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Investigation of the prevalence of *tetQ*, *tetX* and *tetX1* genes in *Bacteroides* strains with elevated tigecycline minimum inhibitory concentrations

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ABSTRACT

In this study, the antibiotic susceptibilities to tigecycline and tetracycline of 35 selected Bacteroides fragilis group strains were determined by Etest, and the presence of *tetQ*, *tetX*, *tetX1* and *ermF* genes was investigated by polymerase chain reaction (PCR). tetQ was detected in all 12 B. fragilis group isolates (100%) exhibiting elevated tigecycline minimum inhibitory concentrations (MICs) ($\geq 8 \mu g/mL$) as well as the 8 strains (100%) with a tigecycline MIC of 4 µg/mL, whilst tetX and tetX1 were present in 15% and 75% of these strains, respectively. All of these strains were fully resistant to tetracycline (MIC \geq 16 μ g/mL). On the other hand, among the group of strains with tigecycline MICs < 4 μ g/mL (15 isolates), tetQ, tetX and tetX1 were found less frequently (73.3%, 13.3% and 46.7%, respectively). All but two strains harbouring the tetQ gene in this group were non-susceptible to tetracycline, with an MIC > 4 μ g/mL. These data suggest that in most cases tigecycline overcomes the tetracycline resistance mechanisms frequently observed in Bacteroides strains. However, the presence of tetX and tetX1 genes in some of the strains exhibiting elevated MICs for tigecycline draws attention to the possible development and spread of resistance to this antibiotic agent among Bacteroides strains. The common occurrence of *ermF*, *tetX*, *tetX1* and *tetQ* genes together predicted the presence of the CTnDOT-like Bacteroides conjugative transposon in this collection of Bacteroides strains.

1. Introduction

Since the introduction of the first tetracyclines, these antibiotics have been used widely in the treatment of bacterial infections because of their low price, oral availability and broad-spectrum antimicrobial activity. As a result of the frequent use of tetracycline, resistance to this antibiotic group has emerged during recent decades. In response to this increasing resistance, a new generation of tetracyclines, the glycylcyclines, has been developed.

Tigecycline is the first member of the glycylcycline family, a semisynthetic derivative of minocycline licensed in 2005 by the USA Food and Drug Administration (FDA). Modification of the basic structure protects tigecycline from frequent resistance mechanisms that inactivate tetracycline. The tetracycline resistance mechanisms are classified as ribosomal protection, activity of efflux pumps and enzymatic inactivation of the drug [1]. It is said that tigecycline evades the efflux pumps, which account for most of the acquired resistance against tetracycline, and binds to bacterial ribosomes that have been modified by ribosomal protection proteins, but remains vulnerable to some chromosomally encoded efflux pumps frequently present in *Pseudomonas* and *Proteus* strains [1,2].

Tigecycline displays good activity against clinically significant Gram-positive and Gram-negative bacteria, including meticillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* and extended-spectrum β -lactamase (ESBL)-producing bacteria. All clinically important anaerobic bacteria are also highly susceptible to tigecycline [3,4]. However, in different resistance surveillance studies, *Bacteroides fragilis* group

strains with tigecycline minimum inhibitory concentrations (MICs) >4 μ g/mL have been found, which are considered tigecycline-non-susceptible according to Clinical and Laboratory Standards Institute (CLSI) breakpoints [5]. Tigecycline is recommended in the treatment of complicated intra-abdominal infections, skin and soft-tissue infections as well as infections where a polymicrobial aetiology, multiresistant pathogens or concomitant anaerobic bacteria are suspected [2,4].

The *tetQ* gene encodes a ribosomal protection protein that is responsible for most of the tetracycline resistance observed in *Bacteroides*. The *tetX* gene encodes a FADand NADPH-requiring oxidoreductase that inactivates tetracycline in the presence of oxygen but has been found only in strict anaerobes such as *Bacteroides* [6,7]. Two orthologues of the original *tetX* gene have been identified: *tetX1*, which is an Nterminal truncate (359 amino acids) with 66% identity to the *tetX* gene; and *tetX2*, which is 99% identical to the original gene [6]. These *tet* genes in *Bacteroides* strains are often associated with plasmids and transposons, which may contain other antibiotic resistance genes. The *tetX* gene was identified on transposons Tn4351 and Tn4400, whilst *tetX1* and *tetX2* were found on a *Bacteroides* conjugative transposon, CTnDOT, together with an *ermF* gene on a specific region of it that may be a mobilisable transposon [8]. It was proposed that *tetX* may mediate glycylcycline, and hence tigecycline, resistance among *Bacteroides* spp. [2,6].

The aim of this study was to look for resistance genes that encode tetracycline and possibly tigecycline resistance in *B. fragilis* group strains with different MICs to tigecycline, including strains considered to be tigecycline-resistant.

