CLOSTRIDIUM DIFFICILE AND ITS CYTOTOXIN IN HOSPITALIZED CHILDREN WITH ACUTE DIARRHEA

S.K. Niyogi
P. Dutta
D. Dutta
U. Mitra
S. Sikdar

ABSTRACT

A total of 498 children, aged 0-14 years, admitted at the B.C. Roy Memorial Hospital for Children, Calcutta, were investigated for the occurrence of Clostridium difficile and its cytotoxin. Of the children in the investigation, 369 suffered from acute diarrhea. Only 8.4% of these children had C. difficile in fecal samples and in vitro cytotoxin was demonstrated in 7%. In 27 (7.3%) of the patients with acute diarrhea C. difficile was isolated as the only pathogen. In contrast, among 129 control children not suffering from acute diarrhea, only 4 (3.1%) harboured C. difficile. Isolation of C. difficile was significantly higher in children under one year of age. None of these patients had any history of prior antibiotic therapy.

Key words: *C. difficile, Acute diarrhea, Hospitalized children.*

From the Division of Microbiology and Clinical Medicine, National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road, Scheme XM, Beliaghata, Calcutta 700 010.

Received for publication November 30, 1990; Accepted February 11, 1991

C. difficile is a major etiological agent of pseudomembranous colitis (PMC) and is often isolated from patients with antibiotic or chemotherapeutic associated diarrhea in adults(1,2). Hall and O'Toole were first to describe asymptomatic carriage of *C. difficile* in neonates(3). However, the significance of *C. difficile* and its toxins in children is still a matter of discussion. It has been demonstrated that *C. difficile* produces at least two toxins, toxin A (enterotoxin) and toxin B (cytotoxin)(4). The aim of this study was to examine the role of *C. difficile* and its cytotoxin in hospitalized children with diarrhea and in a control group.

Material and Methods

During the period from January through December, 1989 a total of 498 fecal specimens collected from children aged 0-14 years admitted at the B.C. Roy Memorial Hospital for Children, Calcutta were screened for the presence of *C. difficile*. Of these patients, 369 were suffering from acute diarrhea and 129 children admitted over the same period with non gastrointestinal illness served as control group. Although it was not possible to precisely match controls for age and sex, an attempt was made to study a number of controls in proportion to the number of patients in the various groups. Detailed clinical information was obtained from the attending pediatrician. Any antibiotic therapy within the previous four weeks was also recorded.

Isolation and detection of C. difficile: All fecal samples were processed immediately after receipt on a selective medium (Cycloserine cefoxitin fructose agar; CCFA; Oxoid, Basingstoke, Hants, England(5) and incubated at 37°C for 48 h in

an anaerobic jar with gas generating kit (oxoid). All colony types with typical morphology were subcultured onto blood agar purity plates and identified using criteria detailed in the Anaerobe Laboratory Manual(6). Fecal specimens were also analyzed for other enteropathogens using standard techniques(7).

Cytotoxicity assay: Cytotoxicity was assayed on VERO (African green monkey kidney) tissue culture monolayers(8). Fecal samples were diluted 1 : 10 in phosphate buffered saline (pH 7.2) vortexed and centrifuged to clarify the resulting suspension and the supernatant were filtered through 0.45 μm membrane filters (Millipore, Corp., Bedford, Mass). Filtered supernatant (0.1 ml) was added in duplicate to the wells of a tissue culture plate (Nunc, Denmark) containing monolayers of vero cells in 0.1 ml of maintenance medium and incubated at 37°C in 5% CO_2 incubator. The cell cultures were observed for cytopathic effect after 24 and 48 h of incubation. The specificity of the cytotoxin was confirmed by neutralization by *C. difficile* antitoxin (VPI Anaerobe Laboratory, Blacksburg, VA). *In vitro* cytotoxin production was determined by growing each strain anaerobically in brain heart infusion broth (Difco Laboratories, Detroit, Mich) for 48 to 72 h at 37°C. The cultures were centrifuged and filtered supernatant were assayed on vero cells just as for the fecal filtrate.

Statistical analysis: The data were analyzed for statistical significance using χ^2 and χ^2 with yates's correction at 5 and 2% level of significance, respectively.

Results

C. difficile was isolated from 35 children, 31 of these were patients with diarrhea. Only 4 children (3.1%) in the control groups harboured *C. difficile* ($p < 0.05$). *In vitro* cytotoxin (toxin B) production was demonstrated in 7% of the strains isolated from children with diarrhea ($p < 0.02$). Extracts of all the 35 *C. difficile* culture positive fecal specimens were tested for their cytotoxicity. None of the fecal extracts from which *C. difficile* was isolated showed cytotoxicity. The results are summarized in *Table I.*

Table II shows the frequency of isolation of *C. difficile* from fecal specimens of children in relation to the age groups of both children with acute diarrhea and the controls. The prevalence of *C. difficile* was significantly higher in infants (<1 year) than in older children.

In 27 patients (7.3%) with acute

TABLE I—*Isolation of C. difficile and its Cytotoxin from Children with Diarrhea and from Controls*

Group	Number	*C. difficile* isolated	Cytotoxin production +ve	
			In stool	*C. difficile (in vitro)*
Diarrhea	369	31 (8.4)	0	26 (7.0)
Control	129	4 (3.1)	0	1 (0.7)

Figures in the parentheses indicates percentages.

