ORIGINAL ARTICLE

New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection

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ABSTRACT

Isolates of *Clostridium difficile* from 159 hospitalized Danish patients (2005) were analysed by a new 5-plex PCR method targeting the toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB*, and 16S rDNA as an internal positive control. Additionally, the toxin-regulating gene *tcdC* was partially sequenced by a new sequencing-based method that revealed genetic changes that may render the gene product inactive. Finally *tcdA* was analysed using a previously published method for the detection of internal deletions. The 5-plex PCR revealed four different toxin gene profiles: 36 $tcdA^+$, $tcdB^+$, $cdtA^+/cdtB^+$; one $tcdA^+$, $tcdB^-$, $cdtA^+/cdtB^+$; 98 $tcdA^+$, $tcdB^+$, $cdtA^-/cdtB^-$; and 24 non-toxigenic $tcdA^-$, $tcdB^-$, $cdtA^-/cdtB^-$. Deletion studies revealed that 26 strains contained a *c.* 700-bp deletion in *tcdA*, and 39 strains contained at least one possible inactivation feature in *tcdC*. The prevalence of the binary toxin genes was 23%. All strains with the $tcdA^+$, $tcdB^+$, $cdtA^+/cdtB^+$ profile were investigated by PCR ribotyping, and this revealed eight different ribotypes, none of which were 027. The 5-plex PCR method offers a one-step, rapid and specific screening method for *C. difficile* toxin genes. This toxin gene profiling, together with deletion studies in *tcdA* and *tcdC*, may allow an evaluation of the pathogenic potential of *C. difficile*.

Keywords *Clostridium difficile*, diagnostics, diarrhoeagenic, multiplex PCR, toxigenic

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INTRODUCTION

Clostridium difficile is the major cause of nosocomial infectious diarrhoea, and toxin-producing *C. difficile* isolates are recognized in 10–25% of cases of antibiotic-associated diarrhoea, and in almost all cases of pseudomembranous colitis [1–3]. *C. difficile*-associated disease in humans may have an animal origin, as *C. difficile* has been found in retail meat for human consumption, and typing studies have revealed an overlap between strains isolated from humans and calves [4]. The two major

C. difficile toxins are the large clostridial toxins; i.e. an enterotoxin, TcdA, and a cytotoxin, TcdB, encoded by *tcdA* and *tcdB*, respectively. The two genes are part of the PaLoc operon, which also contains *tcdR*, *tcdE* and *tcdC*, of which *tcdC* is a putative negative regulator of *tcdA* and *tcdB* [5]. Some *C. difficile* strains contain an additional toxin, the *Clostridium difficile* binary toxin (CDT), expressed from the *cdtA* (enzymatic component) and *cdtB* (binding component) operon. *C. difficile* can be diagnosed by culturing faecal samples on selective media, and toxigenic strains producing TcdA and/or TcdB may subsequently be identified using tissue culture cytotoxin assays or enzyme immunoassays (EIAs). Both of these methods may also be applied directly to stool filtrates. However, methods based on stool filtrates do not allow the isolation of *C. difficile* colonies, which is crucial for

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further characterization and epidemiological surveillance, and they may display reduced sensitivity, due to the loss of cytotoxin activity during transport and storage of the faecal sample [6]. Furthermore, neither the tissue culture cytotoxin assays nor the EIAs are currently adequate for detection of the binary toxin [7–10]. Diagnostic strategies targeting nucleic acids, including PCR methods [11–13] and real-time PCR methods [14,15], have been developed for the detection of the genes encoding TcdA and/or TcdB.

