Al-Mustansiriyah Journal of Science
ISSN: 1814-635X (print), ISSN: 2521-3520 (online)
Volume 29, Issue 2, 2018
DOI: http://doi.org/10.23851/mjs.v29i2.345

Research Article **Open Access**

ERIC-PCR Typing, RAPD-PCR Fingerprinting and Quorum Sensing Gene Analysis of *Pseudomonas aeruginosa* Isolated from Different Clinical Sources

Ruqaia S. Sallman*, Suzan S. Hussein, Munim R. Ali
Department of Biology, College of Science, Mustansiriyah University, IRAQ.
**Correspondent author email: ruqaia.s1988@yahoo.com*

ArticleInfo

Submitted
18/11/2017

Revised
11/01/2018

Accepted
16/01/2018

Abstract

Recently, *Pseudomonas aeruginosa* infections proportions have increased significantly. Molecular typing and virulence analysis are good techniques, which can lead us to know *P. aeruginosa* infections. *P. aeruginosa* isolates were identified by using housekeeping gene (16S rDNA gene) via PCR technique for accurate identification. The highest percent 41.26% of *P. aeruginosa* bacteria was found in the burn infections followed by 28.57% in wound swabs, 17.46% in ear discharge and lowest percentage were obtained from sputum samples. All isolates classified into six groups (A-F) according to classes of antibiotics. Of the 63 bacterial isolates, 100% were resistant to carbencillin, whereas 31.74% were resistant to ticarcillin and all isolates susceptible to imipenem. In addition all of clinical isolates indicated multidrug resistant (MDR) patterns, the highest rate of MDR was observed with pattern C these isolates were able to resist (9-12) antibiotics. All isolates were typed genotypically by using two methods of amplification, ERIC and RAPD-PCR. The results of the ERIC-PCR typing of *P. aeruginosa* bacteria that 96.82% showed amplification bands ERIC-PCR also revealed 17 groups of genotypes (A-R) and 4 unique isolates. The results of RAPD-PCR fingerprint revealed 12 groups of genotypes (A-M) of 40–90% similarity according to coefficient values and 4 unique isolates, except 7.93% were untypeble. QS genes (*lasI*, *lasR*, *rhlI*, *rhlR*), screen showed all isolates 100% were positive for one or more QS genes, in the other hand 82.53% carrying *lasI*, *lasR*, *rhlI*, and *rhlR*, while the 15.87% carrying *lasI*, *rhlI*, and *rhlR* and 1.58% carrying *lasI*, *lasR*, and *rhlR* genes. ERIC genotyping significantly correlated resistance patterns but not with virulence control QS genes. RAPD genotyping significantly correlated with source of infection, resistance patterns and virulence control QS genes. These results can help initial diagnosis MDR *P. aeruginosa* outbreaks associated with specific genotyping patterns.

Keywords: *Pseudomonas aeruginosa*, ERIC-PCR typing, RAPD-PCR fingerprinting, Quorum sensing genes.

الخلاصة

ازدادت في الآونة الأخيرة عدوى الزائفة الزنجاريه (*Pseudomonas aeruginosa*) بشكل ملحوظ لذلك جاءت هذه الدراسة ,حيث أجري التنميط الجزيئي وتحليل عوامل الضراوة التي تعتبر تقنيات جيده ومتطورة يمكن ان تقودنا لمعرفة عدوى *P. aeruginosa* , تم تشخيص عزلات *P. aeruginosa* باستخدام جين 16S rDNA عن طريق استخدام تقنية تفاعل البلمرة المتسلسل (PCR) للتشخيص الدقيق, حيث وجد أعلى نسبة إصابة من 41.26% *P. aeruginosa* والتي تم العثور عليها في الالتهابات الحروق تليها 28.57% من مسحات الجروح , 17.46% من خراج الاذن وأقل نسبة إصابة تم الحصول عليها من عينات البلغم. اعتمادا على نتائج فحص الحساسية جميع العزلات تم تصنيفها الى ست مجموعات (A-F) وفقا لفئات المضادات الحيوية , من بين 63 عزلة , 100% كانت مقاومة Carbenicillin , في حين أن 31.74% كانت مقاومة Ticarcillin وجميع العزلات حساسة Imipenem , وبالإضافة إلى ذلك ، جميع العزلات السريرية أشارت إلى أنماط المقاومة المتعددة (MDR)، وقد لوحظ أن أعلى معدل للمقاومة المتعددة هو النمط C حيث كانت هذه العزلات قادرة على مقاومة (9-12) من المضادات الحيوية. من جانب أخر تم تنميط جميع العزلات جينيا باستخدام طريقتين مختلفتين ERIC-PCR وRABD-PCR, حيث أظهرت النتائج بتقنية ERIC-PCR للعزلات السريرية من *P. aeruginosa* أن 96.82% أظهرت ناتج

