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Estimation of faecal carriage of *Clostridium difficile* in patients with ulcerative colitis using real time polymerase chain reaction

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***Background & objectives*: Ulcerative colitis (UC) is a disease of unknown aetiology in which exacerbations are sometimes linked to intestinal colonization by toxin-producing *Clostridium difficile*. We undertook this study to detect and quantitatively assess *C. difficile* in the stool of patients with UC using real time polymerase chain reaction (RT-PCR), and to compare it with healthy individuals.**

***Methods*: A total of 37 consecutive patients with UC (26 male, mean age 41.3 yr) and 36 healthy adult volunteers (20 male, mean age 36.4), none of whom had received antibiotics within two months prior to faecal collection, were included in the study. Faecal DNA was extracted, quantitative PCR (qPCR) carried out using primers to amplify species-specific segments of 16S rDNA of *C. difficile*, and expressed as relative fold difference against amplification of highly conserved (universal) segments. Toxins A and B were assayed by ELISA.**

***Results*: Quantitative PCR detected *C. difficile* sensitively, and spiking with increasing numbers of the organism resulted in linear increase in amplification (R^2=0.974). *C. difficile* was detected by qPCR in faeces of 20 of 36 healthy volunteers and 34 of 37 patients with UC. Relatively greater amplification of *C. difficile* (fold difference) was noted in UC compared to controls (P<0.0001). There was no significant difference in *C. difficile* amplification between patients with proctitis, left sided colitis and pancolitis, or between active and quiescent colitis. Toxin was detected in the faeces of 8 of 37 patients with UC compared to 2 of 36 healthy volunteers.**

***Interpretation & conclusions*: Findings of this study showed overgrowth of *C. difficile* in the stool of Indian patients with UC. However, its relevance to disease pathogenesis and severity in a tropical country like India needs to be investigated further.**

Key words *Clostridium difficile* - real-time PCR - ulcerative colitis

The pathogenesis of ulcerative colitis (UC) remains unclear. The disease is characterized by exacerbations and remissions and runs a prolonged course. In addition to possible genetic factors concerned with immunoregulation and altered mucosal barrier function, dysbiosis which is an abnormal ratio of beneficial to detrimental commensal microbial agents has also been implicated in disease pathogenesis and relapse[1]. Studies

in germ-free mono-contaminated experimental animals indicate that commensal bacteria may be involved in the pathogenesis of colonic inflammation[2]. The intestine and colon are host to a very large number of bacteria, most of which are anaerobic. The normal resident intestinal flora prevents the overgrowth of pathogenic bacteria. *Clostridium difficile* is a Gram-positive anaerobic bacterium that is present as a very minor constituent of the colonic flora. The use of broad-spectrum antibiotics disrupts the ecosystem of the normal colonic flora, and may predispose to dense colonization with *C. difficile*, and lead to *C. difficile*-associated disease (CDAD). Patients on broad-spectrum antibiotics, hospitalized patients, those with malignancies, immunosuppressed patients and the elderly are all prone to develop CDAD[3]. In western countries, this organism is now implicated in both nosocomial and community acquired diarrhoea[4]. The organism produces two toxins, toxin A and toxin B, which are both cytotoxic. In healthy infants, *C. difficile* has been cultured from stool in numbers ranging from 10^3 to 10^7 per g, and a significant number of the isolates produced toxin *in vitro*[5]. In older individuals, *C. difficile* colonization becomes less common, and can be detected by stool culture in 4-15 per cent of healthy individuals[6]. In different studies *C. difficile* has been cultured from the stool (or toxin has tested positive) of 12, 28 and 32 per cent of patients with UC[7-9]. Recently there has been a renewed focus on the role of *C. difficile* in ulcerative colitis and inflammatory bowel disease[10,11]. CDAD may account for up to 20 per cent of flares of the disease in western countries[12].

Several previous studies in inflammatory bowel disease or ulcerative colitis have only included participants with disease[8-12] and did not study healthy individuals as controls. The advent of molecular techniques has enabled us to better examine the complex intestinal flora, especially to detect and quantitate fastidious and poorly cultivable anaerobic bacteria such as *C. difficile*. This study was undertaken to determine the rate of faecal carriage of *C. difficile* in patients with ulcerative colitis and to compare this carriage with that in healthy individuals using a sensitive quantitative PCR employing primers to amplify species-specific segments of the 16S rDNA, and to reassess the rate of detection of *C. difficile* toxins in the stool in these study groups.

