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Determination of Transposition Property of Neomycin , Streptomycin and Trimethprim Resistance Genes in Clinical Isolates of *Escherichia coli* and *Proteus mirabilis*

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ABSTRACT

Twenty bacterial isolates were collected from human diarrheal samples and then identified. Seven out of twenty isolates were *E. coli* and three *P. mirabilis* . These isolates were checked for their resistance to six antibiotics and two heavy metals (HgCl2 and Cdcl2). The bacterial isolates showed variation in their resistance to antibiotics and heavy metals.and only one *E. coli* but all *P. mirabilis* isolates showed high level of resistance. Transposition induction of genes in the two chosen bacterial isolates was carried out.. Our results suggested that in *E. coli* isolate, neomycin resistance gene was induced to jump and insert itself into chloramphenicol and tetracyclin resistance genes causing them to mutate .in percents reaching 80% and 8% respectively. On the other hand, the streptomycin resistance gene transposition appear to be specific for generating mutations in the heavy metals resistance genes at rates 48% and 100 % respectively. In addition, transposition induction of the above genes in the chosen *P. mirbilis* isolate seemed to cause mutations in ampicilin, chloramphenicol and tetracyclin resistance genes ranging between 2 and 4 % .

تعيين صفة القفز في المورثات مانحة المقاومة لنيومايسين ،ستربتومايسين وتراي ميثبريم في عزلات من جرثومة *Proteus mirabilis* و *Escherichia coli*

الملخص

تم جمع عشرين عزلة جرثومية من عينات الإسهال للانسان ثم شخصت .إن سبعة من عشـرين عزلة كانت تعود إلى *E.col* وثلاثة كانت *P. mirabilis* . اختبرت مقاومة هذه العـزلات الجرثومية لستة من المضادات الحيوية وكذلك للمعادن الثقيلة (كلوريد الزئبق وكلوريد الكادميوم) . إن العزلات الجرثومية أظهرت اختلافا في مقاومتها لهذه المضادات الحيوية وجميعها كانت مقاومة للمعادن الثقيلة وتبين إن عزلة واحدة من *E.coli* وجميع عزلات*P. mirabilis*. أظهرت مقاومة عالية ليعض هذه المضادات الحيويـة . حفزت عملية القفز في المورثات مانحة المقاومة للنيومايسين ,ستربتومايسين وترايمثبـريم فـي العزلـة الجرثومية المختارة من *E.coli* وكانـت النتائج كالأتي : المورث مانح المقاومة للنيومايسين استطاع

مهاجمة واقتحام المورث مانح المقاومة للكلوروفينيكول والتتراسايكلين مسببا طفرات جينية وبنسبة80% و 8% على التوالي. من جهة أخرى فان المورث مانح المقاومة للستربتومايسين كان ذو خصوصية في مهاجمة المورثات مانحة المقاومة للمعادن الثقيلة (الزئبق والكادميوم) عند تحفيزه حيث وصلت النسبة إلى 100% و 48% على التوالي. أضف إلى ذلك فان تحفيز القفز في المورثات مانحة المقاومة للنيومايسي وستربتومايسين و ترايمثبريم في العزلة الجرثومية المختارة من *P. mirabilis* اظهر حدوث تطفيرا في المورثات مانحة المقاومة للأمبسلين وكلوروفنيكول والتتراساكلين وبنسب تتراوح بين 2-4%.

INTRODUCTION

Most bacteria possess transposable elements which are capable of moving from one replicon to another by recA-independent recombination systems (Freifelder, 1987).

Transposons have not yet been shown to exist autonomously. They need to reside in a functional bacterial replicon (Lewin, 1997). Various genes on plasmids that specify antibiotics or heavy metals resistance (Cohen,1976), toxin production, lactose frementation or hydrocarbon degradation (Chakrabarty, 1978), are known to be located in discrete DNA sequences, named transposons.

Transposable elemente are important agents of mutations. They cause mutations in two principle ways. Most transposable elements are present in nonessential regions of the genome and usually do little or no harm. However, when an element transposes , it can insert itself into an essential region and disrupt its function. Because most transposable elements contain coding region of their own, transcription of these transposons will interfere with transcription of the gene into which it is inserted or transcription of the gene will terminate within the transposable element. Another mechanism of transposable elements mutagenesis results from recombination . Transopsable elements are present in multiple copies, often with two or more in the same chromosome . If the copies are near enough they can pair during synapsis and undergo crossing over, with the result that the region of DNA between elements is deleted (Hartl, 1991).

