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Evaluation of Rosco Neo-Sensitabs for phenotypic detection and subgrouping of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae

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The increasing prevalence of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae necessitates reliable phenotypic tests for detection and categorization. The main objective of this study was to evaluate ROSCO Neo-Sensitabs with different β-lactam-β-lactam inhibitor combinations for phenotypic detection and categorization of β-lactamases in Enterobacteriaceae. Using standard CLSI/EUCAST methodology, differences in zones of inhibitions between a β-lactam alone compared with the combination with a β-lactamase inhibitor as well as subjective synergy observations were determined for 172 well characterized Enterobacteriaceae strains with defined resistance mechanisms. The results showed that for all ESBL-positive strains (n = 66), combinations of clavulanic acid synergy with cefotaxime, ceftazidime or cefepime, were observed. All acquired AmpC β-lactamases (n = 17) were detected using cloxacillin combined with cefotaxime and/or ceftazidime (both combinations were required). Carbapenemase producers (n = 59) with the exception of one KPC-producer were correctly grouped using the combination of meropenem ± aminophenylboronic acid (APBA) or dipicolinic acid (DPA). Ethylene diamine tetraacetic acid (EDTA) also inhibited all metallo-β-lactamases, but as with DPA, one false positive result was observed. Based upon these data, we propose a tablet layout for 14 cm agar plates, which could be used as a whole or in a targeted approach for detection and categorizing of relevant acquired β-lactamases in Enterobacteriaceae.

Key words: Antibiotic resistance; enterobacteriaceae; susceptibility tests; cephalosporins..

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Isolates belonging to Enterobacteriaceae are important causes of hospital- and community-acquired infections such as urinary tract infections, lower respiratory tract infections and blood stream infections. In hospitals, these infections are often treated with extended-spectrum cephalosporins or carbapenems. The emergence and global dissemination of extended-spectrum β-lactamase (ESBL), acquired AmpC

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and carbapenemase-producing Enterobacteriaceae is therefore of great concern (1). Efficient and reliable phenotypic tests are thus important for implementation of relevant infection control measures and for surveillance purposes. Various confirmatory phenotypic methods for detection of β-lactamases have been described using β-lactam-β-lactam inhibitor combinations (2, 3). For detection of ESBLs the combination of clavulanic acid (CLA) and cephalosporins [cefotaxime (CTX) and/or ceftazidime (CAZ)] is widely used and also recommended by the CLSI (4). Combinations of boronic acid (APBA) or cloxacillin (CLOX) with cephems [cefotetan or cefoxitin (FOX)] have been described as useful for the detection of AmpC β-lactamases (3). For carbapenemases combination tests using carbapenems in combination with boronic acids or metal chelators like ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA) have been described for the detection of class A serine carbapenemases (KPC) and metallo-β-lactamases (MBLs) respectively (3).

In the present study, different combinations of diagnostic Neo-Sensitabs (Rosco Diagnostica A/S, Taastrup, Denmark) were evaluated for phenotypic detection of ESBL, acquired or chromosomally hyperproduced AmpC, and carbapenemases in Enterobacteriaceae. The aim of the study was to design a tablet layout for 14 cm agar plates, which could be used for detection and categorization of relevant β-lactamase(s) in Enterobacteriaceae with reduced susceptibility to extended-spectrum cephalosporins. The tablet layout could be used as a whole or in a targeted approach based on resistance profile and local epidemiology. This may be cost efficient compared to genotyping that is also limited by available technology and their inherent inability to detect new resistance mechanisms.

MATERIALS AND METHODS

Bacterial strains

A total of 172 characterized strains from several countries (5–16) producing various β-lactamases were included in this study. The panel consisted of ESBL-producing *Escherichia coli* (n = 23), *Klebsiella pneumoniae* (n = 26, including *K. pneumoniae* ATCC 700603), *Enterobacter cloacae* (n = 2) and *Citrobacter freundii* (n = 1, also hyperproducing an AmpC enzyme), AmpC-producing *E. coli* (acquired n = 11 and chromosomally hyperproducing n = 5), *K. pneumoniae* (n = 2), *Salmonella* spp. (n = 2), *Hafnia alvei* (n = 1) and *Proteus mirabilis* (n = 1), AmpC hyperproducing *E. cloacae* (n = 1), OXY-hyperproducing *Klebsiella oxytoca* (n = 22), KPC-producing *K. pneumoniae* (n = 26), OXA-48-producing *E. coli* (n = 2) and *K. pneumoniae* (n = 1), VIM-producing *K. pneumoniae* (n = 18) and *E. coli* (n = 1), IMP-producing *K. pneumoniae* (n = 1), NDM-1-producing *E. coli* (n = 1) and *K. pneumoniae* (n = 6, including *K. pneumoniae* NCTC 13443) and SME-producing *Serratia marcescens* (n = 3). Also *K. pneumoniae* (n = 13) and *E. coli* (n = 1) producing ESBL in combination with reduced permeability and *E. cloacae* (n = 1) and *Enterobacter aerogenes* (n = 1) hyperproducing AmpC in combination with reduced permeability, were tested.

