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Evaluation of the Carba NP Test for Carbapenemase Detection

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The Carba NP test was evaluated against a panel of 61 carbapenemase-producing bacterial species (15 producing class A carbapenemases, 15 producing class D carbapenemases, and 31 producing metallo-β-lactamases) and against 111 isolates with non-wild-type carbapenem susceptibility but not producing carbapenemase. Carbapenemase production was verified by PCR and UV-spectrophotometric measurement of imipenem hydrolysis. No false positives were seen, but there were consistent problems with the detection of OXA-48-like enzymes and also some rarer class A enzymes.

Carbapenemase-producing bacteria are spreading through the world and seem set to follow the rise of those producing extended-spectrum β-lactamases (ESBLs). Although many of the species are the same as in the ESBL epidemic, the carbapenemase genes are more diverse, and laboratory detection is immensely more challenging. Many strains have carbapenem MICs in the susceptible range, and different phenotypic methods such as the modified cloverleaf test, and disk tests with different inhibitors, lack specificity and sensitivity. Detection of carbapenemase genes and the ability of a strain to hydrolyze carbapenems remain the gold standard methods of identification. Gene detection is, however, out of reach for many clinical laboratories. Therefore, the recently published Carba NP method (1–3) has the potential to fill a gap. Without specialized equipment, it detects the pH change caused by the breakdown of imipenem in a solution containing lysed test bacteria (thus bypassing confounding factors caused by membrane changes). We have tested the sensitivity and specificity of this method in our reference laboratory.

(Preliminary results were presented at the 23rd European Congress of Clinical Microbiology and Infection, 27 to 30 April 2013, Berlin, Germany.)

Strains tested and routine carbapenemase detection methods. We routinely test isolates suspected to be carbapenemase producers that are sent from locations throughout the whole country. PCR was used to detect KPC, NDM, VIM, OXA-48, IMP, GES, OXA-23, -24, and -58, GIM, and SPM and UV spectrophotometry to detect hydrolysis of imipenem to confirm carbapenemase production or to exclude its presence in gene-negative isolates with non-wild-type carbapenem susceptibility, as described previously (4). From 12 April to 16 August 2013, all routine isolates ($n = 101$) were tested with the UV-spectrophotometric method and the Carba NP test in parallel. In addition to this, a number of strains with identified carbapenemases or carbapenemase activity were tested retroactively with Carba NP. In total, the following strains were tested ($n = 172$), all of which were isolated in Finland (Table 1): 61 carbapenemase-producing strains (26 described previously [4]), 51 carbapenem-resistant *Pseudomonas aeruginosa* strains with no carbapenemase activity, and 46 *Enterobacteriaceae* strains with reduced susceptibility to carbapenems due to different mechanisms but no carbapenemase activity (CTX-M or ESBL-SHV genes [$n = 20$], transferable *ampC* genes [$n = 7$], CTX-M and *ampC* genes [$n = 1$], and no common extended-spectrum-β-lactamase genes [$n = 18$]). The method was also challenged with 14 *Enterobacteriaceae* strains with (mostly low-to-moderate) carbapenemase activity in the UV-spectrophotometric test and no known carbapenemase genes. They were 5 *Klebsiella oxytoca* strains, 4 *Enterobacter cloacae* strains, 3 *Escherichia coli* strains, 1 *Citrobacter freundii* strain, and 1 *Enterobacter aerogenes* strain, collected between February 2010 and August 2013. In addition to PCR and hydrolysis experiments, phenotypes were determined with methods (including the use of imipenem/EDTA tablets, oxacillin inhibition, modified Hodge test, and sequencing by another laboratory) that were refined over the 3.5 years of collection: overproduced or mutated chromosomal β-lactamases with extended activity toward carbapenems were deemed to represent the most probable mechanism in all cases.

The carbapenemase producers in this material are not dominated by any single clone (4), with the exception of the IMP-positive *P. aeruginosa* strains (data not published). They consist mostly of isolates imported from very diverse locations (4); thus, the collection avoids the clonal and enzyme class bias seen in some studies.

Disk diffusion was done using the EUCAST disk diffusion methodology and EUCAST breakpoints (v. 3.1; www.eucast.org).

