Clostridium difficile virulence factors as the cause of antibiotic-associated diarrhea (AAD): a literature review

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ABSTRACT

Clostridium difficile is an anaerobic gram-positive bacillus, capable of forming spores and toxins, transmitted to humans by the faecal-oral route *C. difficile* infection (CDI) is recognized as a typical cause healthcare-associated infections (HAIs) and contributes to a significant proportion of morbidity and mortality of hospitalized patients. *C. difficile* culture and toxin examinations are still minimal in many hospitals in various Asian countries. As a result, reports of *C. difficile* in Asia are still rare, while reports of cases of CDI in Indonesia are still rare. Several risk factors including advanced age, antibiotic exposure, and hospitalization are strongly associated with CDI. *C. difficile* has the ability to colonize the large intestine, then release exotoxin proteins (TcdA, TcdB) causing colitis in people with risk factors. A diagnosis of suspected *C. difficile* infection in a patient with diarrhea without a clear alternative explanation, with relevant risk factors (including long antibiotic consumption, hospitalization events, and elderly age), was then performed microbiological examination to carry out proper management and control of infection. This study aims to review *C. difficile* virulence factors as the cause of antibiotic-associated diarrhea.

Keywords: *Clostridium difficile*, virulence factors, antibiotic-associated diarrhea.
Cite This Article: Wedari, N.L.P.H., Budayanti, N.N.S., Darwinata, A.E. 2022. *Clostridium difficile* virulence factors as the cause of antibiotic-associated diarrhea (AAD): a literature review. *Bali Medical Journal* 11(3): 1277-1281. DOI: 10.15562/bmj.v11i3.3696

INTRODUCTION

Clostridium difficile infection or known as CDI has been recognized as a typical HAIs and contributes to a significant proportion of morbidity and mortality among hospitalized patients with case-fatality rates of multiple infections varying from mild diarrhea to pseudomembranous colitis (PMC), especially in the elderly patients on antibiotic treatment.[1-5] In addition, the high health costs associated with CDI increase the government's financial burden on health spending. It was noted that half a million infections were associated with CDI in the United States in 2011 with an incidence rate of 8.75 cases/1,000 adult admissions in 2009.[1] In study of Saito *et al.*, 2019 reported the results of *C. difficile* ribotyping without information on the prevalence or incidence of CDI in Japan.[2] The incidence of CDI increased from 1.7/1,000 to 2.7/1,000 adults in Korea and 17.1/10,000 hospitalized patients in Shanghai were associated with CDI.[6] Meanwhile, about 44% and 14% of colitis positive patients were diagnosed positively with *C. difficile* toxin in the Philippines

and Malaysia, respectively.[6] A more recent study showed that the prevalence of CDI was 9.2% in Thailand.[7] There are few reports of CDI incidence or prevalence in Indonesia. There were eight types of *C. difficile* strains that appeared in healthy people, while another study showed the prevalence of *C. difficile* (TcdA) was 1.3% in communities and hospitals in Jakarta. Recent reports from Central Java indicate the prevalence of CDI will be 20.6% in 2017.[4-10]

Several risk factors including advanced age, exposure to antibiotics, and hospitalization are strongly associated with CDI. Regulation of the use of antibiotics in Asian countries is considered poor. There has been a review in Southeast Asian countries describing 47% of pneumonia cases not receiving appropriate antibiotics whereas 54% of diarrheal patients received unnecessary antibiotics, with 40% of antibiotics being prescribed under-dose.[9,10] Elderly individuals on recent antibiotic treatment are at the highest risk for CDI because they lack beneficial gut microbiota and have low immunity due to age and other comorbidities. This group

was severely affected and had the highest mortality rate due to CDI with a 2% risk increasing every year after the age of 18. A report describes approximately one in ten deaths from CDI in the elderly in the United States in 2010.[11,12] There is no data on CDI in the elderly in Indonesia, perhaps because of the lack of supervision in cases of CDI followed by limited laboratory modalities. facilities in hospitals that are capable of diagnosing CDI. In addition, cases of relapse (relapse/reinfection) and death from CDI in the elderly will be higher due to inappropriate treatment.[11-16]

