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 hundred 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 hundred twenty specimen 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- Antibi0tic 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: Na2HPO4 (6g) ; KH2PO4 (3g) ; NaCl (0.5g) ; NH4Cl (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 CaCl2 (0.03g/10 ml) ; 10 ml Glucose (2g/10ml).

The components are sterilized by filtration 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 separated colonies on M9 medium [8].

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

| Component       | Amount |
|-----------------|--------|
| mannitol        | 10 g   |
| sodium gluconate| 2 g    |
| K2HPO4          | 0.5g   |
| MgSO4           | 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\textsubscript{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**

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

R:Resistant S:Sensitive I:Intermediate PI:Piperacillin 100 µg TI:Ticarcillin 75 µg CAF:Cefazidime 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 |
|---------|------|------|------|------|------|------|------|------|------|
| Type | NP | NP | NP | NP | P | NP | NP | NP | NP |
| Isolate | Ab11B | Ab12B | Ab13B | Ab14B | Ab15U | Ab16U | Ab17U | Ab18TS |
| Type | P | NP | NP | NP | P | NP | NP | NP |
| P:Producer | NP:Non produce | S:sputum | W:wound | B:blood | U:urine | TS: tracheal secretion |
The highest 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):

![Fig.1 Colonies of Siderophore Producing Isolates on M9 Minimum Medium](image)

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 occurs 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.FeCl₃ 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 occurring 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.
References:
[1] Ali, S. S. and Vidhale, N. N. 2013. Bacterial Siderophore and their Application: A review Int. J. Curr. Microbiol. App. Sci. 2(12): 303-312.
[2] Aguado- Santacruz, G. A. A.; Moreno-Gómez, B. A.; Jiménez-Francisco, B. B.; García-Moya E. B. and Preciado-Ortiz, R. E. 2012. Impact of the microbial siderophores and phytosiderophores on the iron assimilation by plants. Rev. Fitotec. Mex.; 35(7):9–21.
[3] Chhibber, S.; Nag, D. and Bansal, S. 2013. Inhibiting biofilm formation by Klebsiella pneumoniae B5055 using an iron antagonizing molecule and a bacteriophage. BMC Microbiol.; 13(1):174–183.
[4] Andrews, S. C.; Robinson, A. K. and Rodriguez-Quinones, F. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. 27(5):215-237.
[5] Faraldo- Gómez, J. D. and Sansom, M. S. 2003. Acquisition of siderophores in Gram-negative bacteria. Nat. Rev. Mol. Cell Biol. 4(1): 105–116.
[6] Mossialos, D. and Amoutzias, G. D. 2008. Role of siderophores in cystic fibrosis pathogenesis foes or friends. Int.J.Med.Microbiol.299 (15):87-98
[7] Actis, L. A.; Tolmasky, M. E.; Crosa, L. M. and Crosa, H. J. 1993. Effect of Iron-Limiting Conditions on Growth of Clinical Isolates of Acinetobacter baumannii. J. Clin. Microbiol.10(2):2812-2815.
[8] Yamamoto, S.; Okujo, N. and Sakakibara, Y. 1994. Isolation and structure elucidation of acinetobactin, a novel siderophore from Acinetobacter baumannii. Arch. Microbiol. 162(4):249-254.
[9] Sambrook, J.; Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning:a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. 29.
[10] Shenker, M.; Oliver, I.; Heimann, M.; Hadar, Y. and Chen, Y. 1992. Modified M9 medium to detection siderophores of Rhizobium ssp in Tomato roots. J. Plant Nutr. 15(9):2173-2178.
[11] Jadhav, R. S. and Desai, A. J. 1992. Isolation and characterization of siderophore from cowpea Rhizobium (peanut isolate). Curr. Microbiol. 24(3): 137-141.
[12] Neilands, J.B. 1981. Microbial iron compounds. Ann. Rev. Biochem. 50(4):715-731.
[13] Sokol, P. A.; Lewis, C. J. and Dennis, J. J. 1992. Isolation of a novel siderophore from Pseudomonas cepacia. J. Med. Microbiol. 36(11): 184-189.
[14] Bnyan, I. H.; Bnyan H. A. and Ali, J. A. 2010. The Siderophore Production of E. coli Isolated from Urinary Tract Infection and Fecal Isolates. J. Babylon University/Pure and Applied Sciences/ 3(18):862-864.
[15] Abass, Z. N.; Habib, K. A. and Abed, Z. A. 2014. Genotypic Study of Two Virulence Factors fimH and kps MTII in Uropathogenic Escherichia coli Isolates from Children Patients with Urinary Tract Infections. Bag. Sci. J. 11(4):1475-1480.
[16] Goel, V. K.; Kapil, A.; Das, B. and Rao, D. N. 1998. Influence of iron on growth and extracellular products of Acinetobacter baumannii. Jap. J. Med. Sci. & Biol. 51(1):25-33.
[17] Hussain, S. S.; Desouky, O. A.; Abdel-Haliem, M. E. F. and El-Mougith, A. A. 2013. Enhancement the Production of Siderophores-Pyoverdine by Pseudomonas aeruginosa SHA 282 and Its Chelation with Thorium (IV). W. Res. J. Biotech. 1(1):17-23.
[18] Schalk, I. 2008. Metal trafficking via siderophores in Gram-negative
bacteria: specificities and characteristics of the pyoverdine pathway. J. inorg. and Biochem., 102(10):1159-1169.

