Identification of enterotoxigenic *Bacteroides fragilis* in patients with diarrhea: a study targeting 16S rRNA, *gyrB* and *nanH* genes

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Research

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Abstract

Background

We aimed to identify the enterotoxigenic *Bacteroides fragilis* (ETBF) and *bft* subtypes among patients with diarrhea. In addition, we assessed whether DNA gyrase subunit B (*gyrB*) and neuraminidase (*nanH*) genes are useful determinants for identification of *B. fragilis* compared to 16S rDNA sequencing as a reference method.

Results

A total of 111 Gram-negative anaerobic coccobacilli were isolated from 530 fecal specimens using BBE agar. Of the 111 isolates, 100 (90.09%) were assumed to be a member of *Bacteroides fragilis* group as they yielded an amplicon through PCR using the group-specific primers (Bfra-F/g-Bfra-R). However, only 28 isolates out of 100 were definitively identified as species of *Bacteroides* using 16S rRNA gene sequencing; of which 15 isolates were *B. fragilis* and the remaining 13 isolates were identified as *B. thetaiotaomicron* (n=6), *Parabacteroides distasonis* (n=3), *B. vulgatus* (***Phocaeicola vulgatus***) (n=1), *B. ovatus* (n=1), *B. congolensis* (n=1) and *B. nordii* (n=1). Among the 15 isolates of *B. fragilis*, 4 were found to be ETBF. Compared to the reference method, the specificity and accuracy of the PCR targeting *gyrB* gene (64.7% and 65%) was higher than of *nanH* (36.4% and 46%, respectively).

Conclusions

This study demonstrated that more than one-fourth of *B. fragilis* isolates harbored *bft* gene and less than 1% of patients with diarrhea harbored ETBF. The slight agreement between the PCR assays -already used for identification of *B. fragilis* which targeting *gyrB* or *nanH*- and 16S rRNA gene sequencing as the reference method was noted.

Background

The members of *Bacteroides fragilis* group are opportunistic pathogens that cause severe infections such as intra-abdominal, pelvic, and brain abscesses, peritonitis and sepsis [1]. Among *Bacteroides fragilis* group, *B. fragilis* is the most important and virulent species [2]. *B. fragilis* isolates are classified into the non-enterotoxigenic *B. fragilis* (NTBF) and the enterotoxigenic *B. fragilis* (ETBF) strains colonizing the human intestinal tract [3]. NTBF strains can cause extra- intestinal infections such as sepsis, abscesses, and necrotizing skin and soft tissue infections [3]. ETBF strains are found to be associated with acute diarrhea, gastrointestinal tract infections, inflammatory bowel disease (IBD) and recently with colorectal cancer [4, 5]. Of virulence determinants, *B. fragilis* toxin (BFT) or fragilysin is a metalloprotease that encoded by a 6-kb pathogenicity island [6]. *bft* gene has three subtypes which have similar function, but the potency of these subtypes seems to be different as follows: BFT-2 > BFT-1 > BFT-3 [7]. The strains harboring *bft-1* gene are often isolated from adults with diarrhea while the strains carrying *bft-2* are predominantly recovered from children suffered from antibiotic-associated diarrhea [7].

The members of *B. fragilis* group are almost indistinguishable phenotypically [8]. The most clinically relevant species is *B. fragilis* and the resistance to antibiotics is not uncommon among the isolates [9]. Of great concern, *B. fragilis* have the ability to express and transfer antimicrobial resistance genes in human gut microbiota [10]. The selective media such as Bacteroides Bile Esculin agar (BBE) is commonly used for isolation of members of the *B. fragilis* group; however, the overgrowth of enterococci and lactobacilli in such media could be an issue. This issue is partly related to the increasing resistance of enterococci to gentamicin which is frequently used as a supplement in BBE agar [11]. In addition, most phenotypic methods are time-consuming, laborious, couldn't clearly distinguish closely related species and require long incubation time [12]. Also, the automated identification methods, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are not affordable in laboratories with limited resources [8]. Therefore, the correct identification and differentiation of this species using the candidate genetic markers is of prime importance.

