

Rapid detection of carbapenemase activity: benefits and weaknesses of MALDI-TOF MS

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Abstract Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been introduced as an identification procedure for bacteria and fungi. The MALDI-TOF MS-based analysis of resistance to β -lactam antibiotics has been applied to detect hydrolysis of carbapenems by different bacterial strains. However, the detection of enzymatic carbapenem degradation by MALDI-TOF MS lacks well-standardized protocols and several methods and models of interpretation using different calculations of ratio-of-peak intensities have been described in the literature. Here, we used faropenem and ertapenem hydrolysis as model compounds. In an attempt to propose a universal protocol, the hydrolysis was regularly monitored during 24 h using well-characterized bacterial strains producing different types of carbapenemases (KPC, IMP, NDM, VIM, and OXA-48). Variable responses and different timing for detectable hydrolysis, depending on the enzyme produced, were observed. KPC degrades its template antibiotics very quickly (15 min for some KPC producers) compared to other types of enzymes (more than 90 min for other enzymes). Prior bacterial lysis was shown to be of no interest in the modulation or optimization of the hydrolytic kinetics. The adequate detection of carbapenem hydrolysis would, therefore, require several MALDI-TOF MS readouts for the timely detection of rapid hydrolysis without missing slow hydrolysis. This

enzymatic constraint limits the implementation of a standard protocol in routine microbiology laboratories.

Introduction

The growing number of antibiotic-resistant microorganisms entails an increasingly important health care problem. Resistance can be based on different biological mechanisms. They include altered influx/efflux systems preventing the antibiotic agents from acting within the cells [1, 2], prevention of the interaction of the antibiotic agent with the target by alteration of the target binding site [3], and enzymatic modification of the antibiotic [4]. Carbapenem resistance is the most recent obstacle in the therapy of Gram-negative bacterial infections. One major underlying resistance mechanism is the production of carbapenemases [5], the detection of which has become a critical challenge in clinical microbiology [6, 7]. Carbapenem resistance can be detected using classical susceptibility testing methods [e.g., minimum inhibitory concentration (MIC) determination, agar diffusion, and application of the modified Hodge test] and DNA-based methods [i.e., polymerase chain reaction (PCR)]. However, the time to results is at least 18 h for susceptibility testing methods and PCR results are available the same day but at an elevated cost per reaction and without quantification of the MIC. Due to its high specificity, PCR can only detect genes encoding known enzymes [8]. More recently, the biochemical RAPIDEC[®] CARBA NP test has been developed and confirms carbapenemase production within 2 h with excellent sensitivity and specificity [9]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been introduced into routine microbiological laboratories for the identification of bacteria and fungi [10–12]. Also, MALDI-TOF MS assays to detect β -lactamase activity have been developed (Table 1).

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Table 1 Conditions used in several studies for carbapenem hydrolysis assay with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS)

	Bacterial inoculum	Assay volume	Carbapenem tested	Antibiotic concentration	Incubation time	Incubation conditions
Hrabák et al. 2011 [13]	8McF in 3 ml and 1 mL centrifuged for the test	50 µL	Meropenem	0.1 mM	3 h	20 mM Tris–HCl buffer (pH 6.8)
Burckhardt et al. 2011 [14]	One loop of 10 µL	1 mL	Ertapenem	0.5 mg/mL	2.5 h	NaCl buffer (0.45 %)
Kempf et al. 2012 [15]	One loop of 10 µL	1 mL	Imipenem	0.25 mg/mL	4 h	NaCl buffer (0.45 %)
Sparbier et al. 2012 [16]	One loop of 1 µL	10 µL	Imipenem, ertapenem, and meropenem	0.5 mg/mL	3 h	?
Hrabák et al. 2012 [17]	3 McF inoculum	50 µL	Meropenem	0.1 mM	2 h	20 mM Tris–HCl buffer (pH 6.8)
Lee et al. 2013 [18]	One loop of 1 µL	20–50 µL	Ertapenem	0.5 mg/mL	2–12 h	Tris–HCl pH7 buffer
Carvalhoes et al. 2013 [19]	One loop of 10 µL	1 mL	Ertapenem	0.12 to 0.5 mg/mL	2–4 h	Tris–HCl pH7 buffer
Hoyos et al. 2013 [20]	Dense suspension	50 µL	Ertapenem	0.25 mg/mL	4 h	20 mM Tris–HCl buffer
Alvarez et al. 2013 [21]	2.5×10^{10} CFU/mL	?	Imipenem	1 mg/ml	1 h	0.46 % NaCl or 20 mM Tris–HCl, pH 6.8, buffers
Johansson et al. 2014 [22]	4McF inoculum	20 µL	Ertapenem	0.5 mg/mL	15 min to 2 h	?
Sauget et al. 2014 [23]	One loop of 1 µL	1 mL	Imipenem	0.25 mg/mL	1 h	NaCl buffer (0.45 %)
Knox et al. 2014 [24]	One loop of 1 µL	100 µL	Imipenem	0.25 mg/mL	4 h	NaCl buffer (0.45 %)

