Original Article

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Prevalence of *Clostridium Difficile* Among Paediatric Patients in a Tertiary Care Hospital, Coastal Karnataka, South India

Microbiology Section

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ABSTRACT

Introduction: The study was intended to analyse the burden of *Clostridium difficile (C. difficile)* and associated intestinal pathogens from children with diarrhoea who were hospitalized in a tertiary care teaching hospital of South India.

Materials and Methods: Stool samples from 138 children with diarrhoea belonging to the age group 0-14 years were analysed by semi quantitative culture, latex agglutination and enzyme immunoassay for *C. difficile*. The associated intestinal pathogens were also detected from the specimens by standard procedures.

Results: Stool samples of 138 children were tested during the period; 21 (15.22%) samples were culture positive for *C. difficile* and the isolates were confirmed by biochemical reactions. 9(6.52%) were positive by latex agglutination. EIA for *C. difficile* toxins A and B was done on all the stool specimens and 15 were found to be positive (10.87 %). According to the reference standard method employed in our study, 4 toxigenic *C. difficile* isolates (2.90%) were obtained from 138 specimens. Among the other intestinal pathogens, *Escherichia coli* predominated (22.46%). Rota virus was detected in 7.27% stool samples of children under the age of five years.

Conclusion: The study shows the prevalence of *C. difficile* in hospitalized children in our locality which highlights the importance of judicious use of antibiotics and strict infection control measures.

Keywords: Antibiotic associated diarrhoea, *Clostridium difficile*, cycloserine cefoxitin fructose agar, semi quantitative culture

INTRODUCTION

Clostridium difficile (*C. difficile*), an anaerobic Gram positive spore forming bacillus is an important pathogen responsible for antibiotic associated diarrhoea (AAD) and pseudomembranous colitis (PMC). Many authors have reported hospital acquired and community based cases of *C. difficile* associated disease (CDAD) [1,2]. Estimates regarding the occurrence of *C. difficile* infection (CDI) in United States per year has been mentioned in a study [3]. A few reports are available regarding the prevalence of CDI in Asian countries [4,5]. The mutant hypervirulent strain, NAP1/BI/027 (North American Pulse-field gel electrophoresis type 1 /restriction endonuclease analysis BI/ribotype 027) which was responsible for the outbreaks of CDAD in many parts of the world has given due significance to the organism [6].

In children the organism has gained much relevance during the past decade and there are documented reports from various parts of the world especially from 1997 to 2006 with an incidence of 21% among children below one year of age [7,8]. Children of age group1-4 y showed the highest incidence of *C. difficile* [9]. Gogate et al., reported that *C. difficile* is an important causative agent for AAD in children of age group 5-12 y [6]. A study conducted among children with cancer revealed that the drugs like aminoglycoside, third generation cephalosporin, cefepime, proton pump inhibitor and chemotherapy had significant role in hospital acquired CDI [10]. Another study performed in paediatric residents of Olmsted County, Minnesota identified that 75% cases of CDI were community – acquired [11].

The reports from India regarding the prevalence of *C. difficile* in children are scanty. The estimates of CDI could be less than the actual picture in our country due to tedious and costly anaerobic techniques required for the detection of *C. difficile*. The present study is intended to assess the burden of *C. difficile* from paediatric patients with diarrhoea by means of semi quantitative isolation, characterization and toxin detection. Anaerobic culture has been done on cycloserine cefoxitin fructose agar (CCFA) for the isolation of *C. difficile* from the faecal samples. Latex agglutination has been performed for the confirmatory identification of *C. difficile* colonies grown on CCFA. Culture and latex agglutination cannot differentiate between toxigenic and non-toxigenic *C. difficile* isolates. The purpose of Enzyme immunoassay (EIA) was to detect the toxins A and B of *C. difficile* which is mainly responsible for its pathogenesis. The study also analyses the associated intestinal pathogens from the children.

MATERIALS AND METHODS

The study was conducted in a tertiary care teaching hospital in coastal Karnataka. Stool samples were collected from patients who were admitted in the various paediatric wards during the period from January 2012 to December 2013.

