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EFFECT OF MINT OIL AND COLISTIN ANTIBIOTIC ON *FLIC* VIRULENCE GENE AMONG *PSEUDOMONAS AERUGINOSA* CLINICAL INFECTION ISOLATES FROM BAGHDAD, IRAQ.

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Abstract

Pseudomonas aeruginosa considered as one of the most dangerous bacteria in the world and the studying of its resistant and virulence mechanisms is as important as the elimination of this bacteria. One hundred and fifteen burn and wound samples were collected, from hospitals in Baghdad, diagnosed and identified by routine tests, API 20E and VITEK-2 system. Also the identification of genus was conducted by detection the specific gene *rpsl* using polymerase chain reaction (PCR) technique. According to cultural and biochemical test results 105 of 115 were showed growth on media, however; only 49 isolates (46.7%) of which were identified as *P. aeruginosa* as a most causative agent in burn. Sensitivity test was performed for eight antibiotics by Kirby-Bauer standard disk diffusion method, the levels of resistance against, colistin 22.4%, Ticarcillin 59.2%, Pipracillin tazobactam 28.6%, Amikacin 49%, Cefepime 49%, Ciprofloxacin 28.6%, Ceftazidime 59.2%, Imipenem 42.9%. Minimum inhibitory concentrations (MICs) of Colistin and mint oil were evaluated by well diffusion method (to identify antimicrobial activity) for 5 isolates most resistance to antibiotic, appearance MIC for Colistin was 32 µg/ ml, and MIC for mint oil is 1/16 ml/ ml. At the molecular level of this study, the results of PCR reaction showed the presence of *rpsl* gene in all isolates (100%) and this confirmed the role of this gene in the identification of *P. aerugionsa* species and its intrinsic in this species, while the *fliC* type a gene presented in 18 isolates (36.7%), while *fliC* type b gene presented in 38 isolates (77.6%). The gene expression of *fliC* gene was conducted by using reverse transcriptase quantitative PCR technique. It was found that the value of gene expression fold was reduced for the burns samples after exposure to mint oil in contrast with the untreated isolates, The *rpsl* gene expression results, which were used as reference gene, demonstrated that this gene was well suited as housekeeping gene because of the minimal variations of expression of this gene whether in Colistin and mint oil treated or untreated isolates.

Key words : *P. aeruginosa* clinical Isolates, *fliC* Virulence Gene, Mint oil, Colistin antibiotic.

Introduction

Burn injury, a globally significant public health issue, is at high risk for nosocomial infections (Church *et al.*, 2006). The burn wound embodies a vulnerable site of opportunistic colonization by organisms such as *Pseudomonas aeruginosa*. The traditional association of *P. aeruginosa* with burn infections is very strong. The most typical answer of physicians or medical students asked to name an infection caused by *P. aeruginosa* is the characteristic response: "burns." Burn infections and associated sepsis are the cause of more than 50% of fatalities in seriously burned patients (Smith, 1994). *P. aeruginosa* is a leading cause of nosocomial infection, and considered the second most common pathogen isolated from patients among the gram negative pathogens reported to National Nosocomial Infectious Surveillance (NNIS) system. (Shenoy *et al.*, 2006). *P. aeruginosa* tends to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, and medical implant materials, and tissues. In developing countries, the opportunistic pathogen *P. aeruginosa* is causing (severe, acute and chronic) nosocomial infections in immunocompromised as well as catheterized and burn patients (Japoni *et al.*, 2009), (Salimi *et al.*, 2009), *P. aeruginosa* infections are a result of its ability to exploit the host immune compromise and

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the secretion a variety of important virulence factors (Gonzalez *et al.*, 2016). *P. aeruginosa* can also overrun the bloodstream from these sites, resulting in a systemic infection called bacteremia (Migiyama *et al.*, 2016). The symptoms of these infections are generalized inflammation and sepsis. Particularly in burns patients where the skin host defenses are destroyed and patient with impaired immune defense including HIV (human immunodeficiency viruses) or cancer infection who are immunosuppressed (Rostamzadeh *et al.*, 2016).

Medicinal plants have been used for centuries in traditional medicine because of their therapeutic value. Mint species have been exploited by man for more than two thousand years. Peppermint itself has been used for more than 250 years (Saharkhiz *et al.*, 2012). *Mentha piperita,* (family Lamiaceae) is a species found in Iraq and many parts of the world which has an economical value for its flavoring, odor, and therapeutic properties in foods and cosmetic industrial products. In addition, the leaves and flowers of *M. piperita* have medicinal properties (Caroviæ-StanKo *et al.*, 2016).

