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ORIGINAL ARTICLE

Use of *tcd*C gene sequencing to prevent misidentification of *Clostridioides difficile* ribotype 176 and 027

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ABSTRACT

Aim To compare the sequences of the *tcdC* gene between *Clostridioides difficile* (*C. difficile*) strains identified as PCR ribotype 176 and the reference strain *C. difficile* PCR ribotype 027 and to evaluate the use of the Xpert *C. difficile*/Epi assay for their differentiation.

Methods A total of 45 strains were grown from storage beads. DNA of sufficient quality and quantity for sequencing was extracted from 9 samples. Single consensus sequences of PCR ribotype 176 strains and PCR ribotype 001, PCR ribotype 070 (a control group) were mapped to a reference genome of strain CDI-01 (PCR ribotype 027).

Results Four strains (out of seven; 57%) characterized as PCR ribotype 176 had 100% identity of the *tcdC* gene with the reference strain. The average length of the *tcdC* gene in these four strains (PCR ribotype 176) was 643 bp, which is 36 bp shorter than the reference genome. Three strains (PCR ribotype 176) had a percentage identity of the *tcdC* gene in the range of 99.37-100%. Strains 25 (PCR ribotype 001) and 28 (PCR ribotype 070) had a similarity in the range of 95.39-95.63% as a result of different ribotype to the reference strain.

Conclusion PCR ribotype 176 strains have almost the same *tcdC* gene sequence as PCR ribotype 027, resulting in misidentification of this PCR ribotype by the Xpert *C. difficile*/Epi assay. Information about presumptive positive results based on deletion in the *tcdC* gene should be treated with caution or disregarded.

Keywords: DNA, mutation, polymerase chain reaction

INTRODUCTION

Clostridioides difficile (C. difficile) is an anaerobic and spore-forming bacterium that could even cause pseudomembranous colitis. It is widespread in the world with high rates of recurrence and mortality and *C. difficile* has become one of the major concerns in the healthcare system (1). The European survey on the incidence of *C. difficile* infection (CDI) (involving 559 hospitals) in 2022 showed 3.48 cases per 10,000 patient days. Hospital-associated CDI accounted for 60.9% of cases, while community-acquired CDI accounted for 32.7% (2). Mortality from CDI within 3 months of diagnosis is 6%, and 13% in patients over 80 years of age. The average length of hospital stay was prolonged to 14 days and the cost per adult patient was approximately 10,000 € (3).

C. difficile transmission in hospital occurs frequently and any time overt outbreaks by high-virulence strains. The good tools for early action to the outbreak are needed for surveillance of *C. difficile* (4). During outbreaks, the usage of high sensitivity tests like nucleic acid amplification test (NAAT) is recommended firstly (5). The first commercially accessible NAAT for *C. difficile* detection in the world was Xpert *C. difficile* (6). Application of GeneXpert as the only molecular method to diagnose *C.difficile* is sufficient, because of high sensitivity 0.97 (0.95-0.98) and specificity 0.96 (0.95-0.97) of the assay (7). It is also capable of identifying the hypervirulent ribotype (RT) 027 based on the detection of deletion in 117nt of *tcdC* gene. *TcdC* gene is located on 19.6 kb chromosomal re-

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gion called pathogenicity locus (PaLoc) in the length of approximately 679 bp. *TcdC* gene is coding for a 26-kDa dimeric protein, which contains C-terminal functional domain and N-terminal transmembrane region (it anchors to the cell membrane). The function of this protein is still unclear (8). There are two possible explanations of protein functions: The first one is that, *tcdC* is a negative regulator of toxin transcription (anti-sigma factor). *TcdC* C-terminus binds to the DNA secondary structures via oligonucleotide/oligosaccharide binding fold (OB-fold), which is necessary for transcriptional toxin repression (8–10). The second one is associated with extracellular localization of *tcdC*. This location is not compatible with the direct binding of OB-fold domain to intracellular nucleic acid or protein, and it suggested the different mechanism function as anti-sigma factor. The localization of the 50-amino-acid N terminal domain is unknown. Further studies are needed to elucidate the role of *tcdC* gene (8).

