

Accepted Manuscript

Evaluation of the carbapenem inactivation method (CIM) for detecting carbapenemase activity in enterobacteria

A. Aguirre-Quinonero, ME Cano, D. Gamal, J. Calvo, L. Martínez-Martínez

PII: S0732-8893(17)30085-8
DOI: doi: [10.1016/j.diagmicrobio.2017.03.009](https://doi.org/10.1016/j.diagmicrobio.2017.03.009)
Reference: DMB 14321

To appear in: *Diagnostic Microbiology and Infectious Disease*

Received date: 28 November 2016
Revised date: 6 March 2017
Accepted date: 13 March 2017

Please cite this article as: Aguirre-Quinonero A, Cano ME, Gamal D, Calvo J, Martínez-Martínez L, Evaluation of the carbapenem inactivation method (CIM) for detecting carbapenemase activity in enterobacteria, *Diagnostic Microbiology and Infectious Disease* (2017), doi: [10.1016/j.diagmicrobio.2017.03.009](https://doi.org/10.1016/j.diagmicrobio.2017.03.009)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



**EVALUATION OF THE CARBAPENEM INACTIVATION METHOD
(CIM) FOR DETECTING CARBAPENEMASE ACTIVITY IN
ENTEROBACTERIA.**

Aguirre-Quiñonero A¹, Cano ME¹, Gamal D², Calvo J¹, Martínez-Martínez L^{1, 3}.

¹University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain.

²Microbiology Department, Theodor Bilharz Research Institute, Cairo, Egypt.

³Department of Molecular Biology, University of Cantabria, Santander, Spain.

Corresponding Author

Amaia Aguirre-Quiñonero

Avda. Valdecilla, Nr. 25; 39008,

Santander (Cantabria),

Spain.

Email Address: amayiaaguirre@gmail.com

Telephone number: 0034942202520.

Fax Number: 0034942203462

ABSTRACT

OBJECTIVES: The objective of this study was to evaluate the accuracy of the CIM test in the detection of carbapenemase activity in 124 strains of Enterobacteriaceae.

METHODS: A panel of 124 previously characterized Enterobacteriaceae was tested: 77 strains producing the following carbapenemase families: KPC (n=14), GES (n=22), NDM (n=19), VIM (n=4), IMP (n=4) and OXA-48 (n=14) and 47 non-carbapenemase producers. For the CIM method, an active susceptibility meropenem disc was exposed to a bacterial suspension of a test strain; when a carbapenemase is produced, the antibiotic is inactivated allowing uninhibited growth of an indicator strain after overnight incubation. A clear inhibition zone (≥ 20 mm) was considered indicative of no-carbapenemase activity.

RESULTS: All KPC, NDM, VIM, IMP or OXA-48 producing strains were unequivocally detected with the CIM test. CIM false negative results were obtained with eleven *Enterobacter cloacae* producing GES-6. Two other *E. cloacae* not producing carbapenemase (one with SHV-12, one hyperproducing AmpC) were positive by the test. The sensitivity and specificity of the assay compared to those of molecular methods were 85.7% and 95.7%, respectively.

CONCLUSIONS: The CIM method proved to be inexpensive and easy to interpret. It provided less than optimal results in the detection of GES-6 activity.

KEYWORDS: Carbapenemase, Carbapenem Inactivation Method, Rapid test, GES-6.

INTRODUCTION

Resistance to carbapenems is mainly due to the production of beta-lactamases inactivating carbapenems (carbapenemases), included in classes A, B, C or D of the Ambler classification. Whereas class A, C and D beta-lactamases share a serine residue in the active site, class B enzymes (also referred to as metallo-beta-lactamases) require the presence of zinc for their activity [1-4].

In Enterobacteria, carbapenemase-encoding genes are often located on plasmids which contributes to a rapid spread among clinically relevant Gram-negative bacteria all over the world [5-7]. The most frequent types of enzymes among carbapenemase-producing Enterobacteriaceae (CPE) include KPC, NDM, OXA-48 and related enzymes, and to a lesser extent, VIM type or IMP carbapenemase types. A large variety of less frequent enzymes (including some GES-type and other carbapenemases) have also been described [1, 8, 9].

