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ORIGINAL ARTICLE

POLYMERASE CHAIN REACTION FOR THE DETECTION OF TOXIN A (*TCD A*) AND TOXIN B (*TCD B*) GENES OF *CLOSTRIDIUM DIFFICILE* ISOLATED FROM DIARRHOEAL CASES AND ANALYSIS OF THE CLINICAL SPECTRUM

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ABSTRACT: BACKGROUND: Molecular methods for detection of toxigenic *Clostridium difficile* have been established in the developed countries though not very common in our country. **AIMS:** The study was intended to determine the presence of toxin A and toxin B genes of *Clostridium difficile* isolates by means of polymerase chain reaction (PCR) and analysis of clinical picture of the patients. **MATERIALS AND METHODS:** The prospective study was conducted in a tertiary care teaching hospital, South India from January 2012 to December 2014. Stool samples were collected consecutively from 563 in patients with diarrhoea from various wards. *Clostridium difficile* was isolated and identified by semi quantitative culture, latex agglutination and biochemical reactions. These isolates were then subjected to PCR for the detection of toxin A and toxin B genes. In addition, enzyme immunoassay was performed on stool samples for the detection of toxins A and B. The clinical spectrum of PCR positive patients was also analyzed. **RESULTS:** From 563 stool specimens, 113 (20.07%) *Clostridium difficile* isolates were grown by culture and identified by latex agglutination and biochemical reactions. Out of 113 isolates, 94 were subjected to PCR. 50 (53.19%) isolates out of 94 were found to be positive. Three toxigenic types obtained were A^+B^+, A^-B^+ and A^+B^- which accounted for 6.38%, 42.55% and 4.26% respectively. A^-B^- isolates were 46.81%. 30 (26.55%) out of 113 stool samples (which were culture positive) was also enzyme immunoassay positive. 32 (64%) out of 50 PCR positive patients exhibited antibiotic usage ($p<0.05$) and 39(78%) revealed the presence of underlying illnesses/conditions ($p<0.01$). **CONCLUSION:** The study highlights the usefulness of PCR for detection of toxigenic *Clostridium difficile* and for determination of its molecular epidemiology.

KEYWORDS: *Clostridium difficile*, polymerase chain reaction, cycloserine cefoxitin fructose agar.

INTRODUCTION: *Clostridium difficile* (*C. difficile*) is an anaerobic, Gram positive spore forming bacillus. The organism has been implicated in 90% of pseudomembranous colitis (PMC) and 20-25% of antibiotic associated diarrhoea (AAD).[1] The main virulence factors of the pathogenic *C. difficile* strains are the toxins A and B of which toxin A is an inflammatory enterotoxin responsible for fluid secretion while toxin B is a cytotoxin.[1] Literature also shows the presence of a third toxin (binary toxin) which could possibly enhance the virulence of the organism.[2,3] *C. difficile*-associated disease (CDAD) ranges from asymptomatic carrier state, diarrhoea, colitis without pseudomembrane formation, PMC, megacolon to death.[4] The main predisposing factors for CDAD are administration of antibiotics, immunosuppressive agents, proton pump inhibitors (PPIs) cancer therapeutics and host risk factors like elderly age, underlying diseases, prolonged hospital stay etc.[5] The epidemiology of the organism has been changing over the last decade from being a nosocomial pathogen to a community- acquired one.[6]

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Cell culture cytotoxicity assay, toxigenic culture, Enzyme immunoassays (EIAs) for the detection of glutamate dehydrogenase (GDH) enzyme, toxins A and B and real-time polymerase chain reaction (RT-PCR) have been the mainstay in the laboratory diagnosis of *C. difficile*.[7]

Polymerase chain reaction (PCR) has gained due significance in detection of toxigenic *C. difficile* in the past few years as it is a highly sensitive, specific and rapid method. Multiplex PCR methods for the detection of *tcd A* (toxin A gene), *tcd B* (toxin B gene), *cdt A* and *cdt B* (both binary toxin genes) and the in-frame *tcd C* gene deletion of *C. difficile* have been presented by various authors.[8,9] A multiplex RT PCR for the detection of toxigenic *C. difficile* which also revealed the presence of mutant hypervirulent strain, NAP1/BI/027 (North American Pulse-field gel electrophoresis type 1 /restriction endonuclease analysis BI/ribotype 027) has been described in a study.[10]

A handful of studies from India have reported the prevalence of *C. difficile*.[11-14] Still the toxigenic profile of the strains prevalent in our country remains unclear. The present study is intended to assess the presence of toxin A and toxin B genes present in *C. difficile* isolates originated from diarrhoeal cases of a tertiary care hospital, South India by means of PCR. The study also analyses the clinical characteristics of the PCR positive patients.

