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RESEARCH NOTE

Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR

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ABSTRACT

A real-time PCR assay for *Clostridium difficile* was developed, based on the *tcdB* gene, which detected all known toxinogenic reference strains (n = 45), within 30 serogroups and 24 toxinotypes. The analytical sensitivity was 1×10^3 CFU/mL, and the detection limit in faeces was 1×10^5 CFU/g. The optimal protocol for DNA extraction from faecal samples involved use of the MagnaPure system with a Stool Transport and Recovery (STAR) buffer pre-treatment. In a 1-month prospective study of 85 patients with diarrhoea, the sensitivity, specificity and positive and negative predictive values of the assay were 100%, 94%, 55% and 100%, respectively, compared with the standard cell cytotoxicity assay.

Keywords Assay, *Clostridium difficile*, faecal samples, real-time PCR, *tcdB* gene

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Clostridium difficile has been recognised as the causative agent of antibiotic-associated diarrhoea (CDAD) and pseudomembranous colitis (PMC). Enteropathogenicity depends on the production of enterotoxin A (TcdA; 308 kDa) and cytotoxin B (TcdB; 270 kDa) [1,2]. TcdA has been regarded as the most important factor causing enteropathogenic disease [3,4], but there have been an increasing number of reports of disease caused by TcdA-negative, TcdB-positive strains [5]. Therefore, the present study designed a real-time PCR assay for *tcdB* to enable rapid diagnosis of CDAD associated with toxinogenic *C. difficile*. An optimal DNA extraction protocol for faecal samples was established, and an internal control was included to verify amplification.

Primers and probe (Table 1) were designed from the non-repeat region of a known *tcdB* sequence (accession no. X53138) using the Primer3 program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplification reactions were performed in a 50-μL final volume, containing 25 μL IQ Supermix (Bio-Rad, Veenendaal, The Netherlands), 5 pmol forward primer, 10 pmol reverse primer, 4 mM $MgCl_2$, 0.2 μM probe, and 5 μL DNA extract. Following an enzyme activation step for 3 min at 95°C, amplification comprised 50 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). The assay was optimised using *C. difficile* strain ATCC43594, and had an analytical sensitivity in saline 0.9% w/v of 1 CFU/PCR, corresponding to 1×10^3 CFU/mL. In addition, ten-fold dilutions of ATCC43594 (1×10^7–1 CFU) were spiked into 1 g of pooled *C. difficile* culture-negative faeces to determine the sensitivity of the real-time PCR assay in comparison with culture.

For *C. difficile* culture, faecal samples, with and without ethanol-shock treatment, were inoculated on to selective media as described previously [6]. Colonies of Gram-positive bacilli with sub-terminal spores were tested for L-proline-aminopeptidase production and aesculin hydrolysis [7]. Two separate experiments revealed that the sensitivity of the real-time PCR assay with faeces was less

Table 1. Sequences of primers and probes used for real-time PCR detection of *Clostridium difficile*

Target	Primers and probes	Nucleotide sequence (5′-3′)	Amplicon size (bp)
C. difficile	398CLDs	GAAAGTCCAAGTTTACGCTCAAT	177
	399CLDas	GCTGCACCTAAACTTACACCA	
	551CLD-tq-FAM	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA	
PhHV [10]	295PhHVs	GGGCGAATCACAGATTGAATC	89
	296PhHVas	GCGGTTCCAAACGTACCAA	
	531PhHV-tq-CY5	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2	

PhHV, phocine herpes virus (internal control).

than that of culture (1×10^5 CFU/g faeces vs. 1×10^4–1×10^5 CFU/g faeces), but was comparable to the sensitivity (5×10^4 CFU/g faeces) reported for a real-time PCR assay described previously [8].

To define the analytical specificity of the assay, all known *C. difficile* serogroups (n = 30; gift of M. Delmee, University of Louvain, Brussels, Belgium) and toxinotypes (n = 24; gift of M. Rupnik, University Goettingen, Goettingen, Germany) were tested. All toxinogenic serogroups (n = 23) and 22 of 24 toxinotypes were detected; two toxinotypes (XIa and XIb) do not harbour *tcdB*, and were not detected by the assay. In contrast, Belanger *et al.* [8] were unable to detect *tcdB* in toxinotypes III, IV and VI. This discrepancy was associated with polymorphisms around the 3′-end of the primers. There were some mismatches between the primers and probe used in the present study and the available sequences for TcdA–/TcdB+ strains 1470 and 8864 (accession nos. CDTOXBA and CDI011301, respectively). Strain 1470, representing toxinotype VIII and serogroup F, showed one mismatch with the forward primer, but was still detected by the real-time PCR. Strain 8864, representing toxinotype X, showed two mismatches for both the forward primer and the probe, and one mismatch in the reverse primer at the 5′-end. This strain was also positive by the real-time PCR, but may be detected with reduced sensitivity when analysing clinical samples. Although degenerate primers could solve this problem, strain 8864 is a naturally occurring isolate that has not yet been detected in clinical samples. Nine *Clostridium* spp., other than *C. difficile*, and 27 other (including enteropathogenic) bacterial species were all negative according to the assay.

