAP1/027 [polymerase chain reaction (PCR) ribotype 027] and PCR ribotype 078 [1–3]. Poor patient outcome, treatment failure, and increased mortality and recurrence rates have been associated with PCR ribotypes 027 and 078 [4–6].

The main causative agents of CDI are two well-known large clostridial toxins, toxin A (TcdA) and toxin B (TcdB), expressed from their respective genes, *tcdA* and *tcdB*, in the pathogenicity locus (PaLoc) [7]. Regulators of *tcdA* and *tcdB* reside within the PaLoc, including a putative negative regulator *tcdC* [7]. The genotypic characteristics of highly virulent strains of *C. difficile* are several mutations in the *tcdC* gene. PCR ribotype 027 harbours an 18-bp in-frame deletion and a single nucleotide (nt) deletion at position 117 (Δ117) in *tcdC*, whereas PCR ribotype 078 (and 066) possesses a 39-bp in-frame deletion and a single nt substitution at position 184

(C184T) in *tcdC* [6, 8]. However, the importance of these genotypic changes in *tcdC* for virulence remains to be determined [9–12]. In addition to *tcdA* and *tcdB*, epidemic strains (including PCR ribotypes 027, 066 and 078) may express a third toxin, the binary toxin (actin-ADP-ribosylating toxin *C. difficile* transferase, CDT), encoded by *cdtA* and *cdtB* located in the Cdt locus [13]. The binary toxin genes have been suggested as an additional cause of excess morbidity, mortality and higher recurrence rates of CDI [14, 15].

The presence of *tcdA*, *tcdB* and *cdtA*/*cdtB* in combination with specific nt mutations in *tcdC*, expressed by epidemic strains of *C. difficile*, can be exploited in the laboratory diagnosis of *C. difficile*. Rapid and reliable diagnosis of CDI is essential for optimal patient management, infection control and understanding the epidemiology. Several fast commercial nucleic acid amplification tests (NAATs) are now available, including seven U.S. Food and Drug Administration (FDA)-approved tests (http://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm330711.htm). Conventional PCR, real-time (RT) PCR and loop-mediated isothermal amplification (LAMP) are established NAATs used in laboratory diagnostics of microorganisms. A limitation to PCR detection, when applied to biological samples, is the presence of inhibitors [16], which can result in indeterminate or false-negative results. However, reagents such as bovine serum albumin (BSA) are capable of buffering inhibitors in biological samples [17]. Therefore, BSA has the potential to optimise RT PCR-based diagnostic methods.

The objective of this study was to evaluate the performance of three commercial NAATs, illumigene® *C. difficile*, GeneXpert® *C. difficile*/Epi PCR and PCRFast® *C. difficile A/B*, and an in-house RT PCR algorithm with comparison to our current method, toxigenic culture.

Materials and methods

Study population and sample collection

A total of 300 consecutive faecal samples from 283 hospitalised patients with diarrhoea were enrolled from February 14th to April 5th 2011. Samples were included upon submission for routine diagnostics of *C. difficile* at the Department of Clinical Microbiology, Slagelse Hospital, Denmark. The criterion for testing was infectious diarrhoea. Multiple samples from the same patient were allowed, with a minimum interval of 14 days between sampling. The samples were sent at ambient temperature without a transport medium. The age inclusion criterion was ≥2 years. However, children <2 years were included if *C. difficile* testing was specifically requested by the physician.

Toxigenic culture and genotyping

On arrival in the laboratory, the faecal samples were kept at 5 °C until cultures were performed, usually within 4 to 5 h of receipt. The majority (>90 %) of samples were tested within 24 h of collection. For spore purification, 1 ml of stool was suspended in (i) 1 ml of 99.9 % ethanol and incubated for 1 h at ambient temperature or (ii) 1 ml of saline, heated at 80 °C for 10 min and cooled for 5 min at ambient temperature. Approximately 50–75 μl of stool suspension was plated on cycloserine cefoxitin fructose agar (CCFA) plates (SSI Diagnostica, Hillerød, Denmark) and incubated under anaerobic conditions at 37 °C for 48 h. Anaerobic culture was performed in duplicate; one plate for the routine and one for the research diagnostics. Presumptive *C. difficile* colonies were identified by standard phenotypic characteristics: yellow colour; flat, filamentous, snowflake-like colony morphology; 'horse-barn' odour (paracresol); and, when indeterminate, positive L-proline aminopeptidase test (Rosco Diagnostica, Taastrup, Denmark). Routine diagnostic results were blinded to the NAATs testing. All negative research plates at 48 h were re-incubated for an additional 7–10 days. Continued culture-negative but NAAT-positive samples were re-cultured with an extended incubation time (7–10 days). Prolonged and/or re-culture results are represented by the 'expanded culture' reference method.

