Instant screening and verification of carbapenemase activity in *Bacteroides fragilis* in positive blood culture, using matrix-assisted laser desorption ionization–time of flight mass spectrometry

Åsa Johansson,[1] Elisabeth Nagy[2] and József Sóki[2]

[1]Department of Clinical Microbiology, Växjö, Sweden

[2]Institute of Clinical Microbiology, Faculty of General Medicine, University of Szeged, Szeged, Hungary

Correspondence
Åsa Johansson
asa.c.johansson@ltkronoberg.se

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Rapid identification of isolates in positive blood cultures are of great importance to secure correct treatment of septicaemic patients. As antimicrobial resistance is increasing, rapid detection of resistance is crucial. Carbapenem resistance in *Bacteroides fragilis* associated with *cfiA*-encoded class B metallo-beta-lactamase is emerging. In our study we spiked blood culture bottles with 26 *B. fragilis* strains with various *cfiA*-status and ertapenem MICs. By using main spectra specific for *cfiA*-positive and *cfiA*-negative *B. fragilis* strains, isolates could be screened for resistance. To verify strains that were positive in the screening, a carbapenemase assay was performed where the specific peaks of intact and hydrolysed ertapenem were analysed with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). We show here that it is possible to correctly identify *B. fragilis* and to screen for enzymic carbapenem resistance directly from the pellet of positive blood cultures. The carbapenemase assay to verify the presence of the enzyme was successfully performed on the pellet from the direct identification despite the presence of blood components. The result of the procedure was achieved in 3 h. Also the Bruker mass spectrometric β-lactamase assay (MSBL assay) prototype software was proven not only to be based on an algorithm that correlated with the manual inspection of the spectra, but also to improve the interpretation by showing the variation in the dataset.

INTRODUCTION

Today it is obvious that matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is an impressive technique for species identification, and it has been fully accepted and introduced in many microbiology laboratories all over the world. It has revolutionized routine work by its rapidness, and the fast recognition of bacteria directly from positive blood cultures is an application proving to be a very useful tool (Martiny *et al.*, 2012). When treating patients with septic shock, a delay in implementing the appropriate antimicrobial treatment will considerably reduce the average survival of the patient (Kumar *et al.*, 2009). Martiny *et al.* (2013) showed in their study that the introduction of direct identification (direct ID) from blood cultures led to modification of therapy in 13.38 % of adult patients, and in 37.50 % of paediatric patients a suspected contaminant could be confirmed.

When performing species identification with MALDI-TOF MS, a unique spectrum for that particular strain is generated, and that spectrum might contain more information other than species identity. There are several publications showing this type of reanalysis or subtyping (Griffin *et al.*, 2012; Josten *et al.*, 2013; Nagy *et al.*, 2013). Nagy *et al.* (2011a) showed the possibility to separate *Bacteroides fragilis* strains that carry the *cfiA* gene from those that do not. By generating *cfiA*-specific main spectra (MSP), strains can easily be screened for the possible presence of a beta-lactamase.

The activity of the carbapenemase can be verified with a hydrolysis assay performed with MALDI-TOF MS, and we have published a successful assay using ertapenem as a substrate for carbapenemase producing *B. fragilis* (Johansson *et al.*, 2014). Recently Jung *et al.* (2014) published a paper showing the possibility to perform hydrolysis assay from the

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; MSBL assay, mass spectrometric β-lactamase assay; MSP, main spectra.

pellet being generated when performing direct ID from blood cultures.

In this paper we are focusing on *B. fragilis*, which is a strictly anaerobic rod found in the gut. It carries several virulence factors and members of the *B. fragilis* group are the most frequent anaerobes found in blood cultures (Lombardi & Engleberg, 1992). Antimicrobial resistance or reduced susceptibility has for a long time been considered rare in *B. fragilis*, but the reported numbers of resistant strains are growing (Nagy *et al.*, 2011b). Multidrug resistant (MDR) *B. fragilis* has been reported (Sherwood *et al.*, 2011; Kalapila *et al.*, 2013), and a study from Justesen *et al.* (2013) showed that 10.2 % of *B. fragilis* strains in blood cultures from several Danish hospitals carried the *cfiA* gene, which encodes a carbapenemase. Since antimicrobial testing of anaerobes is more time-consuming and often more expensive, a rapid and accurate method to detect carbapenemase production in these strains is of great importance.

We present here an optimal scenario where *B. fragilis* is rapidly identified from a positive blood culture and instantly screened for the presence of *cfiA*-gene within an hour, followed by the verification of carbapenemase activity, by performing an ertapenem hydrolysis assay in less than 3 h.