للتنميط, كما كشفت عن17 مجموعة من التراكيب الوراثية (A - R) و 4 عزلات فردية (B, C, F, R) , أضافة الى ذلك فأن نتائج التنميط بطريقةRAPD-PCR كشفت عن 12 مجموعة من التراكيب الوراثية (A - M) من 40-90٪ كنسبة تشابه وفقا لقيم coefficient values و 4 عزلات فرديه (A، C، D، H)، أضافة الى ذلك 7.93% لم تظهر نتائج للتنميط, في هذه الدراسة أيضا تم التحري عن جينات تحسس الزحام (QS genes) حيث أظهرت النتائج 100% موجبة لجين واحد أو أكثر, كما بينت النتائج 82.53% حاملة للجينات الاربعة (*lasI, lasR, rhlI, rhlR*) بينما 15.87% حاملة لثلاث جينات (*lasI, rhlI, rhlR*) في حين 1.58% حاملة لهذه جينات (*lasI, lasR, rhlR*). أظهر ارتباطا كبيرا مع أنماط المقاومة ولكن ليس مع عوامل الضراوة المسيطر عليها من قبل جينات تحسس الزحام (QS genes), في حين RAPD-PCR ERIC-PCR يرتبط بشكل كبير مع مصدر العدوى وأنماط المقاومة و أيضا مع عوامل الضراوة المسيطر عليها من قبل جينات تحسس الزحام (QS genes). هذه النتائج يمكن أن تساعد التشخيص الأولي لتفشي *P. aeruginosa* المتعددة المقاومة المرتبطة بأنماط جينيه محددة.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and one of the main causes of nosocomial infections, including surgical wound infections, bloodstream infections, urinary tract infections and pneumonia. It is frequently isolated from immunocompromised individuals and intubated patients, and causes chronic lung infections in cystic fibrosis (CF) patients, as well as in adults with bronchiectasis and chronic obstructive pulmonary disease [1]. *P. aeruginosa* virulence factors are coordinated by a cell density monitoring mechanism termed quorum sensing system (QS). The two well-defined QS systems in *P. aeruginosa* bacteria, namely rhl and las, depend on the N-acyl homoserine lactone (AHL) signal molecules, also termed autoinducers (AI) [2]. QS is responsible for the regulation of a large number of genes, for instance, around 10% of genes in the genome of *P. aeruginosa* are regulated by QS [3]. Typing of this nosocomial pathogen like *P. aeruginosa* is very important for detecting the source of nosocomial outbreaks and to implement effective control methods for the prevention the spread of the pathogen, a good typing technique should be highly discriminatory, easy to use, inexpensive, reproducible, and fast. Phenotypic and biochemical methods based on antimicrobial susceptibility, serotyping, and bacteriophage typing lack discriminatory power and stability and were replaced with molecular based techniques. Many molecular typing methods have been developed for typing *P. aeruginosa* [4], like pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism analysis (RFLP), random amplification of polymorphic DNA (RAPD-PCR), and enterobacterial repetitive intergenic consensus (ERIC-PCR) [5], have been used for the typing of strains. ERIC-PCR and RAPD-PCR, rapid and inexpensive PCR-based typing methods, can be used to screen, discriminate, and to determine genetic relatedness among the strains [6]. Molecular typing represents a tool to elucidate the genetic diversity underlying important phenotypic features such as host specificity, pathogenicity, and antibiotic resistance and virulence factors [7]. So, because of the pathogenesis of *Pseudomonas* is complex and multifactorial, since the bacterium is both invasive and toxigenic, can cause a range of infections in humans. The study came to study the characterization the genetic diversity of *P. aeruginosa* isolates obtained from different clinical sources using ERIC and RAPD-PCR fingerprinting and to compares between ERIC, RAPD-PCR to demonstrate close concordance among these methods, and to investigate the QS molecules in *P. aeruginosa* to understanding the relation between multidrug resistant and pathogenesis.

Materials and Methods

Sample collection

In a present study, 63 bacterial isolates of *P. aeruginosa* were collected from different clinical specimens from three hospitals in Baghdad city including: Baghdad Teaching Hospital and Burn Hospital in Medical City during and Al-Yarmuk Teaching Hospital, a period between (January to March 2016). The bacterial isolates were isolated

from different of clinical samples including: swab samples were taken from patients with burn, wound and ear infections, sputum and trachea samples from respiratory tract infection (RTI), mid-stream urine samples were taken from patients suffering from urinary tract infection (UTI).

Antibiotic sensitivity assay

Resistance tested for the isolates towards 12 antibiotic agents related to six categories including: Cephalosporin class (Ceftazidime & Cefepime); Aminoglycoside (Amikacin, Gentamycin & Tobramycin); Quinolones (Ciprofloxacin & Levofloxacin); Monobactam (Aztreoname); carbapeneme (Imipenem); Penicillins (Carbencillin, piperacillin, and Ticarcillin). Susceptibility was determined based on the interpretative criteria recommended by the CLSI [8]. Sensitivity test was analyzed from the identified strains by using Mueller Hinton agar and incubated at 37°C for 24 h. Standard strain of *P. aeroginosa* ATCC 27853 was used as a quality control strain (Department of Biology / College of Science / Mustansiryah University).

DNA extraction

Chromosomal DNA was extracted using by boiling method [9]. Briefly, few isolated bacterial colonies of overnight growth bacteria suspended in 1ml distilled water and boiled in water bath, for 10 min. After centrifugation at 12,000×*g* for10 min at 4°C, supernatant were recovered and 5µl was directly used as the template for PCR.

Application of PCR

In order to confirm the isolates as *P. aeruginosa*, PCR assay that based on housekeeping gene (16S rDNA gene) sequence with specific primers F: (5´-GGGGGATCTTCGGACCTCA-3´) and R: (5´- TCCTTAGAGTGCCCACCCG-3´) [10], was carried out in 25µl reaction volumes composed from 12.5µl of GoTaqGreen Master Mix, template DNA 4µl, forward & reverse primers 0.75µl for each, and 7µl of Deionized Nuclease Free water was added to PCR mixture. PCR mixture without template DNA was used as a negative control. PCR was run under the following conditions: primary denaturation step at 95°C for 2 min, 30 repeated cycles start with denaturation step at 94°C for 20 sec, annealing at 58°C for 20 sec, and 40 sec at 72°C as extension step followed by final extension step at 72°C for 7 min.

ERIC- and ARPD-PCR primers and conditions

The clonality of *P. aeruginosa* isolates was determined by using the ERIC-PCR "Enterobacterial repetitive intergenic consensus". Specific primer of ERIC 1 (5´-ATGTAAGCTCCTGGGGATTCAC-3´) and ERIC 2 (5´-AAGTAAGTGACTGGGGTGAGCG -3´) [11]. Amplification was carried out with an initial denaturation (94°C, 7min), followed by 35 cycles of denaturation (94°C, 30sec), annealing (48°C, 2min), and extension (72°C, 4min) with a single final extension (72°C, 15 min). ERIC-PCR were carried in 25µl, volume comprising of 5µl of *P. aeruginosa* DNA, 1µl of each primer (10 Pmol/µl) and 12.5µl master mix. Filtered water was added to the mixture to make a final volume. The second primer, RAPD-PCR fingerprinting "Random amplification of polymorphic DNA" was performed using primer sequence (5´-ACGGCCGACC-3′) [12], amplification was carried out with an initial denaturation step was omitted, followed by 50 cycles of denaturation (94°C, 1min), annealing (44°C, 1min), and extension (72°C, 1min) with a single final extension (72°C, 10 min). The same mixture concentration in ERIC-PCR were used for RAPD-PCR, except that the 2µl of primer.

DNA sequence analysis of lasI, lasR, rhll, and rhlR genes

lasI, *lasR*, *rhll*, and *rhlR* genes from all isolates were PCR amplified using the primers sets

described by Cotar *et al*. [13]. For PCR amplification the following primers were used as shown in Table 1.

