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Temocillin and piperacillin/tazobactam resistance by disc diffusion as antimicrobial surrogate markers for the detection of carbapenemase-producing Enterobacteriaceae in geographical areas with a high prevalence of OXA-48 producers

Te-Din Huang[1]*, Laurent Poirel[2,3], Pierre Bogaerts[1], Catherine Berhin[1], Patrice Nordmann[2,3] and Youri Glupczynski[1]

[1]National Reference Laboratory for Monitoring of Antimicrobial Resistance in Gram-negative Bacteria, CHU Mont-Godinne, Université Catholique de Louvain (UCL), 1 Avenue Docteur Gaston Therasse, 5530 Yvoir, Belgium; [2]Service de Bactériologie-Virologie, INSERM U914 'Emerging Resistance to Antibiotics', Hôpital de Bicêtre, Faculté de Médecine et Université Paris-Sud, 94275 Kremlin-Bicêtre, France; [3]Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Switzerland

*Corresponding author. Tel: +32-81-423212; Fax: +32-81-423204; E-mail: te-din.huang@uclouvain.be

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Objectives: To assess the performance of the agar disc diffusion method for the detection of carbapenemase-producing Enterobacteriaceae (CPE) referred to the national reference laboratories (NRLs) in Belgium and France.

Methods: All Enterobacteriaceae isolates referred to the NRLs for the confirmation of CPE in 2012 were included. The inhibition zone diameters of meropenem, piperacillin/tazobactam and temocillin using CLSI disc diffusion methodology were recorded. Phenotypic and molecular detection of carbapenemases was performed on all isolates.

Results: A total of 1354 Enterobacteriaceae isolates, including 435 (32.1%) confirmed CPE isolates [OXA-48 ($n=323$), KPC ($n=60$), VIM ($n=32$) and NDM ($n=20$)] and 919 carbapenemase-negative isolates, were tested. Using recommended interpretative criteria, non-susceptibility to meropenem had poor sensitivity (52.0% by CLSI susceptibility breakpoint and 80.0% by EUCAST screening breakpoints), while non-susceptibility to piperacillin/tazobactam (according to CLSI breakpoint) or to temocillin (according to Fuchs, Barry, Thornsberry *et al. Eur J Clin Microbiol* 1985; **4**: 30–3) was highly sensitive (99.8% and 98.2%, respectively) but poorly specific (29.4% and 42.9%, respectively) for the detection of CPE. Temocillin diameters <12 mm alone had high specificity (90.0%) and the combination of temocillin diameters ≥12 mm with piperacillin/tazobactam diameters ≥16 mm observed in 40% of all referred isolates displayed excellent negative predictive value (99.2%).

Conclusions: In geographical areas with a high prevalence of OXA-48 producers, recommended meropenem susceptibility or screening breakpoints failed to detect CPE in a large proportion of isolates. The combination of modified zone diameter cut-offs for piperacillin/tazobactam (≥16 mm) and temocillin (≥12 mm) can be used to rule out the presence of carbapenemase and avoid unnecessary additional testing for confirmation of CPE.

Keywords: meropenem, breakpoints, OXA-48

Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) isolates have been increasingly reported worldwide.[1] *Klebsiella pneumoniae* is by far the most frequently encountered CPE species and OXA-48 the most frequent carbapenemase in Belgium and in France.[2,3] Rapid detection of CPE is essential for the control of their dissemination. Ertapenem non-susceptibility has been proposed as the most sensitive marker for the detection of CPE strains, but has shown poor positive predictive value, particularly in geographic areas with a low-prevalence setting.[4] Furthermore, phenotypic methods for the confirmation of carbapenemase production based on the use of inhibitor-supplemented antimicrobial discs allow differentiation between Ambler class A and B carbapenemase producers, but fail to confirm Ambler class D OXA-48-producing CPE. In this study, we evaluated in two different national reference laboratories (NRLs) the ability to detect CPE in carbapenem-non-susceptible Enterobacteriaceae (CNSE) by using the disc diffusion method, with a particular focus on OXA-48-producing Enterobacteriaceae isolates.

