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Phenotypic Detection and Differentiation of Carbapenemase Classes Including OXA-48-Like Enzymes in *Enterobacterales* and *Pseudomonas aeruginosa* by a Highly Specialized Micronaut-S Microdilution Assay

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ABSTRACT The objective of this study was to evaluate the Micronaut-S carbapenemase detection microtiter plate assay for the detection of carbapenemases and Ambler class determination. The Micronaut-S carbapenemase detection microtiter plate was tested using a challenging collection of 154 carbapenemase-producing and 150 carbapenemase-negative clinical strains of *Enterobacterales* and *Pseudomonas aeruginosa*. The Micronaut-S carbapenemase detection assay was able to detect 148/154 carbapenemase producers correctly, whereas 5/150 non-carbapenemase-producing isolates tested as false positive. This resulted in an overall sensitivity of 96% and a specificity of 97%. Regarding the detection of the carbapenemase class, the sensitivities and specificities were 93%/100%, 96%/100%, and 97%/99% for class A ($n = 27$), class B ($n = 54$), and class D ($n = 73$) carbapenemases, respectively. The Micronaut-S carbapenemase detection microtiter plate represents an easy-to-use and valuable tool for accurate and reliable detection of carbapenemases. In addition, it provides identification of the class of carbapenemase in most cases which can provide significant therapy guidance.

KEYWORDS multidrug resistance, OXA-48, KPC, metallo-β-lactamases, Micronaut, carbapenemases, detection of resistance mechanism, microdilution

Carbapenem resistance in clinically relevant Gram-negative bacteria has become one of the most threatening developments in clinical microbiology worldwide. Carbapenem resistance can be caused by four mechanisms which can also occur in combination: (i) the loss of porins in the outer membrane, (ii) expression of exporter pumps, (iii) mutations of penicillin binding proteins, and (iv) the production of carbapenemases (1–6). Based these mechanisms, the production of carbapenemases is of utmost importance due to the large potential of horizontal spread and the large substrate spectrum of most carbapenemases (7–9). Carbapenemases are found in the molecular Ambler classes A, B and D and are characterized by different hydrolysis and inhibition properties: While class A carbapenemases such as KPC or GES are inhibited by boronic acid, class B metallo-β-lactamases are inhibited by chelators such as EDTA (7). Class D carbapenemases on the other hand are a very diverse class of enzymes, with OXA-48-like being the most important ones in *Enterobacterales*, since they are inhibited by avibactam (10, 11). Despite the introduction of various tests for carbapenemase detection, fast and reliable detection of carbapenemase producers is still a challenge for microbiological laboratories. The commercial tests currently existing are either phenotypic nonspecific tests for any carbapenemase, such as the modified carbapenem inactivation method or biochemical methods (12, 13), immunochromatographic assays, or molecular methods based on real-time-PCR detection of carbapenemase genes (14,

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15). However, each of these approaches has shortcomings: most phenotypic assays do not give any information on the type of carbapenemase, which can be important due to therapy guidance or epidemiological purposes. Furthermore, genotypic or immunodetection-based methods fail to detect rare or novel carbapenemases that are not included in their respective DNA or protein targets.

In this study, we evaluated a novel microdilution assay, the Micronaut-S carbapenemase detection, for nonspecific detection of carbapenemases in *Enterobacterales* and *Pseudomonas aeruginosa* that can also provide determination of the class of carbapenemase.

MATERIALS AND METHODS

The Micronaut-S carbapenemase detection microtiter plate (MERLIN Gesellschaft für Mikrobiologische Diagnostika mbH, Bornheim-Hersel, Germany) is a CE-IVD-labeled commercial assay that contains doubling serial dilution ranges of meropenem as a monocompound (0.06 to 128 μg/ml) and in combination with the β-lactamase inhibitory substances avibactam, EDTA, and boronic acid (0.031 to 32 μg/ml each). Furthermore, temocillin high-level resistance (i.e., MIC of >32 mg/liter) was also tested. The plate can be used to test two bacterial isolates in parallel. The plate was evaluated using a well precharacterized strain collection of 304 clinical strains of *Enterobacterales* ($n = 277$) and *Pseudomonas aeruginosa* ($n = 27$) producing various carbapenemases ($n = 154$) or no carbapenemase ($n = 150$).

