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ORIGINAL ARTICLE

Antibiotic associated diarrhea due to *Clostridioides difficile* in a tertiary care teaching hospital, central India

Sunandini Kapoor[1], Shashwati Nema[1*], Debasis Biswas[1], Sagar Khadanga[2], Saurabh Saigal[3], Mahesh Maheshwari[4]

[1]Department of Microbiology, All India Institute of Medical Sciences, Bhopal, India
[2]Department of General Medicine, All India Institute of Medical Sciences, Bhopal, India
[3]Department of Anesthesiology and Critical Care, All India Institute of Medical Sciences, Bhopal, India
[4]Department of Pediatrics, All India Institute of Medical Sciences, Bhopal, India

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ABSTRACT

Background and Objectives: The misuse of antibiotics in recent years has led to an increase in antibiotic associated diarrheas (AAD). Out of several implicated pathogens, *Clostridioides difficile* is responsible for causing 15-25% of all cases of AAD. However, it has remained under diagnosed for a long time. The current study is planned to explore prevalence of *C. difficile* amongst AAD patients and to study clinical presentation and associated risk factors.

Materials and Methods: Hospital based cross sectional study conducted in patients above 2 years of age. Diagnosis of *C. difficile* was done by two modalities i.e. glutamate dehydrogenase test followed by toxin detection using enzyme immunoassay and stool culture followed by toxin gene detection.

Results: Twelve of 65 patients (18.4%) were positive for *C. difficile.* Maximum cases were found in younger age group. Abdominal pain and fever were most common complaints. 12 (18.4%) out of 65 study subjects were found to be positive by ELISA. 2/65 (3%) patients were positive for culture with presence of only *tcdB* gene. Ceftriaxone was the most commonly used antibiotic (25%).

Conclusion: *C. difficile* is significant pathogen implicated in AAD with a prevalence rate of 18.4%. GDH antigen detection followed by Toxin A/B ELISA for *C. difficile* yielded better detection rate as compared to stool culture.

Keywords: *Clostridioides difficile*; Diarrhea; Antibiotics

INTRODUCTION

Antibiotic associated diarrhea (AAD) is among the commonest intestinal complications of antibiotic use. An overuse of antibiotics alters the gut flora thereby facilitating the germination and vegetative growth of the pathogenic organisms such as *Clostridioides difficile, Clostridium perfringens, Staphylococcus aureus, Klebsiella oxytoca,* and *Candida albicans.* Out of these, *Clostridioides difficile* is the most important and most commonly implicated pathogen (1).

Clostridioides difficile (formerly known as *Clostridium difficile*) (2) is a Gram positive, spore forming, anaerobic bacillus. It causes disease in humans by the production of two protein exotoxins- toxin A and toxin B. It is responsible for causing 15-25% of

*Corresponding author: Shashwati Nema, MD, Department of Microbiology, All India Institute of Medical Sciences, Bhopal, India.
Tel: +91-8085850616 Email: snema.microbiology@aiimsbhopal.edu.in

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all cases of AAD (3). Over the past few years, there has been a significant rise in cases of *C. difficile* infections. The proportion of patients who had complicated cases (defined as development of megacolon, perforation, colectomy, shock requiring vasopressor support, mortality) has increased from 7.1 to 18.2 percent and 30-day mortality has increased from 4.7 to 13.8 percent (4). In 2019, this bacterium was listed as an urgent antibiotic resistant threat by Centre for Disease Control (CDC) making it an emerging cause of concern worldwide (5). AAD due to *C. difficile* has remained underdiagnosed for a long period of time owing to the limited availability of diagnostic facilities. Being an emerging cause of morbidity and mortality among patients, it is becoming increasingly important to study the disease burden of this organism and device measures to control it. Therefore, the present study was planned to explore the prevalence of *C. difficile* amongst AAD patients and to study the clinical presentation and associated risk factors. Efforts were made to compare the available diagnostic modalities for detection of *C. difficile viz.* ELISA for toxin A and toxin B detection and culture followed by toxin gene detection.