Rapid and reliable detection of carbapenemase production among clinical isolates is needed for therapeutic and control reasons. Potential carbapenemase production is usually screened first by conventional susceptibility testing; however, this screening is hampered since susceptibility to carbapenems may vary significantly among carbapenemase producers. Some enzymes exhibit weak activity against carbapenems, and can determine MIC values of carbapenems below the clinical susceptibility breakpoint and even below the screening breakpoint proposed by the EUCAST for detection of carbapenemase producers [10-14]. Over the last years, an increasing number of non-molecular assays for rapid detection of carbapenemase activity have been described. These tests are designed to provide preliminary information before confirmatory assays are performed either by molecular or by UV spectrophotometry methods. In the modified Hodge test (MHT) the carbapenemase activity is

detected when the clinical isolate producing carbapenemase allows growth of a full susceptible strain towards an imipenem disc [15]. Although this method has been extensively used, its interpretation is not always easy and both false positive and false negative results have been reported [15-17]. Other methods of carbapenemase detection include colorimetric assays based on the enzymatic hydrolysis of the beta-lactam ring of a carbapenem leading to a pH decrease and consequent colour shift of the pH indicator (phenol red or bromothymol blue) in the presence of carbapenemase activity [18, 19]. These tests have recently been commercially launched into the market as RAPIDEC[®] CARBA-NP (bioMérieux, France) [20-23] or Rapid CARB Blue kit (Rosco Diagnostica A/S, Denmark) [24, 25] and have both shown promising results [20-25]. Moreover, combined-disc tests containing a carbapenem plus specific inhibitors, such as EDTA or boronic acid have also been commercialized for the detection of class B and class A carbapenemases, respectively [26-28]. Lately, mass spectrometric assays are being developed for detection of carbapenem degradation products due to carbapenemase activity [29-34].

The recently described carbapenem inactivation method (CIM) [35] has shown promising results in previously published reports [35, 36]. CIM is based on the enzymatic hydrolysis of a meropenem disc after its exposure to a carbapenemase producing strain and its consequent inactivation which allows uninhibited growth of a full susceptible indicator strain. This new phenotypic test can detect carbapenemase activity irrespective of the coding genes in Gram-negative bacteria; according to the authors who described it, the method can provide results within eight hours, is easy to interpret and inexpensive.

The objective of this study is to evaluate the accuracy of the CIM test.

MATERIALS AND METHODS

Strain collection

The evaluation of the test was carried out with 124 Enterobacteriaceae strains obtained from different centres and countries. One isolate per patient was included in this study. All isolates had been previously characterized as carrying *bla*_{KPC}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{IMP}, *bla*_{OXA-48} or *bla*_{GES}; 77 strains produced the following carbapenemases: KPC-2 (n=14), GES-6 (n=22), NDM-1 (n=18), NDM-5 (n=1), VIM-1 (n=4), IMP-13 (n=4) and OXA-48 (n=14). Forty-seven strains proved to be genotypically carbapenemase-negative, among which 28 had decreased susceptibility to at least one carbapenem (imipenem, meropenem, ertapenem) (Table 1).

The species identification was confirmed for this study using MALDI-TOF Vitek-MSTM (v2 SARAMIS MS -ID, bioMérieux, France) and corresponded to *Citrobacter freundii* (n=3), *Enterobacter aerogenes* (n=4), *E. cloacae* complex (n=44), *Escherichia coli* (n= 15), *Klebsiella oxytoca* (n=4), *K. pneumoniae* (n=51), *Providencia stuartii* (n=2) and *Raoultella ornithinolytica* (n=1). In order to ensure genetic diversity of the studied organisms, pulse-field gel electrophoresis (PFGE) was performed and interpreted as described elsewhere for organisms producing GES.

