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Antibiotic Associated Diarrhea with Special Reference to *Clostridium difficile*

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author RB designed the study and managed the analyses of the study. Author SP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Antibiotic associated diarrhea (AAD) is a common side effect of treatment with antibiotics. Depending on the antibiotic used, up to 25% of patients experience such unpleasant bowel conditions. The normal flora is replaced by pathogenic organisms like *Candida species*, *Clostridium difficile* etc which play an important role in the causation of AAD. Almost all the antibiotics have been associated with diarrhea. *C. difficile* is known to occur as an outbreak in health care settings in cases of AAD. However reports from India are in-frequent. Aims: Our present study was undertaken to assess the role of different pathogen in causation of AAD and to evaluate the role of various antimicrobials in causation of AAD.
Study Design: Prospective observational study.
Place and Duration of Study: Department of Microbiology, B.J. Government Medical College and Sassoon General Hospital, Pune from January 2014 to December 2015.
Materials and Methods: Stool samples of 70 patients developing AAD were included in the study. Individuals from all age groups were included in the study. Majority of the patients were from the age group 31 to 40 years with female preponderance Outdoor as well as Indoor patients from all

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faculties like medicine, surgery, obstetrics and gynecology and pediatrics were included in the present study. Samples were subjected to aerobic, anaerobic and fungal culture. *C. difficile* toxin was detected in stool by ELISA. PCR was also performed to look for the presence of *C. difficile* DNA in stool sample.

Results: Ceftriaxone (55.7%) was the commonest antibiotic causing AAD followed by amoxicillin (44.3%) and amoxicillin plus clavulanic acid (41.4%). *Candida species* was the major pathogen isolated from 58.6% of patients. *Clostridium difficile* associated diarrhea either by culture, PCR or toxin detection was seen in 18.6% of patients. Aerobic pathogens were detected in 18.6% cases of AAD. *Klebsiella pneumonia* (8.6%) was the commonest. No pathogens were detected in 4.3% cases.

Conclusion: *Candida species* (58.6%) is the major pathogen in AAD in our tertiary care center. However *Clostridium difficile* (18.6%) seems to be an emerging pathogen in our health care setting. It has been reported from the developed countries in epidemic proportions but seems to be just gaining foothold in India. Apart from stoppage of antibiotics, it is important to identify the pathogen in AAD so that appropriate management may be instituted.

Keywords: Clostridium difficile; antibiotic associated diarrhea; candida; ceftriaxone; amoxicillin; proton pump inhibitors; clostridium difficile associated diarrhea.

1. INTRODUCTION

Advances in medicine have resulted in increase in the use of antibiotics in health care settings. Indiscriminate use of antibiotics has resulted in the emergence of problems like antibiotic-associated diarrhea (AAD). About 5%–25% patients undergoing antibiotic treatment often develop AAD as a side effect of therapy ranging from a mild illness to a serious complication like pseudomembranous colitis [1]. Almost all antibiotics have been associated with diarrhea. However some antibiotics are more commonly associated with AAD such as ampicillin, amoxicillin, clindamycin and cephalosporins [2]. Apart from antibiotic use other risk factors such as use of proton pump inhibitors (PPI) and immunosuppressive agents and chemotherapeutic agents can also give rise to a similar presentation [3].

Exposure of the colonic ecosystem to antimicrobial agents results in a shift in population of the microbes as a result of suppression or elimination of some microorganisms or overgrowth by microorganisms not susceptible to these agents and establishment of antimicrobial-resistant members that are normally excluded. The normal flora is replaced by pathogenic organisms like *Clostridium difficile, Clostridium perfringes, Staphylococcus aureus, Klebsiella pneumonia, Klebsiella oxytoca, Pseudomonas species, Salmonella species, Candida species* etc which play an important role as an etiological agent of AAD [4]. *C. difficile* has resulted in nationwide outbreak in the developed world in health care settings. Over the last decade there has been a marked increase in *C. difficile* infection incidence and mortality across the United States, Canada, and Europe [3,4]. However reports of this pathogen from India are limited.

So, a prospective cross sectional study was planned to identify the microorganisms associated with AAD with the following Aims & objective-

1) To evaluate the role of various antimicrobials and proton pump inhibitor in causation of AAD.
2) To determine the prevalence of *Clostridium difficile* in AAD in a tertiary care center.
3) To study the role of aerobes and fungus in causation of AAD.