The types of ESBLs produced in isolates used for this study cover a broad variety of ESBL subtypes including various combinations thereof (e.g. CTX-M-15 + SHV-28). CTX-M-1, -2 and -9 group ESBLs, as well as SHV, OXA and TEM ESBLs are well represented, with less frequently encountered types like VEB and GES represented in a lower number. Plasmid-mediated AmpC ß-lactamases are dominated by the CMY group, but also DHA-, ACC-, ACT- and FOX- ß-lactamases were included. Table 1 illustrates the profiles of acquired β-lactamases produced by the isolates included in this study.

Phenotypic detection

Phenotypic detection of the various β-lactamases present in the strains, was approached using two methods: (i) double tablet synergy testing testing, using subjective observations of synergy, or (ii) a combination tablet method, focusing on the differences in the zones of inhibition. A difference of ≥ 5 mm in zone of inhibition between a given β-lactam alone as compared with the same agent combined with a β-lactamase inhibitor was used as a break point, except for imipenem (IMP) ± EDTA where a difference of ≥7 mm was used due to the effect on some strains of this inhibitor in itself.

Neo-Sensitabs (ROSCO), double tablet synergy tests and combination tablet tests included in the study were initially selected from recommendations in the User's Guide Neo-Sensitabs. 19th Edition and later supplemented with recommendations from the online/continuously updated version of the User's Guide (http://rosco.dk/default.asp?mainmenu = 20&submenu = 21&webmanage = Users Guide). Table 2 gives an overview of the selected tablets. The tablets had the following potencies: ceftazidime 30 µg, ceftazidime/clavulanic acid 30 µg/10 µg, ceftazidime/cloxacillin 30 µg/750 µg, ceftazidime/APBA 30 µg/

Table 1. Profile of acquired β-lactamases tested in this study and species distribution

Acquired β-lactamases	Species	Subgroups tested
ESBL		
CTX-M	*E. coli* (n = 15), *K. pneumoniae* (n = 6) *E. cloacae* (n = 2)	(CTX-M:) 1, 2, 3, 9, 10, 14, 15, 27, 51, 57
CTX-M, SHV	*E. coli* (n = 2), *K. pneumoniae* (n = 10)	
CTX-M, SHV, TEM	*K. pneumoniae* (n = 1)	
CTX-M, SHV, OXA	*K. pneumoniae* (n = 1)	
CTX-M, OXA	*E. coli* (n = 1), *K. pneumoniae* (n = 1)	
CTX-M, TEM	*E. coli* (n = 1)	
SHV	*E. coli* (n = 1), *K. pneumoniae* (n = 5), *C. freundii* (n = 1)	(SHV:) 2, 5, 12, 18, 28, 32, 33, 36, 69, 71, 83, 85, 108
OXA-10	*E. coli* (n = 1)	
TEM	*E. coli* (n = 1), *K. pneumoniae* (n = 1)	(TEM:) 52, 104, 158-like
VEB-1	*E. coli* (n = 1)	
GES-1	*K. pneumoniae* (n = 1)	
AmpC		
CMY	*E. coli* (n = 1)	2, 7, 22
DHA-1	*E. coli* (n = 2), *K. pneumoniae* (n = 2), *P. mirabilis* (n = 1)	
ACC-1	*H. alvei* (n = 1), *S. bareilly* (n = 1)	
ACT-1	*E. coli* (n = 1)	
FOX-1	*Salmonella* spp. (n = 1)	
KPC/SME		
KPC, SHV	*K. pneumoniae* (n = 25)	
KPC, SHV, CTX-M	*K. pneumoniae* (n = 1)	
SME	*S. marcescens* (n = 3)	
MBL		
VIM, SHV	*K. pneumoniae* (n = 16)	
VIM, SHV, CTX-M	*E. coli* (n = 1), *K. pneumoniae* (n = 2)	
IMP	*K. pneumoniae* (n = 1)	
NDM-1, CTX-M	*K. pneumoniae* (n = 4)	
NDM-1, CMY	*E. coli* (n = 1), *K. pneumoniae* (n = 1)	
NDM-1, CTX-M, CMY	*K. pneumoniae* (n = 1)	
OXA-48		
OXA-48	*E. coli* (n = 1)	
OXA-48, CTX-M	*E. coli* (n = 1)	
OXA-48, CTX-M, SHV	*K. pneumoniae* (n = 1)	

