Genetic study to bacteria *Acinetobacter baumannii* β- Lactamase producer

Rasah Abid Ali
Biotechnology Department, College of Science, Baghdad University.

Abstract

Six isolates were collected from different clinical sources from laboratory in medicine century. These isolates were belonging to the genus *Acinetobacter* depending on morphological and biochemical tests. The antibiotic susceptibility tests against 8 antibiotics were examined, and it was found that the all isolates have multiple resistant to antibiotic, all isolates were resistant to ampicillin, ceftriaxone, cefotaxime, 50% were resistant to augmentin, 20% were resistant to ciprofloxacin ,while 10% from isolates show resistant to amikacin and trimethprim. All isolates have sensitivity to imipenem. The ability of *Acinetobacter* isolates to produce β-lactamase enzymes was, tested using iodometric method, and the results showed that all isolates produced this enzyme. The ability of these isolates to produce extended spectrum β-lactamase (ESBLs) were also determined by double disc synergy test, only one isolates produced these enzyme. Agarose gel electrophoresis showed that *Acinetobacter* isolates β-lactamase producer have two small plasmid bands. Transformation experiments revealed that these plasmids were capable to transform *E. coli* MM294, an observation which indicates the ability of these plasmids to show their expression in more than one host.

Acinetobacter, β-lactamase, plasmid, transformation

Introduction

The genus *Acinetobacter spp*. are group of G-ve cocobacilli, oxidase-negative, catalase positive ,stictly aerobic, they are non motile and non spore former .they can use a various carbon sources for growth then can be cultured on relatively simple media (1). *Acinetobacter* can be found widespread in nature, that have been isolated from soil, water, various sample of animal and human (2). *Acinetobacter baumanni* recognized as an important human pathogen that causes sever infections in hospitalized patients as well as deadly cases of community acquired pneumonia. This bacterium is capable of causing septicemia, endocarditic, meningitis and skin wound, respiratory tract and urinary tract infections (3). *Acinetobacter baumannnii* have acquired resistance to all commonly antibiotics , therefore can cause serious therapeutic failure. Within the last decades these strain become resistant to β-lactam antibiotics, resistance to β-lactam can be due to enzyme that inactivate the antibiotic, altered pencillin-binding protein or combination of altered pencillin binding protein and reduced outer membrane permeability (4).In this study, we examined the different β-lactam resistance pattern and related this to β-lactamase expression, and detection of genetic factors controlling ESBLs production.

Material and Method

Selection of Strain

Six bacterial isolates were obtained from clinical sample of patient with wound infection which collected from Baghdad hospital medical city, these isolates are belonging to genus *Acinetobacter baumanni* after doing morphological and biochemical tests (5).

Antibiotic Susceptibility Testing

Antibiotic test was preformed by Vandipitte disk diffusion method using Mueller –hinton agar (6), The following antibiotics were tested ampicillin, ceftriaxone, cefotaxime, Augmentin, ciprofloxacin, amikacin ,trimethprim, and imipenem. The results were interpreted according to current NCCLs (7) .

Detection β-lactamase enzyme

β-lactamase production was tested for all bacterial isolates using iodometric method, as described by WHO(8).

Detection of ESBLs production

ESBLs production was preformed for all bacterial isolates that were positive in β-lactamase production test, using the Double

Disk Synergy Test (DDST) as described by Benedic (9).

- Muller-Hinton agar plate was inoculated with the bacterial isolates as recommended for a standard disk diffusion susceptibility test.
- Disk containing 30 μg of cefotaxime were placed 15 mm (edge to edge) from a disk of augmentin (20 μg amoxicillin plus 10 μg of clavulanic acid).
- Following incubation for 16-20 hours at 35C, any enhancement of the zone of inhibition between a beta-lactam disk and augmentin disk, was indicative of the presence of an ESBL.

Isolation of plasmid DNA

ESBLs producing isolates were subjected for plasmid DNA isolation following the protocol of salting out (10).

Agarose gel electrophoresis

Agarose gel electrophoresis of isolated plasmid DNA was carried out according to maniatis (11).

Transformation

Transformation experiment was preformed as described by mainatis (11). *Acinetobacter* isolate (A3) was selected for study bacterial transformation by transformed *E.coli*MM294 (plasmid free –riphampicin resistant strain).

Result and Discussion

Identification of Isolates

Six isolates were identified as *Acinetobacter baumannii*, depending on morphological and biochemical tests according to Forbes (5).

