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The occurrence of antibiotic resistance genes in drug resistant *Bacteroides fragilis* isolates from Groote Schuur Hospital, South Africa

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ABSTRACT

Bacteroides fragilis, an anaerobic gut commensal and opportunistic pathogen, is a leading cause of anaerobic abscesses and bacteraemias. Treatment of infections is complicated by the emergence of resistance to several of the antibiotics used in the clinical setting. Genetic analysis of 23 *B. fragilis* isolates found that none of the metronidazole resistant strains carried the *nimA-J* genes, and no *cfxA* or *ermF* genes were detected. All of the tetracycline resistant isolates contained the *tetQ* gene and were sensitive to tigecycline. The *cfiA* gene was found in 3 of the strains, one of which was imipenem resistant and contained an upstream IS*4351* insertion sequence. Another resistant strain had a unique G to A substitution in the promoter region of the *cfiA* gene, while the third was imipenem sensitive. Thirty percent of the isolates contained at least one plasmid, however, *tetQ* gene was located on the chromosome and not on any of the plasmids.

1. Introduction

Bacteroides fragilis is one of the leading causes of intra-abdominal infections and bacteremias, which can prove fatal, if not treated with antibiotics [1]. Recent surveillance data has revealed that the incidence of *B. fragilis* antibiotic resistance is increasing worldwide. This phenomenon is largely driven by the ability of *B. fragilis* to transfer antibiotic resistance determinants efficiently and effectively in the form of conjugative transposons, integrated genetic elements and conjugative and mobilisable plasmids [2,3].

Surveys performed in Europe found that between 24.2 and 28.5% of isolates were clindamycin (Cln) resistant [2,3], while a similar USA survey observed only 6% resistance in 1980 but 35% in 2007 [4]. Although *B. fragilis* is resistant to most common β-lactam antibiotics, a few of the cephalosporins and carbapenems such as cefoxitin (Cef) and imipenem (Imp), respectively, remain effective [2,5]. Carbapenem resistance has been shown to be due to the presence of the *cfiA* gene encoding a metallo-β-lactamase. The *cfiA* gene may also be expressed to various extents or may remain completely silent, resulting in various levels of resistance [5]. Both of the USA [4] and European studies [3] reported less than 1% resistance to Imp. In comparison resistance to Cef was slightly higher for both Europe (13.7% resistance) and the USA (9%).

One of the most effective antibiotics used for treating *B. fragilis* infections is metronidazole (Mtz) [6]. Mtz is a 5-nitroimidazole agent which is often used as the last line of treatment when other antibiotics have failed to eliminate an infection. Worldwide, the incidence of Mtz resistance has been reported to be between 1 and 5% [7]. Both the European study and the survey performed in the USA observed less than 1% resistance, while a Japanese study was unable to detect any Mtz resistance in their *B. fragilis* isolates [3,4,8].

In comparison to the rest of the world, very few antibiotic resistance surveys have been undertaken in South Africa and those that have been performed have not included an in-depth analysis of the genetic mechanisms underlying the observed antibiotic resistance. An initial antibiotic screening of the strains used in this study was performed by our group in 2011 on 23 *B. fragilis* clinical isolates from patients admitted to Groote Schuur Hospital (GSH) Cape Town, South Africa [9]. This showed that 8% of isolates were resistant to Mtz and Imp. Only 4% resistance to Cef was observed, while no Cln resistance was detected. In contrast 65% of the strains were found to be Tet resistant [9].

The aim of the study reported here was, therefore, to expand the scope of the initial investigation to determine of the resistance of these strains to additional clinically important antibiotics, as well as to identify the genes responsible for the observed resistance. It was

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hoped that this would provide further information regarding the susceptibility patterns and genetic makeup of *B. fragilis* isolated from clinical infections at a major South African hospital.

2. Materials and methods

2.1. Media and growth conditions

The 23 experimental strains used in this study were isolated at the Microbiology NHLS at Groote Schuur Hospital (GSH) in Cape Town, South Africa [9]. The strains and plasmids used are described in Table 1. The *B. fragilis* strains were cultured on supplemented Brain Heart Infusion (BHIS) and incubated at 37 °C in an anaerobic chamber (Model 1024 Forma Scientific Inc., Marietta, Ohio).

2.2. Minimum inhibitory concentration (MIC) determination

The susceptibility of the strains to metronidazole (Mtz), tetracycline (Tet), cefoxitin (Cfx), imipenem (Imp) and Clindamycin (Cln) was previously described by Galvão et al. [9]. In the current study, sensitivity to erythromycin (Erm) and tigecycline (Tig) was similarly determined on the same strains by measuring the minimum inhibitory concentration (MIC) on BHISA plates using E-test strips according to the manufacturer's instructions (AB Biodisk). The standard breakpoints for clinical resistance were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (document M11-A7).

2.3. Polymerase chain reaction (PCR) techniques

All PCR reactions used Kapa Ready Mix and 1 μl of genomic DNA (Inqaba Biotech) with 2.5 mM $MgCl_2$ as per manufacturer's instructions, and the thermal cycling was performed using a GeneAmp® PCR system 9700 (Applied Biosystems). The standard PCR parameters were initial 5 min denaturation at 95 °C, primer pair specific number of cycles of denaturation (Table 2) for 30 s at 95 °C, annealing for 30 s at primer pair specific temperatures, and elongation at 72 °C for 1 min. The thermal cycling was then concluded with a 7 min elongation step at 72 °C.

