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Molecular Detection of the *exoU* and *toxA* genes among *Pseudomonas aeruginosa* of patients with burn and wound infection in Baghdad City

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Abstract:

Background: *Pseudomonas aeruginosa*, a human opportunistic Gram-negative pathogen, is one of the most important nosocomial pathogens and is a major health problem, primarily in immunocompromised individuals. Aims: to isolate *Pseudomonas aeruginosa* of clinical samples (Burns, wounds) swab, in order to diagnose *P. aeruginosa* using culture media and VITEK2 device. To study of the resistance of *P. aeruginosa* towards some antibiotics with VITEK2. To detect *exoU* and *toxA* genes in *Pseudomonas aeruginosa* isolates with PCR techniques. Materials and Methods: The current study included a collection of (210) swabs from patients suffering burns and wound infections in both genders, (93) males and (117) females, with ages ranged from 5-78 years were included, for the period from November 2019 to the end of February 2020, samples were collected from Burns departments at different hospitals within Baghdad\ Iraq. Results: After final diagnosis of samples, 50(23.80%) isolates of *P. aeruginosa* were obtained from 16 (32%) burn swabs, and 34 (68%) of wound swab. The highest resistance of *P. aeruginosa* to antibiotics was against antibiotics Levofloxacin, Ceftazidime, Imipenem, Tobramycin and Aztreonam; 88%, 88%, 78%, 70%, 68% respectively by using Modified Kirby-Bauer method. The highest MIC of the antibiotics was 90% for Ticarcillin 64µg/mL, 96% for Ticarcillin/clavulanic acid 64µg/ mL, 96% for Piperacillin 64µg/ mL, and 96% for Piperacillin/Tazobactam 64µg/ mL. The bacterial DNA was extracted from 50 isolates of *P. aeruginosa*. The PCR was carried out for all bacterial isolates, using specialized primers, to detect these two genes (*toxA* and *exoU*). The results showed that the 20 *P. aeruginosa* isolates have the *toxA* gene in a percentage 40% from all isolates. The results also showed that the same 20 *P. aeruginosa* isolates have the gene *exoU* in a percentage 40% from all isolates. The presence of *exoU* and *toxA* virulence genes in wounds is high positive rate of burn infection, where they were 60% for burns and 40% for wounds. Conclusion: current study found that the bacterial isolates carrying the *exoU* gene were also containing the *toxA* gene, so their pathogenic potential would be higher than if they carried one of the two genes.

key words: *Pseudomonas aeruginosa, exoU, toxA, burn, wound infection.*

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Introduction:

Pseudomonas aeruginosa, is Gram-negative bacteria, is one of the most important nosocomial pathogens and is a major health problem, primarily in immunocompromised individuals. It causes a wide spectrum of infections in multiple organs, such as the respiratory, urinary and gastrointestinal tracts, in addition to skin infections (Rasmussen *et al.*, 2015). This organism is highly tolerant of harsh conditions and has the ability to survive in various environments, including hospital environments, on medical equipment, such as mechanical ventilators, urinary or dialysis catheters and endoscopes, and in sinks. Stability in these environments causes contamination (Pachori *et al.*, 2019). It is considered the most common cause of ventilator associated pneumonia and burn wound infections, both of which have a mortality rate >30% (Kidd *et al*., 2015). This pathogen is the main reason for infections in burn patients owing to the destruction of skin as the first line of the innate immunity. In addition, it constitutes approximately 77% of mortality in burn patients during the past 25 years (Mahmmudi *et al*., 2016). Although therapeutic approaches for burns have greatly developed, infections remain one of the major causes of death in these patients, particularly critically ill burn patients. Compared with other hospitalized individuals, skin shortage, long hospital stays, and multiple invasive operations characterize burn patients; therefore, they are more susceptible to infection, due to its inherent resistance to different antibiotics or chemotherapeutic agents, *P. aeruginosa* can only be eliminated with difficulty and leads to a high mortality rate (Rowe *et al.*, 2005). Several studies have also underscored the link between multidrug resistance and increased morbidity and mortality (Morris & Cerceo, 2020). The worldwide spread of resistant *Pseudomonas aeruginosa* clones poses a threat to public health and therefore new treatment alternatives are urgently required (Tacconelli *et al*., 2018).

