

RESEARCH ARTICLE

Study on carbapenemase-producing bacteria by matrix-assisted laser desorption/ionization approach

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Abstract

The development of new techniques for the detection of carbapenemase activity is of great importance since the increased incident of resistance against carbapenems represents a serious threat to global public health. In this context, the **matrix-assisted laser desorption/ionization approach** already demonstrated to be a reliable tool for rapid carbapenemase detection. As a newly developed test, there is still a lack of in-depth analysis of its robustness and possible wider application. The main goal of this study was to evaluate the potential for using the design MBT STAR-Carba assay as the pre-characterization method for *Enterobacteriales* and *P. aeruginosa* strains in terms of the produced classes of carbapenemases using modified procedure parameters—various suspension densities and incubation times. Moreover, its usefulness for the in-depth analysis and characterization of metallo- β -lactamases (MBL) was tested by applying inhibition assays. In this study, the designed assay proved to be a sensitive tool for the detection of carbapenemase hydrolytic activity, which can be successfully used to partially classify the class of carbapenemase present. Additionally, the use of defined high concentration suspensions would allow to shorten the incubation time to 1 minute for certain strains. Considering that the assay was also suitable to investigate the effect of different inhibitors on the MBL activity, it demonstrates far higher discriminatory potential than only a rapid routine carbapenemase detection tool and could be used as a susceptibility assay.

Introduction

The increasing prevalence of drug-resistant bacteria is a serious threat to human health and life around the world. Carbapenems are amongst the latest developed β -lactam antibiotics still possessing a broad spectrum of bactericidal activity, therefore often used as last resort medication for the treatment of severe bacterial infections [1–3]. Therefore, the global rise of carbapenemase-producing bacteria (CPB) incidence represents an increasing threat to the healthcare system and patient safety and is of great concern worldwide [4,5]. Reported increase in resistance to carbapenems among *Enterobacteriales* as well as *Pseudomonas aeruginosa* isolates is

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linked to their high consumption during treatment of infections caused by extended-spectrum- β -lactamases (ESBLs) harboring microorganisms [6,7].

Although carbapenem resistance can occur in several ways, enzymatic hydrolysis of an antibiotic (carbapenemase expression) is considered of more clinical concern since CPB demonstrate the greatest potential for rapid dissemination within hospital settings via horizontal transfer of the carbapenemase-encoding genes as well as clonal expansion [8,9]. Regarding Ambler classification, carbapenemases are classified into 3 main groups—class A serine β -lactamases mostly represented by KPC (*Klebsiella pneumoniae* carbapenemase), class B metallo- β -lactamases—MBL (e.g. NDM-, VIM-, IMP-, and GIM-types) as well as class D, another serine β -lactamase class, including OXA-48 group like OXA-48, OXA-162, or OXA-181 [10].

Detection of carbapenemases is still challenging for clinicians since interpretative criteria recommended by the EUCAST or the Clinical and Laboratory Standards Institute (CLSI) lead to nearly 20% of false negative results in classic antibiotic susceptibility testing using breakpoint values for carbapenems even with low screening cut-offs [1,10]. Therefore, various phenotypic tests for detection of carbapenemase producers have been developed, e.g. Carbapenem Inactivation Method, gradient MIC strips (Etest) or combined disc inhibitory tests for carbapenemase activity [11–13]. Although growth-based phenotypic tests are inexpensive and easy to perform, they, nevertheless, require additional incubation thus significantly extending the time needed to receive results to 24–72 hours and very often are characterized by lack of sensitivity and specificity [10,14]. These drawbacks could be overcome using molecular methods such as targeted PCR assays receiving results within a few hours (<4 h); however, with higher costs, the need for significant expertise along with the limitation to a predefined range of known carbapenem-resistance genes [3,13].

In view of the mentioned drawbacks, rapid biochemical assays based on carbapenem-hydrolyzing activity detection are considered as the most powerful methods which facilitate the indication of the presence of all classes and variants of carbapenemases, including the rarest ones, with high specificity and sensitivity [15,16]. Recently, special attention has been paid to the use of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), as an approach that shortens the time-to-result for antibiotic susceptibility testing [17]. This technique proved to produce also reliable same-day results for detecting β -lactamase activity [18,19]. So far, several publications have demonstrated the feasibility of MALDI-TOF MS analysis to detect carbapenemase activity [20–23]. They relate to the data analysis performed based on the manual calculations and self-developed algorithms for peak patterns investigation, thus difficult to implement in routine workflows.

Currently, MBT STAR-Carba assay is extensively used as a fast qualitative test of the carbapenemases presence. Therefore, the main goal of this study was to evaluate the potential for using the design MBT STAR-Carba assay also as the pre-characterization method of the bacterial strains in terms of the produced classes of carbapenemases using modified procedure parameters, here: different bacterial suspension densities and various incubation times. In total twelve (12) different *Enterobacterales* and three (3) *Pseudomonas aeruginosa* strains harboring various classes of carbapenemases (A, B, D) were investigated using both manufacturer's and modified test protocols to compare results of antibiotic hydrolysis activity and resulting strain classification. Moreover, β -metallo-carbapenemase inhibitors were used to evaluate the usefulness of MBT STAR-Carba test as a susceptibility assay.

Materials and methods

Bacterial strains

For this study, 12 *Enterobacterales* and 3 *Pseudomonas aeruginosa* strains (15 in total) belonging to the bacterial collection of the Department of Application Development Microbiology &

Table 1. List of investigated carbapenemase-producing bacteria with marked type of β -lactamases and hydrolytic activity value obtained using routine protocol of MBT STAR-Carba assay.

Strain	Type of β -lactamases	Ambler class*	Activity value (According to MBT STAR-Carba Kit protocol)
<i>P. aeruginosa</i> 1	GES-5, GES-20	A	0.43
<i>K. oxytoca</i> 2	KPC 3	A	1.09
<i>E. coli</i> 3	KPC 3	A	1.03
<i>P. aeruginosa</i> 4	IMP	B	0.87
<i>P. aeruginosa</i> 5	GIM 1	B	0.51
<i>K. pneumoniae</i> 6	GIM	B	1.18
<i>E. coli</i> 7	NDM-1	B	1.03
<i>C. freundii</i> 8	GIM	B	1.00
<i>C. freundii</i> 9	VIM	B	0.98
<i>Salmonella</i> sp. 10	IMP-4	B	1.07
<i>E. kobei</i> 11	IMP-22	B	0.96
<i>K. pneumoniae</i> 12	NDM 1, SHV-28, TEM-1, CTX-M-15, CMY 6, OXA-1, rmtC	B/C	0.94
<i>K. pneumoniae</i> 13	OXA-48	D	1.02
<i>S. marcescens</i> 14	OXA-1, OXA-181	D	1.03
<i>E. cloacae</i> 15	OXA-48	D	0.96
<i>E. coli</i> ATCC 25922 (Negative control)			-0.11
<i>K. pneumoniae</i> ATCC BAA-1705 (Positive control)			1.06

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Diagnostics, Bruker Daltonik GmbH in Bremen (Germany), were used. All strains were previously characterized as β -lactamase-producing bacteria (BLPB) using validated routine PCR methods (TaqMan-probe realtime-PCR using the KAPA™ PROBE FAST qPCR MasterMix Universal (peqlab), Primer and TaqMan-Probes (both metabion) using an ABI 7500 Fast System) and showed the presence of different classes and types of β -lactamases (BLs) coding genes—including Ambler class A & D serine carbapenemases as well as class B β -metallo-carbapenemases (Table 1).