Methods

All Enterobacteriaceae isolates referred by local laboratories to the NRLs for non-susceptibility to at least one carbapenem (ertapenem, imipenem, meropenem) according to local routine practice were included for the period from January to December 2012 in Belgium and from March to September 2012 in France. In France, the majority of the laboratories adhered to the recommendations issued by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM),[5] while in Belgium the CLSI[6] and EUCAST[7] guidelines were variably used. All isolates were tested for imipenem hydrolysis by the Carba NP assay as previously reported[8] and those with a positive test result underwent multiplex PCR targeting bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC} and bla_{OXA-48} for the detection of carbapenemase-encoding genes.[9] Inhibition zone diameters of meropenem 10 μg, piperacillin/tazobactam 100/10 μg and temocillin 30 μg paper discs (Bio-Rad) tested according to CLSI disc diffusion methodology[10] were measured for all isolates. The recommended susceptibility zone diameter breakpoints chosen in this study were meropenem ≥23 mm, piperacillin/tazobactam ≥21 mm (according to CLSI guidelines)[10] and temocillin ≥19 mm (based on disc diffusion interpretative criteria proposed by Fuchs *et al.*[11]). To ensure the relationship between disc diffusion inhibition zone diameters and microdilution MICs, 76 randomly selected isolates underwent in parallel MIC determination for meropenem and piperacillin/tazobactam using broth microdilution panels (Sensititre®, Thermo Fisher Scientific, Cleveland, USA). The MIC value of temocillin was determined by the Etest method (bioMérieux, Marcy-l'Étoile, France).

Results and discussion

In 2012, 635 Enterobacteriaceae isolates referred to the Belgian NRL and 719 referred to the French NRL were analysed. Species and carbapenemase type distribution of the isolates are detailed for each NRL in Table 1. Overall, from a total of 1354 Enterobacteriaceae isolates screened, 435 (32.1%) were confirmed as carbapenemase producers [OXA-48 (n=323), KPC (n=60), VIM (n=32) and NDM (n=20)]. While the proportions of each species referred to the NRLs were slightly different (a higher number of *Enterobacter cloacae* isolates was analysed in France), the proportions of each carbapenemase type and species among confirmed CPE isolates were similar. In *Enterobacter* spp. decreased susceptibility to ertapenem is common due to extended-spectrum β-lactamase or cephalosporinase production in conjunction with permeability defects.[12] Therefore, the higher number of *E. cloacae* isolates referred in France may be attributed to the systematic testing of ertapenem by laboratories and the higher screening breakpoint recommended by the CA-SFM (<28 mm) in comparison with the CLSI and EUCAST guidelines used in Belgium.

The distributions of meropenem, piperacillin/tazobactam and temocillin inhibition zone diameters as well as the carbapenemase-encoding genes are shown in Figures 1–3, respectively. Scattergrams for meropenem, piperacillin/tazobactam and

Table 1. Species and carbapenemase enzyme distribution of Enterobacteriaceae isolates referred to the NRLs in 2012 for confirmation of CPE (n=1354)

			Carbapenemase				
Country of NRL	Species	Total	OXA-48	KPC	VIM	NDM	negative
Belgium	*Klebsiella pneumoniae*	273	138	35	3	1	96
	Enterobacter cloacae	97	13		8	2	74
	Escherichia coli	69	21	1	2	2	43
	Enterobacter aerogenes	82	1				81
	Klebsiella oxytoca	37	8	2	3		24
	Citrobacter freundii	20	7		3		10
	miscellaneous[a]	57	2		2	1	52
	total	635	190	38	21	6	380
France	*K. pneumoniae*	266	94	21	4	6	141
	E. cloacae	237	11		1	2	223
	E. coli	90	23			4	63
	E. aerogenes	46	1				45
	K. oxytoca	5					5
	C. freundii	29	1	1	5		22
	miscellaneous[b]	46	3		1	2	40
	total	719	133	22	11	14	539
Total		1354	323	60	32	20	919

[a]*Proteus mirabilis* (n=6), *Serratia marcescens* (n=13), *Hafnia alvei* (n=3), *Morganella morganii* (n=2), *Enterobacter* spp. (n=28), *Citrobacter* spp. (n=1), *Raoultella* spp. (n=1), *Providencia* spp. (n=2) and *Salmonella* spp. (n=1).
[b]*P. mirabilis* (n=12), *S. marcescens* (n=4), *H. alvei* (n=7), *M. morganii* (n=6), *Enterobacter* spp. (n=8), *Citrobacter* spp. (n=5), *Raoultella* spp. (n=3) and *Salmonella* spp. (n=1).

