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Review

A Focus on Carbapenemase-Producing Pathogens Detection by Phenotypic Tests

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Abstract

Over the past decade the medical community has witnessed the severe compromise of last resort antibiotics such as carbapenems, widely used in the treatment of severe infections, due to multidrug-resistant *Enterobacteriaceae*. A variety of carbapenem-hydrolyzing enzymes, located on diverse mobile genetic elements, attainable via horizontal transfer, have been associated with the resistant profiles conferred. As the therapeutic pallet diminishes prompt identification forms the cornerstone for effective containment and epidemiological surveillance, in an attempt to hinder dissemination in the hospital and community setting. Nevertheless, issues regarding detection and accurate characterization of the enzyme at hand persist due not only to the variety of proposed methodologies, but also because of the necessity for each laboratory to tailor its workflow to its individual needs and resources. We aim to describe the recent status in phenotypic detection and characterization of carbapenem-hydrolyzing enzymes, with a focus on inhibitor-based tests, which can offer a reliable, cost-effective, easily applicable alternative to other more expensive, in need of specialized personnel and equipment methods.

Keywords: carbapenemases, carbapenemase-producing *Enterobacteriaceae*, phenotypic tests, KPC, MBL, OXA-48

Резюме

През последното десетилетие медицинската общност е свидетел на падането и на последните „крепости" на антибиотиците, каквато е групата на карбапенемите, широко прилагани за лечение на остри инфекции, причинени от мултирезистентни Enterobacteriaceae. Профилите на резистентността са свързани с многообразни карбапенем-хидролизиращи ензими, гените за които са локализирани върху мобилни елементи, придобивани чрез хоризонтален трансфер. С намаляването на терапевтичните възможности, тяхното точно идентифициране се очертава като крайъгълен камък за ефективното потискане и епидемиологичния контрол за спиране на разпространението на инфекциите в болниците и сред населението. Въпреки разнообразните методологии и необходимостта за съобразяване на изследванията с възможностите на отделните лаборатории, разпознаването и прецизното охарактеризиране на ензимите си остава актуално. Целта на настоящата работа е да опишем съвременния статус на фенотипната детекция и характеризиране на карбапенем-хидролизиращите ензими. Ударението е върху тестовете за инхибиране, които биха предложили надеждни, икономически изгодни и лесно приложими алтернативи, които да заместят по-скъпите методи, изискващи специализиран персонал и оборудване.

Introduction

Over the past decade the medical community has witnessed the severe compromise of last resort antibiotics such as carbapenems, widely used in the treatment of severe infections, due to multidrug-resistant *Enterobacteriaceae* (Nordmann *et al*., 2012a; Tängdén and Giske, 2015). Carbapenem resistance does not encompass a uniform entity of potential mechanisms but may be caused by a variety of factors and their combinations. Acquisition via horizontal transfer of diverse mobile genetic

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elements bearing genes responsible for the production of carbapenem-hydrolyzing enzymes and decreased antibiotic uptake due to outer membrane permeability issues, either in the form of efflux pump up-regulation coupled with complete loss of porin-expressing genes or debilitating mutations, in association with AmpC β-lactamase hyperproduction or ESBL expression, constitute the main mechanisms leading up to a carbapenem resistant profile (Cornaglia and Rossolini, 2010; Nordmann *et al*., 2012a). As the available therapeutic pallet diminishes and given the complexity and diversity of the underlying resistance mechanisms, the need for timely, accurate and cost effective detection/ characterization of the molecular background at hand becomes even more pressing, in an attempt to tailor the administered individual patient regimens (Nordmann, 2014; Jean *et al.,* 2015; Tängdén and Giske, 2015).

Therefore, focusing on the acquired carbapenem-hydrolyzing enzyme aspect of a carbapenem resistant phenotype, it is essential to bear in mind the diversity of the enzymes at hand as previously grouped, based on sequence and structural homology, in the Ambler classification(Ambler, 1980; Queenan and Bush, 2007). To date, in regards to the *Enterobactericeae* species, carbepenem resistance has been linked to class A enzymes of the KPC, SME, IMI, NMC, GES types, class B carbapenemases of the IMP, VIM, NDM, GIM, SPM, SIM category and class D oxacillinases such as the OXA-48 and its variants, with the KPC, VIM, IMP, NDM and OXA-48 types constituting the most effective carbapenemases in terms of hydrolytic potential, dissemination and geographical spread (Tängdén and Giske, 2015).

