Genetic aspects of Ambler class C, extended spectrum and metallo-beta-lactamases among beta-lactam resistant Escherichia coli and Klebsiellae pneumoniae

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الخلاصة

ان الهدف من هذه الدراسة هو لتحديد دور انزيمات البيتالاكتاميز من نوع امبلر صنف C والميتالو في بكتريا الايشيرشيا القولونية المقاومة لمضادات البيتالاكتام والعناصر الوراثية المشفرة لانتاجها . انجزت اختبارات المسح الاولية والاختبارات المظهرية التاكيدية المعتمدة من قبل الجمعية الدولية للمقاييس المختبرية السريرية ل25 عزلة للايشريشيا القولونية و15 عزلة للكلبسيلا الرئوية . تم اجراء تقنيات عزل البلازميدات وتحييدها. اجريت عملية نقل البلازميدات بطريقة الاقتران الوراثي. اظهرت النتائج بان 17(68٪) 11(61٪) من عزلات الايشيريشيا القولونية والكلبسيلا الرئوية على التوالي تاثير حامض الكلافيولانك بطريقة تازر الاقراص الثنائية فيما اظهرت 15 (60٪) و10(55.6٪) هذا التاثير بواسطة التخافيف الدقيقة في المرق المغذي. ان الجينات المسؤولة عن انتاج انزيمات البيتالاكتميز واسعة النطاق ل13 (86.7٪) و 9(90٪) من عزلات الايشريشيا القولونية والكبسيلا الرئوية المنتجة لهذا النوع من الانزيمات كان محمولا على بلازميدات اقترانية فيما شفرت هذه البلازميدات ل3(30٪) ، 3(37.5٪) من العزلات اعلاه المنتجة لانزيمات امبلر صنف C وعزلة واحدة من الايشيريشيا القولونية المنتجة لانزيم الميتالوبيتالاكتاميز. تستنتج الدراسة بان انزيمات البيتالاكتاميز الواسعة النطاق ، امبلر صنف C تلعب دورا اساسيا في مقاومة للايشيريشيا القولونية والكلبسيلا الرئوية لمضادات البيتالاكتاميز كذلك فقد وجد بان البلازميدات الاقترانية كانت العنصر الوراثي الرئيسي المشفر لانزيمات البيتالاكتاميز واسعة النطاق وبدرجة اقل لانزيمات امبلر المنتجة من قبل عزلات الدراسة. اظهرت الدراسة بروز عزلة مقاومة لمضادات البيالاكتام منتجة لانزيمات الميتالولاكتاميز بواسطة بلازميد اقتراني .

Objective: To detect the role of Ambler class C (AmpC), extended spectrum (ESBL) and metallo-B-lactamases in the resistance of *Escherichia coli* and *Klebsiellae pneumoniae* to P-lactams and whether the genetic expression of these enzymes are encoded by conjugative plasmids DNA or chromosomal DNA.Methods: The National Committee for Clinical Laboratory Standard AmpC and ESBLs screening and phenotypic confirmatory tests were performed for 25 isolates of *Escherichia coli* and 18 isolates of *Klebsiellae pneumoniae.* Plasmid profile analysis and curing experiments were performed. Plasmid DNA recombination for resistant genes was carried out by conjugation.Results: Initially, 17 (68.0%),11 (61.1%) isolates of£ *coli* and *K pneumoniae* respectively demonstrated a clavulanic acid (CA) effect by double disk synergy and 15 (60.0%), 10 (55.6%) isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively demonstrated a CA effect by broth microdilution tecnhique.The genes encoding for ESBL in 13 (86.7%), 9 (90.0%) isolates of ESBL producing *Escherichia coli* and *Klebsiellae pneumoniae* respectively were carried by conjugative plasmids while this genetic elements were encoded for 3 (30.0%), 3 (37.5%) of AmpC producing isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively. Further, the resistant genes encoding for metallo-p-lactamase in one isolate of *Escherichia coli* was carried by conjugative plasmid.Conclusions: The study concluded that ESBLs and AmpC cephalosporinases play an essential role in the resistance of *Escherichia coli* and *Ktebsiellae pneumoniae* to P-lactams and in the lesser extent metallo-p-lactamase. Further, isolation of plasmid DNA revealed that most of the isolates examined harbored more than one plasmidFurthermore, conjugative plasmids were found to be the main genetic elements encoding for ESBLs produced by *Escherichia coli* followed by *Klebsiellae pneumoniae* and in the lesser extent to AmpC producing strains of the same bacteria respectively while the B-lactamases production for the remaining isolates is suggested to be under non-conjugative plasmids or chromosomal control.

