

Surveillance for Antibiotic Resistance in *Clostridium difficile* Strains Isolated from Patients in a Tertiary Care Center

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Abstract

Clostridium difficile is the major etiological agent of nosocomial diarrhea primarily precipitated by antimicrobial therapy. We prospectively investigated the antibiogram profile of *C. difficile* strains isolated from patients reporting to a tertiary care hospital in North India. Fecal samples obtained from 1110 suspected cases of *C. difficile* infection were cultured for isolation of *C. difficile*. Colonies suspected as those of *C. difficile* were identified by phenotypic and molecular methods. Antimicrobial susceptibility of *C. difficile* isolates for different classes of antibiotics was determined using the Epsilon test for vancomycin, metronidazole, clindamycin and ciprofloxacin. The fecal samples cultured for *C. difficile* belonged to 709 (63.9%) males and 401 (36.1%) females. The mean age of the patients was 38.7 years. *C. difficile* was cultured from 174 (15.7%) of the total samples. Antibiotic resistance was largely observed towards clindamycin (57.5%) and ciprofloxacin (38.5%) but was significantly low towards metronidazole (1.72%) and nil (0%) towards vancomycin. *C. difficile* isolates had a high degree of resistance towards clindamycin and ciprofloxacin with low level of resistance to metronidazole and none towards vancomycin. Antibiogram surveillance of *C. difficile* will help for clinical practice and add to the epidemiological data of the organisms.

Keywords

Clostridium difficile, Cultural Identification, Antimicrobial Susceptibility, E-Test

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1. Introduction

Clostridium difficile, a Gram-positive spore bearing, anaerobic toxigenic bacterium is the major etiological agent of nosocomial diarrhea and is frequently precipitated with antimicrobial therapy [1]. *C. difficile* infection (CDI) is associated with considerable morbidity, mortality and relapse among hospitalized patients across the globe [2] [3]. Metronidazole is the first line of therapy for CDI. This drug has been found to be effective in mild to moderate disease on the basis of its efficacy, cost and antimicrobial stewardship. In more severe cases of CDI, vancomycin has been shown to be superior to metronidazole [4].

According to a European survey of diagnostic methods for *C. difficile* identification, culture of the organism is performed only in a few countries [5]. This is because *C. difficile* is a fastidious organism and requires selective medium for its isolation. A variety of culture media like ceftioxin-cycloserine-fructose agar (CCFA) or *C. difficile* moxalactam norfloxacin [6] [7] are available for its isolation. CCFA was the initial formulation developed for isolation of *C. difficile* [6]. But *C. difficile* also grows easily on Columbia blood agar (CBA) at 37°C by 48 hours [8]. The addition of lysozyme or bile salts such as taurocholate to the medium facilitates the outgrowth of spores [9] [10].

Acquisition of resistance to clindamycin is considered as one of the mechanism whereby clonal strains of *C. difficile* emerge and predominate in healthcare environments. Historically, fluoroquinolone antimicrobial agents were considered as low risk for CDI. However, studies carried out later on indicated a shift in the risk associated with their use [11] [12]. Furthermore outbreaks in Canada and the United States during the last decade have been associated with fluoroquinolone exposure and the emergence of the fluoroquinolone resistant hypervirulent NAP1/BI/027 (North American Pulse Field type 1, Restriction Endonuclease Assay BI, Ribotype 027) strain [13].

Studies on antibiotic resistant pattern of *C. difficile* from developing countries are sparse. This is due to the lack of funding and facilities for anaerobic culture. Initially, the disk diffusion method was used for antibiotic susceptibility by workers in the field. Later on Epsilon test (E-test) became available and was found to be a reliable and easy-to-perform method for minimal inhibitory concentration (MIC) determination of *C. difficile* antibiotic susceptibility testing in diagnostic laboratories [14]. We performed a prospective study to investigate the antibiogram profile of *C. difficile* strains cultured from the fecal samples of patients suspected to have CDI.

