Bacteriophages effects on antibiotic sensitivity of *Staphylococcus aureus*

تأثير العاثيات على حساسية بكتريا المكورات العنقودية الذهبية تجاه مضادات الحيوية

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Abstract

A total of 50 swab samples were collected from patients suffering from different infections. Morphological and biochemical characterization showed that 46% isolates were characterized as *Staphylococcus aureus.* All these isolates were able to grow on mannitol salt agar medium and ferment mannitol and sucrose. β-hemolysin, coagulase production and antibiotics sensitivity of *S.aureus*, before and after treatment with phage, was examined. It showed there is no change in the β- hemolysin and in the coagulase production but lysogenic phage effect the antibiotic sensitivity of *S. aureus* isolates significantly. In addition plasmid DNA was isolated from bacteria whose showed multiple antibiotic resistances. Large and small bands were obtained .Thereafter curing process had done to evaluate the plasmids content, it was found entire losing for the small plasmids, while the large plasmids remain intact. From this study, it can be concluded that *S.aureus* isolates found in wound at high percentage; moreover, *S.aureus* treated with phages effect the sensitivity patterns against antibiotics.

المستخلص

جمعت 50 مسحة من مرضى يعانون من اصابات مختلفة لاجراء مسح للمحتوى البكتيري لغرض التقصي عن وجود بكتريا المكورات العنقودية في هذه العينات . اظهر التوصيف المظهري والكيموحيوي بأنّ 46 % من العزلات التي تم الحصول عليها هي بكتريا المكورات العنقودية الذهبية واظهرت جميع هذة العزلات قدرتها على النمو على وسط Mannitol salt agar كما لها القدرة على تخمير المانتول وسكر السكروز.تم اختبار عينات من العاثيات لمعرفة أيّ واحد منها له القابلية على عمل تحلحلي لبكتريا المكورات العنقودية. كما تم دراسة عامل الضراوة للبكتريا المعزولة في أنتاج انزيم تحلل الدم من النوع بيتا على وسط اكار الدمّ قبل وبعد المعاملة بالعاثي وأيضا إختبار مساعد الخثرة قبل وبعد المعاملة بالعاثي كما تم اجراء اختبارقابلية العزلات على مقاومة ستة انواع مختلفة من اقراص مضادات الحيوبة.اذ كانت النتائج عدم وجود تغاير في صفة أنتاج انزيم تحلل الدم وكذالك عدم وجود تغاير في إختبار مساعد الخثرة بينما اظهرت النتائج تأثير فعال للعاثي على مقاومة العزلات قيد الدراسة لمضاد الحيوية اذ جعلت هذة العزلات حساسة لهذة المضادات بعد معاملتها بالعاثي , فضلا عن ذلك عزل الدنا البلازميدي من العزلات التي تمتلك مقاومة متعددة لمضادات الحيوية , وتم الحصول على حزم بلازميدية كبيرة الحجم وحزم صغيرة الحجم . أجريت عملية التحييد فتبين اختفاء البلازميدات الصغيرة مع بقاء البلازميدات الكبيرة لكل منها. نستنتج من هذة الدراسة وجود بكتريا المكورات العنقودية بنسبة عالية في اصابات الجروح وللعاثيات تأثير فعال على تغيير صفة المقاومة لبكتريا المكورات العنقودية تجاه مضادات الحيوية المستعملة في الدراسة.

Key words: Antibiotic sensitivity; *Staphylococcus aureus*, Phages.

Introduction:

Staphylococcus aureus is the most important clinical member of Staphylococcus genus. It can be isolated from the skin or mucous membranes of the body. It can cause various infections (e.g., carbuncles, abscesses, pneumonia, endocarditis, food poisoning, toxic shock syndrome) throughout the body [1]. In addition, some of these *Staphylococci* are resistant to penicillin, this resistance comes about when the bacteria produce penicillinase (β-lactamase), which hydrolyzes the β-lactam ring of penicillin, *Staphylococcal* resistance to penicillin is mediated by penicillinase (a form of β-lactamase) production: an enzyme which breaks down the β-lactam ring of the penicillin molecule, antimicrobial resistance has been noticed as one of the paramount microbial threats of the twenty first century , *S. aureus* has been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of pathogens [2]. *S. aureus* strains posses a large number of cell-associated and extracellular factors, some of which contribute to ability of the organism to overcome the body's defenses and to invade, survive in and colonize the tissue [3].