Key words: Extended spectrum P-lactamases, metallo- P-lactamases, plasmids *

Introduction:

The resistance pattern to third generation cephalosporins in *E coli* and *K pneumoniae* is most commonly attributed to the production of AmpC, extended-spectrum and metallo-B-lactamases . These enzymes are frequently encoded by multi-drug resistance conjugative plasmids and evolve from the more common TEM-1 and SHV-1

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Table 1. The distribution of clinical isolates of Escherichia coli and Klebsiellae pneumoniae according to the type of specimen and type of infection.

Study isolates number (%)	Typ e of specimen	Type of infection
A- Escherichia coli 25 (58.1 %)		
3(12.0%)	Ear swab	Chronic suppurative otitis media
8(32.0%)	Wound aspirate	Diabetic foot infection
4(16.0%)	Wound aspirate	Osteomyeilitis
6(24.0%)	Mid stream urine	Significant urinary tract infection
4(16.0%)	Knee aspirate	Septic arithritis
B- Klebsiellae pneumoniae 18 (41.9%)		
7(38.9%)	Mid-stream urine	Significant urinary tract infection
5(27.8%)	Ear swab	Chronic suppurative otitis media
6(33.3%)	Woundeaspirat	Diabetic foot infection

to production of other type of enzymes like metallo- (β -lactamases. Carbapenem hydrolyzing enzymes producing bacteria are resistant to penicillins, cephalosporins, and carbapenems and are poorly inhibited by all classical β -lactamase inhibitors except EDTA and p-chloromercuribenzoate (pCB). Up to my better simple knowledge, no studies in our country have assessed the occurrence of AmpC, extended spectrum and metallo-β -lactamases at the same time among *E coli* and *K pneumoniae* and their genetic coding. Thus, this study has been undertaken to detect the occurrence of AmpC cephalosporinase, extended spectrum and metallo-β -lactamases at the same time among P-lactam resistant *E coli* and *K pneumoniae* and the possibility of their differentiation *in vitro.* Further, to detect whether the genetic control for their production are encoded by plasmids (conjugative or not) or chromosomes.

penicillinases through point mutations in regions important for (β -lactam binding and/or hydrolysis. Conjugative dissemination of ESBL-encoding plasmids is thought to facilitate the spread of resistance in the clinical setting between DNA replicons and bacterial strains. As well as extended spectrum cephalosporinase (ESCs), group 1 AmpC cephalosporinase are clinically significant because they also confer resistance to a wide variety of (β -lactam drugs, including methoxy-lactams, such as cefoxitin, narrow-, expanded-, and broad-spectrum cephalosporins, β -lactam- β -lactamase inhibitor combinations, and aztreonam. Unlike ESCs, they are poorly inhibited by clavulanic acid. Genes for AmpC beta-lactamases are commonly found on the chromosomes of several members of the family of Enterobacteriaceae. Recently, It is found that these genes have been found on plasmids that transfer non-inducible cephalosporins resistance to *E coli* and *K pneumoniae*. On the other hand, the resistance pattern of gram negative bacteria may be due

Table 2. MICs and inhibition zone criteria for the detection ofESBLs in K pneumoniae (NCCLS)

Antibiotic	Zone diameter for susceptible Strains	Zone diameter for possible ESBL producing strains	MICs for susceptible strains	MICs for possible ESBL producing strains
Cefotaxime	≥23 mm	≤ 27 mm	≤ 8 mg/l	≥ 2 mg/l
Ceftriaxone	≥21 mm	≤ 25 mm	≤ 8 mg/l	≥ 2 mg/l
Ceftazidime	≥18mm	≤ 22 mm	≤ 8 mg/l	≥ 2 mg/l

Patients and Methods

Clinical isolates: Out of Fourty-three well-characterized study isolates, 25 (58.1%) were *E coli* while the remaining 18 (41.9%) represent *K pneumonia.* The study isolates were taken from different sites of infection during the period from April 2001-May 2002. Clinical data concerning the isolates (type of specimen and type of infection) are presented in table 1. Bacteriological identification and confirmatory tests were performed following methods mentioned by **Baron, Peterson, and**

Table 3. The result of screening test by antimicrobial susceptibility test for imipenem cefoxitin and third generation cephalosporins against 25 β lactamase producing isolates of Escherichia coli

Antimicrobial agents	Symbol	Concentration (mcg/disk)	Resistant (%)	Intermediate (%)	Susceptible (%)
Cefotaxime	CTX	30	25(100)	0(0.0)	0(0.0)
Ceftriaxone	CRO	30	25(100)	0(0.0)	0(0.0)
Ceftazidime	CAZ	30	25(100)	0(0.0)	0(0.0)
Cefoxitin	FOX	30	10(40.0)	0(0.0)	15(60.0%)
Imipenem	IPM	10	1(4.0)	0(0.0)	24(96.0)

enzymes by the local clinical isolates of *K pneumoniae* and *E coli,* the cell suspension rapid iodometric method described by Miles and Amyes[8] was used as follows: from an overnight culture of the test organism, a heavy suspension was made (containing 10^7 colony-forming units/ml) in 100 mM sodium phosphate buffer at pH 7.3 containing penicillin at 6 g/liter. A negative control test had been done without the organisms. An organism known to produce beta-lactamase was often tested in parallel as a positive control. The test and controls in parallel were setting up by placing 0.1 ml aliquots into the wells of a microtiter plate. After incubation for 1 hr. at 37°C, two drops of the freshly prepared 1% soluble starch solution were added to each well. A drop of the iodine reagent was added. If the blue colour is lost within 10 min., the presence of a beta-lactamase is inferred. If, however, the blue colour persists, the culture is considered to be beta-lactamase negative