MATERIALS AND METHODS

Ethical considerations. The study was conducted after approval of Institutional Human Ethics Committee (Ethical clearance reference no. IHEC-LOP/2019/MD0113). Written informed consent of all recruited participants was obtained. For participant below 18 years, informed assent was obtained.

Study setting. A hospital based cross sectional study was conducted in anaerobic bacteriology laboratory, Department of Microbiology of our institute from October 2019 to March 2021.

Study participants. Patients more than 2 years of age, passing at least 3 unformed stools over a period of 24 hours, those who had received antibiotics within 8 weeks of onset of diarrhea and visiting the OPD/admitted in IPD/ICU of our institute were included in the study (6). Patients on laxatives or any other form of stool softeners and patients on chemotherapy were excluded from the study (7).

Sample collection and storage. Stool samples from the recruited participants were collected in a sterile wide mouthed container and were immediately transported to the laboratory for further processing.

Microbiological analysis. After assigning the laboratory number, the stool sample was divided into 2 equal parts for *C. difficile* toxin detection and stool culture for *C. difficile* followed by PCR for toxin gene detection.

***C. difficile* toxin detection.** It was performed by two step approach as per CDC protocol i.e. Glutamate Dehydrogenase (GDH) test followed by *C. difficile* toxin A and toxin B detection.

GDH test. The stool sample was screened for GDH by immunochromatography based EpiTuub®Fecal *C. difficile* Antigen Rapid Tes Kit (Epitope Diagnostics, Inc., San Diego, California, United States) by strictly following manufacturer's instructions (Fig. 1).

Enzyme linked immunosorbent assay (ELISA) for toxin detection. All GDH positive samples were tested for *C. difficile* toxin A and toxin B by ELISAusing Fecal *C. difficile* ToxinA and BELISAkit (Epitope Diagnostics, Inc., SanDiego, California,

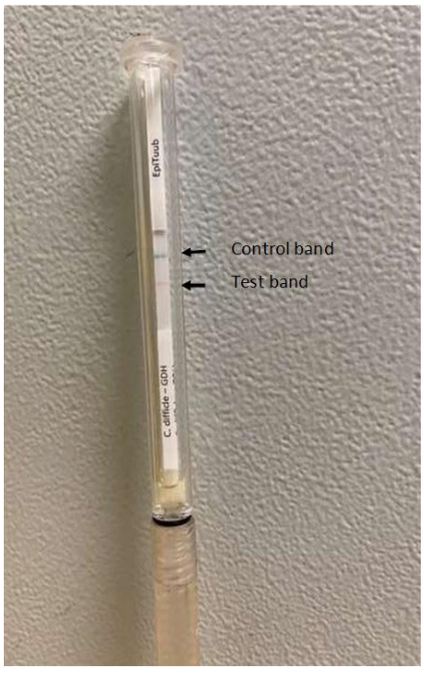

Fig. 1. Glutamate Dehydrogenase (GDH) antigen detection test showing positive test result. The controlline is shownin-green and the test line is shown in pink.

United States) as per manufacturer's instructions (8).

Stool culture. The stool sample was pre-treated with absolute ethanol for 1 hour at 37°C and inoculated on Robertson Cooked Meat (RCM) medium. After 48 hours, subcultures from RCM were made on cycloserine cefoxitin fructose agar (CCFA) and Brucella blood agar (BBA). Suspected *C. difficile* isolates showing grey, flat to slightly raised colonies with 2-3 mm diameter, rugose margins and typical "horse-barn" odour on Brucella Blood Agar (Fig. 2), were identified by both standard conventional biochemical tests (catalase, gelatin liquefaction, Indole test, nitrate reduction, lecithinase test, lipase test and carbohydrate fermentation test) and Vitek2 automated system (BioMerieux, USA).