The difficulties noted in carbapenemase detection and characterization, arise from the fact that due to the diversity observed in the active sites of these enzymes and the coexistence or not of additional resistance mechanisms, the hydrolytic profile conferred is variable not only in regards to the specific carbapenem substrate affected but also the minimum inhibitory concentration (MIC) conferred (Queenan and Bush, 2007; Poirel *et al*., 2012). Furthermore, as these resistance genes are mostly harbored on transposon-and/or integron-encoded determinants, prone to inter/intra-species dissemination, further changes in host range and epidemiological distribution can be anticipated, thus rendering prompt identification the cornerstone for effective containment, in an attempt to hinder further dissemination following an introduction in the hospital or community setting (Naas *et al*., 2008; Poirel *et al*., 2012; Tängdén and Giske, 2015). This review aims to present in a comprehensive manner the current status in regards to the available phenotypic tools for detecting and characterizing carbapenemase-producing *Enterobactericaeae* (CPE), which can be applied in the clinical laboratory setting and give insight into those limitations associated with sensitivity and cost.

Detection of carbapenemase-producing *Enterobacteriaceae* (CPE)

Screening tests

The lingering controversy behind which isolates could be characterized as carbapenem resistant was addressed with the implementation of the novel 2010 CLSI breakpoints and guidelines, followed however by a debate on the necessity of further confirmatory and phenotypic testing (CLSI, 2010). Although currently further testing is not routinely recommended neither by the CLSI nor EUCAST accurate characterization of carbapenem resistance mechanisms in the clinical laboratory remains crucial not only for the implementation of infection control measures but also for epidemiological surveillance, especially in areas with preexisting endemicity where various genes are likely to have simultaneously disseminated.

The clinical laboratory is called upon to answer two vital questions. The former addresses the presence of a carbapenem-hydrolyzing enzyme in a carbapenem resistant *Enterobacteriaceae* (CRE) and the latter the nature of the enzyme detected.

Currently in regards to the first question three main techniques for detecting carbapenemase activity are receiving considerable attention, the modified Hodge test (MHT), the Carba NP test and the carbapenem inactivation method (CIM) all based on indirectly witnessing carbapenem hydrolysis.

The MHT, based on the inactivation of a carbapenem by a carbapenemase-producing strain, therefore enabling a susceptible indicator strain to extend growth towards the carbapenem containing disk, is currently recommended by the CLSI as a confirmatory test for the detection of diffusible carbapenemases (Lee *et al.*, 2001). However, in its original form, this test although simple and easily applicable in the laboratory setting, is fraught not only by false positive results in the presence of other β-lactamase genes such as AmpCs and ESBLs when presented with coexisting permeability defects but also a low sensitivity of 50% for NDM-producers (Pasteran *et al*., 2009; Carvalhaes

et al., 2010; Girlich *et al.*, 2012). In an attempt to address issues regarding NDM-producers improved protocols have been presented with the addition of supplements such as ZnSO(4) to the culture media or of a nonionic surfactant (Triton X), capable of releasing the membrane bound NDM carbapenemases (Girlich *et al.,* 2012; Pasteran *et al.*, 2016). Detection of class A carbapenemases with a reduction in false positives is also feasible with the addition of inhibitors such as boronic acid (BA) and oxacillin (OXA) (Pasteran *et al.*, 2010).