The *P. aeuroginosa* produces many toxins such as exotoxin A, pyocyanin, elastase and type III secretion system proteins. The virulence factors exotoxin A are secreted through Type II secretion mechanism, which use a pilus-like apparatus to secrete proteins into the extracellular environment, including lipase, phospholipase, alkaline phosphatase and protease, Besides, exotoxin A has been demonstrated to be involved in local tissue damage and invasion (Antônio *et al.*, 2019). During the acute phase of infection, *P. aeruginosa* uses T3SS, a macromolecular syringe-like structure, to inject toxins that ultimately destroy the host cell. The T3SS effector toxins ExoU, ExoS, ExoT, and ExoY induce cell death by disrupting the host cell cytoskeleton, cell membrane, and cAMP levels thereby enhancing disease severity (Scheetz *et al.*, 2009). So, the objectives of the study are: Isolation of *P. aeruginosa* from clinical samples (Burns, wounds swabs); Diagnosis of *P. aeruginosa* using culture media, Biochemical methods and the VITEK2 device; and then study of the resistance of *P. aeruginosa* to some antibiotics with Modified Kirby-Bauer method and VITEK2 system. Detection of the *exoU* and *toxA* genes in *P. aeruginosa* isolates with Real time PCR techniques.

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Methods:

A. Samples collection: The current study included a collection of (210) swabs from patients suffering from burns and wound infections in both genders, (93) males and (117) females, whose ages ranged from 5-78 years, for the period from November 2019 to the end of February 2020, from Burns departments at Teaching Medical City Hospital; Martyr Ghazi Al-Hariri Hospital for Specialized surgery; Baghdad Teaching Hospital; Burns Hospital Specialized; Educational laboratories\ Medical City Hospital; Al-Karama Teaching Hospital and Al-Yarmouk Teaching Hospital-Baghdad\ Iraq.

B. Identification of bacterial isolates: *Pseudomonas aeruginosa* isolates were identified and confirmed by utilizing conventional microbiological methods. The samples were cultivated on Blood agar, MacConkey agar and Cetrimide agar to study the phenotypes of *P. aeruginosa* colonies. King A medium (Oxoid, England) and King B medium (Oxoid, England) were prepared as described in (MacFaddin, 2000) to detect *P. aeruginosa*. As well as, The Gram stain was used for identification of *P. aeruginosa* in the samples (Baron *et al*., 2007). Catalase test, Oxidase test, IMVIC test as described by (Collee *et al*., 1996). Motility test was done by using motility medium (Collee *et al*., 1996). The bacterial growth test at 42°C was done by using nutrient agar plates, the positive result is it growth at this temperature (Collee *et al*., 1996). VITEK 2 system was used for identification and study the resistance to the antibiotics. Antimicrobial susceptibility test was carried out on the identified isolates of *P. aeruginosa* using commercially prepared antibiotic disks on Mueller Hinton agar plates by disk diffusion method.

C. DNA Extraction: The bacterial DNA extracted from the isolates of *P. aeruginosa* which grown in culture media, according to the protocol of the supplying company (ZR Fungal/ Bacterial/ Yeast DNA MiniPrep^{TM} D6005). The primer solutions were prepared according to the manufacturer's instructions (Integrated DNA Technologies company; IDT, Canada) by dissolving them in deionized distilled water (ddH_2O) to obtain a final concentration of 100 pmol\µl as stock solution. Stock solutions of the primers were kept at -20 °C until use (Table 1 and 2). To prepare 10 pmol/µl concentration as work primer suspended; 10 µl of stock solution was taken and added to 90 µl of ddH_2O, then it mixed well with a vortex device to homogenize before use and then kept at a temperature of -20 °C until use. The *tox*A primer pairs forward 5'- GGTAACCAGCTCAGCCACAT - 3', *tox*A reverse 5'-TGA TGT CCA GGT CAT GCT TC -3' (Ulla *et al*., 2017). The primer sequences for *exoU* gene used in this study, *exoU* forward 5'-ACC AGC TCA GCC ACA TGT C - 3', *exoU* reverse 5'-CGC TGG CCC ATT CGC TCC AGC GCT -3'(Elmouaden *et al*., 2019).

