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RESEARCH ARTICLE
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Molecular identification of enterotoxigenic Bacteroides fragilis and the Distribution 16S rRNA gene, bft gene subtypes and gyrb gene in Iraqi patients with diarrhea

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ABSTRACT

This study includes 50 stool: 21 patients with diarrhea and 29 healthy control samples were collected from different hospitals in Baghdad city (Baghdad teaching hospital, AL-yarmok teaching hospital and Pediatric teaching Hospital at the beginning of March/2020 to the end April/2021 . DNA was extracted directly from stool specimens and conventional PCR targeting 16S rRNA gene gyrb gene used for B.fragilis detection, Bacteroides fragilis toxin (bft) gene used for detection of ETBF. The results showed that presence of 16S rRNA gene was detected in 17(32%) stool samples included 7(41.17%) of patients and 10 (58.8%) of control group . Presence of gyrb gene was detected in 9(52.9%) stool samples included 4 (44.4%)of patients and 5 (55.5%) of control group: the incidence of bft gene in 4(23.5%) stool samples included : 3 of patients and 1 control .bft2 and bft3 were found in 1 and 2 samples from 3 diarrheic patients with ETBF respectively.bft1 present in one sample in control group.

Keywords: Molecular, Gene, Diarrhea

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INTRODUCTION

Bacteroides fragilis is a ubiquitous member of the human gut microbiota. It belongs to the phylum Bacteroidetes, which together with the phylum Firmicutes constitutes about 80% of the total human gut microbiota [1, 2]. Bacteriod fragilis can be classified into two subtypes based on their pathogenic potential 1) non-enterotoxigenic strains that do not encode the B.fragilis toxin, and 2) enterotoxigenic B.fragilis strains that have the bft genes that encode the toxin [3]. BFT is among the most studied virulence factors of B. fragilis and current evidence suggests that this toxin may be a driver for chronic colitis and colorectal cancer [4]. BFT is a 397 amino acid pre-proprotein which is eventually secreted as a 20 kDa mature protein [5, 6]. BFT exists in three isoforms, BFT 1–3, with BFT-2 having the greatest potential to elicit tissue damage. Among isolates from humans, BFT-1 is the most common toxin variant, while the BFT-3 has a geographical propensity for Southeast Asia [7,8]. Enterotoxigenic B. fragilis (ETBF) were discovered in the 1980s and were found to be associated with diarrhea in lambs, calves, pigs, foals, and humans and also found to be present in sewage waters [9]. Conventional PCR assays detecting BFT have been successful in determining the specific isotype of toxin present in culture by attempting to identify the presence of bft gene. Detection of the bft gene without characterization of the toxin isotype has been described using boiled bacterial DNA obtained from cultured isolates [10].

Aim of study

The present work aimed for direct detection of B.fragilis bacteria from stool samples with the screening of,gyrb ,bft,bft1,bft2 and bft3 genes using PCR technique.

MATERIAL AND METHOD

Collection of Samples

In this study 50stool sample including 21 patients with diarrhea and 29 healthy control were collected from different hospitals in Baghdad city: Baghdad teaching hospital, AL-yarmok teaching hospital and Paediatric teaching Hospital from the beginning of March/2020 to the end April/2021 . Stool samples were examined microscopically if contained pus cell, red blood cell or any parasite.

Detection of B.fragilis by molecular methods

DNA extraction

DNA extracted directly from 50 stool samples according to the protocol of QIAamp® Fast DNA Stool Mini Extraction Kit, Qiagen company .Detection of DNA bands using Agarose gel electrophoresis (1.5%). DNA was Stored at -80 °C until use.

Primers

Primers used in this study are listed in Table(1). 16S rRNA gene and gyrB gene for the identification and discrimination of B.fragilis from other species belonging to B.fragilis group ,bft gene and subtypes (bft-1, bft-2, bft-3) used for detection of ETBF.

The mixture of PCR was prepared as follows; 10 µl Go Taq® Green Master Mix supplied by (Promega Company), 1 µl Forward Primer (10 pmol), 1 µl Reverse Primer (10 pmol), 4 µl DNA template, and 4 µl nuclease-free water to complete the volume up to 20 µl.PCR conditions illustrated in Table-2. used for 16S rRNA,gyrb,bft,bft1,bft2 and bft3 genes.

