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Original Article

Clostridium difficile Induced Diarrhoea Among Hospitalized Patients of Tertiary Care Hospitals in Dhaka

Kakali Halder[1], Maherun Nesa[1], Nusrat Noor Tanni[1], Sharmeen Ahmed[2], Shaheda Anwar[2] Sanjida Khondakar Setu[2], Ahmed Abu Saleh[2]

[1]Department of Microbiology, Dhaka Medical Collage, [2]Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University

Abstract

Clostridium difficile (C. difficile) has become a global public health challenge as *C. difficile* associated-diarrhea (CDAD) is increasing in incidence and severity of disease in several countries during recent years. This cross sectional study evaluated the frequency of CDAD among 100 adult patients who were clinically diagnosed as nosocomial diarrhoea in various clinical wards of Bangabandhu Shiekh Mujib Medical University (BSMMU) and Dhaka Medical College and Hospital (DMCH). CDAD diagnosis was based on detection of *C. difficile* along with clinical symptoms of diarrhea. Stool microscopy was done for cytology followed by anaerobic culture in cycloserine cefoxitin fructose agar (CCFA) media, confirmed by latex agglutination of culture isolates. Toxin genes (both A and B) were detected by multiplex Polymerase chain reaction (PCR) from culture isolates. Out of 100 diarrhoeal stool samples collected, 25% samples were pus cell positive in microscopy, culture yielded growth of *C. difficile* in 10% samples and all isolated *C. difficile* were confirmed by both latex agglutination and PCR. Out of 10 isolates, 7 were only *tpi* (triose phosphate isomerase) gene positive which is species-specific for *C. difficile* indicating the presence of non-toxigenic *C. difficile* and 3 isolates had both tpi and toxin genes (both *tcd*A and tcdB gene) on PCR indicative of toxigenic *C. difficile* respectively. *C. difficile* toxin gene detection by PCR along with culture is highly specific and sensitive diagnostic modality for CDAD. Differentiation between toxigenic and non-toxigenic strains by PCR may facilitate the appropriate patient management.

Key words: *Clostridium difficile* associated-diarrhea (CDAD), Nosocomial diarrhoea, Toxin, Cycloserine cefoxitin fructose agar (CCFA), Triose phosphate isomerase (tpi), Polymerase chain reaction (PCR).

Introduction:

Nosocomial diarrhoea (develops ≥3 days after hospitalization) is a common complication in hospitalized patients, especially in those who receive antibiotics ranging from 3% to 29%.[1] Approximately 15-25% of all cases of nosocomial antibiotic-associated diarrhoea (AAD), *C. difficile* is the primary cause and also of documented antibiotic-associated pseudomembranous colitis in 95-100%[2]. Exposure to this organism may lead to asymptomatic gastrointestinal tract infection, but can also lead to symptoms ranging from mild diarrhoea to severe colitis and rarely pseudomembranous colitis, intestinal perforation, toxic megacolon, sepsis and death.[3,4] About 10-20% of CDAD recur after an initial episode of *C. difficile* usually within 8-10 weeks, but when a patient has had one recurrence, rates of further recurrences increase to 40-65%.[5]

Correspondence:
Dr. Kakali Halder
Assistant Professor
Department of Microbiology
Dhaka Medical College, Dhaka.
Mobile: 01733252444, E-mail: dr.kakali7@gmail.com

Primary risk factors of nosocomial *C. difficile* infection (CDI) includes antibiotic treatment, nosocomial transmission, underlying chronic disease, prolonged hospital stay, gastrointestinal and transplant surgeries, chemotherapeutic agents, immunosuppressant, old age, nasogastric intubation and enteral tube feeding.[6,7] Secondary risk factors include vitamin D deficiency, inflammatory bowel diseases.[8] Up to 3 -5% of healthy adults may be colonized asymptomatically in their gut with *C. difficile*. The colonization rate increases markedly in the health-care setting. Within the first week of hospitalization, 13%–20% and by 4 weeks, 50% of patients are colonized with *C. difficile*.[9] Ten percent elderly patients (defined as greater than 65 years of age) are especially at risk with colonized with *C. difficile* at hospital admission.[10]