D. PCR for genes detection: The concentration and purity of extracted bacterial DNA of *P. aeruginosa* isolates were determined according to Nakayama *et al*., 2016. Then, the reaction mixture was prepared using a Maxime PCR pre-Mix kit (i-Taq) 20l

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according to the manufacturer's instructions (iNtRON). The optimum conditions of *exoU* and *tox*A gene detection were (initial denaturation 95 °C, 5 min., 1 cycle; denaturation 95 °C, 45sec., 35 cycle; annealing 60 °C, 45sec., 35 cycle; extension 72 °C, 45sec., 35 cycle; final extension 72°C, 7 min., 1 cycle).

E. Detection of amplified DNA bands: the presence of the amplified DNA bands was investigated (Al-Ahmadi and Roodsari, 2016).

F. Statistical Analysis: Descriptive statistics used for categorical data were presented in the form of frequency and percentages. Chi-square test used to find-out the association between categorical data. P-values less than 0.05 were considered statistically significant throughout this study.

Results and discussion

(50) isolates of *P. aeruginosa* were obtained (23.8%), as shown in table (1).

Table 1: The percentages of *P. aeruginosa* isolates isolated from wound and burn swabs by using selective and enriched culture media

P-value	Number of *P. aeruginosa* isolates (%)	Number of samples (%)	Source of isolates
0.907	16 (32)	58 (27.61)	Burns swabs
	34 (68)	152 (72.38)	Wound swabs
	50 (100)	210 (100)	Total

The percentage of infection with *P. aeruginosa* bacteria was higher in females than males, 27(54%) and 23(46%), respectively (Table 2). The highest rate of bacterial infection was within the age group of 41-50 years, followed by the age group 31-40, 18(36%) and 10(20%), respectively (Table 3). The results of the current research agree with the results of Hussien *et al*., 2012 where the researchers found that the percentage of females that suffering from burn inflammation was more than that of men; (70% and 30%) respectively, and the highest age group suffering from burn inflammation was within 21−30 years. The researcher Muhammad *et al*., 2014 found that the highest rate of infection with bacteria which caused wound infection in

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Baghdad City was within the age group of 11−20 years, and *P. aeruginosa* formed an infection rate of 53 (36.05%).

Table 2: The percentage of *P. aeruginosa* in both males and females suffering from wounds and burns inflammations

Gender	Positive cases with *P. aeruginosa* (%)	Negative cases with *P. aeruginosa* (%)	Total (%)	p- value
Male	**23 (46)**	**70 (43.75)**	**93 (100)**	**0.907**
Female	**27 (54)**	**90 (56.25)**	**117 (100)**	
Total	**50 (100)**	**160 (100)**	**210 (100)**	

Table 3: Distribution of *P. aeruginosa* according to age groups in wound and burn swabs.

Age ranges (Years)	Number of *P. aeruginosa* isolates		Total (%)	P-value
	Male (%)	Female (%)		
1-10	**1 (4.34)**	**2 (7.40)**	**3 (6)**	**P = 0.937**
11-20	**3 (13.04)**	**3 (11.11)**	**6 (12)**	
21-30	**4 (17.39)**	**5 (18.51)**	**9 (18)**	
31-40	**5 (21.73)**	**5 (18.51)**	**10 (20)**	
41-50	**8 (34.78)**	**10 (37.03)**	**18 (36)**	
51-60	**1 (4.34)**	**1(3.70)**	**2 (4)**	
61-70	**1 (4.34)**	**0 (0)**	**1 (2)**	
71-80	**0 (0)**	**1 (3.70)**	**1 (2)**	
Total	**23 (100)**	**27 (100)**	**50 (100)**	

Chi-square test

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The results of the current study are compatible with many studies which showed that *P. aeruginosa* bacteria are the most isolated bacteria from burn injuries and wounds in higher percentages, 52.5% of all swabs had *P. aeruginosa* isolated from patients in Baghdad hospitals (AL-Kaisse *et al*., 2015). The present results were agreed with other researchers in Saudi Arabia (El-Ageery *et al*., 2016), as they found that 31.6% of swabs had *P. aeruginosa*. In India, the infection of wounds and burns with *P. aeruginosa* reached to 46% (Augustine *et al*., 2015). The diagnosis of *P. aeruginosa* was based on the morphology of bacterial colonies on both enriched and selective culture media (figure 1, 2), and this compatible with the results of previous researches (Forbes *et al*., 2002; Tadess and Alem, 2006; Todar, 2011).

