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Expression of virulence factors of *Clostridioides difficile* at sub-minimum inhibitory concentration of antibiotics: A review

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Abstract*---Clostridioides difficile*** (*C. difficile*) generally colonizes in the colon of hospitalized patients who receive antibiotics for a long time. *C. difficile* expresses its virulence factors which are associated with pathogenesis. Naturally, the expression of these virulence factors may be influenced by antibiotics. The effect of antibiotics at the sub-minimum inhibitory concentration (MIC) on virulence factors has been investigated and it is varied and depends on the type of antibiotic and *C. difficile* isolate. Some of the antibiotics at sub-MIC upregulate virulence factors, while others downregulate. Meanwhile, some antibiotics have no detectable effects on the regulation of virulence factors. Nearly, all investigations have surveyed a few numbers of *C. difficile* isolates in terms the expression of virulence factors at sub-MIC. Most of the antibiotics at sub-MICs regulate gene expression of virulence factors, toxin production, spore formation, and germination by several mechanisms especially the SOS response system. However, to achieve a clear understanding of the effect of antibiotics at sub-MIC on the expression of genes of virulence factors, which are related to the pathogenesis of *C. difficile*, further and wider investigations are needed, especially on the issue of the numbers of isolates that have been discussed in the present review article.

***Keywords*---**virulence factors, sub-MIC, clostridioides difficile, antibiotics, gene expression.

Introduction

Clostridioides difficile (*C. difficile)* is an anaerobic, spore-forming, bacterium isolated from the stools of healthy infants at 1935 years as a member of microbiota (1). In 1978, it was isolated from the stools of patients treated with clindamycin and hence the disease was known as clindamycin colitis (2). The early works showed that *C. difficile* produces at least two toxins and in the next years, it became the most antibiotic-associated- diarrhea (AAD) worldwide (3). Nowadays, Sixty-six percent of AAD is healthcare-associated and the incidence of

C. difficile AAD reaches 453000 cases in the USA hospitalized patients, annually (3).

Among the factors associated with *C. difficile* infection (CDI), antibiotics are the most important agents. Nearly, all antibiotics are associated with CDI and among them, aminoglycosides, macrolides, quinolones, clindamycin, penicillin, and sulfonamides/trimethoprim are very important (4,5). Induction of AAD takes place in the presence of antibiotics in the gut environment. Therefore the effect of *C. difficile* has been considered in the presence of antibiotics in sub-minimum inhibitory concentration (sub-MIC). The effect of antibiotics on some virulence factors such as toxins genes expression, toxin production, spore formation, and colonization factor genes expression has been investigated. In the present review, the effect of antibiotics at sub-MIC on their genes expressions, toxin production, and spore formation will be discussed.

Virulence genes expression in sub-MIC of Antibiotics

The environmental condition, nutritional factors, and antibiotics in sub-MIC influence the expression of *C. difficile* virulence factors (6). In 1982, for the first time, the effect of antibiotics was investigated at sub-MIC on toxin production in the toxigenic and nontoxigenic isolates of *C. difficile*. From 62 toxigenic isolates, only six isolates produced more toxins in the ranges of 16-64 folds at ½ × MIC to 1/32 × MIC of antibiotics. The minimum toxin production was observed when antibiotics were added to the media before inoculation of toxigenic *C. difficile* isolates (7).

Decreasing or increasing the virulence genes expression have performed in the sub-MIC in other bacteria such as *Salmonella enterica*, *Pseudomonas aeroginosa,* and *Escherichia coli* (8). Polymyxin B down-regulates flagellar genes which are associated with invasion and up-regulate the exopolysaccharide in *Salmonella enterica* serovar Typhimurium (9,10). *Pseudomonas aeroginosa* enhanced the beta-lactamase, alginate, and peptidoglycan biosynthesis genes at sub-MIC of imipenem while it suppresses flagellum and pilus genes (11). Regardless of their effects on CDI, different antimicrobial agents have been used to investigate their roles in toxin production, spore formation and germination as follows.