2. MATERIALS AND METHODS

2.1 Study Design

A prospective observational study was conducted after ethical approval. 70 patients were enrolled in the study after taking informed consent.

2.1.1 Inclusion criteria

Individuals from all age groups were included in the study. Outdoor as well as indoor patients from all faculties like medicine, surgery, obstetrics and gynecology and pediatrics were included in the present study. A detailed history regarding age, sex, duration of antibiotic

treatment, frequency of diarrhea and associated symptoms was obtained in each case.

Patients were identified as having AAD if they develop diarrhea in 3 or more days of antibiotic usage and within 4 weeks after discontinuation of antibiotics and with no other identifiable cause of diarrhea.

2.1.2 Exclusion criteria

Patients presenting to the hospital with diarrhea without history of antibiotic therapy.

2.2 Laboratory Processing of the Stool Samples

2.2.1 Collection of sample

Stool samples were collected from the patients giving history suggestive of 'antibiotic associated diarrhea'. Samples were collected in sterile wide mouth leak proof tightly lidded container. For anaerobic bacteriology 5 loops full of the sample were immediately transferred to robertson's cooked meat medium. The stool specimens were processed immediately without delay.

2.2.2 Macroscopy and microscopic examination

- Macroscopically the colour, consistency, presence of blood and mucous was noted.
- Microscopic examination of thin smear of the stool sample was prepared and stained with gram stain. Smear was examined for the presence of polymorph nuclear leukocytes, yeast cells, presence of pseudohyphae, and morphology of bacteria seen were described.

A saline and iodine wet mount of the fecal sample was examined for the presence of parasites, eggs and cysts. Patients showing parasitic infestation were excluded from the study [5].

2.2.3 Follow up of sample for aerobes

Stool sample was inoculated on MacConkey agar and Blood agar and incubated directly and after enrichment. All plates were incubated at 37°C overnight. Colonies obtained were identified by standard techniques [5].

2.2.4 Follow up for fungi

Stool sample was inoculated on Sabourauds dextrose agar which was incubated at 37°C. Growth of creamy white moist colonies was noted. Gram stain was made from these colonies to look for the presence of yeast cells i.e. round or oval cells showing budding with presence or absence of pseudo hyphae. Further processing and identification of the organisms was done by standard microbiological techniques [5].

2.2.5 Follow up of stool sample for anaerobes

Stool sample from robertson's cooked meat medium after overnight incubation at 37°C were then inoculated on to

a) Neomycin blood agar
b) Kanamycin – vancomycin blood agar
c) Willis and hobbs medium
d) Cycloserine Cefoxitin Fructose Egg yolk Agar (CCFA)

All different type of colonies obtained were then tested for aero tolerance. Organisms which did not grow on aero tolerance plate were regarded as anaerobes. Strict anaerobes obtained were identified by standard protocol [6].

C. difficile was identified by its colony appearance on CCFA (Fig. 1), chartreuse fluorescence (Fig. 2) and biochemicals.

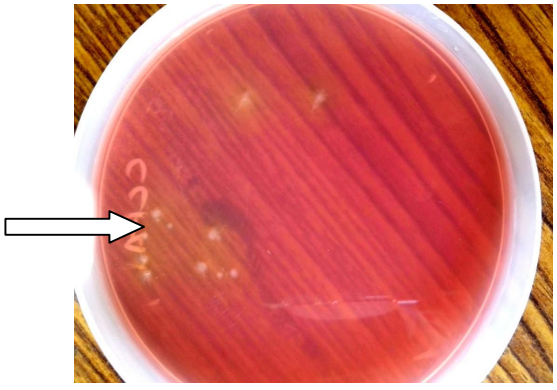

Fig. 1. Growth of *C. difficile* on cycloserine cefoxitin fructose egg yolk agar. Colonies are large flat to low umbonate, yellow with a ground glass appearance and a slightly filamentous edge. The medium has changed from orange pink to yellow around the colony

