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Production and Extraction of Siderophores-Catecholate- from -MDR-*Acinetobacter baumannii*

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Abstract:

Siderophores are low molecular weight organic compounds produced by microorganisms growing under low iron concentration.In this study we describe the detection, production and extraction of siderophores secreted by *Acinetobacter baumannii* (Multiple-drug resistant) pathogens.

One hundered twenty Gram –negative non lactose fermenter bacilli isolates have been collected from three hospitals at Baghdad city over three months. Primary identification of these isolates is performed by standard diagnostic methods (biochemical tests and API 20 NE); 19 clinical isolates of *A. baumannii* are cultured on CHROMagar (highly selective medium for detection of MDR Acinetobacter) as well as diagnoses is documented by using Vitek 2 system. Isolates are examined towards 11 different antibiotics. High resistance is recognized for most isolates. Detection of siderophore has been done by examining the isolates on M9 minimum medium; 5 isolates (26%) are producers for siderophore, the highest producing one is isolated from sputum and chosen to extract siderophore catecholate . (Ab5S) isolate is examined on specific synthetic medium for production then siderophore molecules are extracted by ethyl acetate .Weight of dried extract is determined (115 mg/ml) and siderophore chemical nature has been assessed which appeared as catecholate.

Key words: *A. baumannii* , Siderophores, Catecholate

Introduction:

Siderophores are low molecular weight (<14 kDa) iron chelating compounds synthesized in large quantity under iron limitation conditions. There are three major types of siderophores; hydroxamate, catecholate and carboxylate [1]. Iron is a necessary element for the growth of bacterial cells because it acts as a catalyst in enzymatic processes, electron transfer, DNA, RNA syntheses and oxygen metabolism [2]. Iron is also essential for biofilm production because it stabilizes the polysaccharide layer and arranges surface motility [3].

One of the most important steps in initiating an infection is the availability of iron [4]. There are different methods to acquire iron by microorganisms; production of siderophores represents the first and more important method, iron adheres to the bacterial cell by specific receptors and moves inside by common transport techniques [5].

Genes of siderophore biosynthesis are responsible for bacterial infection in

mouse, they activate exotoxins formation, affects cell movement and biofilms maturity [6].

Few studies show the ability of clinical isolates of *A.baumannii* to grow and produce siderophore compounds under iron-deficient condition [7].

A novel siderophore, called acinetobactin, with both catecholate and hydroxamate functional groups are isolated from low-iron cultures of *A. baumannii* ATCC 19606 [8].

Aim of the study: Siderophores have many medical applications , the most important one when act as avechile for transport antibiotics inside bacterial cell so extraction of these molecules should be investigated.

Materials and Methods:

1-Bacterial Isolates : One hundered twenty specimens belonging to non fermenter Gram-negative bacilli have been collected from three hospitals at Baghdad city over three months, 19 clinical isolates have been identified as *A. baumannii*, the diagnosis is confirmed by using highly selective medium CHROMagar Acinetobacter and Vitek 2 system .

2-Antibiotic Susceptibility: *A. baumannii* isolates are tested against 11 different antibiotic discs which have been provided by Bioanalyse (Turkey).

3-Detection of Siderophore: All the isolates are cultured on M9 minimum solid medium which is prepared according to [9] Sambrook *et al*.,(1989) and modified by [10] Shenker *et al*.,(1992) as follows:

A-Dissolving : Na_2HPO_4 (6g) ;KH_2PO_4 (3g) ;NaCl (0.5g) ; NH_4Cl (1g) ; agar-agar(15g) in one liter D.W,adjusted pH to 7.2, autoclaved,cooled to 45°C.

B- The following components are added to the medium prepared in (A):

20ml of MgSo4 (0.5g/20ml); 1ml Dipyridine (0.005g/10ml); 1ml $CaCl_2$ (0.03g/10 ml); 10 ml Glucose (2g/10ml). The components are sterilized by filteration using 0.22µM millipore filters. The medium is then supplemented with 0.1g thiamine.

C- The components are well mixed and poured in disposable sterile plates,.

D-After being solidified, the plates are inoculated with tested isolates (touch by sterile woody stick) and incubated at 37°C for 24 hrs.

E- If the isolate is siderophore producing, the growth will appear as small,single and seperated colonies on M9 medium [8].

4-Siderophore production: A synthetic medium with the following components per liter is used:

mannitol	10 g
sodium gluconate	2 g
K_2HPO_4	0.5g
$MgSO_4$	0.2g
NaCl	0.1g

pH was adjusted to 7 and autoclaved [11].