Culture-positive samples were analysed for their toxigenic nature by toxin profiling, PCR ribotyping and toxinotyping (TT). These tests were performed blinded to the NAATs results. One to five (I–V) *C. difficile* colonies from the research plate were sub-cultured individually onto 5 % horse blood agar plates (SSI Diagnostica) and incubated anaerobically at 37 °C for 24–72 h. Isolates were stored in beef broth with 10 % glycerol (SSI Diagnostica) at −80 °C. Following the inclusion period, one isolate per episode was recovered and genotypic toxin profiling of *tcdA*, *tcdB* and *cdtA*/*cdtB* was performed as previously published [18, 19]. In addition, the in-frame deletions of *tcdC* were detected according to Persson et al. [19]. PCR ribotyping was carried out according to O'Neill et al. [20] and Stubbs et al. [21]. Unknown PCR ribotypes (no profile match in the reference strain collection) were designated SLAxxxx. TT, based on the restriction patterns of *tcdB* and *tcdA* using restriction fragment length polymorphism PCR, was done according to Rupnik et al. [22]. When the obtained genotypic results from GeneXpert and/or the in-house PCR diverged in comparison to the genotypic toxin profiling and PCR ribotyping, additional isolates from the specific sample were analysed, if available.

NAATs

Two automated FDA-approved tests with integrated DNA extraction (provided in the kit) and nucleic acid amplification

(NAA) protocols and two manual tests with independent DNA extraction and PCR protocols were evaluated. The automated tests were illumigene (Meridian Bioscience, Milan, Italy) and GeneXpert (Cepheid, Sunnyvale, CA, USA); the manual tests were PCRFast (ifp, Institut für Produktqualität, GmbH, Berlin, Germany) and one in-house RT PCR two-step algorithm [23, 24]. Toxigenic culture, anaerobic culture followed by genotypic toxin profiling combined with PCR ribotyping, was used as the reference method to evaluate the performance of the four NAATs.

illumigene and PCRFast detect *tcdA* and *tcdB* only and, thus, provide a toxigenic *C. difficile*-positive or -negative answer. According to the absence or presence of *cdt* and Δ117*tcdC*, GeneXpert can differentiate toxigenic *C. difficile*-positives in non-027 or presumptive PCR ribotype 027. The in-house PCR was designed to classify toxigenic *C. difficile*-positive samples in non-027, presumptive PCR ribotype 027 or potential 066 or 078 (066/078) on the basis of $cdtA^{+/-}$ and specific single nt mutations in the *tcdC* gene.

Automated NAATs and faecal suspension preparation for independent DNA extraction were performed daily, and that for the manual NAATs in batches weekly.

For the three commercial tests, execution, results interpretation and resolving of indeterminate results (including inhibited samples) were performed according to the manufacturers' guidance and by the visual inspection of raw data (amplification curves, Ct values and level of fluorescence).

Automated NAATs

illumigene is based on LAMP, whereas GeneXpert is a multiplex RT PCR assay. For illumigene, external quality control was performed once a day and for GeneXpert for each lot of kits using a negative control (NC) (w/o stool) and a positive control (PC) (toxigenic *C. difficile* faecal sample). Inhibited samples were repeated with a reduced faecal load until a valid result was recorded.

Independent DNA extraction for manual NAATs: NucliSENS® easyMAG®

Nucleic acid extraction from stool was performed on the NucliSENS® easyMAG® platform (bioMérieux, Marcy I'Etoile, France), according to the manufacturer's protocol optimised for stool samples ("Extraction Protocol for the NucliSENS easyMAG BTL039444 rel. 1.0 for stool samples" in combination with "Specific B" protocol). Stool samples were transferred to NucliSENS® Lysis Buffer (bioMérieux) [1:2 (wt/vol)], vortexed and homogenised for 1 min at 7,000 rpm using the MagNA Lyser Instrument (Roche Applied Science, Penzberg, Germany). Faecal suspensions were centrifuged for 2 min at 16,000 g and stored at −20 °C until batch testing. The automated extraction was conducted in batches of 24 samples. 100 μl of thawed and re-spun supernatant was incubated in 2 ml of NucliSENS® Lysis Buffer for 10 min at room temperature, including phocine herpesvirus (PhHV) [25] serving as the internal extraction and amplification control (IC) in the in-house algorithm. This total volume of 2.1 ml together with 140 μl of magnetic silica was loaded onto the platform. The output eluates (110 μl) were stored at 5 °C until weekly manual RT PCR analysis.

