Curing Analysis of Drug Resistance Plasmid in Proteus Mirabilis

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Abstract

Forty five isolates of Proteus mirabilis were identified among 600 samples taken from patients with urinary tract infection from different hospitals in Erbil City. Isolates were identified from urine sample by using cultural, morphological and biochemical characteristics and confirmed confirmed by VITEK 2 systems. Antibiotic sensitivity testing was done for all isolates by using fifteen antibiotic types which included Amikacin (AK), Ampicillin (AMP), Aztreonam (AT), Chloramphenicol (C), Cephalothin (CEP), Ciprofloxacin (CIP), Ceftriaxone (CTR), Cefotaxime (CTX) Fosfomycin (FOX), Gentamicin (GEN), Imipenem (IMP), Nalidixic acid (NA), Norfloxacin (NOR), Piperacilin (PI) and Tobramycin (TOB). IMP was the most effective antibiotic against isolated of P. mirabilis. The resistance rate of the isolates toward these antibiotics were 8.8% for CIP, 11% for AT, 11% for FOX, 15.5% for NOR, 17.7% for TOB, 20% for AK, 22% for PI, 26% for CTR, 28.8% for CTX, 33% for NA, 35.5% for GEN, 44% for C, and 62% for AMP respectively. To control the antibiotic resistance of the tested P. mirabilis isolates, curing of plasmid DNA was conducted using tetracycline and elevated temperature at 46 ^{o}C, two most resistance isolates were chosen for this purpose P32, and P40, then treated with different concentrations of tetracycline (0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4) µg/ml separately. The MIC for tetracycline was 2.5µg/ml and SMIC 2µg/ml was used as a curing agent. The genes encoded resistance to ampicillin, chloramphenicol, cephalothin, ceftriaxone, cefotaxime, gentamicin and tobramycin were cured from P32 and the percentage of curing was (46.6%), while amikacin, chloramphenicol, ciprofloxacin and gentamicin resistance genes were cured from P40 isolate and the curing percent was (26.6%).The results confirmed by conducting gel electrophoresis and revealed that tetracycline

DOI: 10.24086/cuesj.si.2017.n2a5

removed plasmid of P32, while it had no effect on plasmid of P40. On the other hand, elevated temperature used also as curing agent and the results revealed that resistance to amikacin, ampicillin, chloramphenicol, cephalothin, ceftriaxone, gentamicin and tobramycin genes were cured from P32 and the curing percent was (46.6%), while genes encoding amikacin, ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, cefotaxime, fosfomycin, gentamicin, nalidixic acid, norfloxacin and tobramycin were cured from P40 isolate with the percentages of (73.3%) after incubating the isolates at 46 ^{o} C. It is clear that elevated temperature is the most efficient method than tetracycline. The results confirmed by conducting gel electrophoresis and showed that both tested isolates lost their plasmids after incubation at 46 ^{o} C.

Key words: Proteus mirabilis, antibiotic resistance, and plasmid curing

Introduction

P. mirabilis identified as an opportunistic pathogen and its major causative agent of complicated UTI, it's rarely associated with UTI in patients that are otherwise considered healthy. These types of infection often referred to as community-acquired or uncomplicated UTIs which are caused by *Escherichia coli* in the vast majority of cases [1]. *P. mirabilis* is associated with complicated UTIs that occur in patients who have functional or anatomical abnormalities in the urinary tract or are undergoing catheterization [2]. Antimicrobial resistance in *P. mirabilis* is of great public health concern in the developing world. The accelerated emergence of antibiotic resistance among the prevalent pathogens is the most serious threat on the management of infectious diseases [3]. Plasmids, which can transfer between genera and species of bacteria, lead to prevalence the resistance by conjugation and transformation [4]. There are other mechanisms were by bacteria confer resistance to the antibiotics including intrinsic impermeability and acquired resistance as transposons and mutations [5]. Plasmid represents extra chromosomal, autonomously replicating elements important for bacterial adaptability and survival [6]. Many plasmids control medically important properties of pathogenic bacteria, including resistance to one or several antibiotics [7]. Plasmid sometime can be eliminated or lost from host cells by various treatments. This process termed curing,. Curing may occur spontaneously or induced. It is greatly increased by application of some physical and chemical factors such as acridine dye, sodium dodecyl sulfate (SDS) and ethidium bromide dye. Using of heavy metals, ultraviolet, ionizing radiation or growth at temperature above the optimum may also result in elimination of the plasmid [8] .So this study try to isolate and identify of *P. mirabilis* from patients suffering from UTIs , study the antibiotic resistance pattern, and attempt to eliminate the antibiotic resistance by elevated temperature and tetracycline.