C. difficile is acquired through ingestion of spores usually transmitted from other patients through the hands of healthcare personnel or the environment and potentially facilitate the spread over greater distances by those in the carrier state.[11] *C. difficile* pathogenesis is associated with the production of two exotoxins, toxins A and B, encoded by their genes, *tcd*A and *tcd*B, which are located,

along with surrounding regulatory genes, on a 21-kilobase section of chromosomal DNA known as the pathogenicity locus (paLoc).[11] Toxin-negative *C. difficile* strains are considered as nonpathogenic.[12] In addition to toxins A and B, some strains also produce a third toxin known as binary toxin C, encoded by *ctd*A and *ctd*B, located outside the paLoc.[13] Toxin A and B act as glucosyltransferases that inactivate small GTPases such as Rho, Rac and Cdc42 within eukaryotic target cells.[14] Rho proteins are important to control cells actin cytoskeleton re-organization. So, the inactivation of Rho leads to disruption of the intracellular actin cytoskeleton, opening of tight junctions and ultimately cell death.[15] The toxins lead to a characteristic inflammatory response, which includes damage to the intestinal epithelial cells, neutrophilic infiltration, and local chemokine and cytokine secretion.[16,15] Both toxins A and B lead to activation, degranulation, and the release of inflammatory mediators from mast cells.[17]

CDAD can be diagnosed by anaerobic stool culture, cell culture cytotoxicity neutralization assay (CCNA), toxigenic stool culture, biochemical tests following culture. Stool culture for *C. difficile* is the most sensitive method, though have highest rate of false positivity and lengthy detection time5. In addition, enzyme immuneassay (EIA), latex agglutination, ICT and PCR are used for the rapid detection of toxin producing *C. difficile* from stool and PCR for detection of toxigenic and non-toxigenic strain, gives the advantages of faster detection with higher sensitivity and specificity.[18]

While an increasing number of studies have been carried out in East Asian countries, limited data are available on CDI from South Asia.[19] The reason might be the lack of suitable diagnostic facility for *C. difficile* in this region.[20] Additionally, comprehensive culture and toxin testing for *C. difficile* are lacking in the majority of hospitals in south Asian country.[21] Due to the high rate of indiscriminate and inappropriate use of antimicrobials and lack of maintenance of proper hygiene, it is conceivable that CDI is relatively common in south Asian country like Bangladesh.[22] Prompt recognition and an accurate diagnosis of CDI is required to alert healthcare providers to implement effective prevention measures, re-evaluate the need for antimicrobials, and implement effective therapy to reduce the nosocomial acquisition of this organism.[23]

Materials and methods:

Place and duration of the study: This prospective observational study was carried out in the Department of Microbiology and Immunology, BSMMU, Dhaka from September, 2016 to August, 2017.

Study population, Inclusion criteria: Stool samples were obtained from 100 adult (>18years) patients clinically diagnosed as nosocomial diarrhoea in clinical wards-ICU, Burn Unit ICU, Oncology, Gastroenterology, Hematology, Orthopedics, Palliative Care Unit, Colorectal Surgery and Internal Medicine of BSMMU and DMCH.

Methodology: Stool samples were immediately processed for microscopy and anaerobic culture and tested within 24 hours of collection. Specimens that could not be tested within this time were frozen at -200C for the further tests to be performed later. Microscopy: Microscopic examination of stool samples were done for cytological examination by saline wet mount and iodine preparation of the samples.

