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Study of *Clostridium difficile* in South Gujarat region of India

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Abstract

Toxin-producing Clostridium difficile strains is a common cause of diarrhoea today. Clostridium difficile presently has been identified as a causative agent of a spectrum of diseases referred to as Clostridium difficile Infections (CDI). CDI can establish as self-limiting antibiotic-associated diarrhoea (AAD) and antibiotic-associated colitis (AAC) to severe and life threatening forms like; pseudomembranous colitis (PMC) and toxic megacolon. Indian studies have reported Clostridium difficile-associated Diarrhoea (CDAD) prevalence rates ranging from 7.1% to 26.6% and its incidence varies considerably from place to place. The present work was conducted with the objective to study Clostridium difficile and its prevalence in the South Gujarat Region of the Gujarat state of India. Alcohol Shock treatment was given to the stool sample, followed by enrichment of spores by inoculating an RCM broth (added with 0.1% sodium taurocholate). After 48 hr., subculture was made on CCFA medium. Plates were incubated anaerobically for 48hrs. The C. difficile isolates were identified by colonial characteristic, fluorescence under UV light and positive Latex Agglutination test. The isolates were further characterized by gram reaction for cellular morphology, spore staining and various biochemical tests as described in Bergey's Manual of Systematic Bacteriology. Among 271 clinical stool investigated, 176 were of male and 95 were of female patients. In total 16 isolates have been obtained from total of 271 clinical samples i.e. the isolation rate is 5.9% during six month. Among this C. difficile has been isolated from 10 males (62.5%) and 6 females (37.5%). A case of PMC has also been reported in present study.

Keywords: *Clostridium difficile* Infections (CDI), antibiotic-associated diarrhoea (AAD), antibiotic-associated colitis (AAC), Pseudomembranous colitis (PMC), toxic Megacolon, *Clostridium difficile*-associated Diarrhoea (CDAD).

Introduction

Clostridium difficile presently has been identified as a causative agent of a range of diseases referred to as *Clostridium difficile* Infections (CDI). CDI can manifest as self-limiting antibiotic-associated diarrhoea (AAD) and antibiotic-associated colitis (AAC) to severe and life threatening forms like; pseudomembranous colitis (PMC) and toxic megacolon[1]. In 1978, *C. difficile* was first identified as a cause of diarrhoea and in the last thirty years it has reached an epidemic state in both healthcare and community settings. There has been reports of increasing incidence and severity of CDI with severe outcomes in this years[2]. The remarkable changes in the epidemiology of CDI during recent years have made CDI a global public health challenge. Emergence of a previously rare and more virulent strain, BI/NAP1/027 has been recognized as a main cause of increase in incidence of CDI. Increased toxin producing ability and high-level of resistance to third generation antibiotics have made this strain a very successful pathogen, particularly in healthcare settings. In addition to this observations says that the populations that were thought to be at low risk previously has now been identified to be acquiring and suffering from severe forms of CDI[3].

Scientific knowledge and information about the epidemiology, pathogenesis, diagnosis, and clinical management of *C. difficile* infections (CDI) has developed immensely in the last decade or so. During the past several years, many newer methods for diagnostic testing of *C. difficile* infections have emerged[4,5,6]. Additionally, several other treatment modalities has also been worked out and available for CDI apart from traditional drugs viz., metronidazole and vancomycin; this include new drugs such as fidaxomicin and other options such as probiotic treatment and fecal microbiota transplantation[2].

Microbiology of *Clostridium difficile* and brief about CDI: *Clostridium difficile* are anaerobic, gram-positive spore-forming bacilli. In 1935, it was initially detected in the fecal flora of healthy newborns by Hall and O'Toole[7].