METHODS: The study was performed in a tertiary care teaching hospital of coastal Karnataka, South India. Diarrhoeic stool samples were obtained from 563 patients who were admitted in the various wards like Medicine, Paediatrics, Surgery, Oncology and Orthopaedics during January 2012 to December 2014.

The approval for the study was obtained from the Institutional Ethics Committee (Ref. No FMMC/ IEC/ 816/ 2012).

Any inpatient with diarrhoea of the above mentioned wards was included in the study. The case history of each patient including the details of age, sex, severity of diarrhoea, usage of antibiotics or other drugs, underlying illnesses, period of hospital stay etc. was extracted from the medical records. Written informed consent was taken from the patients or the guardians of the patients in case of minors.

The faecal samples were collected in sterile wide mouthed containers and were processed without delay on receipt to the lab. The stool samples were cultured on cycloserine cefoxitin fructose agar (CCFA) and anaerobically incubated for the isolation of *C. difficile*. Colonies grown on the plate were identified as *C. difficile* by Gram stain, morphology and characteristic odour and then further subjected to latex agglutination and biochemical reactions. [15, 16] The purpose of latex agglutination was to confirm the identification of *C. difficile* colonies. All the stool samples were subjected to EIA for detection of the toxins A and B of *C. difficile*.

The *C. difficile* colonies grown on CCFA were then analyzed by PCR for the detection of toxin A and toxin B genes. Two primer pairs were employed to detect toxin A gene. Primers NK3 and NK2 (derived from the nonrepeating portion of the *C. difficile* toxin A gene); Primers NK11 and NK9 (derived from the repeating portion of toxin A gene). [17] NK9 and NK11 were employed to detect the deletion of the 3' end of the toxin A gene.[18] Toxin B gene was detected by using primers NK104 and NK105 (derived from the nonrepeating sequence of the *C. difficile* toxin B gene).[17]

C. difficile ATCC 43593 was employed as a control strain throughout the study.

Procedure of the tests employed are summarized below:

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Isolation and identification of *C. difficile*:

Culture: A semi quantitative culture of stool was performed on CCFA plate.[15] The plate was incubated anaerobically at 37^0 C for 48 hours in Hi gas-pak jar using BD GasPak EZ Anaerobe container system with Indicator or otherwise in individual sachets (BD GasPak™ EZ Gas Generating Pouch Systems). Circular, yellow, fimbriate colonies of 4mm size or larger, Gram positive bacilli with subterminal oval spores having characteristic horse stable odour was indicative of *C. difficile*.

Stool samples were inoculated on Brucella blood agar and also in Robertson's cooked meat (RCM) broth which served as a supplemental medium.

Latex Agglutination: Latex agglutination was performed on presumptively identified colonies of the organism with Oxoid *C. difficile* Test Kit (DR 1107A), UK according to manufacturer's instructions.

Biochemical Reactions: Standard biochemical tests were also done using the typical colonies. [15, 16] The isolates of *C. difficile* were stored in skimmed milk in screw capped 5ml vials at -70^0C until further use. The vials were thawed whenever needed. Frequent freezing and thawing was avoided. The isolates were also preserved in RCM vials in the refrigerator.

Toxin Detection: EIA for the detection of toxins A and/or B of *C. difficile* from the faecal samples was done using Premier Toxins A & B (*C. difficile*) EIA kit M/S Meridian Bioscience, Europe according to the manufacturer's instructions.

Detection of toxin genes *tcd A* and *tcd B* of *C. difficile* by PCR: Isolation of genomic DNA was done from the bacterial colonies using AMpurE Bacterial gDNA Mini isolation kit from Amnion according to manufacturer's instructions. The DNA extracted was further subjected to electrophoresis for quantitative analysis. Briefly, agarose gel electrophoresis was performed by 0.8% agarose gel with ethidium bromide (5µl) in Tris acetic acid EDTA buffer. The bands were visualized on UV transilluminator after the electrophoresis was completed.

