Published in "Antimicrobial Agents and Chemotherapy doi:
10.1128/AAC.01239-13, 2014"
which should be cited to refer to this work.

Strategy for a rapid detection of carbapenemase-producing Enterobacteriaceae

Laurent Dortet,[1] Ludivine Bréchard,[1] Gaëlle Cuzon,[1] Laurent Poirel,[1,2] and Patrice Nordmann[1,2]*

[1]INSERM U914, South-Paris Medical School, K.-Bicêtre, France, and [2]Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Switzerland

*Corresponding author. Mailing address: Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, rue Albert-Gockel 3, CH-1700 Fribourg, Switzerland. Phone: 41-26-300-9581. E-mail: patrice.nordmann@unifr.ch

Keywords: Carbapenem, multidrug resistance; antibiotic; Gram negatives; ß-lactam

Running title: Strategies for early detection of carbapenemases

A prospective survey was conducted on 862 *Enterobacteriaceae* with reduced susceptibility to carbapenems. The Carba NP test, UV spectrophotometry and DNA microarray were used for detecting carbapenemase producers, and results compared to PCR and sequencing. The 172 carbapenemase producers were detected using the Carba NP test and UV spectrophotometry whereas DNA microarray failed to detect IMI producers. Use of the Carba NP test as first screening, followed by the use of molecular techniques has been defined as an efficient strategy for identification of carbapenemase-producing Enterobacteriaceae.

Carbapenemases lead to the ultimate evolution of resistance in Enterobacteriaceae, leaving virtually very few efficient antibiotics left (1, 2). The most clinically-significant carbapenemases in Enterobacteriaceae are: (i) Ambler class A enzymes including KPC, IMI and SME enzymes (1, 3, 4), (ii) metallo-β-lactamases (MBL) of VIM-, IMP- and NDM-types (5, 6), and (iii) OXA-48-like enzymes (7). Detection of carbapenemase producers include screening of patients at risk to be carriers of carbapenemase producers including patients who have been hospitalized abroad and implementation of efficient isolation procedures for carriers are the main features for limiting the spread of this emerging resistance trait (8-10).

The biochemical Carba NP test, based on detection of carbapenem hydrolysis has been recently developed (11). Molecular methods such as simplex and multiplex PCRs, DNA hybridization and sequencing are also used for the identification of carbapenemase genes.

The aim of this study was to evaluate prospectively an efficient and cost-effective strategy for detection and characterization of carbapenemase-producing Enterobacteriaceae.

From June 2011 to July 2012, 862 non-duplicate clinical *Enterobacteriaceae* isolates from worldwide origin were tested for characterization of the mechanisms leading to reduced susceptibility to carbapenems (Figure 1). The isolates were identified using MALDI-TOF

mass spectrometry (Vitek MS, bioMérieux, La Balme-les-Grottes, France). Susceptibility testing was performed by determining MIC values using the Etest® (bioMérieux) on Mueller-Hinton agar plates at 37°C and results were recorded according to US guidelines (CLSI), as updated in 2013 (12). All tested isolates were non susceptible to at least one of the three carbapenem molecules, imipenem, meropenem, or ertapenem.

Detection of *bla*$_{KPC}$, *bla*$_{IMI}$, *bla*$_{SME}$, *bla*$_{GES}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{GIM}$, and *bla*$_{OXA-48}$ carbapenemase genes was performed by simplex PCR followed by sequencing (13). Results of PCR and sequencing were used as standards for the evaluation of the other detection techniques. A molecular detection of the β-lactamase genes was also performed for all carbapenemase producers (n=173) using a DNA hybridization array approach (Check-MDR CT103 array; Check-Points, Wageningen, The Netherlands) following the manufacturer instructions (16).

Detection of carbapenemase production was performed by UV spectrophometry as previously described (14). It was also performed by using the Carba NP test (11). An improved version (faster and easier) of this test was performed with isolates grown on Mueller-Hinton agar plates (Becton Dickinson, Le Pont-de-Chaix, France) at 37°C for 18-22 h, as previously described (see Supplementary data) (1, 15).

