Rapid detection of sequence variation in *Clostridium difficile* genes using LATE-PCR with multiple mismatch-tolerant hybridization probes

Kenneth E. Pierce a,*, Huma Khan b,1, Rohit Mistry b,1, Simon D. Goldenberg c, Gary L. French c, Lawrence J. Wangh a

**A R T I C L E   I N F O**

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**A B S T R A C T**

A novel molecular assay for *Clostridium difficile* was developed using Linear-After-The-Exponential polymerase chain reaction (LATE-PCR). Single-stranded DNA products generated by LATE-PCR were detected and distinguished by hybridization to fluorescent mismatch-tolerant probes, as the temperature was lowered after amplification in 5 °C intervals between 65 °C and 25 °C. Single-tube multiplex reactions for *tcdA*, *tcdB*, *tcdC*, and *cdtB* (binary toxin) sequences were initially optimized using synthetic targets and were subsequently done using genomic DNA; each target was detected and characterized by hybridization to one or more probes of a different fluorescent color. In the case of *tcdC*, three probes, each labeled with a Quasar fluorophore, hybridize to different locations with known mutations, including the deletion at nucleotide 117 in ribotype 027 strains and the premature stop codon mutation at nucleotide 184 in ribotype 078 strains, each of which is associated with hypervirulent infections. These and other *tcdC* mutations were distinguished from the reference sequence, as well as from each other by changes in the fluorescent contour generated from the combined Quasar-labeled probes. Specific variations in *tcdA* and *tcdB* were also identified in the multiplex assay, including those that identified strains lacking toxin A production. This single closed-tube assay generates substantially more information about virulent *C. difficile* than currently available commercial assays and could be further expanded to provide strain typing.

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1. Introduction

Toxigenic *Clostridium difficile* is the most common cause of antibiotic-associated diarrhea and the cause of virtually all cases of life-threatening pseudomembranous colitis, a severe inflammation of the colon (Sebaihia et al., 2006). The genomic and extrachromosomal DNA of *C. difficile* encodes several antibiotic resistance genes. Moreover, the bacterium forms resistant spores that can germinate and cause the disease to recur even after treatment with appropriate antibiotics. Spores are also a major concern in hospitals and other healthcare facilities, because they can survive on surfaces for prolonged periods, are resistant to many disinfectants, and are easily transmitted from patient to patient. Rapid detection and characterization of *C. difficile* are vital for determining patient treatment and reducing the possible spread of the disease within health care facilities.

Significant improvement in diagnostic sensitivity for virulent *C. difficile* has been obtained through commercially available PCR-based diagnostics (Babady et al., 2010; Chapin et al., 2011; Karre et al., 2010; Zidarić et al., 2011). Those methods compare favorably to immunoassay-based diagnostics and are faster and less labor-intensive compared with the cell toxicity assay which is currently regarded as the diagnostic “gold standard”. The toxin genes *tcdA* and *tcdB*, and the regulatory gene *tcdC* make up part of the Pathogenicity Locus (PaLoc) and PCR methods include the detection of one or more of those genes. Genes *cdtA* and *cdtB* outside the PaLoc encode a separate, binary toxin and are also detected in some PCR-based assays. Binary toxin has been detected in 6% to 28% of cases and although it is not believed to cause virulence, it may contribute to host symptoms and inflammatory response (Geric et al., 2006; Goldenberg and French, 2011; Gonçalves et al., 2004; Stubbs et al., 2000).