2. Materials and Methods

The study was carried out at Post Graduate Institute of Medical Education and Research, a tertiary care center of North India. This hospital caters to different parts of North India viz. Chandigarh, Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir, western parts of Uttar Pradesh and some parts of Rajasthan. The study was approved by the Institute Ethics Committee and was carried out from June 2012 to May 2014. A total of 1110 patients suspected to have CDI formed the basis of investigation. Informed consent was taken from all the patients or their wards.

2.1. Laboratory Chemicals and Media Used in the Study

All chemicals used were of analytical grade. Robertson's cooked meat media (RCM), Columbia blood agar, urea agar base, Brucella agar, esculin agar, esculin, urea, sodium taurocholate, sodium borohydride, citric acid and glucose were procured from HiMedia, Mumbai, India. Sodium bicarbonate, methylene blue, crystal violet, iodine, safranin, hydrochloric acid and p-dimethylaminobenzaldehyde were purchased from Central Drug House, New Delhi, India.

2.2. Isolation and Phenotypic Identification of *C. difficile*

Single fecal specimens collected from the patients in sterile containers with spoons (Stericol, HiMedia, India) were subjected to initial enrichment in RCM media overnight at 37°C in an anaerobic condition. Culture of *C. difficile* was done on CBA containing 0.1% sodium taurocholate and incubated anaerobically for 48 h at 37°C in an anaerobic jar. The fecal samples were also cultured on CBA after alcohol shock treatment to allow the survival of sporulating bacteria. Colonies suspected as *C. difficile* were further identified by Gram staining, ultra-violet fluorescence and by conventional biochemical reactions like indole test, urease test, lecithinase test, lipase test and bile esculin hydrolysis.

2.3. Molecular Identification of *C. difficile*

The identity of isolates was further confirmed using polymerase chain reaction (PCR) primers for amplifying triose-phosphate isomerase (*tpi*) gene which is specific for *C. difficile*. *C. perfringens* 13124 MTCC (Microbial Type Culture Collection) obtained from Institute of Microbial Technology, Chandigarh, India served as the negative control. Briefly, DNA was isolated by phenol-chloroform method and PCR amplification was performed in a mastercycler (Eppendorf, Germany). The 20 µl reaction mixture contained 1× PCR buffer (50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM MgCl₂), 0.2 mM of 10 mM each of dNTP, 1pM of 10 pM oligonucleotide forward and reverse primers and 0.5 µl of 1.0 U Taq polymerase. Amplifications were carried out by initial denaturation at 94°C for 5 min followed by 25 cycles of 30sec at 94°C, 30 sec at 50°C, 30 sec at 72°C, and a final extension step of 5 min at 72°C. After amplification, the products were electrophoresed in 1.8% agarose gel containing 0.5 µg/ml ethidium bromide. The amplification gave a single product of 230-bp fragment in all *C. difficile* isolates as visualized under Gel Documentation System (Alpha Image, San Diego, USA).

2.4. Antibiogram Profile

Antimicrobial susceptibility of *C. difficile* isolates was determined using the E-test for vancomycin, metronidazole, ciprofloxacin and clindamycin. Breakpoint for vancomycin and metronidazole was defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and for clindamycin and ciprofloxacin by Clinical Laboratories Standards Institute (CLSI). Briefly, the E-test was performed by inoculating the surface of pre-reduced Brucella Agar plates containing 5% defibrinated sheep blood with 1 McFarland standard matched *C. difficile* inoculum. The inoculation was done with sterile cotton-tipped swabs dipped into the inoculum, pressed against the inside of the tube to remove excess fluid and streaked three times at 90°C. The plates were incubated at 37°C for 48 h under anaerobic conditions, and the MIC were read directly from the test strip point where the growth inhibition zone intersected with the test strip in accordance with the manufacturer's instruction. Quality controls used were *Clostridium perfringens* MTCC 13124 and *Streptococcus* sp. MTCC 689 strains.

3. Results

During analysis the patients from whom fecal samples were obtained were categorized into the following four groups according to their age.