For precharacterization, species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany). Carbapenemase detection was performed at the German National Reference Centre for Multidrug-resistant Gram-Negative Bacteria (NRC) using the modified Hodge test (16), the mCIM (13), and combined disk tests with EDTA (17) and boronic acid (18). PCRs for KPC (19)-, VIM (17, 20)-, IMP (17)-, NDM (21)-, and OXA-48 (22)-encoding genes were performed routinely. If the phenotypic tests suggested a carbapenemase that was not detected by PCR, the isolates were analyzed by a microbiological bioassay (23), and additional PCRs for rarely occurring carbapenemases, such as FRI (primers 5′-GTCTTCCATTGAACTCATTCGCC-3′ and 5′-TGATTAGGACGTGTTGTGTAGAC-3′), GES (primers 5′-ATCGCTCACTCTGCATATGC-3′ and 5′-GAGTTGTG TAATAACTTGACCGAC-3′) and IMI (24), were performed. Detection of ESBLs/AmpCs and/or porin loss/impermeability was performed according to EUCAST guidelines for detection of resistance mechanisms (25).

The *Enterobacterales* species were *Escherichia coli* ($n = 116$), *Klebsiella pneumoniae* ($n = 89$), *Enterobacter cloacae* ($n = 18$), *Klebsiella oxytoca* ($n = 16$), *Serratia marcescens* ($n = 12$), *Citrobacter freundii* ($n = 7$), *Proteus mirabilis* ($n = 6$), *Klebsiella aerogenes* ($n = 4$), *Morganella morganii* ($n = 3$), *Providencia rettgeri* ($n = 2$), *Salmonella* spp. ($n = 2$), *Hafnia alvei* ($n = 1$), and *Providencia stuartii* ($n = 1$). The carbapenemases produced by the 154 respective isolates were OXA-48 ($n = 44$), KPC-2 ($n = 12$), VIM-1 ($n = 10$), OXA-244 ($n = 9$), VIM-2 ($n = 7$), NDM-1 ($n = 6$), OXA-181 ($n = 6$), OXA-232 ($n = 5$), GIM-1 ($n = 4$), KPC-3 ($n = 3$), OXA-162 ($n = 4$), GES-5 ($n = 3$), IMP-1 ($n = 3$), OXA-204 ($n = 3$), VIM-4 ($n = 3$), FIM-1 ($n = 2$), IMI-1 ($n = 2$), FRI-3 ($n = 1$), IMI-2 ($n = 1$), IMI-4 ($n = 1$), IMI-9 ($n = 1$), IMI-12 ($n = 1$), IMI-14 ($n = 1$), IMI-16 ($n = 1$), IMP-7 ($n = 1$), IMP-8 ($n = 1$), IMP-13 ($n = 1$), IMP-14 ($n = 1$), IMP-28 ($n = 1$), IMP-31 ($n = 1$), IMP-50 ($n = 1$), NDM-5 ($n = 1$), NDM-7 ($n = 1$), OXA-245 ($n = 1$), OXA-370 ($n = 1$), VIM-5 ($n = 1$), VIM-11 ($n = 1$), VIM-17 ($n = 1$), VIM-19 ($n = 1$), VIM-26 ($n = 1$), VIM-28 ($n = 1$), VIM-31 ($n = 1$), VIM-46 ($n = 1$), VIM-52 ($n = 1$), and VIM-56 ($n = 1$), resulting in 73 OXA-48-like producers, 54 metallo-β-lactamase producers, and 27 class A carbapenemase producers. Testing was performed according to the manufacturer's instructions by preparing a 0.5 McFarland suspension of the test strain grown overnight on Columbia blood agar in 0.9% saline solution. Then, 50 μl of this bacterial suspension was transferred into Mueller-Hinton broth (final bacterial concentration of approximately 5×10^5 CFU/ml). Subsequently, 100 μl of this suspension was transferred into each well of the microtiter plate. After incubation at 35 $\pm$ 2°C for 18 to 24 h, bacterial growth was detected using a Multiskan EX microplate photometer (Thermo Fisher Scientific, Cleveland, OH) and Micronaut6 (MCN6) software (Merlin Diagnostika GmbH, Bornheim, Germany). Based on specific growth cutoff values, the MCN6 software determines and reports MIC values for all substances (meropenem, meropenem/EDTA, meropenem/boronic acid, meropenem/avibactam, and temocillin) and synergistic effects (≥3 dilution steps difference of meropenem alone and in combination with the respective β-lactamase-inhibitors) of the different selective inhibitor combinations are investigated. By calculating MIC differences between meropenem and the respective inhibitor-combinations, the MCN6 software detects, differentiates, and reports the presence of class A, MBL, and/or OXA-48-like carbapenemases. In case the synergistic effects results in only two dilution steps difference between meropenem alone and in combination with the respective β-lactamase inhibitors, the software reports the isolate as class A and/or MBL "suspicious." In case no synergistic effects are detected but the meropenem screening cutoff value is exceeded or when meropenem MIC values are >2 μg/ml or out-of-range results occur, the MCN6 software provides information that a carbapenemase and/or porin loss/impermeability might be present in the isolate without further differentiation.