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Screening for AmpC cephalosporinase, extended_spectrum B-lactamase, and Metallo- B-lactamase production:Standard disk diffusion technique: The beta-lactamase producer isolates were screened for their susceptibility to ceftazidime, cefotaxime, ceftriaxone, cefoxitin, aztreonam as a monobactam and carbapenem represented by imipenem. Screening tests were performed by the **standard disk diffusion method, (kirbev-Bauer method) mentioned by Bauer, Kirby, Sherris, and Turck ; WHO**[9] with Muller-Hinton agar and antibiotic disks from **Bioanalyse company, Ankara-Turkey.** Further, **broth microdilution method** following methods mentioned by NCCLS[10] was performed with the same third generation cephalosporins mentioned previously. *Escherichia coli* 25922, *Escherichia coli* MM 294 were used as the reference strains. With regard to ESBL production, the result of MICs and inhibition

Table 4. Screening test by antimicrobial susceptibility test for imipenem, cefoxitin and third generation cephalosporins against 18 - ß Lactamase producing isolates of Klebsiellae pneumoniae.

Antimicrobial agents	Symbol	Concentration (mcg/disk)	Resistant (%)	Intermediate (%)	Susceptible (%)
Cefotaxime	CTX	30	18(100)	0(0.0)	0(0.0)
Ceftriaxone	CRO	30	17(94.4)	1(5.6)	0(0.0)
Ceftazidime	CAZ	30	17(94.4)	1(5.6)	0(0.0)
Cefoxitin	FOX	30	8 (44.4)	0(0.0)	10(55.6)
mipenem	IIPM	10	0(0.0)	0(0.0)	18(100)

Fig. 5 The result confirmatry terst (the interaction between cefotaxime, and cetazidine with clavulanic acid) against potential AmpC csphalosporinase and extended spectrum B-lactamase producer isolates of *Escherichia coli* and *Klebsiella pneumoniae*

Study isolate (No.)	MIC of cefotaxime	MIC after combination With clavulanate	Fic_{index}	MIC of ceftazidime	MIC after combination with clavulanate	Fic_{index}
Escherichia coli						
Amp C producer (10)	57.6 ±41.4	51.2 ±33.56	0.89	43.2 ±34.6	44.0 ±36.8	1.02
ESCase producer (15)	76.0 ±85.4	2.9 ±4.1	0.038	69.9 ±66.4	3.7 ±5.4	0.05
Klebsiellae pneumoniae						
AmpC producer (8)	64.0 ±53.4	66.0 ±53.7	1.03	42.0 ±38.2	41.0 ±39.1	0.98
ESCase producer (10)	56.4 ±42.9	5.5 ±6.0	0.097	57.6 ±41.4	4.7 ±4.8	0.08

zone criteria for detection of these enzymes were interpreted according to the criteria published by NCCLS[11] (see table 2).

Phenotypic confirmatory testing: (Part I):A-For AmpC production:1-Three dimensional extract method:The inducible cephalosporinase producer isolates were tested for AmpC activity by a three-dimensional extract method. Briefly, 50 a of a 0.5 McFarland bacterial suspension prepared from an overnight blood agar plate was inoculated into 12 ml of brain heart infusion broth and the culture was grown for 4 hr. at 35°C. After that, the cells were concentrated by centrifugation, and crude enzyme preparation was made by freezing-thawing the cell pellets five times. The surface of a Muller-Hinton agar was inoculated with *Escherichia coli* 25922. A 10u,g imipenem disk was placed on the inoculated agar. With a sterile scalpel blade, a slit beginning 5 mm from the edge of the disk was cut in the gar in an outward radial direction. By using a pipet, 25 to 30 ul of enzyme preparation was dispensed into the slit, beginning near the disk and moving outward. Slit overfill was avoided. The inoculated media were incubated overnight at 35°C. Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition was considered a positive three-dimensional test result and was interpreted as evidence for the presence of AmpC beta-lactamase[2]. **B-For extended spectrum cephalosporinase production: 1-Double disk synergy test:** All isolates that showed negative result for AmpC production test were submitted to this test. **The double disk synergy test** was used to demonstrate the production of extended spectrum cephalosporinase. In this test, Muller-Hinton agar plates and disks containing 30 ag ofceftazidime and 30 u-g ofcefotaxime with and without 10 u-g ofclavulanic acid, were used for testing. Disks containing clavulanic acid were prepared by applying 10 u.1 of a 1,000 u-g/ml clavulanic stock solution to each disk (NCCLS)[11]. The prepared disks were allowed to dry for 30 min before use. Disks placement and the distances between them were done according to the validated method mentioned by **Canadian External Quality Assessment**[12]. After overnight incubation at 35°C, the results were interpreted according to the criteria established by the **NCCLS**[11]. A > 5 mm increase in zone diameter for cefotaxime and ceftazidime tested in combination with clavulanic acid versus its zone where tested alone, was considered indicative of ESBL production (i.e., the presence of a clavulanic acid effect). This test was repeated for strains showing discrepant results. *E coli* ATCC 25922, and *E coli*MM 294 were used as the quality control isolates.