Biochemical tests such as the Carba NP, have also been introduced and in current CLSI guidelines are recommended for confirmatory testing (CLSI, 2015) . They have been designed to identify the disruption in the β-lactam ring of the carbapenem substrate (imipenem), based on the produced variation in color, due to the acidic pH alteration from the enzymatic hydrolysis, of a phenol indicator, which is rendered either yellow or orange from an initial red (Nordmann *et al.*, 2012b; Poirel and Nordmann, 2015). Results are readily available within 2 hours following sample preparation (Nordmann *et al.*, 2012b). The second version of the test, Carba NP II, can differentiate class A and B and indirectly predict class D enzymes due to the addition of EDTA and tazobactam, with EDTA and tazobactam inhibiting MBLs and class A carbapenemases respectively (Dortet *et al.*, 2012). Another interesting aspect of the assay is that in biological samples which tend to yield single bacterial isolates such as blood cultures the test can be applied directly using the liquid culture media, thus further reducing the time to detection and effective antibiotic treatment (Dortet *et al.*, 2014). The introduced commercial version of the Carba NP test, Rapidec Carba NP (BioMérieux, La Balme-les-Grottes, France) comes to report a specificity and sensitivity of 96% while considerably reducing the time to detetion to <15 min for class A and B producers and <30min for class C producers, while suggesting an additional simple step prior to testing for isolates which present a mucoid phenotype (Poirel and Nordmann, 2015). Other tests also based on the concept of biochemical detection of carbapem hydrolysis, have been put forward, such as the commercial Rapid CARB Screen Kit (Rosco-Diagnostica A/S Taastrup, Denmark) and the more cost effective Blue-Carba test, yet both have withstanding issues to address regarding either lower sensitivity in comparison to the Carba NP as the former or need for further evaluation studies as the latter (Pires *et al.*, 2013; Yusuf *et al.*, 2014; Pasteran *et al.*, 2015).

Finally, the CIM test has recently been proposed as an effective yet cheaper alternative to the Carba NP test (van der Zwaluw *et al.*, 2015). The concept behind the test relies on the inactivation of the carbapenem substrate harbored in a single 10μg meropenem disk following its inoculation in a bacterial suspension and subsequent incubation for a minimum of two hours. In the event that the isolate being screened does produce a carbapenemase the substrate is hydrolyzed and therefore the application of the disk on a Mueler-Hinton (MH) agar plate streaked with a susceptible reference strain fails to produce an inhibition zone. Despite the fact that in its initial description the method yields, at a fraction of the cost of the Carba NP test, a similar high performance (96,6% concordance), further validation is warranted (van der Zwaluw *et al.*, 2015).

Inhibitor-based tests for carbapenemase characterization

The principle applied in these tests takes advantage of the properties of specific β-lactamase inhibitors, which are class dependent and exhibit specificity in rendering their hydrolytic effect, therefore enabling the characterization of the carbapenemase class of the enzyme under investigation.

Single inhibitor-based tests

EDTA-based tests inhibit β-lactam hydrolysis by exploiting the dependence of metallo-enzymes on zinc ions and discriminate MBL-producing *Enterobacteriaceae* from CRE due to ESBL and/or AmpC hyper-production in light of permeability defects (Yong *et al.*, 2002). Others inhibitors for

Table 1. Class A, B and D carbapenemases and their respective inhibitors

β-lactamase	Class	Genes	Inhibitor Clavulanate	Inhibitor EDTA/DPA	Inhibitor PBA/CLO*
Carbapenemase	**A**	**KPC**	**-**	**-/-**	+/-
	B	IMP, VIM, NDM	**-**	+/+	**-/-**
	D	OXA-48-like	-	-/-	-/-

*Cloxacillin

class B type enzymes include EDTA plus 1,10-phenanthroline, thiol compounds and dipicolinic acid (DPA) (Arakawa *et al*., 2000; Lee *et al*., 2001; Kimura *et al*., 2005) (Table 1).

Furthermore, in attempting to optimize the sensitivity and specificity of the methods used, a variety of potentiation tests, based either on disk diffusion or broth dilution, have been proposed questioning the optimal concentration of inhibitor applied, the distance between disks at which the effect is potentiated and the best β-lactam substrate to be utilized (Yong *et al*., 2002; Migliavacca *et al.*, 2002; Pitout *et al.,* 2005; Franklin *et al*., 2006). In retrospect for the detection of MBLs in *Enterobacteriaceae* two tests are noted to perform better, the combined-disk test using either imipenem/imipenem with 0.5 M EDTA or ceftazidime/ceftazidime with 0.2 M EDTA, while applying a 7 mm difference in inhibition zone cut-off and the double-disk synergy test where an imipenem disk is placed at a 10 mm distance from a 0.5M EDTA supplemented disk (Galani *et al.*, 2008).

