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Sensitivity and Specificity of Various β-Lactam Antibiotics and Phenotypical Methods for Detection of TEM, SHV and CTX-M Extended-Spectrum β-Lactamases

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Summary

The aim of this study was to compare the sensitivity and specificity of six different β-lactam antibiotics using five phenotypical tests for detection of extended spectrum beta-lactamases (ESBLs) based on synergism of β-lactam antibiotics and clavulanate.

Experiments were performed on a set of 80 *Klebsiella pneumoniae* strains and 105 *Escherichia coli* strains with previously characterized ESBLs (SHV, TEM and CTX-M). ESBLs were detected by five different phenotypical methods: MIC (minimum inhibitory concentration) determination of β-lactam antibiotics with and without clavulanate, double-disk synergy test (DDST), inhibitor-potentiated disk-diffusion test (IPDDT), CLSI-Clinical and Laboratory Standard Institution (former NCCLS) combined-disk-test, and modified MAST-disk-diffusion test (MAST-DD-test). Seven antibiotics were tested as indicators of ESBL production: ceftazidime, cefotaxime, ceftriaxone, aztreonam, ceftibuten, cefpodoxime and cefepime. Ceftazidime and aztreonam were the best indicators for SHV-5, SHV-12 and TEM β-lactamases whereas cefotaxime and ceftriaxone were the most sensitive in detection of SHV-2 and CTX-M β-lactamases in DDST, IPDDT and CLSI test. MIC determination of β-lactam antibiotics with and without clavulanate was the most sensitive method. DDST was the least sensitive test. Double-disk synergy test, which is the most frequently used test for detection of ESBLs in routine laboratories, was the least sensitive independently of the indicator antibiotic. Since MIC determination is a very laborious and time consuming method, we would recommend the NCCLS combined disk test or IPDD test for detection of ESBLs in routine laboratories with 5 mm zone augmentation breakpoint.

Key words: **Extended-spectrum β-lactamases, detection, sensitivity, specificity, ceftazidime**

INTRODUCTION

The plasmid-mediated extended-spectrum β-lactamases (ESBL) confer resistance to oxyminocephalosporins such as cefotaxime, ceftazidime, and ceftriaxone, and to the monobactam, aztreonam. Resistance to expanded-spectrum cephalosporins through the acquisition and expression of extended-spectrum β-lactamases (ESBLs) is increasing[1]. Most ESBLs are mutant TEM and SHV enzymes but a

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few have different ancestors [1]. Apart from widely distributed TEM and SHV- ESBL, new types such as CTX-M, PER, GES, BES and IBC β-lactamases are being reported with increasing frequency [1-2]. The clinical implications of ESBLs are extremely serious, and sensitive diagnostic methods are urgently needed to guide therapy, monitor resistance development and implement intervention strategies [3]. Although ESBLs were associated with typical nosocomial pathogens in the past, recent data indicate that infections caused by ESBL-producing organisms may be an emerging problem in outpatient settings in various parts of the world [4]. CTX-M β-lactamases are the most prevalent in community specimens [5]. A key problem in detection of ESBL producers is the possibility of low-level expression of the enzyme and of an inoculum effect, resulting in variable MIC values and zone diameters by disk-diffusion testing [6]. Consequently, isolates may be reported by the laboratory as sensitive, whereas treatment failure may occur with this group of antibiotics in the *in vivo* situation [7]. For that reason quick detection of ESBLs in routine laboratories is necessary. The increased prevalence of Enterobacteriaceae producing ESBLs creates a great need for laboratory testing methods that will accurately identify the presence of this enzyme in clinical isolates [1].

The sensitivity and specificity of a susceptibility test to detect ESBL vary with the cephalosporin tested [1]. Many investigators have suggested that either dilution or diffusion test performed with cefpodoxime detected more ESBLs than other cephalosporins such as ceftazidime, cefotaxime and ceftriaxone [8]. However, recent data suggest that susceptibility testing with cefpodoxime can lead to a high number of false positives if the current CLSI criteria are applied [1]. The procedure currently recommended by Clinical and Laboratory Standard Institution (CLSI) to detect ESBL-producing *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Escherichia coli* involves an initial disk-diffusion or broth dilution screening test with one or more oxyimino β-lactams, followed by a confirmatory test to measure susceptibility to ceftazidime and to cefotaxime alone and in combination with clavulanic acid which inhibits ESBLs. Automated procedures have also been developed [9]. Molecular methods such as PCR are very sensitive and specific, but are expensive and time consuming and require well equipped laboratories [10-11]. Recently a new DNA microarray test for rapid detection of TEM β-lactamases has been introduced [12].

It is a well known fact that ESBLs differ regarding the substrate profile, for instance SHV-5, SHV-12 and some TEM β-lactamases are ceftazidimases, whereas SHV-2, SHV-7 and CTX-M β-lactamases prefer cefotaxime as a substrate [1]. It was hypothesized that ceftazidimases would be detected more accurately with ceftazidime and aztreonam contrary to cefotaximases, which are expected to be better identified with cefotaxime and ceftriaxone. The aim of this study was to compare sensitivity and specificity of seven different β-lactam antibiotics in five phenotypical tests for detection of ESBLs based on synergism of β-lactam antibiotics and clavulanate. One dilution-based method and four disk-diffusion based methods were evaluated.