Table 1: The sequences of the primers and the amplicon sizes for *lasI, lasR, rhlI,* and *rhlR* genes.

Primers	Sequence (5′ - 3′)	Amplicon size (bp)
lasI	F: 5′-TCGACGAGATGGAAATCGATG-3′ R: 5′-GCTCGATGCCGATCTTCAG-3′	402
lasR	F: 5′-TGCCGATTTTCTGGGAACC-3′ R: 5′-CCGCCGAATATTTCCCATATG-3′	401
rhll	F: 5′-CGAATTGCTCTCTGAATCGCT-3′ R: 5′- GGCTCATGGCGACGATGTA-3′	182
rhlR	F: 5′-TCGATTACTACGCCTATGGCG-3′ R: 5′- TTCCAGAGCATCCGGCTCT-3′	208

Cycling conditions for *lasI*, *lasR* and *rhlL* genes are as follows: an initial denaturation (95°C, 5min), followed by 35 cycles of denaturation (95°C, 30sec), annealing (59°C, 1min), and extension (72°C, 30sec) with a single final extension (72°C, 15 min). The same cycling conditions were used for *rhlR* gene except that the annealing temperature (54 ºC. 30 min).

Gel electrophoresis

After PCR, the amplified products were subjected to electrophoresis in 1.5% (w/v) agarose gels (Promega, USA), stained with 5 g/100 ml ethidium bromide in 1x TBE buffer (40 mMTris, 20mM boric acid, 1 mM EDTA, pH 8.3). The gels were photographed after electrophoresis under UV light to record results.

Statistical analysis

The DNA banding patterns generated by ERIC and RAPD-PCR methods were used in generating dendrograms using the software PAleontological STatistics version 3.11. The relatedness between the profiles was derived based on unweighted pair group method with arithmetic mean (UPGMA) using the Dice correlation coefficient method and expressed as percentage similarity. The discriminatory index was calculated for RAPD-PCR and ERIC-PCR by using the Simpson's index of diversity [14]. Average similarity value was calculated on the basis of the similarity matrix [15].

Results and Discussion

Isolation and identification of Pseudomonas aeruginosa

P. aeruginosa has emerged as one of the most problematic nosocomial pathogens; it is considered an opportunistic pathogen that causes infections [16]. It is the leading cause of wound, surgical wound infections, and urinary tract and ear infections [17]. A total of 100 samples have been subjected for isolation and identification phenotypically by api20E and Geno-typically by polymerase chain reaction (PCR) based on 16S rDNA gene of bacteria *pseudomonas aeruginosa* was isolated from 63 (63%) samples, Figure 1. From these positive cases, the isolation rate of *P. aeruginosa* was the highest in burn swab samples 26(41.26%), followed by 18(28.57%) in wound swabs, 11(17.46%) in ear discharge and lowest percentage were obtained from sputum samples as shown in Table 2. An accurate and rapid system for the identification of *P. aeruginosa* is important to prevent further spreading of the diseases. In current study, *P. aeruginosa* bacterial isolates were identified by using molecular method (PCR) [34]. *P. aeruginosa* isolates were identified using 16S rDNA gene via PCR technique as it was developed as an alternative ways for accurate identification and classification of bacterial species; especially when amplifying bacterial housekeeping genes [18]. *P. aeruginosa* infections were most common in burn and

wound infections. The highest percent of *P. aeruginosa* was found in burn infections. In concordance with our result, in Egypt, Mahmoud *et al*. [19], who founded that 32.3% of infections in burn unit causing by *P. aeruginosa* bacteria, also Pathmanathan *et al*.[20], reported that *P. aeruginosa* bacteria is a nosocomial pathogen oftentimes isolated from burn infections.

Table 2: Percentage and number of *P. aeruginosa* isolates according to isolates sources.

Type of specimen	Number of isolates	*P. aeruginosa* isolates, (%)
Burn discharge swabs	26	41.26%
Wound discharge swabs	18	28.57%
Ear discharge swabs	11	17.46%
Urine samples	4	6.34%
Trachea samples	3	4.76%
Sputum sample	1	1.58%
Total	63	100%

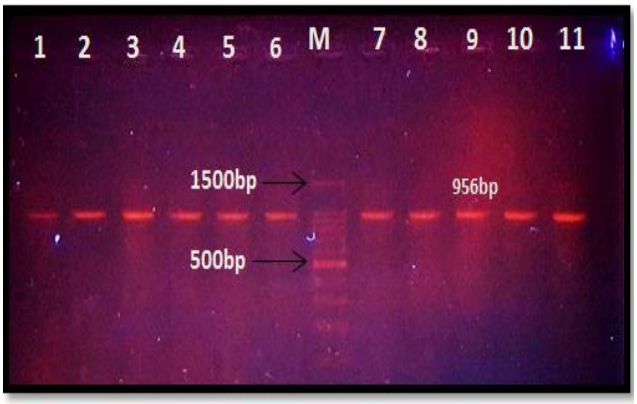

Figure 1: Agarose gel electrophoresis 1% agarose of 16S rDNA gene (956bp amplicon). Lane M 100bp DNA ladder, lanes1-11 showed positive PCR bands of *P. aeruginosa* isolates.

Phenotypically Method by Antimicrobial Susceptibility Test

In the present study, *P. aeruginosa* bacterial isolates were typed phenotypically by using antibiotic susceptibility test (Antibiotype). The antimicrobial resistance patterns of the bacterial isolates are shown in Table 3, were constructed according to the resistance patterns. All isolates classified into six groups (A-F) according to classes of antibiotics (Cephalosporin, Aminoglycoside, Quinolones, Monobactam, Penicillins and Carbapeneme classes). Of the 63 isolates, 63(100%) were resistant to carbencillin, whereas 20(31.74%) were resistant to ticarcillin. The 18 isolates from wound swabs, 7(38.88%) were resistant to ticarcillin. In addition, lowest resistance 1(1.58%) to amikacin contain one isolate from burn swab and all isolates susceptible to imipenem. The antimicrobial susceptibility results showed that the most active drug against *P. aeruginosa* bacterial isolates was carbencillin (100%), also Mahmoud *et al*. [19], reported that isolates were completely resistant to carbencillin. Whereas, in our study, resistance to ticarcillin was 31.74%, which is high as compared to an earlier study from Saudi Arabia by Khan and Faiz. [21]. However, a very high rate of resistance to ticarcillin (93%) was reported from Turkey [22], while 25.39% of *P. aeruginosa* isolates resistant to aztreoname, also Khan and Faiz. [21] reported 32.6% of isolates resistant to aztreoname. Whereas, also in Saudi Arabia Al-Zahrani. [23] study reported that aztreoname exhibited high susceptibility pattern. This variation in drug resistance rate may be correlated with the inappropriate use of relevant antibiotics. On the other hand, results in current study showed completely sensitive to imipenem and lowest resistance to amikacin (1.58%). In Iraq, Hassan *et al*. [24] agreement with this results showed that all isolates were completely susceptible to imipenin and 13.95% of the isolates were resistant to amikacin.