Materials and Methods

A. Patients and sample collection

Six hundred urine samples were collected from patients with symptomatic urinary tract infection attended from different hospital in Erbil City (Rapareen Padiatric, Rizgary, Hawler Teaching, West Emergency and Zheen International hospitals), during the period of 1st April to15th August 2012 .Bacterial isolates were identified by performing morphological, cultural, biochemical tests, and Vitek 2 system.

B. Antibiotic susceptibility test

To study the effect of different antibiotics on the isolates of *P. mirabilis*, Mueller-Hinton agar was used as growth media [9].Antibiotic resistance patterns of the isolates were determined by using the Disc diffusion (Kirby Bauer) method; bacterium inoculate was adjusted to 0.5McFarland standard of Clinical and Laboratory standards institute [10]. The tested inoculums were spread onto Mullur-Hinton agar using a sterile cotton swab. The tested antimicrobial agents were aseptically placed on the inoculated Muller Hinton agar and incubated overnight. The zones of inhibition were measured and interpreted according to [10].

C. Plasmid Curing minimum inhibitory concentration (MIC) for tetracycline

The MIC of tetracycline were determined by turbidity method (spectrophotometer method) at wave length 600 nm and the following dilutions were prepared (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4) µg/ml and the sub-minimum inhibitory concentration (SMIC) was used as a curing agent [11].

D. Tetracycline as a curing agent

To investigate the elimination power of tetracycline, a method mentioned by [12] was followed. Five ml of LB broth inoculated with single colony of *P. mirabilis*. After incubation at 37^{o} C with 100 rpm for 24 hours, 0.1 ml of an overnight culture were inoculated into 5 ml LB broth containing SMIC (2µg/ml) of tetracycline incubated at 37 ^{o} C for 24 hours with 100 rpm in shaker incubator. Serial dilutions were prepared up to 10^{-6}, 0.1 ml from the last three dilutions were spreaded onto nutrient agar plates and incubated at 37 ^{o} C for 24 hrs. After incubation time transferring 50 colonies to nutrient agar plates and incubated over night at 37 ^{o} C representing the master plate. Ten colonies were randomly chosen, transferred to Muller Hinton agar plates and tested for antibiotic sensitivity pattern, incubated at 37 ^{o} C for 24 hrs. After overnight culture incubation the percentage of curing colonies for antibiotic sensitivity pattern were calculated.

E. Curing of plasmid DNA by elevated temperature

This method was conducted according to [13]. Ten ml of LB broth inoculated with single colony of P. mirabilis isolate and incubated at 37 ºC overnight, after incubation time, 0.2 ml of bacterial culture transferred to 10 ml LB broth and incubated with 100 rpm at 46 ºC for 24 hrs. Serial dilutions were prepared up to 10-6 0.1 ml from the last three dilutions were spreaded onto nutrient agar plates and incubated at 37 ° C for 24 hrs. After incubation time, 50 colonies were transferred to nutrient agar plates and incubated over night at 37 ºC representing the master plate.Ten colonies were randomly chosen, transferred to Muller Hinton agar plates and tested for antibiotic sensitivity pattern, incubated at 37 ºC for 24 hrs. After an overnight incubation, the percentages of cured colonies for antibiotic sensitivity pattern were calculated.