Vancomycin

Vancomycin (VAN) is one of the best choices for treatment or prevention of CDI and other gram-positive related infections in ICU and hospitalized patients (12,13). Therefore, the effect of sub-MIC of VAN has been evaluated on toxins genes expression and production. Several investigations have shown that VAN had no detectable effect on toxin production at sub-MIC of VAN (14-20). VAN at ½, 1/8, and 1/16 MIC, except ¼ MIC, had no effect on toxin A and B genes expression in hypervirulent, NAP1/027, ATCC 5325 strain (21). However, some investigators have reported that VAN at sub-MIC increased the level of the produced toxin and gene expression. The level of toxin A and B genes expression and toxin production has been variable depends on the types of strain (21-23). For example, Aldape and colleagues reported that the level of toxin and genes expression was increased in the historically strain ATCC 9689 at ¼ to 1/16 MIC

but not in hyper virulent strain ATCC 5325 at ½, 1/8, and 1/16 MIC (21). Few studies showed that the level of produced toxin and gene expression was decreased at sub- and supra- MIC (19,24).

The sporulation of *C. difficile* takes place in the presence of some antibiotics. However, few studies have addressed this issue. Based on many studies, VAN at sub-MIC levels had no detectable effects on spore production (25,26). In one research, the VAN reduced the number of produced spores in the NAP1 strain and without any reduction or increase in the historical strain *C. difficile* ATCC 9689 (21).

Metronidazole and related agents

Metronidazole (MTR) with a similar effect to vancomycin has been used for the treatment of CDI for a long time (27). Studies, although fewer than vancomycin, shows that upregulation or downregulation of toxin A and B genes induced by MTR is similar to the pattern of VAN and depends on the type of strain (*16, 22*). For example, in one study, the copies of toxins mRNA achieved to 10^6 fold in comparison to the free antibiotic medium. However, the level of mRNA copies was reduced within 48 hours although, it was still high compared to the control medium (22). There is another study showing that *C. difficile* did not enhance the level of produced toxin at ½ MIC and supra-MIC (8 and 80 MIC) in comparison to the VAN (*19*). Also, Aldape et, al. reported that MTR had a paradoxical effect on toxin A and B genes expression in two strains at sub-MIC. While toxin gene expression was increased in *C. difficile* ATCC 9689, hypervirulent NAP1/027 and ATCC 5325 showed a converse response (21).This inconsistency effect was reported for sporulation as well. At least two studies are evincing that MTR had not had a tangible effect on spore production (25,26). However, Aldape and colleagues reported that spore production was suppressed in the ATCC 9689 strain and it was enhanced in two of the three studies, have performed on the sporulation of *C. difficile* in the sub-MIC (25,26). But in one study MTR have suppressed the spore production in the ATCC 9689 strain and enhanced it in the NAP1/027 ATCC 5325 strain at ¼-1/16 MIC (21).

Protein inhibitors

Among protein synthesis inhibitors, clindamycin (CLI) markedly has reduced the level of toxins in the supernatant of culture (7). Studies are indicating that CLI did not affect toxin A and B gene expression and toxin production in comparison to antibiotic-free medium (15,20,22). However, Baines and coworkers demonstrated that CLI at sub-MIC had a positive effect on toxin A and B genes expression and toxin production (28). Fidaxomicin (FDX) is an antibiotic that belongs to the transcription inhibitors antimicrobial agents and it's now approved by FDA for treating *C. difficile* AAD. FDX has no systemic absorption and is superior to VAN for the treatment of CDI (29). Also, FDX has markedly reduced the risk of recurrence of CDI (30). FDX and its analog, OP-1118 compound, can reduce toxin A and B genes expression approximately 80% in two strains, NAP1/027 UK-14 and ATCC 43255, in comparison to the control medium. *C. difficile* UK-14 and ATCC 43255 with 10 and 24 hours delay have achieved to stationary phase (17). The effect of tigecycline at ¼ MIC to 1/16MIC on the two strains was different.