The mutation in *tcdC* gene applied in the Xpert *C. difficile*/Epi test to identify *C. difficile* PCR ribotype 176 led to misidentification with PCR ribotype 027. The reason is that PCR ribotype 027 and PCR ribotype 176 share all the genetic characteristics regarding toxin production and *tcdC* mutations (11). This finding was also confirmed by another study which found a similarity between these two RTs (12). Furthermore, another study described the case where RT 591 (diagnosed in the USA) and RT 244 (diagnosed in Australia) were identified by this assay as presumptive positive RT 027. RT591 is also clustered in clade 2 as RT 027 and RT 176. These three RTs share the same virulence factors (13).

The aim of this study was to analyse the *tcdC* gene sequences of *C. difficile* strains classified as RT 176 to evaluate the differences in the *tcdC* gene between RT 176 and 027, and to draw conclusions about the application of the presumptive positive RT 027 result of the Xpert *C. difficile*/Epi assay in everyday laboratory use.

MATERIALS AND METHODS

Materials and study design

Data on 45 *C. difficile* strains (30 RT 176, 8 RT 001, 3 RT 017, 1 RT 070, 1 RT 020, 1 RT 027, 1 RT 049) were obtained. Thirty strains (RT 176 only) were selected for Sanger sequencing of *C. difficile* strains, but after DNA extraction the quality of the DNA obtained from most strains was insufficient for sequencing. We selected nine strains based on the quality and quantity of DNA for further processing. Strains characterized as RT 001 and RT 070 were used as a control group, assuming that the *tcdC* gene in their genome would have greater differences with the reference genome than PCR RT 176 strains. *C. difficile* RT 176 strains were tested on the Cepheid GeneXpert system in the laboratory of the Department of Microbiology and Immunology, Jessenius Faculty of Medicine in Martin, Slovakia. The Xpert *C. difficile*/Epi test reagent assay detects the following targets: 1) *binary toxin* gene, 2) *tcdb* gene and 3) deletion in 117 nucleotide (nt) of the *tcdC* gene characteristic of RT 027. We collected data from Xpert *C. difficile*/Epi assays of *C.difficile* RT 176 strains previously tested in 2016 (12–14). Previously tested strains were stored in VIABANK beads at -80 °C for 6 years. A total of 30 tested strains were identified as RT 176 by PCR ribotyping. However, the Xpert *C. difficile*/Epi assays gave presumptive positive results for 027.

For this type of the study, an approval of the Ethics Committee was not required.

Methods

All strains were cultivated on blood agar from storage beads at 37 °C for 48 hours in anaerobic conditions. DNA isolated using the ISOLATE II Genomic DNA kit (Bioline/MeridianBioscience, Ohio, USA). The lysis process (Lysis Buffer GL and Proteinase K) of ISOLATE II Genomic DNA kit was adjusted for 7 hours at 70 °C. Phusion polymerase for the amplification was used. The target of PCR was *tcdC* gene, that was amplified using primer pair 5' TTAATTAATTTTCTCTACAGCTATCC 3' (forward primer) and 5' TCTAATAAAAGGGAGATTGTATTATG 3' (reverse primer) (length of amplicon 718 bp) (9). Amplification condition was 98 °C for 5 minutes, followed by 30 cycles of 98 °C for 20 seconds, 52 °C for 20 seconds and 72 °C for 1 minute, then 72 °C for 10 minutes and held at 4 °C. Electrophoresis was performed on 1.5% agarose gel, at 60 V, for 50min.

Nine PCR products were cleaned with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Cleaned PCR products were amplified with BigDye Terminator kit (Applied Biosystems™), then PCR products were purified with SigmaSpin Sequencing Reaction Clean-up (Sigma Aldrich, USA). Cleaned forward and reverse sequences of the *tcdC* gene in strains RT 176 were aligned using the BioEdit Sequence Alignment Editor software. These consensus sequences were compared by BLAST analysis to the reference genome of *C. difficile* RT 027 (CDI-01, taxid: 1496, GenBank: CP126076.1 in NCBI GenBank) (Figure 1).