Table (4): The morphological and biochemical results for *P. aeruginosa* identification

TEST	RESULT
Gram stain	**Gram –Ve bacilli**
Motility	**+ Ve**
MacConkey agar	**Non-lactose fermenting bacteria**
Blood agar	**β-hemolysis**
Growth on Cetrimide agar	**+ Ve**
King A medium	**Greenish Blue Color**
Growth at 4 °C	**W**
Growth at 42 °C	**+ Ve**
Catalase	**+ Ve**
Oxidase	**+ Ve**
Indole, Methyl red, Voges- Proskauer, Citrate utilization	**– Ve, – Ve, – Ve, + Ve respectively**
Kligler iron agar	**K / K (slant and bottom)**

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+ Ve: positive; - Ve: Negative; K: alkaline; W: Weak

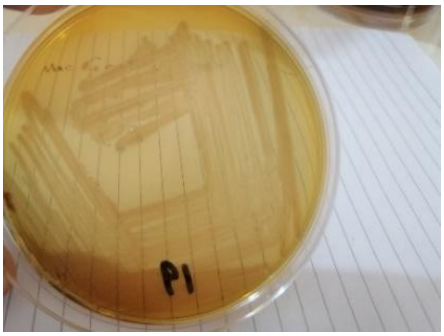

Figure 1: The pale-yellow colonies of *P. aeruginosa* on MacConkey agar plates

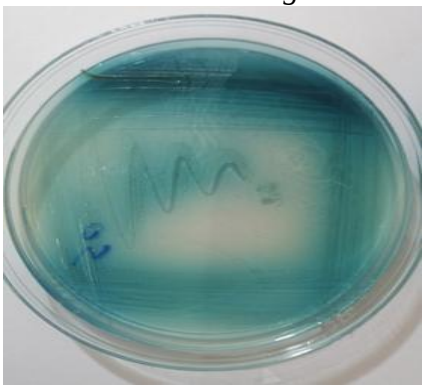

Figure 2: The greenish blue colonies of *P. aeruginosa* on King A agar

In the current study, the highest resistance to antibiotics was to the antibiotics Levofloxacin, Ceftazidime, Imipenem, Tobramycin and Aztreonam; 88%, 88%, 78%, 70%, 68% respectively by using Modified Kirby-Bauer method (disk diffusion method) (Figure 3). The results of the current study are consistent with Al-Shwaikh and Alornaaouti, 2018; as the isolates of *P. aeruginosa* showed high resistance to Ceftazidime (81%), Cefotaxime (78%), Piperacillin (76%) and was resistant to both Ciprofloxacin and Tobramycin (74%) and Gentamicin (72%); Amikacin and Meropenem were (70%) for each and resistant to both Ofloxacin (66%) and Imipenem (65%). In another study, *P. aeruginosa* were isolated in percentage of 31.46% from burn swabs at four hospitals in Baghdad, and the isolates were 100% resistant to Cefotaxime, Cephalothin, Gentamycin, Trimethoprim; while 55% were recorded for Amikacin and 88% for Ciprofloxacin (Al-Taie *et al*., 2014). These ratios are close to the resistance rates of *P. aeruginosa* isolates in the current study.

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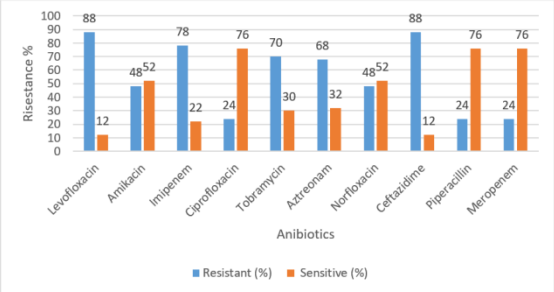
Figure 3: The percentages of antibiotic resistance and sensitivity of *P. aeruginosa* isolates (disk diffusion method)



