

Isolation of anaerobic bacteria of the *Bacteroides fragilis* group from environmental samples

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Abstract. The aim of this study was to analyze the effectiveness of identification of *Bacteroides fragilis* group (BFG) strains isolated from human and rat feces, hospital wastewater, and untreated and treated sewage from a wastewater treatment plant (WWTP). BFG strains were plated on Bacteroides Bile Esculin (BBE) agar. Characteristic colonies were isolated from the culture medium, tested for antibiotic resistance and identified by PCR and MALDI-TOF MS. A total of 319 strains that formed characteristic colonies were isolated from BBE agar, of which 250 were resistant to kanamycin, colistin and vancomycin. PCR revealed that only 135 strains harbored the *bfr* gene specific to the BFG. In MALDI-TOF MS analysis, only 123 isolates were classified as members of the BFG. The most frequently isolated species was *Parabacteroides distasonis* (51.22% of strains). *B. fragilis*, *B. ovatus* and *B. thetaiotaomicron* were less frequently encountered in the analyzed environmental samples (30.01%, 8.13% and 5.69%, respectively). The strains isolated from human and rat feces on BBE agar were most reliably identified, and 100% of the isolated strains were classified as members of the BFG. The effectiveness of isolation of BFG strains from hospital wastewater and untreated and treated sewage from the WWTP was relatively low (20%, 19%, 40%, respectively). The results of this study suggest that the method for isolating BFG strains from environmental samples on BBE agar requires modification. Additional methods, such as PCR and MALDI-TOF MS, can be used to more effectively identify BFG strains isolated from different environmental samples.

1 Introduction

Bacteria of the genus *Bacteroides* and the closely related genus *Parabacteroides* are Gram-negative obligate anaerobes. These immobile and non-sporulating bacteria are part of normal gut microbiota in humans and animals [1]. Species of the genera *Bacteroides* and *Parabacteroides* include the *Bacteroides fragilis* group (BFG) of 44 potentially pathogenic species which are the most frequently isolated anaerobes in human infections [1, 2]. BFG strains are the most antibiotic-resistant anaerobic pathogens [3,4]. Clinical BFG strains are presumptively identified on Bacteroides Bile Esculin (BBE) agar, a selective and differential culture medium [5, 6]. The medium is used to differentiate bacteria capable of growing in the

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presence of 20% bile and gentamycin, bacteria hydrolyzing esculin and bacteria capable of growing in the presence of hemin, which can be used in the catalase test. Vitamin K is added to the medium to stimulate the growth of many *Bacteroides* spp. [6]. Due to its specific composition, BBE agar stimulates the growth of BFG strains only which are identified based on the blackening of the medium around the formed colonies. Most BFG species are resistant not only to gentamycin but also to colistin, kanamycin and vancomycin. This trait is used to differentiate BFG strains from other anaerobic bacteria. However, BFG species can differ in resistance to the above antibiotics. Isolates have to be rapidly and accurately identified for effective treatment of bacterial infections. BFG strains are characterized by high phenotypic similarity, which is why they are often incorrectly identified in routine biochemical tests [2]. In addition, the existing methods for the identification of BFG strains based on phenotypic traits do not account for recent taxonomic changes and new species of the genus *Bacteroides*.

The progress in molecular biology and the availability of genotyping techniques have contributed to the development of new methods for the identification of the BFG. Bacterial species, including BFG strains, can be identified in hybridization tests with DNA and RNA probes [7–9], and in polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays with the use of specific primers [10, 11]. 16S rRNA gene sequencing is regarded as the most effective method of bacterial identification [2]. However, this method is costly and time consuming, and it is not widely used. Genotyping techniques support the identification of bacterial strains based on the presence of conserved nucleotide sequences that are specific to a given genus, species or a group of species, mostly in the region of 16S rRNA and 23S rRNA genes. Alternative methods of microbial identification involve spectroscopic techniques [12]. Raman spectroscopy and Matrix Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) are regarded as the most promising techniques. Microbiological laboratories rely on MALDI-TOF MS to identify bacterial and fungal species [13–15]. This method has also been effectively deployed to identify BFG strains [12, 14]. MALDI-TOF MS generates characteristic mass spectral fingerprints that are unique for every bacterial genus or species. However, reliable identification of bacteria requires an expanded database [12].