A severe form of *C. difficile* (CDI) infection caused by a hypervirulent strain identified as North American type 1, class B1 from restriction-endonuclease analysis, ribotype 027 as presented by PCR. The hypervirulent strain caused national CDI outbreaks in European countries, Canada, and the United States. The first reported outbreak of type 027 CDI occurred in Canada where the most severe infection was Quebec in 2005. In the United States, type 027 CDI affects 38 states.[17-19] Meanwhile, based on the European Centre for Disease Prevention and Control, there

were infections in 16 countries due to CDI type 027.[17,18] The hypervirulent toxinotype III strain had the toxin genes TcdA and TcdB, had an 18-bp deletion in TcdC of the toxin regulatory gene, and a deletion in area 117. This causes a premature stop codon and a frame shift, leading to truncation of the TcdC protein.[19] The increase in cases of type 027 virulence is associated with more excessive toxin production which is associated with a lack of regulatory control of TcdC.[20,21] The cohort study estimates that approximately 40% of cases of CDI are community-acquired CDI (CA-CDI). CA-CDI occurs in younger people, less severe symptoms, shorter hospital stay, lower recurrence rate and no deaths have been reported due to CA-CDI. In addition, CDI was also exacerbated by the discovery of hypervirulent strains and the quinolone-resistant antibiotic, gatifloxacin as a substitute for levofloxacin.[20–23]

Epidemiologically, since 2000, mortality and greater severity of CDI have been attributed to the hypervirulent form of *C. difficile*. The BI/NAP1/027 strain is widespread and vigorous over the past 10 years and has been associated with the CDI epidemic. The most common ribotype in the Middle East is 140, 126, 078, 046, 014, 002, 001, while the more common ribotype in Asia is 018, 017, 014, 002, 001. In North America and Europe, the ribotype 078, 027 , 020, 014, 001 has been the utmost strain. Ribotype 027 has been found to have reduced sensitivity to chloramphenicol, imipenem, clindamycin, moxifloxacin, rifampin, and metronidazole.[23,24] These characteristics have implications for the presentation of more severe disease, higher morbidity and mortality due to antimicrobial resistance compared to other strains. Spores of ribotype 027 grow stronger and easier in hospitals because they are resistant to disinfectants, cleaning and hospital environments. An observational study of diarrheal patients at the Veterans Affairs Medical Center, US showed that approximately 22% were positive for the BI/NAP1/027 strain, its mechanism of pathogenesis, risk factors, currently available treatment options, along with proposed infection prevention and control measures.[25] Based on those mentioned above, this review aims to further evaluate virulence factors of *C. difficile* as the cause of antibiotic-associated diarrhea (AAD).

Clostridium difficile Infection (CDI)

Clostridium difficile is an anaerobic gram-positive bacillus, capable of forming spores and toxins, transmitted to humans by the faecal-oral route. In the United States, *C. difficile* is the most commonly reported pathogen of HAIs. The 2011 surveillance study found 453,000 cases of CDI, 29,000 associated deaths; while about a quarter is obtained from the community.[6] HAIs by *C. difficile* quadruple the cost of hospitalization leading to an estimated $1.5 billion increase in spending in the US each year. It was recorded that half a million infections were associated with CDI in the United States in 2011 with an incidence rate of 8.75 cases/1,000 adult admissions in 2009.[8] In Hong Kong, there were more than fifteen thousand cases of CDI from 2006 to 2014 of which most cases were identified as HAIs. A national study in Korea revealed the total incidence of CDI was 2.7 cases/1,000 adult admissions in 2008. CDI is also known for its tendency to relapse among 35% of patients on antibiotic therapy and more than half of CDI recurrences are identified as relapses or recurrences.[26,27]

Due to CDI, about $1.1 billion is used in healthcare costs annually in the US, while around €3 million is associated with healthcare costs in Europe. Compared with reports from countries in Europe and the United States, the prevalence of CDI in Asia is not fully known.[9] In Korea, a survey of 17 tertiary hospitals from 2004 to 2008 found that the incidence of CDI jumped from 1.7/1,000 to 2.7/1,000 adults. The proportion of community-acquired CDI (CA-CDI) to the total CDI cases in a hospital in Busan was 7.1%, while 59.4% of CDI cases in the emergency department of Seoul Hospital were CA-CDI.[11,12] Based on a comprehensive study in Shanghai, China from March 2007 to April 2008, the overall incidence of CDI was 17.1/10,000 of the number of hospitalized patients; Mild CDI due to a younger mean age (62.8 years) compared with 63% of patients aged 65 years in a comprehensive European study. In addition, a survey in 13 Asia-Pacific countries showed the proportion of CDI associated with health facilities was 53.6% and CA-CDI was 16.5%. Case reports of CDI in Indonesia are still rare. *C. difficile* was identified in 1.3% of stool samples from Indonesian children. However, these data are insufficient to reflect the global prevalence in Asia. Furthermore, data on the prevalence of CDI in the elderly is still not available to date.[28,29]