Strains were stored frozen in tryptic soy broth with 10% glycerol at -70°C. For the present studies, strains were re-cultured on Columbia agar + 5% sheep blood (bioMérieux) and incubated at 37°C for 24 hours prior to performing the CIM test.

Molecular testing for carbapenemase genes

For all strains, the presence of the following genes: *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{SPM}, *bla*_{AIM}, *bla*_{DIM}, *bla*_{GIM}, *bla*_{SIM}, *bla*_{KPC}, *bla*_{BIC} and *bla*_{OXA-48} was studied as described Poirel et al. [37]. We also investigated the presence of *bla*_{GES} using the following primers: GES-F: 5'-ATGCGCTTCATTACGCACT-3' and GES-R: 5'-CTATTTGTCCGTGCTCAGGA-3'. Bacterial DNA was extracted using InstaGene matrix kit (Bio-Rad) according to the manufacturer's recommendations and 1 µl was added to 1x ready mix PCR Kit KAPA2G fast Hotstart (Kapa Biosystems) along with 0.5 µM each primer, in a final volume of 20 µl. The amplification conditions were 95°C for 2 min, and then 35 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s. Amplicons were purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Both strands were sequenced (Macrogen Europe, Amsterdam, The Netherlands) and the BLAST program was used to compare the nucleotide and protein sequences to those available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Phenotypic testing by CIM test

The CIM method was performed as stated by Van der Zwaluw K. et al. [35]: an active susceptibility meropenem disc (10 µg) was immersed in a suspension composed by a full 10 µL inoculation loop of culture homogenised in 400 µL of water. After two hours of incubation at 35°C the disc was removed from the suspension and placed on a Mueller-Hinton agar plate previously streaked with a susceptible *E. coli* indicator strain (ATCC 25922) and subsequently incubated at 35°C. Results were read after overnight incubation: the bacterial suspension of strains harbouring a carbapenemase resulted in an inactivation of the antibiotic allowing uninhibited growth of the susceptible indicator strain; conversely, a clear inhibition

zone appeared in strains without carbapenemase activity. Tijet N et al. [36] defined the inhibition zone diameter defining non-carbapenemase producers in ≥ 20 mm.

Sensitivity and specificity

PCR analysis detecting genes encoding carbapenemases (*bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{SPM}, *bla*_{AIM}, *bla*_{DIM}, *bla*_{GIM}, *bla*_{SIM}, *bla*_{KPC}, *bla*_{BIC}, *bla*_{OXA-48} and *bla*_{GES}) was considered as the reference method for carbapenemase characterization.

In addition, and because novel carbapenemases may not be detected since only specific genes were examined, when discrepancies were observed between PCR-sequencing results and CIM, the CMI test was repeated and the following additional assays were also performed: (i) RAPIDEC[®] CARBA-NP (bioMérieux, France) and (ii) Rapid CARB Blue kit (Rosco Diagnostica, Denmark).

RESULTS

A summary of the obtained data is presented in Table 1.

All *bla*_{IMP}⁻, *bla*_{NDM}⁻, *bla*_{KPC}⁻ and *bla*_{OXA-48}⁻ positive isolates were unequivocally positive according to the CIM test with inhibition zone diameters of 6 mm.

Eight of the 22 (36.4%) isolates with the gene coding for GES-6 displayed an inhibition zone diameter of 6 mm, while eleven strains exhibited inhibition zone diameters of ≥ 20 mm, considered negative according to Tijet et al. [36]. Among those strains with inhibition zone diameters ≥ 20 mm, five featured a diameter of 20-24 mm and six presented an inhibition zone of 25-30 mm. MICs of carbapenems for these 11 isolates are presented in Table 2.

Additionally, both CARBA-NP and Rapid CARB Blue assays yielded positive results for these 11 isolates. Therefore, the overall sensitivity of the CIM test was 79.3%. These false negative strains harboured ertapenem minimum inhibitory concentrations (MICs) values of > 0.12 (Table 2.) defined by the EUCAST as the screening cut-off and yielded positive results with the RAPIDEC[®] CARBA-NP (bioMérieux, France) and Rapid CARB Blue kit (Rosco Diagnostica, Denmark).

