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Prevalence of Colistin Resistance in *Pseudomonas aeruginosa* Isolated from Burn Patients in Sulaymaniyah City

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Abstract: *P. aeruginosa* a major cause of nosocomial infections; it is increasingly being associated with various epidemics and has become a widespread concern in a variety of hospitals worldwide. Multidrug-resistant (MDR) *P. aeruginosa*, is now recognized to be of great clinical significance. Through the period from December 2021 to April 2022, 265 specimens were collected from patients referring to the Sulaimani Burns and Plastic Surgery Hospital (the Burn Centre) in Kurdistan/Iraq and private laboratory. The strains were isolated from clinical specimens: 183 burn swabs, 44 Tissue, 18 Blood specimens from burn patient, 13 Urine sample from burn patient, 5 wound swabs and 2 swabs from environmental of hospital. The majority of patients burned were from female 152 (57.3%) out of 265 samples while 113 males were burn (42.6%), The most patients who visited the center and private labs were found to be between 20-30 years old. According to burn center the *Staphylococcus aureus* have a highest rate with 49 strains from 210, then follow by *P. aeruginosa* with 37 strains, *Acinetobacter baumannii* with 30 strains, *E. coli* with 19 and *Klebsiella pneumonia* with 13 strains, while data from private labs the Staphylococcus *aureus* have a highest rate with 30 strains from 55 samples, then follow by *P. aeruginosa* with 20 strains and 5 sample didn't show any growth of any microorganism. 23 isolates were submitted to PCR technique for detection to 16SrRNA (housekeeping gene) and virulence genes *toxA*, *pmrA* and *pmrB* genes. The results showed that all samples were positive for 16S_rRNA while *tox*A positive in 14 (60.8%) sample. *pmrA* positive in 17 (73.9%) isolates and *pmrB* positive in 16 (69.5%) isolates.

Keywords: *Pseudomonas aeruginosa*, Colistin , burn, MDR.

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Introduction

Pseudomonas aeruginosa is a gram-negative aerobic bacterium can be isolated from most environments, including soil, plants, and mammal tissue. *P. aeruginosa* has ability to continue in live on medical devices and different surfaces by using its important binding factors, like flagella, pili, and biofilms. It has become an important cause of nosocomial infections and antibiotic resistance (1).

Pseudomonas aeruginosa can be specified as a bacteria related to healthcare infections, including ventilator-associated pneumonia (VAP), intensive care unit infections, central line-related blood stream infections, surgical site infections, urinary tract infections, burn infections, keratitis, and otitis media. It also causes a high mortality rate in patients diagnosed with these infections and refer to ability of *P. aeruginosa* to adapt with changes in the environment, rapidly developing resistance to antibiotics (1).

Therefore, antibiotic selection for patients with *P. aeruginosa* infections is challenging because of the pathogen's intrinsic resistance to many commercially available antibiotics. Multidrug-resistant strains are prevalent, and often require treatment with novel or "last resort" agents like Colistin (2, 3).

Resistance to the Colistin is dependent on many of factors primarily chromosomally mediated by reduced permeability of cell wall (lipopolysaccharide modification), Overexpression of efflux pump systems and overproduction of capsule polysaccharide, while resistant acquired from other organisms occur by plasmids mediated (24).

Colistin resistance in Gram-Negative bacteria particular in *P.aerginosas* is most commonly due to decreased binding to the bacterial outer membrane because of lipopolysaccharide modification that is caused by changes in *PmrAB* regulatory systems, Which results in a less anionic lipid A, So lead to stop or reduce interaction (25).

Pmr is an auto-regulated two-component signal transduction system, Which in addition to a sensor kinase and response regulator also transferase which contributes to colistin resistance by adding moieties to the lipid A component of LPS which lead to reduces the negative charge of the bacterial membrane and decreases binding of positively charged in colistin, So these modifications lead remove negative charges, lowering the affinity of LPS, thereby increasing resistance to colistin, *PmrAB* expressed the highest at the stationary phase in the absence of colistin, but reduced in growth with amount of colistin (23).