The *tcdC* gene produces a protein that can inhibit the transcription of the large toxin genes, *tcdA* and *tcdB* (Carter et al., 2011; Matamoros et al., 2007). Mutations in *tcdC* have been associated with recent outbreaks of “hypervirulent” *C. difficile*. The deletion of a single nucleotide at position 117 of *tcdC* has been found in the ribotype 027 strain responsible for a high percentage of cases in Europe and North America (McDonald et al., 2005). In other cases, absence of a functional *tcdC* protein is caused by a single nucleotide substitution at nucleotide 184 that produces a premature stop codon and has been identified in hypervirulent strains of ribotype 078 (Goorhuis et al., 2008). The hypervirulence of those strains...
may be due in part to higher expression of the toxins, although a direct link between the mutations and clinical severity has been questioned (Cartman et al., 2012; Sirard et al., 2011). Several other tcdC sequence variations have been identified, including deletions of 18 or more nucleotides in the repeat region of the gene (Curry et al., 2007), but the effect of these mutations on protein function is unknown. Some of the commercially available PCR assays detect the presence of the nucleotide 117 deletion, but none of the other sequence variations. Improved diagnostics are needed that can detect different mutations and provide other indications of strain identity.

LATE-PCR is a non-symmetric PCR with expanded detection capability that offers the potential for rapid diagnosis and strain typing. LATE-PCR generates an abundance of single-stranded DNA which can be detected at endpoint with mismatch tolerant probes over a broad range of temperatures (Pierce et al., 2005; Rice et al., 2007; Sanchez et al., 2004; Sanchez et al., 2006). We have taken advantage of this feature of LATE-PCR to construct an assay for C. difficile capable of identifying sequence differences in multiple genes. Such an assay could provide both clinical diagnosis and rapid characterization that can be detected at endpoint with mismatch tolerant probes over a broad range of temperatures (Pierce et al., 2005; Rice et al., 2007; Sanchez et al., 2004; Sanchez et al., 2006). We have taken advantage of this feature of LATE-PCR to construct an assay for C. difficile capable of identifying sequence differences in multiple genes. Such an assay could provide both clinical diagnosis and rapid characterization that are needed for tracking hospital-acquired and community-acquired infections.

2. Materials and methods

2.1. Preparation of synthetic C. difficile sequence targets and genomic DNA

Amplification sequences for tcdA, tcdC, and cdtB were cloned into plasmid vectors using a custom gene synthesis service (Epoch Biolabs, Missouri City, TX, USA). The modified pBluescript II SK vectors contained confirmed sequences for nucleotides 73 through 521 of tcdC coding regions of the reference strain, VPI 10463 or nucleotides 73 through 439 for other strains (http://pubmlst.org/cdifficile/). Similar synthetic gene targets were made for tcdA nucleotides 1 through 502 of the reference sequence (GenBank Accession X92982), and for cdtB nucleotides 1 through 438 of strain CD3 (GenBank Accession DQ117053). Custom DNA oligonucleotide synthesis was done by Sigma-Aldrich (St. Louis, MO, USA) to generate the shorter targets for tcdB nucleotides 5673 to 5775 of the reference strain. Rough estimates of target concentration were made from C₅ values following amplification by single primer pairs and detection by the double-strand-DNA-binding dye, Eva Green (Biotium, Hayward, CA, USA).

C. difficile was cultured from clinical isolates or was purchased from Health Protection Agency Culture Collections (Salisbury, UK) and DNA was extracted as described by Goldenberg and French (2011). Sequencing of tcdC was done using primers C1 and C2 as described by Spigaglia and Mastrantonio (2002). The study was approved by the London Stanmore Research Ethics Committee, formerly North London REC 2 (09/H0724/26).