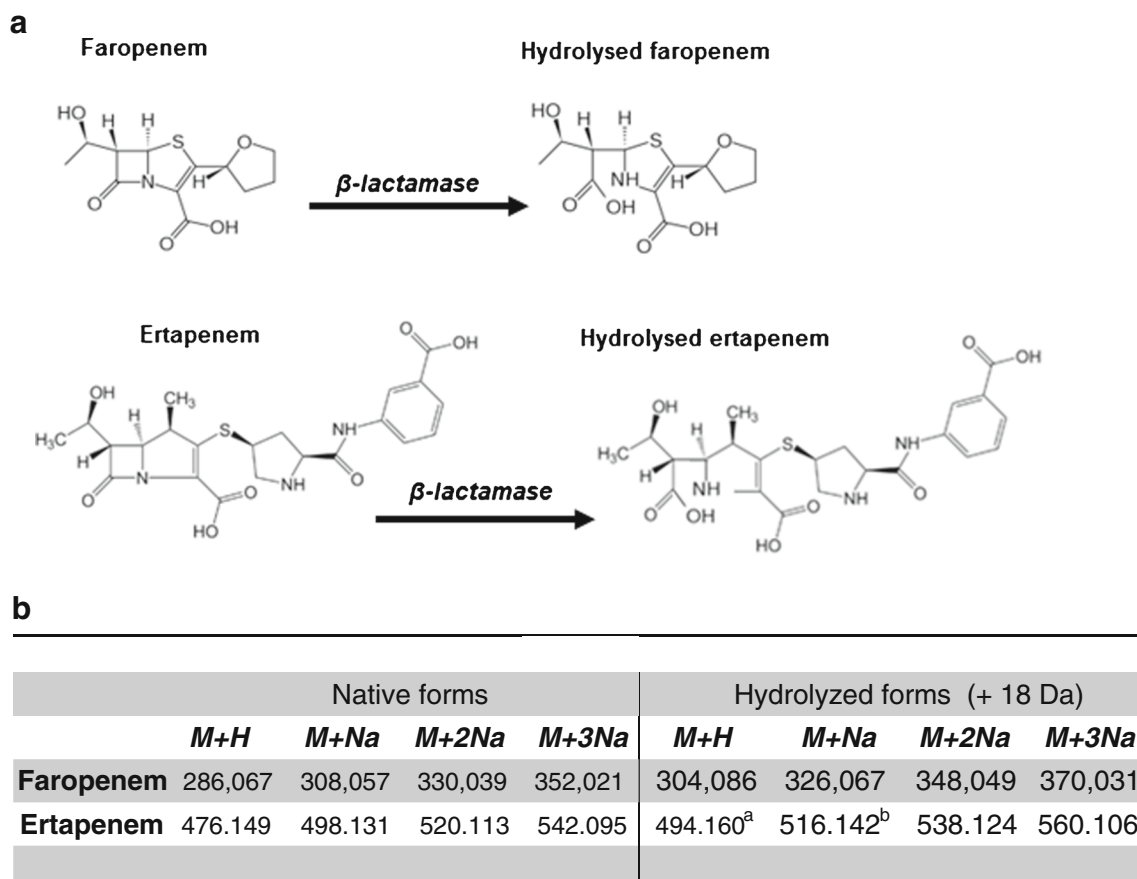
These studies rely on the mass-based detection of native β -lactams and their related degradation products following bacterial enzymatic activity. The β -lactam hydrolysis can be monitored by MS because the hydrolysis of the β -lactam ring by β -lactamases results in an increase of the molecular mass by 18 Da and in disappearance, total or partial, of the original mass peak (Fig. 1). Several protocols have been described and demonstrate the proof of concept regarding the detection of carbapenemase activity by MALDI-TOF MS (Table 1). Nonetheless, the variability in the protocols described also shows the difficulty to define a universal method for antibiotic hydrolysis assays on MALDI-TOF MS. Indeed, all studies have used different methodologies and different sets of bacteria/antibiotic combinations, buffers, and incubation times for hydrolysis assays. We discuss here the strengths and weaknesses of the MALDI-TOF MS approach with respect to applicability and practicability in routine settings of clinical microbiology laboratories. Our study is focused on detection of the hydrolysis of ertapenem and faropenem using the VITEK[®] MS RUO system with a set of well-characterized strains, monitored during 24 h to try to understand the kinetics of hydrolysis of the antibiotics tested. We chose two antibiotics with different molecule masses. Faropenem is an oral penem antibiotic with efficacy for the treatment of community-acquired infections caused by Enterobacteriaceae and has not yet been tested with a MALDI-TOF approach (Table 1). Faropenem was recently described as an excellent indicator of carbapenemase activity in the Enterobacteriaceae [25, 26], with interesting sensitivity and specificity in disk diffusion assays. Ertapenem is widely used in various phenotypic susceptibility tests and several publications report various

MALDI-TOF MS-based protocols to monitor its enzymatic degradation [14, 16, 18, 22].

Materials and methods

Bacterial strains, characterization, and antibiotic susceptibility testing

Clinical bacterial isolates (bioMérieux collection, La Balmeles-Grottes, France) classified by standard methods as carbapenemase-producing strains were used in this study (Tables 2 and 3). The production of carbapenemases was evaluated by VITEK[®]2 (bioMérieux, France) with the antimicrobial susceptibility cards AST-N233 and AST-XN05, by PCR, and with the RAPIDEC[®] CARBA NP test (bioMérieux, France). The type of enzymatic variant produced was identified by sequencing. The extended-spectrum β -lactamase (ESBL) and high-level cephalosporinase (HL-Case) phenotypes indicated by automated microbiology systems (VITEK[®]2, bioMérieux) were further corroborated by a confirmatory PCR test and sequencing. Non-carbapenemase-producing strains from different bacterial species were also added for control purposes (Tables 2 and 3). The MICs for imipenem, meropenem, and ertapenem were determined using Etest[®] strips (bioMérieux, France) and interpreted according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI, <http://www.clsi.org>). The MICs for faropenem were obtained using the microdilution method described by the CLSI (Tables 2 and 3). Bacteria were cultured on blood agar plates (COS medium, bioMérieux,



^{a,b} Ertapenem hydrolyzed and decarboxylized corresponding forms (- 44 Da): *M+H* 450.170, *M+Na* 472.152.