The aim of this study is isolation and identification of *P. aeruginosa* with colistin resistance collected from burn patient samples.

Materials and methods

-Specimens collection

Through the period from December 2021 to April 2022, 265 specimens were collected from patients referring to the Sulaimani Burns and Plastic Surgery Hospital (the Burn Centre) in Kurdistan/Iraq and private laboratory. The strains were isolated from clinical specimens: 128 burn swabs, 44 Tissue, 18 Blood specimens from burn patient, 13 Urine sample from burn patient, 5 wound swabs and 2 environmental swabs, while 55 burn swab were collected from patients attended to private labs.

Bacterial identification

The colonies morphology on Nutrient agar, MacConkey agar and Cetrimide agar was depended initially to identify bacterial isolates, colony shape, texture, color of colony was examined (Figure 1 and Figure 2).

Antibiotic susceptibility test

-Disc diffusion

The susceptibilities of the isolates to 15 antibiotics (Himedia, India) by Kirby-Bauer disk diffusion method (4): Ceftazidime 30µg, Gentamycin 10µg, Tobramycin 10µg, Imepenem 10µg, Ciprofloxacin 5µg, Levofloxacin 5µg, Amikacin 30µg, Streptomycin 10µg , Cefepime 30µg, Tetracycline 30µg , Meropenem 10µg, Colistin 25µg, Ampicillin 10µg, Piperacillin-Tazobactam 30µg, CoTrimoxazole 25µg .The zone of inhibition diameter was measured and the

results were interpreted based on the guidelines by the Clinical and Laboratory Standards Institute (5).

-Microtitter plate

A single colony was transferred into broth and incubator overnight at 37 °C. After 12–18 h of incubation, then broth was spun down using a centrifuge set at 4000 rpm for 5 min.

Culture was dilution by using 20 ml of normal saline and centrifuged again at 4000 rpm for 5 min, the final concentration was equal to 5 × 106 cfu/ml. The resazurin solution was prepared by dissolving a 270 mg in 40 ml of distilled water, then vortex to ensure that it was a well-dissolved and homogenous solution.

Preparation of 96 well plate was labelled, volume of 100 µl of stock solution was added and pipetted into the first column of the plate and all other wells 50 µl of muller hinton broth from 2 to 8 well, then serial dilutions were added were discarded after use such that each well had 50 µl of the stock solution in serially descending concentrations. To each well 10 µl of resazurin indicator solution was added.

Finally, 10 µl of bacterial suspension (5 × 106 cfu/mL). Each plate had a set of controls: a column with a colistin antibiotic as positive control and a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 µl of nutrient broth instead. (6&7).

-Broth serial dilution

Five hundred µl from working stock solution were added to 500 µl MHB medium in tube and perform two fold serial dilutions to get drug concentrations as 256 µg/ml , 128 µg/ml, 64 µg/ml , 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml , 1 µg/ml, and 0.5 µg/ml.

Fifty µl of MHB broth to were added to all wells of column 1 to 10 in prepared 96 well plate, add 75 µl in column 11 and 100 µl in column 12 of the microtittre plate**,** Add 25 µl of Colistin dilution 256µg/ml to column 1, and make folding till 0.5µg/ml in column 10 of the microtiter plate. Column 11 will be growth control containing only media and bacterial inoculum while column 12 will be media control containing only media 100µl. Each well of the microtiter plate should finally contain total volume of 100µl. (8).

Genetic identification

DNA was extracted from pure culture of *P. aeruginosa* bacteria using protocol of ABIOpure Genomic kit. PCR products were visualized by electrophoresis using a 1.2% agarose gel stained with Red Safe.

Primers used in this study was designed using the *P. aeruginosa* NCBI as a reference. These primers were supplied by macrogen Company in a lyophilized form. The genes with a amplicon size and its genetic sequence used in this study shown in Table (1).