Material & Methods

Subjects: A total of 37 consecutive patients (26 male) with ulcerative colitis ranging in age from 22-67 yr (mean $\pm$ SD 41.3 $\pm$ 12.7 yr) were recruited from attendees at the outpatient clinic of the Department of Gastrointestinal Sciences, Christian Medical College and Hospital, Vellore, Tamil Nadu during October 2004 to July 2005. Of these, 29 patients hailed from urban areas and 8 from villages. Ulcerative colitis was diagnosed on the basis of a clinical history of blood and mucus diarrhoea, colonoscopy showing diffuse mucosal oedema, granularity or ulceration at time of diagnosis, and biopsy confirming the presence of crypt branching or deformity along with cryptitis or crypt abscesses. Patients with UC included 16 with pancolitis, 14 with left-sided colitis, and 7 with proctitis. The average duration of disease in patients with UC was four years (range 0.5 to 12 yr). The shortest time of follow up of these individuals was 6 months which was sufficient to exclude infective colitis. Disease severity (Truelove and Witts criteria)[13] was judged as quiescent in 8 patients, mild in 13, moderate in 11 and severe in 5. Patients receiving antibiotics at the time of study, or any time in the two months preceding the study, were excluded from the study. At the time of study, three of the patients had never received prior medication. All the remaining 34 patients with UC received aminosalicylates (mesalazine); 20 received steroids and 3 received azathioprine in addition to aminosalicylates. Faecal microscopy did not reveal parasites in any of the patients, and standard faecal culture (for *Salmonella*, *Shigella*, and related pathogens) did not reveal any pathogens. Thirty six healthy adult volunteers (20 male) in the age group 25-53 yr (mean $\pm$ SD 36.4 $\pm$ 8.3 yr) were recruited from the community. These community volunteers were derived from Vellore town (29) or villages (7) in this district and were matched for socio-economic status with the UC group. Any participant receiving antibiotics in the two month period preceding the study were excluded. Informed written consent was obtained and the study was approved by the institutional Research Committee.

Sample collection: For the quantitative PCR (qPCR) and toxin studies, fresh faeces was collected and transported within half an hour to the laboratory, where samples were labelled and stored in aliquots at -80°C till processing.

DNA extraction: Samples were processed in batches. Approximately 250 mg (wet weight) of faecal sample was added to 2 ml sterile tubes containing 1.4 ml of ASL buffer from the QIAamp DNA stool mini kit (QIAgen, Germany), and DNA isolated according to

Table. Details of primers used in the study

Target organism	Primer	Sequence (5'-3')	Tm (°C)	Amplicon size
C. difficile[14]	Forward	TTG AGC GAT TTA CTT CGG TAA AGA	58	
	Reverse	CCA TCC TGT ACT GGC TCA CCT	58	157 bp
Universal[15]	Forward	TCCTACGGGAGGCAGCAGT	59	
	Reverse	GGACTACCAGGGTATCTAATCCTGTT	58	466 bp

Superscript numerals denote reference numbers

manufacturer's instructions. The DNA was eluted in a final volume of 200 µl and was stored at -20°C.

Primers: Primers used for this study amplified species-specific regions of the 16S rDNA of *C. difficile*. These primers have been characterized and their specificity established earlier[14]. Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria, the universal primer set[15], the amplification of which served as the denominator against which amplification of other bacterial nucleic acid was compared (Table). The primers were once again compared to all available 16S rDNA sequences using the BLAST database search program (*www.ncbi.nlm.nih.gov/blast*). Primers were purchased from Sigma Genosys (Bangalore, India).

PCR: The PCR amplification and optimal annealing temperatures of the PCR primers were initially optimized with gradient PCR in a Chromo 4 system (MJ Research/Biorad, USA) using SYBR Green master mix (RT-SN2X-O3+WOUN, Eurogentec, Belgium) which contained also the Hot Gold Star Polymerase. All PCRs were performed in duplicate in a volume of 20 µl, using high profile tubes and ultraclear sealing caps (MJ Research/Biorad, USA). The amplified products after a gradient PCR were subjected to electrophoresis and checked for the single, expected band and also were compared with the melting curve analysis.