Clostridium difficile was considered to be nonpathogenic till 1978, when Bartlett and Chang *et al*[8] identified it as the source of cytotoxin in the stools of patients suffering from pseudomembranous colitis (PMC). *Clostridium difficile* infection- an intestinal disorder often associated with antimicrobial usage is now established as a leading cause of hospital-associated gastrointestinal infections. CDI is a tricky problem for the health-care system because the patients with CDI usually have longer stay in hospitals and at the same time it is also reported to be frequently associated with cause of large hospital outbreaks of diseases[9].

Clostridium difficile is excreted in the faeces of patients as well as carriers. Also reported that 15% to 75% of healthy neonates acts as reservoirs for *C. difficile*. Additionally, the rate of asymptomatic carriage of *C. difficile* in healthy adults is 3% to 5% [10] and is prevalent in up to 30% of patients on antibiotics or those who are hospitalized[11]. The *Clostridium difficile* is transmitted via feco-oral route. It is mostly acquired from the hospital environment, by touching the inanimate objects or surfaces contaminated with faeces (spores of *C. difficile*). It can also be transmitted by health care workers through contact with contaminated patients or infected patients faeces. The incubation period of CDI is not precisely known. In most patients, infection is evident within 7 days after ingestion of spores, however the incubation period can extend up to 4 weeks[12].

Resistant *C. difficile* spores survive the stomach acidity and reach the small intestine where it germinates to give rise to the vegetative forms. In small intestine *C. difficile* proliferates in a situation of disturbed normal colonic flora, usually by exposure to antimicrobial agents. This leads to appearance of a broad spectrum of clinical conditions that ranges from 'asymptomatic carriage' to 'diarrhea of varying severity' to 'fulminant colitis' and even death[13].

Two large exotoxins, toxins A and B are released by proliferating *C.difficile* cells in the large intestine (*C.difficile* toxins i.e. toxin A and B are encoded by genes *tcd*A and *tcd*B respectively. These genes along with regulatory genes are located on a 21kb segment of chromosomal DNA referred to as the pathogenicity locus or shortly as paloc region). These toxins are responsible for the virulence and pathogenesis of *C. difficile*. Toxin-negative i.e. toxin non-producer *C. difficile* strains are considered nonpathogenic. In addition to toxins A and B, some *C. difficile* strains also produce a third toxin called binary toxin (encoded by genes *ctd*A and *ctd*B, located outside the paloc region). The role of binary toxin in the pathogenesis of *C. difficile* has remained unclear; however, it has been detected in epidemic strains (e.g. BI/NAP1/027) and thus suggests its probable synergistic role in causing severe colitis along with two exotoxins[14].

Prevalence of CDI: Over recent decades, several epidemics of CDI have been reported in North America and Europe and it has demanded the need for surveillance of *C. difficile* strains all over the world and efforts should be put in force by the international associations working for the CDI[15]. Since 2005 outbreaks *by C. difficile* ribotype 027 has occurred in England and Wales, Ireland, Netherland, Belgium, Luxemburg and France and the same strain has also been detected in several countries like Austria, Scotland, Switzerland, Poland and Denmark[16]. As reported by Scroeder M.S.[17], *Clostridium difficile* infections results in approximately 3 million cases of diarrhoea and colitis each year in United States alone and the case mortality rate is about 1 to 2.5%.

In Asia, the consciousness and surveillance of CDI have remained poor. However, available reports from the limited studies executed throughout Asia indicate that CDI is also a significant nosocomial pathogen in this region, however, the true prevalence of CDI has remained unrevealed. In the situation of unregulated use of antibiotics in many Asian countries, the prevalence of CDI may be relatively high. In addition to this, molecular studies have showed that ribotypes 027 and 078, which have caused significant outbreaks in other region of the world, are rare in Asia. However, variant strains of ribotype 017 (toxin A-negative/toxin B-positive) have caused epidemics across a number of Asian countries[18].

Indian Scenario: Repeated occurrence of *Clostridium difficile* Associated Diseases in an infected patient, as evident by the sudden recurrence of diarrhoea and other symptoms frequently within a week of discontinuation of treatment has made it a difficult clinical problem to manage[12]. There has been limited documentation on incidence of culture or toxin proven CDAD in India, probably due to the lack of technology and facilities for culturing anaerobic pathogens[19, 20].

