Indian J Med Res 122, December 2005, pp 518-524

Diagnostic role of stool culture & toxin detection in antibiotic associated diarrhoea due to *Clostridium difficile* in children

A. Gogate, A. De, R. Nanivadekar, M. Mathur, K. Saraswathi, A. Jog* & M.V. Kulkarni*

*Departments of Microbiology & *Pediatrics, L.T.M. Medical College, Mumbai, India*

Received November 4, 2004

***Background & objectives*: Mere diagnosis of *Clostridium difficile* by culture does not help in the diagnosis of antibiotic associated diarrhoeae (AAD) due to *C. difficile*. Detection of toxins A and B form the mainstay in the diagnosis of AAD due to *C. difficile*. This study was undertaken to find out the role of stool culture and toxin detection in the diagnosis of AAD due to *C. difficile*. As there are very few documented reports from India about AAD due to *C. difficile* in children in the age group of 5-12 yr, this age group was selected.**

***Methods*: Faecal samples were collected from 250 hospitalized children in the age group of 5-12 yr who developed diarrhoea on receiving antibiotics for different medical problems for more than five days duration. Also faecal samples of 250 age and sex matched controls were collected. Culture for *C. difficile* was done on cycloserine cefoxitin fructose egg yolk agar (CCFA) and colonies were identified by standard laboratory techniques. ELISA for toxins A and B detection and tissue culture on HeLa cells for toxin B detection were also done.**

***Results*: Overall positivity was 18 per cent in this study group compared to the controls ($P<0.001$). Maximum positive cases were in 5-8 yr age group (84.4%). Severe diarrhoea, liquid stool with mucus and blood, faecal leucocytes >5/high power field, altered flora and presence of Gram-positive bacilli with oval subterminal spores on Gram stain were sensitive predictors for diagnosis of AAD due to *C. difficile*. Amongst positive cases, 68.9 per cent responded to discontinuation of antibiotics and 31.1 per cent to metronidazole therapy.**

***Interpretation & conclusion*: *C. difficile* was an important pathogen responsible for antibiotic associated diarrhoea (AAD) in children of 5-12 yr age group. Conservative use of antibiotics would be beneficial to decrease the incidence of AAD.**

Key words Antibiotic associated diarrhoea - *Clostridium difficile*

Since the inception of the term PMC (Pseudomembranous colitis) by Finney in 1893[1,2] and description of *Clostridium difficile* and its toxigenicity by Hall and O'Toole in 1935[3], several investigators had come up with the varied aetiology of PMC. It was Larson *et al*[4] in 1978, who associated *C. difficile* with PMC or clindamycin associated colitis. Since then a lot of work has been done on *C. difficile* and its pathogenicity, and toxins A and B have been detected[5-7].

Diarrhoea still remains a major problem in the South East Asian countries, including India. With the discovery of newer antibiotics, cases of antibiotic associated diarrhoea (AAD) are also on a rise. But the faecal specimens from these patients are usually sent for culture of routine enteropathogens and are therefore, reported as negative. Most of the laboratories do not have facilities for *C. difficile* culture. Moreover, culture positivity is also very low. Therefore, detection of toxin A and B by enzyme linked immunosorbent assay (ELISA) and detection of toxin B by tissue culture form the mainstay in the diagnosis of *C. difficile*. Though many reports are available from western countries[8-12], very few documented reports about toxin detection are available from India in children in the age group of 5-12 yr[13,14]. Thus we undertook this study to detect the toxins produced by *C. difficile* by ELISA and diagnostic role of tissue culture in cases of antibiotic associated diarrhoea in the 5-12 yr age group children.

Material & Methods

A total of 250 stool samples collected in sterile wide-mouthed containers from all children with diarrhoea (5-12 yr), who were admitted in Paediatric wards of Lokmanya Tilak Municipal General Hospital, Sion, Mumbai, during February 1999 and August 2001. Inclusion criteria in this study were antibiotic treatment within two months with significant diarrhoea (>3 stools of changed consistency); and no other aetiology or diarrhoea inducing factors.