In order to avoid iron contamination ,inoculation of the of producer isolate is performed by sterile woody stick and incubating the culture for 20hrs. at 35°C.

Note: All the flasks and glassware materials are soaked with acid, rinsed several times with water before using to minimize iron concentration (8).

5-Extraction of siderophore: According to the method of **Jadhav** and Desai (1992); bacterial suspension is centrifuged at 8000 rpm/20 min. The supernatant is acidified to pH=2 ,and immediately siderophore is extracted by adding equal volume of ethyl acetate ,shaked in 50°C water bath to evaporate ethyl acetate layer, then the extract is placed in an oven at 50°C in open petri dishes to obtain dried extract.

6-Estimation of the dry weight of crude extract.

7-Determining the chemical nature of siderophore molecules; bacterial supernatant is used for assay by adding

1 ml of 2% of aqueous $FeCl_3$ to 1ml of sample. The result is positive by appearance of wine color absorbed at 490 nm in UV spectrophotometer [12].

Results and Discussion:

Identification and Antimicrobial Susceptibility

Nineteen isolates of *A. baumannii* from several clinical sources; (5 from sputum; 5 from wound swab; 4 from blood; 3 from urine and two isolates from tracheal secretion) are identified by growing of red colonies on CHRO Magar and depending on the identification results of Vitek 2 system (7). The results of the antimicrobial susceptibility are shown in Table(1).

Table . 1 Antimicrobial Susceptibility of *A.baumannii* Isolates

	Antibiotic										
Isolate	**PI**	**TI**	**CAZ**	**FEP**	**CRO**	**CTX**	**MEM**	**TE**	**CIP**	**LEV**	**SXT**
Ab1S	S	S	R	R	R	R	S	I	R	R	I
Ab2S	R	R	R	S	R	R	R	R	R	R	R
Ab3S	S	S	R	S	R	R	S	S	R	R	R
Ab4S	R	R	S	S	I	I	R	R	R	R	R
Ab5S	R	R	R	R	R	R	R	R	R	R	R
Ab6W	S	R	R	R	R	R	R	R	R	R	I
Ab7W	S	S	R	R	R	R	S	R	R	R	I
Ab8W	S	S	R	R	R	R	S	I	R	R	R
Ab9W	S	S	R	R	R	R	S	I	R	R	R
Ab10W	S	S	R	R	R	R	S	I	R	R	R
Ab11B	R	R	R	R	R	R	R	R	R	R	I
Ab12B	S	S	R	S	R	R	S	I	R	R	R
Ab13B	R	S	R	R	I	R	S	R	R	R	R
Ab14B	R	R	R	R	R	R	R	R	R	R	R
Ab15U	S	S	R	R	R	R	R	R	R	R	R
Ab16U	R	S	R	R	I	R	S	R	R	R	I
Ab17U	S	S	R	R	S	R	R	I	R	R	I
Ab18TS	R	R	R	R	R	R	R	R	R	R	R
Ab19TS	R	S	R	R	S	R	R	R	R	R	I

R;Resistant S;Sensitive I;Intermediate PI;Piperacilin 100µg TI;Ticarcilin 75 µg CAF;Ceftazidime 30 µg; FEP;Cefepime 30 µg CRO;Ceftriaxone 30 µg ;CTX;Cefotaxime 30 µg; MEM; Meropenem 10 µg TE ; Tetracycline 30 µg CIP; Ciprofloxacin 5 µg LEV; Levofloxacin 5 µg SXT ; Trimethoprim-Sulfamethoxazole 1.25/23.75 µg

Detection, Production and Extraction of Siderophores:

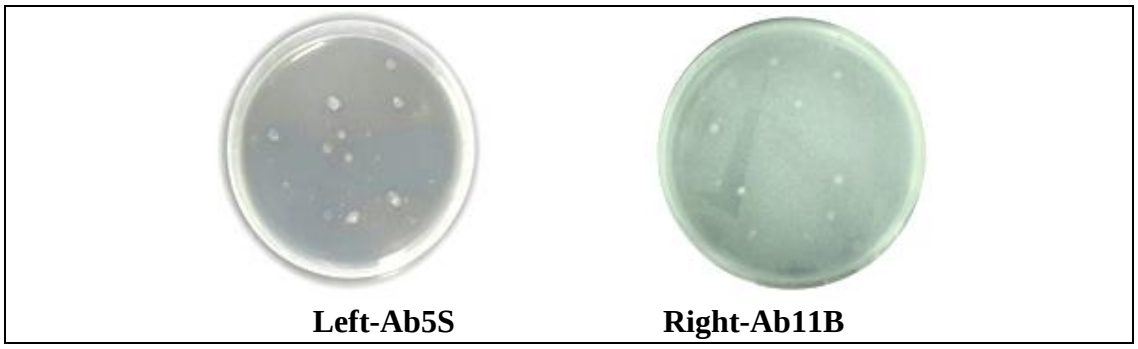
Investigation of iron chelating molecules (catecholate) secreted by MDR-*A.baumannii* under iron restricted conditions has been carried out in this study. Five isolates (26%) are siderophore producers when tested on M9 medium as in Table (2):