Culture conditions

For each analysis, bacterial isolates grown overnight on BD™ Columbia Agar on 5% Sheep Blood plates (Becton Dickinson, Germany) incubated at 35 ($\pm 2^\circ\text{C}$) under aerobic conditions were used. To obtain single bacterial colonies from primary cultures, the quadrant streaking technique was applied.

MBT STAR-Carba standard assay

For testing BLs activity in standard mode, the MBT STAR-Carba assay was performed according to the manufacturer's instructions provided with MBT STAR-Carba IVD Kit (Fig 1A).

For each sample and controls, one inoculation loop (1 μL) filled with sufficient material of bacterial colonies was suspended in the antibiotic solution (MBT STAR-Carba Antibiotic Reagent dissolved in 50 μL MBT STAR Buffer) and then incubated for 30 min. at 35 $\pm 2^\circ\text{C}$ with shaking (900 rpm) using a Thermomixer (Eppendorf AG, Germany). Subsequently, 1 μL of each sample was deposited on the MALDI Biotarget 96 (Bruker Daltonics) in duplicate, air-dried, and overlaid with 1 μL of MBT STAR Matrix. For each analyzed MALDI Biotarget 96 prepared, one spot with 1 μL MBT STAR-ACS (antibiotic calibration mass standard containing dedicated masses below $m/z = 1000$) as well as negative (dedicated β -lactam non-hydrolyzing quality control strain—*E. coli* ATCC 25922) and positive controls (dedicated β -lactam hydrolyzing quality control strain—*K. pneumoniae* ATCC BAA-1705) were used.

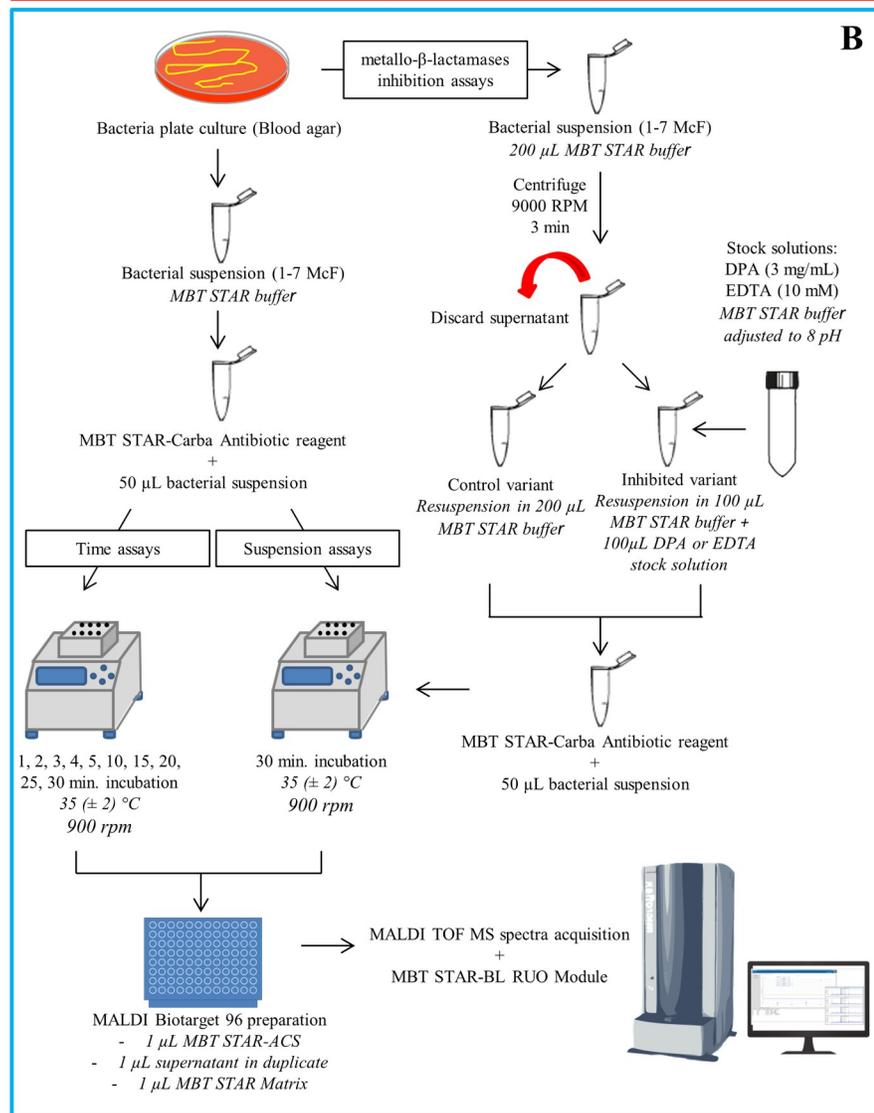
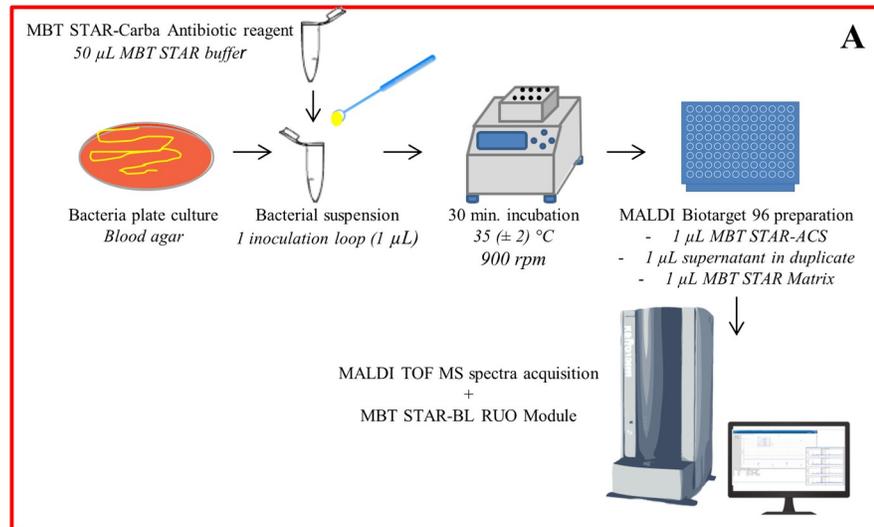


Fig 1. Scheme illustrating the MBT STAR-Carba routine (A) and experimental in-house (B) workflow. DPA—dipicolinic acid; EDTA—ethylenediaminetetraacetic acid.

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Effect of suspension density and incubation time on the MBT STAR-Carba results—in-house protocols

Multiple bacterial suspensions of different densities (1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7 McFarland Units—McF) were prepared by transferring and suspending sufficient amounts of respective bacterial colonies from overnight cultures into MBT STAR Buffer. Density was measured by McFarland densitometer DEN-1B (Grant Instruments Ltd, United Kingdom). Subsequently, 50 μ L of individual bacterial suspensions were added to the tubes containing dissolved MBT STAR-Carba Antibiotic Reagent and, after mixing, were subjected to (a) 30 min. incubation–suspension assay–and (b) 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 min–incubation time assay–at $35 \pm 2^\circ\text{C}$ with shaking (900 rpm) using Thermomixer (Eppendorf AG, Germany) (**Fig 1B**). Afterwards, MALDI Biotarget 96 was prepared according to the procedure described in the previous paragraph.

