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*Original Article*

# Prevalence of C*lostridium difficile* toxinotypes in infected patients at a tertiary care center in Lebanon

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## Abstract

Introduction: Due to the increase in the incidence of *Clostridium difficile* associated diseases at a tertiary care center in Lebanon, this study was undertaken to determine the prevalent *C. difficile* toxinotypes.

Methodology: The immunocard method was used to test for toxins A and B in 88 collected stool samples, followed with API 20A to confirm for *C. difficile*. PCR amplification of the triose phosphate isomerase (*tpi*) gene, the toxin encoding genes *tcd*A, and *tcd*B, followed by toxinotyping, were performed on recovered isolates and stool specimens.

Results: Out of the 88 stool samples obtained, 30 (65.2%) were Immunocard positive, culture and or *tpi* positive for *C. difficile.* Of the 30 isolates, 4 were PCR negative for the *tcd*A and *tcd*B genes (A-B-), and 26 were PCR positive for the *tcd*A and / or *tcd*B genes with 4 being A+B+, 1 A+B-, and 21 A-B+. The results of toxinotyping showed that 2 isolates belonged to toxinotype 0, 4 to toxinotype XI, 2 to toxinotype XII, 1 to toxinotype XVI, 1(A+B-) and twenty (A-B+) designated as toxinotype 0-like. *C. difficile* was detected in 65.2% of patients' stools with prevalence of toxinotype 0-like.

Conclusion: Identification of toxinotypes of *C. difficile* is important to determine the virulence potential of strains and control their spread.

**Key words:** *Clostridium difficile*; toxinotyping; *tcd*A; *tcd*B; RFLP.

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## Introduction

*C. difficile* infection (CDI) is the number one cause of infectious diarrhea among healthcare workers in healthcare facilities [1-3]. In addition, *C. difficile* can cause toxin-mediated diseases including post-antibiotic diarrhea and extending to severe pseudomembranous colitis (PMC). Pseudomembranous colitis is an inflammation of the colon often caused by the bacterium *C. difficile*, and is directly associated with simultaneous prolonged hospitalization of infected people who will be put on antibiotics. The pathogenesis of *C. difficile* is due to the release of toxins that lead to mucosal damage and inflammation. Two main virulence factors are involved, toxins TcdA and TcdB encoded by the toxin genes, *tcd*A and *tcd*B on the PaLoc (pathogenicity locus) region [4].

The toxigenic strains of *C. difficile* are characterized by a 19.6 Kb chromosomal region, the PaLoc or pathogenicity locus, which encodes the main toxin genes *tcdA* and *tcdB*, as well as the accessory genes *tcdC*, *tcdD, and tcdE* [5,6].

Toxinotyping is performed by amplifying this locus to produce ten PCR fragments and comparing with the reference strain VPI 10463, denoted as toxinotype 0. For simplification purposes, only two of these fragments, B1 and A3, are amplified to produce a specific pattern which allows us to classify a specific strain into a given toxinotype [4].

Variant *C. difficile* toxinotypes are currently present. Changes in *tcdA* and *tcdB* genes sequences on the Paloc region led to the emergence of 27 groups of variant toxinotypes till now (I to XXVII). Variations at the level of the PaLoc region can result in the production of various toxins with different properties leading to increased virulence of *C. difficile* [7].

As a result to the increasing incidence of *C. difficile* associated infections at a tertiary care center in Beirut, Lebanon, this study was undertaken to determine the prevalence of toxigenic *C. difficile* and their various toxinotypes. To achieve this objective *C.*

**Table 1**. Primers used for PCR-based assays

| Targeted gene | Primer | Sequence 5'-3' | Expected product size (bp) | Reference |
|---|---|---|---|---|
| *tcdA* | A3C<br>A4N | TATTGATAGCACCTGATTTATATACAAG<br>TTATCAAACATATATTTTAGCCATATATC | 3100 | 16 |
| *tcdB* | B1C<br>B2N | AGAAAATTTTATGAGTTTAGTTAATAGAAA<br>TATTGATAGCACCTGATTTATATACAAG | 3100 | 16 |
| *TPI* | *Tpi*-F<br>*Tpi*-R | AAAGAAGCTACTAAGGGTACAAA<br>CATAATATTGGGTCTATTCCTAC | 230 | 15 |

*difficile* was detected by the immunocard test, culture and PCR of the *tpi* gene in clinical stool specimens, and further toxinotyped by restriction fragment length polymorphism (RFLP) analysis of the A and B toxins to determine the prevalence toxinotype(s) at the medical center.

## Methodology

During the period extending between September 2011 through April 2012, 88 stool samples were collected anonymously by the Department of Pathology and Laboratory Medicine (PLM) from patients admitted to different hospital units including Pediatrics, Medicine and Surgery at the Medical Center in Beirut. All collected samples were tested for the presence of toxins A and B using the Immunocard test (Meridian Bioscience, Cincinnati, USA).