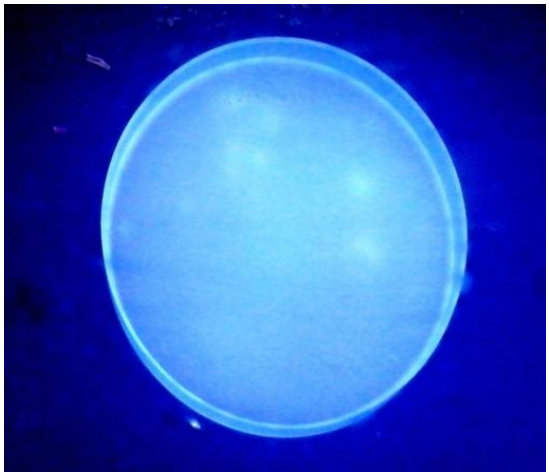

Fig. 2. Colonies of *C. difficile* showing yellow green or chartreuse fluorescence when exposed to ultraviolet light (360 nm)

MIC for metronidazole and vancomycin was detected for all *C. difficile* isolate by using the E test method (Himedia).

Gram stain, culture results and clinical presentation of patients were all taken into consideration before deciding the causative organism of antibiotic associated diarrhea.

2.2.6 Toxin detection by ELISA

All stool samples were processed for the presence of *C. difficile* toxin A and B by Enzyme immunoassay which was performed [3]. In the present study the kit used was Premier Toxins A & B in vitro Diagnostic Medical Device, USA. The assay was performed according to the manufacturer's instructions.

A positive result was taken to be a test positive for presence of *C.difficile* toxin A and /or B [3].

2.2.7 PCR

Since *C. difficile* is difficult to grow an attempt was also made to detect *C. difficile* in stool by PCR. DNA extraction was done by silica gel method [7,8]. PCR was performed for detection of species specific regions of the 16S rDNA of *C. difficile* on all stool samples [9]. PCR products were analyzed by electrophoresis using 2% agarose gel. The amplified product was visualized on an UV trans-illuminator. Appearance of expected size band of 157 bp for *C. difficile* was noted as PCR positive as shown in Fig. 3.

3. RESULTS

The study was carried on 70 patients developing diarrhea after antibiotic use.

Table 1. Shows age wise distribution of the patients included in the study

Age (years)	No. of patients (n= 70)	Percentage (%)
<1 year	5	7.1
1 to 10	3	4.3
11 to 20	4	5.7
21 to 30	10	14.3
31 to 40	17	24.3
41 to 50	13	18.6
51 to 60	10	14.3
61 to 70	5	7.1
71 to 80	3	4.3

Majority of the patients were from the age group 31 to 40 years (24.3%) followed by age group 41 to 50 years (18.6%). 7.1% of patients were below 1 year of age. 42 patients (60%) were female while 28 patients(40%) were males. So, female preponderance (60%) was seen.

Distribution of patients by hospital department/ services is shown in Table 2.

Table 2. Shows distribution of patients by hospital department/ services

Department	No. of patients	Percentage (%)
In patients	65	92.9
1)Surgery	22	31.4
2)Medicine	19	27.1
3)Obstetrics and Gynecology	14	20
4)Pediatrics	10	14.3
Out patients	5	7.1
Total	70	100

AAD was more common in indoor patients (92.9%) as compared to outdoor (7.1%). Majority of patients (31.4%) in present study were from Faculty of Surgery.

The antibiotics which resulted in causation of AAD are shown in Table 3.

Patients receiving ceftriaxone more commonly developed AAD, followed by amoxicillin and amoxicillin plus clavulanic potassium. 47.1% of the patient received multiple antibiotics and 4 (5.7%) patients in addition received proton pump

inhibitors. However, no patient in the current study was receiving only proton pump inhibitors.

Table 3. Shows distribution of antibiotic intake in the study group

No.	Antibiotic	No. of patients	Percentage (%)
1	Ceftriaxone	39	55.7
2	Amoxicillin	31	44.3
3	Amoxicillin + clavulanate potassium	29	41.4
4	Amikacin	22	31.4
5	Metronidazole	14	20
6	Norfloxacin	9	12.9
7	Ciprofloxacin	6	8.6
8	Trimethoprim-Sulfomethoxazole	3	4.3
9	Erythromycin	2	2.9
10	Penicillin	1	1.4

The predominant organisms isolated from stool in patients of AAD are shown in Table no. 4.