Control group consisted of 250 healthy children of same age group. They were school children who came to our hospital for routine health check-up and immunization clinic; and healthy children from a nearby municipal school. A detailed proforma was filled up for every study case, which included age, sex, severity of diarrhoea, association with other symptoms like fever, abdominal pain, vomiting, antibiotics used, any other illness, *etc*.

All stool samples were processed within 2 h of collection. Saline and iodine mounts and Gram stain were prepared and methylene blue stain was done for presence of faecal leucocytes[15]. For routine bacterial pathogens, enrichment was done in alkaline peptone water and Gram-negative broth overnight at 37^0C and then plated onto MacConkey agar and Xylose lysine deoxycholate agar plates (Hi-Media, Mumbai) respectively, incubated at 37^0C overnight and bacterial enteropathogens were identified by standard laboratory methods[16].

For *C. difficile*, stool samples were inoculated in Robertson's cooked meat (RCM) broth (Hi-Media, Mumbai) for enrichment, and incubated at 37^0C for 24-48 h. Direct plating of each sample was done on Cycloserine cefoxitin fructose egg yolk agar (CCFA) (Hi-Media, Mumbai) and subculture on CCFA was also done from 48 h incubated RCM broth; all the plates were incubated anaerobically in McIntosh Fildes jar (Equitron, Mumbai) for 48-72 h[17]. Suspected colonies were identified as *C. difficile* by Gram stain and standard biochemical tests like sugar fermentations, gelatin hydrolysis, hydrogen sulphide production, *etc*[18]. After 48 h of incubation on CCFA, colonies of *C. difficile* were 4 mm or larger, flat to slightly raised yellowish rhizoid colonies which had a speckled opalescence and strong horse manure-like odour. These emitted yellow-green fluorescence under long wave U-V light.

For toxin assays, stool filtrates were prepared by suspending the stool in phosphate buffer saline (PBS), *p*H 7.0. The suspension was centrifuged at 3000 *g* for 20 min, and the supernatant was then filtered through Millipore membrane filter (Sartorius-Goettingen, Germany) of pore size 0.45 μm for study of toxin A/B by ELISA and toxin B by tissue culture assay. All the filtrates were preserved at -70^0C until tested.

ELISA was performed using Ridascreen® *Clostridium difficile* Toxin A/B kit manufactured by R-Biopharma GmbH, Darmstadt, Germany[19]. This is an enzyme immunoassay for qualitative detection of toxins A and B of *C. difficile*. The absorption was measured at 450 nm wavelength using a microtitre plate reader (Spectra II, Austria).

For toxin B, tissue culture was performed using human carcinoma of cervix (HeLa) cell line. The cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, Maharashtra. The cell line was grown in minimum essential medium (MEM) with foetal calf serum (FCS). The growth was observed everyday under inverted microscope and by change in colour of medium from red to yellow. For trypsinization, the medium was aspirated from monolayer culture of cells. First, the cells were washed with trypsin to remove FCS. Then 2 ml trypsin was added and kept for one minute. The flask was rocked so that trypsin coated the cells in the flask; excess trypsin was aspirated out. Flask was placed in incubator for 5-10 min. Cells were checked for detachment and resuspended in growth medium. Cells were counted in haemocytometer (Rohem, India) using trypan blue dye for excluding the dead cells. Bottles were prepared according to the number of cells and incubated in CO_2 incubator. $1x10^6$ cells per ml were inoculated in one bottle with 10 ml of MEM and 1 ml of FCS. Bottles were incubated in CO_2 incubator and growth was observed up to 7 days. In microwell plate $1x10^5$ cells/ml were put. After full growth, 1 ml media was taken out and 50 µl stool filtrate was added and incubated at 37^0C for 30 min. Same quantity of medium was again added to the wells, incubated at 37^0C and observed after 24, 48, 72 and 96 h, for rounding of cells under inverted microscope. Results were interpreted as positive (>90% rounding of cells); weak positive (50-70% rounding); and negative (<50% rounding).

Data were analysed using Chi square test.