We determined the percentage of similarity or difference in the strains tested. We tried to detect 18 nt deletion and a deletion at position 117 nt in PCR ribotypes 001 and 070, but we assumed that these types of mutations were not present in these ribotypes. PCR ribotypes 001 and 070 are not misidentified with PCR ribotype 027 by the Xpert *C. difficile*/Epi assay should not contain these mutations in this particular gene.

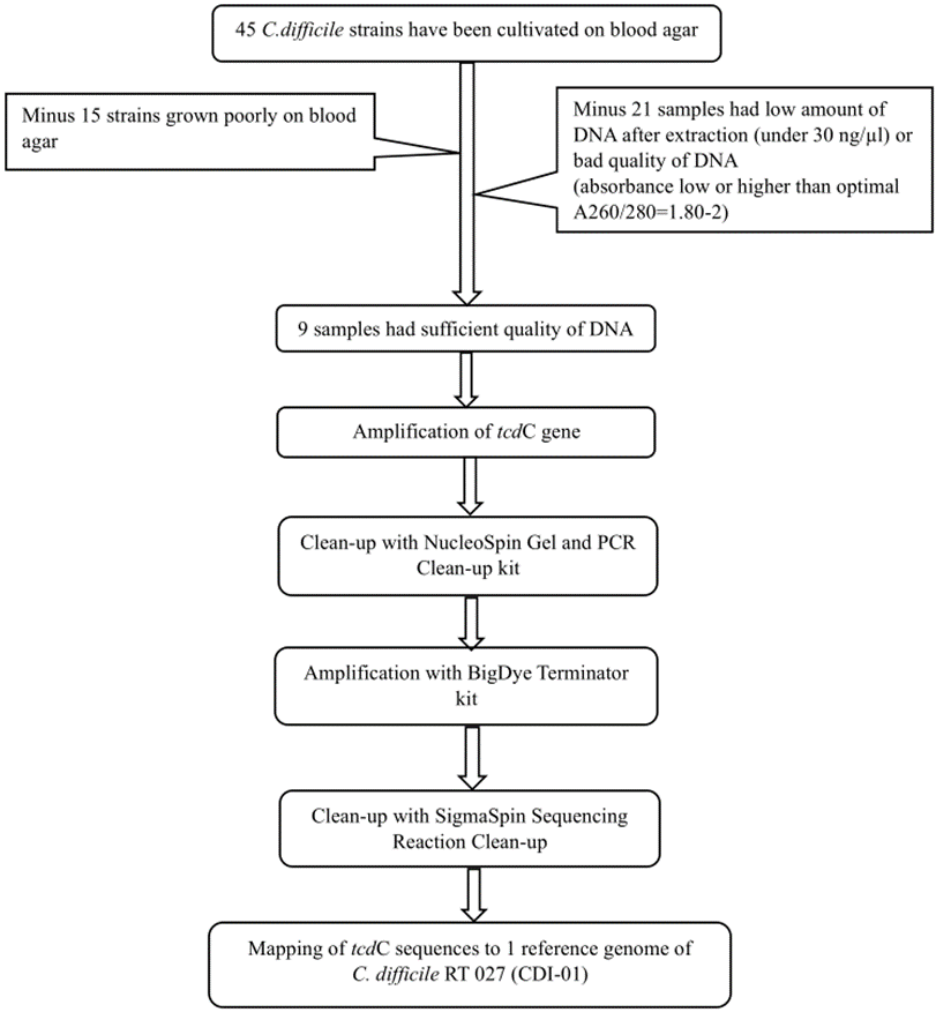

Figure 1. Sample processing workflow

RESULTS

A total of 45 strains were cultivated: 15 strains from VIABANK beads grown poorly on blood agar, 21 samples had low amount of DNA (under 30 ng/µl) or the quality of DNA was not sufficient for further processing (absorbance lower or higher than optimal A260/280=1.80-2). We picked 9 DNA samples for sequencing according to the quality of DNA (Table 1).

