

Modification of the three-dimensional method for the detection of AmpC β -lactamase in *Enterobacter* spp. and *Escherichia coli*

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ABSTRACT

Laboratory failure in the detection of Ambler class C (AmpC) β -lactamases in Gram negative bacteria represents a major cause for its spreading and may be associated with low therapeutic outcome. The aim of this study was to develop a more practical and simplified laboratory technique by modifying the three-dimensional method for the detection of AmpC β -lactamase in *Enterobacter* spp and *Escherichia coli*. Twenty eight clinical isolates including 11 isolates of *Enterobacter cloacae*, 2 isolates of *Enterobacter aerogenes* and 15 isolates of *Escherichia coli* tested by standard disk diffusion methods against cefoxitin 30 μ g disk as primary screening for detection of AmpC β -lactamase then all isolates tested for its production of AmpC β -lactamase by a modified three dimensional method (3DM) with cefoxitin. *Escherichia coli* ATCC 25922 was used as negative controls (non-AmpC producer). In addition, the susceptibility test of 15 *Escherichia coli* isolates was performed against selected beta-lactam antibiotics. All *Enterobacter* isolates and one *Escherichia coli* isolate showed resistance to cefoxitin and showed positive result with the modified 3DM (M3DM) by enhanced growth of the surface organism (*E. coli* ATCC 25922) at the point where the slit intersected the zone of inhibition, while all cefoxitin-susceptible *E. coli* isolates showed M3DM negative results. A high percentage of *Escherichia coli* isolates were non-susceptible to β -lactam antibiotics used but they showed good susceptibility to imipenem (100%). The M3DM provides a cost-effective alternative for AmpC β -lactamase testing in clinical microbiology laboratories, thus negating the need for tedious bacterial lysis procedures required in the 3DM.

Introduction

AmpC β -lactamases are important resistance mechanisms encoded by chromosomal gene in some Gram-negative bacteria including *Enterobacter*, *Serratia marcescens*, *Citrobacter freundii*, *Morganella morganii* and *Escherichia coli* (1, 2). These enzymes have a broad substrate profile that includes penicillins, cephalosporins, and monobactam; therefore, they show resistance to all β -lactam antibiotics except carbapenem and cefepime and in contrast to extended spectrum β -lactamases (ESBLs), they hydrolyze cephamycins and are not inhibited by commercially available β -lactamase inhibitors (3, 4).

Enterobacter spp. are common pathogens frequently associated with infections in the intensive care unit (5), in which expression of chromosomal AmpC β -lactamase are inducible and exposure to certain β -lactam antibiotic results in increased

synthesis of AmpC β -lactamase and induction of resistance to broad spectrum cephalosporins (6, 7, 8), but certain genetic mutation lead to constitutive expression and production (7).

Usually low amounts of enzymes are produced constitutively in *Escherichia coli* because the chromosomal AmpC gene is regulated by a weak promoter and a strong attenuator. These *Escherichia coli* isolates are sensitive to the cephamycins (9). However, cephamycin-resistance in clinical isolates of *Escherichia coli* can be due to promoter or attenuator gene mutations that lead to hyper-production of AmpC enzymes or to the acquisition of plasmids with AmpC genes which believed to be originated from the chromosomes of *Enterobacter*, *Citrobacter*, and *Pseudomonas* spp, in addition the decrease in outer membrane permeability can also contribute to cephamycin resistance (9, 10). Occurrence of plasmid-mediated AmpC β -lactamases in *Klebsiella*

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pneumoniae and *Escherichia coli* has been documented (2, 10).

Infection caused by AmpC β -lactamase producing bacteria usually associated with adverse clinical outcomes because this enzyme shows resistance to all β -lactam antibiotics except carbapenems and cefepime (3). Phenotypic tests for AmpC β -lactamases detection are not well defined and organisms that produce these types of enzymes often go undetected and have been responsible for several nosocomial outbreaks (11); thus, the aim of this study was to develop a more practical and simplified laboratory technique by modifying the three-dimensional method for the detection of AmpC β -lactamases in *Enterobacter* spp. and *Escherichia coli*.