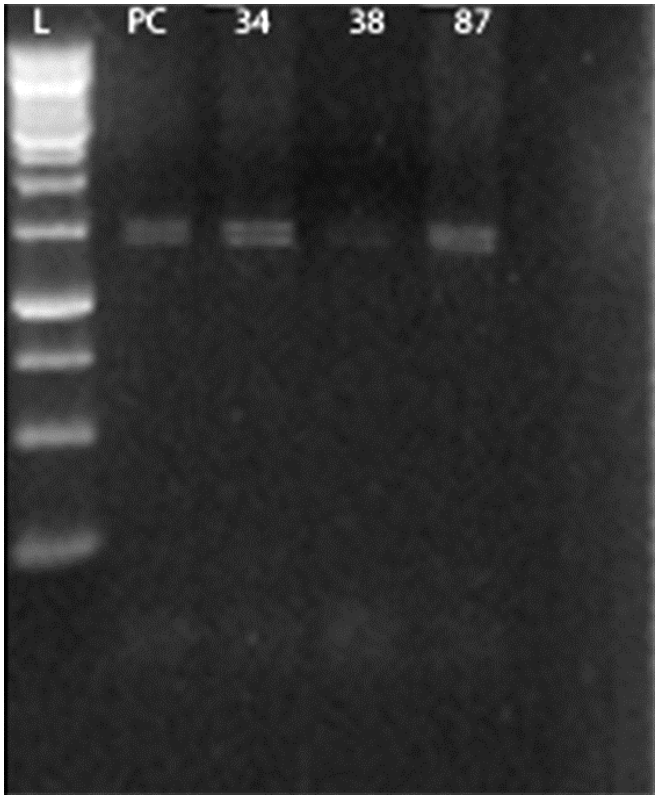
The stool specimens were immediately cultured on Cycloserine Cefoxitin Fructose Agar (CCFA) (Amresco, Solon, USA), enriched with 5% horse blood and incubated at anaerobic conditions using GasPak jar (Becton Dickinson, Franklin Lakes, USA), at 37°C for 48-72 hours. Gram staining and API20A kit (Biomérieux, Marcy-l'Étoile, France) were used for the confirmation of the isolated colonies. Total DNA was extracted directly from the stool samples and from culture positive *C. difficile* isolates using the QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's specifications. This was followed by the polymerase chain reaction (PCR) adopting the PCR Sprint Thermal Cycler (Thermo electron Corporation, Waltham, USA) to amplify the *tpi* gene, using a previously described protocol [8].

Confirmed *C. difficile* strains were tested for the presence of toxins A and B to differentiate between toxigenic and non-toxigenic strains. This was done by detecting the genes, encoding for the toxins (*tcd*A and *tcd*B) respectively according to Rupnik *et al* [9] .The PCR mix consisted of 2.5 µl of each primer (10pmol/µl), 5µl 10X Taq buffer (1.5mM), 0.25 µl Taq polymerase enzyme (5U/µl), 3µl$MgCl_2$ (25mM), 2 µldNTPs (5mM), 24.75µl nuclease free water (Amresco, USA). For the *tcd*A gene 10 µl$10^{-3}$M TMA were also added to the PCR mix (Table 1).

The amplification products were analyzed by gel electrophoresis, on 1% agarose gel (Seakem LE agarose, Lonza, Basel, Switzerland), to detect the presence of the 310 bp band of *tcd*A and *tcd*B genes encoding toxins A and B respectively.

Gels were stained with ethidium bromide (Amresco, USA) and visualized under UV illumination (Transilluminator, Haakebuchler Instruments Inc, USA) using Olympus digital camera, and the DigiDoc-ItProgram (UVP, CA, UK).

**Figure 1.** PCR-RFLP patterns of the A3 fragments which were used for toxinotyping. EcoRI (E) restrictions were tested. Samples 34, 38, and 87 showed restriction patterns similar to the positive control (toxinotype 0: ATCC 9689)



*C. difficile* strains DNA that harbored toxin genes were further tested by RFLP-based toxinotyping using restriction enzymes *Acc*I and *Hinc*II to cut B1 fragments, and restriction enzyme *Eco*RI to cut *A*3 fragments, followed by PCR and Gel electrophoresis.

## Results

The distribution of C. difficile in the 88 analyzed stool samples was as follows: 30 (65.2%) were Immunocard positive, 24 (27%) were culture positive and *tpi* gene positive and 6 (6.9%) were culture negative and *tpi* gene positive. Selected according to sex, 15 (50%) were males and 15 (50%) were females. Stratified according to age, three main groups were classified: children (6-17 years old): n = 5 (16.66%), adults (18-64) years old: n = 13 (43.33%) and elderly (> 64 years old): n = 12 (40%).

Toxinotyping of the 24 isolates revealed 2 (7.69%) to be of toxinotype 0, 2 (7.69%) of toxinotype XII, 1 (3.84%) of toxinotype XVI, 1 (3.84%) of a new toxinotype having a phenotype (A+B-) where the restriction patterns of A3 is of type 1 and finally 20 (76.92%) were of a new toxinotype having a phenotype of (A-B+) where the restriction pattern of B1 is of type 1, this group was designated as toxinotype 0-like (Figures 1 and 2).