In this study 23 isolates were submitted to PCR technique for detection to 16SrRNA (housekeeping gene) and virulence genes *toxA, pmrA* and *pmrB* genes (Fig 4,5 and 6) , PCR was performed with a final volume of 25 µl. Each reaction contained 12.5 µl from master max, 1.5 µl from forward primer, 1.5 µl from reverse primer, 4.5 µl from free nuclease free water and 5 µl from template DNA. Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker (Neogen/USA) was run with each gel

and determined by the size of the amplified product**.** The reaction was submitted to the PCR condition as mentioned in Table (2).

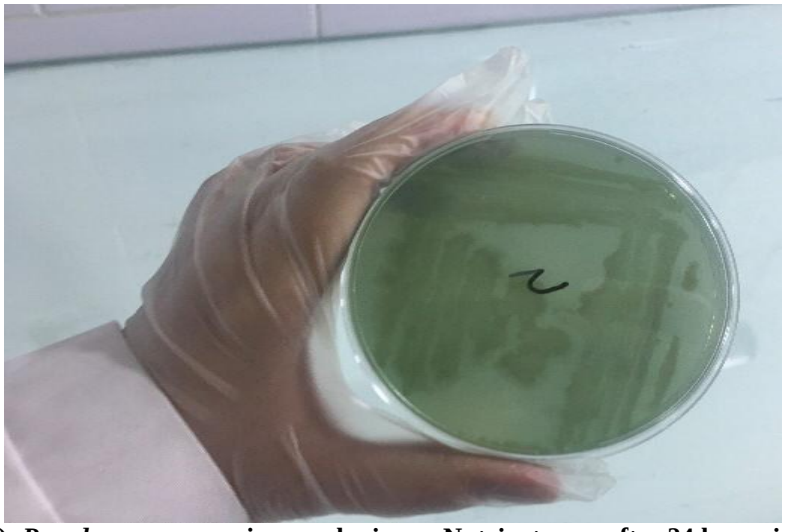

Figure (1): *Pseudomonas aeruginosa* colonies on Nutrient agar after 24 hours incubated.

Figure (2): *Pseudomonas aeruginosa* colonies on Cetrmide agar after 24 hours incubated.

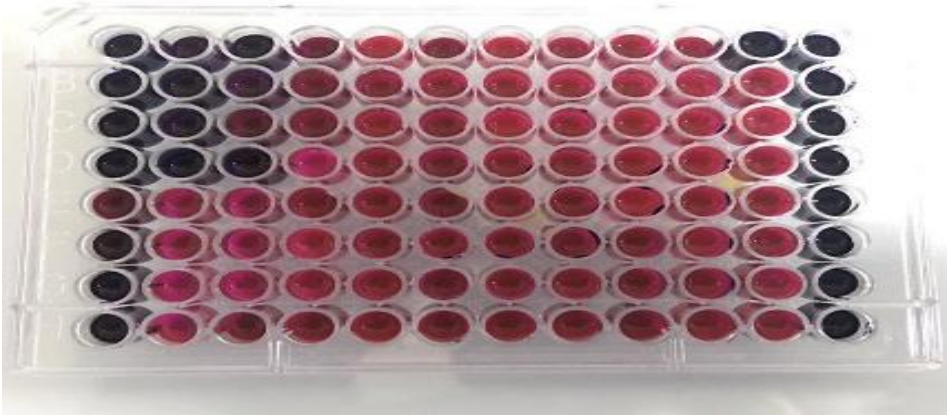

Figure (3): Antibiotic Susceptibility Test estimated by Microtitter plate 96-well.

