Plasmid Profiling, Genetic Site Determination of Antibiotic Resistance Gene of *Pseudomonas Aeruginosa* Isolated from Burned Patients

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***Abstract*— In this study forty isolates of *Pseudomonas aeruginosa* were recorded from burn patients admitted to West Emergency Hospital in Erbil city. Isolates from burn samples were identified using cultural, morphological, biochemica1 tests. Antibiotic sensitivity testing was performed for all isolates using l4 types of antibiotics including Ampicillin (AMP), Chloramphenicol (C), Amoxicillin-clavulanic acid (AMC), Cefotaxime (CTX), Penicillin (P), Aztreonam (ATM), Meropenem (MEM), Tobramycin (TOB), Gentamycin (CN), Amikacin (AK), Ciprofloxacillin (CIP), Ceftazidim (CAZ), Tetracycline (TE) and Imipenem (IMP). The antibiotic imipenem was the most effective against P. aeruginosa isolates. The resistance rates of the isolates to these antibiotics were l00% for AMP, CTX, P, AMC, and C. For ATM 32.5%, for MEM 42.5%, for TOB, AK and CN 45%, for CIP 62.5%, for CAZ 67.5%, and for TE 80%. The plasmid profile of 5 antibiotic-resistant isolates (Pl, P3, Pl3, P29 and P38) were screened, results showed a single band with a molecu1ar weight more than l0 kb for a1l 5 isolates. Moreover five isolates (Pl, P3, Pl3, P29, and P38) were chosen for the transformation process and the identification of antibiotic resistance genes, using 1aboratory strain *Escherichia coli DH5-α* with plasmid DNA purified from these isolates. The transformation process was completed successfully, and the results revealed that the resistance genes for (Amikacin, Ampicillin, Cefotaxime, Tobramycin, Gentamycin, Chloramphenico1, Amoxicillin-cluvanic acid, Ciprofloxacin, Tetracycline, Penicillin, Ceftazidime, Azetreonam, and Merpenem) 1ocated on plasmid DNA for Pl isolate, whi1e for P3 (Amoxicillin- cluvanic acid, Ampicillin, Amikacin, Ciprofloxacin, Merpenem, Tobramycin, Chloramphenicol, Gentamycin, Tetracycline, Cefotaxime, and Penicillin) 1ocated on plasmid DNA. On other hand the resistant genes for (Amoxicillin- cluvanic acid, Ampicillin, Amikacin, Ciprofloxacin, Tobramycin, Chloramphenico1, Gentamycin, Tetracycline, Cefotaxime, and Penicillin) were located on plasmid DNA for Pl3. Moreover (Amoxicillin-cluvanic acid, Ampicillin, Amikacin, Ciprofloxacin, Merpenem, Tobramycin, Chloramphenicol, Gentamycin, Tetracycline, Cefotaxime, and Penicillin) for P29 were located on plasmid DNA, lastly for the P38 the resistant genes were located on plasmid for (Amikacin, Ampicillin, Cefotaxime, Tobramycin, Gentamycin, Chloramphenicol, Amoxicillin-cluvanic acid, Ciprofloxacin, Tetracycline, Penicillin, Ceftazidime, Azetreonam, and Merpenem). Transformation results confirmed by gel electrophoresis and demonstrated that plasmids (Pl, P3, Pl3, P29, and P38) had been successfully transformed.**