TABLE II—*Age Distribution of Children with Diarrhea and Controls*

Age (years)	Children with diarrhea			Control group		
	n	*C. difficile*	(%)	n	*C. difficile*	(%)
<1	168	19	11.3	43	2	4.6
1-4	120	7	5.8	69	1	1.4
5-14	81	5	6.1	17	1	5.8

diarrhea *C. difficile* was isolated as the only pathogenic bacteria, in a further 4 cases other enteropathogens, *i.e., S. typhimurium, V. cholerae,* EPEC and *Sh. flexneri* in addition to *C. difficile* were observed.

There was no definite history of prior administration of antimicrobial agents among the patients from whom *C. difficile* was isolated. All the *C. difficile* positive patients recovered spontaneously.

Discussion

C. difficile is a major cause of PMC in adults and often the reason for antibiotic associated diarrheas(1). The association of *C. difficile* with enteric diseases in children is less clear. The carrier rate in neonates and young children is high and rates upto 64% have been reported(9). Therefore, we investigated the occurrence of *C. difficile* and the toxin production amongst hospitalized children with acute diarrhea and in a control group. The rate of isolation was age dependent. The highest rates occurred in children of less than one year of age in both groups and the rate of isolation decreased in older children. This finding is in accordance with the results of other investigators(9-11). Only 7% of the strains isolated from diarrheal cases produced cytotoxin (toxin B). Although we did not specifically search for toxin A (enterotoxin), the detection of toxin B (cytotoxin) can be assumed to signal the simultaneous presence of toxin A, as no strain of *C. difficile* has yet been detected that produces either of the toxins alone(12). In the present study none of the fecal extracts showed cytotoxicity. The *in vitro* findings that *C. difficile* cytotoxin is inactivated by the myeloperoxidase system of neutrophils and H_2O_2 from *Lactobacillus acidophillus*(13), may explain the absence of cytotoxin in fecal samples though toxin producing *C. difficile* was isolated from the same specimens. All the children hospitalized with acute diarrhea and growth of *C. difficile* as the only demonstrable pathogen had no history of prior antibiotic treatment. Most of the *C. difficile* positive patients in this study recovered completely within a week without any antibiotic treatment. This suggest that *C. difficile*, like the most of the other intestinal pathogens should not be treated with antibiotics unless severe symptoms are present.

The results of the present study indicate that *C. difficile* may cause infection and diarrhea in the same way as other enteropathogenic bacteria and could be an important etiological agent of acute diarrhea amongst hospitalized children and must be looked for routinely.

REFERENCES

1. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic associated pseudomembranous colitis due to toxin producing clostridia. N Engl J Med 1978, 298: 531-536.
2. Miller SD, Koornhof HJ. *Clostridium difficile* colitis associated with the use of anti neoplastic agents. Eur J Clin Microbiol 1986, 3: 10-13.
3. Hall IC, O'Toole E. Intestinal flora in new-born infants with description of a new pathogen anaerobe, *Bacillus difficiles*. Am J Dis Child 1935, 49: 390-402.
4. Taylor NS, Thorne GM, Bartlett JG. Comparison of two toxins produced by *Clostridium difficile*. Infect Immun 1981, 31: 1036-1043.
5. George WL, Sutter VL, Citron D, Finegold SM. Selective and differential medium for isolation of *Clostridium difficile*. J Clin Microbiol 1979, 9: 214-219.
6. Holdeman LV, Cato EP, Moore WEC. Anaerobe Laboratory Manual, 4th edn. Blacksburg VA, Virginia Polytechnic Institute, 1977.
7. World Health Organization. Programme for Control of Diarrheal Diseases. Manual for Laboratory Investigations of Acute Enteric Infections. Geneva, World Health Organization, 1987, pp 4-22 (CDD/83.3 Rev 1 1987).
8. Bowman RA, Riley TV. Laboratory diagnosis of *Clostridium difficile* associated diarrhea. Eur J Clin Microbiol Infect Dis 1988, 7: 476-484.
9. Holst E, Helin I, Mardh PA. Recovery of *Clostridium difficile* from children. Scand J Infect Dis 1981, 13: 41-45.
10. Camoralinga-Ponce M, Gamboa M, Barragan JJ, *et al*. Epidemiological aspects of *Clostridium difficile* in a pediatric hospital and its role in diarrheal diseases. Eur J Clin Microbiol 1987, 6: 542-546.
11. Merida V, Moerman J, Colaert J, Lemmens P, Vandepitte J. Significance of *Clostridium difficile* and its cytotoxin in children. Eur J Pediatr 1986, 144: 494-496.
12. Lyerely DM, Wilkins, TD. Characteristics of the toxins of *Clostridium difficile*. *In:* Antibiotic Associated Diarrhea and Colitis. Ed. Borreillo SP. Boston, Den Haag: Martinus Nijhoff, 1984, pp 89-102.
13. Qoi W, Levine HG, LaMont JT, Clark RA. Inactivation of *Clostridium difficile* cytotoxin by the neutrophil myeloperoxidase system. J Infect Dis 1984, 149: 215-219.