Print ISSN 2710-0952 Electronic ISSN2790-1254

Molecular Identification and Phenotypic Analysis of Bacterial Species Associated with Burns Infection in AL-Anbar Governorate.

التعريف الجزيئي والتحليل الظاهري للأنواع البكتيرية المرتبطة بعدوى الحروق في محافظة الأنبار.

Riyam[1]* T. Salih , Saad [1]* T. Mutlk.

سعد طه مطلك.*[1] ريام طارق صالح ،*[1]

[1] **University of Anbar, College of Science, Biology Department, Ramadi, Iraq**
Email,riy23s1014@uoanbar.edu.iq
Phone No, 07806315700
Email, saad.t.mutalk@uoanbar.edu.iq
Phone No.07821688893

Abstract*:*

In order to molecularly and phenotypically identify the bacteria causing burn infections in the Anbar Governorate. Pathogenic bacteria were recovered from samples taken from burn wounds in hospitals in the Anbar Governora The isolates were collected and identified by Clinical specimens were cultured on a differant medium as nutrient agar, maconky,blood agar, mannitol salt aga .Growing bacterial were then studied under a microscope via Gram stain to examine their morphological features and biochemical tests such as catalase, coagulase, IMVC, and oxidase. Then they were identified by VITEK_2 Compact system. Polymerase chain reaction (PCR) and other molecular identification methods, Conventional PCR was performed to detect the presence of housekeeping genes (*16srRNA*) in all *S. aureus*, *K. Pneumonia*, *Ps.aeroginosa* isolates. The PCR product was detected by electrophoresis. The study samples were molecularly identified by the detection of the *16srRNA* gene. shows that all isolates (100%) contained the *16srRNA* gene, which confirmed the identification of the bacterial isolates as *S. aureus*, *K. Pneumonia and Ps.aeroginosa*.. also by sequences, , and phenotypic analysis was employed to evaluate the ability to produce biofilms and the resistance to antibiotics. Numerous bacterial species, such as Pseudomonas aeruginosa, Klebsiella pneumoniae, and Enterobacter spp., that are frequently found in burn infections were isolated, Many of the isolates had numerous antibiotic resistances, especially to cephalosporins and penicillin's. The isolates' capacities to form biofilms varied, and treatment resistance and

delayed wound healing are significantly influenced by biofilm development.

الملخص.

من أجل التعرف جزيئيًا وظاهريًا على البكتيريا المسببة لالتهابات الحروق في محافظة الأنبار. تم استرداد البكتيريا المسببة للأمراض من العينات المأخوذة من جروح الحروق في المستشفيات في محافظة الأنبار. تم جمع العزلات وتحديدها بواسطة العينات السريرية التي تمت زراعتها على وسط مختلف مثل أجار المغذيات، ماكونكي، أجار الدم، أجار ملح المانيتول. ثم تمت دراسة البكتيريا النامية تحت المجهر باستخدام صبغة جرام لفحص سماتها المورفولوجية والاختبارات الكيميائية الحيوية مثل الكاتالاز، الكواغيولاز، IMVC تفاعل. والأوكسيديز. ثم تم التعرف عليها بواسطة نظام VITEK_2 Compact وطرق التعريف الجزيئية الأخرى، تم إجراء تفاعل البوليميراز المتسلسل PCR البوليميراز المتسلسل (التقليدي للكشف عن وجود جينات التدبير المنزلي (١٦ srRNA في جميع عزلات (S. aureus و K. Pneumonia و Ps.aeroginosa . تم الكشف عن منتج تفاعل البوليميراز المتسلسل عن طريق الرحلان الكهربائي. تم التعرف على عينات الدراسة جزيئيًا عن طريق الكشف عن جين ١٦ srRNA. يُظهر أن جميع العزلات (١٠٠%) تحتوي على جين ١٦ srRNA، مما يؤكد تحديد العزلات البكتيرية على أنها S. aureus و K. Pneumonia و Ps.aeroginosa أيضًا من خلال التسلسلات، واستُخدم التحليل الظاهري لتقييم القدرة على إنتاج الأغشية الحيوية ومقاومة المضادات الحيوية. تم عزل العديد من الأنواع البكتيرية، مثل Pseudomonas aeruginosa و Klebsiella pneumoniae و Enterobacter spp، والتي توجد بشكل متكرر في حالات عدوى الحروق. أظهرت العديد من العزلات مقاومات عديدة للمضادات الحيوية، وخاصة السيفالوسبورينات والبنسلينات. تفاوتت قدرات العزلات على تكوين الأغشية الحيوية، وتتأثر مقاومة العلاج وتأخر التئام الجروح بشكل كبير بتطور الأغشية الحيوية.