The reaction mixture (20µl) for the PCR contained 2.5 µl of Taq buffer, 2µl of dNTPs (deoxynucleotide triphosphate), 0.5 µl of Taq polymerase enzyme (GBiosciences, USA), 1µl of each primer of the different primer pairs for each reaction, 2µl of genomic DNA extract and 11µl of sterile distilled water. All the PCR reactions were performed along with positive and negative controls. The PCR was run as per the instructions of the manufacturer (Applied Biosystems Simpli Amp Thermal Cycler by Life technologies). The primer pairs were employed according to previously described protocols.[18] The primer details are given in Table1 and the thermal profile for primer pairs are given in Tables 2 and 3.

The PCR products were visualized in 1. 5% agarose gel and were compared with respect to the 1kb DNA ladder.

STATISTICS: Data was analyzed by frequency percentage and Chi-square test.

RESULTS: Stool samples were collected from 563 inpatients having diarrhoea from various wards during the study period. Out of 563 specimens, 113(20.07%) *C. difficile* isolates were identified by culture, latex agglutination and biochemical reactions. One isolate per sample was considered. 30 (26.

55%) out of 113 stool samples (which were culture positive) was also EIA positive. Thus 30 isolates were regarded as toxigenic *C. difficile* according to toxigenic culture.[7] The remaining 83 isolates were non- toxigenic.

From 113 *C. difficile* isolates, 94 isolates (27 toxigenic isolates out of 30+67 non- toxigenic isolates out of 83) were subjected to PCR. PCR confirmed the toxigenicity of 26(96.30%) of the 27 toxigenic isolates. PCR was positive also in 24 (35.82%) of the 67 non- toxigenic isolates. Thus the presence of toxin A and/or toxin B genes was detected in a total of 50 (53.19%) isolates out of 94.44 (46.81%) isolates out of 94 did not show the presence of toxin A or toxin B genes. The toxigenic profile of *C. difficile* according to the presence or absence of toxin A and toxin B genes is presented in table 4. Clinical features and risk factors of the PCR positive patients are given in table 5. Table 6 gives the list of antibiotics used by these patients.

DISCUSSION: *C. difficile*, an anaerobic Gram positive bacillus is an established pathogen in the Western countries.[19,20] Literature has also revealed a few studies on the pathogen from Asia.[21,22] The organism is reported to be responsible for mere asymptomatic colonization to life threatening complications.[4] The presence of the organism in the community as well as in animals and foods in addition to hospital settings is a matter of great concern.[23,24] Still a diagnostic method for *C. difficile* which is highly sensitive, specific, rapid, economical and less cumbersome is lacking.

The present study includes the detection of *tcd A* and *tcd B* from *C. difficile* isolated from the stool specimens of patients with diarrhoea who were admitted in various wards with different medical problems. The advantages of PCR assay in detecting toxigenic *C. difficile* from stool specimens has been evident in literature.[25] 94 isolates of *C. difficile* were tested by PCR. Out of 94 isolates, 50 (53.19%) were found to be positive. Isolates were classified depending on the presence of toxin A and/or toxin B genes as A^+B^+, A^-B^+ and A^+B^- which accounted for 6.38%, 42.55% and 4.26% respectively. A^-B^- isolates which did not contain both the toxin genes were 46.81%. Toxin gene profile of *C. difficile* has been reported by different authors.[8,25-29] The presence of A^+B^- strains though rare in literature was reported earlier by some authors.[8,27]

Analysis of clinical spectrum of the PCR positive patients was also attempted as a part of the study. The clinical features and risk factors usually encountered in patients with toxigenic *C. difficile* were observed in our patients also. Treatment with antibiotics has been regarded as the main predisposing factor for *C. difficile* infection (CDI).[5] 32 (64%) out of 50 patients had documented antibiotic usage which was statistically significant ($p<0.05$). Cephalosporins, fluoroquinolones, carbapenems and penicillins were used by 17(34%), 16(32%), 3(6%) and 8(16%) of our patients respectively. This is in complete agreement with the fact that these antibiotics have been associated with CDI.[5,30,31]