Prevalence of CDAD in India as mentioned in available reports estimates to 15-30% of paediatric and adult patients taking antibiotics[21-27]. *C. difficile* was isolated from 25.3% patients (of all age groups) with diarrhoea in a study by Gupta and Yadhav. Niyogi and Dutta *et al.*[28] reported *C. difficile* in 8.4% and cytotoxin in 7% fecal samples from children between 0-14 year of age group. In 7.3% of acute diarrhea patients *C. difficile* was identified as the sole pathogen in a study by Dutta *et al.*, of which 82.4% were cytotoxin producers. In one study, Niyogi and Bhattacharya *et al.* isolated *C. difficile* from fecal samples from 11% hospitalized patients with diarrhoea and 2.9% from non-diarrheic controls. 87% of these isolates reported to produce cytotoxin; however the patients with diarrhoea had no history of antibiotic usage. Kochhar *et al.*[29] verified that *C. difficile* was responsible for some of the exacerbations in ulcerative colitis patients even though there was no history of recent antimicrobial exposure or hospitalization. Vaishnavi *et al.* in 2011 reported that *C. difficile* toxin test was positive for 30% of hospitalized patients receiving single to multiple antibiotics for different diseases (of all age group), but only for 7% patients not receiving antibiotics. Chaudhry *et al.* reported that number of *C. difficile* positive cases have decreased during a 5 year study period and have ascribed this reduction in CDI cases to rigorous surveillance and an better antibiotic policy implemented in the hospital.

Diagnosis of CDI: The clinical diagnosis of CDI is based on presence of appropriate clinical symptoms which includes watery diarrhea (defined as ≥3 loose stools in 24 hours) with or without abdominal pain, fever or ileus[30,9]. This clinical picture should be supplemented by a positive laboratory test for *C. difficile* or the presence of pseudomembranes in endoscopy both of which are highly suggestive of CDI[2].

Among laboratory tests, Fecal leucocyte testing is not sensitive for CDI diagnosis as the test is positive in less than 30% of patients with CDI[31]. Stool culture is the most sensitive test and hence considered the "gold standard" for detecting *C. difficile*, but its acceptance in clinical laboratories is limited due to its slow

turnaround time. Enzyme immunoassay (EIA) to detect toxins A and B produced by *C. difficile* is a rapid test but it lacks sensitivity. EIA for glutamate dehydrogenase enzyme is very sensitive but not very specific and so it is adopted by some laboratories as a screening test in combination with another more specific confirmatory test[32]. Another advanced test is Polymerase chain reaction (PCR), used to detect genes *tcdB*, which encodes the toxin and/or *tcdC* (which negatively regulates the toxin produced by *C. difficile*) is considered an alternative gold standard based on studies reporting its excellent sensitivity, specificity and test-retest reliability[33]. Due to its fast turnaround time the PCR test is more widely accepted by different laboratories in place of toxin EIA[32].

The purpose of the present study is to have an overview of the prevalence of *Clostridium difficile* in South Gujarat region of Gujarat state of India. The work has been undertaken with our best knowledge that none of such work has yet been done in the South Gujarat region of India.

Material and Methods

Sample Collection: Diarrhoeal stool samples were collected from two civil hospitals and four private hospitals and laboratories receiving clinical samples for analysis from these hospitals of South Gujarat Region of the state Gujarat. The hospitals are located in Surat, Navsari and Valsad districts of Gujarat. Samples received for analysis were left-over samples. Ethical clearance was taken from respective hospital committee for the processing of Clinical samples collected. 271 samples were collected and processed during the period of July-2013 and December-2013. Month-wise distribution of sample collected is; 1 in July, 4 in August, 25 in September, 162 in October, 69 in November and 10 samples in December.