C. difficile strains have been found to contain various alterations in the PaLoc genes. These include internal deletions and premature stop codons in *tcdC* that may render the gene product inactive, and registered polymorphisms in the PaLoc genes, which to date have led to the identification of 24 profiles (toxinotypes) after PCR–restriction fragment length polymorphism analysis [16–18]. Antibiotic resistance profiling has also attracted much attention, as *C. difficile*-associated disease may be a consequence of antibiotic treatment itself, and strains with varying and extensive resistance profiles have been observed [19–21]. Both genetic analyses and antibiotic susceptibility testing have been used to characterize the various clones isolated from specific outbreaks and severe infections. Epidemic strains of *C. difficile* associated with severe disease have been observed in Canada, the USA and Europe, with the following characteristics: (i) positive for the binary toxin; (ii) containing an 18-bp deletion in *tcdC*; and (iii) resistant to fluoroquinolones. These strains have been typed as PCR ribotype 027, pulsed-field gel electrophoresis type NAP1, restriction endonuclease analysis (REA) type B1 and toxinotype III [22]. TcdA-negative/TcdB-positive *C. difficile* strains have also been involved in nosocomial outbreaks [23–25]. These variants harbour a deletion in the 3'-end of *tcdA*, which encodes the ligand-binding domain [12,25,26], and for this reason they are difficult to detect by culture cytotoxin assays and they are non-detectable by EIAs directed towards toxin A only.

The aim of the present study was to develop new molecular methods for the detection of pathogenic *C. difficile* isolates, including: (i) a multiplex PCR for detecting the genes encoding TcdA, TcdB and CDT; and (ii) a sequencing-based method for investigating deletions and premature stop codons in *tcdC*. These methods, in addition to a previously published method for *tcdA* analysis [27], were applied to bacterial isolates from 159 hospitalized Danish patients.

MATERIALS AND METHODS

Strains and DNA preparation

The following *C. difficile* reference strains (kindly provided by M. Rupnik) were used for validation (toxinotype/PCR ribotype, if determined, in parentheses): EX623 (I/102), AC008 (II/103), 44027 (III/075), 55767 (IV/023), SE881 (V/066), 51377 (VI/066), 57267 (VII/063), 1470 (VIII/017), 8864 (X/036), as previously described [17], R12087 (IIIb/027), as previously described [28], IS58 (XIa/033), R11402 (XIb/033), IS25 (XII/056), R9367 (XIII/070), R10870 (XIV/111), R9385 (XV/122), as previously described [29], SUC36 (XVI), J9965 (XVII), GAI00166 (XVIII), TR13 (XIX), TR14 (XX), as previously described [30], 6223 (XXI), as previously described [31], and 8785 and 597B, as previously described [16]. In total, 159 *C. difficile* isolates were obtained from hospitalized Danish patients with diarrhoea during the period April–October 2005. The strains were collected at hospitals located in five different counties in Denmark, covering the major eastern areas of the country.

Primary identification of *C. difficile* was conducted by boiling faecal samples in phosphate-buffered saline (pH 7.4), followed by culturing on cycloserine cefoxitin fructose agar medium (SSI Diagnostica, Hillerød, Denmark) in an atmosphere composed of 86% N_2, 7% H_2 and 7% CO_2 at 37°C for 48 h. Bacterial colonies were prepared for PCR in the following way. Three to five colonies (the equivalent to that contained in a 1-μL sterile loop) were transferred to 200 μL of 10% Chelex 100 (Bio-Rad, Hercules, CA, USA) in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8), boiled for 15 min, and then centrifuged briefly. The supernatant was diluted 1 : 10 in TE, and 5 μL was used directly in PCRs. DNA was purified for PCR ribotyping using a ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen Carlsbad, CA, USA).

tcdA, *tcdB*, *cdtA* and *cdtB* analysis

A 5-plex PCR was developed for the detection of *tcdA*, *tcdB*, *cdtA*, *cdtB* and 16S rDNA. The PCRs were run in total reaction volumes of 25 μL containing the following reagents: 1× PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8.3), 2.6 mM $MgCl_2$, 260 μM each of dATP, dCTP, dGTP and dTTP, 1.25 U of FastStart *Taq* polymerase (Roche Diagnostics, Mannheim, Germany), and the 12 primers shown in Table 1, at the concentrations given. Thermocycler conditions were 10 min at 94°C, followed by 35 cycles of 50 s at 94°C, 40 s at 54°C and 50 s at 72°C, and a final extension of 3 min at 72°C. As a confirmatory analysis of *tcdB*, the PCR method of Spigaglia *et al.* (2002), using primers TB1 and TB2, was applied.