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Fig.6 Extended spectrum B-lactmase production and potential AmpC enzyme production of B-lactmase producer isolates of *Escherichia coli* and *Klebsiella pneumoniae* isolated from different sites of infection

Study isolates	No (%) of study isolates		
	lactamase producer isolates	Extended spectrum cephalosporinase producer isolates	AmpC cephalosporinase producer isolates
Escherichia coli	25(100.0)	15 (60.0%)	10(40.0)
Klebsiella pneumoniae	18(100.0)	10 (55.6%)	8(44.4)

Phenotypic confirmatory testing:(Part II) A-For ESBLs and AmpC production: The NCCLS ESBLs and AmpC pheotypic confirmatory tests with cefotaxime and ceftazidime were performed with each organism with the standardized bacterial suspension by broth microdilution technique (NCCLS)[11]. In this test, MIC tubes containing ceftazidime or cefotaxime with and without clavulanic acid were prepared in Muller Hinton broth. Ceftazidime and cefotaxime (in concentrations of 0.5 to 256 ag/ml) were tested alone and in combination with 4 u-g ofclavulanic acid per ml. The results were interpreted according to the criteria established by the NCCLS[11]. A >: 3 twofold-concentration decrease in a MIC for ceftazidime tested in combination with clavulanic acid versus its MIC when tested alone was considered indicative of ESBL production (i.e., the presence of a clavulanic acid effect). The data were analyzed by constructing a fractional inhibitory concentration index (FICjndex) which is mathematically equivalent to minimal inhibitory concentration (MIC) of the antimicrobial in combination / MIC of the antimicrobial alone. The results were expressed as synergy, addition, indifference and antagonism when the values of the FICindex were ^ 0.5, 0.5-<1, 1-<2, and ^2 respectively[11].

Plasmid profile: Plasmid profile analysis was performed in small-scale procedure by alkaline extractionmethod of **Birnboim and Doley**[14] mentioned with some modifications by **Sambrook, Fritsch, Maniatis**[15].

Conjugation process: Resistance transfer was carried out by conjugation. *Escherichia coli* MM 294 was used as the recipient strain. Recipient and donors were separately inoculated into brain heart infusion broth and were incubated at 37 °C for 4 hr. They were then mixed at a volume ratio of 1:1 for overnight incubation at 37 °C. A 0.01 ml volume of the overnight broth mixture was then spread into a selective media containing ceftazidime (1 u-g/ml)[16].

Plasmid curing; Curing experiments were performed by using Ethidium Bromide and Sodium Dodecyi sulphate. After that selection of cured cells was performed on the selective medium containing appropriate concentration of Ceftazidime or Cefotaxime[17].

Statistical analysis; Results were expressed as mean ±SD. Statistical analysis of the data was made using Student's t-test. The 0.05 level of probability was regarded as the lowest limit of significance. The data were analyzed by constructing a fractional inhibitory concentration index (FICindex) which is mathematically equivalent to minimal inhibitory concentration (MIC) of the antimicrobial in combination / MIC of the antimicrobial alone. The results were expressed as synergy, addition, indifference and antagonism when the values of the FICindex were <_0.5, 0.5-<1, 1-<2, and >2 respectively.

Results:

Initially, 17 (68.0%), 11 (61.1%) isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively demonstrated a clavulanic acid (CA) effect by double disk synergy (i.e., an increase in CAZ or CTX zone diameters of>5 mm in the presence ofCA), and 15 (60.0%), 10 (55.6%) isolates *of Escherichia coli* and *Klebsiellae* 3.4 and

