Single fluorophore melting curve analysis for detection of hypervirulent *Clostridium difficile*

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This study demonstrates a novel detection assay able to identify and subtype strains of *Clostridium difficile*. Primers carefully designed for melting curve analysis amplify DNA from three *C. difficile* genes, *tcdB*, *tcdC* and *cdtB*, during quantitative (q)PCR. The *tcdB* gene allows for confirmation of organism presence, whilst the *tcdC* and *cdtB* genes allow for differentiation of virulence status, as deletions in the *tcdC* gene and the concurrent presence of the *cdtB* gene, which produces binary toxin, are associated with hypervirulence. Following qPCR, subtyping is then achieved by automated, inline melting curve analysis using only a single intercalating dye and verified by microchip electrophoresis. This assay represents a novel means of distinguishing between toxigenic and hypervirulent *C. difficile* strains NAP1/027/BI and 078 ribotype, which are highly prevalent hypervirulent strains in humans. This methodology can help rapidly detect and identify *C. difficile* strains that impose a significant health and economic burden in hospitals and other healthcare settings.

INTRODUCTION

Melting curve analysis following quantitative (q)PCR is a sensitive technique that leverages differences in melting temperature of PCR amplicons to identify multiple PCR products from within a single reaction. With up to single-nucleotide resolution, melting curve analysis has been shown to be a robust sensing method for a variety of diagnostic platforms (Guion *et al.*, 2008; Lyon, 2001; Ririe *et al.*, 1997; Tsiatis *et al.*, 2010; van der Stoep *et al.*, 2009; White *et al.*, 2007; Yeh *et al.*, 2004). More specifically, real-time qPCR has revolutionized microbiological testing, providing a huge improvement in time-to-answer, as well as in the sensitivity and specificity of assays for bacteria and viruses. Real-time assays for cytomegalovirus, human immunodeficiency virus and mycobacteria species typing (Cockerill, 2003; Espy *et al.*, 2006) have been developed using melting curve analysis methodologies and have proved to be of great clinical utility. Additionally, this methodology has been incorporated into diagnostic techniques for species-level identification of common staphylococcal species (Skow *et al.*, 2005) and for the detection of *Escherichia coli* (Guion *et al.*, 2008) with high sensitivity/specificity and a total run time of <2 h. This detection paradigm is also readily incorporated into high-throughput workflows and is faster than other methods of PCR product analysis (Tsiatis *et al.*, 2010; van der Stoep *et al.*, 2009). Thus, qPCR assays represent a more sensitive and rapid means of detecting and quantifying micro-organisms in patient samples as well as for detecting genes and mutations in pathogens that confer antimicrobial resistance, including the identification of such single nucleotide polymorphisms in *C. difficile* (Espy *et al.*, 2006; Pecavar *et al.*, 2012). We leveraged these advantages in designing our strain typing assay for the detection of *C. difficile*.

*C. difficile* is a healthcare-associated pathogen that opportunistically infects the gastrointestinal tract, often after antibiotic administration perturbs the indigenous microbiota. *C. difficile* in the colon may lead to only asymptomatic carriage or, particularly after antibiotic exposure, can manifest as severe diarrhoea and toxic megacolon. The health burden of *C. difficile* has increased dramatically over the past decade, in both North America and Europe (Lessa *et al.*, 2015; Wiegand *et al.*, 2012). Specifically, in the USA, there were ~453 000 cases per year leading to 29 300 deaths in 2011 (Lessa *et al.*, 2015). This rapid increase in *C. difficile* infection is thought to be the result of emerging hypervirulent strains of the bacterium (Goorhuis *et al.*, 2008; McDonald *et al.*, 2005). Correspondingly, there is a critical need for a highly sensitive and rapid method of detection and strain typing for discriminative diagnosis and epidemiological research (Knetsch *et al.*, 2013; Tenover *et al.*, 2010).