Table 4. Shows predominant organism isolated from patients of AAD

Organism	Total	Percentage (%)
Candida Spp.	**41**	**58.6**
• *Candida albicans*	31	44.3
• *Candida non albicans*	10	14.3
C. difficile (Culture/PCR/Toxin)	**13**	**18.6**
Aerobic organisms		
• *Klebsiella pneumonia*	6	8.6
• *Pseudomonas aeruginosa*	4	5.7
• *Klebsiellaoxytoca*	1	1.4
• *S.aureus*	2	2.9
Anaerobic organism	**2**	**2.9**
No known pathogen	**3**	**4.3**

Candida species was the major pathogen isolated from 58.6% cases of AAD. *Candida albicans* in 44.3% and *non albicans Candida* in 14.3% of AAD cases.

Aerobic pathogens were isolated from 18.6% of the patients. Among these *Klebsiella pneumonia* was the predominate isolate in 8.6% of patients followed by *Pseudomonas aeruginosa* (5.7%).

Overall 13/ 70 (18.6%) patients gave evidence of *C. difficile* infection either by culture, PCR or toxin detection.

Table 5. Patients demonstrating *C. difficile* in stool by culture / PCR / Toxin (n =13)

Patient no.	Culture	PCR for *C. difficile* DNA	Toxin by ELISA
1	+	+	+
2	+	+	+
3	-	+	-
4	-	+	-
5	-	+	-
6	-	+	+
7	-	+	+
8	-	+	+
9	-	+	+
10	-	-	+
11	-	-	+
12	-	-	+
13	-	-	+

+ positive, - negative

E test showed MIC of 0.19 (ug/ml) and 0.064 (ug/ml) for vancomycin in the two isolates respectively. MIC for metronidazole was 256 (ug/ml) and 128 (ug/ml) for the two isolates respectively. Both the isolate were resistant to metronidazole.

In the present study the only complication encountered was Pseudomembranous colitis in one (7.7%) patient of *Clostridium difficile* associated diarrhea.

4. DISCUSSION

Antibiotic usage is a common cause of diarrhea in health care centers. It is a growing nosocomial and public health problem. The prevalence of *C. difficile*-associated disease is global and the incidence varies considerably from place to place.

In present study the maximum number of AAD cases (24.3%) occurred in the age group of 31 to 40 years. Mean age of the patients was 36.9. Our results are in agreement with Swartzberg et al. [10] who also observed that antibiotic associated diarrhea occurred nearly twice as often in patients over twenty years than in younger patients and the mean age observed was 35.7. Gurwith et al. [11] have reported AAD to be more common in older age group. In their study 46% of the patients were 60 years and above. There was female (60%) preponderance seen in the present study. Leigh and Simmons [12] have reported AAD to be more common in age group over 60 years and it was seen more in women (19%) than men (13%).

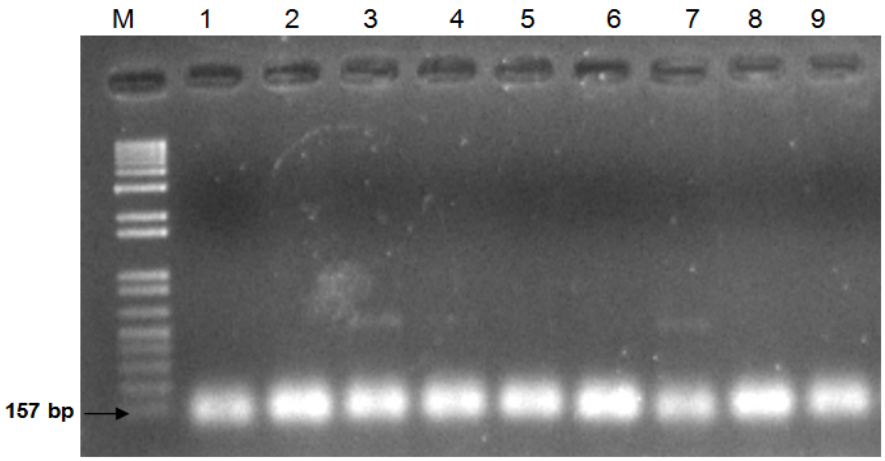

Fig. 3. Bacterial PCR for *C. difficile*. 2 percent agarose gel electrophoresis of conventional PCR product (~157 bp) for *C. difficile*. Lane M: Molecular weight marker (1 kbp ladder). Lanes 1 to 9 shows positive band for *C. difficile* in stool sample of AAD cases

Swartzberg et al. [10] and Wistrom et al. [13] also reported AAD cases more common in females. However there have been studies which have reported male predominance [3,14,15]. Chaudhry et al. [16] in their study reported that there was no gender predilection.