Essential oils are valuable natural products used as raw materials in many fields including perfumes, cosmetics, aromatherapy, phototherapy, spices, and nutrition. Peppermint (*M. piperita*) oil is one of the most popular and widely used essential oils, mostly because of its main components, Menthol, and menthone (Afridi *et al.*, 2016). Previous studies have shown antiviral, antibacterial, antifungal, antibiofilm formation, radioprotective, antioedema, analgesic and antioxidant activities of the EO and methanolic extracts of herbal parts and callus cultures of *M. piperita* (Kizil *et al.*, 2010).

The spread of antibiotic resistant bacteria is considered an international problem, and due to the importance of *P. aeruginosa* as a multidrug resistant bacterium, this study aimed to assess the effect of mint oil on *fliC* gene expression as antibacterial agent instead of antibiotics.

Materials and Methods

Bacterial strains

The samples were collected from clinical burn infection patients, during the period from November 2017 until end of February 2018. From hospitals in Baghdad including, burn center in medical city, burn center in Al-Yarmuk, central health laboratory. The isolates were identified by means of traditional tests: colony morphology and pigment formation on selective medium (Cetrimide agar), catalase test, oxidase reaction, and identify by Api20E system, VITEK2 device (Biomerieux/ France). Finally, all strains were identified by a molecular detection.

Molecular detection

DNA was extracted from activated pure culture of *P. aeruginosa* bacteria using protocol of Wizard kit (Promega, USA). Detection of DNA bands using Agarose gelelectrophoresis 1%. The primers used in this study are shown in table 1. PCR amplification of *rpsl* gene for the detection of *P. aeruginosa* species, and amplification of *flic* gene for detection the flagellin virulence gene shown in table 2. Components of PCR reaction was made in a total volume 20µl**,** extracted DNA 2 µl, each primer (Alpha DNA, USA) 1 µl, Go Taq® Green Master Mix (Promega, USA) 10 µl and nuclease-free water 6 µl, was thawed and vortexed to have homogenous contents. A negative control contained all materials with the substitute of distilled water instead of template DNA.

After amplification, 5 µl of each sample was removed and separated by electrophoresis on a 1.5% agarose (Sigma, St. Louis, Mo.) gel to confirm the presence of an amplified product.

Table 1: Primers used in this study.

Gene	Primer	Sequence 5'to 3'	Product size (bp)	References
rpsl	Rpsl- F	52 -GCAAGCGCATGGTCGACAAGA-32	201	(Dumas *et al.*, 2006).
	Rpsl- R	52 -CGCTGTGCTCTTGCAGGTTGTGA-32		
flic	CW45-F	5'-GGCAGCTGGTTNGCCTG-3'	1020 and1250	(Dumas *et al.*, 2006).
	CW45-R	5'-GGCCTGCAGATCNCCAA-3'		

Table 2: PCR amplification Program for genes used in this study.

	Steps	Temperature ℃	Time: m:s	No. of Cycle
rpsl gene	Initial Denaturation	95	05:00	1
	Denaturation	95	00:45	35
	Annealing	60	00:45	
	Extension	72	00:45	
	Final extension	72	07:00	1
fliC gene	Initial Denaturation	95	05:00	1
	Denaturation	95	00:30	30
	Annealing	60	00:30	
	Extension	72	01:00	
	Final Extension	72	07:00	1

Gene Expression

Analysis and Calculation of gene expression levels of one or more genes depend on RNA /miRNA concentration after conversion it to cDNA. All processes including total RNA purification, qPCR amplification and data analysis. RNA was isolated from sample according to the protocol of TRIzol™ Reagent. The primers used in this study are shown in table 1.

The components of the reaction mixture of one Step RT-PCR shown in table 3, and Real Time PCR program shown in Table 4.

Table 3: The components of the reaction mixture of One Step RT-PCR, final volume is 10µl.

Components	Volume
qPCR Master Mix	5 µl
RT mix	0.25 µl
$MgCl_2$	0.25 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Nuclease-free water	2 µl
RNA	1.5 µl
Final volume	10 µl

Table 4: Real Time PCR Program.

