

Evaluation of Phenotypic and Genotypic Approaches for the Detection of Class A and Class B Carbapenemases in *Enterobacteriaceae*

Simone Ambretti,* Paolo Gaibani,* Andrea Berlingeri, Miriam Cordovana, Maria Vittoria Tamburini, Gloria Bua, Maria Paola Landini, and Vittorio Sambri

The spread of carbapenemases in *Enterobacteriaceae* is among the most important issues in the antimicrobial resistance. The rapid and recent diffusion of class A and B carbapenemases determined the need of specific diagnostic tests able to detect with high sensitivity this type of resistance and to discriminate between the different enzymes. The aim of this study was to test two carbapenemase detection assays, the Rosco Synergic and the Hyplex polymerase chain reaction–enzyme-linked immunosorbent assays for screening carbapenemase-producing *Enterobacteriaceae*. The phenotypic and genotypic tests were evaluated among 108 clinical isolates, including *Klebsiella pneumoniae* carbapenemase (KPC) ($n=50$) and metallo- β -lactamase- (MBL) ($n=20$), and AmpC- ($n=10$) producing *Enterobacteriaceae*. The commercial phenotypic assay showed a high sensitivity performance detecting all KPC and MBL producers, including New Delhi MBL 1 (NDM-1) strains. In addition, the Rosco Synergic assay was able to distinguish specifically between the different mechanisms that confer resistance to carbapenems in *Enterobacteriaceae*. We also demonstrated that the genotypic test was able to detect all the class A and B carbapenemases showing high sensitivity (100%) and specificity (98%) in a fast and reliable time. Based on these results, both the commercial phenotypic and the genotypic assays could be helpful as confirmatory and discriminatory tests for the detection of class A and class B carbapenemases.

Introduction

IN THE LAST YEARS, spread of carbapenem-resistant *Enterobacteriaceae* (in particular, *Klebsiella pneumoniae*) was observed worldwide. Hospital-acquired infections caused by *Enterobacteriaceae* producing different types of carbapenem-hydrolyzing enzymes (carbapenemases) are now commonly observed in several countries and represent a great limitation for antimicrobial therapy.^{3,12,17} Recently, spreading of different types of carbapenemases, such as *K. pneumoniae* carbapenemase (KPC) and the New Delhi metallo- β -lactamase 1 (NDM-1), was reported in Italy and elsewhere.^{9,10,12,15,17} Different tests have been proposed to detect carbapenemases, using either phenotypic or genotypic techniques.^{2,11,19} Among the phenotypic techniques, the cloverleaf test or modified-Hodge test (MHT) has been proposed as a confirmatory test for detection of carbapenemase-producing *Enterobacteriaceae*.^{4,14} Previous studies showed that MHT has a high efficiency for detection of class A and D carbapenemases, while its effectiveness is lower when used for detec-

tion of metallo- β -lactamases (MBL): in this case, addition of supplementary reagents, such as $ZnSO_4$, could improve the test performance.^{11,14} An important limitation of MHT is the interpretation of the results that requires well-trained personnel and cannot be straightforward among the types of carbapenemase producers.¹⁴ To overcome these limits, growth media were recently proposed to detect carbapenemase producers.¹⁴ Significant limitations of these media are the growth of nonenterobacterial gram-negative rods and the inability to discriminate among different types of carbapenemases, which is important for an efficient surveillance of the spread of these bacteria.

Molecular techniques are nowadays considered the gold standard for the optimal identification of different types of carbapenemase-producing *Enterobacteriaceae*.¹⁵ Among the commercially available tests, Hyplex multiplex polymerase chain reaction–enzyme-linked immunosorbent assay (PCR-ELISA) is capable to reliably identify different bacterial pathogens directly from blood culture or resistance mechanisms in *Enterobacteriaceae*.^{2,18,20}

Operative Unit of Clinical Microbiology, S. Orsola-Malpighi University Hospital, Bologna, Italy.

*These two authors equally contributed to this work.