METHODS

Twenty-six isolates of *B. fragilis* were analysed (see Table 1). All strains were examined for the presence or absence of the *cfiA* gene by end point PCR (previously described by Sóki *et al.*, 2004a, 2006). The set-up consisted of 15 *cfiA*-positive and 11 *cfiA*-negative isolates with various ertapenem MICs. To confirm correct species all isolates were cultivated on fastidious anaerobic agar (LAB-M) overnight in 35 °C in an anaerobic environment and identified to species level using the Microflex and the MALDI Biotyper 3.1 software (Bruker Daltonics) according to the manufacturer's instructions. A score value of $\geqslant$2.0 was considered as a secure species level identification. Susceptibility testing was performed for ertapenem using Etest (bioMérieux) and results interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. Carbapenemase production was verified with spectrophotometric assay (Sóki *et al.*, 2006).

Blood culture and direct identification (direct ID). To simulate a true blood culture, with all blood components that might disturb the analysis, BD BACTEC Plus/Anaerobic/F bottles (Becton Dickinson) were inoculated with 5 ml of human blood (outdated blood components donated from Transfusion medicine at Central hospital Växjö) and 10 µl of bacterial suspension of McFarland 0.5 in 0.9 % NaCl. Bottles were incubated in a BD BACTEC FX (Becton Dickinson) until positive signalling from the system was obtained. As control, a bottle containing blood without bacteria was used and incubated for 24 h. Direct identification was performed according to the published protocol by Martiny *et al.* (2013) with the modification of adding 1 µl of 70 % formic acid on the spotted target plate before applying 1µl HCCA matrix (2.5 mg α-cyano-4-hydroxycinnamic acid dissolved in 50 % acetonitril, 47.5 % HPLC-pure H_2O and 2.5 % trifluoroacetic acid; Bruker Daltonics) and left to dry. Identification was performed according to the manufacturer's instructions in the MALDI Biotyper 3.1 software (Bruker Daltonics). Scores generated from the direct ID are presented in Tables 1 and 2.

Screening for cfiA-positive isolates. Each of the spectra generated at identification was uploaded in the MALDI Biotyper OC software (Bruker Daltonics) and blasted against the previously published *cfiA*-positive and *cfiA*-negative MSP (Nagy *et al.*, 2011a). Score values from this BLAST are presented in Tables 1 and 2. Getting the *cfiA*-positive MSP as first best match and with a log score difference of >0.3 to the second best match was sufficient to consider the isolate *cfiA*-positive in the screening.

Ertapenem hydrolysis assay. The pellet generated when performing direct ID was resuspended in 20 µl 10 mM ammonium hydrogen citrate buffer (pH 7.1) dissolved in water (Sigma-Aldrich) containing 0.05 mg ertapenem ml^{-1} (Invanz, MSD). As controls, ertapenem-containing (0.05 mg ml^{-1}) cell-free buffer solutions were applied (ertapenem only), as well as pellets generated from bacteria-free blood-containing blood culture bottles (ertapenem and blood). All suspensions were incubated at 35 °C on a shaker for 2 h. After incubation, the suspensions were centrifuged at 13 400 ***g*** for 2 min. Two microlitres of the supernatant were spotted on a polished steel target plate (Bruker Daltonics), left to dry and then overlaid with 1 µl HCCA matrix (Bruker Daltonics).

All measurements were performed on a Microflex (Bruker Daltonics) mass spectrometer. The parameter settings were: ion source 1, 19.0 kV; ion source 2, 17.2 kV; lens, 6.0 kV; detector gain, 2.5 kV. Spectra were recorded in the mass range of 0–1000 Da with 60 Hz laser frequency. Each spectrum was obtained from 240 laser shots. For calibration, the peptide calibration standard II (Bruker Daltonic) was used. The peaks employed for calibration were the HCCA peaks $[M+H]^+$ at 190.05 Da, $[2M+H]^+$ at 379.09 Da and the bradykinin (1–7) peak $[M+H]^+$ at 757.40 Da.

The analysis of MALDI-TOF MS spectra was performed using the Flexanalysis 3.3 software (Bruker Daltonics). The spectra were smoothed and baseline subtracted and then manually examined for the specific ertapenem related peak patterns in the mass range of 4–600 Da. The hydrolysis spectra were also tested against the prototype software developed by Bruker Daltonics.

RESULTS AND DISCUSSION

Since blood components might disturb the identification as well as the hydrolysis assay, we chose to inoculate the blood culture bottles with human blood, to simulate a true blood culture. The spiked blood cultures were incubated in the BD BACTEC FX and became positive in the range of 12–30 h. Bottles were then removed from the BACTEC FX at various time points resulting in different total incubation times. There was no correlation with log score value from direct ID and incubation time in this set-up.

