Comparison of two commercial molecular tests for the detection of *Clostridium difficile* in the routine diagnostic laboratory

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Two commercial real-time PCR assays for the detection of *Clostridium difficile*, BD GeneOhm Cdiff assay (BD Diagnostics) and Xpert *C. difficile* assay (Cepheid), were compared to each other and to toxigenic culture, which was used as a gold standard, on a set of 194 clinical stools submitted for routine diagnostic analysis. Of 28 (14.4 %) toxigenic culture positive samples 23 were positive with both assays, the BD and the Cepheid real-time PCR assays, 4 were positive only by Cepheid Xpert *C. difficile* assay and 1 sample was negative by both PCR assays, resulting in sensitivity, specificity, positive predictive value and negative predictive value of 82.1, 98.2, 88.5 and 97.0 %, respectively, for the BD GeneOhm Cdiff assay, and 96.4, 97.3, 87.1 and 99.3 %, respectively, for the Cepheid Xpert *C. difficile* assay. Altogether 26 out of 194 (13.4 %) samples were reported invalid by Cepheid. Toxigenic *C. difficile* positive samples contained 15 different PCR ribotypes distributed into toxinotype 0 and 2 different variant toxinotypes (III, IV). Clinical data were available for 24 out of 28 (85.7 %) toxigenic *C. difficile* positive patients and 18 (75.0 %) of them were diagnosed with diarrhoea, while others had other symptoms or risk factors related to possible *C. difficile* infection (antibiotics, bloody stool, peritonitis, Crohn's disease).

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INTRODUCTION

Clostridium difficile infection (CDI) is one of the most important causes of nosocomial diarrhoea and is also becoming important in the community and in populations with low or no risk factors (Rupnik *et al.*, 2009). CDI can result in asymptomatic colonization or can result in clinical symptoms ranging from mild diarrhoea to severe colitis, leading to complications such as toxic megacolon or sepsis. There are a variety of commercially available diagnostic tests for the diagnosis of *C. difficile*, differing in turnaround time, sensitivity, specificity, costs, workload and availability (Crobach *et al.*, 2009). ELISAs are the most commonly employed tests in *C. difficile* diagnosis. They are rapid and inexpensive but lack sensitivity (Planche *et al.*, 2008).

Especially during outbreaks and during the spread of strains with increased virulence it is important that the laboratory diagnosis is rapid and sensitive to ensure

appropriate therapy and quick implementation of hospital hygiene measures. Therefore several groups have reported in-house real-time PCR assays. All had very good sensitivity, specificity and short turnaround times but special procedures for elimination of PCR inhibitors during DNA extraction were needed (Alonso *et al.*, 1999; Bélanger *et al.*, 2003; Van den Berg *et al.*, 2005; Eastwood *et al.*, 2009; Wroblewski *et al.*, 2009). Currently several real-time PCR assays that detect toxigenic *C. difficile* directly from stool are commercially available. Published reports cover only three of them: BD GeneOhm Cdiff assay (BD Diagnostics), ProGastro Cd assay (Prodesse) and Cepheid Xpert *C. difficile*, which detect the toxin B gene (*tcdB*) (Barbut *et al.*, 2009; Huang *et al.*, 2009; Stamper *et al.*, 2009; Terhes *et al.*, 2009; Babady *et al.*, 2010; Doing *et al.*, 2010; Knetsch *et al.*, 2011; Kvach *et al.*, 2010; Novak-Weekley *et al.*, 2010; Swindells *et al.*, 2010; Tenover *et al.*, 2010). Cepheid Xpert *C. difficile* also gives information about the binary toxin gene and 117 bp *tcdC* deletion, both features of the NAP1/BI/027 strain, a causative agent for epidemics of CDI in countries worldwide. A fourth

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval.

commercially available assay is based on the ligase chain reaction (illumigene *C. difficile*; Meridian) and detects the *tcdA* gene (Norén *et al.*, 2011).

The aim of this study was to compare two commercial molecular tests to the toxigenic culture method performed as a part of routine diagnostics. In addition, detection of *C. difficile* was correlated with clinical data and with the genotype (PCR ribotype and toxinotype) of the strain.