**Abbreviations:** ATCC, American Type Culture Collection; NAP, North American Profile; q, quantitative.
Current methods of diagnosing *C. difficile* include cell culture cytotoxicity neutralization, enzyme immunoassays, glutamate dehydrogenase detection and nucleic acid amplification. Cell culture cytotoxicity neutralization assays are expensive and time-consuming, requiring maintenance of cell cultures and 24–48 h incubation periods (Lyerly *et al.*, 1988). Enzyme immunoassays for *C. difficile* toxin A and toxin B, which until recently were the most common diagnostic assays in clinical laboratories, are relatively inexpensive, but produce an abundance of false-positives, resulting in poor specificity and positive predictive value (de Jong *et al.*, 2012; Dubberke *et al.*, 2011; Eastwood *et al.*, 2009). Glutamate dehydrogenase is an enzyme produced at high levels in all strains of *C. difficile*, including toxigenic and non-toxigenic isolates. As such, this assay has a high negative predictive value, but positive results must be confirmed by an additional diagnostic method (Burnham & Carroll, 2013). Methods reliant upon nucleic acid amplification include loop-mediated isothermal amplification (Kato & Arakawa, 2011), capillary gel electrophoresis (Janezic *et al.*, 2011) and multiplex PCR (Persson *et al.*, 2011). Multiplex qPCR, whilst often providing high sensitivity and specificity, requires specialized instrumentation with multiple distinct colour channels for gene probes (Jensen *et al.*, 2015; Pallis *et al.*, 2013). Some studies have followed PCR with melting curve analysis, sometimes referred to as high-resolution melt, for *C. difficile* detection (Grando *et al.*, 2012). Others have demonstrated high-resolution melt assays, often with single-nucleotide resolution, but not in a single-tube reaction that simultaneously targets the requisite genes for confirming organism presence, determining virulence status and differentiating hypervirulent strains (de Boer *et al.*, 2010; Wolff *et al.*, 2009).

We present a multiplex PCR assay with melting curve analysis allowing for the simple, rapid detection of *C. difficile* with strain-specific resolution. Our assay targets three *C. difficile* virulence genes, *tcdB*, *cdtB* and *tcdC*, which code for toxin B, one of two unlinked proteins that compose binary toxin, and a negative regulator of toxin A and B production, respectively. These genes were selected as targets for our assay due to their roles in conferring a virulent phenotype and impacting disease severity, as described below. Additionally, our assay has been designed to require just a single fluorescence colour channel despite the detection of three separate genes, thus making the method much more accessible.

**METHODS**

**Target assay design.** Hallmarks of hypervirulent strains of *C. difficile* include production of a toxin known as binary toxin, as well as elevated toxin A and toxin B production. Binary toxin is encoded by the gene *cdtB*, which does not occur in all toxigenic *C. difficile* and is correspondingly used as an indicator of virulence. Within the genome of *C. difficile*, the pathogenicity locus (PaLoc) encodes genes for the protein toxins A (*tcdA*) and B (*cdtB*) as well as three accessory genes (*tcdC, tcdD* and *tcdE*), of which *tcdC* is thought to be a negative regulator of *tcdB* gene expression (Curry *et al.*, 2007; Lyras *et al.*, 2009). *tcdA* and *cdtB* are typically used as markers for the presence of the organism, but in order to detect the two prominent hypervirulent strains [NAP1/027/BI (also known as VA17) and 078 ribotype], other molecular markers are required. The VA17 strain carries distinct 1 and 18 bp deletions in the *tcdC* gene, both of which are thought to play a role in the hypervirulent phenotype (Dupuy *et al.*, 2008; Loo *et al.*, 2005; O’Connor *et al.*, 2009). Reports suggest that higher infection rates and increasingly negative outcomes of *C. difficile* infection are associated with the emergence of the VA17 strain (Blossom & McDonald, 2007; Carter *et al.*, 2007; Fletcher & Cinalli, 2007; McDonald *et al.*, 2005; Vonberg *et al.*, 2008), which displays increased production of toxins A and B and binary toxin generation. 078-ribotype strains carry a larger 39 bp deletion in the same region of *tcdC*. Strains that are designated as 078 ribotype are genetically similar to the VA17 strain and have recently emerged as similarly hypervirulent, but do not appear to be as prevalent and pervasive as their counterpart (Goorhuis *et al.*, 2008). In this assay we used one such 078-ribotype strain, henceforward referred to as 5325. A more in-depth view of these deletions is provided in Fig. 1, which displays the 630 reference genome with annotations highlighting the

![Fig. 1. *tcdC* 630 sequence and annotated deletions in VA17 and 5325 strains. Deletions in the VA17 strain are in red and deletions in the 5325 strain are underlined. *tcdC* primers are highlighted in blue. The bold C represents a C\rightarrow T substitution creating a stop codon in the 078-ribotype strain.](http://jmm.microbiologyresearch.org/63)
deletions for the hypervirulent strains, as well as other strain-specific markers.