tcdA 3'-end deletion analysis

The *tcdA* gene was amplified using the PCR method of Kato *et al.* (1999), with minor modifications: The total volume of the PCR was 30 μL, containing the following reagents: 1× PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 200 μM each of dATP, dCTP, dGTP and dTTP, 2.8 mM $MgCl_2$, 1 U of Platinum *Taq* polymerase (Invitrogen), and the primers as

Table 1. Primers used in the present analysis

Analysis	Gene target	Primer name	Sequence (5′–3′)	Primer concentration (μM)	Amplicon size (bp)
5-plex PCR	*tcdA*	tcdA-F3345	GCATGATAAGGCAACTTCAGTGGTA[a]	0.6	629
		tcdA-R3969	AGTTCCTCCTGCTCCATCAAATG	0.6	
	tcdB	tcdB-F5670	CCAAAARTGGAGTGTTACAAACAGGTG	0.4	410
		tcdB-R6079A	GCATTTCTCCATTCTCAGCAAAGTA	0.2	
		tcdB-R6079B	GCATTTCTCCGTTTTCAGCAAAGTA	0.2	
	cdtA	cdtA-F739A	GGGAAGCACTATATTAAAGCAGAAGC	0.05	221
		cdtA-F739B	GGGAAACATTATATTAAAGCAGAAGC	0.05	
		cdtA-R958	CTGGGTTAGGATTATTTACTGGACCA	0.1	
	ctdB	ctdB-F617	TTGACCCAAAGTTGATGTCTGATTG	0.1	262
		cdtB-R878	CGGATCTCTTGCTTCAGTCTTTATAG	0.1	
	16S rDNA	PS13	GGAGGCAGCAGTGGGGAATA	0.05	1062
		PS14	TGACGGGCGGTGTGTACAAG	0.05	
tcdC analysis	*tcdC*	tcdC-F(−17)	AAAAGGGAGATTGTATTATGTTTTC	0.2	475[c]
		tcdC-R(+462)	CAATAACTTGAATAACCTTACCTTCA	0.2	
tcdA 3′-end deletions	*tcdA*	NK9	CCACCAGCTGCAGCCATA[b]	0.17	2535[c]
		NKV011	TTTTGATCCTATAGAATYTAACTTAGTAAC[b]	0.17	

[a]The first 23 nucleotides are the same as in primer YT28 from [50].
[b]From [27], except for one degenerate nucleotide (Y) added at position 18 of primer NKV011.
[c]Amplicon size when no deletion was present.

listed in Table 1. Thermocycler conditions were: 6 min at 94°C followed by 37 cycles consisting of 20 s at 95°C, 30 s at 55°C and 120 s at 60°C.

tcdC gene analysis

The *tcdC* gene was amplified in a 25-μL PCR containing the following reagents: 1× PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$, pH 8.3), 260 μM each of dATP, dCTP, dGTP and dTTP, 2.6 mM $MgCl_2$, 1.25 U of FastStart *Taq* polymerase (Roche Diagnostics) and the primers as listed in Table 1. Thermocycler conditions were: 6 min at 94°C, followed by 35 cycles consisting of 50 s at 94°C, 40 s at 47°C and 50 s at 72°C, and a final extension at 72°C for 3 min. The PCR product was subsequently sequenced with the reverse primer (tcdC-R1(+462)) using the BigDye Terminator Sequencing kit v1.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3100 Genetic Analyzer. All gels were run under standard conditions on 1.5% agarose and stained with ethidium bromide.

PCR ribotyping

PCR ribotyping was performed according to Bidet [32], with strains positive for the binary toxin ending the reference strains with known PCR ribotypes. The resulting band patterns were compared and named according to the PCR ribotype of the reference strains. When no match was found, new and unique PCR ribotypes were named arbitrarily.

Toxinotyping

A number of strains were kindly toxinotyped [16] at the laboratory of M. Rupnik.