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

Thirty-five *Bacteroides* strains were selected, comprising 21 *B. fragilis*, 4 *Bacteroides ovatus*, 3 *Bacteroides thetaiotaomicron*, 2 *Parabacteroides (Bacteroides) distasonis*, 2 *Bacteroides vulgatus*, 1 *Bacteroides uniformis*, 1 *Bacteroides salyersae* and 1 *Bacteroides* sp. The selected isolates were from a strain collection compiled for a recent Europe-wide antibiotic resistance surveillance of *B. fragilis* group [5] and a national tigecycline resistance surveillance for anaerobes [9]. Selection of the strains was based on their MIC values for tigecycline, including fully susceptible strains (MIC $\leq 4 \mu g/mL$) and strains with elevated MICs (8 $\mu g/mL$ and 16 $\mu g/mL$).

The strains were stored in Cryobank vials at -70 °C until use. Pre-reduced Columbia agar (Oxoid Ltd., Basingstoke, UK) supplemented with 5% sheep blood, 10 µg/mL haemin and 1 µg/mL vitamin K1 was used for susceptibility testing. Etest gradient strips (AB BIODISK, Solna, Sweden) were applied to measure the MICs of tetracycline and tigecycline [10]. The plates were incubated for 48 h in an anaerobic chamber (Concept 400; Ruskinn Technology Ltd., Bridgend, UK). MIC values were interpreted according to CLSI breakpoints [11].

2.2. Detection of different resistance genes and calculation of their correlation with resistance levels

For the molecular genetics examinations, one colony of each strain was suspended in 100 μ L of sterile distilled water and the mixture was incubated at 100 °C for 10 min and then centrifuged at 14 000 rpm for 2 min. The primers and polymerase chain

reaction (PCR) conditions used in this study are presented in Table 1. The primers were designed by Primer3 software (http://frodo.wi.mit.edu/primer3/). The *tetX* and *tetX1* genes were detected by conventional PCR. The composition of the PCR reaction mix in this case was as follows: $1 \times \text{ReadyMix}^{TM}$ (Sigma-Aldrich, St Louis, MO; 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP and 0.6 U *Taq* polymerase per reaction), 0.7 µM primer and 1/10 volume DNA extract in 20 µL final volume. The products were detected by 1.2% agarose gel electrophoresis using 0.5× TBE buffer [45 mM Tris–borate, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.2]. The *tetX1*, *tetQ* and *ermF* genes were detected by real-time PCR methods. The real-time PCR reactions involved $1 \times iQ^{TM}$ RT-PCR Master-Mix (Bio-Rad, Hercules, CA), 0.7 µM primer, 1/20 volume EvaGreen[®] (Biotium, Hayward, CA) as a double-stranded DNA-binding fluorescent detection dye and 1/10 volume DNA extract in 20 µL final volume. *Bacteroides vulgatus* strain CLA 341 containing *tetQ* and *B. fragilis* strain BF8 containing *tetX*, *tetX1* and *ermF* (unpublished confirmed results) were used as controls.

Statistical analysis was carried out using the SPSS program package (SPSS Inc., Chicago, IL) using Pearson correlation calculations. The significance level was set to P < 0.05.

3. Results and discussion

Of the 35 selected *Bacteroides* strains, 12 had elevated tigecycline MICs ($\geq 8 \mu g/mL$) and were considered intermediate-susceptible or resistant, whilst 23 had low MICs (MIC $\leq 4 \mu g/mL$) placing them in the susceptible range. This latter group included 8

strains with tigecycline MICs of 4 μ g/mL. The distributions of tigecycline and tetracycline MICs of the *B. fragilis* group strains are presented in Fig. 1. The resistance phenotypes of the strains for tetracycline and tigecycline are summarised in Table 2. All strains with a tetracycline MIC $\geq 8 \mu g/mL$ (intermediate or resistant) harboured the *tetQ* gene, whereas only 2 (tetracycline MIC = 4 μ g/mL) of the 6 strains that were susceptible to tetracycline did so. The tetQ gene was present in all 12 isolates with elevated tigecycline MICs (100%), whilst tetX was found in 2 cases (16.7%) and *tetX1* in 9 cases (75%) (Table 3). Among the strains with a tigecycline MIC of 4 μ g/mL, tetQ was found in 8 (100%), tetX in 1 (12.5%) and tetX1 in 6 (75%). In the group of strains with tigecycline MICs between 0.064 μ g/mL and 2 μ g/mL, the genes were found to be present as follows: *tetQ* in 11 (73.3%); *tetX* in 2 (13.3%); and *tetX1* in 7 (46.7%). The calculated correlation values between resistance and the presence of *tetQ* and *tetX1* genes is contained in Supplementary Table 1. Presence of the *tetQ* gene correlated highly both with tetracycline and tigecycline resistance, and presence of the tetX1 gene also correlated with tigecycline resistance although to a lesser extent. The *ermF* gene was detected in 13 (37.1%) of the 35 strains. In seven strains (six with tigecycline MICs \geq 4 µg/mL and 1 with an MIC of 2 μ g/mL) the *ermF* gene was present together with the *tetQ* and *tetX1* genes.