Methods

A total of twenty eight clinical isolates including 11 isolates of *Enterobacter cloacae*, 2 isolates of *Enterobacter aerogenes* and 15 isolates of *Escherichia coli* were identified by API 20 E system (bioMerieux Vittek). The isolates, all from wound patients, obtained from previous work conducted in AL-Ramadi General Hospital in 2008. This study was performed in the Microbiology Laboratory/ College of Education/ University of Sulaiman/ Kalar.

The susceptibility of isolates to cefoxitin disk 30 μ g (from bioanalyse company. Ankara-Turkey) were tested by standard disk diffusion method on Mueller-Hinton agar (from Himedia. India) with 24-h incubation at 35°C (12), this test was used as a primary screening for detection of AmpC β -lactamase and isolates with zone inhibition diameter <18 mm considered to be positive screen according to the criteria used by Coudron et al (7). The test repeated three times with different occasions then the mean was calculated. Susceptibility testing of *Escherichia coli* isolates to β -lactam antibiotics was extended further to include piperacillin (PIP 75 μ g), cefotaxime (CTX 30 μ g), ceftazidime (CAZ 30 μ g), ceftriaxone (CRO 30 μ g), aztreonam (ATM 30 μ g), imipenem (IMP 10 μ g), the results were interpreted according to the standard zone diameter recommended by Soussy et al. (12).

Isolates then tested for its production of AmpC β -lactamase by the herein (in this study) modified three dimensional method which was originally described by (2). The surface of Muller-Hinton agar plate was inoculated with *E. coli* ATCC 25922 standard strain

(surface organism, 106 cfu/ml), 30 μ g cefoxitin disk was placed on the inoculated agar in the centre, with a sterile scalpel blade a slit beginning about 5 mm from the edge of the cefoxitin disk was cut in the agar in outward radial direction (25 mm length,); in the original 3DM description, the bacterial preparation had to undergo a procedure for bacterial lysis then the suspension placed directly into the slit (2). While, the herein modification was at the end of the slit a circular well with a diameter of 5 mm was made in the agar and filled with 30 μ l of freshly prepared bacterial suspension in 0.9% saline adjusted the density to equal a McFarland 4 turbidity standard, ~109 cfu/ml (13), of the test isolate (omitting the bacterial lysis procedure required in the original method, 3DM, and the bacterial suspension was not placed directly into the slit from the surface). Plates were incubated overnight aerobically at 35 oC. *Escherichia coli* ATCC 25922 was used as negative control.

Results

In the screening test for AmpC β -lactamase all *Enterobacter* isolates 13/13(100%), and one *Escherichia coli* isolate 1/15(7%) yielded cefoxitin zone diameters less than 18 mm, screen positive (2) (Table 1), and showed positive result with the M3DM by clearly enhanced growth of the surface organism (*E. coli* ATCC 25922) at the point where the slit intersected the zone of inhibition, while all cefoxitin-susceptible *Escherichia coli* isolates 14/15(93%) showed M3DM negative results (figure 1). The results of disk diffusion test of *Escherichia coli* isolates were non-susceptible 11/15(73%) to PIP and CAZ, 10/15(67%) to CTX, CRO, and ATM, and 1/15 (6.7%) to FOX, while all isolates (100%) were susceptible to imipenem (IMP) as shown in table 2.

Discussion

It is important for clinical microbiology laboratories to be able to detect the AmpC β -lactamase producing isolates because these enzymes have a risk for developing resistance during treatment of infected patients with broad-spectrum penicillins and cephalosporins (14), hence production of these enzymes in clinically significant *Enterobacteriaceae* represents an increasing problem resulting in higher morbidity and mortality (15). Cefoxitin was used as screening agent for AmpC β -lactamase because this

antibiotic is stable against the activity of multiple β -lactamase like TEM-1,

-2, SHV-1 and ESBLs but hydrolyzed by AmpC enzyme (16). Therefore, the resistance to cefoxitin may be due to the presence of AmpC enzyme or reduction in outer membrane permeability (2, 11, 17).

