Role of Conjugative Plasmids in Antibiotic Resistance between Two Strains of *E. coli*

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Abstract

Two isolated bacterial strains (*E. coli* 64, *E. coli* MM294), were purified, they are: gram-negative bacteria, circular, regular edged, thick somewhat glitter, viscous, and lactose fermenter. They belong to the family of *Enterobacteriaceae E. coli* 64 showed the resistant to all used antibiotics except Rifampicin (RA), Cephotaxime (CTX), Nitrofurantion (FT), Tabramycin (TM), and Cephalexin (RX) (Sensitive to). While *E. coli* MM294 showed the sensitivity to all used antibiotics except: Rifampicin (RA), Lincomycin (L), Clindamycin (CM), and Erythromycin (E), (resistantt to) Conjugative experiments showed that *E. coli* strain 64 harbored a self transmissible plasmid carrying resistant genes for: Tetracycline (TE), Amoxicillin (AMX), Neomycin (N), Chloramphenicol (C), Ampicillin (AM), and Trimethoprime + Sulfamethorazole (SXT) It seems that all these resistant genes may be carried on one self transmissible plasmid, or they may be carried on one self transmissible plasmid (except Neomycin, resistant gene may carry on other self transmissible plasmid)..

Keywords: *E. coli*, conjugative plasmid, conjugation, antibiotic resistance.

الخلاصة

أجري البحث على عزلتين من بكتريا *E. coli* (*E. coli* 64, *E. coli* MM294) و بعد تنفيتها وجد ان صفات كل منها كانت : كرام السالبة, مدورة الشكل ذات حافات منتظمة و متألقة المظهر و سميكة بعض الشيء و لزجة و لها القابلية على تخمير سكر الاكتوز تعود هاتان السلالتان الى عائلة Enterobacteriaceae.

أظهرت نتائج اختبار الحساسية ضد المضادات الحيوية بأن *E. coli* 64 كانت مقاومة لكل المضادات الحيوية المستعملة عدا الرفامبسين (Rifampicin (RA)) و السيفوتاكسين (Cephotaxime (CTX)) و النتروفرانشن (Nitrofurantion (FT)) و التبرامايسين (Tabramycin (TM)) و السيفالاكسين (Cephalexin (RX)) كانت حساسة لكل المضادات الحيوية المستعملة عدا الرفابسين (Rifampicin (RA)) و اللينومايسين (Lincomycin (L)) و الكلنداماسين (Clindamycin (CM)) و الارثرومايسين (Erythromycin (E)) كانت مقاومة لهذه الاجناس. و قد بينت تجربة الاقتران البكتيري التة أجريتبين السلالتين بأن سلالة 64 *E. coli* تحمل بلازميدا قابل للانتقال حاملا جينات مسؤولة عن مقاومة هذه السلالة ضد التتراسايكلين (Tetracycline (TE)) و الاموكسسلين (Amoxicillin (AMX)) و النيومايسين (Neomycin (N)) و بينما *E. coli* MM294 كانت حساسة لهذه الاجناس.

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الكلورامفنيكول ((Chloramphenicol (C) و الامبسلين ((Ampicillin (AM) و الترايمثبريم + السالفمثرازول ((Trimethoprime + Sulfamethorazole (SXT).
اوضحت النتائج ان جينات المقاومة ضد المضادات الحيوية قد تكون محمولة جميعها على بلازميد واحد او على بلازميدين احدهما يحمل كل كل الجينات التي تعطي المقاومة ضد كل المضادات الحيوية المستعملة عدا المضاد نيومايسين الذي قد يكونموقع جينه محمولعلى البلازميد الثاني.

