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Rapid Molecular Characterization of *Clostridium difficile* and Assessment of Populations of *C. difficile* in Stool Specimens[▽]

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Our laboratory has developed testing methods that use real-time PCR and pyrosequencing analysis to enable the rapid identification of potential hypervirulent *Clostridium difficile* strains. We describe a real-time PCR assay that detects four *C. difficile* genes encoding toxins A (*tcdA*) and B (*tcdB*) and the binary toxin genes (*cdtA* and *cdtB*), as well as a pyrosequencing assay that detects common deletions in the *tcdC* gene in less than 4 h. A subset of historical and recent *C. difficile* isolates ($n = 31$) was also analyzed by pulsed-field gel electrophoresis to determine the circulating North American pulsed-field (NAP) types that have been isolated in New York State. Thirteen different NAP types were found among the 31 isolates tested, 13 of which were NAP type 1 strains. To further assess the best approach to utilizing our conventional and molecular methods, we studied the populations of *C. difficile* in patient stool specimens ($n = 23$). Our results indicated that 13% of individual stool specimens had heterogeneous populations of *C. difficile* when we compared the molecular characterization results for multiple bacterial isolates ($n = 10$). Direct molecular analysis of stool specimens gave results that correlated well with the results obtained with cultured stool specimens; the direct molecular analysis was rapid, informative, and less costly than the testing of multiple patient stool isolates.

Clostridium difficile is one of the leading causes of infectious antibiotic-associated diarrhea and pseudomembranous colitis worldwide (2, 16). This is illustrated by the increased incidence and severity of *C. difficile* infection, suggesting the emergence of a new hypervirulent strain (5, 13–15, 17, 25, 32).

While TcdB, a cytotoxin, is the known established virulence factor of *C. difficile*, toxin A (TcdA), a cytotoxic enterotoxin, works synergistically with TcdB, causing damage to the intestinal mucosa in cases of *C. difficile* infection (17). The genes that encode these toxins are located on the pathogenicity locus of *C. difficile* (4, 10, 24). Additionally, several deletions in the *tcdC* gene, a putative negative regulator of the expression of the toxin A (*tcdA*) and the toxin B (*tcdB*) genes, have been identified, and these deletions result in higher levels of cytotoxin expression (11). Furthermore, research has shown that some *C. difficile* strains produce another toxin, known as the binary toxin (19, 22, 28). The genes that encode this toxin, *cdtA* and *cdtB*, together produce an actin-specific ADP-ribosyltransferase that induces damage to the actin skeleton, leading to cytopathic effects in cell lines (1). It has been suggested that the binary toxin genes and deletions in the *tcdC* gene are potential virulence factors in the recent emerging hypervirulent strain (22, 29).

The "gold standard" for the detection of *C. difficile* toxin production is a cytotoxin assay with stool specimens or isolates from anaerobic culture. The cytotoxin assay is difficult to perform and time-consuming, and it is often less sensitive than molecular assays (20, 23, 26). Enzyme immunoassays (EIAs) are used most often, and recent reports suggest that manufacturers have improved the performance of EIA kits since their introduction; however, the disadvantages of EIAs include the lower levels of sensitivity and specificity compared to those of the gold standard methods. More importantly, culture is not specific for the identification of toxigenic strains. The laboratory at the Wadsworth Center has developed a multiplex real-time PCR assay and a *tcdC* gene pyrosequencing assay that rapidly identify potential virulence factors of *C. difficile* strains and that can be used to directly test patient stool specimens for *C. difficile*.

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MATERIALS AND METHODS

Bacterial strains, clinical specimens, and specimen processing. The bacterial strains utilized in this study included a specificity panel of 55 *Clostridium difficile* isolates, gastrointestinal flora, and other bacterial pathogens, which are listed with their sources in Table 1. Included in this panel were 30 strains of *C. difficile* with known toxin gene profiles. Ten strains were positive for only the clostridial toxins (*tcdA*, *tcdB*), 10 strains were positive for all four toxin genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*), and 10 were nontoxigenic. Archived frozen specimens from several hospitals were also tested. Thirty-six isolates from stool specimens received by our laboratory between 1990 and 1992 were from a Veterans Affairs facility in Albany, NY (18, 21), and 113 stool specimens from 2005 to the present came from several different hospitals in New York State. In total, 149 specimens were examined.