β -metallo-carbapenemases inhibition assay

For investigation of β -metallo-carbapenemases inhibition: (a) 3 mg/mL dipicolinic acid (DPA, Sigma Aldrich, Germany) and (b) 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, Germany) stock solutions in MBT STAR Buffer adjusted to 8 pH were used. From overnight cultures of 9 investigated strains characterized by the presence of MBL, bacterial suspensions were prepared as described in the previous paragraph—the McF range used for each strain was selected individually based on previous suspension test results. 200 μ L of each suspension were centrifuged (900 rpm, 3 min.), supernatants were discarded and remaining cell pellets were resuspended in (a) 200 μ L of MBT STAR Buffer—control variant–and (b) 100 μ L of MBT STAR Buffer + 100 μ L of respective inhibitor stock solutions: DPA (final conc. 1.5 mg/mL) or EDTA (final conc. 5 mM)—inhibited variants (**Fig 1B**). Subsequently, 50 μ L of prepared suspensions were added to the tubes containing dissolved MBT STAR-Carba Antibiotic Reagent, vortexed, incubated (30 min., $35 \pm 2^\circ\text{C}$, 900 rpm) and transferred onto MALDI Biotarget 96 according to procedure described in the paragraph 2.3. Additionally, all tested MBL strains were subjected to control test using non-MBL inhibitor addition—phenyl boronic acid (PBA) at final concentration 2 mg/mL.

MALDI-TOF MS analysis

Prepared MALDI Biotarget 96 plates were analyzed using a Microflex LT/SH instrument (Bruker Daltonics) and the MBT Compass platform with MBT STAR-BL Module (Bruker Daltonics). Each spot was analyzed in duplicate, finally obtaining 4 spectra for each sample. All the processes, spectra collection, data acquisition, and processing, were performed in a fully automated manner by the software using predefined methods provided by the manufacturer. Obtained results are finally expressed as normalized logRQ values, meaning the calculated logarithm of the ratio of the summed signal intensities of hydrolyzed forms to the summed signal intensities of non-hydrolyzed forms of the antibiotic, normalized using the respective positive (hydrolyzing) and negative (non-hydrolyzing) control strains. The results are understood as a measure of hydrolysis efficiency (higher normalized logRQ = higher degree of antibiotic hydrolysis), with normalized logRQ values ≤ 0.2 threshold—negative result; ≥ 0.4 threshold—positive results; values between these thresholds—unclear results. In this study, all result values described or depicted as “logRQ” refer to normalized values.

Ranking the strains according to carbapenemases activity

For classification of the tested strains according to antibiotic hydrolyzing activity, principal component analysis (PCA) using the Statistica software package (Statistica ver. 12, Statsoft, Poland) was performed. As variables used: (1) the average values of normalized logRQ obtained for different bacterial suspensions (1–7 McF); (2) the incubation time point in which noted first positive results of hydrolyzation at OD 4 McF (H 4 McF) and at OD 7 McF (H 7 McF) as well as the maximum of hydrolyzation level at OD 4 McF (Hmax 4 McF) and at OD 7 McF (Hmax 7 McF). Before PCA, all investigated data have undergone standardization using Z normalization method in order to avoid outlier issues. Assigning ranks corresponding to the level of carbapenemase activity was performed based on the grouping of the strains on the first two PCs-plane.

Results

Results of MBT STAR-Carba standard protocol

The principle of designed approach relies on the mass spectrometric evaluation of the carbapenem molecule after a short co-incubation with the bacterial strains, monitoring the distinct mass peaks of the hydrolyzed and non-hydrolyzed forms of the antibiotic resulting from carbapenemase-dependent β -lactam ring degradation [24,25]. Due to the low signal intensities of the typically observed hydrolyzed molecular forms (254 m/z), the internal standard provided with the MBT STAR Matrix is used as hydrolysis mass reference to calculate the hydrolyzation ratio. On presented spectra (Fig 2) it can be observed signals (green lines) representing native imipenem (300.1 m/z) and its adducts with matrix (489 m/z) which intensity vary depend on carbapenemase activity—the lower signal the higher hydrolytic activity.

The design MALDI TOF assay gave correct results for all investigated strains, however, the analysis of results did not reveal a grouping of the investigated strains because of the class of carbapenemases. Mostly, *Enterobacterales* strains representing the same genus had similar activity values—two *Enterobacter* strains, *E. kobei* and *E. cloacae* (0.96, each), two *C. freundii* strains (0.98 and 1.00), and two *E. coli* strains (1.03, each). More variance was observed for the *Klebsiella* genus (0.94 to 1.18) (Table 1). The use of standard protocol allowed division of hydrolyzing strains into two main groups in view of their carbapenemases activity: (1) strains with considerably lower logRQ values—3 *Pseudomonas aeruginosa* and (2) those demonstrated higher logRQ values—*Enterobacterales* members. Within the first group logRQ values ranged from 0.43–0.87 obtaining the lowest values for GES carbapenemase-producing *P. aeruginosa* 1 strain. LogRQ values in the second group ranged from 0.94 (multi-carbapenemase-producers *K. pneumoniae* 12) to 1.18 (GIM carbapenemase-producing *K. pneumoniae* 6).

Impact of suspension density on the MBT STAR-Carba results

The tested McF density series of bacterial suspensions ranging from McF 1 to 7 enabled the indication of a present carbapenemase activity in all respective strains except for GES producing *P. aeruginosa* 1, for which an increase in logRQ values with increasing suspension density has been observed; however, none exceeded the 0.2 threshold (Table 2; S1 Data).

Examples of graphs obtained using MBT Biotyper Prototype software during results generating are presented in Fig 3A and 3B. The analysis of the results revealed a different effect of bacterial density on the logRQ values depending on the bacterial order and class of carbapenemase.

Regarding *Enterobacterales*, division of the strains into two groups could be observed (Fig 4A).

Group I was mostly comprised of β -metallo-carbapenemase-producing strains (6 out of 7) demonstrating hydrolytic activity in the whole McF range and recognized as the most active group among investigated bacteria. In 4 cases—*E. coli* 7, *K. pneumoniae* 6/12 as well as *E. kobei*