In addition the results showed lowest resistance percent among tested antibiotics; (23.8%) isolates to cefepime, (22.22%) isolates to gentamicin, (14.28%) isolates to ciprofloxacin and tobramycin, (15.87%) isolates to levofloxacin and ceftazidime. Also, in Iraq, Jaafar *et al*. [25] reported that isolates were low

resistance to these antibiotics; (24.14%) isolates resistant to cefepime, ciprofloxacin, levofloxacin, and tobramycin, (27.59%) isolates resistant to ceftazidime and gentamicin. In the same manner 15.78% of *P. aeruginosa* isolates resistant to piperacillin, that agreement with Jamunadevi *et al*. [26], reported 20% of isolates showed resistance to piperacillin. The results showed the QS genes positive isolates showed high antibiotics resistance between experimental isolates and these results may be indicated the correlation between QS signal and bacterial resistance, as well as the classical role of bacterial resistance.

Table 3: Antimicrobial resistance properties in *P. aeruginosa* isolated from clinical infections.

Groups	Classes of Antibiotics	Antibiotics	NO. of isolates	Source of isolates	*P. aeruginosa* isolates, no. (%)
A	Cephalosporin class	Ceftazidime	P4,P8,P20,P2,P25, P28,P41,P46, P48,P49	WP=4 BP=2 UP=2 EP=2	10(15.87 %)
		Cefepime	P4,P8,P11,P20,P25, P26,P28,P29,P39, P40,P41,P42,P46, P48, P49	WP=6 BP=6 EP=2 UP=1	15(23.8%)
B	Aminoglycoside Class	Gentamycin	P4,P8,P11,P16,P25, P26,P28,P29,P41, P46, P48,P49,P56, P57	WP=5 BP=4 EP=4 UP=1	14(22.22%)
		Tobramycin	P4,P8,P11,P16,P28, P29,P48,P49,P56	W=5 EP=2 BP=1 UP=1	9(14.28 %)
		Amikacin	P46	BP=1	1(1.58 %)
C	Quinolones class	Ciprofloxacin	P4,P8,P10,P11,P28, P37,P48,P49,P59	WP=6 BP=1 UP=1 EP=1	9(14.28 %)
		Levofloxacin	P4,P8,P10,P11,P20, P28,P37,P48,P48, P59	WP=6 BP=2 UP=1 EP=1	10(15.87 %)
D	Monobactam Class	Aztreoname	P4,P8,P11,P16,P26, P27,P28,P29,P39, P40,P41,P42,P46,P48,P49 P57	WP=6 BP=7 EP=2 UP=1	16(25.39%)
E	Penicillins Class	Ticarcillin	P4,P8,P10,P11,P16, P20,p21,P25,P26, P28, P29,P37,P38, P41,P45, P48,P49, P57,P58,P61	WP=7 BP=7 EP=4 UP=2	20(31.74 %)
		Piperacillin	P4,P8,P11,P21,P28, P30,P39,P41,P48, P49	WP=5 UP=2 EP=1 BP=1 TP=1	10(15.87 %)
		Carbencillin	All isolates resist	BP=26 WP=18 EP=11 UP=4 TP=3 SP=1	63(100%)
F	Carbapeneme Class	Imipenem	All isolates sensitive	---	0

*W= Wound, B= Burn, E=Ear swab, U= Urine, T= Trachea, S= Sputum, P = *P. aeruginosa*.

The multi-drug resistant pattern (MRD) of *P. aeruginosa* differentiated into 3 pattern (A, B, C), the highest rate of MDR was observed with pattern C include six isolates (P4, P8, P11, P28, P48, P49) in which this isolates were able to resist (9→12) antibiotics. On the other hand the lowest MDR were noticed with pattern A which the total number of resist antibiotics were only (1→4) (see Table 4). In current study, the *P. aeruginosa* bacterial isolates were investigated for MDR, found low prevalence of MDR *P. aeruginosa* (9.52%). In Iran, Tavajjohi and Moniri [28] reported that 30% of *P. aeruginosa* bacterial isolates were MDR. The risk for acquiring MDR microorganisms may be related with the number of carriers in the same ward as well as to individual risk factors, such as patient characteristics and in-hospital events such as antibiotic treatments and invasive devices [29]. Through the current research results most of the isolates were observed resistant isolated from wound infections in different groups of antibiotype, except group E (carbencillin resistance) and group F. As well as the case for MDR, included five isolates from wound infections and one isolate from urine sample.

QS genes (*lasI, lasR, rhlI, rhlR*), were screened by two PCR technique: uniplex and multiplex. The results showed that all isolates 100% (63 /63) isolates were positive for one or more QS genes. Uniplex PCR technique, the results was 100 % (63/63) isolate were positive for *rhlR*, 84.12% (53/63) isolates were positive for *lasR*. While the results of the multiplex PCR, 100 % (63/63) isolate positive for *lasI* and 98.41% (62/63) positive for *rhll*. Also results showed 52 isolates (82.53%) carrying *lasI*, *lasR*, *rhlI*, and *rhlR*, while the 10 isolates (15.87%) carrying *lasI*, *rhlI*, and *rhlR* and one isolate (1.58%) carrying *lasI*, *lasR*, and *rhlR* genes, Figures (2, 3, and 4). Our results indicated the pathogenicity of bacteria controlled by signals produced from QS genes. In this manner the local isolates showed positive for one or more QS genes.

Table 4: The multidrug resistance in *P. aeruginosa* isolated from clinical infections.