Toxin gene expression and production in the ATCC 9689 strain was enhanced at all sub-MIC (1/4, 1/8, and 1/16) concentration. However, a negative effect has been observed in NAP1/027 strain at 1/8 and 1/16 but not in 1/4MIC. Therefore both strains were able to increase the level of toxins at 1/4MIC and the maximum increase was seen in the ATCC 9689 (2.3 fold in the tcdA gene) (21).

Cephalosporins and other cell wall inhibitors

Cephalosporins as a relatively large family and with an extended-spectrum activity against bacterial infections (31,32). The third generation of cephalosporins is now one of the most important antibiotics which are associated with CDI in hospitalized patients (33). The effect of ceftriaxone, cefotaxime, and ceftazidime are investigated on toxin A and B genes expression and production (24,34). Freeman and colleagues have shown that ceftriaxone and its derivative form, des acetyl ceftriaxone, increased the level of toxin in the triple-stage chemostat gut model. These antibiotics were added to the systems at a high dosage (20 mg/L) for a week. The amount of produced toxins was more in one chemostat system and moderate in two others. However, cefotaxime, as a member of third-generation cephalosporins had no detectable effect on toxin production in *C. difficile* (34). Notably, ceftazidime alone and even in combination with vancomycin and clindamycin increased the level produced toxin at ½×MIC and ½× FIC (24). Drummond and associates reported that cefoxitin as a selective agent for isolation of *C. difficile* from clinical samples can inhibit the toxin production at ½ and ¼ MIC. It has caused a 24-hour delay in the achieving of the *C. difficile* in culture into stationary growth phase (15). Penicillin in the sub-MIC has increased 100 folds the level of toxin in comparison to the control medium, without reduction or variation in the viable cell density (23).

Other antibiotics

Although it has been reported that there are many antibiotics involved in the CDI (5), their effects on the toxin production or virulence factors genes expression have not yet been fully investigated. Ridinilazole, an unabsorbed antimicrobial agent with narrow-spectrum inhibitory properties against *C. difficile*, at sub-MIC ranges from the ½ MIC to 40 × MIC, has decreased the amount of produced toxin A and B from 75% t0 100%. The maximum reduction was seen at ½ MIC which was 91% for toxin A and 100% for toxin B (18). Surotomycin is another antibiotic with a lipopeptide structure that disrupts the normal function of the cell membrane. This antibiotic has suppressed the toxin production in the *C. difficile* about 77% (toxin A) and 68% (toxin B) at supra-MIC (4,40 and 80 fold) (16,19). Quinolones including ciprofloxacin are now important antibiotics for induction of CDI (35). Ciprofloxacin has enhanced toxin A and B genes at ¼ MIC about 40 folds in NAP1/027 strain. It has upregulated the tcdA and tcdB after 6 hours of inoculation. Notably, two couriers have observed the level of expression of the toxin B gene at 6 and 24 hours after exposing the NAP1/027 strain to the sub-MIC of ciprofloxacin. Contrary to the NAP1/027 strain ciprofloxacin have suppressed the expression of toxins in the hypervirulent strain ATCC 5325 at 0.0625-0.25 × MIC (36).

Mechanism of Action

The effects of antibiotics of different classes on the virulence factors of *C. difficile* at sub-MIC were investigated in several studies. Tigecycline as a protein inhibitor agent down-regulates mRNA transcription and finally the level of toxin (21). Babakhani and colleagues believed that the main cause of the reduction in spore formation is the reduction in mRNA transcription since the mRNA copies are accumulated in the cytoplasm of *C. difficile* cells. They show that FDX as protein inhibitors at sub-MIC did not alter the viability of cells, however, the number of spores has declined in the medium at sub-MIC of FDX (25). Some investigators mentioned that the reason for the enhancement of the level of toxin at sub-MIC of antibiotics is associated with the releasing of toxin caused by disrupting of the cell by cell wall inhibitors agents, especially in stationary phase (15,16).