2.2. LATE-PCR primers

Primers were designed using LATE-PCR criteria for efficient production of single-stranded DNA. Each limiting primer has a concentration-adjusted melting temperature (Tₘ) that is at least as high as that of the excess primer, but preferably a few degrees higher in order to obtain the highest amplification efficiency (Pierce et al., 2005). Visual OMP (DNA Software, Ann Arbor, MI, USA) was used to design limiting primers with the desired Tₘ of 67–68 °C at 50 nM and excess primers with the desired Tₘ of 64–65 °C at 1000 nM, and to avoid extendible self dimers and heterodimers. The predicted Tₘ values are based on the PCR buffers described below and take into account the contribution from 3 mM magnesium. Primers were custom synthesized by Biosearch Technologies (Novato, CA, USA). Primer and probe sequences are listed in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdtB Limiting primer</td>
<td>ATGTCGATTTGGAACAGCAGATTGGATAAC</td>
</tr>
<tr>
<td>cdtB Excess primer</td>
<td>AAACGGATCCCTCCCACTAATCT</td>
</tr>
<tr>
<td>tcdA Limiting primer</td>
<td>AATTCAGATTTGGAACAGCAGATTGGATAAC</td>
</tr>
<tr>
<td>tcdA Excess primer</td>
<td>AAACGGATCCCTCCCACTAATCT</td>
</tr>
<tr>
<td>tcdB Limiting primer</td>
<td>ATGTCGATTTGGAACAGCAGATTGGATAAC</td>
</tr>
<tr>
<td>tcdB Excess primer</td>
<td>AAACGGATCCCTCCCACTAATCT</td>
</tr>
<tr>
<td>tcdC Limiting primer</td>
<td>ATGTCGATTTGGAACAGCAGATTGGATAAC</td>
</tr>
<tr>
<td>tcdC Excess primer</td>
<td>AAACGGATCCCTCCCACTAATCT</td>
</tr>
<tr>
<td>Lactococcus limit primers</td>
<td>AATTCGATTTGGAACAGCAGATTGGATAAC</td>
</tr>
<tr>
<td>Lactococcus excess primer</td>
<td>AAACGGATCCCTCCCACTAATCT</td>
</tr>
<tr>
<td>cdtB (Binary toxin gene)</td>
<td>AAGAACAGATACATCGGCCTCACTT</td>
</tr>
<tr>
<td>cdtB2 Probe</td>
<td>Cal-Orange-ACCAAGAGATCCCTCTTATTATACACT-BHQ1</td>
</tr>
<tr>
<td>tcdC Probe</td>
<td>Cal Red 610-TTCGAGGCAAAATAFATATATCAGTCA-BHQ2</td>
</tr>
<tr>
<td>tcdC Probe C1</td>
<td>Quasar 670-TACAACAGTACATCGGCCTCAG - BHQ2</td>
</tr>
<tr>
<td>tcdC Probe C2</td>
<td>BHQ2-TACAACAGTACATCGGCCTCAG - Quasar 670</td>
</tr>
<tr>
<td>tcdC Probe C3</td>
<td>Quasar 670-TACAACAGTACATCGGCCTCAG - BHQ2</td>
</tr>
<tr>
<td>Lactococcus probe</td>
<td>Cal Red 610-AGAAAGATCCCTCCCACTAATCT</td>
</tr>
</tbody>
</table>

* Modified tcdB primers were used with genomic DNA.

2.3. Low-Tₘ mismatch-tolerant probes

Probes were designed to have a Tₘ with any known target at least 10 degrees below the Tₘ of the limiting primer. This ensures high amplification efficiency during the exponential phase of amplification (double-strand DNA production) and absence of probe hydrolysis during the extension of the limiting primer. Estimates of the Tₘ of each probe with sequences of the different strains were made using Visual OMP software. The ends of each probe include 2 or 3 complementary bases that can form a stem in the absence of target. This stem enhances interaction of the fluorophore and quencher and reduces background fluorescence. Probe sequences are derived from the same strand as used for the limiting primer so the probe will hybridize to the single stranded product generated by the extension of the excess primer.