The aim of this study was to analyze the effectiveness of isolation of BFG strains from different environmental samples, including human feces, laboratory rat feces, hospital wastewater, and untreated and treated sewage from a municipal wastewater treatment plant (WWTP) in Olsztyn. The analysis was carried out in four consecutive stages: bacteria were cultured on selective and differential BBE agar, the isolated strains were tested for resistance to colistin, kanamycin and vancomycin, the presence of the *bfr* gene specific to BFG was determined by PCR, and the strains were identified to the species level by MALDI-TOF MS.

2 Materials and methods

2.1 Sample collection

Bacteroides fragilis group strains were isolated from human and lab rat feces, from wastewater from a hospital in Olsztyn and from untreated and treated sewage from the WWTP in Olsztyn. The sampling sites in the WWTP and the hospital were described in detail in our previous studies [16, 17]. Thirteen grab samples from hospital wastewater and untreated and treated sewage were acquired in the spring and summer of 2016. Bacterial strains from nine samples of human feces from healthy volunteers were isolated by a private microbiological laboratory in Olsztyn. Three samples of laboratory rat feces were obtained from the Department of Industrial and Food Microbiology of the University of Warmia and

Mazury in Olsztyn. Immediately after collection, all samples were transported on ice to the laboratory and analyzed.

2.2 Isolation of *Bacteroides fragilis* strains

Bacteroides fragilis group bacteria were isolated from rat feces, hospital wastewater, and untreated and treated sewage from the WWTP by serial dilution in saline (1 g of feces or 1 mL of wastewater in 9 mL of 0.85% NaCl). Then, 0.1 mL of each solution was transferred to BBE agar and incubated for 48 h at 37°C under anaerobic conditions. BBE agar was used for the initial identification of BFG strains [5]. Bacteria were cultured under anaerobic conditions in the BACTRON I-2 chamber (Sheldon Manufacturing Inc., USA). BFG strains formed small, regularly spherical, gray colonies on BBE agar where esculin hydrolysis resulted in the blackening of the medium.

All of presumptive *Bacteroides fragilis* group strains isolated from HWW, UWW, TWW and RF samples as well as the strains isolated from HF samples in a private laboratory were streaked onto Brucella Blood agar (BD) (Merck) and incubated for 24 hours at 37°C under anaerobic conditions. Subsequently, the strains were tested for resistance to kanamycin (1000 µg), colistin (10 µg) and vancomycin (5 µg) by the disk diffusion method on Brucella Blood agar (24 h/37°C, anaerobic conditions). BFG strains are resistant to these antibiotics. All strains presumptively identified as members of the BFG were stored in LB-Broth (Miller, Merck) with 10% addition of glycerol, at a temperature of -80°C.

2.3 Genomic DNA extraction

Genomic DNA was extracted by suspending a loopful of bacterial colonies from Brucella Blood agar in 0.5 mL of sterile water. The suspension was heated at 95°C for 10 min and centrifuged at 5000 rpm for 5 min at 4°C according Harnisz et al. [18]. The concentration and quality of extracted DNA was determined with the NanoDrop spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Willmington, DE). DNA was extracted in duplicate and stored at -20°C for further analysis.

2.4 Identification of *Bacteroides fragilis* group strains by PCR

Isolates were identified by PCR using an oligonucleotide primer pair flanking conserved regions of the 16S rRNA *bfr* gene (5'-CTGAACCAGCCAAGTAGCG-3' and 5'-CCGCAAACCTTTCACAACCTGACTTA3') based on the method described by Liu et al. [10]. PCR amplification was carried out in a total reaction volume of 15 µl containing 1.0 U of Run polymerase DNA (A&A Biotechnology, Poland), 1.5 µl of 10 x PCR buffer, 0.5 mM of each primer, 0.2 mM of dNTPs and 1.5 µL of template DNA. The reaction protocol was as follows: initial denaturation at 95°C for 5 min, 35 denaturation cycles at 95°C for 20 s, annealing at 52°C for 1 min, extension at 72°C for 30 s. A 5 min cycle at 72°C was added to the final extension step.

After amplification, DNA was separated by electrophoresis in agarose gel stained with ethidium bromide (0.5 µg/mL). A DNA marker (100-1000 bp, A&A Biotechnology, Poland) was used to determine the size of amplification products. Electrophoresis was performed in a buffer (1x TBE) for 60 minutes at 100 V. After electrophoresis, gels were visualized with Gel Doc™ EZ Imager and Image Lab™ v.6.0.0 2017 software (Bio-Rad Laboratories Inc).

