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High prevalence of division II (*cfiA* positive) isolates among blood stream *Bacteroides fragilis* in Slovenia as determined by MALDI-TOF MS

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ABSTRACT

Bacteroides fragilis can be classified into division I (*cfiA* negative) and division II (*cfiA* positive) isolates. Division II isolates have a silent chromosomal carbapenemase gene (*cfiA*) that can become overexpressed by an insertion of a mobile genetic element and thus develop a phenotypic resistance to carbapenems. Aims of our study were (i) to determine the prevalence of *B. fragilis* division II (*cfiA* positive) isolates among blood stream and non-blood stream isolates from two major Slovenian tertiary-care hospitals and (ii) to assess its influence on phenotypic resistance to imipenem. Consecutive non-duplicate *B. fragilis* isolates from blood stream and non-blood stream specimens were included in the analysis from 2015 to 2017 period. Data from laboratory information system were matched with mass spectra obtained with Microflex LT instrument and MALDI Biotyper 3.1 software (Bruker Daltonik, Bremen, Germany). All mass spectra were reanalyzed using Bruker taxonomy library. Spectra with a log(score) > 2.0 were further analyzed with *cfiA* library that separates *B. fragilis* division I and II isolates based on a log(score) value difference of >0.3. Minimal inhibitory concentrations (MICs) for imipenem were determined with Etest (bioMérieux, Marcy l'Étoile, France), using supplemented Brucella agar and EUCAST breakpoints (S ≤ 2 mg/L, R > 8 mg/L). Altogether 623 consecutive *B. fragilis* isolates were included in the analysis; 47 (7.5%) were isolated from blood stream and 576 (92.5%) from non-blood stream specimens. Among all study isolates, 51 (8.2%) proved to belong to division II (*cfiA* positive). The proportions of division II isolates among blood stream and non-blood stream isolates were 14.9% and 7.6%, respectively ($p = 0.081$, ns). In total, 1.3% (n = 8) were non-susceptible to imipenem (MIC >2 mg/L); 4.3% (n = 2) among blood stream and 1% (n = 6) among non-blood stream isolates. All imipenem resistant isolates belonged to division II. Modal MICs (MIC range) were 0.064 mg/L (0.016 mg/L-2 mg/L) and 0.125 mg/L (0.064 mg/L-≥32 mg/L) for division I and II isolates, respectively.

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1. Introduction

Bacteroides fragilis is an important human gut commensal bacterium which is also the single most common anaerobic isolate from clinical specimens and can cause a plethora of different human infections such as skin and soft tissue, intraabdominal infections and sepsis [1–3]. This organism is inherently poorly susceptible to a majority of β-lactam antibiotics but was until recently almost fully susceptible to β-lactam/β-lactamase inhibitor combinations and carbapenems [4,5]. It can be classified into division I (*cfiA* negative) and division II (*cfiA* positive) isolates.

The existence of carbapenem-resistant *B. fragilis* was first detected in the late 1980s, soon after the introduction of carbapenems into clinical practice [6,7]. These strains produced Zn^{+} dependent metallo-β-lactamase that was able to hydrolyze a majority of β-lactams, including cephamycins and carbapenems [6], and was coded by the chromosomally mediated *cfiA* (also known as *ccrA*) gene [8,9]. It is mostly regarded as a silent gene with a low level of constitutive expression, but its expression can be upregulated following the insertion of an insertion sequence (IS) element