2.4. tcdC primer and probe design

A single limiting and single excess primer were chosen to amplify a 354 nucleotide segment of the tcdC gene, including the nucleotide 117 single base deletion, the nucleotide 184 stop codon mutation, and the 18 bp (or larger) deletion in the repeat region of the gene. Each of these regions served as the target for a different mismatch-tolerant probe (C1, C2, and C3, respectively, Table 2) labeled with Quasar 670 on one end and Black Hole Quencher 2 (BHQ2) on the other end. Quasar 670 was placed on the 5′ end of probes C1 and C3. Quasar 670 was placed on the 3′ end of probe C2 to reduce the possibility of quenching from BHQ2 of probe C1 when both are hybridized to the same target. Each probe was designed to have a Tₘ with the reference sequence of approximately 50 degrees. Sequence variants have a different Tₘ to that of strains containing the nucleotide 117 deletion and therefore exhibits relatively high fluorescence with that target at temperatures above 50 °C. Most other variations from the reference sequence have a lower Tₘ with the probe for that target and therefore lower fluorescence at 50 °C. Probes C1 and C2 are each purposefully mismatched at all targets in one position in order to minimize the fluorescence change due to known silent mutations. Probe C3 hybridizes to targets with the 18 bp or larger deletion at lower temperatures, as the probe has partial homology to adjacent repeats.
Table 2
tcdC Sequence variations targeted by mismatch-tolerant probes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Example strain</th>
<th>Nucleotide variations targeted by tcdC probes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Probe C1</th>
<th>Probe C2</th>
<th>Probe C3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>107</td>
<td>117</td>
<td>120</td>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td>tcdC 19</td>
<td>VP110463</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>47.2</td>
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<tr>
<td>tcdC 47</td>
<td>Pd3</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>47.2</td>
</tr>
<tr>
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<td>KK613</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>49.3</td>
</tr>
<tr>
<td>tcdC 30</td>
<td>758292</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>49.3</td>
</tr>
<tr>
<td>tcdC 22</td>
<td>Pd5</td>
<td>C</td>
<td>T</td>
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<td>52.7</td>
</tr>
<tr>
<td>tcdC 35</td>
<td>AIP2005162</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>52.7</td>
</tr>
<tr>
<td>not listed</td>
<td>KK312</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>52.7</td>
</tr>
<tr>
<td>tcdC 16</td>
<td>1083</td>
<td>C</td>
<td>del</td>
<td>T</td>
<td>57.5</td>
</tr>
<tr>
<td>tcdC 1</td>
<td>R20291</td>
<td>C</td>
<td>del</td>
<td>T</td>
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<td>tcdC 8</td>
<td>sc8</td>
<td>C</td>
<td>del</td>
<td>T</td>
<td>57.5</td>
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<tr>
<td>not listed</td>
<td>8864</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>45.8</td>
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<tr>
<td>tcdC 7</td>
<td>M68</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>44.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genotype numbering based on full length sequences from the Clostridium difficile MLST database at http://pubmlst.org/cdifficile/.

<sup>b</sup> Nucleotide numbers represent positions in the coding sequence of reference strain VPI10463. Variations are shown in bold. G at nucleotide 117, T at 120, and T at 183 do not alter amino acid sequence (silent mutations); T at nucleotide 117 results in an L to F amino acid substitution with unknown consequence; T at nucleotide 184 and 191 give stop codons; del = deletion, del [number] = deletion of the specified number of nucleotides in the region targeted by probe C3.

<sup>c</sup> Melting temperature prediction by Visual OMP software at the salt concentrations of the reaction mixture and 300 nM probe and target.

2.5. tcdA primer and probe design

One goal of the tcdA design was to distinguish strains which do and do not produce toxin A. However, absence of the A toxin can be due to different size deletions in tcdA and not all deletions result in the absence of toxin A protein by immunoassay tests (Rupnik et al., 2003). Moreover, the deletions are within the series of repeats near the 3′ end of the gene and there is a risk that a DNA probe will fail to detect a deletion because it hybridizes to remaining repeat segments. In order to circumvent these issues, primers were designed to hybridize with sequences near the 5′ end of the gene. The probe was designed to hybridize to a region that shows heterogeneity between strains. The probe has a predicted T<sub>m</sub> of 49 °C and 50 °C with the A+ reference strain and C34 strain (GenBank Accession AJ131844), respectively, and 53 °C and 57 °C with the A-strains 8864 (GenBank Accession AJ011301) and 1470 (GenBank Accession AJ132669), respectively.