The statistical analyses were performed using χhi^2 test. *P* values of <0.05 were considered as statistically significant.

Among the 862 enterobacterial isolates tested, the PCR-based techniques followed by sequencing identified 172 carbapenemase producers. As compared to the PCR-based detection method, the UV spectrophotometry method and the Carba NP test were found to be 100% sensitive and 100% specific for detecting carbapenemase producers (Table 1). Since the bla_{IMI} gene was not included in the panel of carbapenemase genes detected by the Check-MDR CT103, this array failed to identify the two *Enterobacter* spp. strains producing the IMI-1 carbapenemase, leading to a 98.8% sensitivity and 100% specificity (Table 1). The positive predictive values (PPV) were of 100 % for all the three techniques and negative predictive values (NPV) were of 100 % for UV spectrophotometry and Carba NP test, and of 99.7 % for the Check-MDR CT103 array (Table 1).

The DNA array was the only technique that could identify additional non-carbapenemase β-lactamases. Indeed, 70% of the carbapenemase-producing Enterobacteriaceae additionally expressed at least one broad-spectrum β-lactamase, such as a plasmid-mediated cephalosporinase and/or an extended-spectrum ß-lactamase (Table 2).

Among the 172 carbapenemase producers, 65% were *Klebsiella pneumoniae*, 15% were *Escherichia coli*, 13% were *Enterobacter* spp., 5% were *Citrobacter freundii*, 1% were *Serratia marcescens* and 1% were *Salmonella enterica* (Table 2). The identified carbapenemases were of the OXA-48- (72%), KPC- (15%), NDM- (6%), VIM- (6%) and IMI-types (1%) (Table 2). The characterization of carbapenemase genes was done by sequencing as listed in Table 2. Regardless of the enterobacterial species considered, OXA-48-like carbapenemases were predominant in our collection (Figure 1). KPC producers were mostly identified in *K. pneumoniae* compared to the other enterobacterial species (96%, $p<0.001$). On the opposite, NDM producers were equally distributed ($p>0.05$) among each type of enterobacterial species (Figure 1).

Overall, this study showed 100% specificity and sensitivity for the Carba NP test and UV spectrophotometry to detect the production of a carbapenemase (Table 1) (11). Additionally, the PPV and NPV of both techniques were also 100 %. The Carba NP test was as efficient as the UV spectrophotometry method to detect carbapenemase producers but with significant advantages, since the Carba NP test is more rapid (<2h versus 24 h for UV spectrophotometry) and does not require any specific training. Its cost is less than 1 US$ per tested strain whereas UV spectrophotometric assay and PCR-based techniques require

additional equipment (UV spectrophotometer and sonicator for the UV spectrophotometric assay, consumables, reagents, and thermocycler for PCR assay). On the other hand, the Check-MDR CT103 failed to detect two IMI-1 producers, leading to 100% specificity, 98.8% sensitivity, 100% PPV and 99.7% NPV (Table 1 and Table 2). Since the Check-MDR CT103 array is designed for its clinical use, it may detect the most clinically-relevant carbapenemase enzymes (KPC, VIM, IMP, OXA-48-like carbapenemase). In addition, it cannot discriminate between the different variants of a given carbapenemase. Additionally, this technique requires several successive steps (DNA extraction, ligation, PCR amplification, hybridization and detection) requiring 8 to 24 h. It also requires additional equipments (DNA extraction kit, thermocycler, thermomixer, Check-Points tube reader including the software) that cost ~ 16,000 $ (16). Additionally, the use of this array on a daily routine basis may be limited by its cost (~ 100 US$) compared to the UV spectrophotometry (2-3 US$), Carba NP test (1 US$) and PCR-based testing (30 US$). However, the microarray technique may help to characterize the entire β-lactamase content of a single isolate by detecting also other broad-spectrum β-lactamase genes.

The diversity of carbapenemases identified here mirrors the worldwide dissemination of the four main described enzymes (KPC, VIM, NDM and OXA-48) (1). Additionally, our

results further highlight the wide dissemination of the OXA-48 carbapenemase in Europe (particularly in France) accounting for 72% of the whole carbapenemases (Figure 1) (7, 17), whereas KPC is the most widespread carbapenemase in the US. Of note, the KPC carbapenemases are nearly restricted to the *K. pneumoniae* species (25/26). On the opposite, OXA-48 and NDM were distributed among all enterobacterial species.