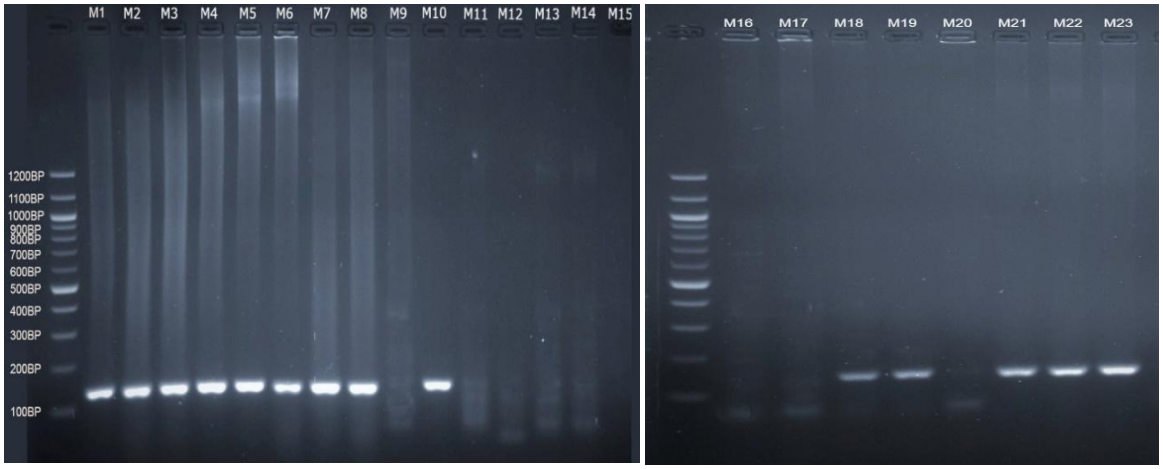

Figure (4): Gel electrophoresis of amplified PCR product of *ToxA* gene in PCR at 85v for 74min and 1.2% agarose stained with Red Safe. DNA ladder (100bp).

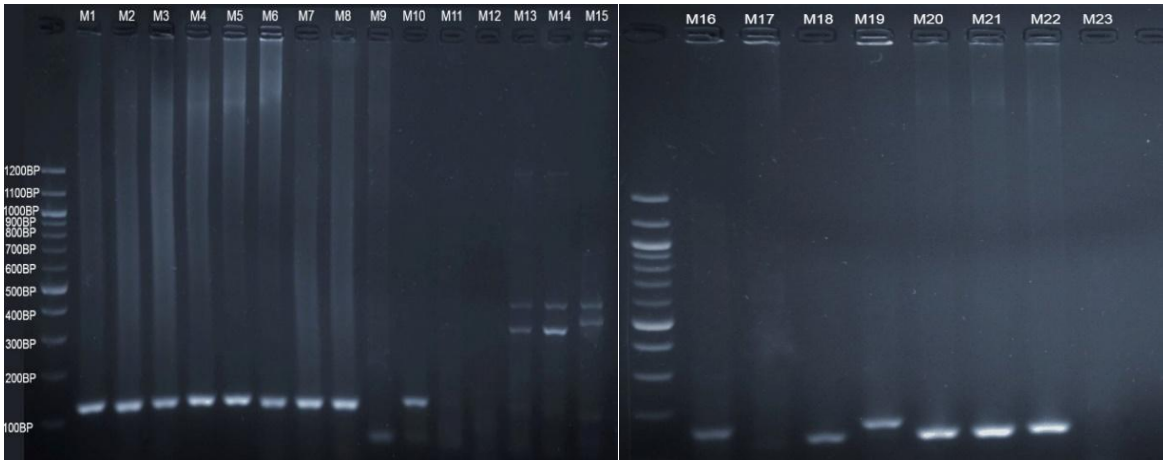

Figure (5): Gel electrophoresis of amplified PCR product of *PmrA* gene in PCR at 85v for 74min and 1.2% agarose stained with Red Safe. DNA ladder (100bp).

