Curing of *Escherichia coli* Antibiotic Resistance Plasmids by Aspirin

تحييد بلازميدات مقاومة مضادات الحيوية من بكترياايشريشيا القولون بواسطة الاسبرين

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Abstract

An isolate of *Escherichia coli* (*E. coli*) was isolated from urine sample due to person infected with urinary tract infection (UTI).The isolate was resistant to the following antibiotics: Ampicillin, Cotrimoxazole, Chloramphenicol and Tetracycline. Agarose gel electrophoresis of its plasmids content has revealed the presence of single large plasmid and two small plasmids bands. The large plasmid was conjugative and contained the resistance genes for four antibiotics. *In vitro* curing of this plasmid was achieved by treatment with salicylic acid (aspirin) with 150,200,250 and 300 µg/ml as indicated by the elimination of resistance, also by absence of large plasmid band following agarose gel electrophoresis. *In vivo* curing was conducted using New Zealand rabbits. UTI was induced by bacterial inoculation via urethral catheterization. The *E. coli* from urine samples of the rabbits, the count of which was proportional to the type of treatment. Minimum number of colonies was associated with group treated with metheprim was aspirin 300mg/kg daily dosage. This result may indicate that the side effect of metheprim was in its maximum with aspirin. Survivor bacteria may indicate incomplete exposure to the drug.

Key words: Antibiotic, Plasmids, Aspirin

المستخلص

تم عزل عزلة بكتريا القولون من عينة ادرار عانده لشخص مصاب بالتهاب المجاري البولية . كانت العزلة مقاومة للمضادات الحيوية : الامبسلين ، الكوتريمكسازول ، الكلورمفنيكول ، التتواسايكلين . اظهر الترحيل الكهربائي في هلام الاكاروز وجود حزم لبلازميد كبير و بلازميد ين صغيرين. كان البلازميد الكبير اقتراني و حاوي على جينات المقاومة لمضادات الحيوية الاربعة . تم اختبار تحييد هذا البلازميد في المختبر بواسطة المعاملة بحامض السالسيلك (الاسبرين) بتركيز 150،200،250،300 مايكروغم/ مليليتر بدلالة فقدان المقاومة، كذلك غياب حزمة اللازميد الكبير في الترحيل الكهربائي بهلام الاكاروز . تم تحييد البلازميدات داخل جسم الكائن الحي بأستعمال ارانب نيوزلندية . تم حث التهاب المجاري البولية لديها بحقن مزروع بكتيري بقسطرة المثانة . أعيد عزل عزلة ايشريشيا القولون من عينات ادرار الارانب ، فكانت اعدادها متناسبه مع نوع المعاملة . ترافق اقل عدد للمستعمرات مع المجموعة المعاملة بجرعة ميثيبريم و اسبرين 300مليغرام/كغم يوميا . قد تشير هذه النتيجه الى ان التأثير القاتل للميثبريم كان عند أعلى مستوى مع الاسيرين . البكتريا الباقية قد تشهر الى عدم تعرضها الكامل للدواء .

الكلمات المفتاحية : مضادات حيوية ، بلازميدات ، اسبرين

Introduction

The horizontal transfer of antibiotic resistance plasmid among bacterial pathogen is not the only health problem. The presence of the second pathogenic bacteria in the locus of infection will be protected from the effect of the use antibiotic [1,2]. Antibiotic resistance plasmids are thus representing accessory hazards. Although some bacterial plasmid undergoes spontaneous segregation and loss, the majorities are stably inherited [3]. It has been always desirable to find practical way to get rid of such resistance plasmids. Curing agent is known to eliminate plasmids. The elimination of plasmids simply means the elimination of accessory factors that contribute to bacterial pathogenicity and resistance [4]. Chemical agents used for plasmid curing interfere with DNA replication such as acridin orange, ethidium bromide and sodium dodecyle sulfate are efficient in curing plasmid in enterobacteriaceae [5]. Mitomycin C and acriflavin are also used [6,7]. However these agents cannot be used *In vivo*. In this paper we present preliminary data of our use of salicylic acid in curing plasmids from pathogenic bacteria *In vitro*, as well as *In vivo*.