Considering all except GES-type carbapenemases the corresponding sensitivity would be 100%. The pulse-field gel electrophoresis (PFGE) analysis after digestion with *Xba*I of the *E. cloacae* GES-6 isolates resulted in 4 pulsotypes (A-D), 73.8% (14/19) being clonally related (pulsotype A) (Figure 1.1.). On the other hand, both *K. oxytoca* GES-6 isolates proved to be subtypes within the same clone (Figure 1.2).

Regarding the 47 carbapenemase negative isolates, all gave negative results except from two *E. cloacae*. Of these, one presented a phenotype of AmpC hyperproduction (and lacked both an ESBL phenotype and genes coding for major ESBL) and another harboured SHV-12. The

inhibition zone diameter with the CIM assay for both isolates was of 6 mm. Both the RAPIDEC® CARBA-NP (bioMérieux, France) and Rapid CARB Blue kit (Rosco Diagnostica, Denmark) assays performed in these two strains displayed positive results.

In the remaining 45 non-carbapenemase producers the inhibition zone diameter was ≥ 25 mm (25 mm (n=2), 26 mm (n=1), 28 mm (n=1), 29 mm (n=2), 30 mm (n= 39)), being 30 mm the most frequent diameter observed among carbapenemase negative strains (80.9%). These results gave a specificity, positive and negative predictive value of 95.7%, 97.8% and 83.0%, respectively.

In view of the results, if the inhibition zone diameter defining non-carbapenemase producing strains is changed from ≥ 20 mm to ≥ 25 mm, the sensitivity would increase from 85.7% to 92.2%.

DISCUSSION

In this study, the CIM test detected unequivocally all strains producing KPC, NDM, VIM, IMP or OXA-48 even if they had low MIC of carbapenems. KPC enzymes usually confer high levels of resistance to carbapenems and to most beta-lactams. In Europe, this type of carbapenemase is almost ubiquitous [1, 6] and in the United States KPC is the predominant enzyme conferring carbapenem resistance [3]. On the other hand, IMP variants have spread worldwide although its frequency is much scarcer than that of KPC producers. Hence, the CIM test proves to be highly accurate for discriminating between isolates with and without these types of carbapenemases.

GES enzymes have been identified worldwide but its occurrence remains scarce. This family currently includes 27 variants (<http://www.lahey.org/studies/other.asp>); all GES variants possess the ability to hydrolyze broad-spectrum cephalosporins, but only a few of them have been described to be able of hydrolyzing carbapenems (GES-2, GES-4, GES-5, GES-6, GES-11, GES-14, GES-16 y GES-18) [38]. The GES-5 variant constitutes the main carbapenem-hydrolyzing GES-type enzyme; it has identified in Enterobacteriaceae and been widely reported in South America, particularly in Brazil [1, 39]. In terms of GES-6 detection, the weak sensitivity (50.0%) we have observed with the CIM assay could be linked to the low hydrolytic profile of GES-producers, but the actual cause is not clear at this moment; additional studies with whole genome sequencing of these isolates would be helpful to understand this observation. From a practical point of view, in scenarios with an increasing prevalence of GES-6-producing Enterobacteriaceae, a negative result with the CIM could require additional confirmation tests.

Recently, several publications on the CIM assay have been published, reinforcing the usefulness and improvements of this phenotypic method [40-42]. In order to improve the

sensitivity and specificity of the CIM an increase of the incubation period to four hours has been proposed [40].

The CIM test proved to have multiple advantages: (i) it is very easy to perform; (ii) it is inexpensive given that only water and a 10 µg meropenem susceptibility-testing disc per isolate is needed and that no specific equipment is required; (iii) on the same Mueller- Hinton agar plate up to four isolates can be tested at the same time; (iv) the results can be objectively interpreted. Its main limitation is that, in our experience, it requires an overnight incubation of the inoculated plates to the results to be read beyond any reasonable doubt, contrasting to the eight hours stated by the authors of the original paper [35]. Not only are the inhibition zones difficult to read after eight hours, but also this protocol might be impractical in some laboratories in their daily routine workflow.