Among the different biochemical and molecular methods which were developed for identification of anaerobic bacteria, 16S rDNA sequencing is considered as a reference method for definitive identification [13–15]. This method is robust, accurate and is useful for identification of new and non-culturable microorganisms. Furthermore this method can be used for taxonomic purpose [13] and distinguishing the related species from one another such as *B. fragilis* group species which are phenotypically similar [15]. In spite of benefits of 16S rDNA sequencing, it was not used routinely in laboratory for identification of bacteria because of the high cost of sequencing [13, 15], particularly in the resource limited laboratories. Here, we aimed to identify ETBF and the *bft* subtypes among the isolates recovered from patients with diarrhea. Also we investigated whether PCR assays of 16S rRNA gene-targeted *Bacteroides fragilis* group, DNA gyrase subunit B (*gyrB*) and neuraminidase (*nanH*) genes are useful determinants for identification of *B. fragilis* compared with 16S rRNA gene sequencing as the reference method.

Materials And Methods

Setting

This study was conducted on 530 patients with diarrhea who were admitted to the pediatrics hospital (n=91) or one of two referral hospitals (n=439) in Tehran, Iran, between June 2016 and April 2017. The demographic characteristics (age and gender) of patients were recorded from the medical files. The feces specimens were cultured on BBE agar and incubated at 37 °C for 24-48 hours in an anaerobic atmosphere using the Anoxomat jar system (MART Microbiology B.V., the Netherlands). Gray colonies with dark background and positive for esculin, were supposed to be a member of *B. fragilis* group. These Gram-negative coccobacilli were cultured on Brucella Blood Agar supplemented with 5 mg/l hemin and 10 mg/l vitamin K1 (Sigma, USA). The isolates were stored at -20 °C until further analysis.

16S rRNA gene sequencing

Following DNA extraction, definitive species-level identification was performed by amplification of the 16S rRNA gene fragment using universal primers E8F and E533R primers [16] and followed by

sequencing. The sequences for the 16S rRNA genes were compared with those of the GenBank (<http://www.ncbi.nlm.nih.gov/blast/cgi>), EZ Bio Cloud (<https://www.ezbiocloud.net/identify>) and leBIBI QBPP (<https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi>). A homology level > 97% was considered for species identification.

PCR assays of 16S rRNA gene-targeted *Bacteroides fragilis* group (BFG), *gyrB* and *nanH*

To identify the isolates of BFG, the group-specific primers (Bfra-F/g-Bfra-R) targeting 16S rRNA gene was used [17]. For identification of the most important species of BFG, *B. fragilis*, genespecific primers were used to amplify *gyrB* (gB904F/gB1272R) [18] and *nanH* (GAI 11/GAI 12) genes [19]. *B. fragilis* ATCC 23745 was used as a reference strain. A representative PCR product for each gene was sequenced and the results were compared with those deposited in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/blast/cgi>).

Detection of BFT

B. fragilis toxin (*bft*) gene was detected using Odamaki primers set [20] and followed by PCR in order to identify the subtypes of *bft* gene, using specific primers (Bft-1F/Bft-1R, Bft-2F/Bft-2R, Bft-3F/Bft-3R primers) [21]. For toxicity assay, bacteria were cultured in BHI broth supplemented with 5 mg/l hemin and 10 mg/l vitamin K1 for 48h. The cytotoxicity assay was carried out using HT-29/C1 cell line as described previously by Pantosti *et al.*[22].

Antimicrobial susceptibility tests (AST)

The minimum inhibitory concentrations (MICs) of metronidazole, cefoxitin, moxifloxacin, piperacilline-tazobactam and tetracycline against ETBF isolates using MIC Test Strips (Liofilchem, Italy) were determined and interpreted as recommended by Clinical and Laboratory Standards Institute [23]. The fresh colonies of ETBF with turbidity of 1.0 McFarland standard was inoculated into Brucella blood agar supplemented with 5 mg/l hemin and 10 mg/l vitamin K1. Plates were incubated at 37 °C anaerobically for 24 h and then the MICs were read and recorded.