Full-genome \textit{C. difficile} gene sequences from 11 strains were acquired from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and aligned to the \textit{C. difficile} 630 reference sequence with multiPipMaker (Schwartz \textit{et al.}, 2000), a multiple alignment tool designed to handle large-scale sequences. The consensus sequences for three target genes (\textit{tcdB}, \textit{cdtB} and \textit{tcdC}) specific to the \textit{C. difficile} genome were analysed by visual inspection and with Primer3 Plus (Untergasser \textit{et al.}, 2007) to identify 100 % conserved regions suitable for amplification by PCR. Care was taken to design the primers in such a way that both the fragment length and melting temperatures would differentiate the strains from each other, as well as from other non-virulent toxigenic \textit{C. difficile} strains similar to the 630 reference strain. Fig. 1 displays the sequences of these fragments with the deletions highlighted compared with the \textit{C. difficile} 630 strain. Table 1 displays the primer sequences utilized for identification of each of the gene fragments, as well as the melting temperatures of the amplicons.

**Melting curve analysis design.** Melting curve analysis utilizes the temperature-dependent dissociation characteristics of dsDNA to infer the presence of multiple PCR products. To obtain a DNA melting curve, the fluorescent signal from a dsDNA-specific dye is measured as temperature increases over time. Controlled heating of amplified dsDNA following qPCR causes a decrease in fluorescence due to the denaturation of the DNA and dissociation of the dsDNA-specific intercalating dye, either SYBR Green I or EvaGreen. Thus, careful design of PCR amplicons is required for proper resolution in a multiplex assay. For each primer pair, primers were optimized to anneal between 53 and 60 °C (Table 1), and the amplicons were designed to melt between 76 and 83 °C. Primer pair melting temperature differences of at least 1 °C were designed to enable differentiation of gene segments, whilst the \textit{tcdC} gene primer pair was designed to enable differentiation between VA17 and 5325 strains. The self- and hetero-dimerization energies of each of the primer pairs were evaluated \textit{in silico} by using an online folding tool DINAMelT (http://unafold.rna.albany.edu/?q=DINAMelT) (Markham & Zuker, 2005) to ensure limited primer-dimer formation. Additionally, the melting temperatures of the amplicons were analysed \textit{in silico}. Several candidate primer pairs for each gene were then obtained from Inte-grated DNA Technologies. Multiple iterations of primer design were completed in order to identify three primer pairs that maximized microchip electrophoresis separation and melting temperature differences in melting curve analysis.

**Cultured sample preparation and control strains.** \textit{C. difficile} culture was performed for both the VA17 and 5325 strains. The VA17 strain was kindly provided by Dr Curtis Donskey (Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, OH, USA) and the 5325 strain was purchased from the American Type Culture Collection (ATCC). Isolated DNA from the \textit{C. difficile} 630 reference strain was also purchased from the ATCC. To culture the VA17 and 5325 strains, the lyophilized culture was suspended in 3 ml pre-reduced Brucella broth (Becton Dickinson Biosciences) and incubated at 37 °C for 24 h in a Whitley MG1000 anaerobic workstation (Microbiology International). A loopful of this culture was streaked for isolation on pre-reduced \textit{C. difficile} selective agar and incubated for 48 h at 37 °C, again in the anaerobic workstation. Following 24 h culture, isolated colonies were inoculated into 3 ml Brucella broth and incubated for 24 h at 37 °C under anaerobic conditions. The culture was centrifuged at 1500 g for 10 min to a cell pellet and the supernatant was removed. Cells were resuspended in PBS to a concentration of 10^7 cells ml^-1 and DNA was extracted using a DNA Mini kit (Qiagen) according to the manufacturer’s instructions.

