Rapid detection of enterotoxigenic *Bacteroides fragilis* in diarrhoeal faecal samples

Nidhi Sharma & Rama Chaudhry

*Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India*

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**Background & objectives:** Enterotoxigenic *Bacteroides fragilis* (ETBF) is an important aetiological agent of acute diarrhoeal illness in humans. It is a fastidious anaerobe and takes 3-5 days when grown in culture, and test for enterotoxin detection requires additional time conventionally. The present study was aimed to standardize a rapid and sensitive diagnostic assay and to screen the presence of ETBF isolates directly in faecal samples using polymerase chain reaction (PCR) technique.

**Methods:** A total of 85 faecal samples were processed for both aerobic and anaerobic bacterial isolation by culture technique and also subjected to PCR, for amplification of neuraminidase and enterotoxin genes of *B. fragilis* using specific primers.

**Results:** PCR was found to be positive for neuraminidase gene in 14/65 (21.5%) and for both neuraminidase and enterotoxin genes in 4/65 (6.1%) faecal samples within 48 h in study group. *B. fragilis* did not grow in any of the faecal samples when cultured anaerobically. However, the delay in transport and processing of faecal samples for anaerobic culture may be responsible for culture negativity.

**Interpretation & conclusion:** PCR assay can be used as a rapid and sensitive diagnostic tool for the identification of ETBF in culture as well as directly from diarrhoeal faecal samples instead of using conventional technique. The association of ETBF in diarrhoeal diseases is unclear and importance of ETBF as a causal agent will require more studies in Indian population.

**Key words** Diarrhoea - ETBF - faecal - PCR
Bacteroides fragilis, the Gram negative anaerobic rod shaped bacteria and normal inhabitants of the large bowel of mammals, are of primary importance to humans as they cause opportunistic and extraintestinal infections. A subgroup of this species which may be important as an aetiological agent of acute diarrhoeal illness in humans has now been identified. B. fragilis carry genes encoding products involved in pathogenicity, such as neuraminidases and an enterotoxin, a zinc containing metalloprotease with molecular weight of 20 kDa^1,2. The enterotoxin of enterotoxic B. fragilis (ETBF) is an important virulence factor inside and outside the intestinal lumen. It produces extensive tissue damage in the intestinal mucosa in vivo and increases bacterial internalization by enterocytes^3,4.

The identification of this organism in clinical microbiology laboratory is sometime difficult, as it is a fastidious organism and takes 3-5 days when grown in culture. Moreover, B. fragilis are often unrecognized in faecal samples because of inappropriate transportation (within 2 h of collection), specific growth requirements and frequent co-existence with aerobic organisms, present in the faecal sample. Further processing for cytotoxic assay and rabbit illial loop assay for enterotoxin test requires additional time. There is no information available from India regarding the detection of ETBF in faecal samples in cases of diarrhoea except a study by Niyogi et al^5, who did cytotoxic assay for ETBF detection. Due to ethical issues involved with animal experiments and non availability of rapid diagnostic technique isolation of ETBF is generally overlooked. Therefore, this study was carried out to apply a rapid and sensitive molecular diagnostic technique to detect neuraminidase gene specific for B. fragilis and enterotoxin gene to detect ETBF directly from faecal samples of patients with diarrhoea.

**Material & Methods**

A total of 65 faecal samples were collected from patients admitted to different wards and those attending outpatients departments at the All India Institute of Medical Sciences, New Delhi, diagnosed with diarrhoea and other gastrointestinal complication for study group, during the period of July 2001 to March 2003. Control group comprised 20 faecal samples collected from patients admitted in the same period with other than gastrointestinal complaints. The mean age of patients in study group was 40.43 ± 13.49 (range 20-72 yr). All faecal samples were processed for aerobic enteropathogens isolation on Mac Conkey agar and for anaerobes samples on Bacteroides bile esculine agar (BBE), brain heart infusion agar (BHIA) supplemented with haemin and vitamin K (Hi-Media Laboratories, Mumbai, India), and simultaneously on blood agar. Plates were incubated aerobically and anaerobically at 37°C for 24 and 48 to 72 h respectively. Anaerobiosis for the growth of B. fragilis and other anaerobes was generated by using ANOXOMAT System (MART Sint-Genesius-Rode, Belgium), programmed for anaerobic gaseous atmosphere (N<sub>2</sub>-80%, H<sub>2</sub>-10%, CO<sub>2</sub>-10%) in anaerobic jar. Conventional biochemical tests were performed to identify both aerobic and anaerobic organisms as per standard microbiological methods^6.