Pattern	No. of antimicrobial which resisted by isolates	No. of the multidrug resistance isolates (%)
A	1→4	50 (79.36%)
B	5→8	7 (11.11%)
C	9→12	6 (9.52%)

*PCR for detection of *lasI, lasR, rhlI, and rhlR* quorum-sensing genes

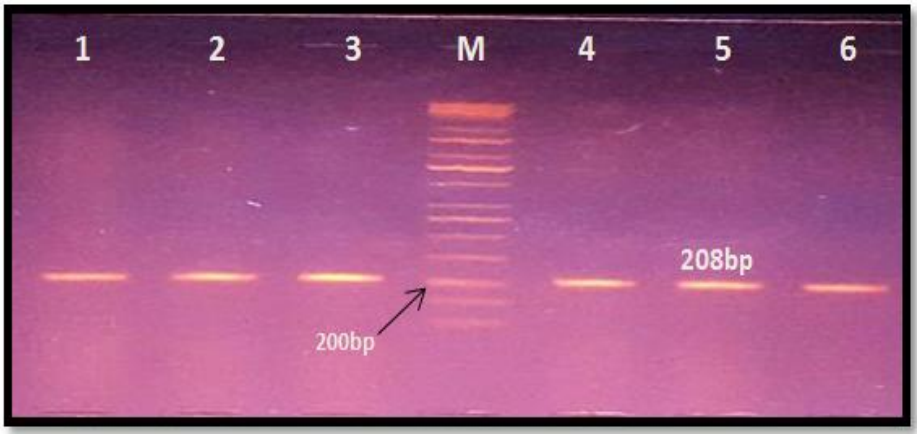

Figure 2: Uniplex PCR : Agarose gel electrophoresis 1% agarose, for *rhlR* gene (amplified size 208bp) of *P. aeruginosa* compared with (100pb) DNA ladder lane (M). Lanes 1-6 were positive results with 208bp *rhlR* gene.

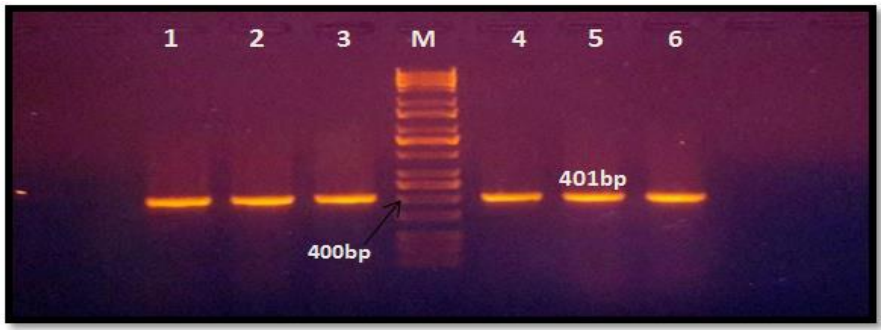

Figure 3: Uniplex PCR: Agarose gel electrophoresis 1% agarose for *lasR* gene (amplified size 401bp) of *P. aeruginosa* compared with (100pb) DNA ladder lane (M). Lanes 1-6 were positive results with 401bp gene.

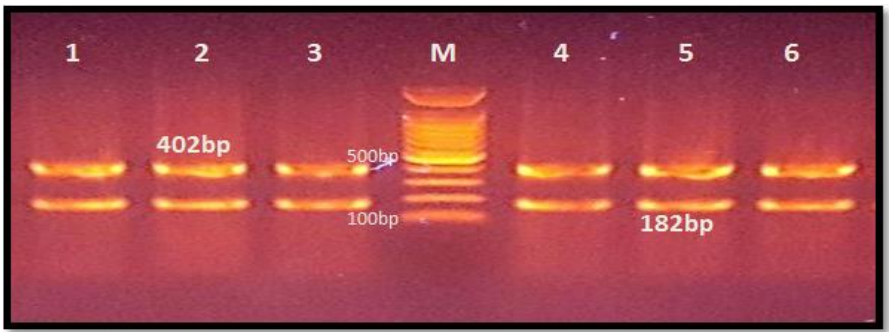

Figure 4 : Multiplex PCR : Agarose gel electrophoresis 1% agarose, of QS genes 1-6 multiplex for (*rhll, lasI*) size products (182bp , 402bp) respectively. Lane M 100bp DNA Ladder.

Genotypically method by ERIC and RAPD-PCR

Different molecular techniques have been developed for typing bacteria strains of *P. aeruginosa* [30]. The molecular typing methods have various advantages over traditional typing methods, including higher discriminatory power, broader application to a variety of bacterial species and speed [31]. *P. aeruginosa* isolates were typed genotypically by using two different methods of amplification, ERIC-PCR and RAPD-PCR typing methods. In the present study, the ERIC primer sequence was used for detecting differences in the distribution and number of this bacterial repetitive sequence in the clinical isolates of *P. aeruginosa* genomes. The results of ERIC amplification of clinical *P. aeruginosa* isolates revealed that 61 isolates (96.82%) showed amplification bands ranging from one to six bands. The PCR products were between 100bp and 1200bp. Also the results show the high frequent band was a 400bp band and low frequent band was 800bp, while two isolates was non-typed by ERIC-PCR. The results of RAPD amplification of *P. aeruginosa* isolates revealed that 58 isolates (92.06%) also showed about one to six amplification bands per isolate, except 5 isolates (7.93%) were untypeble. The most frequent bands was a 400bp band, while low frequent band was a 200bp band in an individual isolates, Table 5.

ERIC-PCR has been successfully applied to *P. aeruginosa* as well as to *Serratia marcescens, Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis, Staphylococcus epidermidis, Acinetobacter baumannii,* and *Escherichia coli* [32].

All 61 isolates examined in this study generated ERIC-PCR banding patterns ranging in size, from100bp to > 1200bp, with between 1 and 6 bands per isolate, some products (e.g. 300, 400, 600, 1000, 1200bp) were common to several bacterial isolates in Figure 5.

Table 5: Frequency of ERIC and RAPD-PCR patterns among *P. aeruginosa* isolates.