2.6. tcdB primer and probe design

Sequences of tcdB gene segments from different strains were aligned using ClustalW2 on-line software and primers were designed to amplify a conserved region within the repeat region of the gene. Following initial tests on genomic DNA, the primers were modified and a second limiting primer was included to improve amplification of ribotype 027 and other strains (Table 1). The sequences chosen for primers are sufficiently distinct from neighboring repeats that only the targeted region should be amplified efficiently. A probe was designed that would hybridize to the reference sequence variant with a predicted T<sub>m</sub> of 53 °C. The probe hybridizes to different sequence variants at different temperatures; some strains, including CD3 (GenBank Accession DQ117279) have a predicted T<sub>m</sub> of 59 °C, while other strains, including the A+B+ strain 8864, have a predicted T<sub>m</sub> of 47 °C.

2.7. cdtB (binary toxin) primer and probe design

Some cases of C. difficile associated diarrhea are due to strains that produce binary toxin in addition to the large toxins A and B. Strains that do not produce the binary toxin have a large deletion that includes parts of cdtA and cdtB (Stare et al., 2007). LATE-PCR primers and a probe were designed to amplify and detect a segment of cdtB that is only present in strains that produce the binary toxin. The probe has a predicted T<sub>m</sub> of 49 °C with the sequence of strains containing intact cdtB, including ribotype 027 strains CD196 and R20291.

2.8. Internal control primer and probe design

A synthetic target containing the nucleotide sequence of a Lactococcus lactis gene (GenBank accession NC_008527) was included in the multiplex amplification assay as an internal control. LATE-PCR primers amplify a 134 nucleotide product that is detected with a probe that hybridizes with a predicted T<sub>m</sub> of 38 °C. The probe is labeled with Cal Red 610, the same fluorophore used on the tcdB probe, but it has a predicted T<sub>m</sub> at least 9 degrees lower than that of the tcdB probe with any known tcdB sequence variant. Samples that contain the control target in the absence of C. difficile DNA generate a low temperature Cal Red 610 signal, but no other signals. The use of Lactococcus cells could provide an internal control for both sample preparation and amplification once those steps are combined.

2.9. LATE-PCR reagents and protocol

Varying concentrations of tcdC synthetic targets were added to a reagent solution with final concentrations of 0.4 mM of each dNTP, 50 nM tcdC limiting primer, 1 μM tcdC excess primer, 500 nM each probes C1, C2 and C3, 250 nM Primase II (Rice et al., 2007), 0.08 U/μl Platinum Tfi exo(−) DNA polymerase (Life Technologies, Grand Island, NY, USA), 1X Tfi Reaction Buffer and 3 mM MgCl₂ in a final volume of 25 μl. An initial denaturation at 95 °C for 1 min was followed by 50 cycles of 88 °C for 5 s, 62 °C for 20 s and 68 °C for 40 s using a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). Temperature was subsequently held at 65 °C for 10 min, then decreased in 5 °C steps and held for 2 min at each step with fluorescence detection (3 detections at endpoint of each step). In order to correct for well-to-well variations in the thermal cycler, raw fluorescence data was exported to Excel (Microsoft, Redmond, WA) and normalized based on fluorescence at 65 °C, at which temperature there is no detectable hybridization between probes and target. The average background fluorescence based on replicate no template control samples was subtracted from normalized fluorescence due to the hybridization of probe with PCR product. Ratio values for each sample were obtained by dividing fluorescent signal at a given temperature by the fluorescent signal 5 degrees lower. Ratio values are included in the graphs at temperatures where fluorescence at the higher temperature is more than 3% over normalized background.

Multiplex amplification of the 5 synthetic gene targets was carried out using the same method as above, but included each of the 5 limiting primers at 50 nM each, the tcdB excess primer at 500 nM, the
other excess primers at 1 μM, and each of the 7 probes at 500 nM. Fluorescence detection for each of the 4 types of fluorophores on those probes was done during each post-PCR temperature step in the Stratagene Mx3005P.