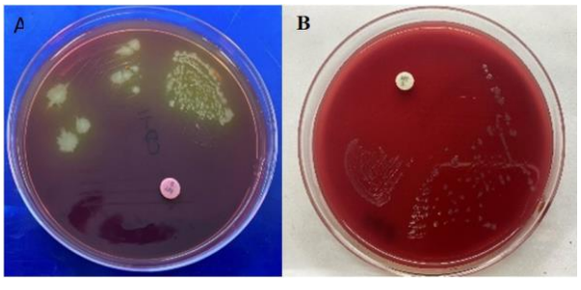

Fig. 2. A: Growth of *C. difficile* on Cycloserine Cefoxitin Fructose Agar showing large, flat, yellow colonies with ground glass appearance and typical "horse-barn" odour. B: Growth of *C. difficile* on Brucella Blood Agar showing grey, flat to slightly raised colonies with 2-3mm diameter, rugose margins

Conventional PCR for toxin gene detection. Bacterial DNA from the confirmed *C. difficile* isolates was extracted using QIA amp DNA kits (QIAGEN, Maryland, United States). A set of forward primer i.e.5'-TGATGCTAATAATGAATCTAAAATGTA-AC-3' and reverse primer i.e.3'-CCACCAGCTG-CAGCCATA-5' targeting 1200 bp region of *tcdA* was used. Another set of forward primer i.e.5'-GT-GTAACCTACTTTCATAACACCAG-3' and reverse primer i.e. 3'-GGTGGAGCTTCAATTGGAGAG-5' targeting 399 bp region of *tcdB* was used (Cella solutions, New Delhi, India). Mastermix preparation was done by using 1× concentration of PCR buffer, 100mmol of extracted DNA, 10 pmol of forward and reverse primer each, 1.2U of Taq polymerase (Thermo Fisher Scientific Inc, USA).

The PCR cycle (Table 1) consisted of initial denaturation at 95°C for 2 minutes followed by 30 cycles each of denaturation at 95°C, annealing at 52°C and extension at 72°C. A final extension at 72°C was provided for 10 minutes. Amplification of target sequence was followed by gel electrophoresis with 1% agarose gel stained with ethidiumbromide for detection of *tcdA* and *tcdB* (9).

Table 1. Steps of PCR reaction

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 minutes	1
Denaturation	95°C	1 minute	30
Annealing	52°C	1 minute	30
Extension	72°C	1 minute	30
Final extension	72°C	10 minutes	30
Whole time	4°C	∞	

Statistical methods. The collected data were transformed into variables, coded and entered in Microsoft Excel. Data were analyzed and statistically evaluated using SPSS-PC-25 version. Quantitative data was expressed in mean ± standard deviation or median with interquartile range. Depending on normality distribution, differences between two comparable groups were tested by student's t-test (unpaired) or Mann-Whitney U test. Qualitative data were expressed in percentage and statistical differences were tested by chi square test or Fisher's exact test. *P* value less than 0.05 was considered statistically significant.

RESULTS

Out of 65 AAD patients recruited in this study, 12 (18.4%) were found to be positive for *C. difficile* infection. The highest numbers of cases were found in the age group of 31-40 years (33.3%) without any gender predilection. Majority of the patients presented with abdominal pain and fever. Two out of 65 patients presented on OPD basis. No statistically significant difference was found in duration of hospital stay in *C. difficile* positive and negative groups (Table 2).

C. difficile detection was compared by two methods i.e. GDH antigen detection followed by toxin detection by ELISA and culture followed by toxin gene detection by PCR. 12 (18.4%) out of 65 study subjects were found to be positive for toxin A and B by enzyme immunoassay. 2 (3%) out of 65 patients were positive for culture. Both culture positive samples were also positive by enzyme immunoassay (Table 3).

Table 2. Demographic and clinical characteristics of study participants

Parameters	*C. difficile* +ve n= 12	*C. difficile* –ve n= 53	*p* value
Mean age	30.58 ± 16.01	36.08 ± 18.22	-
Gender distribution			
Males	6	36	0.24
Females	6	17	
Clinical Presentation			
Fever	7	23	0.35
Abdominal pain	7	29	0.82
Abdominal distention	6	36	0.24
Nausea/Vomiting	4	23	0.75
Risk Factors			
Use of Proton Pump Inhibitors (PPI)	7	29	1.0
Diabetes	5	13	0.28
Steroid use	2	5	0.60
Surgical procedure	2	8	1.0
Hypertension	1	8	1.0
Congestive Heart Disease	0	1	1.0
Irritable Bowel Disease	0	1	1.0
Pancreatic disease	0	1	1.0
Liver disease	0	4	1.0
Previous hospitalization	0	8	0.33
Median duration of hospital stay (days)	5	6.5	0.63

