ASCORBIC ACID INDUCED LOSS OF AN ANTIBIOTIC RESISTANCE PLASMID IN Serratia marcescens

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Abstract
Four bacterial isolates Serratia marcescens (S. m.1, S. m.2, S. m.3, S. m.4) were tested for their resistance toward ten Antibiotics which consider the most common usage in the hospitals in the country by antibiotic disc method. The isolate S. m.1 was selected according to the antibiotic resistance results since it was resist for several antibiotics and four of these antibiotics were chosen because of the previous researches referred to that these features are carried by plasmid which is Ampicillin, Streptomycin, Tetracyclin and Chloramphenicol and used as a plasmid markers, the DNA of the isolate S. m.1 was contain a plasmid band , the Ascorbic acid used in different concentrations as a curing agent for the bacteria S. marcescens plasmids, the results of the curing experiment showed that the curing of S. marcescens with ascorbic acid was succeeded in 0.67 %, the cured bacteria isolate have lost its ability to resist the four antibiotics which the mother isolate was resist, and the plasmid DNA extraction for the cured bacteria result showed that the cured bacteria have lost the plasmid band which confirm that this plasmid which coded for Antibiotic resistance feature against the four antibiotics mentioned above.

Serratia marcescens تحفز فقدان بلازما المقاومة للمضادات الحيوية في بكتريا

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الخلاصة
تم اختبار اربعة غزلوت من بكتريا Serratia marcescens (S. m.1, S. m.2, S. m.3, S. 4 ) في المقاومة للأمراض الحيوية، حيث اخترتarena 1.eps تجاه عشرة من المضادات الحيوية الأكثر استخداماً في القطر بطريقة الأقراص حيث m.1

S. m.1

تضحية عدة مرات ل gọn السلسلة السابقة، حيث كونها ذات طيفاً بلازميدي ومسبباً للامراضة، وتكونت مجموعة مخاطر بلازميدي، تم استخدام الدنا البلازميدي في كل الدلال، وتشمل عملية التوجيه الكهروالفا في هلام الأكازور الحواء للعزلة. S. m.1

Serratia marcescens

استخدام مادة الأكسكورية مرت شنَّى لمستخدمات بلازميدي بكثرة

ظهرت النتائج نجاح عملية التحديد للعزلة في الدلال بنسبة 67.6% . ان العزلة المختلطة المعتادة فقدت

القدرة في محاكمة المضادات الحيوية الأربعة التي كانت العزلة الام تقاومها كما تم استخلاص الدنا

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Introduction

The spp. Serratia marcescens. Contains many of bacterial types belong to the family of enterobacteriaceae which show negative staining with the gram stain. These genus members considered in the past from the normal flora in the environment and represents no threat to the human and animals health.

S. marcescens is the most known type of this species and because of its non pathogenic behavior also for its ability to produce a specific red pigment, the researchers in the early last century used this bacteria to determine the ability of bacteria for penetration and distribution inside the human body [1, 2] but at the last decades of the last century, the ability of S. marcescens for localization in the hospitals causing outbreaks and sever infections in the different body organs to the human had been proven like sever urinary tract infection, bacteremia and septicemia [3, 4, 5, 6, 7, 8] Also cause sever Dermatitis and Endocarditis, infection of soft tissue like post operation wounds, inflammation and infection of bone and arthritis [9].

In addition to detected as a causative agent for sever meningitis, brain abscess, Otitis media and cause sever infection in the Abdominal organs like liver and pancreas abscesses, accumulate the infectious exudates in the periton [10, 11, 12] also detected as the causative agent for recurrent infection in the newly born babies and intensive care unites [13, 14, 15] many S. marcescens infection cases recorded for the patients with chronic and exhausted diseases like diabetes, cancers and renal failure causing sever systemic infection [16, 17].