Since effective DNA extraction from faecal samples and removal of inhibitory factors is a key factor in successful application of PCR [9], polyvinylpolypyrolidone (PVPP) pre-treatment, followed by isolation using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) was compared with pre-treatment using the Stool Transport and Recovery (STAR) buffer, followed by automated extraction with the MagnaPure LC DNA isolation Kit III (Roche, Almere, The Netherlands) in the MagnaPure System (Roche). A fixed amount of Phocine Herpes Virus (PhHV), to give a crossing point (Ct) value of *c.* 33–34, was spiked into clinical samples before DNA extraction to serve as an internal control [10]. Primers used for PhHV detection are listed in Table 1. Although no differences in Ct values could be observed between the two extraction methods with faeces spiked with 2.2×10^6–22 CFU/g, and the internal control was amplified efficiently in all assays, the STAR/MagnaPure method was deemed optimal because of its automated format.

For clinical evaluation of the assay, faecal samples were obtained from 28 patients with CDAD. Primary diagnosis and selection was made by detection of TcdA with an enzyme-linked fluorescent immunoassay (ELFA) (VIDAS CDA2; bioMérieux, Boxtel, The Netherlands) according to the manufacturer's recommendations. All 28 ELFA-positive faecal samples were also positive by real-time PCR and culture for *C. difficile*. When a control group of 43 faecal samples from 43 patients without gastrointestinal symptoms was analysed, three (7%) were positive

Table 2. Prospective comparison of the cell cytotoxicity assay (indicative of *Clostridium difficile*-associated diarrhoea), enzyme-linked fluorescent assay, real-time PCR and culture with 85 faecal samples from patients with diarrhoea

		No. of positive samples	
Clinical manifestation	No. of patients	ELFA	Real-time PCR
CDAD	6	5	6
Non-CDAD	79	1	5
Total	85	6	11[a]

CDAD, *Clostridium difficile*-associated diarrhoea; ELFA, enzyme-linked fluorescent assay.
[a]All real-time PCR-positive results were confirmed by culture of toxinogenic *C. difficile*.

by real-time PCR and culture, indicating asymptomatic carriage of toxinogenic *C. difficile* [11,12]. These three samples were confirmed as positive following re-extraction and re-testing by real-time PCR.

For further clinical evaluation, 85 faecal samples received from adult patients with a request for *C. difficile* testing or from patients with diarrhoea admitted to the hospital for ≥3 days were investigated prospectively (Table 2). These samples were tested initially by ELFA, and were then stored at −80°C within 24 h pending further analysis by the cell cytotoxicity assay and real-time PCR. Of six (7%) samples positive by the cell cytotoxicity assay (Table 2), five were positive by ELFA, and six by real-time PCR. Of 79 cytotoxicity-negative samples, one was positive by ELFA and real-time PCR, and five were positive only by real-time PCR. Thus, 11 (13%) of 85 samples were positive by real-time PCR; *C. difficile* was cultured subsequently from all these samples. Using the cytotoxicity assay as the standard, the sensitivity, specificity and PPV and NPV for the real-time PCR assay were 100%, 94%, 55% and 100%, respectively.

In conclusion, detection of the *C. difficile tcdB* gene in faecal samples by real-time PCR, using an automated DNA extraction protocol and an internal control, can be used as a rapid method for diagnosing CDAD and for detecting carriage in asymptomatic patients.

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RESEARCH NOTE

Prevalence of methicillin-resistant *Staphylococcus aureus* in infected and uninfected diabetic foot ulcers

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ABSTRACT

This study investigated the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in infected and uninfected diabetic foot ulcers of 84 patients with the two types of diabetes. *S. aureus* was the most common pathogen among the Gram-positive bacteria isolated from ulcers, and almost 50% of *S. aureus* isolates were MRSA. The

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