The VITEK 2 Compact system was used to determine the minimum inhibitory concentrations (MICs) of 16 antibiotics (Ticarcillin, Ticarcillin/clavulanic acid, Piperacillin/Tazobactam, Piperacillin, Ceftazidine, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Tobramycin, Pefloxacin, Minocycline, Colistin, Trimethoprim\ Sulfamethoxazole) by using AST card. The results showed that the minimum inhibitory concentration (MIC) of the antibiotics: 90% for Ticarcillin 64µg/mL, 96% for Ticarcillin/clavulanic acid 64µg/ mL, 96% for Piperacillin 64µg/ mL, 96% for Piperacillin/Tazobactam 64µg/ mL,62% for Ceftazidime 16-32µg/ mL, 56% for Cefepime 32µg/ mL, 54% for Imipenem 8-16µg/ mL, 56% for Meropenem 8-16µg/ mL, 52% for Amikacin 32µg/ mL, 50% for Gentamicin 8µg/ml, 60% for Ciprofloxacin 2- ≥4 µg/ mL, 70% for Tobramycin ≤0.25-0.5 µg/ mL, 50% for Pefloxacin 8µg/ mL, 48% Minocycline 4-8µg/ mL, 20% for Colistin ≤0.5-2µg/ mL, 46% for Trimethoprim\ Sulfamethoxazole ≥128µg/ mL, as shown in table (5). Researcher Al-Shwaikh, 2018 indicated in her study using VITEK 2 to determine MIC values on 69 isolates of bacterium *P. aeruginosa* isolated from patients' wounds and burns in Baghdad city, in which resistance was determined. 87% for Ticarcillin (8µg\ mL); 85.5% for Tazobactam/ Piperacillin (8-16µg\mL), Piperacillin (≤4-16µg\mL) and Ticarcillin/Clavulanic acid (16-46µg\mL); 76.8% Cefepime (2-8µg\mL). 1% for Ciprofloxacin (≤0.25-0.5µg\mL); and 63.8% Ceftazidime (2-8 µg\mL).

Table 5: The values of the minimum inhibitory concentration (MIC) in (µg/ mL) for some antibiotics used against 50 *P. aeruginosa* bacteria isolated from Cases of wounds and burns swabs using the VITEK 2 device.

Antibiotics	**R (%)**	**I (%)**	**S (%)**
Ticarcillin	45 (90)	0 (0)	5 (10)
Ticarcillin/clavulanic acid	48 (96)	0 (0)	2 (4)
Piperacillin	48 (96)	0 (0)	2 (4)

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Piperacillin/Tazobactam	48 (96)	1 (2)	1 (2)
Ceftazidine	31 (62)	4(8)	15(30)
Cefepime	28 (56)	2(4)	20(40)
Imipenem	27 (54)	1(2)	22(44)
Meropenem	28 (56)	2(4)	20(40)
Amikacin	26 (52)	1(2)	23(46)
Gentamicin	25(50)	0(0)	25(50)
Ciprofloxacin	30(60)	0(0)	20(40)
Tobramycin	35(70)	0(0)	15(30)
Pefloxacin	25(50)	0(0)	25(50)
Minocycline	24(48)	0(0)	26(52)
Colistin	10(20)	0(0)	40(80)
Trimethoprim\ Sulfamethoxazole	23(46)	0(0)	27(54)

R: Resistance; S: Sensitive; I: Intermediate; MIC: Minimum Inhibitory Concentration

The bacterial DNA extracted from 50 isolates of *P. aeruginosa* were grown in nutrient agar medium, according to the protocol of the supplying company (ZR Bacterial DNA MiniPrep™ D6005). Purity and concentration were confirmed with Nanodrop spectrophotometer system. The results were that the concentrations of all 50 DNA bacterial isolates (figure 4) were between 54 – 337.5 ng/µL, these concentrations were sufficient to use DNA for amplify PCR; and the DNA purity (That was measured by reading the Absorbance at the wavelength 260/280 nm) of all 50 bacterial isolates ranges from 0.76 - 2. The results of the current study were almost agreed with the results of (Al-Azzawi, 2018), where the concentration of DNA in the samples ranged between 39.8 ng/µL and 264.5 ng/µL, and the range of purity was 0.84 - 1.94. As for the results of the study by Al-Shamaa *et al*., 2016, the purity of the DNA of the bacterial isolates in this study ranged between (1.8-2) and the concentration of the DNA in the samples were (60-110 ng/µL) (Al-Shamaa *et al*., 2016).