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TABLE 1: primers used in the study

Primers		Primer sequence	PCR Product	References
16S rRNA	F	TCRGGAAGAAAGCTTGCT	162	[11]
	R	CATCCTTTACCGGAATCCT		
bf -904	F	GGCGGTCTTCCGGGTAAA	55	[12]
bf -958	R	CACACTTCTGCGGGTCTTTGT		
bft	F	GACGGTATGTGATTTGTCTGAGAGA	294	[13]
	R	ATCCCTAAGATTTTATCCCAAGTA		
Bft-1	F	GGGATGTCCTGGT TCA	142	[14]
	R	AATTATCCGTATGCTCAGCG		
Bft-2	F	CTTAGGCATATCTTGGCTTG	219	
	R	GCGATTCTATACATGTTCTC		
Bft-3	F	TTTGGGCATATCTTGGCTCA	145	
	R	ATCATCCGCATGGTTAGCA		

F (forward), R (Reverse), bp (base pair).

TABLE 2: Polymerase Chain conditions used in this study

NO	steps	Temperature(^{0}C)	Time	Number of cycle
1	Initial denaturation	95	5 min	1
2	Denaturation	95	30sec	30
3	Annealing	a-56 b-60 c-52 d-58	30sec 30sec 30sec 30sec	
4	Extension	72	30sec	
5	Final extension	72	7min	1

A= Annealing temperature for 16S rRNA gene,b = Annealing temperature for gyrb gene,c= Annealing temperature for bft gene, d= Annealing temperature for bft-1, bft-2 and bft-3 genes.

RESULTS AND DISCUSSION

Molecular method

Molecular Detection of 16S rRNA gene in stool sample

PCR technique was used to detect housekeeping genes of B.fragilis in 50stool samples and began with the DNA extraction as a first step using specific primers for the gene responsible for determining the B.fragilis (16SrRNA). Results exhibited that presence of 16S rRNA gene with an amplified size(162 bp) as shown in fig (1) . 17(32%) in stool samples,10 in diarrheal patients and 7 in control . The results of the current study were in contrast to the results of study done by [15] that claimed all 84 fecal samples tested positive for the 16S rRNA gene. Moreover, current study were in line with study[16]who mentioned that the detection and sequencing of this gene is an effective means for the identification of of B.fragilis from clinical samples [16]. 16SrRNA gene is one of the structural RNA components of the ribosome, could be used to identify Bacteriodes to the genus level.The 16S rRNA gene has highly conserved region of 16S rRNA gene in all prokaryotic organisims. Many studies used 16S rRNA gene for further confirmation of most bacteria, such as E.coli , P.auroginosa and C .freundii in clinical samples[17,18,19]

