

Combined disc methods for the detection of KPC- and/or VIM-positive *Klebsiella pneumoniae*: improving reliability for the double carbapenemase producers

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Abstract

Klebsiella pneumoniae strains co-producing *Klebsiella pneumoniae* carbapenemase (KPC) and verona integron-encoded metallo-beta-lactamase (VIM) are frequently isolated in Greece and have also occurred in other European countries. Conventional combined disc tests exhibit low sensitivity against these emerging pathogens. We have evaluated modifications of the KPC/Metallo- β -Lactamase Confirmation kit (ROSCO) exhibiting high diagnostic value against KPC, VIM and KPC + VIM producers. The key changes were the inclusion of additional combined tablets containing meropenem plus two inhibitors (dipicolinic acid (1000 μ g per tablet) for metallo- β -lactamases and a boronic acid derivative for KPCs) and the replacement of aminophenylboronic acid by phenylboronic acid (400 μ g per tablet).

Keywords: Carbapenemase, combined disc test, *Klebsiella pneumoniae*, KPC, VIM

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Routine detection of carbapenemase-producing *Klebsiella pneumoniae* and other enterobacteria in the clinical laboratory is not included in the latest recommendations of CLSI [1] and EUCAST (www.eucast.org/clinical_breakpoints/). Neverthe-

less, application of reliable detection methods is essential in surveys performed to delineate the epidemiology of these pathogens and to control their spread [2,3]. There are various carbapenemase-detecting methods grouped as phenotypic [based on the synergy between carbapenems and inhibitors of metallo- β -lactamases (M β Ls), e.g. EDTA and dipicolinic acid, as well as boronates active against *Klebsiella pneumoniae* carbapenemases (KPCs)], molecular (mainly PCR-based assays), and biochemical (e.g. determination of imipenem hydrolysis) [3,4].

Combined disc synergy tests have been extensively used because of their convenience and low cost. However, changes in carbapenemase-producing bacterial populations may compromise the performance of these assays. Previous studies have documented the spread of *K. pneumoniae* strains co-producing KPC and verona integron-encoded metallo-beta-lactamase (VIM) β -lactamases in Greek hospitals [5–8]. It has been estimated that the double carbapenemase producers (DCPs) in this setting comprise approximately 5.5% of all carbapenemase-positive *K. pneumoniae* isolates [9]. Also, hospital infections caused by *K. pneumoniae* co-producing KPC and VIM or IMP β -lactamases have been described in Germany, Italy, China and Colombia [10–14]. Double carbapenemase producers may ‘deceive’ the conventional combined disc tests, frequently appearing negative for one or even both carbapenemases [5,6,12].

We report here on two modifications (Methods A and B) of the KPC/M β L kit (ROSCO Diagnostica, Taastrup, Denmark) aiming to increase the reliability of DCP detection. The latter kit includes four tablets: meropenem (10 μ g), meropenem + aminophenylboronic acid (600 μ g), meropenem + dipicolinic acid (1000 μ g) and meropenem + cloxacillin (750 μ g) (www.rosco.dk) [15,16]. In Method A, an additional tablet containing meropenem + aminophenylboronic acid + dipicolinic acid was included. Method B differed from Method A only in that phenylboronic acid (400 μ g) was used instead of aminophenylboronic acid. Performance of Methods A and B was compared with that of the original KPC/M β L kit, as well as a recently described assay using four discs: meropenem, meropenem + phenylboronic acid, meropenem + EDTA and meropenem + phenylboronic acid + EDTA (Method C) [17].

Methods were tested against a challenge set of 125 *K. pneumoniae* isolates from the collection of the Hellenic Pasteur Institute. Isolates had been characterized as described previously [5,6,18]. The set included 26 KPC-positive, 35 VIM-positive and 40 isolates positive for both VIM and KPC enzymes. The remaining isolates were OXA-48 plus extended spectrum β -lactamase ($n = 5$) and extended spectrum β -lactamase and/or AmpC producers ($n = 19$) (Table 1). The range of imipenem and meropenem MICs for VIM and/or KPC

TABLE 1. Performance of combined disc methods for detection of KPC-producing and/or VIM-producing *Klebsiella pneumoniae*

β -Lactamase content (No. of isolates)	KPC/Metallo- β -lactamase kit		Modifications of KPC/Metallo- β -lactamase kit					
			Method A		Method B		Method C	
	Correctly classified	Misclassified	Correctly classified	Misclassified	Correctly classified	Misclassified	Correctly classified	Misclassified
KPC-2(4)	3	1	3	1	4	0	3	1
KPC-2 plus AmpC and/or ESBL(22)	20	2	20	2	21	1	22	0
VIM-1 (20)	20	0	20	0	20	0	20	0
VIM-1 plus AmpC and/or ESBL(15)	15	0	15	0	15	0	15	0
KPC-2 + VIM-1(15)	3	12	5	10	14	1	7	8
KPC-2 + VIM-1 plus AmpC and/or ESBL(25)	5	20	17	8	25	0	19	6
OXA-48 + ESBL(5)	4	1	4	1	4	1	4	1
AmpC and/or ESBL(19)	19	0	19	0	19	0	19	0
Total(125)	89	36	103	22	122	3	109	16