Anaerobic culture: Enrichment culture was performed from all stool samples to enhance the germination of *C. difficile* spores on CCFA culture media with 5% defibrinated sheep blood containing cycloserine (500mg/dl), cefoxitin (16mg/dl) and lysozyme (5 mg/dL).[24] 1 -2ml of Stool samples were inoculated on 10 ml of brain heart infusion (BHI) broth supplemented with 0.1% sodium taurocholate (Bile salt) and incubated at 37°C for 48 hours in an anaerobic jar with an AnaeroGen sachet (Oxoid, Hampshire, England) and an anaerobic indicator.[25] Alcohol shock consisted of mixing an aliquot of a stool sample with an 100-150ul of 70% ethanol for 25 to 30 minutes, followed by thorough mixing and incubation (room temperature for 1 h).[26] After incubation, each specimen was thoroughly mixed and 50 µl of the homogeneous solution was plated to CCFA (Anaerobe Systems, Morgan Hill, CA) media and incubated anaerobically at 35°C for up to 5 days. CCFA plates were read on day 3 and day 5. *C. difficile* colonies were of 4mm size or larger and typically appeared as grayish-white, circular, flat, fimbriate with irregular edged ground-glass appearance and characteristic odor was produced resembling fresh horse manure or cow dung.[23]

Latex agglutination: *C. difficile* isolates were confirmed from culture by latex agglutination test. One drop of saline, suspected colony and one drop of *C. difficile* Oxoid Latex Reagent (*C. difficile* test kit, Oxoid Microbiology Products, Thermo Scientific, Hampshire, England) were placed in the same circle of the reaction card and gently mixed by inverting the card. The reagent and the colony suspension were mixed with a clean mixing stick for 30 seconds. Then the circle in the card was checked for agglutination or clumping after 2 minutes. Each batch of samples was processed with a positive and a negative control.

Multiplex conventional PCR: *tpi* gene was detected by PCR which is species-specific for *C. difficile* and also toxin A (*tcdA*) and toxin B (*tcdB*) genes from the isolated strains. Bacterial DNA was extracted by boiling method.[26] At first, one loopful (4mm) organism (approximately 10µl) of each strain from CCFA plate was collected and suspended in 1 ml of distilled water and boiled for 20 minutes in heat-block machine (Incublock, Denville scientific inc. USA). Then it was rapidly cooled in ice for 5-10 minutes. The cell lysate was centrifuged

at 6000 rpm for 5 min. Around 200 µl supernatant was taken in a fresh microfuge tube and stored at -70°C until before further analysis via PCR. Amplification was performed in an automated DNA thermal cycler (Applied bio-system 2720) in a final 25ul reaction volume by adding free deionized water including 2 µl template DNA added to 23 µl of reaction mixture to make a final volume of 25 µl. The reaction mixture contained 2.5 µl of 10x PCR buffer (1x buffer components 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl2, pH 8.3, 0.5 µl of 10 mM deoxynucleoside triphosphate (dNTP), 0.5 µl *tpi*, 1 µl *tcdA* and 1.30 µl t*cdB* encoded primer (forward and reverse) together with 0.5 unit of Taq DNA polymerase (5U/l). *tpi*, *tcd*A and *tcd*B genes were detected from isolates by using a multiplex PCR containing 6 primers specific to the one *tpi* and two toxin genes (*tcd*A *tcd*B).[26]

Genes	Primers	Primer sequence (5'-3')	Amplicon length (bp)
tpi	tpi-F tpi-R	AAAGAAGCTACTAAGGGTACAAA CATAATATTGGGTCTATTCCTAC	230
*tcd*A	tcdA-F tcdA-R	AGATTCCTATATTTACATGACAATAT GTATCAGGCATAAAGTAATATACTTT	369
*tcd*B	tcdB-F tcdB-R	GGAAAAGAGAATGGTTTTATTAA ATCTTTAGTTATAACTTTGACATCTTT	160

F=Forward, R=Reverse

A total of 40 cycles were performed where the PCR mixers were denatured at 95ºC for 30 sec, annealing at 55ºC for 30 sec and final extension at 72ºC for 30 sec by using *C. difficile* ATCC 43255 as positive control and *E .coli* ATCC 25922 as negative control.[19, 26] The amplified products were detected by electrophoresis in 1.5% agarose gel with ethidium bromide to detect the specific DNA bands. A 100-bp DNA ladder was run alongside the specimens to verify the correct bp size of the DNA amplified (Figure-I). Following electrophoresis of the amplified products, the DNA bands of the samples was visualized by using a UV trans-illuminator.

