Iraqi Journal of Science

ISSN: 0067-2904

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 diarrheal and 22 (75.9%) in non-diarrheal subjects, while that of *bft* gene was only 3 (3.1%) including 2 (2.9%) in diarrheal and 1 (3.4%) in non-diarrheal 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.

انتشار وجود ذيفان لعزلات *Bacteroides fragilis* في عينات الخروج المجموعة من الاطفال تحت سن الخامسة من العمر في العراق

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الخلاصة

على الرغم من أن *B. fragilis* عبارة عن بكتيريا موجودة داخل ميكروبات الأمعاء ، إلا أن سلالة بكتريا *B. fragilis* المنتجة للسموم مرتبطة بالإسهال لدى الأطفال الذين تقل أعمارهم عن 5 سنوات. تتضمن هذه الدراسة 98 عينة من البراز تم جمعها من الأطفال أقل من 5 سنوات. تم استخراج الحمض النووي مباشرة من عينات البراز وتوجيهه إلى PCR التقليدية من خلال استهداف جين بيتا أيزوبروبيلمات ديهيدروجينيز (*leuB*) للكشف عن *B. fragilis* و *bft* للكشف عن ETBF. أظهرت النتيجة أن معدل انتشار المرض كان 78 (79.6 ٪) بما في ذلك 56 (81.2 ٪)) في الإسهال و 22 (75.9 ٪) في غير الإسهال للجين *leuB* ، في حين أن الجين *bft* كانت فقط 3 (3.1 ٪) بما في ذلك 2 (2.9 ٪) في الإسهال و 1 (3.4 ٪) في غير الإسهال. بناءً على تسلسل العينات الموجبة من *bft* ، تم توضيح أن *bft*–1 و *bft*–2 يمثلان في عينات الإسهال ولكن *bft*–1 الموجودة في عينات السيطرة. في الختام ، سجلت هذه الدراسة الجين *bft* في عينة من

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الأطفال العراقيين المصابين بالإسهال لأول مرة ولم تظهر أي فروق ذات دلالة إحصائية بين مجموعات الإسهال والسيطرة في كلا الجينات.

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-proprotein 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, HEXXHXXGXXH [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	GGCTACTGGCTATGCGTAAA	440 bp	NCBI
R:leuB	CTCCGTCACCATCACATCAA		
F: bft	CGCGGCATTATTAGCTGCATGTTCTAATG	992 bp	[21]
R: bft	GATACATCAGCTGGGTTGTAGACATCCCA		

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	40 Cycles	94 ºC	0:45
	Annealing		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	40 Cycles	94 ºC	1:00
	Annealing		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	1>year	
Diarrhea	Count	49	20	69
	% within Diarrhea	71.0%	29.0%	100.0%
Control	Count	21	8	29
	% within Control	72.4%	27.6%	100.0%
Total	Count	70	28	98
	% 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	Count	56	13	69
	% within Diarrhea	81.2%	18.8%	100.0%
Control	Count	22	7	29
	% within Control	75.9%	24.1%	100.0%
Total	Count	78	20	98
	% within both	79.6%	20.4%	100.0%

