Prevalence of Enterotoxigenic Bacteroides Fragilis in Stool Specimens Collected from Children Less Than 5 Years Of Age in Iraq

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Received: 10/1/2020 Accepted: 15/3/2020

Abstract

Although Bacteroides fragilis is a bacterium present within gut microbiota, the toxin producer strain, known as enterotoxigenic B. fragilis (ETBF), is associated with diarrhea in children less than 5 years of age. This study includes 69 diarrheal and 29 non-diarrheal (control) samples collected from children less than 5 years old. DNA was extracted directly from stool specimens and directed to conventional PCR targeting beta-isopropylmalate dehydrogenase (leuB) gene, used for detection of B. fragilis, and Bacteroides fragilis toxin (bft) gene, used for the detection of ETBF. The results showed that the prevalence of leuB gene was 78 (79.6%) including 56 (81.2%) in diarrhea and 22 (75.9%) in non-diarrhea subjects, while that of bft gene was only 3 (3.1%) including 2 (2.9%) in diarrhea and 1 (3.4%) in non-diarrhea subjects. Based on sequencing of bft-positive specimens, both bft-1 and bft-2 isoforms were represented in diarrheal specimens, whereas only bft-1 was found in the control specimens. In conclusion, this study examined for the first time the leuB and bft gene in a specimen of Iraqi children with diarrhea and showed no significant differences between diarrheal and control groups in both genes.

Keywords: Bacteroides fragilis, Diarrhea, Children.
Introduction

*B. fragilis* is a gram-negative, nonmotile, non-spore forming, and strictly anaerobic bacteria, although it can grow in nanomolar levels of oxygen concentration [1, 2]. It is an opportunistic pathogenic bacteria and its infection usually occurs when the wall of the gastrointestinal tract is disrupted or perforated, usually following surgery, when the contents of the GI-tract can enter the sterile peritoneal cavity [3]. The range of *B. fragilis* infection includes abscess formation, intra-abdominal and gynaecological sepsis, soft tissue infection, bacteremia, and abscess in the abdomen, brain, liver and lungs [2, 4, 5].

*B. fragilis* can be classified into two groups; the first is the enterotoxigenic *B. fragilis* (ETBF) and the second is the non-enterotoxigenic *B. fragilis* (NTBF) [3, 6]. The ETBF strain secretes *B. fragilis* Toxin (BFT) that is encoded by *bft* gene located within the 6-Kb pathogenicity island (BfPAI), in addition to a 18-Kb flanking DNA region [7, 8]. *BFT* is a 397 amino acid pre-protein which is eventually secreted as a 20 kDa mature protein [9, 10]. There are three isoforms of *bft* gene (*bft-1, bft-2 and bft-3*) that can be distinguished by *sau3AI* digestion of the PCR-product of *bft* gene into 848 and 294 bp for *bft-1*, 571, 461 and 110 bp for *bft-2*, and 848, 184 and 110 bp for *bft-3* [11, 12]. The mature toxin domain of each *bft* isotype contains an extended zinc-binding metalloprotease motif, HEXXXHXGXHH [13, 14].

ETBF strains were isolated for the first time in 1987 from diarrheal patients in Bangladesh [15]. Sack *et al.* identified the toxic strains as a causative agent for human diarrheal illness [16]. In addition, Sack *et al.* and Durmaz *et al.* determined that ETBF causative agent affects diarrhea patients less than 5 years of age [17, 18]. Another study published in in 2008 found that ETBF is an etiologic agent of inflammatory diarrhea [19].

**Aim of study**

The present work aimed at assessing the prevalence of *bft* among *B. fragilis* isolates in stool samples collected from Iraqi children.

**Materials and Methods**

**Collection of Specimens**

In this study, 98 stool specimens, including 69 diarrheal and 29 non-diarrheal (control), were collected from children less than 5 years of age. The specimens were collected in sterile cups at Al-Alwaiya hospital for children and the children hospital in Baghdad Medical City. The specimen-related information included age and sex of participants and color and shape of specimens. Diarrheal indicators of the stool, i.e. green color, watery, loose, mucoid or bloody stool, mentioned by Shen *et al.*, were taken into consideration when collecting the specimens. The specimens were examined microscopically for the presence of pus cells, red blood cells, or any parasite [20].

**Extraction of DNA**

The extraction of DNA was performed in the laboratory of the biotechnology department / University of Baghdad. Solid stool or watery stool specimens were used for DNA extraction by using Presto™ Stool DNA Extraction Kit, Geneaid Company.

**Primers**

Primers used in this study are listed in Table-1. *leuB* gene which encodes β-isopropylmalate dehydrogenase was used for the detection of *B.fragilis* [8] whereas *bft* gene was used for the detection of ETBF in fecal specimens.