Step	Temperature	Time (m:s)	No. of cycle
RT. Enzyme Activation	37 ºC	15:00	1
Initial denaturation	95 ºC	10:00	
Denaturation	95 ºC	00:30	40
Annealing	60 ºC	00:30	
Extension	72 ºC	00:30	

Gene Expression Calculation, Relative quantification (Schmittgen *et al.,* 2008)

$$\text{Folding} = 2^{-\ddot{A}\ddot{A}CT}$$

$$\ddot{A}\ddot{A}CT = \ddot{A}CT_{\text{Treated}} - \ddot{A}CT_{\text{Control}}$$

$$\ddot{A}CT = CT_{\text{gene}} - CT_{\text{House Keeping gene}}$$

Antimicrobial susceptibility test against *P. aeruginosa* strains

Susceptibility tested to antibiotics was accure by disk diffusion method (Kirby-Bauer method) (Vandepitte *et al.*, 2003), through measured the zone inhibition around a disk of antibiotic. The antibiotics used in this study was Amikacin (AK), Ceftazidim (CAZ), Cefepim (CPM), Ciprofloxacin (CIP 5µg), Colistin (CT), Piperacillin/ Tazobactam (PTZ), Ticarcillin (TC) and Imipenem (IMI).

Mint Oil

Extraction of mint oil

The oil from mint was extract by using steam distillation (Hanbali *et al.,* 2005) Characterization of essential oil by HPLC analysis of mint, the oil extract in closed tube were separated on FLC (Fast Liquid Chromatographic) column under the optimum condition. Calculation concentration of sample (AOAC *et al.,* 1995):

Sample

$$\text{concentration} = \frac{\text{Area of sample}}{\text{Area of standrd}} \times \text{concentration of standard} \times \text{dilution factor.}$$

Minimum Inhibitory Concentrations (Antimicrobial Activity)

Agar well diffusion method is widely used to evaluate the antimicrobial activity of antibiotics and/ or plants extracts (Magaldi *et al.,* 2004), (Valgas *et al.,* 2007). *P. aeruginosa* isolates were subjected to determine antimicrobial activity against Colistin antibiotic and Mint plant extract.

Statistical Analysis

Data were presented as a percentage, cross-tabulation and ANOVA one-way test. The statistical analysis system IBM SPSS Statistics 23 (2015), was used to analyze the effect of different factors in this study. *P value* $\leq$ 0.05 was considered statistically significant.

Results and Discussion

Isolation of Sample

One hundred and fifteen burn samples were collected from patients. Out of 115 collected samples, only 105 samples (91.3%) samples gave bacterial growth on Brain Heart Infusion (BHI) Agar.

The incidence of infection in burn accuording to gender was high percentage of infected patients 68 samples (59.1%) in male and 47 samples (40.9%) in female. The results of the percentage distribution of the incidence according to the gender of patients it agree with (Abdulameer *et al.*, 2018), but did not agree with the results of (AL-Salihi *et al.*, 2014) (female 52.97 %) and male (49.01%) and; (Othman *et al.*, 2014) (females 57%) and (males 43%).

This variation infection according to gender du to type of work, the female long time in the kitchen, while the male are prone to work in baker, carpenter, and blacksmith and other, or battle related burns, this also cannot be generalized as the study.

Also, according to age was highest percentage of infected patients 36 samples (31.3%) in 15-25 year 32 samples (27.8%) in 26-35 year and 27 samples (23.5%) in 36-45 year while was least 20 samples (17.4%) in 46-

55 year. This variation infection according to age du to this age group is considered the working class in Iraq and non-compliance with safety instructions. The distribution differences may be attributed to the differences in immunity as children are known to have weaker immunity, the difference in hygienic practices, and the length of hospital stay; However, this cannot be generalized as this study was conducted over a few months and on few hospitals in one city (Bekele *et al.*, 2015), (Iduh *et al.*, 2015).

Distribution of *P. aeruginosa* in samples

After all tests were applied for all 105 isolates to identification in burn samples by traditional, biochemical test, API 20E, VITEK 2, the result for distribution of bacterial isolates appearance the *P. aeruginosa* 49 isolates (46.7%), was more distributed in patients than other bacterial isolates infection, that confirmation identification genetically by amplify the *rpsl* gene.

In Iraq similar results are mentioned when they isolated *P. aeruginosa* from the same source, (AL-Shamaa *et al.*, 2016) in Baghdad. The another study Iraqi investigation by (Neamah *et al.*, 2017), from Al-Diwanyia hospital, Both studies showed the number and percentage of *P. aeruginosa* isolates were highest from burns.

The isolation rates of bacteria *P.aeruginosa* was 49 isolates (46.7%), it could be said that *P.aeruginosa* comprises the major cause of burn patients. In addition, relaxation in general hygienic measures are associated with increasing infections with these bacteria. The universal nature to survive in the moist environment and resistance to many antibiotics makes *P. aeruginosa* a common pathogen in burn and wound of the patients (Alhasnawy *et al.*, 2013).

