Detection of Insertion Sequence *ISAba1* Among Clinical Isolates of Carbapenems-Resistant *Acinetobacter baumannii*

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Abstract:

Acinetobacter baumannii is an opportunistic pathogen with increasing clinical importance, especially in immunocompromised patients, causing nosocomial infections of the lungs, urinary tract and surgical wounds. Carbapenems are important antibiotics for treating multidrug resistant *A.* baumannii infections. Insertion sequence *ISAba1* has been detected in correlation with many antimicrobial agent resistance genes in *A. baumannii*. The aim of this study was to investigate existence of insertion sequence (*ISAba1*) in clinical isolates of carbapenem-resistant *A. baumannii* isolated from hospitals in Baghdad city.

Seventy Acinetobacter baumannii isolates were obtained from clinical samples (42 from sputa and 28 from blood) between September 2012 and March 2013.Antibiogram towards several groups of antimicrobial agents including: β- Lactams; aminoglycosides; fluoroquinolone ; and sulfa; as well as the combination of amoxicillin / β-Lactamase inhibitor using disc diffusion method was carried out. Multi-drug resistant isolates were observed in almost all the studied isolates .Minimum inhibitory concentration values were demonstrated using agar dilution method for imipenem antibiotic. Forty eight (68.57%) of 70 isolates were resistant to imipenem with values of MIC ranging between (16-256) µg/ml.ISAba1 sequence among imipenem resistant isolates using PCR technique were identified in 44 isolates (91.66%). Sequencing of amplified product (549 bp) was performed to ensure the occurrence of the ISAba1 sequence in the studied carbapenem-resistant *A.baumannii*.

الخلاصة:

تعد بكتريا *Acinetobacter baumannii* احدى الممرضات الانتهازية التي تزايدت اهميتها من الناحية السريرية,خصوصاً بالنسبة للمرضى الذين يعانون من مشاكل مناعية مسببة اصابات المستشفيات التنفسية والمجاري البولية وجروح العمليات.ان مجموعة الكاربابنيم تعد من المضادات المهمة لعلاج الاصابات المتسببة عن بكتريا *A. baumannii* ذات المقاومة المتعددة للادوية .لقد تم التحري عن كون التسلسل المقحم *ISAba1* له علاقة بالعديد من جينات مقاومة المضادات الجرثومية في بكتريا *A.baumannii* .

الهدف من هذه الدراسة هو للتحري عن وجود التسلسل المقحم *ISAba1* في عزلات سريرية من بكتريا *A.baumannii* مقاومة للكاربابينيم المعزولة من أثنين من مستشفيات مدينة بغداد.

تم الحصول على سبعين عزلة من بكتريا *A.baumannii* من عينات سريرية (42 عينة قشع و 28 عينة دم) للفترة مابين أيلول 2012 الى آذار 2013 .تم إجراء فحص حساسية العزلات البكتيرية تجاه العديد من مجاميع العوامل المضادة للجراثيم التي تشمل مجموعة البيتالاكتم و الامينوكلايكوسيدية والفلوروكوينولونات والسلفا فضلا عن توليفة الاموكسيسلين/مثبط انزيم البيتالاكتاميز وذلك باستعمال طريقة انتشار الاقراص. تم ملاحظة أن الاعم الاغلب من العزلات قيد الدراسة كانت ذات مقاومة متعددة للادوية. تم التحري عن قيم التركيز المثبط الادنى للمضاد الحياتي الاميبنيم تجاه العزلات باستعمال طريقة التخفيف بالاكار, حيث اظهرت نتائج هذا الفحص مقاومة 48 عزلة (68.57%) للاميبنيم من بين مجموع العزلات (70عزلة) وبقيم تتراوح مداها مابين (16-256) مايكروغرام/مل. تم الكشف عن وجود التسلسل المقحم *ISAba1* في 44 عزلة (91,66%) من بين العزلات المقاومة للاميبنيم باستعمال تقنية PCR . تم اجراء معاينة تسلسل ناتج عملية الاكثار بوساطة تقنية PCR الذي كان بطول 549 زوج قاعدة للتأكد من وجود التسلسل المقحم *ISAba1* في بكتريا *A.baumannii* المقاومة للكاربابينيم قيد الدراسة.