***Keywords*: Pseudomonas aeruginosa, Burns, Antibiotics, Antibiotic resistance Plasmid DNA, Transformation.**

I. INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa)* is an opportunistic pathogen that infects humans; it is a bacterium found in the environment that causes a variety of opportunistic human infections. In recent decades, many researchers discovered multi-drug-resistant isolates that are resistant to nearly all antimicrobials used in hospita1 patients [1]. Because of its low outer membrane permeability, chromosomally encoded AmpC, and extensive efflux pump system, the bacterium has intrinsic antimicrobial resistance and has played an important role in the development of acquired resistance mechanisms [2]. The pathology of *P. aeruginosa* is related to different metabolic abilities, numerous resistance mechanisms and a wide range of virulence factors and adaptation, and gene expression organizes all these activities in a very similar way [3]. It is one of the most common causes of burn-infection [4]. It is found that one of the most serious causes of burn wound infections [5]. Burning wound infection is a problem since it delays healing, and promotes scarring which may progress to bacteremia, sepsis (or failure of an organ), whereby organ from several systems

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can not sustain homeostas is on its own and require instant medical attention [6]. Plasmid DNA is a finite-sized extra chromosome element which is typically stable within a bacterial cell line and may be transferable between strains, species or genera. It was observed first in enteric bacteria and was associated with antibiotic resistance progressively in the late 1950's. Its main function is to transport resistant antibiotics genes that increase the pathogenicity of most bacteria [7]. Each plasmid contains only a few genes, and its size ranges from 1 to more than 10 kbp. Plasmids frequently contain genes that are required for organism survival as well as those that are generally beneficial to the host organism, such as antibiotic resistance [8]. Plasmids facilitate the transfer of genetic material, including antimicrobia1 resistance genes, between bacteria1 species and genera [9]. They are a key factor in the development of bacteria, functions like virulence, resistance, metabolism and/or other beneficial functions coded with the plasmid can promote bacterial health [10]. Besides its inherent resistance, *P. aeruginosa* also has an issue with external resistance due to plasmids. Resistance to plasmid mediated enzymes is specifically linked with the topical use of antibiotics and to the production of AmpC-like β-lactamases in areas where high levels of antibiotics are achieved [11]. Transformation is one of three horizontal gene transfer processes in which exogenous genetic material moves from one bacterium to another; the other two are conjugation (transfer of genetic material between two bacterial cells in direct contact) and transduction (injection of foreign DNA into the host bacterium by a bacteriophage). The process of transformation involves the introduction of foreign DNA into a cell. Transformation of plasmid bacteria is important not only in bacterial studies, but also because bacteria are used as a means of storing and replicating plasmids. This means that almost all plasmids (also those designed for the expression of mammalian cells) have both a bacterial replicative origin and an antibiotic resistance gene to be used as a selectable marker in bacteria [12]. In transformation, the genetic material passes through the medium and uptake depends entirely on the bacterium of the recipient [13]. This study was carried out to determine Plasmid DNA profile pattern characterization of the antibiotic resistant isolates, on other hand determination the site of antibiotic resistance gene through transformation process.

II. MATERIALS AND METHODS

A. Collection of Specimens

Fifty samples were collected from wounded burn patients obtained from children, young people, adults attending (West Erbil Emergency Hospital in Erbil city) and the relevant data have been collected from every patient, Age and Gender included. A swab containing a transportation media then transferred directly to the 1aboratory and processed all burn samples within ha lf an hour of collection.

B. Bacterial culture

A1l samples were inoculated on (Cetrimide agar and Pseudomonas agar), for this purpose all disposable swabs have been spread on these two media in a disposable petri plates, then incubated overnight at 37 °C.

C. Antibiotic susceptibility test

The isolates were tested for antibiotic sensitivity on Mueller-Hinton agar using the disc diffusion method, as recommended by the Nationa1 Committee for Clinica1 Laboratory Guidelines and the antimicrobial susceptibility testing protocols [14]. The bacteria1 inoculum were optimized to the Clinical and Laboratory Standards Institute's 0.5 McFarland standard [15]. The sample inoculum was dispersed on Mueller-Hinton agar with a sterile cotton swab. The antimicrobial products that were evaluated, including: Amoxicillin-clavulanic acid, Ampicillin, Aztreonam, Amikacin, Cefotaxime, Gentamycin, Imipenem, Ceftazidime, Ciprofloxacin, Tetracycline, Tobramycin, Penicillin, Chloramphenicol, and Meropenem were placed in an aseptic manner and incubated overnight, The inhibition zones were then interpreted and measured [16].