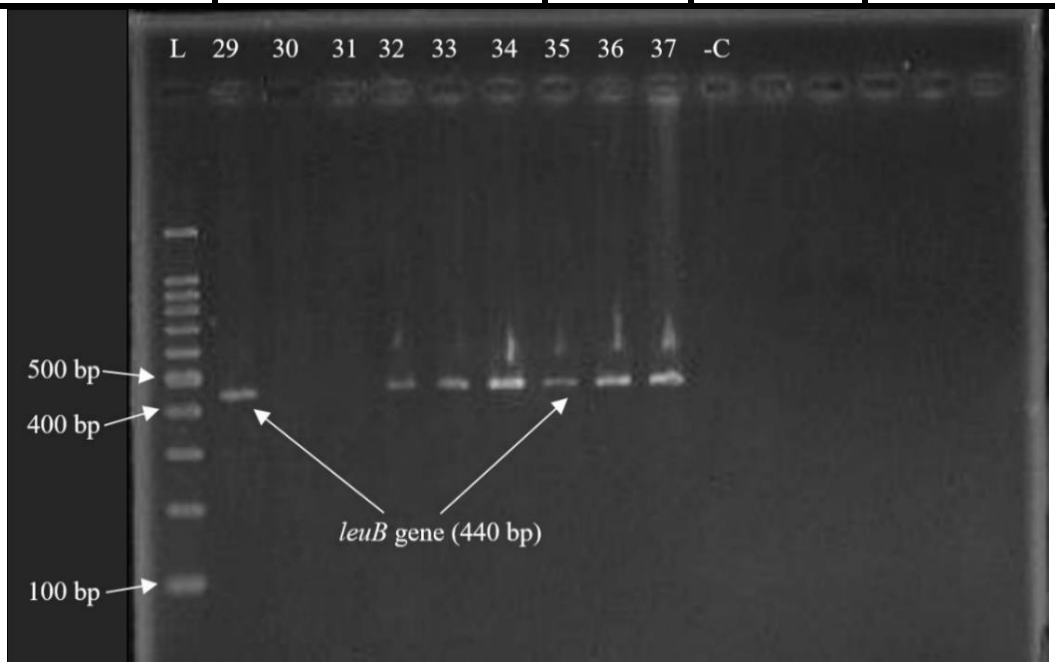

Figure 1- Gel electrophoresis of amplified *leuB* gene (440 bp) from *B. fragilis* using conventional PCR. Agarose 2 %, 70 V/cm for 75 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane L: 100 bp DNA ladder. Lanes 1-9: Amplicons *leuB* gene for *B. fragilis* (Specimens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

		bft gene		Total
		Present	Absent	
Diarrhea	Count	2	67	69
	% within Diarrhea	2.9%	97.1%	100.0%
Control	Count	1	28	29
	% within Control	3.4%	96.6%	100.0%
Total	Count	3	95	98
	% within both	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.

