Evaluation of Stability of Cefamandol and Ceftazidime with Clavulanic Acid Against Extended Spectrum β- Lactamase

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Abstract

The aim of this study is to evaluate in-vitro activity of Cefamandol (Cfm) and Ceftazidime (Cfz), in combination with Clavulanic acid (CA) against ten complicated multiresistant uropathogenic *E.coli* .One hundred clinical strains were isolated from patients with chronic urinary tract infections (UTIs), these isolates were identified by the Api identification systems. The antimicrobial susceptibility tests were determined by Kirby-Bauer method, all of them were sensitive to Imipenem (Imp). Ten strains were chosen for the present study, they were resistant to Ampicillin (Amp), Amoxicillin (Amo), Carbenicillin (Cb), Ticarcillin (Tic), Azlocillin (Azl), Amoxicillin\ Potassium Clavulanate {Augmentin(Amc)}, (Amo\CA), Ticarcillin\ Potassium Clavulanate {Timentin} (Tic\CA) ,Cefazolin (Cfo) ,Cefaloridin (Cfr), Cefamandol, (Cfm),Cefoxitin, Ceftazidime (Cfz), Cefixime (Cxm), Cefoperazone(Cfp) and Aztreonam (Atm), also resistant to other antibiotics, Tetracycline(Tc),Cloramphenicol(Cm),Gentamycin(G),Amikacin (Amk), Ciprofloxacin (Cip) and Trimethoprim. 50% of the isolates were resistant to Nalidixic acid and Rifampicin. The minimum inhibitory concentrations of Cefamandol and Ceftazidime were determined, by tube method. Transfer of plasmids were done by direct conjugation test to sensitive standard *E.coli* ,cell free β- lactamases were prepared and detected by macro-iodometric method. The activity of each cell free ß– lactamases extract against Cfm and Cfz were determined by disks diffusion method (microbiological Masuda method) .Excellent activities were obtained against these strains when Cfm and Cfz, combined with CA, therefore complete zones of inhibition were obtained indicated the prevalence of extended spectrum β- lactamases in *E.coli*. The stability of Cfm and Cfz in the presence of CA were useful in the treatment of chronic urinary tract infections caused by multiresistant β- lactamase (ESBL) producer *E.coli*.

Key words: Extended spectrum β- lactamases, Imipenem, Aztreonam, Ceftazidime.

الخلاصة

يهدف البحث الى دراسة فعالية السيفامندول (Cfm) والسيفتازديم (Cfz) باضافة حامض الكلافجولانك تجاه (E-Coli) المقاومة لمجموعة مضادات البيتالاكتام معزولة من مرضى يعانون من التهاب المجاري البولية المزمن وقد بينت الدراسة ان الفعالية المثلى لكل من السيفاماندول والسيفتازديم تكون بوجود حامض الكلافجولانك وان ثباتية كل من السيفاماندول والسيفتازديم بوجود الحامض تشير الى امكانية استخدامهما في معالجة التهاب المجاري البولية الناتجة عن (E-Coli) المقاومة لمضادات البيتالاكتام من خلال طرائق التقييم والتشخيص التي استخدمت في البحث .

Introduction

Clavulanic acid is a β- lactam; structurally it differs from Pnicillins in two respects, the replacement of sulfur in the Penicillin thiazolidine ring with oxygen in the clavam oxazolidine ring and the absence of the side chain at position 6. Clavulanic acid a naturally occurring clavam isolated from *Streptomyces clavuligerus* has poor antibacterial activity but exerts a potent and irreversible inhibitory effect on β- lactamases especially penicillinase by blocking the active sites of these enzymes and is strongly synergistic with most of the β- lactamines in vitro[1]. Due to this combination, Amoxicillin is protected from degradation and its spectrum is therefore extended to include bacteria normally resistant to amoxicillin and other β-lactam antibiotics[2]. In the case of β- lactam resistant bacteria a bacterial enzyme, β-lactamase, cleaves the β- lactam ring and renders the antibiotic inactive. β-lactamases are a large and diverse group of enzymes in which four clinically relevant classes are known [3]. β-lactamase continues to be the leading cause of resistance to β- lactam antibiotics among Gram-negative bacteria. In recent years there has been an increased incidence and prevalence of extended-spectrum β-lactamases (ESBLs), enzymes that hydrolyze and cause resistance to Oxyimino-Cephalosporins and Aztreonam.The majority of ESBLs are derived from the widespread broad - spectrum β- lactamases TEM-1 and SHV-1.