The study was approved by the Institutional Ethics Committee (Ref. No FMMC/ IEC/ 816/ 2012).

Any paediatric patient with diarrhoea in the age group less than 14 years was included in the study. A detailed case history which included age, sex, severity of diarrhoea, usage of any antibiotic, any other illnesses etc. were taken from the medical records of each patient. Written informed consent was taken from the parents of the patients.

The stool samples were collected in sterile wide mouthed containers. The samples were processed immediately. Anaerobic culture on CCFA for the isolation of *C. difficile* and EIA for the toxins A and B of *C. difficile* were performed on all the stool samples. Colonies grown on CCFA plate were presumptively identified as *C. difficile* which were subjected to latex agglutination and biochemical reactions [12,13]. The associated intestinal pathogens were also identified from the specimens using standard procedures [14,15]. In addition to this, detection of Rota virus was also performed. Standard reference strain ATCC 43593 of *C. difficile* was employed in parallel as a control strain throughout the study. Detection of toxigenic *C. difficile* was based on Toxigenic culture [16].

Isolation and detection of *C. difficile*

Culture: Stool samples were directly plated onto CCFA, Brucella blood agar and were also inoculated in Robertson's cooked meat (RCM) broth as a supplemental medium. Approximately 0.1 gm or 2-3 drops of stool was inoculated on 4 quadrants of a CCFA plate to perform a semi quantitative culture. The plate was incubated in Hi gas-pak jar at 37^{0}C for 48 h using BD GasPak EZ Anaerobe container system with Indicator or in individual sachets (BD GasPak™ EZ Gas Generating Pouch Systems). The grading system applied was 1+ to 4+ (4+ corresponds to 10^5 colony forming units/ml) [12].

On CCFA, circular, yellow, fimbriate colonies of 4mm size or larger, Gram positive bacilli with subterminal oval spores having horse stable odour was presumptively identified as C. difficile.

Colonies morphologically resembling the organism was tested by latex agglutination with Oxoid C.difficile Test Kit (DR 1107A), UK according to manufacturer's instructions. Briefly, saline suspension of the suspected colony was mixed with Oxoid C.difficile Latex Reagent on the reaction card. Appearance of agglutination was examined for a maximum of two minutes, employing appropriate negative and positive controls. Colonies were also confirmed by biochemical reactions [12,13].

Toxin Testing by EIA

Qualitative and quantitative detection of toxins A and B of *C. difficile* was performed using Premier toxins A and B (*C. difficile*) EIA kit M/S Meridian Bioscience, Europe according to the manufacturer's instructions. The results were read spectrophotometrically at 450 nm wavelength.

Detection of Associated Faecal Pathogens

For the detection of bacterial enteropathogens, the stool samples were cultured onto Mac Conkey agar and Xylose lysine deoxycholate agar both directly and after enrichment in Selenite F broth. After overnight aerobic incubation at 37^{0}C, the plates were examined and the colonies were identified by standard laboratory techniques [14]. The detection of parasites was performed by saline wet mount, iodine wet mount and Kinyoun acid –fast stain (modified acid-fast stain) [15].

Detection of Rota Virus

Rota virus was detected by a rapid latex agglutination assay using Rota virus Latex Test Kit from Plasmatec, UK. Briefly, the stool specimen was mixed with the extraction buffer, vortexed, centrifuged and latex agglutination was carried out according to manufacturer's instructions.

Total number of Samples	Culture on CCFA		Latex agglutination		Enzyme immunoassay	
	Number of positive samples	Percentage (%)	Number of positive samples	Percentage (%)	Number of positive samples	Percentage (%)
138	21	15.22	9	6.52	15	10.87

[Table/Fig-1]: Number of samples positive by various tests for the identification of *C. difficile*

STATISTICAL ANALYSIS

Data was analysed by frequency percentage. Age correlation of occurrences of positivity in culture, latex agglutination and EIA was done by Chi-square test.