Antibiotic Susceptibility Test of *P.aeruginosa*.

Forty nine isolates went through the susceptibility test, for eight different of antibiotics previously mentioned, by the disc diffusion method recommended by the clinical and laboratory standards institute (CLSI, 2017) guidelines, the results shown in fig. 1 that showed varied levels of resistance.

In the other study 1.08 % of the isolates were resistant to colistin, which shows that these antibiotic the last choice of therapy for these infections (Bialvaei *et al.*, 2015).

The antibiotic resistant profile of isolates showed increasing resistance, especially in wound and ear isolates. *P. aeruginosa* isolates while sensitive for colistin (polymyxin E). This indicates the importance of antibiotic superintendence development and control of infection in hospital settings (Al-Khafaji *et al.*, 2015), The present results indicate a speciûc relationship among the presence exotoxin genes and antibiotic resistance.

The results were in agreement with the results of (Azimi *et al.*, 2015), who reported that most *P. aeruginosa* strains isolated from patients at Imam Reza, Shaheed Madani, and Sina hospitals in Tabriz, Iran.

Minimal Inhibitory Concentration Test for *P. aeruginosa*

The results of antibiotic Colistin and mint oil by using Minimum inhibition concentration (MIC) was tested for

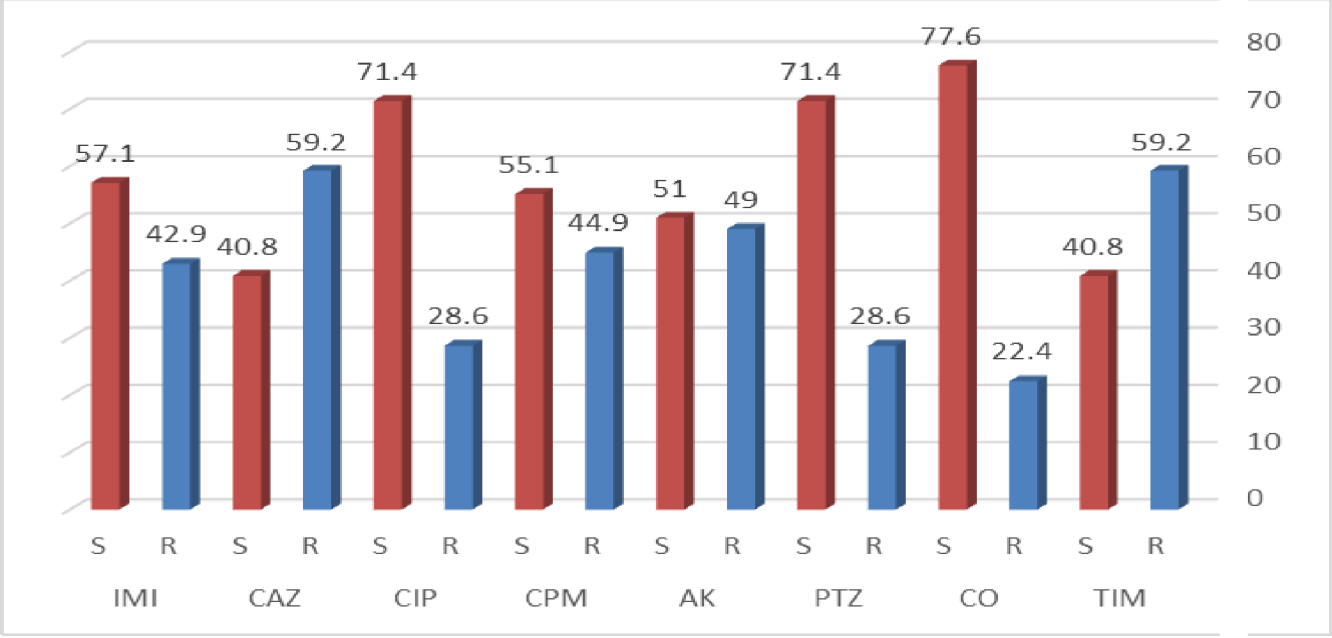

Fig. 1: Percentage of Susceptibility test of *P. aeruginosa* isolates.

five isolates, which were resistance to all antibiotics used in this study. The result appearance high antimicrobial activity for both, 32 µg/ ml for Colistin, and 1/16 ml/ ml for mint oil.