METHODS

Bacteria

A set of 80 non-duplicate *K. pneumoniae* strains with previously characterized SHV-ESBLs (52 -SHV-5 producers, 21- SHV-2, 7- SHV-12), 94 non-duplicate *E. coli* strains producing TEM ESBLs and 11 non-duplicate *E. coli* strains harboring CTX-M group 1 ESBLs (6 CTX-M-3 and 5 CTX-M-15) were included in the study. SHV and CTX-M ESBLs were identified by sequencing of bla_{SHV} [13-14]and bla_{CTX-M}genes [15]. TEM β-lactamases were identified by isoelectric focusing on polyacrylamide gel and polymerase chain reaction with primers specific for TEM β-lactamases (TEM-A-5'-CGC-CGG-GTT-ATT-CTT-ATTTGT-CGC-3' and TEM-B-5'-TCT-TTC-CGA-TGC-CGC-CGC-CAG-TCA-3'), but the exact type was not determined. All strains had higher MICs of ceftazidime and aztreonam compared to cefotaxime and ceftriaxone and according to that the enzymes were classified as ceftazidimases. Twenty-six non-duplicate fully susceptible *E. coli* strains which yielded no amplicon with either TEM, SHV or CTX-M specific primers were used as negative control. *K. pneumoniae* strains were collected from various clinical specimens in Sisters of Mercy University Hospital and Dubrava University Hospital in Zagreb during 1994-1997 from various clinical specimens. *E. coli* strains were obtained from University Hospitals Split and Zagreb during 2001-2003. Bacteria were identified with conventional biochemical tests.

Antibiotics

Seven different antibiotics were tested: ceftazidime, cefotaxime, ceftriaxone, ceftibuten, cefpodoxime, aztreonam and cefepime. Antibiotics were tested at the potency of 30 μg/disk except for cefpodoxime (10 μg/disk). Disks were supplied by Becton Dickinson (BBL). Ceftazidime and clavulanic acid powders were obtained from Pliva, Zagreb; cefotaxime from Belupo, Zagreb; ceftriaxone, cefepime, ceftibuten and aztreonam from American Pharmacopeia, Rockville, Maryland, USP reference standard.

Methods for detection of ESBLs

Dilution method: Minimum inhibitory concentrations of ceftazidime, cefotaxime, ceftriaxone, aztreonam, ceftibuten and cefepime with and with-

out clavulanic acid (4 mg/L, fixed rate) were determined by a twofold microdilution technique using microtiter plates and Mueller-Hinton broth inoculated with 5 x 10^5 CFU/ml according to CLSI -Clinical Laboratory Standard Institution- (formerly NCCLS) [16]. The test was considered positive if the MIC against the β-lactam was reduced 7-8 fold by clavulanate (at least 3 dilutions).

Double-disk-synergy test (DDST): An overnight broth culture of the test organism was diluted in saline to match the turbidity of Mc Farland 0.5 standard. The suspension was swabbed on Mueller-Hinton (MH) agar. A central disk of amoxicillin/clavulanate (20/10 μg) was surrounded by disks of cefotaxime, ceftriaxone, ceftazidime, aztreonam, ceftibuten, cefpodoxime and cefepime at the distance of 2.5 cm on MH agar plate previously inoculated with the test organism [17]. Distortion of the inhibition zones around cephalosporin and aztreonam disks towards central disk was considered as a positive result. Since clavulanic acid disks are not available in Croatia, co-amoxiclav disks were used as a source of clavulanic acid.

Inhibitor potentiated disk-diffusion test (IPDDT): An overnight broth culture of the test organism was diluted in saline to mach the turbidity of McFarland 0.5 standard. The suspension was swabbed on MH agar. Six cephalosporins: ceftazidime, cefotaxime, ceftriaxone, ceftibuten, cefpodoxime and cefepime and monobactam aztreonam were tested for synergy with clavulanate as described previously [18]. MH agar supplemented with 4 mg/L of clavulanate was prepared the day before testing. Clavulanic acid was added to the medium after cooling to 50°C. Antibiotic disks containing ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), aztreonam (30 μg), ceftibuten (30 μg), cefepime (30 μg) and cefpodoxime (10 μg), were placed on clavulanate-containing and clavulanate-free MH agar plates. After overnight incubation at 37°C, the diameters of the inhibition zones were measured. Augmentation zone widths were obtained by subtracting inhibition zone diameters produced by the β-lactam disks on clavulanate-free medium with those in clavulanate-containing medium. Two different zone augmentations breakpoints were applied: 5 mm and 10 mm.

CLSI combined disk test: The inoculum was prepared in the same way as for the IPDD test. The suspension was swabbed on MH agar. Each of the test antibiotic disks was tested against each test organism individually and also in combination with clavulanic acid. 10 μl of clavulanic acid (1000 μg/ml) was dropped with the pipette on the surface of disks [16]. Augmentation zone widths were obtained by subtracting inhibition zone diameters produced by the β-lactam disks without clavulanate with those with clavulanate. Two different zone augmentation breakpoints were applied: 5 mm and 10 mm.

Modified MAST-DD test: The original method described by M'Zali *et al*. uses the commercial disks containing cephalosporins alone or combined with 10 μg clavulanic acid. In our modification, 10 μl of clavulanic acid (1000 μg/ml) was dropped on disks, as described above. Zone diameters produced by combination of β-lactams were divided with the zone diameters produced by a β-lactam alone. A ratio ≥1.5 was taken to signify the presence of ESBL activity [19].

Statistical analysis

Sensitivity and specificity for each test and antibiotic were calculated. Sensitivity is a measure of the success of a test in detecting true positives, and specificity is a measure of the success of a test in excluding negatives [3,20]. Sensitivity was calculated by formula: true positives / true positives + false negatives x100 The specificity was calculated by the following formula: true negatives / true negatives + false positives x100.

Student's *t*-test was used to evaluate the statistical significance of any differences in the results. P value of ≤ 0.05 was set as statistically significant. An alpha value of 0.05 (95%) was used to calculate the confidence. Predictive values differ from the above parameters in that they describe the value of the tests when the actual prevalence is taken into account [20]. The positive predictive value (PPV) is the number of correctly classified positive results compared to the total number of positive results. The negative predictive value (NPV) is the number of correctly classified negative results compared to the total number of negative results. Positive predictive value was calculated by the formula:

%Positive predictive value = true positives / true positives + false positives x100. Negative predictive value was calculated by the formula: % Negative predictive value = true negatives / true negatives + false negatives x 100.