RESULTS

A 5-plex PCR was developed for the detection of the four *C. difficile* toxin genes, *tcdA*, *tcdB*, *cdtA*, and *cdtB*, with 16S rDNA as an internal PCR control (Fig. 1). Primers were chosen to cover all

genetic variants present in GenBank, and amplicon sizes were chosen to be distinguishable on agarose gels. The method was validated on 24 reference strains, each representing a unique toxinotype, and all genes were identified correctly according to previous genetic analysis of the reference strains [16–18,29–31]. The primers used to amplify *tcdA* are located upstream of the repetitive region in the 3′-end which, in some strains, contains various deletions that render the gene product non-detectable by EIA methods. Therefore, strains that are TcdA-negative due to 3′-end deletions are still *tcdA*-positive according to the present 5-plex PCR. This phenomenon is also illustrated by the four reference strains, 1470 [17], SUC36 [30], IS58 and R11402 [29], which are *tcdA*-positive according to the multiplex PCR,
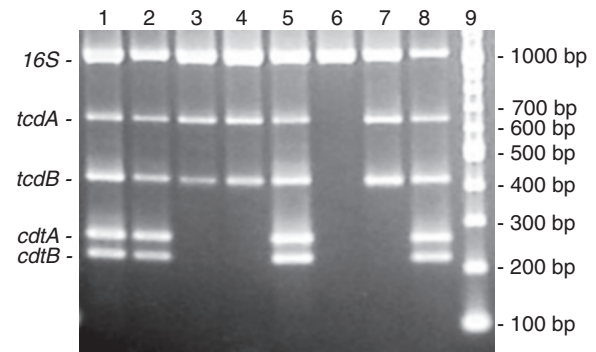

Fig. 1. Toxin gene profiles of eight selected *Clostridium difficile* strains. Lanes 1, 2, 5 and 8: *tcdA*+, *tcdB*+, *cdtAB*+. Lanes 3, 4 and 7: *tcdA*+, *tcdB*+, *cdtAB*−. Lane 6: non-toxigenic *C. difficile*. Lane 9: 100-bp DNA marker.

despite the fact that they all contain 3'-end deletions according to their original references.

The 5-plex PCR was applied to 159 *C. difficile* strains isolated from Danish patients with diarrhoea collected over a period of 7 months. Four different toxin gene profiles were found, as listed in Table 2. Thirty-seven strains (23%) possessed the genes encoding the binary toxin (*cdtAB*), and 36 of these strains also harboured *tcdA* and *tcdB*, whereas one strain was *tcdA*-positive and *tcdB*-negative. As this latter profile is rather uncommon, the absent *tcdB* gene was confirmed by an alternative PCR method [33].

Ninety-eight strains contained *tcdA* and *tcdB*, and no genes encoding binary toxins, and 24 strains were non-toxigenic. In order to investigate 3'-end deletions in *tcdA*, a supplemental PCR was performed, according to Kato *et al.* (1999), and a *c.* 700-bp deletion was found in 26 strains, all harbouring the genes encoding the binary toxin (*ctdAB*), combined with *tcdA* and *tcdB* (25 strains), and one strain positive for *tcdA* and negative for *tcdB*. The *tcdC* gene was investigated by partial sequencing. Thereby, premature stop codons and internal deletions could be identified. This revealed that 39 strains contained a gene deletion of 18, 39 or 54 bp, and that 36 of these strains contained a C → T transition at position 184 bp, which introduces a premature stop codon. The 39-bp deletion was the most frequently observed deletion, in 34 strains, whereas 18-bp and 54-bp deletions were found in one and four strains, respectively (Table 2).

All of the 37 strains that possessed the binary toxin (except three strains that died during the experiments) were subjected to PCR ribotyping, which revealed eight different types. By comparing the band patterns with those of the available reference strains, it was possible to assign two profiles (019 and 023) according to Stubbs *et al.* (1999); the remaining were named arbitrarily (dk1, dk2, dk3, dk10, dk11 and dk12). PCR ribotype dk2 accounted for 21 strains among the Danish isolates, and was therefore by far the most predominant. Among the 21 strains with PCR ribotype dk2, eight different resistance profiles were found. Selected strains were analysed by toxinotyping, and they all belonged to known toxinotypes. When the strain profiles were compared according to geography and patient age, it was found that no particular gene profile was associated with geographical origin or patient age (data not shown).