Introduction

E. coli is a member of the genus *Escherichia* that includes in the family of *Enterobacteriaceae* (*Ewing, W.H., 1986*). It is a gram-negative, nonsporing, facultative rod that ferments lactose with gas formation within 48hr. at 35°C (*Feng, P., et al., 2002*). *E. coli* is the best studied bacterium and the experimental organism of choice for many microbiologists. It is a major inhabitant of the colon of human and other worm-blooded animals (*Conway, P.L., 1995; Madigan, M.T., and Martinko, J.M., 2006)*. It is the major causative agent of urinary tract infection (UTI). UTI is inflammatory disease that involves areas ranging from the kidney to the urethra, with the urethra and bladder most commonly affected. The incidence of UTI is higher among women than men (*Todar, K., 2007*). Water contaminated with infected bacteria (*E. coli*) is a major source of UTI therefore *E. coli* is quite useful in the analysis of water for fecal contamination (*Brenner, K. P., et al., 199*6). UTI is treated by antimicrobial drugs that destroy pathogenic microorganisms at low concentration called Minimum Lethal Concentration (MLC) or inhibit their growth at low concentration called Minimum Inhibitory Concentration (MIC) (Stanley H. Kleven, and David P. Anderson, 1971;*Andrews, J. M., 2001*). Antibiotic sensitivity is variable among bacterial strains due to the presence of plasmids (*Salyers, A.A., et al., 2004*). Plasmids are relatively small, circular DNA molecules that can exist independently of host chromosomes and are found in many bacteria including *E. coli* (they are also present in some yeasts and other fungi). Plasmids are responsible for resistance and transmitting the resistance to specific sensitive strain through a bridge formed by F-factor during a process called conjugation (*Lederberg, J., 1952*). Conjugation is happened between two mating types: donors or males, and recipients or females. The determinant of maleness is F-factor which is denoted by F and called the sex plasmid, genetically a male cell is designated F^+, a female cell lacks the F plasmid and is designated F^-. F-factor is responsible for cell attachment between specific bacterial strains. During conjugation male-female pairs are formed and in a way that is not completely understood, paring signals the replication of F and one copy of F is transferred to female (recipient) in about one minute. After conjugation the recipient cells called transconjugants (Lederberg, J., and Tatum, E., 1946; *Holmes, R.K., and Jobling, M.G., 1996; Ryan, K.J., and Ray, C.G., 2004*).

Materials and Methods

Purification of Bacterial Strains: Two isolated bacterial strains were obtained (*E. coli* 64 was isolated previously from patients with UTI, and *E. coli* MM294 was obtained from Genetic Engineering Institute/Baghdad University), and

one colony of each was cutivated in Macconkey agar (Oxide) plate, and then cultivated in Nutrient agar (Oxide), and then its shapes were identified. (*Jose L. Alonso, et al.,1999*)

Antibiotic Sensitivity (Disk Diffusion Methhod): Antibiotic sensitivity for two isolated bacterial strains was done by using a combination of antibiotics disks (Sigma) including (Clindamycin (CM) 2µg, Erythromycin (E) 15µg, Tetracycline (TE) 30µg, Ampicillin (AM) 10µg, Gentamycin (GM) 10µg, Nalidixic Acid (NA) 30µg, Gephalothin (CF) 30µg, Neomycin (N) 30µg, Cephalexin (KF) 30µg, Cloxacillin (CX), Lincomycin (L) 15µg, Cephotaxime (CTX) 30µg, Trimethoprime 1.25µg + Sulfamethorazole 23.75µg (SXT) Chloramphenicol (C) 30µg, Nitrofurantion (FT) 300µg, Amikacin (AN), Rifampicin (RA) 5µg, Tobramycin (TM) 10µg, Amoxicillin (AMX) 10µg (Sigma) these disks were stored at 4°C) in disks diffusion method as the following: 0.1ml of each strain was cultured by spreading on the suface of nutrient agar plate, and then antibiotic disks were placed on the surface of agar using sterile forceps and incubated at 37°C for 24 hr. The results were indicated according to formation of inhibition zone around the disk for sensitive or not formation of inhibition zone around the disk for resist. (*Report of the Working Party on Antibiotic Sensitivity Testing on the British Society of Antimicrobial Chemotherapy, 1991*).

Minimum Inhibitory Concentration (MIC): Different concentrations of rifampecin (stock solution was prepared by dissolving 0.1g of refampicin powder in 10ml of Dimethyl Sulfoxide (DMSO)) and tetracycline (stock solution was prepared by dissolving 0.1g of tetracycline powder in 10ml of ethanol/distilled water (50% v/v) store at -20°C in the dark) were prepared (10, 20, 30, 40, 50 µg/ml) and each concentration added to the 25ml of LB (Lluris-Bertani) media (Trypton 10g, Yeast Extact 5g, NaCl 5g, D-glucose 1g dissolved in 1000ml D.W., pH adjusted to 7.5, 15.9 agar (Biolife)) and left to solidify, and then each strain cultivated by streaking, and left to incubate at 37°C for 24 hr. Then the minimum antibiotic concentrations that prevent bacterial growth were detected and chosen to be considered as the MIC. (*Winstanley, T., et al., 1994; National Committee for Clinical Laboratory Standards, 1997*).