C. difficile strains were plated on CDC anaerobe agar and grown at 35°C under anaerobic conditions for 48 h. For molecular testing, isolated colonies were suspended in 1× PCR buffer (Applied Biosystems, Foster City, CA) at a level equivalent to a 1 McFarland standard and heat treated at 95°C for 20 min. The specimens were either used directly in the PCR or stored at −20°C until use. Direct, uncultured stool specimens were subjected to nucleic acid extraction with

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TABLE 1. Bacterial organisms used for specificity testing of multiplex real-time PCR for *C. difficile* toxin genes

Species	Source[a]
Bacteroides fragilis	ATCC 25285
Campylobacter coli	NYSDOH
Campylobacter jejuni	NYSDOH
Clostridium botulinum type A	NYSDOH
Clostridium botulinum type B	NYSDOH
Clostridium botulinum type E	NYSDOH
Clostridium botulinum type F	ATCC 35415
Clostridium difficile[b]	NYSDOH
Clostridium difficile (binary toxin gene negative)[c]	NYSDOH
Clostridium difficile (nontoxigenic)[d]	NYSDOH
Clostridium histolyticum	NYSDOH
Clostridium perfringens	ATCC 13124
Clostridium sordellii	NYSDOH
Clostridium sporogenes	NYSDOH
Clostridium tetani	NYSDOH
Enterobacter aerogenes	ATCC 13048
Enterobacter cloacae	ATCC 13047
Enterobacter lentum	ATCC 43055
Enterococcus faecalis	ATCC 51299
Enterococcus faecium	ATCC 12477
Escherichia coli	ATCC 12799
Klebsiella pneumoniae	ATCC 700603
Pseudomonas aeruginosa	NYSDOH
Salmonella enterica serovar Enteritidis	NYSDOH
Shigella flexneri	NYSDOH
Staphylococcus aureus	NYSDOH
Staphylococcus epidermidis	NYSDOH
Vibrio parahaemolyticus	ATCC 275519

[a] ATCC, American Type Culture Collection; NYSDOH, New York State Department of Health.
[b] Ten *C. difficile* strains containing all four toxin genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*) were tested.
[c] Ten *C. difficile* strains containing only the clostridial toxins (*tcdA*, *tcdB*) were tested.
[d] Ten nontoxigenic *C. difficile* strains were tested.

the QIAamp DNA stool minikit by use of a QIAcube (Qiagen, Valencia, CA), according to the manufacturer's instructions.

Real-time PCR primer and probe design. Unique primer and probe sets targeting *C. difficile* enterotoxin A (*tcdA*), cytotoxin B (*tcdB*), and binary toxin genes A and B (*cdtA* and *cdtB*, respectively) were designed by using Primer Express (version 2.0) software (Applied Biosystems) on the basis of the sequences of strains *Clostridium difficile* VPI 10463 (NCBI accession number X92982, ATCC 43255) and *Clostridium difficile* CD196. The *tcdA* and *tcdB* primers and probes were selected so that they did not overlap known variable regions. The *cdtA* and *cdtB* primers and probes were designed to bind to the full 4.3-kb fragment which encodes the functional binary toxin gene, avoiding areas of homology to box 1, box 2, and box 3 found in all *C. difficile* strains (22). The primers and probe sequences are summarized in Table 2. BLAST (the *B*asic *L*ocal *A*lignment *S*earch *T*ool) searches were performed with all selected potential primer and probe oligonucleotides to test that no cross-reaction would occur with other human or microbial pathogens. The Amplify (version 3.0) program was utilized to determine any possible primer-dimer interactions among the oligonucleotides. Probes TcdA-P, CdtB-P, and CdtA-P and all primers were synthesized by Integrated DNA Technologies (Coralville, IA). Probes TcdB-P and Inhib-P were obtained from Applied Biosystems.

Real-time multiplex PCR assay. All isolates were tested in duplicate, and the DNA from stool specimens was tested in triplicate. The real-time PCR assay was performed on an ABI 7500 Fast real-time PCR system (Applied Biosystems) in a 96-well optical plate format with a LightCycler-FastStart DNA master hybridization probes kit (Roche Diagnostics Corp, Indianapolis, IN). For testing of the isolates, each 25-μl reaction mixture consisted of 1× LightCycler-FastStart DNA master hybridization probes, 3.0 mM $MgCl_2$, 900 nM of all eight primers (Table 2), 250 nM of all four probes (Table 2), sterile water, and 10 μl DNA template. For the testing of stool samples, probes TcdA-P, TcdB-P, and CdtA-P were initially added to the mixture. The master mixture was then split, and probe CdtB-P was added to half of the master mixture for the testing of the DNA isolated from the stool and probe Inhib-P was added to the other half of the master mixture; the latter portion was then seeded with the internal inhibition control, for the assessment of inhibition. The thermal cycling conditions were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles (45 cycles for testing of stool specimens) at 95°C for 15 s and 60°C for 1 min. The data analysis was performed according to the manufacturer's instructions, with no passive reference dye being used.