Since the management of patients requires a rapid identification of carbapenemase producers (regardless of its type) (18), a diagnostic strategy for detection of carbapenemase producers in Enterobacteriaceae is proposed here (Figure 2). This strategy is based on: (i) the Carba NP test as the primary screening test for detection of a carbapenemase production, followed by (ii) a specific molecular characterization of the carbapenemase genes by simplex PCRs or DNA microarray. The initial step (susceptibility testing and Carba NP test) may be developed in any laboratory worldwide. Molecular identification of the carbapenemase genes may be also performed locally depending on the molecular techniques available, however it is not required for antibiotic stewardship or infection control purposes.

In case of a negative result obtained with the Carba NP test, the mechanism responsible for carbapenem decreased susceptibility is not related to the production of a carbapenemase (e.g. reduced permeability of the outer membrane associated with over-

expression of chromosomal or acquired AmpC and/or ESBL), therefore no additional test is required (17). In case of positive result with the Carba NP test, the use of a set of five simplex PCR (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMI}$ and *bla*$_{OXA-48-like}$) may then identify all carbapenemase genes of our collection (Figure 1, Table 2). However, this screening may be adapted to the local epidemiology as recently proposed for the detection of carbapenemase SME in the US (4). The DNA microarray may be more useful for epidemiological purposes or for infection control studies when high numbers of isolates have to be rapidly characterized (16). Additionally, this procedure may also detect potential new carbapenemases. Indeed, although molecular techniques are currently considered as "gold standard" for detection of carbapenemase producers, they are only able to detect known carbapenemase genes. With the proposed strategy, a positive Carba NP test followed by negative results using molecular thechniques may correspond to a novel carbapenemase that may be further characterize using cloning experiments (Figure 2).

This is the first prospective study evaluating at an international level the value of the different techniques for detecting carbapenemases. The strategy proposed for detection of carbapenemase producers presents several advantages for treating infected patients and for isolation of carriers. Indeed, it will lead to a rapid identification of carbapenemase producers

(< 2 h) using the Carba NP test, allowing a better antibiotic stewardship (18). This strategy may also have a significant impact for preventing the development of nosocomial outbreaks by acting rapidly on the management of carriers (isolation, cohorting) as demonstrated for KPC outbreaks at least in Israel (19). Finally, since the first step of this strategy which includes susceptibility testing and the Carba NP test is based on cheap techniques, it may be followed worldwide, and therefore contribute to limit the spread of what has been recently termed the new Red Plague (20).

Acknowledgments

This work was partially funded by a grant from the INSERM (UMR914), by the Université of Fribourg, Switzerland, and by grants from the European Community (R-GNOSIS, FP7/HEALTH-F3-2011-282512 and MAGIC-BULLET, FP7/HEALTH-F3-2001-278232).

Transparency declarations

An international patent form for the Carba NP test has been filed on behalf of INSERM Transfert (Paris, France).

REFERENCES

1. **Nordmann P, Dortet L, Poirel L.** 2012. Carbapenem resistance in *Enterobacteriaceae*: here is the storm! Trends Mol. Med. **18:**263-272.

2. **Schwaber MJ, Carmeli Y.** 2008. Carbapenem-resistant *Enterobacteriaceae*: a potential threat. JAMA **300:**2911-2913.

3. **Nordmann P, Cuzon G, Naas T.** 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect. Dis. **9:**228-236.

4. **Bush K, Pannell M, Lock JL, Queenan AM, Jorgensen JH, Lee RM, Lewis JS, Jarrett D.** 2013. Detection systems for carbapenemase gene identification should include the SME serine carbapenemase. Int. J. Antimicrob. Agents **41:**1-4.

5. **Nordmann P, Poirel L, Walsh TR, Livermore DM.** 2011. The emerging NDM carbapenemases. Trends Microbiol. **19:**588-595.

6. **Walsh TR, Toleman MA, Poirel L, Nordmann P.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306-325.