RESULTS

Dilution method

This method was the most sensitive and managed to detect all SHV-ESBL-producers with all antibiotics including ceftibuten and cefepime. Ceftazidime, cefotaxime, ceftriaxone and aztreonam recognized all TEM producers, whereas ceftibuten and cefepime yielded less positive results (*Table 1*). Cefotaxime, ceftriaxone and cefepime were good indicators of CTX-M β-lactamases as shown in *Table 1*. Aztreonam and ceftibuten were not so successful in recognizing CTX-M producers. One false positive result was observed with ceftazidime among ESBL-negative strains (*Table 1*).

The greatest reduction in MIC in the presence of clavulanate was found with aztreonam for SHV-2 producers, with ceftazidime and aztreonam for SHV-

TABLE 1 - *Sensitivity and specificity of various methods and β-lactam antibiotics in detection of SHV, TEM and CTX-M β-lactamases.*

Dilution method							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime[1]**
SHV-2	21/21 (100%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	ND
SHV-5	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	ND
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	ND
TEM	94/94 (100%)	94/94 (100%)	94/94 (100%)	94/94 (100%)	84/94 (89%)	89/94 (95%)	ND
CTX-M[4]	11/11 (100%)	11/11 (100%)	11/11 (100%)	10/11 (90.9%)	3/11 (27.2%)	11/11 (100%)	ND
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	96	100	100	100	100	100	100
Double-disk synergy test							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	17/21 (80.9%)	20/21 (95.2%)	21/21 (100%)	16/21 (76%)	5/21 (23.8%)	13/21 (61.9%)	20/21 (95.2%)
SHV-5	52/52 (100%)	44/52 (84.6%)	48/52 (92.3%)	52/52 (100%)	30/52 (57.6%)	36/52 (69.2%)	47/52 (90.%)
SHV-12	7/7 (100%)	5/7 (71.4%)	6/7 (85.7%)	7/7 (100%)	5/7 (71.4%)	7/7 (100%)	7/7 (100%)
TEM	91/94 (97%)	89/95 (95%)	85/94 (90)	90/94 (96%)	48/94 (51%)	72/94 (76%)	93/94 (98.9%)
CTX-M[4]	0/11 (0%)	10/11 (90.9%)	10/11 (90.9%)	0/11 (0%)	0/11 (0%)	9/11 (81.8%)	10/11 (90.9%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	100	100	100	100	100	100	100
Inhibitor potentiated disk-diffusion test (10 mm)[2]							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	21/21 (100%)	20/21(95.2%)	21/21 (100%)	21/21 (100%)	9/21 (42.8%)	17/21 (80.9%)	20/21(95.2%)
SHV-5	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	33/52 (63.4%)	43/52 (82.6%)	52/52 (100%)
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	5/7 (71.4%)	7/7 (100%)	7/7 (100%)
TEM	89/94 (95%)	74/94 (79%)	70/94 (74%)	88/94 (94%)	17/94 (18%)	51/94 (54%)	89/94 (94.6%)
CTX-M[4]	5/11 (45.4%)	11/11 (100%)	11/11 (100%)	6/11 (54.5%)	5/11 (45.4%)	11/11 (100%)	11/11 (100%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	100	100	100	100	100	100	100
Inhibitor potentiated disk-diffusion test (5 mm)[3]							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	21/21 (100%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	17/21 (80.9%)	21/21 (100%)	21/21 (100%)
SHV-5	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	50/52 (96.1%)	52/52 (100%)	52/52 (100%)
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)
TEM	91/94 (97%)	90/94 (96%)	90/94 (96%)	91/94 (97%)	48/94 (51%)	75/94 (80%)	92/94 (97.8%)
CTX-M[4]	7/11 (63.6%)	11/11 (100%)	11/11 (100%)	7/11 (63.6%)	6/11 (54.5%)	7/11 (63.6%)	11/11 (100%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	100	100	100	96	100	100	100

TABLE 1 - *Continued*

CLSI combined disk test (10 mm)[2]							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	21/21 (100%)	20/21(95.2%)	21/21 (100%)	21/21 (100%)	6/21 (28.5%)	17/21 (80.9%)	19/21 (90.4%)
SHV-5	52/52 (100%)	51/52 (98%)	51/52 (98%)	52/52 (100%)	28/52 (53.8%)	42/52 (80.7%)	45/52 (86.5%)
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	4/7 (57.1%)	7/7 (100%)	7/7 (100%)
TEM	85/94 (90%)	72/94 (76%)	62/94 (66%)	85/94 (90%)	2/94 (2%)	36/94 (38%)	77/94 (81.9%)
CTX-M[4]	5/11 (45.4%)	11/11 (100%)	11/11 (100%)	4/11 (36.3%)	6/11 (54.5%)	10/11 (90.9%)	10/11 (90.9%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	100	100	100	100	100	100	100
CLSI combined disk test (5 mm)							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	21/21 (100%)	21/21 (100%)	21/21 (100%)	21/21 (100%)	16/21 (76.1%)	21/21 (100%)	21/21 (100%)
SHV-5	52/52 (100%)	52/52 (100%)	52/52 (100%)	52/52 (100%)	45/52 (86.5%)	52/52 (100%)	50/52 (96.1%)
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)
TEM	91/94 (96%)	88/94 (94%)	89/94 (95%)	92/94 (98%)	31/94 (33%)	66/94 (70%)	88/94 (93.6%)
CTX-M[4]	6/11 (54.5%)	11/11 (100%)	11/11 (100%)	9/11 (81.8%)	6/11 (54.5%)	11/11 (100%)	11/11 (100%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	96	100	100	100	96	96	100
MAST double- disk test							
Sensitivity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	21/21 (100%)	20/21(95.2%)	21/21 (100%)	21/21 (100%)	5/21 (23.8%)	16/21 (76%)	17/21 (80.9%)
SHV-5	52/52 (100%)	50/52 (96.1%)	51/52 (98%)	52/52 (100%)	28/52 (53.8%)	40/52 (76.9%)	50/52 (96.1%)
SHV-12	7/7 (100%)	7/7 (100%)	7/7 (100%)	7/7 (100%)	4/7 (57.1%)	7/7 (100%)	7/7 (100%)
TEM	84/94 (89%)	67/94 (71%)	61/94 (65%)	85/94 (90%)	0/94 (0%)	29/94 (31%)	79/94 (84%)
CTX-M[4]	5/11 (45.4%)	11/11 (100%)	11/11 (100%)	4/11 (36.3%)	5/11 (45.4%)	10/11 (90.9%)	11/11 (100%)
Specificity							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
	100	100	100	100	100	100	100

