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Laboratory Detection and Neutralizing Activity of Exocellular AmpC β-lactamases by Anti bla-CMY

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Abstract

Detection of AmpC β-lactamases (AmpC-bls) is important for infection control purposes and therapeutic options. Here, we provided a diagnostic anti β-lactamase neutralization test (bla-NT); modified from broth microdilution (BM) for the detection of bls-AmpC, CMY, in multidrug resistant *Escherichia coli* and *Klebsiella pneumoniae*. Anti-bla neutralizing activity against these two bacteria was tested. Anti bla-CMY was prepared in rabbits and used in: bla-NT; investigating effect on bacterial colony forming unit (CFU); and in ELISA. In bla-NT, the anti-bla-CMY neutralized exocellular bls produced by the tested bacterial strains and resulted in an increase in the bacterial sensitivity to the tested antimicrobials and reduction in minimum inhibitory concentration. Interestingly, the anti-bla-CMY decreased the CFU and its morphology when added to the tested bacteria. ELISA-OD was significantly correlated with the drop in minimum inhibitory concentration and CFU counts at P-value ≤ 0.05 and 0.01, respectively. It could be concluded that, bla-NT could detect bls-AmpC and run parallel to BM in microbiology laboratory. Investigations are running to develop the test for quantitative detection of bls-AmpC.

Keywords: *E. coli*, *K. pneumoniae*, Multidrug resistant bacteria, β-lactamases, AmpC

Introduction

AmpC β-lactamases is β-lactmase antibiotics destroying enzyme that has a special clinical interest since late 1980s. It is associated with multiple antibiotic resistance to cephalothin, cefazolin, cefoxitin, most penicillins and bla- inhibitor/β-lactam combinations. Bls enzymes over expression confer resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone [1]. The plasmid-determined enzymes are related, sometimes very closely to chromosomally determined bls-AmpC. Several varieties from the following enzymes, CMY, FOX, ACC, LAT, MIR, ACT, MOX and DHA were detected [2-4]. *Escherichia coli* and *Klebsiella pneumoniae* have a great concern when produce Extended Spectrum β-Lactamases (ESBLs) associated with plasmid mediated bls-AmpC, which should be taken seriously and should be early diagnosed in clinical laboratory. Therefore, it is essential that clinical laboratories accurately detect strains producing bls-AmpC [5]. There are presently no Clinical and Laboratory Standards Institute (CLSI) or other approved criteria for bls-AmpC detection [6].

Some Gram-negative bacteria producing both AmpC and ESBL give positive results in ESBL screening test but fail the confirmatory test involving increased sensitivity with clavulanic acid [7,8]. This phenotype give false positive results in stains producing high level bla TEM-1, OXA-type ESBLs and carbapenemases and that with complex mutation of TEM [9]. Cefoxitin resistance for detection of AmpC enzymes is not specific since cefoxitin resistance can also be produced by certain carbapenemases [10], few classes A- bla [11] and by decreased levels of production of outer membrane porins in both *K. pneumoniae* and *E. coli* [12]. Limited results interpretation in the aforementioned techniques was recorded. Meanwhile, detection by antibody based methods was used with success. Specific antibodies against β-lactamases can neutralize the enzyme reaction and neutralization tests have led to classification of β-lactamases of Gram-negative bacteria into different classes [13-15]. ELISA and immunoblotting could screen and quantify AmpC, SHV and TEM bls with high sensitivity and specificity. In addition, ELISA

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can permit careful study of the effects of point mutations on bls and exploration of issues of induction and regulatory mechanisms affecting bls production [16-19]. ELISA is costly and laborious and could not be routinely used for the detection of bls in clinical laboratories, in addition, DNA based methods does not always indicate enzyme production.

In the present work, we provide bla-NT as modified test from broth microdilution test for accurate detection of bls-AmpC and for determining the possible activity on the multidrug resistant bacterial strains.