Molecular Assay

Extraction, and measured the Concentration and Purity of DND

The DNA of 49 isolates was successfully extracted, with (Wizard ® Genomic DNA Purification Kit, Promega, USA), concentration and purity were confirmed with nanodrop, the concentration ranging between 26.4–110.2 ng/µl, and the purity ranging between 1.71-2.

Conventional PCR

Genetically Identification of *P. aeruginosa*

In this study use specific primers for *rpsl* gene previously mentioned, for identification *P. aeruginosa* isolates by using Conventional PCR was give product size at 201 bp after the optimum condition for this primers in PCR reaction did the PCR reaction on 49 DNA extraction of *P. aeruginosa* isolates shown in Fig. 2. Also, For confirmation, the primers didn't anneal with another type of infection bacteria did control by did the PCR reaction for DNA extracted from deferent bacteria isolates (*K. pneumoniae*, and *E.coli*) at same reaction condition.

The results appearance the *rpsl* gene found in all *P. aeruginosa* isolates (49 isolates). This is evidence of the efficiency of this method of diagnosis by using the DNA extracted from the samples other than bacteria, were *K. pneumoniae* and *E. coli*; however, the gene did not appear after the end of the PCR reaction except in the isolates of *P. aeruginosa* bacteria. In our study proved that PCR amplification using *rpsl* gene was a suitable method for the detection of the isolates of *P. aeruginosa*. (Al-Jubori *et al.,* 2015) used *rpsl* gene for detection the same bacteria and reported positive result for all isolates, these genes should be highly conserved in the particular species.

The *rpsl* considered housekeeping gene because it works as ribosomal binding protein. Molecular detection of *rpsl* gene by PCR is one of the most commonly used techniques for bacterial identification. Although the *rpsl* gene had the ability to identify most of the isolates at the species level but it is not polymorphic enough to give a clear and specific differentiation among all *Pseudomonas* species (Cattoir *et al.,* 2010). The housekeeping genes is the most conserved of the *rRNA* genes. Therefore, sequencing of these genes has been established as a universal standard for identification of bacterial species (Boucher *et al.,* 2009).

Detection of *fliC* virulence Genes of *P. aeruginosa*

In this study use specific primers for *fliC* gene previously mentioned, for identification *P. aeruginosa* isolates by using Conventional PCR was give variable product size for two type a at 1020bp, and type b at 1250pb, after the optimum condition for this primers in PCR reaction did the PCR reaction on 49 DNA extraction of *P. aeruginosa* isolates shown in fig 3. *fliC* type a gene presented in 18 isolates (36.7%), while *fliC* type b gene presented in 38 isolates (77.6%).

