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Changing pattern of *Clostridium difficile* associated diarrhoea in a tertiary care hospital: A 5 year retrospective study

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***Background & objectives*: Frequent use of broad spectrum antibiotics in hospitalized patients has increased the incidence of *Clostridium difficile* diarrhoea in recent years. In our tertiary care hospital in north India, *C. difficile* was responsible for 15 per cent of cases of nosocomial diarrhoea in 1999. A retrospective study was carried out to determine the frequency of *C. difficile* associated diarrhoea (CdAD) in our hospital, and to assess the effect of awareness among the hospital personnel and control measures taken to present *C. difficile* infection following the previous report.**

***Methods*: A retrospective chart review of all suspected cases of CdAD diagnosed at the hospital from January 2001 to December 2005 was done. Clinical specimens comprised 524 stool samples. All the samples were analyzed for *C. difficile* using culture and ELISA for toxin A and B. Attempts were made to type isolates using antibiogram, SDS-PAGE, gas liquid chromatography (GLC), PCR for toxin A and B gene fragments and restriction fragment length polymorphism (RFLP).**

***Results*: A total of 37 (7.1%) specimens were positive for *C. difficile* toxin (11.2% in 2001, 9.4% in 2002, 8.6% in 2003, 5% in 2004 and 4% in 2005). The highest number of *C. difficile* toxin positive cases were from stool samples of patients hospitalized in the haematology/oncology ward (67.5% of all positive cases) followed by gastrointestinal surgery, neurology and nephrology wards. Of the *C. difficile* toxin positive samples, 15 (41%) were also positive for *C. difficile* culture. The isolates were grouped in to one, 3 and 5 groups using antibiogram, SDS-PAGE and PCR RFLP respectively. We observed an increase in the number of stool specimens tested for *C. difficile* infection but a decrease in *C. difficile* positives.**

***Interpretation & conclusions*: A decrease in the number of *C. difficile* positive cases were noted during the 5 year study period though number of samples tested was increased. This may be due to stringent surveillance and an improved antibiotic policy followed in the hospital.**

Key words *C. difficile* - culture - toxin A - toxin B - typing

Diarrhoea is one of the most frequent side effects of antibiotic treatment. The symptoms may vary from slight abdominal discomfort to severe diarrhoea to colitis[1]. The aetiology of antibiotic associated diarrhoea

(AAD) varies. The disruption of normal enteric flora caused by antibiotics may lead to overgrowth of pathogens and functional disturbances of the intestinal carbohydrate and bile acid metabolism, resulting in osmotic diarrhoea[1]. Allergic, toxic and pharmacological effects of antibiotics may also affect the intestinal mucosa and motility[2]. Cytotoxin producing *Clostridium difficile* has been reported to be the causative agent of approximately 20 per cent of AAD and of nearly all cases of pseudomembraneous colitis, the most severe manifestation of AAD[1]. Because of the frequent use of broad spectrum antibiotics, the incidence of *C. difficile* diarrhoea has risen dramatically in recent years[3,4]. Established guidelines should be followed to minimize exposure to the pathogen which include judicious use of antibiotics, rapid detection of *C. difficile* by immunoassays for toxin A and B, isolation of patients who had *C. difficile* associated diarrhoea (CdAD), proper disinfection of objects and education of staff members[5]. In our hospital which is a tertiary care hospital in north India, *C. difficile* was responsible for 15 per cent of cases of nosocomial diarrhoea in 1999[6]. Standard control measures were implemented in our hospital to minimize the spread of this nosocomial pathogen after this report. This retrospective analysis was carried out in continuation of our earlier study[6] to determine the effect of awareness and control measures taken to contain *C. difficile* infection in our hospital during the subsequent years.

Material & Methods

The study comprised retrospective analysis of faecal specimens from 524 patients suspected on clinical grounds to have CdAD[2]. The patients were hospitalized in All India Institute of Medical Sciences, New Delhi, India, over a period of 5 yr (January 1, 2001 - December 31, 2005). These included 80 patients in 2001, 96 in 2002, 92 in 2003, 106 in 2004 and 150 patients in 2005 respectively. Of these, 53 per cent were males and 82.4 per cent were in all age group >12-60 yr (Table I).

Table I. Age and sex distribution of the patients (n=524)

	No. of patients (%)
Male	279 (53)
Female	245 (47)
Age group (*yr*):	
0-12	60 (11.5)
>12-6	432 (82.4)
>60	32 (6.1)

Clinical information about the cause of diarrhoea underlying disease and antimicrobial therapy was obtained by reviewing the patient charts. A patient was considered to have CdAD if AAD was present and a stool specimen was positive in a toxin dependent *C. difficile* assay.