[1] ND-not determined; [2] 10 mm-zone augmentation breakpoint; [3] 5 mm-zone augmentation breakpoint; [4] CTX-M-3 and CTX-M-15 are shown together.

5 and TEM producers, and with ceftriaxone and cefepime for SHV-12 producers (*Table 2*). With CTX-M producers a marked reduction in MIC was shown when cefepime and cefotaxime were combined with clavulanate (*Table 2*).

DDST

Ceftazidime and aztreonam performed well with SHV-5, SHV-12 and TEM producers (*Table 1*). Contrary to that, cefotaxime, ceftriaxone and cefpodoxime were reliable indicators for SHV-2 and CTX-M β-lactamases. Ceftazidime, aztreonam and ceftibuten did not detect any CTX-M producers. (*Table 1*). Ceftibuten was markedly less sensitive than other antibiotics regardless of the type of ESBL. There were no false positive results.

IPDDT

IPDDT showed high sensitivity with ceftazidime and aztreonam for SHV-2, SHV-5 and SHV-12 and

TABLE 2 - *MIC reduction in the presence of clavulanate for various antibiotic-enzyme combinations.*

Antibiotic	Log_2 reduction in MIC by clavulanate Range	Median
SHV-2 (n=21)		
ceftazidime	3-10	5
cefotaxime	5-9	6
ceftriaxone	5-9	7
aztreonam	3-9	8
ceftibuten	3-10	6
cefepime	4-9	6
SHV-5 (n=52)		
ceftazidime	6-11 (16 off scale)	9
cefotaxime	5-10 (2 off scale)	7
ceftriaxone	6-10 (3 off scale)	7
aztreonam	6-11 (41 off scale)	9
ceftibuten	5-8	6
cefepime	5-8	6
SHV-12 (n=7)		
ceftazidime	5-8 (1 off scale)	7
cefotaxime	6-9 (1 off scale)	7
ceftriaxone	6-9 (5 off scale)	8
aztreonam	6-9 (2 off scale	7
ceftibuten	5-8	7
cefepime	5-8	8
TEM (n=94)		
ceftazidime	2-12 (9 off scale)	10
cefotaxime	3-11 (2 off scale)	7
ceftriaxone	5-11 (3 off scale)	8
aztreonam	2-11 (30 off scale)	10
ceftibuten	0-10	6
cefepime	2-9	7
CTX-M (n=11)[4]		
ceftazidime	3-5	4
cefotaxime	7->11 (1 off scale)	9
ceftriaxone	9->12 (4 off scale)	10
aztreonam	2-6	4
ceftibuten	1-4	2
cefepime	5-11	8
ESBL (negative)		
(n=26)		
ceftazidime	-2-3	0
cefotaxime	-1-2	0
ceftriaxone	-2-2	0
aztreonam	-1-3	0
ceftibuten	-1-2	0
cefepime	-1-2	1

TEM producers at both breakpoints. Ceftibuten and cefepime were less sensitive at the 10 mm breakpoint, but the sensitivity was increased when the breakpoint was reduced to 5 mm. Lower breakpoints (5 mm) yielded more positive results. CTX-M producers were best detected with cefotaxime, ceftriaxone, cefpodoxime and cefepime (*Table 1*). Ceftazidime, aztreonam and ceftibuten did not perform well against CTX-M producers. One false positive result was found with aztreonam as shown in *Table 1*. The largest increase in the inhibition zone by clavulanate was observed with aztreonam followed by ceftazidime against SHV-5 and TEM producers. Ceftriaxone produced the largest increase in the inhibition zone against SHV-2 and SHV-12 producers (*Table 3*). Cefotaxime and ceftriaxone produced the largest increase in inhibition zones in CTX-M producers (*Table 3*).

The differences in the inhibition zones in the medium with and without clavulanate were significant for ESBL-positive strains and not significant for ESBL-negative strains apart from cefepime (*Table 3*).

CLSI combined-disk test

Ceftazidime and aztreonam scored positive with all SHV-5 and SHV-12 producers at both breakpoints (*Table 1*). Cefotaxime, ceftriaxone and cefepime were 100% sensitive for all SHV-producers at 5 mm breakpoint and slightly less at higher breakpoints. All antibiotics were less sensitive in recognizing TEM-producers compared to those possessing SHV-ESBLs. Cefotaxime, ceftriaxone and cefepime performed best against CTX-M producers (*Table 1*). One false-positive result was found with ceftazidime, ceftibuten, cefepime among ESBL-negative strains at 5 mm breakpoint as shown in *Table 1*. The largest increase in inhibition zone by clavulanate against SHV-5 and TEM producers was seen with aztreonam followed by ceftazidime similarily as in IPDDT (*Table 4*). Ceftazidime produced the largest enlargement of the inhibition zone against SHV-2 producers followed by ceftriaxone. Cefotaxime and ceftriaxone demonstrated the largest increase in inhibition zones in CTX-M producers in the presence of clavulanate (*Table 4*). The differences in the inhibition zones in the medium with and without clavulanate were significant for ESBL-positive strains and not significant for ESBL-negative strains for most antibiotics except for ceftazidime, ceftibuten and cefepime (*Table 4*).