Multiplex amplification from genomic DNA was done similarly, but included the following changes. PrimeSafe II was used at 150 nM; Excess primers for tcdB and tcdC were used at 500 nM; modified tcdB primers were used including a second tcdB limiting primer for improved amplification of ribotype 027 strains; probe concentrations were 150 nM for tcdB and tcdC, and 300 nM for tcdA, tcdB and Lactococcus. Thermal cycling was done in an Applied Biosystems 7500 (Life Technologies, Grand Island, NY, USA) using a similar protocol to that above, except that the first 4 PCR cycles included a lower annealing temperature of 58 °C.

3. Results

3.1. Multiple probes and ratio analysis for tcdC mutation detection

Each of the eleven synthetic tcdC sequences was amplified using a single pair of LATE-PCR primers and was detected with three mismatch-tolerant probes labeled with Qasar 670. These probes hybridize with the amplified target at regions of several known mutations in tcdC. The tcdC allele designation, a strain example with that sequence, and the predicted Tm with each probe are listed in Table 2. The left side of Fig. 1 shows the post-PCR fluorescent profiles for four different tcdC sequences amplified from approximately 100,000, 1000, or 10 copies of plasmid DNA. Allele tcdC19 sequence (reference strain VP10463) generated a profile consistent with hybridization of all three probes with Tm around 46 to 49 degrees (Fig. 1A, left). The intensity of the signal was proportional to the number of starting targets, although there was greater variation among the 4 replicates containing 10 copies, consistent with stochastic variation in the actual number of templates. Amplified tcdC47 sequence (strain Pd3) containing the 18 nucleotide deletion shows a shift in fluorescence signal toward lower temperatures (Fig. 1B, left), consistent with the reduced Tm to the probe that hybridizes in the region of the large deletion. Amplified tcdC22 sequence (strain Pd5) with the stop codon mutation at nucleotide 184 in addition to a large deletion and other mutations showed relatively low fluorescence above 40 degrees (Fig. 1C, left), consistent with only one probe hybridizing to the target at higher temperatures, and the other two probes hybridizing only at lower temperatures due to the mutations. Two of four samples containing an estimated 10 plasmid targets with tcdC22 failed to generate signal, possibly due to absence of a template. Amplified tcdC1 sequence (strain R20291, ribotype 027) containing both the deletion of nucleotide 117 and the large deletion show a more gradual signal change over a broad temperature range (Fig. 1D, left), consistent with the presence of the 18 nucleotide deletion having a relatively low Tm with probe C3, and the sequence with the nucleotide 117 deletion having a higher Tm with probe C1. Two of four samples containing an estimated 10 plasmid targets with tcdC failed to generate signal. Amplifications of 100,000 copies, 1000 copies, or 10 copies were done for each of the other 7 sequences identified in Table 1. Fluorescent signal profiles were generated similar to those in Fig. 1 and were consistent with the mutations present (not shown).

Ratios of the fluorescent signal at one temperature to that at a second temperature can effectively eliminate effects of amplicon concentration and provide a fluorescent “ratio profile” that distinguishes most sequence variations. The signal at each detection temperature was divided by the signal observed 5 degrees lower and the results are plotted on the right side of Fig. 1. Each of the 4 allele sequences gives a ratio profile that is different from the others. The profiles show almost no variation with the initial target concentration or the level of signal observed in the anneal profiles, since the rate of fluorescence change depends on the thermodynamic properties of the probe-amplicon hybrid, with only minor variation due to differences in the concentration of the PCR product.