CDI occurs mostly in the elderly, which may be explained by several risk factors including frequent exposure to health care, age-related physiological changes, increased use of antibiotics, changes in gut flora composition, and increased comorbidities.[11] Frequent health exposures increase the likelihood of contact with an environment contaminated with *C. difficile* endospores and frequent use of antimicrobials. Carrier patients of *C. difficile*, both with and without symptoms, can harbor spores on their skin and transmit them to the environment.[10,11] Age-related physiological changes also increase the risk of CDI, especially changes in the immune system. The development and recurrence of CDI has been associated with the ability to produce an immune response, and the ability to produce antibodies to the toxin may influence the progress of colonization and active infection. Aging is accompanied by a decline in the immune system – degeneration of the immune system associated with old age – and has been associated with a decline in the adaptive immune system.[11,12]

C. difficile has the ability to colonize the large intestine, then release exotoxin proteins (TcdA, TcdB) causing colitis in people with risk factors. TcdA and TcdB cause diarrhea associated with *C. difficile*, which inactivates a member of the Rho family, Rho GTPase (guanosine triphosphatase). This is followed by neutrophilic colitis, colonocyte death, functional loss of the gut barrier, and colonocyte death. CDI disease expression is clinically related to the host immune response and *C. difficile* strains.[17–19] Dramatic increases in severe CDI in hospitals were initially reported in the early 2000s.[17,18] Centers for Disease Control and Prevention (CDC) isolates described are restriction endonuclease BI group, North American gel electrophoresis (NAP1), polymerase chain reaction (PCR)

027; therefore as BI/NAP1/027. The characteristics of this strain are high level of resistance to fluoroquinolones, strong toxin production, efficient sporulation rate, and very high mortality compared to less virulent *C. difficile*. The BI/NAP1/027 strain first originated in North America and Western Europe, but is now spreading to hospitals around the world.[21,30-32]

Although hospital-acquired CDI is the majority, CA-CDI has increased significantly contributing to 1/3 of new CDI cases. CA-CDI occurs when disease onset begins within 12 weeks in individuals who are not hospitalized or in other health care facilities.[30,31] CA-CDI may occur in younger patients, who have unclear antibiotic exposure and unknown risk factors. Therefore, the main modes of CA-CDI acquisition are currently being investigated. CA-CDI-related morbidity and mortality remain lower than hospital-acquired CDI. Nonetheless, 40% of CA-CDI patients require hospitalization and recurrence rates are similar to those of HA-CDI.[31] The effect of gastric acid levels on CDI is still unclear. Theoretically, decreasing stomach acid allows more vegetative organisms to reach the large intestine. However, *C. difficile* produces spores that are resistant to acidic pH.[33-35]

Virulence Factors of *C. difficile*

The main virulence factors of *C. difficile* are TcdA and TcdB which are encoded by the *tcdA* and *tcdB* genes. *C. difficile* locus has three other genes: tcdR, encoding an alternative sigma factor required for toxin production, *tcdC*, which encoding an anti-sigma factor, with direct interaction with *tcdR* and *tcdE*. PaLoc can be transferred by horizontal pathway to non-pathogenic types.[36,37] Although PaLoc has several characteristics of mobile genetic elements, it appears immobile within every toxigenic *C. difficile* types.[36-41]