Sensitivity and specificity. The analytical sensitivity of the real-time PCR was determined for each target through the use of 10-fold dilutions of DNA from a hypervirulent strain of *C. difficile* (strain NYS BAC-05-3671) with known densities and by the generation of standard curves. The sensitivity of detection in stool specimens was determined by spiking stool specimens known to be negative with known densities of *C. difficile* cells, processing them according to the protocol, and testing the purified DNA by real-time PCR. The specificity of the assay was determined by testing the DNA from 55 organisms, as shown in Table 1.

Internal inhibition control. The DNA isolated from stool specimens was also tested for the presence of PCR inhibitors. An internal control plasmid was constructed from a PCR product that contained a portion of heterologous DNA flanked by the primer binding sites of the *cdtA* gene. This PCR product was cloned into a plasmid vector (pCR2.1-TOPO) with a TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA). The plasmid was diluted to yield a threshold cycle (C_T) value of approximately 30. The control plasmid was added to the PCR mixtures containing DNA from the stool specimen and was detected by the use of a different fluorescent probe (Table 2). The C_T value obtained from this reaction was compared with that obtained from a second reaction containing only the control plasmid. The reaction was considered to be inhibited if the C_T value of the reaction was 3 C_T units or more greater than that for the reaction containing only the control plasmid or if no amplification was found.

***tcdC* sequence analysis by Sanger sequencing.** To determine whether deletions were present or absent, we performed a conventional PCR and then sequenced the amplified products. A primer pair was adapted to target the *tcdC* gene (24); it is shown in Table 2. Each 100-μl amplification reaction mixture consisted of 1× PCR buffer; 2.5 mM $MgCl_2$; 10 mM each of dATP, dCTP, dTTP, and dGTP; sterile water; 50 μM of primers C1 and C2; and 2.5 U of AmpliTaq Gold (Applied Biosystems). The PCR products were analyzed on a 2% E-gel (Invitrogen Life Technologies) with a 1-kb DNA marker (Invitrogen Life Technologies) and were visualized under UV light by using a Bio-Rad Laboratories (Hercules, CA) Gel Doc XR apparatus. The PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) and were sequenced by the Applied Genomic Technologies Core facility at the Wadsworth Center. The resultant sequences were compared to the sequence of the *tcdC* gene (NCBI accession number X92982 for *C. difficile* strain VPI 10463) to determine the presence or absence of *tcdC* deletions.

***tcdC* sequence analysis by pyrosequencing.** A pyrosequencing protocol was developed to determine whether the 1-bp, 18-bp, and/or 39-bp deletions within a portion of the *tcdC* gene were present or absent. Amplification and sequencing primers were designed with Assay Design software (Qiagen; previously Biotage). Briefly, a 378-bp PCR product was amplified with forward primer tcdC-F, which was labeled with biotin for immobilization onto streptavidin-coated Sepharose beads. Two separate sequencing primers (primers TcdC-S1 and TcdC-S2) were used to assess the regions where the 1-bp and 18-bp deletions may be present. If the 18-bp pyrosequencing reaction failed, a third sequencing primer (primer TcdC-S3) was used to assess the sample for the 39-bp deletion, since the binding region of the 18-bp sequencing primer falls within the region of the 39-bp deletion. Primers TcdC-F and TcdC-R were synthesized by Integrated DNA Technologies and are shown in Table 2. The locations of the sequences of the PCR primers and the sequencing primers within the *tcdC* gene are shown in Fig. 1. The amplification reaction mixtures consisted of 1× PCR buffer; 2.0 mM $MgCl_2$; 10 mM each of dATP, dCTP, dTTP, and dGTP; sterile water; 200 nM of primers TcdC-F and TcdC-R; 2.5 U of AmpliTaq Gold (Applied Biosystems); and 10 μl of template DNA in a final volume of 100 μl. The thermocycling conditions in a Bio-Rad Laboratories iCycler apparatus were as follows: 1 cycle of 5 min at 95°C, followed by 50 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C and a final extension for 5 min at 72°C. Pyrosequencing was performed with Pyro Gold reagents on a PyroMark vacuum prep workstation and a PyroMark ID instrument according to the manufacturer's instructions (Qiagen). The specific dispensation order for the 1-bp, 18-bp, and 39-bp deletions was GCTGAATAT, GTTAGCTCTCAGCTAGCT, and GCTCTCTCTCTCT, respectively, with single nucleotide polymorphism analysis of the nucleotides.