The average sequence length of all 9 strains was approximately 638 bp. Seven samples were classified as RT 176 and other two as RT 001 and 070. The BLAST analysis revealed a percentile range of 99.37-100% for the identity between the reference strain and those identified as RT 176. We got four (out of seven; 57%) strains characterized as RT 176, which had the percentage identity of 100% with reference strain.

Two PCR RT 176 (strain 6, 37) contained 1 deletion of G at positions 47 and 57. Strain 36 contained two deletions at position 12 nt (A) and at position 60 (G). The length of *tcdC* gene in reference strain was 679 bp (737784-738463 bp). However, average length of *tcdC* gene in strains 6, 36, and 37 was around 610 bp (619,5 bp, 620,5

Table 1. Quality of DNA

Tested strain	Amount of DNA (ng/µl)	Quality of DNA A260/280
6	53.9	1.90
25	131.5	1.90
28	37.0	1.89
32	60.9	1.89
36	50.9	1.87
37	104.3	1.88
118	30.2	1.87
123	65.1	1.92
137	50.1	1.88

bp and 611,5 bp). Strains 6, 36, and 37 had 1-2 deletions in *tcdC* gene. Strains 25 (RT 001) and 28 (RT 070) had similarity in the range of 95.39-95.63%. Lower percentage similarity of strains 25 and 28 is a consequence of the different RT than reference strain's RT (Table 2).

Strains 25 and 28 showed 18 nt insertion in *tcdC* gene, which showed in the reference genome as deletion. Strain 25 had insertions at position 285-303 bp. Strain 28 had insertion at position 290-308 bp (Figure 2).

Deletions such as 18 nt deletion and probably deletion at position 117 nt in the *tcdC* gene present in PCR ribotype 027 were the same as in RT 176, namely in strains 32, 118, 123, 137, where the percentage identity reached 100%. The average length of the *tcdC* gene in these 4 strains was 643 bp, which is shorter (36 bp) than the corresponding gene in the reference strain. The closest length of the *tcd*C gene to the reference strain was strain 25 with the average length of 662 bp (Table 3). We cannot prove the presence of the deletion at position 117 nt to 100% in 4 *C. difficile* strains RT 176, but we assume that 100% identity with the reference strain correlates with the presence of the particular deletion.

DISCUSION

Application of the GeneXpert assay is usually used to detect the presence of toxigenic *C. difficile* in hospitalised patients (15). In addition, for this application, the major advantage of this assay is the lower limit of detection for the toxin gene in samples (16). These are the reasons why we selected this assay and tried to apply it with other method as the primary method applied during outbreak. The processes, which could affect our testing was connected with DNA extraction of samples and it could influence the quality of samples. We extracted DNA from the small amount of material. Successful DNA extraction in more samples could be gained with the application of lysozyme in lysis process according to the DNA isolation kit manual (ISOLATE II Genomic DNA kit, Bioline/Meridian Bioscience Inc., USA). In the case of *C. difficile*, application of lysozyme in lysis step during DNA extraction could not be applied, due to the presence of S-layer outside of *C. difficile* cell wall, which is resistant to lysozyme (17). Another factor responsible for resistance to lysozyme is extracytoplasmic

Table 2. Percentage identity of tested strains with reference strain CDI-01 (PCR ribotype 027)

Tested strain	Percentage identity with strain CDI-01		PCR ribotype
	Forward primer	Reverse primer	
6	99.52 %; 622/625bp, 2 gaps	100%;	176
25	95.56 %; 624/653bp, 19 gaps	95.39%; 641/672bp, 19 gaps	001
28	95.59 %; 628/657bp, 20 gaps	95.63%; 641/672bp, 19 gaps	070
32	100 %;	100%;	176
36	99.37 %; 630/634bp, 2 gaps	99.84%; 606/607bp, 1 gap	176
37	99.68 %; 617/619bp, 1 gap	99.83%; 603/604bp, 1 gap	176
118	100%;	100%;	176
123	100%;	100%;	176
137	100%;	100%;	176