DISCUSSION

Pathogenic strains of *C. difficile* produce TcdA and/or TcdB and, in addition to these toxins, several strains isolated from outbreaks and severe infections have been shown to harbour the genes encoding the binary toxin CDT. Therefore, *tcdA*, *tcdB*, *cdtA* and *cdtB* were incorporated

Table 2. Genetic profile of the 159 clinical strains of the present study

No. of strains found	*tcdA*/*tcdB*	*cdtA*/*cdtB*	*tcdA* deletion (*c.* bp)	*tcdC* deletion (bp)	*tcdC* stop codon at 184 bp	PCR ribotype[a]	Toxinotype
1	+/+	+/+	–	–	–	019	IX
2	+/+	+/+	–	39	+	dk3	V
1	+/+	+/+	–	39	+	dk3	V
1	+/+	+/+	–	39	+	dk3	ND
4	+/+	+/+	–	54	+	023	IV
1	+/+	+/+	–	39	+	dk11	V
1	+/+	+/+	–	39	+	dk1	ND
3	+/+	+/+	700	39	+	NA	NA
1	+/+	+/+	700	39	+	dk10	ND
7	+/+	+/+	700	39	+	dk2	ND
1	+/+	+/+	700	39	+	dk2	VI
4	+/+	+/+	700	39	+	dk2	ND
2	+/+	+/+	700	39	+	dk2	ND
2	+/+	+/+	700	39	+	dk2	ND
1	+/+	+/+	700	39	+	dk2	ND
2	+/+	+/+	700	39	+	dk2	ND
2	+/+	+/+	700	39	+	dk2	VI
1	+/–	+/+	700	39	+	dk12	XIa
2	+/+	–/–	–	39	–	ND	ND
1	+/+	–/–	–	18	–	ND	ND
95	+/+	–/–	–	–	–	ND	ND
4	–/–	–/–	–	–	–	ND	ND
Total 159							

[a]PCR ribotypes 019 and 023 named according to [28]; the remaining types were arbitrarily named in this study.
NA, not available; ND, not determined.

in the present multiplex PCR, as they were considered to be the most relevant genes for primary characterization of pathogenic *C. difficile*. The templates to be analysed were easily prepared by boiling bacterial colonies, and the PCR conditions were validated by the control band directed towards a universal 16S rDNA sequence.

Variant forms of both *cdtAB* and *tcdA* have been found to contain internal deletions. Therefore, when interpreting the results obtained with the present 5-plex PCR, several considerations should be taken into account. (i) Primers for *cdtA* and *ctdB* are located within the region that has been truncated in strain 630 (accession no.: AM180355) and, therefore, a negative result does not exclude the presence of the truncated genes. However, strains harbouring such deletions do not produce a functional binary toxin and, therefore, are considered to be less relevant to identification from a clinical point of view [18,34]. (ii) Primers for *tcdA* are located upstream of the 3′-end repetitive region, and strains harbouring deletions in this region will therefore produce a positive result. However, more extensively truncated *tcdA* genes, such as that found in strain ATCC 8864 (accession no.: AF134592) with a 5.9-kb deletion of *tcdA* and *tcdC*, resulting in a *tcdA* of only 2.8 kb, will produce a negative result [35,35,36]. Although the clinical significance of strain ATCC 8864 is still unknown, strains with deletions restricted to the repetitive region of *tcdA* have been isolated in the context of several outbreaks [23–25,37–39] and severe infections [26,40,41]. A recent survey of European laboratories has revealed that more than half of the laboratories use ELISA tests that detect only TcdA [42]. As these tests are based on the recognition of the ligand-binding domain encoded by the repetitive region of *tcdA*, they will not be detected. Therefore, in order to detect such strains, methods, such as the method described here, must target *tcdB* and/or the 5′-end of *tcdA*.

The *tcdC* gene has attracted much attention, because it is a presumed negative regulator of the two major *C. difficile* toxins, TcdA and TcdB, and because several potential gene inactivations in this gene have been identified in strains from severe infections and epidemic outbreaks. Therefore, it could be presumed that defects in this gene may be related to elevated toxin expression,

which would explain the relatively high pathogenicity of these strains.