The aim of this report is to determine the transposition property of neomycin, streptomycin and trimethprim resistance genes in the genome of pathogenic bacterial isolates. Also, it was aimed at studying the ability of these antibiotic resistance genes after there transposition induction to create genetic mutations in the other genes of these isolates.

MATERIALS AND METHODS

Collection of the Bacterial Isolates

Twenty isolates were collected from human clinical specimens of stool from patients suffering from diarrhea. These isolates brought from Al-khanzia and Ibn-senia teaching hospitals in Mosul city. The referance bacterial strains were *E. coli* K12 JM83 and *E. coli* JMP294 supplied by Dr. George M. Weinstock, Univ. of Texas, U.S.A .and Dr. Abdul khoa, College of Medicine, Univ.of Baghdad., Iraq, respectively.

Media, Antibiotics and Heavy metals

Various culture media (nutrient broth, nutrient agar, MacConkey agar and EMB agar) were used for bacterial cultivation. Stock solutions of ampicillin, chloramphenicol, neomycin, streptomycin, tetracyclin and trimethprim were prepared and used at the following final concentrations 50, 10, 10, 25, 10 and 15 µg/ml respectively, The heavy metals, mercuric chloride and Cadmium chloride were used at the final concentrations of 25 µg/ml in the culture media.

Identification of the Bacterial Isolates

Morphological characters of the isolated bacterial colonies were studied on different culture media. Then, microscopic examination of the prepared bacterial smears carried out after staining with Gram stain. Finally, Api 20E system was applied to confirm identification of the isolates to species level according to Atlas et al. (1995).

Study of Some Phenotypic Traits of the Bacterial Isolates
Antibiotics and Heavy metals Resistance

Nutrient agar plates containing the used antibiotics and heavy metals with their final concentrations were prepared separately accordig to method of Ahmad (1989). The plates were streaked with bacterical isolates, incubated at 37°C overnight and the results of bacterial growth were recorded.

Dosage Effect of Neomycin, Streptomycin And Trimethprim Resistance Genes

The final concentrations of neomycin, streptomycin and trimethprim were doubled in nutrient agar plates to determine the tolerance of the isolated bacteria to these increased concentrations. Then, these plates were inoculated with bacterial isolates, incubated at 37°C overnight and the growth results were recorded.

Local Determination of Neomycin, Streptomycin And Trimethprim Resistance Genes in the Bacterial Isolates

This was achieved through two steps. In the first step the plasmid DNA content from chosen bacterial isolates that revealed high level of resistance to above antibiotics, were extracted according to Birnboim and Doly (1979). Then, the prepared plasmid DNA was used to transform the referance strains of *E. coli* by heat shock using the technique of Mandel and Higa (1970).

Inducing mutations in Chosen Bacterial isolates After transposition Induction of Their Neomycin , Streptomycin And Trimethprim Rresistance Genes.

The procedure of Klipp and Puhler (1984) was followed. The bacterial isolates were grown in nutrient broth in presence of the highest concentrations of neomycin, streptomycin and trimethprim separately that the isolates tolerate them. Serial dilutions down to 10-9 were prepared and the last three dilutions were plated on nutrient agar plates containing these high concentrations of antibiotics, incubated at 37°C overnight. On the next day, master plates with 100 bacterial colonies were prepared on similar nutrient agar plates. Then these colonies were replicated onto other plates containing ampicillin, chloramphenicol, tetracycline, $HgCl_2$ or $CdCl_2$ at the final concentrations specified above. The percents of colonies failed to grow on these plates were calculated.

RESULTS AND DISCUSSION

Diagnosis of the Collected Bacterial Isolates

Seven out of twenty bacterial isolates appear with small, smooth, entire and convex colonies on blood agar. They were red pink, lactose fermenting on MacConkey agar and on EMB agar plates the colonies of these isolates were blackish with green metallic sheen. These characteristics of the isolates appeared to be related to *E. coli*. On the other hand, the colonies of the remaining isolates showed swarming activity on nutrient agar plates and seemed to be *P. mirabilis*. The stained bacterial smears of all isolates revealed Gram negative– spore non-forming rods. These identifcations were confirmed by the Api 20E system.

Antibiotics and Heavy Metals Resistance Patterns of the Bacterial Isolates

The patterns of resistance of the bacterial isolates to antibiotics and heavy metals used are shown in the table (1).

Table 1: Antibiotics and heavy metals resistance patterns of the bacterial isolates.