F. Antibiotic resistance pattern after plasmid curing

The colonies were screened for antibiotic resistance by the disk diffusion method after curing. Cured markers were determined by comparison between the pre- and post- curing resistance pattern of isolates. Loss of resistance markers gave an indication that those markers were probably located on plasmid and not on the chromosome.

G. Plasmid DNA Extraction and electrophoresis

Laboratory Protocol

Plasmid DNA was extracted and purified from 5ml overnight culture of the selected isolates of the *P. mirabilis* grown in LB broth medium containing 100µg/ml Ampicillin using a plasmid DNA purification kit, according to the manufacturer's instructions (Intron). The extracted plasmids were electrophoresed in 0.7% agarose gel with Tris-borate ethylene diamine tetra-acetic acid (TBE), Agarose gel electrophoresis was used to separate DNA fragments according to size [14].

Results and Discussion

A. Identification of P. mirabilis isolates

It grows well on MacConkey agar with optimum growth at 37˚C and form several sorts of discrete light brown colony indicating of not lactose fermentation .Also shows characteristics of swarming on the blood agar surface which appears as concentric rings of growth emanating from a single colony or inoculum .Under the light microscope, *P. mirabilis* appears as straight Gram-negative rods. Its cells are appearing as non-capsulated and non-spore forming .The biochemical tests for all bacterial isolates were positive for citrate, catalase, motility and urease production test, but they were negative for oxidase test. On Kligler Iron Agar medium, all isolates of *P. mirabilis* under study produced a red (alkaline) slant and a yellow (acidic) butt reaction due to the

non-lactose fermentation and glucose fermentation with H2S production [15]. Furthermore, Vitek 2 system was performed to support above results and to be confirmed that the isolates were *P. mirabilis*.

B. Antibiotic resistance profile

Antibiotic resistance test was performed against 15 antibiotics (AK, AMP, AT, C, CEP, CIP, CTR, CTX, FOX, GEN, IMP, NA, NOR, PI and TOB). Table (1) indicated that all the isolates of P. mirabilis revealed different resistance rate to most antibiotics used and resistance percent were 62 % for AMP, 44% for C, 40% for CEP, 35.5% for GEN, 33% for NA, 28.8% for CTX, 26% for CTR, 22% for PI, 20% for AK, 17.7% for TOB, 15.5% for NOR, 11% for AT and FOX, while the lowest percent 8.8% was to CIP, and the most effective antibiotic was IMP. High resistance pattern for AMP reported with a percentage of 62%, [16] found the resistance to AMP was 56% which is near to our results, [17] reported 80%. This was explained by the overuse of antibiotics especially AMP in Erbil Hospitals, and also to the missuses of these antibiotic as they are prescribed without sensitivity test [18]. In the present work, 33% of the isolates were resistant to NA, this result was in agreement with [19] who stated that 29 % of *P. mirabilis* isolates were resistant for NA, also [17] found that 20 % of *P. mirabilis* isolates were resistant to NA. [20] revealed that 17.5% of *P. mirabilis* isolates were resistant to NA, while these results disagree with [21] and [22] who reported that 52% and 60% of isolates resistant to NA respectively. This may be due to different antibiotic prescribed and subsequently different percentages of sensitivities.

Table I: Number and percent of resistance *E.coli* to antibiotics used

Antibiotics Used	**No. of Resistant isolates**	**Resistance %**
*AK	18	27
AMP	67	95.7
AMC	64	91.4
CIP	23	32.8
CEP	66	94.2
NA	52	74.2
SXT	64	91.4
CRO	46	65.7
IPM	0	0
F	31	44
C	21	30
CTX	61	87
GM	48	68.5

Regarding to C resistance and the obtained results, 44% of isolates was resistant to it. This result is in agreement with [21] who mentioned that 36% of the isolates were resistant to C, while in a study conducted by [23], it was found that 59% *P. mirabilis* isolates were resistant to C.