D. Extraction of plasmid DNA Protoco1

The P1asmid DNA has been extracted then purified by using a plasmid DNA extraction kit. Five ml of overnight cultivation of selected *P. aeruginosa* isolates managed to grow in LB broth medium with 100 ug/ml ampicillin.

1. Single colony of bacteria was used for the incubation of 5 m1 LB, over nights incubated at 37 °C in a shaker incubator. Centrifuged at 13, 000 rpm for 30 seconds, and 3 or 5 m1 supernatant were disposed in the Eppendorf tube of every isolate.

2. The bacterial pellet was re-suspended in 250 ml of the suspension tube by vortexing until there were no cell pellets clumps remaining.

3. Two hundred and fifty µ1 1ysis buffer has been added to the re-suspended cells, and then the tube closed and gent1y mixed several times by inverting the tube.

4. The tube was twisted several times by the addition of 350 µl of neutralization buffer.

5. The Centrifuged cells re-suspended for 10 min at 4 °C at 13 000 rpm, then a column inserted into a tube.

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6. The filtrate has been removed in the co llection tube after centrifugation at l3 000 rpm for 60 sec and immediately transferred to the column. The supernatant is removed from this collection tube. The spin co1umn has been p laced in the same tube.

7. Five hundred µl of washing buffer A added, then centrifuged at l3000 rpm, discarded the flow-through and returned to the collection tube with a purification column.

8. Seven hundred µ1 of washing buffer B was added centrifuged at l0 000 rpm for l min. The filtrate was disposed off in a collection tube and column in the same tube.

9. Again centrifuged at l3, 000 rpm for l min to dry filter membrane.

10. In the upper column reservoir, the column was placed in a sterile and clean Eppendorf, 50 µl elution buffer, and given 1 min to stand, then the tube centrifuged for 1 minute at 13, 000 rpm.

11. The purified DNA was subsequently removed or deposited at -20 C.

E. Location determination of genes in P. aeruginosa conferring antibiotic resistance

This is accomplished by isolating plasmid DNA content from bacterial isolates and then transforming it into standard strains, which serve as bacterial hosts for DNA uptake. The transformation procedure entails:

F. Preparation of competent cells (Heat Shock)

Five ml of LB broth inoculated with a single colony of *E. coli*, to make the cultures competent. *E. co1i DH5α* (plasmidless, whose plasmid has been genetically altered), incubated with shaker incubator, (l00 rpm) at 37 °C for 24 hours, then l m1 of bacterial culture was added to 50 m1 of LB broth, incubated at 37 °C for 3-4 hours, then the cells were extracted by centrifugation at 5, 000 rpm for l0 min, after that supernatant was discarded and then re-suspended. A pellet contained l m1 of ice cooled 0.l M $CaCl_2$ followed by the addition of 39 ml of the same solution. The re-suspended ce1ls 1eft on the ice for 30 minutes, the bacteria1 pe llet re-suspended in 2,5 m1 $CaC1_2$, centrifuged for l0 minutes at 5000 rpm.

G. Competent cell Transformation

Ten µ1 of plasmid DNA prepared from *P. aeruginosa* isolated then cultured on LB broth tubes to guarantee that they are free of bacterial cells, and incubated at 37 °C for 24 hours. The sterile Eppendorf tube containing 5 µl of plasmid DNA added to 200 µl of bacterial suspension and gently mixed. A negative controls consisting of competent cells without DNA on the LB agar plate were prepared with AMP (Ampicillin) for each sequence of transformations. The mixture pl aced on the ice for about 30 minutes then was put in a 42 °C water bath for 1.5 min without moving the tubes. The mixture was moved to a suitable tube containing 1.5 ml of broth after the heat shock, and then put in shaking incubator at 37 °C for 1 hour. After that, the mixture was put on the ice for 5 minutes. Upon incubation, the mixture was shifted aseptically onto Eppendorf tube and centrifuged for about 1 minute at 14000 rpm. The supernatant was discarded and the pellet re-suspended to a small extent. The re-suspended pellet was eventually spread on select LB agar plates. Then the antibiotic disks were applied. All plates have been incubated for 24 hours at 37 ° C [17].

