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.

تحفيز فقدان بلازميد المقاومة للمضادات الحيوية في بكتريا *Serratia marcescens* باستخدام حامض الاسكوريك

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الخلاصة

تم اختبار اربعة عزلات من بكتيريا Serratia marcescens (S. m.1, S. m.2, S. m3, S. 4) واختبرت حساسيتها تجاه عشرة من المضادات الحيوية الاكثر استخداماً في القطر بطريقة الاقراص حيث انتخبت العزلة S. m.1 على ضوء المقاومة للمضادات الحيوية ,وكانت مقازمو لعدد من المضادات الحيوية انتخب منها لؤبعة لكون الدراسات السابقة اشارت لكونها ذات منشأ بلازميدي وهي الامبسلين والستربتومايسين والتتراسايكلين والكلورامفينيكول واستخدمت كمؤشرات بلازميدية, تم استخلاص الدنا البلازميدي للعزلة قيد الدراسة واكدت عملية الترحيل الكهربائي في هلام الاكاروز احتواء العزلة S. m.1 على حزمة بلازميدية, تم استخدام مادة الاسكوربك اسد بتراكيز مختلفة كمادة محيدة لبلازميدات بكتيريا Serratia marcescens اظهرت النائج نجاح عملية التحييد للعزلة قيد الدراسة بنسبة 0.67 % , ان العزلة البكتيرية المحيدة فقدت القدرة على مقاومة المضادات الحيوية الاربعة التي كانت العزلة الام تقاومها كما تم استخلاص الدنا

البلازميدي للبكتيريا المحيدة واظهرت نتائج الترحيل الكهربائي ان البكتيريا المحيدة فقدت الحزمة البلازميدية مما يؤكد ان هذا البلازميد يحمل صفة المقاومة للمضادات الحيوية الاربعة المذكورة اعلاه.

Introduction

The spp. *Serratia marcescens*. Contains many of bacterial types belong to the family of enterobacteriacae 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 ß-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 ºC.

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 *etal.*,[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 ºC 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 $NaHCO_3$. 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 10^8 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 ºC.

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 ºC 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 ºC 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 1 : shows the antibiotic resistance pattern for the Serratia marcesnces stains

Antibiotic Disks	*S. m.* 1	*S. m.* 2	*S. m.* 3	*S. m.* 4
Am (10 µg)	**R**	**R**	**R**	**R**
S (10 µg)	**R**	**R**	**R**	**R**
C (30 µg)	**R**	**R**	**S**	**S**
Tc(30 µg)	**R**	**S**	**S**	**S**
CN(10µg)	**S**	**S**	**R**	**S**
CIP(**5µg)**	**S**	**S**	**R**	**S**
TOB(10µg)	**S**	**S**	**R**	**S**
NA(30 µg)	**R**	**R**	**S**	**S**
CL(30 µg)	**R**	**R**	**R**	**R**
CTX(30µg)	R	**S**	**S**	**S**

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, Tetracyclin, 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 redicals by autoxidation and lipid peroxidation of membrane components (36, 37,38,39).

The reactive O_2 species such as OH^- and H_2O_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).

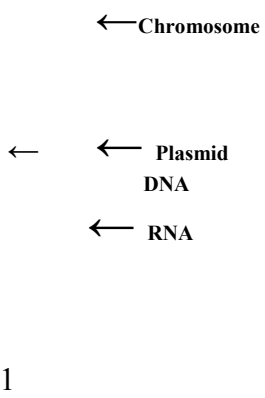

Figure 1: The 0.8 % agarose Gel electrophoresis picture (Time: after 2 hours and Volt: 5V /cm) which shows:

1- **The DNA isolated from the mother strain.**
2- **The DNA isolated from the cured strain.**

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_2O_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

1. Paine,T.F. **1946.** Illness in man following inhalation of *Serratia marcescens* , *J. of Infectious Disease*, **79** :226- 230.