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ESBLs have become widespread throughout the world and are now found in a significant percentage of *E.coli* and *Klebsiella pneumoniae* strains in certain countries [4, 5, 6, 7]. There are also new families of ESBLs, including the Cefotaximase (CTX-M) and OXA- type enzymes, Ceftazidimase, as well as novel unrelated β- lactamases [8, 9, 10]. The stability of different Cephalosporins to the most important β- lactamases was assessed and many clinical studies have shown that up to 75% of the β- lactamases responsible for β-lactam resistance in G-negative bacteria were R-plasmid mediated [11]. Recently, new fourth generation cephalo-sporins, such as Cefepime, Cefpirome, Cefoselis, Cefditoren, Cefozopran [12], were introduced into antibacterial chemotherapy and their activities were compared with other β-lactams such as Ceftazidime,Imipenem and Carbapenem, against..*P.aeruginosa*, *Enterobacteriaceae* (*E.coli, Klebsiella pneumoniae*) and G-positive bacteria. In addition several drug combinations have been produced which contain both a β- lactam antibiotic and a β-lactamase inhibitor; the inhibitor has high affinity for β- lactamase, irreversibly binds to it, and thereby preserves the activity of the β-lactam. Currently, four penicillin inhibitor combinations are in clinical use: Ampicillin-Salbactam (Unasyn), Amoxicillin- Clavulanate (Augmentin), Ticarcillin –Clavulanate (Timentin) and Pipracillin- Tazobactam (Zosyn) [12]. Urinary tract infections (UTIs) are cause a significant health problem and *E.coli* has been reported to be the primary pathogen in approximately 80% of cases. *E.coli*, express structures called adhesins fimbriae or pili that help them bind to specific tissue [13]. The aims of the study are:

1. To know the prevalence of extended spectrum β- lactamase (ESBL) in multi drug resistant (MDR) strains of *E.coli* isolated from complicated urinary tract infections.
2. To evaluate the following combinations: Cefamandol/Clavulanateand Ceftazidime / Clavulanate for their in vitro antimicrobial activity against complicated urinary tract infections caused by ESBLs β- lactamases.

Materials and Methods

Standard strains with plasmid – mediated beta – laectamases were used:
1-E.coli K12 (TEM-1 type β- lactamase with isoelectric point 5.4) confer plasmid(R 111) and E.cloacae P99). 2-E.coli K12 (SHV-1 type β- lactamase Pitton (type II) I.p 7.7 (10).3-E.coli K12 600 Rif and E.coli K12 600 Nal Sensitive to antibiotics [10]. 4-Clinical isolates of E.coli. 5-Pure enzyme of Med Labs. 6- E.coli ATCC 25922 provided by Medical city Identification of E.coli. A total of 100 strains of E.coli were selected and identified by Api 20 E . System (Biomerieux vitek, Inc)[14].

Antibiotic susceptibility test (Disk diffusion method [10])

The resistance pattern for antibiotics were determined by Kirby/Bauer diffusion assay on Mueller – Hinton agar (20ml / plate) the inoculum was $10^4 - 10^5$ CFU / ml, of 6 hours cultures at 37ºC for 24 hours.The antibiotics used were as follow: Amoxicillin (Amo) 30 µg, Augmentin(Amc) (Amo 20µg + CA10µg),(Tic),Azlocillin 100µg, Timentin (Tim) (75µgTic+CA 10 µg), Cefaloridin(Cfr) 30µg ,Cefamandol (Cfm) 30µg and Ceftazidime (Cfz) 30µg, Cefixime(Cxm) 30µg, Ceftriaxone (Ctr) 30µg, Cefoperazon (Cfp) 30µg ,Aztreonam (Atm) 30µg Rifampicin (Rif) 30µg, Nalidixic acid (Nal)30µg, Ciprofloxacin(Cip) 10mcg, Amikacin (Amik)10µg (Tc)30µg, Chloramphenicol (Cm)30µg, Gentamicin (Gm)30µg, and Cotrimoxazole (Trimethoprime 2.5 µg + Sulfamethaxazole 22.5 µg) (Tm).

Minimum inhibitory concentrations (MICs)

MICs were determined by dilutions of different concentrations of Cfm, Cfz, alone and in the presence of Clavulanic acid (CA). According to the method recommended by the National committee for microbiology Laboratory standards (FRANCE) Powders of β -lactam antibiotics were obtained from (Russell and Beecham). [15]

Transfer of genetic information by direct conjugation method

Conjugal transfer of 3GC resistant ESBL producing strains was done at 35°C -37°C in liquid medium {Brain heart infusion (B.H)} or in solid media {Trypticase Soya agar (T.S.A) or Mueller – Hinton (M.H)} using E. coli K12 600 Rif and E.coli K12 600 Nal as recipient. Equal volumes (1 mL) of culture of the donor and the recipient strain (108-109 CFU/mL) grown with agitation in tryptic soya broth were mixed and incubated statically for 18 hours at 35°C. Transconjugants were selected on M.H agar containing 64-µg/mL Nalidixic acid to inhibit the growth of donor and 2.5 µg/mlCfz to inhibit the growth of recipient strain [11].