In conclusion, the CIM test can be efficiently used for screening of carbapenemase production before PCR confirmation of the genes involved. The CIM test can play an important role in the early detection of carbapenemase-producing Enterobacteriaceae and, consequently, in the implementation of infection prevention and control measures, above all in low-income countries.

ACKNOWLEDGEMENTS

We are grateful to Álvaro Pascual, Jesús Mingorance and Patrice Nordmann for kindly some of the carbapenemase-producing isolates used in this study.

DECLARATIONS

Funding: Our research did not include any funding.

Competing Interests: We have read and understood JAA policy on declaration of interests and declare that we have no competing interests.

Ethical Approval: Not required

ACCEPTED MANUSCRIPT

REFERENCES

1. Nordmann P, Poirel L. The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. Clin Infect Dis 2014; 20: 821-830.
2. Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2011; 17: 1791-1797.
3. Queenan AM, Bush K. Carbapenemases: the versatile β -lactamases. Clin Microbiol Rev. 2007; 20: 440-458.
4. Bush K. Carbapenemases: partners in crime. J Glob Antimicrob Resist. 2013; 1: 7-16.
5. Martínez-Martínez L, González-López JJ. Carbapenemases in Enterobacteriaceae: types and molecular epidemiology. Enferm Infecc Microbiol Clin. 2014; 32: 4-9.
6. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, et al. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect. 2012; 18: 413-431.
7. Pitout JD, Nordmann P, Poirel L. Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. Antimicrob Agents Chemother. 2015; 59: 5873-5884.
8. Viau R, Frank KM, Jacobs MR, Wilson B, Kaye K, Donskey, et al. Intestinal carriage of carbapenemase-producing organisms: current status of surveillance methods. Clin Microbiol Rev. 2016; 29: 1-27.
9. Tängdén T, Giske CG. Global dissemination of extensively drug-resistant carbapenemase-producing Enterobacteriaceae: clinical perspectives on detection, treatment and infection control. J Internal Med. 2015; 277: 501-512.
10. Giske CG, Martínez-Martínez L, Cantón R, Stefani S, Skov R, Glupczynski Y, et al. 2013. EUCAST guidelines for detection of resistance mechanisms and specific

resistances of clinical and/or epidemiological importance.

http://www.eucast.org/resistance_mechanisms/.

11. Dortet L, Cuzon G, Plésiat P, Naas T. Prospective evaluation of an algorithm for the phenotypic screening of carbapenemase-producing Enterobacteriaceae. J Antimicrob Chemother. 2016; 71: 135-140.
12. Miriagou V, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski M, et al. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. Clin Microbiol Infect. 2010; 16: 112-122.
13. Nordmann P, Gniadkowski M, Giske CG, Poirel L, Woodford N, Miriagou V, et al. Identification and screening of carbapenemase-producing Enterobacteriaceae. Clin Microbiol Infect. 2012; 18: 432-438.
14. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 26th Ed. CLSI supplement M 100-S, Wayne, PA: Clinical and Laboratory Standards Institute: 2016.
15. Girlich D, Poirel L, Nordmann P. Value of the Modified Hodge Test for detection of emerging carbapenemases in Enterobacteriaceae. J Clin Microbiol. 2012; 447-449.
16. Takayama Y, Adachi Y, Nihonyanagi S, Okamoto R. Modified Hodge test using Mueller-Hinton agar supplemented with cloxacillin improves screening for carbapenemase-producing clinical isolates of Enterobacteriaceae. J Med Microbiol. 2015; 64: 774-777.
17. Carvalhaes CG, Picão RC, Nicoletti AG, Vavie D, Gales A. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. J Antimicrob Chemother. 2010; 65: 249-251.
18. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2012; 18: 1503-1507.

