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Prevalence of Antibiotics Associated Diarrhoea by Clostridium Difficile Through Detection of Toxins in a Tertiary Care Hospital, Patna

[1]Randhir Kumar, [2]Samir Kumar, [3]Namrata Kumari, [4]Shailesh Kumar, [5]Kumar Saurabh, [6]Richa Sinha

[1,5,6]Assistant Professor, [3,4]Professor Department of Microbiology, IGIMS, Patna, Bihar, India

[2]Assistant Professor, Department of General Medicine, IGIMS, Patna, Bihar, India

Correspondence:

Randhir Kumar

Assistant Professor, Department of Microbiology, IGIMS, Patna, Bihar, India

ABSTRACT

Background: Clostridium difficile (C. difficile) is the most common antibiotic-associated diarrhoea agent in selected patients. C. difficile strains are classified as toxigenic or non-toxigenic based on their ability to produce toxins.

Aim: The frequency of C. difficile and CDAD among patients in a tertiary hospital in Patna, India was investigated in this study.

Methods: From the patients, a total of 233 diarrheal samples were extracted. The samples were cultivated on Clostridium difficile medium with cycloserine (500 mg/L), cefoxitin (16 mg/L), and lysozyme (5mg/L) and 5 per cent defibrinated sheep blood. Polymerase chain reaction (PCR) of the 16s rRNA gene identified the isolates as C. difficile, as did the presence of toxins genes (tcdA, tcdB, cdtA, and cdtB). The toxin production of isolates was then assessed using Rapid Card (CerTest BioTech SL Spain).

Results: C. difficile was identified from 49 (21%) of the 233 samples tested. The total isolates were classified as $A^-/B^-/GDH^-$ (48.97%), $A^+/B^-/GDH^-$ (28%), $A^+/B^+/GDH^-$ (20.4%) and $A^+/B^+/GDH^+$ (2%) types. Both types of C.difficile, $A^-/B^-/GDH^-$and $A^+/B^-/GDH^-$, which account for 77.5 per cent of all isolates, were both unable to generate the toxin (nontoxigenic). However, $A^+/B^+/GDH^+$ and $A^+/B^+/GDH^-$ (22.5%), were able to manufacture toxin or were toxigenic.

Conclusion: The prevalence of C. difficile was approximately 21%, and only 22.4 percent of C. difficile isolates were capable of producing toxins. C. difficile $A^+/B^+/GDH^\pm$ are likely to be toxigenic and linked to C. difficile-associated diarrhoea (CDAD). Furthermore, approximately 4.7 percent of hospitalised patients had CDAD, which is greater than the rates reported in developed countries. Notably, 28% of the isolates were C. difficile $A^+/B^-/GDH^-$, which only possesses tcdA genes but does not produce toxin.

Keywords: Clostridium difficile, Intensive Care Unit, CDAD**.**

INTRODUCTION

Clostridium difficile (C.difficile), a rod-shaped gram-positive anaerobic spore-forming bacterium, is found in 1-3 percent of healthy people and 15-20 percent of babies [1, 2]. Clostridium difficile is the most common infectious cause of nosocomial diarrhoea, as well as a significant cost burden on health-care systems [3]. It is responsible for around 25% of reported antibiotic-associated diarrhoea cases and almost all instances of pseudomembranous colitis (PMC) worldwide [4, 5]. C. difficile infection (CDI) progresses from asymptomatic carriage to self-limited, mild, watery diarrhoea, intestinal perforation, toxic megacolon, sepsis, fulminant colitis, and death [6, 7, 8].Antibiotic use, advanced age, and unsafe exposure to healthcare facilities have all been identified as major CDI risk factors. Since the introduction of the C. difficile 027/NAP1/BI strain, which has been linked in big outbreaks, the

epidemiology of CDI has shifted, with a significant increase in the disease's incidence and severity. Clindamycin, penicillins, sulfonamides/Trimethoprim, cephalosporins, aminoglycosides, macrolides, and quinolones are examples of broad-spectrum antibacterial drugs that can promote CDAD [10, 11].

Antibiotics disturb microbiome, promoting C. difficile colonisation and overgrowth. The bacterium causes diarrhoea by producing toxins A and B, which promote gut inflammation. Some strains also produce binary toxin, commonly known as C. difficile toxin, which is an actin-ADP-ribosylating toxin (CDT) [12]. CDT positive strains are more frequently associated with severe illness. In hospitalised patients, the risk of CDAD is approximately 20-25 percent, with 3-5 percent suffering from severe diarrhoea [13]. In the patients, the mortality rate with fulminant CDAD might range from 34.7 to 57 percent [14].