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with an efficient promotor immediately upstream of the gene. This insertion is a one-step genetic event that can occur following antibiotic therapy with carbapenems and is considerably stable even without further selection pressure with carbapenems [10–12]. However, the IS element insertion can explain only about one half of high-level carbapenem resistance in *cfiA* positive strains while the other half may have other genetic background such as mutations leading to porin loss, upregulation of drug efflux, reduced affinity of penicillin-binding proteins or by some other less well characterized mechanisms [12,13]. Furthermore, it was shown that *cfiA* positive isolates belong to a distinct homology group of *B. fragilis* also referred to as division II isolates and that division I *B. fragilis* are *cfiA* negative [14,15]. These two groups of *B. fragilis* isolates are not formal taxonomic entities so far, however they may represent two distinct genospecies or may even represent two groups of *B. fragilis* isolates not yet separated at the species level [15]. Other chromosomal β-lactamases have been described in *B. fragilis*. CepA is the most widespread among them and confers resistance to penicillins and cephalosporins (except cephamycins). It is inhibited by several β-lactam inhibitors including clavulanic acid and tazobactam and is the reason why β-lactam/β-lactamase inhibitor combinations retain their activity against most *B. fragilis* isolates. CepA is mainly associated with division I isolates [14,15]. CfxA is a cephalosporinase that is most active against cephaloridine and can be found in several other *Bacteroides* species in addition to *B. fragilis* [16,17].

It is unclear whether there are any clinical consequences following an infection with specific division I or II of *B. fragilis*. Furthermore, there is a paucity of data regarding the epidemiology and antimicrobial resistance of *B. fragilis* in Slovenia and in general. Aims of our study were (i) to determine the prevalence of *B. fragilis* division II (*cfiA* positive) isolates among blood stream and non-blood stream isolates from two major Slovenian tertiary-care hospitals and (ii) to assess its influence on phenotypic antimicrobial resistance to imipenem.

2. Methods

2.1. Clinical setting and Bacteroides fragilis isolates

The study was performed at the Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, the largest Slovenian microbiology laboratory that serves bacteriological samples from two major tertiary-care hospitals in the area, (i) the University Medical Centre Ljubljana and (ii) the Institute of Oncology Ljubljana, comprising in total around 2400 beds.

The study period was 18 months long, from 07/2015 to 12/2017. From that period, we retrospectively extracted data for all *B. fragilis* isolates from the Institute's laboratory information system. The initial spreadsheet included 1163 isolates with basic patient demographic data, information on collection date, specimen type and antimicrobial susceptibility results, including the MIC values for 5 antimicrobial agents (penicillin, co-amoxiclav, imipenem, clindamycin and metronidazole). From this spreadsheet, we excluded all duplicate isolates, which were defined as isolates from the same patient and specimen type isolated within 30 days period and isolates with identification log(score) < 2.0. We further labeled all isolates from blood as *blood stream* and isolates from other specimen types, majority of which were from wounds and abdominal region, as *non-blood stream*. The final spreadsheet included 623 consecutive non-duplicate *B. fragilis* isolates.

2.2. Identification and antimicrobial susceptibility testing

All isolates were identified with MALDI-TOF mass spectrometry (MS), using Microflex LT instrument and MALDI Biotyper 3.1 software (Bruker Daltonik, Bremen, Germany). We recovered all *B. fragilis* spectra from the Institute's archive and reanalyzed them with (i) the regular taxonomy library and, furthermore, spectra that corresponded to *B. fragilis* with a log(score) > 2.0 with (ii) the "*cfiA* library" that separates *B. fragilis* division I and II isolates based on a log(score) value difference of >0.3, following the recommendations of the manufacturer. The "*cfiA* library", provided to us by the Bruker, has two entities (*cfiA* positive and *cfiA* negative) and, was validated previously [15]. The delineation between the two divisions of *B. fragilis* isolates thus involved manipulation and reanalysis of the initially obtained MALDI spectra.

Minimal inhibitory concentrations (MICs) were determined with Etests (bioMérieux, Marcy l'Étoile, France) using supplemented Brucella agar as previously described [3]. EUCAST breakpoints (version 7.1) for penicillin (S ≤ 0.25 mg/L, R > 0.5 mg/L), co-amoxiclav (S ≤ 4 mg/L, R > 8 mg/L), imipenem (S ≤ 2 mg/L, R > 8 mg/L), clindamycin (S ≤ 4 mg/L, R > 4 mg/L) and metronidazole (S ≤ 4 mg/L, R > 4 mg/L) were used for category interpretation. *B. fragilis* ATCC 25286 was used for quality control of the entire susceptibility setting on a weekly basis.