TcdA and TcdB are in clostridial glycosylation toxin group, also as well as lethal toxin (TcsL), and also hemorrhagic toxin (TcsH) in *Clostridium sordellii*, alpha toxin (TcnA) in *Clostridium perfringens* large cytotoxin TpeL.[21] The size of the toxin is huge, 250 and 308 kDa, with a high degree of sequence identity. Three main domains: N-terminal domain (active catalytically), translocation domain, and
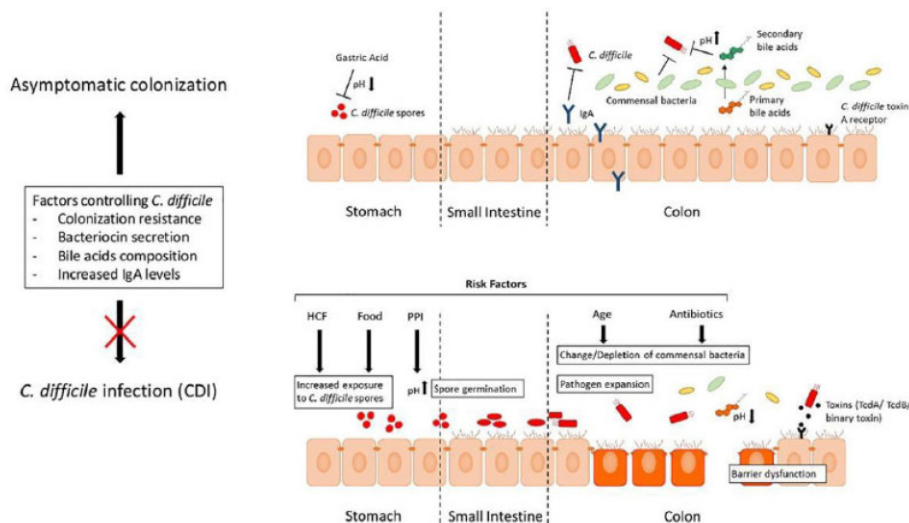

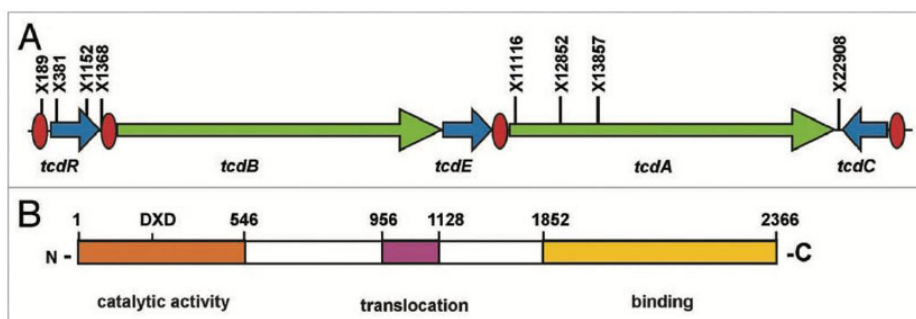**Figure 1**. *C. difficile* colonization to infection.[1]

Figure 2. PaLoc genetic organization and toxin structure. (A) PaLoc genetic structure of *C. difficile*. PaLoc coordinates are shown, and the red color of ovals indicate promoters of gen. (B) TcdB structure. The coordinates depicts number of amino acid.[41]
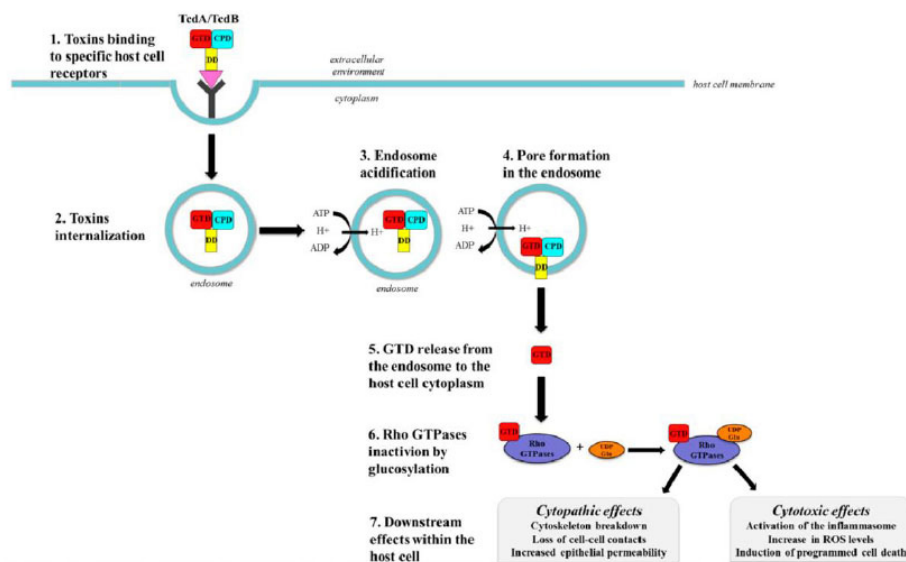