Alcohol Shock Treatment: Spore selection technique i.e. Alcohol shock treatment was applied to the fecal samples before culturing it. For this, 1ml of sample in sterile micro-centrifuge tube was mixed with 1ml of absolute alcohol, vortexed to mix content well and then allowed to stand for 1hr.

Culturing and Isolation of *Clostridium difficile*: 100µl of the alcohol shock treated sample is inoculate in Reinforced Cooked Meat Medium (RCM broth) (Hi Media), added with 0.01% Sodium Taurocholate in a screw capped tube and incubated at 37°C for 48hr. with the aim of enriching the growth *Clostridium difficile* and its sporulation. After 48hr. of incubation the RCM broth culture was inoculated on *C. difficile* selective agar medium- Cefoxitin Cycloserine Fructose Agar (CCFA) (Hi Media) containing 0.1% sodium taurocholate. Then the culture plates were incubated anaerobically, in an Anaerobic jar for 48hr. Isolates were identified based on colonial characteristic, fluorescence under UV light and positive Latex Agglutination test [HiC.difficile™ Latex Test, Hi Media]. Each isolate was described in terms of gram reaction, cellular morphology, spore staining, colonial characteristics and various biochemical tests as described in Bergey's Manual of Systemetic Bacteriology , 2nd Edition (2001)[34].

Latex Agglutination Test: Test was performed using HiC.difficile™ Latex agglutination Test (HiMedia). Latex particles coated with rabbit IgG antibodies specific for *C. difficile* cell wall antigens (20µl) when mixed with a suspension of *C. difficile* colonies (20µl prepared in isotonic saline) from selective CCFA agar medium, the finely dispersed latex particles agglutinated rapidly into aggregates that are visible to the unaided eye.

Biochemical Characterization: The identified colonies were phenotypically further characterised by several biochemical tests viz. Lecithinase production, Lipase production, Aesculin Hydrolysis, Gelatine (2%) Liquefaction Test, Nitrate Reduction Test, Meat Digestion test, Indole test, Urease Test, Milk Reaction (for Lac Fermentation and Casein Coagulation), Starch Hydrolysis, Sugar Fermentation test for- Fructose, Mannose and Mannitol and Motility Test by Tetrazolium Reduction Method.

Sequencing: One of the isolate was sent for partial sequencing to Yaazh Xenomics, Mumbai, India.

Results and Discussion

Total 271 diarrhoeal stool samples were analysed for *C. difficile*, collected from six big hospitals and associated laboratories of South Gujarat Region of India during the period of July-2013 and December-2013. A total 16 number of *C. difficile* isolates were identified by the growth characteristic on CCFA agar medium viz. large, creamy-yellow to gray, flat to slightly raised colony with irregular edge, showing yellowish green fluorescence under UV light. And 255 cases of diarrhoea were reported due to other organisms; may be other gram negative or gram positive bacteria as observed by gram reaction of RCM broth culture inoculated with alcohol shock treated fecal samples.

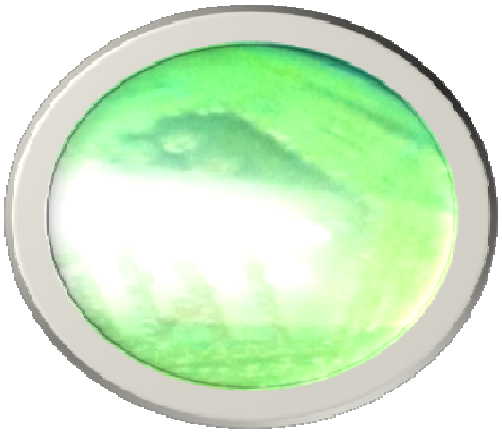


Figure-1
Growth of C.difficile isolate on CCFA medium (Exposure under UV light)

Only 16 isolates showed gram positive rods with terminal or subterminal spores in their RCM broth culture. All of them showed positive latex agglutination reaction when tested from growth on CCFA medium using HiC.difficile™ Latex agglutination Test kit (HiMedia).