Material and methods

Bacterial isolates

Eighty-eight samples were collected from different sources. The samples included nineteen human urine samples; 19 specimens from livers of freshly dead broilers and 50 diarrheic fecal samples from sheep. Samples were inoculated onto MacConkey's and Eosine Methylene Blue agar media (Oxoid, UK) and then incubated at 37ºC for 24 h. Isolates were identified to the species level with API 20E system (BioMe´rieux, Marcy l'Etoile, France.

An *E. coli* control positive strain (code No. HE1) was previously known as bla-CMY producer after testing with the specific primers [20]. Periplasmic extracts of these strains were used for the preparation of anti-CMY serum in rabbits. It was kindly supplied by Microbiology Department, Faculty Veterinary Medicine, Zagazig University, Egypt.

Extraction of β-lactamases

Periplasmic extracts were prepared from *E. coli* and *K. pneumoniae* as previously described [21]. Briefly, the bacterial strains were inoculated into tryptic soya broth with ampicillin at concentration of 100 mg/L, incubated at 37ºC for 24 h. At the end of incubation period; bacterial sediments were centrifuged at 3000 r.p.m. for 15 min. Bacterial pellets were suspended in 500 µL (Tris HCl, 50 mM, pH 7.4) and 2 µL of lysozyme solution (40 mg/mL) for 30 min. Periplasmic crude extracts were obtained after 14,000 r.p.m for 15 min. Periplasmic extracts protein concentration of the test and control strains were determined (0.66-1.04 mg/ml) and the extracts were examined for 38 KD band by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Production of anti-β-lactamases polysera in rabbits

Polyclonal antisera against periplasmic extracts containing bla-CMY were produced in rabbits [18], these extracts were obtained from the *E. coli* control strain (code No. HE1) previously known and confirmed as bla-CMY producer. Four female New Zealand white rabbits each weighing approximately 1.5 kg were used in the study. Three rabbits were injected in multiple sites I.M and S.C with one mL complete Freund's adjuvant (CFA) and periplasmic extract with protein content at concentration of 500 µg and one rabbit received CFA and served as control. One week from the initial dose, rabbits were given two booster doses each of one mL incomplete Freund's adjuvant containing 250 µg periplasmic extract at interval of 2 weeks in between each injection. A final dose of 100 µg purified extract suspended in sterile PBS was injected I.V. The control rabbit was injected at the same intervals with sterile PBS during the course of experiment. Anti-bls polysera development was monitored before each inoculation by double immunodiffusion [21]. Two weeks after the final injection, the four rabbits were bled, blood was collected and the sera were harvested. Sera were heat-inactivated, divided into aliquots and stored at -20ºC.

Disc diffusion

Resistance phenotypes were detected using β-lactam antibiotic discs: amoxicillin (25 µg), amoxicillin/ clavulanate (3/30), cephalexin (30 µg), aztreonam (30 µg), cefuroxime (30 µg), cefepime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), imipenem (10 µg) and piperacillin (75 µg). Strains with multiple resistances to β-lactams were included in this study. Zone size interpretation of antimicrobial agents was according to Clinical Laboratory Standards Institute (CLSI) M100-S24 [22].

Broth microdilution (BM)

Broth microdilution was run as previously done [23], 100 µL of serial double-fold

dilution of ceftazidime in MHI were added to equal amount of MHI containing 2.5 X 10^6 CFU/mL of the examined strains. Microtiter plates were incubated for 18 h at 35ºC. MIC of ceftazidime and CFU counts in each MIC well were examined.