**Standardization of PCR assay:** PCR assay were performed for amplification of neuraminidase gene and enterotoxin gene by using published primers^3,4. Standard strain of ETBF (B45) was provided by A. Pantosti, Laboratory of Bacteriology and Mycology, isituto superiore di santa, Rome, Italy, which was used for standardization of PCR.

**Preparation of bacterial strain (B45) for PCR analysis:** Standard strains of enterotoxigenic B. fragilis were grown on BBE and BHIA. Colonies were picked up with sterile loop and suspended in sterile phosphate buffer saline (PBS) pH 7.2, washed
the cells with 1 x PBS by giving spin at 4000 g for 1 min (2-3 times). Pellet was resuspended in 50 µl sterile double distilled water and DNA was extracted by boiling method to perform the PCR.

Isolation of DNA from spiked faecal and diarrhoeal samples: Briefly, 100 µl of faecal sample was suspended in 100 µl of TE buffer [10 mM Tris HCl (pH 7.5); 1 mM EDTA] and 200 µl of lysis buffer

Fig. 1. PCR sensitivity with neuraminidase gene of *B. fragilis*. Lane M-marker (100 bp), lane 1-7 (10⁹ - 10⁴ cfu/g), lanes 8-11 (10² - 12.5 cfu/g), lane 12 negative control and lane 13 positive control.

Fig. 2. PCR sensitivity with enterotoxin gene of *B. fragilis*. Lane M-marker (100 bp), lane 1-6 (10⁹ - 10⁴ cfu/g), lane 7-8 (10¹ - 10² cfu/g), lanes 9 positive control and lane 10 negative control.
[200 mM NaCl+20 mM EDTA+20 mM Tris HCl pH 8.0, 4% (w/v) SDS, 1 mg/ml Proteinase K] was added and incubated at 60°C water bath for 2 h. After cell lysis, DNA was extracted by phenol-chloroform method\(^7\) and then subjected to the PCR/electrophoresis.

**PCR assay for neuraminidase gene of B. fragilis:**
PCR assay for detection of neuraminidase gene of *B. fragilis* was carried out by using primers, designed to amplify a 262 bp sequence, specific for neuraminidase gene as reported by Jatwani *et al*.\(^3\)

Forward primer GAI 11 (5’ > GCC GGT CAG AAT

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Fig. 3. PCR products from faecal samples of patients with diarrhoea to detect a segment of neuraminidase gene. Lane 1 marker, lanes 2 to 7 DNA from diarrhoeic stool samples, lane 8 blank, lane 9 negative control, lane 10 positive control. (262 amplification).

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262bp

Fig. 4. PCR products from faecal samples of patient with diarrhoea to amplify enterotoxin gene from the samples. Lane 1 marker, lanes 2-9 diarrhoeic faecal samples, lane 10 positive control, lane 11 negative control. (294 amplification).

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294 bp
GGG AGT AGG AGA CC 3'). Reverse primers GAI 12 (5' > CCC GAC GAG CCG GAC CTT GCA ACA GA 3') were synthesized by BIO BASIC Inc. Canada.

The PCR reaction mixture consisted of 50 pM of each primer, 100 µM each deoxynucleotide triphosphate (dNTPs) (Banglore Genei, India), 1 U Taq DNA polymerase (Banglore Genei), 1X PCR buffer containing 1.5 MgCl₂ (Bangalore Genei) and 5 µl of DNA template in 25 µl of reaction volume. PCR cycle consisted of 35 cycles of initial denaturation at 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The amplified PCR product (262bp) was detected under UV, after staining with ethidium bromide.