2.5 Identification of *Bacteroides fragilis* group strains by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS)

Each isolate was cultured overnight on Brucella Blood agar, and it was identified in the Vitek MS Plus system (bioMérieux, Marcy l'Etoile, France). The isolates were plated and incubated in a BD GasPak™ EZ container under anaerobic conditions generated with the use of BD GasPak EZ anaerobe container system sachets. The samples were prepared on barcode-labeled target slides (Vitek MS-DS; bioMérieux, Inc.). An overnight culture was transferred and smeared directly onto the plate to inoculate the spots. The preparations were overlaid with 1 µl of α -cyano-4-hydroxycinnamic acid matrix solution (Vitek MS-CHCA; bioMérieux, Inc.) and were air dried for 1-2 min at room temperature. The calibration spots of each acquisition group were inoculated with the *Escherichia coli* ATCC 8739 strain as a calibrator and internal ID control, according to the manufacturer's recommendations.

The mass spectrum (2,000-20,000 Da) was generated with the Vitek MS Plus mass spectrometer (bioMérieux, Marcy l'Etoile, France). For each bacterial sample, mass fingerprints were processed by Compute Engine and the advanced spectrum classifier (ASC) algorithm of the Vitek MS system. The strains were identified to the species level by comparing the obtained spectra against the spectra typical of each claimed species (Vitek MS IVD version 3.0.0). The RUO mode (Vitek MS Saramis database) was used when an isolate could not be identified by Vitek MS IVD. In line with the manufacturer's instructions, a confidence interval of 60-99% was considered acceptable for species identification (ID).

3 Results and discussion

A total of 319 bacterial strains were isolated from selective and differential BBE agar. The appearance of all colonies was characteristic of the BFG: they were small ($\varnothing = 1-2$ mm), gray and spherical, with visible blackening of the medium around the colonies. The highest number of 157 strains were isolated from untreated wastewater flowing into the WWTP in Olsztyn, followed by hospital wastewater (56 strains), treated wastewater evacuated from the WWTP (40 strains), and human and rat feces (33 strains each). All strains isolated from BBE agar tested positive for catalase.

During preliminary identification, the isolated strains were tested for resistance to colistin, kanamycin and vancomycin on Brucella agar. All strains isolated from samples of human and rat feces were resistant to the above antibiotics (100%, 33 strains each). Lower levels of resistance to the tested antibiotics were noted in the strains isolated from hospital wastewater (83.93%, 47 out of 56 strains) and treated sewage (77.50%, 31 out of 40 strains), whereas the lowest percentage of resistant strains was noted in raw sewage (67.52%, 106 out of 157 strains). Strains resistant to colistin, kanamycin and vancomycin were presumptively identified as members of the BFG and were subjected to further analyses.

In the following two stages, bacterial strains were analyzed with the use of molecular methods. In the first step, the species identity of the isolated strains was resolved by PCR. The presence of the *bfr* gene specific to the BFG was analyzed in the DNA from the studied isolates. Only 135 out of the 250 strains identified as members of the BFG with the use of the culture-dependent method harbored the *bfr* gene (PCR product with a size of 126 bp). The *bfr* gene was detected in all strains isolated from human and rat feces (100%, 33 strains each). The above gene was also identified in 17 out of 31 strains (54.84%) from treated sewage evacuated by the WWTP, in 21 out of 47 strains (44.68%) from hospital wastewater, and in 31 out of 106 strains (29.25%) from untreated sewage. Liu et al. [10] demonstrated that PCR was a highly effective technique for identifying BFG strains. The application of additional primers, which were not described in this study, could facilitate rapid and reliable identification of ten species belonging to the BFG: *B. caccae*, *B. distasonis*, *B. eggerthii*,

B. fragilis, *B. merdae*, *B. ovatus*, *B. stercoris*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus* [10].

Strains that presented a characteristic growth profile on BBE agar, were resistant to colistin, kanamycin and vancomycin and harbored the *bfr* gene were identified to the species level by MALDI-TOF MS. Ultimately, 119 out of 135 strains were classified as members of the BFG, and another 4 isolates, despite failed attempts to resolve their species identity, were also classified as members of the BFG. The percentage of strains from environmental samples which were identified as BFG species is presented in Figure 1. Hong et al. [9] demonstrated that *B. fragilis*, *B. thetaiotaomicron*, *B. caccae*, *B. eggerthii*, *B. vulgatus*, *B. uniformis* and *B. massiliensis* were frequently encountered in samples of human feces. In the present study, the species composition of the strains isolated from human feces differed from published data. In human feces isolates, the predominant BFG species were *B. fragilis* (61%) and *P. distasonis* (21%). The remaining strains were identified as *B. thetaiotaomicron* (9%), *B. ovatus* (6%) and *B. caccae* (3%). In the clinical trials described by Snyderman et al. [19, 20], Eitel et al. [21] and Nagy et al [22], *B. fragilis* was also most frequently isolated from human feces (up to 80% isolates).