Based on these considerations, the aim of this study was to evaluate the performance of commercial phenotypic (Rosco Synergy Test; Rosco Diagnostica) and genotypic (Hyplex; Amplex Diagnostics) diagnostic tests, in comparison with MHT, to detect and discriminate specifically various classes of carbapenemases.

Materials and Methods

One hundred eight various β -lactamase-producing strains obtained from four different Italian Hospitals were included in this study. The list of the isolates is presented in Table 1. Routinely, the minimum inhibitory concentrations (MIC) were determined by using a Vitek2 automated system (Biomérieux). A reduced susceptibility to Ertapenem (MIC of ≥ 2 mg/L) is considered the most sensitive marker to detect *Enterobacteriaceae* that express resistance mechanism affecting the carbapenem efficiency, and for this reason, we used this value as an inclusion criterion for the bacterial strains used in this study.^{5,8,14} Moreover, we collected all the isolates that showed MIC values for imipenem and/or meropenem higher than epidemiological cut-off as defined by the EUCAST documents (available on line at www.eucast.org/documents/rd/; lastly accessed on October 18th, 2012).

To identify ESBL, AmpC, and carbapenemases following Ambler classification,⁸ different PCR tests targeted at the genes *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA48} were performed as previously reported.^{1,6,9,10,13,16}

Among the 108 clinical isolates, the presence of class A and class B carbapenemases was detected, respectively, in 50 and 20 clinical isolates. In particular, carbapenemase producers consisted of KPC2/3-producing ($n=50$) *K. pneumoniae*, NDM-1-producing ($n=7$) *K. pneumoniae* and ($n=1$) *Escherichia coli*, VIM-1-producing ($n=6$) *K. pneumoniae*, ($n=3$) *Citrobacter freundii*, ($n=1$) *Klebsiella oxytoca*, and ($n=2$) *Enterobacter cloacae*, as shown in Table 1. The remaining 38 carbapenemase-negative *Enterobacteriaceae* were 28 ESBL and 10 AmpC producers.

The modified cloverleaf test was performed as the phenotypic confirmatory test for carbapenemase production as suggested by the CLSI.⁴ A modification of MHT with the addition of 100 μ g/ml of ZnSO₄ in Mueller Hinton agar was performed on the same isolates, to improve the limit of detecting carbapenemase producers and in particular MBL, as previously described.¹¹

Commercial diagnostic tablets from Rosco (Rosco Diagnostica) were evaluated following the manufacturer's instructions. The results were interpreted as follows: an increase ≥ 5 mm of inhibition zone diameter around tablets containing boronic acid and dipicolinic acid, in comparison with the diameter of tablet with meropenem alone, was considered a positive result, respectively, for KPC and MBL production, while an increase ≥ 5 mm in diameter around both tablets containing boronic acid and Cloxacillin suggests AmpC production.

A multiplex PCR-ELISA was tested among the 108 clinical isolates, for the detection of ESBL-producing (Hyplex[®] ESBL

TABLE 1. SUMMARY OF RESULTS OBTAINED ON 108 ISOLATES OF *ENTEROBACTERIACEAE* CLASSIFIED BY PCR AS PRODUCERS OF CLASS A AND CLASS B CARBAPENEMASES, ESBL, AND AMPC, TESTED BY MHT [\pm ZnSO₄], ROSCO SYNERGIC TEST, AND HYPLEX PCR-ELISA