600 μg, cefotaxime 30 μg, cefotaxime/clavulanic acid 30 μg/10 μg, cefotaxime/cloxacillin 30 μg/750 μg, cefotaxime/APBA 30 μg/600 μg, cefepime 30 μg, cefepime/clavulanic acid 30 μg/10 μg, cefoxitin 30 μg, meropenem 10 μg, meropenem/cloxacillin 10 μg/750 μg, meropenem/APBA 10 μg/600 μg, meropenem/DPA 10 μg/1000 μg, imipenem 10 μg, imipenem/EDTA 10 μg/750 μg, cloxacillin 500 μg, APBA, 250 μg and DPA 250 μg.

Test conditions were standard EUCAST (The European Committee on Antimicrobial Susceptibility Testing)/CLSI (Clinical and Laboratory Standards Institute) conditions for disc diffusion testing, as described for non-fastidious microorganisms (4, 17). Briefly, Mueller Hinton agar plates (Becton Dickinson, Sparks, MD, USA) were inoculated with a 0.5 McFarland adjusted suspension in 0.9% NaCl, streaked with cotton swabs using a plate rotator (bioMérieux S.A., Marcy l'Etoile, France), and incubated over night at 35 °C for 16–18 h, in aerobic atmosphere. *E. coli* ATCC 25922 was used as quality control strain.

RESULTS AND DISCUSSION

The results are listed in Table 2 and discussed below.

Clavulanic acid based tests for detection of ESBL

Combination tablet testing with cefotaxime-clavulanic acid (CTX-CTX/CLA) in combination with ceftazidime-clavulanic acid

Table 2. Tested combinations of β-lactam-β-lactam inhibitors and number of positive isolates in the respective tests

Test	Number of positive isolates						
	ESBL (n = 66[1])	acqAmpC (n = 17)	cAmpC (n = 9[1])	KPC/SME (n = 29)	MBL (n = 27)	OXA-48 (n = 3)	*K. oxytoca* (n = 22)
Detection of ESBL							
CTX-CTX/CLA (CT)	**62**	2	3	18	9	0	2
CAZ-CAZ/CLA (CT)	**36**	9	6	18	17	2	1
FEP-FEP/CLA (CT)	**58**	0	0	3	3	0	2
Detection of AmpC							
FOX/CLOX (DTST)	10	**16**	**6**	0	0	0	0
CAZ/CLOX (DTST)	4	**17**	**8**	0	1	0	2
CTX-CTX/CLOX (CT)	11	**13**	**8**	0	1	0	0
CAZ-CAZ/CLOX (CT)	7	**16**	**9**	0	0	0	0
MEM-MEM/CLOX (CT)	0	**0**	**2**	0	0	0	0
Detection of serine carbapenemases (KPC/SME)							
CAZ/APBA (DTST)	6	16	8	**25**	5	0	3
CTX/APBA (DTST)	5	15	9	**26**	0	0	4
CAZ-CAZ/APBA (CT)	15	14	9	**8**	0	1	0
CTX-CTX/APBA (CT)	11	12	9	**17**	0	0	1
MEM-MEM/APBA (CT)	0	0	2	**29**	0	0	0
Detection of MBL							
CAZ/DPA (DTST)	2	0	0	0	**12**	0	1
MEM/DPA (DTST)	9	0	0	7	**27**	0	0
IPM-IPM/EDTA (CT)	0	0	0	1	**27**	0	0
MEM-MEM/DPA (CT)	1	0	0	0	**27**	0	0

CT, combination tablets; DTST, double tablet synergy test; acqAmpC, acquired AmpC; cAmpC, hyperexpressed chromosomal AmpC; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; IPM, imipenem; CLA, clavulanic acid; CLOX, cloxacillin; APBA, aminophenylboronic acid; DPA, dipicolinic acid; ETDA, ethylenediaminetetraacetic acid. Results of specific relevancy are numbered in bold types.