In the European Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) study, tigecycline exhibited the lowest MICs against anaerobes and was superior to clindamycin and cefoxitin, with activity comparable with that of metronidazole or imipenem against *Bacteroides* [3]. Tigecycline resistance was also very low (1.7%) in a Europe-wide antibiotic resistance surveillance involving 824 *B. fragilis* group strains, with some differences between the different species (1.8% for *B. fragilis* and

4.8% for *B. vulgatus*). In a Hungarian multicentre antibiotic resistance study in 2009, only 3 of 280 *Bacteroides* strains were found to be resistant (MIC \ge 16 μ g/mL), whilst 8 strains had elevated MICs (8 µg/mL) to tigecycline [9]. Using a lower resistance breakpoint for tigecycline (≥8 µg/mL), 8% of 141 B. fragilis group strains were found to be resistant during a recent study in Belgium [12]. It was reasonable to start to look for possible resistance genes among *Bacteroides* isolates with different MICs for tigecycline. It has been shown that the presence of *tetX*, originally found in Bacteroides strains, transferred to Escherichia coli dramatically increased MIC values of different tetracycline derivatives, including minocycline [6]. The current study revealed the presence of the *tetX* and *tetX1* genes in *Bacteroides* strains exhibiting elevated MICs for tigecycline, but a direct predictive value for tigecycline resistance could only be observed for the presence of the *tetX1* gene. In the cases of the *tetQ* and *tetX1* genes, a higher prevalence was found in strains exhibiting MICs \geq 4 μ g/mL to tigecycline (Table 3). Detection of the *tetX1* gene was included in this study despite the fact that it has been considered a pseudo-gene because we expected redox activity due to the 66% homology to the *tetX* gene. The unexpectedly high prevalence (46.7–75%) of *tetX1* is noteworthy and in our view is in contrast to it being a pseudo-gene. Presence of the ermF gene was also investigated because it might furnish information on the presence of genetic elements, e.g. the CTnDOT (tetracycline resistance conjugative transposon) that carries the tetQ, tetX1, tetX2 (99% homology to *tetX*) and *ermF* genes coincidentally [8]. In this way, we found three strains that harboured the *tetQ*, *tetX* and *tetX1* genes besides the *ermF* gene. These strains could harbour CTnDOT-like conjugative transposons, and thus the prevalence of this element could be around 8.6% in this *Bacteroides* population, similar to earlier findings (10%) [13]. These studies are important from the

perspective that the plasmids and transposons carrying these resistance genes are highly likely to promote the dissemination of these antibiotic resistance genes among bacteria. A recent study revealed that >80% of *Bacteroides* isolates currently carry conjugative transposons [13]. It is not yet clear whether the high prevalence of *tetX* and *tetX1* genes in the chromosome of *Bacteroides* strains with elevated MICs for tigecycline is a real threat, but the presence of *tetX* and *tetX1* on mobile elements emphasises the possible spread of tigecycline resistance both in *Bacteroides* and other anaerobic and aerobic strains.

Further studies are needed to transfer the tigecycline resistance from a highly resistant strain to a susceptible recipient by conjugation and to determine the possible transfer of the genes investigated in this study.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Distribution of (A) tigecycline and (B) tetracycline minimum inhibitory concentrations (MICs) among 35 selected *Bacteroides* strains.





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Table 1

Primers and cycling parameters for polymerase chain reaction (PCR) experiments

Primer	Position	Sequence (5'→3')	PCR conditions		
	а				
tetX-1	247–	TTAGCCTTACCAATGGGTGT	94 °C for 2 min 30 s, 94 °C for		
b	267		15 s, 55 °C for 30 s, 72 °C for		
tetX-2	470–	CAAATCTGCTGTTTCACTCG	30 s, 35×, 72 °C for 7 min		
b	489				
tetX1-	106–	TCAGGACAAGAAGCAATGAA	94 °C for 10 min, 94 °C for 10		
1	126		s, 63 °C for 30 s, 72 °C for 30		
tetX1-	236–	TATTTCGGGGTTGTCAAACT	s, 35×, 72 °C for 1 min 30 s,		
2	255		melting curve 72 °C to 94 °C		
tetQ-3	678–	ATCGGTATCAATGAGTTGTT	94 °C for 10 min, 94 °C for 15		
	697		s, 50 °C for 1 min, 72 °C for		
tetQ-2	718–	GACTGATTCTGGAGGAAGTA	30 s, 35×, 72 °C for 1 min,		
	737		melting curve 72 °C to 95 °C		
ermF1	113–	TAGATATTGGGGCAGGCAAG	94 °C for 10 min, 94 °C for 15		
	132		s, 58 °C for 1 min, 72 °C for		
ermF2	271–	GGAAATTTCGGAACTGCAAA	30 s, 35×, 72 °C for 1 min 30		
	290		s, melting curve 72 °CX to 95		
			°C		