H. Ge1 electrophoresis Protoco1

Ge1 electrophoresis method of [16] was followed with minor modifications. For this purpose 0.7 g for plasmid agarose was added to 100 ml 1x TBE buffer, then by using microwave oven, the mixture melted for l-2 minutes, or unti1 it was clear and completely dissolved and allowed to cool around 50 ° C, With a gentle swirling motion, The agarose solution was carefully mixed with l0 u1 of prime safe dye. The tray's edges were filmed together, and the appropriate comb was inserted into the tray. At room temperature, the agarose solidified after it was gradually poured into the tray (15-30 min) and any remaining bubbles were pushed away with a disposab le tip. After removing the tape from the tray, the tray was placed in the electrophoresis tank. More TBE buffers were added to the tank, bringing the gel completely under buffer. PCR product 1oaded into the wells l5 ul, based on the size of the PCR sampl e, the first well 5 u1 of the ladder l Kb for plasmid was used. The gel operates at 100 V for 50 minutes. Finally, the UV transilluminator was used and the gel was photographed.

III. RESULT AND DISCUSSION

A. P. aeruginosa isolates Collection

A Tota1 of Fifty samples were collected from burned patients admitted to West Erbil Emergency Hospita1. Twenty isolates presumptively diagnosed as *P. aeruginosa*, representing 40 % of total, a group of confirming tests were carried out to ensure that all of the reclaimed bacterial isolates belonged to the *P. aeruginosa* species.

B. Culture media

Pseudomonas colonies appeared pale and non-lactose fermenters on MacConkey agar. The colonies of *P. aeruginosa* on 5% blood agar were typically pale colonies

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and exhibited β-haemolytic activity, and also there were some isolates exhibited α- hemolytic activity [18].

C. *Pseudomonas aeruginosa antimicrobial sensitivity test*

Fifty isolates of P. aeruginosa were tested for resistance to fourteen commonly used antibiotics, incl uding (Cefotaxime, amoxicillin-clavu1anic acid, Aztreonam, Amikacin, Ampicil1in, Meropenem, Penicil lin, Ciprofloxacin, Chloramphenicol, Gentamcin, Imipenem, Tetracyclin, Ceftazidime, and Tobramycin).

Table l revealed the percentage of resistance for bacteria1 isolates to different antibiotics understudy. The resistance percentage for Ciprofloxacillin 62.5 %, for Gentamycin 45 %, for Amikacin 45 %, Meropenem 42.5 %, Aztreonam 32.5 %, for Tetracyclin 80 %, Tobramycin 45 %, Ceftazidime 67.5 %, whi1e the highest percentage of resistance (100 %) was to (Ampicillin, Amoxicillin-clavulanic acid, Cefotaxime, Chloramphenicol, Penicillin) Imipenem was effective against all isolates.

Table l: *The percentage of bacteria1 isolates resistant to various antibiotics.*

Antibiotics Name	Symbol	No. of resistant Isolates	% of Resistant
Amikacin	AK	22	45 %
Ampicillin	AM	50	100 %
Amoxicillin-clavulanic acid	AMC	50	100 %
Aztreonam	ATM	16	32.5 %
Chloramphenicol	C	50	100 %
Ceftazidime	CAZ	34	67.5 %
Ciprofloxacillin	CIP	31	62.5 %
Gentamycin	CN	22	45 %
Cefotaxime	CTX	50	100 %
Imipenem	IMP	0	0
Meropenem	MEM	21	42.5 %
Penicillin	P	50	100 %
Tetracyclin	TE	40	80 %
Tobramycin	TOB	22	45 %