Strains (No. of studied)	MHT No. of positive		Rosco disc synergic assay No. of positive			Hyplex PCR assay No. of positive		
	[- ZnSO ₄]	[+ ZnSO ₄]	Boronic acid	Dipicolinic acid	Cloxacillin	KPC	NDM	VIM
Class A carbapenemases	50/50	50/50	50/50	—	—	50/50	—	—
KPC								
<i>Klebsiella pneumoniae</i> (50)	50	50	50	—	—	50	—	—
Class B carbapenemases	11/20	17/20	—	20/20	—	—	8/8	12/12
NDM								
<i>K. pneumoniae</i> (7)	2	5	—	7	—	—	7	—
<i>Escherichia coli</i> (1)	1	1	—	1	—	—	1	—
VIM								
<i>K. pneumoniae</i> (6)	2	5	—	6	—	—	—	6
<i>Citrobacter freundii</i> (3)	3	3	—	3	—	—	—	3
<i>Enterobacter cloacae</i> (2)	2	2	—	2	—	—	—	2
<i>Klebsiella oxytoca</i> (1)	1	1	—	1	—	—	—	1
AmpC	4/10 ^a	4/10 ^a	6/10	—	6/10	—	—	—
<i>Enterobacter cloacae</i> (9)	3 ^a	3 ^a	5	—	5	—	—	—
<i>K. pneumoniae</i> (1)	1 ^a	1 ^a	1	—	1	—	—	—
ESBL	6/28 ^a	6/28 ^a	—	2/28 ^a	—	2/28 ^a	—	—
<i>K. pneumoniae</i> (18)	—	—	—	2 ^a	—	1 ^a	—	—
<i>E. cloacae</i> (3)	3 ^a	3 ^a	—	—	—	1 ^a	—	—
<i>Enterobacter aerogenes</i> (3)	1 ^a	1 ^a	—	—	—	—	—	—
<i>Proteus mirabilis</i> (1)	1 ^a	1 ^a	—	—	—	—	—	—
<i>Proteus vulgaris</i> (1)	1 ^a	1 ^a	—	—	—	—	—	—
<i>E. coli</i> (1)	—	—	—	—	—	—	—	—
<i>Providencia stuartii</i> (1)	—	—	—	—	—	—	—	—

^aNumber of strains that gave false-positive results.

PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; MHT, modified-Hodge test; NDM, New Delhi metallo- β -lactamase; VIM, Verona integron-encoded metallo- β -lactamase.

ID) and KPC-MBL-OXA-48 producing (Hyplex Super Bug) bacteria, following the manufacturer's instructions.^{17,19}

The performances of both genotypic and phenotypic commercial tests for detection of different carbapenemases were compared to gene-specific PCR assays, considered as the reference standard.

Results

The reference PCRs results showed that 50 isolates were positive for *bla*_{KPC} (vast majority of KPC3),¹⁰ while 20 strains were MBL producers (8 positive for *bla*_{NDM} and 12 positive for *bla*_{VIM}) (Table 1). Among the other 38 isolates, 28 expressed an the *ESBL* gene, and 10 strains were found as AmpC producers (Table 1).

The MHT and MHT added with ZnSO₄ showed a positive result, respectively, for 71 and 77 of the 108 *Enterobacteriaceae* isolates. The specificity of MHT and MHT (ZnSO₄) compared with PCR targeting single resistance was 70%, while sensitivity increased since 87% to 96%. Among 38 carbapenemase-negative strains, false-positive results were obtained by MHT for 4 out of 10 isolates producing AmpC (Table 1).

Afterward, the isolates were evaluated by using the phenotypic assay (Rosco), which combines the meropenem activity in association with specific inhibitors (boronic acid, dipicolinic acid, and Cloxacillin). As shown in Table 1, the concordance between the results obtained with Rosco Synergic assay and those found with reference PCRs was 100% for all KPC- and MBL-producing isolates (20/20), including all NDM-1 producers, while two *ESBL* producers gave false-positive results with dipicolinic acid (Table 1). Similar results were observed in a previous study performed with a combined-disc test consisting of meropenem alone and with phenylboronic acid (PBA), EDTA, or both PBA and EDTA to detect carbapenemase production.¹⁷ Among the 34 carbapenemase-negative strains, 6 out of 10 isolates resulted as AmpC producers, showing a concordance of 60% with reference PCR. In summary, our results showed that sensitivity of Rosco Synergic assay was 100% for KPC and MBL and 60% for AmpC, while specificity was, respectively 100%, 98%, and 100%.