Table.2 Siderophore Producing Isolates

Isolate	Ab1S	Ab2S	Ab3S	Ab4S	Ab5S	Ab6W	Ab7W	Ab8W	Ab9W	Ab10W
Type	NP	NP	NP	NP	P	NP	NP	NP	NP	NP
Isolate	Ab11B	Ab12B	Ab13B	Ab14B	Ab15U	Ab16U	Ab17U	Ab18TS	Ab19TS	
Type	P	NP	NP	P	NP	NP	P	P	NP	

P:Producer NP;Non produce S:sputum W:wound B:blood U:urine TS: tracheal secretion

The highlest producing isolate is Ab5S from sputum. Clear correlation is noticed between antimicrobial resistance and siderophore formation. Table (1) shows that the producer isolates are; Ab5S, Ab6W, Ab11B, Ab14B, Ab18TS with high resistance to antimicrobials. On M9 medium as mentiond earlier, the producer isolates colonies appear circular wrinkled and dried (Fig.1):



Left-Ab5S **Right-Ab11B**

Fig.1 Colonies of Siderophore Producing Isolates on M9 Minimum Medium

Each colony in the Figure above represents the growth resulted from the stick touch, little and weak growth may be attributed to the poor and limited nutrients and depleted iron in M9 minimum medium.

Not all the isolates are siderophore producers and this agreed with the results of Yamamoto *et al*.,(1994) who report that 4 of 12 clinical *A.baumannii* strains examined are siderophore producers, indicative of strain –to-strain variation in the ability of acinetobactin production. [13] Sokol *et al*.,(1992) describe a novel siderophore from *Pseudomonas cepacia* (recently *Burkholderia cepacia*) cultures named azurechelin, and indicat that 88% of pathogenic isolates produced it. This compound correlates to bacterial virulence and may increase morbidity and mortality in patients of cystic fibrosis.[14] Bnyan *et al*.,(2010) showing that siderophore production is in 76.6% of uropathogenic *Escherichia coli* (UPEC) compared to 5% in *E.coli* fecal isolates thus siderophore production has been shown to be more frequent in *E. coli* from patients with UTI than in fecal isolates and is suggests that siderophore production positive isolates can be considered as UPEC. [15] Abass *et al*.,(2014) demonstrate the role of two other genes in the virulence of UPEC; fimH (90.0)% and kpsMTII (72.0)% of *E.coli* isolated from UTI.

Other isolates have shown no growth on M9 minimum medium that may suggest variation in efficiency of siderophore production or they may form different type of siderophore other than catecholate according to Yamamoto *et al*.,(1994) who detect and extract the acinetobactin (catecholate and hydroxamate functional groups) from *A.baumannii* .

The isolate Ab5S is inoculated in a specific minimum liquid medium and care is taken to use metal-free glass ware. Flasks and other glassware are kept in acid to remove all traces of metals from medium, inoculation have been done by sterile woody stick. The optimum conditions for maximum production occurs after 20hrs.at 30°C, pH 7, where no iron contamination was found. Previous studies indicate that the presence of iron can inhibit siderophore production as well as results indicating that iron-binding proteins, which may play a role in chelating the siderophore-bound iron, are produced under iron-starved conditions [16,17]

Iron-binding proteins are present in membranes of cultures grown under iron

limitation. Siderophores chelate iron and supply to bacterial cell by outer membrane receptors, iron is an important nutrient element for growth and maintenance, hence the siderophore molecules after 20 hrs. Become outside the bacterial cells –in the medium. The concentration of siderophores in the culture supernatant is maximal after 20 hrs. of growth which means that siderophore production occurrs in parallel with growth. Therefore extraction of these molecules should be done on bacterial filterate after precipitating of bacterial cells.

Because ethyl acetate layer is evaporated to dryness by shaking water –bath at 50° C, care should be taken from high temperatures which may denature the amino acids conjugated with the phenolates in catecholate siderophores.