After the discovery of Bacteriophage 90 years ago, it was hoped that they would be useful in the treatment of bacterial infections. Bacteriophage therapy was initiated in 1921 by Bruynoghe and Maisin [4] in the treatment of *Staphylococcal* infections. Bacteriophage therapy was abandoned after the introduction of sulphonamides and then antibiotics into medical practice. However, the lytic action of Bacteriophage *in vitro* enabled some investigators to use specific Bacteriophage for the differentiation of various species of bacteria [5].Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics , particularly in the state of Georgia [6].They tend to be more successful where there is a biofilm covered by a polysaccharide layer, that antibiotics typically cannot penetrate [7].

A clear benefit of phage therapy is that it does not have the potentially very severe adverse effects of antibiotics [8].Also it can be fast-acting, once the exact bacteria are identified and the phages administered. Another benefit of phage therapy is that although bacteria are able to develop resistance to phages the resistance is much easier to overcome [9]. Thus, the aim of this research is to study the therapeutic effect of phage on these isolates and determination of *S. aureus* prevalence percentage in different source, and the plasmid profile of *S. aureus* isolates from individuals clinical samples.

Materials and Methods:

Sample Collection:

Fifty swabs were collected from patient's suffering from different infections from Al-yarmook General hospital during the period 5/11/2007-15/1/2008. These samples were processed in the laboratory within 30min of collection.

Isolation and identification of *S.aureus* isolates:

All isolates were obtained using the procedure described by Mandell *et al.* [10]. Biochemical assays were carried out such as : Catalase production, Growth at 45ºC, Growth in 7-9% NaCl, Coagulase production ,Haemolysis patterns on blood agar, and Sugar fermentation, identification of isolates was done following

diagram described by Cowan[11].Maintenance of bacterial isolates was performed according to Maniatis *et.al.*, [12].

Antibiotic Sensitivity:

The antibiotic susceptibility pattern for β-lactamase producing *S. aureus* was determined using standard disk diffusion method [12] for 6 antimicrobial agents: Penicillin G, Amoxicillin, Cephalexin, Ciprodar, Tetracyclin and Nutronidazol as follows: 5 ml sterile BHI broth was inoculated with 0.1 ml of a young culture of tested *S. aureus* (rapid β-lactamase producer), incubated to mid log phase (O.D about 0.3-0.4). 0.1 ml of the above culture was spreader on Mullar Hinton agar plates. By rotating the plate approximately 60º each time to allow absorption of excess moisture. With sterile forceps the selected antibiotic disk were placed on inoculated plates, incubated for 18 hr. at 37ºC in an inverted position. The diameter of inhibition zones were measured by a ruler.

Plasmid DNA Isolation:

All the isolates were subjected to plasmid DNA extraction by alkali lysis method as described by Bimboim [13], the bacterial pellet obtained from single bacterial colony into 2 ml of Lural broth medium in 100µl of ice-cold Solution (50 mM glucose, 25 mM TrisCl,10 mM EDTA,10 mg/ml of lysozyme was Powdered in Solution I) was resuspend by vigorous vortex ,then let stand at room temperature for 5 min. and 200 µl of freshly prepared Solution includes (0.2 N NaOH ,1% SDS) was added and tubes was closed tightly, then the contents mixed by inverting the tube rapidly five times ,then 150 µl of ice-cold solution was added which consisted 5 M potassium acetate , Glacial acetic acid, and H_2O. The tube was closed and vortex gently,centrifuged at 12,000g for 5 minutes at 4C° in a microfuge .After that the supernatant was trranferred to a fresh tube. The double-stranded DNA was precipitated with 2 volumes of ethanol at room temperature, mixed by vortex. The extracted plasmid DNA was electrophoresed on 1.2% agarose gel stained with ethidium bromide. about 20 µL of plasmid DNA preparation was loaded into each well and electrophoresed at 50 volts for 1 hr. Plasmid DNA bands were observed with UV transilluminator and photographed. Molecular weight of the DNA bands was calculated by comparing with Lambda DNA Hind III digest (promega,USA) as standard marker.