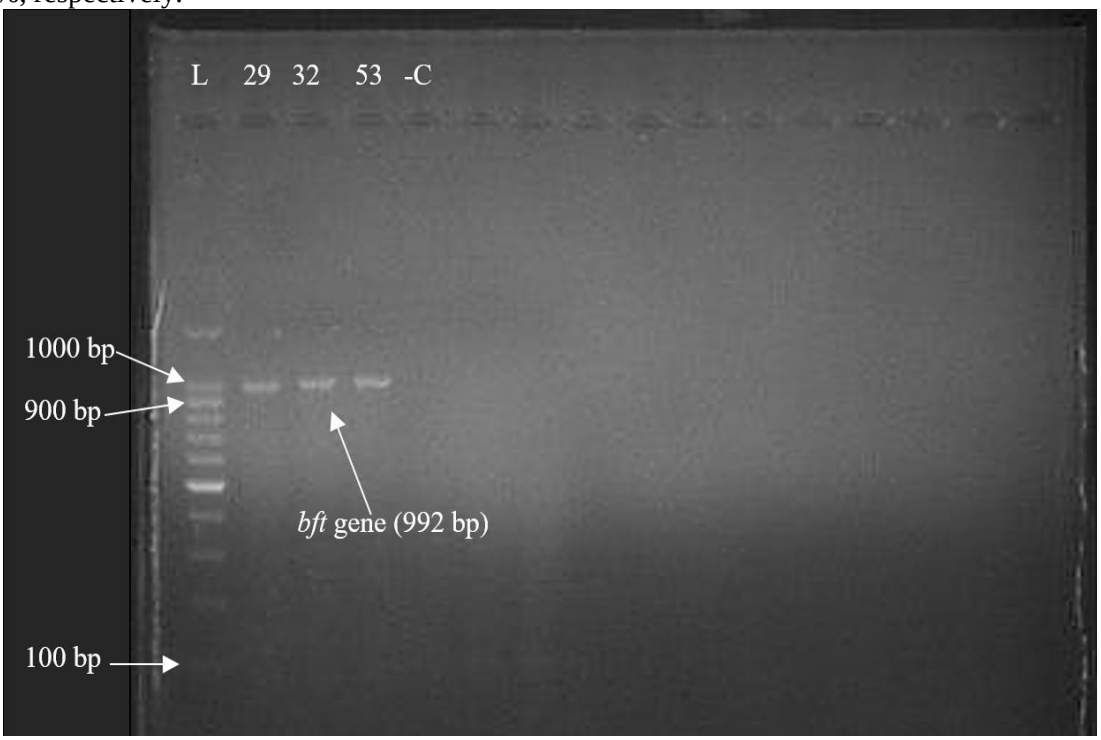

Figure 2- Gel electrophoresis of amplified *bft* gene (992 bp) from ETBF using conventional PCR. Agarose 1 %, 70 V/cm for 75 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane L: 100 bp DNA ladder. Lanes 1-3: Amplicons *bft* gene for ETBF (Specimens 29, 32, and 53). 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, Sãu 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.

References

1. Sears, C. L., Geis, A. L. and Housseau, F. **2014**. Bacteroides fragilis subverts mucosal biology: from symbiont to colon carcinogenesis. *The Journal of clinical investigation*, **124**(10): 4166-4172.
2. Kowal, M. T. **2017**. Characterisation of proteins secreted in the outer membrane vesicles of Bacteroides fragilis.
3. Wexler, H. M. **2007**. Bacteroides: the good, the bad, and the nitty-gritty. *Clinical microbiology reviews*, **20**(4): 593-621.
4. Sherwood, J. E., Fraser, S., Citron, D. M., Wexler, H., Blakely, G., Jobling, K. and Patrick, S. **2011**. Multi-drug resistant Bacteroides fragilis recovered from blood and severe leg wounds caused by an improvised explosive device (IED) in Afghanistan. *Anaerobe*, **17**(4): 152-155.
5. Meggersee, R. and Abratt, V. **2015**. The occurrence of antibiotic resistance genes in drug resistant Bacteroides fragilis isolates from Groote Schuur Hospital, South Africa. *Anaerobe*, **32**: 1-6.
6. Wick, E. C. and Sears, C. L. **2010**. Bacteroides spp. and diarrhea. *Current opinion in infectious diseases*, **23**(5): 470.
7. Franco, A. A., Cheng, R. K., Chung, G. T., Wu, S., Oh, H. B. and Sears, C. L. **1999**. Molecular evolution of the pathogenicity island of enterotoxigenic Bacteroides fragilis strains. *Journal of bacteriology*, **181**(21): 6623-6633.
8. Fathi, P. and Wu, S. **2016**. Suppl-1, M3: Isolation, Detection, and Characterization of Enterotoxigenic Bacteroides fragilis in Clinical Specimens. *The open microbiology journal*, **10**: 57.
9. Sears, C. L. **2009**. Enterotoxigenic Bacteroides fragilis: a rogue among symbiotes. *Clinical microbiology reviews*, **22**(2): 349-369.
10. Remacle, A. G., Shiryaev, S. A. and Strongin, A. Y. **2014**. Distinct interactions with cellular E-cadherin of the two virulent metalloproteinases encoded by a Bacteroides fragilis pathogenicity island. *PLoS One*, **9**(11): e113896.
11. Chung, G. T., Franco, A. A., Wu, S., Rhie, G. E., Cheng, R., Oh, H. B. and Sears, C. L. **1999**. Identification of a third metalloprotease toxin gene in extraintestinal isolates of Bacteroides fragilis. *Infection and immunity*, **67**(9): 4945-4949.
12. Mootien, S. and Kaplan, P. M. **2017**. Monoclonal antibodies specific for Bacteroides fragilis enterotoxins *BFT*1 and *BFT*2 and their use in immunoassays. *PloS one*, **12**(3): e0173128.
13. Goulas, T., Arolas, J. L. and Gomis-Rüth, F. X. **2011**. Structure, function and latency regulation of a bacterial enterotoxin potentially derived from a mammalian adamalysin/ADAM xenolog. *Proceedings of the National Academy of Sciences*, **108**(5): 1856-1861.
14. Shiryaev, S. A., Aleshin, A. E., Muranaka, N., Kukreja, M., Routenberg, D. A., Remacle, A. G. and Strongin, A. Y. **2014**. Structural and functional diversity of metalloproteinases encoded by the Bacteroides fragilis pathogenicity island. *The FEBS journal*, **281**(11): 2487-2502.

