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Short Communication

Determinants of resistance in *Bacteroides fragilis* strain BFR_KZ01 isolated from a patient with peritonitis in Kazakhstan

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ABSTRACT

Objectives: Bacteroides fragilis is one of the most important human anaerobic pathogens often found in various clinical infections. The purpose of this study was to determine the susceptibility of a *B. fragilis* clinical strain (BFR_KZ01) from Kazakhstan to the most commonly used anti-anaerobic drugs at the local level and to detect genes associated with resistance to these antibiotics.
Methods: Species identification of the bacterial isolate was performed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) and 16S rRNA gene sequencing. Susceptibility to broad-spectrum antibiotics (metronidazole, meropenem, ciprofloxacin, clindamycin and tetracycline) most commonly used for the treatment of intra-abdominal infections (IAIs) was determined. Mass spectra groups essential for identifying *cfiA*-positive strains among clinical isolates were studied using ClinProTools 3.0.22 software. An Ion Torrent PGM™ platform was used for whole-genome sequencing (WGS) of the studied isolate.
Results: The resulting WGS data of strain BFR_KZ01 was submitted to GenBank. In total, 5300 coding sequences (CDSs) and 69 RNA genes were determined. Analysis of the whole-genome data revealed that the studied strain harbours *cfiA*, *nimB*, *tetQ* and *gyrA* genes conferring resistance to key drugs used in treatment of the IAIs. MALDI-TOF/MS analysis assigned strain BFR_KZ01 to Group II (*cfiA*-positive); however, BFR_KZ01 was phenotypically sensitive to meropenem (mean MIC, 1.3 mg/L).
Conclusion: Determinants of drug resistance in strain BFR_KZ01 were identified. It was revealed that *B. fragilis* strain BFR_KZ01 from Kazakhstan is multidrug-resistant since it carries *nimB*, *tetQ* and *gyrA* genes conferring resistance to metronidazole, tetracycline and ciprofloxacin.

1. Introduction

One of the most common anaerobic infections of the abdominal cavity is peritonitis, which is widespread in surgery departments around the world. *Bacteroides fragilis* group bacteria are the major contributors to these infections. *Bacteroides fragilis* is a non-spore-forming, Gram-negative, anaerobic, rod-shaped bacterium that constitutes 0.5–1% of all micro-organisms in the large intestine. Owing to damaged intestinal barrier integrity, bacterial contamination during the surgical intervention and the weak immune system of patients, *B. fragilis* can be found in 30–60% of cases of purulent-septic infections. They are particularly common in intra-abdominal wounds, multiorgan and non-organ abscesses of the abdominal cavity, endometritis, salpingitis and urological infections [1]. Treatment of anaerobic intra-abdominal infections (IAIs) requires immediate action. Carbapenems are considered as the most active agents against *B. fragilis*.

Bacteroides fragilis group strains have a silent *cfiA* gene that encodes a metallo-β-lactamase enzyme responsible for carbapenem resistance [2]. Thus, *B. fragilis* is divided into two subgroups: Group I (*cfiA*-positive) and Group II (*cfiA*-negative) [3,4]. Although not all *cfiA*-positive *B. fragilis* strains are resistant to carbapenems,

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there is a possibility of becoming resistant to this group of antibiotics by acquisition of an appropriate insertion sequence element for full expression of the *cfiA* gene, leading to possible treatment failure [2].

Metronidazole is particularly useful against *Bacteroides* spp. Panresistance to metronidazole in the *B. fragilis* group is associated with the presence of the nitroimidazole resistance genes *nimA* to *nimG* [5].

Use of empirical therapy has caused the emergence of antibiotic resistance in *B. fragilis*. Antibiotic resistance is a global issue in treating infectious diseases caused by these micro-organisms. Low treatment efficacy is confirmed by the results of studies in different countries that report little effectiveness of empirical therapy and note the importance of susceptibility testing of anaerobes for locally prescribed antibiotics [6]. Recent studies have reported regional variations in antimicrobial resistance to some of these antibiotics in several countries [7]. Takesue et al. investigated the antimicrobial susceptibility of pathogens isolated from post-operative IAIs in Japan [8]. Among *B. fragilis* group species, low levels of susceptibility were observed to cefoxitin, moxifloxacin and clindamycin, whereas high susceptibility rates were observed for piperacillin/tazobactam, meropenem and metronidazole [8]. Boyanova et al. reported that the resistance patterns in multidrug-resistant (MDR) strains could vary widely across different centres and countries [9]. Currently, no data are available on the susceptibility of *B. fragilis* group bacteria in patients with anaerobic IAIs to broad-spectrum antibiotics in Kazakhstan.