β-lactamase neutralization test (bla-NT)

β-lactamase neutralization test is a modified broth microdilution. We hypothesized that rabbit polysera containing anti-bla-CMY added in the test vicinity may neutralise the produced AmpC-bls by the bacteria under the test conditions and may protect the β-lactam (ceftazidime) in the test conditions, and hence may raise sensitivity of that bacteria to the β-lactam leading to detectable decrease in MIC. Briefly, 100 µL of serial double-fold diluted ceftazidime in Muller Hinton broth (MH) were added to equal amounts of MH containing 2.5 X 10^6 CFU/mL of the examined bacteria. After being mixed well, 10 µL of rabbit polysera containing anti-bla-CMY were added to each well. Control positive wells without polysera and contain instead 10 µL periplasmic bls extracts (0.01 mg to each well of the assay) were included. Microtiter plates were incubated for 18 h at 35ºC. MIC of ceftazidime and CFU counts in each MIC well were detected.

Percent drop of ceftazidime MIC and CFU counts in bla-NT were calculated as follows:

- % drop of ceftazidime MIC= Ceftazidime MIC in BM - ceftazidime MIC in bla-NT/ ceftazidime MIC in BM X100.

- % drop of CFU counts = CFU counts in MIC well of BM - CFU counts in MIC wells of bla-NT/ CFU counts in MIC well of BM X100.

Indirect ELISA

Indirect ELISA was done according to the protocol of Tijssen [23]. Ninety-six-well immunoassay plates (Fisher) were coated overnight with 2 µg/mL of periplasmic extracts of the examined strains suspended in fresh carbonate-bicarbonate buffer (pH 9.6). Plates were washed 3x times with PBS containing 0.05% tween 20, and then blocked with 5% bovine serum albumin in PBS for 2 h. Sera dilutions of immunized and non-immunized rabbits were done and 100 µL/well were added from each dilution and the plates were incubated for 2 h at room temperature. After being washed 3x in PBS, diluted horse radish peroxidase (HRP) conjugated goat-anti-rabbit IgG (KPL, San Antonio, CA, USA) was added at volume of 100 µL/well in PBS and the plates were incubated for an hour. Checkerboard titration of primary antibody and conjugate was done to determine OD values of periplasmic extracts. Plates were incubated with ABTS substrate solution for 15-45 min at room temperature, color development was terminated by the addition of 0.5 M of H_2SO_4. OD was determined at 405 values using ELISA plate reader (Cerus Corporation, CA, U.S.A).

Results

Strains resistance profile to β-lactams

E. coli and *K. pneumoniae* were resistant to amoxicillin, amoxicillin/ clavulanate, aztreonam, cephalexin, cefuroxime, cefotaxime, ceftriaxone and cefoxitin, with variable resistance to piperacillin and cefepime according to disc diffusion. All strains isolated from chickens, sheep and humans were extremely resistant to β- lactams, and they were resistant to seven or more of β- lactams but sensitive to ceftazidime and imipenem. Except an *E. coli* isolated from human, all the strains were resistant to cefepime. All strains were sensitive to ceftazidime, therefore, this antimicrobial was used in bla-NT and broth microdilution (Table 1).

Table 1: Minimal inhibitory concentrations (MIC) of ceftazidime (CAZ) in β-lactamase neutralization test (Bls NT) and broth microdilution (BM) against *E. coli* and *K. pneumoniae* resistant phenotypes

Strain	Strain source	Strain code	β-lactam resistant phenotype	BM	bls NT		
						% drop in	
				CAZ MIC	CAZ MIC	CAZ MIC	CFU counts
E. coli	Human	HE1*	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, PRL	1.0	0.125	87.5	100
		HE6	AX,AMC,CL,CXM,CTX,FOX,AZ	4.0	0.50	87.5	100
	Broiler	BE1	AX,AMC,CL,CXM,CTX,CRO, FOX,AZ, PRL, FEB	1.0	0.25	87.5	100
		BE12	AX,AMC,CL,CXM,CTX,CRO, FOX,AZ, PRL, FEB	2.0	0.50	75.0	100
		BE18	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, FEB	1.0	0.25	87.5	100
	Sheep	SE12	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, PRL, FEB	0.25	0.125	50.0	40
K. pneumoniae	Human	HK1	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ	1.0	0.50	50.0	50
		HK3	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, PRL, FEB	0.5	0.25	50.0	40
		HK6	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, PRL, FEB	1.0	0.50	50.0	66
		HK7	AX,AMC,CL,CXM,CTX,CRO,FOX,AZ, FEB	0.5	0.25	50.0	50