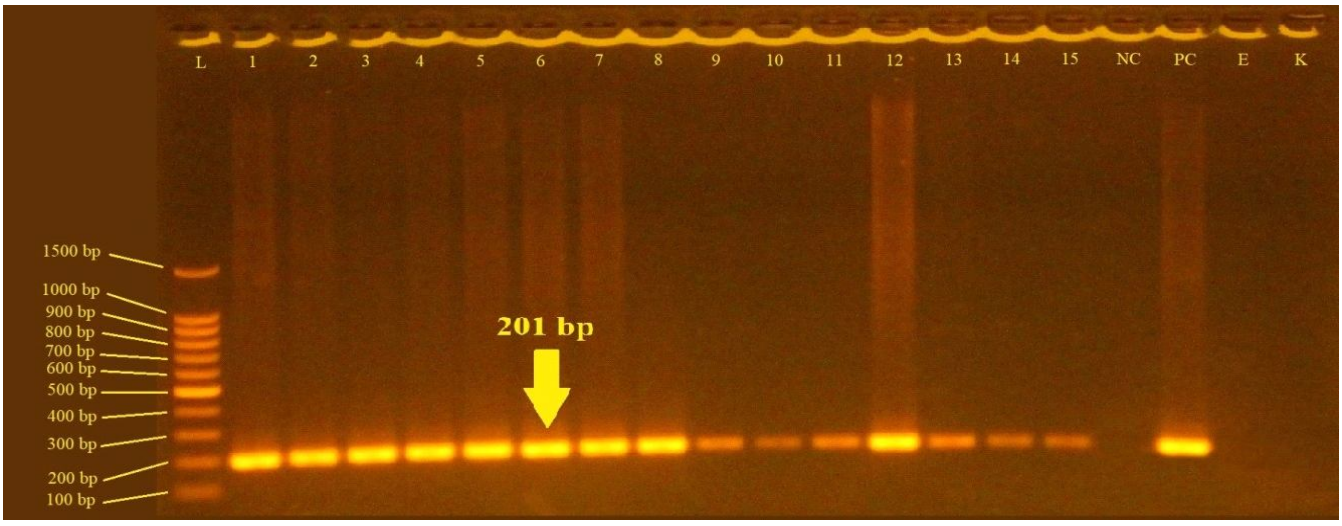

Fig. 2: Gel electrophoresis of amplified *rpsl* (201 bp), from *P. aeruginosa* using conventional PCR. Agarose 1.5%, 70 V/cm for 1 hrs. . and 20 min, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane L: 100 bp DNA ladder. Lane 1-15: Amplicons *rpsl* gene for *P. aeruginosa*. Lane E-K: Control, DNA extracted from deferent bacteria isolates (*K. pneumoniae, E.coli*). Lane NC: Negative control (had all PCR mixture with the substitution of water for DNA template).

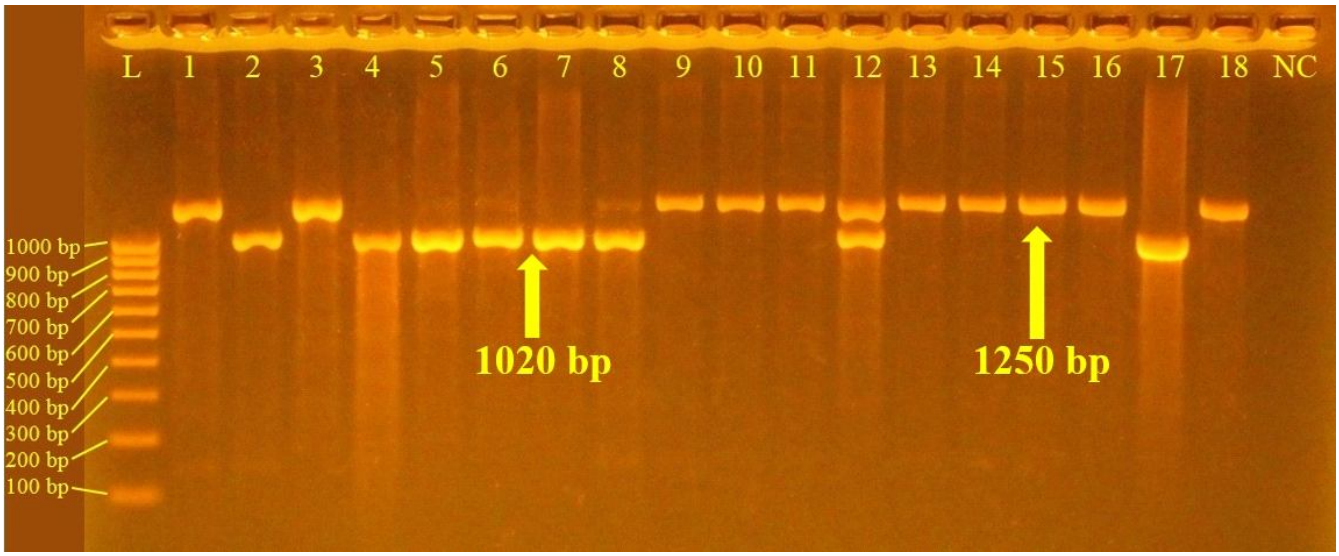

Fig. 3: Gel electrophoresis of amplified *fliC* (type a 1020bp, and type b 1250pb) from *P. aeruginosa.* using conventional PCR. Agarose 1.5%, 70 V/cm for 1 hrs. and 20 min, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane (L): 100 bp DNA ladder. Lane (1-18): Amplicons of the *fliC* (type a and type b) gene for 1-18 No. of isolates. Lane (NC): Negative control (had all PCR mixture with the substitution of water for DNA template).

Apart from extracellular factors, the initial attachment mediator (flagella) plays a significant role in initiation of infection. Two types of flagellin proteins have been identified in *P. aeruginosa*, type a and type b, which can be detection on the basis of molecular sizes. *P. aeruginosa* flagellin Type a and b do not exhibit phase variation; a single strain produces single type of flagellin, and no switching between types a and b has been observed. Oligonucleotide primers specific for N-terminal (CW46) and C-terminal (CW45) conserved regions of flagellin gene were used for PCR amplification of the flagellin gene of *P. aeruginosa* (Allison *et al.,* 1985).

