A new type of toxin A-negative, toxin B-positive *Clostridium difficile* strain lacking a complete *tcdA* gene

Sandra Janezic1, Mercedes Marín 2,3, Adoracion Martín 2,3, Maja Rupnik 1,4,5

1National Laboratory for Health, Environment and Food, Maribor, Slovenia
2Servicio de Microbiología Clínica-Enfermedades Infecciosas. Hospital General Universitario Gregorio Marañón, Madrid, Spain
3Instituto de Investigación Sanitaria Gregorio Marañón. Madrid. Spain
4University of Maribor, Faculty of Medicine, Maribor, Slovenia
5Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Ljubljana, Slovenia

**Running Head**: New type of A-B+ *C. difficile*

**Correspondent footnote**

#Address correspondence to Sandra Janezic; sandra.janezic@nlzoh.si
Abstract

Toxins A and B are the main virulence factors of *Clostridium difficile* and are the targets for molecular diagnostic tests. Here we describe a new A-B+CDT- toxinotype (XXXII) characterized by a variant type of pathogenicity locus (PaLoc) without *tcdA* and with an atypical organization of PaLoc integration site.

*Clostridium difficile* is the main cause of community and nosocomial diarrhea associated to antibiotic treatment and has great healthcare and economic impact (1–3).

Three toxins, toxin A (*TcdA*, enterotoxin), toxin B (*TcdB*, cytotoxin) and binary toxin CDT, are produced by *C. difficile*. The latter is present only in a subset of strains, and its role in pathogenesis is increasingly recognized but still unclear (4). Toxins A and B are the main virulence factors causing damage to intestinal epithelium, producing diarrhea and inflammation, and are also a main target for enzyme-based or molecular diagnostic tests (5, 6). Genes encoding TcdA and TcdB are located on the chromosome and together with a three additional genes (*tcdR, tcdE* and *tcdC*) form a 19.6 kb pathogenicity locus (PaLoc). The genes for CDT toxin are located elsewhere on the chromosome (CdtLoc).

*C. difficile* strains can be differentiated based on different patterns of toxin production. Strains which do not produce any of the toxins are non-toxinogenic and do not cause disease. The majority of toxigenic strains produce both toxins, TcdA and TcdB (A+B+), but some strains produce only TcdB (A-B+). Toxigenic strains can be also further differentiated into toxinotypes based on changes (deletions, insertions, SNPs) in the PaLoc.

By 2008 24 different toxinotypes were published (7) and currently 31 toxinotypes are differentiated designated by roman numbers from I to XXXI (http://www.mf.uni-mb.si/tox/).
Here we describe a case history, and the isolation and characterization of a new A-B+ variant of *C. difficile*.

**Case Report.** A 68-year-old Spanish male followed as an outpatient in the Gregorio Marañon University hospital of Madrid because of ischemic cardiomyopathy, angina and chronic pancreatitis was admitted to the emergency department in October 2011 with a 21-day history of diarrhea. The patient did not have fever, his general condition was good but he reported a recent weight loss of 5 kg. Stool microbiological studies were requested at the emergency department for enteropathogens, antigens of rotavirus and adenovirus, intestinal parasites and *C. difficile* culture and toxin detection. A direct cytotoxicity assay was performed by centrifuging stool specimen dilutions (1/40) made with phosphate-buffered saline and filtering 500 μl of supernatant onto monolayers of human MRC-5 fibroblasts. A test result was not considered negative until after 48 h of incubation at 37°C. Specificity of the cytopathic effect was confirmed using a neutralizing high-titre *C. difficile* antitoxin (TechLab) following the manufacturer’s instructions.

Rapid detection of glutamate dehydrogenase (GDH) and toxin A & B (Techlab C. diff Quik Chek Complete, Blacksburg Va) were also performed in stools showing positive results for GDH but negative for antigens of toxins A&B. Following international recommendations (5, 6) GeneXpert C. diff (Cepheid, California, USA) was then performed and results for toxigenic *C. difficile* was negative.

As his condition was considered as non urgent, the patient was discharged with a diagnosis of subacute diarrhea and it was arranged that he be controlled by the digestive medicine department as an outpatient.

On the next day, the direct stool cell culture cytotoxicity neutralization assay was positive and after 48 hours of incubation of feces in CLO agar (bioMérieux, Marcy l’Etoile, France) C.
Difficile was isolated. The isolate was then tested positively for both GDH and toxin A&B, however GeneXpert C. diff was negative once again. A report was then issued as “isolation of toxigenic C. difficile” and “direct cytotoxicity positive”. Parasitological examinations, rotavirus and adenovirus antigen detection gave negative results.

The patient was treated with metronidazole 500 mg/8h for 12 days but diarrhea persisted although it was less intense, probably related to chronic pancreatitis. Abdominal ultrasound and colonoscopy performed a few days later only showed diverticulosis. In following microbiological examinations no C. difficile or other pathogens were isolated from stool.

The patient had only occasional contact with health care settings and his medical history did not reflect antibiotic consumption. Although the toxigenic C. difficile was the only detected pathogen, the diarrhea had not resolved after treatment and the clinical significance of C. difficile in this particular case is unknown.