Fig. 1 Native and hydrolyzed carbapenem structures (a) and related masses (b). Calculated masses were given as mono-isotopic masses computed from relative atomic masses as available from the National

Institute of Standards and Technology (NIST) website; for example, <http://www.nist.gov/pml/data/comp.cfm>

France) without selective agents overnight at 37 °C. Colonies from fresh overnight cultures were used for the antibiotic MS hydrolysis assays.

MALDI-TOF MS analysis

Mass profiles were acquired using a MALDI-TOF mass spectrometer VITEK® MS RUO and the Launchpad 2.9 software. The instrument operated in linear mode in the mass range of 200 to 700 Da (Fig. 1), with parameter settings optimized for this range. Over the full mass range, isotope peaks of detected compounds were resolved to the baseline (resolution \gg 1000). With Launchpad 2.9 software, the spectra were processed by deisotoping and applying peak filters to reduce non-informative data. All individual spectra were calibrated using the mono-isotopic peaks of $(\text{CHCA}+\text{Na})^+$ and $(\text{CHCA}_2+\text{H})^+$ at m/z 212.032 and m/z 379.093, respectively, as internal calibrators. The m/z values of measured mono-isotopic faropenem and ertapenem peaks showed a coefficient of variation of 0.005 % and the maximum absolute deviation from the calculated m/z was 0.05 Da.

Optimization of carbapenem detection on VITEK® MS RUO

To determine the optimal antibiotic concentration detectable on the VITEK® MS RUO system, a range of ertapenem and faropenem concentrations was tested. Each antibiotic solution (from 0.001 to 1 mg/ml) in sterile water or NaCl solution (0.45 %) was spotted (2 μL) on MALDI disposable target plate (bioMérieux, France) and overlaid with the 2 μL matrix solution [α -cyano-4-hydroxycinnamic acid (CHCA) in ethanol, acetonitrile, and water, 1:1:1]. To study the variability of detection, six spots per concentration were analyzed.

Faropenem hydrolysis assay with *K. pneumoniae* KPC producers

Nine *K. pneumoniae* clinical isolates classified as KPC carbapenemase-producing strains were tested with faropenem. Six carbapenemase-negative isolates (two ESBL, two high-level cephalosporinase producers, and two susceptible strains) were added for comparison purposes (Table 2). Freshly

Table 2 Characteristics of KPC *Klebsiella pneumoniae* producers and other strains used for the initial faropenem hydrolysis tests. All strains were held in the bioMérieux clinical strains collection (La Balme-les-Grottes, France)

Strains type	Resistance mechanisms ^a	Bacterial species	API no.	MIC (mg/L)				RapidEC [®] Carba NP		MALDI-TOF MS faropenem test		
				Erta ^b	Imi ^b	Mero ^b	Faro ^c	30 min	2 h	T0	T15min	T3h
Carbapenemase-producing isolate	KPC-2	<i>K. pneumoniae</i>	1108001	16	≥32	≥32	128	+	/	-	+	+
	KPC-3	<i>K. pneumoniae</i>	0601009	≥32	≥32	≥32	>256	+	/	-	-	+
	KPC-3	<i>K. pneumoniae</i>	0505049	≥8	8	≥16	128	+	/	-	-	+
	KPC-2	<i>K. pneumoniae</i>	0601007	8	16	≥32	256	+	/	-	-	+
	KPC-2	<i>K. pneumoniae</i>	1108008	≥32	≥32	≥32	>256	+	/	-	-	+
	KPC-3	<i>K. pneumoniae</i>	1108003	4	≥32	≥32	128	+	/	-	+	+
	KPC-3	<i>K. pneumoniae</i>	1108002	≥32	≥32	≥32	>256	+	/	-	-	+
	KPC-2	<i>K. pneumoniae</i>	0505034	4	≥32	≥32	256	+	/	-	-	+
	KPC-2	<i>K. pneumoniae</i>	0603004	≥32	≥32	≥32	>256	+	/	-	-	+
Carbapenemase-negative isolate	ESBL TEM-23	<i>E. coli</i>	0607051	0.25	0.19	0.047	0.8	-	-	-	-	-
	ESBL CTX-M2	<i>P. mirabilis</i>	0607045	0.032	1.5	0.125	2	-	-	-	-	-
	Non-ESBL ^d	<i>E. coli</i>	0804151	0.047	0.25	0.032	1.6	-	-	-	-	-
	Non-ESBL ^d	<i>M. morgani</i>	0504063	0.023	1.5	0.064	2	-	-	-	-	-
	None	<i>S. aureus</i> ^d	7509008	NA	NA	NA	0.05	-	-	-	-	-
	None	<i>E. coli</i> ^d	7308009	0.004	0.125	0.012	0.8	-	-	-	-	-

