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Detection of Carbapenemase Production in a Collection of *Enterobacteriaceae* with Characterized Resistance Mechanisms from Clinical and Environmental Origins by Use of Both Carba NP and Blue-Carba Tests

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Rapid-screening methods to confirm the presence of resistance mechanisms in multidrug-resistant bacteria are currently recommended. Carba NP and Blue-Carba tests were evaluated in carbapenemase-producing *Enterobacteriaceae* from hospital (n = 102) and environmental (n = 57) origins for detecting the different molecular classes among them. Both methods showed to be fast and cost-effective, with high sensitivity (98% to 100%) and specificity (100%), and may be easily introduced in the routine laboratory.

Carbapenemase-producing *Enterobacteriaceae* (CPE) constitute a critical medical and public health issue (1). Moreover, plasmids responsible for carbapenemase transmission could carry other resistance mechanisms, making the antibiotic choice for the treatment of infections caused by these isolates very limited (2, 3). Rapid identification of these isolates colonizing or infecting patients is crucial to improve infection control measures with adequate antibiotic therapy and to minimize the spread of these resistant microorganisms. The variable hydrolysis spectrum of carbapenemases and the simultaneous presence of other β-lactam resistance mechanisms usually make their identification difficult (4).

Colorimetric methods to detect carbapenemase production directly in bacterial isolates recovered from microbiological cultures are easy to perform and reliable enough to be used in routine laboratory work. They are based on the enzymatic hydrolysis of the β-lactam ring of a carbapenem (usually imipenem) causing the acidification of an indicator solution (namely phenol red for Carba NP, pH 7.8, or bromothymol blue for Blue-Carba, pH 7.0, respectively) that changes its color due to pH modification. Detection of CPE takes a maximum of 2 h according to described protocols (5, 6).

In this study, we evaluated the performance of two different colorimetric methods to detect CPE: the Carba NP (5) and the Blue-Carba (6) tests. Both tests, using in-house prepared indicator solutions and imipenem, were performed in a 96-well tray format first and/or in 1.5-ml Eppendorf tubes. It should be emphasized that indicator solutions require strict adjustment of pH and addition of the carbapenem immediately before using. Studied isolates (n = 229) included (i) CPE from various clinical samples (n = 102) recovered at the Hospital Universitario Ramón y Cajal (Madrid, Spain), (ii) CPE from environmental samples (sewage waters, n = 57) from the Centro de Vigilancia Sanitaria Veterinaria (VISAVET; Madrid, Spain), and (iii) noncarbapenemase producers (NCPE, n = 70) (either susceptible or nonsusceptible to carbapenems) from various clinical sources (n = 48) and from sewage water (n = 22). This group included wild-type isolates (n = 34), extended-spectrum β-lactamases (ESBL) (TEM, SHV, CTX, and OXA variants; n = 11), and AmpC (chromosomally encoded or plasmid-mediated) β-lactamase producers either associated or not with porin loss (n = 25). *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1705 (a *Klebsiella pneumoniae* carbapenemase-2 [KPC-2] producer) were used as controls. Isolate identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany).

A preliminary detection and/or confirmation of the presence of a carbapenemase was assessed with the modified Hodge test (MHT) (7). MICs (of imipenem, ertapenem, and meropenem) were determined using gradient strips (MIC test strip; Liofilchem, Roseto degli Abruzzi, Italy). EUCAST clinical breakpoints and the screening cutoff values for CPE were considered (8, 9). The characterization of carbapenemase genes was performed by conventional PCR assays and sequencing, as previously published (10–12). Carba NP and Blue-Carba tests were simultaneously performed in duplicate (2 separate days) in all strains, following previously published instructions. Two independent readers interpreted the results (5, 6). When equivocal results were obtained, testing were repeated and included the following modifications: (i) for the Carba NP test, the volume of lysis buffer was increased (200 μl instead of 100 μl) and (ii) for the Blue-Carba test, a 10 μl-loop full of pure culture instead of a 5 μl-loop was used.