3.2. Multiplex amplification of synthetic targets

To test whether additional C. difficile genes could be co-amplified for diagnostic confirmation without affecting the consistency of the tcdC ratios, each of the eleven synthetic tcdC targets was amplified in a multiplex that contained primers, probes, and targets for the binary toxin gene cdtB, the large toxin genes tcdA and tcdB, and a Lactococcus gene sequence that served as an internal control for the assay. For this test, tcdA and tcdB sequences were those of the reference strain in all samples. The cdtB sequence was that of strain CD3 in all samples, as the reference strain does not contain the intact binary toxin genes. Fluorescent signals were generated for each of the gene targets and the internal control (not shown). tcdC signal ratios were calculated for 4 replicate samples of each of the eleven tcdC synthetic sequence variants in the multiplex amplification. The ratio profiles were virtually the same as those obtained in the monoplex amplifications and showed low variation among replicates. The fluorescence ratios at two pairs of temperatures for each of the eleven tcdC allele sequences are shown in Fig. 2. The ratios at those temperatures were the most informative for all strains. The ratios of all four replicates form a tight cluster for each allele sequence and most replicate groups are well separated from one another. The exception was the grouping of 3 different alleles (tcdC16, tcdC35, and strain KK312) that included the nonsense mutations at nucleotides 184 or 191. Fluorescence ratios at other temperatures provided further characterization of the sequence variants. For example, tcdC1 and tcdC8 (sc8) showed a much higher ratio value at 55 degrees to 50 degrees due to the higher Tm of probe C1 with those targets containing the deletion of nucleotide 117; a result that could potentially be used to identify some of the strains that have been associated with outbreaks of hypervirulent C. difficile.

3.3. Multiplex amplification and sequence analysis of genomic DNA

Following confirmation that the C. difficile genes could be co-amplified and still generate highly reproducible tcdC signal ratios, a multiplex amplification and probe hybridization analysis was done using C. difficile genomic DNA. Each of the 21 samples was tested in triplicate and was also subjected to sequencing in a separate reaction. Nine different tcdC alleles were identified, including tcdC7, tcdC3 and tcdC9 that were not tested with synthetic targets. The latter two have sequences identical to tcdC19 (reference strain) in the regions targeted by the probes and are presented as a single group in Figs. 3 and 4. That group and the other samples produced tcdC signal ratio profiles consistent with the tests on synthetic targets, although specific values differed slightly between the different tests, presumably due to modifications of the protocol and the use of different thermal cyclers (Figs. 3A and 4). Most importantly, the alleles with the deletion of nucleotide 117, the stop codon mutation at nucleotide 184, any of the large deletions, or a combination of those mutations could be clearly distinguished from one another and from the reference sequence on the basis of one or more tcdC signal ratios.

The presence or absence of the binary toxin gene cdtB with particular tcdC alleles was consistent in most cases with previously published results (Goldenberg and French, 2011). For example, cdtB was detected in all samples with tcdC1, tcdC22, and tcdC35, none of the samples with tcdC7 and tcdC19, and in three of four samples with tcdC47 (previously reported as variable) (Fig. 3B). In contrast to that report, a positive cdtB signal was observed in a sample with tcdC15 and could be a different strain. The possibility of a false positive signal is unlikely, as the LATE-PCR assay detected the binary toxin gene in all 3 replicates.

The tcdA signal ratio profiles reflected the presence of each of the 4 known sequence variations targeted by the single probe (Fig. 3C). The tcdA profiles were closely correlated with specific tcdC alleles. A high Tm for the tcdA sequence was observed with samples containing tcdC7 allele present in toxin A negative strains and consistent with the tcdA

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probe design. Thus, those strains can be easily distinguished from toxin A positive strains containing \( \text{tcdC}19 \) or similar alleles, even though the \( \text{tcdC} \) signal ratios show only minor differences.

The signal ratios in Fig. 3D reflect the combined fluorescence from the \( \text{tdcB} \) probe with relatively high \( T_m \) of 49 °C, 53 °C, or 59 °C and the internal control probe with the lower \( T_m \) of about 37 °C. The \( \text{tcdB} \)
allele was easily identified in the combination and correlated with the tcdC allele identification. The presence of fluorescent signal limited to 45 degrees and lower provided a useful control for amplification in the absence of C. difficile DNA.