Attempts to phenotypically detect class A carbapenemases were initially based on the use of clavulanate, which inhibits weakly resistance to ertapenem and imipenem (Yigit *et al*., 2001) . Phenylboronic acid (PBA) was latter directly associated with the inhibition of class A type carbapenemases ,although the mechanism of action still remains unclear (Tsakris *et al*., 2008) (Table 1). Following this observation different boronic acid compounds have been used as inhibitors at various concentrations (Pasteran *et al*., 2009; Tsakris *et al*., 2009, 2011). Comparative evaluation of different combinations of aminophenylboronic acid (APBA) (at 300 μg or 600 μg respectively) and PBA at 400 μg, for KPC-producing *Enterobacteriaceae*, highlight PBA at a concentration of 400 μg as the most effective KPC carbapenemase inhibitor using meropenem as a substrate and evaluating an increase in zone diameter inhibition equal or greater than 5 mm between meropenem alone and meropenem plus PBA (Tsakris *et al*., 2011).

Combined inhibitor-based tests

The challenge however, remains the exact identification with an easy to apply method, of these resistance traits, in isolates likely to be simultaneously harboring multiple carbapenemases as well as other β-lactamase genes. Integrating compounds with different inhibitor properties into a single test has led to the proposal and evaluation of a variety of phenotypic tests.

In this direction Tsakris *et al.* impregnated 292 μg EDTA and 400 μg PBA separately and in combination onto 10 μg meropenem disks and applied a minimum 5 mm increase in inhibition zone diameter as an indicator of a positive result (Tsakris *et al*., 2010). This protocol yielded a sensitivity and specificity of 96.8% and 100%, respectively, in detecting simultaneous production of KPC and VIM carbapenemases, with the 100% sensitivity and specificity ratio for isolates harboring either VIM or KPC enzymes, deterred only in regards to the specificity for KPC detection (98.8%), compromised by two ESBL and AmpC producers with positive tests to meropenem/ PBA and meropenem/ PBA/EDTA (Tsakris *et al*., 2010).

Giske *et al.* validated a carbapenemase detection test based on a five disk principal with the use of meropenem alone and supplemented with 730 μg EDTA, 1000 μg DPA, 600μg APBA and 750 μg cloxacillin (Giske et al., 2011). In contrast to EDTA, DPA and cloxacillin for which a 5 mm cut-off was applied, the accurate detection of all KPC producing isolates mandated that for APBA a 4 mm increase in zone inhibition diameter should be considered as indicative of KPC production. AmpC hyper-producers with porin loss were positive in the APBA test but, unlike KPC producers, showed cloxacillin synergy. Furthermore DPA performed better than EDTA as far as specificity was concerned in MBL-producing isolates (Giske *et al*., 2011).

In a significantly more complicated and technically demanding approach Birgy et al. evaluated the simultaneous preparation and use of a combination of inhibitor-supplemented (EDTA, PBA, cloxacillin) MH agar disks in an attempt to also characterize carbapenemase and ESBL producers (Birgy *et al.*, 2012).

Accurate detection of OXA-48-like-possessing *Enterobacteriaceae* isolates remains problematic in the absence of a reliable inhibitor and therefore the optimal screening agents and methods are yet to be defined (Poirel *et al*., 2012). However, class D carbapenemase detection has been recently been addressed by a novel single phenotypic test, the OXA-48 disk test (Tsakris *et al.*, 2015). The test is based on the use of EDTA to permeabilize the bacterial cell and inhibit MBL carbapenemase production while a solution of PBA inhibits KPC production. A 10μg imipenem is placed on a MH agar plate, inoculated with a lawn of a susceptible strain, adjacent to two blank disks supplemented with 292 μg EDTA and 292 μg EDTA/600 μg PBA,

respectively. The isolate under investigation is then applied onto the imipenem disk and following incubation resulting distortions were assessed and interpreted (Tsakris *et al*., 2015). Pending further validation the sensitivity of the method was estimated at 96.3% (Tsakris *et al.*, 2015). Also, temocillin MIC testing on Mueller-Hinton agar supplemented with cloxacillin was found specific for detecting OXA-48 production (Maurer *et al*., 2015).