[1]One isolate counts in both the ESBL- and cAmpC-group.

(CAZ-CAZ/CLA) detected all ESBL-producing strains, including 14 with reduced permeability and the presence of an ESBL. Cefepime-clavulanic acid (FEP-FEP/CLA) tested negative with eight strains of *E. coli* and *K. pneumoniae* producing CTX-M group 1 (n = 2), SHV-ESBLs (n = 4), GES-1 (n = 1), and VEB-1 (n = 1), including two and three strains that tested negative with CTX-CTX/CLA and CAZ-CAZ/CLA respectively. Thus, the results indicate that for detection of ESBL in *E. coli* and *K. pneumoniae*, the combination of CTX-CTX/CLA and CAZ-CAZ/CLA are sufficient despite the fact that 30 strains tested negative with CAZ-CAZ/CLA. The high number of strains with a negative result could suggest that the CAZ-CAZ/CLA tablets should be recalibrated. The use of FEP-FEP/CLA in combination with only CTX-CTX/CLA or CAZ-CAZ/CLA is suboptimal in this context. However, for strains co-producing ESBL and AmpC, inhibition with clavulanic acid could theoretically be masked. Consequently, FEP-FEP/CLA could be useful, as cefepime is only minimally affected by AmpC-expression, apart from the - until now – rare examples of isolates producing an extended spectrum AmpC β-lactamase (ESACs) (18). For the same reason, inducible or derepressed, chromosomal AmpC-producers (e.g. *Enterobacter* spp., *Citrobacter* spp. and *S. marcescens*) could also be tested with FEP-FEP/CLA, theoretically enhancing ESBL detection. However, in our study, a single *C. freundii* strain expressing ESBL (SHV-12) and derepressed AmpC, did not display FEP-FEP/CLA synergy.

These FEP-FEP/CLA negative strains illustrate that the sensitivity of this combination is dependent on the actual ESBL-substrate profile. For our strain collection, FEP-FEP/CLA did not add definitive, diagnostic information.

The OXY-hyperproducing *Klebsiella oxytoca* also represents a challenge with respect to detection of ESBLs. False positive ESBL tests

were observed for 3 of the 22 *K. oxytoca* strains with one strain being positive in all ESBL tests. Thus, for *K. oxytoca*, close investigation of the antibiogram and correct species identification is necessary for correct interpretation of ESBL test results. High level resistance to cefuroxime, piperacillin/tazobactam and aztreonam, in combination with borderline MICs/zones of inhibitions for cefotaxime and cefepime, should be interpreted as hyperproduction of the chromosomal OXY β-lactamase rather than ESBL production (19). Conversely, resistance to ceftazidime and inhibition with clavulanic acid should lead to additional investigation (i.e. genotyping) of ESBL production.

Detection of AmpC

To detect all strains with acquired AmpC using the combination tablet method, both cefotaxime-cloxacillin (CTX-CTX/CLOX) and ceftazidime-cloxacillin (CAZ-CAZ/CLOX) were required, as four and one strains remained undetected for each of the combinations respectively. Double tablet synergy testing with CAZ/CLOX detected all strains with acquired AmpC whereas the same approach with cefoxitin and cloxacillin (FOX/CLOX) detected 16/17 strains with an acquired AmpC. The test negative strain was a DHA-1-producing *K. pneumoniae*. All strains overexpressing their chromosomal AmpC β-lactamase (n = 9) showed a positive combination tablet test result, when including both CTX-CTX/CLOX and CAZ-CAZ/CLOX. Moreover, all five chromosomal AmpC hyperproducing *E. coli* strains tested positive in the double tablet synergy test with either CAZ-CLOX or FOX-CLOX. This observation was in contrast to the double tablet synergy test results for the four depressed AmpC strains, *Enterobacter* spp. (n = 3) and *C. freundii* (n = 1), as this test only detected one with FOX-CLOX and three with CAZ-CLOX.