**PCR.** Target sequences were amplified by qPCR by adding extracted DNA to a final reaction volume of 10 µl. Unless otherwise specified, the reaction mix contained: 1 × Taq polymerase buffer (New England Biolabs), 12.0 mM MgCl₂ (Invitrogen), 0.1 µg BSA µl⁻¹, 0.2 mM each dNTP (New England Biolabs), 0.267 µM each \textit{tcdC} primer, 0.267 µM each \textit{cdtB} primer and 0.167 µM each \textit{tcdB} primer. After an initial heating step of 94 °C for 5 min, the samples were amplified for 40 cycles at 94 °C for 30 s, 65 °C to 53 °C for 30 s decreasing 0.5 °C per cycle, and 72 °C for 1 min. The samples were then held at 72 °C for 5 min for a final extension step. Singleplex reactions were carried out under the same conditions except 1.5 mM MgCl₂ was utilized, and the annealing temperatures were 55 °C for \textit{tcdB} and \textit{tcdC} and 57 °C for \textit{cdtB}. Target sequences were amplified using a conventional thermal cycler (Bio-Rad). The qPCR product size was determined using DNA 1000 chips on an Agilent 2100 Bioanalyzer (Agilent Technologies). To perform sequencing analysis, the PCR products from the singleplex reactions were first purified using a Wizard SV Gel and PCR Clean Up System (Promega) and then inserted into pGEM-T easy vector (Promega). After transformation in XL1 cells, the DNA was purified using the Miniprep kit (Qiagen). Sequencing was performed by Genewiz.

**Melting curve analysis and data processing.** To generate melting curves enabling the identification of multiple \textit{C. difficile} gene fragments from within a sample, raw fluorescence data (PikoReal24; ThermoFisher) were collected at 0.05 °C s⁻¹ intervals using Eva-Green dye (Biotium). The derivative of fluorescence with respect to temperature was smoothed (factor 0.05) through local regression and plotted against temperature to generate the amplicon melting curve; this curve reflects the specific nucleic acid content of the amplicon and has a peak corresponding to the melting temperature of the

### Table 1. PCR primers for amplification of \textit{C. difficile} \textit{tcdB}, \textit{tcdC} and \textit{cdtB} genes, and corresponding amplicon melting temperatures (\textit{T}_m)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Primer \textit{T}_m (°C)*</th>
<th>Strain(s)</th>
<th>Amplicon length (bp)</th>
<th>Amplicon \textit{T}_m (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{tcdB} FWD†</td>
<td>CTGGAGAATCTATATTGTAG</td>
<td>54.9</td>
<td>630/VA17/5325</td>
<td>328</td>
<td>79.3</td>
</tr>
<tr>
<td>\textit{tcdB} REV</td>
<td>GCAGTTGATCATATTCAAC</td>
<td>56.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{tcdC} FWD</td>
<td>CTCAAAAAACAGAAAATAGAAC</td>
<td>56.7</td>
<td>630/VA17/5325</td>
<td>301/283/262</td>
<td>83.0/82.0/81.8</td>
</tr>
<tr>
<td>\textit{tcdC} REV†</td>
<td>ACCTCCTACCACTTTC</td>
<td>56.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{cdtB} FWD</td>
<td>TCTTAAATGCAAGTAAATCTGA</td>
<td>59.1</td>
<td>VA17/5325</td>
<td>150</td>
<td>76.9</td>
</tr>
<tr>
<td>\textit{cdtB} REV</td>
<td>AATTCCAATAAGAGATTGTG</td>
<td>59.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated at 12 mM MgCl₂.
†Primer sets published in Angione \textit{et al.} (2014).
amplicon. Multiple peaks within a sample therefore indicate the presence of different gene fragments, the identity of which can be identified based on peak location. All melting curve experiments were done in triplicate and no-template controls were included in each run to monitor for contamination.

**Microchip electrophoresis.** Quantitative analysis for DNA was completed using an Agilent 2100 Bioanalyzer (Agilent Technologies) with the DNA 7500 and DNA 1000 labchip kits as per the manufacturer’s instructions.

**Statistical analysis.** Two-tailed, unpaired t-tests of unequal variance were applied with a Bonferroni correction to assess significance between independent samples. The significance threshold used was a stringent $\alpha = 0.01/3 = 0.0033$, which includes the multiple comparisons correction.