Strain characterization Because of the discrepancies in microbiological results, the C. difficile isolate was further characterized. Several approaches were used to amplify parts of the toxin genes. By means of a multiplex PCR to detect tcdA, tcdB, cdtA and cdtB (8, 9) positive results were obtained only for tcdB gene. PCR amplification of tcdC was negative (10). These results were further confirmed by PCRs covering the entire PaLoc as a part of toxinotyping scheme (11). All three PCRs covering tcdA gene were negative and only B1 and B2 fragment covering the tcdB gene were positive. Whole genome sequencing (genomic sequencing and analysis was performed as described previously) (12) has finally clarified the structure of the PaLoc region in this strain, and it represents a new toxinotype (XXXII) (Figure 1A). In this toxinotype genes tcdR and tcdE are conserved. Also present is a complete tcdB gene, but several SNPs were found in this region aligning to primers used for toxinotyping, explaining the negative PCR result for B3 fragment and possibly also the negative GeneXpert toxin B based molecular
test. However, toxin B is produced and was detected with EIA toxin test and on cell culture cytotoxicity assay. The other two genes within the PaLoc, tcdA and tcdC, are completely absent and were not detected even in truncated form in the PaLoc or anywhere else in the genome. Hence, toxinotype XXXII is one of the A-B+ toxinotypes. Up until recently all known A-B+ toxinotypes had at least a part of the tcdA gene present and the entire or deleted version of tcdC (7). An Australian group described the first A-B+ variants lacking the complete tcdA and two accessory genes tcdE and tcdC (toxinotypes XXX and XXXI), however all were binary toxin positive (A-B+CDT+) (13, 14). Toxinotype XXXII in addition to lacking tcdA and tcdC also has no binary toxin CDT genes (A-B+CDT-). The strain was PCR-ribotyped using agarose-based and capillary-based approaches. The PCR-ribotype profile was not previously recognized in the Leeds collection (typed in October 2013) nor in WEBRIBO collection and was designated by the internal designation; SLO 148 (Figure 2). In-silico MLST demonstrated that the strain belong to the sequence type ST200.

Analysis of regions upstream and downstream of the PaLoc indicated that the PaLoc in toxinotype XXXII could be inserted at a genomic location that is different from other strains studied to date (Figure 1A). Furthermore, five genes that have been previously identified inserted between cdu1 and cdd1 in strains WA12 (15) and Cgriifficile_H5078 (12) have been found inserted upstream of cdd2 gene (Figure 1B). In toxinotype XXXII ORF1 is fragmented and was found on two different contigs. Subsequent linking of the contigs by PCR and sequencing demonstrated insertion of mobile element (IStron) within the ORF1 (Figure 1A).

In addition, a shorter (86 bp) 115 bp-stretch that is normally present in non-toxinogenic strains replacing the PaLoc region was found adjacent to cdu1. To date, both regions have been described only in non-toxinogenic strains (12, 15, 16).
**Summary.** Here we report a new type of A-B+ *C. difficile* strain (toxinotype XXXII) with a variant form of PaLoc and atypical organization of PaLoc integration site. The strain is characterized by the complete absence of *tcdA* and *tcdC* genes, while *tcdR*, *tcdB* and *tcdE* are present and TcdB is produced and detected by diagnostic toxin specific tests. Conserved toxin genes are located within a so far undescribed region of *C. difficile* genome. The known boundaries of PaLoc are conserved (cdu1/cdd2) and genes between the boundaries were already described in at least two other (non-toxinogenic) *C. difficile* strains. The strain was isolated from diarrheic patient, and despite the unknown clinical significance, it is important to keep in mind that new variants of *C. difficile* strains might be present in patients, and because of the changes in PaLoc might not be detected by molecular *C. difficile* tests.

**Acknowledgements.** The authors would like to thank Dr. Kate Dingle and Dr. Derrick Crook for the genomic sequencing. SJ and MR are supported by Slovenian Research Agency grant P3-0387. SJ was supported by FEMS Research Grant.

**Literature**


Figure legends

Figure 1. Schematic representation of organization of PaLoc and flanking regions. A) PaLoc and flanking genes in toxinotype XXXII (strain 173070). B1C, B2N, B2C, B3N, B3C and B4N primers used to amplify the B1, B2 and B3 fragment of tcdB gene, respectively. Putative function of predicted genes were identified by Blast search of NCBI. B) From top to bottom: PaLoc and flanking region of toxinotype 0 (strain CD630); reference strain of toxinotype XXX (strain ES130) (12), first variant toxinotype with PaLoc characterized by complete absence of tcdA gene; PaLoc insertion site in non-toxigenic strains; the 7.2 kb region (shaded in grey) inserted in the PaLoc insertion site in non-toxinogenic isolates; left Cgifficile_H5078 (HG002397.1) (12) and right WA12 (HG002390.1) (15).
Figure 2. PCR-ribotyping (agarose based) profiles of representative A-B+ strains. PCR-ribotypes from our library belonging to new toxinotype XXXII, similar toxinotypes XXX and XXXI and most prevalent toxinotype VIII are shown.
A 173070 (toxinotype XXXII) (A-B+CDT-)

B CD630 (toxinotype 0) PaLoc and border regions (A+B+CDT-)

ES130 (toxinotype XXX) PaLoc region (A-B+CDT+)

115 bp PaLoc integration site in non-toxinogenic strains

Insertion site between cdu1 and cdd3 in non-toxinogenic strain C. difficile H5078

Insertion site between cdu1 and cdd3 in non-toxinogenic strain WA12