RESULTS

Stool samples of 138 children aged less than 14 years were collected during the study period and analysed for the presence of *C. difficile* by culture, latex agglutination and EIA. The results are given in [Table/Fig-1].

Positivity obtained in culture for *C. difficile*, latex agglutination and EIA for the toxins among the various age groups of children was compared and the results are given in [Table/Fig-2].

The number of specimens positive in various combinations when subjected to culture for *C. difficile,* latex agglutination and EIA for the toxins is given in [Table/Fig-3].

Clinical features and risk factors seen in 15 children who were positive by EIA for the toxins A/ or B were analysed from the patient data and they are summarized in [Table/Fig-4].

The other intestinal pathogens identified from the stool samples were: *Escherichia coli* (22.46%), *Proteus* spp (0.72%), *Citrobacter freundii* (1.45%), *Shigella flexneri* (2.90%), *Klebsiella* spp (3.62%), *Enterobacter* spp (0.72%), *Salmonella typhimurium* (0.72%), *Shigella sonnei* (1.45%), *Pseudomonas aeruginosa* (1.45%), *Candida* spp (1.45%) and Rota virus (7.27%).

DISCUSSION

C. difficile, an anaerobic Gram positive spore forming bacillus causes a variety of clinical conditions ranging from asymptomatic carrier state, diarrhoea, PMC, prolonged ileus, megacolon to death [6]. It is reported that the two main toxins; toxin A (enterotoxin) and toxin B (cytotoxin) are responsible for the pathogenesis of *C. difficile* [17]. The presence of another toxin (binary toxin) has also been described by some authors which enhances virulence of *C. difficile* [18,19]. In the present study, 10.87% positivity was obtained when toxin detection (toxins A and B) was done by EIA.

The scope of the present study includes the isolation of *C. difficile* by culture, confirmatory identification of *C. difficile* colonies by latex agglutination, detection of toxins A and B by EIA among paediatric patients with diarrhoea and thereby detecting the prevalence of CDI in the particular group.

Inter laboratory comparison of data in India regarding the prevalence of CDI was cumbersome because detection methods of *C. difficile* was based on different criteria in different studies. Toxigenic culture was employed as the reference standard method for the detection of toxigenic *C. difficile* in the present study. Toxigenic culture is the culture on selective media like CCFA followed by in vitro toxin detection by EIA/ cell culture cytotoxicity assay/ polymerase chain reaction to analyse the toxigenicity of the isolated strain [16]. A total of 4 toxigenic *C. difficile* isolates (2.90%) was obtained from 138 stool samples during the study period.

Tests performed	Positivity in different age groups				Total number of positives	χ^2 test
	<1 year (n=30)	1-5 years (n=80)	6-10 years (n=21)	11-14 years (n=7)		
Culture	4 (19.05%)	15* (71.43%)	2 (9.52%)	0 (0%)	21	χ^2=14.00 p=0.001, HS
Latex agglutination	2 (22.22%)	6 (66.67%)	1 (11.11%)	0 (0%)	9	χ^2=4.667 p=0.097, NS
Enzyme immunoassay	4 (26.67%)	8* (53.33%)	1 (6.67%)	2 (13.33%)	15	χ^2=7.95 p=0.047, significant

[Table/Fig-2]: Positivity in various tests for *C. difficile* among different age groups of 138 children
HS = highly significant, NS = not significant.
*When the occurrence of positivity of culture, latex agglutination and EIA was compared among different age groups, the culture and EIA positivity were significant in the age group 1-5 years when compared to other age groups

Group	Positivity in various combinations of tests	Total number of samples = 138
I	Number of samples positive in all the three tests ie. culture, latex agglutination and EIA	1 (0.72%)
II	Number of samples positive in only two tests ie. culture and EIA	3 (2.17%)
III	Number of samples positive in only two tests ie. culture and latex agglutination	9 (6.52%)
IV	Number of samples positive by only EIA	11 (7.97%)
V	*Toxigenic culture (I + II)	4 (2.90%)

[Table/Fig-3]: Positivity in various combinations of tests for *C. difficile.* *According to European society of clinical microbiology and infectious diseases (ESCMID) criteria

Total number of children = 15		Number of children with relevant clinical features and risk factors
Clinical features	Abdominal pain	1
	Fever	6
	Vomiting	6
Risk factors	Antibiotic treatment	7
	Prolonged hospital stay	2
	Underlying diseases	7
	Surgery	1

[Table/Fig-4]: Clinical features and risk factors encountered in patients who demonstrated the presence of toxins A and/ B.

