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Heterogeneity in resistant fecal *Bacteroides fragilis* group collected from healthy people

T. Narimani [a], M. Douraghi [b], P. Owlia [c], A. Rastegar [a], M. Esghaei [d], B. Nasr [e], M. Talebi [a, *]

[a] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Microbiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[c] Molecular Microbiology Research Center(MMRC), Shahed University, Tehran, Iran
[d] Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[e] Department of Microbiology, Isfahan University of Medical Sciences, Isfahan, Iran

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ABSTRACT

Normal nonpathogenic flora would represent a constant lake of resistance genes potentially transferable to human pathogens. To assess the prevalence of resistance genes and genetic variability of *Bacteroides fragilis* group (BFG) from normal flora, 177 Bacteroides isolates obtained from the fecal samples of healthy individuals. These isolates were subjected to antibiotic susceptibility testing and pulsed field gel electrophoresis (PFGE). The isolates were further tested for the presence of *erm*F, *tet*Q and *bft* genes by PCR. Our results indicated the presence of different clonal strains (1 common type and 57 single types) among the resistant isolates. The resistance rate for the six antibiotics in this study was between 1% and 95%. Most of the isolates (99%) were susceptible to metronidazole. *erm*F and *tet*Q were detected in all erythromycin and tetracycline resistant isolates. None of the isolates were carried *bft* gene. These data suggest dissemination of heterogenic clonal groups in healthy persons and resistance to 5 high commonly used antibiotics.

1. Introduction

Among phylum Bacteroidetes, Bacteroides is the predominant bacterial genus and normally commensal bacteria in human gut [1,2]. Human intestine is an important location for horizontal gene transfer (HGT) due to the attendance of an obtusely populated community of microorganisms which are necessary to maintain good health [3]. HGT has the potential to influence the development of microbial community and cause to spread of antibiotic resistance genes from commensal organisms to potential pathogens in the human intestine [3,4]. However, some studies show that even as a gut commensal *Bacteroides fragilis* could be harming the host by acting as a reservoir of resistance determinants which can then be passed on to other species of the *Bacteroides fragilis* group as well as to much more virulent bacteria [5]. They may also be the recipients of resistance genes from other species of *Bacteroides fragilis*, carry a variety of resistance genes such as the tetracycline and erythromycin resistance genes, *tet*Q and *erm*F respectively. These genes can be transferred within the population or to other related bacterial species through the use of mobile genetic elements [3]. In BFG isolates, clindamycin resistance is basically due to a macrolide-lincomycin-streptogramin (MLS) mechanism that encoded by *erm*F and *erm*G genes [6]. Also, *erm*F gene is often coexisted with the *tet*Q gene on tetracycline resistance conjugative transposons [6]. A growing problem in anaerobe infections is clindamycin resistance. Resistance to clindamycin can became a major prediction for treatment of anaerobic infection since this drug is often prescribed for anaerobic complications [7].

Metronidazole, the other common antibiotic, is an important anti-microbial agent in the anaerobic infections therapy. While Bacteroides strains often are susceptible to metronidazol, recent data has shown an emergence of resistance in the *Bacteroides fragilis* [7]. Different and increasing antimicrobial resistance patterns of BFG bacteria are reported from different geographical areas [7].

It has been showed that the pathogenic potential and antimicrobial susceptibility of *Bacteroides fragilis* vary among different strains [8]. This species has less phenotypic variability, but comprised of genetically heterogeneous strains. Because of heterogeneity, comparative genomics is essential for understanding

* Corresponding author.
E-mail addresses: talebi.m@iums.ac.ir, talebi_25@yahoo.com (M. Talebi).

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the antimicrobial resistance of this species Pulsed-field gel electrophoresis (PFGE) is a tool for analyzing of the differences in genome structure of many strains [8].

We analyzed fecal specimens from humans for both presence and frequency of Bacteroides spp. with resistance to one or more of six different antimicrobial agents and for their ability to produce cytotoxic changes in HT-29 cells and detection of *bft* gene.

2. Method and material

2.1. Sample collection, isolation and identification

From April 2014 until March 2015, fecal samples were collected from 230 healthy individuals 105 female and 125 male patients; mean age, 39 years(ranging from 12 to 83 years old) in Tehran. All volunteers had normal bowel activity and those with a history of antibiotic usage during the last three months prior to the study were excluded. All procedures were done in accordance to Declaration of Helsinki (1975) amended in 2013 [9]. Information including age, sex, underlying disease and history of antibiotic use were collected from each individual.