7. **Poirel L, Potron A, Nordmann P.** 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. **67:**1597-1606.

8. **Mitka M.** 2013. Indian public health leaders move to reduce antimicrobial resistance. JAMA **309:**531-532.

9. **Spellberg B, Blaser M, Guidos RJ, Boucher HW, Bradley JS, Eisenstein BI, Gerding D, Lynfield R, Reller LB, Rex J, Schwartz D, Septimus E, Tenover FC, Gilbert DN.** 2011. Combating antimicrobial resistance: policy recommendations to save lives. Clin. Infect. Dis. **52 Suppl 5:**S397-428.

10. **Walsh TR, Toleman MA.** 2011. The emergence of pan-resistant Gram-negative pathogens merits a rapid global political response. J. Antimicrob. Chemother. **67:**1-3.

11. **Nordmann P, Poirel L, Dortet L.** 2012. Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg. Infect. Dis. **18:**1503-1507.

12. **Clinical and Laboratory Standards Institute.** 2013. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement, M100-S23, vol. M100-S23. CLSI, Wayne (PA), USA.

13. **Poirel L, Dortet L, Bernabeu S, Nordmann P.** 2011. Genetic features of $bla_{\text{NDM-1}}$-positive *Enterobacteriaceae*. Antimicrob. Agents Chemother. **55:**5403-5407.

14. **Bernabeu S, Poirel L, Nordmann P.** 2012. Spectrophotometry-based detection of carbapenemase producers among *Enterobacteriaceae*. Diagn. Microbiol. Infect. Dis. **74:**88-90.

15. **Dortet L, Bréchard L, Poirel L, Nordmann P.** 2014. Comparison of diverse growing media for further detection of carbapenemase production using the Carba NP test. J. Med. Microbiol. In press.

16. **Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P.** 2012. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J. Antimicrob. Chemother. **67:**1865-1869.

17. **Dortet L, Cuzon G, Nordmann P.** 2013. Dissemination of carbapenemase-producing *Enterobacteriaceae* in France, 2012. J. Antimicrob. Chemother. In press.

18. **Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, Polsky B, Adams-Haduch JM, Doi Y.** 2012. Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: superiority of combination antimicrobial regimens. Antimicrob. Agents Chemother. **56:**2108-2113.

19. **Schwaber MJ, Lev B, Israeli A, Solter E, Smollan G, Rubinovitch B, Shalit I, Carmeli Y.** 2011. Containment of a country-wide outbreak of carbapenem-resistant

Klebsiella pneumoniae in Israeli hospitals via a nationally implemented intervention. Clin. Infect. Dis. **52:**848-855.

20. **Looke DF, Gottlieb T, Jones CA, Paterson DL.** 2013. Gram-negative resistance: can we combat the coming of a new "Red Plague"? Med. J. Aust. **198:**243-244.

FIGURES LEGENDS

Figure 1. Distribution of the different carbapenemase types among carbapenemase-producing Enterobacteriaceae.

Figure 2. Chart flow for detection and characterization of carbapenemase producers among Enterobacteriaceae. The Carba NP test is used for a rapid differentiation between carbapenemase and non-carbapenemase producers. The second step includes molecular techniques (PCRs or DNA microarray) for precise identification of carbapenemase genes. Bold arrows indicate the preferred way for identification of carbapenemase genes. This second step may be followed only in University hospitals or large-size microbiology laboratories.

Table 1. Main characteristics of Carba NP test, UV spectrophotometry method and DNA microarray for the detection of carbapenemase-producing Enterobacteriaceae

Characteristics of the tests	Detection of carbapenemase-producing *Enterobacteriaceae*			
	PCR + sequencing	**Carba NP test**	**UV spectrophotometry**	**DNA microarray**
Test efficiency				
Sensitivity	100%	100%	100%	98.8%
Specificity	100%	100%	100%	100%
PPV	100%	100%	100%	100%
NPV	100%	100%	100%	99.7%
Other characteristics				
Rapidity	24-48h	< 2h	12-24h	8-24h
Cost	$$	$	$	$$$
Expertise needs	++	+	+++	++
Complete gene identification	+	-	-	+/-