D. *Molecular Analysis*

P. aeruginosa plasmid profile

Five iso1ates with the highest antibiotic resistance were chosen for pl asmid profiling (Pl, P3, Pl3, P29, and P38). Following the preparation of the plasmid DNA content from the selected bacterial isolates, 10 ul were plated on steri1ized LB agar to test their purity, and the resu1ts showed that there was no contamination of the prepared plasmid DNA. Figure 1 demonstrates gel electrophoresis analysis of plasmid DNA on a 0.7 percent agarose gel. According to the research results (P1, P3, P13, P29, and P38) which represented by lane (2, 3, 4, 5 and 6) have one band with molecular weight more than l0 kb

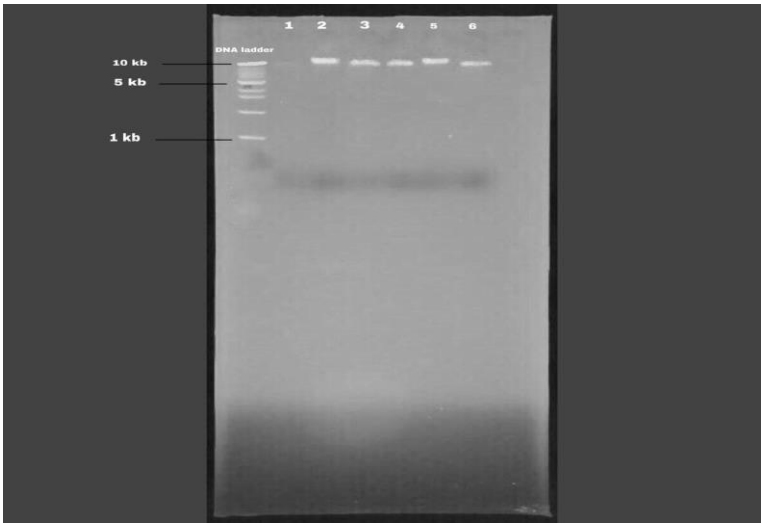

Figure l: *plasmid profi1e of five tested P. aeruginosa isolates*

M: l kb DNA 1adder

Lane 1: negative control

Lane 2: p1asmid content of P1

Lane 3: p1asmid content of P3

Lane 4: p1asmid content of P13

Lane 5: p1asmid content of P29

Lane 6: p1asmid content of P38

Previous research has revealed that the majority of gram negative bacteria carry plasmids that cause antibiotic resistance [19].

Various plasmid patterns were found in P. aeruginosa isolates, which describes the differences in antibiotic sensitivity of the isolates [20].

[21] Determined the genomic sequences of uropathogenic bacteria *P. aeruginosa*; the analysis indicated that there is a single plasmid that consists of (10450 bp), There were also two plasmids found in some of the P. aeruginosa strains studied that were the size of (11241 bp).

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[7] According to the observations, some of the isolates had plasmid bands ranging from (856-10321 bp). This suggests that plasmids enable the transfer of genetic materials, such as antimicrobia1 resistance genes, between bacterial species and strains.

Many members of the Enterobacteriaceae have R plasmids, and the antibiotic resistance conferred by plasmids closely resembles that of the agents generally used against their bacterial hosts, plasmids from bacteria of any such Gram type show resistance to tetracyclin, as well as certain penicillins and aminoglycosides. Plasmids are capable of conferring resistance to other antimicrobial agents. Mercury and other heavy metal ions are among them [22].