In the current study, it has been shown that the resistance raised to the third generation of cephalosporin 3GC such as CTX and CTR, and the results were 28.8% and 26% for CTX and CTR respectively. These results were agreed with [24] who mentioned that 31.25% of the isolates resistant for the 3GCs. It was illustrated from table (1) that 40% of the isolates were resistant to CEP and this was agreed with [16] and [25] who found that the resistance for CEP was 33% and 35.2% respectively, while these results disagree with [24] who found that the resistance percent for CEP was 100%. Low resistance pattern to CIP reported with a percentage of 8.8%. This result is in agreement with [19] who found that the resistance to CIP was 10% and 6.25%, respectively. This may be due to the efficiency of this antibiotic in eradication of infections caused by Gram negative bacilli, also 15.5% of isolates were resistant to NOR, this result agreed with [20] who revealed that 19.6% of the isolates were resistant to NOR, while disagree with [16] who found that 52% of the isolates were resistant to NOR. Twenty-two percent of the isolates were resistant PI; these results disagree with [21] who found that 44 % of *P. mirabilis* isolates were resistant to PI. Also it was recorded that addition of sulbactam or tazobactam to PI increased the sensitivity to 100% [26].The resistance of *P. mirabilis* isolates to GEN was 35.5%. [17] found that the resistance to GEN was 20%. These results disagree with [19] and [20]. Which they reported the resistance to GEN as 13% and 16.5% respectively, also [16] found that 86% of *P. mirabilis* isolates were resistant to GEN. On detecting the resistance of *P. mirabilis* for FOX and AT, it was found that 11% of the isolates were resistant to both, [19] reported that 25% of *P. mirabilis* isolates showed resistance to FOX, while the same researcher showed that the resistance of the isolates to AT was 100%. In our work, 20% of the isolates were resistant to AK, this result agree with [25] who found that 16.6% of the isolates resistance to AK. Finally 17.7% of the isolates were resistant to TOB, and this result disagree with [19] who detected that the resistance for TOB was 100%. IMP was the most effective antibiotic against isolates of *P. mirabilis* and 100% of these isolates were susceptible to IPM, and this result agree with [21], and [27] who they reported that all *P. mirabilis* isolated from different clinical source were sensitive for IMP. This may be attributed to the inability of *P. mirabilis* to produce enzymes that degrade or inactivate this antibiotic. Therefore, IMP is the most active drug for the treatment of UTIs causing by *P. mirabilis*. The widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance [28]. The high resistance of the bacterial isolates in this study to different antibiotics may be related to the presence and dissemination of plasmids within heterogeneous

population of these bacteria [3], which can transfer between genera and species of bacteria lead to the prevalence of resistance by conjugation and transformation [4]. In addition to weakling immunity system in some human due to poor nutrition or heredity factors makes bacteria to be more resistant [29]. Another reason of resistance in *P. mirabilis* may be attributed to mutation in the structure of bacterial cell wall and leads to lacking of porins, also consider as reason for resistance of bacteria to antibiotic especially to β- lactam antibiotics [30].