METHODS

Stool specimens. Samples from hospitalized patients and patients from other institutions (<5% of all samples; sent by general practitioners and long-term care facilities) submitted to the Institute of Public Health, Maribor, for routine *C. difficile* testing between November 2008 and April 2009 were included in the study. Samples were either liquid (62.4%), semi-solid (12.4%) or solid (14.4%), while for 10.8% of samples the consistency was not determined. Samples with sufficient stool available were tested with two commercial real-time PCRs within 2 to 3 days after collection, and were stored during this time at 4 °C. If testing in this time frame was not possible samples were stored at -25 °C until use.

Isolation of *C. difficile*. During the routine isolation of *C. difficile*, stool samples were subjected to alcohol shock and cultured on commercially available CLO agar plates (bioMérieux). Initial identification was based on the morphology of the colonies and typical odour, and was confirmed by the VITEK system (bioMérieux). The VIDAS *C. difficile* toxin A and B assay (bioMérieux) was used for the detection of toxins directly from stool samples or from multiple colonies from positive primary cultures if the toxin result from the sample was negative.

A sample with isolated *C. difficile* that produces toxin A and/or B according to a positive toxin test was defined as a positive routine toxigenic culture. If the isolated strain did not produce toxins, the sample was defined as a routine nontoxigenic culture.

In the case of a negative routine culture result and a positive result by at least one molecular test, enrichment from the stool sample was performed in CCFB (cycloserine-cefoxitin-fructose broth) (which has the same ingredients as Oxoid *C. difficile* agar minus the agar), supplemented with D-cycloserine and cefoxitin (*C. difficile* selective supplement; Oxoid) for 5 days in anaerobic jars at 37 °C. After incubation, an equal amount of ethanol was added to 500 µl enrichment culture, this was incubated for 30 min at room temperature and centrifuged at 10 000 g for 5 min. The entire pellet was inoculated onto CLO agar plates (bioMérieux). One or more recovered *C. difficile* colonies were subcultured on blood agar plates.

Characterization of *C. difficile* isolates. Toxinotyping and PCR ribotyping were performed on strains subcultured for 24–48 h on Columbia blood agar as described by Rupnik (2008) and Bidet *et al.* (1999). Binary toxin genes were detected by PCR, as described by Stubbs *et al.* (2000). The PaLoc-negative genotype was confirmed by PCR using Lok1/Lok3 primers (Braun *et al.*, 1996). PCR ribotype profiles were analysed by BioNumerics software and PCR ribotypes were designated either with standard Cardiff nomenclature (001, ...) if the profile corresponded to one of 25 reference PCR ribotype strains, including the current most frequent PCR ribotypes (including among others 001, 002, 014, 015, 023, 027, 078, 106, 126, ...), or with internal nomenclature (SLO 001, ...).

Molecular tests. Both molecular tests were performed directly on stool samples. Molecular testing and routine culture/toxin testing

were each carried out independently by different members of the laboratory. If commercial PCR failed to detect *tcdB* directly from toxigenic culture positive stool, isolates were also analysed by the same commercial PCR to test the detection of the isolated strains by these assays.

BD GeneOhm Cdiff assay. The BD GeneOhm Cdiff assay was performed according to the manufacturer's protocol. Lysis of the specimen (faeces or 1–5 *C. difficile* colonies cultured for 24 h on blood agar plates) was performed by vortexing the sample with glass beads, heating and cooling. The lysate was added to a SmartCycler tube containing reconstituted master mix. Every PCR run included a PCR positive control (reconstituted DNA from the manufacturer's kit). Uninoculated sample buffer served as a negative control. Every sample assay also included an internal control. The specific reaction tubes were placed in the SmartCycler I-CORE module (Cepheid) and run using Cepheid SmartCycler software with the BD GeneOhm Cdiff amplification protocol. The interpretation of the assay results was reported as: 'negative' where no *tcdB* gene was detected, 'positive' where *tcdB* gene was detected, 'unresolved' in case of inhibition of internal control or reagent failure, 'invalid assay run' where one of the PCR controls (positive or negative) failed and 'not determined' when there was an I-CORE module malfunction.