Samples from each person were collected in sterile plastic containers and were immediately transported to the laboratory and cultured on anaerobic agar plates (BBE: *B. fragilis*-bile-esculin agar) (Himedia Laboratories Pvt. Ltd, India). Then plates were incubated in an anaerobic atmosphere for 72 h at 37 °C. From each plate were selected three black colonies and each colony were cultured on blood agar supplemented with 0.005 g/L hemin and 0.0 1 g/L vitamin K1 separately and incubated in the formerly mentioned condition (an anaerobic atmosphere for 72 h at 37°C) [10]. In further subcultures, some of colonies were missed and we could save 177 isolate finally.

The identification of anaerobic gram negative bacilli was done using API-32A system (bioMerieux, Marcy l'Etoile, France). The isolates were stored in Brain Heart Infusion (BHI) broth with 20% glycerin at −80 °C.The final identification of Bacteroides spp. were performed by PCR [10].

2.2. Antibiotic susceptibility test

Minimal inhibitory concentration (MIC) was performed by E-test method according to the protocol proposed by European Committee on Antimicrobial Susceptibility Testing (EUCAST). Isolates were evaluated for following antibiotics: ampicillin, clindamycin, tetracycline, ciprofloxacin, erythromycin and metronidazole.

2.3. PCR detection for resistance and toxin genes

DNA was extracted by boiling method [11]. In brief, colonies was picked up with sterile loop and suspended in buffer saline phosphate and centrifuged at 4000 rpm for 1 min [11]. Pellet was suspended in 250ul of molecular biology-grade water, was boiled at 100 °C in a water bath for 10 min, cooled on ice, and centrifuged at 14,000 rpm for 10 min. The supernatant was used for performing the PCR:

For confirming of *Bacteroides fragilis* isolates, multiplex PCR was done. Then the isolates were further tested for the presence of the *bft* and *ermF* and *tetQ* resistance genes. PCR was performed in a total volume of 25 μl reaction mixtures including: 1.25U Taq DNA polymerase, 50 ng DNA template, 1X PCR buffer, 2.5 mmol/l MgCl2, 200 μmol/l dNTP, 30 pmol primer.

The PCR for amplification of *bft* was performed under the following condition: denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 1 min annealing at 52 °C for 1 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min [12].

Detection of resistant *tetQ* gene was performed as follows: an early cycle at 95 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min (4), and in the case of *ermF* procedure was as follows: an early cycle at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min [13].

The amplification of multiplex PCR for detection of species was performed under the following condition: denaturation at 94 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 1 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min (11). All primer sequences are listed in Table 1.

2.4. Cytotoxicity assay

A tissue culture assay using HT- 29 cell line was used for cytotoxicity assay. Brifly each Bacteroides isolate was cultured in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke),supplemented with yeast extract (Oxoid) 0.5% and haemin 0.005%, and then it was placed in anaerobically condition for 24 h at 37 °C. The cultures were centrifuged at 8000 rpm for 5 min and the supernates were frozen immediately and kept at −20 °C until used. HT-29 cells were grown in RPMI 1640 medium (Biosera, England) supplemented with pensterp 100 IU/ml (Biosera, England) and heat inactivated fetal bovine serum, at 37 °C in air with CO_2, 5% [14]. Cells were transferred to fresh medium with a 1 in 2 dilution three times a week (18). For the cytotoxicity assay, HT-29 cells from one flask were divided (200 ul/well) into a 96-well microtitration plate and allowed to grow for 2−3 days. Then the medium was removed and 180 ul of fresh medium without serum was added to each well; 20 u1 of bacterial culture supernates were inoculated into the wells in

Table 1
Primers sequences used in this study.

Gene name	Sequence	Product size	Reference
tetQ	F1:5′-GGC TTC TACGAC ATC TAT TA-3′ F2: 5-CAT CAA CATTTA TCT CTC TG-3′	758 bp	4
ermF	F1: 5′-CGG GTC AGC ACTTTA CTA TTG-3′ F2:5′GGA CCT ACC TCA TAG ACAAG 3′	466 bp	13
bft	F1:5′ACGGTGTATGTGATTTGTCTGAGAGA-3′ F2:5′-CCCTAAGATTTTATTATCCCAAGTA-3′	294 bp	12
Species Identification (Mutiplex PCP)	Bfr-F:5′-CTGAACCAGCCAAGTAGCG-3′ Bfr-R:5′-CCGCAAACTTTCACAACTGACTTA-3′	230 bp	11
	Bfr-G2:5′-ATCAGGTTCGACTCTTGCT-3′ Bfr-G3:5′-CCGTCAGCTGGCAGGA-3′	450 bp	
	G23S-1:5′-GTTGGCTTAGAAGCAGC-3′ G23S-2:5′-CATTTTGCCGAGTTCCTT;-3′	400 bp	