Evaluation of Carbapenemase Detection Methods Using a Challenging Collection of Enterobacteriaceae Isolates

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ABSTRACT

Background: The increase in carbapenemase-producing isolates has highlighted the need for reliable methods for detecting those strains. We evaluated a collection of 100 isolates carrying carbapenemase encoding genes and/or other carbapenem resistance mechanisms against both methodologies recommended by the CLSI documents for epidemiological detection of carbapenemases, hydrolysis assays for different carbapenems using a spectrophotometer and MALDI-TOF.

Methods: 100 Enterobacteriaceae isolates [9 bacterial species] were tested, including isolates known to produce carbapenemases (CARB; n=60), susceptible isolates carrying carbapenemase encoding genes (carbS-CARB; n=10), isolates with efflux pumps hyper-expression or porin loss/decreased expression (Acr/OMP; n=15) and a combination of the resistance mechanisms (CARB-Acr/OMP; n=15). Isolates were susceptibility tested with an extended MIC range for imipenem (IMI), meropenem (MER) and ertapenem (ERT). Modified Hodge test (MHT) was performed with 3 carbapenems, Carba-NP test was performed using an in house method and commercial tablets. Additionally, hydrolysis assays were carried out using IMI, MER, ERT in the spectrophotometer and the MALDI-TOF.

Results: MIC values for carbapenems ranged from susceptible (<1 µg/mL) up to 1024 µg/mL. Different methods provided sensitivity and specificity varying from 44.7 to 88.5% and 71.4 to 100.0%. The highest sensitivity was observed for the MHT (84.2 – 88.5%) followed by the Carba-NP (81.0 – 82.5%) and the highest specificity was noted for IMI hydrolysis in the spectrophotometer and CARBA-NP (in house and commercial; all 100.0%). Among the CARB-producers, 16-21/21 KPC-producers were detected by the various methods, but NDM, OXA-48- and VIM-producing strains had variable results. CARB-Acr/OMP strains were all detected by all methods but ERT MALDI hydrolysis (11 positive/15). Different methods were able to detect 50.0 to 90.0% of the carbS-CARB isolates. Acr/OMP isolates had positive MHT and MER hydrolysis results, but all CARBA-NP were negative.

Conclusions: CLSI recommended methods that include CARBA-NP displayed good specificity and sensitivity. Isolates carrying NDM-encoding genes yielded negative results for various methods raising concerns to the ability of clinical laboratories to early detect these isolates.

INTRODUCTION

A large number of acquired carbapenemases have been identified and characterized and among these diverse enzymes, KPC-, VIM- and NDM-variants seem to have spread in various continents and bacterial species. Genes encoding carbapenemases are associated with mobile genetic elements that allow for rapid dissemination in the clinical setting. Therefore, detection and surveillance of carbapenemase-producing organisms have become matters of major importance for the selection of appropriate therapeutic schemes and implementation of infection control measures.

Various screening methods for carbapenemases have been described with varying sensitivity and specificity results. The Clinical and Laboratory Standards Institute (CLSI) now recommends either testing methods for detection of carbapenemases for Enterobacteriaceae with epidemiological purposes, the modified Hodge test (MHT) or the Carba-NP method that uses a pH indicator to detect imipenem hydrolysis. Additionally, methods using Matrix Assisted Laser Desorption/ionization – Time of Flight (MALDI-TOF) mass spectrometry to detect carbapenem hydrolysis have been described and are appealing for clinical laboratories that use this instrument for bacterial identification.

In this study, we evaluate the performance of MHT for imipenem, meropenem and ertapenem disks, Carba-NP using in house and commercial reagents and hydrolysis methods performed in the spectrophotometer and the MALDI-TOF for imipenem, meropenem and ertapenem tested against a collection of 100 Enterobacteriaceae clinical isolates, 85 of which produce carbapenemases and 15 isolates were carbapenem-susceptible or -resistant due to intrinsic mechanisms of resistance.