In the case of a reported 'invalid' or 'unresolved' assay run the assay should, according to the manufacturer's protocol, be repeated with the same lysate (crude DNA). No such results were obtained during this study.

Cepheid Xpert *C. difficile* assay. The Cepheid Xpert *C. difficile* assay was performed according to the manufacturer's protocol. A sterile Copan swab was dipped into the stool specimen and material was resuspended in the sample buffer and transferred to the single-use disposable cartridge. The amplification and detection was run in the GeneXpert Dx module. Every run included the SPC (sample processing control), the control for adequate processing of the target bacteria and to monitor inhibition of PCR, and the PCC (probe check control), the control that verified reagent rehydration, PCR tube filling in the cartridge, probe integrity and dye stability. Results were automatically interpreted by the software as: '*C. difficile* positive', '*C. difficile* 027 NAP1 presumptive positive', '*C. difficile* negative', 'invalid', 'error' or 'no result'. Also, the Cepheid assay enabled the viewing of amplification curves and hence interpretation by the technician. In the case of 'invalid', 'error' or 'no result', the reported test was repeated. Retesting was performed using a new swab sample taken from the original stool sample.

RESULTS

Results of both molecular tests compared to routine toxigenic culture

A total of 194 samples were analysed by both molecular tests (BD and Cepheid) and the results compared to toxigenic culture. Of all 194 tested samples, 28 (14.4%) were toxigenic culture positive in the routine testing (without enrichment) (Table 1). Initially, 23 (11.9%) samples were positive by all three assays and 141 (72.7%) were negative by all three assays. Of the 28 routine toxigenic culture positive samples, 4 were positive only by Cepheid test and not by BD test.

At initial testing 26 (13.4%) invalid (reported as invalid or error) results were obtained by the Cepheid assay. Of them

Table 1. Comparison of two molecular tests to toxigenic culture obtained in the routine laboratory

Toxigenic culture	Xpert <i>C. difficile</i> (Cepheid)			GeneOhm Cdiff (BD)		
	Positive	Negative	Total no. of samples	Positive	Negative	Total no. of samples
Positive	27	1	28	23	5	28
Negative	4	146	150	3	163	166
Total no. of samples	31	147	178*	26	168	194

*Samples that gave an invalid result by molecular methods were repeated. If the result was positive or negative, the sample was included in the analysis; if the result was invalid again the sample was excluded from the analysis ($n=16$ samples).

two were routine toxigenic culture positive and one was positive after *C. difficile* enrichment (all three were BD positive). After repeated testing Cepheid resolved the result in 10 out of 26 samples. The remaining 16 'invalid' samples were excluded from further analysis (Table 1). None of the samples was invalid by BD assay.

Compared to the routine toxigenic culture, and after exclusion of invalid results, the sensitivity (95 % confidence interval) (95 % CI), specificity (95 % CI), positive predictive value (95 % CI) and negative predictive value (95 % CI) for the BD GeneOhm assay ($n=194$) were: 82.1 % (67.9–96.3), 98.2 % (96.2–100.0), 88.5 % (76.2–100.0) and 97.0 % (94.4–99.6), respectively, and for the Cepheid Xpert *C. difficile* assay ($n=178$) 96.4 % (89.5–100.0), 97.3 % (94.7–99.9), 87.1 % (75.3–98.9) and 99.3 % (98.0–100.0), respectively (Table 1).

In a total of 12 samples, the results of one or both molecular tests were not in agreement with routine toxigenic culture, but all discrepant results were clarified after repeated testing or after enrichment (Table 2). In cases where retesting of the false negatives with BD and/or Cepheid assay resulted in a negative outcome, isolated strains from that sample were also tested with the same molecular test and were all recognized. The outcome for six out of seven routine toxigenic culture negative samples was a positive result with at least one of the molecular tests (Table 2) and one was indicated to be positive only by Cepheid assay.