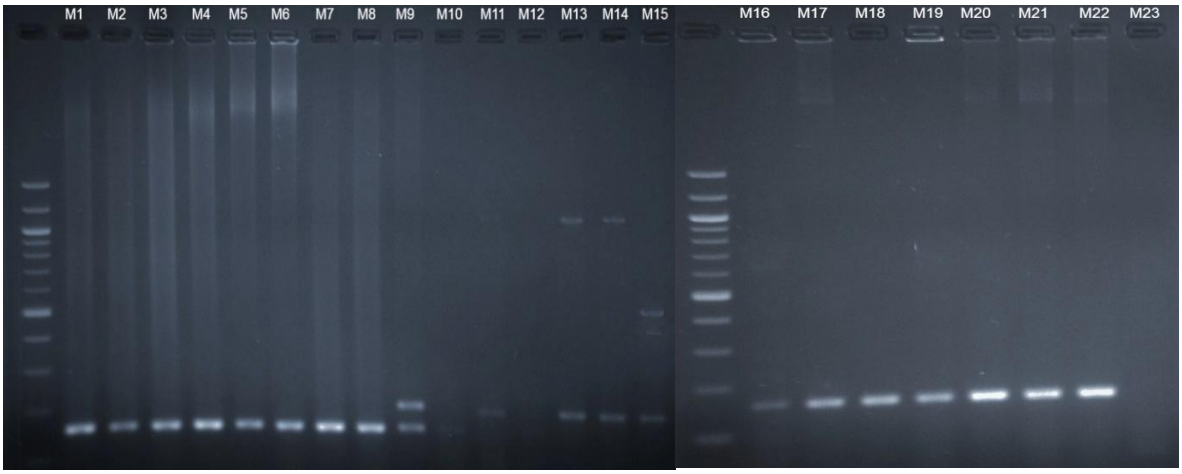

Figure (6): Gel electrophoresis of amplified PCR product of *PB* gene in PCR at 85v for 74min and 1.2% agarose stained with Red Safe. DNA ladder (100bp).

Table (1): Primers used in this study

Genes	Sequence 5`to 3`	Product Size bp
***tox*A**	F-5'-AGCCCTCGAACATCAAGGTG-3'	138
	R-5'-CCTGACGAAGAAGGTGGCAT-3'	
***pmr*A**	F-5'GGAAGGCGATACCGTGGAAT-3'	138
	R-5'-AGGTTGCGCAGGATGTCC-3'	
***pmr*B**	F-5'-AGCGGATCAGCACCTTGATG-3'	138
	R-5'-CGACAGCTCGTGTCCTTGT-3'	

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

Steps	Temperature	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	94	30 ses	30
Annealing	55	30 sec	30
Extension	72	30 sec	30
Final extension	72	7 min	30
Hold	4	10 min	1

Results and discussion

Out of 265 specimens that were collected from hospital and private laboratories, 46 isolates were diagnosed as *P. aeruginosa*. The second most prevalent pathogen percentage among burn infections may be related to the increasing numbers of the weaken immune patients due to infect with diseases or expose to contaminations from the environment or stay in hospital for long time.

In the current study, the results showed that the majority of patients with burn were female 152 (57.3%) out of 265 samples while 113 males were burn (42.6%). This record similar to study done in Sulaimaniyah city/Iraq who showed female accounted 57% and male 43% (9). Also, similar to results in two studies done in Iran; they found male patients were 53 female 26% (10), and 52% male and 41% for female patients (12).

However, this study disagrees with study done in Basra city (11) recorded 54.8% male and 45 % female also disagree with study conduct In Duhok and Erbil Hospitals/Iraq recorded 63.5% male and 36.4 female (13).

According to our study burn center the *Staphylococcus aureus* have a highest rate with 49 strains from 210 *Staphylococcus aureus* (20), *Staphylococcus aureus* Coagulase + (27) ,*Staphylococcus aureus* MRSA (2)), Then follow by *Pseudomonas aeruginos*a with 37 strains, *Acinetobacter baumannii* with 30 strains, *E. coli* with 19 and *Klebsiella pneumonia* with 13 strains , 68 sample didn't showed any growth of any microorganism while 4 sample have normal flora , 2 sample have candida and 15 sample were contaminated (Figure 7).