Maximum patients (92.84%) in the current study were indoor patients as compared to outdoor patients. Various other studies like Gogate et al. [15], Lusk et al. [17], Neu et al. [18], Biddle et al. [19] and Gerding et al. [20] have also reported AAD to be more in hospitalized patients. The reason for this is long term exposure to antibiotics of these patients. Among the hospitalized patients in the current study majority of them belonged to Surgical units (31.4%) followed by medical (27.1%), obstetrics and gynecology (20%) and pediatric units (14.28%) Gerding et al. [20] observed that 75% of patients in their study belonged to surgical services. However Vishwanath et al. [21] reported that most of their patients (72%) with AAD were from general medical wards, followed by oncology (12%); surgery (8%), and pediatric wards (8%).

Faculty wise prevalence of AAD may differ from center to center based on antibiotic prescribing practices. Incidence of antibiotic associated diarrhea is probably seen more in surgical units because of long term antibiotic treatment are used as a part of surgical prophylaxis after major contaminated or clean contaminated surgery.

In the present study, patients developing AAD had received ceftriaxone (55.7%) followed by amoxicillin (44.3%) and amoxicillin plus clavulanate potassium (41.4%). High rates of diarrhea with these drugs probably reflect their common use in our hospital. Other studies have also reported use of cephalosporin to be commonly associated with AAD [10,18,19]. All the cases of *C. difficile* associated diarrhea were on ceftraixone alone or in combination. Among other cephalosporin Gogate et al. [15] reported that cefotaxime in combination with amikacin were responsible for 8.4% cases and cefotaxime and ampicillin in 7.2% and only cefotaxime in (3.2%).

The other two antibiotics commonly associated with AAD in study were amoxicillin (44.3%) and amoxicillin plus clavulanate potassium (41.4%). Turck et al. [22] have shown that the incidence of AAD cases in their study was particularly high after administration of amoxicillin /clavulanate (23%). Also, our study showed that quinolones such as norfloxacin was associated with 12.9% and ciprofloxacin was associated with 8.6% cases of AAD. Vaishnavi et al. [23] reported that quinolones including fluoroquinolones were associated with AAD in 14.2% of their patients.

Proton pump inhibitor (PPI) as a risk factor have been implicated in the causation of *C. difficile* diarrhea because the survival of spores is facilitated by elevated gastric pH levels and due to the effect of PPI on the toxin production of the organism or on immune function of the host. Our study showed that 4 (30.7%) patients diagnosed as *C. difficile* associated diarrhea were on proton pump inhibitor. Kaneria and Paul [14] reported

that all the patients with *C. difficile* associated diarrhea in their study were on PPI. However McFarland et al. [24] in 2007 reported no association between PPI use and nosocomial *C.difficile* associated diarrhea.

As conflicting data exist and till further evidence is available PPI should be used cautiously.

Our research showed that *Candida* species was the major pathogen, accounting for 58.6% of AAD cases. *C. albicans* was isolated from 44.3% and non *Candida albicans* from 14.3 % patients. In a study carried out by Nkuo-Akenji TK et al. [25] *C. albicans* was identified in 35.9% of samples. Similar study done by Brad et al. [26] found that *Candida albicans* was responsible for 20% of AAD cases. Vaishnavi et al. [27] have observed that the predominant isolates were *C. tropicalis* followed by *C. albicans* and *C. krusei*. Hyun Joo Song, et al. [28] isolated *Candida albicans* in 5.2% and *Candida tropicalis* in 2.6% AAD cases. The results of the present study suggest that *C. albicans* is an important cause of antibiotic associated diarrhea in our settings. Thus, this fungus should be routinely checked in individuals presenting with diarrhea in patients on prolonged or frequent antibiotic therapy.

In addition, our study showed 18.6% of aerobic pathogens isolated in patients with AAD. Among these *Klebsiella pneumonia* was isolated in 8.6% of patients followed by *Pseudomonas aeruginosa* (5.7%), *Staphylococcus aureus* (2.9%) and *Klebsiella oxytoca* (1.4%). Leigh and Simmons [12] observed increased counts of Enterobacteriaceae in 64% of their patients with antibiotic associated diarrhea. A study carried out by Hyun Joo Song et al. [28] reported that *K. pneumoniae* accounted for 15.8% cases of AAD.