Conjugation (in Liquid Media): single colony of the donor and recipient strains was inoculated into nutrient broth (Biolife) separately and incubated at 37°C for 24 hr., the two strains were mixed in sterilized tube and incubated at 37°C for 2 hr. without shaking, sample was taken from conjugated tube and diluted properly and spread on a selective media (Nutrient agar containing 60 µg/ml tetracycline + 20 µg/ml refampicin) then incubated at 37°C for 24 hours. Controls were done by spreading each donor and recipient strains separately on Nutrient agar containing 60 µg/ml tetracycline + 20 µg/ml refampicin for identification of transconjugants. (*William R. Will and Laura S. Frost 2006*). The growth of transconjugant, donor, and recipient cells has been recorded by plate count method (*Brenner, K. P., et al., 199*6).

Identification of Transconjugants: Nine colonies from plate that contains trasconjugants were inoculated on the

surface of nine plates of Nutrieint agar (each colony was cultivated in one plate). Numbers of antibiotic disks were added to the surface of Nutrient agar of each plate (the antibiotic disks include tetracycline, and antibiotics that *E. coli* 64 resist and *E. coli* MM294 sensitive to it). Plates were incubated at 37°C for 24 hr. (*William R. Will and Laura S. Frost 2006*).

Results and Discussion

Purification of Bacterial Strains: The two strains (*E. coli* 64, and *E. coli* MM 294) were purified by cultivation For this reason this strain can be found in the urine sample of UTI patients. The results for *E. coli* MM294 indicate the sensitivity of this strain to 15 and resistance to 4 antibiotics (19 types of antibiotics were tested) (Table 1). For this reason this strain can't be isolated from urine samples of UTI patients but it can be found in lab as experimental strain, and here it can be considered as recipient strain in conjugation experiment while *E. coli* 64 can be considered as a donor strain. (*Vidal, O., et al., 1998; Fux, C.A., et al., 2005*).

The tetracycline and refampicin were selected to be used as selectable markers in conjugation between *E. coli* 64 and *E. coli* MM294 because *E. coli* 64 is sensitive to reifampicin and resist to tetracycline while *E. coli* MM294 is opposite (Table 1).

Minimum Inhibitory Concentration (MIC): Each of *E. coli* 64 and *E. coli* MM294 was cultivated in different concentrations of tetracycline and refampicin agars after 24 hr. of incubation at 37°C (Table 2).

Antibiotic Sensitivity of Transconjugants: to check whether cells in the media containing (60 µg/ml tetracycline + 20 µg/ml on Macchonkey agar media, their morphological characteristics and properties were determined as: circular, regular edge, thick somewhat, glitter pink, viscous, and lactose fermenter. (*Feng, P., et al., 2002*).

Antibiotic Sensitivity: The sensitivity to antibiotic was determined and the results indicate that *E. coli* 64 was sensitive to 5 antibiotics and resist to 14 antibiotics from 19 types of antibiotics used (Table 1).

The study found that *E. coli* 64 grew at all tetracycline concentrations because *E. coli* 64 is resistant to it (Table 1) while it didn't grow at any concentrations of refampicin even at lowest concentration (10µg/ml). In contrary *E. coli* MM294 grew at 10. 20, 30, 40 µg/ml (Table 2) so this concentration of tetracycline can inhibits the growth of *E.coli* MM294, while it grew at all rifampicin concentration (resists to refampicin, Table 1). According to these results 20µg/ml of refampicin and 60 µg/ml tetracycline can be considered as MICs that prevent the growth of *E. coli* 64 and *E. coli* MM294. (*Andrews, J. M., 2001*).

Conjugation: results in Table 3 illustrate the growth of cells in a petridish containing 60 µg/m. of tetracycline and 20 µg/ml of rifampicin after mixing the two bacterial strains (*E. coli* 64 and *E. coli* MM294) while there are no growth when each of bacterial strains has been cultivated separately in medium contains both quantity of antibiotics mentioned previously. rifampicin) grew as a result of spontaneous mutation (mutant cells) or as a result of transfer of drug resistant plasmid from donor cells to

recipient cells during conjugation (transconjugants), this can be done by making antibiotic sensitivity of transconjugants by selecting randomly 9 colonies and making antibiotic sensitivity test for each one of nine colonies to 6 types of antibiotic: Tetracycline, Amoxicillin, Neomycin, Chloramphenicol, Ampiciline, and Trimethoprim + Sulfamethorazole. Where *E. coli* 64 (donor cell) was resistant to each one of these 6 types of antibiotics while *E. coli* MM294 (recipient cell) was sensitive to all these 6 types of antibiotics. As shown in the Table 4 there are 7 colonies can resist to all these 6 types of antibiotics while there are 2 colonies from 9 colonies can resists to all six types of antibiotics except neomycin that inhibit the growth of each of colony number 2 and colony number 6.