Manual NAAT: PCRFast® *C. difficile*

PCRFast is an RT PCR test amplifying *tcdA*, *tcdB* and an IC. All input eluates were initially diluted 1:4 in nuclease-free water (Qiagen) in order to prevent total inhibition (this is in accordance with the protocol). NC (water) and PCs (supplied in the kit) were included in each batch. Inhibited samples (no signal from the IC) were resolved by further dilution of the DNA input, until a valid result (signal from the IC) was obtained.

Manual NAAT: in-house multiplex RT PCR two-step algorithm

The in-house algorithm consists of two RT PCR steps; a 'toxin' followed by a '*tcdC* genotypic' reaction. The algorithm was adapted from previously published primer and probe designs [23, 24], with optimisations and modifications compatible with our facility, including the introduction of new probes.

The toxin reaction detects *tcdA*, *tcdB* and *cdtA*, and serves as a screening assay [23]. The toxin reaction was performed on all 300 faecal samples, whereas *tcdC* genotyping for three *tcdC* alleles [wild type (wt), Δ117 deletion and an A117T point mutation] was carried out on toxin gene-positive samples only, although including NAAT-negative but culture-positive samples.

In our hands, the original probe designs by de Boer et al. [24] and Hoegh et al. [23] produced low fluorescence and linear amplification curves in pilot testing (data not shown). However, for the toxin reaction, the addition of a master mix optimised for multiplex RT PCR in combination with BSA produced conclusive results.

For the *tcdC* genotyping reaction, new probes were designed to improve specificity and enable the identification of presumptive *C. difficile* PCR ribotypes 066 and 078. Sequence analysis of *tcdC* gene sequences available from GenBank identified an A117T mutation in *tcdC*, which results in a truncated open reading frame. In previously *tcdC* sequenced strains, we found this variant in epidemic PCR ribotypes as 066 and 078, although not exclusively. The binary toxin-positive PCR ribotype 023 was seen to carry this mutation too. The new probe set makes it possible to distinguish the A117T signal from the Δ117 signal. Both mutation

probes will generate a signal in the presence of either the Δ117 deletion or the A117T point mutation in *tcdC*; however, the 'correct' probe will yield the strongest signal (lowest Ct value), enabling discrimination. Usually, wt samples will yield a single *tcdC* signal exclusively. Thus, the *tcdC* genotyping, in combination with the toxin reaction, separates *C. difficile* non-027, presumptive 027 and potential 066/078 PCR ribotypes. The primer and probe sequences and final concentrations are given in Table 1. PCR reactions contained 5 μl of eluate in a total volume of 20 μl (toxin reaction) or 25 μl (*tcdC* genotyping) and 0.2 μg/μl BSA (Fermentas, Ontario, Canada). The toxin reaction contained the 2× TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), whereas the *tcdC* genotyping reaction contained the originally reported 2× TaqMan Universal PCR Master Mix (Applied Biosystems) [24]. DNA extracts from a negative (NC), a non-027 and a PCR ribotype 027-positive *C. difficile* faecal sample (PCs) served as controls in each batch test. PCR was performed on an Mx3005P thermocycler (Stratagene, La Jolla, CA, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C; followed by 42/40 cycles of 15 s at 95 °C and 1 min at 60 °C. Batch RT PCR was considered valid when the NC generated the IC signal and the two PCs generated signals according to their genotypes. Patient specimens were considered positive when two or more signals, toxin and *tcdC* genotyping combined, reached a fixed threshold of 0.05. An RT PCR was considered inhibited when no signal from the IC was recorded.