PPV, Positive predictive value
NPV, Negative predictive value
The number of $ correlates with the effective price of the test
The number of + correlates with the expertise and training needed to perform and interpret the test

Table 2. Molecular characterization of carbapenemase-producing Enterobacteriaceae using sequencing and DNA microarray

Carbapenemase type	Carbapenemase variant	Species	n	DNA microarray			
				Acquired penicillinase	ESBLs	Acquired cephalosporinase	Carbapenemase
KPC	KPC-2	*K. pneumoniae*	7	TEM-type	None	None	KPC
			2	TEM-type	CTX-M type 1	None	KPC
			1	TEM-type	CTX-M type 9	None	KPC
			10	TEM-type	SHV-type	None	KPC
		C. freundii	1	TEM-type	None	None	KPC
	KPC-3	*K. pneumoniae*	4	TEM-type	None	None	KPC
			1	TEM-type	CTX-M type 1	None	KPC
VIM	VIM-1	*K. pneumoniae*	2	TEM-type	SHV-type	None	VIM
		E. cloacae	1	TEM-type	None	None	VIM
			1	TEM-type	SHV-type	None	VIM
		C. freundii	1	TEM-type	None	None	VIM
	VIM-2	*C. freundii*	4	TEM-type	TEM-type	None	VIM
	VIM-4	*K. pneumoniae*	1	TEM-type	None	None	VIM
NDM	NDM-1	*E. coli*	1	None	CTX-M type 1	None	NDM
			1	TEM-type	CTX-M type 1	None	NDM
			1	TEM-type + SHV-type	CTX-M type 1	None	NDM
		K. pneumoniae	1	None	None	CMY-2-like	NDM
			1	None	CTX-M type 1	None	NDM
			1	TEM-type	CTX-M type 1	None	NDM
		E. cloacae	1	TEM-type	CTX-M type 1	None	NDM
			1	TEM-type	CTX-M type 1 + SHV-type	None	NDM
		Salmonella spp.	1	TEM-type	None	DHA-type	NDM
IMI	IMI-1	*E. cloacae*	1	TEM-type	None	None	None
		E. asburiae	1	None	None	None	None

OXA-48-like	OXA-48	*E. coli*	4	None	None	None	OXA-48
			7	TEM-type	None	None	OXA-48
			1	TEM-type	None	CMY-2-like	OXA-48
			1	None	CTX-M type 1	None	OXA-48
			7	TEM-type	CTX-M type 1	None	OXA-48
			1	TEM-type + SHV-type	CTX-M type 1	None	OXA-48
			1	TEM-type	CTX-M type 9	None	OXA-48
		K. pneumoniae	5	None	None	None	OXA-48
			1	TEM-type	None	None	OXA-48
			1	None	None	DHA-type	OXA-48
			5	None	CTX-M type 1	None	OXA-48
			64	TEM-type	CTX-M type 1	None	OXA-48
			1	TEM-type	CTX-M type 1	CMY-2-like	OXA-48
			1	None	CTX-M type 9	None	OXA-48
		E. cloacae	1	None	None	None	OXA-48
			1	None	CTX-M type 1	None	OXA-48
			10	TEM-type	CTX-M type 1	None	OXA-48
			4	TEM-type + SHV-type	CTX-M type 1	None	OXA-48
		E. hormacchei	1	TEM-type	CTX-M type 1	None	OXA-48
		C. freundii	1	TEM-type	SHV-type	None	OXA-48
		C. freundii	1	SHV-type	CTX-M type 1	None	OXA-48
		S. marscescens	1	None	None	None	OXA-48
			1	TEM-type	CTX-M type 1	None	OXA-48
	OXA-162	*C. freundii*	1	None	SHV-type	None	OXA-48
	OXA-181	*E. coli*	1	None	CTX-M type 1	None	OXA-48
		K. pneumoniae	2	TEM-type	CTX-M type 1	None	OXA-48
NDM + OXA-48-like	NDM-1 + OXA-181	*K. pneumoniae*	1	TEM-type	CTX-M type 1	None	NDM + OXA-48