The 3DM with cefoxitin can be used to detect the presence of AmpC β -lactamase in isolates with multiple β -lactamases, the positive result in this test inferring the presence of AmpC enzyme, and not reduction in the outer membrane permeability, to be responsible for the resistance to cefoxitin, in addition the test with specific AmpC inhibitor more appropriate when available (3, 18).

All the 13 *Enterobacter* isolates (100%) and one isolate of *Escherichia coli* 1/15 (7%), showed decrease susceptibility to cefoxitin and gave positive results in the M3DM by enhanced growth of surface organism inside the inhibition zone of cefoxitin disk near the slit, demonstrating hydrolysis of cefoxitin by AmpC β -lactamase and excluding the possibility of decreased permeability to cefoxitin (11), while the cefoxitin-susceptible *Escherichia coli* and *Escherichia coli* ATCC 25922 showed negative results Fig.1.

The occurrence of AmpC β -lactamase enzyme in *Escherichia coli* is less frequent (1/15, 6.7%) than in *Enterobacter* spp. (12/12, 100%), because all *Enterobacter cloacae* and *Enterobacter aerogenes* have the chromosomal gene for the production, inducible and/or constitutive, of AmpC enzymes which cause cefoxitin resistance; while the chromosomal gene in *Escherichia coli* is usually expressed constitutively at low level due to weak promoter and strong attenuator (2, 10). The apparently hyperproduction of AmpC enzymes, in this *Escherichia coli* isolate may be due to mutation in the AmpC chromosomal gene or acquisition of plasmid-mediated AmpC β -lactamases (9). Thus, upon using cephalosporins, resistant variants (mutants) of this *Escherichia coli* isolate may be selected and eventually emergence can be overwhelming and consequently resulting in life-threatening treatment failure (11). Further, the cefoxitin resistant isolate showed resistance to all β -lactam antibiotics used except imipenem as it is not hydrolyzed by AmpC β -lactamases (1, 16) and it is recommended to be used in this clinical condition.

The susceptibility testing of *Escherichia coli* isolates (table 2) showed low susceptibility to third generation cephalosporin in the presence of high

susceptibility to cefoxitin, is strongly indicative of ESBLs "extended spectrum β -lactamases" production which further requires confirmatory tests with combination of β -lactam and β -lactamase inhibitor clavulanate (12).

An important clinical feature of ESBLs is that they are capable of breaking down β -lactam antibiotics such as CTX, CAZ and CRO as well as the monobactams such as ATM, but they usually remain susceptible to carbapenems, cephamycin, and β -lactamase inhibitors, like clavulanic acid (16). Further, the 100% susceptibility exhibited by imipenem is consistent with the notion that it is the most active one of all β -lactam antibiotics and remains very stable to the action of AmpC β -lactamase and ESBL enzymes (1).

In this study, a reasonable standardization was used with a constant inoculum size, and constant dimensions measurement uniformed for all isolates, evading the bacterial lysis procedure, instead a bacterial suspension of the test isolate is placed in a well open into the slit allowing the suspension to seep inside the slit by capillary effect, and thus, avoiding applying the suspension from the top surface of the plate that otherwise may cause contamination of the surface with the test bacteria that the growth of which may interfere with the response of the surface standard bacteria making reading the results more difficult.

Also the use of crude enzyme preparation from individual isolate if available are more appropriate to differentiate between the presence of AmpC β -lactamase or reduced outer membrane permeability (16), but the simple technique in this study with minimal cost is useful and can be used in clinical laboratories for detection of AmpC β -lactamase in all Gram-negative bacteria.

It is concluded that the M3DM provides a cost-effective alternative for AmpC β -lactamase testing in clinical microbiology laboratories, thus negating the need for bacterial lysis procedures required in the 3DM.