Percentage *fliC* occurrence was found to be *fliC* type a gene presented in 36.7%, while *fliC* type b gene presented in 77.6%. Absence of flagellin in *P. aeruginosa* obtained from patients with burn infection in this study, the organism must become non-motile to chronically persist. Phagocytic cells respond directly to flagellar motility. This represents a novel mechanism by which the innate immune system facilitates clearance of bacterial pathogens, and provides an explanation for how selective pressure may result in bacteria with down-regulated flagellar gene expression and motility as is evident in isolates causing chronic infections. Thus, variation in the flagellin gene distribution among *P. aeruginosa* isolates from burn infection patients may be due to the selective pressure of the disease (Lovewell *et al.,* 2011).

Gene expression of *fliC* analysis by Quantitative Real Time PCR (qRT-PCR)

The purpose of this step is to measure the expression of *rpsl* and *fliC* genes and compare the gene expression in the presence of Mint oil, Colistin antibiotic, and Mint combination with Colistin and in the absence of them in order to improve the role of this gene in the resistance of *P. aeruginosa* to antibiotics by using the concentration below the dose of MIC for each sample. The calculation of gene expression fold change was done by using relative quantification (RQ) from delta delta Ct value. Shown in Fig. 4 and table 5 for *fliC*.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is distinguished from other methods for gene expression due to the accuracy, sensitivity and fast results. This technology is the golden standard for gene expression analysis. It is important to realize that in a relative quantification study, the experiments are usually interested in comparing the expression level of a particular gene among different samples (Derveaux *et al.,* 2010).

All genes expression were compared with *rpsÅ* gene expression as the housekeeping gene. The assay was performed three times for each sample and the mean and stander division (SD) of three obtained quantities was considered as quantity of given gene expression for that sample. The $\Delta\Delta C_T$ was used for determining gene expression. The $\Delta\Delta C_T$ was gained by subtracting ΔC_T of sample from ΔC_T of reference gene. Based on data of relative gene expression is the comparative C_T method also referred to as the fold change= $2^{-\Delta\Delta C_T}$ method of [14], which is golden equation technique to compare the gene expression in different samples. Each sample is related to an internal control gene in both treated and untreated cases.

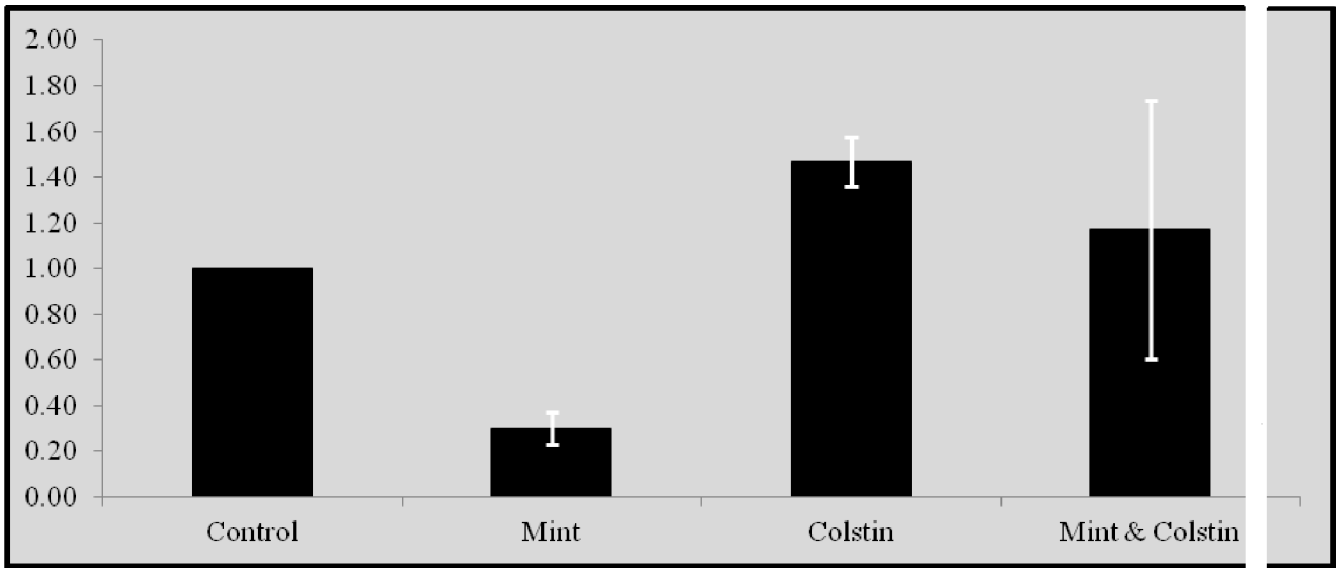

Fig. 4: The mean folding for *fliC* gene in different status.