In the case of discrepant results, the respective isolate was recharacterized by the phenotypic and genotypic methods described before. *E. coli* ATCC 25922 (carbapenem-susceptible), *P. aeruginosa* ATCC 27853 (carbapenem susceptible, temocillin resistant), *K. pneumoniae* ATCC 700603 (carbapenem susceptible), and *K. pneumoniae* ATCC BAA-1705 (carbapenem resistant, KPC positive) served as quality control strains.

The MIC_{50} and MIC_{90} values were calculated as previously described (26).

TABLE 1 Micronaut-S carbapenemase detection microplate results for 154 carbapenemase-positive Gram-negative isolates[a]

Species	Total no.	Carbapenemase(s) detected (no. of isolates)			No. of test results					Carbapenemase(s) not detected
		Class A	MBL	OXA-48-like	Class A	MBL	OXA-48-like	Negative	Other	
C. freundii	6	2	4		1	4		1		GES-5
E. cloacae	16	9	6	1	8	5	1	1	1[a]	IMI-16,[a] IMP-14
E. coli	30	2	8	20	1	8	19	1	1[a]	GES-5,[a] OXA-244
K. aerogenes	1			1			1			
K. oxytoca	5	1	3	1	1	3	1			
K. pneumoniae	62	10	8	44	9	8	44	1		KPC-2
M. morganii	1	1			1					
P. aeruginosa	22	1	21		1	19		1	1[b]	IMP-7,[b] IMP-31
P. mirabilis	1		1			1				
P. stuartii	1			1				1		OXA-204
S. marcescens	9	1	3	5	1	3	5			
Total	154	27	54	73	23	51	71	6	3	

[a]Isolates categorized as "carbapenemase positive" but for which Ambler class determination was not possible due to low inhibition by boronic acid.
[b]The isolate was categorized as "MBL suspicious" due to low inhibition by EDTA.