Weight of crude extract is estimated which is equal to 115mg/l, it is considered low when compared to the result got by Hussien *et al*.,(2013) where the weight of pyoverdin extract from *P.aeruginosa* is equal to 235 mg/l that may be attributed to the difference in the extraction medium and other experiment parameters as well as the producing microorganism. In another study [18], the weight is 200 mg/l also they extracted siderophores from *P.aeruginosa* .Chemical nature of the extracted molecules indicates catecholate (phenolates) structure because of wine colour of extract after adding 2% aq.$FeCl_3$ indicator absorbed at 490 nm in UV spectrophotometer.

Actis *et al.,* (1993) show in their work that different *A. baumannii* isolates are able to grow under iron-depleted conditions. The bacterial growth is accompanied by the formation of iron-regulated catechol siderophores, independently of the bacterial plasmid content.

Goel *et al*.,(1998) report that *A. baumannii* under iron restricted conditions develop four high molecular weight outer membrane proteins (OMPs) of 88, 84, 80 and 77 kDa in iron depleted medium CDM-Fe which were absent in CDM + Fe medium, expressing iron regulated outer membrane proteins (IROMPs) along with production of catechol type siderophore is necessary to acquire iron from the external medium.

[19] Fukushima *et al*.,(2013) have documented that under iron starvation, siderophores are excreted, scavenge ferric ions and the complex is shuttled inside the cell. The microbial hydrophobicity decreases if Fe concentration is restricted which alters the surface protein receptors and leads to limitation of biofilm secretion [20].

[21] Pal and Gokarn (2010) have concluded that there is no significant difference occuring in the production of siderophore in commensal and clinical bacterial isolates. They suggested that siderophore production may be a necessary factor of virulence but not a determinant of virulence.

[22] Al- Muhanna *et al*., (2014) have discussed the correlation between siderophore and *aerobactin* gene. Isolates of *K. pneumoniae* that produce *aerobactin* are more virulent, but non siderophore producing isolates are less virulent. Also, they find that *K. pneumoniae* isolates totally produce siderophores are expressed *aerobactin* genes.

[23] **Naik** and Dubey (2011) document that low lead nitrates concentrations up to 0.5mM may enhance siderophore production in *P. aeruginosa*.

We conclude from our study that MDR-*A. baumannii* could produce siderophores but in variable amounts among isolates. It is apparent that highly antimicrobial resistant isolates are siderophore producers.The extracted siderophore compound is from catecholate type.

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إنتاج وإستخلاص مركبات السايدروفور- نوع الكاتيكولات- من بكتريا *Acinetobacter baumannii* متعددة المقاومة للمضادات الحيوية

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الخلاصة:

السايدروفورات (المركبات الحاملة أو الناقلة للحديد) هي جزيئات عضوية ذات أوزان جزيئية واطئة تنتجها الأحياء المجهرية عند نموها في ظروف يكون فيها عنصر الحديد قليل أو معدوم . تم في الدراسة الحالية التحري عن مركبات السايدروفور وظروف انتاجها واستخلاصها من عزلات سريرية لبكتريا *A.baumannii* متعددة المقاومة للمضادات الحياتية. جمعت 120 عزلة لبكتريا سالبة لصبغة غرام غير مخمرة لسكر اللاكتوز من ثلاث مستشفيات في مدينة بغداد ولمدة ثلاثة شهور. شخصت العزلات مبدئيا بالاختبارات التشخيصية القياسية: الاختبارات الكيموحيوية واشرطة (API 20 NE) وظهر أن 19 عزلة منها تعود لبكتريا *A.baumannii*متعددة المقاومة للمضادات الحيوية والتي تم تأكيد تشخيصها بأستخدام الوسط الملون CHROMagar الخاص بالاسينيتو فضلا عن نظام Vitek 2 . اخضعت جميع العزلات لفحص الحساسية للمضادات الحياتية . تم التحري عن العزلات المنتجة للمركبات الناقلة للحديد بتنميتها في الوسط المتدني M9 minimum medium الصلب وتبين أن 26% منها كانت منتجة لجزيئات السايدروفور أذ كانت العزلة الأوفر إنتاجاً هي AbS5 مصدرها هو القشع (Sputum). نميت هذه العزلة في وسط تركيبي خاص لأنتاج الجزيئات الناقلة للحديد وأستخلصت هذه الجزيئات بواسطة مركب خلات الإيثيل وتم تقدير وزن المستخلص الخام بما يساوي 115 ملغم/ مليلتر وتحديد طبيعته الكيميائية والتي ظهر أنها من الكاتيكولات (فينولات).

الكلمات المفتاحية: *A.baumannii*، السايدروفورات، الكاتيكولات .