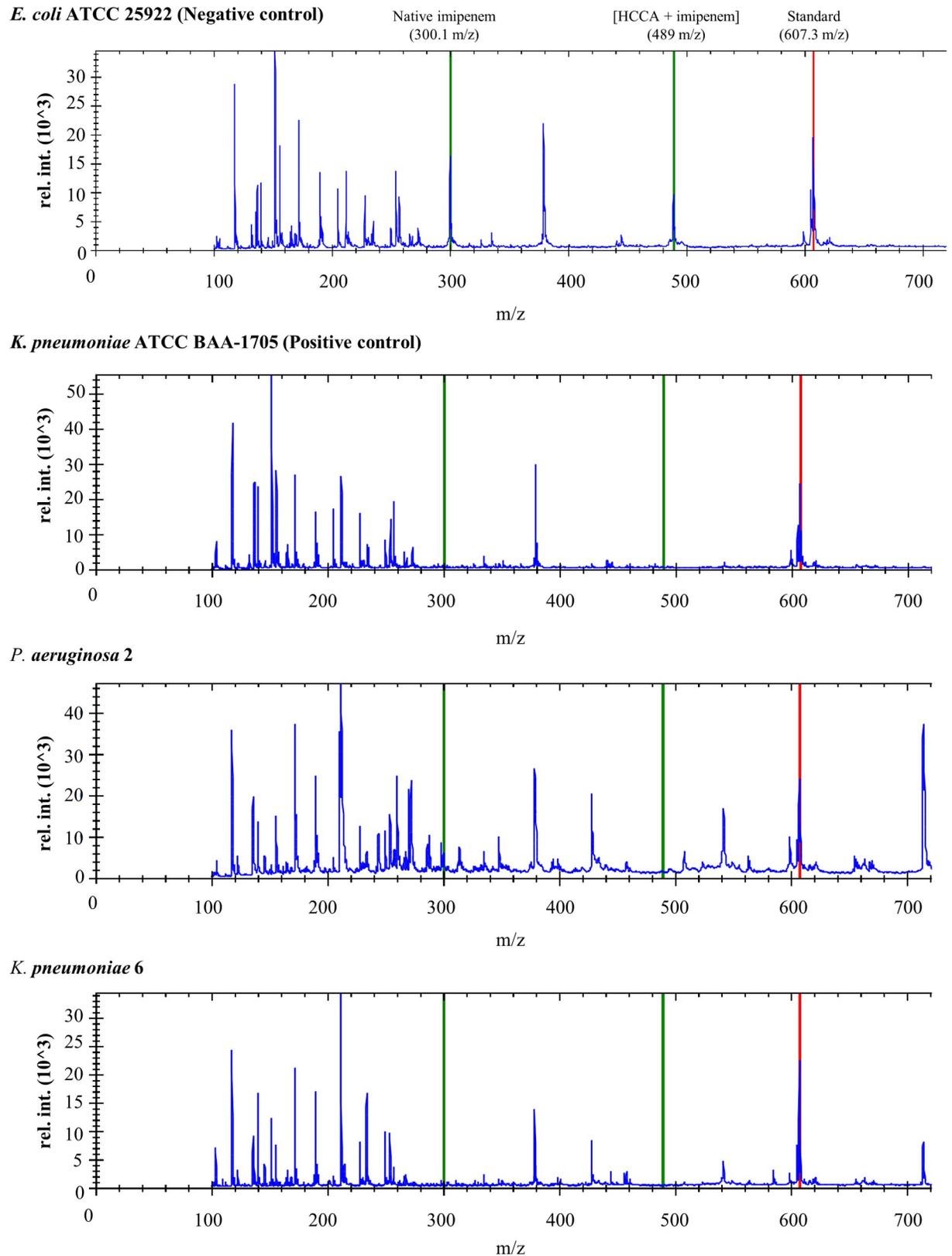


Fig 2. Exemplary MS spectra obtained during detection of carbapenemase activity via design MALDI TOF approach. Green lines represent signals for nonhydrolyzed antibiotic (native and adducts with HCCA) and the red line marks internal standard used for hydrolyzation rate calculation.

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Table 2. The hydrolytic activity levels (normalized logRQ) noted for the investigated bacteria depending on the cell's suspension densities (1–7 McF).

	Strain	McF	Hydrolytic activity level [normalized logRQ]									Δcontrols	
			1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0		7.0
A	<i>P. aeruginosa</i> 1		-0.40	-0.40	-0.43	-0.41	-0.40	-0.37	-0.28	-0.27	-0.06	-0.06	1.12
	<i>K. oxytoca</i> 2		0.08	0.10	0.13	0.18	0.12	0.30	0.39	1.02	1.05	0.99	1.90
	<i>E. coli</i> 3		0.24	0.73	0.85	0.86	0.90	0.94	0.90	0.97	1.00	1.00	1.67
	<i>P. aeruginosa</i> 4		-0.36	-0.34	-0.36	-0.29	0.44	0.74	0.98	0.73	0.58	0.55	1.12
	<i>P. aeruginosa</i> 5		0.10	0.74	1.00	1.02	0.97	0.92	0.80	0.63	0.48	0.37	1.40
	<i>K. pneumoniae</i> 6		0.83	0.80	0.82	0.91	0.97	0.91	0.97	1.00	1.08	1.06	1.71
	<i>E. coli</i> 7		0.80	0.87	0.88	0.95	0.83	0.92	0.89	0.93	0.96	0.97	1.90
B	<i>C. freundii</i> 8		0.02	0.46	0.55	0.60	0.86	0.93	0.96	0.96	0.98	1.09	1.67
	<i>C. freundii</i> 9		0.10	0.07	0.05	0.06	0.13	0.15	0.26	1.02	1.04	1.02	1.81
	<i>Salmonella</i> sp. 10		0.16	0.86	0.90	0.91	0.93	0.84	0.99	1.04	1.06	1.03	1.77
	<i>E. kobei</i> 11		0.82	0.85	0.86	0.90	0.95	0.90	0.92	1.00	1.03	1.04	1.71
	<i>K. pneumoniae</i> 12		0.88	0.91	0.96	0.95	0.90	0.98	1.03	1.12	1.04	0.98	1.77
D	<i>K. pneumoniae</i> 13		0.06	0.05	0.06	0.11	0.03	0.99	1.05	1.05	1.12	1.10	1.60
	<i>S. marcescens</i> 14		0.01	0.06	0.00	0.06	0.13	0.33	1.04	1.03	1.03	1.02	1.67
	<i>E. cloacae</i> 15		0.12	0.08	0.21	0.32	0.68	0.99	1.03	1.02	1.03	1.04	1.81
non-hydrolyzed													
unclear results													
hydrolyzed			0.41–0.65		0.66–0.89		>0.90						

Δ controls—quality control of the results calculated by subtracting the highest calculated original logRQ value of the acquired negative control spectra from the lowest calculated original logRQ value of the acquired positive control spectra; ≥ 0.7 means that the assay results are evaluated as accepted.

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11—positive detection of carbapenemases activity was possible already with the lowest suspension density (1 McF) and differences between 1 and 7 McF suspensions did not exceed 0.25 logRQ. Group II was mostly represented by class A and D carbapenemase producers (4 out of 5 strains) characterized by high hydrolytic activity only at a higher McF range—positive results of the test started from 3–5 McF depending on the strain (Table 2).

For all investigated *Enterobacteriales* strains, elevated logRQ values were observed as suspension density increased. In turn, for two β -metallo-carbapenemase-producing *P. aeruginosa* strains (4 and 5), a considerably different behavior was observed. For both strains, the increase of hydrolytic activity in the lower suspension density range was followed by a gradual activity decrease for respective higher McF values. For *P. aeruginosa* 4, positive results started with a 3 McF suspension achieving the highest logRQ value at 4 McF before decreasing again, while *P. aeruginosa* 5 strain demonstrated first positive carbapenemase activity at 1.5 McF and maximum values in the range of 2–3 McF.

Regardless of the family investigated, as the bacterial suspension density increased, the logRQ values of the tested strains approached the levels obtained when using the standard protocol, except for the strain *P. aeruginosa* 4 in which standard method normalized logRQ values placed between results noted for 4 and 5 McF suspension (Table 2).

Effect of incubation time on the MBT STAR-Carba results

Based on the suspension experiments, bacterial suspensions with McF 4 and 7 were chosen for investigation of the incubation time. Strain *P. aeruginosa* 1 was excluded from investigation due to lack of positive results in investigated suspensions range.

