

TOXIGENIC *CLOSTRIDIOIDES DIFFICILE* AND THEIR ANTIBIOTIC SUSCEPTIBILITY IN BULGARIAN PATIENTS DURING AND POST-COVID-19 PANDEMIC

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Received on February 23, 2025

Presented by B. Petrunov, Member of BAS, on March 25, 2025

Abstract

The COVID-19 pandemic boosted antibiotic use in Bulgaria. The aim of the study was to evaluate for the first time in our country, the influence of the COVID-19 pandemic on both characteristic, prevalence and antibiotic susceptibility of *Clostridioides difficile*. We investigated 75 *C. difficile* strains isolated since the pandemic compared with 52 strains from 2013–2019. In 2020–2023, the toxigenic strains (TCDs) were 92.0% of all strains vs. 57.7% of those isolated beforehand. In 2020–2023, TCD resistance was metronidazole 5.8%, vancomycin 1.4%, and fidaxomicin 1.6%, without significant differences between the two periods. We observed a 5.5-fold increase in the prevalence of hypervirulent (HCD) strains. In HCD strains in 2020–2023, metronidazole resistance was 7.9%. Notably, resistance to levofloxacin 1 and 32 mg/l was 91.9% and 75.8%, respectively, in TCD strains from 2020–2023. Briefly, the increase in both TCD and HCD strains and the presence of resistance to all three antibiotics for treating *C. difficile* infections imply the need for susceptibility testing of all TCD isolates. The strikingly high levofloxacin resistance was linked to fluoroquinolone consumption in Bulgaria. The results stress the need for regular monitoring of antibiotic susceptibility of TCD strains, and strict adherence to the antibiotic policy.

Key words: *Clostridioides difficile*, prevalence, toxigenic, hypervirulent, antibiotic resistance

Introduction. *Clostridioides difficile* is a gram-positive sporeforming anaerobe and a causative agent of severe infections (CDIs) such as antibiotic-associated diarrhoea, pseudomembranous colitis, and toxic megacolon [1]. Only toxigenic *C. difficile* strains are clinically important [2]. Toxigenic *C. difficile* strains produce up to three exotoxins: *tcdA*-encoded toxin A, *tcdB*-encoded toxin, and *cdtA/cdtB*-encoded binary toxin. TcdA and TcdB are glucosyltransferases which produce transmembrane pores in the intestinal cells, modify human Rho/Ras GTPases, and lead to epithelial cell destruction and inflammation [3].

Binary toxin (CDT), which is frequent in hypervirulent strains, disrupts the F-actin, rearranges the actin cytoskeleton and forms microtubule protrusions [4]. CDI can be life-threatening, but antibiotic choice for CDI therapy is strongly limited. Fidaxomicin is the drug of choice, with vancomycin as an alternative, whereas metronidazole is acceptable only for mild infections [1]. Due to the limited treatment options, antibiotic resistance of the strains should be monitored. Moreover, the COVID-19 pandemic has sharply boosted antibiotic use in Bulgaria, and in Varna, nearly half of COVID-19 patients were prescribed antibiotics [5].

The aim of the study was to assess the prevalence and antibiotic susceptibility of toxigenic and hypervirulent *C. difficile* strains since the start of the COVID-19 pandemic in Bulgaria in 2020, and to compare the results to those in the period 2013–2019.

Material and methods. Bacterial strains. In 2013–2023, we studied 127 *C. difficile* strains from faecal samples of 127 patients with diarrhoea and loose stools, clinically suspected of having CDI. We tested toxigenic strain susceptibility to fidaxomicin, vancomycin, metronidazole and levofloxacin. Informed written consent was taken from all patients or the children's parents by the clinicians. Among the 99 patients with toxigenic strains, eight had inflammatory bowel disease, three had irritable bowel syndrome, and four patients had malignant diseases. The patients with toxigenic strains in 2013–2019 and 2020–2023 were 13 and 13 children, 17 and 56 adults, 15 and 33 males, and 15 and 36 females, respectively.