pneumoniae respectively demonstrated a CA effect by broth microdilution (reduction of CAZ or CTX MICs by > 3 dilutions). The combination of clavulanic acid and cefotaxime or ceftazidime yielded low potentiation or frank indifferent response against potential AmpC cephalosporinase producing isolates of *E coli* (The MICs of cefotaxime 57.6 ±41.4, and ceftazidime 43.2 ±34.6 before combination were 51.2 ±33.56 and 44.0 ±36.8 for both antibiotics respectively after combination with clavulanic acid). The FICindex for cefotaxime-clavulanic acid was 0.89 and 1.02 for ceftazidime-clavulanic acid combination. Also with regard to *K pneumoniae.* The MIC of cefotaxime, 64.0 ±53.4 and ceftazidime 42.0 ±38.2 before combination were 66.0±53.7and ±39.1 for both antibiotics respectively after combination with clavulanic acid. The FICindex tor cefotaxime-clavulanic acid was 1.03 and 0.98 for ceftazidime-clavulanic acid combination. The MIC of cefotaxime (76.0 ±85.4 and ceftazidime 69.9±66.4 for ESBL producing isolates *ofE coli* were reduced after combination with clavulanic acid to 2.9 ±4.1 and 3.7 ±5.4 respectively. The FICindex tor cefotaxime-clavulanic acid was 0.038 and 0.05 for ceftazidime-clavulanic acid combination. Also with regard to *K pneumoniae.* The MIC of cefotaxime, 56.4 ±42.9and ceftazidime 57.6±41.4 before combination were 5.5 ±6.0 and 4.7 ±4.8 for both antibiotics respectively after combination with clavulanic acid. The FICindex tor cefotaxime-clavulanic acid was 0.097and 0.08 for ceftazidime-clavulanic acid combination (see **table 5).**Result revealed that all extended spectrum cephalosporinase producing isolates of *E coli* and *Klebsiellae pneumoniae* gave zones at least 5mm larger with third generation cephalosporins-clavulanate containing disks than non-clavulanate containing disks (mean difference, 8.5mm, standard deviation, 2.9mm for *E coli* and 7.5mm difference, standard deviation 0.7mm for *Klebsiellae pneumoniae}.* On the other hand, *E col* ATCC 25922 exhibited < = 3mm increase in zone diameter for an antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid.The cefoxitin zone diameters for 10 (40.0%), 8 (44.4%) isolates of *Escherichia coli* and *Klebsiellae pneumoniae* that failed to show a CA effect by broth microdilution were < 14 mm (see table 3 and4).The ESBL genes for 13 (86.7%), 9 (90.0%) isolates of ESBL producing *Escherichia coli* and *Klebsiellae pneumoniae* respectively were carried by conjugative plasmids while this genetic elements were encoded for 3 (30.0%), 3 (37.5%) ofAmpC producing isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively (see figure 1**).** Our result showed one isolate of *E coli* was carbapenem hydrolyzing producer. This isolate was resistant to cefotaxime, Ceftriaxone, ceftazidime, aztreonam, and imipenem considering the zone of inhibition of carbapenem was 8 mm in diameter. In plasmid profile analysis, it was found that the resistant genes encoding for this enzyme was carried by conjugative plasmid*pneumoniae}*. On the other hand, *E col* ATCC 25922 exhibited < = 3mm increase in zone diameter for an antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid.The cefoxitin zone diameters for 10 (40.0%), 8 (44.4%) isolates of *Escherichia coli* and *Klebsiellae pneumoniae* that failed to show a CA effect by broth microdilution were < 14 mm (see table 3 and4).The ESBL genes for 13 (86.7%), 9 (90.0%) isolates of ESBL producing *Escherichia coli* and *Klebsiellae pneumoniae* respectively were carried by conjugative plasmids while this genetic elements were encoded for 3 (30.0%), 3 (37.5%) ofAmpC producing isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively (see figure 1**).** Our result showed one isolate of *E coli* was carbapenem hydrolyzing producer. This isolate was resistant to cefotaxime, Ceftriaxone, ceftazidime, aztreonam, and imipenem considering the zone of inhibition of carbapenem was 8 mm in diameter. In plasmid profile analysis, it was found that the resistant genes encoding for this enzyme was carried by conjugative plasmid.

Discussion within that infections due to ESBL- and AmpC producing organisms present a major therapeutic dilemma as the choice of antibiotics is extremely limited.