The original Carba NP protocol was followed (1–3). Briefly, bacteria were grown overnight on Mueller-Hinton II agar (Becton Dickinson BBL, Le Pont de Claix, France) with an ampicillin disk. Bacterial mass was scraped off with a 10-μl loop and suspended in lysis buffer, mixed using a vortex device, incubated at room temperature for 20 min, centrifuged at +4°C to remove cell wall debris and DNA, and kept on ice to preserve enzymatic activity. A 30-μl volume of this lysate was mixed with 100 μl of indicator solution: phenol red with 0.1 mmol/liter $ZnSO_4$ and 6 mg/ml Tienam (imipenem-cilastatin) and, as a control well, the phenol red solution without antibiotic. This was incubated at 37°C for up to 2 h, after which a color change to yellow in the antibiotic-containing well was read as a positive result.

Due to the prospective nature of part of the study, BugBuster

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TABLE 1 Isolates tested and Carba NP results[a]

Group	No.	Species	Carbapenemase gene (additional β-lactamase gene)	Carba NP test result		Rate of hydrolysis of imipenem measured UV-spectrophotometrically[b]
				No antibiotic	IPM + Zn^{2+}	
Carbapenem nonsusceptible		*Pseudomonas aeruginosa* ($n = 51$)	Not found	−	−	Negative
		Enterobacteriaceae ($n = 46$)	Not found (various)	−	−	Negative
Imipenem hydrolysis positive/ambiguous (spectrophotometry method)		*Enterobacteriaceae* ($n = 14$)	Not found	−	−	Varying positive
Metallo-β-lactamases	2,174	*P. aeruginosa*	IMP	−	+	Moderate
	2,186	*P. aeruginosa*	IMP	−	+	Moderate
	2,219	*P. aeruginosa*	IMP	−	+	Moderate
	2,269	*P. aeruginosa*	IMP	−	+	Moderate
	2,342	*P. aeruginosa*	IMP	−	+	Moderate
	2,422	*P. aeruginosa*	IMP	−	+	Moderate
	1,648	*P. aeruginosa*	IMP-15	−	+	Moderate
	1,871	*P. aeruginosa*	IMP-15	−	+	Moderate
	2,003	*P. aeruginosa*	IMP-15	−	+	Moderate
	2,310	*P. aeruginosa*	IMP-15	−	+	Moderate
	3,413	*E. coli*	NDM	−	+	High
	2,347	*E. coli*	NDM (CTX-M, TEM)	−	+	High
	2,491	*E. coli*	NDM (CTX-M, TEM)	−	+	High
	3,582	*E. coli*	NDM (CTX-M, TEM, CMY)	−	+	High
	2,775	*E. coli*	NDM (TEM, CIT)	−	+	High
	4,049	*E. coli*	NDM (CIT)	−	+	ND
	4,091	*E. coli*	NDM (TEM, SHV)	−	+	High
	3,583	*K. pneumoniae*	NDM (CTX-M, TEM, CMY)	−	+	High
	2,050	*K. pneumoniae*	NDM-1 (CTX-M)	−	+	High
	4,047	*K. pneumoniae*	NDM (CTX-M, CIT, DHA, TEM)	−	+	High
	2,098	*K. pneumoniae*	VIM	−	+	High
	2,187	*K. pneumoniae*	VIM	−	+	ND
	2,398	*K. pneumoniae*	VIM	−	+	High
	2,089	*K. pneumoniae*	VIM	−	+	Moderate
	2,126	*P. aeruginosa*	VIM	−	+	Moderate
	2,203	*P. aeruginosa*	VIM	−	+	ND
	2,506	*P. aeruginosa*	VIM	−	+	Moderate
	3,327	*K. oxytoca*	VIM (ACC)	−	+	High
	3,127	*A. baumannii*	VIM, OXA-58	−	+	High
	1,653	*P. aeruginosa*	VIM-2	−	+	ND
	1,872	*P. aeruginosa*	VIM-2	−	+	Moderate
Class A carbapenemase	3,697	*E. cloacae*	GES-5	−	−	Low
	2,049	*K. pneumoniae*	GES-14	−	−	Low
	2,168	*E. cloacae*	IMI-1	−	+	Moderate
	1,468	*E. cloacae*	IMI-2	−	−/+	High
	3,810	*E. cloacae*	KPC	−	+	High
	2,015	*K. pneumoniae*	KPC (CTX-M)	−	+	High
	3,523	*K. pneumoniae*	KPC (CTX-M, TEM)	−	+	High
	4,013	*K. pneumoniae*	KPC (TEM)	−	+	High
	2,801	*K. pneumoniae*	KPC (TEM, CTX-M)	−	+	High
	1,592	*K. pneumoniae*	KPC-2 (TEM)	−	+	High
	1,597	*K. pneumoniae*	KPC-2 (TEM)	−	+	High
	1,618	*K. pneumoniae*	KPC-2 (TEM)	−	+	High
	1,620	*K. pneumoniae*	KPC-2 (TEM)	−	+	High
	2,247	*E. cloacae*	NMC-A	−	+	Moderate
	2,779	*Serratia marcescens*	SME	−	−	High