Antimicrobial resistance of *Acinetobactor* isolates

Antibiotic sensitivity test was done to show the ability of *Acinetobactor* isolates to resistance the antibiotics by use 8 antibiotics disc, result was taken after determine the diameter of antibiotic inhibition zone. Table (1), illustrate most clinical isolates developed multiple drug resistance .The result show that all isolates exhibit highly resistant to ampecillin, cefotaxim, ceftriaxone, the prevalence of resistance to these antibiotic due to the routine use of these antibiotics in medicine resulted in widespread antibiotic resistance and in the development of genetic mechanisms efficient for the dissemination of antibiotic gene .most resistance to ampecillin, cefotaxim and ceftriaxone in *Acinetobactor* associated with production of pencillinase (12,13).The increased resistance to different β-lactam antibiotics is due to somebacterial strategies to avoid the bacteriocidal effects of these compounds by production of hydrolyzing enzyme (β-lactamases) , alteration the permeability of bacterial outer membrane proteins, and pencillin binding protein, (14). Table (1) illustrated the sensitivity to impenem can be found in all isolates rather than in many studies which concerned the evolution resistance to imipenem among *Acinetobacter* was due to various mechanisms, including class B and D carbapeneamse production enzymes (15,16). *Imp* genes are horizontally transferable because they are inserted in integrons, and some of these integrons are located on conjugative plasmids (17). And about the Augmentin, results show that 50%from isolates resistance to augmentin, while Urban and Rahal demonstrated that Augmentin was bactericidal in vitro and was effective therapeutically for patients infected with *Acinetobactor* strains (4). The low level of resistance to aminoglycoside present in this study, only 16.5% of isolates were resistant to Amikicin, one of aminoglycoside drug. there are three type of aminoglycoside–modifying enzymes have been identified within clinical *Acinetobacter* strains: acetylating, adenylating and phosphorylating enzyme, These enzyme are responsible of *Acinetobacter* resistance to aminoglycoside but it is geographic variations in the incidence of particular genes has been observed (14). 33%from isolates were resistance to (4-quinolone antibiotics) represented by ciprofloxacin, The extensive use of these antimicrobial led to increasing incidence of ciprofloxacin-resistant isolates, 4-quinolone resistance can also be conferred by outer membrane changes that result in decreased uptake(18). Result also showed that only one isolate has resistance to trimethoprime. The resistance to trimethoprime due to genes encoding such resistance are often associated with multiple other resistance gene transposone structures on large conjugative plasmid (15, 19).

Table (1)
Show the result of antibiotic sensitivity for Acinetobacter isolates in Mueller–hinton agar, 37°C, 24hr.

A 6	A 5	A 4	A 3	A 2	A 1	اسم المضاد
R	R	R	R	R	R	*Ampicillin*
R	R	R	R	R	R	*Ceftriaxon*
R	R	R	R	R	R	*Cefotaxime*
S	S	S	S	S	S	*Imipenem*
S	S	S	R	R	R	*Augmentin*
S	S	S	R	S	S	*Trimethoprim*
S	S	S	R	S	S	*Amikacin*
S	S	S	R	S	R	*Ciprofloxacin*

R :resistant ***S: sensitive***

Detection of β-lactamase production

Rapid iodometric method was used for detection β-lactamase production in *Acinetobacter* isolates .This method depends on detection of pencillioc acid, resulted from break down of amide bond in β-lactam ring for pencillins (20). Iodine reacts with starch for formation of dark blue complex, which stay without changing in absence of β-lactamase enzyme (Fig.(1)).In the case of β-lactamase producing bacteria,the resulting pencillioc acid will reduce iodine into iodide, consequently, decolorization of starch–iodine complex occurs (changing the color directly to white) (21). Results show that all isolates were give rapid positive results for production β-lactamase enzyme (5-15 sec). Many studies referred to β-lactamase production is one mechanisms of resistance to β-lactam in *Acinetobacte*r. Furthermore, the β-lactamase encoding gene in *Acinetobacter* isolates which clustered into three groups : the first one is TEM-1 and CARB-5 pencillinases plasmid–mediated enzyme, and the seconde one is class D oxacillinases (integron –located) and the third one is enzyme belonging to class C β-lactamase which chromosomal–encoding gene (14, 22,23).