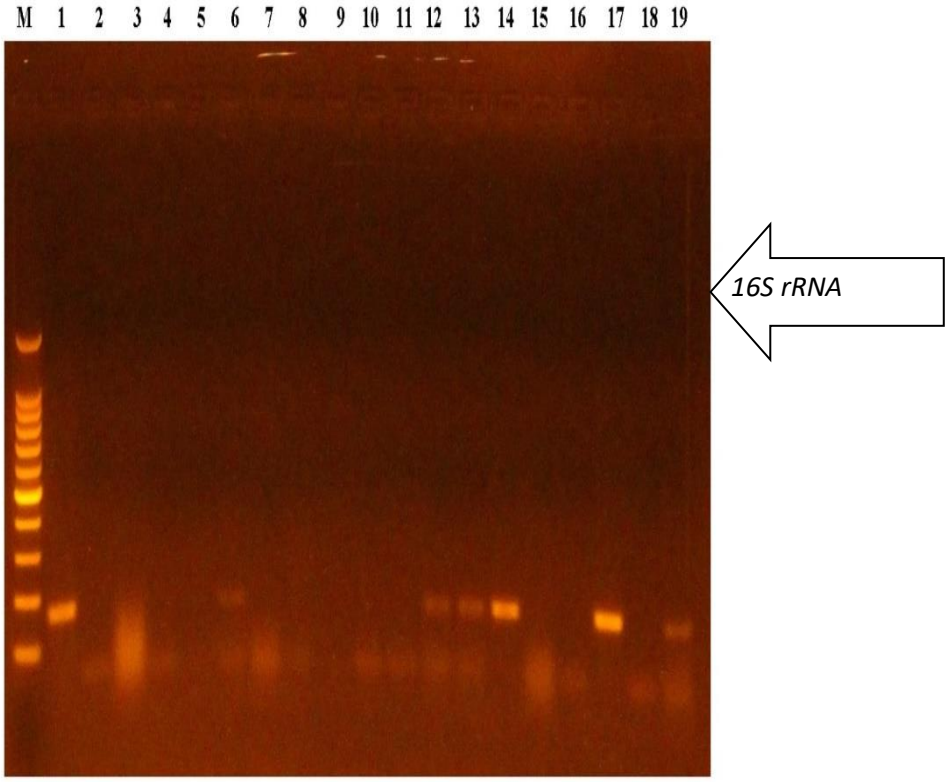

FIGURE 1: Gel electrophoresis of amplified 16S rRNA housekeeping gene (162bp), B. fragilis isolates using conventional PCR. Agarose 1.5 %, 100 V for 60 minutes stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder.Lanes:1,12,13,14,17, 19 showed positive results.Lanes2,3,4,5,6,7,8,9,10,11,15,16,18 showed negative results.

Molecular Detection of gyrB gene in stool sample

The gyrB gene was tested in the 17 stool samples that had positive 16S rRNA gene results. As shown in Fig2, the findings showed that 9(52.9%) samples of B.fragilis tested positive for the gyrB gene (55 bp) (table2). 4 samples in patients and 5 samples in control group. No local research has been done to our knowledge to screen for and detect this gene. gyrB gene 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 [12]. The result of present study showed that rate of gyrB gene was slightly higher than what was observed in the results of the study by[16].

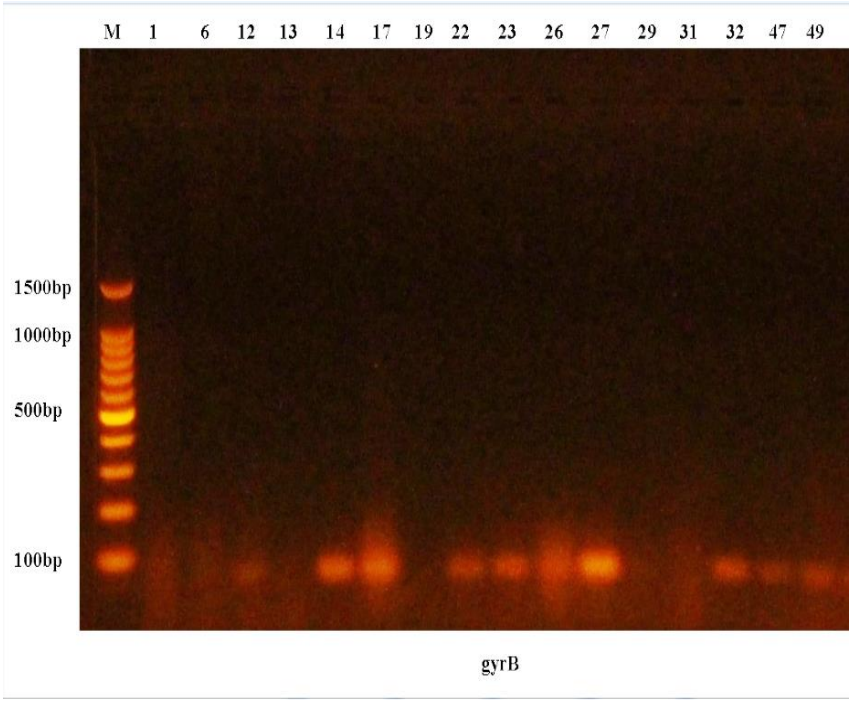

FIGURE 2 :Gel electrophoresis of amplified gyrb gene (55bp) from ETBF using conventional PCR. Agarose 1.5 %, 100 V/cm for 60 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder. Lanes:,14,17,22,23,26,27,32,47,49 showed positive result .Lanes:1,6,15,12,13,31 showed negative result .

Molecular Detection of bft gene in stool sample

Bft gene was used for ETBF detection, the bft gene was tested in the 17 stool samples that had positive results for 16S rRNA gene results.