Statistics

For the statistical comparison of routine toxigenic culture-negative samples, a regression method for paired samples was used [26]. The outputs were relative positive fractions with 95 % confidence intervals (CIs). For tabulated data with low or zero counts, Fisher's exact test and McNemar's test were used. A *p*-value of 0.05 was considered significant. The statistical calculations were performed in the open access software R. The Fisher test command in R allows for tables larger than 2×2.

Ethical approval

This study obtained approval from the Regional Ethics Committee of Zealand, Denmark (SJ-208). Informed patient consent was waived.

Table 1 Primers and probes for the in-house real-time (RT) polymerase chain reaction (PCR) two-step algorithm

Gene target	Sequence name	Sequence (5′-3′)[a, b]	Final concentration (nM)	Reference
Toxin reaction				
tcdA	*tcdA*-F	AATTTAGCTGCAGCATCTGACATAGT	300	[23]
	tcdA-R	TTCCCAACGGTCTAGTCCAATAG	300	[23]
	tcdA-P	VIC-TGTTGATATGCTTCCAGGTAT-MGB	100	[23]
tcdB	*tcdB*-F	ATAATGGTAGATTTATGATGGAACTAGGAA	300	[23]
	tcdB-R	TCTTGATAAGCTGCCGCATATG	300	[23]
	tcdB-P	FAM-AGAGTTGGTTTCTTCCCAG-MGB	100	[23]
cdtA	*cdtA*-F	ATGTAAATGATTATATGCGTGGAGGAT	100	[23]
	cdtA-R	GGTTCACGTTTTAATGCATTTTCA	300	[23]
	cdtA-P	NED-TCAAATGGTCCAGTAAAT-MGB	100	[23]
PhHV	PhHV-F	GGGCGAATCACAGATTGAATC	80	[25]
	PhHV-R	GCGGTTCCAAACGTACCAA	100	[25]
	PhHV-P	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BBQ	100	[25]
tcdC genotyping				
tcdC	CD-*tcdC*-F	GCACAAAGGRTATTGCTCTACTGG	300	[24]
	CD-*tcdC*-R1	AGCTGGTGAGGATATATTGCCAA	300	[24]
	CD-*tcdC*-R2	CAAGATGGTGAGGATATATTGCCA	300	[24]
	tcdC-wtAP	FAM-CACGCCTAAAATAA-MGB	100	This study
	tcdC-wtGP	FAM-ACGCCCAAAATA-MGB	100	This study
	tcdC-Δ117	VIC-AACACACCAAAATAA-MGB	100	This study
	tcdC-A117T	NED-ACACACCAAAAATAA-MGB	100	This study

[a] *MGB* minor groove binder; *BBQ* Blackberry quencher

[b] R = A or G

Results

Routine toxigenic culture as the reference method

A total of 300 consecutive faecal samples from 283 patients were tested for *C. difficile* by duplicate anaerobic culture and four NAATs. One patient contributed three samples and 15 patients contributed two samples. Two patients under the age of 2 years were included. The median age of the 161 female and 122 male patients was 66 years (range 4 months to 97 years).

In routine testing, anaerobic culture yielded 42 *C. difficile*-positive samples from 39 patients (median age 69.8 years; range 6–95 years). *C. difficile* isolated from three samples were non-toxigenic by genotypic PCR, and equally found negative by the four NAATs and were classified as negative. One isolate (F-5830) could not be recovered and was excluded from analysis. Hence, the prevalence of toxigenic *C. difficile* estimated by routine culture was 12.7 % (38 out of 299).

Forty-nine faecal specimens yielded an initially invalid (inhibited) test result in one or two NAAT assays: none for in-house PCR, two with GeneXpert, eight with illumigene and 40 with PCRFast (Table 2). One specimen (F-4350) gave an invalid result in both illumigene and PCRFast, whereas the other 48 invalid results were recorded in one assay only. The majority (98.0 %) of invalid results were recorded from *C. difficile* culture-negative samples. The total inhibition rate of PCRFast (13.4 %) was significantly ($p<0.001$) higher than the rate of inhibition produced by the other assays (Table 2). Specimens yielding an initially invalid result were re-tested with diluted template or reduced faecal load until a valid result was recorded, and this result was used in performance characterisation.