While data from private labs the *Staphylococcus aureus* have a highest rate with 30 strains from 55 samples, then follow by *Pseudomonas aeruginosa* with 20 strains and 5 sample didn't showed any growth of any microorganism (Figure 8).

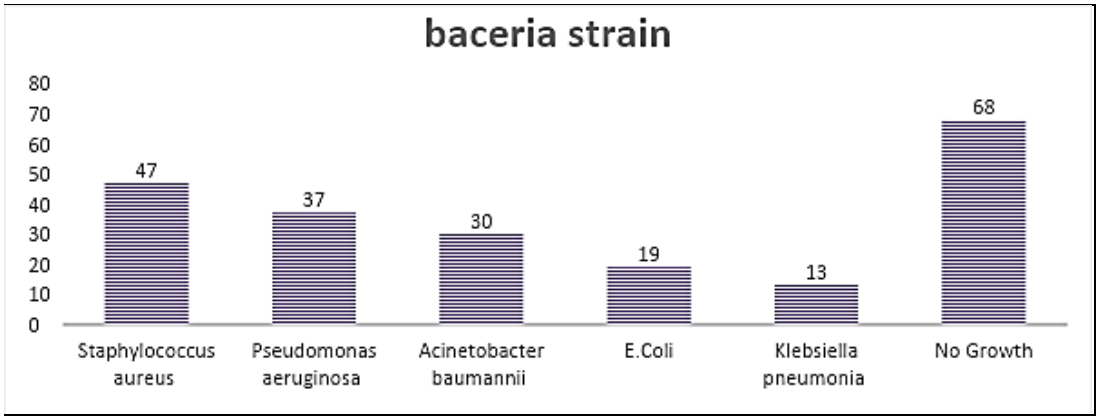

Figure (7): Percentage of bacterial isolates from burn patients attended Burn Center.

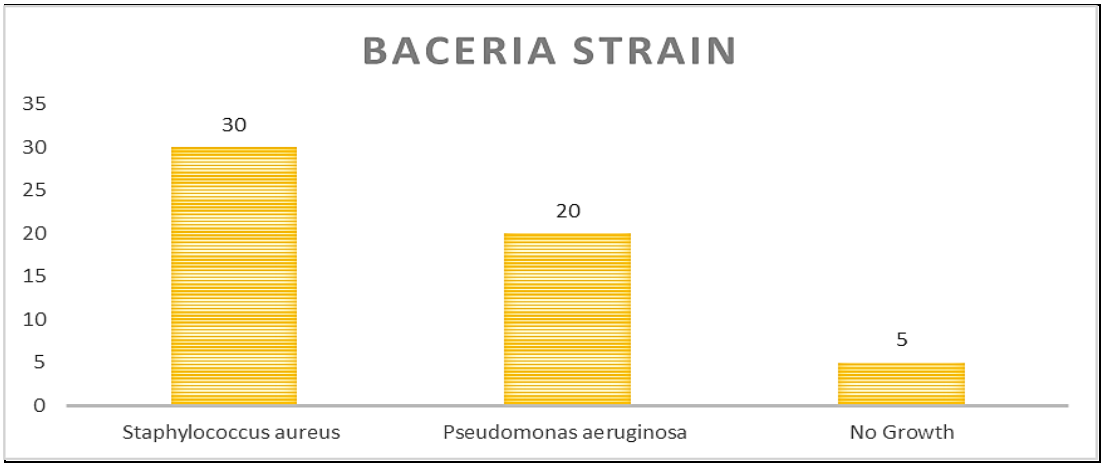

Figure (8): Percentage of bacterial isolates from burn patients attended private labs.

Minimum inhibitory Concentrations for Colistin

- Micro titter plate method

Minimum inhibitory concentrations for colistin antibiotic were determined for 23 isolates using the micro titter plate method. The results were interpreted after incubation at 37 °C after 24 hours according to the Clinical and Laboratory Standards Institute CLSI depending on break point of colistin antibiotic.

