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**Combined disk 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 KPC and VIM are frequently isolated in Greece and have also occurred in other European countries. Conventional combined disk tests exhibit low sensitivity against these emerging pathogens. We have evaluated modifications of the KPC/Metallo- $\beta$ -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  $\mu$ g per tablet] for metallo- $\beta$ -lactamases and a boronic acid derivative for KPCs) and the replacement of aminophenylboronic by phenylboronic acid (400  $\mu$ g per tablet).

Routine detection of carbapenemase-producing *Klebsiella pneumoniae* and other enterobacteria in the clinical laboratory is not included in the latest recommendations of the CLSI [1] and EUCAST ([www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)). Nevertheless, 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- $\beta$ -lactamases (M $\beta$ LS) (e.g. EDTA and dipicolinic acid) as well as boronates active against KPCs, molecular (mainly PCR-based assays) and biochemical (e.g. determination of imipenem hydrolysis) [3, 4].

Combined disk (CD) synergy tests have been extensively used due to their convenience and low cost. However, changes in carbapenemase-producing bacterial populations may compromise performance of these assays. Previous studies have documented the spread of *K. pneumoniae* strains co-producing KPC and VIM  $\beta$ -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 due to *K. pneumoniae* co-producing KPC and VIM or IMP  $\beta$ -lactamases have been described in Germany, Italy, China and

Colombia [10-14]. DCPs may “deceive” the conventional CD 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 $\beta$ L kit (ROSCO) aiming to increase reliability of DCP detection. The latter kit includes four tablets: meropenem (MEM 10  $\mu$ g), MEM + amino-phenyl-boronic acid (APBA 600  $\mu$ g), MEM + dipicolinic acid (DPA 1000  $\mu$ g) and MEM + cloxacillin (CLOX 750  $\mu$ g) ([www.rosco.dk](http://www.rosco.dk)) [15, 16]. In method A, an additional tablet containing MEM+APBA+DPA was included. Method B differed from method A only in that phenyl-boronic acid (PBA 400  $\mu$ g) was used instead of APBA. Performance of methods A and B was compared with that of the original KPC/M $\beta$ L kit, as well as a recently described assay utilizing four disks, MEM, MEM+PBA, MEM+EDTA and MEM+PBA+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 ESBL and (n=5) ESBL and/or AmpC producers (n=19) (Table 1). The range of imipenem and meropenem MICs for VIM and/or KPC carbapenemase-producing isolates as well as for the OXA-48-positive isolates was 1->32 mg/L. MIC50 and MIC90 values for both carbapenems were 32 and >32 mg/L, respectively). The respective values for carbapenemase-negative isolates (ESBL 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 (ST) 258 and the remaining were scattered into STs 133 (N=2) and 383 (N=3). The VIM-positive isolates as well as DCPs were distributed into STs 147 (N=56, 22 DCPs), 323 (N=5, 5 DCPs), 383 (N=12, 12 DCPs) and 945 (N=2, 1 DCP).

The algorithm utilized to interpret results obtained by methods A and B is described in Fig. 1. An isolate was classified as KPC-positive when APBA or PBA caused a >4 mm increase in the

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inhibitory zone diameter of meropenem. A difference of >5 mm between the inhibitory zone diameters around disks containing MEM+DPA and MEM alone indicated M $\beta$ L production. To facilitate detection of DCPs, inhibition by the MEM+DPA+ APBA/PBA disks was considered for all isolates appearing positive for either carbapenemase type or negative for both. Thus, a >4 mm difference in inhibition zone by the “triple” disc as compared to MEM+DPA was regarded as indicative of KPC production. Also, a similar inhibition zone difference between the “triple” and MEM+APBA/PBA disks indicated M $\beta$ 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 $\beta$ 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 ESBL producers) were not observed. However, one out of the five OXA-48-positive isolates was falsely classified as an M $\beta$ L producer. Also, all four methods performed well against the 61 single carbapenemase producers. The KPC/M $\beta$ L kit and method A failed to detect carbapenemase in three KPC producers while method B correctly classified all but one single carbapenemase producer. Method C misclassified a KPC-positive isolate as a producer of both carbapenemase types. Significant problems, however, were encountered with the subset of the 40 DCPs. The lowest sensitivity was observed with the KPC/M $\beta$ L kit that 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 APBA with PBA. Indeed, method B correctly classified 39 DCPs. Most of the misclassified DCPs by the above assays appeared as M $\beta$ L producers. Performance of method C was comparable with that of method A.