Sample collection and isolation of C. difficile: All the stool specimens were processed immediately for culture of *C. difficile* and stool aliquots were stored at -20^{0}C for <72 h till they were tested for *C. difficile* toxin A and B. Spore selection was performed using 95 per cent ethanol and culture for *C. difficile* was done on cycloserine cefoxitin fructose agar (CCFA) and brain heart infusion agar (BHIA) as described elsewhere[6]. Concurrently, a loopful of stool specimen was inoculated into Robertsons cooked meat broth and incubated at 37^{0}C for 48 h.

The plates were incubated anaerobically at 37^{0}C in an anaerobic jar for 48 h. After incubation, the plates were examined and colonies which resembled *C. difficile* were Gram stained and identified by biochemical reactions using standard methods[7].

When culture plate were negative for *C. difficile*, subcultures were made from cooked meat broth onto CCFA and BHIA and incubated anaerobically at 37^{0}C up to 5 days before being discarded as negative.

Enzyme immunoassay for toxin A and B: Detection of enterotoxin and cytotoxin (toxin A and toxin B) of *C. difficile* was performed on the stool specimens by a double sandwich enzyme-linked immunosorbent assay technique using a commercial kit (Premier toxins A & B; Meridian Diagnostics, Inc., Cincinnati, Ohio, USA). The assay was performed according to the manufacturer's instructions.

Characterization of C. difficile isolates: All the *C. difficile* isolates were characterized phenotypically using antibiogram, SDS-PAGE[6], gas liquid chromatography (GLC)[7], and genotypically using PCR for toxin A gene and RFLP[8,9].

Antibiogram typing - Antibiogram patterns were determined by disc diffusion method[10]. The antibiotics tested were chloramphenicol (30 µg), penicillin G (10 units), clindamycin (2 µg), vancomycin (5 µg), metronidazole (5 µg), tetracycline (30 µg) and erythromycin (10 µg). The results were expressed as susceptible or resistant.

Analysis of volatile fatty acids by gas liquid chromatography (GLC): All isolates were inoculated

to cooked meat broth and incubated anaerobically for 48 h or more for GLC analysis to detect volatile fatty acids produced as metabolic end products. 1 ml of RCM broth was acidified with 0.2 ml of 50 per cent sulphuric acid and extracted with 1ml of diethyl ether. The mixture was shaken vigorously and centrifuged at 176 *g* for 3 min; 1.5 µl of the extracted ether layer was injected to the injection port of preconditioned GLC column with a 10 µl Hamilton syringe. Chromatography was performed on a Nucon Series 5700, fitted with a flame ionization detector (FID)[7]. Operating conditions were as follows: carrier gas (oxygen free nitrogen): 60 ml/min oven temperature: detector 240°C, column 175°C, injector 240°C, attenuation 4X, sensitivity of the detector was set at 1000X. Fatty acids were identified by comparing the retention times of peaks in the test samples with those of known standard solutions which were examined each day[7].

PCR assay for toxin gene fragments: The presence of toxin A gene in all isolates of *C. difficile* was determined by specific PCR using published primers[8]. PCR to detect the toxin B gene was performed in the *C. difficile* isolates using primers that had been developed and validated by Gumerlock *et al*[9] to yield a 399-bp fragment for toxin B gene. PCR was performed in a 25 µl reaction volume. Each reaction tube contained 1 X buffer (10 mm Tris HCl, *p*H 8.3, 50 mm KCl, 2.5 mm $MgCl_2$, 0.001% gelatin), each deoxynuclotide at a concentration of 100 mm (MBI, Fermentas, USA), each primer at a concentration of 20 pmol, 1.25 U Taq polymerase (MBI, Fermantas, USA) and 10 µl of DNA. PCR was performed for 2 min at 95°C followed by 30 cycles of 1 min of denaturation at 95° C, 1 min of annealing at 52°C and 1 min of extension at 72°C. After the 30th cycle, extension was continued for an additional 10 min. 10 µl of the amplified product was analyzed in 1 per cent agarose gel stained with ethidium bromide.

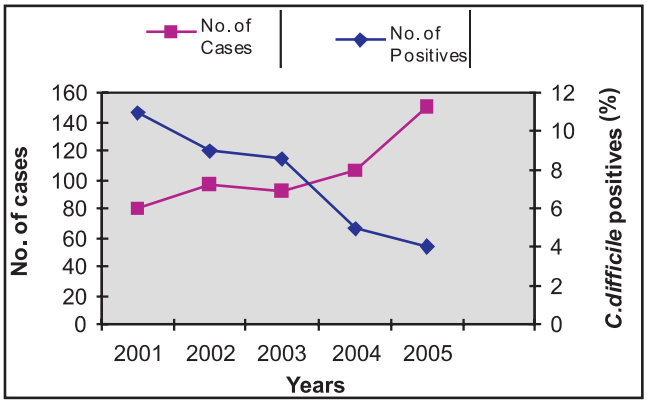

Fig. Percentage of *C. difficile* infection from year 2001 to 2005.