The antibiotic resistance genes in ETBF isolates such as *nim*, *cfiA*, *cepA*, *ermF* and *tetQ* were detected by PCR using gene-specific primers [24-26].

Nucleotide sequence accession number

The nucleotide sequences of 16S rRNA gene fragment for the all isolates tested were deposited in GenBank as indicated in Table S1. The nucleotide sequences of the representative amplicon of *gyrB* and 16S rRNA for BFG were deposited in GenBank under the accession number MG252858 and MG388289, respectively.

Results

A total of 111 Gram-negative anaerobic coccobacilli were isolated from 530 fecal specimens using BBE agar. Of the 111 isolates, 100 (90.09%) were assumed to be a member of BFG as they yielded an amplicon through PCR using the group-specific primers (Bfra-F/g-Bfra-R). However, only 28 isolates out of 100 were definitively identified as species of *Bacteroides* using 16S rRNA gene sequencing; of which 15 isolates were *B. fragilis* and the remaining 13 isolates were identified as *B. thetaiotaomicron* (n=6), *Parabacteroides distasonis* (n=3), *Bacteroides vulgatus* (*Phocaeicola vulgatus*) (n=1), *B. ovatus* (n=1), *B. congdonensis* (n=1) and *B. nordii* (n=1). According to Figure 1, there were 13 non-*B. fragilis* (Figure 1). Considering the 16S rRNA gene sequencing as the reference method, the specificity of PCR assay of 16S rRNA gene-targeted BFG was 11.9% (95% CI: 5.86-20.8).

Of these 100 isolates, 77 produced amplicons using PCR primers targeting *gyrB* or *nanH* genes. As shown in the Venn diagram (Figure 2); a subset of 32 isolates generated amplicons using the primer sets for *gyrB* as well as *nanH*. A subset of eight isolates was exclusively positive for *gyrB* and was missed by the primer targeting *nanH*. A subset of 37 isolates was only *nanH*-positive. Overall, 40 isolates yielded an amplicon using primers targeting *gyrB* gene while 69 were positive for *nanH*. Compared to the reference method, the specificity and accuracy of the PCR targeting *gyrB* gene was higher than of *nanH* (Table 1).

Table 1
Specificity, accuracy and agreement of PCRs targeting *gyrB* or *nanH* for identifying *B. fragilis* compared to 16S rRNA gene sequencing as the reference method.

Gene	16S rRNA gene sequencing		Specificity % (95% CI)	Accuracy % (95% CI)	Agreement %
	No. of <i>B. fragilis</i> identified				
	Positive	Negative			
gyrB	10	30	64.7 (53.5-74.7)	65 (54.8-74.2)	65
Positive					
Negative	5	55			
nanH	15	54	36.4 (26.2-47.6)	46 (35.9-56.2)	46
Positive					
Negative	0	31			

PCR amplification of *bft* gene in the 15 isolates that definitely identified *B. fragilis* by 16S rRNA gene sequencing showed that 4 isolates contained BFT. In other words, 26.6% of the *B. fragilis* isolates were ETBF (one from an adult and three from children) and the remaining isolates were classified as NTBF. All ETBF isolates were positive for *gyrB* as well as *nanH*. The ETBF isolates harbored *bft-1* and *bft-2* subtypes: three were *bft-1* positive (one from an adult and two from children) and only one was *bft-2* positive from a child). All of the isolates which were toxin positive with PCR showed cytopathic effect in cell culture assay. The properties of the 100 isolates in relation to the presence of *nanH* and *gyrB* genes

are shown in Table S1. The antibiotic susceptibility profile of ETBF and their antibiotic resistance genes were shown in Table 2.

Table 2
The MIC of antibiotics tested against ETBF and the antibiotic resistance genes.