Table 3. Length of t*cd*C gene sequence in tested strains

Strain	Length of sequence (reverse primer)	Length of sequence (forward primer)
6	614 bp	625 bp
25	672 bp	653 bp
28	663 bp	657 bp
32	655 bp	635 bp
36	607 bp	634 bp
37	604 bp	619 bp
118	638 bp	634 bp
123	657 bp	638 bp
137	648 bp	638 bp

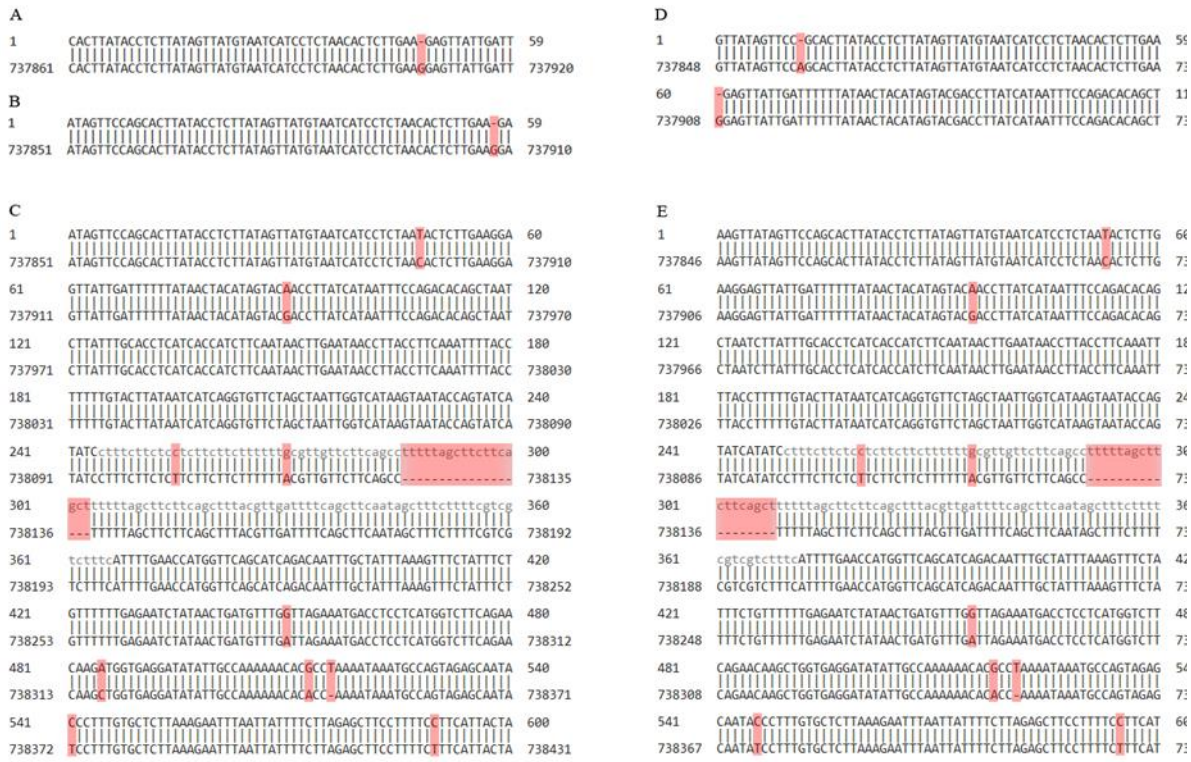

Figure 2. Mutations in the *tcd*C gene of tested strains are compared to the reference strain, with a specific focus on the 18nt deletion and deletion at position 117 nt. A) Single consensus sequence of the *tcd*C gene of strain 6 with deletion at position 47 nt (G); B) Single consensus sequence of the *tcd*C gene of strain 37 with deletion at position 57 nt (G); C) Single consensus sequence of the *tcd*C gene of strain 25 with insertion and substitutions; D) Single consensus sequence of the *tcd*C gene of strain 36 with deletion at position 12 nt (A) and at position 60 (G); E) Single consensus sequence of the *tcd*C gene of strain 28 with insertion and substitutions