Results

Percentage positivity of *C. difficile* by culture was 7.2 per cent (18) in the study group as compared to none in the control group, the difference was statistically significant ($P< 0.001$) (Table). One hundred forty nine (59.6%) of the study group had moderate diarrhoea (7-10 motions/day) and 42 (16.8%) had severe diarrhoea (>10 motions/day). Liquid stool was observed in 102 (40.8%) cases and liquid stool with mucus and blood in 30 (12%) cases. Apart from diarrhoea, the main presenting symptoms in the study group were fever in 173 (69.2%) cases, abdominal pain in 89 (35.6%) and vomiting in 28 (11.2%).

Ampicillin was taken by 77 (30.8%) cases, followed by combination of ampicillin and amikacin in (36) 14.4 per cent, ampicillin and gentamicin in (23) 9.2 per cent, cefotaxime and amikacin in (21) 8.4 per cent, only amikacin in (20) 8 per cent, cefotaxime and ampicillin in (18) 7.2 per cent, and only cefotaxime in (8) 3.2 per cent cases. Combination of cefotaxime, amikacin and ampicillin was taken by (13) 5.2 per cent cases. Children in control group had not been on any antibiotics during the two months.

Gram-positive bacilli with oval subterminal spores were seen in 95 (38%) cases, faecal leucocytes >5/high power field (hpf) in 57 (22.5%) and altered flora in 50 (20%) cases. Other enteropathogens isolated included *Escherichia coli*, *Proteus morganni*, *Pseudomonas aeruginosa*, cysts of *Entamoeba histolytica* in children of both the groups, and *Aeromonas hydrophila*, *Shigella boydii*,

Table. Age and sex distribution and isolation of *Clostridium difficile* from cases and controls

	Study group (%)	Control group (%)
Male	134 (53.6)	131 (52.4)
Female	116 (46.4)	119 (47.6)
Age group 5-8 yr	165 (66.0)	141 (56.4)
Age group >8-12 yr	85 (34)	109 (43.6)
C. difficile grown on CCFA	18	0
Percentage positivity	7.2	0

n=250 in each group
CCFA, cycloserine cefoxitin fructose egg yolk agar

Cryptosporidium parvum, *Trichomonas huminis*, *etc*., in study group only. 178 (71.2%) patients responded to stoppage of antibiotics and only 70 (28%) patients required oral metronidazole therapy. Two patients in the study group expired. In one case, death was due to renal failure (*C. difficile* was not isolated in culture, and ELISA and tissue culture for *C. difficile* toxins were also negative in this patient). In another case, ELISA for *C. difficile* toxin A/B was positive, but culture and tissue culture were negative and the cause of death was hepatoblastoma.

ELISA for toxin A/B was positive in 35 cases giving a positivity of 14 per cent in the study group. None of the control cases were positive by ELISA. In the study group, tissue culture for toxin B was positive in 48 cases (19.2%). Of these, 41 were positive and seven weak positive. Weak positives were negative by ELISA as well as by bacterial culture.

Of the 250 samples in the study group, 45 were true positive, giving an overall positivity of 18 per cent. Of these, 13 samples were positive for all the three *i.e.*, culture for *C. difficile*, tissue culture and ELISA for toxin A/B and 4 were positive for culture for *C. difficile* and tissue culture, 18 were positive for ELISA and tissue culture and 4 were positive only for ELISA and 6 samples were strongly positive for tissue culture. The presence of *C. difficile* in the study group was statistically significant ($P<0.001$) compared to the control group.

Amongst 45 positive cases, 24 (53.33%) were males and 21 (46.67%) females; 38 children (84.44%) were in the age group of 5-8 yr, while 7 (15.56%) were >8-12 yr old. Of these 45 positive cases, 10 had taken ampicillin, 4 amikacin, 2 each took amoxycillin and co-trimoxazole. A combination of amikacin and cefotaxime was taken by 13 ampicillin and amikacin by 8 cases. The remaining 6 cases took different combinations of two or more antibiotics.