Using routine toxigenic culture as the reference method for comparison, the sensitivity and specificity of the four NAATs are given in Table 3. The overall agreement (accuracy) with routine toxigenic culture was similar for all four NAATs; 92.6 %, 93.3 %, 95.3 % and 95.7 % for the in-house PCR, GeneXpert, PCRFast and illumigene assay, respectively (Table 3). The specificity ranged from 92.0 % to 98.1 %, with no significant difference between tests. The highest specificity was recorded for the PCRFast assay. However, this assay had a significantly ($p<0.001$) lower sensitivity (76.3 %). illumigene, in-house PCR and GeneXpert had sensitivities of 94.7 %, 97.4 % and 100 %, respectively. The false-negative samples (routine toxigenic culture-positive samples that failed to be detected by an NAAT) are given in Table 4. One sample failed to be detected in three NAATs, one sample failed in two NAATs and seven samples failed in PCRFast only.

As presented in Table 3, it is evident that routine toxigenic culture missed some *C. difficile*-positive samples. Three of the four NAATs identified more *C. difficile*-positive samples than routine toxigenic culture; illumigene, GeneXpert and the in-house PCR. The two multi-target assays were significantly more sensitive than routine toxigenic culture. Overall, for all 299 samples, 38 (12.7 %) were positive in routine toxigenic culture. The GeneXpert and in-house PCR gave 58 (19.4 %) positive results, significantly increasing the diagnostic yield with a relative positive fraction of 1.53 (95 % CI 1.05–2.24, $p=0.03$). Using the positive fraction of expanded toxigenic culture as the reference, no significant difference in specificity was found (Table 3).

'Expanded toxigenic culture' as the reference method

Besides identifying all routine positive samples as *C. difficile*-positives, expanded culture (duplicate plating, prolonged incubation time and re-culture of NAAT-positive but routine culture-negative samples) resolved an additional 12 samples as *C. difficile* culture-positives (Table 5). Two strains were excluded; one was non-cultivable after storage (F-3618) and one was non-toxigenic (F-5508) by genotypic PCR, giving a prevalence of 16.1 % when the expanded culture is included (48 out of 299). Seven samples remained culture-negative but NAAT-positive by at least two NAATs. In all, 16 routine culture-negative samples were resolved as true-positives (TPs) by the use of a composite reference (positive in at least two NAATs) (Table 5).

Table 2 Inhibition rate (recorded from the initial test) of the four nucleic acid amplification tests (NAATs) for *Clostridium difficile* diagnostics: illumigene, GeneXpert, PCRFast and in-house RT PCR

Assay	Routine toxigenic culture-negative ($n=261$)		Routine toxigenic culture-positive ($n=38$)		Total number of inhibition (%)
	Number of invalid results (%)	Number of valid results (%)	Number of invalid results (%)	Number of valid results (%)	
illumigene *C. difficile*	8 (3)	253 (97)	0 (0)	38 (100)	8 (2.7)
GeneXpert *C. difficile*	2 (1)	259 (99)	0 (0)	38 (100)	2 (0.7)
PCRFast *C. difficile*	39 (15)	222 (85)	1 (3)	37 (97)	40 (13.4)*
In-house PCR	0 (0)	261 (100)	0 (0)	38 (100)	0 (0.0)

*$p<0.001$, Fisher's exact test

Table 3 Comparison of routine toxigenic culture with expanded toxigenic culture and NAATs

Assay	Routine toxigenic culture-negative (n=261)			Routine toxigenic culture-positive (n=38)		Total number of positives (%)	Accuracy (%)
	Number of negatives (%)[a]	Number of positives (%)	Relative positive fraction (95 % CI)	Number of negatives (%)	Number of positives (%)[b]		
Expanded toxigenic culture	250 (95.8)	11 (4.2)	1	0 (0)	38 (100)	49 (16.4)	288 (96.3)
illumigene *C. difficile*	250 (95.8)	11 (4.2)	1.00 (0.44–2.30)	2 (5.3)	36 (94.7)	47 (15.7)	286 (95.7)
GeneXpert *C. difficile*	241 (92.3)	20 (7.7)	1.82 (0.91–3.86)	0 (0)	38 (100)	58 (19.4)*	279 (93.3)
PCRFast *C. difficile*	256 (98.1)	5 (1.9)	0.45 (0.14–1.23)	9 (23.7)	29 (76.3)**	34 (11.4)	285 (95.3)
In-house PCR	240 (92.0)	21 (8.0)	1.91 (0.96–4.03)	1 (2.6)	37 (97.4)	58 (19.4)*	277 (92.6)