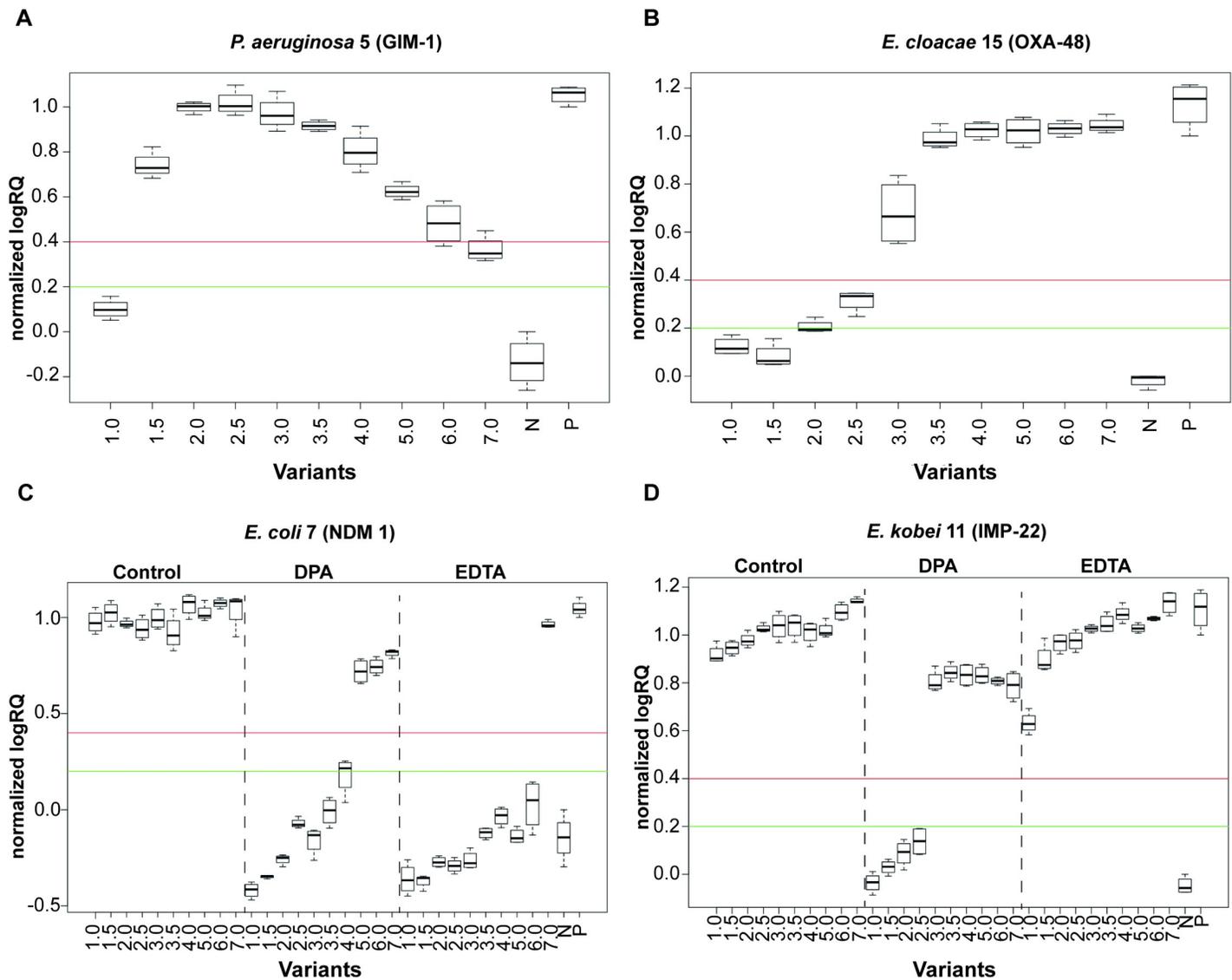
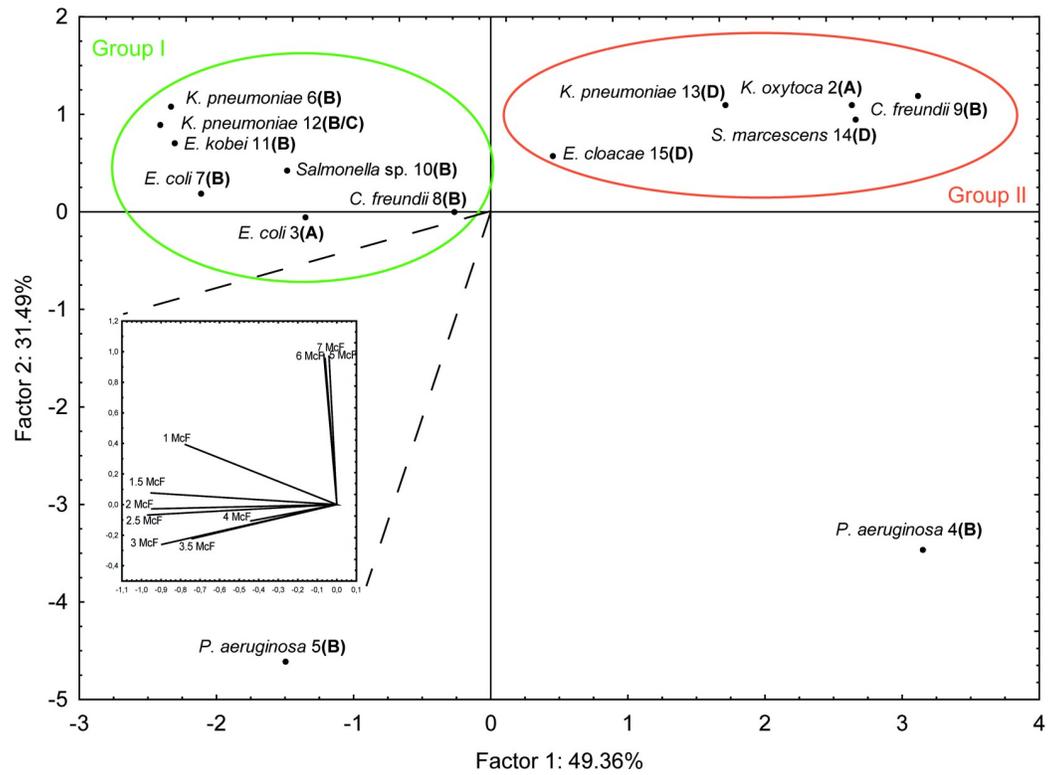


Fig 3. Examples of graphs showing the dependence of the determined carbapenemase activity level (normalized logRQ) on the density of the cell suspension used (1–7 McF) (A, B) as well as the addition of the inhibitor (DPA and EDTA) (C, D) created using MBT Biotyper Prototype software. N- negative control; P- positive control. The graph area between the green and red lines (0.2–0.4 normalized logRQ values) determines the range of the unclear results of hydrolytic activity.

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Regarding lower suspension density, the analysis revealed that in case of all OXA-like carbapenemase producers (class D) as well as *P. aeruginosa* 3 strain time needed to achieve positive results (H 4McF) was longer compared to the class A and B producers except for *C. freundii* 9 (no positive results at 4 McF noted) (Group II, [Fig 4B](#); [S2 Data](#)). The same applies to time to obtain maximum hydrolyzation level (H_{max} 4 McF). Strains clustered as Group I on the PCA graph were characterized by the shortest time needed to achieve positive as well as maximum results of hydrolytic activity and was represented by all class A producers as well as almost half of the β -metallo-carbapenemases producing strains (4 out of 9). Analysis revealed that those strains required no more than 3 minutes of incubation time to give positive results when OD 4 McF was used while in the case of class D producers 10 and more minutes were needed ([S1 Table](#); [S2 Data](#)).