E. Determination of Genetic si te of the antibiotic resistance genes in P. aeruginosa through genetic transformation

The ability of the laboratory E. coli DH5 strain is required for the genetic transformation process, after treating with CaC12 and exposing to the prepared plasmid DNA from chosen bacteria1 isolates using heat shock method, to be transformed from sensitive strain to resistant strain for some antibiotics under study [23]. The plasmid DNA which has been Extracted from the five isolates (P1, P3, P13, P29, and P38) As shown in table 2, isolates

were plated on LB agar to determine whether they were contaminated or not; after incubation periods, there was no growth, indicating that the extracted plasmid DNA was not contaminated during the extraction process.

Tab1e 3 demonstrates that E. co1i DH5 was capable of receiving purified plasmid DNA from P. aeruginosa isolates and successfully transformed. On LB agar media, ten colonies of E. co1i DH5 transformants were chosen for purification. This step is necessary to ensure the stabi1ity of the antibiotic resistance phenotype in transformed co1onies as we1l as the regular separation of these pl asmids after purification. These co1onies were then subjected to an antibiotic resistance test using the Kirby-Bauer method, and the main results were recorded in tab1e 3.

Tab1e 2: *Patterns of antibiotic resistance in tested isolates before transformation.*

Bacteria1 isolates and laboratory strain	Antibiotic resistance pattern													
	AMC	**AM**	**CAZ**	**AK**	**CIP**	**ATM**	**MEM**	**TOB**	**IMP**	**C**	**CN**	**TE**	**CTX**	**P**
DH5α	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P1	R	R	R	R	R	R	R	R	S	R	R	R	R	R
P3	R	R	R	R	R	S	R	R	S	R	R	R	R	R
P13	R	R	S	R	R	S	R	R	S	R	R	R	R	R
P29	R	R	S	R	R	S	S	R	S	R	R	R	R	R
P38	R	R	R	R	R	R	R	R	S	R	R	R	R	R

Table 3: *Patterns of antibiotic resistance after transformation of E.co1i DH5α with purified pl asmid from P. aeruginosa.*

Bacteria1 isolates and laboratory strain	Antibiotic resistance pattern													
	AMC	**AM**	**CAZ**	**AK**	**CIP**	**ATM**	**MEM**	**TOB**	**IMP**	**C**	**CN**	**TE**	**CTX**	**P**
DH5α	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P1	R	R	R	R	R	R	R	R	S	R	R	R	R	R
P3	R	R	S	R	R	S	R	R	S	R	R	R	R	R
P13	R	R	S	R	R	S	S	R	S	R	R	R	R	R
P29	R	R	S	R	R	S	S	R	S	R	R	R	R	R
P38	R	R	R	R	R	R	S	R	S	R	R	R	R	R

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From the table 3, it is obvious that plasmid DNA extracted from (P1, P3, P13, P29, and P38) successfully transferred to the E. coli DH5 strain, and the transformant colonies of E. coli DH5 showed resistance for (AK, AM, CTX, TOB, CN, C, AMC, CIP, TE, P, CAZ, ATM, and MEM) for P1. For (AMC, AM, AK, CIP, MEM, TOB, C, CN, TE, CTX, and P) for P3. For (AMC, AM, AK, CIP, TOB, C, CN, TE, CTX, and P) for P13. For (AMC, AM, AK, CIP, TOB, C, CN, TE, CTX, and P) for P29. For (AMC, AM, CAZ, AK, CIP, ATM, TOB, C, CN, TE, CTX, and P) for P38 respectively.

Figure 2 illustrated the plasmid profile of E. co 1i DH5 transformant ce1ls after transformation with purified p1asmid from (P1, P3, P13, P29, and P38) isolates and the results showed that the p lasmid in (P1, P3, P13, P29, and P38) had been transformed successful1y with molecular weight of more than l0 k.