ERIC Band Nb.	M.W	(%)	RAPD Band Nb.	M.W	(%)
ERIC1	100	9(14.75%)	**RAPD1**	200	1(1.72%)
ERIC2	200	10(16.39%)	**RAPD2**	300	31(53.44%)
ERIC3	300	18(29.5%)	**RAPD3**	400	52(89.65%)
ERIC4	400	27(44.26%)	**RAPD4**	500	4(6.89%)
ERIC5	500	8(13.11%)	**RAPD5**	600	5(8.62%)
ERIC6	600	12(19.67%)	**RAPD6**	700	16(27.58%
ERIC7	700	7(11.47%)	**RAPD7**	800	9(15.51%)
ERIC8	800	2(3.27%)	**RAPD8**	900	26(44.82%)
ERIC9	900	9(14.75%)	**RAPD9**	1200	14(24.13%)
ERIC10	1000	16(26.22%)	**RAPD10**	1400	20(34.48%)
ERIC11	1200	14(22.95%)	**RAPD11**	1500	12(20.68%)

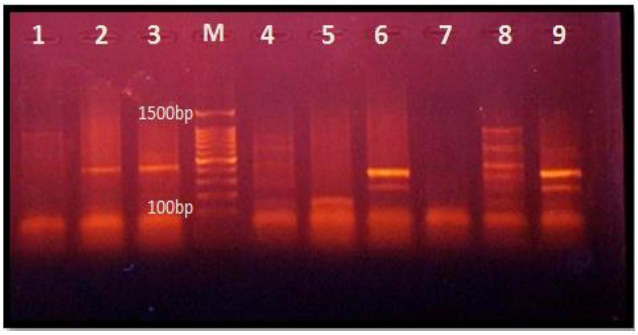

Figure 5: ERIC Agarose gel electrophoresis (1% agarose) of ERIC-PCR Lane M 100bp DNA ladder, lanes 2-3-4-5-6-8-9 showed positive PCR bands; lanes 1-7 negative.

In order to evaluate the genetic relations of the bacterial strains tested, the ERIC typing obtained were recorded and subjected to computerized analysis. The DNA relatedness, which was based on the Dice coefficients, was shown in Figure 6. Two main groups (I–II) at the level over 5% of Dice coefficient were observed. ERIC-PCR typing revealed 17 groups of genotypes (A-R) of 25–92% similarity according to coefficient values and 4 unique isolates (B, C, F, and R). Genotype Q was the most frequent cluster with 10 (10/61; 16.39% of total isolates) isolates, which were isolated from different sources. It was followed by genotypes M and J (9/61; 14.75%, 7/61; and 11.47% isolates respectively), D and K (each 6/61; 9.83% isolates), G (4/61; 6.55% isolates), and P (each 3/61; 4.91% isolates). These seven clusters accounted for 45/61 isolates (73.77%) examined in the study. Of the remaining genotypes observed, there were six genotypes (A, E, H, L, N and O) each containing two members (2/61; 3.27% isolates), genotype I consisted of 3 strains (3/61; 4.91%) and four genotypes (B, C, F, and R) represented were by a single isolate. The results indicated that there was no association between a specific cluster and the origin. The ERIC-genetic distance (ERIC-GD) value ranged from lowest (0.22) and highest (0.88). ERIC-PCR analysis, A and D groups have one bands different in product size and antibiotic resistance (D group contain MDR isolate), while all isolates in these groups isolated from different infections and contain all QS genes (*lasI*, *lasR*, *rhlI*, and *rhlR*). H, L, M, P and Q groups, all isolates similar in number of bands (different in products size) and penicillin resistance, these isolated from different source of infections except H, M and Q groups contain MDR isolates and H, L and M groups have all QS genes. B, C, E, F, G, N and O groups also share in number of bands (different in products size) and penicillin resistance, while most groups

(except N and O) isolated from burn infections, as well as F, G, and N groups have all QS genes. J and k groups, all isolates in these groups share in number of bands and have the same products size of bands but isolated from different source of infections. Either for antibiotic resistance both groups similar in penicillin and monobactam resistance and some isolates have three QS genes (*lasI*, *rhlI*, and *rhlR*). Finally, R group consist from one MDR isolate from wound infection and has all QS genes.

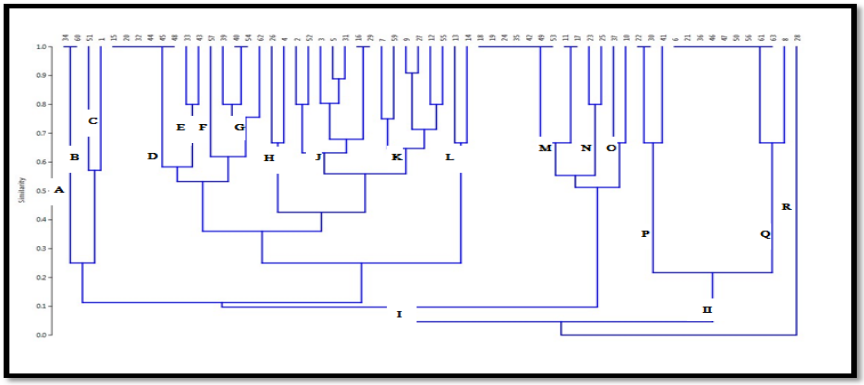

Figure 6: ERIC-PCR generated dendrogram indicating the genetic relatedness of the *P. aeruginosa* isolates.

On the other hand the RAPD-PCR method could be an additional rapid typing method for studying the molecular characterization and epidemiology of *P. aeruginosa* bacterial isolates [33]. The results of RABD-PCR, include banding pattern ranging in size, from 200bp to > 1500bp, with between 1 and 6 bands per isolate, some products (e.g. 300, 400, 900, 1400bp) were common to several isolates, Figure 7.

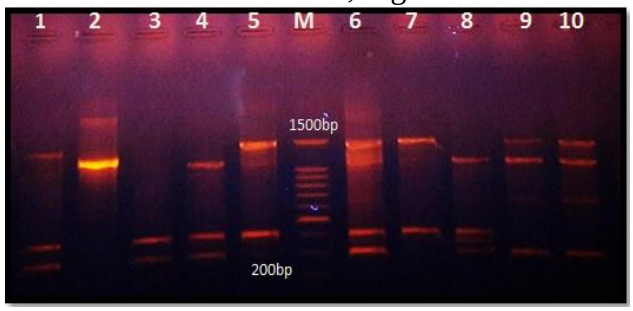

Figure 7: Agarose gel electrophoresis (1% agarose) of ERIC-PCR Lane M 100bp DNA ladder, lanes 1-10 showed positive PCR bands.