Conclusion

Carbapenemase-possessing bacteria have arisen as a serious health associated issue over the past decade and their detection and accurate characterization has been the focus of extended investigations (Nordmann *et al*., 2012a; Tängdén and Giske, 2015). Infections due to CPE not only compromise patient outcomes but also add a significant financial burden on national health care systems (Daroukh *et al.*, 2014; Biehle *et al*., 2015; Patel and Nagel, 2015).

Unfortunately the issue of cost has created a gap between those with ample means and those financially challenged. Novel methodologies such as MALDI-TOF-MS and molecular methods such as microarrays, multiplex- PCRs and even next-generation sequencing platforms are associated with either a high initial acquisition cost or become demanding in their introduction to the laboratory workflow since they require specialized personnel and run at significantly higher cost (Voulgari *et al*., 2013). Regardless of these issues however, prompt detection and identification remain the cornerstones to establishing a successful intervention in regards to the dissemination of these genes.

In an attempt to meet this need in a reliable and inexpensive manner, phenotypic tests have been established as an effective alternative at a fraction of the cost. Inhibitors based tests performed along with confirmatory methods, despite their limitations; provide a reliable, easily implemented, low cost option in carbapenemase enzyme typing.

As the medical community is currently glimpsing but the tip of the iceberg, as far as carbapenemase-producing isolates are concerned, the tools necessary for coping with this ominous future have to be readily available and need to cater for all budgets. Confirmatory testing and enzyme characterization remain cornerstones in effective active surveillance protocols. Given that our options in administering effective antibiotic regimens are already limited and likely to diminish in the future, prevention and containment are vital. In this context inhibitor based tests offer the advantage of bypassing in most cases the need for more expensive tests and can be implemented successfully in everyday laboratory practice.

References

Ambler, R. P. (1980). The structure of beta-lactamases. P*hilos. Trans. R. Soc. Lond. B. Biol. Sci.* **289**: 321-331.

Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, M. Goto (2000). Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. *J. Clin. Microbiol.* **38**: 40-43.

Biehle, L. R., J. M. Cottreau, D. J. Thompson, R. L. Filipek, J. N. O'Donnell, T. M. Lasco, M. V. Mahoney, E. B. Hirsch (2015). Outcomes and risk factors for mortality among patients treated with carbapenems for *Klebsiella* spp. bacteremia. *PloS One* **10**: e0143845.

Birgy, A., P. Bidet, N. Genel, C. Doit, D. Decré, G. Arlet, E. Bingen (2012). Phenotypic screening of carbapenemases and associated β-lactamases in carbapenem-resistant *Enterobacteriaceae. J. Clin. Microbiol.* **50**: 1295-1302.

Carvalhaes, C. G., R. C. Picão, A. G. Nicoletti, D. E. Xavier, A. C. Gales (2010). Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae:* be aware of false positive results. *J. Antimicrob. Chemother.* **65**, 249-251.

CLSI, C. and L.S.I. (2010). Performance standards for antimicrobial susceptibility testing; twentieth informational supplement (June 2010 update) Document M02-A10.CLSI, Wayne, PA.

CLSI, C. and L.S.I. (2015). Performance standards for antimicrobial susceptibility testing; Twenty-fourth Informational Supplement Document M100-S24. CLSI, Wayne, PA.

Cornaglia, G., G. M. Rossolini (2010). The emerging threat of acquired carbapenemases in Gram-negative bacteria. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. *Clin. Microbiol. Infect. Dis.* **16**: 99-101.

Daroukh, A., C. Delaunay, S. Bigot, J. M. Ceci, N. Siddhoun, I. Bukreyeva, J. Raisin, H. Porcheret, L. Maisonneuve, M. A. Bouldouyre (2014). Characteristics and costs of carbapenemase-producing enterobacteria carriers (2012/2013). *Médecine Mal. Infect.* **44**: 321-326.

Dortet, L., L. Poirel, P. Nordmann (2012). Rapid identification of carbapenemase types in *Enterobacteriaceae* and *Pseudomonas* spp. by using a biochemical test. *Antimicrob. Agents Chemother.* **56**: 6437-6440.