15. Myers, L. L., Shoop, D. S., Stackhouse, L. L., Newman, F. S., Flaherty, R. J., Letson, G. W. and Sack, R. B. **1987**. Isolation of enterotoxigenic Bacteroides fragilis from humans with diarrhea. *Journal of clinical microbiology*, **25**(12): 2330-2333.
16. Sack, R. B., Myers, L. L., Almeido-Hill, J., Shoop, D. S., Bradbury, W. C., Reid, R. and Santosham, M. **1992**. Enterotoxigenic Bacteroides fragilis: epidemiologic studies of its role as a human diarrhoeal pathogen. *Journal of diarrhoeal diseases research*, 4-9.
17. Sack, R. B., Albert, M. J., Alam, K., Neogi, P. K. and Akbar, M. S. **1994**. Isolation of enterotoxigenic Bacteroides fragilis from Bangladeshi children with diarrhea: a controlled study. *Journal of Clinical Microbiology*, **32**(4): 960-963.
18. Durmaz, B., Dalgalar, M. and Durmaz, R. **2005**. Prevalence of enterotoxigenic Bacteroides fragilis in patients with diarrhea: a controlled study. *Anaerobe*, **11**(6): 318-321.
19. Sears, C. L., Islam, S., Saha, A., Arjumand, M., Alam, N. H., Faruque, A. S. G. and Sack, R. B. **2008**. Association of enterotoxigenic Bacteroides fragilis infection with inflammatory diarrhea. *Clinical Infectious Diseases*, **47**(6): 797-803.
20. Shen, H., Zhang, J., Li, Y., Xie, S., Jiang, Y., Wu, Y. and Hu, Q. **2016**. The 12 gastrointestinal pathogens spectrum of acute infectious diarrhea in a sentinel hospital, Shenzhen, China. *Frontiers in microbiology*, **7**: 1926.
21. Franco, A. A., Buckwold, S. L., Shin, J. W., Ascon, M. and Sears, C. L. **2005**. Mutation of the zinc-binding metalloprotease motif affects Bacteroides fragilis toxin activity but does not affect propeptide processing. *Infection and immunity*, **73**(8): 5273-5277.
22. Ji, D. D., Huang, I. H., Lai, C. C., Wu, F. T., Jiang, D. D. S., Hsu, B. M. and Lin, W. C. **2017**. Prevalence and characterization of enterotoxigenic Bacteroides fragilis and toxigenic Clostridium difficile in a Taipei emergency department. *Journal of Microbiology, Immunology and Infection*, **50**(1): 83-89.
23. Ignacio, A., Fernandes, M. R., Avila-Campos, M. J. and Nakano, V. **2015**. Enterotoxigenic and non-enterotoxigenic Bacteroides fragilis from fecal microbiota of children. *Brazilian Journal of Microbiology*, **46**(4): 1141-1145.
24. Albert, M. J., Faruque, A. S. G., Faruque, S. M., Sack, R. B. and Mahalanabis, D. **1999**. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *Journal of clinical microbiology*, **37**(11): 3458-3464.
25. Nguyen, T. V., Le Van, P., Le Huy, C. and Weintraub, A. **2005**. Diarrhea caused by enterotoxigenic Bacteroides fragilis in children less than 5 years of age in Hanoi, Vietnam. *Anaerobe*, **11**(1-2): 109-114.
26. Akpınar, M., Aktaş, E., Cömert, F., Külah, C. and Sümbüloğlu, V. **2010**. Evaluation of the prevalence of enterotoxigenic Bacteroides fragilis and the distribution *bft* gene subtypes in patients with diarrhea. *Anaerobe*, **16**(5): 505-509.
27. Akhi, M. T., Seifi, S. J., Asgharzadeh, M., Rezaee, M. A., Oskuei, S. A., Pirzadeh, T. and Sofla, H. S. Y. **2016**. Role of Enterotoxigenic Bacteroides fragilis in Children Less Than 5 Years of Age With Diarrhea in Tabriz, Iran. *Jundishapur journal of microbiology*, **9**(6).
28. Ramamurthy, D., Pazhani, G. P., Sarkar, A., Nandy, R. K., Rajendran, K., Sur, D.and Ramamurthy, T. **2013**. Case-control study on the role of enterotoxigenic Bacteroides fragilis as a cause of diarrhea among children in Kolkata, India. *PloS one*, **8**(4): e60622.
29. Krzyzanowsky, F., and Avila-Campos, M. J. **2003**. Detection of non-enterotoxigenic and enterotoxigenic Bacteroides fragilis in stool specimens from children in São Paulo, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, **45**(4): 225-227.
30. Pantosti, A., Menozzi, M. G., Frate, A., Sanfilippo, L., D'Ambrosio, F. and Malpeli, M. **1997**. Detection of enterotoxigenic Bacteroides fragilis and its toxin in stool specimens from adults and children in Italy. *Clinical infectious diseases*, **24**(1): 12-16.
31. San Joaquin, V. H., Griffis, J. C., Lee, C. and Sears, C. L. **1995**. Association of Bacteroides fragilis with childhood diarrhea. *Scandinavian journal of infectious diseases*, **27**(3): 211-215.
32. Pathela, P., Hasan, K. Z., Roy, E., Alam, K., Huq, F., Siddique, A. K. and Sack, R. B. **2005**. Enterotoxigenic Bacteroides fragilis–associated diarrhea in children 0–2 years of age in rural Bangladesh. *The Journal of infectious diseases*, **191**(8): 1245-1252.