Detection of variant and nontoxigenic strains by molecular tests

C. difficile was cultured from 38 out of 194 stool samples. In addition to 28 toxigenic strains, 5 nontoxigenic strains were isolated by routine culture (SLO 010, SLO 028, SLO 030, SLO 055, SLO 057), and an additional 5 samples yielded toxigenic *C. difficile* after enrichment. Neither of the commercial molecular tests reported the *tcdB* marker in five stool samples that yielded nontoxigenic strains.

From 38 samples a total of 39 strains were obtained. The majority (94.9 %) of isolated toxigenic strains belonged to toxinotype 0 (014/020, SLO 009, SLO 022, SLO 026, 002,

SLO 011, SLO 050, SLO 007, 029, SLO 071, SLO 016, SLO 068, SLO 074). Only two strains were variant toxinotypes: toxinotype IV (binary toxin positive; PCR ribotype 023) and toxinotype III (binary toxin negative; PCR ribotype SLO 049). Both molecular tests detected the *tcdB* gene in toxinotype 0, toxinotype III and toxinotype IV strains. In the latter strain (IV/023), binary toxin gene was also correctly identified by Cepheid test. Out of 15 different PCR ribotypes, 7 were not always detected by one or both molecular tests (Table 2). None of the samples contained all three genetic markers (*tcdB*, binary toxin and *tcdC* deletion) and no PCR ribotype 027 was recovered from any of the culture positive samples.

Clinical data and positive samples

Clinical data were available for 28 out of 38 (73.7 %) *C. difficile* positive samples, including toxigenic and nontoxigenic strains. *C. difficile* was detected at least once in 24 out of 170 (14.1 %) sampled patients. During the study period 14 patients were sampled only once, 4 patients were sampled twice, and for a small number of patients three samples ($n=3$), four samples ($n=2$) and five samples ($n=1$) were taken. For most of patients with multiple samples the time frame between the first and last sample was from 1 to 3 months. Selected patients with more than one sample are shown in Table 3.

Clinical signs (diarrhoea, colitis or pseudomembranous colitis) correlated with toxigenic culture in 18 out of 24 (75.0 %) toxigenic culture positive samples, where clinical data were available. Other patients did not have signs of diarrhoea at the time of sampling but were tested for *C. difficile* due to other reasons, like severe abdominal pain, previous antibiotic treatment, an episode of inflammatory bowel disease and vomiting.

DISCUSSION

The aim of our study was to compare the detection of *C. difficile* in samples from patients suspected of having CDI with two commercial molecular *C. difficile* diagnostic assays. Valid results of both tests were in congruence with

Table 2. Resolution of discrepant results

1st testing			Repeat testing			Toxinotype/PCR ribotype of isolate	Sample assignment
Routine culture	1st BD test	1st Cepheid test	Enrichment	2nd BD test	2nd Cepheid test		
Neg	Pos	Neg	Neg	Neg	ND	NA	True negative
Neg	Neg	Pos	Neg	ND	Neg	NA	True negative
Neg	Neg	Pos	Pos	Neg	ND	0/029	True positive
Neg	Neg	Pos	Pos	Neg	ND	0/014/020	True positive
Neg	Pos	Pos	Pos	ND	ND	0/029	True positive
Neg	Pos	Invalid	Pos	ND	Invalid	0/SLO 009	True positive
Pos	Neg	Neg	ND	Neg	Pos	0/014/020	True positive
Pos	Neg	Pos	ND	Neg	ND	0/014/020	True positive
Pos	Neg	Pos	ND	Neg	ND	0/002	True positive
Pos	Neg	Pos	ND	Neg	ND	0/SLO 068	True positive
Pos	Neg	Pos	ND	Pos	ND	0/SLO 074	True positive
Neg	Neg	(Neg)*	Pos	Neg	Pos	0/SLO 016	True positive

NA, Not applicable; ND, not done; neg, negative; pos, positive.

*Cepheid test reported as negative, but the amplification curve was rising after the cut-off and hence the test was repeated.

toxigenic culture results obtained in the routine laboratory. In some cases the molecular diagnostic test was even more sensitive than the usual routine culture, and *C. difficile* was isolated from these samples only after enrichment. Most of the 28 *C. difficile* toxigenic culture positive cases with obtained clinical data were presented as with either diarrhoea or some other gastrointestinal symptoms.