sigma factor σV, which causes the expression of peptidoglycane deacetylases PgdA and PdaV. As a result, the interaction between active site of lysozyme and acetyl groups of peptidoglycane is not possible (17). However, a combination of lysozyme (10 mg/ml) and proteinase K (20 mg/ml) to isolate DNA from *C. difficile* was used in other studies: in the first step, cells are incubated with lysozyme solution at 37 °C overnight, then proteinase K is added and incubated at 56 °C for 30 minutes (18). These findings do not correlate with information about lysozyme resistance of *C. difficile*, but prolonging the lysis process during the DNA extraction was sufficient in some samples also in our process of DNA extraction and it could be the way how to improve the yield of the DNA extraction.

Results from the sequencing revealed that in more than 50% of our samples, the PCR ribotype was assessed incorrectly - RT 027 instead of 176. Both RT 027 and 176 cause severe disease and increased mortality; however, RT is not a clear predictor of infection severity (19). Epidemic clones such as RT 027 have also 18 nt deletions and deletion at position 117 in *tcdC* gene (20). Deletion at position 117 nt in *tcdC* gene is associated with recurrent CDI and overall higher mortality rate. This deletion correlated with the detection of *C. difficile* RT 027 (21). Toxigenic strains possess *tcdC* gene and non-toxigenic strains do not express it (22). Emergent RTs (RT 027, 176 and 4 – belong to clade 2) caused severe disease and increased morbidity and mortality (23). However, RT 181 from outbreak in Greece caused mild to moderate disease, and it also belongs to clade 2. The clade 2 includes sequence type group 1 consisting of RT 027, RT 176, RT 016 and RT 181. These strains are called as 027-like types and bear resemblance with 027 according to the whole genome sequencing (23). The similarity of RT 027 and RT 176 correlated with what we found regarding the similarity of the *tcdC* gene in these ribotypes. In some strains, the disrupted *tcdC* gene is similar to the N-terminal truncated *tcdC* gene of hypervirulent strains such as RT 027 (24). PCR-ribotyping revealed RT 027 instead of RT 176 in our tested samples. PCR ribotyping patterns of RT 027 differentiate from RT 176 pattern only in one fragment in length of 546 bp (14). We gained 4 strains, which have 18 nt deletion and possible deletion at position 117 nt according to comparison with reference strain. We obtained 4 strains (toxigenic positive), which could be detected by Xpert *C. difficile*/Epi assays as presumptive positive 027.

Hand hygiene contributes to an effective way to reduce the incidence of nosocomial infections (CDI being one of the most dangerous). It is also considered the primary measure in preventing the spread of microorganisms (25).

Our results suggest that most of the PCR ribotype 176 in our study may be hypervirulent strain 027. The result of the Xpert *C. difficile*/Epi assay on the positive presumptive RT 027 could be considered an error and the true strain is actually RT 176. In the event of an outbreak, this result should be confirmed by capillary gel electrophoresis-based PCR ribotyping and further information on transmission should be obtained by whole genome sequencing.

In conclusion, most *C. difficile* RT 176 strains had the same sequence of the *tcdC* gene as RT 027 according to Sanger sequencing and BLAST analysis. We have shown that the Xpert *C. difficile*/Epi assay is not sufficient to diagnose RT 027 based on the deletion at position 117 nt in the *tcdC* gene alone, even though the result is just informative and not precise. We recommend that a note be added to this assay warning all laboratory users that the "presumptive positive" result has a high percentage of false positives (100% of all strains tested) and that they should consider using other methods to verify this result.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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