Modified MAST disk-diffusion test (MAST-DD-test)

The highest sensitivity against SHV-2, SHV-5, SHV-12 and TEM producers was achieved with ceftazidime and aztreonam followed by cefotaxime and ceftriaxone (*Table 1*). All SHV-12 producers were recognized with all antibiotics except for ceftibuten

TABLE 3 - *Inhibition zones of K. pneumoniae and E. coli strains producing particular types of β-lactamases in IPDDT.*

Antibiotic	Mean zone diameter ± SD (mm) ± confidence MH agar	MH agar + clavulanate	Mean zone augmentation	P value
SHV-2 (n=21)				
ceftazidime	17.09±4.22±1.80	34.04±3.45±1.47	16.95	<0.0001
cefotaxime	18.33±3.96±1.69	35.00±4.15±1.77	16.67	<0.0001
ceftriaxone	16.33±3.16±1.35	33.38±4.38±1.87	17.05	<0.0001
aztreonam	15.42±3.5±1.49	32.09±3.47±1.48	16.67	<0.0001
ceftibuten	23.38±1.68±0.71	33.00±3.06±1.30	9.62	<0.0001
cefepime	24.19±1.86±0.79	38.42±3.41±1.45	14.23	<0.0001
cefpodoxime	16.38 ±4,14±1.77	31.52±3.84±1.64	15.14	<0.0001
SHV-5 (n=52)				
ceftazidime	8.13±3.14±0.85	26.73±4.04±1.09	18.6	<0.0001
cefotaxime	13.80±4.01±1.08	32.36±2.61±0.70	18.56	<0.0001
ceftriaxone	13.26±3.16±0.85	32.00±3.19±0.86	18.74	<0.0001
aztreonam	6.96±1.87±0.50	28.17±3.42±0.92	21.21	<0.0001
ceftibuten	21.71±4.08±1.10	34.36±2.89±3.96	13.27	<0.0001
cefepime	23.57±2.99±0.81	37.55±3.26±0.88	13.98	<0.0001
cefpodoxime	11.44±3.87±1.05	29.13±4.71±1.28	17.69	<0.0001
SHV-12 (n=7)				
ceftazidime	11.57±2.69±1.99	26.00±1.15±0.85	14.43	<0.0001
cefotaxime	19.42±2.43±1.80	35.85±3.53±2.61	16.43	<0.0001
ceftriaxone	16.85±1.46±1.08	36.71±2.81±2.08	19.86	<0.0001
aztreonam	9.57±1.95±1.44	27.42±2.76±2.04	17.85	<0.0001
ceftibuten	20.28±1.79±1.32	31.85±2.85±2.11	11.57	<0.0001
cefepime	20.71±0.75±0.55	35.71±2.36±1.74	15	<0.0001
cefpodoxime	11.57±3.20±2.37	30.00±2.51±1.85	18.43	<0.0001
TEM (n=94)				
ceftazidime	12.04±5.56±1.12	30.93±2.61±0.52	18.89	<0.0001
cefotaxime	19.25±4.66±0.94	33.60±3.67±0.74	14.35	<0.0001
ceftriaxone	19.46±4.52±0.91	33.46±3.20±0.64	14.00	<0.0001
aztreonam	11.18±5.67±1.14	32.27±3.80±0.76	21.09	<0.0001
ceftibuten	30.11±4.41±0.89	35.88±3.30±0.66	5.77	<0.0001
cefepime	25.70±4.74±0.95	37.00±3.52±0.71	11.3	<0.0001
cefpodoxime	12.75±5.13±0.95	32.57±3.86±0.78	19.82	<0.0001
CTX-M (n=11)				
ceftazidime	22.80±6.32±3.73	32.45±2.69±1.58	9.65	0.000419
cefotaxime	10.00±4.79±2.83	32.81±3.91±2.31	22.81	<0.0001
ceftriaxone	9.81±4.04±2.38	32.54±3.80±2.24	22.73	<0.0001
aztreonam	22.20±4.31±2.54	31.81±2.08±1.22	9.61	<0.0001
ceftibuten	33.25±3.40±2.00	35.90±3.08±1.82	2.65	0.004003
cefepime	17.63±2.29±1.35	34.90±3.59±2.12	17.27	<0.0001
cefpodoxime	6.00±0±0	23.36±4.34±2.56	17.36	<0.0001
ESBL-negative (n=26)				
ceftazidime	30.96±3.19±1.22	32.19±2.20±0.84	1.23	0.11315
cefotaxime	34.00±3.28±1.26	35.07±3.12±1.19	1.07	0.231518
ceftriaxone	33.65±2.88±1.10	35.03±2.27±0.87	1.38	0.060448
aztreonam	35.34±2.84±1.09	36.46±2.54±0.97	1.12	0.144789
ceftibuten	36.57±2.11±0.81	37.57±2.11±0.81	1	0.095186
cefepime	34.26±1.56±0.59	35.69±1.54±0.59	1.43	0.001772
cefpodoxime	31.00±2.00±0.76	32.07±1.93±0.74	1.07	0.001

TABLE 4 - *Inhibition zones of K. pneumoniae and E. coli strains producing particular types of β-lactamases in CLSI combined-disk test.*