Curing experiments:

Curing process had done according to Shittu *et al*. [14] by selecting one of resistant isolates for curing experiments based on their antibiotic resistance profile. The isolates were sub cultured on Brain Heart Infusion Agar (BHIA, Biolab) and incubated at 43.5°C for 24 hr. then sub-culturing on freshly prepared BHIA was performed twice and incubated as stated above, colonies were screened for loss of the resistance determinants by plasmid analysis and visualized after electrophoresis on the same conditions described in plasmid profile

Phage Isolation:

Phage isolation was obtained by centrifugation 25 ml of raw sewage for 10 minutes and passed the supernatant through a 0.45 µm membrane filter to remove bacterial contaminants. 8.0 ml portion of the filtrate was transferred into sterile, screw-cap

tubes. Then 0.2 ml of chloroform added to each screw-cap tubes with a sterile pipette, and mixed thoroughly. The virus stock can be used immediately or kept in the refrigerator until it can be analyzed.*S. aureus* was cultured on the brain heart infusion agar by spreading, and then a drop was taken from stock phage which isolated by pipette and put it on the bacteria. The plates were incubated at 37ºC for 24 hours .Thereafter, the plates stored in the refrigerator after incubation and examined if there was any plaque [4].

Results and Discussion:

Fifty swab samples were collected from patients suffering from different infections. The isolates were characterized, morphologically and biochemically [7].

Forty six isolates (46%) were identified as *S.aureus* all these isolates were able to grow on mannitol salt agar media. Fermentation of mannitol and sucrose are a very useful aid in identification of *S.aureus* [9]. *Staphylococcus aureus* mostly selected from colonies surrounded by yellow zones due to fermentation of mannitol. These colonies were round, concaves smooth with glistering surface, batyrous consistency. They were also able to grow at high salt medium (7-9%) and showed good growth at 45ºC. On blood agar medium they were able to produce golden yellow pigment and were surrounded by a zone of clear (beta) hemolysis. Microscopical examination after gram staining demonstrated that these were gram-positive and grouped in irregular clusters that resemble bunches of grape [9]. From results shown in Table (1) Its clear that patients with surgical wound infection, middle ear infection , bacterimia were at particularly high risk for infection with *S. aureus* 60%, 43%, 33%, respectively.

Table (1): Number and Percentages of *S. aureus* obtained from different specimens

Source of specimens	No. of specimens	No. of *S.aureus* isolate	Percentage (%)
Surgical wound infection	15	9	60
Middle ear infection	23	10	43
Sputum	9	3	33
Bcterimia	3	1	33
Total	50	23	

These results were agreement with De-lalla, [15] who found that *S.aureus* was the most common pathogen responsible for high percent of simple and surgical wound infections. Furthermore, Cheung [16] was found that *S.aureus* as important pathogen of human and their animals that causing bacterimia.

Then, the standard disk diffusion method [12] was used to determine the antibiotic sensitivity pattern of *S. aureus* isolates. Six antibiotic disks were used which include different groups of antibiotics table (2).It was revealed that all *S.aureus* isolates were resistant to penicillin and amoxicillin. Also, 60% of isolates were resistant to cephalexin when 3 of 5 isolates showed resistant to it, as well as, 40% of them showed resistant to tetracycline and nutrianidazol when 2 of 5 isolates showed resistant. While all the *S. aureus* isolates were susceptible to ciprodar.