**Table 1- Primers sequence and the PCR product**

| Primers  | Sequence          | PCR Products | References |
|----------|-------------------|--------------|------------|
| F: *leuB* | GCCTCTGGGTATGCGTAAA | 440 bp       | NCBI       |
| R: *leuB* | CTCCGTCACCATCAGTCAA |             |            |
| F: *bft*  | CGCGGGCATATTAGCTGATTTGTAATG | 992 bp       | [21]       |
| R: *bft*  | GATACATCGCTGGTTGAGACATCCCA |             |            |

F (forward), R (Reverse), bp (base pair).
Detection of *B. fragilis* and enterotoxin

The mixture of PCR was prepared as follows; 12.5 µl Go Taq® Green Master Mix supplied by (Promega Company), 1 µl Forward Primer (10 pmol), 1 µl Reverse Primer (10 pmol), 5 µl DNA template, and 6.5 µl nuclease-free water to complete the volume up to 25 µl. The negative control was prepared by using the same ingredients without DNA template. The program of PCR used for both *leuB* and *bft* genes [21] is illustrated in Table-2.

**Table 2-** PCR program for *leuB* and *bft* genes

| Gene | Steps | No. of Cycles | Temperature | Time (M:S) |
|------|-------|---------------|-------------|------------|
| *leuB* | Initial Denaturation | 1 Cycle | 94 ºC | 5:00 |
| | Denaturation | | 94 ºC | 0:45 |
| | Annealing | 40 Cycles | 58 ºC | 0:45 |
| | Extension | | 72 ºC | 0:45 |
| | Final Extension | 1 Cycle | 72 ºC | 10:00 |
| *bft* | Initial Denaturation | 1 Cycle | 94 ºC | 5:00 |
| | Denaturation | | 94 ºC | 1:00 |
| | Annealing | 40 Cycles | 58 ºC | 1:00 |
| | Extension | | 72 ºC | 1:00 |
| | Final Extension | 1 Cycle | 72 ºC | 10:00 |

Detection of *bft* gene subtypes

PCR products of *bft* positive specimens were stored at – 20 ºC and delivered with the forward *bft* primer to Macrogen Company, Korea for sequencing. The data received were analyzed by basic local alignment search tool (BLAST) provided by the National Center of Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the homology with published sequences.

**Statistical analysis**

Fischer’s exact test and $x^2$ tests were used to compare the rates of prevalence for ETBF in patient and control groups.

**Results and Discussion**

A total of 98-stool specimen (69 diarrheal and 29 control) were collected from two hospitals in Baghdad. The age distribution of the patients was as follow; 20 patients (29.0%) were less than one year of age and 49 (71.0%) were in the age range of 1-5 years. For the non-diarrheal subjects, 8 (27.6%) were less than 1 year of age, while 21 (72.4%) were in the range of 1-5 years. Significant age differences were not present between patients and control (P > 0.05) (Table-3). According to gender distribution, the patients group included 41 males and 28 females while the control included 23 males and 6 females.

**Table 3-** Distribution of specimens according to age.

| Age/year | Total |
|----------|-------|
|          | 1-5year | >year |
| Diarrhea | 49       | 20     | 69     |
| Count    | % within Diarrhea | 71.0% | 29.0% | 100.0% |
| Control  | 21       | 8      | 29     |
| Count    | % within Control | 72.4% | 27.6% | 100.0% |
| Total    | 70       | 28     | 98     |
| Count    | % within both | 71.4% | 28.6% | 100.0% |

**Detection of *leuB* gene**

The *leuB* gene was used for the detection of *B. fragilis*. Conventional PCR revealed the presence of *leuB* gene (440 bp) in stool specimens from 78 participants (79.6%), with an increased gene expression in the patients (56, 71.8%) as compared to the control (22, 28.2%) (Table-4 and Figure-1).
It was not surprising to find insignificant gene expression differences because this bacterial species is a common inhabitant of human colon. A previous study used another gene, namely gyrB (B-subunit of DNA gyrase), for the detection B. fragilis, using both 16s rRNA or 16s-23s rRNA. Also, Ji et al. used gyrB gene for the same purpose [22] and found a prevalence of 39.44% in 513 stool specimens. Another study used 16s-23s rRNA for the detection of B. fragilis [23].

Table 4 - leuB gene in diarrheal and non-diarrheal specimens

|                           | leuB gene | Total |
|---------------------------|-----------|-------|
|                           | Present   | Absent|       |
| **Diarrhea**              | 56        | 13    | 69    |
| Count                     |           |       |
| % within Diarrhea          | 81.2%     | 18.8% | 100.0%|
| **Control**               | 22        | 7     | 29    |
| Count                     |           |       |
| % within Control           | 75.9%     | 24.1% | 100.0%|
| **Total**                 | 78        | 20    | 98    |
| % within both              | 79.6%     | 20.4% | 100.0%|

Figure 1- Gel electrophoresis of amplified leuB gene (440 bp) from B. fragilis using conventional PCR. Agarose 2 %, 70 V/cm for 75 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane L: 100 bp DNA ladder. Lanes 1-9: Amplicons leuB gene for B. fragilis (Specimens from 29 until 37). Lane -C: negative control (all PCR mixture with the substitution of water for DNA template).