Antibiotic	Mean zone diameter ± SD (mm) ± confidence MH agar	MH agar + clavulanate	Mean zone augmentation	P value
SHV-2 (n=21)				
ceftazidime	17.09±4.22±1.80	35.57±3.59±1.53	18.48	<0.0001
cefotaxime	18.33±3.96±1.69	36.09±3.65±1.56	17.76	<0.0001
ceftriaxone	16.33±3.16±1.35	34.42±2.54±1.08	18.09	<0.0001
aztreonam	15.42±3.50±1.49	32.42±2.90±1.24	17	<0.0001
ceftibuten	23.38±1.68±0.71	31.52±2.52±1.07	8.14	<0.0001
cefepime	24.19±1.86±0.79	38.85±2.95±1.26	14.66	<0.0001
cefpodoxime	16.38±4.14±1.77	30.19±3.47±1.48	13.81	<0.0001
SHV-5 (n=52)				
ceftazidime	8.13±3.14±0.85	28.88±2.81±0.76	20.75	<0.0001
cefotaxime	13.8±4.01±1.08	32.07±3.19±0.86	18.27	<0.0001
ceftriaxone	13.26±3.16±0.85	31.71±2.54±0.69	18.45	<0.0001
aztreonam	6.96 ±1.87±0.50	28.61±2.87±0.78	21.65	<0.0001
ceftibuten	21.71±4.08±1.10	33.40±3.35±0.91	11.69	<0.0001
cefepime	23.53±2.99±0.81	37.84±3.07±0.83	14.31	<0.0001
cefpodoxime	11.44±3.90±1.06	27.09±4.69±1.27	15.65	<0.0001
SHV-12 (n=7)				
ceftazidime	11.25±2.69±1.99	28.80±1.25±0.92	17.23	<0.0001
cefotaxime	19.42±2.43±1.80	36.42±2.99±2.21	17	<0.0001
ceftriaxone	16.85±1.46±1.08	35.00±1.73±1.28	18.15	<0.0001
aztreonam	9.57±1.71±1.26	27.85±1.95±1.44	18.28	<0.0001
ceftibuten	20.28±1.79±1.32	32.28±2.42±1.79	12	<0.0001
cefepime	20.71±0.75±0.55	37.85±1.21±0.89	17.14	<0.0001
cefpodoxime	11.57±3.20±2.37	27.85±1.86±1.37	16.28	<0.0001
TEM (n=94)				
ceftazidime	12.04±5.56±1.12	29.10± 2.40±0.48	17.06	<0.0001
cefotaxime	19.25±4.66±0.94	32.45± 2.46±0.49	13.20	<0.0001
ceftriaxone	19.56±4.63±0.93	31.69± 2.45±0.49	12.13	<0.0001
aztreonam	11.80±5.64±1.14	30.42± 2.83±0.57	18.62	<0.0001
ceftibuten	30.11±4.41±0.89	33.52± 2.84±0.57	3.41	<0.0001
cefepime	25.70±4.74±0.95	33.84± 2.64±0.53	8.14	<0.0001
cefpodoxime	12.73±5.12±1.03	26.60± 5.21±1.05	13.87	<0.0001
CTX-M (n=11)				
ceftazidime	22.18±6.32±3.73	31.00± 3.92±2.31	8.82	0.001174
cefotaxime	9.90± 4.88±2.88	29.00± 3.49±2.06	19.1	<0.0001
ceftriaxone	9.54± 4.13±2.44	28.72± 3.19±1.88	19.18	<0.0001
aztreonam	22.27±4.31±2.54	29.72± 2.05±1.21	7.45	0.000135
ceftibuten	28.45±6.25±3.69	35.63± 3.66±2.16	7.18	0.003342
cefepime	17.81±2.08±1.22	29.45± 4.10±2.42	11.64	<0.0001
cefpodoxime	6.00±0±0	17.36±1.56±0.92	11.36	<0.0001
ESBL negative (n=26)				
ceftazidime	30.96±3.19±1.22	32.57±2.43±0.93	1.61	0.045922
cefotaxime	34.00±3.28±1.26	35.19±3.07±1.18	1.19	0.182708
ceftriaxone	33.65±2.88±1.10	35.11±2.93±1.12	1.46	0.07588
aztreonam	35.34±2.86±1.09	36.76±2.95±1.13	1.65	0.084368
ceftibuten	36.57±2.11±0.81	38.07±1.99±0.76	1.5	0.011465
cefepime	34.26±1.56±0.59	36.34±1.80±0.69	2.08	<0.0001
cefpodoxime	31.00±2±0.76	31.46±2.35±0.90	0.46	0.069388

which was markedly less sensitive. All antibiotics were less sensitive in recognizing TEM-producers compared to SHV-positive strains. Cefotaxime, ceftriaxone and cefepime performed best against CTX-M producers whereas ceftazidime, aztreonam and ceftibuten detected only one-half of CTX-M-positive strains. No false positive results were observed.

Statistical analysis

PPVs of the tests for detection of ESBLs are shown in *Table 5* and NPVs in *Table 6*. PPVs were high for all the tests used in this study. The exception was DDST with ceftazidime, aztreonam and ceftibuten against CTX-M producers. NPVs were significantly lower for TEM compared to SHV and CTX-M producers. DDST displayed lower NPVs compared to other tests due to the high number of false negative results.

DISCUSSION

This study attempted to address some of the problems associated with the detection of ESBLs. Although molecular methods seem to be sensitive, they are expensive, time consuming and require specialized equipment and expertise [10-11]. Our results show that ceftazidime and aztreonam were the best indicators for SHV-5, SHV-12 and TEM β-lactamases, whereas cefotaxime, ceftriaxone and cefpodoxime were the most sensitive in detection of SHV-2 β-lactamase and CTX-M producers in DDST, IPDDT and CLSI combined-disk-test. Ceftibuten and cefepime have been shown not to be reliable indicators of ESBL production, probably because they are more stable to hydrolysis by ESBLs than oxyminocephalosporins, and for that reason they did not perform well in all methods tested in this study except in dilution method. It is difficult to explain the high sensitivity of these two antibiotics in the dilution method and poor performance in disk-diffusion methods.