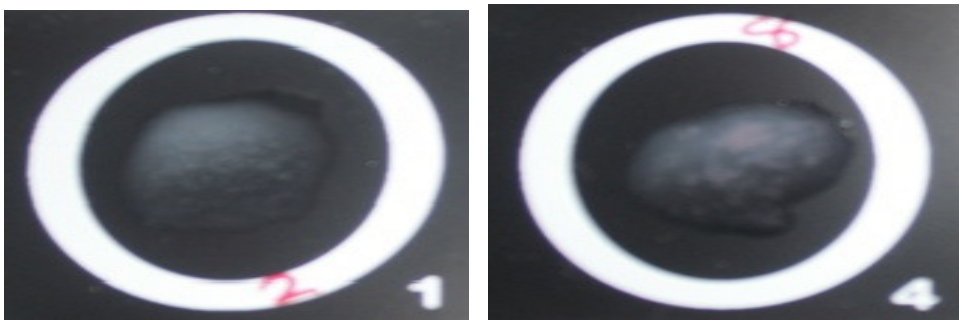


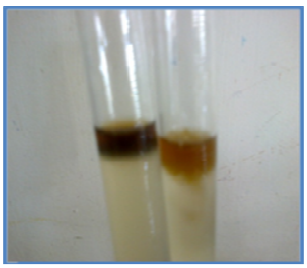
Figure-2
Latex Agglutination test result of C.difficile isolate grown on CCFA medium

Figure-3
Gram stain of RCM broth culture of *C. difficile* Isolates

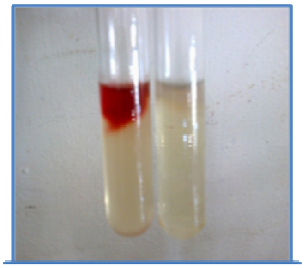
Table: Biochemical Tests of *C. difficile* isolates

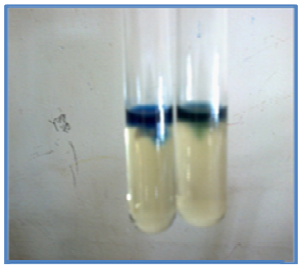
S. No.	Isolate No.→ / Biochemical Test ↓	2	8	14	34	37	51	55	56	96	122	137	153	157	158	222	261
1	Motility Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Aesculin Hydrolysis	+	d	d	+	–	+	–	+	+	+	+	+	d	+	+	+
3	Indole test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	Urease Test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	Nitrate Reduction Test	–	w	+	–	+	–	+	–	–	–	–	w	+	+	+	+
6	Gelatine Liquefaction Test (2%)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Meat Digestion Test	+	+	+	+	+	+	+	+	+	+	+	+	+	–	w	w
8	Milk Reaction; Lac Fermentation	+	w	–	+	+	–	w	w	+	w	–	–	–	+	w	+
	Casein Coagulation	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
9	Lecithinase Production	–	+	–	+	+	+	+	w	–	+	–	–	+	–	+	+
10	Lipase Production	–	+	–	+	–	+	+	–	–	–	–	–	+	–	–	–
11	Starch Hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	Sugar Fermentation; Fructose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
	Mannitol	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G
	Mannose	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G	A G