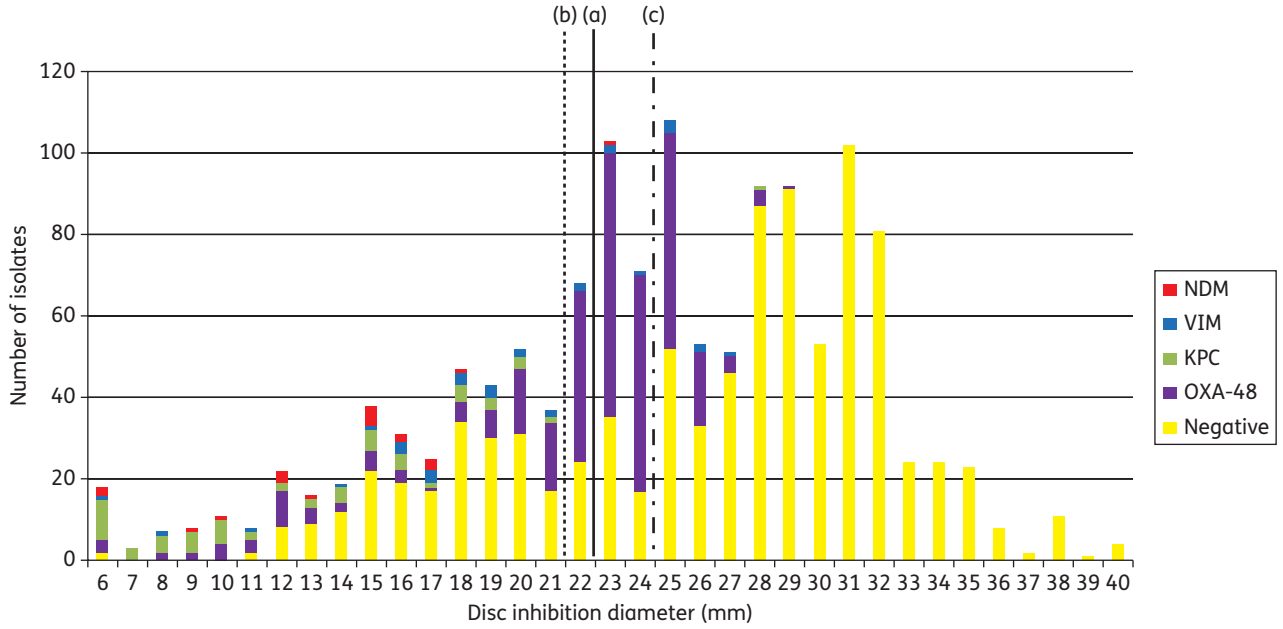

Figure 1. Distribution of meropenem 10 μg disc inhibition zone and of carbapenemases for Enterobacteriaceae isolates referred to the NRLs in 2012 (n=1354). (a) 2013 CLSI meropenem disc diffusion susceptibility zone diameter breakpoint (≥23 mm). (b) 2013 EUCAST meropenem susceptibility zone diameter breakpoint (≥22 mm). (c) 2013 EUCAST meropenem disc diffusion screening cut-off for the detection of CPE (<25 mm).