DDST, which is the most frequently used test for detection of ESBLs in routine laboratories, was the least sensitive, independently of the indicator antibiotic, contrary to the results obtained by other investigators [3]. This could be explained by the fact that a critical issue with DDST is placing the indicator disks at the optimal distance from the central co-amoxiclav disk. In our study disks were placed at a fixed distance of 2.5 mm, which was probably not optimal for all the strains. The reason why this test is generally recommended for detection of ESBLs is because it is easy to perform, there is no need to measure zone sizes and it can be read easily based on the presence or absence of synergy [3]. MIC determination of β-lactams alone and combined with clavulanate, IPDDT and CLSI combined-disk test have shown to be more sensitive. However, with the dilution method there were a lot of off scale results particularly with ceftazidime and aztreonam against SHV-5 and SHV-12 producers due to very high MICs of those antibiotics which often exceeded 1024 mg/L. The IPDDT and CLSI-combined-disk tests were highly sensitive when a breakpoint of 5 mm was applied. However, a few false positive results were observed at that breakpoint value. Increase of the breakpoint value to 10 mm improved specificity, but at the expense of sensitivity. In our investigation, the CLSI combined-disk test and IPDDT achieved higher sensitivity compared to the results of other authors [3,18]. The methods evaluated in this study detected SHV- producers better than TEM-producers. This observation was previously reported by MacKenzie *et al* [3]. Particularly ceftibuten and cefepime were unsuccessful in recognizing TEM-ESBLs.

Since MIC determination is a very laborious and time-consuming method we would recommend using the CLSI combined-disk method for detection of ESBLs in routine laboratories with a 5 mm zone augmentation breakpoint because sensitivity is more important than specificity for the screening test. Lack of sensitivity is a major drawback, whereas lack of specificity is a minor drawback [3].

The problem with the CLSI test that was used in this study is that fresh solution of clavulanic acid has to be prepared on the day of the test and cannot be stored. Co-amoxiclav disks could also be used as a source of clavulanic acid. In this modification, desribed as disk on disk test, co-amoxiclav disks are placed on the top of the cephalosporin or aztreonam disk [3]. Commercial disks containing ceftazidime and cefpodoxime combined with clavulanate (Oxoid) are also available [21]. IPDDT showed slightly higher sensitivity than the CLSI combined disk test, because it produced a larger increase in the inhibition zones in the presence of clavulanate, especially with TEM producers, but it requires incorporation of clavulanic acid into the medium. None of the methods tested in this study were 100% sensitive and specific if only one antibiotic was used. In order to increase sensitivity it is important to place at least two disks: ceftazidime and cefotaxime as recommended by CLSI, to avoid false negative results due to variable substrate profiles of ESBLs. CLSI recommends screening of all *Klebsiella* spp and *E. coli* strains first by DDST, and confirmation of ESBL production by one of the disk-diffusion or dilution based confirmatory tests. Some authorities suggest limitation of confirmatory tests only to *Klebsiella* spp. and *E. coli* strains with inhibition zone diameters ranging between the CLSI recommendations for ESBL screening and the intermediate category breakpoints [22]. Detection of CTX-M β-lactamases poses a great challenge since they are recognized with acceptable sensitivity only with cefotaxime, ceftriaxone, cefpodoxime and cefepime disks. Livermore *et al* recommend a cefpodoxime disk as a single indicator disk, because it detects well all three types of ESBLs:

TABLE 5 - *Positive predictive values of various tests for ESBL detection with the following antibiotics (%).*

	ceftazidime	cefotaxime	ceftriaxone	aztreonam	ceftibuten	cefepime	cefpodoxime[1]
Dilution method							
SHV-2	95	100	100	100	100	100	ND
SHV-5	98	100	100	100	100	100	ND
SHV-12	88	100	100	100	100	100	ND
TEM	99	100	100	100	100	100	ND
CTX-M[4]	92	100	100	100	100	100	ND
Double-disk synergy test							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	100	100	100
SHV-5	100	100	100	100	100	100	100
SHV-12	100	100	100	100	100	100	100
TEM	100	100	100	100	100	100	100
CTX-M[4]	0	100	100	0	0	100	100
Inhibitor potentiated disk-diffusion test (10 mm)[2]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	100	100	100
SHV-5	100	100	100	100	100	100	100
SHV-12	100	100	100	100	100	100	100
TEM	100	100	100	100	100	100	100
CTX-M[4]	100	100	100	100	100	100	100
Inhibitor potentiated disk-diffusion test (5 mm)[3]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	95	100	100	100
SHV-5	100	100	100	98	100	100	100
SHV-12	100	100	100	88	100	100	100
TEM	100	100	100	99	100	100	100
CTX-M[4]	100	100	100	87	100	100	100
CLSI combined disk test (10 mm)[2]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	100	100	100
SHV-5	100	100	100	100	100	100	100
SHV-12	100	100	100	100	100	100	100
TEM	100	100	100	100	100	100	100
CTX-M[4]	100	100	100	100	100	100	100
CLSI combined disk test (5 mm)[3]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	95	100	100	100	94	95	100
SHV-5	98	100	100	100	98	98	100
SHV-12	88	100	100	100	87	87	100
TEM	99	100	100	100	97	98	100
CTX-M[4]	86	100	100	100	86	92	100
MAST double- disk test							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	100	100	100
SHV-5	100	100	100	100	100	100	100
SHV-12	100	100	100	100	100	100	100
TEM	100	100	100	100	100	100	100
CTX-M[4]	100	100	100	100	100	100	100

[1] ND-not determined; [2] 10 mm-zone augmentation breakpoint; [3] 5 mm-zone augmentation breakpoint; [4] CTX-M-3 and CTX-M-15 are shown together.