^a Confirmed by PCR (KPC, TEM, and CTX-M genes)

^b MIC evaluated using Etest[®] strips (bioMérieux, France). Susceptible and resistant breakpoints are ≤0.5; ≥2 mg/ml for ertapenem (*Erta*), ≤1; ≥4 mg/ml for imipenem (*Imi*), and ≤1; ≥4 mg/ml for meropenem (*Mero*) (CLSI)

^c MIC evaluated using the microdilution method according to the CLSI for faropenem (*Faro*)

^d Non-ESBL strains (confirmed by PCR) but resistant to third-generation cephalosporins

^e ATCC strains used as negative control: *S. aureus* (ATCC 25923) and *E. coli* (ATCC 11775)

Table 3 Characteristics of bacterial strains tested and data summary of kinetics with faropenem and ertapenem during 24 h at 37 °C (bioMérieux clinical strains collection, La Balme-les-Grottes, France)

Bacterial species	API no.	Resistance mechanism ^a	MIC (mg/L)			RapidEC [®] Carba NP		Hydrolysis (time ^d), Faropenem		Hydrolysis (time ^d), Ertapenem	
			Imi ^b	Erta ^b	Faro ^c	30 min	2 h	Without lysis	Lysis	Without lysis	Lysis
<i>E. coli</i>	7308009	None	0.125	0.004	≤0.5	-	-	-	-	-	-
<i>E. coli</i>	9401005	None	0.19	0.008	≤0.5	-	-	-	-	-	-
<i>E. aerogenes</i>	0502112	ESBL CTX-M2	0.25	0.38	≤0.5	-	-	-	-	-	-
<i>K. pneumoniae</i>	1108001	KPC-2	≥32	16	≥32	+	+	+(1.5 h)	+(3 h)	+(1.5 h)	+(3 h)
<i>K. pneumoniae</i>	0603004	KPC-2	≥32	≥32	≥32	+	+	+(3)	+(3 h)	+(5 h)	+(8 h)
<i>K. pneumoniae</i>	0709121	IMP-1	3	8	≥32	+	+	+(24 h)	+(5 h)	+(5 h)	+(8 h)
<i>E. cloacae</i>	1011172	NDM-1	4	8	≥32	+	+	-	+(3 h)	-	+(3 h)
<i>K. pneumoniae</i>	1103201	OXA-48	≥32	≥32	≥32	-	+	-	-	-	-
<i>P. aeruginosa</i>	0509057	VIM-2	≥32	≥32	≥32	+	+	+(24 h)	+(24 h)	-	+(24 h)
<i>K. pneumoniae</i>	1008057	VIM-1	≥32	≥32	≥32	+	+	+(8 h)	+(1.5 h)	+(24 h)	+(3 h)

^a Confirmed by PCR

^b MIC evaluated using Etest[®] strips (bioMérieux, France). Susceptible and resistant breakpoints are ≤1; ≥4 mg/ml for imipenem (*Imi*) and ≤0.5; ≥2 mg/ml for ertapenem (*Erta*) (CLSI)

^c MIC evaluated using the microdilution method according to the CLSI for faropenem (*Faro*)

^d Time necessary for 50 % of antibiotic hydrolysis in the conditions tested (ratio of intensity antibiotic/metabolite ≤0.5 or ratio metabolite/antibiotic ≥0.2)

prepared faropenem (Sigma-Aldrich, Germany) stock solution (0.125 mg/mL in NaCl 0.45 % buffer) was tested with the different bacterial strains. One 1 µl loop-sized amount of bacteria was re-suspended in 50 µL of antibiotic solution and incubated at 37 °C for 15 min and 3 h. After incubation, the tubes were immediately centrifuged for 2 min at 13,000 g at room temperature. The cell-free supernatant was analyzed by MALDI-TOF MS. 2 µL of the supernatant was directly spotted onto a MALDI disposable target plate (bioMérieux) and overlaid with 2 µl of CHCA matrix (10 mg/ml in acetonitrile/ethanol/water, v/v/v). On mass spectra obtained, the carbapenem hydrolysis was evaluated looking at specific peaks and their relative intensity. According to the literature, and for comparison, several ratios of peak intensity were calculated at T0, 15 min, and 3 h: faropenem/CHCA-dimer [$I(m/z\ 304)/I(m/z\ 379)$] [16]; faropenem/major metabolite ($I(m/z\ 304)/I(m/z\ 308)$) [15]; and major metabolite/faropenem ($I(m/z\ 308)/I(m/z\ 304)$) [27]]. Results interpretation was done according to other authors: a result was considered positive when 50 % of hydrolysis was observed after 15 min or 3 h of incubation (Table 2).

Monitoring of faropenem and ertapenem hydrolysis during 24 h with different types of carbapenemase producers

Seven clinical isolates producing different types of carbapenemase were tested together with three carbapenemase-negative isolates (Table 3). All strains were tested with faropenem and ertapenem (0.125 mg/mL in NaCl 0.45 % buffer). For kinetic analysis, the bacterial inocula were standardized at 2McF (equivalent to 6.10^8 CFU/ml), in a 2 mL assay volume. During 24 h, the kinetics of hydrolysis were monitored. From the same bacterial inoculum, two protocols were tested with or without previous cell lysis at T0. Cell lysis was performed using a mix of glass beads of several sizes (3 mm, 500 µm, 100 µm) and mixed during 5 min with a vortex system. After different incubation times (T0, T0.5h, T1h30, T3h, T5h, T8, and T24h), 50-µL suspensions were centrifuged for 2 min at 13,000 g at room temperature and the supernatant analyzed using VITEK® MS RUO.