Figure 3. Delivery of toxins into the host cell cytosol.[46]

C-terminal (Figure 2).[41] The toxins are mono glycosyltransferases with similar substrate specificity; both catalyze transfer process of glucose to GTPases in the Rho group (Cdc42, Rho, Rac) in aimed cells of target.[24,37-47]

Mechanism of Action of TcdA and TcdB

Main steps divided into seven paths: (1) binding of the toxin to host cell surface receptors; (2) toxin internalization via endocytosis mediated by receptor; (3) acidification of endosome; (4) subsequent pore formation; (5) divulge of GTD in endosomes to cytoplasm of host cell; (6) Rho GTPases inactivation by process of glycosylation; and (7) cytopathic and toxin-induced cytotoxic effects.[46]

The intoxication pathway starts from endocytic absorption of toxins via a clathrin- and dynamin-dependent mechanism.[44,45] Both toxins enter the cell due to their receptor binding surface binding to one or more receptors present on the target cell surface. The receptors are sucrase-isomaltase and glycoprotein 96, on apical membrane of human colonocytes and cytoplasm, leads to inflammatory cascade and facilitate TcdA cytotoxicity.[45-46]

The process that proceeds from the endosome to the cytosol is called translocation and is determined by the mechanism of pore formation capable of penetrating the endosome membrane and by allowing the release of toxins into the cytosol of the host cell. Once in the cytosol, the toxin undergoes Insp6-dependent autocatalytic cleavage with consequent release of a glucosyltransferase domain (GTD) targeting Rho proteins in the cytosol.[45,46]

Finally, in autocatalytic process, TcdA and TcdB pathways into the host cell cytosol, then TcdA and TcdB glucosylate some elements of the Rho subfamily by transporting the glucose moiety detached from uracil diphosphate glucose (UDP-glucose) to the Thr35/37 residue of the Rho protein. Glucosylation process of Rho protein leading to inactivation therefore glucosyltransferase activity of *C. difficile* toxin is very important for CDI pathogenesis.[45-49]

CONCLUSION

C. difficile infection (CDI) has been recognized as a typical cause of HAIs and contributes to a significant proportion of morbidity and mortality among hospitalized patients. Extensive *C. difficile* culture and toxin examinations are still minimal in many hospitals in various Asian countries. Subsequently, reports of *C. difficile* in Asia are still rare, while reports of cases of CDI in Indonesia are still rare. Several risk factors including advanced age, antibiotic exposure, and hospitalization are strongly associated with CDI. The main virulence factors of *C. difficile* are TcdA and TcdB causing cytopathic and toxin-induced cytotoxic effects.

CONFLICT OF INTEREST

No competing interests regarding manuscript.

ETHICAL CONSIDERATION

Not applicable.

FUNDING

No financial support from any parties regarding manuscript.

AUTHOR CONTRIBUTION

Wedari NLPH conducted literature searches and wrote manuscript. Budayanti NNS conceptualized ideas and framework. Darwinata AE reviewed conceptual framework and final draft of manuscript.