All the 108 isolates were evaluated by the genotypic assay Hyplex *ESBL* ID that permitted the identification of all main known carbapenemase determinants (*bla*_{KPC}, *bla*_{OXA-48}, *bla*_{NDM}, *bla*_{VIM}, and *bla*_{IMP}) within ~4.5 to 6 hr, by a PCR amplification followed by reverse hybridization.⁵ A previous study reported excellent results for the Hyplex MBL Multiplex PCR-ELISA on *bla*_{VIM}- and *bla*_{IMP}-harboring *Enterobacteriaceae*, while sensitivity and specificity against NDM producers were not investigated.² This test showed in our study overall values of 100% and 98%, for sensitivity and specificity, respectively, when compared with PCR findings. This genotypic method was able to identify screened class A and B carbapenemases (Table 1). These results are in accordance with those previously published.² The Hyplex method gave two false-positive results, but these isolates had a very low optical density (OD) value, strictly closed to the cut-off (0.4 OD).

Conclusion

Increasing and rapid diffusion of mobile genetic elements that determined acquired resistance to carbapenems and all other β -lactams in *Enterobacteriaceae* is now observed in

several countries.³ Carbapenems represent the preferential treatment for severe infections caused by multidrug-resistant gram-negative bacteria showing an *ESBL* production.^{12,17} Accurate detection of carbapenemases-producing strains by phenotypic and genotypic assays has an important clinical and epidemiological value.^{14,15} The MHT is probably the best-known and most-used confirmatory assay, but its analytical performance is not optimal, also due to difficult interpretations.¹⁴ Moreover, MHT cannot discriminate among different types of carbapenemases.¹¹

We showed that Rosco Synergic test was able to detect both MBL, including NDM, and KPC producers with higher specificity and sensitivity than MHT. The use of Synergic assay allows to discriminate between different types of carbapenemases and can be suggested for routine diagnostic application because of its low cost, reliability, and very good discriminatory potential among different resistance mechanisms. However, this assay cannot detect OXA-48 producers such as other phenotypic assays, by the fact that this enzyme is not inhibited by boronic or clavulanic acid or any zinc chelators.¹⁴ Noteworthy, this is a clear limitation of the practical use of the Synergic assay, since OXA-48 producers are rapidly emerging and disseminating in Europe,⁷ regardless of the limited number of cases so far reported in Italy.

Moreover, our data indicate that Hyplex PCR-ELISA may be a useful complementary method in the clinical laboratory for timely identification and high specificity and sensitivity both for KPC and MBL (*bla*_{VIM} and *bla*_{NDM}). In addition, to improve the specificity of the Hyplex method, we hypothesize that it could be useful to increase the established cut-off value.

Based on these findings, this genotypic assay could be considered in the diagnostic workflow as confirmatory method for carbapenemase production and/or as an identification tool for the most important different carbapenemase genes.

Acknowledgments

This study was supported by funds RFO 2010 and 2011 from the University of Bologna to VS.

Disclosure Statement

All authors report no conflicts of interest relevant to this article.

References

1. Ambretti, S., P. Gaibani, F. Caroli, L. Miragliotta, and V. Sambri. 2010. A carbapenem-resistant *Klebsiella pneumoniae* isolate harboring KPC-1 from Italy. *New Microbiol.* 33: 281–282.
2. Avlami, A., S. Bekris, G. Ganteris, E. Kraniotaki, E. Malamou-Lada, M. Orfanidou, O. Paniara, A. Pantazatou, C.C. Papagiannitsis, E. Platsouka, I. Stefanou, E. Tzelepi, H. Vagiakou, and V. Miriagou. 2010. Detection of metallo- β -lactamase genes in clinical specimens by a commercial multiplex PCR system. *J. Microbiol. Methods.* 83:185–187.
3. Cantón, R., M. Akóva, Y. Carmeli, C.G. Giske, Y. Glupczynski, M. Gniadkowski, D.M. Livermore, V. Miriagou, T. Naas, G.M. Rossolini, Ø. Samuelsen, H. Seifert, N. Woodford, P. Nordmann, and European Network on