Based on the type of toxin produced, C. difficile isolations are categorised as follows: A- /B- /CDT, which is the non-toxin-producing type, while A- /B- /CDT+, A- /B+/CDT+, A- /B+/CDT-, A+/B+/CDT-, A+/B+/CDT+, A+/B- /CDT+ and A+/B- /CDT- are considered as toxin production types [15]. The strains' frequency in clinical samples varies. The majority of the literature links CDAD to all strains, while some findings show that C. difficile A- /B- /CDT- may be normal flora and not associated with diarrhoea [16]. Furthermore, there have been few studies that show the involvement of C. difficile A+/B- /CDT in human infectivity and its relationship to CDAD [17]. As a result, the goal of the study was to assess the frequency of C. difficile toxin production types and CDAD in diarrhoea hospitalised patients among the selected patients, which could be relevant given that there are few publications in this regard from India.

METHODS

PATIENTS AND SAMPLES

During March 2020- January 2022, 233 diarrheal stool samples were collected from the patients of a selected tertiary hospital in Patna, India. All patients who had more than three bowel motions per day and who had received antibiotics were included in the trial; those who had not received antibiotics were excluded. The patients were admitted to the ICU and wards from different departments. For future research, the stool samples were frozen at -20ºC. Sufficient volumes of thawed samples were cultivated on Clostridium difficile medium with 5 percent defibrinated sheep blood containing cycloserine (500 mg/L), cefoxitin (16 mg/L), and lysozyme (5mg/L) after being heated at 80 ºC for 10 minutes. Cultured plates were incubated at 37 ºC in anaerobic jars for 48-72 hours, and suspected colonies with a distinct odour, non-hemolytic activity, and spore stain (sub-terminal spores) were identified as C. difficile.After cultivation on brain heart infusion (BHI) blood agar for 72-96 hours (excellent sporulation), isolates were identified as C. difficile and kept at -70 ºC in BHI broth with 40% glycerol. As mentioned subsequently, suspected isolates were confirmed by PCR based on 16s rRNA gene amplification.

DNA EXTRACTION

Before DNA extraction, the isolates were taken from the -70 ºC freezer and cultured on BHI agar with 5% defibrinated sheep blood for 24 hours in an anaerobic jar. For DNA extraction, fresh colonies were employed. For Gram positive bacteria, DNA extraction was carried out using the CinnaPureDNA extraction kit. Several colonies were chosen and dissolved in 200 L of distilled water. The bacterial suspension was stirred for 5 seconds before being centrifuged at 8000 rpm for 5 minutes. The pellet was dissolved in G prelysis buffer containing 20 L (500 g/mL) lysozyme and incubated at 37 ºC for 45 minutes before being heated to 55 ºC. The suspension was then treated with 10 L ributinase and incubated at 55 ºC for 45 minutes.The suspension was used for DNA extraction according to the kit's instructions and kept at -20 ºC for future use.

PCR ASSAY AND ELECTROPHORESIS

The PCR experiment was carried out in accordance with earlier research. Amplification of the 16S rDNA gene verified the C. difficile isolates. The thermocycler was used to do the PCR, and the amplicon was run in 1 percent agarose gel electrophoresis for 45 minutes. Green viewer stain was used to stain the gel, which was then read by gel document.

TOXIN PRODUCTION EVALUATION

To confirm toxin-producing isolates, the Clostridium difficile Toxins A&B Rapid Card (CerTest BioTech SL Spain) Kit was utilised, which detects both toxins A and B in the same sample. In brief, 106 CFU/mL fresh bacteria (cultured for 24 hours) were injected into boiled BHI broth containing 0.05 percent L-cysteine and 0.5 percent yeast extract. After 48 hours in an anaerobic jar, 1 mL of culture was taken from the tube and centrifuged for 5 minutes at 10000 rpm. According to the manufacturer's instructions, the supernatant was used to detect the toxin.

RESULTS

FREQUENCY OF C. DIFFICILE IN DIARRHEA SAMPLES

In all, 49 (21 percent) of the isolates from 233 diarrheal samples were identified as C. difficile. Twenty-four isolates (49%) lacked toxin genes, whereas 25 (51%) tested positive for toxin A. (tcdA). Eleven isolates (24.5 percent) possessed the toxin B gene (tcdB), and one isolate (cdtA and cdtB) gene were positive. In contrast, 48.97% of all isolates lacked any toxin genes ($A^-/B^-/GDH^-$), while 28.57% were positive for only $A^+/B^-/GDH^-$. However, 20.4 percent of total isolates carried the tcdA and tcdB genes ($A^+/B^+/GDH^-$) and 2.04 percent carried the tcdA, tcdB, and CDT genes ($A^+/B^+/GDH^+$) (Table 1).

Table 1: Frequency of nontoxigenic and toxigenic C. difficile in diarrhea samples

Toxin production type	No. (%)
$A^-/B^-/GDH^-$	24 (48.97 %)
$A^+/B^-/GDH^-$	14 (28.57 %)
$A^+/B^+/GDH^-$	10 (20.4 %)
$A^+/B^+/GDH^+$	1 (2.04 %)

FREQUENCY OF TOXIGENIC AND NONTOXIGENIC C. DIFFICILE ISOLATES

Toxin generation by the isolates was evaluated, and it was discovered that both kinds of C. difficile, $A^-/B^-/GDH^-$ and $A^+/B^-/GDH^-$, which account for 77.5 percent of all isolates, were unable to manufacture the toxin (nontoxigenic). The remaining C. difficile isolates, $A^+/B^+/GDH^+$ and $A^+/B^+/GDH^-$ (22.5 percent), could generate toxin or were toxigenic (Table 1).