Due to the broad-spectrum of the beta-lactamases produced by these organisms, ESBL and AmpC producing enterobacteriaceae are typically resistant to beta-lactam antibiotics including broad-spectrum cephalosporins, aztreonam, and extended-spectrum penicillins. Furthermore antibiotics such as trimethoprim-sulfamethoxazole and aminoglycosides especially gentamicin are often co-transferred on a resistance plasmid, resulting in multiple drug resistance . ESBLs and AmpC cephalosporinase are increasing sources of resistance to oxyimino-aminothiazolyl cephalosporins [1,20]. The later-generation cephalosporins were developed to overcome beta- lactamases which conferred resistance to earlier beta-lactam drugs. Within two years of their clinical introduction, the ubiquitous TEM and SHV plasmid- encoded beta-lactamase genes underwent simple point mutations that changed key amino acids around the active site of the protein and enabled the enzyme to bind and hydrolyse these new drugs. Successive mutations interacted in concert, radically increasing the enzymes abilities to bind and confer resistance to later-generation cephalosporins . The investigation of (3-lactamases production for all study isolates was carried out by using rapid iodometric method. In this test, all the study isolates were produced this enzymes. It is well documented that methods used for detecting ESBL-producing bacteria have been evolving for more than a decade, beginning with the description of the disk approximation test by Jarlier, Nicolas, Fournier, and Philippon".This test, also known as the 'double synergy disk' test. Based on the finding that the common extended spectrum P-lactamase all remained sensitive to inhibition by clavulanic acid, therefore, any strain that is resistant to ceftazidime or cefotaxime but becomes sensitive in the presence of clavulanic acid is likely to contain an extended spectrum P-lactamase[8]. Thus, the **double disk synergy test** was used in this study as phenotypic confirmatory test for detection the synergy between extended-spectrum cephalosporins and clavulanic acid considering in mind that this test has served as the reference method for detecting ESBL-producing strains for a number of years[25] While laboratory detection for these enzymes can be problematic, because some ESBLs do not confer obvious resistance to all their substrates *in vitro* and up to 35% ofESBL producers continue to be reported as susceptible to cefotaxime and ceftriaxone [19,20]. Thus, accurate detection nevertheless is important because clinical failures arise even when the MICs of cephalosporins for ESBL producers are only 1 ag/ml. Thus, this study has been undertaken to detect accurately the occurrence of these enzymes *in vitro*. It is must be stressed that precise placement of the disks, correct storage of the clavulanate-containing disks, and performance of appropriate control tests are critical to the sensitivity of the disk approximation method[24] .In the double disk synergy test, extended spectrum cephalosporins, cefotaxime, ceftazidime and cefoperazone were used. Ceftazidime gave more sensitive and predictable result in contrast with cefotaxime and cefoperazone that give false negative results in some isolates considering the interpretation of results was dependent on the reading the differences in the study third generation cephalosporins diameters[2] rather than using the ratio of the zone diameters with and without clavulanate to infer ESBL production. This result is in agreement with the same results obtained by **Katsanis, Sprago, Ferraro and Jacoby**

generation cephalosporins diameters[2] rather than using the ratio of the zone diameters with and without clavulanate to infer ESBL production. This result is in agreement with the same results obtained by **Katsanis, Sprago, Ferraro and Jacoby** and therecommendation laid down by NCCLS[26] that ceftazidime proved to have adequate sensitivity to detect the ESBL and AmpC cephalosporinase producing isolates. The high resistance rates to this antibiotic (100%, 92.3%,) for *E coli* and *Klebsiellae pneumoniae* respectively suggesting the high possibility of these isolates to be potential ESBLs or AmpC cephalosporinase producers. This suggestion was confirmed laterly by