- Carbapenemases.** 2012. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. *Clin. Microbiol. Infect.* **18**:413–431.
4. [CLSI] **Clinical and Laboratory Standards Institute.** 2009. Performance standards for antimicrobial susceptibility testing. 19th Informational Supplement. CLSI document M100-S19. Wayne, PA. Available at www.cdc.gov/HAI/pdfs/labSettings/HodgeTest_Carbapenemase_Enterobacteriaceae.pdf (Online.)
 5. [CLSI] **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing. 19th Informational Supplement. CLSI document M100-S22, Wayne, PA.
 6. **Dallenne, C., A. Da Costa, D. Decré, C. Favier, and G. Arlet.** 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* **65**:490–495.
 7. **Dimou, V., H. Dhanji, R. Pike, D.M. Livermore, and N. Woodford.** 2012. Characterization of *Enterobacteriaceae* producing OXA-48-like carbapenemases in the UK. *J. Antimicrob. Chemother.* **67**:1660–1665.
 8. **European Committee on Antimicrobial Susceptibility Testing.** 2012. Breakpoint tables for interpretation of MICs and zone diameters. Version 2.0, 1 Jan 2012. Available at www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf (Online.)
 9. **Gaibani, P., S. Ambretti, A. Berlingeri, M. Cordovana, P. Farruggia, M. Panico, M.P. Landini, and V. Sambri.** 2011. Outbreak of NDM-1-producing *Enterobacteriaceae* in northern Italy, July to August. *Euro. Surveill.* **16**:pii:20027.
 10. **Gaibani, P., S. Ambretti, A. Berlingeri, F. Gelsomino, A. Bielli, M.P. Landini, and V. Sambri.** 2011. Rapid increase of carbapenemase-producing *Klebsiella pneumoniae* strains in a large Italian hospital: surveillance period 1 March–30 September 2010. *Euro. Surveill.* **16**:pii:19800.
 11. **Girlich, D., L. Poirel, and P. Nordmann.** 2012. Value of the modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*. *J. Clin. Microbiol.* **50**:477–479.
 12. **Gupta, N., B.M. Limbago, J.B. Patel, and A.J. Kallen.** 2011. Carbapenem-resistant *Enterobacteriaceae*: epidemiology and prevention. *Clin. Infect. Dis.* **53**:60–67.
 13. **Mendes, R.E., K.A. Kiyota, J. Monteiro, M. Castanheira, S.S. Andrade, A.C. Gales, A.C. Pignatari, and S. Tufik.** 2007. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. *J. Clin. Microbiol.* **45**:544–547.
 14. **Nordmann, P., M. Gniadkowski, C.G. Giske, L. Poirel, N. Woodford, V. Miriagou, and European Network on Carbapenemases.** 2012. Identification and screening of carbapenemase-producing *Enterobacteriaceae*. *Clin. Microbiol. Infect.* **18**:432–438.
 15. **Nordmann, P., and L. Poirel.** 2010. Emerging carbapenemases in Gram-negative aerobes. *Clin. Microbiol. Infect.* **8**: 321–331.
 16. **Pérez-Pérez, F.J., and H.D. Hanson.** 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J. Clin. Microbiol.* **40**:2153–2162.
 17. **Queenan, A.M., and K. Bush.** 2007. Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.* **20**:440–458.
 18. **Treviño, M., L. Moldes, L. Martínez-Lamas, C. Varón, and B.J. Regueiro.** 2009. Carbapenem-resistant *Enterobacter cloacae* and the emergence of metallo-beta-lactamase-producing strains in a third-level hospital (Santiago de Compostela, NW Spain). *Eur. J. Clin. Microbiol. Infect. Dis.* **28**:1253–1258.
 19. **Tsakris, A., A. Poulou, S. Pournaras, E. Voulgari, G. Vrioni, K. Themeli-Digalaki, D. Petropoulou, and 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.
 20. **Wellinghausen, N., B. Wirths, A. Essig, and L. Wassill.** 2004. Evaluation of the Hyplex blood screen multiplex PCR-enzyme-linked immunosorbent assay system for direct identification of gram-positive cocci and gram-negative bacilli from positive blood cultures. *J. Clin. Microbiol.* **42**:3147–3152.

Address correspondence to:

Paolo Gaibani, PhD

Operative Unit of Clinical Microbiology

S. Orsola-Malpighi University Hospital

9 via G. Massarenti

Bologna 40138

Italy

E-mail: paolo.gaibani@unibo.it