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Table 1. Diameters of inhibition zone of cefoxitin disk diffusion test for the study *Enterobacter* spp. and *Escherichia coli* isolates.

Isolate	Diameters of inhibition zone (mm, n=3) Mean \pm SD	Isolate	Diameter of inhibition zone (mm, n=3) Mean \pm SD
<i>Enterobacter cloacae</i> 1	6.0 \pm 0.0	<i>Escherichia coli</i> 2	25.0 \pm 0.0
<i>Enterobacter cloacae</i> 2	8.3 \pm 0.57	<i>Escherichia coli</i> 3	28.3 \pm 0.57
<i>Enterobacter cloacae</i> 3	8.3 \pm 0.57	<i>Escherichia coli</i> 4	22.3 \pm 1.52
<i>Enterobacter cloacae</i> 4	9.3 \pm 0.57	<i>Escherichia coli</i> 5	30.3 \pm 0.57
<i>Enterobacter cloacae</i> 5	7.3 \pm 0.57	<i>Escherichia coli</i> 6	33.0 \pm 1.0
<i>Enterobacter cloacae</i> 6	7.3 \pm 0.57	<i>Escherichia coli</i> 7	26.3 \pm 0.57
<i>Enterobacter cloacae</i> 7	6.3 \pm 0.57	<i>Escherichia coli</i> 8	23.3 \pm 0.57
<i>Enterobacter cloacae</i> 8	6.0 \pm 0.0	<i>Escherichia coli</i> 9	30.0 \pm 0.0
<i>Enterobacter cloacae</i> 9	6.0 \pm 0.0	<i>Escherichia coli</i> 10	24.0 \pm 1.0
<i>Enterobacter cloacae</i> 10	7.0 \pm 0.0	<i>Escherichia coli</i> 11	27.0 \pm 0.0
<i>Enterobacter cloacae</i> 11	7.0 \pm 0.0	<i>Escherichia coli</i> 12	25.0 \pm 1.0
<i>Enterobacter aerogenes</i> 12	7.3 \pm 0.57	<i>Escherichia coli</i> 13	32.3 \pm 0.57
<i>Enterobacter aerogenes</i> 13	6.3 \pm 0.57	<i>Escherichia coli</i> 14	26.3 \pm 0.57
<i>Escherichia coli</i> 1	10.3 \pm 0.57	<i>Escherichia coli</i> 15	23.3 \pm 0.57

Table 2. The susceptible and non-susceptible number and percentage of *Escherichia coli* isolates to the tested antibiotics.

Antibiotic	PIP	CTX	CAZ	CRO	ATM	FOX	IMP
Susceptibility							
Non-susceptible (%)	11/15 73%	10/15 67%	11/15 73%	10/15 67%	10/15 67%	1/15 6.7%	0/15 0%
Susceptible (%)	4/15 27%	5/15 33%	4/15 27%	5/15 33%	5/15 33%	14/15 93.3%	15/15 100%

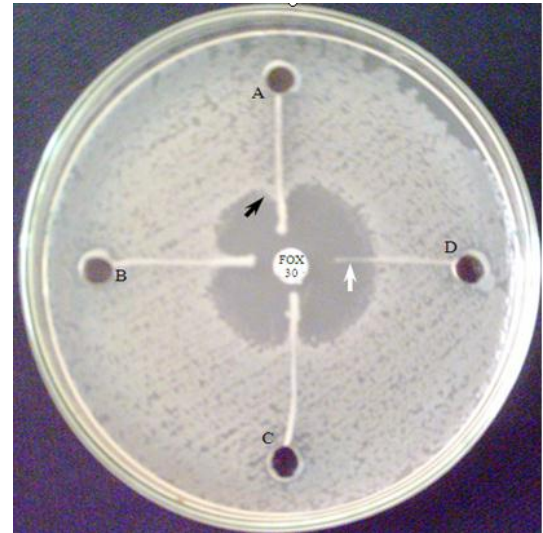
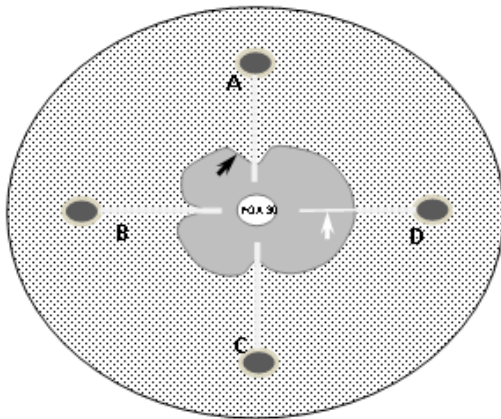