Overexpression of AmpC in combination with porin loss may result in reduced susceptibility or resistance to carbapenems. Thus, to distinguish AmpC with porin loss from serine carbapenemases like KPC, which are also inhibited with boronic acids, the combination of meropenem and cloxacillin (MEM-MEM/CLOX) could be used. Positive results for both meropenem-boronic acid (MEM-MEM/APBA) and MEM-MEM/CLOX would indicate AmpC with porin loss, rather than production of KPC (as would negative results for both tests). As expected the *E. aerogenes* (n = 1) and *E. cloacae* (n = 1) strains with AmpC and porin loss showed zone of inhibition differences ≥5 mm for both combinations. In one recent study including five AmpC-producers with porin loss, 4/5 isolates were positive using in-house prepared MEM-MEM/CLOX-discs, whereas only 2/5 were positive with MEM-MEM/CLOX Neo-Sensitabs. However, the three MEM-MEM/CLOX negative isolates were also negative with MEM-MEM/APBA, leaving no isolates falsely categorized as KPC producers (20).

Boronic acid and metal chelator based tests for detection of serine carbapenemases and metallo-β-lactamases

All KPC-producing strains were detected with MEM-MEM/APBA combination tablet testing and cefotaxime/boronic acid (CTX/APBA) double tablet synergy testing. The latter test method, using ceftazidime/boronic acid (CAZ/APBA), failed to detect one KPC-3 producer. Combination tablet testing, making use of boronic acid synergy with either cefotaxime or ceftazidime, was unreliable for KPC detection as only 17/26 and 8/26 strains gave positive results, respectively. Further, SME-producing *S. marcescens* were only detected with MEM-MEM/APBA and not CTX/APBA or CAZ/APBA. Based on this, combination tablet testing with MEM-MEM/APBA seems preferable for serine carbapenemase detection in combination with MEM-MEM/CLOX to exclude AmpC-producers with porin loss.

Detection of ESBLs among KPC-producing isolates is perhaps of minor clinical significance but could be of potential interest for epidemiological purposes as well as for limiting the spread of the underlying resistance mechanisms (20). An additional trial with the KPC-producers was performed with MEM/APBA placed between CTX/CLA and CAZ/CLA (distance 10–15 mm). Using this approach, clavulanic acid inhibition of an ESBL in all 26

KPC-producing strains was observed (data not shown). However, given the subjective nature of this double tablet synergy test-type of observation, a negative result should not lead to a final exclusion of the possibility of an ESBL being present.

All MBL-producing strains were detected with both combination tablets of meropenem-dipicolinic acid (MEM-MEM/DPA) and imipenem- ethylenediaminetetraacetic acid (IMP-IMP/EDTA). However, one KPC-producer displayed a false positive result with IMP-IMP/EDTA. EDTA has previously been shown to be unspecific for the detection of MBLs (8). Another KPC-producer was false positive with MEM-MEM/DPA (repeatedly tested to a zone of inhibition difference of 5 mm) indicating that DPA could also have an unspecific effect in some cases. Synergism was observed with the double tablet synergy test meropenem/dipicolinic acid (MEM/DPA) not only for all 27 MBL-producers, but also for 9 ESBL (plus reduced permeability)-, 5 KPC-, and 2 SME-producers. Double tablet synergy test using ceftazidime/dipicolinic acid (CAZ/DPA) was unreliable. It was observed that the distance between the tablets was important. However, even with a meticulous placing of the tablets, only 10 of the 19 VIM-producers were detected using this method.

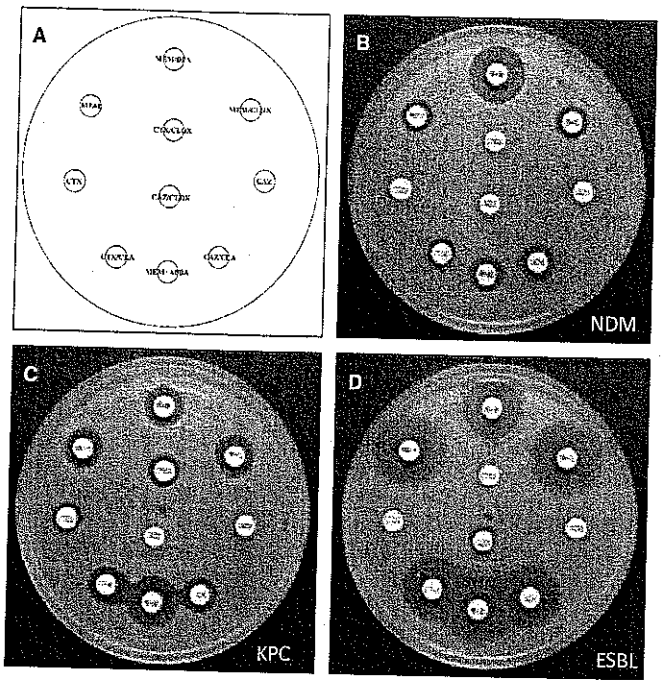
Overall, the use of both MEM-MEM/APBA and MEM-MEM/DPA detected and subgrouped all the KPC and MBL-producers correctly in this study.