Direct ID from positive blood cultures by MALDI-TOF MS is routinely performed at the Department of Clinical Microbiology at Central hospital in Växjö, and the spiked bottles were treated as any other positive blood culture according to the previously described protocol (Martiny *et al.*, 2013). Score values from direct ID of the *B. fragilis* strains are presented in Tables 1 and 2, and ranged between 1.865 and 2.414. A local algorithm allows score values >1.7 to be considered as secure level species identification when performing direct ID; however 24 of 26 *B. fragilis* strains had a score value >2, which is considered a secure level for species identification by the assay manufacturer. This high score value despite the disturbing blood components fits

Table 1. *cfiA*-positive *B. fragilis* strains used in the study with direct ID scores from positive blood cultures, *cfiA*-specific MSP BLAST scores and hydrolysis assay results

Increased MIC values (due to growth of micro-colonies in the ellipse) after 48 h incubation are shown in parentheses.

B. fragilis	PCR detection of *cfiA* gene	IS element*	Direct ID (log score)	*cfiA* + specific MSP (log score)	*cfiA* – specific MSP (log score)	Ertapenem hydrolysis	Ertapenem MIC (mg l^{-1})	Reference
TAL3636	+	IS942	2.199	2.396	1.621	+	>32	Rasmussen *et al.* (1990)
1672	+	IS1186	2.12	2.39	1.792	+	>32	Sóki *et al.* (2004a)
2944	+	IS614B	2.022	2.046	1.782	+	>32	Sóki *et al.* (2004a)
1776	+	IS1187	2.034	2.239	1.397	+	>32	Sóki *et al.* (2004a)
2685	+	IS614B	2.04	2.25	1.289	+	>32	Sóki *et al.* (2004a)
4729	+	IS1187	2.142	2.147	1.312	+	>32	Sóki *et al.* (2004a)
6712	+	IS612B	2.163	2.314	1.205	+	>32	Sóki *et al.* (2004a)
16997	+	–	2.129	2.272	1.56	+	16	Sóki *et al.* (2004a)
388	+	–	2.027	2.112	1.493	+	2	Sóki *et al.* (2004a)
BF8 (BFr81)	+	–	2.173	2.281	1.554	+	4	Podglajen *et al.* (1992)
72	+	–	2.169	2.262	1.703	+	2	Nagy *et al.* (2001)
55474/1	+	–	1.995	2.188	1.76	+	2	Johansson *et al.* (2014)
22	+	–	2.239	2.298	1.889	+	2	Nagy *et al.* (2001)
AA-137-TH	+	–	1.865	1.826	1.431	+	2 (32)	Johansson *et al.* (2014)
12-538566	+	–	2.115	2.338	1.539	+	1 (8)	Johansson *et al.* (2014)

*IS element upstream of the *cfiA* gene.

Table 2. *cfiA*-negative *B. fragilis* strains used in the study with direct ID scores from positive blood cultures, *cfiA*-specific MSP BLAST scores and hydrolysis assay results

B. fragilis	PCR detection of *cfiA* gene	Direct ID (log score)	*cfiA* + specific MSP (log score)	*cfiA* − specific MSP (log score)	Ertapenem hydrolysis	Ertapenem MIC (mg l^{-1})	Reference
638R	−	2.254	1.726	2.259	−	0.125	Nagy *et al.* (2001)
43	−	2.336	1.614	2.291	−	0.125	Johansson *et al.* (2014)
363	−	2.123	1.732	2.378	−	0.125	Johansson *et al.* (2014)
51981/1	−	2.414	1.485	2.449	−	0.25	Johansson *et al.* (2014)
38860/1	−	2.215	1.472	2.365	−	0.5	Johansson *et al.* (2014)
56001/2	−	2.336	1.505	2.317	−	0.125	Johansson *et al.* (2014)
38360/2	−	2.388	1.525	2.465	−	0.5	Johansson *et al.* (2014)
664	−	2.189	1.638	2.252	−	0.25	Johansson *et al.* (2014)
480	−	2.117	1.486	2.163	−	0.125	Johansson *et al.* (2014)
461	−	2.065	1.476	2.163	−	0.125	Johansson *et al.* (2014)
76240	−	2.102	1.5	2.234	−	0.5	Johansson *et al.* (2014)
Blood only	NA	1.299	NA	NA	−	NA	This study

NA, Not analysed.

well with a local evaluation of direct ID of rapidly growing Gram-negative rods (data not shown). The direct ID performed from the bacteria-free blood-containing blood culture bottle generated a score value of 1.299 proving that blood components give rise to spectra that might have a negative impact on generated spectra from direct ID. However, in this particular case, the score values from the tested *B. fragilis* strains were undisputable.

