

APPLICATION OF MOLECULAR-BIOLOGICAL METHODS
IN THE TOXIN-TYPING OF *CLOSTRIDIUM DIFFICILE*

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Abstract

Clostridium difficile is both a member of the normal human flora and a human pathogen with clinical significance, a causative agent of *Clostridium difficile*-associated diarrhoea. Determination of the strains and related information about their virulence is very important for the correct diagnosis and therapy of patients, the prophylaxis and the control of the spreading of the infection in society. The precise phenotyping of this organism is complicated because of the relatively low sensitivity of the classical microbiological methods and a low vitality of the anaerobe *Clostridium difficile* in an atmosphere of oxygen. The methods developed in the last decade for PCR-techniques directly from clinical samples have specific disadvantages.

That was why we genotyped *C. difficile* using primers for the genes tcdA, tcdB and tcdC for a direct DNA isolation from pure culture suitable for work in our lab.

Key words: *C. difficile*, genotyping, toxin A, B, PCR

Introduction. *Clostridium difficile* is both a human pathogen and a member of the normal human intestinal flora with clinical significance, causative agent of *Clostridium difficile*-associated diarrhoea. Determination of the strains and related information about their virulence is very important for the correct diagnosis and therapy of patients, the prophylaxis and the control of the spreading of the infection in society [6]. Precise phenotyping of *Clostridium* is complicated because of the relatively low sensitivity of the classical microbiological methods and a low vitality of the anaerobe *Clostridium difficile* in an atmosphere of oxygen. The methods developed in the last decade for PCR-techniques directly from clinical samples are not useful for sequencing and need special equipment and transport conditions to be taken, kept and preserved until their genotyping.

Clinically the most important types of *C. difficile* are included in several PCR ribotypes [2]. Sufficient diagnostic methods need an update in accordance with the current data of epidemiology, drug resistance and sequencing. The best

investigated *C. difficile* factors of virulence are toxin A, toxin B and a binary toxin. The genes encoding the toxins A and B are named *tcdA* and *tcdB*. Negative and positive regulators of toxin expression are encoded by the genes *tcdC* and *tcdR*. These 4 genes – *tcdA*, *tcdB*, *tcdC* and *tcdR* are situated on the locus of pathogenicity (PaLoc). The binary toxin is encoded by the genes *cdtA* and *cdtB* with common operon with the positive regulator *cdtR*. Several alterations in the *tcdC* gene are described, such as the in-frame deletion of 18, 39 and 54 bp, the mutation at position 117 (1-bp deletion) and position 184 (C3T transition). PCR ribotype 027 is responsible for the most known outbreaks in the world. It has an 18-bp in-frame deletion and deletion $\Delta 117$ [14]. Such deletions in the *tcdC* are responsible for the high level of toxin production and the hypervirulence of the strains which care for them [4, 8]. Their association with clinical manifestations has not been determined [3, 7, 9, 11]. *tcdC* deletions – 18 bp and $\Delta 117$, are markers of screening for the *C. difficile* PCR ribotype 027 [1, 5, 10, 12]. In this study we proved toxins A and B on gene level and estimated 20 bp deletion in a PCR amplicon with specific primers for *tcdC* (British article).

Methods. DNA from 25 clinical strains from the Bulgarian National Referent Laboratory of Anaerobes and Ed Kuujper's laboratory from Leiden University Medical Center, the Netherlands. They were stored in skim milk at -70°C . The revival was done with the help of inoculating on Brucella blood agar dishes cultivated in a jar Gaspack in anaerobic conditions at 37°C for 48 h. DNA was extracted using QIAamp[®] DNA Mini Kit of Qiagen[®]. One loop of bacterial culture was dissolved in 180 μl ATL buffer + 20 μl proteinase K, incubated 2 h on 56°C water bath and every 15–20 min the samples were vortexed. After that 200 μl AL buffer was added to the sample, and it was incubated for 10 min in water bath at 70°C , then 200 μl ethanol was added, this DNA/buffer mixture was vortexed and loaded on spin column, washed with 500 μl AW1 on micro-centrifuge of 8000 rpm 1 min and for a second time with 500 μl AW2 – 14 000 rpm for 3 min. DNA was eluted with 200 μl elution buffer. The last elution step was done three times in a final volume of 600 μl . The approximate yield of clostridial DNA with this protocol is 100 μg – 1 mg per isolation. Classical phenol-chlorophorm extraction method gave no yield. The amplification was completed as described in [13]), using 1 μl of extracted DNA as a template in a final volume of 20 μl with Invitrogen[®] Taq polymerase, 1 mM MgCl_2 . The amplicons were visualized on a 3% agarose gel stained with ethidium bromide and run on electrophoresis of 7 V/cm for 2.5 h. The amplification of specific sequences was done in the following conditions:

- 1 μl DNA template
- 0.25 μl Taq polymerase Invitrogen[®]
- 2 μl 10 \times PCR Buffer
- 0.6 μl MgCl_2

0.25 µl dNTP
0.5 µl 10 pM primer tcdA,B or C forward
0.5 µl 10 pM primer tcdA,B or C reverse

Sequences of the primers:

tcdA: 5'-AGATTCCTATATTTACATGACAATAT-3'
5'-GTATCAGGCATAAAGTAATATACTTT-3'

tcdB: 5'-TGATGAAGATACAGCAGAAGC-3'
5'-TGATTCTCCCTCAAAATTCTC-3'

tcdC: 5'-AAAAGGGAGATTGTATTATGTTTTTC-3'
5'-CAATAACTTGAATAACCTTACCTTCA-3'

Conditions of amplification of tcdA:

95 °C– 15 min
{94 °C– 30 s
52 °C– 30 s
72 °C– 40 s} – 35 times
72 °C– 5 min

Conditions of amplification of tcdB and tcdC:

95 °C– 15 min
{94 °C– 30 s
50 °C– 40 s
72 °C– 1min 10 s} – 35 times
72 °C– 5 min

The sequencing was completed by Macrogen. The results were compared versus *C. difficile* genomic databases on <http://blast.ncbi.nlm.nih.gov/>. BLASTN 2.2.26 + programme was used [16].

Results and discussion. The protocol for standard DNA-isolation from plates in aerobic conditions gave no detectable results. The yield of DNA with the help of QIAamp[®] DNA Mini Kit of Qiagen[®] is in the range of 100 ng – 1 µg in final volume of 60 µl of the elution buffer of the kit. Distribution of proven genes into 2 different allele modifications using this primer pair for tcdC was estimated. Sequencing of tcdC amplicons showed two different variations 480 bp. (expected) and 460 bp. with deletion of 20 bp. The region with the different sequence within tcdC sequence is:

(1) 5'-CTGAAAAT C AA C G T AAAGCT-3'
(2) 5'-CTGAAAATCAACGTAAAGCTGAAGAAGCTAAAAAAGCT-3'

Both sequences showed 100% similarity to tcdC of different strains of *Clostridium difficile*. Sequence (1) is identical to a region of tcdC of 72 strains of *Clostridium difficile*, sequence (2) is identical to 28 strains (these data are valid for July 2012) with described genomes in the bioinformatics databases All GenBank, EMBL, DDBJ and PDB. The distribution of tcdA, tcdB and tcdC within the

investigated strains: tcdA + tcdB + tcdC + 16 strains; tcdA - tcdB + tcdC + 1 strain; tcdA + tcdB + tcdC - 2 strains; tcdA + tcdB - tcdC + 3 strains; tcdA + tcdB + tcdC - 3 strains.

Our aim was to optimize a protocol for DNA extraction from *Clostridium difficile* working without specialized anaerobe equipment. The best results were obtained using Qiagen Kit as described above. Classical phenol-chloroform method and an automated isolation of DNA was applied without success. The observed results gave us suitable protocols for work with *C. difficile* using molecular methods in aerobic atmosphere without special equipment using modifications of a commercial protocol of Qiagen®. Shifts of amplicons of tcdC was estimated for the first time with the primers pair used before and confirmed by sequencing and BLAST analysis.

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