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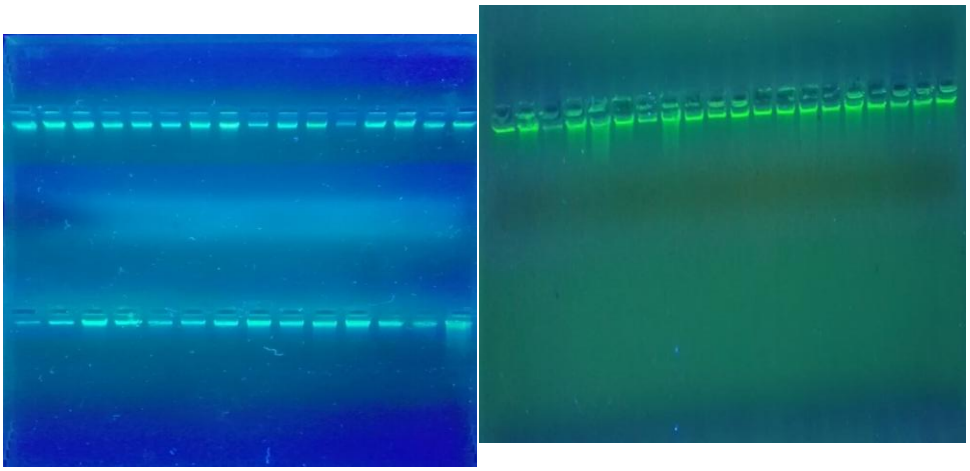

Figure 4: A and B: Gel electrophoresis of genomic DNA extracted from 50 bacterial isolates, 1% agarose gel at electric current of 7 volts/cm for 30 minutes

In this study, *toxA* and *exoU* genes were used for detection in 50 *P. aeruginosa* isolates under study. The two genes (*toxA* and *exoU*) possessed by *P. aeruginosa* were detected using thermocycler for PCR to perform DNA amplification according to the Thermocycling conditions. PCR was carried out for all bacterial isolates, using specialized primers, to detect these two genes. The results showed that the 20 *P. aeruginosa* isolates (8 Burn, 12 wound) under study have the *toxA* gene in a percentage 40% from all isolates. When comparing the amplified bands with the volume band index of DNA ladder, the resulting bands have a molecular weight (352 base pairs), as shown in the figures 5 A and B.

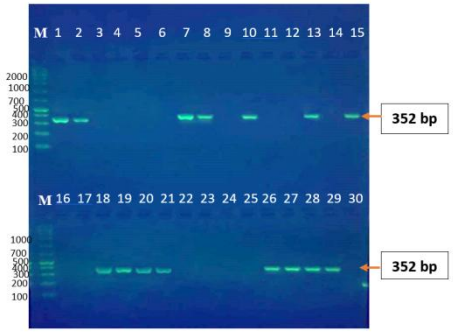

Figure 5 A: Electrophoresis of the polymerase chain reaction products for *toxA* gene, the band size is 352 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 1, 2, 7, 8, 10, 13, 15,18, 19,20, 21, 26, 27, 28, 29 are the *P. aeruginosa* isolates having *toxA* gene.

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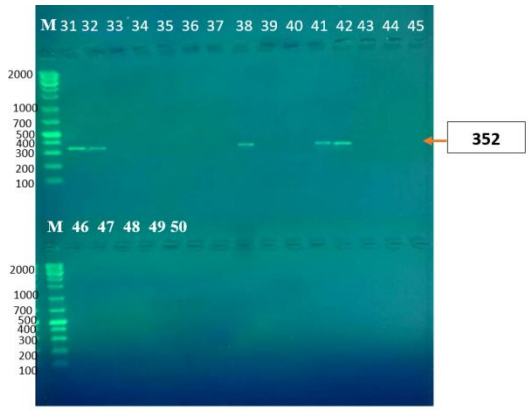

Figure 5 B: Electrophoresis of the polymerase chain reaction products for *toxA* gene, the band size is 352 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 31, 32, 38, 41, 42 are the *P. aeruginosa* isolates having *toxA* gene.

The results also showed that the same 20 *P. aeruginosa* isolates (8 Burn, 12 wound) under study have the gene *exoU* in a percentage 40% from all isolates. When comparing the amplified bands with the volume band index of DNA ladder, the resulting bands have a molecular weight (400 base pairs), as shown in the figures 6 A and B.