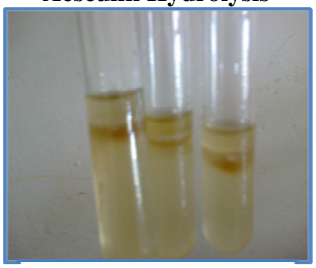
A- Acid detected, G- Gas detected

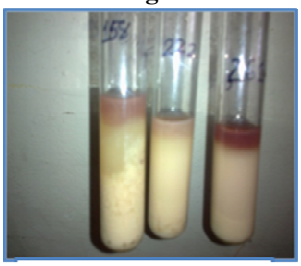

Aesculin Hydrolysis


Meat Digestion


Nitrate Reduction


Urease Test


Indole Test


Litmus Test


Motility Test

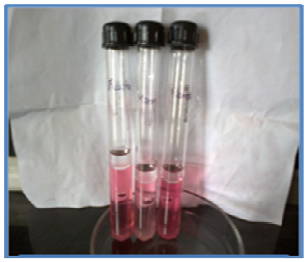
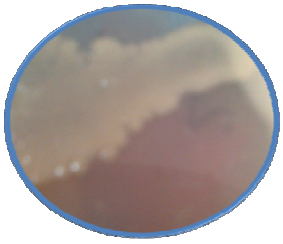

Fermentation of Sugars: Fructose, Mannose, Mannitol

Figure-4a
Biochemical Tests


Lecithinase Test


Lipase Test


Starch Hydrolysis


Gelatin Liquefaction

Figure-4b
Biochemical Tests-Enzyme Profile

Sequencing: Isolate No. 8 was sent for partial sequencing and has been identified as *Clostridium*.

Discussion: *C. difficile* infections has become a global health challenge due to increase in its incidence rates as well as severity. Populations that were considered to be at low risk are now contracting severe forms of CDI[35]. CDAD has become a major public health problem with significant medical and economic consequences. Its occurrence is no longer confined to hospital and can occur in the community. However, hospitalized patients receiving antibiotics are at increased risk as the hospitalized CDAD patients and medical as well as paramedical staff involved in patients care may become vehicles for transmitting *C. difficile* unless infection control procedures are carefully employed. Additionally, *C. difficile* spores can exist in a hospital environment in dormant state and are difficult to eradicate and thus act as a reservoir for new infection[20].

Indian literatures are available for work done on *Clostridium difficile* and CDI in India at several geographic regions including; New Delhi[19,24,36], Calcutta[23,28,37], Mumbai[38,39], Chandigarh[21,25,40] and Manipal- Karnataka[41]. It has been found that no work has been done so far on *Clostridium difficile* and CDI in the South Gujarat Region of Gujarat state of India to our knowledge. So, in this work, we have tried to study *Clostridium difficile* and checking its prevalence in our region.

In India, CDAD prevalence rates reported to be ranging from 7.1% to 26.6% in previous studies[21,22,24,25,38], however, cases of overwhelming infections are less in number as compared to western literatures[35]. We have found isolation rate of 5.9% (16 *C.difficile* isolates from 271 samples analysed) in six month period, which is comparatively less than the reported in above mentioned literatures. The incidence of CDAD varies considerably from place to place[41].

Indian studies have reported varying male-female ratios for CDAD[22,37,42,43]. There were 176 male and 95 female in the present study and *C. difficile* has been isolated from 10 males (62.5%) and 6 females (37.5%). Older age and long duration

hospital stay have been identified as significant risk factors in hospitalized CDAD patients[44]. In present study, no such age related prevalence has been observed.

Symptoms like fever, cramping abdominal pain and diarrhoea have been reported to be more common in patients with positive test for *C. difficile* toxin[45,46]. In our study group, diarrhoea was present with majority of patients but in few suspected patients semisolid to formed stools was also found. Stool samples were also positive for Occult blood test, showed presence of mucus and pus cells as examined in chemical and physical analysis. Based on these observations, we recommend that *C. difficile* should be considered as a cause of gastrointestinal infections in high-risk hospitalized patients even if the typical symptoms viz., fever or cramping abdominal pain or loose watery diarrhoea are not present.

Fulminant colitis leading to ileus, toxic megacolon, perforation, and death have been reported in 1% to 3% of cases[47]. Only one case of Pseudomembranous colitis (PMC) was found in this six month study. No outbreak of CDAD has occurred in the South Gujarat Region (Gujarat, India) during the period of present work.