Results and discussion

Carbapenem detection on the VITEK® MS RUO system

In view of the different protocols and the many different antibiotic concentrations used by other authors (Table 1), we optimized the conditions for the detection of antibiotics and their hydrolysis products on the VITEK® MS RUO system (Fig. 2). A range of antibiotic concentrations was analyzed to determine the limits of detection of the system. High

concentrations were suboptimal because the relative peak height was beyond the linear range. Faropenem was well detected in solutions containing NaCl (0.45 %), but for the peak intensity ratio faropenem/CHCA-dimer, a linear response was only observed for antibiotic concentrations lower than 0.125 mg/mL (Fig. 3). Furthermore, using six sample spots per condition, reproducibility was lower when concentrations exceeded 0.25 mg/mL (especially for ertapenem). Therefore, concentrations of faropenem and ertapenem of 0.125 mg/mL in 0.45 % NaCl were chosen for the subsequent assays.

MS tests with *K. pneumoniae* carbapenemase (KPC) producers and faropenem

We first analyzed faropenem degradation, using nine KPC *K. pneumoniae* producers and control strains. The KPC producers chosen were isolated from different geographical origins, between 2005 and 2011 (Table 2), and presented different antimicrobial resistance profiles regarding non-carbapenem molecules (data not shown). Based on the methods described by other authors [16, 18, 24], a rapid preparation of bacterial inoculum was performed with one 1 µl loop-sized amount of bacteria in a 50-µL volume. The protonated, native faropenem ion ($m/z\ 286.3$) was not detected as a predominant peak, while the versions with sodium adduct ions, $(M+Na)^+$ and $(M+2Na)^+$, were clearly detected at $m/z\ 308.3$ and 330.3 , respectively (Figs 1 and 2). After the incubation of faropenem with carbapenem-resistant *K. pneumoniae* strains, these two peaks disappeared and two major degradation products were detected at $m/z\ 304.3$ ($[\text{faropenem}+H_2O+H]^+$) and 326.3 ($[\text{faropenem}+H_2O+Na]^+$). In the presence of a carbapenemase-negative strain, no faropenem degradation was observed. Interestingly, we obtained the same interpretation regardless of the ratio calculation used. Concerning the nine KPC-producing strains tested, two of them were positive after 15 min of incubation at 37 °C (API nos. 1108001 and 1108003). After 3 h, all of the KPC producers tested were positive. The carbapenemase-negative isolate did not hydrolyze the faropenem at neither 15 min nor at 3 h (Table 2). The question of optimal timing of the hydrolysis assay was, therefore, seen as critical. For KPC strain analysis, Johansson et al. considered the optimal incubation time to be 15 min [22]. In our study, we show that, with such a time of analysis, 7 out of 9 positive KPC strains would not have been identified. Only strains with high hydrolytic activity give a rapid positive result. This finding underlines the need for a well-standardized protocol with relevant incubation time to avoid false-negative results.

Ertapenem versus faropenem hydrolysis

To follow ertapenem and faropenem hydrolysis during 24 h, two ratios were calculated. The ratio of peak intensity