2.3. Molecular confirmation of cfiA gene and IS element

During the study period, we prospectively collected *B. fragilis* isolates with imipenem MIC ≥0.25 mg/L for further molecular characterization and thus for confirmation of MALDI-TOF MS *cfiA* typing results. Isolates were cryopreserved and transported to the Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary, for *cfiA* gene and activating IS element detection. These experiments were performed as previously described [15], except for *B. fragilis* ID 7, where the upstream activating IS was detected by inverse PCR. This procedure has three steps: (i) digestion of genomic DNA, (ii) ligation to form circular DNAs and (iii) PCR of the inversely ligated DNA. This inverse PCR was essentially carried out as described previously with the following specificities: *Bsu* RI restriction digestion of chromosomal DNA, I-cfiA-1 (5'-CCA-TACCCCATCCTTCGATT) and I-cfiA-2 (5'-ATTCACCGATTCACTGACCG) inverse primers and 94 °C 3 min; 94 °C 15 s, 56 °C 30 s, 72 °C 3 min, 35x; 72 °C 7 min inverse PCR cycles [26].

2.4. Statistical methods

Basic descriptive statistics was used to describe the sample parameters. Pearson Chi-square test was used for comparison of *cfiA* positivity prevalence between *blood stream* and *non-blood stream B. fragilis* isolates. SPSS Statistics version 23 (IBM Corporation, Armonk, New York, USA) and Microsoft Excel version 16 (Microsoft Corporation, Redmond, Washington, USA) were used for data manipulation and statistical analysis. Statistical significance was set to p-value of <0.05.

3. Results

Altogether, 623 consecutive non-duplicate *B. fragilis* isolates were included in the final analysis, belonging to 596 patients; mean age 66 years (standard deviation: 19 years, range: 0–99 years) of which 48.7% (n = 290) were females. 7.5% of the isolates (n = 47) were from blood stream specimens (blood cultures) and 92.5% (n = 576) were from non-blood stream specimens: 74.2% (n = 462) from wounds and 18.3% (n = 114) from abdominal specimens.

With regard to division I and II distribution, 91.8% (n = 572) of our *B. fragilis* isolates were division I (*cfiA* negative) and 8.2% (n = 51) were division II (*cfiA* positive). The proportions of division II isolates among blood stream and non-blood stream isolates were

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Table 1
B. fragilis isolates and phenotype distribution.

B. fragilis isolates	Number	Proportion	Division II[b]
	n	%	n (%)
Blood stream	47	7.5	7 (14.9)[c]
Non-blood stream	576	92.5	44 (7.6)[c]
Abdominal[a]	114	18.3	5 (4.4)
Wound[a]	462	74.2	39 (8.4)
Total	623	100	51 (8.2)

[a] Non-blood stream isolates.
[b] Division II (*cfiA* positive) based on MALDI-TOF MS typing.
[c] The difference between blood stream and non-blood stream (abdominal+wound) isolates is statistically non-significant ($p = 0.081$).

14.9% (n = 7) and 7.6% (n = 44), respectively. The difference was substantial; however, it did not reach statistical significance ($p = 0.081$). Table 1 shows the distribution of division II isolates among blood stream and non-blood stream *B. fragilis* isolates.

