Evaluation of a Double Synergy Differential Test (DSDT) for differential detection of ESBL and AmpC-type β-lactamases in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*

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SUMMARY

This work describes a simple and practical double synergy differential test (DSDT) that couples the detection of ESBLs and AmpC-type enzymes by means of a combo-disk approach using cefotaxime and ceftazidime as indicator substrates, and clavulanate and boronic acid as enzyme inhibitors. The DSDT was tested with a collection of 118 *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* strains with different beta-lactamase profiles, and proved to be highly sensitive and specific for the detection of ESBL and AmpC-producing isolates.

KEY WORDS: AmpC β-lactamase, ESBL, Enterobacteriaceae, Boronic acid, Laboratory detection

Received August 24, 2011 *Accepted* December 21, 2011

AmpC beta-lactamases, class C classified according to Ambler and to group 1 by Bush-Jacoby-Medeiros, are well defined enzymes with a broad substrate specificity (Babic *et al*., 2006). These enzymes, both chromosomal and plasmid mediated, show an action spectrum similar to beta-lactamase ESBLs. However their activity is normally limited to the fourth-generation cephalosporins and aztreonam. Chromosome-mediate AmpC β-lactamase have been described in a wide variety of Gram-negative bacilli (Barlow *et al.*, 2002; Korfman *et al*., 1991; Linderberg *et al*., 1986; Normark *et al*., 1986), such as *Pseudomonas aeruginosa, Enterobacter spp*., *Acinetobacter spp*., *Aeromonas spp*., *Citrobacter freundii*, *Escherichia coli, Serratia marcescens,* etc. Overproduction of their chromosomal AmpC- β-lactamase by mutation is probably responsible for the resistance in these organisms (Jan *et al*., 2002). In most genera of the Enterobacteriaceae family AmpC is inducible (Bush *et al*., 1995). Unlike chromosome-mediated AmpC, most plasmid-mediated AmpC genes, such as MIR-1, are expressed constitutively even in the presence of a complete system for induction (Phillipon *et al*., 2002).

The transfer of chromosomal genes to plasmids allowed the expression of AmpC-β-lactamases in *Klebsiella* spp., *E.coli*. *Proteus mirabilis* and *Salmonella* spp. (Jacoby, 2009).

The accurate detection of plasmid-mediated AmpC is important to improve the clinical management of infection and to provide epidemiological data. There are currently no standardized phenotypic methods for the screening and detection of AmpC enzymes. In recent years, several laboratory tests have been described for the detection of AmpC: the three-dimensional test (Blank *et al.,* 2005; Lee *et al*., 2005) cefoxitin-agar medium (CAM) (Nasim *et al*., 2004), β-lactam inhibitors (Jacoby *et al*., 2006; Song *et al*., 2007) and the molecular method (Cheng *et al*., 2007; Zhu *et al.,* 2007). However, these tests are not standardized and time-consuming, not being convenient for routine work in clinical microbiology laboratories.

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In a previous work (Sabia *et al.*, 2010), the performance of a new simple and practical Double Synergy Differential Test (DSDT) to simultaneously detect production of ESBLs and AmpC-type β-lactamase was described; such test was characterized by sensitivity and specificity in *P. mirabilis*. According to these results in this study was to investigate the possible use of this test also against clinical isolates of *Escherichia. coli* and *Klebsiella pneumoniae*.

We analysed a total of 118 consecutive non-duplicate strains of *E. coli*, *K. pneumoniae* and *P. mirabilis* isolated at the Clinical Microbiology Laboratory of "S. Agostino - Estense" Hospital (Modena, Italy) during May-December 2010 and fulfilled the following criteria: cefotaxime (CTX) or ceftazidime (CAZ) MIC >1 mg/L. Four additional strains, previously characterized for production of ESBLs, acquired AmpC-type β lactamases and hyperproduction-AmpC, were included as control.

Species identification and antimicrobial susceptibility testing were carried out using the Vitek2 system and the AST-N089 cards (bioMérieux Italia S.p.A.).

A simple disk-diffusion test, DSDT, was designed to simultaneously detect the production of ESBL and AmpC-type β-lactamase activity. Using a single plate, the test measures susceptibility to CTX and CAZ (BBL, Becton Dickinson Italia) in a conventional disk-diffusion format, and the susceptibility to the same agents in the presence of clavulanate (inhibitor of ESBLs) or boronic acid (inhibitor of AmpC-type enzymes) (Figure 1).