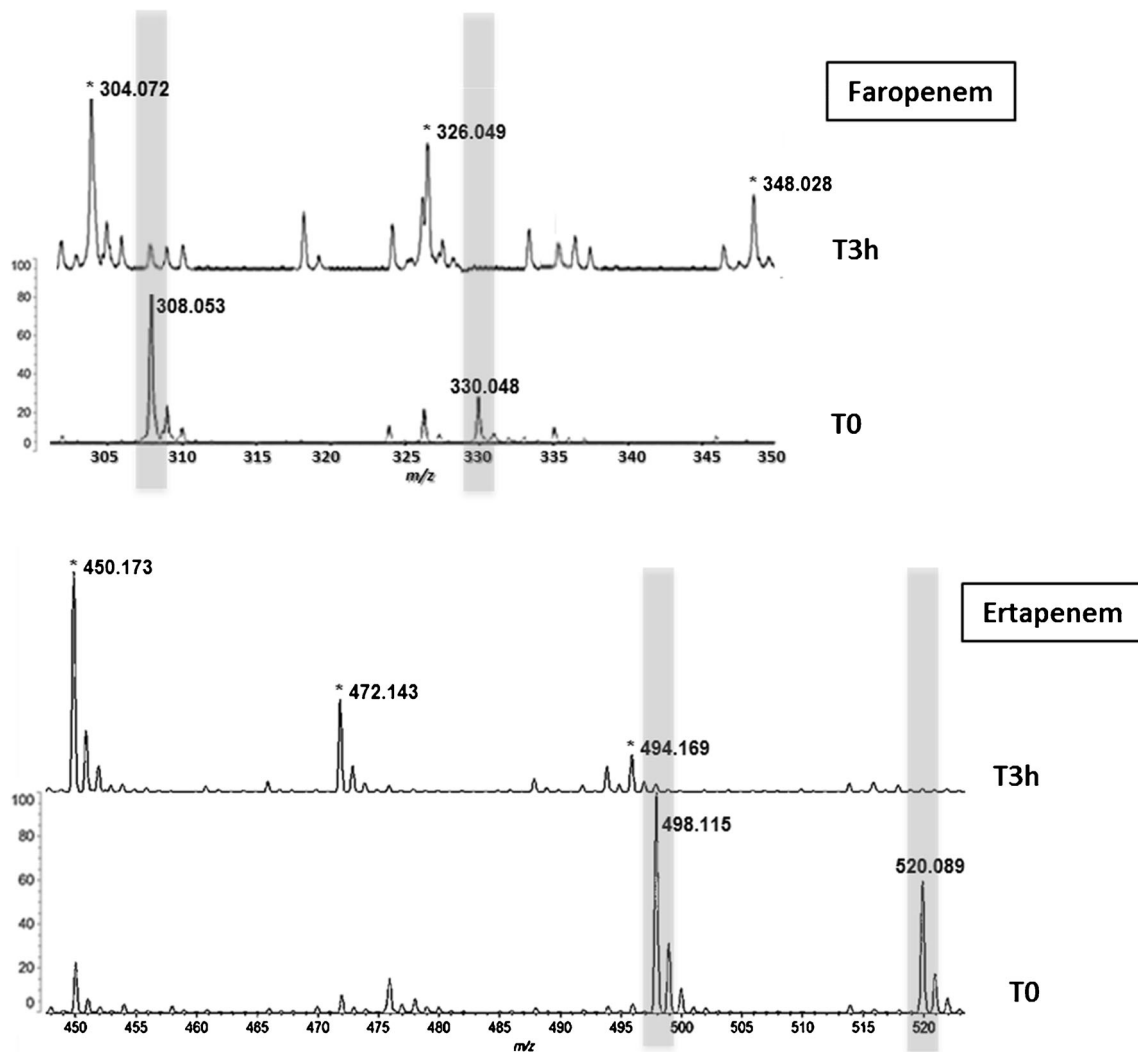


Fig. 2 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectra of faropenem and ertapenem. Hydrolysis assay with a resistant strain (KPC-producing isolate *Klebsiella pneumoniae* API

no. 1108001). The asterisks indicate the peaks of degradation products with respective measured monoisotopic m/z

“hydrolyzed product/native form” and “native form/hydrolyzed product” was calculated for comparison and to determine the time necessary for 50 % of antibiotic hydrolysis under the conditions tested (Table 3). In parallel, Fig. 4 shows the monitoring of hydrolysis for each strain by appearance of the major product of degradation of faropenem or ertapenem. The ratio $[I(m/z\ 304)/I(m/z\ 308)]$ was used for faropenem. For ertapenem, the predominant native form at 498 m/z was chosen for ratio calculation. Indeed, three peaks of degradation products of ertapenem are visible at m/z 450, 472 and 494 (Fig. 2). However, the peak at m/z 494 was chosen to calculate the ratios, because the two other peaks correspond to decarboxylated products (Fig. 1) and we observed that these compounds were not sufficiently stable and disappeared during the enzymatic reactions, complicating an analysis using the sum of intensities of peaks of products of degradation. By contrast, the peak at m/z 494 (corresponding to the M+H hydrolyzed form) is stable and

does not disappear even if hydrolysis is total. Experiments performed with carbapenem-sensitive controls (*E. coli* API 7308009, 9401005 and *E. aerogenes* API 0502112) confirmed that these strains do not contain carbapenemases (Fig. 4). The negative control without bacteria demonstrates the stability of faropenem and ertapenem for 24 h under the conditions tested. The results were the same for ertapenem and faropenem: resistant strains hydrolyzed both antibiotics, showing that faropenem is a good indicator for carbapenemase activity. These results are in agreement with disk susceptibility testing using faropenem as described by Day et al. [25]. The faropenem disk test showed an equivalent sensitivity (99 %) and superior specificity (94 %) to any of the recommended screening indicators of potential carbapenemase activity [imipenem, ertapenem, or meropenem as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)].