Material and methods

Pathogenic *E. coli* isolate urine sample of UTI case. Characterization included biochemical character, antibiotic profile, plasmids screening and conjugation experiments. Standard procedures were followed [8,9].

Curing plasmid by salicylic acid *in vitro*:

Curing plasmid by salicylic acid (Aspirin) *in vitro* was accomplished as followed [10]: A single colony of *E. coli* was inoculated into M9 minimal broth medium supplemented with 0.1% casamino acid, 0.1% glucose and $CaCl_2$ was added to final concentration of 0.054 µg/ ml and $MgCl_2$ in 2.4 µg/ ml, incubated over night at 37ºC. Salicylic acid was prepared by dissolving 0.4 gram of salicylic acid powder (BDH-England) in 12ml of sterile de-ionized water and the pH was adjusted to 7.5 using of 10M of NaOH. The volume was completed to 20 ml (final concentration 20mg/ml). Salicylic acid was added to test tubes contain M9 broth to prepare the following concentration: 150,200,250 and 300 µg/ ml. Each was inoculated with 0.1 ml of the culture with proper control 0.1 ml (10^{-6}-10^{-7}) serial dilution was spread on nutrient agar plates and incubated over night at 37º C [10].The aerobic plates count (APC) was performed and replica plating of 100 isolated colonies were subjected to different salicylic acid concentrations on indicator plates supplemented with each of the antibiotics Ampicillin, Tetracycline and Chloramphenicol with final concentration 10, 30, 30µl/ml respectively which consider plasmids markers and incubated at 37º C over night [5].

Curing plasmid by salicylic acid *in vivo*:

Group of 18 healthy New Zealand rabbits were experimentally infected by the *E. coli* isolate using 4×10^8 (colony forming unit) CFU/ml via urethral catheterization. The rabbits were distributed to six groups each group contained three rabbits, which they were: group A was negative control (no infected rabbits), group B was positive control (infected rabbits leave without any treatment). UTI was induced in group C, D, E and F treated orally in daily dosage for five days with aspirin and/ or cotrimoxazole (metheprim) suspension each 5ml contain: 200mg Sulfamethoxazole and 40mg Trimethoprim (Asia pharmaceutical lab.-Syria). Group C was treated with metheprim only, group D was treated with aspirin 300µg/ml only, group E was treated with metheprim and aspirin 150 µg/ml, and group F was treated with metheprim and aspirin 300 µg/ml. *E coli* was re-isolated and screened for its antibiotic markers. **Results and Discussion:**

The results of curing with 150,200,300 µg/ml of salicylic acid *in vitro* revealed total loss of resistance of Metheprim, Choramphenicol and Tetracycline accompanied by plasmid loss.

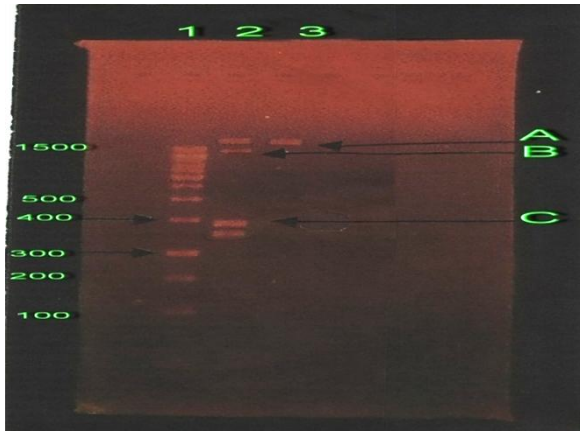

Fig. (1): Gel electrophoresis of DNA content of *E. coli* isolate before and after salicylic acid treatment using 0.8%, agarose gel 70 volt and 4 h. A: chromosomal band; B: large plasmid band; C: small plasmid bands

- **Lane 1 show size marker 1500 bp.**
- **Lane 2 show A,B and C content of isolate DNA before salicylic acid treatment .**
- **Lane 3 show A only content of isolate DNA after salicylic acid treatment.**