PFGE. A selection of 31 strains of *C. difficile* was additionally examined by pulsed-field gel electrophoresis (PFGE) to preliminarily assess the North American pulsed-field (NAP) types in our collection and for comparison of the NAP types to potential virulence characteristics. These strains were chosen from

TABLE 2. Primers and probes used for real-time PCR, Sanger sequencing, and pyrosequencing

Gene target	Primer or probe	Probe sequence (5′–3′)[a]	Amplicon size (bp)	Assay	Region
tcdA	TcdA-F	CAGGGCTAATAGTTTGTTTACAGAACA	143	Real-time PCR	
	TcdA-R	CAACATCTAAATATACTCCGCCAAAA	143	Real-time PCR	717–859[b]
	TcdA-P	Texas Red-TTATAGTCAGCAGCTAAATTTCCAC GATTTAACAACTCC–BHQ-2	143	Real-time PCR	
tcdB	TcdB-F	AGCAGTTGAATATAGTGGTTTAGTTAGAGTTG	144	Real-time PCR	
	TcdB-R	CATGCTTTTTTAGTTTCTGGATTGAA	144	Real-time PCR	6552–6695[b]
	TcdB-P	VIC-CATCCAGTCTCAATTGTATATGTTTCTCC A-MGB	144	Real-time PCR	
cdtA	CdtA-F	GATCTGGTCCTCAAGAATTTGGTT	103	Real-time PCR	
	CdtA-R	GCTTGTCCTTCCCATTTTCGATT	103	Real-time PCR	1051–1153[c]
	CdtA-P	FAM-AACTCTTACTTCCCCTGAAT-MGB	103	Real-time PCR	
cdtB	CdtB-F	AAAAGCTTCAGGTTCTTTTGACAAG	132	Real-time PCR	
	CdtB-R	TGATCAGTAGAGGCATGTTCATTTG	132	Real-time PCR	837–968[c]
	CdtB-P	Cy5-CAAGAGATCCGTTAGTTGCAGCATATCCA ATTGT–BHQ-2	132	Real-time PCR	
Inhibition control	Inhib-P	NED-CGATCCCCCCAGCTT-MGB	231	Real-time PCR	
tcdC	C1[d]	TTAATTAATTTTCTCTACAGCTATCC	718	Sanger sequencing	
	C2[d]	TCTAATAAAAGGGAGATTGTATTATG	718	Sanger sequencing	
tcdC	TcdC-F	Biotin-AGGGTATTGCTCTACTGGCATTTATT	378	Pyrosequencing	
	TcdC-R	CCCCTTCAAATTTACCTTTTGTACTATAATCA	378	Pyrosequencing	
tcdC	TcdC-S1	CGTTGTTCTTCAGCC	NA[e]	Pyrosequencing	
	TcdC-S2	TTGCCAAAAAACACG/A	NA	Pyrosequencing	
	TcdC-S3	TCATAAGTAATACCAGTATCATAT	NA	Pyrosequencing	

[a] BHQ-2, Black Hole Quencher 2; MGB, Minor Groove Binder; FAM, 6-carboxyfluorescein; NED, 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein.
[b] On the basis of sequences in GenBank with accession number X92982 for *tcdA*, *tcdB*, and *tcdC*.
[c] On the basis of sequences in GenBank with accession number L76081 for *cdtA* and *cdtB*.
[d] As described previously (24).
[e] NA, not applicable.

among strains submitted between 1980 and 2008 and included strains that we had determined harbored the *tcdA* and *tcdB* toxin genes or the *tcdA*, *tcdB*, *cdtA*, and *cdtB* toxin genes. DNA was prepared from *C. difficile* cells grown for 7 h in reduced peptone-yeast-glucose broth at 37°C, according to established protocols (12). DNA was digested with restriction endonuclease SmaI (New England Biolabs, Beverly, MA), and the fragments were separated in 1.0% agarose gels on a clamped homogeneous electric field apparatus (CHEF Mapper; Bio-Rad Laboratories, Richmond, CA). The initial pulse time of 5.0 s was increased to 40.0 s over 19.5 h. The gels were stained with ethidium bromide, destained in distilled water, and visualized with a Gel Doc 2000 gel analysis system (Bio-Rad Laboratories). The PFGE patterns were analyzed with Bionumerics software (Applied Maths, Belgium). Dendrograms were created by use of unweighted pair group similarity and arithmetic mean with Dice coefficients, and the position tolerance was set at 1.1%. NAP types were determined by comparison with