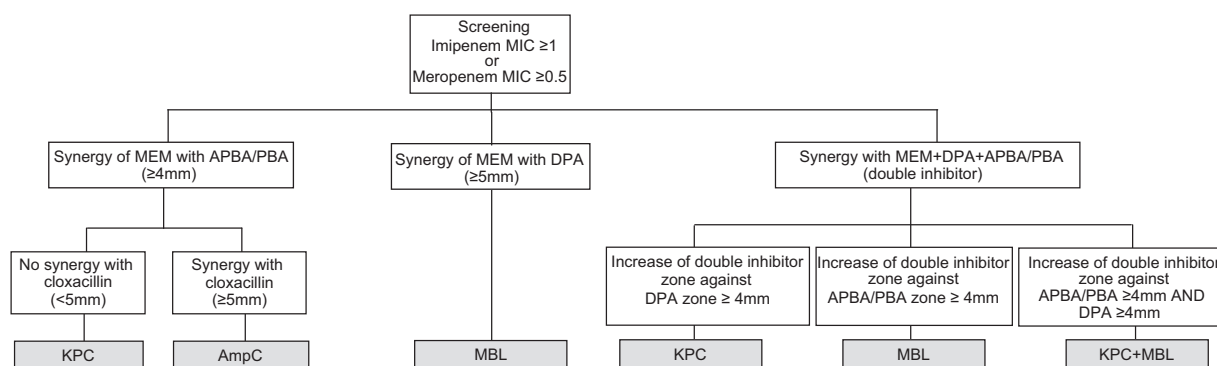
ESBL, extended spectrum β -lactamase

carbapenemase-producing isolates as well as for the OXA-48-positive isolates was from 1 to >32 mg/L. The MIC₅₀ and MIC₉₀ values for both carbapenems were 32 and >32 mg/L, respectively. The respective values for carbapenemase-negative isolates (extended spectrum β -lactamase and/or AmpC producers) were significantly lower (MIC ranges for imipenem and meropenem were 1–2 and 0.5–8 mg/L, respectively). Twenty-one (80.89%) of the KPC producers were classified into sequence type 258 and the remaining were scattered into sequence types 133 ($n = 2$) and 383 ($n = 3$). The VIM-positive isolates as well as DCPs were distributed into sequence types 147 ($n = 56$, 22 DCPs), 323 ($n = 5$, 5 DCPs), 383 ($n = 12$, 12 DCPs) and 945 ($n = 2$, 1 DCP).

The algorithm used to interpret results obtained by Methods A and B is described in Fig. 1. An isolate was classified as KPC-positive when aminophenylboronic acid or phenylboronic acid caused a >4 mm increase in the inhibitory zone diameter of meropenem. A difference of >5 mm between the inhibitory zone diameters around discs containing meropenem + dipicolinic acid and meropenem alone indicated M β L production. To facilitate detection of DCPs, inhibition by the meropenem + dipicolinic acid + aminophenylboronic acid/phenylboronic acid

discs was considered for all isolates appearing positive for either carbapenemase type or negative for both. Hence, a >4-mm difference in inhibition zone by the 'triple' disc compared with meropenem + dipicolinic acid was regarded as indicative of KPC production. Also, a similar inhibition zone difference between the 'triple' and meropenem + aminophenylboronic acid/phenylboronic acid discs indicated M β L production. In cases of negative results with both 'double' discs as well as the 'triple' disc, other carbapenem-resistance mechanisms, most importantly OXA-48 production, must be considered.

Results are summarized in Table 1. The KPC/M β L kit, as well as Methods A, B and C exhibited excellent specificity; false positives among the isolates lacking either VIM or KPC carbapenemases and possessing AmpC and/or extended spectrum β -lactamase producers) were not observed. However, one out of the five OXA-48-positive isolates was falsely classified as an M β L producer. Also, all four methods performed well against the 61 single carbapenemase producers. The KPC/M β L kit and Method A failed to detect carbapenemase in three KPC producers whereas Method B correctly classified all but one single carbapenemase producer. Method C misclassified a KPC-positive isolate as a producer of both carbapenemase

**FIG. 1.** Algorithm used for interpretation of results obtained by the KPC/Metallo- β -Lactamase Confirmation-based Methods A and B that included an additional double inhibitor disc.

types. Significant problems, however, were encountered with the subset of the 40 DCPs. The lowest sensitivity was observed with the KPC/M β L kit, which missed 32 DCPs. Method A displayed low sensitivity—detecting production of both carbapenemase types in only 22 of these isolates. Sensitivity was dramatically improved by replacing aminophenylboronic acid with phenylboronic acid. Indeed, Method B correctly classified 39 DCPs. Most of the DCPs misclassified by the above assays appeared as M β L producers. The performance of Method C was comparable with that of Method A.

It has been shown previously that phenylboronic acid is more effective than aminophenylboronic acid in detecting KPC producers by boronate-based combined disc assays [19]. Hence, the better diagnostic value of Method B over Method A against DCPs can be attributed to the use of phenylboronic acid instead of aminophenylboronic acid. Apparently, phenylboronic acid reduced the masking effect of the simultaneously produced VIM more efficiently than aminophenylboronic acid. We did not systematically pursue the sources of DCP classification errors of Method C. Specificity problems caused by EDTA, as reported previously [15], were not observed, probably because relatively low amounts of the inhibitor were used [17]. Yet, the proposed algorithm in Method C implies a questionable term that may partly explain DCP misclassifications: results of the meropenem + phenylboronic acid + EDTA-containing discs were taken into account only in *K. pneumoniae* isolates that were found to be negative for both carbapenemase types by the respective meropenem + single inhibitor discs [17]. Apparently, in Method C, the fact that a DCP may appear as a single carbapenemase producer was overlooked.

The main limitation of this study is that it includes isolates from a single enterobacterial species, *K. pneumoniae*, carrying only two carbapenemase types, KPC and VIM. Yet, the proposed modification (Method B) of the KPC/M β L kit resulted in a clear improvement in detection of the emerging group of double carbapenemase-producing *K. pneumoniae*.

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Transparency Declaration

J. Bou Casals is an employee of ROSCO Diagnostica. The other authors have no conflicts of interest to declare.

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