Out of the 20 isolates of the toxinotype (A-B+), 4 came from children, 8 came from each of adults and elderly. They constituted of 11 females and 9 males. The toxinotype XVI was from an adult male. The toxinotype 0 isolates included 1 child and 1 elderly male. The toxinotype XII included 1 adult male and 1 elderly male. The A+B- toxinotype was from an adult female.

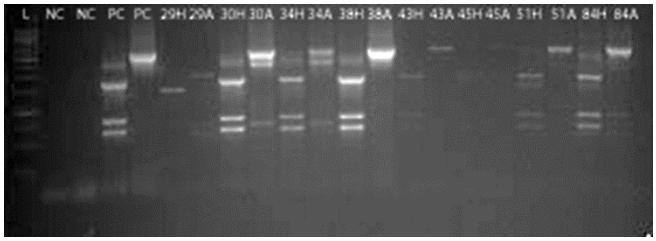
## Discussion

In the present study, *C. difficile* was detected in clinical stool specimens using the Immunocard test, culture and PCR of the *tpi* gene. Out of the 88 stool samples, 30 were identified positive.

There was no difference in gender stratification, which is in accordance with previous reports. However stratification based on age groups indicated that a higher percentage of the infected patients were elderly (40%) and adults (43.33%) compared to children (16.66%). *C. difficile* infections affects elderly patients (> 65 years of age), with previous hospitalization and recent exposure to antibiotics [10,11].

Toxinotypes are not associated with particular forms of disease or patient populations, however, some toxinotypes, such as III and VIII, are associated with increased pathogenicity and recurrent outbreaks

**Figure 2**. PCR-RFLP patterns of the B1 fragments, which were used for toxinotyping. Hinc*II* (H) *Acc*I(A) restrictions were tested. A comparison of eight 8 representative types to the main restriction patterns in this study is presented. Lane L: 1 Kb DNA ladder, NC: negative control, PC: positive control (toxinotype 0; ATCC 9689).




worldwide [11]. Our results indicated that toxinotyping by RFLP analysis allowed the identification of potentially rare variant strains including; the toxinotype 0-like and the toxinotype A+B- strains. In toxinotype 0, restriction patterns of both toxin genes are of type 1; similar patterns are observed in toxinotype 0-like for the B1 fragment but *tcdA* is deleted [11-13]. Indeed, deletions are found most commonly in *tcd*A and to date no form of significant deletions in *tcdB* is found [4,13].

*C. difficile* toxins TcdA and TcdB belong to large clostridial toxins (LCT), which are known for their large size (260-308 kDa) and on the basis of these two toxins *C. difficile* is classified into toxinotypes I-XXVII [4].

Furthermore *C. difficile* can be allocated to different toxinotype groups, based on the combination of three toxins possibly produced by the bacterial strain: A+B+CDT-, A-B+CDT-, A-B+CTD+, A+B-CDT+, A+B+CDT+, A-B-CDT+, A-B-CDT-. While TcdA and TcdB phenotypes are determined by gene amplification and toxin production, CDT is determined only by the occurrence of the gene. CDT toxin is rarely analyzed, thus CDT screening was not performed in this study [4].

There are currently four known toxinotypes exhibiting the A-B+ phenotype of two potentially rare variant strains, the toxinotype 0-like and the toxinotypes A+B- strains. In 2003, Johnson *et al.* identified a rare strain having a phenotype A-B+ CDT+ that was designated as toxinotype 0-like. Further analysis is needed to confirm that we are dealing with the same strain [14].

The second potentially new toxinotype, the A+B- strain is the opposite of the toxinotype 0-like strain. It has no *tcdB* gene, but the restriction fragment of A3 is of type 1; also similar to toxinotype 0. Therefore, a similar conclusion could be deduced where a deletion

of the *tcdB* gene could have occurred in the toxinotype 0 strain.

The toxinotypes found in this study were of little significance in Europe and America with the exception of toxinotype 0, which is the most prevalent toxinotype worldwide. According to Maja Rupnick, toxinotype 0 is the most prevalent toxinotype worldwide; toxinotype XII is considered a variant minor toxinotype, having a serogroup S1 and a ribotype 056. It is one of five toxinotypes most likely to be isolated from patients. While toxinotype XVI is of serogroup C and considered to be a major toxinotype as well. Both toxinotypes XII and XVI were never associated with any outbreaks [4].

Some *C. difficile* toxinotypes are recently found having hypervirulent potential and spreading in many countries leading to several diseases and outbreaks. It follows that further studies on *C. difficile* toxins and the mechanisms leading to the generation of variability in the PaLoc region may be helpful in understanding their evolution and role in pathogenesis. Thus the identification of *C. difficile* toxinotypes is important in order to determine the virulence potential of the strains, control their spread in the medical center and reduce their potential in causing outbreaks of PMC.

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