2.4. Screening of GSH isolates for antibiotic resistance genes

Known antibiotic resistance genes were amplified using published primers (Table 2). The following genes were analysed: *nimA-J* genes (Mtz), *tetQ* gene (Tet), *cfxA* (Cfx), *cfiA* (Imp), *ermF* (Erm/Cln). The following bacterial strains and plasmids were used as positive controls: *B. fragilis* Gbr7 (*nimA*), *Bacteroides vulgatus* CLA341 (*cfx*A), pMCL140:616*nimJ* (*nimJ*), pLYL01 (*tetQ*) and pYT646 (*ermF*) (Table 1).

Table 1
Strains and plasmids used in this study.

Strain/plasmid	Genotype/phenotype
Plasmids	
pLYL01 [19]	Mob^+, $Tet^R Amp^R$, *tetQ*
pYT646 [20]	Mob^+, $Tet^R Amp^R Erm^R$, *tetX1*, *ermF*
pMCL140:616*nimJ* [21]	Mob^+, Amp^R, *nimJ*
Strains	
Bacteroides vulgatus	
CLA341 [5]	Clinical Strain, Tet^R Cfx^R, *tetQ cfxA*
Bacteroides fragilis	
638R [22]	Clinical Strain, Rif^R
Gbr7 [23]	Clinical Strain, Mtz^R, *nimA*
GSH2, 3, 5 to 28, 30 [9]	Clinical Isolates

Table 2
Strains and plasmids used in this study.

Primers	PCR conditions
F27 5′-AGAGTTTGATCITGGCTCAG-3′ R5 5′-ACGGITACCTTGTTACGACTT-3′	55 °C and 25 cycles [24]
nim3 5′-ATGTTCAGAGAAATGCGGCGTAAGCG-3′ nim5 5′-GCTTCCTTGCCTGTCATGTGCTC-3′	57 °C and 25 cycles [25]
615-616nimJ-qRT-F 5′- TGACAAGGCTTCGTTCTGTG-3′ 615-616nimJ-qRT-R 5′- GTCGAAACGAATCATCAGCA-3′	55 °C and 31 cycles [21]
tetQ1 5′-GGCTTCTACGACATCTATTA-3′ tetQ2 5′-CATCAACATTTATCTCTCTG-3′	50 °C and 30 cycles [26]
cfiA1 5′-CCATGCTTTTCCCTGTCGCAG-3′ cfiA2 5′-GGGCTATGGCTTTGAAGT-3′	50 °C and 35 cycles [27]
cfxA1 5′-ATCGTAGTTTTGAGTATAGCT-3′ cfxA2 5′-TAAAAGCACTCCGATAACGAT-3′	57 °C and 30 cycles [28]
ermF1 5′-CCTTATGGCATTACTTCCGA-3′ ermF2 5′-GGACCTACCTCATAGACAAG-3′	55 °C and 30 cycles [29]
IS942B 5′-AGAAAAGCATGGTCTTTAACCAAAGTC-3′ IS1186A 5′-GAGAATCAAGCTTCTCGCC-3′ IS4351C 5′-AACCGAGGATCCAAGGTATGCAATTTCT-3′ IS1169/1 5′-TGAGTCAGAGAATCGTG-3′ IS1170/1 5′-CTTCTGTGTGTCATGAG-3′	50 °C and 35 cycles [10]
Up2 5′-TACGCTTTTCTGTGCCATAACTGC-3′ G 5′-CGCCAAGCTTTGCCTGCCATTA-3′	52 °C and 35 cycles [10]

2.5. Identification of insertion sequences (IS) upstream of cfiA

The PCR strategy of Soki et al. [10], was followed. Briefly, to determine whether the IS element was upstream of the *cfiA*, PCR was performed with a forward primer specific to the IS elements IS942B, IS1186A, IS4351C, IS1169/1 or IS1170/1, and a reverse primer to either the 5′ (primer Up2) or 3′ (primer cfiA2) end of the *cfiA* (Table 2). To identify novel IS elements, PCR was done using a forward primer annealing to the upstream region of *cfiA* gene (primer G) and a reverse primer recognising the 5′ region of *cfiA* (primer Up2). This strategy is depicted in Fig. 1a. The PCR amplification protocol for the IS elements included 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 1 min at annealing temperature (Table 2), 3 min at 72 °C and 10 min at 72 °C. PCR products were sequenced (Macrogen Inc., Seoul, Korea) and bioinformatics analysis was performed using the National Centre for Biotechnology Information (www.ncbi.nih.gov). Multiple sequence alignments were carried out with DNAMAN version 4.13 (Lynnon BioSoft).

2.6. Total DNA extraction and detection of the presence of plasmids

Total *B. fragilis* DNA was extracted using the high-salt buffer total DNA extraction method [11]. The DNA was subjected to gel electrophoresis on a 1% agarose gel at 60 V for 2 h. Southern blotting