Isolate number	Species	Nutrient agar plates containing antibiotics and heavy metals in µg/ml							
		Ap 50	Cm 10	Neo 10	Str 25	Tet 10	Tri 10	HgCl2 25	CdCl2 25
E1	*E. coli*	S	R	S	R	R	R	R	R
E2	*E. coli*	R	R	R	R	R	R	R	R
E3	*E. coli*	R	S	S	R	S	S	R	R
E4	*E. coli*	R	R	R	R	R	R	R	R
E5	*E. coli*	R	S	R	S	S	R	R	R
E6	*E. coli*	R	R	S	S	R	R	R	R
E7	*E. coli*	S	R	R	R	R	R	R	R
P1	*P. mirabilis*	R	R	R	R	R	R	R	R
P2	*P. mirabilis*	R	R	S	R	R	R	R	R
P3	*P. mirabilis*	R	R	S	R	R	R	R	R
JM83	*E. coli* K-12	R	S	S	R	S	R	S	S
JMP294	*E. coli* K-12	R	R	R	S	R	S	S	S

R: refers to resistance, S: refers to sensitive.

It is clear from table 1 that the bacterial isolates showed multi-resistance phenomena to most antibiotics used. The isolates of *E. coli* (E2 and E4) and all *P. mirabilis* isolates were completely resistant to all antibiotics tested.. Also , all isolates except the laboratory strains can grow in presence of the heavy metals HgCl2 and CdCl2 at concentration 25 µg/ml. So the prevalance of resistanance to antibiotics and heavy metals is very clear.among isolates. This antibiotics resistance may be related to many mechanisms, these include mutations in the target site at which antibiotic bind in the bacterial genome, reducing the permeability of the cell membran., degradation of the antibiotics by the enzymes encoded by antibiotic resistance genes on plasmid DNA molecules in the bacterial cell or the presence of efflux system that pumps the antibotics outside the bactreial cell (Spratt, 1994). On the other hand , the resistance of our bacterial isolates to

HgCl2 may be due to presence of the *mer*operon carried on plasmid DNA molecules. The *mer* determinants RTPCDAB in bacteria (cluster of genes) are often located on plasmid or transposon and can also found in chromosome (Andrea et al., 2003*)*. While the resistance CdCl2 may be related to presence of plasmid DNA encoding Cd efflux system in the bacterial cells (Horitsu et al., 1986*)* .

Our results agree with those reported by Mustifa (2002) who demonstraed that the isolates of *P. mirabilis* from different clinical sources were resistant to ampicillin, chloramphenicol, tetracyclin, streptomycin and other antibiotics. Also, Al-delemy (2005) mentioned that the *E. coli* isolates from diarrhea samples were multi-resistant to many antbiotics and heavy metals used in her research.

Dosage Effect of Neomycin, Streptomycin And Ttrimethprim Resistance Genes in the Bacterial isolates

The bacterial isolates of *E. coli* and *P. mirabilis* that showed resistance to neomycin, streptomycin and trimethprim at final concentrations 10, 25, 10 µg/ml respectively were exposed to increased concentrations of these antibiotics (2, 4, 8 , 16, 32 folds) in the nutrient agar plates. Only one among bacterial isolates of *E. coli* can tolerate the above antibiotics at concentrations 80, 200 and 160 µg/ml respectively and this isolate labeled as E4 (Table 1). On the other hand, all *P. mirabilis* isolates showed resistance to 160, 300, 320 µg/ml of neomycin, streptomycin and trimethprim respectively and one of them chosen and labeled as P1 (Table 2). The isolates E4 and P1 were chosen for transposition induction Studies..

The ability of our bacterial isloates to tolerate these high concentration of neomycin, stretomycin and trimethprim may be related to presence of high copy number of genes encoding these antibiotics resistance. These genes located on plasmid DNA molecules with a high copy number in the bacterial cell. Also, it is possible that these antibiotic resistance genes found in chromosomal DNA of the bacterial cell and may be expressed constitutively (Nordstrom et al., 1972).

Detection the location of Neomycin, Streptomycin And Trimethprim Resistance Genes in the Chosen bacterial isolates

After extraction of the plasmid DNA from the isolates E4 and P1 and transferring one microgram of prepared DNA into the laboratory strains JM83 and JMP294 and plating 0,1 ml of transformation mixure (10 cells) on nutrient agar plates containing neomycin , streptomycin and trimethprim separately. No transformant colonies were obtained on these plates after incubation them at 37C overnight. These findings might indicate that the genes encoding these antibiotics resistance are located in chromosoml DNA of the chosen bacterial isolates E4 and P1 and not in their plasmid DNA.