Table 3. Diagnostic modalities used for *C. difficile* detection

Diagnostic modality	*C. difficile* +ve N (%)	*C. difficile* -ve N (%)
Toxin detection		
• GDH antigen	12 (18.4%)	53 (81.5%)
• ELISA for toxin A and B	12 (18.4%)	53 (81.5%)
Culture followed by toxin Gene detection		
• *C. difficile* isolation	2 (3%)	63 (96.9%)
• *tcdA* gene	0 (0%)	65 (100%)
• *tcdB* gene	2 (3%)	63 (96.9%)

Among the confirmed cases of AAD due to *C. difficile*, ceftriaxone was found to be the most commonly used antibiotic (25%) followed by amoxicillin clavulanic acid (16.6%), meropenem (16.6%) and norfloxacin (16.6%).

Leukocytosis and thrombocytosis was noted among the *C. difficile* infected patients. Statistically significant difference was observed in serum potassium levels among *C. difficile* infected group (p value<0.01). Other biochemical parameters were found within normal limits.

DISCUSSION

C. difficile infection is regarded as the most important cause of antibiotic associated diarrhea, especially in those who have undergone recent hospital stay. *C. difficile* infection rate in our study was found to be 18.4% (Table 2). The prevalence of AAD due to *C. difficile* across the world and in India is very variable. Various studies in India have shown a prevalence rate between 1.2% and 29% (10-12). The difference in prevalence of *C. difficile* in various regions may be

attributed to the variability in hospital infection control practices and diagnostic modalities being used in different settings.

In the present study, the highest numbers of cases were found in younger population (Fig. 3). In a study done by Ingle et al. the mean age of patients with *C. difficile* associated diarrhea was 45.2 years which represents a relatively younger population group (13). Several other studies have reported a higher rate of *C. difficile* positive cases in elderly population of the age group 51-60 years (14, 15). The rate of isolation of *C. difficile* is different in adult and pediatric population. In our study, 3 out of 65 cases were from pediatric age group and 9 out of 65 cases were from adults. Elgendy et al. have reported similar finding in their study with a higher rate of isolation of toxigenic *C. difficile* from adult population (16).

Among the *C. difficile* positive cases, abdominal pain (58.3%) and fever (58.3%) were observed as the most commonly associated complaints (Table 2). Study by Chaudhary et al. in 2017 supports our findings where the most commonly associated clinical features were fever (50%) followed by abdominal pain (39.6%) (14). The higher incidence of fever and abdominal cramps point towards the inflammatory nature of *C. difficile* diarrhea.

Majority of *C. difficile* infected patients had history of Proton Pump Inhibitors (PPI) intake (Table 2). This finding is supported by several studies that have shown PPI use as a major risk factor developing *C. difficile* associated diarrhea (17). Diabetes mellitus and steroid use were found as the other most common risk factor in our study. Previous study conducted by Elgendy et al. (16) has reported similar finding, however, the exact mechanism underlying the increased incidence of AAD due to *C. difficile* in patients of diabetes mellitus type 2 is yet to be fully understood. Presence of IgG antibody against toxin A offers protection against *C. difficile* infection. This function is hampered in steroid use leading to increase in its incidence in patients on chronic steroid therapy (18).