Table (2): Antibiotic Resistance Pattern of *S. aureu* β-hemolysin produceing isolates

Antibiotic group	Antibiotics	No. of resistant isolates	Percentage of resistance
Penicillin group	Penicillin G	5	100
Amino penicillin	Amoxicillin	5	100
First generation cephalosporin	Cephalexin	3	60
Thired generation cephalosporin	Ciprodar	–	–
Amionglycoside	Tetracyclin	2	40
5-nitroimidazole	Nutronidazol	2	40

Development of antibiotic resistance may be explained by different mechanism; it may either due to possing of β-lactamase by the isolate which may be encoded by transferable plasmids or could be due to apparent of tolerant strains [17].

Our results were agreement with previous study [18] who found that all *S. aureus* isolates were resistant to penicillin and amoxillin and Staphylococcal hospital cultures produce high amounts of β-lactamase enzymes.

Analysis of plasmid profiles demonstrated the presence of one to three plasmids in all of the isolates (Fig. 1) included similar contains of large plasmid while showed different bands of small plasmids. All the plasmid profiles of isolates were compared with the Lambda DNA *Hind III* digest and standard strain (*Escherichia coli* MM294)

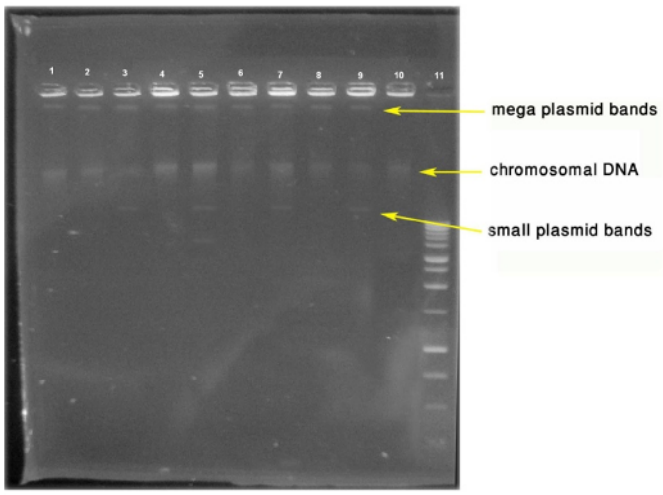

Fig.(1): Plasmid profile of *Staphylococcus aureus* isolates analyzed by ethedium bromide–stained agarose gel electrophoresis (1%, 5v/cm).
Lane 1-9: represents isolates harboring large and small plasmid.
Lane 10: standard strain *Escherichia coli* MM294 (without plasmid).
Lane 11: represents Lambda DNA *Hind III* digest, as size marker.

Rahman *et al.* [19] reported the presence of 23 kb plasmid in *S. aureus* strains isolated from skin lesion sample which is in agreement with the present study and he was confirmed that the same plasmid might be carrying the genes coding for multidrug resistance and the plasmids were transferable, and the same study showed that multiple drug resistance of *S. aureus* is due to several drug resistant genes in a single

plasmid methicillin (100%), amoxicillin (91.3%), bacitracin (73.91) and novobiocin (86.95%). The variation in the drug resistance may be well related to the type of antimicrobial agents prescribed for treating various diseases in different geographical areas and low doses of antibiotics are used against bacteria, they inhibit the growth of susceptible bacteria, leaving the smaller number of already resistant bacteria to thrive and grow.

Also the curing experiments had done to evaluate the plasmids content were selected based on their antibiotic resistance profile by using physical approach (heat) it was found entire losing for the small plasmids, while the large plasmids remain intact as shown in figure (2) .