Antimicrobial susceptibility results are shown in Table 2. Non-susceptibility rates (i.e. resistant and intermediate category combined) among all isolates for penicillin, co-amoxiclav, imipenem, clindamycin and metronidazole were 99.8%, 8.3%, 1.3%, 16.7% and 0%, respectively. In the subgroup analysis, there was significant difference in clindamycin non-susceptibility rates between blood stream (29.8%) and non-blood stream (15.6%) isolates ($p = 0.012$) and significant difference in non-susceptibility rates among division I and II isolates for co-amoxiclav (6.8% vs 25.5%; $p < 0.001$) and imipenem (0% vs. 15.7%; $p < 0.001$), respectively. All imipenem resistant isolates belonged to division II. Modal imipenem MICs and MIC ranges were 0.064 mg/L and ≤0.016 mg/L-2 mg/L for division I isolates and 0.125 mg/L and 0.064 mg/L-≥32 mg/L for division II isolates. Fig. 1 shows relative MIC distribution for both division I and II isolates.

A selection of 30 non-duplicate *B. fragilis* isolates with imipenem MIC ≥0.25 mg/L was available for further molecular characterization and detection of *cfiA* gene and IS element, and thus confirmation of MALDI-TOF MS *cfiA* typing results. Among them, 24 isolates had MIC ≤2 mg/L and were thus susceptible, two isolates had MIC = 4 mg/L (intermediate) and four had MIC ≥32 mg/L (resistant). Seventeen isolates belonged to division II based on MALDI-TOF MS typing and all were confirmed to also harbor the *cfiA* gene. The concordance between MALDI-TOF MS typing and molecular detection of *cfiA* gene was 100% among the selected isolates. IS elements were detected in 2 of the imipenem resistant division II (*cfiA* positive) isolates. They were identified as IS*942* (after inverse PCR of *B. fragilis* ID 7, since the amplification of the upstream fragment of the *cfiA* gene gave two non-specific fragments) and IS*1187* (*B. fragilis* ID 232) following sequencing. In other 2 imipenem resistant isolates (ID 75 and ID 154), as well as in all other non-resistant isolates, we could not confirm the presence of IS elements. See Table 3 for details.

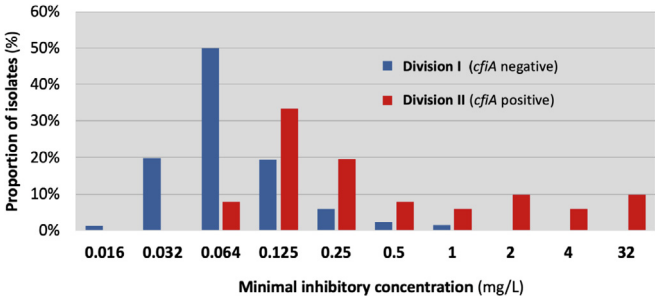

Fig. 1. Relative imipenem MIC distribution among division II (*cfiA* positive) and division I (*cfiA* negative) B. fragilis isolates (n = 623).

4. Discussion

In this study, we have successfully screened a large collection of routine *B. fragilis* isolates (n = 623) for phylogenetic division using MALDI-TOF MS. Overall, 8.2% prevalence of division II isolates was detected in the two largest Slovenian tertiary care hospitals with a marked difference in proportion of division II (*cfiA* positive) isolates between blood stream and non-blood stream specimens (14.9% vs. 7.6%; $p = 0.081$), respectively. All imipenem resistant isolates belonged to division II. One double dilution higher, but still in the susceptible range, modal imipenem MIC was observed among division II isolates. Imipenem and co-amoxiclav non-susceptibility were linked to division II isolates, while clindamycin non-susceptibility was more common among blood stream isolates. Perfect concordance between MALDI-TOF MS typing for division I/II discrimination and *cfiA* gene detection was confirmed in a subset of *B. fragilis* isolates from the study.