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TABLE 1 (Continued)

Group	No.	Species	Carbapenemase gene (additional β-lactamase gene)	Carba NP test result		Rate of hydrolysis of imipenem measured UV-spectrophotometrically[b]
				No antibiotic	IPM + Zn^{2+}	
Class D carbapenemase	2,279	*K. pneumoniae*	OXA-181 (CTX-M, TEM)	−	+/−	Moderate
	2,485	*K. pneumoniae*	OXA-181 (CTX-M, TEM)	−	−	Moderate
	3,328	*K. pneumoniae*	OXA-181 (CTX-M, TEM)	−	−	Moderate
	2,287	*E. coli*	OXA-181 (TEM)	−	−	Moderate
	2,536	*K. pneumoniae*	OXA-181 (TEM, CTX-M)	−	−	Moderate
	3,993	*Acinetobacter* sp.	OXA-23	−	−	Low
	3,977	*E. coli*	OXA-48	−	+	High
	2,350	*K. pneumoniae*	OXA-48	−	+	High
	3,979	*K. pneumoniae*	OXA-48	−	+	High
	2,005	*Raoultella planticola*	OXA-48	−	+	High
	2,270	*E. coli*	OXA-48 (CTX-M)	−	+	High
	1,604	*E. coli*	OXA-48 (TEM)	−	+/−	Low
	2,663	*E. coli*	OXA-48 (TEM, CTX-M)	−	−/+	Moderate
	2,493	*K. pneumoniae*	OXA-48 (TEM-1)	−	+	High
	3,728	*E. coli*	OXA-48-like (CTX-M, TEM)	−	+/−	Moderate

[a] IPM, imipenem; −, negative; +, positive; +/− and −/+, weakly positive and very weakly positive, respectively; ND, not determined.
[b] The relative rates of hydrolysis were arbitrarily defined as high (all imipenem broken down by all three dilutions of bacterial lysate in less than 10 min), moderate (all imipenem broken down by all dilutions in less than 30 min), and low (imipenem not cleared completely in 30 min). Negative, drop in (lysate-plus-imipenem) OD of <0.25 to 0.3 over 30 min.

(Novagen/Merck Millipore, Espoo, Finland) was used instead of the B-PER II lysis buffer (Thermo Fisher, Rockford, IL) recommended by Dortet et al. (2, 3); we have used this routinely since 2008 in our hydrolysis assay, and, as far as we were able to determine, it was quite similar (both are Tris-buffered nonionic detergent solutions). We tested the B-PER buffer on 17 of the carbapenemase producers, including all that gave a negative Carba NP result, and we saw no difference in performance. A different buffer was used also by Vasoo et al., with excellent results (5).

Pure imipenem was replaced by the clinical substance Tienam (doubling the amount to compensate for the cilastatin component), weighed fresh each day. This alternative has recently been published by the inventors (6).

Not a single false positive was found, but the 100% sensitivity reported by the Nordmann group could not be reproduced by us (Table 1). Consistent problems were seen with OXA-181. Four of five isolates (tested 4 times) were repeatedly negative, and the fifth was positive only weakly. Several weak results were seen also with OXA-48. We use an OXA-48-positive *Raoultella terrigena* strain as a positive control in UV spectrophotometry; it was used also in the Carba NP test and gave a weak reaction in 4/19 tests, despite always giving a clear spectrophotometry result. We discuss below some factors that have been suggested to be behind the negative results.