19. Pires J, Novais Â, Peixe L. Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol.* 2013; 51: 4281-4283.
20. Tijet N, Boyd D, Patel SN, Mulvey MR, Melano RG. Evaluation of the Carba NP test for rapid detection of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2013; 57: 4578-4580.
21. Dortet L, Poirel L, Nordmann P. Further proofs of concept for the Carba NP test. *Antimicrob Agents Chemother.* 2014; 58: 1269.
22. Garg A, Garg J, Upadhyay GC, Agarwal A, Bhattacharjee A. Evaluation of the Rapidec Carba NP test kit for detection of carbapenemase-producing Gram-negative bacteria. *Antimicrob Agents Chemother.* 2015; 59: 7870-7872.
23. Lifshitz Z, Adler A, Carmeli Y. Comparative study of a novel biochemical assay, the Rapidec Carba NP test, for detecting carbapenemase-producing Enterobacteriaceae. *J Clin Microbiol.* 2015; 54: 453-456.
24. Novais Â, Brihante M, Pires J, Peixe L. Evaluation of the recently launched Rapid CARB Blue kit for detection of carbapenemase-producing Gram negative bacteria. *J Clin Microbiol.* 2015; 53: 3105-3107.
25. García-Fernández S, Morosini MI, Gijón D, Beatobe L, Ruiz-Garbajosa P, Domínguez L, et al. 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. *J Clin Microbiol.* 2015; 54: 464-466.
26. Pournaras S, Poulou A, Tsakris A. Inhibitor-based methods for the detection of KPC carbapenemase-producing Enterobacteriaceae in clinical practice by using boronic acid compounds. *J Antimicrob Chemother.* 2010; 65: 1319-1321.

27. Van Dijk K, Voets GM, Scharringa J, Voskuil S, Fluit AC, Rottier WC, et al. A disc diffusion assay for detection of class A, B and OXA-48 carbapenemases in Enterobacteriaceae using phenyl boronic acid, dipicolinic acid and temocillin. *Clin Microbiol Infect.* 2014; 20: 345-349.
28. Tsakris A, Poulou A, Bogaerts P, Dimitroulia E, Pournaras S, Glupczynski Y. Evaluation of a new phenotypic OXA-48 disk test for differentiation of OXA-48 carbapenemase-producing Enterobacteriaceae clinical isolates. *J Clin Microbiol.* 2015; 53: 1245-1251.
29. Bernabeu S, Poirel L, Nordmann P. Spectrophotometry-based detection of carbapenemase producers among Enterobacteriaceae. *Diag Microbiol Infect Dis.* 2012; 74: 88-90.
30. Hrabák J, Walková R, Študentová V, Chudáčková E, Bergerová T. Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol.* 2011; 49: 3222-32
31. Burckhardt I, Zimmermann S. Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. *J Clin Microbiol.* 2011; 3321-3324.
32. Hrabák J, Chudáčková E, Walková R. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis. *Clin Microbiol Rev.* 2013; 26: 103-114.
33. Knox J, Jadhav S, Sevier D, Agyekum A, Whipp M, Waring L, et al. Phenotypic detection of carbapenemase-producing Enterobacteriaceae by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and Carba NP test. *J Clin Microbiol.* 2014; 52: 4075-4077.