* = Control positive strain *E. coli* standard strain. AX: amoxicillin, AMC: amoxicillin/clavulanate, CL: cephalexin, AZ:aztreonam, CAZ:ceftazidime, CXM:cefuroxime, CTX: cefotaxime, CRO: ceftriaxone, FEP:cefepime , FOX:cefoxitin, PRL: piperacillin.

β-Lactamases were detected in the tested antibiotics resistant bacteria

Periplasmic extracts of the tested and control strains were examined for bla-CMY. A band of 38 KD was seen which is characteristic for CMY β-lactamase and was at the same level of the standard and the positive control (Figure 1A).

Neutralization of exocellular β-lactamases in bla-neutralization test

Anti-bla CMY reduced the MIC of the multidrug resistant strains

Anti-bla-CMY sera neutralizing activity against *E. coli* and *K. pneumoniae* strains confirmed that they are multidrug resistant. MIC of ceftazidime was detected in bla-NT and broth microdilution (Table 1). MIC of ceftazidime against the *E. coli* and *K. pneumoniae* bacterial strains ranged 0.25-4.0 μg/mL and 0.125-1.0 μg/mL, respectively, in broth microdilution. The anti-CMY-bla neutralized exocellular β-lactamases produced from the bacteria under the induced ceftazidime conditions in bla-NT test raised sensitivity of the bacteria to ceftazidime with 50-87.5% drop of ceftazidime MIC. Control wells with periplasmic extracts instead of polysera showed raised ceftazidime MIC 1-2-fold among the examined strains (Table 1).

Anti-bla CMY reduced the bacterial colony forming units (CFU)

Colony counts were determined in the anti-CMY treated wells. Interestingly, a drop of CFU counts in MIC wells of bla-NT ranged 40-100% (Table 1). The drop in the CFU was not restricted to the sample source where all the tested strains revealed drops in the CFU regardless their origin (Table 1).

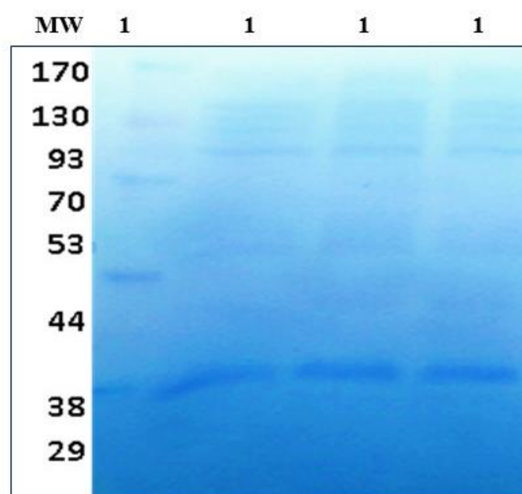

Figure 1: Molecular weight of the purified extracts. After extraction of Bla-CMY and protein quantification, three tested periplasmic extracts showed 38 kb bands (2-4) that were comparable to the control (1).

Anti-bla-CMY changed the bacterial colony phenotypes

Subculture of the strains from MIC wells of bla-NT was done. After being cultured overnight, the bacterial colonies were examined. The bacterial colonies of the MIC antisera treated wells were pin point and flat colonies; meanwhile colonies from MIC wells of broth microdilution were normal elevated and circular (Figure 1B). The colonies were dense and pasty in exposed to anti-bla neutralizing sera compared with few and dry colonies of the same bacterial strain that not exposed to antisera.