Immediately after direct ID the generated ID-spectra were uploaded in the Biotyper OC software and blasted against the two *cfiA*-specific MSPs already described in the paper from Nagy *et al.* (2011a). All the *cfiA*-positive strains were correctly assigned to the *cfiA*-positive MSP as first best match with score values ranging between 1.826 and 2.396 (Table 1). Naturally, the *cfiA*-negative MSP were assigned as second best match with score values ranging between 1.205 and 1.889 (Table 1). The log score difference between first and second best match ranged between 0.4 and 1.11. All the *cfiA*-negative strains were correctly assigned to the *cfiA*-negative MSP as first best match with score values ranging between 2.163 and 2.465 (Table 2). As second best match, the *cfiA*-positive MSP were presented with score values ranging between 1.472 and 1.732 (Table 2). The log score difference between first and second best match ranged between 0.53 and 0.96. This simple and fast reanalysis of generated spectra acts as a screening of the strains and made it possible to sort out potential carbapenemase producers. At this stage it would also be a great advantage to alert the clinician about the possible presence of a carbapenemase producing *B. fragilis* in positive blood cultures.

To verify the positive screening results, the presence of carbapenemase needed to be proven. All strains were tested in a hydrolysis assay with MALDI-TOF MS where the intact or hydrolysed forms of ertapenem were studied. Initially the strains were tested according to our published protocol using an ertapenem concentration of 0.5 mg ml^{-1} (Johansson *et al.*, 2014). However this concentration was probably too high compared with the amount of bacteria in the direct ID pellet since only partial hydrolysis was seen after 2 h incubation (data not shown). All strains were then tested with a 10-fold lower concentration, 0.05 mg ml^{-1}, and hydrolysis was clearly seen in all the *cfiA*-positive strains by the presence of the following peak pattern (m/z): 450.5, 472.5, 494.5, 516.5 and 438.5 Da, proving the activity of the carbapenemase. No hydrolysis was seen in the *cfiA*-negative strains or blood only, which was proven by the presence of intact ertapenem with the following peak pattern (m/z): 476.5, 498.5, 520.5 and 542.5 Da.

Notable is the fact that some of the *B. fragilis* strains had a quite low MIC for ertapenem (MIC 2 mg l^{-1}) but showed hydrolysis of ertapenem in the hydrolysis assay. After prolonged incubation (48 h) of the Etests, the MIC increased due to micro-colonies in the ellipse. The presence of similar micro-colonies has been described by Sóki *et al.* (2004b). In Table 1, these variable MICs are shown in parentheses.

Fig. 1. Results of the ertapenem hydrolysis assay calculated according to the logRQ algorithm used in the mass spectrometric β-lactamase assay (MSBL assay) prototype software from Bruker Daltonics, and correlated with the MIC of ertapenem. LogRQ=log (sum of hydrolysed peak intensities)/(sum of non-hydrolysed peak intensities).

The hydrolysis spectra were manually and qualitatively evaluated, giving a result of hydrolysis or no hydrolysis. However, the spectra were also tested with prototype software from Bruker Daltonics where the spectra were calculated with the following formula logRQ=log (sum of hydrolysed peak intensities)/(sum of non-hydrolysed peak intensities). This approach visualized a much more dynamic result of the hydrolysis assay (Fig. 1). The *cfiA*-negative strains (n=11) as well as blood only and ertapenem only ended up with a logRQ <0.25, which clearly separated them from the other strains, comparable with manual examination of the spectra. But within the *cfiA*-positive population there was a separation that correlated with the MICs. Strains (eight isolates) with lower MICs (2–16 mg l^{-1}) had lower logRQ values (between 0.25 and 0.75), which means slower hydrolysis. All strains (seven isolates) with MICs >32 mg l^{-1} had the highest logRQ values (>0.75), with one exception. Strain 1776 showed a lower logRQ, comparable with strains with lower MICs. When repeating the experiment, the strain ended up with a similar logRQ score. One can only hypothesize that this strain might have a porin defect that prevents ertapenem from entering into the periplasmatic space, lowering the logRQ value. The software prototype was only tested with this small collection of strains, and it is difficult to fully draw any conclusions regarding the dynamics within the *cfiA*-positive population, even though it was repeatable with these particular strains.

To summarize, the method presented here not only allows the direct ID of the *B. fragilis* strains from positive blood cultures, but combined with of the use of the MALDI Biotyper OC software and the ertapenem hydrolysis assay with manual or automated evaluation, but also can very rapidly answer the question of whether carbapenems can be used for the treatment of an infection leading to sepsis.

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