Phenotypic confirmatory disc diffusion test (PCDDT) for ESBL [18]

Ten µl of CA solution was added to discs of Cfz and Cxm one hour before culture, these were applied to the surface of a Muller Hinton agar, seeded with a suspension of 10^4-10^5/CFU of bacteria under test.. An increase in zone diameter for either antimicrobial agent tested in combination with CA versus its zone when tested alone was observed. For Cfz an increase in zone diameter of> 5mm and for Cxm > 3 mm was considered as an ESBL producer.

Extraction of ß– lactamase

Cell free beta –lactamases were prepared from strains known to be good producers of the desired enzymes, (ß–lactamases, type TEM-1 and SHV-1, R-plasmid mediated enzymes) and ß–-lactamase from *E.cloacae* P99 (cephalospornase) as references. Crud enzymes also prepared from test isolates of *E.coli* [10].

Detection of ß– lactamase by Macro - iodometric method.

This test is based on the reaction of the (oic) acid of penicillin with iodine. ß–lactamasehydrolyze penicillin to penicilloic acid, which in turn react with iodine, the presence of ß– lactamase in a test system was shown by decolorization of starch-iodine complex, observed in 1-18hours at 4°C [16].

Assessment of stability of ß– lactams to cell-free ß– lactamases [17]

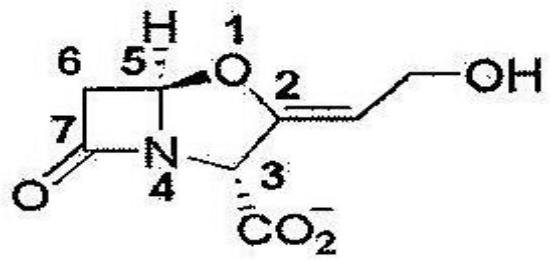
The surface of Muller Hinton agar was seeded with a suspension of sensitive indicator *E.coli* ATCC. Four discs containing ß– lactams under test were placed near filter papers discs; each of them was impregnated with 30µl of the extract enzymatic. The plates were incubated at 37°C for 18hours, the ß–lactamase activity was observed like half moon zone of inhibition.

Masuda microbiological method [17]

Ten clinical isolates were screened for ß–lactamase inhibitors using 10µl CA in combination with 30 µl of Cfz or Cfm. Sensitivity discs containing Cfz or Cfm and a filter disc incorporated with10µl enzyme and 10µl (CA) as potassium clavulanate were placed on agar plate on which a bacterial suspension of sensitive *E.coli* ATCC (standard) was spread the inoculum was 10^4 – 10^5 CFU / ml, of 6 hours cultures at 35 C^0-$37C^0$ for 24 hours. Unchangeable inhibition zones demonstrate stability of the antibiotic to the enzyme.

Results and Discussion

Extended –spectrum ß– lactamases (ESBLs) are derivatives of enzymes such as SHV-1 and TEM-1 that have undergo site specific mutation that enable them to hydrolyze , and thus inactivate , oxyimino – cephalosprins such as, cefotaxime and ceftazidim.[19]. All clinically important reactions of ß– lactamase inhibitors , such as tazobactam , sulbactam, and calvulanic acid , involve ß– lactam ring cleavage during acylation of an active site . Although other clavams produced in nature may possess antibacterial and antifungal properties, clavulanic acid is the only one known clavam with potent ß– lactamase inhibitory activity owing in part to its 3R ,5R stereochemistry , it is a potent inhibitor of ß–lactamase enzymes produced by many strains of Staphylococcus aureus , E.coli , Klebsiella , Proteus , Sheglla , Pseudomonas , and Haemophilus influenzae [20,21,].