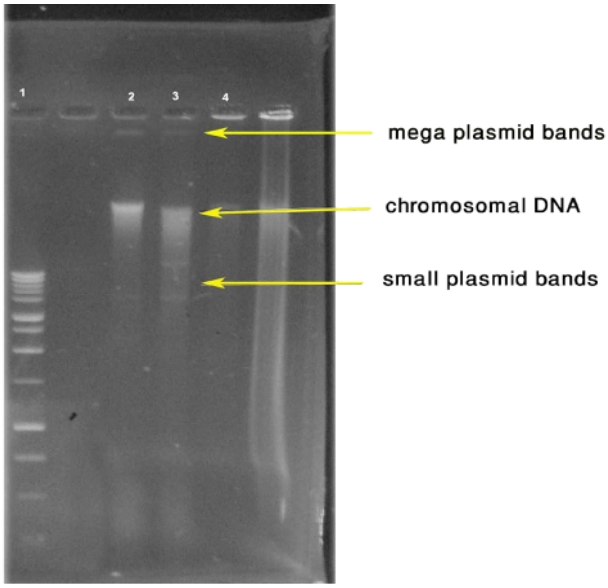

Fig.(2): Plasmid curing profile of *Staphylococcus aureus* isolates analyzed by ethedium bromide–stained agarose gel electrophoresis (1%, 5v/cm).
Lane 1: represents Lambda DNA *Hind III* digest, as size marker
Lane 2, 3: represents isolates before and after curing treatment.
Lane 4: standard strain *Escherichia coli* MM294 (without plasmid).

Resistance to Amoxicillin and Penicillin in the cured isolate was lost together with small plasmid absence, this indicates that antimicrobial resistant genes were plasmid located and this result in agreement with loss of resistance determinants (plasmids) that was investigated as previously reported [20].

Phage infection was obtained by cultured of *S. aureus* on the brain heart infusion agar by spreading then a drop was taken from stock phage which isolated and mixed thoroughly. The results showed increasing of susceptibility toward all used antibiotics and this referred to incorporated of genome of this phage inside the DNA of *S. aureus* and cause inhibition to the genes which responsible for antibiotic resistant property so that its became more sensitive to antibiotic and this can be noticed in table (3) in which the diameter of inhibition zones which formed due to the effect of antibiotic disks will increase after treatment the bacteria with phage ,this deal with Ogston, A. [21].

Table (3): Antibiotic Resistance Pattern of *S. aureu* before & after treatment with Phage

Antibiotics Types	Diameter of Inhibition Zones (mm)	
	Before Treatment with phage	After Treatment with phage
Amoxicillin	_	10
Ciprodar	_	30
Penicillin	_	10
Nitronidazol	15	16
Tetracycline	14	25
Cephalexin	14	16

García *et al*. [22] found that the dairy environment is an extensive source of staphylococcal phages, which may be effective in certain milk types to inhibit staphylococcal strains that often cause mastitis and contaminate milk.

Bacteria are most likely to modify the molecule that the phage targets, such as a cell surface glycoprotein, which is usually a bacterial receptor. In response to this modification, phages will evolve in such a way that counteracts this change, thus allowing them to continue targeting bacteria and causing cell lysis. As a consequence phage therapy is devoid of problems similar to antibiotic resistance and much of the evidence shows that appropriately administered phage therapy is very effective for treatment and prevention of many kinds of bacterial infectious diseases, especially those caused by multidrug-resistant bacteria. Currently, many pathogenic bacteria have acquired multiple drug resistance, which is a serious clinical problem.Bacteriophages are often very specific, targeting only one or a few strains of bacteria. Traditional antibiotics usually have more wide-ranging effect, killing both harmful bacteria and useful bacteria such as that facilitating food digestion and a new mechanism has been proposed recently in which a bacteriophage in either donor, recipient or indeed as part of a plasmid itself can effect plasmid transfer. Since cell to cell contact is necessary for transfer, the bacteriophage may cause alterations in the surface of the cell so that its adhesiveness is changed, enabling one or more plasmids to pass directly from the donor to recipient, the attraction of this hypothesis is that it requires neither death of the donor nor exposure of the recipient to large numbers of lytic phage particl [9].

References

1. David MD, Kearus AM, Gossain S, Ganner M, Holmes A (2006).Community – associated methicillin – resistant *Staphylococcus aureus*: nosocomial transmission in a neonatal unit. J. Hosp. Infect.64: 244-250
2. Johnson A.; Aucken H.; Cavendish S.; Ganner M.; Wale M.; Warner M.; Livermore D. and Cookson B. (2001). "Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS)". J Antimicrob. Chemother. 48 (1): 143–144.