PCR-restriction fragment length polymorphism (RFLP) analysis: The amplified toxin A gene fragment was then digested with *Alu* I (10 units) restriction enzyme, under conditions recommended by the supplier (MBI-Fermentas). These digests were then subjected to electrophoresis on 2.5 per cent agarose gel at 60 V, along side a PCR size marker (100 bps, Sigma, USA).

Comparisons of patterns were performed visually. Strains with patterns differing alteast by one band were assigned to different types.

Results

A total of 524 stool specimens were analyzed for *C. difficile* from suspected cases of CdAD. The maximum number of *C. difficile* suspected cases were from oncology ward (378 cases, 72%), followed by other wards such as gastrointestinal surgery, neurology, nephrology and other medical wards. A total of 95 per cent of the analyzed group were on multiple antibiotics which included, 65 per cent on cephalosporins, 35 per cent on quinolones, 43 per cent on aminoglycosides, 12 per cent on macrolides, 69 per cent in vancomycin and metronidazole.

Of the analyzed group, 37 (7.1%) patients were positive for *C. difficile* infection by the toxin dependent assay. Of these, 9 samples (11.2%) were positive in 2001, 9 (9.4%) in 2002, 8 (8.6%) in 2003, 5 (5%) in 2004 and 6 patients (4%) in 2005 (Fig. 1). Fifteen (41%) of the 37 toxin positive stool samples were also positive for *C. difficile* by culture. Eight of the 37 toxin positive cases expired, the cause of death was not directly related to *C. difficile* diarrhoea, although this might have been a contributory factor. Other pathogenic clostridia isolated from the patient group included *C. perfringens* (2.5%).

The highest number of *C. difficile* toxin positive cases were from stool samples of patients hospitalized in the haematology/oncology ward (25 samples, 67.5% of all positive cases), followed by gastrointestinal surgery, neurology and nephrology wards. Recovery rates of *C. difficile* in patient populations surveyed and summarized in Table II.

Of the 37 positive cases, 19 (51%) were males; 32 patients (86%) experienced diarrhoea during antibiotic

Table II. Recovery rates of *C. difficile* in the study populations

Age group (yr)	Culture & ELISA positive	Culture negative, ELISA positives	Cases expired
0-12 (n=60)	1 (1.6)	3 (5)	1 (1.3)
12-60 (n=432)	10 (2.3)	27 (6.2)	5 (1.2)
>60 (n=32)	4 (12.5)	7 (21.8)	2 (6.2)
Total	15	37	8

Values in parentheses are percentages

treatment or within 15 days after the start of antibiotic treatment. The median time of occurrence of symptoms was 7 days (ranges 0-16 days) after start of antibiotic treatment and 8 days after admittance to hospital. All the patients were on multiple drugs and 50 per cent of the positive cases were on 3rd generation cephalosporins. None of the positive cases was on clindamycin. *C. difficile* positive cases were treated with metronidazole or vancomycin.

Antibiogram grouped all 15 isolates together as all were sensitive to erythromycin, chloramphenicol, penicillin, tetracyclin, clindamycin, vancomycin and metronidazole.

The identical fatty acid producers were grouped into 2 groups based on the production of isocaproic acid. Except one, all the isolates were producing isocaproic acid.

Based on the protein profiles observed on SDS-PAGE, the isolates were placed into 3 groups; 12 isolates in group A, 2 in group B and 1 in group C.

PCR and RFLP analysis: All the isolates were positive for toxin A (1.2 kb fragment) and B (399 bp) gene by PCR. Five different restriction profiles were obtained using *Alu* I endonucleases. The isolates were classified into five RFLP groups. The most frequent RFLP type was group I (6 isolates) group II, III, IV and V had 5, 2, 1 and 1 isolates respectively.

Discussion

C. difficile is considered as the most frequent aetiological agent of nosocomial diarrhoea occurring in hospitalized patients, spreading easily to the environment, the hands of health care workers and subsequently to other patients, particularly in large hospitals[12]. A trend of increasing prevalence of *C. difficile* has been reported in Europe and USA during the past 10 years[13].

In our hospital *C. difficile* was found to be responsible for 15 per cent of the cases of nosocomial diarrhoea in our earlier study[6]. In this study, *C. difficile* was isolated mainly from patients in the haematology/ oncology wards. This points to the high risk areas for nosocomial spread of *C. difficile* isolates[14]. However, the percentage of infection showed a gradual decrease during the recent years.