34. Lasserre C, De Saint Martin L, Cuzon G, Bogaerts P, Lamar E, Glupczynski Y, et al. Efficient detection of carbapenemase activity in Enterobacteriaceae by matrix-assisted laser desorption ionization-time of flight mass spectrometry in less than 30 minutes. *J Clin Microbiol*. 2015; 53: 2163-2171.
35. Van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling J, Schouls LM. The carbapenemase 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*. 2015.10: e0123690.
36. Tijet N, Patel SN, Melano RG. Detection of carbapenemase activity in Enterobacteriaceae: comparison of the carbapenem inactivation method versus the Carba NP test. *J Antimicrob Chemother*. 2016; 274-276.
37. Poirer L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis*. 2011; 70:119-123.
38. Bebrone C, Bogaerts P, Delbrück H, Bennink S, Kupper MB, Rezende de Castro R, et al. GES-18, a new carbapenem-hydrolyzing GES-Type β -lactamase from *Pseudomonas aeruginosa* that contains Ile80 and Ser170 residues. *Antimicrob Agents Chemother*. 2013; 57: 396-4041.
39. Ribeiro VB, Falci DR, Rozales FP, Barth AL, Zavascki AP. Carbapenem-resistant GES-5-producing *Klebsiella pneumoniae* in Southern Brazil. *Braz J Infect Dis*. 2014; 18: 231-232.
40. Yamada K, Kashiwa M, Arai K, Nagano N, Saito R. Evaluation of the modified carbapenem inactivation method and sodium mercaptoacetate-combination method for the detection of metallo- β -lactamase production by carbapenemase-producing Enterobacteriaceae. *J Microb Methods*. 2017; 132: 122-115.

41. Aktas E, Malkoçoğlu G, Otlu B, Çopur Çiçek A, Külâh C, Cömert F, Sandalli C, Gürsoy NC, Erdemir D, Bulut ME. Evaluation of the Carbapenem Inactivation Method for Detection of Carbapenemase-Producing Gram-Negative Bacteria in Comparison with the RAPIDEC CARBA NP. *Microb Drug Resist.* 2016 Aug 30..
42. Saito K, Nakano R, Suzuki Y, Nakano A, Ogawa Y, Yonekawa S, Endo S, Mizuno F, Kasahara K, Mikasa K, Kaku M, Yano H. Suitability of the Carbapenem Inactivation Method (CIM) to detect IMP metallo- β -lactamase-producing Enterobacteriaceae. *J Clin Microbiol.* 2017 Jan 18. pii: JCM.02275-16. doi: 10.1128/JCM.02275-16.

Figure 1.1. PFGE pulsotypes of GES-6-producing *E. cloacae* isolates.

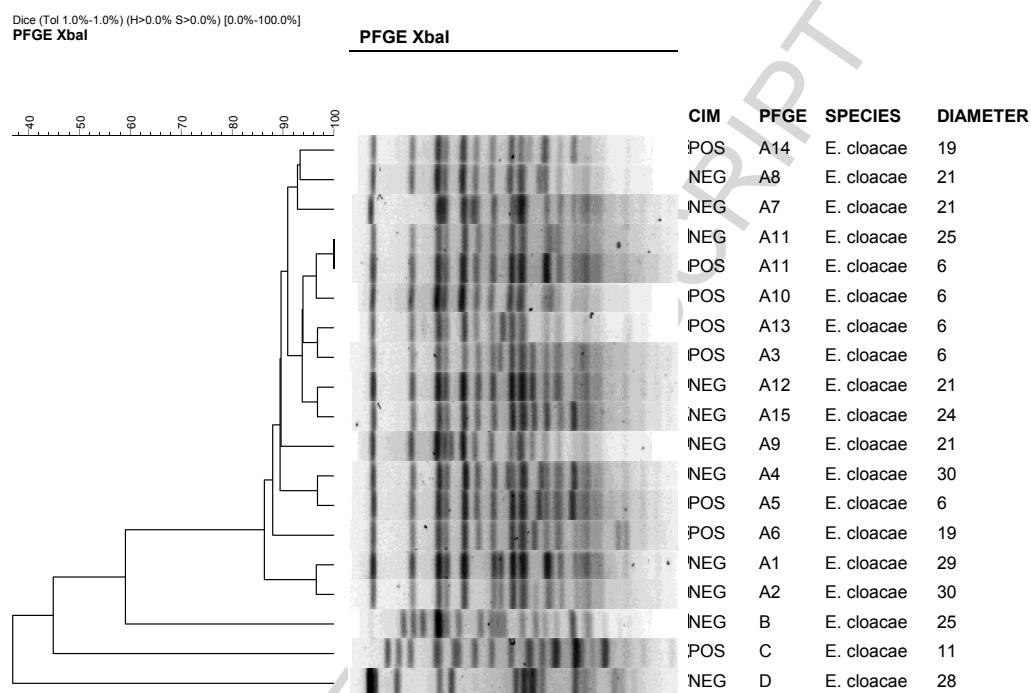


Figure 1.2. PFGE pulsotypes of GES-6-producing *K. oxytoca* isolates.