In order to evaluate the genetic relations of the strains tested, the fingerprints obtained were recorded and subjected to computerized analysis. The DNA relatedness, also which was based on the Dice coefficients, was shown in figure (8). Two main groups (I–II) at the level over 20% of Dice coefficient were observed. RAPD-PCR fingerprint revealed 12 groups of genotypes (A-M) of 40–90% similarity according to coefficient values and 4 unique isolates (A, C, D, H). Genotype G was the most frequent cluster with 12 (12/58; 20.68% of total isolates) isolates, which were isolated from burn swabs infections. It was followed by genotypes F and K (each 10/85; 17.24% isolates), M (8/58; 13.79% isolates), E and L (each 5/58; 8.62% isolates). These six clusters accounted for 50/58 isolates (86.2%) examined in the study. The remaining genotypes observed: B and J, each contains two members (2/58; 3.44% isolates), genotype I consisted of 3 strains (3/58; 5.17%) and three genotypes (A, C, D, and H) represented were by a single isolate. The results indicated that there was no association between a specific cluster and the origin. The RABD-genetic distance (RABD-GD) value ranged from lowest (0.22) and highest (0.88). The results of RAPD-PCR analysis, include A, C and H groups, these groups consist from isolates contain one band different in size and most isolated from burn infections, which have resistance to quinolones, cephalosporin & penicillin classes. A group have all QS genes, C group have three QS genes (*lasI*, *lasR*, and *rhlR*), while H group have three QS genes (*lasI*, *rhlI*, and *rhlR*). E, G and L groups share in number of

bands (different in products size) and resistance to quinolones, cephalosporin, aminoglycoside and penicillin classes. As well as most isolates have all QS genes and isolated from burn infections. B and J groups, each one consist from isolates has four bands different in products size but similar in antibiotic resistance (penicillin resistance), isolated from different infection and have all QS genes. D, F, K and M groups, all these groups different in isolation of source, antibiotic resistance (except F and K groups contain all MDR isolates) and percentage of QS genes. In addition share in number of bands (different in products size). In addition the results from this study appear the relationship between QS genes, antibiotics resistance, MDR and ERIC-PCR, all MDR isolates in D, H, M, and R groups contain all QS genes (expect Q group contain MDR isolate but have some isolates contain three types of QS genes) but different in number of bands, products size, source of isolation and antibiotic resistance. While for RAPD-PCR only F and K groups contain all MDR isolates share in number of bands and consist from most isolates have four and some have three QS genes. As well as these isolates different in products size, source of isolation and antibiotic resistance.

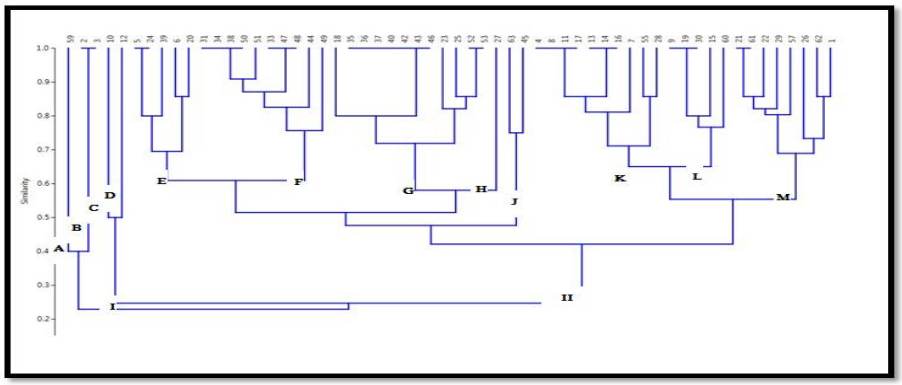

Figure 8: RABD-PCR generated dendrogram indicating the genetic relatedness of the *P. aeruginosa* isolates.

The high Simpson's index of diversity for RAPD, and ERIC typing indicates greater diversity. The ERIC genotyping Simpson's index of diversity was 0.08. On the other hand, the RAPD genotyping Simpson's index was 0.12. Both RAPD and ERIC genotyping methods have high evenness. The results indicted to relationship between QS genes encoded to virulence factors responsible of pathogenesis of *P. aeruginosa*, antibiotic resistance (MDR) and ERIC- PCR typing, RAPD-PCR fingerprinting.

Conclusion

ERIC genotyping significantly correlated resistance patterns but not with virulence control QS genes. RAPD genotyping significantly correlated with source of infection, resistance patterns and virulence control QS genes. These results can help initial diagnosis MDR *P. aeruginosa* outbreaks associated with specific genotyping patterns.

Acknowledgment

We thank the staff of the Education Baghdad Hospital, Ghazi Al-Hariri Hospital for Specialized Surgery and Burns Specialist Hospital for their helping in the collection of different samples from patients. We thank all patients and their families for their cooperation with us in obtaining samples for completing our study.

References

[1] Valderrey, A. D., Pozuelo, M. J., Jime´nez, P. A., Macia´, M. D., Oliver, A., Rotger, R. Chronic colonization by *Pseudomonas*

aeruginosa of patients with obstructive lung diseases: cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. Diagn. Microbiol. Infect. Dis. 68, 20–27, 2010.

[2] Schuster, M. and Greenberg, E.P., A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int. J. Med. Microbiol. 296, 73-81, 2006.

[3] Williams, P. and Cámara, M., Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: at ale of regulatory networks and multifunction al signal molecules. Curr. Opin. Microbiol. 12, 182–191, 2009.

[4] Naze, F., Jouen, E., Randriamahazo, R. T., *Pseudomonas aeruginosa* outbreak linked to mineral water bottles in a neonatal intensive care unit: fast typing by use of high-resolution melting analysis of a variable-number tandem-repeat locus. J. Clin. Microbiol. 48(9), 3146-52, 2010.

[5] Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L., Hauser, A. R., Microbiol. 147, 2659–2669, 2001.

[6] Kidd, T. J., Grimwood, K., Ramsay, K. A., Rainey, P. B., Bell, S. C., Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples from patients with cystic fibrosis. J. Clin. Microbiol. 49(1), 263-8, 2010.

[7] Li, W., Raoult, D., Fournier, P. E., Bacterial strain typing in the genomic era. FEMS Microbiol. 33(5), 892–916, 2009.

[8] CLSI, (Clinical & Laboratory Standards institute), Performance standard for antimicrobial susceptibility testing, 2014.

[9] Ruppé, E., Hem, S., Lath, S., Gautier, V., Ariey, F., Sarthou, J.L., Monchy, D., Arlet, G., CTX-M β-Lactamases in *Escherichia coli* from Community-acquired Urinary Tract Infections. J. Emerg. Infect. Dis. 15(5), 741-8, 2009.