RESULTS

The meropenem MIC_{50}/MIC_{90} values were 32/128 mg/liter, 32/128 mg/liter, and 1/32 mg/liter, respectively, for the isolates producing class A, B, and D carbapenemases. For the carbapenemase-negative isolates, the MIC_{50}/MIC_{90} values were 0.0625/0.125 mg/liter, with 11 isolates showing values above the EUCAST screening cutoff for carbapenemase detection of >0.125 mg/liter (25). The Micronaut-S carbapenemase detection assay was able to detect 148/154 carbapenemase producers correctly, while 5/150 non-carbapenemase-producing isolates were tested false positive (Table 1; see also Table 2). This resulted in an overall sensitivity of 96% and a specificity of 97%, as well as positive and negative predictive values of 0.97 and 0.96, respectively. The carbapenemase producers not detected were a GES-5-producing isolate of *C. freundii* (meropenem MIC of 64 mg/liter), an IMP-14-producing isolate of *E. cloacae* (meropenem MIC of 4 mg/liter), an IMP-31-producing isolate of *P. aeruginosa* (meropenem MIC of 128 mg/liter), a KPC-2-producing isolate of *K. pneumoniae* (meropenem MIC of >128 mg/liter), an OXA-204-producing isolate of *P. stuartii* (meropenem MIC of 1 mg/liter), and an OXA-244-producing isolate of *E. coli* (meropenem MIC of 0.25 mg/liter). The isolates producing GES-5, IMP-14, IMP-31, and OXA-204 were not efficiently inhib-

TABLE 2 Micronaut-S carbapenemase detection microplate results for 150 carbapenemase-negative Gram-negative isolates

Species	Total no.	No. of isolates demonstrating resistance mechanism(s)						No. of test results[b]		
		ESBL	AmpC	ESBL + AmpC	AmpC + porin loss	OXY HP[a]	No β-lactamase	−	Potential	+
C. freundii	1		1					1		
E. cloacae	2	2						2		
E. coli	86	45	8	8	1		24	86		
H. alvei	1		1					1		
K. aerogenes	3		1		2			1	1	1[c]
K. oxytoca	11	4		1		5	1	11		
K. pneumoniae	27	21	4				4	24	3	
M. morganii	2		2					2		
P. mirabilis	5	2	3					5		
P. rettgeri	2	2						2		
P. aeruginosa	5						5	5		
Salmonella spp.	2	1	1					2		
S. marcescens	3		3					3		
Total	150	77	24	9	3	5	34	145	4	1

[a]OXY HP, OXY hyperproduction. OXY-type cephalosporinases are intrinsic to *K. oxytoca*.
[b]−, negative; +, positive; the "potential" heading refers to potential carbapenemase or potential impermeability and/or porin loss.
[c]The isolate was misclassified as class A carbapenemase positive.

ited by the respective β-lactamase inhibitors avibactam, EDTA, or boronic acid, resulting in failed detection. The KPC-2-producing isolate of *K. pneumoniae* showed out-of-range results for meropenem (MIC >128 mg/liter) and all inhibitor-combinations (>32 mg/liter). Of the five isolates that tested false-positive, four were classified as potential class D carbapenemase producers or to be potentially affected by increased membrane impermeability. Of these, three were *K. pneumoniae* strains producing ESBLs ($n = 2$) or a plasmid-mediated AmpC-β-lactamase ($n = 1$), and one was a *K. aerogenes* isolate. One *K. aerogenes* isolate overexpressing the intrinsic *ampC* gene as determined by phenotypic pre- and recharacterization was misclassified as a class A carbapenemase producer due to inhibition by boronic acid and avibactam.

Regarding the detection of the carbapenemase class, the sensitivities/specificities were 93/100%, 96/100, and 97/99% for class A, class B, and class D carbapenemases, respectively.

From the class A carbapenemase strain set, a GES-5-producing isolate of *C. freundii* and a KPC-2-producing isolate of *K. pneumoniae* were misidentified as OXA-48 producers, since meropenem plus boronic acid hat no effect, whereas meropenem plus avibactam inhibited growth. Two strains producing IMI-16 and GES-5 were detected as carbapenemase positive, but no clear β-lactamase class identification was possible due to a low inhibition by boronic acid (0/1 dilution steps difference of meropenem versus meropenem/boronic acid, respectively). As already described, from the class B strain set two IMP-14- and IMP-31-producing isolates were not detected since they showed no growth inhibition by meropenem plus EDTA (0/1 dilution steps difference of meropenem versus meropenem/EDTA, respectively). One *P. aeruginosa* isolate producing IMP-7 was not detected due to little inhibition by EDTA but was categorized as MBL suspicious by the MCN6 software (two dilution steps difference of meropenem versus meropenem/EDTA). Phenotypic and genotypic retesting confirmed the MBL presence in these three isolates. From the OXA-48-like strain set, two strains producing OXA-204 and OXA-244 showed no high-level temocillin resistance (i.e., MIC of ≤32 mg/liter). Consequently, the strains were not classified as OXA-48-like producers by the MCN6 software. Recharacterization showed that one of the strains was tested as temocillin resistant by disk diffusion with an inhibition zone diameter of 10 mm, while the other showed an inhibition zone diameter of 17 mm, which is not typical for an OXA-48-like producer (27).