The DSDT was performed as follows: the surface of a Mueller Hinton (MH) agar plate (diameter 9 cm, bioMérieux Italia S.p.A) was evenly inoculated using a cotton swab with a McFarland 0.5 bacterial suspension in normal saline, prepared from an overnight culture. After inoculation, three sets of disks were placed onto the medium. One set included a cefotaxime (CTX) (30 µg) and a ceftazidime (CAZ) (30 µg) disk; the second set included a cefotaxime plus clavulanate (CTX-CLA) and a ceftazidime plus clavulanate (CAZ-CLA) disk; the third set included a cefotaxime plus boronic acid (CTX-BA) and a ceftazidime plus boronic acid (CAZ-BA) disk. Results were recorded after incubation of the plates at 35°C for 16-20 hrs. For ESBL detection results were interpreted as recommended by the CLSI, i. e. a ≥5 mm increase of inhibition zone diameter around CTX and/or CAZ disks was tentatively adopted as a cut-off value for synergy with boronate indicating AmpC production. The disks containing boronic acid were prepared by adding to each antibiotic-containing disk, immediately after deposition on plate, 10 µl of a 60 mg/ml solution of benzo(β)thiophene-2-boronic acid (BZBTH2B) in dimethyl sulphoxide (Sigma Italia).

For the detection of ESBL genes PCR for TEM, SHV, CTX and PER was performed (Al Naiemi *et al.*, 2006 Perilli *et al.*, 2002) and AmpC genes were detected using family-specific primers developed by Perez and Hanson (2002).

For the amplification of the promoter region of *E. coli* chromosomal AmpC, the primers and the

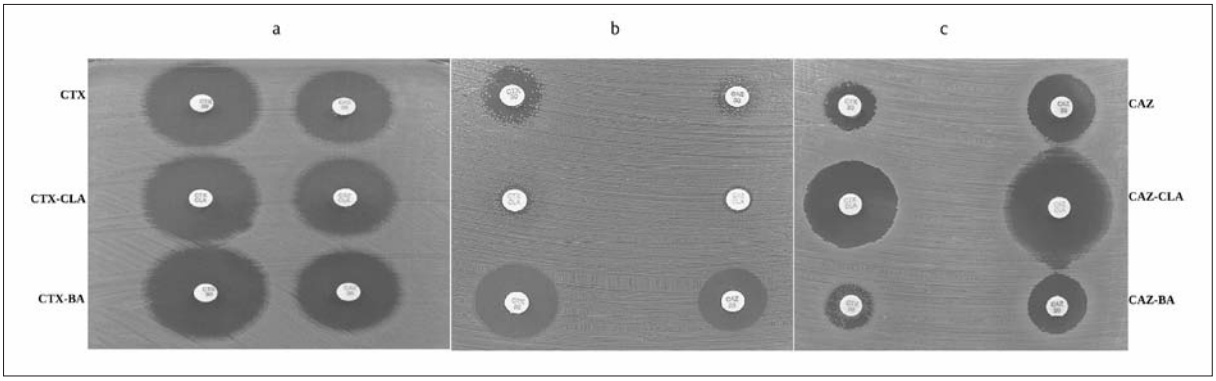

FIGURE 1 - *Representative results using the DSDT a - Wild-type, b - AmpC positive and c - ESBL positive. (CTX cefotaxime, CAZ ceftazidime, CTX-CLA cefotaxime with clavulanic acid, CAZ-CLA ceftazidime with clavulanic acid, CTX-BA cefotaxime with boronic acid and CAZ-BA ceftazidime with boronic acid).*