CI confidence interval

[a] Specificity and [b] sensitivity using routine toxigenic culture as the reference method

*p<0.05, Fisher's exact test

**p<0.001, Fisher's exact test (p<0.01, McNemar's test)

Seven of the ten additional toxigenic culture-positive strains belonged to PCR ribotype 027, two were other PCR ribotypes (SLA0014 and 078) and one sample (F-5957) was polymicrobial, containing two PCR ribotypes, 014/020/077 and SLA0011 (non-toxigenic strain) (Table 5). GeneXpert detected all additional samples as positives. In-house PCR detected all but the polymicrobial sample. illumigene and PCRFast detected only 50 % and 20 % of the additional samples, respectively.

Screening for presumptive 027 and potential 066/078 PCR ribotypes

To assign the GeneXpert and in-house PCR assays' ability to identify presumptive 027 and 066/078 PCR ribotypes, the typing results from the expanded culture was used as the reference method (Supplemental Table S1). Two specimens (F-5568 and F-5957) were polymicrobial and, therefore, excluded in this context. Fifty-four percent of the total of 46 toxigenic strains belonged to PCR ribotype 027; 17.4 % were 014/020/077 and the remaining were various other PCR ribotypes, including ten previously unknown. The study included a total of two 066 and 078 PCR ribotypes. The GeneXpert and in-house PCR assays identified all 027 faecal samples as positive for toxigenic *C. difficile*, although the in-house PCR incorrectly assigned one sample as non-027. PCRFast classified 40.0 % (10) of 027 samples as negative. All non-027-066-078 PCR ribotype samples were correctly identified by the GeneXpert assay. One, three and six of the non-027-066-078 PCR ribotype samples failed to be detected by the in-house PCR, illumigene and PCRFast assays, respectively. These samples represented six different PCR ribotypes (014/020/077, 095, SLA0005, SLA0008, SLA0010, SLA0014) belonging to toxinotype 0 (Supplemental Table S1). The in-house PCR design's ability to recognise a mutation in *tcdC* characteristic of presumptive 066 and 078

Table 4 Discrepant results between routine toxigenic culture-positive samples and NAATs results

Sample ID	Routine culture	PCR ribotype	Toxinotype	illumigene *C. difficile*[a]	GeneXpert *C. difficile*[b]	PCRFast *C. difficile*[a]	In-house PCR[b]
F-2407	*C. difficile*	027	III	Pos	027	Neg	027
F-2859	*C. difficile*	027	III	Pos	027	Neg	027
F-4036	*C. difficile*	027	III	Pos	027	Neg	027
F-4771	*C. difficile*	027	III	Pos	027	Neg	027
F-2266	*C. difficile*	SLA0008	0	Pos	Non-027	Neg	Non-027
F-4347	*C. difficile*	SLA0005	0	Pos	Non-027	Neg	Non-027
F-5108	*C. difficile*	095	0	Pos	Non-027	Neg	Non-027
F-4685	*C. difficile*	SLA0010	0	Neg	Non-027	Neg	Neg
F-4698	*C. difficile*	014/020/077	0	Neg	Non-027	Neg	Non-027

[a] Toxigenic *C. difficile*: *Neg* negative; *Pos* positive

[b] Toxigenic *C. difficile*: *027* potential 027 PCR ribotype; *Non-027* potential non-027 PCR ribotype; *Neg* negative

Table 5 Additional true-positive samples: positive by expanded culture and/or positive in at least two NAATs (composite reference)