Pediatric Group: This group comprised of 189 patients in the pediatric age group of 2 - 18 years.

Young Adult Group: This group included 504 young adults in the age group >18 - 45 years.

Middle Age Group: This group involved 342 patients belonging to middle age of >45 - 65 years.

Geriatric Group: In the geriatric group, 75 patients above 65 years of age were included.

3.1. Phenotypic Identification of *C. difficile* Isolates

Colonies suspected as *C. difficile* had a characteristic horse-manure odor and appeared white to grey in color with irregular margins enhancing towards the streaked line (**Figure 1(a)**). The colonies gave yellow-green fluorescence under ultraviolet illumination (**Figure 1(b)**). Biochemical reactions showed 100% of the isolates were indole negative, urease negative, lecithinase negative, lipase negative and bile esculin positive (**Figure 2**). Thus by phenotypic identification, *C. difficile* was isolated from 174 (15.7%) of the 1110 fecal samples.

3.2. Genotypic Confirmation of *C. difficile* Isolates

All of the microbiologically identified *C. difficile* isolates (100%) were further confirmed genotypically by the presence of housekeeping *tpi* gene, which is more discriminatory than 16S ribosomal DNA for the identification of various species within the genus *Clostridium* (**Figure 3**). *C. difficile* positivity was 13.8% in Pediatric Group, 17.0% in Young Adult Group, 15.0% in Middle Age Group and 14.7% in Geriatric Group.

3.3. Antibiogram Profile of Clinical Isolates

The MIC breakpoint for isolates resistant to vancomycin and metronidazole was taken as >2 mg/L according to EUCAST guidelines. Strains with MIC breakpoint of ≥0.025 mg/L for ciprofloxacin and MIC breakpoint of

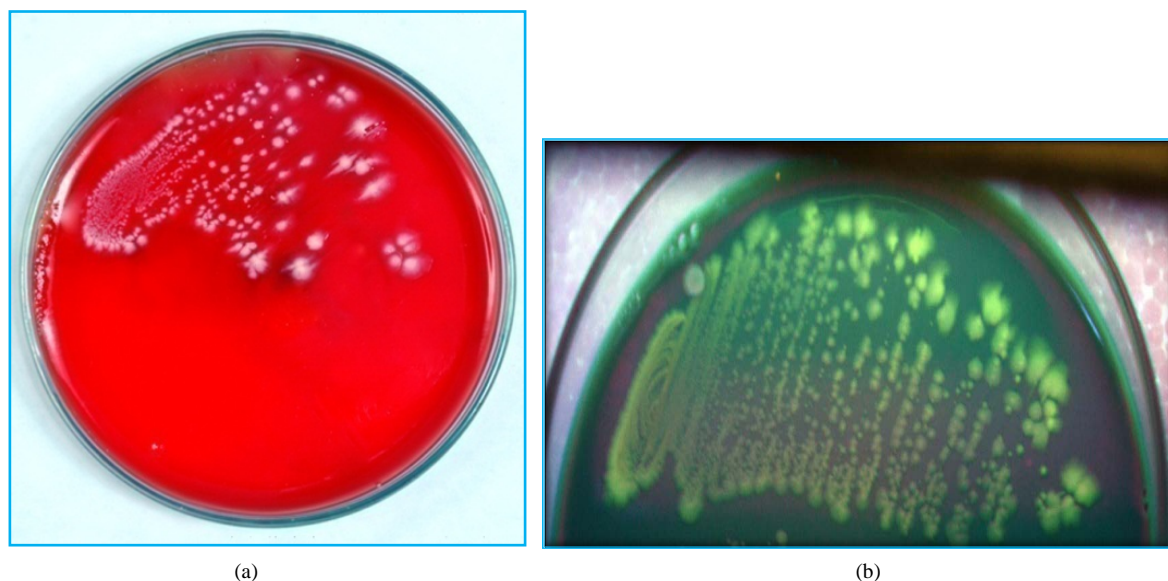


Figure 1. (a) Colonies of *C. difficile* on CBA; (b) Colonies of *C. difficile* fluoresce under UV light.