The appearance of new strains of S. marcescens which show a wide resistance against the antibiotics may classify this bacteria as a highly virulence pathogen toward the human and animals in the near future and many researches refer to the reason of increasing the clinically importance of S. marcescens which comes from the Plasmid – encoded resistance against some of the important antibiotics which belong to the stable β-lactamic antibiotic like Ceftazidime, Cefotaxime, Aztreonam, Imipenem, Carbapenem also which belong to the group of Aminoglycoside like Amikacin [18] which are currently wide used in the hospitals, this type of resistance can be transmitted among the bacterial populations via conjugation and transformation which happens normally in the nature [19, 20, 21, 22, 23, 24]. Many method used to cure the bacterial plasmid like chemical curing agents such as: Novobiocin, Ethidium bromide, Acriflavine, Acridine orange also physical agents like growth at elevated temperature in order to study the traits carried on the plasmids [25, 26].

However, use of an intercalating dye such as Ethidium bromide is hazardous and may lead to rearrangement of the DNA molecule rather than actual loss of plasmid [27] also there are several articles have demonstrated the physical loss of a plasmid from a strain of Staphylococcus aureus [28, 29, 30] also demonstrated the physical loss of a plasmid from a strain of Pediococcus acidilactici [31].

In our investigation we have used ascorbic acid (vitamin C) which is a non-hazardous, cheap, available curing agent and safe to handle in order to test its efficacy in curing of Serratia marcescnes's plasmid

Materials and methods

Bacterial isolates

four strains of serratia marcescens were obtained from the Biotechnology department /College of Science- Baghdad University ,and tested to determined there resistance for 10 types of Antibiotic disks as described in Vandepitte [32].

Plasmid profile

Colonies of the selected strain was purified individually, transferred into nutrient broth and grow for 18 h. at 30 °C. Plasmid DNA was isolated from the selected strain according to the method described by Heijazi [33] were analyzed by agarose (0.8%) gel electrophoresis, stained with ethidium bromide [34].

Curing experiment

The curing experiment done as described by Ramesh et.al.[31] with few modifications. A
single colony of *S. marcescens* which showing a resistance toward the antibiotics: Ampicillin, Tetracycline, Chloramphenicol and Streptomycin. A single colony was selected and incubated into Nutrient broth and incubated at 30 °C for 18 h. this culture was used as an inoculum for the curing experiment. The L-ascorbic acid (sigma) was used by preparing a freshly prepared stock solution with a concentration of 50% by dissolving 5 g of ascorbic acid into 10 ml of Distilled water whose pH was adjusted to 7.2 by addition of NaHCO₃ and sterilized by the filtration through 0.22 µl Millipore filters.

Tubes contains 1 ml of Nutrient broth supplemented with ascorbic acid with following addition: 100 – 900 µl /ml from the freshly prepared stock also used a control tube which composed of Nutrient broth without ascorbic acid, then these tubes were inoculated with 50 µl of 10⁸ cells over night growth (the grown culture was determined approximately by comparing with Mcferland tube [32], and the tubes were grown for 18 hour at 30 °C. The tube prior the one with no growth (contains no turbidity) comparing with the control tube (in which case the cells where grown in Nutrient broth without ascorbic acid) chosen was and many dilutions prepared from this tube and the appropriate dilutions of the culture were pour plated and well spread by sterile spreader on Nutrient agar plates and incubated for 18 hour at 30 °C for colonies to appear.

The plate contained from 30 – 300 single colonies was chosen and the single colonies were transported to two nutrient agar plates (duplicates) the first one contains Amoxicillin with final concentration 10 µl /ml and the plates was incubated at 30 °C for 18 h., the colony failed to grow on the Nutrient agar plate with Amoxicillin was picked up from its replica in the duplicate plate without Amoxicillin and its ability to resist the antibiotics under study (Streptomycin, Tetracycline, Chloramphenicol, were tested.

the plasmid profile of the parental strains and its cured derivative were isolated as described by Heijazi etal.,[33] were analyzed by agarose (0.8%) gel electrophoresis, stained with ethidium bromide [34].