Isolate	MIC (µg/ml)				<i>nim/ cfiA/cepA/ermf/tetQ</i>
	Cefoxitin	Metronidazole	Moxifloxacin	Tetracycline	
BF100	1.5	0.125	0.094	12	+/+/+/+/+
BF122	1.5	0.094	0.19	2	-/-/-/+/+
BF165	8	0.25	0.19	6	+/-/+/+/+
BF194	≥ 256	≥ 256	0.38	24	+/-/+/+/+

Discussion

There is a dearth of data on distribution of ETBFs in patients with diarrhea in Iran- the second largest country in the Middle East. Accordingly, we conducted the current study to assess the distribution of ETBF in children and adults who suffered from diarrhea. In the present study, we found that more than one-fourth of *B. fragilis* isolates harbored *bft* gene and altered HT-29/C1 morphology. The sequence of *bft* gene was identical to those deposited at the GenBank. Of four ETBF isolates, three were recovered from children and one from an adult. In the current study, three ETBF isolates harbored *bft-1* and only one isolate was positive for *bft-2*. All but one of ETBF isolates tested were susceptible to cefoxitin, metronidazole, moxifloxacin and tetracycline. Previous studies pointed to the association between ETBF and diarrhea, particularly in children. In addition, *bft-1* is identified as the most prevalent subtypes of *bft* in patients with diarrhea [27–29]. Due to the small number of ETBF tested in this study, we could not reach a clear conclusion about the distribution of *bft* subtypes in children and adults as demonstrated previously by others [30, 31]. Another finding of this study is that the majority of *B. fragilis* isolates obtained from patients with diarrhea did not harbor the *bft* gene. A possible explanation for this finding is that the virulence factors other than BFT might be implicated in pathogenesis of *B. fragilis* or these isolates might be found as a part of microbiota in these patients. In other words, the diarrhea causes might not be associated with the *B. fragilis* and its etiology remains unknown. Narimani *et al.* (2016) demonstrated that none of the *B. fragilis* isolates harbored *bft* gene as their isolates were obtained from healthy individuals in Tehran, Iran [32]. In a study recently conducted in the north-west of Iran, four ETBF isolates were recovered from diarrheal fecal samples of the 100 outpatient and hospitalized children under the age of 5 years [33]. A study by Zamani *et al.* (2017) conducted in Tehran and aimed to provide evidence of association between *B. fragilis* and ulcerative colitis (UC) and non-inflammatory bowel disease (nIBD). They detected *bft* genes in 51.4% and 1.6% of UC and nIBD samples, respectively: all *bft* positive isolates harbored the *bft-1* [34]. Another study was conducted by Rashidan *et al.* (2018) in Tehran and they isolated *B. fragilis* group strains from biopsy specimens of IBD (38%) and nIBD (25%) cases.

ETBF was isolated from 6/19 IBD (31.5%) and 2/5 nIBD (40%) cases [35]. Several studies showed that the distribution of ETBF was uneven in various regions around the world. For instance; Ramamurthy *et al.* (2013) isolated 7.2% ETBF from < 5 years children (32 of 446 diarrheal cases and 31 of 428 controls; 7.2% for each group) from Kolkata, India, and the isolates carried *bft-1* and *bft-3* [28]. Akpinar *et al.* (2010) isolated 15% ETBF from 200 patients with diarrhea and the isolates were positive for *bft-1* and *bft-2* [29]. Merino *et al.* (2011) identified 9.1% *bft* gene (*bft-1* and *bft-3* subtypes) in children < 10 years with diarrhea [27]. Obuch-Woszczatyński *et al.* (2004) identified 15% and 13% ETBF among the Dutch and the Polish strains, respectively.