Chemotherapy is regarded as another established risk factor.[5,32] In our study 14(28%) patients out of 50 had undergone chemotherapy. PPI use is also indicated as predisposing to CDI according to some authors.[5,33] 17(34%) patients were found to use PPIs in our study. Prolonged hospital stay and presence of underlying diseases/conditions which have been regarded as the main host risk factors for CDI were exhibited by 24(48%) and 39(78%) of our patients respectively. Presence of underlying illnesses/conditions in our group of PCR positive patients was highly significant statistically ($p<0.01$, HS). It has been reported that prolonged length of stay in the healthcare settings and presence of other illnesses contribute to CDAD.[5]

Among our patients, 19(38%) suffered from various types of carcinoma. The association between CDI and cancer had already been established.[32] It has been shown that previous surgery could also act as a risk factor for CDI.[34] 6(12%) of our patients had undergone different types of surgery before diagnosis of CDI. Out of 50 patients, 22(44%) were males and 28(56%) were females.

Ours was a prospective study and the results we obtained could be correlated with the clinical picture. Molecular methods for detection of *C. difficile* are yet to be popularized in our country. More studies are required in future to establish the toxigenic profile of *C. difficile* in our geographical area.

CONCLUSION: *C. difficile* has emerged as a powerful pathogen not only in Western countries but also in Asian subcontinent. The detection methods for the pathogen are still not uniform throughout the globe. A standard criterion for the diagnosis of CDAD is yet to be determined. Molecular methods for identification of *C. difficile* especially PCR assays have been popular since the past few years. PCR methods which are economical and requires less expertise need to get established in every parts of the world for quick detection of the organism and thus to rapidly prevent outbreaks.

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Gene	Primer	Sequence (5'→3')	Length	Annealing temp (°C)	Product size (bp‡)
*tcdA**	NK11 NK9	F (5'TGATGCTAATAATGAATCTAAAATGGTAAC -3') R (5'CCACCAGCTGCAGCCATA -3')	30 18	55°C	1200bp
tcdA†	NK3 NK2	F (5'GGAAGAAAAGAACTTCTGGCTCACTCAGGT-3') R (5'CCCAATAGAAGATTCAATATTAAGCTT-3')	30 27	55°C	250bp
tcdB†	NK104 NK105	F (5'GTGTAGCAATGAAAGTCCAAGTTTACGC -3') R (5'CACTTAGCTCTTTGATTGCTGCACCT -3')	28 26	55°C	200bp

Table 1: Primer details

* Repeating region, †Nonrepeating region, ‡base pair.

STEP		TEMPERATURE	TIME
Initial Denaturation		95°C	5min
35 cycles	Final Denaturation	95°C	30 seconds
	Annealing	55°C	1 minute
	Initial elongation	72°C	1 minute
Final Extension		72°C	8 minutes
Hold		4°C	

Table 2: PCR Program for primers NK11, NK9 and NK104, NK105

The PCR reactions were kept for 35 cycles.

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STEP		TEMPERATURE	TIME
Initial Denaturation		95°C	5min
35 cycles	Final Denaturation	95°C	20 seconds
	Annealing	55°C	2 minutes
	Initial elongation	72°C	1 minute
Final Extension		74°C	5 minutes
Hold		4°C	

Table 3: PCR Program for primers NK3 and NK2

The PCR reactions were kept for 35 cycles.

Types	Total no. of isolates = 94
A^+B^+ (both *tcd A* and *tcd B* are present)	6 (6. 38%)
A^-B^+ (only *tcd B* is present)	40 (42. 55%)
A^+B^- (only *tcd A* is present)	4 (4. 26%)
A^-B^- (both *tcd A* and *tcd B* are absent)	44 (46. 81%)

Table 4: Types of *C. difficile* according to the presence or absence of toxin A and toxin B genes

Clinical features and risk factors		Total number of patients
Abdominal pain	11	50
Fever	14	
Vomiting	14	
Antibiotic treatment	32	
Proton pump inhibitors	17	
Pseudomembranous colitis	1	
Chemotherapy	14	
Prolonged hospital stay	24	
Sex	Male : Female = 22:28	
Patients with underlying diseases/conditions*	39	

Table 5: Clinical features and risk factors of the PCR positive patients

*Underlying diseases/conditions were mainly various types of Carcinoma (19), Diabetes mellitus (4), Hypertension (5), Heart disease (3), Kidney diseases (5), Pneumonia (1), Anaemia (2), Bronchial asthma (2), Inguinal hernia (1), Urinary tract infection (2), lung abscess (1), Irritable bowel syndrome (1), Grade 1 protein energy malnutrition (1), Myelodysplastic syndrome (1) , Antral gastritis (1), Osteoarthritis (1), Ulcerative colitis (1), Liver disease (1), Surgery (6).