Introduction:

Acinetobacter spp. has appeared as one of the most significant pathogens involved in health care associated infections in recent decades. The species *Acinetobacter baumannii* is frequently involved in an intensive care settings where it is an etiology of severe infections such as ventilator-associated pneumonia, bacteremia, urinary tract infections,

meningitis and wound infections [1,2]. It affects mainly the severely immune-compromised, and is typically selected by prior antimicrobial therapy [3].

Currently, one of the most concerns in medicine is enhancement of anti-microbials resistance of bacterial patho-gens. This truth is correlated with higher mortality and morbidity rates, extended hospital stays and increased treatment-related costs [4-6]. Such negative modes have also been noticed in Acinetobacter spp. strains.

Carbapenems are considered sub-stantial antimicrobial agents for healing infections due to multidrugresistant *Acinetobacter* spp. However, several reports have been shown the emergence of resistance to these drugs, with increasing frequency, among *Acinetobacter* spp. clinical isolates [7,8].

Among various mechanisms may award carbapenem resistance in *Acinetobacter* spp., production of carbapenemases is considered the most important one, most often by those belonging to Ambler's class D,these enzymes are called carbapenem-hydrolyzing class D betalactamases (CHDLs), and less frequently by mettallo-betalactamaes (MBLs) [9].

There are four main OXAtype carbapenemases subgroups correlated with A. baumannii. OXAs emerge such weak hydrolysis of carbapenems that they should not allow the development of resistance; however, they are sometimes coupled with insertion elements that can develop carbapenemase expression [10,11]. Insertion sequences (IS) are the smallest and the most generous transposable elements with the ability to independent transposition in microbial genomes. They may lead to several changes in the genetic materials of the microbes such as insertion mutations, genome rearrangements; and increase the dissemination of resistance and virulence determinants among species [12-14]. IS*Aba1* is flanked by 15-bp short inverted repeat sequences, and is bound by 9-bp short direct repeats that correspond to target site duplications likely generated upon transposition [15,16].

Several promoters-containing IS elements play a role in the expression of downstream genes of antimicrobial resistance [17]. Earlier studies identified ISABA-1 neighboring to a β-lactamase resistance gene (ampC) in A. bauman-nii[18,19], and it has been shown that transcription of ampC was dependent on promoter sequences within the element [19], also several authors were reported that increase carbapenem hydrolysis rates may arise due to the acquisition of the IS*Aba1* elements upstream of the naturally existing OXAtype carbapenemase (*bla*OXA-51-like) in addition to acquired (*bla*OXA-23, *bla*OXA-58) encoding genes[20,21]. Mugnier *et al.* [22] demonstrated that IS*Aba1* and the composite transposon Tn2006 were have the ability to transposition in *E. coli* strains, also the capability of IS*Aba1* to mobilize gene of an antimicrobial resistance.

Materials and Methods:

Bacterial isolates:

Seventy Acinetobacter baumannii isolates were collected from clinical specimens (42 from sputa and 28 from blood) of inpatients in Baghdad Teaching hospital and Martyr Gazi Al-Hariry hospital between September 2012 and March 2013.All strains were identified using non-fermenting bacteria identify-cation cards by Vitek-2 compact system (bioMerieux, France)

Antimicrobial susceptibility testing:

The disk diffusion method was used to assess susceptibility to the following antimicrobial agents: Amikacin (30µg/disc); gentamicin (10µg/disc); amoxicillin/clavulanic acid (20/10µg/disc); cefotaxime (30µg/disc); ceftazidime (30µg/disc); cefepime (30µg/disc); aztreonam (30µg/disc); imipenem (10µg/disc); meropenem (10µg/disc); ciproflo-xacin (5µg/disc); and sulfam-

ethoxazole / trimethoprim (23.75/1.25 µg/ disc) (Himedia, India).

Minimum inhibitory concentration (MIC) values of imipenem were determined using agar plate dilution. Two-fold serial dilutions of imipenem were added to molten Mueller-Hinton agar base (Oxoid, England). The resulting plates were inoculated with 10^4 cfu/spot of bacteria and incubated at 37^0C for 24hrs. *Escherichia coli* ATCC 25922 (Obtained from Central Health Laboratories\ Baghdad) was used as control. Antimicrobial susceptibility was counted using break point criteria as defined by the Clinical and Laboratory Standards Institute[23] .

