Detection of *Clostridium difficile* toxin A/B genes by multiplex real-time PCR for the diagnosis of *C. difficile* infection

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Toxigenic *Clostridium difficile* culture is considered to be the standard diagnostic method for the detection of *C. difficile* infection (CDI). Culture methods are time-consuming and although enzyme immunoassay is rapid and easy to use, it has low sensitivity. In the present study, the AdvanSure CD real-time (RT)-PCR kit (LG Life Sciences) was evaluated for its ability to detect *C. difficile* toxin A (*tcdA*) and B (*tcdB*) genes, simultaneously. A total of 127 fresh diarrhoeal stool specimens, submitted to the clinical microbiology laboratory for *C. difficile* culture, were tested. *C. difficile* toxins and toxin genes were detected with a VIDAS *C. difficile* A&B (VIDAS-CDAB) enzyme-linked fluorescent immunoassay (ELFA) and the AdvanSure RT-PCR kit, respectively, according to the manufacturers' instructions. Their performance was compared with a standard toxigenic culture method as a reference. The sensitivity, specificity and positive and negative predictive values using the AdvanSure RT-PCR kit were 100 %, 98.3 %, 84.6 % and 100 %, respectively, while those of the VIDAS-CDAB system were 63.6 %, 100 %, 100 % and 96.6 %, respectively. Four *tcdA*⁺/*tcdB*⁺ strains of *C. difficile* were detected with the AdvanSure RT-PCR kit, which offers comparable sensitivity and specificity to the reference method with a turnaround time of ~3 hours.

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INTRODUCTION

Clostridium difficile usually produces two toxins, toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin), and is responsible for a range of diseases from mild diarrhoea to pseudomembranous colitis. This micro-organism is the most common cause of healthcare-associated diarrhoea (Bartlett, 2002). In recent years, the incidence of *C. difficile* infection (CDI) has rapidly increased as has disease severity associated with the emergence of hypervirulent strain BI/NAP1/027 (McDonald *et al.*, 2005; Kuijper *et al.*, 2008).

The gold standards for CDI diagnosis, currently in use, are the cytotoxicity assay and toxigenic culture method. Two-step testing, in which a negative result with one test is considered to be negative but a positive result is subjected to further testing, has also been proposed to combine the benefits of greater sensitivity, rapid turn-around time and reduced cost (Crobach *et al.*, 2009; Cohen *et al.*, 2010). *C. difficile* culture has the demerit of being time-consuming, taking up to 72 h to produce results. Although various enzyme immunoassays (EIA) have proven to have sensitivities that are less than optimal as diagnostic tests, they

are the assays that are most commonly used due to their rapidity and ease of use (Ticehurst *et al.*, 2006; Eastwood *et al.*, 2009). Thus, a single, rapid and accurate diagnostic test is needed.

Real-time (RT)-PCR for the detection of the *tcdB* gene and multiplex RT-PCRs for the detection of *tcdB* gene deletions and *tcdC* gene mutations at nucleotide 117 have been developed, especially for the presumptive identification of epidemic strain BI/NAP/027. (Eastwood *et al.*, 2009; Huang *et al.*, 2009; de Boer *et al.*, 2010; Barbut *et al.*, 2011; Knetsch *et al.* 2011). The sensitivities and specificities of these tests have been reported to be 88.5–100 % and 88.0–97.7 %, respectively. Recently, a new RT-PCR kit, the AdvanSure CD RT-PCR kit (LG Life Sciences) was developed. The kit can simultaneously detect *tcdA* and *tcdB* genes and easily recognize *tcdA*[−]/*tcdB*⁺ strains of *C. difficile*. To our knowledge, this is the first study to evaluate the AdvanSure multiplex RT-PCR kit for diagnosing CDI.

METHODS

Stool specimens. A total of 127 fresh diarrhoeal stool specimens, submitted to the clinical microbiology laboratory of the Severance Hospital for *C. difficile* culture from June to July 2010, were analysed. The specimens were obtained consecutively and with no duplicates.

Abbreviations: CDI, *Clostridium difficile* infection; EIA, enzyme-linked immunoassay; ELFA, enzyme-linked fluorescent immunoassay; RT, real-time.

Toxigenic culture (anaerobic culture followed by detection of *tcdA* and *tcdB* genes by PCR), VIDAS *C. difficile* Toxin A&B (VIDAS-CDAB; bioMérieux) ELFA and AdvanSure RT-PCR methods were performed on the day of specimen reception by independent laboratory staff. Assay performance (sensitivity, specificity and positive and negative predictive values) of the AdvanSure RT-PCR and the VIDAS-CDAB kits were estimated and compared, using the results of the toxigenic culture as a reference method.