clavulanic acid

100% of the isolates were found to be resistant to Amp, Amo,Cb,Tic, Azl, Cfr,Cfo, Tc,Cm and Tm , 10% were resistant to Cfm Cxm, Cfz,Cfp,Ctr , Atm Tim, and Amc.Also resistant to G,Amk,Cip and Tm , 50% of the isolates were resistant to Nal and Rif as shown in Table 1. ESBL was detected in 10 isolates by PCDDT the zone of inhibition increased in presence of CA. For Cfz >10mm, and for Cfm and Cxm>5mm, potentiation of the inhibiton zone of 3GC in the presence of CA was observed.. indicated ESBL production in ten strains; the diameters zone of inhibition for Amc and Tim were range from 0 - 5mm while the normal diameters zones of inhibition were for Amc 14-21mm and for Tim is 13mm. The critical normal MICs for Tim and Amc were (4-16) and (128) respectively. The MICs were studied for ten clinical isolates of *E.coli* in comparison with standard resistant strains, the range of MICs for Cfm was 512 - 2048 µg/ml and for Cfz 32-64 µg/ml, while for non ESBL producer it ranged from 0.02-8 µg/mL. After the addition of CA eight-fold reduction or more in MICs (Table 2) . These results was in

agreement with the investigation of Chaudhary ,U, Aggarwal-R[17] indicated ESBL producers. All the isolates were sensitive to Imp, but among the non ß– lactam antibiotics Cip and Amk were most effective drugs 90 strains were sensitive. Resistance to Cfz was transferred to recipient *E. coli* K12 C_{600} Rif or *E. coli* K12 C_{600} Nal strains, along with resistance to other ß– lactam antibiotics, ESBL production is coded by genes on conjugation plasmids which are easily transmitted among different members of Enterobacteriaceae, all ESBLs have serine at their active sites. The results of detection of ß– lactamases by iodometric method were positive for 10 strains comparing with standard negative and positive ß– lactamases R111 (TEM-1) and *E.coli* ESBL producer. The inhibition of beta-lactamase production by CA has been demonstrated with many strains of bacteria, this effect potentiates the action of many beta lactams, such as Amp, Amo, Cb and Azl. Many clinical reports of combination of Amo with CA have been encouraging, in urinary tract infections due to ß– lactamase-producing organisms type TEM and SHV, whilst Amo alone had no effect, the addition of CA (as salt) dramatically change the half moon inhibition zone to complete inhibition zone [3,4,11]. Figure 1 indicate the activity of ß–lactamase extracts against ß-lactams antibiotics, figure 2 indicate the Antibiotic – enzyme Interaction by the highly sensitive double disks technique, demonstrated their hydrolysis, however ß-lactamase of *E.cloacae* not effected by Amc and inhibited by Azl and hydrolyzed all cephalosporins. [5,6,20] .

Table 1: Sensitivity Tests of Ten Strains Determined by Disk Diffusion Test .

NO of isolates	Diameters of zone of inhibition / MM												
	Amo	**Amc**	**Tic**	**Tim**	**Cxt**	**Cfm**	**Cfz**	**Cxm**	**Ctr**	**Amk**	**Cip**	**Rif**	**Nal**
1	0	4	0	5.5	16	2	3	10	11	10	12	19	0
2	0	4.5	0	7	16	3	3	12	10	11	10	0	18
3	0	3.5	0	8	18	2	5	8	5	14	12	19	0
4	0	4	0	8.5	17	0	4	4	13	12	11	10	0
5	0	5	0	9	17	6	14	15	12	12	11	19	0
6	0	5.5	0	9	19	10	14	15	13	12	18	0	0
7	0	6	0	7	20	11	11	15	12	18	20	0	18
8	0	6	0	6.5	20	5	10	13	10	18	24	0	19
9	0	6	0	9.5	20	5	11	12	10	15	22	19	19
10	0	7.5	0	9	17	7.5	13	10	10	12	10	19	18
E.coli (ESBL)	0	0	0	5	17	7	13	12	10	12	18	11	17
E.coli ATCC 25922	21	21	13	13	22	22	21	21	21	32	40	32	33

Abbreviation's : Amo: Amoxicillin Amc:Amoxiclave; Cb: Carbenicillin;Azl: Azlocillin; Tim: Timentin; Cxt Cefoxitin ; Cfm: Cefamandol; Cfz: Ceftazidime; Cxm:Cefixime,Ctr:Ceftriaxone, Cfp :Cefoperazon; Amk: Amikacin, Cip: Ciprofloxacin , Rif: Rifampicin; Nal: Nalidixic acid; the diameters of zone of inhibition for Ampicillin, Amoxicillin, Carbenicillin,Ticarcillin, Azlocillin,Cefazolin(Cfo), Cefaloridin(Cfr),Cefoperazone(Cfp) Aztreonam(Atm) , Tetracycline ; Chloramphenicol; Trimethoprim and Gentamicin were zero. All of them sensitive to Imipenem (Imp) 17-23mm and Cefoxitin 15-22mm .Normal zone for Amc: 14-21mm; Tim:13mm; Cfz,Ctr,Ctx: 15-21mm; Cfm:15-22mm. Amk:25-32mm; Rif :19-32mm;Cip:30-40mm.