Dortet, L., A. Boulanger, L. Poirel, P. Nordmann (2014). Bloodstream infections caused by *Pseudomonas* spp.: how to detect carbapenemase producers directly from blood cultures. *J. Clin. Microbiol.* **52**: 1269-1273.

Franklin, C., L. Liolios, A. Y. Peleg (2006). Phenotypic detection of carbapenem-susceptible metallo-beta-lactamase-producing gram-negative bacilli in the clinical laboratory. *J. Clin. Microbiol.* **44**: 3139-3144.

Galani, I., P. D. Rekatsina, D. Hatzaki, D. Plachouras, M. Souli, H. Giamarellou (2008). Evaluation of different laboratory tests for the detection of metallo-beta-lactamase production in *Enterobacteriaceae. J. Antimicrob. Chemother.* **61**: 548-553.

Girlich, D., L. Poirel, P. Nordmann (2012). Value of the modi-

fied Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae. J. Clin. Microbiol*. **50:** 477-479.

Giske, C. G., L. Gezelius, Ø. Samuelsen, M. Warner, A. Sundsfjord, N. Woodford (2011). A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis*. **17**: 552-556.

Jean, S.-S., W.-S. Lee, C. Lam, C.-W. Hsu, R.-J. Chen, P.-R. Hsueh, (2015). Carbapenemase-producing Gram-negative bacteria: current epidemics, antimicrobial susceptibility and treatment options. *Future Microbiol.* **10**: 407-425.

Kimura, S., Y. Ishii, K. Yamaguchi (2005). Evaluation of dipicolinic acid for detection of IMP- or VIM- type metallo-beta-lactamase-producing *Pseudomonas aeruginosa* clinical isolates. *Diagn. Microbiol. Infect. Dis.* **53**: 241-244.

Lee, K., Y. Chong, H. B. Shin, Kim, Y. A., Yong, D., Yum, J. H. (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. C*lin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **7**: 88-91.

Maurer, F. P., C. Castelberg, C. Quiblier, G. V. Bloemberg, M. Hombach (2015). Evaluation of carbapenemase screening and confirmation tests with *Enterobacteriaceae* and development of a practical diagnostic algorithm. *J. Clin. Microbiol.* **53**: 95-104.

Migliavacca, R., J.-D. Docquier, C. Mugnaioli, G. Amicosante, R. Daturi, K. Lee, G. M., Rossolini, L. Pagani (2002). Simple microdilution test for detection of metallo-beta-lactamase production in *Pseudomonas aeruginosa. J. Clin. Microbiol.* **40**: 4388-4390.

Naas, T., G. Cuzon, M.-V. Villegas, M.-F. Lartigue, J. P. Quinn, P. Nordmann (2008). Genetic structures at the origin of acquisition of the beta-lactamase bla KPC gene. *Antimicrob. Agents Chemother.* **52**: 1257-1263.

Nordmann, P. (2014). Carbapenemase-producing *Enterobacteriaceae:* overview of a major public health challenge. *Méd. Mal. Infect.* **44**: 51-56.

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

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

Pasteran, F., T. Mendez, L. Guerriero, M. Rapoport, A. Corso (2009). Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae. J. Clin. Microbiol.* **47**,: 1631-1639.

Pasteran, F., T. Mendez, M. Rapoport, L. Guerriero, A. Corso (2010). Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of *Enterobacteriaceae.* by incorporating boronic *Acid. J. Clin. Microbiol.* **48**: 1323-1332.

Pasteran, F., O. Veliz, P. Ceriana, C. Lucero, M. Rapoport, E. Albornoz, S. Gomez, A. Corso, ReLAVRA Network Group (2015). Evaluation of the Blue-Carba test for rapid detection of carbapenemases in gram-negative bacilli. *J. Clin. Microbiol.* **53**: 1996-1998.

Pasteran, F., L. J. Gonzalez, E. Albornoz, G. Bahr, A. J. Vila, A. Corso (2016). Triton Hodge test: improved protocol for modified Hodge test for enhanced detection of NDM and other carbapenemase producers. *J. Clin. Microbiol*. **54**: 640-649.