Several factors have been introduced as the real role of antibiotics on decreasing or increasing of virulence factors genes expression at sub-MIC of antibiotics. These factors include growth, nutrition, amino acids, and oxidation-reduction potential, glucose concentration, and temperature (22,37). Most investigations insist on destroying of microbiota by antibiotics (which permit *C. difficile* to colonize in the colon) and SOS response is a regulatory system that permits *C. difficile* to manage the expression of their genes depending on their requirements (15,16,21,22,36). VAN and FDX inhibit the germination of spore to vegetative form. Because the interstitial form or outgrowth form is not a fully vegetative form, it is more sensitive to VAN, FDX, and oritavancin compare to the fully vegetative cell (38).

Discussion

Among nosocomial pathogens, *C. difficile* is of paramount clinical importance, since it causes antibiotic-related diarrhea mostly in hospitalized patients (39). Some antibiotics disturb normal flora facilitating spore germination and as a result, virulence factors of the bacteria are expressed and produced leading to colitis and diarrhea by destructive effects on epithelial cells of the large intestine (40). These antibiotics not only have a dose-related detrimental effect on gut microbiota but also *C. difficile per se* can be affected by them and based on their requirements, bacteria may down- or up-regulate their virulence factors. To date, most antibiotic-related studies have been conducted in vitro and in vivo, or even animal-model investigations are of limited numbers. *In vitro* studies are commonly focused on the effects of sub-MIC concentrations of some antibiotics on the gene expression of toxins, adhesins, sporulation, and germination in certain strains of the bacteria including ribotype 027, ATCC 9686, ATCC 5325, ATCC 43255, UK-14, and more rarely on few clinical strains (17,20,21,23,34,41).

While some antibiotics such as metronidazole, clindamycin, tigecycline, ceftazidime, penicillin, ampicillin, ciprofloxacin, and moxifloxacin induce expression of virulence-related genes such as toxin production, surface proteins A, CWP66, FBP, CWP84 at sub-MIC concentrations (14,15,19-22,34,36,41-43). other antibiotics including vancomycin, metronidazole, clindamycin, fidaxomicin, tigecycline, ciprofloxacin, cefoxitin, surotomycin, and ridinilazole reduce expression of similar genes such as tcdA, tcdB, toxin production, and sporulation

(7,15-17,25,26,36,41). The more interesting issue is that some antibiotics including metronidazole, ciprofloxacin, clindamycin, and tigecycline had a contradictory effect on the expression of some virulence-related genes involved in the production of toxins A or B (7,17,19,21,22,42). Also, some studies revealed that vancomycin, metronidazole, clindamycin, cefotaxime, piperacillin/tazobactam, norfloxacin, and ofloxacin had no effects on these genes (14,15,19-22,25,26,28,34). Although limited isolates were investigated in these studies, it may be concluded that based on the type of antibiotic and even of isolate there is a variation in expression of virulence-related genes in *C. difficile* (21,36). However, several studies indicated that vancomycin and in some degree fidaxomicin had no effect or may reduce the expression of these genes. Therefore, it can be concluded that vancomycin is prioritized over metronidazole for the treatment of *C. difficile-related infections.*

Many of these studies have not yet presented any convincible reasons for these paradoxical effects although, factors such as type of antibiotic, nutritional status, oxidation/reduction potential, and also SOS response affected by antibiotic stress are responsible for variable expression of some virulence-related genes particularly toxins (15,16,19,21,22,25,37,38). Furthermore, some studies reported that protein-inhibitor antibiotics reduce gene expression (28), However, Aldape and associates reported that cell wall synthesis-inhibitor antibiotics induce the afore-mentioned genes (21). Some studies stated that intracellular accumulation of toxins secreted at the stationary growth phase is a reason for the enhancement of toxin production. Since the CodY protein is involved in toxins, secretion is produced in this phase and after bacterial and or endospore death or by the effect of antibiotics, more toxins are produced (44). While some studies claiming that the presence of antibiotics is necessary for *C. difficile*-related infections (19,28), more studies are reporting that gut microbiota has a more significant role in pathogenesis and gene expression than antibiotics (15,16).