Transposition Induction of Neomycin, Streptomycin and Trimethprim Resistance Genes in the Chosen Bacterial Isolates

A Master plate with 100 bacterial colonies from cultures which induced for transposition by high concentrations of neomycin, streptomycin and trimethprim, were prepared then these colonies were tested for their growth on nutrient agar plates containing ampicillin, chloramphenicol, tetracyclin and heavy metals separately, incubated at 37℃ overnight and the percents of colonies failed grow on the latter plates calculated and the following table demonstrate the results.

Table 2: Transposition Induction of Neomycin, Streptomycin and Trimethprin Resistance Genes in the chosen bacterial isolates.

Isolate number and species	Antibiotic used for transposition induction	Percents of colonies growth failure on nutrient agar plates containing antibiotics and heavy metals in µg/ml				
		Ap 50	Cm 10	Tet 10	HgCl2 25	CdCl2 25
E4 *E. coli*	80 µg/ml neomycin	0	80	8	0	0
	200 µg/ml streptomycin	5	0	4	100	48
	160 µg/ml trimethprim	0	0	0	0	0
P1 *P.mirabilis*	160 µg/ml neomycin	2	0	4	0	0
	300 µg/ml streptomycin	1	0	3	0	0
	320 µg/ml trimethprim	4	0	3	0	0

As shown in Table 2, *E. coli* isolate (E4) gave a high percent of chloramphenicol resistance inactivation of master plate colonies after transposition induction of their neomycin resistance gene, reaching 80%. This result indicate that neomycin resistance gene in these colonies may be induced to jump from its location in chromosomal DNA and attack the chloramphenicol resistance gene and prevent its expression. The high percent obtained may refer to specificity in the behavior of neomycin resistance gene during its transposition. In addition, the tetracyclin resistance was removed from induced master plate bacterial colonies at rate reaching 8%. On the other hand, no effects were observed on ampicillin and heavy metals resistance genes in the tested bacterial colonies. Furthermore, the presence of 200 µg/ml streptomycin cause the streptomycin resistance gene to transpose from its position in chromosomal DNA and inserted into Hg and Cd resistance genes with high percents reaching 100% and 48% respectively. No remarkable effects of streptomycin resistance gene transposition on ampicillin, chloramphenicol and tetracyclin resistance. Also, the presence of high concentration of trimethprim has no effect on transposition induction of trimethprim resistance gene in the chosen *E. coli* isolate.

Table (2) also shows that the percents of colonies failed to grow on nutrient agar plates containing ampicillin, chloramphenicol and tetracyclin separately and heavy metals are too low after induction of P1 isolate. with high concentrations of neomycin, streptomycin and trimethprim separately. From these results it appear, that neomycin, streptomycin and trimethprim resistance genes may transpose and insert into different genes randomly in the bacterial genome but at low rates or may be due to spontaneous mutations in genes encoding ampicillin, chloramphenicol and tetracyclin as a result of repeated bacterial cultivation..

Conclusion can be suggested from our results that the neomycin resistance gene in the *E. coli* isolate (E4) may be transposon, probably Tn5, but more investigation are needed. Similar results were obtained by Klipp and Puhler (1984) who demonstrated that in *E. coli* strain S605, the Tn5 transposon encoding neomycin resistance exist in chromosomal DNA and has the ability to jump from its original site and insert into chloramphenicol resistance gene causing inactivation of its expression and not randomly jumped to the genes of bacterial genetic content.. On the other hand, transposition of genes could occur between plasmid DNA molecules in the bacterial cell. McConnell et al. (1979) reported that there was transposition from the small ampicillin plasmid to the enterotoxin plasmid in a strain of *E. coli* serotype 0159 H34 of human origin. Also, Rubens *et al*. (1979) demonstrated that in the two plasmid DNA there are sequences mediating multiple antibiotic resistance transposed *in vivo* between coexisting plasmids in clinical isolates of *Serratia marcescens* and this event resulted in the evolution of of a transferable multi-resistance plasmid. Both sequences, designated as Tn1699 and Tn1700 and could transpose between replicons independently of the *E. coli* recA function. Alyas (2006) demonstrated that the neomycin resistance gene located in chromosomal DNA and has the ability to generate mutation in chloramphenicol resistance gene at rate reaching 60% and also causing different rates of inactivation in many antibiotics and heavy metals resistance genes in the clinical bacterial isolates of *Serratia marcescens*.

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