PCR assays and sequencing:

Genomic DNA was extracted by standard DNA extraction kit (Bioner, Korea). *A. baumannii* strains were examined for occurrence of IS*Aba1* sequence by PCR with primers *ISAba1*F: CAC GAA TGC AGA AGT TG and *ISAba1*R: CGA CGA ATA CTA TGA CAC (Accession No. EU604835) giving rises to a 549 bp fragment. The PCR was performed in a thermo cycler (TECHNE, USA). Reaction mixes contained 20 pmol of each primer, 800µM dNTPs, and 25µ Taq DNA polymerase (Bonier, Korea)in a final volume of 50µl. The amplification conditions were as following: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 3 min and final elongation at 72°C for 5 min[24]. The amplified products were noticed after electrophoresis on a 1% agarose gel with ethidium bromide staining; purified PCR products were then sequenced with the dye termination cycle sequencing technique (Macrogene DNA sequencing, South Korea). Searches and alignments for the nucleotide sequences were carried out with the Blast Program http://www.ncbi.nih.gov/Blast.

Results and discussion:

Among 70 multi-drug resistant (MDR) *A. baumannii*, forty five (64.28%) were resistant to all studied antimicrobial agents. The resistance pattern of these MDR isolates were as follow: 100% for cefepim, cefotaxime, and amoxicillin/ clavulanic acid; 97.14% for aztreonam; 94.28% for cotrimoxazole; 91.42% for gentamicin; 90% for ceftazidime; 87.14% for ciprofloxacin; 72.85%for amikacin; and 68.57% for imipenem and meropenem (Table-1). Results of antibiogram have been shown that *A. baumannii* which found in studied hospitals were highly resistant toward antimicrobials used, and this phenomenon may indicate to clinical problematic to conflict this nosocomial pathogen. Previous studies stated that increasing frequencies of MDR *A. baumannii* among the etiology of nosocomial infections causing a perplexing trouble for clinical treatment of this microorganism [25,26].

Adams et, al.[27]. showed that *A. baumannii* were resistant to several groups of antimicrobials including carbabenems, also *A. baumannii* with highly resistance to carbapenems and amino glycosides was reported, the mechanisms of these resistance were due to production of both the OXA-23 carbapenemase and the ArmA 16SrRNA methylase respectively[28]. On the other hand, the resistance to floroquin-olones was found among Gram-negative bacteria including *A. baumannii*, *and* this type of resistance has been shown that correlated with substitutions in guinolone resistance-determining region of DNA gyrase and topoisomerase IV [29,30].

Carbapenem-resistant *A. baumannii* (48 isolates) were subjected to PCR amplification technique to investigate the existence of ISAba1 sequence. Forty four (91.66%) isolates were given positive results with 549 bp amplified product of ISAba1 sequence (Figure-1). The data of amplified product sequencing was revealed the percentage of identity of *ISAba*1

sequence (Figure-2) with the sequence references available in http://www.ncbi.nih.gov/Blast site.

Many authors were identified numeral of putative promoters in *ISAba-1*[19,31]. and it is likely that a various, or more than one, promoter is used in the expression of an adjacent gene. In this respect, based on RTPCR analysis, increased transcription from a promoter located in *ISAba-1* was proposed to be accountable for the hyperproduction of AmpC and ceftazidime resistance in A. baumannii [18].

Segal *et al.*[24].were reported that ISAba1is one of several promotors-containing IS elements that play a role in the expression of genes that encode for antimicrobials resistance, also several studies have been suggested that insertion of ISAba1 upstream of the *bla* OXA-51-like genes may supply the promoter to increase gene expression potentially offering increased levels of carbapenems resistance [10,32,33].

Bratu *et al*. [34]. also mentioned that there is a correlation between the existence of promoter sequence IS*Aba1* and the *bla*OXA-51-like carbapenemase among carbapenems-resistant *A. baumannii*.

Nowak *et al.*[35]. showed by using PCR analysis the presence of *bla*OXA-51-like gene and IS*Aba1* in all carbapenem-resistant *A. baumannii* isolates in this study, as well as all of these isolates were PCR positive for IS*Aba1* sequence.