result revealed that all extended spectrum cephalosporinase producing isolates of *E coli* and *Klebsiellae pneumoniae* gave zones at least 5mm larger with third generation cephalosporins-clavulanate containing disks than non-clavulanate containing disks (mean difference, 8.5mm, standard deviation, 2.9mm for *E coli* and 7.5mm difference, standard deviation 0.7mm for *Klebsiellae pneumoniae).* On the other hand, *E coli* ATCC 25922 exhibited < = 3mm increase in zone diameter for an antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid.Unlike, ESBLs, however, AmpC cephalosporinase are not inhibited by clavulanic acid or other similar compounds. Accordingly, if an ESBL confirmation test using clavulanic acid is not performed, many AmpC producing strains may be presumed to be ESBLs producing strains[7]. For this reason, in addition to ESBL production 4-est (double disk synergy test), a **three-dimensional extract method** test was used in parallel in this study to detect the production of inducible AmpC cephalosporinase. In this study, out of 25 P-lactamase-producing isolates of *E coli,* 10(40.0%) isolates were AmpC cephalosporinase producers while the remaining 15(60.0%) produced an extended spectrum cephalosporinase. Also, out of 18 (3-lactamase-producing isolates of *Klebsiellae pneumoniae* 8 (44.4%) were AmpC producers and 10(55.6%) were ESBLS producers (table 4). It is well known that AmpC cephalosporinases were grouped according to **Bush, Jacoby, and Medeiros classification**[4]. These enzymes are not well inhibited by clavulanic acid, but were often inhibited by a low concentration of aztreonam or cloxacillin. Further. AmpC cephalosporinase may also mediate resistance to extended spectrum cephalosporins and aztreonam in addition to cephamycins such as cefoxitin[27] Based on the fact that clavulanate does not effectively inhibit class 1 enzyme (essentially AmpC type), and so does not potentiate antibiotic against strains that are stably derepressed for these enzymes[6,18]. Thus, the combination ofclavulanic acid and cefotaxime or ceftazidime yielded low potentiation or frank indifferent response against inducible AmpC cephalosporinase producing isolates of *E coli* (The MIC of cefotaxime 57.6 ±41.4, and ceftazidime 43.2 ±34.6 before combination were 51.2 ±33.56 and 44.0 ±36.8 for both antibiotics respectively after combination with clavulanic acid. The FICindex tor cefotaxime-clavulanic acid was 0.89 and 1.02 for ceftazidime-clavulanic acid combination. Also with regard to *K pneumoniae,* The MIC of cefotaxime, 64.0 ±53.4 and ceftazidime 42.0 ±38.2 before combination were 66.0 ±53.7and 41.0 ±39.1 for both antibiotics respectively after combination with clavulanic acid. The FICindex tor cefotaxime-clavulanic acid was 1.03 and 0.98 for ceftazidime-clavulanic acid combination. In contrast with ESBLs producing isolates of *E coli,* synergistic interaction was observed which is reflected by the value of FICindex which indicates the degree of synergism and it is expressed as synergy when the values of FICindex was < 0.5[13]. The MIC of cefotaxime (76.0 ±85.4 and ceftazidime 69.9 ±66.4 for ESBL producing isolates *ofE coli* were reduced after combination with clavulanic acid to 2.9 ±4.1 and 3.7 ± 5.4 respectively. The FICjndex for cefotaxime-clavulanic acid was 0.038 and 0.05 for ceftazidime-clavulanic acid combination. Also with regard to *K pneumoniae.* The MIC of cefotaxime, 56.4 ± 42.9and ceftazidime 57.6 ±41.4before combination were 5.5 ±6.0 and 4.7 ±4.8 for both antibiotics respectively after combination with clavulanic acid. The FICindex tor cefotaxime-clavulanic acid was 0.097and 0.08 for ceftazidime-clavulanic acid combination.It is well documented that metallo-P-lactamase are classified as group 3 in the functional classification scheme for P-lactamases laid down by **Bush, Jacoby and Medeiros .** These enzymes are characterized by hydrolyzing of penicillins, cephalosporins, and carbapenems. Carbapenem hydrolyzing enzymes are poorly inhibited by all classical P-lactamase inhibitors except EDTA and p-

chloromercuribenzoate (pCMB)[11] Thus, in this study, carbapenem represented by imipenem was used in the screening test for metallo-P-lactamase production.In this study, Plasmid profile analysis was performed in small scale procedure by alkaline extraction method. This method was designed to prevent generation of the "irreversibly denatured" form; at the same time, the extracts must be alkaline enough for denaturation of chromosomal DNA to occur[14]. Curing experiments were performed by using ethidium bromide and sodium dodecyi sulphate to confirm that the resistance property was carried by plasmids. It is well known that the extra-chromosomal piece of DNA, plasmid may simply be refractory to the curing agents, or appear so because it carries functions vital for cells viability. This is perhaps most likely for large plasmid The ESBL genes for 13 (86.7%), 9 (90.0%) isolates of ESBL producing *E coli* and *Klebsiellae pneumoniae* respectively were carried by conjugative plasmids while this genetic elements were encoded for 3 (30.0%), 3 (37.5%) of AmpC producing isolates of *Escherichia coli* and *Klebsiellae pneumoniae* respectively. Our result showed one isolate of *E coli* was carbapenem hydrolyzing producer. This isolate was resistant to cefotaxime, Ceftriaxone, ceftazidime, aztreonam. and imipenem considering the zone of inhibition of carbapenem was 8 mm in diameter. In plasmid profile analysis, it was found that the resistant genes encoding for this enzyme was carried by conjugative plasmid. With the exception of this isolate, all the study isolates were sensitive to imipenem. The high resistance rates to ceftazidime and all theother antimicrobials studied leave imipenem as the only reliable agent for the empirical treatment of life threatening infections caused by ESBL and AmpC cephalosporinase producers. The study concluded that AmpC, extended spectrum, and metallo-P-lactamases production play an essential role in the resistance of *E coli* and *K pneumoniae* to expanded spectrum p-lactams, and aztreonam. Further, the isolation of plasmid DNA revealed that most of the isolates examined harbored more than one plasmid.

Furthermore, conjugative plasmids were found to be the main genetic elements encoding for ESBLs produced by *E coli* followed by *K pneumoniae* and in the lesser extent to AmpC producing strains of the same bacteria respectively while the (3-lactamases production for the remaining isolates is suggested to be under non-conjugative plasmids or chromosomal control. On the other hand, the study suggested that imipenem is the only reliable agent for the empirical treatment of life threatening infections caused by ESBL and AmpC cephalosporinase producers when high resistance rates to ceftazidime and other extended spectrum (3-lactam and aztreonam is observed.The study recommended that accurate detection for ESBLs and AmpC production (involved standardization for all test variables like precise placement of the disks, correct storage of the clavulanate-containing disks, and performance of appropriate control tests) are important because clinical failures arise even when the MICs of cephalosporins for ESBL and AmpC producers are only 1 ug/ml. Also, the study recommended introduction of ESBL and AmpC production tests in the laboratory sensitivity routine procedures to give the clinicians the broader impression in the treatment of life threatening infections due to ESBL and AmpC harboring gram negative isolates and the last, ESBLs and AmpC cephalosporinase production should be tested continuously in parallel becaus if an ESBL confirmation test using clavulanic acid is not performed, many AmpC producing strains may be presumed to be ESBLs producing strains.