Thirty eight of the 45 positive cases (84.44%) had fever, which was statistically significant ($P<0.05$) compared to the negative cases 250-45=205 cases with in study group. Abdominal pain, mild and moderate diarrhoea was seen in 40, 13.3 and 60 per cent cases respectively. Severe diarrhoea was present in 12 (26.67%) cases which was significantly ($P<0.001$) higher as compared to the negative cases in the study group (30/205=14.63%). Only liquid stool and semiformed stool were present in 17 (37.78%) cases each and the difference was not significant. However, liquid stool with mucus and blood was seen in 11 (24.44%) cases and was significantly ($P<0.01$) more than the negative cases in the study group (19/205=9.27%). Gram-positive bacilli with oval subterminal spores were seen in 39 (86.67%) positive cases. Presence of faecal leucocytes >5/hpf and altered flora in 19 (42.22%) and 26 (57.78%) cases respectively was significantly ($P<0.001$) higher than the negative cases in the study group (38/205=18.54% and 24/205=11.71% respectively).

Amongst the positive cases, 31 (68.9%) responded to discontinuation of antibiotics and 14 (31.1%) to oral metronidazole therapy. No death was encountered amongst the true positive cases.

Discussion

The laboratory diagnosis of AAD due to *C. difficile* is based on culture and toxin detection in faecal specimens. Culture is very sensitive but, when used alone without toxin detection, leads to low specificity and underdiagnosis of cases when high rates of asymptomatic carriage exist. Culture is also difficult and time consuming[12]. *C. difficile* was isolated in 7.2 per cent cases in this study. Dutta *et al*[20] had isolated from 3.6 per cent cases, whereas another study reported 20 per cent culture positivity[12].

Overall positivity in this study was 18 per cent which was similar to that reported by Dhawan *et al*[13]. Similar findings were reported in other studies[12,14,21].

Mere isolation of *C. difficile* on culture is not sufficient to establish the pathogenic role of these isolates. One case in our study was only culture

positive. This can be due to colonization of *C. difficile* in younger children or diarrhoea caused by non-toxigenic strains of *C. difficile* as reported by Sultana *et al*[12].

Overall ELISA positivity was 14 per cent in this study. This was similar to the study by Dhawan *et al*[13], who reported 13.6 per cent positivity, but lower than that reported by Sultana *et al*[12] (28.8%). Dutta *et al*[20] reported 2.7 per cent isolation of *C. difficile* from control group, but in our study, none of the control stool samples grew *C. difficile*. All control samples were negative for toxins A/B by ELISA also. This was similar to another study[14] showing no association of *C. difficile* with carriage rate.

C. difficile is responsible for 15-25 per cent cases of AAD[22]; and was found to be an important enteropathogen responsible for AAD in 18 per cent cases in our study. An Indian study reported 15 per cent prevalence[13] and Oguz *et al*[23] identified *C. difficile* in 16 per cent cases of AAD.

Combination of antibiotics was responsible for AAD more frequently than single antibiotic treatment in this study. Most patients with *C. difficile* infection develop diarrhoea during antibiotic therapy or within 6-8 wk of treatment[24]. Antibiotics responsible are clindamycin, cephalosporins, or the extended spectrum penicillins[25].

Ampicillin in AAD is well documented[20,26], aminoglycosides and cephalosporins are also known to produce AAD[14,20,26]. In the present study, combination of cefotaxime and amikacin was responsible for diarrhoea in 28.9 per cent, and amikacin and ampicillin in 17.8 per cent positive cases. In an Indian study, combination of antibiotics was responsible in 23 per cent cases[20].

Severe diarrhoea and fever were significant predictors in our study, while abdominal pain was not. Thompson *et al*[27] had reported fever, abdominal pain and distension as the predominant symptoms. Liquid stool with mucus and blood was also a sensitive predictor for AAD in our study. Alvarez *et al*[28] have also reported presence of mucus in stools and a temperature over 37.8^0C as significant predictors of *C. difficile* infection. Presence of spores in Gram stain was also a definite predictor of *C. difficile* diarrhoea, though Fekety *et al*[29] reported on the contrary.