Figure 1. M3DM patterns for four isolates shown on the left, actual photograph of the Petri dish, on the right, a drawing depicting the essential details. Enhanced growth of the surface organism *Escherichia coli* ATCC 25922, is seen near agar slits (black arrow) that contain bacterial suspensions of test *Enterobacter* isolates (A, B cefoxitin non-susceptible *Enterobacter*) and (C) contain cefoxitin non-susceptible *Escherichia coli* isolate, all AmpC producers showed the positive results, while (D) contain a bacterial suspension of cefoxitin-susceptible *Escherichia coli* isolate showed the negative results. Note: poor growth along in slit D and in the region lying within the inhibition zone of cefoxitin disk (white arrow) reflecting susceptibility of the isolate.

تحويل طريقة إختبار ثلاثي الأبعاد للكشف عن أنزيمات AmpC بيتالاکتاميز في *Enterobacter* و *Escherichia coli* spp.

سرور مصطفى محمد

الخلاصة:

الغفل المختبري في الكشف عن انزيمات بيتالاکتاميز نوع امبلر صنف C في البكتريا السالبة لملون غرام يمثل السبب الرئيسي لانتشار البكتريا المنتجة لهذه الأنزيمات وربما يؤدي الى انخفاض في النتائج العلاجية. استهدفت الدراسة الحالية إيجاد طريقة مختبرية عملية بسيطة عن طريق تحويل إختبار ثلاثي الأبعاد للكشف عن انزيمات بيتالاکتاميز امبلر صنف C في عزلات مرضية للانتروبيكتريا والايشريشيا القولونية. تم اخضاع ٢٨ عزلة مرضية تضمنت ١١ عزلة من الانتروبيكتريا كلويكيا، عزلتان من الانتروبيكتريا ايروجينز و ١٥ عزلة من الايشريشيا القولونية لاختبار الحساسية تجاه مضاد السفوكستين وبطريقة انتشار الاقراص القياسية كمنح اولي للكشف عن انزيمات بيتالاکتاميز امبلر صنف C. ثم اخضعت العزلات للاختبار ثلاثي الأبعاد والمحور باستخدام السفوكستين للكشف عن انتاج انزيمات بيتالاکتاميز امبلر صنف C. وكذلك اختبرت حساسية عزلات الايشريشيا القولونية تجاه عدد من مضادات البيتا لاکتام. اظهرت جميع عزلات الانتروبيكتريا وعزلة واحدة من الايشريشيا القولونية مقاومة تجاه مضاد السفوكستين وأعطت نتيجة موجبة للاختبار ثلاثي الأبعاد والمحور المتمثلة بزيادة نمو البكتريا المزروعة على سطح الوسط الزرع والحساسية للسفوكستين في منطقة تقاطع الشق مع منطقة التثبيط؛ بينما أظهرت جميع العزلات الحساسية للسفوكستين نتيجة سالبة للاختبار، نسبة عالية من عزلات الايشريشيا القولونية ظهرت غير حساسة تجاه مضادات البيتا لاکتام المستخدمة باستثناء الامينيم اذ اظهرت جميع العزلات حساسة تجاهها. نستنتج ان طريقة الإختبار الثلاثي الأبعاد المحور يمثل بديلا بسيطا وغير مكلف للكشف عن إنزيمات بيتالاکتاميز امبلر صنف C في مختبرات الإحياء المجهرية السريرية.