Isolation and susceptibility testing. Strain isolation was carried out as previously described [6]. Isolates were identified by RapID ANA II (Oxoid) or MALDI TOF MS (Vitek MS, bioMérieux). Metronidazole, levofloxacin and vancomycin susceptibility was tested by E tests (Liofilchem) and that of fidaxomicin was assessed by a breakpoint susceptibility testing method. The medium used was Anaerobe Basal Agar (Oxoid) with hemin, vitamin K, and 5% horse blood, and the incubation of the plates was at 37 °C for 2 to 3 days. Fidaxomicin (Glentham Life Sciences Ltd) was solubilized in dimethyl sulphoxide and further diluted to obtain final concentrations in the agar of 0.5, 1, and 2 mg/l. Susceptibility to fidaxomicin, metronidazole and vancomycin was read according to EUCAST breakpoints [7]. Strains were considered levofloxacin resistant when MICs were > 1 mg/l, since there are no EUCAST breakpoints for *C. difficile* resistance to fluoroquinolones.

Genetic analysis. PCR was performed using primers for *gluD* (glutamate dehydrogenase as a marker for *C. difficile*) [8], *tcdA* (toxin A), and *tcdB* (toxin B) genes [9,10]. For the binary toxin, a multiplex PCR was used for *cdtA/cdtB* genes according to the protocol of PERSSON et al. [11].

Statistical analysis. Differences between the groups were detected by chi square with Fisher's exact test.

Results and discussion. An alarming result in our study was a significant 1.6-fold increase in toxigenic *C. difficile* strains along with a sharp 5.5-fold increase of hypervirulent strains since 2020. Before and after 2020, toxigenic strains were 57.7% (30/52 strains) and 92.0% (69/75, $p = 0.0001$), respectively, of the strains. Hypervirulent strains were detected in 10.0% (3/30 strains) in 2013–2019, and 55.1% (38/69, $p = 0.0001$) of the strains in 2020–2023 (Fig. 1).

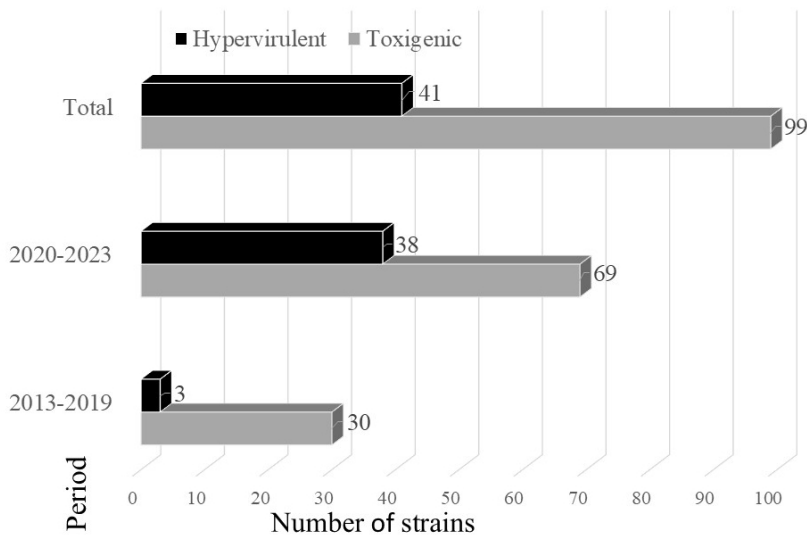


Fig. 1. Hypervirulent *C. difficile* strains as a part of all toxigenic strains studied

Overall resistance rates in the toxigenic strains were vancomycin 3.0%, metronidazole 7.1%, fidaxomicin 1.4%, levofloxacin 91.9% at MIC of 1 mg/l, and 75.8% at MIC of ≥ 32 mg/l (Table 1). Among the 38 hypervirulent strains isolated in 2020–2023, no resistance to fidaxomicin and vancomycin was detected, whereas 7.9% (3/38 strains) of the strains were metronidazole resistant, and 34 (89.5%) were resistant to levofloxacin at MIC of 1 mg/l.

The COVID-19 pandemic in Bulgaria boosted antibiotic use. Two European countries (Bulgaria and Romania) registered the highest increase in antibiotic consumption in 2020 compared to 2019 [12]. It is known that antibiotic use and hospitalization are risk factors for CDI. In our study, the sharp increase (5.5-fold) in the frequency of hypervirulent strains in 2020–2023 could be due to the high