In order to investigate *tcdC*, PCR and sequencing primers that allowed the identification of the first 425 bp were designed, covering previously identified possible inactivation features, including: (i) C-terminal deletions of 18 or 39 bp [22,33,43]; (ii) a premature stop codon introduced by a single base-pair deletion at position 117, characteristic of the Canadian 027 strain [44]; (iii) a single base-pair deletion in a stretch of adenines between positions 10 and 17 [35], introducing a frameshift and, hence, a premature stop codon resulting in a 22 amino acid product; and (iv) a C → T transition at position 184 leading to a premature stop codon and a truncated protein of 61 amino acids [33]. The Danish strains contained only one type of premature stop codon, resulting from the C → T transition at position 184, whereas three different C-terminal deletions of 18, 39 and 54 bp were observed. To the best of our knowledge, a 54-bp deletion, revealed by sequence analysis, has not previously been described, whereas the three different deletion sizes (18, 39 and 54 bp) may correspond to what was also observed by Stare *et al.* (2007) after PCR– restriction fragment length polymorphism analysis, where restriction pattern type 4 may correspond to a 54-bp deletion. Also, restriction pattern 4 was found in a toxinotype IV strain, as it was in all strains with a 54-bp deletion in this study. The meaning of the different alterations in *tcdC* remains to be investigated, and it will be interesting to see whether future experiments will be able to link different *tcdC* variants to different levels of *tcdA* and *tcdB* expression and, hence, to a gradation in the pathogenicity. A recent study [45] has shown that a *tcdC* harbouring an 18-bp deletion does indeed encode an active TcdC that is able to downregulate toxin expression, and it was pointed out that the Δ117 mutation, observed in the 027 strains, is more likely to be responsible for the highly pathogenic phenotype. In light of the different genetic changes that may affect the functionality of *tcdC*, the present sequencing-based method is considered to be a valuable strategy for investigation of different genetic characteristics in one analysis.

Thirty-seven strains (23%) contained the genes encoding the binary toxin (*cdtAB*) and, among these, 36 strains harboured a possibly inactivated *tcdC* and 26 strains a 700-bp deletion

in *tcdA* (Table 2). The prevalence of the binary toxin genes among the Danish strains is relatively high, as compared to those of between 2.8% and 8.6% that have been reported in other studies [31, 46–49]. Only one type of *tcdA* deletion of 700 bp (estimated after agarose gel electrophoresis) was observed, by use of the primer system originally developed by Kato *et al.* (1999). This primer system amplifies a stretch of 2535 bp if no deletion is present, and, in their study, truncated genes were reduced by 1821 bp, due to two different 3′-end deletions. This primer system has also been used by van der Berg *et al.* (2004), who observed deletions of 1.8, 1.7 and 0.8 kb. The 0.8-kb deletion was found in a strain of serogroup S3, and may correspond to the 0.7-kb deletion observed in the Danish strains.

Strains that were positive for the binary toxin were also subjected to PCR ribotyping in order to investigate relatedness to the highly pathogenic strain of PCR ribotype 027. No strains in the present study matched the 027 PCR ribotype. The Danish strains revealed eight different PCR ribotypes, with dk2 being the predominant type, accounting for 21 strains, all with a 700-bp deletion in *tcdA*, a 39-bp deletion and a premature stop codon in *tcdC*. The PCR ribotype dk2 strains were not associated with any particular geographical area, or with any particular patient age group. For unknown reasons, this particular PCR ribotype, with a very homogeneous genetic profile, is the most prevalent among the Danish isolates. Unfortunately, it was not possible to label the different PCR ribotypes according to the nomenclature of Stubbs *et al.* (1999), as this would require the extensive strain collection for complete band comparison. The initiative taken by the ESCMID Study Group for *C. difficile*, to make a remote-access server available for PCR ribotype band comparison, seems to be a promising strategy for future strain identification and epidemiological surveillance [22].

We conclude that the new 5-plex PCR method presented in this article is a valuable tool for primary characterization of *C. difficile*. Strains with relevant toxin profiles may subsequently be assayed for *tcdA* and *tcdC* deletions and subjected to PCR-based ribotyping. This will allow further evaluation of their pathogenic potential and produce data that are important for epidemiological studies.

TRANSPARENCY DECLARATION

None of the authors has any commercial relationships or conflicting interests with respect to the present article.

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