Antibiotic-associated-hemorrhagic-colitis (AAHC) is a distinct form of antibiotic-associated-colitis (AAC) caused by *K. oxytoca.* In our study *K. oxytoca* was isolated from 1 (1.4%) patient .Högenauer et al. [29] reported that 83.3% patients suffering AAHC tested positive for *K. oxytoca*. A prospective study of hospitalized patients with AAD identified cytotoxic *K. oxytoca* in 16.7% patients with bloody diarrhea [30].

Brad et al. [26] isolated *Pseudomonas aeruginosa* in 72.7% of AAD cases. Kim, Peck, Jung, et al. [31] isolated *P. aeruginosa* from stool cultures of seven patients who developed diarrhea after the administration of antibiotics. Chandra et al. [32] observed *Pseudomonas aeruginosa* in 3% cases of hospital-acquired diarrhea.

Staphylococcus aureus is known to cause antibiotic associated diarrhea and colitis [28,33-36]. In the present study *Staphylococcus aureus* was isolated from 2.9% of the cases. Various studies have also reported cases of *S. aureus* associated with antibiotic- associated diarrhea [28,33,34,35,36]. In fact in two patients it was obtained in pure culture in our patients where no other pathogen was detected.

4.1 *Clostridium difficile* Associated Diarrhea

C. difficile is considered as the most frequent etiological agent of nosocomial diarrhea occurring in hospitalized patients in developed countries, spreads easily to the environment, to the hands of health care workers and subsequently to other patients.

Clostridium difficile was associated in 18.6% of cases of AAD.

Toxin was demonstrated in 10 (14.9%) patients and PCR demonstrated presence of organism in additional 3 patients.

Prevalence *C. difficile* associated diarrhea varies from 7.3% to 17.5% according to different geographical areas [3,14,15,16,21,37, 38,39]. The prevalence of *C. difficile* associated diarrhea in our study is almost similar to study conducted by Gogate et al. [15] from Mumbai. However some authors like Chaudhry et al. [16] (7.1%) and Bhattacharya et al. [37] (7.3%) have shown lower prevalence of *C. difficile* associated diarrhea from New Delhi and Kolkata respectively. Manabe et al. [40] from Baltimore, USA reported positivity rate of 14.55% whereas a higher prevalence rate was reported by Wistron et al. [13] from Sweden. He observed 62.5% positivity rate of *C. difficile* infection among AAD patients.

In the present study the only complication we encountered was Pseudomembranous colitis (PMC) in one patient (7.7%).Various studies have suggested Pseudomembranous colitis as the most severe manifestation of *C. difficile* associated diarrhea [2,4].

Gurwith et al. [11] reported PMC in 2.1% cases of clindamycin associated diarrhea and 0.3% in ampicillin associated diarrhea. However Ingle et

al. [3] have observed no complications in their study group.

Drug of choice for *C. difficile* infection is metronidazole and vancomycin. Both isolate were resistant to metronidazole and sensitive to vancomycin. In a study carried out by Nils Henning Zaiß et al. [41] all isolates were susceptible to metronidazole and vancomycin by E test method. So, metronidazole resistance in *C. difficile* seems to be an emerging phenomenon, though resistance to metronidazole has been reported in other anaerobes [42].

In 4.3% cases no known pathogen was isolated. The cause of AAD in these patient could be due to other mechanisms which include decreased metabolism of carbohydrates, reduced break down of primary bile acids, antibiotic having allergic or toxic effects on intestinal mucosa and their effect on gut motility or increase in the amount of un-degraded fiber in the feces, which increases the bulk of the feces leading to diarrhea [2].

5. CONCLUSION

Thus to conclude *Clostridium difficile* is an emerging pathogen in AAD in health care settings. It has been reported from the developed countries in epidemic proportions but seems to be just gaining foothold in India. However, *Candida species* remain the major pathogen in AAD. It is important to identify the pathogen in AAD so that appropriate management may be instituted. However restricting the use of antibiotics would go a long way in preventing antibiotic associated diarrhea.

CONSENT

All authors declare that written informed consent was obtained from the patient.

ETHICAL APPROVAL

Ethical clearance was obtained from the institutional ethics committee with reference no. BJMC/IEC/ Pharmac/D-1113131-131.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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