Agarose gel electrophoresis of the strain DNA content has revealed the presence of a large plasmid and two plasmids bands in figure (1). The large plasmid was conjugative and harbored the resistance gene of Ampicillin, Chloramphenicol and Tetracycline and this result agreed with [5], and this result corresponded with study of [11], which mentioned that *E. coli* are known to harbor plasmids of different molecular size ranging from 2-3kb to 6.5kb and maximum 26kb. Salicylic acid has chelating effect on the calcium and magnesium, important ions for bacterial outer membrane and its flexible permeability [12], and leakage may be resulted [13]. The curing may have resulted from the

effect of salicylic acid in increasing negative potential of plasmic membrane or disturbing plasmid segregation during bacterial division [14]. However, despite the curing of two antibiotics markers, the third, Ampicillin was not loss. This may indicate the prescience of resistance gene for this antibiotic in two copies (a second chromosomal or transposable copy) [5,15].

Plasmid curing by aspirin *in vivo*:

Within seven days of inducing UTI by E. coli, which consider the common cause of UTI [16] in rabbits. The ascending route to get infection by catheterization was depended, because it is the most important way causing UTI cases [17].

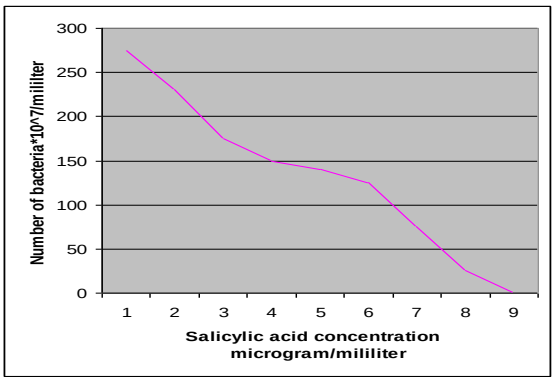

Fig. (2): Relationship between salicylic acid concentrations with viable count of the bacterial cell in the broth medium.

Figure (2) show a reverse relationship between acid concentration and bacterial growth density measured by aerobic plate count (APC). This indicates the ability of salicylic acid to increase permeability of the bacterial plasmic membrane and this compatible with study of Leive [15]. The medical parameters such as pyuria and bacteriuria was depended, because they are very good marker to indicate UTI [18]. Bacterial culturing was performed from urine samples of rabbit, which also collected by catheterization, to obtain pure culture of *E coli*, where the presence of one germ at every immersion microscopically field equal to 10^5 CFU/ml by bacterial culturing [19], then susceptibility test toward same antibiotics used before enter the animals, was performed. The results showed a decrease in pus cells/H.P.F. (high power field) and CFU/ml of urine samples salicylic-treated rabbits table (1).

Table (1): Effect of deferent treatments of aspirin or/and metheprim dosage on Pus cells/H.P.F. and CFU/ml of animals urine samples

Group	Rate of Pus cells/H.P.F.	Rate of 10^5 CFU/ml
A	**0**	**0**
B	**21**	**5**
C	**10**	**3**
D	**19**	**4**
E	**10**	**3**
F	**6**	**2**

A: Negative control group, B: Positive control group, C: Group treated with metheprim only, D: Group treated with aspirin 300 mg/kg only, E: Group treated with aspirin 150 mg/kg metheprim together and F: Group treated with aspirin 300 mg/kg metheprim together .

Analysis method was compared with experimental groups and negative control (group A) figure (3) and positive control (group B) figure (4) in addition to the histological examinations. There was a similar result between group C (treated with metheprim only) and group E (treated with metheprim and salicylic acid 150mg/kg daily, both showed slight decrease in bacteriuria and pyuria. This could be reasoned by irregular absorption of oral drugs in herbivorous animals [20] (like rabbits). Curing effect of aspirin (which is hydrolyzed to salicylic acid and acetic acid) in 150 mg /kg *in vivo* dosage had no curing effect as it was *in vitro*, and this contrast with positive results of experimental study *in vitro* curing effect of salicylic acid with 150 µg/ml concentration on antibiotic resistance encoding plasmid of *E. coli* isolated from UTI case [21]. The results of group D figure (5) (treated with aspirin 300 mg/kg daily) showed significant results. Bacteriuria and pyuria were reduced to 75% in comparison to group C, which treated with metheprim only (still used as drug of choice for UTI in