Table l.The isolated microorganisms from ear discharge specimens of patients suffering from chronic suppurative otitis media.

Microorganism	No. of isolates	Percentage (%)
1-Bacteria		
A: Gram positive	78	89.7
- Staphylococcus aureus	19	21.8
- Strepococcus Pneumoniae	3	3.5
- Peptostreptococcus spp.	3	3.5
B: Gram negative		
- Pseudomonas aer uginosa	26	30
- Proteus vulgaris	4	4.6
- Klebsiellae pneumoniae	8	9.2
- Haemophilus influenzae	3	3.5
- Bacteroides fragilis	12	13.8
2 Fungi	9	10.3
- Aspergillus niger	5	5.7
- Candida albicans	4	4.6
3-No growth	4	5.5
Total	87	100%

together has been suggested this is because the aerobic organisms create an environment in which the anaerobes can grow in mixed infections by lowering the local oxygen concentration[11]. As the presence of resistant microorganisms to commonly used antimicrobial agents is an international problem[12]. the antimicrobial susceptibilities need to be evaluated periodically to guide clinician in choosing the appropriate medications. Thus, this study had been undertaken to determine the microbiological causative agents of CSOM and the antimicrobial susceptibility to the most frequently used antimicrobial agents among patients in Ramadi city.

Patients and methods

Seventy two patients visited E.N.T. unite of Saddam General Hospital in Ramadi during the period from June- October 2002 .These patients were clinically diagnosed with CSOM, the specimens were collected by introducing a cotton ear swab and rotating it in the external auditory canal. The ear swabs were delivered immediately and cultured within one hour. The ear swabs were inoculated on 1. Two plates of blood agar for aerobic and anaerobic culture. 2. Chocolate agar plate. 3. MacConkey's agar plate and 4. Sabourad's agar plate for 24 hours . If negative the anaerobic and Sabourad's cultures were incubated for a further 24 hours. All isolates were bacteriologically identified and confirmed by biochemical test following methods mentioned by Baron, Peterson, Finegold[13]. The antibiotics susceptibilities testing was performed by the standardized Kirbey-Bauer disc diffusion method using the Muller Hinton agar. Antibiotic discs with the following potencies were used: Ampicillin (lOmcg) , Cloxacillin (5mcg), ampiclox (lOmcg), gentamicin (lOmcg), tobramycin (30mcg), cefotaxime (30mcg), ceftazidime (30mcg), ceftriaxone (30mcg), co-trimoxazole (25mcg), vancomycin (30mcg), ciprofloxacin (5mcg), cephalexin (30mcg), piperacillin (lOOmcg) and rifampicin(30mcg). If the organism was sensitive to two or more antibiotics no more test were carried out, otherwise more test were performed.

Results: The age of the patients ranged between 1- 75 years (mean age 30.6). The female to male ratio was 1.4:1. Bacterial isolates encountered in 78(89.7%) and fungal isolates occurred in 9(10.3%). The aerobic isolate 63(72.4%) was more predominant than anaerobes 15(17.3%). The most common aerobic isolates were *Pseudomonas aeruginosa* 26(30%), *Staphylococcus aureus* 19(21.8%), *Klebsiellae pneumoniae* 8(9.2%), *proteus vulgaris* 4(4.6%), and to less extent *Streptococcus pneumoniae* 3(3.5%) and *Haemophilus influenzae* 3(3.5%). The most common anaerobic isolates were *Bacteroides fragilis* 12(13.8%) and to less extent *Peptostreptococcus* 3(3.5%) (see table1).Among the 9 fungal isolates, 5(5.7%) were *Aspergillus niger* and the remaining 4(4.6%) were *Candida albicans*.

Out of 72 patients, 4(5.5%) specimens showed no growth of microorganisms (see table 1).With regard to antimicrobial susceptibility test; *Pseudomonas aerugenosa* were found to be sensitive to ciprofloxacin (96%), ceftazidime (88%), tobramycin (82%) and gentamicin (78%), on the other hand it was resistant to ampicillin (100%), cephalexine (96%), co-trimoxazole (94%) and ampiclox (93%), while *Staph. aureus* was sensitive to vancomycine (96%), ciprofloxacin (93%), ceftazidime (89%), Rifampicin(84%), ceftriaxone (83%), cefotaxime(82%), Co-trimoxazole (78%) and cloxacillin (75%), but it