REFERENCES

1. Schäffler H, Breitrück A. *Clostridium difficile* - From Colonization to Infection. Front Microbiol. 2018;9:646.
2. Saito R, Usui Y, Ayibieke A, et al. Hypervirulent clade 2, ribotype 019/sequence type 67 *Clostridioides difficile* strain from Japan. Gut Pathog. 2019;11:54.
3. Riley TV, Collins DA, Karunakaran R, et al. High Prevalence of Toxigenic and Nontoxigenic *Clostridium difficile* Strains in Malaysia. J Clin Microbiol. 2018;56(6):e00170-18.
4. Chrisnanda RH, Wardhani P. The incidence of *Clostridium difficile* infection in diarrhea patients after receiving antibiotics at Dr. Soetomo Hospital Surabaya. Bali Medical Journal. 2019;8(2):342-346.
5. Collins DA, Gasem MH, Habibie TH, et al. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Indonesia. New Microbes New Infect. 2017;18:34-37.
6. Collins DA, Riley TV. *Clostridium difficile* in Asia: Opportunities for One Health Management. Trop Med Infect Dis. 2018;4(1):7.
7. Shaw HA, Preston MD, Vendrik KEW, et al. The recent emergence of a highly related virulent *Clostridium difficile* clade with unique characteristics. Clin Microbiol Infect. 2020;26(4):492-498.
8. Collins DA, Hawkey PM, Riley TV. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrob Resist Infect Control. 2013;2(1):21.
9. Borren NZ, Ghadermarzi S, Hutfless S, Ananthakrishnan AN. The emergence of *Clostridium difficile* infection in Asia: A systematic review and meta-analysis of incidence and impact. PLoS One. 2017;12(5):e0176797.
10. Burke KE, Lamont JT. *Clostridium difficile* infection: a worldwide disease. Gut Liver. 2014;8(1):1-6.
11. Balsells E, Shi T, Leese C, et al. Global burden of *Clostridium difficile* infections: a systematic review and meta-analysis. J Glob Health. 2019;9(1):010407.
12. Rodriguez-Palacios A, Mo KQ, Shah BU, et al. Global and Historical Distribution of *Clostridioides difficile* in the Human Diet (1981-2019): Systematic Review and Meta-Analysis of 21886 Samples Reveal Sources of Heterogeneity, High-Risk Foods, and Unexpected Higher Prevalence Toward the Tropic. Front Med (Lausanne). 2020;7:9.
13. Davies K, Lawrence J, Berry C, et al. Risk Factors for Primary *Clostridium difficile* Infection; Results From the Observational Study of Risk Factors for *Clostridium difficile* Infection in Hospitalized Patients with Infective Diarrhea (ORCHID). Front Public Health. 2020;8:293.
14. Miranda-Katz M, Parmar D, Dang R, Alabaster A, Greenhow TL. Epidemiology and Risk Factors for Community Associated *Clostridioides difficile* in Children. J Pediatr. 2020;221:99-106.
15. Eze P, Balsells E, Kyaw MH, Nair H. Risk factors for *Clostridium difficile* infections - an overview of the evidence base and challenges in data synthesis. J Glob Health. 2017;7(1):010417.
16. Cui Y, Dong D, Zhang L, et al. Risk factors for *Clostridioides difficile* infection and colonization among patients admitted to an intensive care unit in Shanghai, China. BMC Infect Dis. 2019;19(1):961.
17. Cookson B. Hypervirulent strains of *Clostridium difficile*. Postgrad Med J. 2007;83(979):291-295.
18. Fatima R, Aziz M. The Hypervirulent Strain of *Clostridium difficile*: NAP1/B1/027 - A Brief Overview. Cureus. 2019;11(1):e3977.
19. Valiente E, Dawson LF, Cairns MD, Wren BW. Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. J Med Microbiol. 2012;61(1):49-56.
20. Yakob L, Riley TV, Paterson DL, et al. Mechanisms of hypervirulent *Clostridium difficile* ribotype 027 displacement of endemic strains: an epidemiological model. Sci Rep. 2015;5:12666.
21. Merrigan M, Venugopal A, Mallozzi M, et al. Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. J Bacteriol. 2010;192(19):4904-4911.
22. Goorhuis A, Bakker D, Corver J, et al. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008;47(9):1162-1170.

23. Muñoz M, Camargo M, Ríos-Chaparro DI, Gómez P, Patarroyo MA, Ramírez JD. Community-acquired infection with hypervirulent *Clostridium difficile* isolates that carry different toxin and antibiotic resistance loci: a case report. Gut Pathog. 2017;9:63.

24. Alcalá HL, Reigadas RE, Bouza SE. *Clostridium difficile* infection. Infección por *Clostridium difficile*. Med Clin (Barc). 2017;148(10):456-463.

25. Goldenberg JZ, Yap C, Lytvyn L, et al. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. Cochrane Database Syst Rev. 2017;12(12):CD006095.

26. Shen NT, Maw A, Tmanova LL, et al. Timely Use of Probiotics in Hospitalized Adults Prevents *Clostridium difficile* Infection: A Systematic Review With Meta-Regression Analysis. Gastroenterology. 2017;152(8):1889-1900.

27. Bouza E, Muñoz P, Alonso R. Clinical manifestations, treatment and control of infections caused by *Clostridium difficile*. Clin Microbiol Infect. 2005;11(4):57-64.