Based on these considerations, and since *C. difficile* is a fastidious and strictly anaerobic bacterium few strains are investigated so it is difficult to present a clear outline regarding the role of antibiotics in genes expression. For shedding more light on this field, the following issues are noteworthy; More in vitro investigations on clinical isolates using cell culture or animal models are warranted although there is an essential difference between human and animal models in terms of number and pattern of the microbiota. In vivo studies on humans are also important although hindered by the lack of enough volunteers or clinical samples. Certainly, more molecular investigations are required to understand the accurate mechanisms of virulence gene expression in the presence of antibiotics in *C. difficile*.

Conclusion

There is an interaction between antibiotics and C.*difficile* for gene expression. While the presence of particular antibiotics in the gastrointestinal tract is effective in C.*difficile*-related infections, C.*difficile per se* can regulate expression of their virulence-related genes using a different mechanism including SOS response. Although gene expression is affected by the type of antibiotic and isolate, limited studies on a few isolates have reported that vancomycin has the most conformity

in terms of its effect on gene expression and even on sporulation, accordingly, it is the best choice for treatment of C.*difficile*-related infections and has priority over metronidazole. Further in vitro and preferably in vivo studies are warranted to develop a better understanding of the exact mechanism involved in the antibiotic effects on virulence-related genes.

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References

1. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. Anaerobe. 2016;40:95-9.
2. Bartlett JG. Clostridium difficile Infection. Infect Dis Clin North Am. 2017;31(3):489-95.
3. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, et al. Burden of Clostridium difficile infection in the United States. New England Journal of Medicine. 2015;372(9):825-34.
4. Dantes R, Mu Y, Hicks LA, Cohen J, Bamberg W, Beldavs ZG, et al., editors. Association between outpatient antibiotic prescribing practices and community-associated Clostridium difficile infection. Open forum infectious diseases; 2015: Oxford University Press.
5. Cho SM, Lee JJ, Yoon HJ. Clinical risk factors for Clostridium difficile-associated diseases. The Brazilian Journal of Infectious Diseases. 2012;16(3):256-61.
6. Willing SE, Richards EJ, Sempere L, Dale AG, Cutting SM, Fairweather NF. Increased toxin expression in a Clostridium difficile mfd mutant. BMC Microbiol. 2015;15(280):280.
7. Nakamura S, Mikawa M, Tanabe N, Yamakawa K, Nishida S. Effect of clindamycin on cytotoxin production by Clostridium difficile. Microbiology and immunology. 1982;26(11):985-92.
8. Davies J, Spiegelman GB, Yim G. The world of subinhibitory antibiotic concentrations. Current opinion in microbiology. 2006;9(5):445-53.
9. Bader MW, Navarre WW, Shiau W, Nikaido H, Frye JG, McClelland M, et al. Regulation of Salmonella typhimurium virulence gene expression by cationic antimicrobial peptides. Molecular microbiology. 2003;50(1):219-30.
10. Goh E-B, Yim G, Tsui W, McClure J, Surette MG, Davies J. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. Proceedings of the National Academy of Sciences. 2002;99(26):17025-30.
11. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, et al. Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and β-lactamase and alginate production. Antimicrobial agents and chemotherapy. 2004;48(4):1175-87.