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Antibiotics used		No. of patients who used the particular antibiotics	Total number of patients = 50
Third generation cephalosporins	Cefixime	3	14 (28%)
	Ceftriaxone	8	
	Cefoperazone	1	
	Cefotaxime	2	
Second generation cephalosporins	Cefuroxime	2	2 (4%)
First generation cephalosporins	Cefadroxil	1	1 (2%)
Aminoglycosides	Amikacin	2	2 (4%)
Fluoroquinolones	Ofloxacin	2	16 (32%)
	Ciprofloxacin	9	
	Levofloxacin	5	
Penicillins	Cloxacillin	1	8 (16%)
	Piperacillin tazobactam	5	
	Amoxicillin clavulanate	2	
Carbapenems	Meropenem	1	3 (6%)
	Imipenem / cilastatin	2	
Macrolides	Azithromycin	2	2 (4%)
Nitroimidazoles	Metronidazole	8	8 (16%)

Table 6: Antibiotics used by the PCR positive patients

Figure 1: Agarose gel picture of toxin A gene (Non-repeating region) PCR products. PC- Positive control, NC – Negative control, 1 to 5 represent different isolates of *C. difficile* positive or negative for toxin A gene, L – DNA ladder.

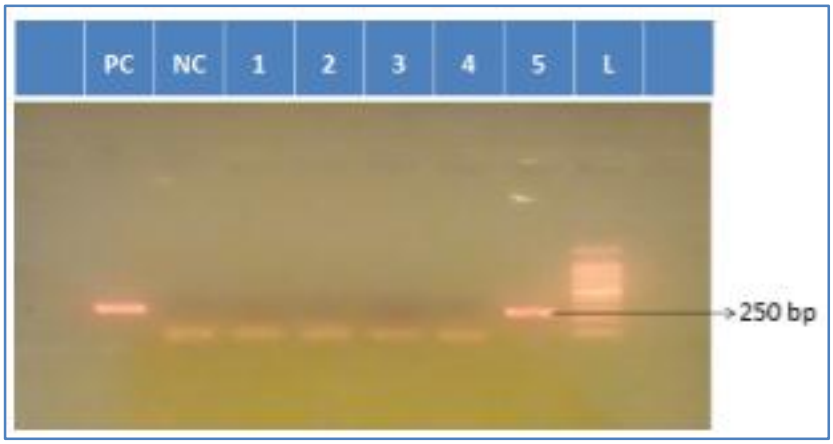

Fig. 1

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Figure 2: Agarose gel picture of toxin B gene PCR products. 1 to 13 represent different isolates of *C. difficile* positive or negative for toxin B gene, PC- Positive control, NC – Negative control, L – DNA ladder.

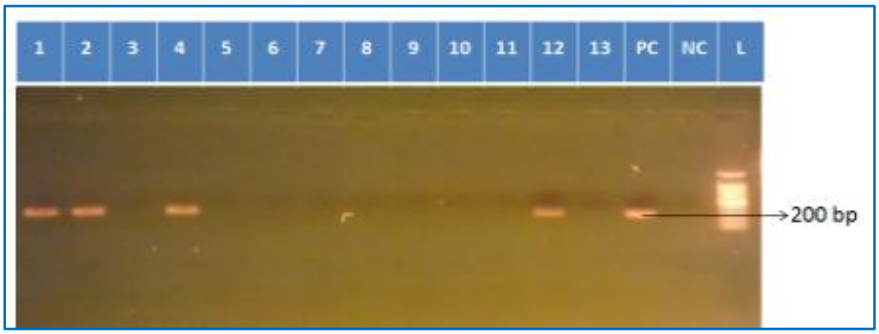

Fig. 2

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