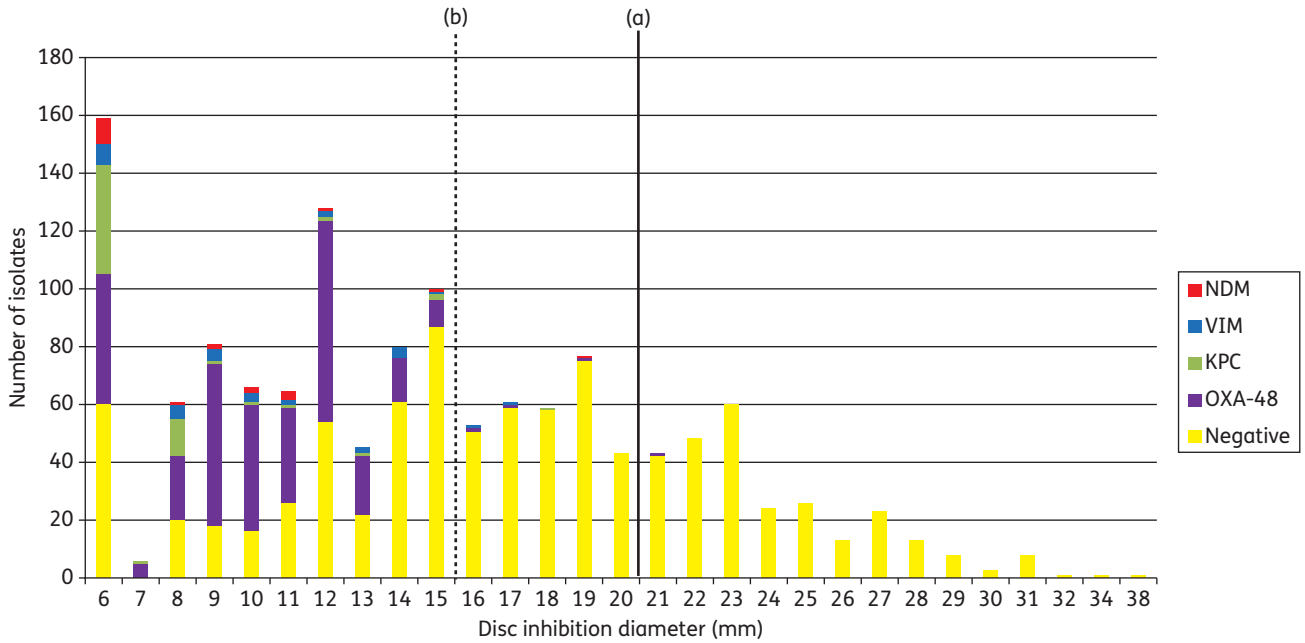

Figure 2. Distribution of piperacillin/tazobactam 100/10 μg disc inhibition zone and of carbapenemase enzymes for Enterobacteriaceae isolates referred to the NRLs in 2012 (n=1354). (a) 2013 CLSI piperacillin/tazobactam disc diffusion susceptibility zone diameter breakpoint (≥21 mm). (b) Piperacillin/tazobactam modified cut-off proposed in this study (<16 mm).

temocillin are presented in Tables S1, S2 and S3 (available as Supplementary data at JAC Online). A significant correlation between zone diameters and MICs was found for the three antimicrobials evaluated (Pearson's correlation coefficient r was −0.859, −0.746 and −0.845 for meropenem, piperacillin/tazobactam and temocillin, respectively; P<0.001).

Sensitivity, specificity and positive and negative predictive values for the detection of carbapenemase production according