12. Kheir MM, Tan TL, Azboy I, Tan DD, Parvizi J. Vancomycin prophylaxis for total joint arthroplasty: Incorrectly dosed and has a higher rate of periprosthetic infection than cefazolin. Clinical Orthopaedics and Related Research®. 2017:1-8.
13. Hung Y-P, Lee J-C, Lin H-J, Chiu C-W, Wu J-L, Liu H-C, et al. Perceptions of Clostridium difficile infections among infection control professionals in Taiwan. Journal of Microbiology, Immunology and Infection. 2017.
14. Deneve C, Bouttier S, Dupuy B, Barbut F, Collignon A, Janoir C. Effects of subinhibitory concentrations of antibiotics on colonization factor expression by moxifloxacin-susceptible and moxifloxacin-resistant Clostridium difficile strains. Antimicrob Agents Chemother. 2009;53(12):5155-62.
15. Drummond LJ, Smith DG, Poxton IR. Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by Clostridium difficile. J Med Microbiol. 2003;52(Pt 12):1033-8.
16. Bouillaut L, McBride S, Sorg JA, Schmidt DJ, Suarez JM, Tzipori S, et al. Effects of surotomycin on Clostridium difficile viability and toxin production in vitro. Antimicrobial agents and chemotherapy. 2015;59(7):4199-205.
17. Babakhani F, Bouillaut L, Sears P, Sims C, Gomez A, Sonenshein AL. Fidaxomicin inhibits toxin production in Clostridium difficile. J Antimicrob Chemother. 2013;68(3):515-22.
18. Bassères E, Endres BT, Khaleduzzaman M, Miraftabi F, Alam MJ, Vickers RJ, et al. Impact on toxin production and cell morphology in Clostridium difficile by ridinilazole (SMT19969), a novel treatment for C. difficile infection. Journal of Antimicrobial Chemotherapy. 2016;71(5):1245-51.
19. Endres BT, Bassères E, Khaleduzzaman M, Alam MJ, Chesnel L, Garey KW. Evaluating the effects of surotomycin treatment on Clostridium difficile toxin A and B production, immune response, and morphological changes. Antimicrobial agents and chemotherapy. 2016;60(6):3519-23.
20. Zarandi ER, Mansouri S, Nakhaee N, Sarafzadeh F, Moradi M. Toxin production of Clostridium difficile in sub-MIC of vancomycin and clindamycin alone and in combination with ceftazidime. Microbial pathogenesis. 2017;107:249-53.
21. Aldape MJ, Heeney DD, Bryant AE, Stevens DL. Tigecycline suppresses toxin A and B production and sporulation in Clostridium difficile. J Antimicrob Chemother. 2015;70(1):153-9.
22. Gerber M, Walch C, Loffler B, Tischendorf K, Reischl U, Ackermann G. Effect of sub-MIC concentrations of metronidazole, vancomycin, clindamycin and linezolid on toxin gene transcription and production in Clostridium difficile. J Med Microbiol. 2008;57(Pt 6):776-83.
23. Onderdonk AB, Lowe BR, Bartlett JG. Effect of environmental stress on Clostridium difficile toxin levels during continuous cultivation. Appl Environ Microbiol. 1979;38(4):637-41.
24. Zarandi ER, Mansouri S, Nakhaee N, Sarafzadeh F, Iranmanesh Z, Moradi M. Frequency of antibiotic associated diarrhea caused by Clostridium difficile among hospitalized patients in intensive care unit, Kerman, Iran. Gastroenterology and hepatology from bed to bench. 2017;10(3):229.
25. Babakhani F, Bouillaut L, Gomez A, Sears P, Nguyen L, Sonenshein AL. Fidaxomicin inhibits spore production in Clostridium difficile. Clin Infect Dis. 2012;55 Suppl 2(2):S162-9.