DISCUSSION

The accurate, fast, and reliable detection of carbapenemases is becoming mandatory for diagnostic laboratories worldwide. Many tests for detection of these spreading enzymes have been developed in the last years using different methodical approaches. The Micronaut-S carbapenemase detection assay is a classical broth microdilution microtiter plate combining serial dilutions of meropenem with different β-lactamase inhibitory substances.

The Micronaut-S carbapenemase detection microplate showed a very good performance and was able to detect almost all tested combinations of different species and carbapenemases, including rare enzymes such as IMI or GES. Furthermore, this method provided reliable determination of the respective molecular Ambler β-lactamase class. Although the general detection of a carbapenemase is more important, fast identification of the type of carbapenemase is also a very useful information for clinicians as it can help to decide if ceftazidime-avibactam should be considered for therapy in case of class A carbapenemase presence or if usage should be avoided in case of MBL production (28). This can also apply to aztreonam or the novel drug combination aztreonam-avibactam, which is not hydrolyzed by MBLs and could be used for therapy when no other resistance mechanisms against this drug are present (29). Other methods, such as the biochemical assays CarbaNP, β-Carba, or Blue-Carba (12, 30, 31), do not provide this information, which also applies to nonspecific disk diffusion assays like the mCIM or its numerous variations (13). However, most of these methods do not need overnight incubation, which is also a major limitation of the Micronaut-S carbap-

enemase detection microplate, since it may prolong or delay the diagnostic process. Immunology-based assays such as the RESIST-4 O.K.N.V. or the Carba5 (14, 32) or PCR-based tests such as the Xpert CarbaR (33) or GenePOC Carba (34) can provide information on the type of carbapenemase in a much shorter time; however, they fail to detect carbapenemases that are not included in their target spectrum. The Micronaut-S carbapenemase detection plate, on the other hand, can identify any carbapenemase producer, even when an as-yet-unknown carbapenemase is present. Furthermore, MIC determination for meropenem as a more or less secondary finding can be interesting, for example, to confirm prior MIC results. However, validation of the meropenem MIC determined by the plate was not part of this study, and we consequently do not recommend usage of the plate for meropenem MIC determination, although internal manufacturer data obtained for 27 quality control strains according to ISO 20776-2 (35) showed repeatability and reproducibility of 100% regarding MIC of meropenem at three different sites (data not shown).

The main limitation of this study and the plate itself is that no isolates producing multiple carbapenemases from different Ambler classes were tested. It must be assumed that the plate will have problems to detect such isolates since the inhibitory effect of the respective substances could potentially be masked by the noninhibited carbapenemase in case these belong to different Ambler classes. In case of carbapenem resistance without significant synergistic effects of inhibitors due to multiple carbapenemase production, the MCN6 software cannot distinguish between the different types, but a general warning that a carbapenemase might be present is displayed.

Another limitation is that only 14/150 carbapenemase-negative strains had meropenem MICs above the EUCAST screening cutoff for carbapenemase detection. Potentially, the number of false-positive results would increase if more such strains would be tested. However, this study was designed according to ISO 20776-2 (35) and the isolates were selected accordingly (approximately 50% carbapenemase-producing and 50% carbapenemase-negative isolates).

In conclusion, the Micronaut-S carbapenemase detection assay showed an excellent rate of detection of carbapenemases when confronted with a challenging set of clinical isolates producing a large variety of different carbapenemases. Furthermore, by reliable identification of the molecular class of the respective carbapenemase, it can provide important information for guidance of antibiotic therapy.

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