Standard laboratory methods for diagnosing these infections include stool culture and identification of bacterial isolate, faecal toxin detection and *C. difficile* antigen detection. The culture lacks specificity due to the possible faecal carriage of non-toxigenic isolates, therefore many laboratories rely on toxin detection rather than culure for diagnosis of *C. difficile* infection[15]. A European survey of diagnostic methods for *C. difficile*, showed that culture of the organism is performed only in few countries. Mostly *C. difficile* toxin EIAs were used for diagnosis of CdAD[16]. In this study we used ELISA for toxin A, B and culture for diagnosing *C. difficile* infection. However, in the previous study[6] we used *C. difficile* toxin A dependent ELISA for the analysis.

There was a gradual decrease in the percentage of *C. difficile* infection during 2001 and 2005. The fact that 14 of the 22 culture negative cases were on metronidazole or vancomycin at the time of sample analysis might be responsible for the decrease in isolation of organism as compared with the ELISA.

Older age, female gender and a prolonged hospital stay have been identified as risk factors in hospitalized CdAD patients[17]. In the current study, there was no gender prevalence among the positive cases and the median age of positive cases were 39 yr. However, highest percentage of culture positives was seen among patients >60 yr of age. Prolonged courses of antibiotic treatment have been related to an increased risk of AAD[18,19]. The median time for occurrence of symptoms was 7 days after the start of treatment in the present study, which was in accordance with other studies[1,20]. This suggests that disturbance of the normal colonic

flora, eventually resulting in diarrhoea, takes place within about one week of antibiotic treatment. Prolonged duration of hospital stay has also been reported to be associated with AAD and CdAD[19, 21]. In the present study, the median time of hospital stay was 8 days.

AAD was found to be frequently associated with cephalosporins, clindamycin and broad spectrum penicillins and quinolones[22-25]. In this study, about 50 per cent of our CdAD cases were on cephalosporins. However, since all the patients were on multiple antibiotics, the association with a particular group was not identified.

Discontinuation of antibiotic therapy withdraws the offending agents but is often not appropriate if the indication for such therapy was correct. An alternative is to change to antibiotics that do not belong to the high risk groups for induction of CdAD, such as quinolones, sulphonamides, parenteral aminoglycosides, co-trimoxazole, *etc*[26]. Metronidazole is suggested as the first line drug for the treatment of *C. difficile* infection[2], and therefore the policy of the use of metronidazole in the treatment of suspected CdAD in our hospital is justified.

No nosocomial outbreak of *C. difficile* was reported during the study period. In this study we found antibiogram was least discriminatory of the typing strategies evaluated. The detection of short chain fatty acids by GLC is commonly utilized in bacteriological laboratories to identify anaerobes[27]. As all the isolates were positive for toxin A gene, we looked forward to analyze the variability of toxin A gene among *C. difficile* isolates by RFLP analysis. As the sequence analysis of the amplified 1.2 kbp toxin A gene fragment does not show any restriction sites for the previously reported restriction enzymes like *Hin*c II, *Hin*d III, *Acc* I, *Eco*R I[28], we decided to examine the amplified gene structure using restriction enzyme *Alu* I (5' AG↓CT 3', 3'TC↑GA 5'), which showed multiple restriction sites (8 sites) in the amplicon.

Although PCR-RFLP types 1 and II clustered some patient isolates, there was no epidemiological association between them. The locations where these patients were housed were different, and were admitted at different time periods. Better discriminatory methods such as pulsed field gel electrophoresis (PFGE) or ribotyping may be used to analyze the epidemiology of the pathogen.

In our recent prospective study, all the *C. perfringens* isolates were analyzed for the presence of enterotoxin by reverse passive later agglutination (RPLA), ELISA and by PCR assay for the presence of enterotoxin gene[29]. Of these, two were positive by PCR, RPLA and ELISA for *C. perfringens* enterotoxin. None of these samples had a co-infection with *C. difficile*.

Prevention of *C. difficile* infection is challenging. A change in antibiotic policy and implementation of standard infection control measures reduce the incidence of *C. difficile* symptomatic infections[30,31]. Combined approach, involving effective control measures, the use of rapid and sensitive techniques for laboratory diagnosis as well as prudent use of antibiotics, is necessary to reduce morbidity and mortality due to *C. difficile* associated infections in hospitalized patients.

In conclusion, we observed a decrease in the number of *C. difficile* toxin positive cases during the 5 yr of the study though there was an increase in the number of stool specimens tested per year for *C. difficile.* This possibly could be a result of stringent surveillance and antibiotic policy followed in our hospital especially in high risk areas such as haematology/oncology wards. Secondly, the use of clindamycin has been minimized in the hospital. Thirdly, antibiotics effective against *C. difficile* such as metronidazole have been included as the first line drugs in suspected CdAD cases. Isolation of the patients having *C. difficile* infection and regular awareness programmes conducted in the hospital might also have contributed.

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