Molecular characterization of toxigenic Clostridium difficile in a northern Italian hospital.

Molecular Characterization of Toxigenic *Clostridium difficile* in a Northern Italian Hospital

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Abstract.

C. difficile is responsible of more than 90% of cases of antibiotic-associated diarrhoea and pseudomembranous colitis. The most important virulence factors are two toxins called enterotoxin A and cytotoxin B,moreover, some *C. difficile* strains contain the C. difficile binary toxin [CDT]. The aim of our study was to prospectively analyze *C. difficile* clinical isolates in a single center to determine the molecular features of collected strains. Among the 252 isolates, 217 were A+ B+ [86.1%], 33 were A+ B+ cdt+ [13.1%] and 2 were A- B+ [0.8%]. There were 15 different ribotypes with a predominance of 018.

Sir,

C. difficile is a major nosocomial pathogen and is responsible of more than 90% of cases of antibiotic-associated diarrhoea and pseudomembranous colitis. *C. difficile*-associated diseases [CDAD] range from uncomplicated mild diarrhea to toxic megacolon, from sepsis to severe sepsis, with prolonged hospital stay and high mortality of more severe forms [2,4]. Each year, CDAD affects an estimated 500,000 persons, accounting for more than $1 billion in costs and 20,000 deaths. Rapid and accurate diagnosis of CDI is essential for decreasing the prevalence of this bacterium and the consequences of its infection, for improving outcomes of patients with CDAD and for reducing horizontal transmission in health care facilities [4].

The most important virulence factors are two toxins called enterotoxin A, coded by tcdA gene, and cytotoxin B, coded by tcdB gene, which are part of Pathogenicity Locus [PaLoc] like tcdC, an accessory gene that is involved in the positive and negative regulation of tcdA and tcdB expression; moreover, some C. difficile strains contain an additional toxin called C. difficile binary toxin [CDT], expressed from the cdtA and cdtB operon [2].

The aim of our study was to prospectively analyze *C. difficile* clinical isolates in a single center to determine the molecular features of collected strains.

Bacterial isolates and DNA extraction. We collected 252 *C. difficile* strains isolated in S. Giovanni Battista – Molinette hospital from January 2008 to April 2010. *C. difficile* toxin detection was performed by Vidas [bioMerieux, Mercy Le Toille, FR] on unformed/liquid stool samples. Positive samples were plated, after alcoholic shock, on CDSA agar [BD Diagnostic, San Diego, CA] and incubated in anaerobic condition at 37 for 48-72h. DNA extraction was performed from colonies grown by using Mo Bio Ultraclean manual kit, in according with manufacturer of commercial kit. All 252 isolates were analyzed with Multiplex Polymerase chain reaction [PCR] for detection of genes tcdA, tcdB, tcdtA and cdtB; PCR Ribotyping; tcdC delection and tcdC sequencing. Regarding genes detection, the tcdA, tcdB, cdtA and cdtB genes were amplified by multiplex PCR like described by Persson et al.[3]; 16S rDNA were amplified as an internal PCR control.

PCR-Ribotyping. PCR ribotyping was performed according to Bidet at al., with some modification in the detection of PCR products [1]; briefly, primer sequences were: 16S 5'-GTGCGGCTGGATCACCTCCT-3' and 23S 5'-CCCTGCACCCTTAATAACTTGACC-3'. 1 µl of the amplified product was separated on a microfluidic chip and was detected by Agilent 2100 Bioanalyzer [Agilent Technologies, Palo Alto, CA]. Data were processed using the DiversiLab Software and reports were automatically generated.

tcdC analysis. tcdC delection and tcdC sequencing were performed using the method described by Spigaglia et al. with primers tcdC-C1 5'-TTAATTAATTTTCTCTACAGCTATCC-3' and tcdC-C2 5'-TCTAATAAAAGGGAGATTGTATTATG-3'[5].

Antibiotic susceptibility testing. The MICs of vancomycin, metronidazole, moxifloxacin, and levofloxacin were determined using Etest [bioMerieux].

Among the 252 toxigenic isolates, 217 were A+ B+ [86.1%], 33 were A+ B+ cdt+ [13.1%] and 2 were A- B+ [0.8%]. There were 15 different ribotypes and the most frequent are reported in Figure 1; the ribotype 018 was predominant.

CDT is produced by the majority of strains with mutation in the CD PaLoc; all ribotypes 078 and 027 strains have the cdtA/cdtB genes. The analysis of the tcdC gene showed a delection of 18 bp in 9 isolates, of which 8 were ribotype 027 and one unknown. A delection of 39 bp was found in all 16 ribotypes 078 and 126 strains. Furthermore, we also found a wild type strains and a strain with a delection of 36 bp. In ribotype 027 is present a delection in position 117 with a stop codon that inactivates the tcdC and the ribotype 078 have the C184T mutation inserting a stop codon that truncates the tcdC protein.

Regarding the antibiotic susceptibility testing, there was a high level of resistance to fluoroquinolones, not only for ribotype 027. All strains were susceptible to vancomycin and metronidazole.

In this study more than 85% of toxigenic isolates were A+ B+, as recently reported by Chen et al.: the toxin gene profiling revealed that 83.2% of clinical isolates were positive for both toxin A

[tcdA] and toxin B [tcdB] [A+B+], followed by toxin A-negative and B-positive [A-B+] [16.8%][2]. However, in that study only three of the toxigenic strains [1.9%] were positive for both the cdtA and cdtB genes, compared to 13% in our study [2].

In our study the ribotype 018 was predominant [41%], followed by ribotype 020 [7%], ribotype 078 [6%] and ribotype 001 [6%]. In another Italian study on 92 toxigenic isolates of C. difficile, 90.1% of the strains belonged to the PCR ribotype 018, 3.3% to PCR ribotype 078 and 2.2% to PCR ribotype 002 and ribotype 106 [4].

All the strains were susceptible to metronidazole and vancomycin and the increased number of *C. difficile* strains resistant to fluoroquinolones in Italy was confirmed. In the aforementioned Italian study, all the ribotype 018 strains were resistant to fluoroquinolones, suggesting that the increased use of these antibiotics has played a determinant role in selection and spread of these strains [4].

In conclusion, we confirmed the high frequency of ribotype 018, the high rate of toxigenic isolates expressing both toxin A and B [86.1%] and the high level of resistance to fluoroquinolones. The use of molecular methods is warranted to study and describe the local epidemiology of *C. difficile*, a potential life-threatening pathogen for hospitalized patients in an era of antimicrobial resistance.

Competing interests: The authors declare that they have no competing interests

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Figure 1. Percentages of ribotypes, San Giovanni Battista hospital.