Correlation of indirect ELISA with bla-neutralization test

ELISA-OD of periplasmic extracts correlated with the results of bla-NT (Table 2). Correlation coefficient revealed strong direct correlation of ELISA with percent drop of both ceftazidime MIC and CFU counts in MIC wells of bla-NT. Correlation coefficient of 0.725 and 0.796 with corresponding P-value less than 0.05 and 0.01 were detected, respectively. Moreover, CMY-bla produced from an *E. coli* strain isolated from sheep (code no. SE12) was only detected in bla-NT. Bla-OD value of this isolate was similar as that of negative control sample (Table 2).

Table 2: ELISA-OD and parameters of β-lactamases neutralization test (Bls NT) for detection of CMY-β-lactamases in *E. coli* and *K. pneumoniae*

Method		Strains code No.									
		E. coli						*K. pneumoniae*			
		HE1*	HE6	BE1	BE12	BE18	SE12	HK1	HK3	HK6	HK7
ELISA	**O.D.**	0.55	0.49	0.42	0.37	0.36	0.03	0.34	0.24	0.37	0.23
Bls NT	**drop % of ceftazidime MIC**	87.5	87.5	87.5	75.0	87.5	50.0	50.0	50.0	50.0	50.0
	% drop of CFU counts	100	100	100	100	100	40.0	50.0	40.0	66.0	50.0

* = Control positive strain O.D=optical density

Discussion

Resistance due to plasmid mediated AmpC enzymes is less common than extended spectrum bls (ESBL) production in some parts of the world but may be both harder to detect and broader in spectrum. Other parts suffer from annual increased incidence of bls-AmpC in *E. coli* and causing mainly urinary tract infections in older women [24]. Detection of plasmid-mediated AmpC-bls by three-dimensional test, cefoxitin agar method, clover leaf test, and double-disk test have disadvantages of difficult results interpretation [25]. Currently, there are no recommendations available from the CLSI for detection of organisms producing plasmid-mediated AmpC-bls where many bls-AmpC producers are not resistant to broad spectrum cephalosporins by CLSI breakpoints. Yet, adverse clinical outcomes occur in these patients when treated with cephalosporins [20]. In vicinity of bla-NT, anti-CMY-bla neutralized the lactamases produced by the bacteria under test, therefore, the sensitivity of the bacteria to ceftazidime with drop of ceftazidime-MIC and CFU counts in MIC wells of bla-NT raised. In the present work, *E. coli* and *K. pneumoniae* strains were resistant to cefoxitin and clavulanate, some of them were resistant to cefepim but sensitive to ceftazidime and imipenem (Table 2).

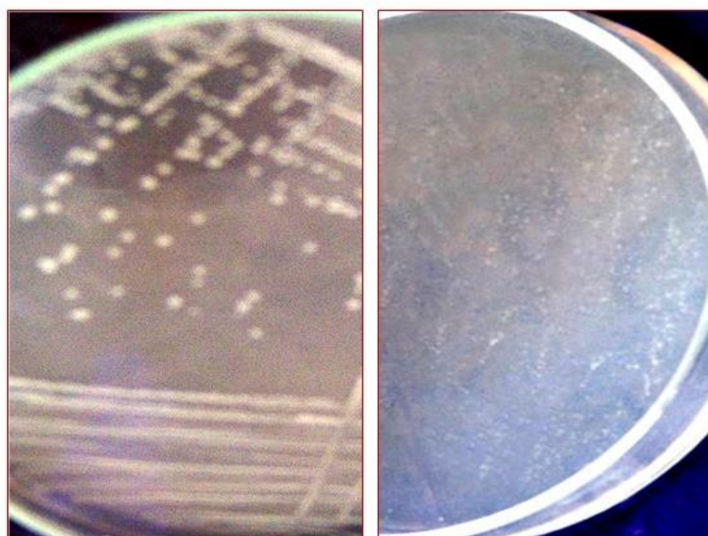

Figure 2: Bacterial colony shape after subculture from MIC wells. Right plate show normal circular pasty colonies (control), left plate show pin point colonies after to neutralization of β-lactamses by anti-CMY serum in vicinity of bla neutralization test.