A few Indian authors have reported the prevalence rates of CDI among children. Gogate et al., reported a culture positivity of 7.2% and enzyme-linked immunosorbent assay (ELISA) positivity of 14% whereas Dutta et al., reported an isolation rate of 3.6% [20, 21]. In our study, 15.22% culture positivity was obtained. Ingle et al reported a prevalence rate of 17% using ELISA for *C. difficile* toxins A and B in a retrospective study of all in-patients and out-patients of their hospital [22]. However, review of literature revealed higher incidence of *C. difficile* in the Western countries [7,8,23].

The sensitivity of culture, latex agglutination and EIA was 100%, 25% and 100% respectively when compared with toxigenic culture which was our reference standard method. The specificity of culture, latex agglutination and EIA was 87.31%, 52.94% and 91.79% when compared with toxigenic culture. The age correlation of occurrences of positivity in culture, latex agglutination and EIA was done by Chi-square test. It was found that positivity of culture and EIA was significant in the age group 1-5 y when compared to other age groups. Previous literature has given similar findings [9]. All the four children in our study, who harboured toxigenic *C. difficile* according to the ESCMID criteria, belonged to the age group 1-5 y. One among the four children had undergone herniotomy. He had loose stools after the surgery which could be associated with cephalosporin administration following the surgery and could be classified as a clear case of AAD. The second patient was treated with a third generation cephalosporin, ceftriaxone for *Shigella sonnei* which would have predisposed to *C. difficile*. The other two patients had Rota virus and *Escherichia coli* respectively in their stool specimens in addition to toxigenic *C. difficile*. The child with *Escherichia coli* was given amoxicillin-clavulanate and a third-generation cephalosporin, cefotaxime for the urinary tract infection which would have led to AAD. Cephalosporins and amoxicillin-clavulanate have long been implicated in CDI [24]. Also, it was shown that majority of health care associated CDAD occur during or after treatment with antimicrobial agents [24]. It is evident that even toxigenic *C. difficile* can be present as a colonizer [25]. This creates a dilemma that the diarrhoea present was attributable to *C. difficile* or to the other intestinal pathogens. Amongst the 15 toxin positive cases, 11 (73.33%) were males and 4 (26.67%) were females.

Eleven children who showed toxin production by ELISA were negative by culture and thus those children cannot be included under the definition of CDI according to the reference standard method employed in our study. Those specimens will be tested for glutamate dehydrogenase antigen before considering them as false positive [26]. Seventeen isolates of *C. difficile* (out of 21 isolates, 4 were toxigenic) which were isolated by anaerobic culture could be regarded only as colonizers because they were non toxigenic [27]. Among the other intestinal pathogens identified from the stool specimens, *Escherichia coli* was predominant (22.46%) and Rota virus was detected in 8 (7.27%) among 110 children who were under five years of age.

Though we were not able to classify our cases as hospital- acquired or community associated CDI, our study has several advantages. It was a prospective study which employed a reference standard method for the detection of toxigenic *C. difficile* and the results were well correlated with the clinical data. Hence we recommend the screening of *C. difficile* in children above one year of age with diarrhoea if other routine enteropathogens are absent.

CONCLUSION

Data regarding CDI from the paediatric population is scanty from India, especially from Southern India. The incidence of CDI in children is rising around the globe. Presence of toxigenic strains among children in our study is an indication that the situation is not much different in our locality too. The actual number of cases of *C. difficile* is being underestimated due to laborious and costly anaerobic techniques. The only way to reduce CDI is to judiciously use antibiotics and to give prime importance to strict infection control measures.

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