**RESULTS**

**Strains utilized for PCR and melting curve analysis**

We optimally targeted three different gene segments for our qPCR and melting curve analysis assay, so as to indicate not only the presence of toxigenic *C. difficile*, but also the presence of the hypervirulent genotypes (Angione *et al.*, 2014). Due to the presence of toxin B in nearly all toxigenic strains, the *tdcB* gene served as our target for the presence of the disease-causing organism (Carter *et al.*, 2007). We also targeted the regulatory gene *tcdC*, with the ability to detect both the 18 bp deletion in the VA17 strain, as well as the larger 39 bp deletion in the 5325 strain (Carter *et al.*, 2007). Our final target was the *cdtB* gene, which encoded binary toxin and was expressed by both hypervirulent strain types. We utilized three distinct strains in this study in order to assess the ability of the assay to detect and differentiate between both non-hypervirulent and hypervirulent *C. difficile* strains. The *C. difficile* 630 strain (ATCC BAA-1382) was utilized as a benchmark for toxigenic non-virulent *C. difficile* and is designated toxinoype 0, ribotype 012 (Rupnik *et al.*, 1998). It is toxin A- and toxin B-positive, but lacks binary toxin (Rupnik *et al.*, 1998). The 630 genome (GenBank accession number AM180355) also encodes the entire *tcdC* regulatory gene and produces a fully functional non-truncated product. This strain was utilized as a representative strain for *C. difficile* that commonly infects the human population, but is not classified as hypervirulent. We utilized a cultured *C. difficile* strain, VA17, for evaluation of the assay to detect the NAP1/027/BI strain. This strain has been confirmed as toxinoype III, designated by PFGE as the North American Profile 1 (NAP1), PCR-ribotype 027 and restriction endonuclease analysis type BI, otherwise known as the NAP1/027/BI strain. It is positive for toxins A and B and binary toxin. Lastly, we utilized the hypervirulent ATCC BAA-1875 (strain 5325), which is toxinoype V, PFGE NAP7, PCR-ribotype 078, restriction endonuclease analysis type BK, and is toxin A- and B-positive, as well as positive for binary toxin.

**Validation of PCR primer specificity**

First, primer amplification of the appropriate gene target following qPCR was confirmed individually using microchip gel electrophoresis and melting curve analysis, as shown in Fig. 2. For a description of how raw fluorescence data were transformed into a melting temperature for a given amplicon, see Methods. Across all three strains, the *tdcB* gene amplicon was confirmed to be consistent in melting temperature (Fig. 2b), as well as in size (Fig. 2a, lane 1). Note that only the 630 strain is shown due to equivalence with other strains. Furthermore, the melting temperature of 79.3 °C agreed exactly with that predicted *in silico* using DINAMelt. The two hypervirulent strains VA17 and 5325 showed expectedly equivalent *cdtB* gene amplicon sizes of 150 bp (Fig. 2a, lanes 5 and 6). The melting temperature for this amplicon was also equivalent between strains and agreed with the predicted melting temperature of 76.9 °C (Fig. 2f, g). The 630 strain does not contain this gene and thus this amplicon is absent after PCR with this primer set (Fig. 2a, lane 7). Lastly, the *tcdC* gene amplicon sizes differed between strains in accordance with the known variation in gene size. The 630 strain amplicon reflected the 301 bp non-truncated product (Fig. 2a, lane 2), whilst the VA17 and 5325 strains had smaller 283 and 262 bp amplicons (Fig. 2a, lanes 3 and 4, respectively), representative of the deletions shown in Fig. 1. The length differences in these amplicons translated into observable differences in melting temperature reflective of *in silico* predictions (Fig. 2c–e). As verification, the identities of all amplicons were confirmed by sequencing. Additionally, no signal (assessed by observation of no change in fluorescence over time) was observed in the no-template controls. Taken together, these results confirm that our primer sets can detect the mutations in the *tcdC* gene associated with virulence in both the VA17 and 5325 *C. difficile* strains (Curry *et al.*, 2007; Hundsberger *et al.*, 1997; Loo *et al.*, 2011). The assay can additionally detect the binary toxin gene *cdtB* in VA17 and 5325 strains, which is not present in strains similar to the strain 630 reference genome.

**Melting curves and microchip electrophoresis**

Following the validation of the individual primer pairs as effective for amplification of the *tdcB*, *tdcC* and *cdtB* gene fragments, we performed the assay in multiplex. The three primer sets were utilized in an experimentally determined ratio (see Methods) for adequate amplification of each region of interest during qPCR. Fig. 3(b) displays normalized –dF/dt melting curve peaks for the three strains derived from the normalized raw fluorescence data shown in Fig. 3(a). The melting temperatures of the *tdcB* amplicons represented by the overlapping central peak in Fig. 3(b) are highly reproducible for the strains of *C. difficile* tested and no significant differences in melting
However, microchip electrophoresis has the resolution to differentiate between all three strains using retention time shifts in the \( tcdC \) gene, as seen with the 301, 283 and 262 bp amplicons in Fig. 4(a–c), and the electropherogram peak shifts between 72 and 77 s. These statistically significant differences (\( P<0.001 \)) are depicted in Fig. 4(e). These results demonstrate that electropherograms are capable of not only categorizing strains as either non-hypervirulent or hypervirulent, but also subtyping these strains.