Streptococcus sp. MTCC 689
negative for bile esculin
hydrolysis test

C. difficile isolate positive for
bile esculin hydrolysis test

Figure 2. Bile esculin test.

≥ 0.002 mg/L for clindamycin were respectively considered clinically resistant according to CLSI guidelines. The MIC range for vancomycin, metronidazole and ciprofloxacin was 0.016 - 256 mg/L while for clindamycin, it was 0.002 - 0.032 mg/L. All of the isolates (100%) were sensitive to vancomycin, 1.72% was resistant to metronidazole, 52.9% were resistant to clindamycin and 33.9% resistant to ciprofloxacin (**Figure 4(a)**, **Figure 4(b)**, **Figure 5**).

4. Discussion

Although the need for *C. difficile* epidemiology is urgent, not much progress has been reported due to the lack of awareness and surveillance against CDI in many parts of India. The main reason to identify *C. difficile* is the emergence of the hypervirulent strains leading to outbreaks of infection associated with an increased severity of disease and significant mortality [15]. Another reason is to know the development of antimicrobial resistance of the pathogen to the commonly used CDI specific drugs. Thus, it becomes necessary to culture the organism and

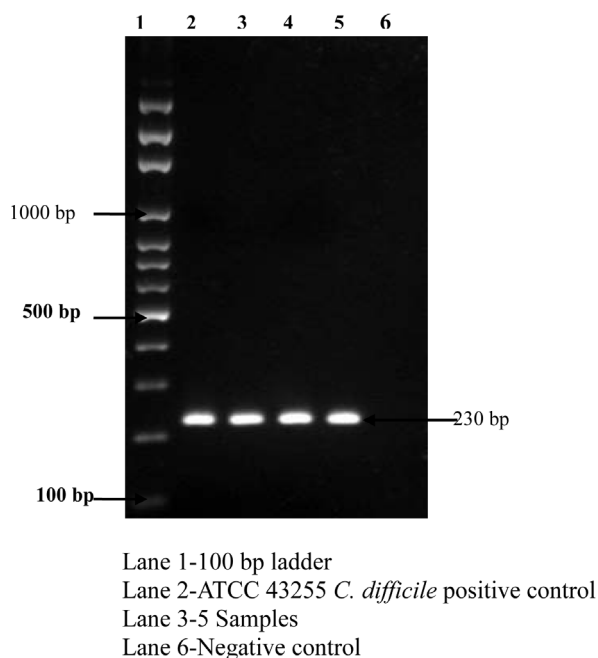


Figure 3. PCR amplification of *tpi* gene with 230 bp amplicon size.