[10] Spilker, T., Coenye, T., Vandamme, P., LiPuma, J. J., PCR-Based Assay for Differentiation of *Pseudomonas aeruginosa* from Other *Pseudomonas* Species Recovered from Cystic Fibrosis Patients. J. of Clin. Microbiol. 42, 2074–2079, 2004.

[11] Tosin, I., Silbert, S., Sader, H. S., The use of molecular typing to evaluate the dissemination of antimicrobial resistance among Gram-negative rods in Brazilian hospitals. Braz. J. Infect. 7, 360-9, 2003.

[12] Auda, I. G.; Al-Kadmy, I. M. S.; Kareem, S. M.; Lafta, A. K.; A'Affus, M. H. O.; Khit, I. A. A.; Kheraif, A. A.; Divakar, D. D.; Ramakrishnaiah, R., RAPD- and ERIC-Based Typing of Clinical and Environmental *Pseudomonasa eruginosa* Isolates. J. of AOAC Inter. 100(2), 2017.

[13] Cotar, A. I., Chifiriuc, M. C., Dinu, S., Pelinescu, D., Banu, O., Lazăr, V., Quntitative Real-time PCR study of the influence of probiotic culture soluble fraction on the expression of *Pseudomonas aeruginosa* quorum sensing genes. Romanian archives of microbial. and immunol. 69(4), 213-223, 2010.

[14] De la Puente-Redondo, V. A., del Blanco, N. G., Gutierrez-Martin, C. B., Garcia-Pena, F. J. & Rodriguez Ferri, E. F., Comparison of different PCR approaches for typing of *Francisella tularensis* strains. J. Clin. Microbiol. 38, 1016–1022, 2000.

[15] Nei, M. and Lei, W. H., Mathematical model for studying genetic variation in terms of vostriction endonucleases. Proc. Natl. Acad. Sci. USA. 76, 5269–73, 1979.

[16] Brooks, E., Melnick, J. L., Adelberg, E. A., Adelberg, Medical Microbiology. 24th . McGraw – Hill companies. N.Y, U.S.A., 189-201, 2007.

[17] Todar, K. "*Pseudomonas aeruginosa*." Online Text Book for Bacteriology. 2008.

[18] Farahani, A., Mohajeri, P., Gholamine, B., Rezaei, M., Abbasi, H., Comparison of Different Phenotypic and Genotypic Methods for the Detection of Methicillin-

Resistant *Staphylococcus aureus*. N. Am. J. Med. Sci. 5(11), 637–640, 2013.

[19] Mahmoud, A. B., Zahran, W. A., Hindawi, G. R., Labib, A. Z., Galal, R., Prevalence of Multidrug Resistant *Pseudomonas aeruginosa* in Patients with Nosocomial Infections at a University Hospital in Egypt, with Special Reference to Typing Methods. J. of Virol. & Microbiol.

[20] Pathmanathan, S. G., Samat, N. A., Mohamed, R., Antimicrobial Susceptibility of Clinical Isolates of *P. aeruginos*a from a Malaysian Hospital. Mal. J. of Med. Sci. 16 (2), 146-152, 2009.

[21] Khan, M.A. and Faizb, A., Antimicrobial resistance patterns of *Pseudomonas aeruginosa* in tertiary care hospitals of Makkah and Jeddah. Ann. Saudi Med. 36(1), 23-28, 2016.

[22] Bayram, A. and Balci, I., Patterns of antimicrobial resistance in a surgical intensive care unit of a university hospital in Turkey. BMC Infect. Dis. 6, 155-160, 2006.

[23] Al-Zahrani, S. H. M., Bacteria isolated from contact and non-contact lens and antibiotic susceptibility patterns of isolated *Pseudomonas aeruginosa*. Afr. J. of Microbiol. Res. 6(47), 7350-7356, 2012.

[24] Hassan, K.I., Rafik, S.A., Mussum, K., Molecular identification of *Pseudomonas aeruginosa* isolated from Hospitals in Kurdistan region. J. of Adva. Med. Res.. 2, 90-98, 2012.

[25] Jaafar, Z.M., Dhahi, M. A. R., Abd, A. H., Jaafar, S. M., Molecular identification and antibiotics resistance genes profile of *Pseudomonas aeruginosa* isolated from Iraqi patients. Afri. J. of Microbiol. Res. 8(21), 2183-2192, 2014.

[26] Jamunadevi, S., Balashanmugam, P., Muralitharan, G. and Kalaichelvan P. T., Molecular Characterization of Pathogenic and Non-pathogenic *Pseudomonas aeruginosa* with Special Reference to Phenazine Gene. J. of Modern Biotechnol. 1(2), 70–74, 2012.

[27] Warnes, S.L., Highmore, C.J., Keevil, C.W. Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. mBio 3(6):00489-12, 2012.

[28] Tavajjohi, Z. and Moniri, R. Detection of ESBLs and MDR in *Pseudomonas aeruginosa* in a Tertiary-Care Teaching Hospital. Iranian J. of Clin. Infect. Dis.. 6(1), 18- 23, 2011.

[29] Carmeli, Y. N., Eliopoulos, G. M., Samore, M. H., Antecedent Treatment with Different Antibiotic Agents. Emerg. Infect. Dis. 8, 802–807, 2002.

[30] Dawson, S. L., Fry, J. C., Dancer, B. N., A comparative evaluation of five typing techniques for determining the diversity of fluorescent pseudomonads. J. Microbiol. Meth. 50, 9–22, 2002.

[31] Tenover, F. C., Arbeit, R. D., Goering, R.V., How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review for healthcare epidemiologists. Infect Control Hosp. Epidemiol. 18, 426–439, 1997.

[32] Patton, T. G., Katz, S., Sobieski, R. J., Crupper SS. Genotyping of clinical Serratia marcescens isolates: a comparison of PCR-based methods. FEMS Microbiol. Lett. 194(1), 19-25, 2001.

[33] Wolska, K. A. and Szweda, P. I. Pol. J. Microbiol. 57, 157-163, 2008.

[34] Salman, R.S., Ali, M.R., Hussin, S.S. A Multiplex PCR for Detection of hlyA, papC, and traT genes in multidrug resistance Escherichia coli isolated from pregnant women. AJPS, 13(2), 129-138, 2013.