2. McEntegart, M. G. and Porterfield, J. S. **1949**. Bacteremia following dental extractions, *Lance*t, **2**: 596 - 600.
3. Okuda, T., Endo, N., Osada, N. and Zen-Yoji, H. **1984**. Outbreak of nosocomial urinary tract infections caused by *Serratia marcescens*, *J. of Clinical Microb.* **20**(4): 691- 695.
4. Franczek, S. P., Williams, R. P. and Jull, S. I. **1986.** A survey of potential virulence factors in clinical and environmental isolates of *Serratia marcescens, J. of Med Microbil*, **22**: 151- 156.
5. Traub ,W. H. and Baner , D. **1987.** Outer membrane protein alterations in *Serratia marcescens marcescens* resistant against aminoglycoside and ß – lactam antibiotics, *Chemo therapy*, **33** (3) : 172- 176.
6. Hawkey,P.M. and Constable, H.K. **1988**. Selection of netilmycin resistance, associated with increased 6- aming glycoside acetyltransferase activity in *Serratia marcescens*, *J. of Antimicrobial chemotherapy*, **21**: 535- 544.
7. Bollmann, R., Halle, E., Sokolowska-Köhler, W., Grauel, E.L., Buchholz, P., Klare, I., Tschäpe, H. and Witte, W. **1989.** Nosocomial infection due to *Serratia marcescens* – clinical finding, antibiotic susceptibility patterns and fine typing, *Infection*, **17** (5): 294- 300.
8. Palomar, J., Leranzo,A.M. and Viñes, M. **1995.** *Serratia marcescens* Adherence: The effect of O- antigen presence, *Microbios*, **81** :107- 113.
9. Svensson, O., Parment, P. A and Blomagen, G. **1987.** Orthopaedic infections by *Serratia marcescens*, *A report of Infectious Disease*. **19**: 69- 75.
10. Graevenitz, A.V. **1980.** Infection and colonization. App: 167. Graevenitz, A., and Rubin, S.J (ed.s), CRC press, Florida.
11. Palomar, J., Montilla, R., Fusttě, M. C. and Viñes, M. **1993.** The role of antigen in susceptibility of *Serratia marcescens* to non – immune serum, *Microbios*, **76**:189- 196.
12. Cursino, L., Chartone - Souza, E. and Nascimento, A. M. A. **2005**. Synergic interaction between ascorbic acid and antibiotics against *Pseudomonas aeroginosa*, *Braz. Arch. Boil. Technol*. **48** (3): 379- 384.
13. Sleigh, J. D. **1983**. Antibiotic resistance in *Serratia marcescens*, *Briti Med J*. **287**: 1651- 1653.
14. Skotmicki, M. L. ,Hall, J. D., Al –Harithy, R. N., Smith, R. and Skotnicki, A. **1987.** Incidance and transfer of R- plasmids of a hospital in Saudi Arabia, *Microbios*, **51**: 97-105.
15. Wilhelmi. I., De Quiros, G. C. L. B, Remero – Vivas, J., Duarte J., Rojo, E. and Bouza, E. **1987.** Epidemilogical outbreaks of *Serratia marcescens* infection in a cardiac surgery unit, *Applied Microbiology*, **25**: 1298- 1300.
16. Black, W. A. and Hodgson,R. **1971.** Search for *Serratia marcescens*, *J. of Clin Patholo,* **24**: 444- 448.
17. Kumagi, Y., Okada, K., Ishimaru, T., Sawai, Y., Kuroiwa, A. and Nomoto, K **1989**. Effects of vaccination against systemic *Serratia marcescens* infection, *J. of Clinical Lab. Immunol.* **29**: 125- 132.
18. katoh-Kanno, R. Kimura, M., Ikeda,Y., and Kimura, S. **1986**. Survey of modifying enzymes and plasmids in amikacin -resistant *Serratia marcescens, Microbiolo and Immunolo,* **30** (60): 509- 519.
19. Arakawa, H. Y.,Ohsuka, S., Wacharotayankun, R., Kato. N. and Ohta, M. **1995.** Plasmid mediated dissemination of the metallo ß – Lactamase gen *bla IMP* among clinically isolated strains of *Serratia macescens*, *Antimicrobial Agent and Chemotherapy,* **39** : 824- 829.
20. Arakawa,Y.,Shibata,N.,Shibayama,K., Kurokawa, H., Yagi, T., Fujiwara , H. and Goto, M. **2000.** Convenient test for screening metallo ß – Lactamaseproducing Gram negative bacteria by using thiol compounds, *J. of Clini Microbiolo***, 38** (1) : 40- 43.
21. Ito**,** H., Ameakawa, Y., OhsuuKka, S., Wacharotayan Kun, R., Kato, N. and Ohta, M. **1995.** Plasmid - mediated dissemination of the metallo – beta lactamase gen *bla IMP* among clinically isolated strains of *Serratia marcescens, Antimicrobial Agent and Chemotherapy,* **93**: 824- 829.
22. Goto, M., Takahashi, T., Yamashita, F., Koreeda, A., Mori, H., Ohta, M., M. and Arakawa,Y. **1997.** Inhibition of the metallo ß – Lactamase produced from *Serratia marcescens* by thiol compounds, *Biol. Pharm. Bull.* **20** (11) :1136- 1140.
23. Perilli, M., Felici, A., Pagani, L., Luzzaro, F., Orator, A., Rossolini, G. M., Knox, J. R. and Amicosane, G. **1997.** Chracterization of a new TEM- derived ß -Lactamase produced

in a *Serratia marcescens* stain, *Anti Microbial Agents and Chemotherapy*, **41**(11): 2374- 2382.