Results denounced that 4(23.5%) of B.fragilis were positive for bft gene called Enterotoxigenic B.fragilis as shown in fig (3) including: 3(17.6%) in patient with diarrhea and 1(5.9%) in healthy control. The remaining of the 14 stool samples that showed negative results for bft gene called Non Enterotoxigenic B.fragilis(NTBF).All 4 isolates of ETBF were positive for gyrb gene. The results of the current study agree with that the study in Iraq by [20] they used bft gene for the molecular identification of Enterotoxigenic B.fragilis from direct stool samples. The findings of current study are closer to the findings of another researches belong to several authors in worldwide, Ignacio et al.,(2015) showed that bft gene rate were 4(4.7%) from 84 fecal samples [15]and also with the study by Amiri et al.,(2022) found that bft gene rate in 4 (26.6%) isolates of ETBF from 513 stool samples[16]. Current study conflict with study by [21] revealed that the presence of bft gene in 29 (15%) of the patients with diarrhea and 27 (14%) of the control group.

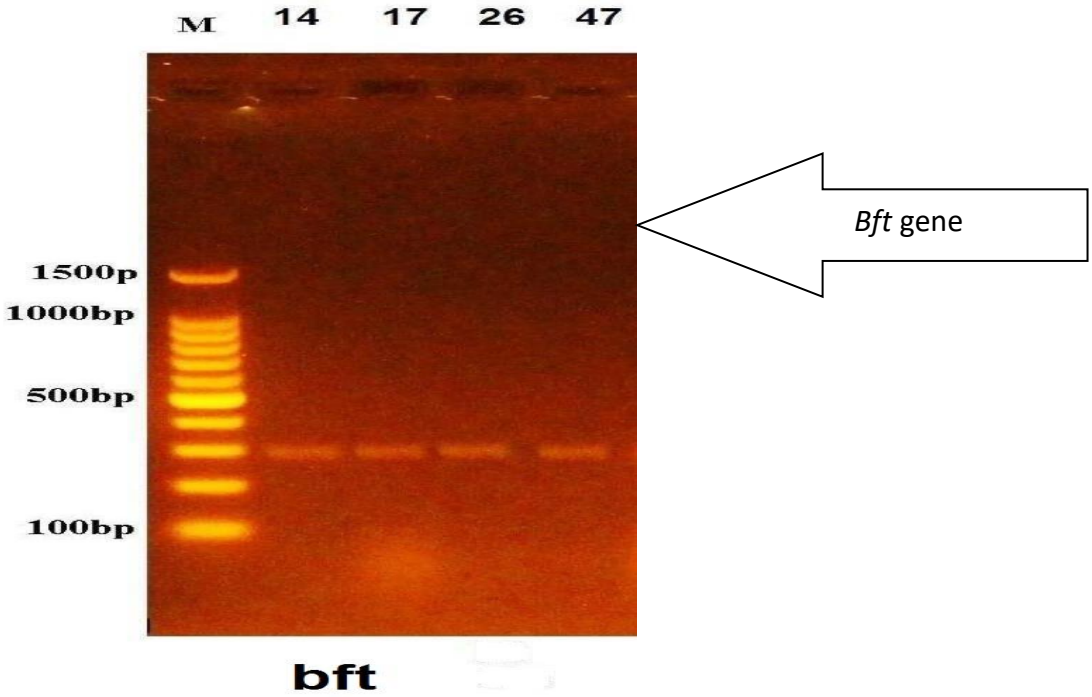

FIGURE 3: Gel electrophoresis of amplified bft gene (294bp), from ETBF using conventional PCR. Agarose 1.5 %, 100 V/cm for 60 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder.Lanes14,17,26 and 47 showed positive results for bft gene.

Detection of (bft-1, bft-2and bft-3) in stool samples

The DNA extracted from 4 isolates of of Entrotoxigenic B.fragilis were examined for bft gene subtypes (bft1,bft2 and bft3).

The results here demonstrated: presence of bft-1 in one sample,bft-2 in 1 sample and bft-3 in 2 sample as shown in fig(4,5,6) respectively . Present results were in line with a result of study by [16] reported that 4 ETBF isolates harbor bft-1 and bft-2 subtypes: three were bft-1 positive and only one was bft-2 positive. The present research disagreed with many studies from other nations that showed that bft-1 gene subtypes were more prevalent than bft-2 and bft-3. Akpinar et al., (2010) reported that the rate of bft-1 was higher than bft-2 in patient and control specimens [21]. Ignacio et al.,(2015) found that bft-1 had the commonest rate, while only one bft-3 positive specimen was recorded [15] . Nguyen et al., (2005) from Vietnam proved that bft-1 (67.4%) had the highest rate than bft-2 (18.6%) and bft-3 (14%) [22].