Results of MIC had confirmed the previous results of antibiotic disc diffusion test and the current study showed that the number of isolates that gave MIC values (4µg/ml) were 7 of clinical isolates, 7 of clinical isolates gave (32µg/ml), 1 of clinical isolates gave (64µg/ml), 6 of clinical isolates gave (128µg/ml) and 2 of clinical isolates gave (256µg/ml).

- Broth serial dilution method

Minimum inhibitory concentrations for 23 isolates for colistin antibiotic were determined using the Serial dilution broth method. The results were interpreted after incubation at 37 °C after 24 hours according to the Clinical and Laboratory Standards Institute CLSI depending on break point of colistin antibiotic.

Results of the current study showed that the number of isolates that gave

MIC values (4µg/ml) were 6 of clinical isolates, 4 of clinical isolates gave (16 µg/ml),4 of clinical isolates gave (32µg/ml), 1 of clinical isolates gave (64µg/ml), 6 of clinical isolates gave (128µg/ml) and 2 of clinical isolates gave (256µg/ml) and this recorded similar to study (18).

The results showed 16 isolates from 46 were MDR and 14 were sensitive to all tests antibiotic, *P. aeruginosa* isolates against following class: Aminoglycosides class (Amikacin) 15.2 % resistance, Tobramycin 15.2%, Streptomycin 17.3% and Gentamicin 39.1% like (20) study recorded 43.75%, Cephalosporins class (Cefepime 13%, Ceftazidime 21.7%), higher than study conducted in Kurdistan region with resistance rate Ceftazidime (77%), Cefepime (78%) (21).

Carbepenems class (Imipenem 21.7 % and Meropenem 23.9%) which less than study in Duhok city (14) who recorded Imipenem 33.3% and Meropenem 31.7%. While study conducted in Baghdad recorded Imipenem resistance was 24.4% while Meropenem was17.24% (16).

Quinolones class (ciprofloxacin 32.6% and Levofloxacin 15.2%), Penicillins subclass of β-Lactamase inhibitors class (Ampicillin 21.7% and Piperacillin/tazobactam 6.5%) lower than study conducted in Kurdistan reigon who recorded 86% resistance towards Ampicillin (21).

Polymyxins class (Colistin 21.7%) like study recorded 28.6% (15) and other study in Baghdad recorded (%22.5) (19). Finding in this study have higher rate than studies recorded in Duhok city (14) which showed resistance rate (8.3%) to colistin. Also, higher than study recorded colistin resistance rate 2.78% in Wasit city (17). and higher than study conducted in Kurdistan region with resistance rate 4% (21). Sulfonamides (CoTrimoxazole 19.5%) and Tetracyclines (Tetracycline 15.2%).

Molecular detection of target genes

In this study all samples were positive for 16S_rRNA, *tox*A positive in 14 sample 60.8% this recorded less than study conduct in two cities Duhok with (88%) and Erbil (84%) (13), *pmr*A and *pmrB* positive in 15 (65.2%) for each one as mentioned in Table (3) and this disagree with study held in Iran founded genes in all isolated (22).

The results of the PCR products were confirmed by comparing their molecular weight with 100bp DNA Ladder by the analysis of the bands on gel electrophoresis to genes present in DNA *P. aeruginosa* isolates, with a PCR product size single amplicon138bp band in the agarose gel, DNA template appear as single band under U.V. light using Red Safe as a specific DNA stain.

Table (3): Result of genes detection by PCR

Gene Name	Result of detection by PCR	No.
toxA	Positive	14
	Negative	9
pmrA	Positive	17
	Negative	6
pmrB	Positive	16
	Negative	7

Conclusion

The present study showed a high prevalence of MDR and *P.* aeruginosa showing colistin resistance among patients admitted to burn center suffering from burn infections. Also, it showed the presence of different mechanisms that can result in colistin resistance. This indicates the urgent need of changing the antibiotic-treatment strategies for humans.

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