Table 5: Ct values and fold of gene expression of *fliC* gene and *rpsl* gene of *P. aeruginosa* that was treated with mint, Colistin, and combination.

Group	Samples	H.K.	CW	ΔCT	ΔΔCT	Folding	Mean	± S.D
Untreated	1	29.32	26.15	-3.17	0.00	1.00	1	0
	2	30.54	26.09	-4.45	0.00	1.00		
	3	32.87	28.79	-4.08	0.00	1.00		
	4	31.79	28.62	-3.16	0.00	1.00		
	5	31.40	26.36	-5.04	0.00	1.00		
Mint	1	30.11	28.99	-1.12	2.05	0.24	0.30	0.071
	2	29.61	27.40	-2.21	2.24	0.21		
	3	32.24	29.64	-2.60	1.48	0.36		
	4	29.71	27.98	-1.73	1.44	0.37		
	5	28.20	24.79	-3.41	1.63	0.32		
Colistin	1	32.17	28.38	-3.79	-0.62	1.54	1.47	0.105
	2	32.75	27.76	-4.99	-0.54	1.46		
	3	31.39	26.74	-4.65	-0.57	1.49		
	4	29.47	25.94	-3.54	-0.37	1.30		
	5	32.88	27.20	-5.68	-0.64	1.56		
Mint and Colistin	1	32.60	28.63	-3.97	-0.80	1.74	1.17	0.567
	2	32.93	29.10	-3.83	0.61	0.65		
	3	33.11	28.23	-4.88	-0.80	1.74		
	4	32.33	30.00	-2.33	0.83	0.56		
	5	31.82	26.57	-5.25	-0.22	1.16		

Table 6: The result of one way ANOVA statically analysis for *fliC* gene expression.

fliC	Mean	±S.D	F value	Sig.	Statically
Control	1	0	14.537	0.0001*	Statically significant
Mint	0.3	0.071			
Colistin	1.47	0.105			
Mint with Colistin	1.17	0.567			

* *p value* $\leq$ 0.05 significant.

One-way ANOVA test was used to estimate the difference with statically significance between groups according to *fliC* gene expression, Table 6 shows test results and statically significance.

From table 6 it can be concluded that there are differences with statically significance between groups according to for folding of *fliC* gene expression, whereas F-value = 14.537 and *P-value* = 0.0001 which is less than 0.05 (statically significant), table 6 also shows that folding of *fliC* gene expression in Mint group was the lowest compared with other groups, whereas mean value = 0.3.

Multiple Comparisons (Scheffe) test was used to clarify the causes of differences with statically significance, as shown in (Table 7).

Table 7: The result of multiple comparisons (Scheffe) for *fliC* gene expression.

(A) group	(B) group	Mean Difference (A-B)	*P-value*	Statically
Control	Mint	0.7*	0.014	Significance
	Colistin	0.47	0.131	No Significance
	Mint with Colistin	0.17	0.836	No Significance
Mint	Colistin	1.17**	0.0001	Significance
	Mint with Colistin	0.87**	0.002	Significance
Colistin	Mint with Colistin	0.3	0.470	No Significance

*: *p value* $\leq$ 0.05, **: *p value* $\leq$ 0.01.

(Table 7) shows that the causes of differences with statically significance belong to the difference between Mint and Colistin with mean difference (1.17) according to Mint and Mint and Colistin with mean difference (0.87) and Control and Mint with mean difference (0.7), *P-value* was (0.0001, 0.014 and 0.002) respectively ($\leq$ 0.05). Whereas the differences between Control and Colistin, Control and Mint and Colistin, and Colistin and Mint and Colistin group were not statically significant, and *P-value* was (0.131, 0.836 and 0.3) respectively (> 0.05).

Therefore, the study hypothesis "There is a statically significance in *fliC* gene expression to groups (Control, Mint, Colistin and Mint with Colistin)" was accepted for Mint group.

Conclusion

PCR is a reliable technique for the diagnosis of *P. aeruginosa* strains isolated from different sources by using primers specific for *rpsl* gene gave ideal results when used a housekeeping gene in the gene expression experiment with the minimal variation in different sources. Detection of target virulence gene *fliC* responsible for attachment and mortality by conventional PCR. The sensitive test to local isolate of *P. aeruginosa* to different antibiotic revealed that (77.6%) isolates sensitive to colistin. Exposure the resistant isolates to mint oil and colistin to evaluate the gene expression of *fliC* target gene. The gene expression of virulence gene reduced after exposed to mint oil more than colistin antibiotic.

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