This study aimed to explore a *B. fragilis* isolate at the molecular and genetic level from a patient with an anaerobic IAI prescribed broad-spectrum antibiotics in Nur-Sultan (Astana) City Hospital, Kazakhstan.

2. Materials and methods

2.1. Isolation, cultivation and identification of Bacteroides spp

Clinical strain BFR_KZ01 was isolated from a 48-year-old male patient diagnosed with 'an acute gangrene perforated appendicitis, peritonitis' from specimen BFR_1 at City Hospital, Nur-Sultan. Organisms were collected using swabs from drainage wounds and subsequent immersion of probes into a tube containing Amies medium. To obtain pure *Bacteroides* culture, all samples were cultivated on esculin-gelatin medium with incubation at 37 °C for 48 h under anaerobic conditions. Thereafter, isolates were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen Germany) as well as by determining the direct nucleotide sequence of a fragment of the 16S rRNA gene using an automatic system (3730xl Genetic Analyzer; Applied Biosystems). Bacterial cultures were stored in 30% glycerin at −70 °C.

2.2. Morphological and culture characterisation

Bacteroides bile esculin agar (Condalab) was used to study the culture and morphological properties of *Bacteroides*. Microscopy was conducted using an Axio Imager Z1 biological microscope (Carl Zeiss, Germany).

2.3. Determination of antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) of the antibiotics ciprofloxacin, metronidazole, meropenem, clindamycin and tetracycline were determined using M.I.C.Evaluator™ strips (Oxoid Ltd., Basingstoke, UK) at the following concentrations: 0.002–32 mg/L for meropenem and ciprofloxacin; and 0.015–256 mg/L for metronidazole, clindamycin and tetracycline. Inocula with a concentration equivalent to a 1.0 McFarland standard were used. MICs were determined in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards for Gram-negative anaerobes (http://www.eucast.org/), except for tetracycline that was interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoints [10,11].

2.4. Whole-genome sequencing (WGS)

DNA concentration was measured using a Qubit® dsDNA HS Assay Kit (Invitrogen) on a Qubit 2.0 fluorimeter (Invitrogen). Approximately 100 ng of total genomic DNA from *B. fragilis* was sheared with a Bioruptor® UCD-200 ultrasonicator (Diagenode). A barcoded library was prepared using an Ion Xpress™ Plus Fragment Library Kit and an Ion Xpress™ Barcode Adapters 1–16 Kit (Thermo) according to the manufacturer's instructions. Sequencing was conducted on an Ion Torrent™ PGM™ Sequencing Platform using a Hi-Q™ Sequencing Kit (Thermo) and a 318 Chip (Thermo) according to the manufacturer's instructions. Initial analysis of the data was carried out using Torrent Suite™ v.5.0.5 software (Thermo).

CSI Phylogeny 1.4 was used (Call SNPs and Infer Phylogeny) for bioinformatics analysis.

2.5. MALDI-TOF/MS analysis

To identify the *cfiA* gene responsible for resistance to carbapenems, the ClinProTools 3.0.22 program for the mass spectrometer (Bruker Daltonik) was used to differentiate *B. fragilis* bacteria into subgroups I and II. All measurements were performed on a microflex® LT MALDI-TOF mass spectrometer in linear positive ion mode with a laser frequency of 60 Hz. The mass range was 2000–20 000 Da. Spectra were captured in automatic mode. To detect *cfiA*-positive and *cfiA*-negative specific peaks, mass spectra groups of specific *cfiA*-positive and *cfiA*-negative strains of *B. fragilis* were imported into ClinProTools 3.0.22 software. The collected spectra were normalised and calibrated. Subsequently, the peak groups as well as peak shifts were found by visualising the spectral groups. The main spectra (MSP) dendrogram was created using MALDI Biotyper® 4.0 (Bruker Daltonik). Isolate BFR_KZ01 was subjected to MALDI-TOF identification. Log score values above 2.0 indicated identification at the species level. The MSP dendrogram was created by matching the collected MSPs to an MSP library. The MSP library comprised 14 *B. fragilis* MSPs from the reference library. The *Bacteroides ovatus* reference MSP was used as an outgroup.

3. Results and discussion

3.1. Identification of the extracted isolate

Using MALDI-TOF/MS and 16S rRNA sequencing, the isolate was identified as *B. fragilis* and was assigned the identification number BFR_KZ01. Sequences were analysed using BLAST (http://blast.ncbi.nlm.nih.gov). The nucleotide sequence of the 16S rRNA fragment of the extracted *B. fragilis* isolate was 99.73% identical to that of the 16S rRNA fragment of *B. fragilis* strain S14

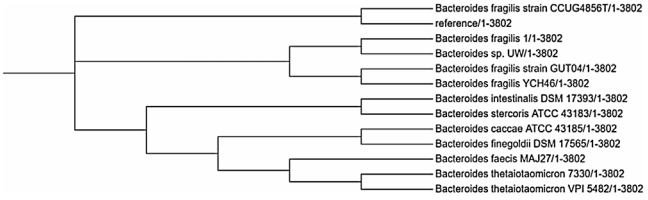

Fig. 1. Phylogenetic tree highlighting the position of *Bacteroides fragilis* BFR_KZ01 strain relative to other species within the *Bacteroides* genus.