Sampling and microbiological technique have a major influence on the isolation of anaerobic bacteria from clinical samples - including *B. fragilis* [18]. For the purpose of anaerobic bacteremia detection, we have used BACTEC Lytic blood culture bottles (Becton Dickinson, Sparks, USA), and for the isolation of anaerobes from other relevant specimen types (i.e. skin and soft tissue and abdominal infections), we have used two anaerobic agar media in combination with thioglycolate broth, namely Schaedler agar (non-selective) and Schaedler Neo. Vanco. agar (selective) (bioMerieux, Marcy-l'Étoile, France, both). A minimum of 48 h and 5 days anaerobic incubation was used for non-blood culture specimens and blood cultures, respectively. All isolates were identified using MALDI Biotyper 3.1 (Bruker Daltonik, Bremen, Germany) and tested for antimicrobial susceptibility as previously described [3]. Thus, we can be sure that the selection of isolates used in this study is representative of *B. fragilis* infections in our clinical setting.

In order to identify and differentiate between division I and II *B. fragilis*, we have used a technique described by Nagy and Wybo that is based on MALDI-TOF MS and uses specific main spectra

Table 2
Antimicrobial susceptibility data among all B. fragilis isolates and among two subgroups: (i) blood stream vs. non-blood stream isolates and (ii) division II vs. division I isolates.

Isolates	All			Blood stream	Non-blood stream	*p*-value	Division II (*cfiA* pos)	Division I (*cfiA* neg)	*p*-value
	(n = 623)			(n = 47)	(n = 576)		(n = 51)	(n = 572)	
Antibiotics	S (%)	I (%)	R (%)	NS (%)	NS (%)		NS (%)	NS (%)	
Penicillin	1 (0.2)	0 (0)	622 (99.8)	47 (100)	(99.8)	0.775	51 (100)	571 (99.8)	0.765
Co-amoxiclav	571 (91.7)	12 (1.9)	40 (6.4)	7 (14.9)	45 (7.8)	0.091	13 (25.5)	39 (6.8)	**<0.001**
Imipenem	615 (98.7)	3 (0.5)	5 (0.8)	2 (4.3)	6 (1)	0.060	8 (15.7)	0 (0)	**<0.001**
Clindamycin	519 (83.3)	0 (0)	104 (16.7)	14 (29.8)	90 (15.6)	**0.012**	6 (11.8)	98 (17.1)	0.325
Metronidazole	623 (100)	0 (0)	0 (0)	0 (0)	0 (0)	n/a	0 (0)	0 (0)	n/a

S susceptible, **I** intermediate, **R** resistant, **NS** non-susceptible (I+R isolates).
n/a not applicable, **pos** positive, **neg** negative.

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Table 3
Concordance between MALDI-TOF MS typing for division I/II discrimination and cfiA gene detection among a selection of n = 30 isolates with imipenem MIC ≥0.25 mg/L.

ID	MIC (imipenem)	MALDI-TOF typing	*cfiA* gene	IS element[a]
	(mg/L)	(division I/II)	(±)	(±)
7	32	II	+	+ (IS942)
75	32	II	+	−
154	32	II	+	−
232	32	II	+	+ (IS1187)
134	4	II	+	−
220	4	II	+	−
100	2	II	+	−
226	2	II	+	−
230	2	II	+	−
190	1	II	+	−
115	0.5	II	+	−
208	0.5	II	+	−
222	0.5	II	+	−
102	0.5	II	+	−
86	0.25	II	+	−
103	0.25	II	+	−
217	0.25	II	+	−
108	1	I	−	nt
228	1	I	−	nt
81	0.5	I	−	nt
147	0.5	I	−	nt
166	0.5	I	−	nt
201	0.5	I	−	nt
91	0.25	I	−	nt
93	0.25	I	−	nt
135	0.25	I	−	nt
227	0.25	I	−	nt
233	0.25	I	−	nt
125	0.25	I	−	nt
213	0.25	I	−	nt

nt not tested.
[a] IS insertion sequence element.

library for division discrimination [15,19]. This technique has been proven in several studies to be perfectly concordant with traditional molecular detection of *cfiA* gene [19–21,24]. It is fast, non-costly, can be applied in *real-time* following bacterial identification and in different diagnostic settings, including as a direct identification from positive blood cultures bottles [24]. We have used this technique on a prospectively collected mass spectra saved in our database with a batch analysis and thus in a high-throughput manner. However, we also prospectively collected *B. fragilis* isolates with imipenem MIC ≥0.125 mg/L for a molecular confirmation of *cfiA* gene and IS element in upstream region position and for validation of the entire dataset.