This antibiogram might be an indicator for bls-AmpC, in addition, anti CMY polyserum used in bla-NT was raised against bls with molecular weight 38 kb, these bls were obtained from PCR confirmed CMY-bla producing strain [1,15]. Anti-bla CMY used in bla-NT does not crossly react with other lactamases. Previously, no cross-reactivity of the anti-CMY-2 antibody was seen against TEM-1, SHV-1, K-1, or OXA- bla but polyclonal rabbit anti-bla CMY-2 could detect CMY-2, P99 and ACT-1 by ELISA [25]. Antibody based methods depended previously on extracted periplasmic bls from bacterial periplasm [18,26], and it was suggested that level β-lactamase expression due to either promoter mutations or gene copy number which may affect the ability of bla-SHV detection in Gram-negative bacilli using fluorescein-labeled anti-bodies [27]. Meanwhile, in this work, exocellular bls-AmpC were encoded and expressed sufficiently from bacteria under the conditions of bla-NT and then detected by specific polyserum. There was a strong direct correlation 0.725 of bla-NT with indirect ELISA, in addition, β-lactamases were expressed sufficiently in bla-NT vicinity that lead to AMPC-bla was detected in one more

isolate (code No.SE12, Table 2). PCR is the "gold standard" for the identification of only bla genes but could not detect expressed enzymes, in addition, PCR/sequencing is less feasible [28]. AmpC-bls expression screening could be detected by bla-NT. By extension, there will be interest to develop bla-NT quantitative detection of produced bls-AmpC and improve antibody-antigen combination in the test vicinity. ELISA could detect picograms of lactamases in periplasmic extracts of Gram-negative bacteria, meanwhile, ELISA is not routinely used to study clinical samples because they are expensive and time consuming. Bla-NT could be used routinely in microbiology laboratory and go parallel to standardized broth microdilution.

Conclusion

AmpC-bls produced from *E. coli* and *K. pneumoniae* strains could be detected by anti-CMY-bla added in vicinity of bla neutralization test. Results of bla-NT correlate with ELISA-OD. More AmpC producing strains could be detected due to sufficient expression of lactamases in bla-NT test conditions, therefore, it could be run parallel to standardized broth microdilution in clinical microbiology laboratory. Further work is required for quantitative detection of bls-AmpC and gene expression in Gram negative bacteria.

Conflict of interest

The authors declare no conflict of interest

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الملخص العربي

استخدام مضادات إنزيمات CMY لتحديد مفرازات إنزيماتAmpC

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تحديد إنزيمات بيتا لاكتاماز ذو اهميه في العلاج والسيطره علي الوبائيات. وفي هذا البحث تم معادله هذه الانزيمات في تجربه معدله من اختبار الحساسيه للمضادات الحيويه (Bla-NT) بطريقه الشوربه المخففهbroth microdilution وذلك لتحديد هذه الانزيمات في ميكروبي الكولاي القولوني والكليبسيلا وتم تحضير مضادات الانزيمات في الارانب ليتم استخدامها في تقنيه الاليزا وفي هذا الاختبار ولقد اظهرت النتائج ان الانزيمات الناتجه في محيط الاختبار تم معادلتها واصبح الميكروب اكثر حساسيه للمضاد الحيوي المضاف في محيط الاختبار مع نقص في قيمه الادني المثبط وقد كانمت نتائج الاليزا مؤكده لهذه النتائج وذلك في المعدل الاحصائي $P\text{-value} \leq 0.05$, 0.01 ومما سبق نستنتج ان التجربه المعدله من اختبار الشوربه المخفف يمكن ان تستخدم في معامل التشخيص لتحديد انواع البيتا لاكتاماز وذلك علي التوازي مع اختبارات الحساسيه للمضادات الحيويه الدارجه.