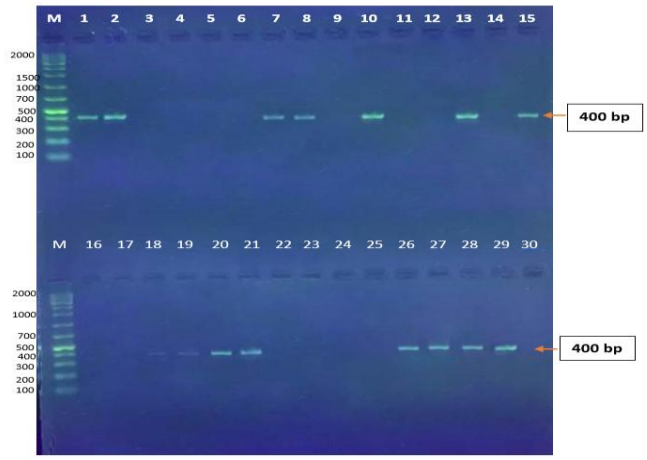

Figure 6 A: Electrophoresis of the polymerase chain reaction products for *exoU* gene, the band size is 400 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 1, 2, 7, 8, 10, 13, 15, 18, 19,20,21, 26,27, 28, 29 are having the *P. aeruginosa* isolates having *exoU* gene.

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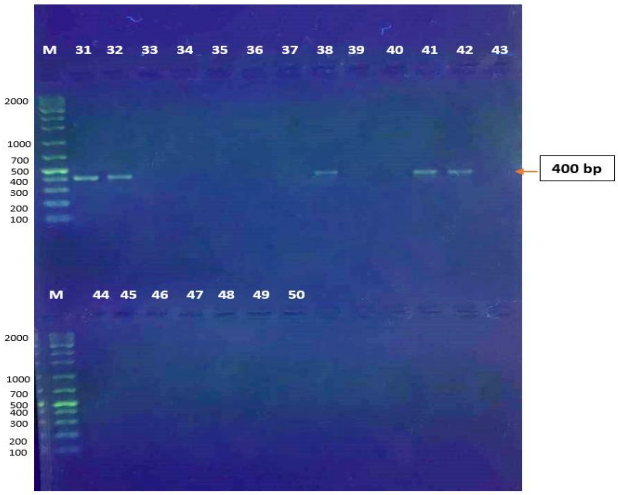

Figure 6 B: Electrophoresis of the polymerase chain reaction products for *exoU* gene, the band size is 400 bp. The product was electrophoresed on 2% agarose at 70 volt/cm^2 for 60min. M: DNA ladder Marker (100bp). The lines 31, 32, 38, 41, 42 are the *P. aeruginosa* isolates having are the *P. aeruginosa* isolates having *exoU* gene.

The results of the present study showed that the presence of *exoU* and *toxA* genes in wounds is high positive rate of virulence genes from burn infection, where they were 60% for burns and 40% for wounds.

The percentages of *tox*A gene in *P. aeruginosa* isolates were 100% from swabs of burned Iraqi patients (Al Rubaye, 2020; Yuosif *et al*., 2015). The study conducted by AL-Shamaa *et al.*, (2016) was used for screening the protein synthesis inhibition genes (*exoA, lasB, exoU*) of the 31 *P. aeruginosa* isolates, 17(55%) isolates were PCR-positive for the *exoU* gene, and 14(45%) isolates were PCR-negative. These isolates were recovered from 25 burn; 15(60%) gave positive result for that gene in PCR, and 6 wound isolates which gave 2(33%) positives result in PCR (AL-Shamaa *et al.*, 2016). In the other study, AL-Mayyahi *et al*., 2018 revealed that 60.31% of *P. aeruginosa* isolates carried *exoU* gene which coding exoenzyme that plays an important role in *P. aeruginosa* pathogenicity. The bacterial isolates were collected from different clinical specimens includind wound, burn, ear swabs, sputum and Cerebrospinal fluid (AL-Mayyahi *et al*., 2018).

Conclusion: The current study found that the bacterial isolates carrying the *exoU* gene were also containing the *toxA* gene, so their pathogenic potential would be higher than if they carried one of the two genes.

Recommendations: Conducting many epidemiological studies on the causes of the spread of resistance to antibiotics by covering the genes responsible for this resistance. Isolation the *P. aeruginosa* from another sources of infection.

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