The results in Table 4 reveal that all these nine colonies are transconjugants (they are result of conjugation) and not mutant cells (result to spontaneous mutation), and it seems that more than one plasmid can participate in transferring drug resistance as following: Plasmid contains neomycin gene, Plasmid contains TE, AMC, C, AM, and SXT genes, and/or Plasmid contains all six genes of tested antibiotics.

These results are in agreement with the study of Carrasco, C. E., and his colleagues in 1997. They reported the acquisition of tetracycline resistnace by *E. coli* HB101, which is sensitive to this antibiotic, and also they suggested that tropical waters exposed to sewage contamination may present a great danger to public health due to the survival of *E. coli* in these environments because of antibiotic resistance that may be readily transferred among the genera of *Enterobacteriaceae* , this fact has been studied extensively in 1983 through the researches of Bell, J. B., and his colleagues, and Hanahan, D.

Conclusion

The experimental results indicate the role of conjugative plasmids in spreading drug resistance among urinary tract infection bacterial strains (*E. coli*), The study predicts the danger role of *E. coli* MM294 on public health in case of discarding such experimental strain directly without killing into sewage leading to poisoning of food (vegetable) after washing with water known to receive sewage contamination. Therefore the course of treatment with antibiotics will be difficult due to antibiotic resistance phenomenon that convert such strains from recipients to transconjugants and make them resistant to wide number of antibiotics.

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Table 1.

Sensitivity of E. coli 64 and E. coli MM294 to Several Antibiotics

Antibiotic	Bacterial Strain	
	***E. coli* 64**	***E. coli* MM294**
TE	R	S
RA	S	R
L	R	R
CTX	S	S
C	R	S
FT	S	S
TM	S	S
CM	R	R
E	R	R
AM	R	S
GM	S	S
CF	R	S
KF	R	S
NA	R	S
N	R	S
CX	R	S
AN	R	S
SXT	R	S
AMX	R	S

R = Resistant, S = Sensitive, TE =Tetracycline, RA = Rifampicin, L = LIncomycin, CTX = Cephotamxime, C = Chloramphenicol, C = Chloramphenical, FT = Nitofurantion, TM = Tobramycin, CM = Clindamycin, E = Erythomycin, AM = Ampicillin, GM = Gentamycin, CF = Cephlothin, KF = Cephalexin, NA = Naldixic Acid, N = Neomycin, CX = Cloxacillin, AN = Amikacin, SXT = Trimethoprime+Sulfamethorazole, AMX = Amoxicillin.

Table 2.

Minimum Inhibitory Concentration of Tetracycline and Rifampicin for *E. coli* 64 and *E. coli* MM294

Antibiotic	µg/ml	Bacterial Strain	
		***E. coli* 64**	***E. coli* MM294**
TE	**10**	**+**	**+**
	20	**+**	**+**
	30	**+**	**+**
	40	**+**	**+**
	50	**+**	**-**

RA	**10**	**-**	**+**
	20	**-**	**+**
	30	**-**	**+**
	40	**-**	**+**
	50	**-**	**+**

+ = growth, - = no growth, TE = Tetracycline, RA = Rifampicin.

Table 3.

Conjugation between *E. coli* 64 and *E. coli* MM294

Conjugation Method	**Donor Strain**	**Recipient Strain**	**Selection for**	**Number of Transconjugants/ml**
In Liquid Media	*E. coli* 64	*E. coli* MM294	TE^r, RA^r	7.3×10^2
Control			**Selection for**	**Growth/ml**
E. coli 64			TE^r, RA^r	-
E. coli MM294			TE^r, RA^r	-

TE^r = Tetracycline resistant gene, RA^r = Rifampicin resistant gene.

Table 4.

Antibiotic Sensitivity of Transconjugants

Antibiotics	**Number of Transconjugants**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
TE	R	R	R	R	R	R	R	R	R
AMX	R	R	R	R	R	R	R	R	R
N	R	**S**	R	R	R	**S**	R	R	R
C	R	R	R	R	R	R	R	R	R
AM	R	R	R	R	R	R	R	R	R
SXT	R	R	R	R	R	R	R	R	R

R = Resistant, S = Sensitive, TE = Tetracycline, AMX = Amoxicillin, N = Neomycin, C = Chloramphenicol, AM = Ampicillin, SXT = Trimethoprime+Sulfamethorazole.