Parabacteroides distasonis (70%) was the predominant species in rat feces isolates. The remaining species, including *B. ovatus*, *B. fragilis* and *B. stercoris*, accounted for 3-15% of BFG strains. In a study analyzing the presence of *Bacteroides* spp. in pig and cow feces, Hong et al. [9] did not detect the above BFG species, but identified *P. distasonis*, *P. goldsteinii* and *P. merdae*.

All isolates from hospital wastewater were classified as *P. distasonis* (100%). The strains isolated from raw sewage in the WWTP belonged to three species, mostly *P. distasonis* (60%), as well as *B. fragilis* (37%) and *B. thetaiotaomicron* (3%). *Bacteroides fragilis* was the predominant species (31%) in the group of strains isolated from treated wastewater which also contained *P. distasonis* (25%), *B. ovatus* (19%) and *B. thetaiotaomicron* (19%). One BFG strain (6%) was not identified to the species level. The remaining strains from hospital wastewater and untreated and treated sewage in the WWTP did not belong to the BFG and were identified by MALDI-TOF MS as *Serratia marcescens* (10 isolates) and *Raoultella ornithinolytica* (2 isolates) of the family *Enterobacteriaceae*. These isolates were phenotypically similar to BFG strains. However, the PCR test screening for the presence of the *bfr* gene produced additional bands on agarose gel, which probably led to the misclassification of the above 12 strains as members of the BFG. The occurrence of the BFG in wastewater has been rarely investigated. Hong et al. [9] relied on fluorescence in situ hybridization (FISH) to detect *Bacteroides* spp. in raw sewage in a municipal WWTP. They identified the following BFG species in sewage samples: *B. fragilis*, *B. caccae*, *B. uniformis*, *B. vulgatus*, *B. massiliensis* and *B. thetaiotaomicron*. Their results are partially consistent with our findings.

In the current study, anaerobic BFG strains were detected in treated wastewater despite the presence of an aeration step in the treatment process. It can be probably attributed to the presence of catalase which enables anaerobes to survive under aerobic conditions [23]. This applies particularly to *B. fragilis* and *P. distasonis*, as well as selected strains of *B. thetaiotaomicron*, *B. ovatus* and *B. eggerthii* [24]. In this study, the above species were

detected in all environmental samples, and they were predominant in samples of treated wastewater.

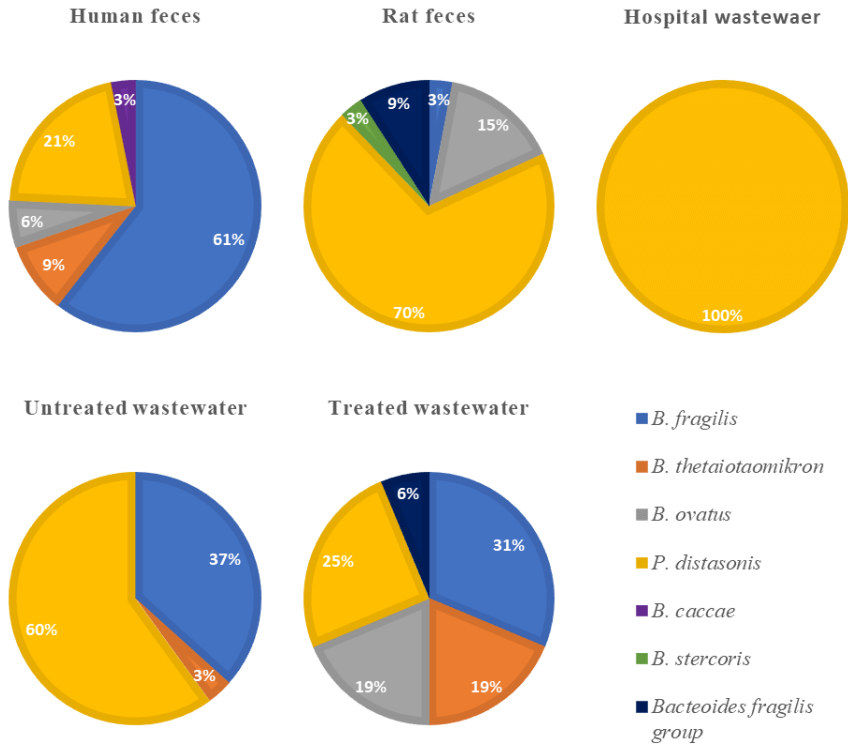


Fig. 1. Percentage composition of *Bacteroides fragilis* group in environmental samples.