Regarding methodology applied: Most laboratories in India presently use the ELISA test for detecting *C.difficile* toxin A and B in fecal samples. Though it is a suboptimal alternative, it is mainly adopted because of speed, affordability and ease of performance. Repeated testing cannot overcome low sensitivity of the EIA test, because it may give false positive result due to its low specificity[35]. To overcome the poor EIA sensitivity, a potential strategy recommended is to carry out a 2-step protocol that employs detection of GDH by ELISA for initial screening followed by performance of cell cytotoxicity assay or toxigenic culture as a confirmatory test for GDH-positive stool specimens[30]. We applied 2-step algorithm as: first, culturing of suspected cases stool samples on modified *C.difficile* selective medium (CCFA- containing 0.1% sodium taurocholate) followed by confirmation of *C.difficile* isolates by Latex Agglutination test [HiC.difficile™ Latex Test, Hi Media]. We observed confluent growth of *C. difficile* on the modified selective medium.

We applied enrichment technique (initial culturing of alcohol shock treated fecal sample in RCM broth containing 0.1% sodium taurocholate, followed by its plating on selective medium) to increase possibility of recovering *C. difficile* from the clinical samples and found it to be a successful strategy. The results are in conformation with the work by O' Farrell *et al.*[48] 1983.

Our study has several limitations. The study includes data of only six month period. Additionally, the data is based on the study conducted on the leftover-residual fecal samples collected and that may not have been preserved in optimal condition (looking to this reality and the fact that resistant *C. difficile* spores can survive in suboptimal condition, as supported by the work of Wilson *et al.*[49], that indicated use of CCFA containing 0.1% sodium taurocholate to achieve good isolation rate particularly when fecal samples are not handled optimally and vegetative forms lose viability from prolonged exposure to air. And so, we directly opted for the culture on selective CCFA medium containing 0.1% sodium taurocholate). J. S. Brazier[50] in 1998 has also reported that as the organism sporulates readily, the cultures of *C. difficile* should be largely unaffected by ambient storage.

It is a matter of consideration that why CDI is less frequent and severe in India as compared to other countries. Possible underlying factors that can be mentioned are; chiefly vegetarian diet among Indians, widespread availability as over the counter drug and frequent use of metronidazole, non-adherence in completing the course of antibiotics, a strong anamnestic antibody response due to repeated infections with *C. difficile* and probably rare occurrence of the virulent (more toxigenic) strains in our country. However, it is highly probable that more cases of CDI will be reported in India too, as the *C. difficile* toxin assays as well as more sophisticated methods of diagnosis will be in use on a larger scale[35] in routine laboratories, which are costing high at present.

Conclusion

C. difficile infections has become a global health challenge due to increase in its incidence rates as well as severity. Comparatively a small number of studies have been reported on CDAD in India and whatever reports available have shown prevalence of *Clostridium difficile* lesser than those have been reported in many other countries. However, the awareness about *C. difficile* infections have been increasing as has been evident by increasing testing of clinical stool samples mainly for *C. difficile* toxins.

Present study with the available resources, was the effort in the same line to study prevalence of *Clostridium difficile* in the South Gujarat Region of the Gujarat state of India. Study results demonstrated that the rate of isolation of C. difficile was little lower than that reported in other Indian literatures. However, the study shows that Clostridium difficile is prevalent in the South Gujarat Region of the Gujarat state of India and it should be considered to be a cause of gastrointestinal infections and found to be involved in causation of PMC too. Suspicion for CDI should be based not only on duration of hospital stay but also on existence of clinically significant diarrhoea with a history of antibiotic therapy or occurrence of acute abdominal disorder may be associated with little or no diarrhoea.

In India, still there is a lack of wide spread availability of *C. difficile* toxin assays, culture facilities for anaerobes like C.difficile and genotypic identification facilities. And to control this emerging intestinal disorder, a good quality of clinical and/or laboratory studies/surveys on *C. difficile* infection are needed to create a pool of local epidemiological data that may prove to be

crucial to increase awareness amongst the medical as well as paramedical staff.

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