3. Wilkinson B.; Crossly K. and Archer G. (1997). The *Staphylococci* in human disease. Churchill Livimgstone, Drug Resistance Updates.1(5): 325-335
4. Ackerman H. and Dubow M. (1987). "Viruses of prokaryotes. I. General properties of bacteriophages". CRC Press Boca Raton. (1)49-76.
5. McGrath S. and vanSinderen D. (2007). Bacteriophage: Genetics and Molecular Biology, 1st edition .Caister Academic Press, Norfolk, UK.pP: 445-514.
6. Thiel, K. (2004). "Old dogma, new tricks—21st Century phage therapy". Nature Biotechnology. 22 (1): 31 - 36.
7. Pirisi A. (2000). "Phage therapy--advantages over antibiotics". Lancet 356:1418-1421.
8. Christine F. and Thomas V. (2003). Non-antibiotic therapies for infectious diseases." Communicable Diseases Intelligence. (27): 127-132.
9. Gulig P.A.; and Duckworth, D. (2002). Bacteriophages: potential treatment for bacterial infections. BioDrugs: clinical immunotherapeutics, biopharmaceuticals and gene therapy .16(1):57-62.
10. Mandell G.; Bennett J. and Dolin R. (1995). "Prenciple of practice & infection disease" churchill livingstone. 4th ed. New York: Churchill Livingstone.2538-2593.
11. Cowan ST (1985). Cowan and Steel Manual for Identification of Medical Bacteria 3 ed, Cambridge University Press, London pp. 54-120.
12. Maniatis T.; Fritsch E. and Sambrook J. (1982)."Molecular cloning, alaboratary manual. Cold Sppring Harbor Laboratory".
13. Bimboim HC. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol, .100: 243 – 255.
14. Shittu A. O.; Udo E. and Lin J. (2009). Phenotypic and molecular characterization of *Staphylococcus aureus* isolates expressing low- and high-level mupirocin resistance in Nigeria and South Africa. BMC Infectious Diseases, 9(10):1-9.
15. De-lalla.F. (1999)."Antimicrobial therapy in control of surgical infection complication".J.chem.Ther.11 (6): 440-450.
16. Cheung K. and Chung E. (1994). "Diminished virulence of a sar agr mutant of *Staphyloccocus aureus* in the rabbit model of eudocarditis".J. Clin. Invest.94: 1815-1822.
17. Bowersox J. (2007). "Experimental *Staph.* Vaccine Broadly Protective in Animal Studies". National Institute of Allergy and Infectious Diseases. (301): 452-463
18. Vancraeynest D. ; Haesebrouck F. ; Deplano A. ; Denis O. ; Godard C. ; Wildemauwe C. and Hermans K. (2006). "International Dissemination of a High Virulence Rabbit *Staphylococcus aureus* Clone". *Journal of Veterinary Medicine* 53(9): 418–422.
19. Rahman, M.; Khan, AH. ; Shahjahan, M.; Paul, DK. and Hasan, P. (2005). Antibiotic susceptibility and R- plasmid mediated drug resistance in *Staphylococcus aureus*. Med J Islamic world academy of sciences. 15(3): 111-116
20. Daini, O. A.; and Akano, S. A.(2009). Plasmid-mediated antibiotic resistance in
21. *Staphylococcus aureus* from patients and non patients. Scientific Research and Essay. 4 (4): 346-350
22. Ogston, A. (1984). Classics in Infectious Diseases. Rev Infect Dis 6 (1): 122–28.
23. García, P.; Madera,C.; Martínez, B.;Rodríguez,A.; and J. Evaristo (2009). Prevalence of bacteriophages infecting *Staphylococcus ureus* in dairy samples and their potential as biocontrol agents. Journal of Dairy Science. 92 (7):3019-3026.