It was reported that the PCR ribotype can affect the sensitivity of some enzymic diagnostic tests and that, for example, PCR ribotype 027 is more likely to be recognized by immunological tests than some other prevalent PCR ribotypes (e.g. ribotype 014) (Tenover *et al.*, 2010). In our study 9 out of 33 (27.3 %) toxigenic culture positive samples (routine and enrichment cultures) resulted in false

Table 3. Collected data and testing results of selected patients sampled more than once

Patient	Routine culture	Enrichment	Cepheid test	Cepheid repeat test	BD test	BD repeat test	Sampling date	Toxinotype/PCR ribotype	Symptoms	Antibiotic treatment
Patient 1	tox	ND	+	ND	+	ND	28.11.2008	0/SLO 009	d/col	No
	tox	ND	Invalid	+	+	ND	15.01.2009	0/SLO 009	d	No data
	–	tox	Invalid	Invalid	+	ND	25.02.2009	0/SLO 009	d	No
Patient 2	–	ND	–	ND	–	ND	12.01.2009	Neg	No data	No data
	–	ND	–	ND	–	ND	15.01.2009	Neg	No data	No data
	–	tox	+	ND	–	–	12.02.2009	0/029	No data	No data
	tox	ND	+	ND	+	ND	17.02.2009	0/029	a	Yes
	tox	ND	+	ND	+	ND	08.03.2009	0/029	d	Yes
Patient 3	tox	ND	+	ND	+	ND	01.12.2008	0/014/020	a	Yes
	tox	ND	+	ND	+	ND	10.12.2008	0/SLO 022	d	Yes
	tox	ND	+	ND	+	ND	07.01.2009	0/014/020	d/pmc	No
	–	ND	+	–	–	ND	26.01.2009	Neg	a	Yes
	tox	ND	+	ND	+	ND	31.12.2008	0/SLO 011	d	Yes
Patient 4	tox	ND	+	ND	+	ND	19.01.2009	0/SLO 011	No data	No data
	tox	ND	+	ND	–	–	30.01.2009	0/SLO 068	a	No
	–	tox	+	ND	+	ND	24.02.2009	0/029	d	Yes
	nontox	ND	–	ND	–	ND	19.01.2009	tox-/SLO 010	No data	No
Patient 5	nontox	ND	–	ND	–	ND	22.01.2009	tox-/SLO 055	a	No
	–	tox	–	+	–	–	07.02.2009	0/SLO 016	d	Yes

a, Asymptomatic; col, colitis; d, diarrhoea; ND, not done; neg, no isolated strains; nontox, nontoxigenic *C. difficile*; pmc, pseudomembranous colitis; tox, toxigenic *C. difficile*.

negatives by at least one of the two molecular assays. These nine isolates were grouped into seven different PCR ribotypes and no association was seen between false-negative results and specific genotypes isolated from the samples.

Although both molecular assays are validated and suggested for use on liquid or unformed stools, the diagnostic laboratory would often receive formed stool samples for testing. These were mainly from patients with symptoms other than diarrhoea, such as abdominal pain, fever or a previous *C. difficile* episode. In our study only 62.4 % samples were unformed, and the results suggest that consistency did not affect sensitivity and was not associated with invalid results.

The BD test did not give any invalid results. A low percentage of invalid results was reported by Stamper *et al.* (2009) and Terhes *et al.* (2009). In our study the Cepheid test had a higher proportion of invalid results and they were rarely resolved after repeating the test. This was not reported previously. However, the Cepheid test allows viewing of the amplification curves. In our study, one sample had all tests reported as negative but because the Cepheid assay showed a very late increase of the amplification curve we repeated the test, as well as performing an enrichment culture, and both resulted in a positive outcome. At this point, the patient was also symptomatic (Table 2; Table 3, patient 5).

Both molecular assays were found to be useful for routine diagnosis of *C. difficile* and could improve detection. In some patients with multiple samples a low detection limit of routine culture testing at the initial or the final sampling points was noticed (Table 3). The higher sensitivity of molecular tests in these cases could therefore contribute to early detection of *C. difficile* and could have an impact on successful treatment of the disease.

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