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TABLE 1 Activity of carbapenems, MHT results, and Carba NP and Blue-Carba performances against the 159 carbapenemase-producing isolates

Carbapenemase (*n*)	Microorganism (*n*)	MIC (μg/ml)[a] of:			No. of positive results for:		
		IMP	MEM	ERT	MHT	Carba NP	Blue-Carba
Class A							
KPC-2 (45)	*Citrobacter freundii* complex (15)	0.38 to >32	0.5 to >32	0.5 to >32	14[b]	14	14
	Enterobacter cloacae complex (11)	0.75 to >32	0.75 to >32	1 to >32	11	11	11
	E. coli (2)	0.5 to 6	1 to 8	1.5 to 2	2	2	2
	Klebsiella oxytoca (5)	0.75 to 4	0.38 to 2	0.75 to 4	4[c]	5	5
	K. pneumoniae (3)	4 to 12	4 to 12	6 to 8	3	3	3
	Kluyvera ascorbata (1)	12	12	6	1	1	1
	Kluyvera cryocrescens (1)	4	4	2	1	1	1
	Raoultella ornithinolytica (7)	0.25 to 12	1.5 to 24	1.5 to 4	7	7	7
KPC-3 (36)	*Citrobacter freundii* complex (2)	4 to 8	2 to 6	4 to 16	2	2	2
	Enterobacter cloacae complex (2)	0.5 to 0.75	0.25 to 1	0.38 to 2	2	2	2
	E. coli (2)	1	0.38	0.25 to 0.5	2	2	2
	K. pneumoniae (28)	0.25 to 6	0.38 to 12	0.19 to 12	28	28	28
	Kluyvera ascorbata (2)	12 to >32	8 to >32	4 to >32	2	2	2
Class B							
VIM-1 (24)	*Citrobacter freundii* complex (3)	0.75 to 8	0.5 to 6	1 to 4	3	3	3
	Enterobacter cloacae complex (7)	1 to 3	0.25 to 8	0.25 to 12	7	7	7
	E. coli (1)	0.5	0.75	1	1	1	1
	Klebsiella oxytoca (4)	0.38 to 2	0.25 to 1	0.047 to 0.5	4	4	4
	K. pneumoniae (5)	1 to 6	0.5 to 3	0.125 to 1	4[c]	5	5
	Raoultella ornithinolytica (2)	1.5 to >32	0.5 to >32	1.5 to >32	2	2	2
	Serratia marcescens (2)	1.5 to 2	0.38	0.19 to 0.25	2	2	2
NDM-1 (1)	*K. pneumoniae*	>32	>32	>32	1	1	1
Class D							
OXA-48 (53)	*Citrobacter amalonaticus* (1)	2	0.19	0.25	1	1	1
	Citrobacter freundii complex (2)	0.5 to 2	0.25 to 0.38	0.38	2	2	2
	Citrobacter koseri (1)	0.38	0.125	0.25	1	1	1
	Enterobacter aerogenes (1)	1	1	6	1	1	0[d]
	Enterobacter cloacae complex (1)	0.75	0.25	1.5	1	1	1
	E. coli (6)	0.19 to 4	0.125 to 1	0.25 to 6	6	6	6
	Klebsiella oxytoca (1)	0.5	0.25	0.75	1	1	1
	K. pneumoniae (37)	0.25 to >32	0.125 to >32	0.75 to >32	34[c,e]	37	35[d]
	Kluyvera ascorbata (2)	0.5 to 0.75	0.38 to 0.5	0.75 to 1.5	2	2	2
	Raoultella ornithinolytica (1)	1	0.38	0.75	1	1	1

[a] IMP, imipenem; MEM, meropenem; ERT, ertapenem.
[b] +, positive result.
[c] MHT equivocal results: *n* = 1 *Klebsiella oxytoca* KPC-2 + CTX-M-15; *n* = 1 *K. pneumoniae* VIM-1; *n* = 1 *K. pneumoniae* OXA-48 + CTX-M-15; *n* = 1 *K. pneumoniae* OXA-48 + SHV.
[d] Blue-Carba false negatives: *n* = 2 *K. pneumoniae* OXA-48 + CTX-M-15; *n* = 1 *E. aerogenes* OXA-48 + CTX-M-15.
[e] MHT negative result: *n* = 1 *K. pneumoniae* OXA-48 + CTX-M-15.