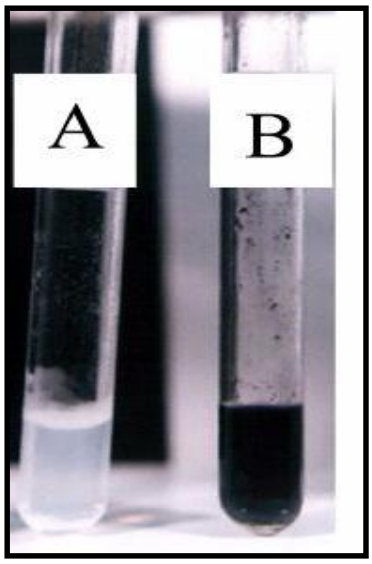

Fig.(1) : Detection of β-lactamase production in Acinetobacter by iodometric assay A-positive resulting Acinetobacter (A3) B-Standard strain E.coliATTC 25922 (negative control).

Detection of ESBLs production

DDST were preformed for detection of ESBLs production, in this test results were determined depending on enhancement of the inhibition zone between beta-lactam disk (cefotaxime) and Augmentin disk (Amoxicillin-clavalanate-20/10mg/ml) was indicative of the presence of ESBL. Out of six –β-lactamase producing strains, only one ESBL-producing isolate (A3) was detected. (Fig.(2)) illustrates the production of ESBL in clinical isolate (A3) ,by enhancement of the inhibition zone between augmentin disk and cefotaxime disk. The detection of ESBL-mediated resistance in G-ve bacteria is one of the major problems in a clinical microbiology laboratories (24). Several researchers mentioned that *Acinetobactor* had ability to produce these type of enzymes, Some of these enzyme are encoded by genes locate on conjugative plasmids which facilitate their transferring rapidly among clinical species and strains in different geographic regions (15,17,20).

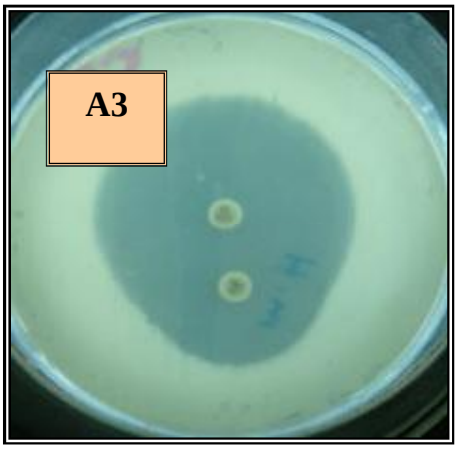

Fig.(2) : Detection of ESBL production in Acinetobacter (A3) byDDST.

Isolation of plasmid DNA

One ESBL producing isolate(A3) was studied in order to determine whether the gene harboed by these plasmids encode for the β-lactamase production and ESBL .Plasmid was extracted using salting out described by Pospiech and Neuman (10). The results (Fig.(3)) revealed that the isolate has two small bands which may be related to two plasmids or may be related to two physiological form of one plasmid and the molecular weight of these plasmids similar to of molecular weight of p BR322 plasmid(4.3kb) in *E.coli* HB101 when electrophoresis was preformed. Agarose gel electrophoresis for detecting plasmid contents is good tool to investigate the epidemiology of nosocomial infections (25). Worldwide showed that *Acinetobacter* isolates harbor more than one plasmid encoded resistance to large number of antibiotics, β-lactamase production and metal ions, most of these markers were associated with plasmids, chromosome, and transposon (26, 27, 15).

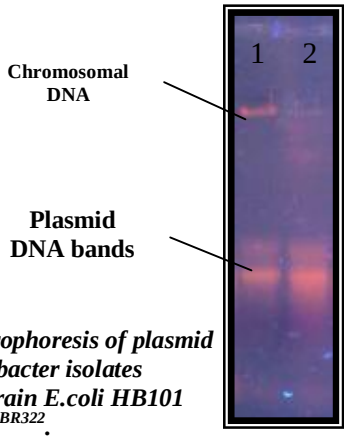

Fig.(3):Agarose gel electrophoresis of plasmid profiles of Acinetobacter isolates
A-Line 1 Standered strain E.coli HB101 contain p ^{BR322}.
B-Line 2 Acinetobacter (A3).