The prevalent of ISABA-1 in acinetobacters containing the element reflects the movability of the element and indicates that transposition events had happened frequently. This proposes plasticity of the acinetobacter genome as transposition of IS elements can cause a different genome rearrangements [24].

On the other hand, our results revealed that four isolates of forty four carbapeneme-resistant isolates were PCR negative for IS*Aba1*(Figure-1), this finding proposes the presence of another mechanisms of resistance to carbapenems such as ESβLs, the alteration of PBPs, changes in porin expression, or efflux of an antibiotic from a cell [36,37].

In conclusion, we have demonstrated the prevalence of *A. baumannii* with highly resistance to several groups of antimicrobials, also we have identified the insertion sequence IS*Aba1* among almost all of carbapenems-resistant *A.baumannii* clinical isolates.

Table-1: Antimicrobial susceptibility in 70 *A. baumannii* clinical isolates to 11 antimicrobial agents.

Antimicrobials	R		S	
	No.	(%)	No.	(%)
Amikacin	51	72.85	19	27.14
Gentamicin	64	91.42	6	8.57
Amoxicillin/Clavulanic acid	70	100	0	0
Cefotaxime	70	100	0	0
Ceftazidime	63	90	7	10
Cefepime	70	100	0	0
Aztreonam	68	97.14	2	2.85
Imipenem	48	68.57	22	31.42
Meropenem	48	68.57	22	31.42
Ciprofloxacin	61	87.14	9	12.85
Sulfamethoxazole/Trimothprim	66	94.28	4	5.71

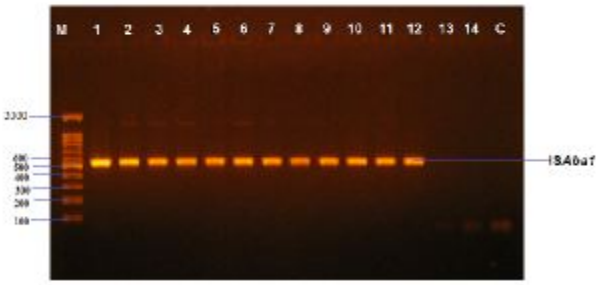

Figure-1: Representative PCR amplification products of *ISAba1* sequence in carbapenems-resistant A. *baumannii* clinical isolates (549 bp product). Lane M, DNA ladder 100-2000 bp; lanes 1-12, *ISAba1*-containing *A baumannii*; lane 13-14, A. *baumannii* isolates with PCR negative to. *ISAba1* sequence; Lane C, Negative control (contain all precursors of PCR mixture without DNA to be amplified). Acinetobacter baumannii strain D46 insertion sequence *ISAba1*, complete sequence; and *AmpC* (ampC) gene, complete cds

Sequence ID: gb|KF030679.1| Length: 2739 Number of Matches: 1
Range 1: 837 to 1006GenBankGraphics

Score:237 bits (128) Expect: 9e-59 Identities:158/172 (92%) Gaps:3/172(1%) Strand:Plus/Plus

Query-3
CACTGCTCACCGATAAACTCTCTGTCTGCGAACCATTCACAATACGGTCTTT ACCAAAA 61
|||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct-837
CACTGCTCACCGATAAACTCTCTGTCTGCGAACACATTCACAATACGGTCTT TACCAAAA 896
Query-62
ATGGCTATAAAGCGTTGAATCATAGCAATAGCGCATCTTTCGAATCTGAACT TCCACGTT-121
|||||||||||||||||||| ||||||| ||| ||||||| ||||||| |||||||||
Sbjct-897
ATGGCTATAAAGCGTTGAATCAAAGCAATACGCTCTTTCGTATCTGAATTTC CACGTT 954
Query-122
AATTAAGCACTGTCCATTGGACAGGTATCGCCATCCCACGATATACGATTGC 173
||||||||| |||||| ||| ||||||||| || |||||||| ||||||||
Sbjct-955
TATTAAGCAATGTCCAAAGGATAGGTATCGCTATTCCACGATAAACGATTGC 1006

Figure-2: Sequencing of insertion sequence ISAba1

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