EVALUATION OF A NEW PHENOTYPIC METHOD TO REVEAL AmpC PRESENCE AND PORIN LOSS IN ENTEROBACTERIACEAE WITH DECREASED SUSCEPTIBILITY TO CARBAPENEMS

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Background

Different Enterobacteriaceae can harbor AmpC β-lactamase genes, that can be either chromosomal (as in *Enterobacter* species, *Citrobacter freundii* etc.), or plasmid-encoded. In many Enterobacteriaceae, AmpC expression is low but inducible in response to β-lactam exposure, with a complex induction mechanism.

The AmpC resistance affects the activity of all the β-lactams, but it is more active on cephalosporins and can hydrolyze cephamycins, oxyiminocephalosporins and monobactams. However, the hydrolysis rates for cefepime, cefpirome, and carbapenems are very low. Carbapenems, in particular, remain almost always susceptible. β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam are ineffective on AmpC β-lactamases, that are instead inhibited by both cloxacillin and boronic acid.

Regarding the resistance to carbapenems, in AmpC producers strains the decrease of the number of porin entry channels or the increase of efflux pump expression can augment enzyme efficiency. Thus, carbapenem resistance in these strains involves various combinations of overproduction of AmpC β-lactamase, porin loss (PL) and activation of efflux systems.

The AmpC detection is not clinically relevant, but it is useful for epidemiological purposes. To our knowledge, there are presently no approved criteria to do it. The molecular approach is cumbersome and can be performed only in reference centers.

Different phenotypical approaches have been proposed. The most commonly used is based on gradient strips with cefotetan or cefoxitin on one half and the same combined with a constant concentration of cloxacillin. Either a reduction in cephamycin MIC of at least three dilutions, deformation of the ellipse of inhibition, or a "phantom zone" mean positive test.

More difficult is to evaluate the contemporary presence of AmpC + PL. To do that, it could be used a disk diffusion synergy test, which compares the activity of meropenem alone compared with those of the combination of both meropenem/boronic acid and meropenem/cloxacillin. However, AmpC-PL+ isolates can show a decreased susceptibility only to ertapenem, whilst the other carbapenems remain susceptible. If so, the above synergy tests result undetermined (figure 1). The performance of a new phenotypic test, product by Liofilchem®, Italy, and consisting in agar diffusion gradient strips of ertapenem (ETP)/ertapenem + boronic acid (ETP/EBO) and ETP/ertapenem + cloxacillin (ETP/ECX) was evaluated.

Figure 1 - Disk diffusion synergy tests (Rosco, Denmark) on an *Enterobacter cloacae* strain: meropenem alone and meropenem with aminophenylboronic acid, dipicolinic acid and cloxacillin. No synergy effect is noted.

Materials and methods

- In the present study 34 different isolates and 6 controls were tested by using the MIC test strips ETP/ECX and ETP/EBO, produced in different formulation by Liofilchem®, Italy. The test was performed according to the manufacturer indications.
- 23 out of the 40 strains were AmpC producers (18 *Enterobacter cloacae*, 4 *Enterobacter aerogenes*, 1 *Citrobacter freundii*), as demonstrated by using MIC test strips (Liofilchem®, Italy) with a gradient of cefotetan/cefotetan+cloxacillin. 11 out of the 40 strains were ESBL+ (6 *Klebsiella pneumoniae* – KP and 5 *Escherichia coli*); the ESBL resistance was confirmed using the procedure suggested by CLSI. All these 34 strains were clinical strains isolated in two different Italian hospitals (Reggio Emilia and Bergamo) and showed a decreased susceptibility to ertapenem (range: 1 to >32 μg/ml), with MICs for imipenem and meropenem less than 2 μg/ml. All the strains were tested for the presence of resistance genes for carbapenemases by using a *in-house* multiplex-PCR able to detect the bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{IMP}, $bla_{OXA-48-like}$ genes, based on a modification of the protocol of Poirel et al.. All the strains were also tested using the disk diffusion synergy tests (Rosco, Denmark).
- As internal controls, 6 different strains of carbapenemase producing Enterobacteriaceae were included in the study. These isolates were 2 bla_{KPC} (1 KP, 1 *E. coli*), 1 bla_{NDM} KP, 1 bla_{VIM} KP, 2 $bla_{OXA-48-like}$ (1 KP, 1 *E. cloacae*). The summary of the tested strains is shown in table 1.

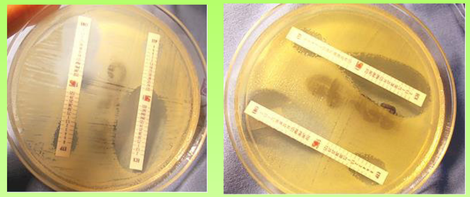
Results

- None of the 34 test strains showed synergistic effect using the meropenem/boronic acid and meropenem/cloxacillin compared with meropenem alone. All these microorganisms did not harbour resistance genes for carbapenemases, except for a *Citrobacter freundii* which resulted positive for $bla_{OXA-48-like}$ gene.
- 22 out of the 23 AmpC producers showed a clear synergistic effect for both ETP/EBO and ETP/ECX combinations (i.e., MICs ratio of ETP vs ETP/EBO and ETP/ECX was of at least three dilutions, figures 2 and 3). One *E. cloacae*, with a ertapenem MIC = 1 mcg/ml, gave an indeterminate result. Among the ESBL producers, none showed synergy for both ETP/EBO and ETP/ECX.
- As regards the control strains, the bla_{NDM}, bla_{VIM}, and the two $bla_{OXA-48-like}$ strains did not show any synergic effect for the combination tested. Among the 2 bla_{KPC} strains, the KP showed a synergy with boronic acid, but not with cloxacillin, as expected. Instead, the *E. coli* strains gave a non determinable result for boronic acid and synergy with cloxacillin).

Strains (nr)	AmpC	ESBL	OXA-48	NDM	VIM	KPC
Enterobacter cloacae (19)	18	0	1	0	0	0
Klebsiella pneumoniae (10)	0	6	1	1	1	1
Escherichia coli (6)	0	5	0	0	0	1
Enterobacter aerogenes (4)	4	0	0	0	0	0
Citrobacter freundii (1)	1	0	1	0	0	0

Table 1 – Summary of the strains tested in the present study

- The above results were obtained after having tested different formulations of the boronic acid, to find the correct concentration that allowed to avoid false synergies, e.g. with ESBL positive strains.



Figures 2-3 – The synergistic effect for both ETP/EBO and ETP/ECX combinations in two *Enterobacter cloacae* strains

Discussion

- Although the detection of AmpC enzymes is not mandatory for clinical purposes, infection control considerations can support the need of this test.
- This phenotypical test can help in revealing AmpC strains with reduced susceptibility to carbapenems. The use of ertapenem instead of meropenem allows to better investigate these strains, which remain often susceptible to meropenem.
- The synergistic effect for both ETP/EBO and ETP/ECX combinations is represented by a MICs ratio of ETP vs ETP/EBO and ETP/ECX of at least three dilutions, by the deformation of the ellipse of inhibition, or through the presence of a "phantom zone".
- The positivity of the test confirms that the analysed microorganism is an AmpC producer and that its decreased susceptibility to ertapenem (and in case to other carbapenems) could be due to overexpression of the AmpC, and/or decrease in the number of porin entry channels.
- The most recent Liofilchem® formulation seems very promising as demonstrated by its very good sensitivity and specificity.
- Further studies with a larger number of strains and with the support of molecular investigations are needed for the final validation of this method.

Acknowledgments

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