**Results and discussion**

The antibiogram of the selected strains of *S. marcescens* were tested toward the Antibiotic disks listed in the table no. 1

<table>
<thead>
<tr>
<th>Antibiotic Disks</th>
<th>S. m. 1</th>
<th>S. m. 2</th>
<th>S. m. 3</th>
<th>S. m. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am (10 µg)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S (10 µg)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>C (30 µg)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Tc(30 µg)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>CN(10µg)</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>CIP(5µg)</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>TOB(10µg)</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>NA(30 µg)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>CL(30 µg)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>CTX(30µg)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

S= Sensitive       CIP=Ciprofloxacin
R=Resistant        C=Chloramphenicol
CN=Gentamycin      S=Streptomycin
TOB= Tobramycin    CL = Cephalexin
CTX=Cefotaxim      Tc = Tetracyclin
NA=Nalidixic acid  Am= Ampicillin

According to the antibiotic resistance pattern, the strain *S. m 1* was chosen because it showed a big resistance to the antibiotics: Ampicillin, Tetracycline, Streptomycin and Chloramphenicol which recorded as plasmid mediated traits in *S. marcescens*, for this they used as markers for curing experiment.

The plasmid DNA isolation of the selected strain showed that it contains a plasmid which may carry the antibiotic resistance features.

The concentration of ascorbic acid play a role in inhibit the bacterial growth which means when the concentration of ascorbic acid the viability of bacterial growth will be decrease dramatically (31), for this when different ascorbic acid added to the tubes were the *S. marcescens* inoculated, the bacterial growth culture was absent (no growth) in the tube with the concentration of 490 µl (from the stock)/ml for this the tube with concentration of ascorbic acid 480 µl (from the stock)/ml was chosen which was contained a weak bacteria growth which represented the utmost effect of ascorbic acid on *S. marcescens* population which not lead to kill the bacteria but put exposure strong pressure which may cause the curing.

The toxic effect of the ascorbate on the cells was clearly dose depended (31). Ascorbic acid causes conformational damage to unprotected
circular covalently closed (CCC) plasmid, L-ascorbic acid inhibits a wide range of biological function and modify the properties of DNA, generation of hydrogen peroxide and hydroxyl radicals by autoxidation and lipid peroxidation of membrane components (36, 37,38,39). The reactive O$_2^-$ species such as OH$^-$ and H$_2$O$_2$ are involved in DNA damage by a Fenton – type reaction which has been shown to occur in bacteria cell (40) also the ascorbic acid increase the effect of other agents to cleavage of plasmid DNA this will lead to an inefficient replication and might eventually lead to its loss during cell division.

When the appropriate diluent , were pour plated on the plates, the single colonies to the nutrient agar plates with Ampicillin in order to check if they curing experiment was succeeded , the selected plate with the dilution of $10^4$ which was contained 150 single colonies which transported to the duplicate plates one contained the Ampicillin as a marker and the second without antibiotic, one cured colony was selected because it couldn't grew on the plate with antibiotics but grew on the plate without it and this may indicates that this colony was loss its plasmid.

The ability of this derivative strain to resist the antibiotics used in this study were tested again and the result showed that this strain became sensitive toward these antibiotics which the mother cell was resistant against them.

The plasmid DNA was isolated from both colony (the mother strain and the derivative strain)and agarose gel electrophoresis revealed the elimination of plasmid from the cured strain (well no. 2) comparing with mother strain which was contains a clear plasmid bands (well no. 1).

This result refer to the fact that ascorbic acid – treated cells have lost the plasmid coding for resistance of antibiotics.

The effect of ascorbic acid in induce loss of plasmid – encoding antibiotic resistance has been demonstrated earlier in _Pediococcus acidilactici_ (31) _Azotobacter chroococcum_ (30) and _Staphylococcus aureus_ (41,29).

Our results refers to that the curing percentage was 0.67 % and this consider low percentage when comparing this result with the other researches which referred to that the result of using ascorbic acid to cure the plasmids of _Pediococcus acidilactici_ was 35% (31) and in case of _Staphylococcus aureus_ the result was 12-35% (41, 29), and this may be due to the ability of _S. marcescens_ to produce Catalase enzyme (42) which is known to be a protective enzyme reduce the effect of H$_2$O$_2$ and OH$^-$ mediated damage to the DNA (26) also the synergic interaction of ascorbic acid with the antibiotics against multi resistance bacteria like _Pseudomonas aeruginosa_ (12) refer to that the ascorbic acid since he is well tolerated from the human and animals bodies, then it may be useful when it giving to the patients concomitant with courses of antibiotics in order to control on the multi resistance bacteria.

References