Table 1
Genes conferring resistance to antimicrobial agents annotated in the genome of *Bacteroides fragilis* strain BFR_KZ01.

Resistance mechanism	Genes
Antibiotic inactivation enzyme	*cfiA*/*ccrA* family, *nimB*
Antibiotic target in susceptible species	*ddl*, *dxr*, EF-G, EF-Tu, *folA*, *dfr*, *folP*, *gyrA*, *gyrB*, *inhA*, *fabI*, Iso-tRNA, *kasA*, *murA*, *rho*, *rpoB*, *rpoC*, S10p, S12p
Antibiotic target protection protein	*tet(Q)*
Gene conferring resistance via the absence	*gidB*
Protein altering cell wall charge conferring antibiotic resistance	*gdpD*
Regulator modulating the expression of genes conferring resistance to antibiotics	*oxyR*

(CP012706.1) and similar to *B. fragilis* strain BOB25 (CP011073.1) and *B. fragilis* strain JCM 17587 (AB618793.1).

Strain BFR_KZ01 identified by MALDI-TOF/MS had a score of 2.059. Grouping spectra from multiple MALDI mass spectrometer measurements allows calculation of the so-called main spectra profiles (MSP). The MSP dendrogram revealed a close association among selected *B. fragilis* MSPs. The BFR_KZ01 main spectra are closely related to the *B. fragilis* ENR_0039 strain from the reference library.

3.2. Morphological and culture characterisation of the extracted isolate

Morphological analysis of the strain revealed that it was a bacillus-like, Gram-negative, pleomorphic bacterium. In the smears, colonies were placed one by one, immobile and non-spore-forming.

On a dense esculin-gelatin medium, *Bacteroides* formed convex small (1–3 mm) black colonies.

3.3. Antimicrobial susceptibility of Bacteroides fragilis *BFR_KZ01*

The antimicrobial susceptibility of BFR_KZ01 clinical strain to five antibiotics (ciprofloxacin, metronidazole, meropenem, clindamycin and tetracycline) widely used to treat IAIs revealed that the strain was susceptible to clindamycin (mean MIC, 0.12 mg/L) and meropenem (mean MIC, 1.3 mg/L) and was resistant to tetracycline (MIC > 16 mg/L), ciprofloxacin (MIC > 32 mg/L) and metronidazole (MIC > 256 mg/L).

Salipante et al. isolated a MDR (resistant to metronidazole and carbapenem) *B. fragilis* and identified it as a novel *Bacteroides* genomospecies based on WGS data [12]. Based on molecular characterisation of *B. fragilis*, Sárvári et al. demonstrated the appearance of MDR strains in Hungary [13]. The authors reported the importance of antimicrobial susceptibility testing and surveillance among *B. fragilis* group isolates. The increasing antibiotic resistance may be responsible for therapeutic failure and higher mortality rates.

3.4. Sequencing, assembly and annotation

The whole-genome sequence of strain BFR_KZ01 was deposited in GenBank and assigned accession number SSKL00000000.1.

Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/), which allowed the identification of 5369 total genes, including 5300 coding sequences (CDSs), 5 rRNA genes (3, 1 and 1 of 5S, 16S and 23S, respectively) and 62 tRNA genes and a GC content of 43.81%.

Salipante et al. performed WGS of a MDR strain (*Bacteroides* sp. UW isolate 1; GenBank accession no. JANI00000000) [12]. In the present study, we carried out a phylogenetic analysis of our strain. The *B. fragilis* strain 'UW' from the aforementioned article was also included. The phylogenetic tree of the complete genomes of *B. fragilis* is depicted in Fig. 1. The analysis included the complete reference genome of *B. fragilis* NCTC 9343 strain as well as representative reference genomes of species in the *B. fragilis* group, *Bacteroides caccae*, *Bacteroides faecis* and *Bacteroides thetaiotaomicron*. As illustrated in Fig. 1, *B. fragilis* UW and BFR_KZ01 strains form one cluster [14]. Salipante et al. identified the organism as a novel genomospecies of *Bacteroides* [12].