The usefulness of conserved genes for molecular identification has been highlighted in previous studies [18, 19, 36], proposing the genetic identification as the gold standard for identification of anaerobic bacteria [37, 38]. 16S rRNA gene sequencing is the reliable method to be used for definitive species-level identification of anaerobic bacteria. However, this method is not at reasonable turnaround time and prices to be used routinely by microbiology laboratories for identification of various species. Hence, we sought the performance of the PCR assays which were previously applied for identification of BFG or the most virulent species, *B. fragilis*. Of them, the PCR assay of 16S rRNA gene-targeted BFG was 32.7% in agreement with 16S rRNA gene sequencing. In other words, this PCR-based method was able to identify only 26 of isolates which belong to BFG according to 16S rRNA gene sequencing and 74 isolates which are not a member of BFG yielded an amplicon by this PCR. We found that this PCR is not good enough to detect members of BFG as the sequencing data obtained in the current study was in slight agreement to PCR assay of 16S rRNA gene-targeted BFG.

Of various candidate genes for identifying *B. fragilis*, we selected *nanH* and *gyrB* as they were found to be potentially reliable gene for the identification and discrimination of *B. fragilis* from other species belonging to *B. fragilis* group. *nanH* gene encodes the neuraminidase that is an enzyme produced by all *B. fragilis* strains and enhances their pathogenicity [19]. We found that the all the isolates which were identified as *B. fragilis*, yielded an amplicon using primers targeting *nanH* genes. However, there were 54 isolates of non-*B. fragilis* either other species of *Bacteroides* or other genera produced amplicons by primers targeting *nanH*. This PCR was also in slight agreement to 16S rRNA gene sequencing and had low specificity to identify *B. fragilis*. Jotwani *et al.* (1995) and also Kuwahara *et al.* (1996) used *nanH* gene for identification of *B. fragilis* and demonstrated that *nanH* could specifically identify *B. fragilis* [19, 36]. Of 45 different species studied by Jotwani *et al.* (1995) using the primers targeting *nanH*, only *B. fragilis* and *Bacteroides merdae* ATCC 43184 yielded an amplicons. All the other species such as *Bacteroides vulgatus* ATCC 29327 and *Bacteroides thetaiotaomicron* ATCC 2974 were negative for *nanH* [19]. The other candidate gene is *gyrB* which encodes the β -subunit of DNA gyrase and it also seems to be a specific gene for *B. fragilis* and able to differentiate this species from other *Bacteroides* [18]. We noted that the PCR assay targeting *gyrB* was 65% in agreement to 16S rRNA gene sequencing which was higher than that of *nanH*. The specificity of this PCR was also higher than that of *nanH*. Lee *et al.* (2010) used the specific primers which could discriminate *B. fragilis* from *Bacteroides ovatus* and *Prevotella melaninogenica* [18]. The major discrepancy between genes in identification of *B. fragilis* might be partly

related to high level of sequence conservation of *nanH* [39]. Of genes examined here, *gyrB* is more specific than *nanH* for identifying *B. fragilis*.

Conclusion

We showed that less than 1% of patients with diarrhea harbored ETBF. Here, we noted the slight agreement between PCR-based methods for identification of *B. fragilis* and 16S rRNA gene sequencing as the reference method.

Declarations

Ethics approval and consent to participate

The study protocol was in accordance with ethic guidelines of Tehran University of Medical Sciences (TUMS) and the study was approved by the review board at TUMS, Tehran, Iran.

Consent for publication

Not applicable.

Availability of data and material

Data sharing is not applicable to this article.

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Authors' contributions

RA performed the microbiologic and molecular experiments and wrote the manuscript. ZN participated in the cell culture cytotoxicity assay. NK performed the bioinformatic analysis. SG performed the experiments. MY performed the statistical analysis of data. AB was clinical coordinator. Besides revising the manuscript, MD designed, coordinated, and supported this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Diagram of the culture method and the PCR assays of 16S rRNA gene-targeted BFG, *gyrB* or *nanH* for identification of *B. fragilis* in fecal specimens from patients with diarrhea.

Figure 2

Venn diagram illustrating the frequency of unique (N= 8 for gyrB and N= 37 for nanH) and shared (N= 32) positive results for gyrB and nanH among the isolates tested.