26. Garneau JR, Valiquette L, Fortier LC. Prevention of Clostridium difficile spore formation by sub-inhibitory concentrations of tigecycline and piperacillin/tazobactam. BMC Infect Dis. 2014;14(29):29.
27. Zar FA, Bakkanagari SR, Moorthi K, Davis MB. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile–associated diarrhea, stratified by disease severity. Clinical Infectious Diseases. 2007;45(3):302-7.
28. Baines SD, Freeman J, Wilcox MH. Effects of piperacillin/tazobactam on Clostridium difficile growth and toxin production in a human gut model. Journal of Antimicrobial Chemotherapy. 2005;55(6):974-82.
29. Manthey C, Eckmann L, Fuhrmann V. Therapy for Clostridium difficile infection–any news beyond Metronidazole and Vancomycin? Expert Review of Clinical Pharmacology. 2017(just-accepted).
30. Fehér C, Rubio EM, Amador PM, Garcia-Campero AD-I, Salavert M, Merino E, et al. The efficacy of fidaxomicin in the treatment of Clostridium difficile infection in a real-world clinical setting: a Spanish multi-centre retrospective cohort. European Journal of Clinical Microbiology & Infectious Diseases. 2017;36(2):295-303.
31. Wu W-x, Liu D, Wang Y-w, Wang C, Yang C, Liu X-z, et al. Empirical Antibiotic Treatment in Diabetic Foot Infection: A Study Focusing on the Culture and Antibiotic Sensitivity in a Population From Southern China. The International Journal of Lower Extremity Wounds. 2017:1534734617725410.
32. Abraham P, Lamba N, Acosta M, Gholmie J, Dawood HY, Vestal M, et al. Antibacterial prophylaxis for gram-positive and gram-negative infections in cranial surgery: A meta-analysis. Journal of Clinical Neuroscience. 2017.
33. Khanafer N, Vanhems P, Barbut F, Luxemburger C, group CS. Factors associated with Clostridium difficile infection: A nested case-control study in a three year prospective cohort. Anaerobe. 2017;44:117-23.
34. Freeman J, O'neill FJ, Wilcox MH. Effects of cefotaxime and desacetylcefotaxime upon Clostridium difficile proliferation and toxin production in a triple-stage chemostat model of the human gut. Journal of Antimicrobial Chemotherapy. 2003;52(1):96-102.
35. Santos A, Isidro J, Silva C, Boaventura L, Diogo J, Faustino A, et al. Molecular and epidemiologic study of Clostridium difficile reveals unusual heterogeneity in clinical strains circulating in different regions in Portugal. Clinical Microbiology and Infection. 2016;22(8):695-700.
36. Aldape MJ, Packham AE, Nute DW, Bryant AE, Stevens DL. Effects of ciprofloxacin on the expression and production of exotoxins by Clostridium difficile. J Med Microbiol. 2013;62(Pt 5):741-7.
37. Karlsson S, Dupuy B, Mukherjee K, Norin E, Burman LG, Åkerlund T. Expression of Clostridium difficile toxins A and B and their sigma factor TcdD is controlled by temperature. Infection and immunity. 2003;71(4):1784-93.
38. Chilton C, Freeman J, Baines SD, Crowther G, Nicholson S, Wilcox M. Evaluation of the effect of oritavancin on Clostridium difficile spore germination, outgrowth and recovery. Journal of Antimicrobial Chemotherapy. 2013;68(9):2078-82.
39. Balsells E, Shi T, Leese C, Lyell I, Burrows J, Wiuff C, et al. Global burden of Clostridium difficile infections: a systematic review and meta-analysis. Journal of global health. 2019;9(1).

40. Czepiel J, Dróżdż M, Pituch H, Kuijper EJ, Perucki W, Mielimonka A, et al. Clostridium difficile infection. European Journal of Clinical Microbiology & Infectious Diseases. 2019:1-11.
41. Zarandi ER, Mansouri S, Nakhaee N, Sarafzadeh F, Moradi M. Effect of sub-MIC of vancomycin and clindamycin alone and in combination with ceftazidime on Clostridium difficile surface layer protein A (slpA) gene expression. Microbial pathogenesis. 2017;111:163-7.
42. Baines SD, Saxton K, Freeman J, Wilcox MH. Tigecycline does not induce proliferation or cytotoxin production by epidemic Clostridium difficile strains in a human gut model. J Antimicrob Chemother. 2006;58(5):1062-5.
43. Deneve C, Delomenie C, Barc MC, Collignon A, Janoir C. Antibiotics involved in Clostridium difficile-associated disease increase colonization factor gene expression. J Med Microbiol. 2008;57(Pt 6):732-8.
44. Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL. Repression of Clostridium difficile toxin gene expression by CodY. Molecular microbiology. 2007;66(1):206-19.