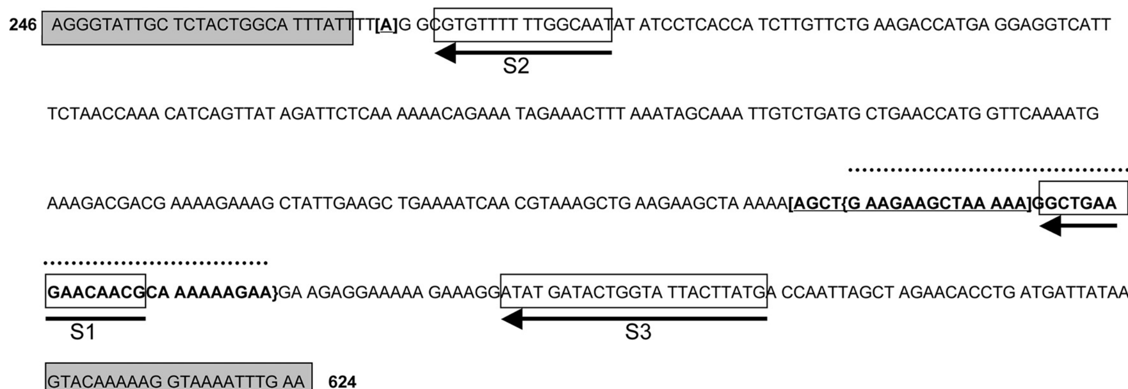

FIG. 1. Design for amplification and pyrosequencing of the *tcdC* gene. A diagram of a portion of the *tcdC* gene used to detect the 1-bp, 18-bp, and 39-bp *tcdC* gene deletions found in strains of *Clostridium difficile* is shown. The sequence from NCBI accession number DQ870674 (*C. difficile* strain ATCC 43594) was used, and the forward and reverse primer locations are shaded in gray. Deletion locations are shown in boldface and in brackets, with the 1-bp and 18-bp deletions being underlined. The 39-bp deletion is shown with a dotted line over it. Sequencing primers S1, S2, and S3 are boxed and have arrows indicating the direction of sequencing.

known NAP types. An isolate with ≥80% similarity to a known NAP type was considered to belong to that NAP type (11).

Assessment of *C. difficile* populations in patient specimens. Twenty-three stool specimens from cases of presumed community-associated *C. difficile* infection were examined to determine whether the patients studied had homogeneous populations of *C. difficile.* The definition of presumed community-associated *C. difficile* infection was a positive EIA result for *C. difficile* toxin with a specimen collected when the patient was as an outpatient or within 72 h of patient admission to a hospital and with no evidence of hospitalization in the preceding 3 months. "Hospitalization" was defined as an overnight stay in a hospital or other skilled nursing facility. The patients were surveyed for antibiotic use preceding *C. difficile* infection, but antibiotic use was not used as an exclusion criterion.

Specimens were collected and stored frozen until culture could be performed. Twenty microliters of thawed stool specimen was inoculated onto in-house-prepared *Clostridium difficile* selective agar and cycloserine-cefoxitin-fructose agar with antibiotic concentrations of 480 μg/ml for cycloserine and 15.4 μg/ml for cefoxitin (9). The media were not prereduced before they were plated. The plates were incubated for 4 days at 37°C under anaerobic conditions. Up to 10 colonies from each specimen were subcultured to CDC anaerobe agar. One colony from each specimen was identified as *C. difficile* by a battery of conventional biochemical tests, including Gram staining and tests for anaerobic growth requirement, motility, catalase production, indole reaction, nitrate reduction, H_2S production, growth in bile, lipase production, lecithinase production, and urease production. The isolates were also assayed for their abilities to ferment carbohydrates; the substrates included glucose, maltose, mannitol, lactose, sucrose, xylose, salicin, arabinose, and glycerol. Cellular fatty acid analysis was also performed by using the Microbial Identification System (Midi Inc., Newark, DE). The remaining isolates from each specimen were identified only by Gram staining, a test for anaerobic growth requirement, and cellular fatty acid analysis. Each isolate was then assessed for the presence of potential virulence factors by the molecular methods described above. In a further prospective analysis, two isolates from each of the 89 patient samples were examined in a continuation of our assessment of *C. difficile* bacterial populations and virulence characteristics.