Iraq), and this result may be due to the antimicrobial effect of aspirin in high dosage [22]. Synergism of metheprim and aspirin at this dose 300 mg/kg may be explained by plasmid curing in the infection foci that led to metheprim exposure. The rate of CFU/ml of re-isolated bacteria was reduced in group F figure (6) in comparison with group B (positive control) figure (4), which may indicate partial plasmid curing effect ,while the results of susceptibility test to antibiotics of re-isolated bacteria from urine samples were same to that before enter the rabbits bodies .

To conclude there was incomplete plasmid curing in experimental group F, that make the metheprim work as bacteriocidal agent, and then reduce the viable count of bacteria. This may be due to the sub effective concentration of salicylic acid, because the aggregation manner of the bacteria on the epithelial cells of urinary system UTI cases, where the pili of bacteria not act to adhesion to mucus membrane onl, but cause pili-pili aggregates[23], so the bacteria in the foci exposure to sub-effective concentration of salicylic acid. It is so difficult obtain same degree of infection in all experimental animals, because the different immunity among them [24].These factors may be additional factor to the sub exposure to salicylic acid. These factors are still need to be examined in future work. The use of aspirin in curing resistance and virulence associated plasmids is a practical approach for drug formulation point of view.

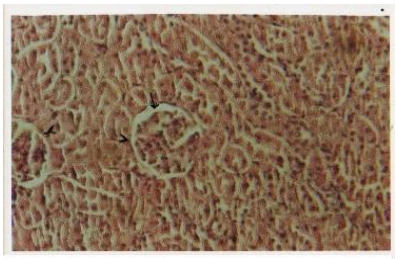

Fig.(3) : Cross section in kidney tissue of uninfected animal (group A). (Colored with hematoxilin-eiosen stain in 250X power). Notice; glumerulum, urinary tubules and intracellular tissue appear normally

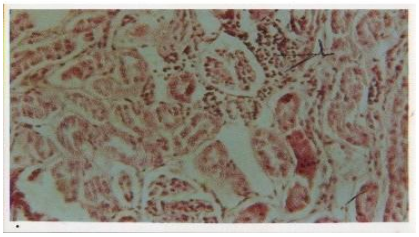

Fig.(4): Cross section in kidney tissue of infected animal leave without any treatment (group B). (Colored with hematoxilin-eiosen stain in 400X power). Notice infiltration of inflammatory cells especially in intracellular tissue.

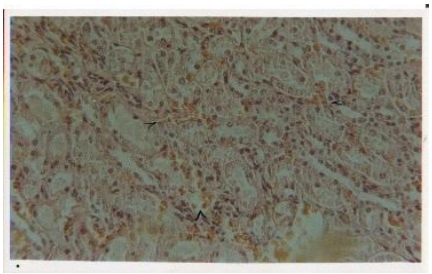

Fig. (5): Cross section in kidney tissue of infected animal treated with aspirin 300mg/kg together (group D),Notice the presence of congestion leading to obvious bleeding within the tissue Colored with hematoxilin-eiosen stain in 400X power).

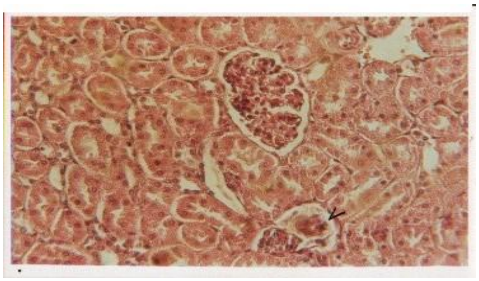

Fig. (6): Cross section in kidney tissue of infected animal treated with metheprim and aspirin 300mg/kg together (group F), Notice the tissue nearer to be normal in comparison with group C (Colored with hematoxilin-eiosen stain in 250X power).

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