In this study, the diarrhoea subsided in about 70 per cent cases of AAD due to *C. difficile*, after withdrawal of the suspected antibiotic/s. Remaining cases responded to oral metronidazole therapy. Effective antimicrobial agents are metronidazole and vancomycin; 20-25 per cent patients experience re-infection or relapse after initial therapy and require retreatment[25]. In rare cases, subtotal or total colectomy is required[30]. Prompt and accurate diagnosis is very important as discontinuation of antibiotic resolve the diarrhoea.

In the control group, children in whom cysts of *E. histolytica* and ova of *Ascaris lumbricoides* were detected were given antiamoebic/antihelminthic drugs. Wet mount of the stool samples of these children were performed after three weeks and no cysts or ova were detected.

Though cytotoxin assay for toxin B is the gold standard, tissue culture facilities are required for accurate diagnosis and time taken is 1-2 days. Enzyme immunoassay for toxin A or B is less sensitive than cytotoxin assay, but it is rapid, easy to perform and has high specificity[31]. Culture, though sensitive is not specific for toxin producing bacteria and takes 2-5 days. Latex agglutination assay for bacterial enzyme (glutamate dehydrogenase) is rapid, easy to perform and inexpensive, but has poor sensitivity and specificity. Polymerase chain reaction (PCR) for toxin A or B genes, though highly sensitive and specific needs appropriate infrastructure and technical expertise[31].

In conclusion, *C. difficile* was found to be an important pathogen responsible for AAD in children

of 5-12 yr age group. Cefotaxime, amikacin and ampicillin were mainly responsible for causing AAD. Therefore, conservative use of antibiotics would be beneficial to decrease the occurrence of AAD.

Acknowledgment

Authors acknowledge the Indian Council of Medical Research (ICMR), New Delhi for providing financial support.

References

1. Finney JMT. Gastroenterology for cicatrizing ulcer of the pylorus. *Bull Johns Hopkins Hosp* 1893; *4* : 53-5.

2. Lyerly DM, Krivan HC, Wilkins TD. *Clostridium difficile*: Its disease and toxins. *Clin Microbiol Rev* 1983; *1* : 1-18.

3. Hall IC, O'Toole E. Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficilis. Am J Dis Child* 1935; *49* : 390-402.

4. Larson HE, Price AB, Honour P, Borriello SP. *Clostridium difficile* and the aetiology of pseudomembranous colitis. *Lancet* 1978; *i* : 1063-6.

5. Moncrief JS, Barroso LA, Wilkins TD. Positive regulation of *Clostridium difficile* toxins. *Infect Immun* 1997; *65* : 1105-8.

6. Tucker KD, Carrig PE, Wilkins TD. Toxin A of *Clostridium difficile* is a potent cytotoxin. *J Clin Microbiol* 1990; *28* : 869-71.

7. Norwood DA Jr, Sands JA. Physical map of the *Clostridium difficile* chromosome. *Gene* 1997; *201* : 159-68.

8. Kader HA, Piccoli DA, Jawad AF, McGowan KL, Maller ES. Single toxin detection is inadequate to diagnose *Clostridium difficile* diarrhoea in pediatric patients. *Gastroenterology* 1998; *115* : 1329-34.

9. Wei SC, Wong JM, Hsueh PR, Shieh MJ, Wang TH, Luh KT, *et al*. Diagnostic role of endoscopy, stool culture, and toxin detection in *Clostridium difficile*-associated disease. *J Formos Med Assoc* 1997; *96* : 879-83.

10. Vargas SO, Horensky D, Onderdonk AB. Evaluation of a new enzyme immunoassay for *Clostridium difficile* toxin A. *J Clin Pathol* 1997; *50* : 996-1000.

11. Pituch H, Obuch-Woszczatynski P, Rouyan GS, Martirosian G, Meisel-MiKolajczyk F. Detection of toxin producing strains of *Clostridium difficile* using rapid diagnostic methods. *Med Dosw Mikrobiol* 1998; *50* : 55-61.

12. Sultana Q, Chaudhry NA, Munir M, Anwar MS, Tayyab M. Diagnosis of *C. difficile* antibiotic associated diarrhoea culture versus toxin assay. *J Pak Med Assoc* 2000; *50* : 246-9.