MATERIALS AND METHODS

Bacterial isolates. One hundred Enterobacteriaceae isolates previously screened by reference PCR/sequencing for the presence of carbapenemases were selected and evaluated against selected carbapenemase phenotypic screening methods. The collection included isolates known to produce carbapenemases (n=60) belonging to the following bacterial species: 22 *Klebsiella pneumoniae*, 15 *Enterobacter cloacae*, 9 *Escherichia coli*, 3 *Citrobacter freundii*, 3 *Klebsiella oxytoca*, 3 *Serratia marcescens*, 2 *Enterobacter aerogenes*, 2 *Proteus mirabilis* and one *Providencia stuartii*. Carbapenem-susceptible isolates carrying carbapenemase encoding genes (n=10) were also included and these were 4 *K. pneumoniae*, 3 *E. cloacae*, 2 *E. coli* and one *K. oxytoca*. Additionally, isolates displaying hyper-expression of efflux pumps (AcrAB-TolC) or outer membrane protein (OMP) loss/decreased expression determined by quantitative real-time PCR in addition to a carbapenemase gene were selected (n=15; all *K. pneumoniae*). Isolates negative for production of carbapenemase-encoding genes (n=15) were included as a negative control group (11 *K. pneumoniae*, two *E. cloacae* and one of each *E. coli* and *K. oxytoca*).

Isolates not carrying carbapenemases (n=15) were considered negative and all isolates carrying these genes (n=85; **Table 1**), including the ones with low carbapenem MIC values, were considered positive for statistical calculations.

Susceptibility testing. Susceptibility testing was performed by reference broth microdilution testing methods according to the CLSI guidelines (M07-A10) for imipenem, meropenem, ertapenem and doripenem with concentrations ranging from 0.5 to 1024 µg/mL. Quality control (QC) testing was performed by testing *E. coli* ATCC 25922 and 35218, *Pseudomonas aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603. All QC results were within published ranges.

CLSI carbapenemase screening methods. Modified Hodge test (MHT) was performed with imipenem, meropenem and ertapenem disks as outlined in the CLSI documents. Carba-NP test was performed using the method described in the CLSI guidelines and by using commercial tablets (Rosco, Denmark) according to the manufacturer's instructions.

Hydrolysis assay. Imipenem, meropenem and ertapenem hydrolysis assays were carried out in the spectrophotometer as described elsewhere.

Hydrolysis of imipenem, meropenem and ertapenem were determined using MALDI-TOF. A 1 µL inoculation loop full of fresh bacterial cultures from agar plate (corresponding to 3-6 x 10^8 cells) was suspended in 30 µL of the carbapenem solution (1 mg/mL in 10mM ammonium citrate). Cell suspensions were incubated under agitation, centrifuged and supernatant was spotted on the target. Antimicrobial solution and standard Peptide calibration standard II were used as controls. After drying in ambient air, spots were overlaid with 1 µL of HCCA matrix (10 mg/mL of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2.5% trifluroacetic acid; Bruker Daltonik, Germany). MALDI-TOF MS measurements were performed with Microflex LT (Bruker Daltonics, Germany). Spectra were recorded in positive linear mode in the mass range of 100- to 1,200-Da with following settings: ion source 1[IS1], 19kV; IS2, 17kV; lens 5.8kV. Samples showing significant reduction in the peaks for each carbapenem (imipenem, 300.4Da; meropenem, 384.4 Da; ertapenem 498.3 and 520.4 Da) compared to the control were considered hydrolysis positive.

Carbapenemase screening quality control. *K. pneumoniae* BAA-1705 (KPC-producing) and BAA-1706 were included as carbapenemases positive and negative controls, respectively.