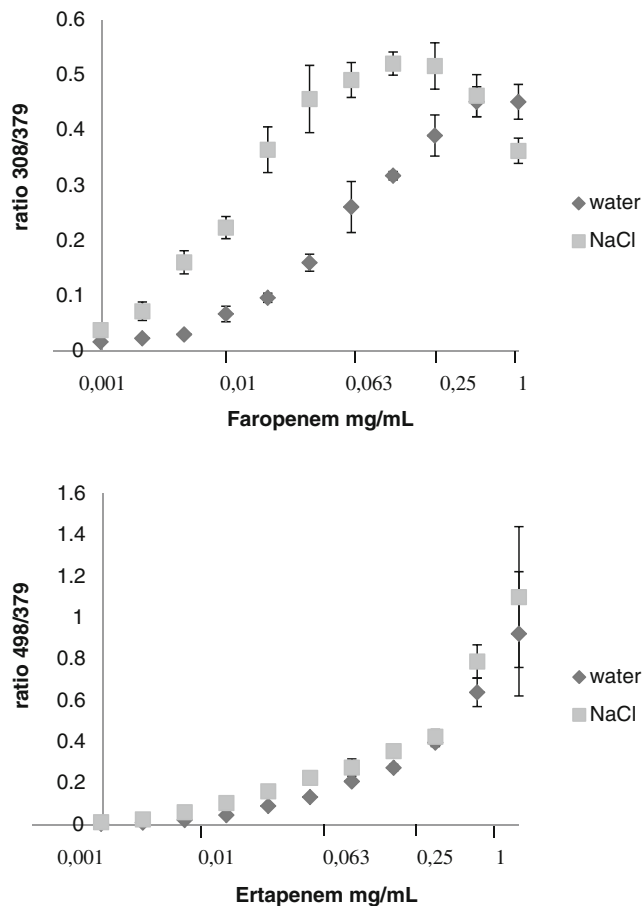


Fig. 3 Detection of faropenem and ertapenem depending on the concentrations and diluent used. Ratios of peak intensity are calculated to estimate the detection of antibiotic: predominant native form [m/z 308 and m/z 498 for (faropenem+Na)⁺ and (ertapenem+Na)⁺, respectively/matrix dimer (CHCA2+H)⁺; m/z 379]. The symbols represent mean \pm standard deviation (SD); $n=6$

Monitoring of faropenem and ertapenem hydrolysis during 24 h with different types of carbapenemase producers

Interestingly, the carbapenemase producers tested did not hydrolyze faropenem and ertapenem with the same rate. This enzymatic property led us to further question the assay duration. Several authors recommended an incubation between 2 h or 4 h [13, 14, 16], but is it really enough or, in contrast, too long for such a test? To address this question, kinetic studies of faropenem and ertapenem degradation were performed for ten bacterial isolates producing different types of carbapenemases (Table 3). The KPC producers degraded antibiotics quickly (1.5–3 h), in contrast to IMP, NDM, or VIM producers (≥ 3 h, Fig. 4 and Table 3). The OXA-48 producer hydrolyzed neither ertapenem nor faropenem under the conditions tested. In Johansson et al.'s study, KPC producers were also the strains that were the first to be detected as positive with

MS hydrolysis assays [22]. Hrabák et al. tried an incubation of 3 h with meropenem and did not observe any degradation products with positive strains [13], and speculated that the products are further decomposed to smaller molecules not visible on mass spectra. False-negative results seem possible if the duration of incubation is not adapted. Many authors used the ratio calculation (metabolite/native antibiotic) for results interpretation. Indeed, the major change during the hydrolysis should be the disappearance of the native form, but, to make the analysis more reliable, it is preferable to monitor also the generation of degradation products (Fig. 4). In our study, the KPC strain producer API no. 1108001 (protocol with lysis) degraded antibiotics in 1 h 30 (Table 2). Illustrating this aspect of metabolites modification, the kinetics with ertapenem seem to show a transformation of the product of degradation after 3 h (Fig. 4b). Although we only tested one strain producing the OXA-48 enzyme, other authors came to the same conclusion, with the OXA-48 assay only becoming positive after a long period of incubation, sometimes even more than 24 h [22]. The MS test seems to be less sensitive for the detection of carbapenem hydrolysis by the OXA-48 enzyme or, more generally, by enzymes with low activity or low expression levels. Indeed, the result of the RAPIDEC® CARBA NP test for the strain OXA-48 producer tested shows a positive result later (2 h versus 30 min) compared to other carbapenemase strains producers included in our study (Table 3).

The MS assay becomes significantly more cumbersome if samples should be repetitively acquired and measured at different incubation times. A MALDI-TOF MS analysis is in need of a sample deposit, a matrix addition, and a target introduction into an instrument under vacuum, before the result can be interpreted. This can cause experimental variability, which will become obvious pretty quickly when carrying out time series.

Lysis effect

We also studied the effect of mechanical cellular lysis with glass beads. This showed a positive effect and decreased the time for hydrolysis with NDM, IMP, and VIM producers (Fig. 4), suggesting active release of enzymes from the periplasmic membrane. On the other hand, the lysis exerted a negative effect on the KPC-positive strains tested. The KPC enzyme seems more fragile than other carbapenemases, but the origin of this phenomenon was not investigated. Anti-protease addition or a chemical lysis could be alternatives, but these approaches would add an additional step in the reaction scheme and could potentially add parasite peaks to the mass spectra. We, therefore, recommend a careful case-by-case evaluation before using a lysis protocol.