TABLE 6 - *Negative predictive values of various tests for ESBLs detection with the following antibiotics (%).*

	ceftazidime	cefotaxime	ceftriaxone	aztreonam	ceftibuten	cefepime	cefpodoxime[1]
Dilution method							
SHV-2	100	100	100	100	100	100	ND
SHV-5	100	100	100	100	100	100	ND
SHV-12	100	100	100	100	100	100	ND
TEM	100	100	100	100	72.22	83.87	ND
CTX-M[4]	100	100	100	96.29	76.47	100	ND
Double-disk synergy test							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	86.6	96.29	100	83.87	61.9	76.47	96.29
SHV-5	100	76.47	86.6	100	54.16	61.9	83.87
SHV-12	100	92.85	96.29	100	92.85	100	100
TEM	89.65	81.25	74.28	86.6	36.11	54.16	96.29
CTX-M[4]	70	96	96	70	70	92	96.29
Inhibitor potentiated disk-diffusion test (10 mm)[2]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	96.29	100	100	68.42	86.6	96.29
SHV-5	100	100	100	100	57.77	74.28	100
SHV-12	100	100	100	100	96.29	100	100
TEM	83.87	56.52	52	81.25	25.24	37.68	92.85
CTX-M[4]	81.25	100	100	83.87	81.25	100	100
Inhibitor potentiated disk-diffusion test (5 mm)[3]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	86.6	100	100
SHV-5	100	100	100	100	92.85	100	100
SHV-12	100	100	100	100	100	100	100
TEM	89.65	86.6	86.6	89.65	36.11	57.77	92.85
CTX-M[4]	86.6	100	100	86.6	83.87	100	100
CLSI combined disk test (10 mm)[2]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	96.29	100	100	63.41	86.6	92.85
SHV-5	100	96.29	96.29	100	52	72.22	78.78
SHV-12	100	100	100	100	89.65	100	100
TEM	74.28	54.16	44.82	74.28	22.03	30.95	78.78
CTX-M[4]	81.25	100	100	78.78	83.87	83.87	96.29
CLSI combined disk test (5 mm)[3]							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	100	100	100	83.87	100	100
SHV-5	100	100	100	100	78.78	100	92.85
SHV-12	100	100	100	100	100	100	100
TEM	89.65	81.25	83.87	92.85	29.21	48.14	81.25
CTX-M[4]	83.87	100	100	92.85	83.87	100	100
MAST double-disk test							
	ceftazidime	**cefotaxime**	**ceftriaxone**	**aztreonam**	**ceftibuten**	**cefepime**	**cefpodoxime**
SHV-2	100	96.29	100	100	61.9	83.87	86.6
SHV-5	100	92.85	96.29	100	52	68.42	92.85
SHV-12	100	100	100	100	90	100	100
TEM	72	49	44	74	22	40	83.87
CTX-M[4]	83.87	100	100	78.78	83.87	96.29	100

[1] ND-not determined; [2] 10 mm-zone augmentation breakpoint; [3] 5 mm-zone augmentation breakpoint; [4] CTX-M-3 and CTX-M-15 are shown together.

TEM, SHV and CTX-M [23]. Recently, the E-test has been introduced as a simple and sensitive technique for the detection of ESBLs [24], but it is very expensive. The cost of consumables is a very important factor to be considered in most routine diagnostic laboratories.

These results suggest that the categorization of isolates as ESBL-producers or non-producers depends on the detection method, on the indicator antibiotic applied to produce synergism with clavulanate and the type of β-lactamase. The spread of CTX-M ESBLs has prompted rethinking of the most appropriate methods for detection of ESBLs in diagnostic laboratories. In geographical areas where CTX-M type of β-lactamases is dominant cefotaxime is expected to be superior to ceftazidime as indicator antibiotic. For that reason it is important to know which ESBLs are predominant in certain countries where ESBL detection is carried out. Furthermore, it is important to emphasize that, in line with CLSI recommendations, both ceftazidime and cefotaxime testing would have to be used if a higher number of distinct ESBLs are to be detected.

The strains with low level enzyme production and expression of resistance usually pose the most serious problem in phenotypic detection of ESBLs. All of our *K. pneumoniae* and most *E. coli* strains had high MICs of expanded-spectrum generation cephalosporins and aztreonam. It would be interesting to examine the sensitivity and specificity of these methods for detection of ESBLs in strains with borderline resistance to expanded-spectrum cephalosporins or those susceptible to them which are often missed by routine susceptibility testing. Genes encoding ESBLs are not always expressed sufficiently to confer phenotypical resistance to cephalosporins, but these mutations can act as precursors to further mutations that result in unsusceptibility at levels sufficient to produce clinical resistance. For that reason early detection of low levels of ESBLs may prevent further problems in the clinical use of cephalosporins and aztreonam [25]. The important drawback of all phenotypical tests based on synergism with clavulanate is their inability to detect inhibitor-resistant, OXA and AmpC extended-spectrum β-lactamases which are of growing importance. False-negative clavulanic acid effect can be observed with ESBL-producers which possess other resistance mechanisms such as protein loss or hyperproduction of chromosomal K1 β-lactamases [26]. Porin changes may mask a clavulanic acid effect in an ESBL strain by failing to allow the entrance of sufficient quantities of cephalosporin into the bacteria, for the effect to be evident [26]. False-positive clavulanic acid effect was reported with strains producing broad-spectrum β-lactamases which are inhibited by clavulanate, but are not formally classified as ESBLs such as Form I and Form II chromosomal enzymes of *Citrobacter diversus*, plasmid mediated OHIO-1 enzyme of *Serratia marcescens* and FPM-1 β-lactamase of *Proteus* spp [26]. The limitation of CLSI guidelines is that they do not apply to *Enterobacteriaceae* other than *K. pneumoniae, K. oxytoca* and *E. coli* [26]. However, ESBLs are being found with increasing frequency in other enteric bacteria such as *Enterobacter cloacae* [27], *Enterobacter aerogenes* [28], *Serratia marcescens* [29] and *Proteus mirabilis* [30]. Difficulties in detection of CTX-M β-lactamases cause concern because they might appear as community pathogens [31-32] contrary to TEM and SHV β-lactamases which are usually associated with nosocomial pathogens.

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