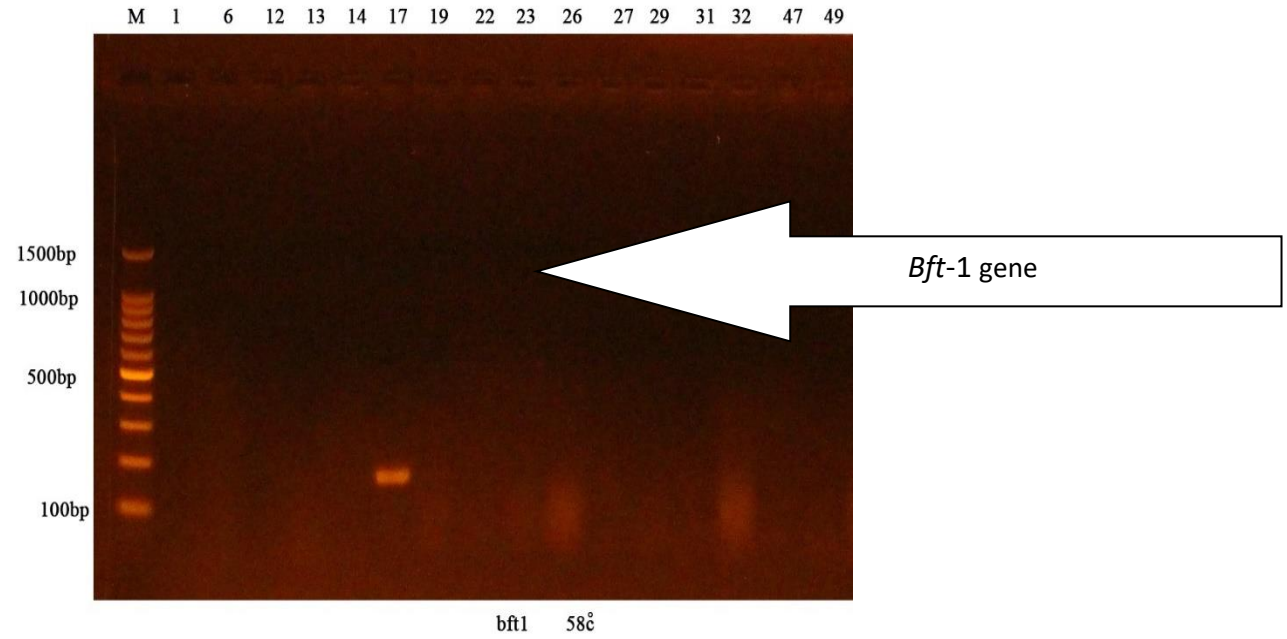

FIGURE 4: Gel electrophoresis of amplified bft1 gene (142bp) from ETBF using conventional PCR. Agarose 1.5 %, 100 V/cm for 60 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder. Lane 17: Amplicons bft-1 gene for ETBF .