A



B

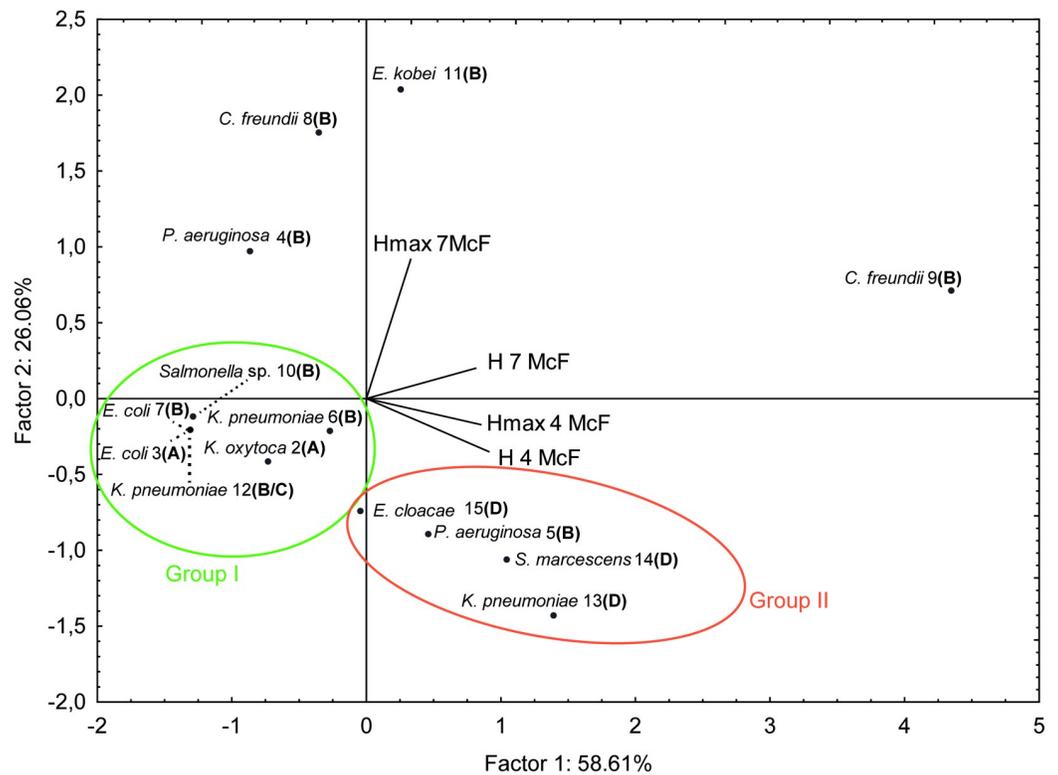


Fig 4. Grouping of the bacterial strains on the factor-plane in view of logRQ values at different suspensions (A) and incubation time needed to observe positive and maximal results of hydrolytic activity (B).

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Considering the highest bacterial density (7 McF), the analysis did not reveal differences between the class of produced carbapenemases since in all cases except for *C. freundii* 9 (4 min) the first time point which revealed positive results was 1 min (S1 Table). Instead, differences were noted in case of time needed to achieve maximum hydrolyzation level, where, 4 of class B producing strains—*P. aeruginosa* 4, *C. freundii* 8/9, and *E. kobei* 11, reached the highest logRQ after at least 15 min. of incubation. In the case of the rest strains H_{mas} 7 McF did not exceeded 5 minutes.

Ranking the strains

Based on the PCA results, strain ranking related to the levels of hydrolytic activity in comparison with results of the standard procedure was performed (Table 3).

Based on the total ranking, the analysis showed good compliance between tested and standard protocol in 53% of cases and decreased to 33% if only time ranking is accounted for. Ranking based on the suspension assay was similar to standard ranking; however, for 40% of the strains only weak compliance was revealed. The best rank matching was obtained for *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella* sp. 10 strains while the highest discrepancies were detected in the case of multi-carbapenemase-producer *K. pneumoniae* 12 ranked as 1 and 12 the most active strain according to the experimental test and standard procedure, respectively.

Results of β -metallo-carbapenemases inhibition assay

The use of the MBT STAR-Carba test to track the effect of carbapenemase inhibition in the tested range of cell suspension density revealed great differences between the type of inhibitor

Table 3. Comparison of strain ranking based on the results obtained during suspension assay and time assay as well as standard protocol. Compliance refers to the similarities with standard method.

Strain	Ranking			
	Suspension	Time	Total	Standard
<i>P. aeruginosa</i> 1(A)	15	na	15	15
<i>K. oxytoca</i> 2(A)	10	5	7	2
<i>E. coli</i> 3(A)	6	3	4	4
<i>P. aeruginosa</i> 4(B)	14	11	13	13
<i>P. aeruginosa</i> 5(B)	13	8	11	14
<i>K. pneumoniae</i> 6(B)	1	7	3	1
<i>E. coli</i> 7(B)	4	2	2	4
<i>C. freundii</i> 8(B)	7	12	10	8
<i>C. freundii</i> 9(B)	12	14	14	9
<i>Salmonella</i> sp. 10(B)	5	4	4	3
<i>E. kobei</i> 11(B)	3	13	8	10
<i>K. pneumoniae</i> 12(B/C)	2	1	1	12
<i>K. pneumoniae</i> 13(D)	9	9	9	4
<i>S. marcescens</i> 14(D)	11	10	11	4
<i>E. cloacae</i> 15(D)	8	6	6	10
% Good compliance (Δ 0–2)	60	33	53	
% Weak compliance ($\Delta \geq 3$)	40	67	47	

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Table 4. Decrease in the level of hydrolytic activity of the metallo- β -lactamases producing strains under DPA and EDTA addition depending on the bacterial suspension density.

Strain McF	Significant inhibition									
	Under DPA addition									
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0	7.0
<i>P. aeruginosa</i> 4 (IMP)	Black	Black	Black	Black	Blue	Blue	Blue	Blue	Blue	Blue
<i>Salmonella</i> sp. 10 (IMP-4)	Blue	Blue	Orange	Orange	Orange	Orange	Yellow	Orange	Orange	Orange
<i>E. kobei</i> 11 (IMP-22)	Blue	Blue	Blue	Blue	Orange	Orange	Orange	Orange	Orange	Orange
<i>P. aeruginosa</i> 5 (GIM-1)	Black	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
<i>K. pneumoniae</i> 6 (GIM)	Black	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Yellow	Yellow
<i>C. freundii</i> 8 (GIM)	Black	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
<i>E. coli</i> 7 (NDM-1)	Blue	Blue	Blue	Blue	Blue	Blue	Yellow	Orange	Orange	Orange
<i>K. pneumoniae</i> 12 (NDM-1)	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
<i>C. freundii</i> 9 (VIM)	Black	Black	Black	Black	Black	Black	Black	Blue	Blue	Blue
Strain McF	Under EDTA addition									
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0	7.0
<i>P. aeruginosa</i> 4 (IMP)	Black	Black	Black	Black	Blue	Blue	Blue	Blue	Blue	Blue
<i>Salmonella</i> sp. 10 (IMP-4)	Blue	Blue	Orange	Orange	Orange	Orange	White	White	White	White
<i>E. kobei</i> 11 (IMP-22)	Orange	White	White	White	White	White	White	White	White	White
<i>P. aeruginosa</i> 5 (GIM-1)	Black	White	White	White	White	White	White	White	White	White
<i>K. pneumoniae</i> 6 (GIM)	White	White	White	White	White	White	White	White	White	White
<i>C. freundii</i> 8 (GIM)	Black	Blue	Yellow	White						
<i>E. coli</i> 7 (NDM-1)	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
<i>K. pneumoniae</i> 12 (NDM-1)	Blue	Blue	Yellow	Blue	Yellow	Yellow	Orange	Yellow	Yellow	Yellow
<i>C. freundii</i> 9 (VIM)	Black	Black	Black	Black	Black	Black	Black	Blue	Blue	Yellow