Transformation

The bacterial transformation were carried out in order to detect the role of plasmids in transferring of drug resistance as well as β-lactamase production. ESBL producing isolates (A3) (which resistant to ceftriaxon, cefotaxim, ampicillin, augmentin, trimethprim, ciprophloxaxin, and amikicin) used to transform the standerd strain *Ecoli*MM294 which resistant to rifampcin so the same standard strain was transformed by p BR322 plasmid DNA of *E.coli* HB101 which resistant to Ampicillin and Tetracyclin as positive control. Result from (Table (2)) reveal that the transferred *Ecoli* MM294 by plasmid DNA of A3 and p BR322 plasmid were successful. The transformation frequency for tow experiment ranged from 1.1 $x10^{-4}$ in *Ecoli*MM294 transferred with A3and 1.5 $x0^{-4}$ in *Ecoli*MM294 transferred with p BR322 plasmid .The transformed *Ecoli*MM294 by A3 expressed the antibiotic resistance when they were grow in selective medium containing ampecillin and ceftriaxon .The acquisition of ampecillin and ceftriaxon resistance in *E.coli* MM294 indicated that this properly was plasmid mediated .These transformed cell were also able to give positive result in β-lactamase production and ESBL test. Transformants cells were examined to agarose gel electrophoresis as demonstrated in (Fig.(4)) results of bacterial transformation revealed that transformed cell line 2and4 possessed same plasmid band that p BR322 line 1and A3 line 3 were possessed .we can be concluded from the results above, that the expression of β-lactamases ,antibiotic resistant to ampecillin and ceftriaxone were plasmid encoded transferred to standard strain, which received these proprieties and revealed that plasmid have ability to express in different host cell.Many studies revealed to Horizontal gene transfer by natural genetic transformation in *Acinetobacter* spp.(28,29).

Table (2)

Transformation frequencies on antibiotic selective media.

Source of DNA	Antibiotic resistance transfer by transformation	Frequency
Acinetobacter(A3)	ampecillin and ceftriaxon	1.1×10^{-4}
*E.coli*HB101 Contain p^{BR322}	ampecillin and tetracyclin	1.5×0^{-4}

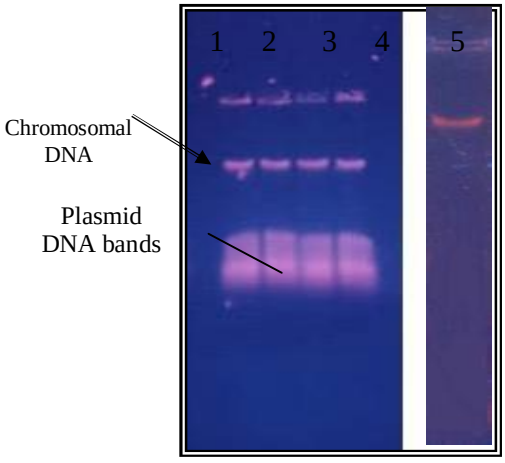

Fig.(4) : Agarose gel electrophoresis of plasmid profiles transformation

1-Standered strain E.coli HB101 contain p^{BR322}

2-Standered strain E.coli MM294 transferred by p BR322 plasmid.

3-plasmid profile of Acinetobacter (A3).

4-Standered strain E.coli MM294 transferred by Acinetobacter (A3 plasmid.

5-Standered strain E.coli MM294.

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الخلاصة

جمعت (6) عزلات من مصادر سريرية من المختبرات التعليمية بمدينة الطب، تم التاكد من عائديتها لجنس *Acinetobacter* اعتمادا على الفحوصات المظهرية والكيموحيوية. ان نتائج فحص الحساسية الدوائية تجاه 8 مضادات حيوية اشارت الى امتلاك العزلات لنمط المقاومة المتعددة، اذ كانت جميع العزلات مقاومة للامبسيلين، والسفترياكسون، والسيفوتاكسيم،، بينما اظهرت 50% من العزلات مقاومة لمضاد الاوكمنتين، اما مضاد السبروفلوكساسين فقد اظهرت 20% من العزلات قدرتها على مقاومة هذا المضاد، بينما اظهرت 10% من العزلات قدرتها على مقاومة كل من الترايمثبيريم والاميكايسين وكانت جميع العزلات حساسة لمضاد الامبينيم. تملك العزلات جميعا القدرة على انتاج انزيمات البيتالاكتاميز باستخدام طريقة اليود القياسية. كما اختبرت قابلية العزلات على انتاج انزيمات البيتالاكتاميز واسعة الطيف باستخدام طريقة الاقراص المزدوجة، وبينت النتائج قابلية عزلة واحدة فقط على انتاج انزيمات البيتالاكتاميز واسعة الطيف. تمت دراسة النسق البلازميدي للعزلة المنتجة لانزيمات البيتالاكتاميز الواسعة الطيف ودلت نتائج الترحيل الكهربائي في هلام الاكاروز ان هذه العزلة تملك حزمتين بلازميدية صغيرة. اظهرت نتائج تجارب التحول ان البلازميدات الصغيرة انتقلت الى بكتريا *E.coli*MM294، مما يشير الى قابلية هذه البلازميدات على التعبير المظهري في اكثر من مضيف.