Incomplete lysis? Another group found the same detection problem with OXA-48-like enzymes (7, 8) and ruled out the possibility of incomplete lysis. We would agree with this; in UV spectrophotometry, it is of utmost importance to extract enough active enzyme. This is done by lysing enough bacteria and by preferably keeping the lysate cold until measurement: both lysate and imipenem solution should be used within a few hours. The Carba NP test differs only in the detection method; otherwise, the same principles should apply. In our spectrophotometric analysis, we measure the relative amounts of lysates of the strains, and poor yield was not a typical problem for OXA-positive isolates. Tijet et al. (7) tested bead beating as an alternative lysis method with no difference in results; we tested this when setting up the UV-spectrophotometric method and found that it could partly destroy β-lactamase activity.

Amount of bacteria? The amount of bacteria recommended, one calibrated 10-μl loopful, is a rather arbitrary measure, and Tijet et al. had better results by increasing this to 3 to 4 loopfuls (7, 8). The number of bacteria that are physically able to stick to a loopful varies depending on the strain, and we have often observed that a large mass of growth does not necessarily correspond to high absorbance in the UV-spectrophotometric test; this is presumably due to the presence of various amounts of polysaccharides. Experience has taught us to use as much bacterial mass as possible, sometimes scraping off the entire plate, when the growth is sparse. This was done here also.

Enzyme class or clonal property? The negative results in fact correlated only with enzyme class, not species, which could be expected if incomplete lysis, caused, e.g., by the presence of mucoid klebsiellas, was the problem. Nor were the negative results correlated to susceptibility, which is logical, since membrane changes are important in the creation of high-level resistance: you could have a highly resistant strain with low enzyme activity. The hydrolysis rate (UV-spectrophotometrically measured) is often lower for OXA-48-like enzymes, and there seems to be some correlation between hydrolysis rate and Carba NP results (Table 1), but only among the OXA enzymes. The OXA-181-positive *Klebsiella pneumoniae* isolates belonged to two multilocus sequence types (MLSTs) (ST14 and ST101), and the fifth undetected OXA-181-positive isolate was an *E. coli* (ST38) isolate; the detection problems were thus not related to a single clone or species.

Inhibition by buffer? Dortet et al. have recently validated a modified Carba NP protocol for *Acinetobacter* spp. (9), replacing the lysis buffer with a hyperosmotic NaCl solution and increasing the amount of bacteria. This they compared to the updated Carba NP test. They found that Carba NP did not detect the OXA class of

enzymes but the CarbAcineto NP did: the buffer apparently hindered the color change. Our results are in line with this: the *Acinetobacter baumannii* isolate with OXA-23 was negative with the Carba NP test, while the *A. baumannii* isolate with VIM was positive. Our results for *A. baumannii* are excluded from the sensitivity calculation below, since the method that was used is not recommended for this species.

All metalloenzymes tested positive; the agar plate used is crucial for this test according to one study (6). All class A-positive isolates were positive except three: the GES enzyme test results were negative, which was not so surprising in light of their slow hydrolysis, but the SME test result also was negative, despite a very steep UV-spectrophotometric curve. All KPC strains were positive: thus, most of the clinically significant enzymes (KPC, NDM, and VIM) were detected.

In our hands, the overall sensitivity of the Carba NP test was thus 88% compared to that of the gold standard method, PCR. Eradication of carbapenemase strains in health care settings would call for a simple detection method that finds all positive strains, and the false positives could then be singled out using molecular tests. UV spectrophotometry can find all or most positives, but the procedure is not simple (from the viewpoint of a routine laboratory). Still, the combined sensitivity and specificity of the Carba NP test are better than for most other phenotypic methods bar UV spectrophotometry. In our opinion, Carba NP test has the potential to be a very useful tool for clinical laboratories. However, using the original Carba NP test, one should keep in mind that isolates with suspicious negative results might have to be further tested by genetic methods. The test should be developed further, to make it more robust in the hands of routine users, and the reason for the problems with OXA-enzyme detection should be confirmed.

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