12 (18.4%) out of 65 suspected AAD cases were found to be positive for toxin A and B by enzyme immunoassay (Table 3). Enzyme immunoassays detect the presence of toxin directly from the sample and are becoming increasingly popular in diagnostic laboratories due to rapid turnaround time, low cost and ease of use. The enzyme immunoassays are found to have sensitivity and specificity of 100% and 89.7% respectively and an accuracy of 91.6% making them a quick and reliable method for diagnosis of *C. difficile* from stool samples (16). Two (3%) out of 65 patients were positive for culture which were also positive by enzyme immunoassay. Similar results were

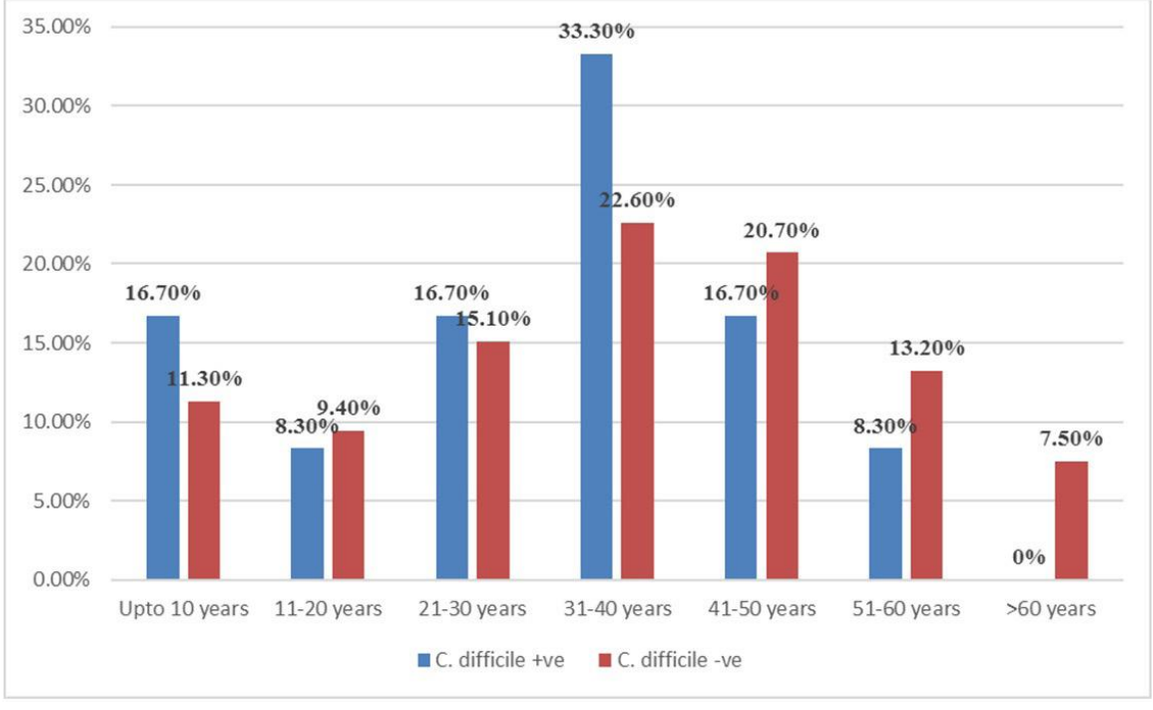

Fig. 3. Age distribution of study subjects

observed in a study by Mirzaei where the frequency of isolation of toxigenic strains of *C. difficile* in stool in hospitals in Babol were found to be 2% (19). Lower culture positivity rates can occur due to the delay transport of sample to the laboratory leading to loss of viability of the *C. difficile* and overgrowth of other organisms, inefficient anaerobiosis, intermittent shedding of *C. difficile* in stool, dilutional effect of liquid stool and lack of standardized methods of stool culture of *C. difficile* (20). However, *C. difficile* culture is required for studying the characteristics, presence of toxin genes and antimicrobial susceptibility of the isolates. In our study, both the culture positive isolates were tested for the presence of *tcdA* and *tcdB* gene. Both isolates showed the presence of only *tcdB* gene. Several studies have reported presence of *toxA-/toxB+*in clinical samples. Predrag et al. reported three toxigenic types in the *C. difficile* isolates out of which Toxin A-/ ToxinB+ accounted for 3% of the total number of isolates (21).