The collection of 229 *Enterobacteriaceae* isolates included *Klebsiella* spp. (*n* = 99), *E. coli* (*n* = 49), *Enterobacter* spp. (*n* = 34), *Citrobacter* spp. (*n* = 25), *Raoultella ornithinolytica* (*n* = 14), *Kluyvera* spp. (*n* = 6), and *Serratia marcescens* (*n* = 2).

The 159 CPE comprised the following variants: OXA-48 (*n* = 53), KPC-2 (*n* = 45), KPC-3 (*n* = 36), VIM-1 (*n* = 24), and NDM-1 (*n* = 1) (Table 1). MHT detected 96.9% (154/159) of CPE. In 8 and 6 isolates of the 159 CPE, ambiguous or false-negative results were obtained with the Blue-Carba test and the Carba NP test, respectively. Finally, after applying the above-cited modifications, a positive result was obtained in all CPE when using the Carba NP test (100% sensitivity). With the Blue-Carba test, 156 of 159 isolates were correctly detected; thus, sensitivity and negative-predictive results were slightly lower, at 98% and 96%, respectively. Blue-Carba detected 100% of KPC, VIM, and NDM enzymes and 94% of OXA-48 enzymes. The 3 OXA-48-producing isolates (*n* = 2, *K. pneumoniae*; *n* = 1, *Enterobacter aerogenes*) with negative results with the Blue-Carba test had hypermucoid phenotypes. Negative results were obtained for all NCPE with both methods, which means specificity and positive predictive values were 100%. Reader results were consistent with each other in all experiments. The carbapenem MICs (in μg/ml) in the NCPE ranged from 0.094 to 0.38 (imipenem), 0.016 to 16 (meropenem), and 0.25 to 4 (ertapenem), and environmental isolates were more resistant than clinical ones (data not shown).

These results confirmed that the Carba NP and Blue-Carba tests are both adequate for the detection of CPE from clinical origin (5). In addition, we demonstrated an adequate performance with CPE from environmental origin. We also noted that unreliable test results when using 96-well microtiter plates could be obtained, as the negative-control exhibited a change to positive color. This could be produced by bacterial components, water

condensation, or interaction with the atmosphere affecting the pH of the solution. This aspect was solved by repeating the assay using 1.5-ml Eppendorf tubes, and no more discrepancies were observed (13). A limitation of both tests is that the in-house indicator solution, simultaneously including imipenem as the substrate, has to be prepared at the time of use to avoid spontaneous degradation of the antibiotic.

Interestingly, when using both methods, it is possible to suspect the type of carbapenemase by attending to the time required to produce the indicator's acidification. Usually, KPC producers required less than 30 min, NDM and VIM producers required less than 1 h, and OXA producers required more than 1 h. Some isolates not producing a clear color change caused indeterminate or false-negative results, particularly those with a low carbapenemase activity, like OXA-48 producers (14), or those with mucoid phenotypes (15). Fortunately, in the case of these conflicting isolates, the stated modifications solved discrepant results. No misidentifications were observed in class A/class B carbapenemase producers, as described previously (15, 16).

Although the Blue-Carba test showed a slightly lower sensitivity than the Carba NP test, Blue-Carba is a simpler test, as a first bacterial protein extraction step is not required, allowing the direct use of colonies. Moreover, the cost of the Blue-Carba test is low, as no extraction buffer is required.

Despite the heterogeneous expression of carbapenemases (MIC range, 0.5 to >32 μg/ml), both methods were able to properly identify CPE. No false positives were observed in isolates in which carbapenem MICs were affected, as was the case of AmpC overproducers and/or ESBL producers coupled with porin loss (17, 18).

In conclusion, both colorimetric tests can be incorporated into the routine workflow of the clinical microbiology laboratory, allowing the rapid and somewhat inexpensive detection of CPE. Moreover, these methods showed similar sensitivity and specificity to molecular methods with the advantage of being accessible to most laboratories. The implementation of these confirmatory tests is particularly important in centers with CPE endemicity, where accurate and rapid infection-control measures, as well as isolation of colonized/infected patients, are a priority in order to limit the spread. Moreover, the tests are also useful in epidemiological studies collecting environmental isolates with complex phenotypes.

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