Table 2: Minimum Inhibitory Concentrations of Ten Uropathogenic E.Coli Comparing with Standard Strains .

No. of Isolate E.coli	MICs mcg/ml				
	Imp	**Cfm**	**Cfz**	**Cfm+CA**	**Cfz+CA**
1,2,3	2	512	64	2	1
4,5,6	1	1024	64	0.5	2
7,8,9,10	4	2048	32	0.25	0.5
E.coli (453)* SHV-1 (7.7) [19]	16	16	0.05	0.25	0.25
E.coli (R111) TEM-1 (5.4)*)	16	32	0.02	0.25	0.25
E.cloacae(P99** (8.3)*	64	512	64	32	64
E.coli (ESBL)	4	128	64	0.25	2

* Isoelectric points. ** Cephalosporinase.

Normal values of MICs : Cfm S<8 mcg\ml R >32mcg\ml

Cfz S<4 mcg\ml R >16mcg\ml

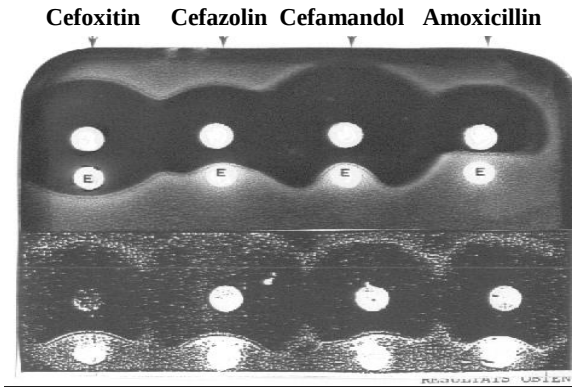


Figure 1: Activity of ß– lactamase against ß-lactams antibiotics

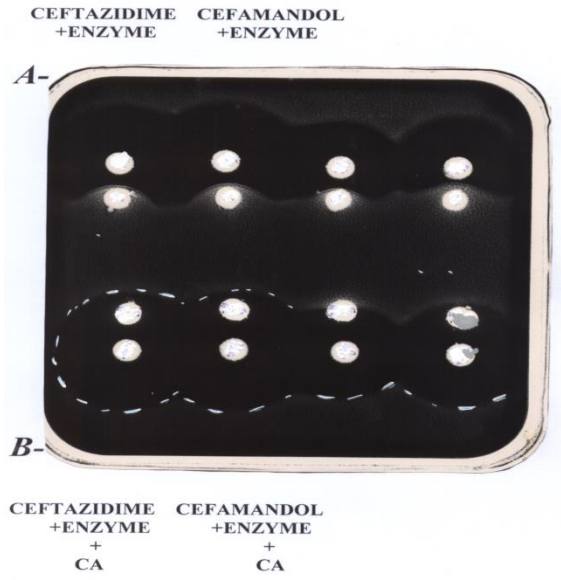


Figure 2: Antibiotic –enzyme Interaction ,by the highly sensitive double disks technique [24] demonstrated
A : hydrolysis of ceftazidime and Cefalmandol by β-Iactamases producing E.coli
B: Inhibition by Clavulanic acid (CA) .

Conclusions

The Ten clinical isolates in this study were very resistant to Amc, Tim,Cfm ,Cfz,Cxm,Cfp, Ctr and Atm, but sensitive to Imipenem comparing with standard TEM-1 and SHV-1 (plasmidic penicillinases) and *E.clocae* P99) (Chromosomic Cephalosporinase) indicating the prevalence of extended-spectrum β-lactamases (ESBLs) enzymes that hydrolyze and cause resistance to oxyimino-cephalosporins and aztreonam. Our study shows presence of ESBL producer *E.coli* in ten clinical isolates. The routine antimicrobial sensitivity test may fail to detect ESBL, mediated resistance against 3GC and detection of ESBL production should be carried out as a routine in diagnostic laboratories by PCDDT as it is a simple and cost effective test, the combination with Clavulanic acid bringing the susceptibility back, confirms the ESBLs. ESBLs have become widespread throughout the world and are now found in a significant percentage of *E.coli* and *Klebsiella pneumoniae* strains in certain countries, [6, 7, 9, 10]. The increasing emergence of cephalosporins resistant *E.coli* has leaded to concern about the use of various combination therapies.

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