28. Predrag S. Analysis of risk factors and clinical manifestations associated with *Clostridium difficile* disease in Serbian hospitalized patients. Braz J Microbiol. 2016;47(4):902-910.

29. Tay HL, Chow A, Ng TM, Lye DC. Risk factors and treatment outcomes of severe *Clostridioides difficile* infection in Singapore. Sci Rep. 2019;9(1):13440.

30. Guh AY, Adkins SH, Li Q, et al. Risk Factors for Community-Associated *Clostridium difficile* Infection in Adults: A Case-Control Study. Open Forum Infect Dis. 2017;4(4):ofx171.

31. Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St Sauver JL, et al. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. Am J Gastroenterol. 2012;107(1):89–95.

32. Kamuju V, Kumar S, Khan WH, Vivekanandan P. Hypervirulent *Clostridium difficile* ribotypes are CpG depleted. Virulence. 2018;9(1):1422-1425.

33. Thornton CS, Rubin JE, Greninger AL, Peirano G, Chiu CY, Pillai DR. Epidemiological and genomic characterization of community-acquired *Clostridium difficile* infections. BMC Infect Dis. 2018;18(1):443.

34. Gupta A, Khanna S. Community-acquired *Clostridium difficile* infection: an increasing public health threat. Infect Drug Resist. 2014;7:63-72.

35. Al Assaad R, Dakessian A, Bachir R, Bizri AR, El Sayed M. Significance of *Clostridium difficile* in community-acquired diarrhea in a tertiary care center in Lebanon. Sci Rep. 2020;10(1):5678.

36. Predrag S, Kuijper EJ, Nikola S, Vendrik KEW, Niko R. Recurrent community-acquired *Clostridium (Clostridioides) difficile* infection in Serbian children. Eur J Clin Microbiol Infect Dis. 2020;39(3):509-516.

37. Oka K, Osaki T, Hanawa T, et al. Molecular and microbiological characterization of *Clostridium difficile* isolates from single, relapse, and reinfection cases. J Clin Microbiol. 2012;50(3):915-921.

38. Napolitano LM, Edmiston CEJ. *Clostridium difficile* disease: Diagnosis, pathogenesis, and treatment update. Surgery. 2017;162(2):325-348.

39. Edwards AN, Suárez JM, McBride SM. Culturing and maintaining *Clostridium difficile* in an anaerobic environment. J Vis Exp. 2013;(79):e50787.

40. Piacenti FJ, Leuthner KD. Antimicrobial stewardship and *Clostridium difficile*-associated diarrhea. J Pharm Pract. 2013;26(5):506-513.

41. Curcio D, Cané A, Fernández FA, Correa J. *Clostridium difficile*-associated Diarrhea in Developing Countries: A Systematic Review and Meta-Analysis. Infect Dis Ther. 2019;8(1):87-103.

42. Vuotto C, Donelli G, Buckley A, Chilton C. *Clostridium difficile* Biofilm. Adv Exp Med Biol. 2018;1050:97-115.

43. Miura M, Kato H, Matsushita O. Identification of a novel virulence factor in *Clostridium difficile* that modulates toxin sensitivity of cultured epithelial cells. Infect Immun. 2011;79(9):3810-3820.

44. Lim SC, Knight DR, Riley TV. *Clostridium difficile* and One Health. Clin Microbiol Infect. 2020;26(7):857-863.

45. Chen S, Sun C, Wang H, Wang J. The Role of Rho GTPases in Toxicity of *Clostridium difficile* Toxins. Toxins (Basel). 2015;7(12):5254-5267.

46. di Bella S, Ascenzi P, Siarakas S, Petrosillo N, di Masi A. *Clostridium difficile* Toxins A and B: Insights into Pathogenic Properties and Extraintestinal Effects. Toxins (Basel). 2016;8(5):134.

47. Janoir C. Virulence factors of *Clostridium difficile* and their role during infection. Anaerobe. 2016;37:13-24.

48. Lyras D, O'Connor JR, Howarth PM, et al. Toxin B is essential for virulence of *Clostridium difficile*. Nature. 2009;458(7242):1176-1179.

49. Taggart MG, Snelling WJ, Naughton PJ, La Ragione RM, Dooley JSG, Ternan NG. Biofilm regulation in *Clostridioides difficile*: Novel systems linked to hypervirulence. PLoS Pathog. 2021;17(9):e1009817.