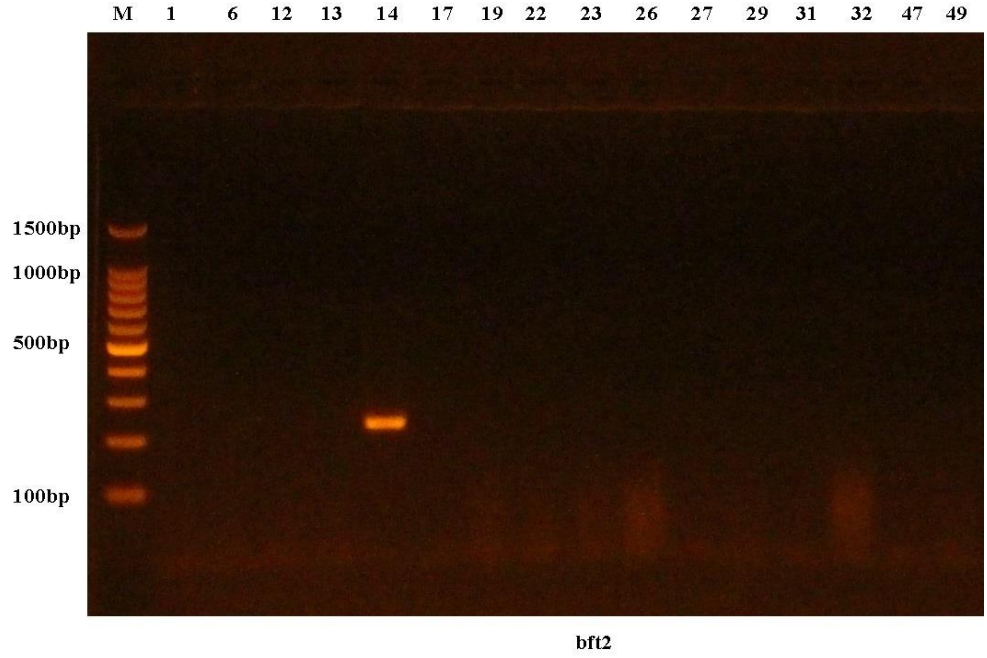

FIGURE 5 :Gel electrophoresis of amplified bft2 gene (219bp) from ETBF using conventional PCR. Agarose 1.5 %, 100 V/cm for 60 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder. Lane 14: Amplicons bft2 gene for ETBF (Sample 14).

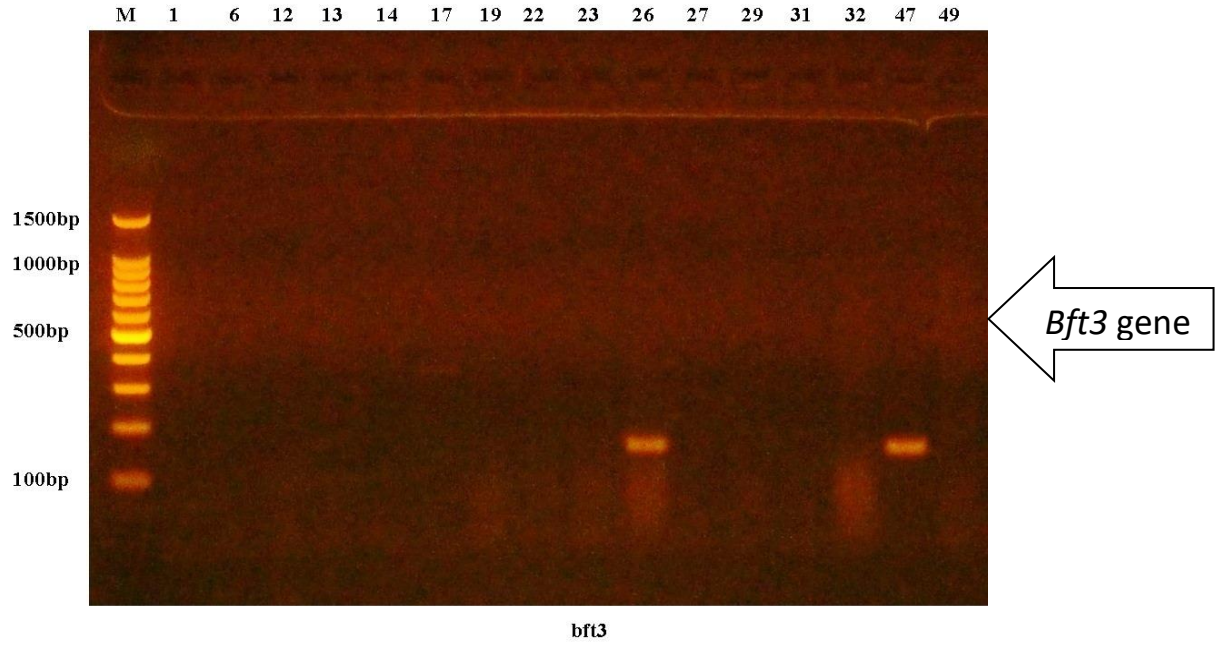

FIGURE 6: Gel electrophoresis of amplified bft3 gene (145bp) from ETBF using conventional PCR. Agarose 1.5 %, 100 V/cm for 60 minutes, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M: 100 bp DNA ladder.All Lanes show negative results except lane 26 and 47 show positive results for bft3.

CONCLUSION

The study showed low number of bft gene was detected in patients with diarrhea. Bft-3 revealed greater values than bft-1 and bft-2 based on the distribution of the bft gene isoforms. There is a need more samples to clarify the involvement of ETBF as a potential cause of diarrhea.

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