It has been shown previously that PBA is more effective than APBA in detecting KPC producers by boronate-based CD assays [19]. Thus, the better diagnostic value of method B over method A against DCPs can be attributed to the use of PBA instead of APBA. Apparently, PBA

reduced the masking effect of the simultaneously produced VIM more efficiently than APBA. 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 here probably due to the use of relatively low amounts of the inhibitor [17]. Yet, the proposed algorithm in method C implies a questionable term that may partly explain DCP misclassification cases: results of the MEM+PBA+EDTA-containing discs were taken into account only in *K. pneumoniae* isolates that were found negative for both carbapenemase types by the respective MEM + 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 $\beta$ 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.

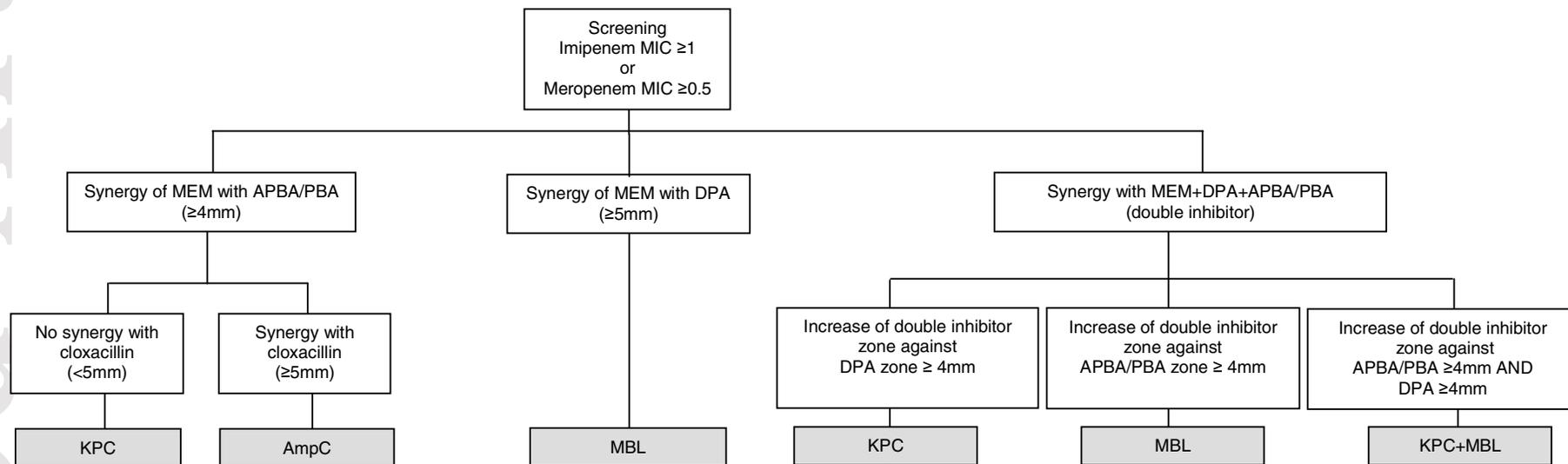
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**TABLE 1. Performance of combined disc methods for detection of KPC- and/or VIM-producing *K. pneumoniae***

<b>β-Lactamase Content</b>		<b>Modifications of KPC/MβL kit</b>					
		<b>KPC/MβL kit</b>		<b>Method A</b>		<b>Method B</b>	
(No. of isolates)		Correctly Classified	Mis-classified	Correctly Classified	Mis-classified	Correctly Classified	Mis-classified
KPC-2	(4)	3	1	3	1	4	0
KPC-2 plus AmpC and/or ESBL	(22)	20	2	20	2	21	1
VIM-1	(20)	20	0	20	0	20	0
VIM-1 plus AmpC and/or ESBL	(15)	15	0	15	0	15	0
<b>KPC-2 + VIM-1</b>	<b>(15)</b>	<b>3</b>	<b>12</b>	<b>5</b>	<b>10</b>	<b>14</b>	<b>1</b>
<b>KPC-2 + VIM-1 plus AmpC and/or ESBL</b>	<b>(25)</b>	<b>5</b>	<b>20</b>	<b>17</b>	<b>8</b>	<b>25</b>	<b>0</b>
OXA-48 + ESBL	(5)	4	1	4	1	4	1
AmpC and/or ESBL	(19)	19	0	19	0	19	0
<b>Total</b>	<b>(125)</b>	<b>89</b>	<b>36</b>	<b>103</b>	<b>22</b>	<b>122</b>	<b>3</b>



**FIG 1.** Algorithm used for interpretation of results obtained by the KPC/Metallo- $\beta$ -Lactamase Confirmation-based methods A and B that included an additional double inhibitor disk.