13. Dhawan B, Chaudhry R, Sharma N. Incidence of *Clostridium difficile* infection: a prospective study in an Indian hospital. *J Hosp Infect* 1999; *43* : 275-80.

14. Rodrigues C, Menon S, Nukala R, Mehta AP. Detection of *Clostridium difficile* toxin A by an enzyme immunoassay. *Indian J Med Microbiol* 1997; *15* : 17-9.

15. Savola KL, Baron EJ, Tompkins LS, Passaro DJ. Fecal leucocyte stain has diagnostic value for outpatients but not inpatients. *J Clin Microbiol* 2001; *39* : 266-9.

16. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC, editors. Introduction to Microbiology. Part II. Guidelines for the collection, transport, processing, analysis and reporting of culture from specific specimen sources. In: *Color atlas and textbook of diagnostic microbiology*. 5th ed. Philadelphia: Lippincott-Raven; 1997 p. 131-6.

17. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC, editors. The anaerobic bacteria. In: *Color atlas and textbook of diagnostic microbiology*. 5th ed. Philadelphia: Lippincott-Raven; 1997 p. 770-4.

18. Sutter VL, Citron DM, Finegold SM, editors. Appendix. In: *Wadsworth anaerobic bacteriology manual*. 3rd ed. London: C.V. Mosby Company; 1980 p. 98, 106, 110, 111.

19. Lyerly DM, Neville LM, Evans DT, Fill J, Allen S, Greene W, *et al*. Multicenter evaluation of the *Clostridium difficile* TOX A/B TEST. *J Clin Microbiol* 1998; *36* : 184-90.

20. Dutta P, Niyogi SK, Mitra U, Rasaidy R, Bhattacharya MK, Chakraborty S, *et al*. *Clostridium difficile* in antibiotic associated pediatric diarrhea. *Indian Pediatr* 1994; *31* : 121-6.

21. Gorenkez L, Dizer U, Besirbellioglu B, Eyigun CP, Hacibektasoglu A, Van Thiel DH. The diagnosis and treatment of *Clostridium difficile* in antibiotic-associated diarrhea. *Hepatogastroenterology* 1996; *46* : 343-8.

22. Barbut F, Petit JC. Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* 2001; *7* : 405-10.

23. Oguz F, Uysal G, Dasdemir S, Oskovi H, Vidinlisan S. The role of *Clostridium difficile* in childhood nosocomial diarrhea. *Scand J Infect Dis* 2001; *33* : 331-3.

24. Giannella RA. Antibiotic-associated diarrhea and *Clostridium difficile*: an update. *Rev Esp Enferm Dig* 2001; *93* : 535-43.

25. Moyenuddin M, Williamson JC, Ohl CA. *Clostridium difficile* associated diarrhea : current strategies for diagnosis and therapy. *Curr Gastroenterol Rep* 2002; *4* : 279-86.

26. Zadik PM, Moore AP, Antimicrobial associations of an outbreak of diarrhoea due to *Clostridium difficile*. *J Hosp Infect* 1998; *39* : 189-93.

27. Thompson CM Jr, Gilligan PH, Fisher MC, Long SS. *Clostridium difficile* cytotoxin in a pediatric population. *Am J Dis Child* 1983; *137* : 271-4.

28. Alvarez M, Gonzalez R, Briceno I, Cofre C, Labarca J, Vial P, *et al*. Diagnosis of *Clostridium difficile* diarrhea: in search of a more efficient clinical focus. *Rev Med Chil* 2001; *129* : 620-5.

29. Fekety R, Shah AB. Diagnosis and treatment of *Clostridium difficile* colitis. *JAMA* 1993; *269* : 71-5.

30. Hurley BW, Nguyen CC. The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. *Arch Intern Med* 2002; *162* : 2177-84.

31. Ayyagari A, Agarwal J, Garg A. Antibiotic associated diarrhoea: Infectious causes. *Indian J Med Microbiol* 2003; *21* : 6-11.

Reprint requests: Dr Anuradha De, Associate Professor, Department of Microbiology, L.T.M. Medical College Sion, Mumbai 400022, India
e-mail: kshitija 234@rediffmail.com