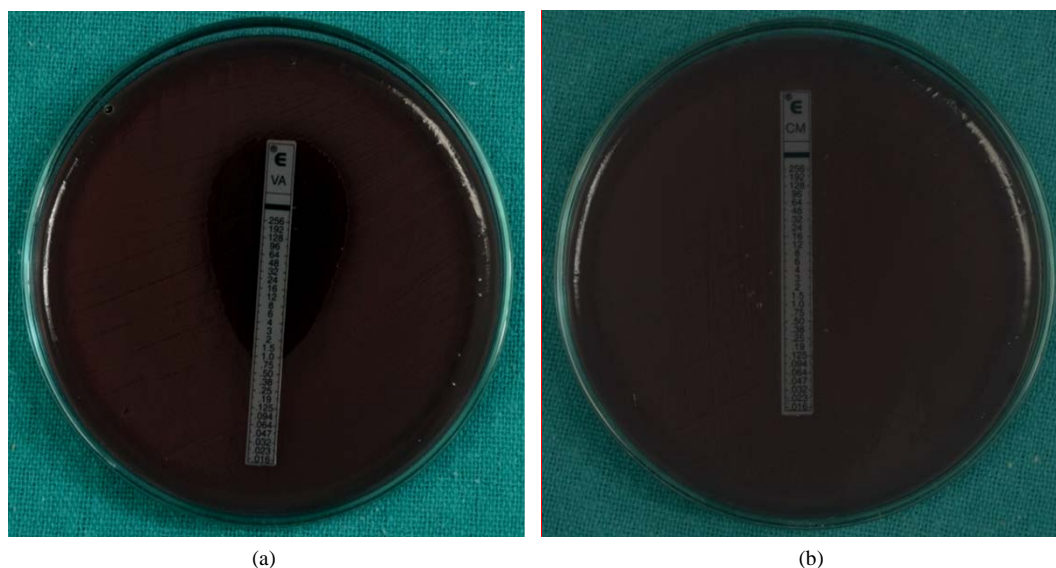


Figure 4. Antibiotic sensitivity by Epsilon test. (a) Vancomycin; (b) Clindamycin.

perform its antibiogram profile. As per the recommendations by both the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America, *C. difficile* culture on CCFA medium should contain a germinant like lysozyme or taurocholate for its isolation [16]. However, *Clostridia* other than *C. difficile* are also able to grow over it [17]. In the present study fecal samples were enriched initially by using Robertson's cooked meat media and further cultured on CBA media along with sodium taurocholate for the germination of *C. difficile* spores. To increase the specificity for growth of spore producing *C. difficile*, fecal samples were also subjected to alcohol shock. Culture enabled the detection of *C. difficile* in 15.7% of a total of 1110 fecal specimens which was further confirmed by molecular method. Toxigenic *C. difficile* was found in 54.6% of the isolates by ELISA. Toxin positive *C. difficile* was present in 53.8% in Pediatric Group, 53.5% in Young Adult

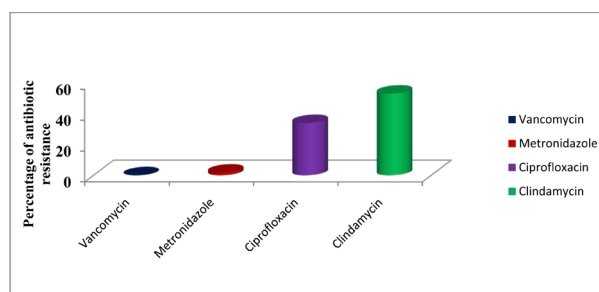


Figure 5. Percentage of antibiotic resistance among different drugs against *C. difficile*.

Group, 55.0% in Middle Age Group and 63.6% in Geriatric Group. However, *C. difficile* toxin positivity was not significant ($p > 0.05$) among any group (data not shown).

There is very little literature of *C. difficile* culture from India due to the lack of technology in culturing this anaerobic pathogen. Gupta and Yadav [18] reported isolation of *C. difficile* from 25.3% of diarrheal patients of all age groups. Ayyagari *et al.* [19] reported the presence of *C. difficile* in 22.6% stool specimens obtained from cases of antibiotic associated colitis. Niyogi *et al.* [20] isolated *C. difficile* from 8.4% fecal samples in pediatric age group. Dutta *et al.* [21] detected *C. difficile* in 8.4% of fecal samples of children between 0 - 14 years of age. Gogate *et al.* [22] isolated *C. difficile* in 7.2% of children in the age group 5 - 12 years with antibiotic associated diarrhea. In general, the prevalence of CDI in India has been reported to be 15% - 30% in pediatric and adult patients receiving antibiotics [21] [23]-[26].