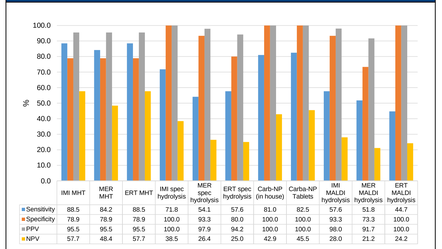
RESULTS

- Sixty carbapenemase producing isolates were tested and among those, only 20 displayed positive results for all screening methods. These isolates were mostly *K. pneumoniae* (16 isolates) and carried genes encoding KPC (12 isolates), IMP (5, including one isolate co-producing OXA-48), and one of each NDM-7, VIM-1 and VIM-32.
- Five carbapenemase-producing isolates were negative for one or two carbapenemase screening tests including meropenem or ertapenem spectrophotometer hydrolysis, MHT for meropenem and IMI MALDI hydrolysis. These isolates were *K. pneumoniae* and one *K. oxytoca* that carried genes encoding four IMP-variants or VIM-4.
- Among 15 isolates carrying a carbapenemase encoding gene (KPC-2 or KPC-3) and decrease/loss of OMP and/or AcrAB-TolC hyper-expression, all isolates had positive results for all screening methods applied. The MIC ranges for these isolates were 4 to 512 µg/mL for imipenem, 1 to 512 µg/mL for meropenem, 1 to >1024 µg/mL for ertapenem and ≤1 to 256 µg/mL for doripenem.
- Three out of ten isolates carrying a carbapenemase encoding gene and displaying a susceptible MIC value for imipenem and/or meropenem, exhibited positive results for all screening methods tested. These isolates were *K. pneumoniae* and carried genes encoding KPC-2 or KPC-3.
- Three other isolates carrying genes encoding KPC-2 (*E. coli*) or OXA-48 (one *E. coli* and one *K. pneumoniae*) and exhibiting susceptible MIC values for carbapenems displayed two or three negative results for: meropenem or ertapenem spectrophotometer hydrolysis (1 isolate each), in-house Carba-NP (1 isolate) and MALDI hydrolysis for all substrates (1 isolate). Additionally, one *E. cloacae* carrying bla_{NDM-1} had positive results for spectrophotometer hydrolysis and MHT, but results were negative for Carba-NP and MALDI hydrolysis methods.
- Among 15 isolates that did not produce carbapenemases and had susceptible carbapenem MIC results (7 isolates) or resistant isolates due to the presence of an intrinsic resistance mechanism (8 isolates), positive (false) results were observed for MHT with all substrates for four isolates, ertapenem spectrophotometer hydrolysis for three isolates and meropenem or imipenem MALDI hydrolysis, for four and one isolate, respectively. All results were negative for imipenem or meropenem spectrophotometer hydrolysis, Carba-NP for both methods and ertapenem MALDI hydrolysis.
- Sensitivity for the different tests ranged from 44.7 to 88.5% and specificity from 7.3 to 100.0% (**Figure 1**). Positive predictive value (PPV) was high and ranged from 91.7% to 100.0% whereas negative predictive value (NPV) ranged from 21.2 to 57.7% (**Figure 1**).
- When evaluating the different enzyme groups (**Figure 2A**), KP-2, KPC-3, IMP variants, NDM-5 and NDM-7 were mostly positive for the different screening methods evaluated and displayed a median value of the percentage of positive values at 100.0%.
- OXA-48, NDM-1 and VIM-1 displayed many negative results for the different screening methods (**Figure 2B**). Only three screening tests were able to detect >50.0% of the NDM-1-producing isolates: MHT using imipenem or ertapenem disks and MALDI imipenem hydrolysis. For OXA-48 and VIM-1, five tests were able to detect >50.0% of the isolates.

Table 1. Carbapenemase-producing Enterobacteriaceae isolates tested in this study by bacterial species.

Organism Carbapenemase	No. of isolates Total	Carbapenemase producers	Carbapenemase producers displaying low carbapenem MIC results	Carbapenemase producers + OMP decrease/loss and/or AcrAB-TolC hyperexpression
Klebsiella pneumoniae	**41**	**22**	**4**	**15**
IMP-1	3	3		
IMP-26	1	1		
IMP-4	2	2		
KPC-2	12		1	11
KPC-3	5		1	4
KPC-4	1	1		
NDM-1	5	4	1	
NDM-1, VIM-1	1	1		
NDM-7	1	1		
OXA-48	6	5	1	
VIM-1	3	3		
VIM-4	1	1		
Enterobacter cloacae	**18**	**15**	**3**	
IMP-1, OXA-48	1	1		
IMP-26	1	1		
IMP-4	2	2		
KPC-2	1		1	
KPC-4	1	1		
NDM-1	4	3	1	
OXA-48	1	1		
VIM-1	2	2		
VIM-23	1	1		
VIM-4	2	2		
VIM-5	2	1	1	
Escherichia coli	**11**	**9**	**2**	
IMP-1	1	1		
IMP-26	1	1		
KPC-2	1		1	
NDM-1	4	4		
NDM-5	1	1		
OXA-48	3	2	1	
Klebsiella oxytoca	**4**	**3**	**1**	
GES-20	1		1	
IMP-1	1	1		
IMP-18	1	1		
OXA-48	1	1		
Citrobacter freundii	**3**	**3**		
NDM-1	1	1		
VIM-1	1	1		
VIM-32	1	1		
Serratia marcescens	**3**	**3**		
IMP-19	1	1		
NDM-1	1	1		
OXA-48	1	1		
Enterobacter aerogenes	**2**	**2**		
NDM-1	1	1		
OXA-48	1	1		
Proteus mirabilis	**2**	**2**		
NDM-1	1	1		
VIM-1	1	1		
Providencia stuartii	**1**	**1**		
VIM-1	1	1		
Total	**85**	**60**	**10**	**15**