[19] Fukushima, T.; Alred, B. E.; Sia, A. K.; Nichiporuk, R.; Andersen, U. N. and Raymond, K. N. 2013. Gram-positive siderophore-shuttle with iron-exchange from Fe-siderophore to apo-siderophore by Bacillus cereus YxeB. Proc. Natl. Acad. Sci. U.S.A. 110(30): 13821–13826.

[20] Simões, L. C.; Simões, M. and Vieira, M. J. 2007. Biofilm interactions between distinct bacterial genera isolated from drinking water. Appl. Environ. Microbiol.; 73(8):6192–6200.

[21] Pal, R. B. and Gokarn, K. 2010. Siderophores and Pathogenicity of Microorganisms. J. Biosci. Tech.1 (3).127-134.

[22] Al- Muhanna, A. S.; Al-Rediany, R. S. and Alzuhairi, M. A. 2014. Molecular characterization of aerobactin gene among Klebsiella isolated from Wound and Burn Infections Int. J. Curr. Microbiol. App. Sci. 3(5): 26-31.

[23] Naik, M. M. and Dubey , S. K. 2011. Lead-enhanced siderophore production and alteration in cell morphology in a Pb-resistant Pseudomonas aeruginosa strain 4EA. Curr. Microbiol.62(2):409-14.

**Acinetobacter baumannii**

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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 ظروف انتاجها واستخلاصها من عزلات سريرية لـAcinetobacter baumannii متعددة المقاومة للمضادات الحيوية. جمعت 120 عزلة لـ Acinetobacter baumannii سالبة لصبغة غرام غير مخمرة لسكر اللاكتوز من ثلاث مستشفيات في مدينة بغداد ولمدة ثلاثة أشهر. شُخصت العزلات مبدئياً بالاختبارات التشخيصية القياسية: الاختبارات الكيميائية وأشرطة API 20 NE وظهر أن 19 عزلة منها تعود لـAcinetobacter baumannii متعددة المقاومة للمضادات الحيوية والتي تم تأكيد تشخيصها باستخدام الوسط الملون CHROMagar البالاسيتيتوفل فضلا عن نظام Vitek 2. أشخضت جميع العزلات فحص الحساسية الخاص بالاستيتو فضلا عن نظام CHROMagar M9 للكريات ومن المتراوحة النسبية في الوسط المغذي minimum medium للعزلات الحساسية. تم التحري عن العزلات المنتجة للحديد بتفتيتهم في الوسط المغذي M9. تم التحري عن مركبات السايدروفور من العزلات الحساسية. أظهرت هذه الدراسة أن النصائح بوضوح أن 26% منها كانت مصدرها من الأحياء المجهرية. تم تأكيد ذلك باستخدام لاصقة Sputum. نتائج هذه الدراسة تؤكد أن Acinetobacter baumannii متعددة المقاومة للمضادات الحيوية والتي تعود لـ Sputum من المصدر مكمل لسواها 1 مل/مليлитر وتحديد طبيعته الكيميائية والتي تدل أنها من الأحياء المجهرية.

الكلمات المفتاحية: السايدروفورات، الكاتيكولات، Acinetobacter baumannii