Detection of OXA-48 in Enterobacteriaceae

In general, phenotypic detection of OXA enzymes represents a more challenging task as compared to the other β-lactamase groups. No single inhibitor for reliable laboratory detection of OXA has been detected until now (21). In recent years the potential of NaCl as a useful inhibitor of OXA enzymes has been investigated, but the conclusion remains that NaCl is inadequate for OXA detection as only some of the variants are inhibited in a visible manner (21). Low-level carbapenem hydrolysis also seems to be a key feature of OXA-48, making this enzyme more challenging to detect with susceptibility testing than other carbapenemases. It should also be mentioned that OXA-48 in itself does not hydrolyze extended spectrum cephaloporins, so if no ESBL or AmpC is co-expressed in a carbapenem low-level resistant isolate, cephalosporin susceptibility could be a helpful information, when assessing an antibiogram for the possible presence of β-lactamase production. Another OXA-48 indicator is high-level resistance to temocillin, which still remains an uncommon phenomenon in Enterobacteriaceae, regardless of ESBL status (22).

In the absence of a useful phenotypic test, a deducing-by-exclusion approach could contribute as an indicator of OXA-48. Reduced susceptibility to carbapenems combined with negative tests for MEM-MEM/CLOX, MEM-MEM/APBA, MEM-MEM/DPA (or IMP-IMP/EDTA), hereby excluding AmpC with porin loss, serine carbapenemases and MBLs, suggest the presence of OXA-48. The 3 OXA-48 producers tested did fit this profile, but accompanying resistance mechanisms, e.g. ESBL (possibly leading to a false conclusion of ESBL plus reduced permeability), could easily obscure the detection of any OXA enzyme in this setup. Thus, genotyping is required to confirm the presence of OXA-48.

In conclusion, the use of various β-lactam-β-lactamase inhibitor combinations is useful for rapid interpretative phenotypic detection of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae. The overall results support the use of a defined plate (shown in Fig. 1) with a simple tablet layout for detection and subgrouping of β-lactamases in Enterobacteriaceae. The tablet layout could be used as a whole or in a targeted approach based on the actual phenotypic profile and local epidemiology. Importantly, correct species identification and investigation of the antibiogram is crucial when interpreting these tests. With the emergence of carbapenemases it is particularly important to apply confirmatory testing of isolates showing reduced susceptibility to carbapenems. However, additional validation of the betalactam/betalactamase-inhibitor combinations should be performed including strains with low prevalence carbapenemases such as NMC-A and IMI as well as an extended panel of derepressed AmpC strains with reduced permeability.



Indicative interpretation	MEM	ZOI difference ≥ 5 mm (combination - target β-lactam alone)				
		CTX/CLA and/or CAZ/CLA	CTX/CLOX and/or CAZ/CLOX	MEM/CLOX	MEM/APBA	MEM/DPA
ESBL	WT	+	–	–	–	–
AmpC		–	+	–	–	–
ESBL + AmpC		+	+	–	–	–
ESBL + impermeability	Non-WT	+	–	–	–	–
AmpC + impermeability or OXA-48		–	+	–	–	–
AmpC + impermeability		–	+	+	+	–
SME (*S. marcescens*)		–	–	–	+	–
KPC (*K. pneumoniae*)		Variable[1]	–	–	+	–
MBL		Variable[1]	–	–	–	+

Fig. 1. Tablet layout (A) on a 14 cm plate with a related indicative interpretation scheme, and three examples of phenotypic outcome when applying the proposed tablet layout on *Klebsiella pneumoniae* producing NDM (B), KPC (C) and ESBL (D). CTX/CLA and CAZ/CLA are positioned with a distance of 10 – 15 mm to MEM+APBA to enhance ESBL detection in KPC producers. Abbreviations: zone of inhibition (ZOI), wild type (WT), cefotaxime (CTX), ceftazidime (CAZ), meropenem (MEM), clavulanic acid (CLA), cloxacillin (CLOX), aminophenylboronic acid (APBA), and dipicolinic acid (DPA). [1]Depending on the presence of co-expressed ESBL enzymes. Note that the ROSCO Neosensitabs codes, differs from the abbreviations used in this article.

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