RESULTS

Sensitivity and specificity of real-time PCR assay. The analytical results obtained with the isolated colonies indicate that the real-time PCR assay evaluated in the present study has a limit of detection of 1 CFU per reaction mixture. The sensitivity of both the real-time PCR assay and pyrosequencing by direct testing of stool specimens was determined to be 10 CFU per reaction mixture (results not shown). In a study that compared the results of the real-time PCR assay to those of the gold standard cytotoxin assay, the real-time assay was found to be 100% sensitive (results not shown). The specificity of the real-time PCR assay was determined to be 100%, as the *C. difficile* strains assessed in the multiplex real-time PCR were positive for the respective genes (*tcdA* and *tcdB* or *tcdA*, *tcdB*, *cdtA*, and *cdtB*) and all non-*C. difficile* bacterial isolates and nontoxigenic *C. difficile* were negative for all four genes, as expected.

Analysis of deletions in *tcdC* gene by Sanger sequencing and pyrosequencing. The *tcdC* gene from 154 isolates was amplified and sequenced by both the pyrosequencing and the Sanger sequencing methods to determine the presence or absence of the 1-bp, 18-bp, and/or 39-bp deletions. Comparison of the results obtained by the two tests revealed a 100% correlation between the results of the two methods (results not shown).

Analysis of *C. difficile* isolates by PFGE. Figure 2 summarizes the results of PFGE analysis of *C. difficile* isolates to obtain a preliminary understanding of the NAP types circulating in New York State. Thirteen NAP type 1 (NAP1) types were identified in the group and possessed all four toxin genes and both the 1-bp and 18-bp *tcd*C gene deletions. Eight additional NAP types were also identified (NAP2, NAP3, NAP4, NAP5, NAP8, NAP10, NAP11, NAP12). All the isolates with NAP types 2, 3, 4, 5, and 10 and undesignated NAP types CldS16015 and CldS16006 were *C. difficile* isolates that contained no binary toxin genes or *tcdC* gene deletions. Interestingly, NAP types 8, 11, and 12, as well as two others, types CldS16002 and CldS16005, were found to have some or all of the potential virulence characteristics. The NAP8 (2008) strain was positive for all toxin genes and the *tcdC* gene 18-bp deletion. The NAP11 (2006) and NAP12 (1990, 1992) strains were negative for the binary toxin genes but positive for the *tcdC* gene 18-bp deletion. Two strains of types CldS16002 and CldS16005 (2006), respectively, differed from the strains designated NAP1 by four bands but were found to be positive for all toxin genes and for the *tcdC* gene 1-bp and 18-bp deletions.

Assessment of *C. difficile* populations in patient specimens. Twenty-three stool specimens were examined by culture, real-time multiplex PCR, as well as Sanger sequencing and pyrosequence analysis for *tcdC* gene deletions. All stool specimens were initially found to be positive for *C. difficile* by culture. One hundred ninety-four colonies from the 23 specimens were examined. The data in Table 3 demonstrate that of the 194 strains isolated, 3 strains (1.5%) exhibited a molecular profile different from that of the other strains isolated from the same individual's stool specimen. Analysis of the stool specimens revealed that three stool specimens (13%) did not contain homogeneous populations. In two of these stool specimens (specimens 4 and 15), the difference was the presence of the binary toxin genes in one or more of the isolates of the patient's stool specimens. For the third specimen (specimen 1), the difference was the absence of both the binary toxin genes and the *tcdC* gene deletion in 1 of 10 isolates from the same patient's stool specimen. Overall, among the 89 stool specimens tested in this prospective analysis (two isolates per stool specimen), no differences in their molecular profiles were identified, as illustrated in Table 4.

Direct molecular analysis of patient stool specimens. The *C. difficile* population data led us to evaluate the potential benefits of direct molecular analysis of stool specimens. A subset of 24 stool specimens was directly tested by the multiplex real-time PCR and pyrosequencing. The preliminary data showed a correlation between the results of direct testing and those of testing following culture. For 23 (95.8%) of the stool specimens tested, identical results and molecular profiles were obtained. However, one stool specimen was negative by direct testing, while culture of that stool specimen identified two colonies of *C. difficile*.