Patel, T. S., J.L. Nagel (2015). Clinical outcomes of *Enterobacteriaceae.* infections stratified by carbapenem MICs. *J. Clin. Microbiol.* **53**: 201-205.

Pires, J., A. Novais, L. Peixe (2013). Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J. Clin. Microbiol.* **51**: 4281-4283.

Pitout, J. D. D., D. B. Gregson, L. Poirel, J.-A. McClure, P. Le, D. L. Church (2005). Detection of *Pseudomonas aeruginosa* producing metallo-beta-lactamases in a large centralized laboratory. *J. Clin. Microbiol*. **43**: 3129-3135.

Poirel, L., P. Nordmann (2015). Rapidec carba NP test for rapid detection of carbapenemase producers. *J. Clin. Microbiol*. **53**: 3003-3008.

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

Queenan, A. M., K. Bush (2007). Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.* **20**: 440-458, table of contents.

Tängdén, T., C. G. Giske (2015). Global dissemination of extensively drug-resistant carbapenemase-producing *Enterobacteriaceae.* clinical perspectives on detection, treatment and infection control. *J. Intern. Med.* **277**: 501-512.

Tsakris, A., I. Kristo, A. Poulou, F. Markou, A., Ikonomidis, S. Pournaras (2008). First occurrence of KPC-2-possessing *Klebsiella pneumoniae* in a Greek hospital and recommendation for detection with boronic acid disc tests. *J. Antimicrob. Chemother.* **62**: 1257-1260.

Tsakris, A., I. Kristo, A. Poulou, K. Themeli-Digalaki, A. Ikonomidis, D. Petropoulou, S. Pournaras, D. Sofianou (2009). Evaluation of boronic acid disk tests for differentiating KPC-possessing *Klebsiella pneumoniae* isolates in the clinical laboratory. *J. Clin. Microbiol.* **47**: 362-367.

Tsakris, A., A. Poulou, S. Pournaras, E. Voulgari, G. Vrioni, K. Themeli-Digalaki, D. Petropoulou, D. Sofianou (2010). A simple phenotypic method for the differentiation of metallo-beta-lactamases and class A KPC carbapenemases in *Enterobacteriaceae* clinical isolates. *J. Antimicrob. Chemother.* **65**: 1664-1671.

Tsakris, A., K. Themeli-Digalaki, A. Poulou, G. Vrioni, E. Voulgari, V. Koumaki, A. Agodi, S. Pournaras, D. Sofianou (2011). Comparative evaluation of combined-disk tests using different boronic acid compounds for detection of *Klebsiella pneumoniae* carbapenemase-producing enterobacteriaceae clinical isolates. *J. Clin. Microbiol.* **49**: 2804-2809.

Tsakris, A., A. Poulou, P. Bogaerts, E. Dimitroulia, S. Pournaras, Y. Glupczynski (2015). Evaluation of a new phenotypic OXA-48 disk test for differentiation of OXA-48 carbapenemase-producing *Enterobacteriaceae* clinical isolates. *J. Clin. Microbiol.* **53**: 1245-1251.

Voulgari, E., A. Poulou, V. Koumaki, A. Tsakris (2013). Carbapenemase-producing *Enterobacteriaceae.* now that the storm is finally here, how will timely detection help us fight back? *Future Microbiol*. **8**: 27-39.

Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-San-

chez, J. W. Biddle, C. D., Steward, S. Alberti, K. Bush, F. C. Tenover (2001). Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother*. **45**: 1151-1161.

Yong, D., K. Lee, J. H. Yum, H. B. Shin, G. M. Rossolini, Y. Chong (2002). Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J. Clin. Microbiol*. **40**: 3798-3801.

Yusuf, E., S. Van Der Meeren, A. Schallier, D. Piérard (2014). Comparison of the Carba NP test with the Rapid CARB Screen Kit for the detection of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*. *Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol.* **33**: 2237-2240.

van der Zwaluw, K., A. de Haan, G. N. Pluister, H. J. Bootsma, A. J. de Neeling, L. M. Schouls (2015). The carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in gram-negative rods. *PloS One* **10**: e0123690.