4. Discussion

Strains of C. difficile, including hypervirulent strains associated with outbreaks of antibiotic-associated diarrhea can be detected and distinguished using fluorescent signal ratios derived from the hybridization of single-stranded PCR product to mismatch-tolerant probes. Specific sequence variations in the pathogenicity locus genes tcdA, tcdB, and tcdC generate reproducible values that do not vary with the concentration of initial bacterial DNA or the final PCR product. This level of strain evaluation is not possible with other rapid molecular diagnostics, including current commercial PCR tests, but is possible with probes and the wide range of detection temperatures characteristic of multiplex LATE-PCR assays.

Fluorescence detection at endpoint over a wide temperature range increases the level of information that can be obtained in a single tube assay. We were able to examine multiple locations of amplified tcdC using three probes all emitting the same color fluorescence by analyzing

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**Fig. 2.** Fluorescence signal ratios for tcdC at two pairs of temperatures following multiplex PCR of synthetic targets. The 4 replicate samples for each tcdC allele sequence show tightly clustered values, illustrating the potential to distinguish variations using quantitative information from the signal ratios. Similar plots can be made using signal ratios at other temperatures.

**Fig. 3.** Signal ratio profiles for tcdC (A), binary toxin gene cdtB (B), tcdA (C), and cdtB plus internal control (D) generated following multiplex amplification of C. difficile genomic DNA samples. Three samples with alleles tcdC19 (reference strain VPI10463), six tcdC3 (sc3), and six tcdC9 (sc9) have identical sequences in the regions targeted by the tcdC probes and are indicated by red lines; nine tcdC1, blue; three tcdC7, dark green; three tcdC15, gold; nine tcdC22, bright green; twelve tcdC35, blue-green; twelve tcdC47, orange. See text for details.

**Fig. 4.** Fluorescence signal ratios for tcdC at two pairs of temperatures following multiplex PCR from C. difficile genomic DNA, illustrating the ability to distinguish tcdC sequence variations. Label 19* includes alleles tcdC1 and tcdC9 as well as the reference sequence tcdC19. Other numbers indicate the corresponding tcdC allele.
fluorescence ratios. Mutations that prevent the production of a functional tcdC protein, such as the deletion of nucleotide 117 or the nonsense mutations at nucleotides 184 or 191 were easily distinguished from the reference strain and other sequence variations. The design is flexible, as probes can be modified or added to the assay to detect new mutations and enable identification of emerging strains. For example, the different nonsense mutations could be better distinguished from each other by modifying the probe to that region to obtain different melting temperatures with each target. Strains containing the tcdC3 and tcd19 alleles could be distinguished by targeting a fourth probe to a variation at nucleotide 148. The only limit to the number of probes used is the fluorescence capability of the detection equipment and the background fluorescence of the combined probes. To overcome these limitations, we have recently investigated the use of “Off Probes” that are labeled only with fluorescent quenching molecules and greatly reduce fluorescence when they hybridize adjacent to “On Probes” that are similar to those in the assay presented here (Rice et al., in press). Traditional PCR assays require subsequent sequencing to evaluate multiple mutation sites within the tcdC gene (Persson et al., 2011), substantially increasing time and labor relative to our multiple probing method.

Although the current assay does not include multiple probes for tcdA, tcdB, or cdtB, it would be possible to include additional probes to those targets as well or to other targets in an expanded multiplex, thereby increasing the capacity of the assay to discriminate between different C. difficile stains. Thus, the LATE-PCR method offers tremendous flexibility for the construction of rapid and reproducible strain typing assays. Such assays achieve amplification and detection in a single, closed tube and avoid possible laboratory contamination associated with other post amplification analysis, thereby offering a potential improvement over current methods that are time consuming and can yield variable results (Kilibore et al., 2008).

The LATE-PCR C. difficile assay described here provides unprecedented levels of sequence information in a rapid, closed-tube test. On-site use of this assay within healthcare facilities could reduce detection times and help limit the spread of the disease. It may also have implications for anticipating individual patient outcomes, as cure and recurrence rates can vary with strain (Petrella et al., 2012). Evaluation of the diagnostic potential for this assay will require testing a large number of clinical samples after direct isolation from stool and is in progress. Accuracy and sensitivity will be compared with currently used diagnostics.

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References