Ceftriaxone was the most commonly used antibiotic in our study group followed by amoxicillin clavulanic acid, meropenem and norfloxacin. A statistically significant difference was found for the use of norfloxacin (p value<0.01) among *C. difficile* infected patients. Administration of broad-spectrum antimicrobial agents impairs the growth of normal flora and promotes proliferation of *C. difficile*. The most commonly implicated antibiotics causing AAD due to *C. difficile* are reported as clindamycin, cephalosporins, fluoroquinolones, ampicillin/amoxicillin which is similar to the findings reported in this study. Vancomycin and metronidazole are the mainstay antibiotics used in the treatment of *C. difficile*. In our study, the patients who were administered vancomycin and metronidazole were also found to be positive for *C. difficile*. In a study by Elgendy et al. the susceptibility of *C. difficile* to vancomycin and metronidazole was 66.7% and 48.2%. This is suggestive of the emerging resistance in *C. difficile* for these antibiotics which is a matter of concern (16).

The mean WBC count of *C. difficile* positive patients was higher as compared to *C. difficile* negative group. In a study done by Bulusu et al. it was found that leukocytosis was common in *C. difficile* positive patients (Mean=15,800/mm^3) as compared to *C. difficile*-negative patients (Mean=7700/mm^3) thereby supporting the finding of the present study (22). Presence of leucocytosis in *C. difficile* positive patients points towards an inflammatory nature of diarrhea. Moreover, thrombocytosis was also observed in *C. difficile* positive patients. In our study, statistically significant difference was observed in the values of serum potassium in *C. difficile* positive and *C. difficile* negative (3.94 +/- 0.88) patients. There is a further need to explore the possibility of association between serum potassium levels and *C. difficile* infection.

The elimination of *C. difficile* spores from the hospital settings poses a major challenge for infection control. Prolonged hospitalization coupled with other risk factors elevates the morbidity and mortality due to *C. difficile* associated diarrhea. Hence, it becomes imperative to take necessary steps to control hospital acquired infections of *C. difficle*. IDSA guidelines suggests measures that must be taken to prevent the spread of this pathogen. Some of these include- contact precaution, hand hygiene, terminal disinfection using sporicidal agents, minimizing the frequency and duration of high-risk therapy etc. The need of the hour to prevent the spread of *C. difficile* infection is to devise a bundle care strategy and implement stringent disinfection measures in the hospital environment.

The present study suffers inherent limitation of small sample size. Since it is single hospital based study, actual prevalence and mortality of AAD due to *C. difficile* in our geographical area could not be commented upon. Moreover, *C. difficile* negative stool samples were not tested for presence of other enteric pathogens like *C. perfringens*, *S. aureus, K. oxytoca, C. albicans* and other intestinal parasites.

CONCLUSION

C. difficile is a significant pathogen implicated in AAD in our hospital with a prevalence rate of 18.4%. Maximum number of *C. difficile* positive cases was found in the younger age group without any gender predilection. GDH antigen detection followed by Toxin A/B ELISA for *C. difficile* yielded better detection rate as compared to stool culture for *C. difficile* followed by toxin gene detection. Leucocytosis, thrombocytosis and hypokalaemia may provide supportive evidence for *C. difficile* infections in resource limited settings. Patients on PPI/steroid use or suffering from diabetes mellitus are at a high risk of acquiring *C. difficile* infection. Judicious use of antibiotics particularly third generation cephalospo-

rins and fluoroquinolones is the need of the hour to prevent AAD due to *C. difficile*.