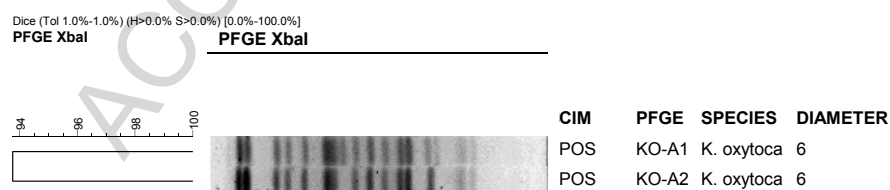


Table 1. Carbapenemase-producing and noncarbapenemase-producing isolates subjected to the CIM test.

		ENZYME	SPECIES ¹	CIM ² ≥ 20 mm	
Carbapenemase producers (77) ¹	Ambler class carbapenemase type (77) ¹				
		KPC-type (14)	KPC-2 (14)	<i>K. pneumoniae</i> (14)	Positive (14)
		GES-type (22)	GES-6 (22)	<i>E. cloacae</i> (19)	Positive (8) Negative (11)
				<i>K. oxytoca</i> (2)	Positive (2)
				<i>K. pneumoniae</i> (1)	Positive (1)
	Class B				
		NDM-type (19)	NDM-1 (18)	<i>E. coli</i> (2)	Positive (2)
				<i>K. pneumoniae</i> (16)	Positive (16)
				NDM-5 (1)	<i>E. coli</i> (1)
		VIM-type (4)	VIM-1 (4)	<i>E. cloacae</i> (3)	Positive (3)
				<i>R. ornithinolytica</i> (1)	Positive (1)
		IMP-type (4)	IMP-13 (4)	<i>E. cloacae</i> (4)	Positive (4)
	Class D	OXA-48 type (14)	<i>E. cloacae</i> (2)	Positive (2)	
			<i>E. coli</i> (1)	Positive (1)	
			<i>K. pneumoniae</i> (11)	Positive (11)	
	Non- carbapenemase producers (47)			<i>C. freundii</i> (3)	Negative (3)
				<i>E. aerogenes</i> (4)	Negative (4)
		<i>E. cloacae</i> (16)	Negative (14) Positive (2)		
		<i>E. coli</i> (11)	Negative (11)		
		<i>K. oxytoca</i> (2)	Negative (2)		
		<i>K. pneumoniae</i> (9)	Negative (9)		
		<i>P. stuartii</i> (2)	Negative (2)		

¹Number of isolates.

² CIM results: The negative results were interpreted using the inhibition zone diameter. of ≥20mm. False-negative and false-positive results are shown in bold.

Table 2. Minimum inhibitory concentration (MIC) results determined with E-test strips for false negative GES-6 producing *Enterobacter cloacae* strains:

NUMBER	PFGE ¹	IZD ²	CIM ³	IMI ⁴	ERT ⁵	MER ⁶
1	A1	23	NEG	0,25	16	2
2	A2	30	NEG	0,5	16	4
3	A4	30	NEG	0,5	1	0,5
4	A7	21	NEG	0,5	2	0,5
5	A8	21	NEG	0,25	0,5	0,5
6	A9	21	NEG	0,25	4	4
7	A11	22	NEG	0,5	>256	0,5
8	A12	22	NEG	0,5	2	0,5
9	A15	24	NEG	0,5	2	0,5
10	B	25	NEG	0,5	2	0,25
11	D	28	NEG	0,5	0,5	0,25

¹PFGE pulsotype.

²Inhibition zone diameter when performed the CIM test.

³CIM test result.

⁴Imipenem.

⁵Ertapenem.

⁶Meropenem.