The following optimized PCR conditions were used for the real-time PCR. Reaction mixtures had 2X SYBR green master mix (200 µM each dNTPs, 5 mM $MgCl_2$, Hot Gold Star Polymerase), 0.5 µM each primer, 2 µl of template and water to make up to the final volume. The thermal cycling conditions started with an initial activation step at 50°C for 2 min and an initial denaturation step at 95°C for 10 min, which was followed by a 45 cycles of denaturation at 95°C for 15 sec, annealing at 61°C for 30 sec, an extension at 72°C for 30 sec and a plate read step. The final extension step at 72°C for 10 min was followed by a melting curve analysis which was performed by increasing the temperature from 40 to 95°C, with a raise in temperature by 1°C every 10 sec with a plate read step to read the fluorescent signal.

The qPCR reads out Ct (cycle threshold) values for universal primer amplification and Ct value for the specific bacterial amplification. The relative difference or fold difference of *C. difficile* amplification compared to universal sequence amplification is automatically calculated by the Opticon 3.1 software of the Chromo 4 real-time detector, using the formula $2^{\Delta Ct}$. The relative difference of *C. difficile* (expressed as the ratio compared to universal rDNA) provides a quantitative comparison between different samples. The ratio calculated for each of the duplicate samples correlated well with the other, with an adjusted R^2 of 0.778.

Clinical isolates of *C. difficile* were obtained and grown on *C. difficile* agar (M836, Hi-media, Mumbai), and were identified after 48 h of growth in blood agar, clostridium agar (Hi-media), and Robertson's cooked meat broth (Hi-media, Mumbai) as colonies 2-3 mm in diameter, slightly raised, white, opaque, circular (irregular margin) with a characteristic horse dung odour, and chemical reactions showing that they were non-proteolytic, egg yolk negative, ferments glucose, but not maltose, lactose or sucrose, and not forming indole or hydrogen sulphide. Colonies were removed, washed with PBS and resuspended in PBS by adjusting against the MacFarland tubes. Resuspended bacteria were serially diluted and added to faeces that was negative for *C. difficile* by PCR such that there were log dilutions from 10^6 to 10^0 bacteria per g of stool. DNA was extracted from spiked faecal samples and used for qPCR.

Toxin testing: The RIDASCREEN® *Clostridium difficile* Toxin A/B test (Cat. No. C801, R-Biopharm AG, Germany), which is a sandwich ELISA using monoclonal antibodies, was used for detection of either toxin in the stool samples.

Statistical analysis: GraphPad Prism V4.03 (*www.graphpad.com*) was used for statistical analysis. Values were expressed as mean ± SEM. Significance of differences was measured using Mann-Whitney test or Kruskal-Wallis test for continuous variables and Fisher's exact test for dichotomous variables. The statistical parameters for the real time PCR (regression analysis) were automatically calculated by the Opticon 3.1 software available with the Chromo4 instrument. Two-sided $P<0.05$ was considered significant.

Results

PCR using the primer set amplified a 157 bp fragment, which was consistently noted with both isolated cultured bacteria and from faeces (Fig. 1). Although specificity of these primers have been earlier established, repeat sequence searches against prokaryotic DNA databases and *in silico* PCR ensured species-specificity. PCR using these primers did not amplify any product from other bacteria including *Bacteroides* species, *Bifidobacterium* species and *C. perfringens* (Fig. 1). Gradient PCR showed an optimum annealing temperature of 61°C for both the universal and the *C. difficile* primer sets and these conditions were used to amplify both universal and *C. difficile* target DNA simultaneously for real time PCR. The size of the amplicon was checked after 45 cycles of PCR amplification, and electrophoretic bands of about 157 base pair and about 466 base pair were produced using the *C. difficile* and the universal primer set respectively. A melt curve analysis was performed on the PCR product, which generated single peaks at 83 and 73°C for the universal primer and *C. difficile* respectively. Stool spiked with increasing concentrations of *C. difficile* organisms showed that the organism was detectable in stool by qPCR to an apparent limit of 10 bacteria/g faeces (Fig. 2). There was a linear relationship between the log numbers of bacteria added to the stool and the Ct value in qPCR ($R^2=0.974$).