Black—excluded from the calculation due to negative results in control; blue—significant decrease compared to the control variant—normalized logRQ values <0.2 (no hydrolysis); yellow—significant decrease compared to the control variant—normalized logRQ values $0.2-0.4$ (unclear results); orange—significant decrease compared to the control variant—normalized logRQ values >0.4 (hydrolysis); white—insignificant differences. DPA—dipicolinic acid; EDTA—ethylenediaminetetraacetic acid.

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used as well as type of β -metallo-carbapenemases (Table 4). Exemplary graphs created via MBT Biotyper Prototype software are presented in Fig 3C and 3D. Moreover, a control test with the use of a non-MBL inhibitor—2 mg/mL PBA—did not reveal any significant inhibitory effects (S2 Table), thereby confirming that the noted observation actually reflects a DPA and EDTA influence and that they are not an artifact.

Considering inhibitors, tested strains proved to be typically more sensitive to 1.5 mg/ml DPA addition—with a significant decrease in logRQ values in all strains regardless of the suspension density used (Table 4; S3 Data). Decline in the hydrolytic activity within the investigated McF range differed depending on the produced type of carbapenemase. GIM producers appeared to be the most sensitive to DPA presence since in almost all cases caused the test result to change from positive to negative except for 6 and 7 McF suspensions of *K. pneumoniae* 6 where unclear results were noted. Simultaneously, the same strains were less sensitive to the addition of EDTA, showing a significant decrease in the logRQ values only in 3 cases, including one negative result. EDTA demonstrated higher inhibitory effect than DPA only in the case of one strain—NDM-1 producing *E. coli* 7—negative results up to 6 McF compared to 3.5 McF that of DPA.

Regarding individual strains, *E. kobei* 11 was the least sensitive to the presence of inhibitors—a decrease in logRQ below 0.2 threshold (which means no hydrolysis) only under supplementation with DPA in the lower McF range (up to 2.5 McF). The greatest inhibitory effect

was observed for NDM-like carbapenemase producing *E. coli* 7 and *K. pneumoniae* 12 as well as in case of *C. freundii* 9; however, the latter one demonstrated relatively low overall hydrolytic activity which was starting only from a 5 McF suspension.

Discussion

Design spectrometric approach proved to be a universal, rapid, and highly sensitive tool for detecting present hydrolytic activity amongst the range of carbapenemases tested, including class A, B, and D. Also, class B GES type carbapenemases were detected, albeit well known to exhibit relatively low hydrolytic activity and therefore to challenge many current colorimetric tests (e.g. β -Carba Test, Biorad) [26,27]. Although the results obtained by the standard procedure revealed great differences in activity levels between individual *Enterobacteriales* and *Pseudomonas* strains—with typically *P. aeruginosa* strains being characterized by lower logRQ values—the test does not allow to ascribe differences in activity to different types or classes of carbapenemases. In this study, carbapenemase-type specific hydrolytic differences were revealed by modifying the standard test procedure regarding the amount of bacterial material, incubation time, and application of inhibitors. In most cases, there is a clear relationship of the modified parameters and the detected activity with the standard procedure being typically in an optimal range for sensitive detection. In most cases, logRQ values of 7 McF variant closed to that obtained using 1 uL inoculation loop which suggests transferring the similar number of cells. Indeed, 7 McF suspension density corresponds to ca. 3×10^8 colony forming units (cfu) and theoretical concentration in 1 uL inoculation loop ranged $3\text{--}6 \times 10^8$ CFU/mL as followed manufacturer guidelines. For GES-harboring *P. aeruginosa* strain, only the standard procedure was detecting the carbapenemase and false-negative results were noted when the modified procedure was applied what resulted from the lower amount of bacterial cells and indicated the reduced activity of this type of carbapenemases. Comparing other types, most metallo-type class B carbapenemase-producing bacteria demonstrated relevant hydrolytic activity also in a wider McF range compared to the class D producers tested, a class of β -lactamases also known to be difficult for rapid detection [28]. Moreover, strains for which positive results were observed already at the lowest density tested (1 McF) and thus classified as the most active, also belonged to the class B producers. Burckhardt et al. [29] noted that strains producing carbapenem-hydrolyzing class D (OXA-type) beta-lactamases demonstrate weaker imipenem hydrolytic activity compared to the MBL producers. Our findings are in agreement with the general statement that strains producing class D β -lactamases, especially OXA-48 producers, are considered as difficult-to-detect strains since they are characterized by low carbapenem MICs and often go undetected by most phenotypic methods [30,31]. Nevertheless, the use of design spectrometric assay can effectively help not only to reliably detect the activity of oxacillinases, but also in their further classification. Similar effect was achieved by Álvarez-Buylla et al. [32] using dipicolinic acid and Zn^{2+} supplementation. Interestingly, similar classification of the strains into the metallo- and OXA-like producers can be performed based on the time needed to present relevant hydrolytic activity, where the oxacillinases-producing strains happened to need longer time-to-result (Fig 4B).

The observed lower hydrolytic activity of *P. aeruginosa* strains compared to that in *Enterobacteriales* has previously been reported in the literature and was associated, among others, with lower quality of carbapenemase producing *P. aeruginosa* spectra [20]. Results of our studies suggest that the explanation of such findings could also be a different behavior of *P. aeruginosa* strains under increasing suspension density compared to *Enterobacteriales* members. Possible explanation of such phenomenon could be the fact that *P. aeruginosa* has the genetic capacity to express a wide repertoire of carbapenems resistance mechanisms—besides

enzymatic antibiotic degradation also expression of efflux pumps and low outer membrane permeability [33,34]. Moreover, Potron et al. [35] claim that carbapenem resistance in *P. aeruginosa* is mostly related to porin deficiency. In this context, the observed gradual decrease of hydrolytic activity with increasing suspension density in higher OD range may be due to the superiority of the efflux resistance mechanism and lowering membrane permeability leading to the limitation of the intracellular penetration of carbapenems. A similar observation was noted in the work of Miltgen et al. [36], in which disruption of the outer membrane by high concentrations of colistin and a freezing-thawing step was proposed as a solution for improving detection performance. *P. aeruginosa* is also known for their ability to synthesize significant amounts of siderophores demonstrating an affinity for Zn^{2+} [37] which could affect the availability of this metal for carbapenemases. However, the available literature lacks any work that addresses the impact of siderophore synthesis on carbapenemases activity detection. Due to restricted number of investigated *P. aeruginosa* strains drawing of general findings is limited, nevertheless, our findings prove a great need to deepen studies on the carbapenemases activity within *Pseudomonas* species conducted on the bigger scale and applying various conditions like different amount of both Zn^{2+} and biomass.