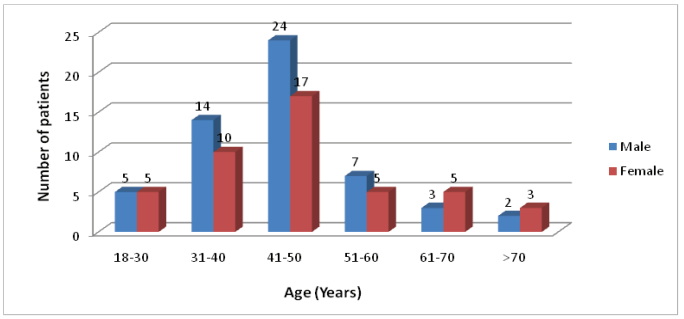
Statistical analysis:

All the data were rechecked, coded and entered in standard statistical software, SPSS software (SPSS, Statistics for Windows, Version-21.0, Armonk, NY).

Results:

A total of 100 adult clinically suspected nosocomial diarrhoea patients were recruited in this study with history of antibiotic, chemotherapy, H_2 blocker, proton pump inhibitor and steroid intake. Majority of the patients (41%) belonged to the age group ranging from 41-50 years and 55% patients were male and 45% were female with a male female ratio of 1.2:1(Table-I).

Table I: Age and sex distribution of the nosocomial diarrhea patients studied (n=100)



Out of 100 diarrhoeal stool samples, 25% samples were pus cell positive in microscopy, culture yielded growth of *C. difficile* in 10% samples and all isolated *C. difficile* were confirmed by both latex agglutination and PCR. Out of 10 isolates, 7 were only tpi gene positive which is species-specific for *C. difficile* indicating the presence of non-toxigenic *C. difficile* and 3 isolates had both *tpi* and toxin genes indicative of toxigenic *C. difficile* respectively. All toxigenic *C. difficile* contains both *tcd*A and *tcd*B genes (Table-II). All the (3%) toxin positive cases were pus cell positive and among them, 33.3% had 1+ pus cell/HPF, followed by 33.3% with 2+ and 33.3% with 4+ pus cell /HPF.

Table II: Results of microscopy, latex agglutination and PCR on isolated culture positive cases (n=10):

Methods		Positive cases
Microscopy (Pus cell)		10
Latex agglutination (cell wall antigen)		10
PCR	Only *tpi* gene (specific for *C. difficile*)	7
	Both *tpi* and toxin genes (*tcd*A and *tcd*B)	3

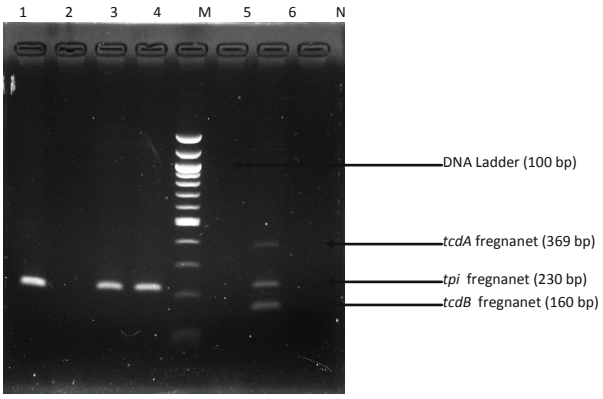


Fig I: Gel electrophoresis showing amplified DNA. 230 bp for *tpi* gene, 369 bp for *tcdA* gene, 160 bp for *tcdB* gene. Lane M: DNA molecular size marker (100bp), Lane N: negative control. Lane 1 (positive control): *tpi*, lane 3: *tpi*, lane 4: *tpi*, lane 6: *tpi* + *tcdA* + *tcdB*.