33. Merino, V. R. C., Nakano, V., Liu, C., Song, Y., Finegold, S. M. and Avila-Campos, M. J. **2011**. Quantitative detection of enterotoxigenic Bacteroides fragilis subtypes isolated from children with and without diarrhea. *Journal of clinical microbiology*, **49**(1): 416-418.

34. Kato, N., Liu, C. X., Kato, H., Watanabe, K., Tanaka, Y., Yamamoto, T. and Ueno, K. **2000**. A new subtype of the metalloprotease toxin gene and the incidence of the three *bft* subtypes among Bacteroides fragilis isolates in Japan. *FEMS microbiology letters*, **182**(1): 171-176.

35. d'Abusco, A. S., Del Grosso, M., Censini, S., Covacci, A. and Pantosti, A. **2000**. The alleles of the *bft* gene are distributed differently among enterotoxigenic Bacteroides fragilis strains from human sources and can be present in double copies. *Journal of clinical microbiology*, **38**(2): 607-612.

36. Ulger, N., Rajendram, D., Yagci, A., Gharbia, S., Shah, H. N., Gulluoglu, B. M. and Soyletir, G. **2006**. The distribution of the *bft* alleles among enterotoxigenic Bacteroides fragilis strains from stool specimens and extraintestinal sites. *Anaerobe*, **12**(2): 71-74.