24. Queenan, A. M., Torres-Viera, C., Golg, H. S., Carmeli, Y., Eliopoulos, G. M., Moellering,R. C. ,Quinn, J. P., Hindler, J., Medeirose, A. A. and Bush,K. **2000.** SME – type Carbapenem-hydrolyzing class A ß – Lactamase from geographically divers *Serratia marcescens* stains, *Antimicrobical Agents and Chemotherapy*, **44** (11): 3035- 3039.
25. Trevors, J. T. 1986. Plasmid curing in bacteria, *FEMS Microbiolo Review,* **32**: 149- 152.
26. Hardy,K **1987**. *Bacterial plasmids.* (2nd edition) American Society for Microbiology. USA.
27. Gupta, R. K. and Batish, V. K. **1992.** Protoplasti - induced curing of bacteriocin plasmid in *Lactococcus lactic* subsp. *Lactis* 484, *J. of Applied Bacteriolo*, **73**: 337 – 341.
28. Cuevas, F. A. A. **1988.** Loss of penicillinase plasmids of *Staphylococcus aureus* after treatment with L- ascorbic acid, *Mitatopm Research Letter***s, 207**: 107 -109.
29. Amábile-Ceuvas, C.F., Pina-Zentella, R.M. and Wah – Laborde,M. E. **1991.** Decrease resistance to antibiotics and plasmid loss in plasmid – carrying strains of *Staphylococcus aureus* treated with ascorbic acid, *Mutation Res*, (264): 119- 125.
30. Garg,F.C., Bharati,R. and Sharma, P.K. **2003.** Isolation of antibiotic - sensitive mutants of *Azotobacter chroococcum* by treatment with ascorbic acid. *letter in Applied Microbiolo,* **24**: 136-138.
31. Ramesh,A., Haami, P. M. and Chandrashekar, A. **2000.** Ascorbic acid – induced loss of a Pediocin – encoding plasmid in Pediococus acidilactivi CFR K7, *World J. of microbiolo. and Biotechnolo.*, **16** :695 – 697.
32. Vandepitte, J., Engba, K. K., Piot, P. and Heuck, C. C. **1991.** *Basic laboratory producers in Clinical bacteriology,* World Health Organiszation Geneva.
33. Hejazi, A., Keane, C. T. and Falkiner, F.R **1997**. The Use of RAPD-PCR as a typing method for *Serratia marcescens*, *J. of Medi Microbiolo*, **46**: 913- 919.
34. Sambrook, J., Fritsch, E. F and Maniatis, T. **1989**. Molecular cloning a laboratory manual,2nd ed. Cold springs Harbor laboratory, New York.
35. Collee, J. G., Fraser, A. G., Marimon, B. P. and Simmons, A. **1996**. *Culture tests and media. in macki and Maccartney Practical Medical Microbilogy*, (14th edition).
36. Morgan, A. R., Cone, R. L. and Elgert, T.M. **1976**. The Mechanism of DNA strand breakage by vitamin C and superoxide dismutase, *Nucleic acid research*, **3**: 1139- 1149.
37. Shamberger, R. J. **1984.** Genetic toxicity of Ascorbic acid*, Mutation research*, **133**: 708 – 714.
38. Sugiyama, M., Tsuzuki, K. and Orgura, R. **1991.** Effect of Ascorbic acid on DNA damage, Cytotoxicity, Glutathione reductase, and formation of paramagnetic chromium in Chinese hamster V-79 cells treated with Sodium Chromate (V.I), *J. Biol – Chem.* **6** (266): 3386- 3383.
39. Halliwell, B. and Aruoma, O.E **1991.** DNA Damage by oxygen – derived species: its mechanism and measurement in mammalian systems, *FEBS letter*,**281**: 9-19.
40. Imlay,J.A. and Linn, S. **1989**. DAN damage and oxygen radical toxicity, *Science*, **240**:1302 -1309.
41. Amábile-Ceuvas, C.F. **1988.** Loss of Pencillinase plasmids of *Staphylococcus aureus* after treatment with L- ascorbic acid, *Mutation research letters*, **207**: 3-4.
42. Grimont, P.A.D and Grimont, F **1984.***Genus Serratia marcescens. In: Bergeys manual of systematic bacteriology*., Baltimore, N. R. K. (ed.s),Williams and Wilkins, **1**: 477- 484.