**DISCUSSION**

Intestinal infection with *C. difficile* is a leading cause of antibiotic-associated diarrhoea and is associated with a variety of symptoms ranging from moderate-to-severe diarrhoea to potentially fatal toxic megacolon. *C. difficile*-associated diarrhoea has been characterized by an increase in both incidence and disease severity, particularly amongst residents and patients in community settings such as nursing homes and healthcare facilities (Lessa *et al.*, 2015), and it is the leading causative pathogen causing healthcare-associated infections in the USA (Magill *et al.*, 2014). The rapid spread of the NAP1/027/BI and 078-ribotype strains has increased the challenges associated with controlling the spread of infection. As a result of the more serious nature point are observed. Similarly for the hypervirulent strains VA17 and 5325, the \( cdh \) amplicons represented by the left-most overlapping peak are expectedly not significantly different. The \( tcdC \) amplicons, however, showed melting temperature differences dependent on strain type in accordance with those predicted in silico and observed in singleplex assays. We observed statistically significant differences (\( P<0.001 \)) between hypervirulent strains and the non-hypervirulent 630 strain, indicating that melting temperature differences due to deletions in the \( tcdC \) gene as well as the presence of \( cdh \) can be utilized to differentiate between hypervirulent and non-hypervirulent strains of *C. difficile*.

In conjunction with melting curve analysis, microchip electrophoresis was performed on the Agilent Bioanalyzer to provide size differentiation of the DNA fragments identified using melting curve analysis. As with melting curve analysis, the \( tcdB \) amplicon was indistinguishable between all three strains. Microchip electrophoresis also reproduced melting curve analysis results with respect to the \( cdh \) gene. This amplicon can be seen for only the VA17 and 5325 strains in both the generated gel plots (Fig. 4b, c, 150 bp) and in the overlapping electropherogram peak (Fig. 4d, 62 s). In contrast to melting curve analysis, however, microchip electrophoresis has the resolution to differentiate between all three strains using retention time shifts in the \( tcdC \) gene, as seen with the 301, 283 and 262 bp amplicons in Fig. 4(a–c), and the electropherogram peak shifts between 72 and 77 s. These statistically significant differences (\( P<0.001 \)) are depicted in Fig. 4(e). These results demonstrate that electropherograms are capable of not only categorizing strains as either non-hypervirulent or hypervirulent, but also subtyping these strains.

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**Fig. 2.** Generated gel plot and singleplex melting curves of amplified DNA from *C. difficile* strains. (a) Generated gel plot. Lane: L, ladder; 1, strain 630 \( tcdB \) 328 bp amplicon; 2–4, 301, 283 and 262 bp \( tcdC \) amplicons from the 630, VA17 and 5325 strains, respectively; 5 and 6, 150 bp \( cdh \) amplicons for VA17 and 5325 strains, respectively; 7, the 630 strain is expectedly negative for \( cdh \); 8, no-template control. (b–g) Individual amplicon melting curves: normalized fluorescence melting signal (grey line) and negative derivative following smoothing (coloured line): (b) \( tcdB \), \( T_m=79.3 \pm 0.20 \) °C; (c) \( tcdC \) (VA17), \( T_m=82.0 \pm 0.06 \) °C; (d) \( tcdC \) (5325), \( T_m=81.8 \pm 0.15 \) °C; (f) \( cdh \) (VA17), \( T_m=76.9 \pm 0.20 \) °C; (g) \( cdh \) (5325), \( T_m=77.0 \pm 0.20 \) °C. Data are presented as mean ± SD.
of *C. difficile* infection, and the relative ease and rapidity with which the disease may be transmitted amongst patients, both the scientific and medical communities alike recognize the need for new methods to rapidly detect the pathogen in potentially afflicted patients (Blossom & McDonald, 2007; Petrella et al., 2012).