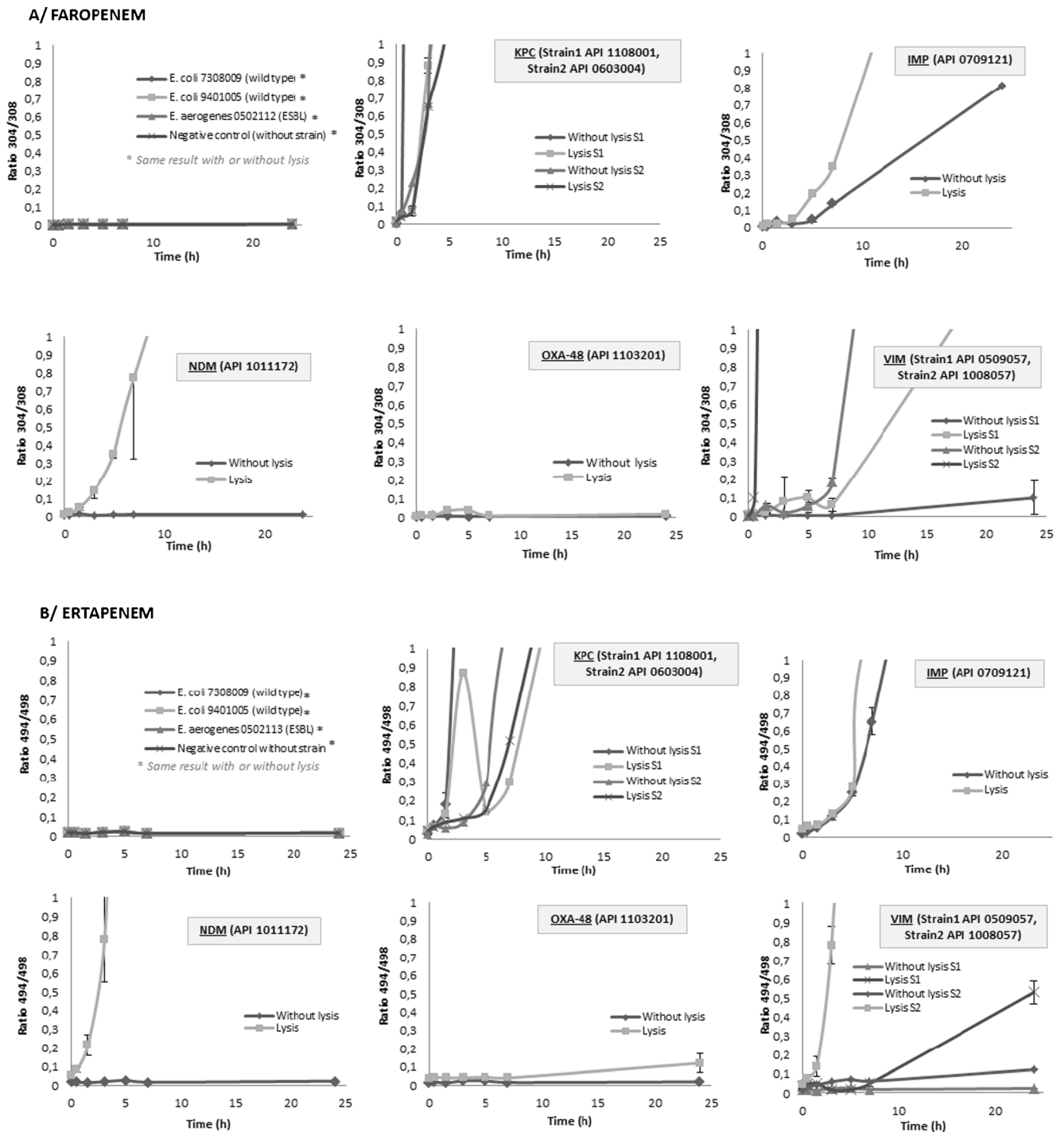


Fig. 4 Monitoring of faropenem and ertapenem hydrolysis during 24 h with different types of carbapenemase producers (S1=strain 1, S2=strain 2). Hydrolysis monitored by apparition of products of degradation with ratio calculation of peak intensities: ratio hydrolyzed product/native form.

Times T0, T0.5h, T1h30, T3h, T5h, T8h, and T24h were studied. The means of four spots were calculated for each time point: two biological replicates (= 2 different bacterial growths) and two spots/replicates

Considerations for routine use in clinical microbiological laboratories

Optimization and standardization of the MALDI-TOF MS assay are required for each drug–enzyme combination

separately, although there have been claims towards multiplexing. A multiplexed assay containing multiple antibiotics in a single test could, therefore, be considered for implementation in clinical laboratories, but given the variability we outlined above, we consider this not prudent at the current

state of technology. Otherwise, the selection of the informative masses needs expert knowledge because each antibiotic has its characteristic masses, with some of these masses being complicated by the existence of (multiple) salt adducts. Manual measurements and analysis of spectra can be cumbersome and difficult for people who are not experienced in MS. Such a manual approach might disturb the routine workflow and, hence, automation is needed. In our study, bacteria were grown on blood agar media without selective agents. A possible improvement of the hydrolysis assay could be to grow bacteria on media with selective antibiotics or ions like zinc to help induce higher levels of enzyme activity [28]. Thereby, the expression of carbapenemase could be enhanced and the time to result could be decreased. It could also provide a way for better detection of OXA-48 producers.

Conclusion

Several studies have been described in the literature and demonstrated proof of concept for the detection of carbapenemase activity by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). We show here that VITEK® MS RUO was successfully used for qualitative measurements of ertapenem and faropenem degradation. However, discrepancies in enzymatic kinetics require multiple readouts and jeopardize the design of a simple and well-standardized laboratory protocol. The time span between test initiation and obtaining actionable information transferable to clinicians may vary between a few minutes to several hours. All previous studies in this field have used different protocols with different incubation times [13–15, 22] and/or lysis steps [29]. This diversity of protocols illustrates the challenges to transfer these research protocols to validated protocols for a user-friendly and accurate use in routine clinical microbiology and public health laboratories. This renders the MS-based assay not really competitive compared to existing phenotypic tests with optical reading [9]. The latter could be read by the naked eye and could be more easily automated. The MALDI-TOF MS assays need to, indeed, automate both the complex deposition and under-vacuum introduction of the target. Due to these technical limitations, it is unlikely that the application of this technique for the determination of resistance mechanisms could replace standard susceptibility testing. Resistance to antibiotics is a complex process. Therefore, the detection of a limited number of specific determinants cannot provide a complete picture of antibiotic resistance.

Conflict of interest The authors declare that they have no conflict of interest.

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