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Antibiotic susceptibility of toxigenic *C. difficile* strains

antibiotic Year	2013–2019			2020–2023			<i>p</i> value	Total		
	No. of strains	No. R	% of R	No. of strains	No. of R	% of R		No. of strains	No. of R	% of R
All toxigenic strains										
Vancomycin	30	2	6.7	69	1	1.4	0.217	99	3	3.0
Metronidazole	30	3	10.0	69	4	5.8	0.430	99	7	7.1
Fidaxomicin	12	0	0.0	62	1	1.6	1.000	74	1	1.4
Levofloxacin (1 mg/l)	30	27	85.7	69	64	92.7	0.695	99	91	91.9
Levofloxacin (32 mg/l)	30	21	70.0	69	54	78.3	0.446	99	75	75.8
Hypervirulent strains only										
Vancomycin	3	1	33.3	38	0	0.0	0.073	41	1	2.4
Metronidazole	3	0	0.0	38	3	7.9	1.000	41	3	7.3
Fidaxomicin	2	0	0.0	38	0	0.0	1.000	41	0	0.0
Levofloxacin (1 mg/l)	3	3	100.0	38	34	89.5	1.000	41	37	90.2
Levofloxacin (32 mg/l)	3	3	100.0	38	28	73.7	0.564	41	31	75.6

R – resistant/resistance; resistance to vancomycin > 2 mg/l, metronidazole > 2 mg/l, fidaxomicin > 0.5 mg/l, levofloxacin > 1 mg/l; hypervirulent-*cdtA/B* positive

antibiotic consumption in our country since the start of the pandemic. Hypervirulent stains display high resistance and multidrug resistance to the antibiotics that can cause CDI [2] and better competitive fitness than other ribotypes [13].

C. difficile resistance to vancomycin and metronidazole can be due to plasmids or chromosomal mutations [14]. To accurately detect metronidazole resistance, the susceptibility testing media have to contain heme [2], therefore, we used a hemin-containing medium to test the susceptibility of toxigenic strains. We used E tests for vancomycin, metronidazole and levofloxacin, since PENG et al. [15] found a high categorical agreement between the results of the E tests and the agar dilution method.

Although infrequently, we found resistance to all the three antibacterials for CDI therapy. EUCAST resistance breakpoint for fidaxomicin is >0.5 mg/l [7]. Considering this breakpoint, no fidaxomicin resistance was detected in Taiwanese, Hungarian, and Japanese isolates [16]. In Germany, SCHWANBECK et al. [17] found one strain (2.0%) with a high level (MIC, > 64 mg/l) fidaxomicin resistance. In our study, fidaxomicin MIC of the resistant strain was lower (2 mg/l) and was detected from the sample of an adult woman who had COVID-19 two months

ago. Given that fidaxomicin is not yet available in the country and the resistance may increase with its impending use, susceptibility to this antibiotic should also be tested.

C. difficile fluoroquinolone resistance can be due to *gyrA* and *gyrB* gene mutations or efflux pumps [18]. We found a strikingly high levofloxacin resistance at MIC of 1 mg/l, reaching 92.7% in 2020–2023. High-level levofloxacin resistance (MICs, ≥ 32 mg/l) was also prevalent in both 2013–2019 (70.0%) and 2020–2023 (78.3%). This can be linked to the high fluoroquinolone (J01M group) consumption in Bulgaria, which was the highest in the European Union in 2017 [19]. Metronidazole resistance in hypervirulent strains was 7.9% in 2020–2023, and was detected in 2022 and 2023.

We did not find any significant evolution ($p > 0.06$) of antibiotic resistance in the toxigenic strains or in the subgroup of hypervirulent strains ($p > 0.07$). This can be explained by the fact that fidaxomicin and metronidazole are not appropriate drugs to treat bacterial infections in COVID-19 patients, and vancomycin was used mainly for methicillin-resistant *Staphylococcus aureus* coinfections.

Conclusion. We observed a sharp increase in the prevalence of both toxigenic and hypervirulent *C. difficile* strains since the start of the COVID-19 pandemic in Bulgaria. This can be explained by the frequent multidrug resistance in the hypervirulent strains to the antibiotics that cause CDI and their better fitness compared with other *C. difficile* strains. Notably, the toxigenic strains exhibited resistance for all three antibiotics for CDI therapy, ranging from 1.4% for fidaxomicin (1.6% in recent years) to 7.1% for metronidazole (5.8% in recent years), although fidaxomicin is still unavailable in the country. Most toxigenic strains ($> 91\%$) were resistant to 1 to 32 mg/l levofloxacin. The results emphasize the need to regularly monitor *C. difficile* antibiotic susceptibility, including for fidaxomicin, and to limit fluoroquinolone misuse and overuse both in hospitals and in the community.

Declaration of competing interest. The authors of this manuscript declare that they have no conflicts of interest regarding this research.

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