DISCUSSION

Although the binary toxin genes and the *tcdC* gene deletions and their contribution to the virulence of *C. difficile* strains causing infections are still poorly understood, characterization may be important for infection control and a proper public health response. Currently, only a few real-time PCR assays are available for the detection of toxins A and B (3, 6, 23, 31). We have developed a sensitive and specific one-tube, real-time PCR assay capable of determining in less than 2 h whether toxins A and B (*tcdA* and *tcdB*) and the binary toxins (*cdtA* and *cdtB*) are present. Pyrosequencing is a novel technique that we have implemented for the detection of the deletions in the

Year	Cell Cytotoxicity Test	Toxin A and B genes[a]	Binary toxin genes[b]	*tcdC* gene deletions	PFGE Pattern type (CldS[c] and NAP[d])	PFGE Results
2006	+	+	-	none	CldS16012/NAP 3	
2007	+	+	-	none	CldS16012/NAP 3	
2007	+	+	-	none	CldS16012/NAP 3	
2006	+	+	-	none	CldS16016/NAP 3	
2008	+	+	-	none	CldS16012/NAP 5	
2008	+	+	-	none	CldS16015	
1980	+	+	-	none	CldS16006	
2008	+	+	-	none	CldS16001/NAP 4	
1990	+	+	-	none	CldS16001/ NAP 4	
2006	+	+	+	1-bp/18-bp	CldS16003/NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2007	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2007	+	+	+	1-bp/18-bp	CldS16003/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16004/ NAP1	
2005	+	+	+	1-bp/18-bp	CldS16009/ NAP 1	
2008	+	+	+	1-bp/18-bp	CldS16009/ NAP 1	
1982[e]	+	+	+	1-bp/18-bp	CldS16017/ NAP 1	
2006	+	+	+	1-bp/18-bp	CldS16002	
2006	+	+	-	18-bp	CldS16011/ NAP 11	
2006	+	+	+	1-bp/18-bp	CldS16005	
2008	+	+	+	18-bp	CldS16013/NAP 8	
2008	+	+	-	none	CldS16014/NAP 10	
2005	+	+	-	none	CldS16010/ NAP 2	
1992	+	+	-	none	CldS16008/ NAP 2	
1990	+	+	-	18-bp	CldS16007/NAP 12	
1992	+	+	-	18-bp	CldS16007/NAP 12	

FIG. 2. Molecular characterization of *Clostridium difficile* from patient specimens (1980 to 2008) by multiplex real-time PCR, sequence analysis of the *tcdC* gene, and PFGE analysis. [a], the *tcdA* and *tcdB* genes were detected by real-time PCR; [b], the *cdtA* and *cdtB* genes were detected by real-time PCR; [c], CldS is the abbreviation used in our database to designate a *Clostridium difficile* subtype; [d], NAP is the abbreviation used for North American pulsed-field type; [e], this strain, received by our laboratory in 1980, was a control strain provided by the CDC for our *C. difficile* cytotoxin assay.

tcdC gene. Studies have previously shown that the deletions that have been described in the *tcdC* gene (7, 17, 23, 27) may be responsible for increases in toxin production. Two deletions, a 39-bp deletion and an 18-bp deletion, were thought to contribute to the enhancement of disease severity and high rates of morbidity; however, newer investigations suggest that a 1-bp deletion in the *tcdC* gene may be the cause of the overproduction of the toxins (7). Utilization of these assays may be informative and may help to provide an understanding of the roles of these genetic factors in *C. difficile* infections.

Following several outbreaks in 2003, further characterization of the hypervirulent strain of *C. difficile* determined that it is PCR ribotype 027 (PCR ribotyping), NAP1 (PFGE), restriction endonuclease analysis type BI, and toxinotype III (toxinotyping) (5, 13, 16). In our analysis of the NAP types circulating in New York State, we found that NAP1 strains were first

TABLE 3. Testing of presumed community-associated *C. difficile*-infectedstool specimens[a]

Stool specimen no.	No. of strains isolated/stool specimen	No. of strains with the following molecular profile:			
		Binary toxin genes, *tcdC* deletions	Binary toxin genes only	No binary toxin genes and no *tcdC* deletion	Homogeneous
1	**10**	**9**	**0**	**1**	**No**
2	10	10	0	0	Yes
3	10	0	0	10	Yes
4	**2**	**0**	**1**	**1**	**No**
5	8	8	0	0	Yes
6	10	0	0	10	Yes
7	10	0	0	10	Yes
8	10	0	10	0	Yes
9	10	0	0	10	Yes
10	10	0	0	10	Yes
11	10	10	0	0	Yes
12	10	10	0	0	Yes
13	10	10	0	0	Yes
14	3	0	0	3	Yes
15	**4**	**0**	**3**	**1**	**No**
16	5	0	0	5	Yes
17	9	9	0	0	Yes
18	9	0	0	9	Yes
19	5	0	0	5	Yes
20	9	0	9	0	Yes
21	10	0	0	10	Yes
22	10	0	0	10	Yes
23	10	0	0	10	Yes

[a] Boldface represents the results for the strains that exhibited a molecular profile different from that of the other strains isolated from the same individual's stool specimen.