Moreover, whole-genome data analysis was carried out using PATRIC: the Pathosystems Resource Integration Center (https://www.patricbrc.org/). A subsystem is a set of proteins that collectively implement a specific biological process or structural complex [15], and PATRIC annotation includes an analysis of the subsystems unique to each genome. PATRIC was used to identify genes. The Genome Annotation Service in PATRIC uses the k-mer-based antimicrobial resistance (AMR) gene detection method, which utilises PATRIC's curated collection of representative AMR gene sequence variants [16] and assigns to each AMR gene functional annotation, broad mechanism of antibiotic resistance, drug class and, in some cases, the specific antibiotic it confers resistance to. A summary of the AMR genes annotated in this genome and the corresponding AMR mechanism is provided in Table 1. Thus, it was found that this strain carried the *cfiA* and *nimB* genes conferring resistance to β-lactam antibiotics and metronidazole, respectively. Moreover, the *tet(Q)* and *gyrA* genes were found, which confer resistance to tetracycline and quinolones.

In the last 5 years, MDR *B. fragilis* strains have gained immense attention in research. Ank et al. characterised a MDR *B. fragilis* isolated from blood of a patient in Denmark using whole-genome analysis [17]. The isolate (JMZX0100000) harboured the *cfiA* gene conferring resistance to carbapenems, the *nimE* gene (metronidazole resistance), the *ermF* gene (clindamycin resistance) and the *tetQ* gene (tetracycline resistance). The authors underestimated the need for routine susceptibility testing of anaerobic isolates instead of merely relying on surveillance reports. Sydenham et al. identified genes conferring resistance to antibiotics based on the sequencing of *B. fragilis* complete genomes [18]. As a result, the full genomes of five MDR isolates were analysed, namely *B. fragilis* O17 (DCMOUH0017B), O18 (DCMOUH0018B), S01 (DCMSKEJBY0001B), O42 (DCMOUH0042B), O67 (DCMOUH0067B) and O85 (DCMOUH0085B) [18]. Strains O17, O18 and S01 harbour the genes *cfiA*, *tetQ* and *nim*, similar to BFR_KZ01. Strain BFR_KZ01 harbours the *nim* gene, but differs in classification. Currently, 11 *nim* genes are known (*nimA* to *nimK*).

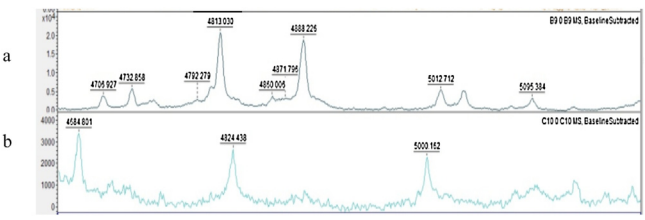

Fig. 2. Differentiation of MS peaks in the range 4500–5500 Da between (a) *cfiA*-negative and (b) *cfiA*-positive strains of *Bacteroides fragilis*.

3.5. Detection of carbapenem resistance genes by mass spectroscopy analysis

Presence of the *cfiA* gene was detected using mass spectrometry analysis. To assess the risk of increased carbapenem resistance in BFR_KZ01, *cfiA* gene carriers were detected using the ClinProTools 3.0.22 program (Fig. 2). Thus, analysing the MS peaks in the range 4500–5500 Da, one can observe their shift from *cfiA*-negative to *cfiA*-positive strains: from 4706 to 4684 Da; from 4813 to 4824 Da; and from 5012 to 5000 Da [3]. Thus, based on MALDI-TOF/MS, strain BFR_KZ01 was assigned to Group II (*cfiA*-positive). However, we assume that in this strain the *cfiA* gene is silent because it lacked phenotypic resistance to the carbapenem meropenem. Jeverica et al. previously reported the silent status of the *cfiA* gene [19].

4. Conclusion

In this study, WGS of *B. fragilis* BFR_KZ01 from Kazakhstan was performed. It was found that *B. fragilis* strain BFR_KZ01 carries the *cfiA*, *nimB*, *tetQ* and *gyrA* genes. BFR_KZ01 can be characterised as a MDR strain taking into account the identified genes, which confer resistance to metronidazole, tetracycline and ciprofloxacin, respectively. However, we assume that in this strain the *cfiA* gene is silent because it lacked phenotypic resistance to meropenem. It is important to note that strain BFR_KZ01 is resistant to metronidazole, one of the key antibiotics in the treatment of IAIs.

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Conflict of interest

None declared.

Ethical approval

Informed consent and questionnaires were approved by RSE Local Ethics Committee of the National Center for Biotechnology (Extract from Protocol No. 4 of 08/29/2017, Kazakhstan). Attending physicians provided data on administered drugs and their concentration. The data used in this work did not contain personal identifiers of the patient.

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