**PCR assay for enterotoxin gene of B. fragilis:** Following sequence of primers were synthesized (BIO BASIC Inc. Canada) on the basis of published gene sequences to amplify a 294 bp product specific for enterotoxin gene coding zinc binding metalloprotease as reported by Pantosti et al. Forward primer BF1 (5' > GAC GGT GTA TGT GAT TTG TCT GAG AGA 3') and reverse primer BF2 (5' > d ATC CCT AAG ATT TAT TAT CCC AAG TA 3') was used for PCR.

The reaction mixture contained d NTPs 100 µM each (Banglore Genei), 50 pM of each primer, DNA polymerase 1U and 10 µl of DNA template in final volume of 25 µl of 1X PCR buffer containing 1.5 MgCl₂ (Bangalore Genei), samples were subjected to 35 amplification cycles carried out in thermal cycler (MJ Research, USA). Each cycle consisted of initial denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, and the last cycle was followed by final extension at 72°C for 5 min 7 µl amplified PCR product was subjected to electrophoresis in 1.6 per cent agarose gel, stained with ethidium bromide and examined under Gel Doc System (Alpha Innotech Inc, San Leandro, CA).

**Sensitivity & specificity of PCR for neuraminidase and enterotoxin gene:** Sensitivity of PCR assay was determined with serial dilution method. Ten-fold serial dilutions were made by spiking the faecal sample. DNA were extracted from each dilution and subjected to PCR (Figs 1, 2). Specificity of PCR was checked by using DNA extracted from panel of aerobic and anaerobic bacterial isolates, such as *Escherichia coli* *Klebsiella pneumoniae*, *Psuedomonas aruginosa*, *Salmonella Typhi*, *Salmonella paratyphi*, *Proteus mirabilis*, *Enterobacter* sp., *Enterococcus faecalis*, *Staphylococcus aureus*, *Clostridium difficile*, *Bacteroides melaninogenicus* and *Bacteroides corporis*, all these species are found to be negatives for enterotoxin gene and neuraminidase gene whereas *B. fragilis* (B45-ETBF) was found to be positive for both genes and *B. fragilis* (non-ETBF) was found to be negative for enterotoxin gene and positive for neuraminidase gene.

**Results**

The sensitivity of PCR was 10³ colony forming unit (cfu) of faeces for neuraminidase gene and 10⁴ cfu of faeces for enterotoxin gene with the spiked samples. It was noted that PCR amplification was obtained only from diluted spiked faecal sample, not from neat spiked sample, which indicated presence of some inhibitors which were removed by dilution. The amplification was not obtained with other organisms. The assay was found to be specific for ETBF.

A total number of 85 samples were subjected to PCR assay for both neuraminidase as well as enterotoxin genes of *B. fragilis* by using specific primers and also processed aerobically and anaerobically for bacterial isolation on their respective media. Common aerobic isolates were *E. coli*, *Klebsiella* spp, *Enterobacter* spp, *Proteus* spp, *Pseudomonas* spp, *Enterococci* and of anaerobes only *Clostridium* spp was isolated. All faecal samples were found to be culture negative for *B. fragilis*. 

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Of the 85 faecal samples, 65 were from patients with diarrhoea and 20 samples were from control (non-diarrhoeic) patients. The anticipated product size was obtained from standard strain (B45), as well as from diarrhoeic samples. The amplification signal was not observed in faecal samples from controls.

PCR was positive for neuraminidase gene in 14/65 (21.5%) and for both genes (neuraminidase and enterotoxin) in 4/65 (6.1%) diarrhoeic samples. (Figs 3, 4). PCR was found to be negative for neuraminidase and enterotoxin gene in all 20 control samples.