The effectiveness of isolation of BFG strains in successive stages of the study varied across the analyzed environmental samples (Fig. 2). All strains isolated from human and rat feces on BBE agar belonged to the BFG (100% accuracy). The percentage of BFG strains isolated from hospital wastewater and untreated and treated sewage which demonstrated traits characteristic of the BFG continued to decrease in successive stages of analysis. BFG strains isolated from treated sewage were identified with 40% accuracy, and those from hospital wastewater and raw sewage – with 19–20% accuracy.

Culebras et al. [2] and Nagy et al. [25] identified *Bacteroides* spp. by MALDI-TOF MS with the use of reference strains and clinical isolates identified by 16S rRNA gene sequencing. Their results revealed that MALDI-TOF MS was an easy, rapid, low-cost and highly effective (87–98%) technique for the routine identification of *Bacteroides* spp. However, the MALDI-TOF MS database is incomplete, and new species such as *B. dorei*, *B. xylanisolvans* and *B. faecis* can be erroneously classified as *B. vulgatus*, *B. ovatus* and *B. thetaiotaomicron*, respectively [2, 26]. The MALDI-TOF MS database should be expanded to increase the accuracy of bacterial species identification.

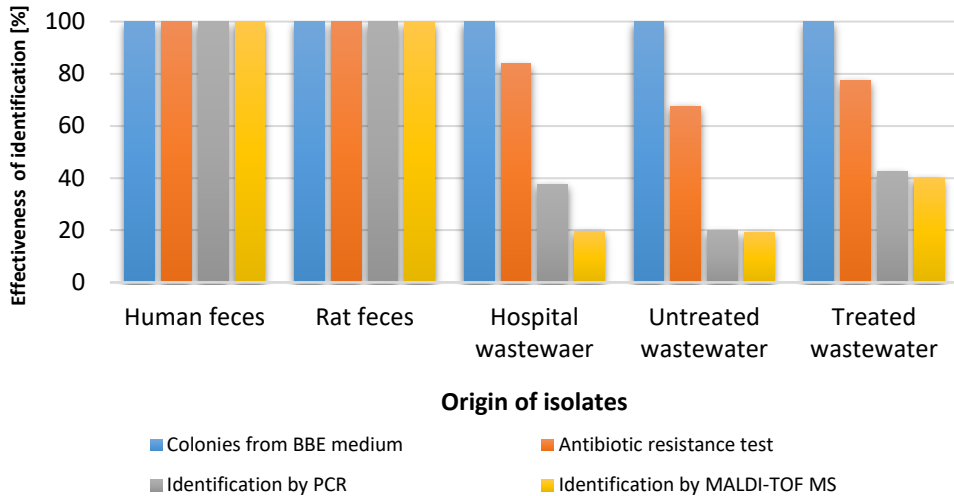


Fig. 2. Effectiveness of isolation of *Bacteroides fragilis* group from environmental samples.

4 Conclusions

All strains isolated from samples of human and rat feces on BBE agar were identified as members of the BFG. The evaluated samples were probably similar to clinical samples for which diagnostic methods have been developed and optimized. The isolates from hospital wastewater and untreated and treated sewage from the WWTP were identified with low accuracy, which can probably be attributed to the different character of those samples, greater bacterial diversity and the presence of bacteria with modified phenotypic traits in these environments. The results of this study suggest that the method for isolating BFG strains from environmental samples on BBE agar requires modification. Additional methods, such as PCR and MALDI-TOF MS, can be used to more effectively identify BFG strains isolated from different environmental samples. However, the MALDI-TOF MS database should be expanded to increase the accuracy of identification.

Human feces were characterized by the highest diversity of BFG strains. Anaerobic BFG strains were detected in treated sewage despite the presence of an aeration step in the treatment process. Treated wastewater is evacuated to surface water bodies, and it can pose a health risk for humans and animals.

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