Sample ID	Routine culture	Expanded culture	PCR ribotype	Toxinotype	illumigene *C. difficile*[a]	GeneXpert *C. difficile*[b]	PCRFast *C. difficile*[a]	In-house PCR[b]	Resolution[c]
F-3920	Neg	*C. difficile*	027	III	Pos	027	Pos	027	TP
F-5272	Neg	*C. difficile*	078	V	Pos	027	Pos	066/078	TP
F-3143	Neg	*C. difficile*	027	III	Pos	027	Neg	027	TP
F-3694	Neg	*C. difficile*	027	III	Pos	027	Neg	027	TP
F-4805	Neg	*C. difficile*	027	III	Pos	027	Neg	027	TP
F-2166	Neg	*C. difficile*	027	III	Neg	027	Neg	027	TP
F-4282	Neg	*C. difficile*	027	III	Neg	027	Neg	027	TP
F-2714	Neg	*C. difficile*	027	III	Neg	027	Neg	Non-027	TP
F-4821	Neg	*C. difficile*	SLA0014	0	Neg	Non-027	Neg	Non-027	TP
F-3468	Neg	Neg	n.d.	n.d.	Pos	027	Pos	027	TP
F-5795	Neg	Neg	n.d.	n.d.	Pos	027	Pos	027	TP
F-2676	Neg	Neg	n.d.	n.d.	Pos	Non-027	Pos	Non-027	TP
F-2594	Neg	Neg	n.d.	n.d.	Pos	027	Neg	027	TP
F-5141	Neg	Neg	n.d.	n.d.	Pos	027	Neg	027	TP
F-4694	Neg	Neg	n.d.	n.d.	Pos	Non-027	Neg	Non-027	TP
F-5793	Neg	Neg	n.d.	n.d.	Neg	Non-027	Neg	Non-027	TP
F-5957	Neg	*C. difficile*	Polymicrobial		Neg	Non-027	Neg	Neg	Inconclusive
F-5957-I/III/IV/V		*C. difficile*, non-toxigenic	SLA0011	n.d.					
F-5957-II		*C. difficile*	014/020/077	0					
F-3618	Neg	*C. difficile*, non-cultivable	n.d.	n.d.	Neg	027	Neg	027	Inconclusive
F-5508	Neg	*C. difficile*, non-toxigenic	SLA0012	n.d.	Neg	Neg	Neg	Neg	Excluded

n.d. not determined

[a] Toxigenic *C. difficile*: *Neg* negative; *Pos* positive

[b] Toxigenic *C. difficile*: *027* potential 027 PCR ribotype; *066/078* potential 066 or 078 PCR ribotype; *Neg* negative; *Non-027* potential non-027 PCR ribotype

[c] *TP* true-positive

PCR ribotypes was 100 % correct ($n=2$). illumigene, GeneXpert and PCRFast identified the 066 and 078 samples as positive; however, GeneXpert incorrectly classified the 078 sample as presumptive 027. The overall agreement between GeneXpert and in-house PCR and PCR ribotyping was high (>95 %). No correlation between discordant results and strain type was found for any of the four NAATs.

Discussion

The optimal laboratory diagnosis of CDI remains an area of controversy [27–29]. In addition, the best suited gold standard method for comparative studies remains to be determined. If the detection of toxigenic strains is the aim, then culture with demonstration of the toxigenic potential of the isolate is the appropriate reference method [27]. The diagnosis of CDI requires both clinical assessment and the laboratory detection of toxigenic *C. difficile*. Thus, laboratory testing should be limited to patients with clinical symptoms compatible with CDI, and the goal of the laboratory is to quickly and accurately ascertain whether toxigenic *C. difficile* is present [27].

Three of the four evaluated NAATs identified more positive samples than routine toxigenic culture; illumigene, GeneXpert and in-house PCR. The two multi-target assays were significantly more sensitive than toxigenic culture. The low sensitivity of our toxigenic culture was surprising, but may be explained by aerobic toxicity, non-cultivable organisms in the sample, our routine spore enrichment method and culture medium (CCFA), which has been reported to be suboptimal [30]. Direct plating of stool samples on a chromogenic agar has been reported to be potentially more sensitive than growth on cefoxitin cycloserine media for *C. difficile* [31, 32]. However, since samples could be resolved by expanded toxigenic culture and high agreement between NAATs was observed, the data suggested that the initial culture-negative but NAAT-positive samples were, indeed, TPs. This is in agreement with the observation that broth enrichment and duplicate culturing may enhance the *C. difficile* culture-positive rate by 30 % [33]. The clinical significance of a positive NAAT as a stand-alone method has been questioned; recently, however, RT PCR detection of toxigenic *C. difficile* by GeneXpert has been reported to correlate with the clinical diagnosis of CDI [29].

Conversely, the inability of an NAAT to detect a culture-positive sample could be a result of poor assay test design, inhibition, limit of detection etc. Furthermore, in the present study, two polymicrobial (multiple strains) samples were found, both yielding inconsistent results between NAATs. Multiple PCR ribotypes of *C. difficile* may be present simultaneously [34, 35], and this might explain deviations between PCR ribotyping and NAAT results, and between different NAATs.