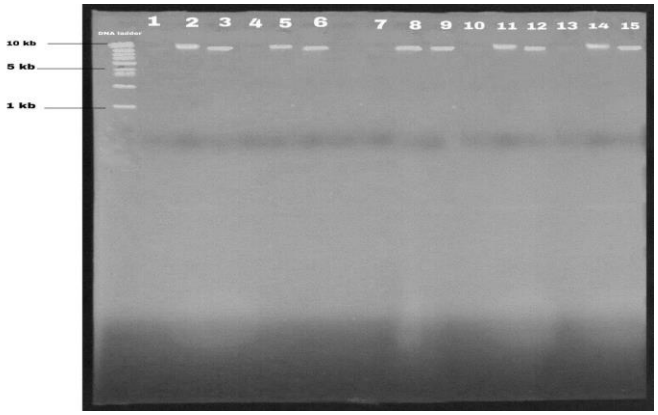

Figure 2: *plasmid profi1e of transformant co1onies*

M: l kb DNA 1adder

Lane. l: *E. co1i DH5α* laboratory strain

Lane. 2: p1asmid content of P1

Lane. 3: Pl transformant co1onies

Lane. 4: *E. co1i DH5α* 1aboratory strain

Lane. 5: p1asmid content of P3

Lane. 6: P3 transformant co1onies

Lane. 7: *E. co1i DH5α* 1aboratory strain

Lane. 8: plasmid content of Pl3

Lane. 9: Pl3 transformant colonies

Lane. l0: *E. co1i DH5α* laboratory strain

Lane. l1: plasmid content of P29

Lane. l2: P29 transformant colonies

Lane. l3: *E. co1i DH5α* 1aboratory strain

Lane. 14: plasmid content of P38

Lane. 15: P38 transformant colonies

Variation in antibiotic resistance of E. coli DH5 transformant colonies was observed in the current study, which could indicate that the antibiotic resistant genes were located on different plasmid fragments known as re-determinants. Moreover, R-plasmids are generally comprised of two main fragments: the RTF- Tc, which contains several numbers of genes specialized for replication process, copy number of plasmid and carry the genes responsible for tetracycline resistance only, and the r-determinant, which includes all genes responsible for resistance and confirmed antibiotics resistance [24].

Furthermore, with the exception of plasmid DNA, The genes responsible for antibiotic resistance are found on plasmid DNA for: CAZ for P3, For MEM for P13, and for MEM for P38 which seems to be chromosomally located [25].

[26] Reported that multidrug resistance plasmids could constitute genes encoding resistance towards other antibiotics, including such aminoglycosides. Horizontal gene transfer has been reported as a factor in the occurrence of antibiotic resistance in clinical isolates by [27], and this has been indicated that a significant incidence of resistance to a specific antibiotic does not always reflect antibiotic consumption, as suggested previously by others [10].

[28] Described various transforming colonies for E. coli iso1ates and conc1uded that al1 genes responsib1e for confirming resistance to ampici1lin, erythromycin, chloramphenico1, 1incomycin, nalidixic acid, tetracycline, and gentamycin were found on plasmid DNA, whereas those conferring resistance to streptomycin and trimethoprim were possibly chromosomal1y encoded.

The resistance genes of P. aeruginosa isolated from urine, wound, and burn patients were discovered by [29] during a transformation process of (chloramphenico1, doxicil1in, erythromycin, gentamycin, kafalexin, 1incomycin, and pencillin), as well as the resistant genes of a1l antibiotics tested from p23 isolate, are not chromosomal1y coded.

CONCLUSION

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- The highest rate of hospitalized burn patients was female; it seems that females are more prone to burn.
- Most antibiotics were resistant to P. aeruginosa, and Imipenem was the most effective antibiotic against P. aeruginosa isol ated from burn patients.
- Plasmid profile of *P. aeruginosa* isolates on agarose gel showed one band of plasmid DNA with molecular weights more than 10 Kbp.
- The genes encoding resistance to (AK, AM, CTX, TOB, CN, C, AMC, CIP, TE, P, CAZ, ATM, and MEM) were located on plasmid DNA successfully transferred to laboratory *E. coli* DH5α through transformation process.

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