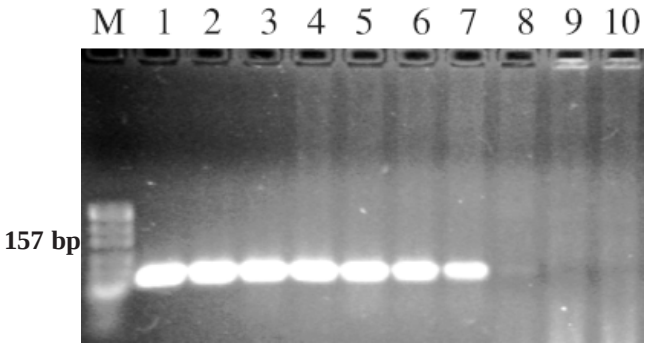

Fig. 1. Bacterial PCR for *C. difficile*. 2 per cent agarose gel electrophoresis of conventional PCR product (~157bp) for *C .difficile*. Lanes 1-3 are colony PCR performed on isolates of *C. difficile* from UC patients, lanes 4&5 are products of *C. difficile* from isolated DNA, Lanes 6-7 are products from amplification of faecal DNA. Lanes 8 and 9 are PCR products from mixed bacterial specimens of *Bacteroides* and *Bifidobacterium*, and Lane 10 is PCR of *C. perfringens*.

C. difficile was detected by qPCR in the faeces of 20 of the 36 healthy volunteers, while it was detected in 34 of 37 patients with UC ($P=0.007$, Fisher's exact test). The quantitative PCR also showed an increase in *C. difficile* in UC patients, with the ratio being significantly higher in UC (0.09527 ± 0.02864, mean ± SEM) compared to healthy controls (8.475E-07 ± 6.456E-07) ($P<0.0001$, Mann Whitney test) (Fig. 3). The *C. difficile* quantitation was not significantly different among patients with pancolitis, left sided

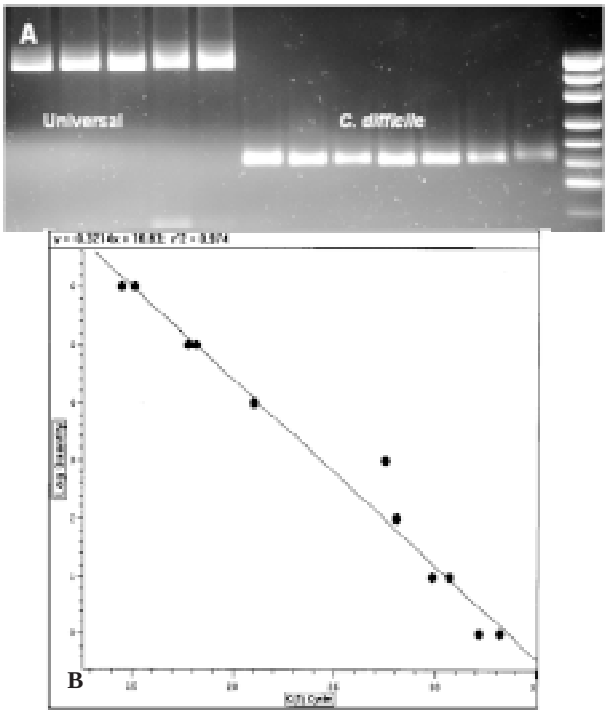

Fig. 2. Faeces were spiked with increasing log concentrations of *C. difficile*, DNA extracted and quantitative PCR carried out. **(A)** Conventional PCR using universal and *C. difficile* primers in the spiked samples. The first five lanes marked "universal" represent PCR product of universal primer amplification. The next seven lanes represent PCR product of *C. difficile* amplification, of serial bacterial dilutions ranging from 10^7 to 10^0. Molecular weight markers shown in the extreme right lane are PUC19DNA/MspI (HpaII) 501-=67 obtained from MBI Fermentas (Germany). **(B)** Linear regression of log bacterial count and Ct value for *C. difficile*.