Compared to enzyme immunoassay (EIA) for glutamate dehydrogenase (GDH), GeneXpert has been reported to have a higher sensitivity for non-027 versus 027 PCR ribotype samples [36], and EIAs to have a higher sensitivity for 027 versus non-027 [36, 37]. However, on cultured isolates, GDH and GeneXpert have been reported not to be affected by specific PCR ribotypes [38]. In accordance, our data showed no difference in the identification of PCR ribotypes, emphasising the importance of a mutual reference method.

The objective of the present study was to evaluate the diagnostic performance on consecutive clinical samples of four NAATs addressing toxin targets compared to toxigenic culture of *C. difficile*. Two of the assays could provide additional information regarding the presence of *cdt* and specific single nt mutations in *tcdC* (Δ117 and A117T) characteristic of potential epidemic strains, such as 027, 066 and 078 PCR ribotypes, and their performance in recognising potential 027 PCR ribotype was high. For GeneXpert, this is in agreement with studies from the United States [39, 40]. In Europe, PCR ribotype 027 is epidemic and 078 is the third most common cause of CDI [41, 42]. Further, the in-house algorithm displayed correct identification of PCR ribotypes 066 and 078. However, the sample size was too small to be conclusive on these PCR ribotypes specifically; hence, further studies on *C. difficile* strains with the A117T *tcdC* allele are warranted. Single nt mutations in *tcdC* (A117T and C184T) have been shown to predict recurrent CDI [12]. Thus, it would be of interest to include clinical data in a study on the A117T *tcdC* allele and its significance to CDI.

In this study, superior *C. difficile* recognition, sensitivity and low inhibition correlated with the presence of the PCR enhancer BSA in the RT PCR reaction. The GeneXpert assay had 0.7 % inhibited samples and the in-house PCR had none. Conversely, PCRFast, not including BSA, displayed a significantly higher rate of inhibition (13.4 %) and a significantly lower sensitivity (76.3 %). Since the in-house PCR utilises the same DNA extract as PCRFast, the high rate of invalid results observed for PCRFast seems to reflect susceptibility to the presence of inhibitors in the template. The protocol for DNA extraction on the easyMAG® platform we used is particularly optimised for stool specimens. In combination with the addition of BSA, the "Specific A" protocol exhibits high efficiency in RT PCR [43]. The "Specific B" protocol used in the present study resembles Specific A, but with six additional washing steps - a further improvement. However, the high inhibition rate of PCRFast indicates that the DNA extraction procedure alone does not manage inhibitors. Faeces are known to contain components that inhibit PCR, e.g. dietary components [44] and heme [45]. BSA and other PCR enhancers have been shown to buffer these [46], an observation which our results seem to support. The PCRFast protocol

recommends resolving inhibited samples by further dilution of input DNA, which, again, was reflected in the significantly lower sensitivity. In comparison, the in-house PCR assay managed twice as much DNA as PCRFast without inhibition.

The lower inhibition rate of illumigene compared to that of PCRFast supports the general finding that the LAMP technique is more tolerant to inhibitors in biological samples compared to PCR [47]. In this study however, the BSA-optimised RT PCR assays exhibited an even lower inhibition rate than the illumigene LAMP-based assay. Indeed, recent findings suggest that the susceptibility of different NAATs to inhibitors depends on the particular inhibitor [48]. To our knowledge, illumigene does not contain BSA. Therefore, we speculate that the addition of an inhibition buffer to the illumigene and PCRFast assays (if possible) might increase the robustness of these assays in the presence of inhibitors and increase sensitivity.

In conclusion, the two best performing assays were a commercial kit-based and fully automated system (GeneXpert) and an in-house RT PCR two-step algorithm, which can be used in different laboratory settings. They offer prompt and proper detection of toxigenic *C. difficile*. Furthermore, they have the ability to differentiate CDI according to the presumptive PCR ribotype. These assays were more sensitive than toxigenic culture, included BSA and were more robust to inhibition compared to the other two NAATs evaluated. With the large number of *C. difficile* NAATs now commercially available, the presence of a PCR enhancer such as BSA may be an additional factor to consider when selecting a reliable laboratory diagnostic test, besides turn-around-time, costs, hands-on time, laboratory facilities, technical skills etc.

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Conflict of interest The authors declare no conflict of interest.

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