C. Plasmid curing

Curing of plasmid in P. mirabilis isolate P32 and P40 by SMIC of tetracycline

To demonstrate whether the drug resistance of the selected isolates was of plasmid or chromosomal origin, the MIC of tetracycline had been used as a curing agent. Minimum inhibitory concentration (MIC) of tetracycline was estimated through the optical density reading at 600 nm by UV light spectrophotometer. The results showed that the MIC of tetracycline was 2.5µg/ml and different concentrations of sub MIC were used as a curing agent. The concentration of 2µg/ml of this antibiotic showed the best results. Table (2) demonstrates the curing percent of plasmid DNA from P. mirabilis isolates by tetracycline. For P32 isolate, tetracycline affect AMP, C, CEP, CTR, CTX, GEN, and TOB genes; the resistance rate of P32 isolate decreased and the percent of curing reached (46.6%), while for P40 isolate, it affect the genes which were responsible for AK, C, CIP, and GEN and the curing percent was (26.6%). Figure (1) shows the plasmid DNA profile for P. mirabilis isolates (P32 and P40) and it is clear that one plasmid DNA exist in the P32 and two plasmid DNA in the P40 isolates before treating with tetracycline . After treating these two isolates with 2µg/ml of tetracycline, this antibiotic removed the plasmid DNA of P32, while for P40; the plasmids were not cured with exceeded incubation time to 48 hours. This inactivity of tetracycline in curing of plasmids of P40 can be attributed to the fact that effectiveness of curing methods depended on the nature of the bacterial host and/or plasmids where some work better than other [31].

Tetracycline reversibly inhibits bacterial protein synthesis by binding to the ribosomal complex, preventing the association of aminoacyl-tRNA with the bacterial ribosome. In Gram negative bacteria, tetracyclines move through membranes via porin channels and accumulate in the periplasmic space. To move across the cytoplasmic membrane, tetracycline requires energy and this energy is obtained by the ΔpH of the proton motive force. Once inside the bacterial cell, the tetracycline molecules bind reversibly with the prokaryotic 30S ribosomal subunit, stopping protein synthesis [32]. Among various plasmids present in bacterial strains, R plasmids are very significant as they confer resistance to one or more antibiotics, thus possess a threat to chemotherapy. Treatment of cells with certain chemical and physical agents enhances the elimination

of plasmids from host cells. This phenomenon is referred to as curing and has been used to ascertain the plasmid associated nature of genes. Susceptibility to curing agent also varies among plasmids (Martinez-martinez *et al*., 2008).At present, tetracycline resistance in bacteria can occur by acquisition of ≥ 1 of the 36 different genes, by mutations to host efflux pumps or in their 16S rRNA sequences, or by alteration in the permeability of the cell [33]. [34] used Ciprofloxacin as curing agent against *Acinetobacter baumannii* isolated from different sources. The result showed that the concentration 0.9 µg/ml of this antibiotic cured two plasmids of this bacterium.[35] employed two methods for plasmid curing, the first one by treatment with 10% SDS against isolates of *P. mirabilis*. This method showed partial curing and did not remove plasmids completely. The second method that used by [35] was treatment with different concentrations of acridine orange. The best concentration of acridine orange used to cure plasmids was 32.5 µg /ml, in which this concentration cured all plasmids. [24] used ethidium bromide for curing experiment of three isolates of *P. mirabilis* that bearing plasmids for detecting the location of antibiotic markers on plasmid or on the chromosomal DNA. As a result, they lost their plasmids and resistant to β-lactam antibiotics as Ampicillin, Amoxicillin, Cephalothin, Cefotaxim, Ceftriaxone, Tetracycline and Gentamycin.

Table II: Curing of plasmid dna from *p. mirabilis* (p32 and p40) isolates by using tetracycline

Antibiotic resistance pattern	**Bacteria +Treatment**			
	P32	Tetracycline 2µg/ml+P32	P40	Tetracycline 2µg/ml+P40
AK*	R	R	R	S
AMP	R	S	R	R
AT	S	S	S	S
C	R	S	R	S
CEP	R	S	R	R
CIP	S	S	R	S
CTR	R	S	R	R
CTX	R	S	R	R
FOX	S	S	R	R
GEN	R	S	R	S
IPM	S	S	S	S
NA	S	S	R	R
NOR	S	S	R	R
PI	R	R	R	R
TOB	R	S	R	R
% of curing	46.60%		26.60%	