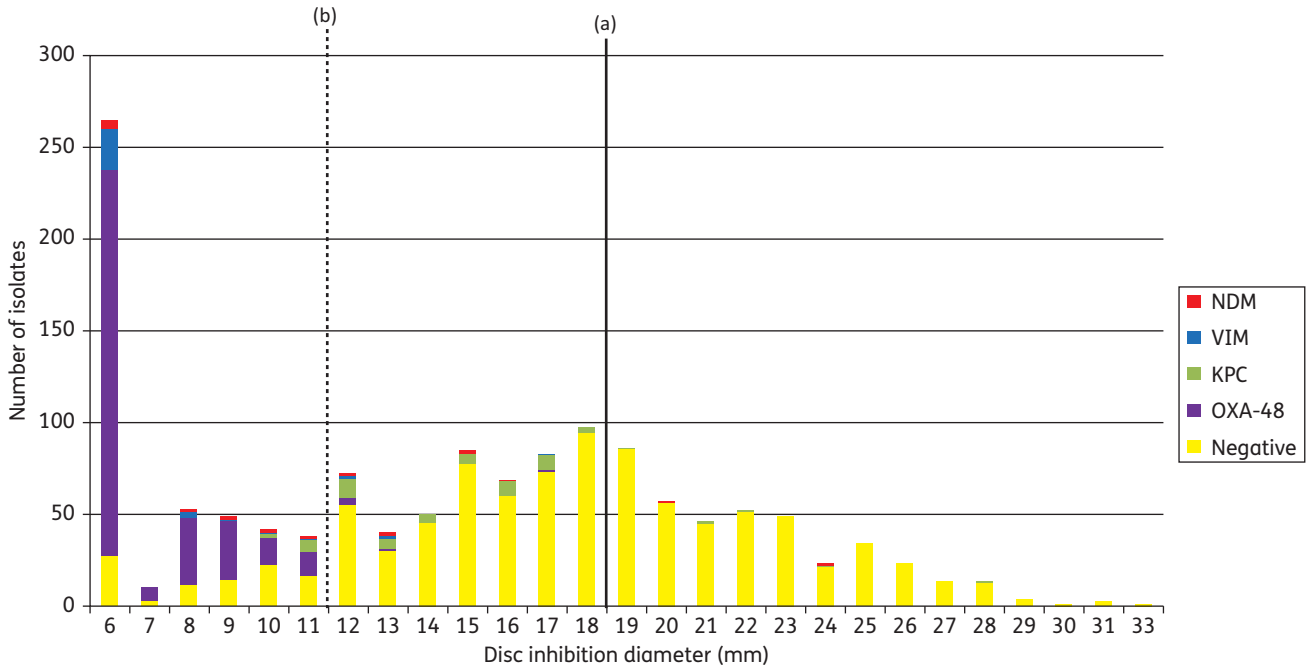

Figure 3. Distribution of temocillin 30 μg disc inhibition zone and of carbapenemase enzymes for Enterobacteriaceae isolates referred to the NRLs in 2012 ($n=1354$). (a) Temocillin disc diffusion susceptibility zone diameter breakpoint according to Fuchs *et al.*[11] (≥19 mm). (b) Temocillin modified cut-off proposed in this study (<12 mm).

Table 2. Carbapenemase distribution according to different inhibition diameters cut-offs and their performance for the detection of CPE among isolates referred to the NRLs in France and in Belgium in 2012 ($n=1354$)

			Number of isolates per carbapenemase enzyme					Performance of the detection of CPE			
	Zone diameter cut-offs (mm)	Total, 1354	OXA-48, 323	KPC, 60	VIM, 32	NDM, 20	negative, 919	sensitivity (%)	specificity (%)	PPV (%)	NPV (%)
Susceptibility breakpoints[a]	MEM <23	452	125	59	23	19	226	**52.0**	75.4	49.9	76.8
	TZP <21	1083	322	60	32	20	649	99.8	29.4	40.0	99.6
	TMO <19	952	323	54	32	18	525	98.2	42.9	44.8	98.0
Modified cut-offs	MEM <29	929	322	60	32	20	495	**99.8**	46.1	46.6	99.8
	TZP <16	790	319	59	30	19	363	**98.2**	60.5	54.0	98.6
	TMO <12	457	317	8	28	12	92	83.9	**90.0**	79.9	92.2
Combination of cut-offs	TZP <16 and TMO <12	426	315	8	26	12	65	83.0	92.9[b]	**84.7**	
	TZP ≥16 and TMO ≥12	533	2	1		1	**529**				**99.2**

MEM, meropenem 10 μg disc; TZP, piperacillin/tazobactam 100/10 μg disc; TMO, temocillin 30 μg disc; PPV, positive predictive value; NPV, negative predictive value. The results shown in bold are discussed further in the text.
[a]According to 2013 CLSI guidelines (for meropenem and piperacillin/tazobactam) and to Fuchs *et al.*[11] (for temocillin).
[b]The specificity of combined cut-offs was calculated for carbapenemase-negative isolates when at least one of the two criteria was not fulfilled.

to the antimicrobial agents tested and zone diameter cut-offs selected are detailed in Table 2. Using the recommended 23 mm diameter breakpoint for meropenem, 209 (48.0%) of the CPE tested were characterized as susceptible [OXA-48 ($n=198$), VIM ($n=9$), NDM ($n=1$) and KPC ($n=1$)]. With the exception of eight carbapenemase producers [KPC ($n=6$) and NDM ($n=2$)], all CPE isolates were resistant to temocillin, including all OXA-48 isolates, which notably exhibited decreased susceptibility to piperacillin/tazobactam in all but one isolate. Recently, a subcommittee of EUCAST proposed tentative guidelines for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance.[13] A screening breakpoint for meropenem <25 mm was suggested. Applying this screening cut-off would have resulted in an improved but still insufficient sensitivity

of 80% for the detection of CPE, mainly missing OXA-48 (80/323) and VIM-positive (6/32) isolates. Day *et al.*[14] reported excellent sensitivities (99%) of different carbapenem (ertapenem, imipenem, meropenem and faropenem) discs and the superior specificity (94%) of faropenem for the screening of CPE isolates. However, this study was conducted in a setting with a different distribution of carbapenemase types, mostly including a large number of NDM producers and a small number of OXA-48-positive strains.