TABLE 4. Prospective *C. difficile* stool specimen characterization[a]

No. of stool specimens	Molecular profile		No. of specimens with the following type of bacterial population:	
	Binary toxin genes	*tcdC* deletions	Homogeneous	Heterogeneous
30	Positive	1-bp, 18-bp	29	0
46	Negative	Negative	46	0
10	Positive	18-bp	10	0
3	Positive	1-bp	3	0

[a] Two isolates from each stool specimen were analyzed.

isolated in 2005. The NAP1 strains that we tested were isolated in recent years, and most contained the four toxin genes in combination with the 1-bp and 18-bp *tcdC* deletions. Retrospectively, we were also interested in assessing the molecular profiles of strains that had caused major outbreaks at a local facility in 1990 and 1992. We found several NAP strains that contained the 18-bp *tcdC* deletion; the other two NAP types were found to be negative for the binary toxin genes and *tcdC* deletions. We also observed other NAP types that had subsets of the genetic factors present; it is unclear whether the strains identified as being unmatched to known NAP designations have been recognized previously or whether they are novel. These data illustrate that these molecular profiles have actually been present in strains for a long time, yet it is only recently that more virulent disease has been appreciated. More work directed toward obtaining an understanding of the NAP types circulating throughout the world and the morbidity and mortality rates associated with each of the strains should continue to shed light on the hypervirulent strain of *C. difficile*.

The assessment of the *C. difficile* populations within individual stool specimens demonstrated that the *C. difficile* isolates recovered from a stool specimen are for the most part homogeneous in a patient with presumed community-associated *C. difficile* infection (described above). However, the isolates in 13% of the stool specimens examined were not homogeneous by our molecular methods, and this raises the question of whether the determination of potential genetic factors from a single *C. difficile* colony isolated from a stool specimen is the best practice. We are not the first to show that more than one strain can be present in a given stool specimen; however, conflicting data have been reported (30). In our analysis, we have shown that for at least one case (stool specimen 1 in Table 3), the possibility exists that the single colony possessing no binary toxin genes or *tcdC* gene deletions could have been the one isolated from the patient's stool specimen, thus resulting in a false-negative result for that patient. The further prospective analysis of 89 additional stool specimens could have shed more light on this question; however, no heterogeneity was identified in that set of specimens.

The results of our testing have led us to conclude that characterization of *C. difficile* directly from stool specimens is the testing route of choice at the Wadsworth Center for the rapid identification of putative genetic factors of *C. difficile*. The idea of testing stool specimens directly is not a novel one (8). Our preliminary data have shown that *C. difficile* could not be detected by the direct method in 4% (1/24) of specimens. The specimen in which *C. difficile* could not be detected by the direct method was found, upon culture analysis, to have only two colonies of *C. difficile*, which was below our limit of detection for stool specimens. We believe, on the basis of our data, that direct testing of stool specimens combined with culture at the start of testing is the most informative approach for laboratories that have the ability to perform all of these methods. If only PCR analysis of stool specimens is utilized, there is a risk that a potentially highly virulent strain may fail to be identified. If culture is routinely performed first, followed by molecular analysis of one or even a few colonies from each patient, there is still the possibility that the hypervirulent strain will be missed among the typical *C. difficile* strains in a given stool specimen. The latter approach can also lead to additional costs because of the need to perform more than one molecular test per specimen. An alternative approach, which we have previously utilized for other pathogens, is to first perform molecular analysis as a screen and to then perform culture only with the specimens with negative or inconclusive results.

In conclusion, the *C. difficile* multiplex real-time PCR assay that we have developed is sensitive, specific, and rapid. Pyrosequencing of the *tcdC* gene is a novel and valuable tool for the further characterization of *C. difficile* isolates that cause infections and that are detected in stool specimens. These molecular assays can be applied directly for the screening of patient specimens and may provide valuable information that helps provide an understanding of these genetic factors and the roles that they may play in *C. difficile* infection.

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