DISCUSSION

C. difficile was found in roughly 21% of the diarrheal samples from the selected hospital in the current study. The findings revealed that 49.57 percent of C. difficile isolates lacked the tcdA, tcdB, and CDT (cdtA & cdtB) genes. About 20.4 percent of the isolates carried tcdA and tcdB genes, and one isolate (2 percent) tested positive for both tcdA and tcdB genes as well as binary toxin genes (cdtA, cdtB). Surprisingly, 77.5 percent of the total isolates were nontoxigenic ($A^-/B^-/GDH^-$ and $A^+/B^-/GDH^-$) and 22.5 percent were toxin generating or toxigenic ($A^+/B^+/GDH^-$ and $A^+/B^+/GDH^+$). Because toxigenic strains are related with CDAD, it is reasonable to conclude that around 4.7 percent of the patients with diarrhoea had CDAD.

Although diarrhoea is common, around 20-25% of these instances are caused by infectious organisms, the most common of which is Clostridium difficile. The prevalence of C. difficile infectivity among CDAD patients varies over the world. The global prevalence of CDAD is 0.9 percent in the general population and 2 percent in the patients, respectively. A similar pattern can be seen in Europe (1%) and Asia (3%) [18, 19]. Furthermore, studies have showed that C. difficile infection is responsible for 3.6 percent, 3.3 percent, 3.3 percent, 0.9 percent,

2.4 percent, and 20 percent of CDAD in hospitalised patients in the United States, Canada, the United Kingdom, France, China, and Taiwan, respectively [20, 21, 22, 23, 24, 25].

In India, the frequency of C. difficile infectivity and CDAD has received little attention, particularly among the patients. Previous Indian investigations looked at the prevalence of C. difficile and CDAD in other regions of the hospital [26, 27]. According to these investigations, the prevalence of CDAD in hospitalised patients and those with gastrointestinal problems was between 6.1- 20% and 5.3 percent, respectively [26, 27]. The current study focuses solely on the epidemiology of C. difficile in patients. Based on the findings and toxin positive strains that are more frequently associated with CDAD, the frequency of CDAD among the patients (4.7 percent) is substantially greater than in other parts of the world, and it appears that C. difficile is the primary cause of CDAD among hospitalised patients in India.

The most common type of toxin generation observed in our investigation was $A^-/B^-/GDH^-$. Several research, including ours, have found that the A-/B-/GDH toxin production type is the most common (42- 50 percent) in clinical data [28, 29]. In some investigations, they were classified as pathogenic, whereas in others, they were classified as non-pathogenic [30]. They are classified as non-pathogenic in this study.

Another toxin producing type, $A^+/B^+/GDH^-$, which is definitely connected with CDAD, has a global prevalence of up to 71.6 percent and a 100% prevalence in India. This strain was abundant in our isolates;however, it was less prevalent than in previous investigations in India and other regions of the world.

Furthermore, the prevalence of C. difficile $A^+/B^-/GDH^-$ was found to be 28.57 percent among admitted patients with diarrhoea. This form of poison manufacturing has also been discovered in India. According to Goudarzi et al. [11], 6.7 percent of C. difficile-related CDAD belonged to this kind. The precise role of these isolates in causing CDAD is unknown. Investigators have rarely reported on them. Rupnik [9] believes this is related to the incorrect primer selection, which may amplify the reaming tcdA gene in Pathogenicity Locus.Monot and colleagues [31], on the other hand, identified this toxin production type from clinical samples and discovered it to be related with CDAD. The presence of the tcdA gene was found in $A^+/B^-/GDH^-$ isolates in this investigation. The toxins were not found in the medium by a commercial Rapid Card kit. It means that $A^+/B^-/GDH^-$ isolates lack the tcdA gene or that the toxin level is too low to be detected by Rapid kit techniques.

The $A^+/B^+/GDH^+$ Toxin producing types can create a third toxin known as CDT. The prevalence of these isolates in clinical samples is increasing, rising from 0% to 45% in the last three decades. CDT has been recorded in India, with a prevalence of 32% in clinical samples [32, 33]. As a result, the prevalence of the $A^+/B^+/GDH^+$ type was low in our study (2%) compared to earlier findings from India and other geographical regions throughout the world.

In clinical samples, non-toxigenic isolates predominate. Only about 28% of isolates possess the tcdA gene, which does not produce toxin A, and their significance in CDAD is unknown. Although the incidence of $A^+/B^+/GDH^-$ and CDT-positive toxin production types is modest in comparison to the global prevalence, it remains the predominant cause of CDAD in patients. Tcd A and tcdB genes are present in 22.4 percent of C. difficile isolates. As a result, around 4.7 percent of total diarrheal patients admitted to suffer from CDAD, which is greater than in other geographical regions of the world such as developed countries [34].

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