Toxigenic *C. difficile* culture and detection of toxins. Stool specimens were cultured anaerobically on *C. difficile* selective agar (CDSA; Becton Dickinson) for 48 h at 37 °C. Species identification was performed on the basis of typical colony morphology on agar plates as well as characteristic odour and ATB 32A system (bioMérieux) results. PCR experiments were performed to detect *C. difficile* toxin genes as described in previous studies (Spigaglia & Mastrantonio, 2004; Terhes *et al.*, 2004). The primer pairs used were NK9-NK11 for the repetitive domain of the *tcdA* gene, NK104-NK105 for the *tcdB* gene, *cdtA* pos-*cdtA* rev for the *cdtA* gene and *cdtB* pos-*cdtB* rev for the *cdtB* gene. *C. difficile* strains VPI 10463 (*tcdA*⁺/*tcdB*⁺/*cdt*⁺), 3608/03 (*tcdA*⁺/*tcdB*⁺/*cdt*⁺), SE844 (*tcdA*⁺/*tcdB*⁺/*cdt*⁺) and 1470 (*tcdA*⁺/*tcdB*⁺/*cdt*⁺) were used as controls for the PCR assays. Stool specimens were examined for toxins with the VIDAS-CDAB kit using mini-VIDAS (bioMérieux) according to the manufacturer's instructions.

Detection of toxin genes by commercial multiplex RT-PCR. Stool specimens were subjected to nucleic acid extraction using the QIAamp DNA stool minikit and a QIAcube (Qiagen), according to the manufacturer's instructions. The AdvanSure RT-PCR kit (LG Life Science) was used for the simultaneous detection of *tcdA* and *tcdB* genes. The primers targeted repeated sequences of the *tcdA* gene and non-repeated sequences of the *tcdB* gene based on TaqMan technology. RT-PCR experiments were performed on a SLAN RT-PCR detection system (LG Life Science) according to the manufacturer's instructions. Results were automatically interpreted using a cut-off value of 0.1. A threshold cycle (Ct) less than 38 was considered positive. Invalid results, which were defined as a negative signal with the internal control for PCR, were retested. To determine the detection sensitivity of the AdvanSure RT-PCR assay, ten-fold serial dilutions of *C. difficile* strains VPI10463 (*tcdA*⁺/*tcdB*⁺) and 1470 (*tcdA*⁺/*tcdB*⁺) were used along with genomic DNA of *C. difficile* ATCC 9689D from 50 ng to 5 × 10⁻¹⁰ ng. To evaluate the specificity of the AdvanSure RT-PCR assay, DNA of clinical isolates of *Clostridium tertium*, *Clostridium septicum*, *Clostridium clostridioforme* and *Clostridium perfringens* were used.

RESULTS AND DISCUSSIONS

The analytical sensitivity for AdvanSure RT-PCR was 1 c.f.u. for *tcdB* detection and 10 c.f.u. for *tcdA* detection per reaction (data not shown). The limits of detection of *tcdA* and *tcdB* genes were 5 × 10⁻⁵ ng and 5 × 10⁻⁶ ng genomic DNA, respectively. This was comparable to the detection limits of 1–10 c.f.u. per reaction using the BD GeneOhm Cdiff PCR assay reported by Stamper *et al.* (2009) and <10 pg of genomic DNA reported by Barbut *et al.* (2011). Clinical isolates of *C. tertium*, *C. septicum*, *C. clostridioforme* and *C. perfringens* showed negative results using AdvanSure RT-PCR.

A total of 17 *C. difficile* isolates were recovered from 127 consecutive specimens in culture. Among them, 11 were toxigenic isolates (8.7 % prevalence); seven had

the genotype *tcdA*⁺/*tcdB*⁺ and four had the genotype *tcdA*⁻/*tcdB*⁺ as determined by single gene specific PCR for the detection of *tcdA* and *tcdB* genes. All *TcdA*⁻/*TcdB*⁺ isolates shared an identical ribotype, 017. Binary toxin-producing (*TcdA*⁺/*TcdB*⁺/*CDT*⁺) strains were not detected in this study.