Discussion

The normal colonic microflora of humans consists of several species of aerobic and anaerobic microorganisms. Gut organisms are involved in numerous metabolic activities in the colon. The anaerobic members play fundamental role in the processing of complex molecule into simpler ones, and through their metabolic activities, the human microflora participates in the complex physiology of host8. Certain isolates of B. fragilis have been shown to be frequently associated with severe infection at extraintestinal sites by producing the enterotoxin9,10. During the last decade, several studies emphasized the possible role of enterotoxin producing B. fragilis in intestinal diseases of animals and human. The enterotoxin of B. fragilis may be important as aetiological agents of acute diarrhoeal illness in human. In 1984, Mayers et al first isolated enterotoxigenic B. fragilis from the faeces of neonatal lambs with diarrhoea. Subsequently similar isolates were obtained from faeces of diarrhoeic calves, piglet’s, foals, and humans11,12.

Many prospective and epidemiological studies were conducted in different countries to understand the pathogenic role of ETBF and its association with diarrhoeal disease by using cytotoxicity and PCR assay. Sack et al13 reported 12 per cent isolation rate of ETBF in native Americans and 9 per cent in Bangladeshi children, compared with 6 per cent of controls. Joaquin et al14 found a strong association between diarrhoeal disease and ETBF in children with the isolation rate of 4.8 per cent, whereas B. fragilis were recovered in 32.1 per cent of children with diarrhoea in an urban setting in United State14.

In the present study, we applied PCR assay to investigate the presence of ETBF directly in faecal samples. In this study only 6.1 per cent faecal samples were found to be positive for ETBF by PCR and all cultures were negative. All 4 faecal samples positive by PCR were also culture negative for C. difficile as well as for other enteropathgens. The presenting symptoms of these patients were watery diarrhoea, pain in abdomen and fever. Identification of different types of diarrhoeagenic E. coli and rotavirus was not done.

Pantosti et al15 reported 17 per cent isolation rate and found that in Italy the rate of ETBF carriage was high in both adults and children and B. fragilis was found in 43 per cent of children, Kato et al16 reported that ETBF was positive in 14.9 per cent of children aged 1 to 14 yr with antibiotic-unassociated diarrhoea and 6.5 per cent of children aged 1-6 yr with antibiotic associated diarrhoea16. In another study10 ETBF was isolated from 13.2 per cent of patients with inflammatory bowel disease. Niyogi et al have also determined the association of ETBF with childhood diarrhoea by cytotoxic assay using human colonic epithelial cell line (HT29/CI) in Indian setting and reported ETBF isolation in 2.6 per cent patient and 1.7 per cent controls in children between 1-5 yr of age. Recently Nguyen et al17 detected 7.3 per cent ETBF by immunoseparation in combination with PCR in children having diarrhoea (less than 5 yr) as compared to 2.4 per cent in controls. These studies showed that ETBF was epidemiologically associated with acute diarrhoeal disease in children and adults17-19. In our study, positivity of ETBF by PCR was similar as reported by other workers but was higher in comparison to previous Indian study5.
The low recovery of ETBF in culture may be due to the delay in sample collection or transportation to the laboratory. Similar results have been reported by Sack et al\textsuperscript{13} who found a marked reduction in the recovery of \textit{B. fragilis} with time. Considering the microbiological methods for isolation and identification of anaerobes takes 7 days and cytotoxic assay requires further 5-6 days to test cytopathic effect on cell lines, PCR assay for amplification of enterotoxin gene directly in the stool of patients with diarrhoea appears to be more rapid and specific detection technique, which can be used in routine clinical laboratories, and within 2 days report can be generated. Since none of the samples was culture positive in the present study, further studies are required both at epidemiological and molecular level for better understanding of the role of ETBF, and to validate the PCR against culture.

In conclusion, 6.1 per cent faecal samples were found to be positive for ETBF, by PCR. PCR represents a rapid and sensitive diagnostic tool for the detection of enterotoxin gene of \textit{B. fragilis} from culture as well as from faecal samples directly, whereas conventional microbiological methods are relatively time consuming or inaccurate for such a fastidious organism. The association of ETBF with diarrhoea is not clear at present due to limited number of positive cases, and importance of ETBF as a causative agent will require further investigations.

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References


Reprint requests: Dr Rama Chaudhry, Professor, Department of Microbiology
All India Institute of Medical Sciences, New Delhi 110029, India
e-mail: rc123@hotmail.com; ramach003@yahoo.com