The overall 8.2% prevalence rate of division II (*cfiA* positive) isolates was expected and is in concordance with other studies from the same geographic area [20,22,23,25]. However, it is difficult to compare those proportions as it is not clear what the published selections of isolates represent (i.e. consecutive vs. selected isolates) and furthermore it has been obtained from a limited number of isolates. Our collection is among the largest published so far (n = 623) and represents consecutive and non-duplicate isolates [26].

Our main question in this study was whether there was any difference in the prevalence of *cfiA* positivity between blood stream and non-blood stream isolates. We found a trend that *cfiA* positive *B. fragilis* are more readily isolated from blood stream samples (14.9% vs 7.6%), yet the difference failed to reach significance ($p = 0.081$). This is in the contrast to the Hungarian study [19], where they surprisingly found no *cfiA* positive blood culture isolates. Other studies did not report on the proportion differences.

Our phenotypic antimicrobial susceptibility results are in line with our previously published data from 2015 [3]. However, it was very interesting to see the influence of either (i) specimen type or (ii) division I/II type on the cumulative resistance of *B. fragilis* for various tested antibiotics. A major difference in non-susceptibility to imipenem and co-amoxiclav was found among division I/II isolates which can be explained, among other reasons, by the fact that division II isolates have *cfiA* gene which is not always expressed and, that this metallo-β-lactamase is not inhibited by clavulanic acid. These results should be read with an empirical antibiotic therapy in mind; it may not be safe to treat division II isolates with imipenem and possibly other carbapenems, but it may be even less safe to empirically treat them with co-amoxiclav since approximately one quarter of them are non-susceptible. Interestingly, we have also found almost 2-times higher proportion of clindamycin non-susceptibility among blood stream *B. fragilis* (29.8% vs. 15.6%). This leaves us with extremely narrow options for empiric therapy of *B. fragilis* bacteremia/sepsis where possibly only metronidazole would be acceptable empiric treatment option out of the five antibiotics included in this study.

Several limitations of our study should be outlined. This was a retrospective analysis based on microbiological data only. We do not have any information on either clinical parameters or antimicrobial therapy of the patients. Most importantly, we have no data regarding carbapenem therapy, which is a known selection factor for expression of *cfiA* gene in division II isolates.

Nevertheless, several implications can be outlined from the results. First, we have confirmed, in the subset of isolates, that division I/II typing is reliable with MALDI-TOF MS and that we can in this way reliably identify *B. fragilis* with silent *cfiA* gene almost simultaneously with species identification. Second, even though there is still paucity of data that therapy with carbapenems is not indicated for division II *B. fragilis*, it is most probably safe to say that clinicians should be at least careful when treating those infections with carbapenems even if the isolates are phenotypically susceptible and that follow up cultures are warranted when they do so. Third, we would discourage empiric therapy with co-amoxiclav or carbapenems for division II isolates at least in our clinical setting.

In conclusion, a trend towards a higher proportion of division II (*cfiA* positive) isolates in blood stream specimens as opposed to non-blood stream specimens was detected (14.9% vs. 7.6%) in Slovenia. One double-dilution higher modal imipenem MIC was noticed among division II isolates; however, no clinical implications can be made. Empiric therapy for division II isolates should be locally adjusted and should not include imipenem and possibly also other carbapenems or co-amoxiclav. Further studies focusing on clinical consequences of infections caused by *B. fragilis* division II strains are needed but until then, rapid identification and careful treatment approach, especially for sepsis cases, is warranted.

Ethical approval and informed consent

Our work did not include any experiments on humans or animals.

Conflicts of interest

Nothing to declare.

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