D. Curing of plasmid DNA by elevated temperature

Elevated temperature at 46°C was used to cure the plasmid DNA that confers resistance to antibiotics in *P. mirabilis* (P32) and (P40) isolates and the main results are represented in table (4). Table (3) indicate that the P32 when treated with elevated temperature at 46 ºC, AK, AMP, C, CEP, CTR, GEN and TOB genes were affected and the curing percent was (46.6%), while when P40 isolate treated with elevated temperature at 46 ºC, genes which responsible for AK, AMP, C, CIP, CTR, CTX, FOX, GEN, NA, NOR and TOB resistance were affected with the percentages of (73.3%). Figure (1) shows that both tested isolates missing their bands of plasmid DNA after incubation at 46°C. These results agree with [36] who studied the effect of elevated temperature at 46o C on plasmid DNA of *P. aeruginosa* isolates and they found sensitivity of isolates to all used antibiotics except lincomycin after incubating at 46 °C. [37] reported the elimination of F factor in *E. coli* when grow at (42-44) ºC.

Table III: Curing of plasmid dna from *p. mirabilis* (p32 and p40) isolates by using elevated temperature

Antibiotic resistance pattern	**Bacteria +Treatment**			
	P32	E. tempt.and 46 ° C+ P32	P40	E. tempt and 46 ° C+ P40
AK*	R	S	R	S
AMP	R	S	R	S
AT	S	S	S	S
C	R	S	R	S
CEP	R	S	R	R
CIP	S	S	R	S
CTR	R	S	R	S
CTX	R	R	R	S
FOX	S	S	R	S
GEN	R	S	R	S
IPM	S	S	S	S
NA	S	S	R	S
NOR	S	S	R	S
PI	R	R	R	R
TOB	R	S	R	S
% of curing	46.6%		73.3%	

From the obtained results, a conclusion can be made that curing by elevated temperature is the most efficient method than tetracycline; this may be due to the fact that the enzymes which contribute to the DNA replication processes are more affected

by this high temperature. The inactivation of these enzymes may be due to the change in the folding of polypeptide at this temperature, i.e. the enzymes are sensitive to elevated temperature [13]. These results also agree with [36] that they concluded that curing by elevated temperature is the most efficient method among others. Furthermore, enzymatic activity declines above the optimum temperature that is characteristic of the heat stability of the particular enzyme [38]. However, plasmids appear to be dependent on host enzymes for their replication, therefore, most of the proteins synthesized during changing of temperature might be utilized for cell division, by that, chance of plasmid replication decreases then curing occurred [36]. [13] found that the 46°C affected the antibiotic resistance plasmids of four *P. aeruginosa* and curing was obtained among them except for the genes responsible for Lincomycin, because they are chromosomally encoded.

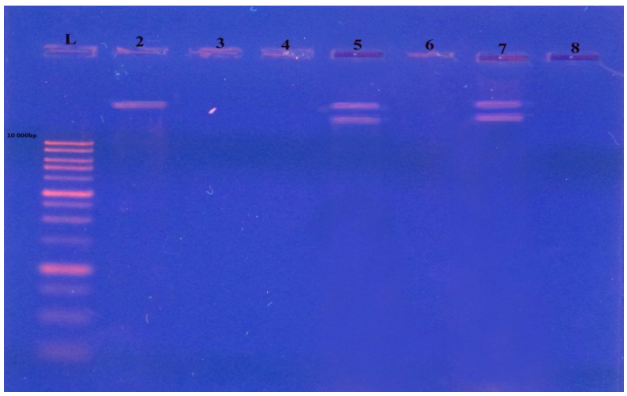

Figure 1. Plasmid profile of cured P32 and P40 by tetracycline and elevated temperature

Lane 1 10 000 bp DNA ladder
Lane 2 Plasmid content of P32
Lane 3 P32 after curing with elevated temperature
Lane 4 P32 after curing with tetracycline
Lane 5 Plasmid content of P40
Lane 6 P40 after curing with elevated temperature
Lane 7 Plasmid content of P40 after curing with tetracycline

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