Figure 1. Comparison of the different carbapenemase-screening methods using sensitivity, specificity, positive-predictive (PPV) and negative-predictive (NPV) values when tested against 100 Enterobacteriaceae isolates.



	IMI MHT	MER MHT	ERT MHT	IMI spec hydrolysis	MER spec hydrolysis	ERT spec hydrolysis	Carb-NP (in house)	Carba-NP Tablets	IMI MALDI hydrolysis	MER MALDI hydrolysis	ERT MALDI hydrolysis
Sensitivity	88.5	84.2	88.5	71.8	54.1	57.6	81.0	82.5	57.6	51.8	44.7
Specificity	78.9	78.9	78.9	100.0	93.3	80.0	100.0	100.0	93.3	73.3	100.0
PPV	95.5	95.5	95.5	100.0	97.9	94.2	100.0	100.0	98.0	91.7	100.0
NPV	57.7	48.4	57.7	38.5	26.4	25.0	42.9	45.5	28.0	21.2	24.2

Figure 2. Evaluation of the carbapenemase screening tests by enzyme. (A) Comparison of the median percentage of positive results for each carbapenemase variant tested; (B) Comparison of the percent of positive results of the enzymes displaying an elevated number of negative results.

Figure 2A

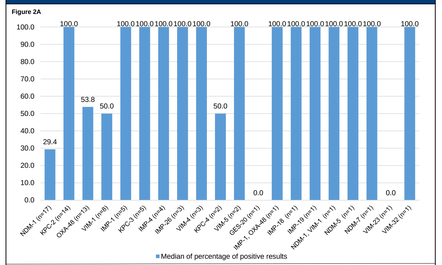
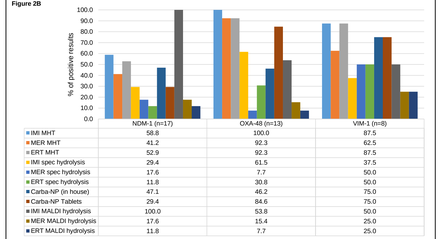


Figure 2B



	NDM-1 (n=17)	OXA-48 (n=13)	VIM-1 (n=8)
IMI MHT	58.8	100.0	87.5
MER MHT	41.2	92.3	62.5
ERT MHT	52.9	92.3	87.5
IMI spec hydrolysis	29.4	61.5	37.5
MER spec hydrolysis	17.6	7.7	50.0
ERT spec hydrolysis	11.8	30.8	50.0
Carba-NP (in house)	47.1	46.2	75.0
Carba-NP Tablets	29.4	84.6	75.0
IMI MALDI hydrolysis	100.0	53.8	50.0
MER MALDI hydrolysis	17.6	15.4	25.0
ERT MALDI hydrolysis	11.8	7.7	25.0

CONCLUSIONS

- An elevated number of negative results for isolates carrying genes encoding OXA-48, NDM-1, VIM-variants and GES-20 were observed for different screening methodologies tested and this generated a low sensitivity and NPV for the various methods evaluated.
- Overall, imipenem hydrolysis in the spectrophotometer, MALDI ertapenem hydrolysis and the two Carba-NP methods analyzed were very specific (100.0%); however, the MHT was more sensitive (84.2-88.5%) to detect carbapenemase-producing isolates.
- Due to the elevated number of false negative results, sensitivity and negative predictive values were low for all screening methods. False negative results could be related to the long term storage of isolates without the presence of selective pressure.

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