conditions previously described have beenemployed (Corvec *et al.*, 2002).
PCR-positive amplicons were purified with the QIAquick PCR Purification Kit (Qiagen Italia) and directly sequenced using amplification primers on the 3130 Genetic Analyzer (Applied Biosystems). Purification and sequencing wer carried out by Genex CZ, s.r.o. Sequence alignment and analysis were performed online using the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).
A total of 118 isolates of belomging to *E. coli* (n=55), *K pneumoniae* (n=35) and *P. mirabilis* (n= 28) strains were tested for both ESBL and AmpC beta-lactamase production by DSDT. Of the, 105 (88,9%) isolates were found to be ESBL positive, 11 (9,3%) isolates were found to be AmpC positive and 2 isolates were foud both (1,6%) both ESBL and AmpC positive (Table 1).
Molecular analysis of ESBL and AmpC determinants is confirmed by DSTD test in all isolates. Also in the isolate with double positivity for ESBL and AmpC in the DSDT, molecular analysis confirmed the presence of either ESBL (TEM-52) and AmpC (CMY-16) gene (Table 1). The other ESBL genes detected included 9 TEM-92, 5 TEM-52, 52 CTX-M-1, 5 CTX-M-3, 24 CTXM-15, 12 SHV-12, 6 SHV-5 and 2 PER-1.
AmpC-type determinants detected in the clinical isolates were mostly represented by CMY-16 and CMY-2 genes. The identification of 3 *E. coli* strains carrying a bla geneCMY-2 highlights the presence of acquired AmpC-type β-lactamases in members of the Enterobacteriaceae, which represents another emerging problem in Italy (Migliavacca e*t al*., 2007). Multifocal detection of *P. mirabilis* isolates carrying the blaCMY-16 AmpC-encoding gene has been reported in dif-

TABLE 1 - *Test results for the 118 strains of Enterobacteriaceae.*

Microrganisms (N° of clinical isolates)	*Genotypes*		*SYN-CLA*	*SYN-BA*
E. coli (55)	ESBL (51) (92,7)		51 (100)	
		TEM-92 (4) (7,2)		
		TEM-52 (2) (3,6)		
		CTX-M-1 (34) (61,8)		
		CTX-M-3(5) (9)		
		SHV- (4) (7,2)		
		SHV-12 (2) (3,6)		
	AmpC (4) (7,2)			4 (100)
		CMY-2 (3) (5.4)		
		AmpC hyperproduction (1) (1,8)		
K. pneumoniae (35)	ESBL (35) (100)		35 (100)	
		TEM-92(1) (2,8)		
		CTX-M-1(4) (11,4)		
		CTX-M-15(24) (68,5)		
		SHV-12 (6) (17,1)		
P. mirabilis (28)	ESBL (19) (67.8)		19 (100)	
		TEM-92 (4) (14,2)		
		TEM-52 (1) (3,5)		
		CTX-M-1 (7) (25)		
		SHV-5 (1) (3,5)		
		PER-1 (2) (7,1)		
		SHV-12 (4) (14,2)		
	AmpC (7) (24,9)		7 (100)	
		CMY-2 (1) (3,5.)		
		CMY-16 (6) (21,4)		
	ESBL+AmpC (2) (3,5)		2 (100)	2 (100)

CTX, cefotaxime; CAZ, ceftazidime; CTX-CLA, cefotaxime- clavulanic acid; CAZ-CLA, ceftazidime- clavulanic acid;CTX-BOR, cefotaxime- boronic acid; CAZ-BOR, ceftazidime-boronic acid; SYN-CLA, synergy with clavulanic acid; SYN-BOR, synergy with boronic acid.

ferent cities in northern Italy (D'Andrea *et al.,* 2006; Luzzaro *et al.,* 2009), in parallel with biofilm-producing strains of *bla* gene presence (Nucleo *et al.,* 2010). Thus, hyperproduction of AmpC gene was confirmed only in one strain of *E. coli*.

In the clinical laboratory, the expression of AmpC enzymes in Enterobacteriaceae is often inferred by resistance to third-generation cephalosporins and cephamycins in the absence of recognisable resistance mechanisms e.g. ESBL.

The recent changes in clinical MIC breakpoints for third-generation cephalosporins, and aztreonam against Enterobacteriaceae, as established by Euopean Committee on Antimicrobial Susceptibility Testing (EUCAST, 2010) and Clinical and Laboratory Standards Institute (CLSI, 2010) decrease the likelihood of interpreting an ESBL producing Enterobacteriaceae as susceptible to third-generation cephalosporins. Thus recognition of ESBL production is supposed not to be necessary for prediction of clinical outcome. However, it is still important for infection control to limit its spread, to measure the evolution of the spread and the impact of control programs. The results of the present study lead us to propose this method phenotypic as a sensitive, economic, easy to perform and to interpret; therefore we recommendsuch method for the routine laboratory with a sensitivity and specificity of 100% of the strains tested.

In conclusion this test is simple enough to be easily integrated into routine diagnostic laboratories and has the potential to greatly simplify the detection of ESBL and of AmpC- type β-lactamases in *E. coli, K. pneumoniae* and *P. mirabilis*.

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