Discussion:

Antibiotic-associated diarrhea is very common in hospitalized patients (3% to 29%), especially in those who receive antibiotics within the last 4 weeks.[27] Although some studies had shown that prior treatment with ciprofloxacin, clindamycin, penicillin and cephalosporins are most frequently associated with CDI; but the use of almost any antibiotic can lead to *C. difficile* infection.[28,29] The main symptom of CDI is diarrhoea which usually starts 5-10 days after continuing the antibiotic therapy. The main virulence factors of the pathogenic *C. difficile* strains are toxin A and B of which toxin A is an inflammatory enterotoxin responsible for fluid secretion while toxin B is a cytotoxin, degrades the colonic epithelial cells.[30] Testing for *C. difficile* is not routinely done in Bangladesh and there is limited data about the prevalence and risk factors for *C. difficile* induced diarrhea. *C. difficile* induced nosocomial diarrhoea in various clinical wards of BSMMU and DMCH were diagnosed in this study.

Majority of the study patients (41%) belonged to the age group ranging from 41-50 years. About 55% patients were male and 45% were female. These findings correlated with a study of India, where they found that among the study participants, 58% were male and 42% female and the mean age of the patients were 46.7 years.[23] Distribution of age and sex among nosocomial diarrhoeal patients in different studies varied which might be due to advanced age, frequent interactions with healthcare system, longer course of antibiotics (>4 weeks) and age-related physiological changes.[31]

Of all the stool samples tested in this study, only 10% samples were culture positive for *C. difficile* and all the isolates were confirmed by both latex agglutination and PCR. A multiplex PCR assay was implemented to facilitate the rapid detection of toxigenic *C. difficile* in stool samples. In culture positive samples, 7 were positive for only *C. difficile* specific tpi gene and 3 were both *tpi* and toxin genes (both *tcdA* and *tcdB* gene) positive. Some previous studies showed the relevant results compared to this study. A total of 16 *C. difficile* isolates were identified in a study in India and were confirmed by PCR.[32] Whereas another study, among nosocomial stool samples, 20% showed growth of *C. difficile* in CCFA plate and identified by latex agglutination and biochemical reactions and 6.38% toxigenic stain was identified by PCR.[33] In Srilanka, culture isolates of *C. difficile* was detected to be 12% and toxin positivity was 3.6% by PCR which was similar to the findings of the present study.[34] Toxin genes were detected from 5 culture isolates and all were both *tcdA* (toxin A) and *tcdB* (toxin B) positive in a study which is consistent with this study.[35] A study in USA, 15.4% samples were positive for *C. difficile* in culture and 8% contained both A and B toxin gene by PCR.[25]

Although anaerobic culture was considered as the most sensitive assay for the detection of *C. difficile*, it lacks specificity for toxigenic strains unless other testing modalities are performed and is also limited by a poor turnaround time, usually 3 to 5 days.[36] In our study, also a relatively lower percentage of culture growth obtained in this study might be due to the variable sensitivity rate (42.6% to 99.6%) for isolation of *C. difficile* in CCFA media. Alcohol pre-treatment and incubation time can also vary the sensitivity results of CCFA media.[37] Growth of *C. difficile* may be inhibited by accidental exposure of specimens to oxygen during transport, processing and while opening the anaerobic jars to check for growth. However, overgrowth of aerobic, facultative organisms or normal flora can also inhibit the growth of *C. difficile*.[38] Culture rate in CCFA media may vary from 7- 20% in different area of India.[39]

Conclusions:

The study observed the frequency of *C. difficile* induced nosocomial diarrhea in 2 tertiary care hospitals and also evaluated different detection methods comprising culture, latex agglutination test and toxin gene detection by PCR. Out of 100 diarrhoeal stool samples, 10 samples showed growth of *C. difficile* and all isolated *C. difficile* were confirmed by both latex agglutination and PCR. Out of 10 isolates, 7 were non-toxigenic *C. difficile* and 3 isolates were toxigenic *C. difficile*. About 3% nosocomial diarrhoea patients were diagnosed as CDAD by PCR from culture isolates. Multiplex conventional PCR may detect toxin genes (*tcdA* and *tcdB*) and *C. difficile* specific *tpi* gene simultaneously and accurately. The rapid and accurate detection of *C. difficile* for the diagnosis of CDAD may prevent the severity and spread of CDAD.

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