REFERENCES

1. Larcombe S, Hutton ML, Lyras D. Involvement of bacteria other than *Clostridium difficile* in antibiotic- associated diarrhoea. *Trends Microbiol* 2016; 24: 463-476.
2. The Lancet Infectious Diseases. *C. difficile*-a rose by any other name. *Lancet Infect Dis* 2019; 19: 449.
3. Barbut F, Petit JC. Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* 2001; 7: 405-410.
4. Depestel DD, Aronoff DM. Epidemiology of *Clostridium difficile* infection. *J Pharm Pract* 2013; 26: 464-475.
5. Kadri SS. Key Takeaways from the U.S. CDC's 2019 antibiotic resistance Threats report for frontline providers. *Crit Care Med* 2020; 48: 939-945.
6. Fekety R. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American college of gastroenterology, practice parameters committee. *Am J Gastroenterol* 1997; 92: 739-750.
7. Neemann K, Freifeld A. *Clostridium difficile*-associated diarrhea in the oncology patient. *J Oncol Pract* 2017; 13: 25-30.
8. Crobach MJT, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, et al. European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for *Clostridium difficile* infection. *Clin Microbiol Infect* 2016; 22 Suppl 4: S63-81.
9. Kato N, Ou CY, Kato H, Bartley SL, Brown VK, Dowell VR, et al. Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. *J Clin Microbiol* 1991; 29: 33-37.
10. Chaudhry R, Joshy L, Kumar L, Dhawan B. Changing pattern of *Clostridium difficile* associated diarrhoea in a tertiary care hospital: a 5 year retrospective study. *Indian J Med Res* 2008; 127: 377-382.
11. Monaghan TM, Biswas R, Sattav A, Ambalkar S, Kashyap RS. Clostridioides difficile epidemiology in India. *Anaerobe* 2022; 74: 102517.
12. Segar L, Easow JM, Srirangaraj S, Hanifah M, Joseph NM, Seetha KS. Prevalence of *Clostridium difficil*e infection among the patients attending a tertiary care teaching hospital. *Indian J Pathol Microbiol* 2017; 60: 221-225.
13. Ingle M, Deshmukh A, Desai D, Abraham P, Joshi A, Rodrigues C, et al. Prevalence and clinical course of *Clostridium difficile* infection in a tertiary-care hospital: a retrospective analysis. *Indian J Gastroenterol* 2011; 30: 89-93.
14. Chaudhry R, Sharma N, Gupta N, Kant K, Bahadur T, Shende TM, et al. Nagging presence of *Clostridium difficile* associated diarrhoea in North India. *J Clin Diagn Res* 2017; 11: DC06-DC09.
15. Rodríguez-Varón A, Muñoz OM, Pulido-Arenas J, Amado SB, Tobón-Trujillo M. Antibiotic-associated diarrhea: Clinical characteristics and the presence of *Clostridium difficile*. *Rev Gastroenterol Mex* 2017; 82: 129-133.
16. Elgendy SG, Aly SA, Fathy R, Deaf EAE, Abu Faddan NH, Abdel Hameed MR. Clinical and microbial characterization of toxigenic *Clostridium difficile* isolated from antibiotic associated diarrhea in Egypt. *Iran J Microbiol* 2020; 12: 296-304.
17. Ghia CJ, Waghela S, Rambhad GS. Systematic literature review on burden of *Clostridioides difficile* infection in India. *Clin Pathol* 2021; 14: 2632010X211013816.
18. Peterson LR, Kelly PJ, Nordbrock HA. Role of culture and toxin detection in laboratory testing for diagnosis of *Clostridium difficile*-associated diarrhea. *Eur J Clin Microbiol Infect Dis* 1996; 15: 330-336.
19. Mirzaei EZ, Rajabnia M, Sadeghi F, Ferdosi-Shahandashti E, Sadeghi-Haddad-Zavareh M, Khafri S, et al. Diagnosis of *Clostridium difficile* infection by toxigenic culture and PCR assay. *Iran J Microbiol* 2018; 10: 287-293.
20. Edwards AN, Suarez JM, McBride SM. Culturing and maintaining *Clostridium difficile* in an anaerobic environment. *J Vis Exp* 2013; 79: e50787.
21. Predrag S, Branislava K, Miodrag S, Biljana M– S, Suzana T, Natasa M– T, et al. Clinical importance and representation of toxigenic and non-toxigenic *Clostridium difficile* cultivated from stool samples of hospitalized patients. *Braz J Microbiol* 2012; 43: 215-223.
22. Bulusu M, Narayan S, Shetler K, Triadafilopoulos G. Leukocytosis as a harbinger and surrogate marker of *Clostridium difficile* infection in hospitalized patients with diarrhea. *Am J Gastroenterol* 2000; 95: 3137-3141.