Several qPCR assays have been developed to provide rapid and sensitive detection of *C. difficile*, and whilst these assays represent an improvement in speed and sensitivity, they require dedicated equipment and multiple fluorescence wavelength excitation and detection capabilities to identify the genes of interest (Houser et al., 2010; Jensen et al., 2015; O’Horo et al., 2012; Pallis et al., 2013). The commercial Xpert *C. difficile*/Epi (Cepheid) assay, which utilizes primers and probes for multiplex detection of *tcdB*, *tcdC* and *cdtB*, is one such assay and is marketed to identify the NAP1/027/BI strain. This assay requires a dedicated and costly system with multiple fluorescence detection capabilities, thus potentially limiting the portability and widespread adoption of the assay. We believe that a single intercalating dye, instead of a multi-fluorophore system, can be a simpler and less expensive method for diagnosing hypervirulent *C. difficile* strains with the speed and sensitivity afforded by qPCR. Such a system, for example, could be adapted for low-cost point-of-care diagnostics using an LED and photodiode, without the need for expensive and complicated optical filters that would be required for a multi-fluorophore system.

Whilst most current laboratory detection methods seek to identify the *tcdB* gene, which codes for the production of protein toxin B, we have demonstrated multiplexed detection of *tcdB*, *tcdC* and *cdtB*. Three sets of PCR primers were designed *in silico* to amplify three specific regions of DNA, each located within a gene with a role in coding for the production of proteins involved in the severity of illness associated with *C. difficile* infection. The strong agreement of *in silico* melting temperature predictions with experimental results suggests such computational methods may be powerful for the rapid design of melting curve assays for other pathogens. Furthermore, with these specific targets we can effectively differentiate between two strains of hypervirulent *C. difficile* and non-hypervirulent strains similar to the *C. difficile* 630 strain using melting curve analysis. Additionally, using DNA electrophoresis, not only can we distinguish between hypervirulent and non-hypervirulent strains, but we can also differentiate between two common hypervirulent strains, the NAP1/027/BI (VA17) strain and the 078-ribotype (5325) strain. Whilst distinguishing between these two strains is not necessary for clinical decisions to direct appropriate treatment, it can be utilized for epidemiological purposes.

One of the major contributions of this work is that it provides several methodologies for detecting three genes associated with hypervirulence. The assay can be performed as a standard PCR with detection using electrophoresis or microchip electrophoresis, as well as performed as a real-time assay. The most important advantage of this assay is that it detects the three genes associated with hypervirulence with one fluorescence reporter molecule (EvaGreen). This greatly simplifies the multiplexing procedure due to primers designed specifically for melting curve analysis.

![Fig. 3. Multiplexed melting curve analysis for three strains of *C. difficile*. (a) Raw fluorescence melting curve: normalized fluorescence melting signal for the 630, VA17 and 5325 *C. difficile* strains. (b) Normalized negative first derivative of melting curves (−dF/dT): the *tcdB* amplicon is present in all three strains as indicated by the overlapping central peak [Tm(630)=79.3±0.07 °C, Tm(VA17)=79.3±0.10 °C, Tm(5325)=79.2±0.08 °C]. The *cdtB* amplicon is appropriately seen with only the VA17 and 5325 strains [Tm(VA17)=76.9±0.06 °C, Tm(5325)=76.8±0.08 °C]. The melting peaks for the *tcdC* amplicon show temperature shifts according to strain [Tm(630)=83.0±0.08 °C, Tm(VA17)=82.0±0.10 °C, Tm(5325)=81.8±0.08 °C]. (c) Amplicon melting temperatures. Melting temperatures for *tcdB* and *cdtB* are consistent between strains, whilst the *tcdC* amplicon melting temperatures differ significantly between the 630 strain and hypervirulent VA17 and 5325 strains (*P*<0.001). Data presented as mean±SD. NS, Not significant.](image-url)
Lastly, the assay has clinical utility by providing a simple and effective means of detecting infection with a virulent strain, which may help physicians to devise specific treatment regimens and may assist in contact isolation strategies necessary to address the challenge of *Clostridium difficile*-associated diarrhoea in healthcare facilities and community settings.

Our results demonstrate a new method to amplify and detect hypervirulent strains of *Clostridium difficile*. Our technique of multiplex gene amplification provides a unique method that is both sensitive and specific to rapidly detect not only the presence of *C. difficile*, but to differentiate between toxigenic *C. difficile* and two different hypervirulent strains. This new means of identifying prevalent strains of *C. difficile* can assist physicians in creating and implementing better treatment regimens for the care of patients with *C. difficile* infections, particularly those with the NAP1/027/BI and 078-ribotype strains.

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