Multiple studies evaluated the time needed for reliable carbapenemases activity detection via MALDI-TOF MS [29,32,38]. However, all of them involve homebrew, non-standardized protocols. Our studies revealed that for the vast majority of bacteria tested (13 out of 15) incubation in the presence of the benchmark antibiotic could be shortened to 1 min if McF 7 suspension is used, a value also reached by the standard procedure. This applies to both *Enterobacteriales* and *P. aeruginosa* strain. Considering that in almost half of the investigated cases, 1 min of incubation was enough to reveal the maximum logRQ value, we can suppose that enzymatic degradation of carbapenem molecules occurred immediately after mixing bacterial suspension with antibiotic solution. Therefore, the spectrometric assay could be potentially used for reliable carbapenemases detection in many cases of *Enterobacteriales* and *Pseudomonas* strains, almost omitting the incubation step making this test even shorter. Similarly, Chong et colleagues [39] revealed complete imipenem hydrolyzation right after reaction mixture preparation ($T = 0$) for 85% of class A carbapenemase-producers, however, the majority of class B and D carbapenemase-producers did not fully hydrolyze imipenem until the 2 h of incubation. Such discrepancies may result from different reaction buffer composition and the applied mass spectrometric detection compared to that in our studies. As Knox and Palombo [30] observed, the addition of zinc ions is essential for consistent detection of many class B carbapenemases producers, like NDM- and VIM-harbouring strains and generally should be a requirement during the investigation of metallo-carbapenemase producers in these assays.

This approach has become particularly important in regard of increasing use of MALDI-TOF MS in clinical microbiology. Results of the works from the last few years regarding the use of MALDI TOF MS analysis for investigation of different carbapenemase activities—under addition of MBL inhibitors (e.g. ertapenem hydrolysis by IMP- and VIM-harbouring enterobacteria and *P. aeruginosa* using 50 mg/mL EDTA [40], by NDM-producing *K. pneumoniae* using 0.05 mg/mL DPA [3] or by VIM- and NDM-like *K. pneumoniae* as well as VIM-like *P. aeruginosa* using 1.5 mg/mL DPA [41]) makes this methodology a good option as a tool for the evaluation of new effective combinations of β -lactams and their inhibitors in any modern laboratory routine. Nevertheless, the authors referred to the use of ertapenem, which implicates the need to conduct a relatively long incubation to obtain correct results—up to 2 hours. Similarly to our work, recent studies of Oviano et colleagues [42,43] showed that the use of imipenem gives the opportunity to obtain reliable results of susceptibility testing in a simple way already after 30 minutes as it was in the case of combinations: imipenem–avibactam and

imipenem-relebactam. Although results of mentioned works showed high sensitivity (up to 98%) and specificity (up to 100%) in detecting KPC- producing *Enterobacterales* susceptible to imipenem/relebactam [43] as well as for detecting KPC and OXA-48-type isolates through avibactam inhibition [42], authors faced the obstacles with the repeated indeterminate results for certain strains or inability to distinguish between the types of carbapenemases. As we suppose, those limitations could be associated with the use of 1 uL microbial loop containing only an approximate number of cells. Contrary to this, in our studies, we used the range of bacterial suspension densities which enabled revealing differences in susceptibility between types of β -metallo-carbapenemases and inhibitors. Thus, conducting tests in a wide range of certain suspension densities opens the possibility of sensitivity comparisons between strains on a given inhibitor depending on the type of carbapenemase produced, solve the problem of indeterminate results as well as has the potential to facilitate interlaboratory comparisons. Considering the fact that special emphasis is currently placed on discovering new clinically significant MBL inhibitors like VNRX-5133 (novel boronic acid, phase I), ANT431 (novel pyridine-2-carboxylic acid, preclinical), or potentially also avibactam (phase III) the development of which is at different phases of clinical trials [44], our proposed use of design assay demonstrates high potential as a surrogate test for susceptibility before the microdilution results become publicly available. In the future investigation, inhibition effect of the other classes of carbapenemases (A, D) using the spectrometric assay is planned, since our findings suggest, as it was in the case of MBL, it may turn out to be a reliable and fast tool for clinical diagnostic laboratories also in this field.

Conclusions

The new spectrometric assay has become known as a highly sensitive and robust tool for a very rapid detection of carbapenemases, including the broad spectrum of β -metallo-carbapenemases. The outcome of our research indicates that, besides using the standard workflow, providing highly sensitive results, procedural modifications of this assay, like varying suspension densities and incubation times, demonstrate great potential in further sub-characterization of samples. The investigation of activity level differences based on normalized logRQ values allowed not only the differentiation of various bacterial species on the order level but also a correlation of different classes of carbapenemases, even within the class B metallo β -lactamases. It also proved that the application of a defined suspension density gives the opportunity to shorten incubation time and thus the total time-to-result of analysis along with further increasing knowledge about differences in the antibiotic hydrolysis level and sensitivity to inhibitors addition of an investigated strain. Such information could be useful during initial adaption towards a proper antibiotic therapy as well as during development of new MBL inhibitors.

Supporting information

S1 Table. The shortest incubation time needed to observe hydrolytic activity using MBT STAR-Carba assay depending on the bacterial suspension density (4 and 7 McF). H—first time point with pronounced hydrolysis (positive results); Hmax- time point with maximum hydrolysis achieved.
(DOCX)

S2 Table. Result of control test of effect of non-MBL inhibitor (phenyl boronic acid -PBA) addition on the level of hydrolytic activity of the investigated strains representing the Ambler class B metallo- β -lactamases depending on the bacterial suspension density. Δ logRQ—differences between the normalized logRQ of the control variants and treated ones. Negative values mean increase compare to control. In case of results recognized as "non-

hydrolyzed” in control variants—values are not shown.
(DOCX)

S1 Data. The results of the hydrolytic activity measurements depending on the cells suspension densities". nhydrInt 300.2"—the signal intensity of the unhydrolyzed imipenem [M+H]⁺; "nhydrInt 489"—the signal intensity of undhydrolyzed imipenem in an adduct form; "calInt"—intensity of the calibrator used (m/z 607.3); "deltaC"—differences between logRQ of positive and negative control.
(CSV)

S2 Data. The result of hydrolytic activity measurements under different incubation time [1–30 minutes]". nhydrInt 300.2"—the signal intensity of the unhydrolyzed imipenem [M+H]⁺; "nhydrInt 489"—the signal intensity of undhydrolyzed imipenem in an adduct form; "calInt"—intensity of the calibrator used (m/z 607.3); "deltaC"—differences between logRQ of positive and negative control.
(CSV)

S3 Data. The result of hydrolytic activity measurements for metallo-carbapenemases producing strains under dipicolonic acid [DPA] and [EDTA] addition depending on the bacterial suspension density". nhydrInt 300.2"—the signal intensity of the unhydrolyzed imipenem [M+H]⁺; "nhydrInt 489"—the signal intensity of undhydrolyzed imipenem in an adduct form; "calInt"—intensity of the calibrator used (m/z 607.3); "deltaC"—differences between logRQ of positive and negative control.
(CSV)

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