The AdvanSure RT-PCR experiments showed *tcdA*⁺/*tcdB*⁺ results in all seven specimens harbouring *C. difficile* with the *TcdA*⁺/*TcdB*⁺ phenotype and *tcdA*⁻/*tcdB*⁺ results in all four specimens harbouring *C. difficile* with the *TcdA*⁻/*TcdB*⁺ phenotype. VIDAS-CDAB showed positive results in seven specimens and false-negative results in four specimens harbouring *TcdA*⁺/*TcdB*⁺ cells. One specimen with no data and one specimen showing equivocal results in VIDAS-CDAB tests were excluded. Compared with the toxigenic *C. difficile* culture method, the sensitivity, specificity and positive and negative predictive values for AdvanSure RT-PCR were 100 %, 98.3 %, 84.6 % and 100 %, respectively, and those for VIDAS-CDAB were 63.6 %, 100 %, 100 %, and 96.6 %, respectively (Table 1). The results showed that AdvanSure RT-PCR offers sensitivity and specificity that is comparable to the reference method. The current study had a low sample size (*n*=127); resulting in only 11 positive specimens. This was reflected especially by the positive predictive values which had large confidence intervals of 65–100 %.

EIA for the detection of toxins A and B are relatively quick but are known to have widely varying sensitivities (38–81.6 %) (Ticehurst *et al.*, 2006; Eastwood *et al.*, 2009; Alcalá *et al.*, 2010). The VIDAS-CDAB test is a new ELFA that detects toxins A and B. The sensitivity and specificity of the VIDAS-CDAB test were reported to be 89.8 and 96.7 %, by Eastwood *et al.* (2009), 65.3 and 93.8 % by Shin *et al.* (2009) and 69.4 and 98.1 % by Alcalá *et al.* (2010), respectively. In the current study, the sensitivity and specificity of the VIDAS-CDAB test were 63.9 and 100 %, respectively. These results were comparable to those from two later studies (Shin *et al.*, 2009; Alcalá *et al.*, 2010). The sensitivity of VIDAS-CDAB is too low to use as a stand-alone test; however, it has some advantages, including rapid turnaround time and ease of use, and could be used in a two-step testing system.

In Korea, *C. difficile* *TcdA*⁻/*TcdB*⁺ strains have emerged recently (Kim *et al.*, 2008, 2010). The prevalence of *TcdA*⁻/*TcdB*⁺ strains increased from 13.0 % in 2002 to 27.1 % in 2006 and reached 39.6 % in 2004. In another Korean study in 2004, the proportion of *TcdA*⁻/*TcdB*⁺ strains was even higher: 45.7 % of 81 isolates (Shin & Kuak, 2006). Unlike other commercially available RT-PCR assays, which target *tcdB* only or *tcdB* and a single nucleotide deletion at position 117 in the *tcdC* gene, used for the presumptive identification of PCR ribotype BI/NAP/027 (Huang *et al.*, 2009; Eastwood *et al.*, 2009), AdvanSure RT-PCR targets *tcdA* and *tcdB* genes, resulting in accurate differentiation between *TcdA*⁺/*TcdB*⁺ and *TcdA*⁻/*TcdB*⁺ isolates. This advantage makes the kit more useful in countries with high

Table 1. Comparison of AdvanSure CD RT-PCR and VIDAS-CDAB methods to the toxigenic culture method for the detection of *Clostridium difficile*

PPV, Positive predictive value; NPV, negative predictive value.

Assay and Result	Toxigenic culture*			Assay Performance (95 % confidence interval)			
	<i>tcdA</i> ⁺ / <i>tcdB</i> ⁺	<i>tcdA</i> ⁻ / <i>tcdB</i> ⁺	<i>tcdA</i> ⁻ / <i>tcdB</i> ⁻	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
AdvanSure RT-PCR				100	98.3 (95.9–100)	84.6 (65.0–100)	100
<i>tcdA</i> ⁺ / <i>tcdB</i> ⁺	7	0	1				
<i>tcdA</i> ⁻ / <i>tcdB</i> ⁺	0	4	1				
<i>tcdA</i> ⁻ / <i>tcdB</i> ⁻	0	0	114				
Total (n=127)	7	4	116				
VIDAS-CDAB				63.6 (35.2–92.1)	100	100	96.6 (93.3–99.9)
Positive	3	4	0				
Negative	4	0	114				
Total (n=125)†	7	4	114				

*Results represent number of isolates testing positive or negative for *tcdA* and/or *tcdB* genes.

†Two specimens were excluded, one of them due to an equivocal result and the other an unavailable result.

prevalence rates of *C. difficile* with the phenotype TcdA⁻/TcdB⁺. Another possible advantage of the AdvanSure RT-PCR system is the ability to monitor the emergence of new genotypes, such as the appearance of *tcdA*⁺/*tcdB*⁻ (Rupnik, 2008). Furthermore, the turnaround time of the AdvanSure RT-PCR was very short (~3 h) compared to the toxigenic culture method (48–72h).

In conclusion, AdvanSure RT-PCR may provide an optimal laboratory method for the diagnosis of CDI with rapid turnaround time and reliable diagnostic accuracy.

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