When modified cut-offs were applied (meropenem <29 mm, piperacillin/tazobactam <16 mm or temocillin <12 mm), sensitivity for meropenem was increased to 99.8% while sensitivity for piperacillin/tazobactam remained high at 98.2%. However, specificities using these cut-offs were low for both meropenem (46.0%) and piperacillin/tazobactam (60.5%). With regard to temocillin, 98.1% (317/323) of the OXA-48-producing Enterobacteriaceae isolates displayed disc inhibition zones of <12 mm compared with only 10.0% (92/919) of the carbapenemase-negative isolates. A more variable distribution of temocillin inhibition zone diameters was found in KPC- and NDM-producing isolates (range 10–28 and 6–24 mm, respectively), although the large majority of KPC-producing isolates (52/60) had inhibition zone diameters of ≥12 mm.

These results support previous data and confirm that high-level resistance to temocillin is a highly sensitive and specific phenotypic surrogate marker of OXA-48 production in strains with decreased susceptibility to a carbapenem, although in itself it could not differentiate OXA-48 from VIM-type carbapenemases.[15–17] When comparing the results obtained for *K. pneumoniae* with those for other Enterobacteriaceae species, no significant difference in sensitivity of CPE detection was found for either meropenem or piperacillin/tazobactam discs regardless of whether the recommended or the modified cut-offs were applied. Using the modified cut-off of temocillin <12 mm, the specificity and the positive predictive value for the detection of carbapenemase production were higher in *K. pneumoniae* (92.0% and 92.9%, respectively) than in other species (89.3% and 61.8%) in which non-carbapenemase mediated resistance to temocillin is more frequently encountered. When applied in geographical areas where OXA-48 represents the predominant carbapenemase, the combination of a piperacillin/tazobactam inhibition zone <16 mm with a temocillin zone <12 mm appeared to be highly predictive of carbapenemase production, especially in *K. pneumoniae* (positive predictive value of 94.6%), while the association of a piperacillin/tazobactam inhibition zone ≥16 mm with a temocillin zone ≥12 mm ruled out the presence of a carbapenemase in ∼40% (529/1354) of all isolates tested (negative predictive value of 99.2%). Since according to the design of our protocol only strains with a positive Carba NP test were selected for molecular identification of carbapenemases, we retrospectively analysed a subset of isolates displaying high-level resistance to temocillin and to piperacillin/tazobactam, but with a negative Carba NP test result. Overall, none of the 26 available isolates tested (out of 65 isolates) yielded a positive multiplex PCR result, hence excluding the presence of carbapenemases (most notably OXA-48) that could have been missed by the Carba NP test-based algorithm.

In conclusion, our study showed that meropenem CLSI susceptibility breakpoints by the disc diffusion method or cut-off diameters recently proposed by EUCAST[10,13,18] fail to detect a substantial proportion of CPE isolates, predominantly OXA-48-producers. This observation is of concern since a large number of microbiology laboratories (50%–60% in Belgium) still rely on the disc diffusion method as the principal method for routine susceptibility testing. Piperacillin/tazobactam and temocillin resistances are highly sensitive surrogate markers for CPE and our study suggests that concomitant susceptibility of CNSE isolates to these two antimicrobials could reliably rule out the presence of a carbapenemase, thus preventing unnecessary additional testing. Finally, in areas where OXA-48 producers are predominant, temocillin testing using a modified zone diameter cut-off of <12 mm and/or an MIC >128 mg/L[16] could be an easy and useful tool for discriminating Ambler class D carbapenemase producers from extended-spectrum β-lactamases and/or AmpC-producing isolates among carbapenem-non-susceptible Enterobacteriaceae isolates that yield a negative result by the inhibitor-based combination disc test.[19] Our findings should, however, be evaluated in areas with different epidemiological backgrounds of carbapenemase enzymes.

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Transparency declarations

None to declare.

Supplementary data

Tables S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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