It is well known that CDI is largely associated with the intake of antibiotics. The widespread use of antibiotics in most Asian countries is poorly regulated. Reports surveyed on Southeast Asian countries found that 47% of pneumonia cases do not receive an appropriate antibiotic, 54% of diarrhea cases are unnecessarily treated with antibiotics, and 40% of antibiotics are prescribed in under-dose [27]. A recent study by Vishwanath *et al.* [26] reported third-generation cephalosporins or beta-lactam/beta-lactamase inhibitor antibiotics as the risk factor for precipitation of CDI. Development of resistance in bacteria is a natural phenomenon, though excessive usage of antibiotic can also exert a selective pressure, leading to reduced susceptibility and ultimately antimicrobial resistance [28]. The two major antibiotics—vancomycin and metronidazole—are the mainstays for the treatment of mild to moderate CDI [29] [30]. Metronidazole is considered to be the drug of choice for CDI because of its low cost, good activity against *C. difficile*, favorable pharmacokinetic and pharmacodynamic properties, and minor adverse effects. Vancomycin is used to treat severe cases of CDI. Due to limited treatment options for CDI, any resistance or reduced susceptibility to these drugs is a significant public health concern [31] [32].

There are several reports from Europe, North America and the Far East on the emergence of multidrug resistance amongst *C. difficile* [33] [34]. Reports of vancomycin and metronidazole resistance are rare and sporadic [35]-[37]. In some studies, resistance towards metronidazole has been reported up to 9% [36] [38]. Pelaez *et al.* [37] [39] reported *C. difficile* isolates with intermediate resistance to vancomycin (3.1%) and metronidazole (6.3%) Several other studies have reported decreased sensitivity of NAP1/BI/027 to metronidazole and increased recurrence of CDI in patients treated with metronidazole and vancomycin [40] [41]. In the present study, no resistance to vancomycin was observed in any of the clinical isolates tested, but resistance to metronidazole was 1.3%. The low resistance seen for metronidazole and none for vancomycin could be due to the absence of NAP1/BI/027 strains in this region of study. Though very little resistance is known against *C. difficile* specific-drugs, there is a definite need for periodic monitoring of any emergence of vancomycin and metronidazole resistance in *C. difficile*.

Apart from vancomycin and metronidazole, the other two drugs investigated for antibiotic profile in the present study were ciprofloxacin and clindamycin as an increase in fluoroquinolone [42] and clindamycin resistant strains [43] have been noted in several other countries. Hypervirulent strains of *C. difficile* particularly NAP1/BI/027 has shown multiple drug resistance towards clindamycin, moxifloxacin and rifampin [44]. Epidemics caused due to NAP1/BI/027 strains have been characterized by recently evolved resistance to fluoroquinolone—the antibiotic currently in wide use [45].

In the present study a high degree of resistance was observed towards ciprofloxacin (33.9%) and clindamycin (52.9%). The high degree of resistance towards ciprofloxacin and clindamycin among the isolates in this study

correlates well with that of others studies. Huang *et al.* [42] reported *in vitro* activity of ciprofloxacin to be moderate or poor against *C. difficile*. High level of resistance against ciprofloxacin was reported against epidemic strains of *C. difficile* [11] [12]. Borgmann *et al.* [46] reported that the increased use of ciprofloxacin by outpatients contributed to increased numbers of CDI. Resistance to clindamycin has been reported to be >50% worldwide [44]. The utility of antibiogram profiling has already been proven useful with previously unseen fluoroquinolone resistance [47]. Resistance to these drugs highlights the emergence of a new strain [48] and a particular profile of antibiogram is also indicative of the hypervirulent PCR ribotype 027 strain [49]. It is also proposed that *C. difficile* may acquire drug resistance genes due to the presence of a high number of transposons (11%) within the genome [33], thereby allowing the genes to be acquired resulting in enhanced survival of the organism both within the gut and outside the gut. Mooyottu *et al.* [50] studied the genome characterization in *C. difficile* isolates and documented the presence of several antibiotic resistance genes and mobile elements that can potentially contribute to generation of multidrug resistant toxigenic *C. difficile* by horizontal gene transfer. Antibiogram profiling for *C. difficile* is thus a valuable tool in the surveillance of antibiotic resistance as shown in the current study.

5. Conclusion

C. difficile isolates had a high degree of resistance towards clindamycin and ciprofloxacin with low level of resistance to metronidazole and none towards vancomycin. Antibiogram surveillance of *C. difficile* will help for clinical practice and add to the epidemiological data of the organisms.

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Transparency Declaration

There is no conflict of interest to report.

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