Detection of enterotoxin (bft) gene

The incidence of bft in the total 98 stool specimens was 3 (3.1%), divided into 2 patients (2.9%) and 1 control (3.4%) (Figure-2, Table-5). All these 3 specimens which were positive for bft belonged to children in the range of 1-5 years of age. No bft positive samples were noticed within the age younger than one years, perhaps because of infant protection from diarrhea and infections by maternal antibodies [18]. Several studies proved that ETBF (which possesses the bft gene) is linked to diarrhea in children 1-5 of age [17, 18, 24, 25]. A previous study from Turkey demonstrated that the bft gene was found in 13 (15%) infants with diarrhea and 13 (8%) infants without diarrhea, younger than one year of age. In the age range of 1-5, bft was found in 31 (39%) diarrheal and 31 (16%) control specimens, although there was no significant difference (P = 0.088) [26].
Table 5- bft gene rates in 98 stool specimens

|               | Count | bft gene |             |         |         |
|---------------|-------|----------|-------------|---------|---------|
|               |       | Present  | Absent      | Total   |         |
| Diarrhea      | 2     | 67       | 69          |         |         |
|               |       | 2.9%     | 97.1%       | 100.0%  |         |
| Control       | 1     | 28       | 29          |         |         |
|               |       | 3.4%     | 96.6%       | 100.0%  |         |
| Total         | 3     | 95       | 98          |         |         |
|               |       | 3.1%     | 96.9%       | 100.0%  |         |

A study from Iran, without control specimens, used anaerobic culture methods of 100 diarrheal specimens only and reported a bft rate of 5.72% [27]. Ramamurthy et al. conducted a case-control study in India and demonstrated an ETBF rate of 7.2% in each the 446 patient and 428 control specimens, with no statistical difference [28]. Krzyzanowsky and Mario used HT-29 Cell-Line method and revealed that 2 (2%) specimens were positive for ETBF in 96 diarrheal specimens from São Paulo, Brazil, with no significant difference to the control group [29]. In Taiwan, Ji et al. analyzed stool samples using PCR and found that bft rate was 1.56% of 513 diarrheal specimens, while the study did not include a control group [22].

The results of the present study are in agreement with those of many studies from different countries [26, 28, 29, 30], whereas they are in disagreement with others [17, 18, 24, 25, 31, 32].

The presence of bft gene in non-diarrheal specimens can be due to the fact that it is located in the 6-Kb BfPAI region in addition to the 18-Kb flanking region on both side of BfPAI. Franco et al. analyzed the BfPAI and its flanking region and suggested that they can be self-mobilized from ETBF strain to NTBF strain [7]. In a previous study, Ignacio et al. elucidated the prevalence of ETBF and NTBF (Pattern III) in 84 non-diarrheal specimens [23]. The rates of ETBF and NTBF was 4.7% and 32.1%, respectively.

Figure 2- Gel electrophoresis of amplified bft gene (992 bp) from ETBF using conventional PCR. Agarose 1 %, 70 V/cm for 75 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane L: 100 bp DNA ladder. Lanes 1-3: Amplicons bft gene for ETBF (Specimens 29, 32, and 53). Lane -C: negative control (had all PCR mixture with the substitution of water for DNA template).
Identification of bft gene subtypes

Based on the sequencing of bft positive specimens, they were analyzed by alignment with reference sequencing in BLAST provided by NCBI. The results showed that the 2 diarrheal specimens were positive for bft-1 and bft-2, while the non-diarrheal specimen was only bft-1. No bft-3 expression was demonstrated in our study. Several studies in different countries elucidated that the commonest rates of bft gene subtypes were for bft-1 rather than bft-2 and bft-3. Akpinar et al. reported that the rate of bft-1 was higher than bft-2 in patient and control specimens from Turkey [26]. In Brazil, São Paulo, bft-1 had the commonest rate, while only one bft-3 positive specimen was recorded [33]. In Vietnam, bft-1 (67.4%) had the highest rate than bft-2 (18.6%) and bft-3 (14%) [25]. In Asia, especially Japan and Korea, bft-3 rates increased in septicemia and diarrheal cases [11, 34], but this bft-3 isoform had the lowest rate in Europe [9, 35, 36].

Conclusions

The study showed no role for bft gene in diarrhea, given that it was located in both groups of patients and control. Based on the distribution of bft gene isforms were bft-1 showed higher values than bft-2. There is a need for additional studies with a higher number of specimens to elucidate the role of ETBF as a possible cause of diarrhea.

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