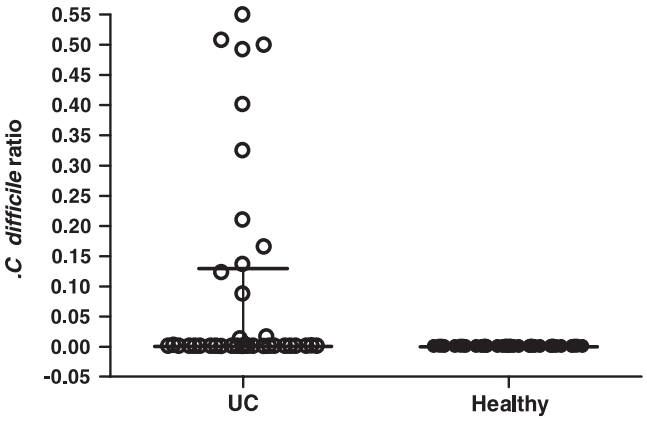

Fig. 3. Ratio of *C. difficile* compared to universal bacterial primer, in control healthy volunteers and in patients with UC. Median and interquartile range are shown. The difference between these two groups was highly significant ($P<0.0001$, Mann Whitney test).

colitis and proctitis. There was no statistically significant difference in *C. difficile* quantitation between patients with inactive colitis, and mild, moderate or severe colitis. Since 34 of the 37 patients with UC were receiving aminosalicylates, the possibility that this drug was responsible for *C. difficile* overgrowth could not be tested. Comparison between 23 patients receiving immunosuppressants and 14 not receiving them did not indicate any significant difference in *C. difficile* numbers between the two groups. *C. difficile* toxin was detected in the faeces of 8 of 37 patients with UC compared to 2 of 36 healthy volunteers.

Discussion

This study was designed to evaluate the occurrence of *C. difficile* in UC patients compared to a healthy control population. Amplification by PCR using specific primers and real time detection was used to express *C. difficile* relative to the total number of bacteria represented by amplification using the universal primer set. *C. difficile* rDNA was detected in the stool of 55.5 per cent of healthy individuals, but was found in almost all (91.9%) patients with UC. Also, *C. difficile* rDNA was present in much higher concentrations in patients with UC than in healthy volunteers.

Molecular methods targeted at bacterial 16S rDNA have come into prominence for detection and quantitation of anaerobic faecal bacteria, particularly those present in relatively small numbers compared to the dominant bacterial flora. Use of real time PCR enabled the detection of even very small numbers of *C. difficile*, thus making it likely that these figures represent the true prevalence of the organism in the human gastrointestinal tract. In this study, the point detection of *C. difficile* in stool of healthy volunteers was found to be 55 per cent, compared to earlier culture-based studies in which the point detection was estimated to be less than 20 per cent[5,6]. Similarly, the present study shows that the organism was present in the stool of over 90 per cent of patients with UC, compared to estimates of up to 32 per cent in earlier studies[7-9]. It is likely that this difference is due to the sensitivity of real time PCR detection. However, it is possible that residence in a tropical and contaminated environment may have caused an increased frequency of faecal carriage of *C. difficile* in both control subjects and patients, as was suggested by an earlier study from northern India[8].

Although *C. difficile* produces two toxins that are cytotoxic, there is controversy as to their exact role in disease pathogenesis in UC. Toxin-producing *C. difficile* have been shown to be associated with CDAD in inflammatory bowel disease patients in the West[10-12], and with disease exacerbations in Indian patients with UC[8]. In the case of UC, where a dysbiosis has been postulated, it is not clear that toxin production is a necessary attribute for these bacteria to induce inflammation. Pathogenic bacteria trigger innate inflammatory responses through a variety of molecules that are recognized by innate immune receptors on epithelial cells or macrophages[1]. Thus, the presence of increased *C. difficile* demonstrated in patients with UC in this study could potentially be of importance in the pathogenesis of inflammation. However, there was no significant difference in this study between patients with active UC and those with quiescent UC, thus suggesting that *C. difficile* carriage in these patients represents an epiphenomenon rather than a causative or contributory factor in the disease. In conclusion, this study demonstrates an increased frequency and increased proportional *C. difficile* intestinal carriage in patients with UC. Its relevance to disease pathogenesis and severity in a tropical contaminated environment deserves to be further investigated.

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