^a Counted from the first nucleotide of the start codons of the respective genes.

^b These primers could detect both *tetX* and *tetX2* genes.

Table 2

Resistance phenotypes of Bacteroides strains selected for detection of resistance

genes tetQ, tetX, tetX1 and ermF

Bacteroides strains	Resistance		Presence of resistance			
	phenotype	notype ^a		genes		
	TET	TIG	tetQ	tetX	tetX1	ermF
Tigecycline MIC 8–16 μg/mL						
<i>B. fragilis</i> (I 46)	R	R	+	-	+	+
<i>B. fragilis</i> (G 23)	R	R	+	+	+	+
<i>B. fragilis</i> (G 91)	R	R	+	-	+	+
<i>B. fragilis</i> (B26)	R	I	+	-	+	-
<i>B. fragilis</i> (H 2)	R	I	+	-	-	_
<i>B. vulgatus</i> (G 87)	R	I	+	-	+	_
<i>B. fragilis</i> (G 1)	R		+	-	+	_
<i>B. fragilis</i> (G 5)	R	1	+	-	+	_
<i>B. fragilis</i> (C 55)	R	I	+	+	+	+
<i>B. fragilis</i> (I 49)	R	I	+	_	_	_
<i>B. salyersae</i> (B 3)	R	I	+	-	-	_
<i>B. fragilis</i> (B 13)	R	I	+	-	+	-
Tigecycline MIC = 4 μ g/mL						
B. fragilis (S 10)	R	S	+	-	+	-
B. fragilis (H 80)	R	S	+	-	+	+
B. fragilis (C 23)	R	S	+	+	+	_
B. uniformis (H 30)	R	S	+	_	_	+
<i>B. fragilis</i> (H 51)	R	S	+	_	_	_
B. thetaiotaomicron (C 19)	R	S	+	-	+	_
<i>B. fragilis</i> (F 41)	R	S	+	_	+	+
<i>B. fragilis</i> (F 63)	R	S	+	-	+	-
Tigecycline MIC 0.064–2 μg/mL						
<i>B. fragilis</i> (B 25)	R	S	+	+	+	+
<i>B. ovatus</i> (H 12)	R	S	+	_	_	+
<i>B. fragilis</i> (H 13)	R	S	+	-	+	-

B. fragilis (H65)	R	S	+	_	+	_
<i>B. vulgatus</i> (H 46)	R	S	+	_	_	_
<i>B. ovatus</i> (H 68)	R	S	+	_	_	+
B. thetaiotaomicron (H 4)	I	S	+	_	+	_
<i>B. ovatus</i> (G 40)	I	S	+	_	_	+
<i>B. ovatus</i> (H 37)	I	S	+	-		-
Bacteroides sp. (H 96)	S	S	+	-	-	
Parabacteroides (B.) distasonis	S	S	-	+	+	+
(H 68)						
<i>B. fragilis</i> (H 73)	S	S	-	-	+	-
P. (B.) distasonis (H 56)	S	S	+	6-	-	+
B. thetaiotaomicron (C 47)	S	S	-	_	+	-
<i>B. fragilis</i> (B 15)	S	S		_	-	-

MIC, minimum inhibitory concentration.

^a MIC breakpoints for tetracycline and tigecycline: susceptible (S), $\leq 4 \mu g/mL$;

intermediate (I), 8 μ g/mL; resistant (R), \geq 16 μ g/mL.

Table 3

Presence of the genes in the examined strains with different minimum inhibitory

concentration (MICs) for tigecycline

Bacteroides strains with tigecycline MIC	No. (%) of strains harbouring the gene				
	tetX	tetX1	tetQ	ermF	
≥8 µg/mL (<i>n</i> = 12)	2 (16.7)	9 (75.0)	12 (100)	4 (33.3)	
4 μg/mL (<i>n</i> = 8)	1 (12.5)	6 (75.0)	8 (100)	3 (37.5)	
<4 µg/mL (<i>n</i> = 15)	2 (13.3)	7 (46.7)	11 (73.3)	6 (40.0)	