Keywords: Burin wound, Molecular Identification, PCR,Sequences, Biochemical test.

INTRODUCTION

Human Skin

The skin, the largest anatomical organ in the human body, is responsible for several physiological functions, such as thermoregulation, proprioception, homeostasis maintenance, and protection from external dangers. Being in close contact with the outside world, the skin plays a significant role in fending off pathogen attacks; it is a man's physical barrier against infections. As a result, disorders that damage the integrity of the skin have numerous serious consequences.(Puca et al., 2021). Burns are more persistent and richer sources of infection than surgical wounds, mostly due to the bigger area involved and the lengthier hospital stay. They also provide an ideal environment for bacterial growth (Agnihotri et al., 2004).For hospitalized burn victims, infection is a leading cause of morbidity and mortality. Two Sepsis has been estimated to be responsible for over 75% of burn injury deaths, particularly in developing nations (Agnihotri, Gupta and Joshi, 2004).Furthermore, bacterial species and antibiotic susceptibilities must be regularly monitored in burns units due to the high rate of cross-infection caused by overcrowding. This is because significant changes in these data may be linked to changes in clinical management regarding the choice of medication for therapy, Understanding how microbial colonization varies over time on burn wounds is crucial for treating infections, particularly in cases of severe burns. Higher rates of morbidity and mortality are also linked to pathogenic resistance to frequently used antibiotics. Few studies have examined burn wounds, although several have examined the evolution of pathogens and bacterial resistance over time in hospital or burn infections. Furthermore, there was no statistical analysis of the time-related trends (Fu et al., 2012).Edema and burn shock come from the inflammatory response that burned eschar stimulates in the living tissues that are immediately deep and peripheral to the eschar, Burn edema, which is unique in that it develops more quickly than other forms of edema, which increases the risk of infection by reducing tissue oxygen transport and perfusion. (Park et al., 2017).Thus, early removal of eschar lowers mortality and length of hospital stay, as well as the incidence of systemic and local infections brought on by colonizing bacteria Temporary (Park et al., 2017).Because many bacterial infections are resistant to antimicrobial medicines, treating them presents significant hurdles. In both industrialized and developing nations, numerous investigations have been conducted to determine the most common multidrug-resistant (MDR) bacteria in burn units. Several coli-form bacilli, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae are among the common pathogenic bacteria found in infected burn victims (El Hamzaoui et al., 2020).

Among burn victims, bacterial infections are among the most frequent and dangerous side effects, increasing mortality and lengthening hospital stays. A significant factor in postponing wound healing and raising the risk of sepsis and systemic consequences is bacteria. To enhance diagnostic and therapeutic approaches, it is therefore essential to identify the bacterial species responsible for these diseases and examine their traits (Ekrami & Kalantar, n.d.).

Given the high frequency of burn infections in the area, this study is very crucial in order to improve our understanding of the primary pathogens and create more potent treatment plans to fight these infections and lower their effects on patients.The purpose of this study is to use genetic techniques to identify the bacterial species linked to burn injuries in the Anbar Governorate and to examine their phenotypic traits. By using contemporary methods to identify the genetic

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makeup of bacteria and investigate their capacity to create virulence factors and antibiotic resistance, this is accomplished.

MATERIALS AND METHODS.

100 samples were collected from individuals with burn wound infections for this study. Swabs were obtained from 100 patients with acute infections aged 12 to 65 years of both sexes. The pathological samples were collected between September 15, 2024 and January 17, 2025. Sterile cotton swabs were used to obtain a swab from the affected area. The samples were then cultured on culture media (MacConkey, Mannitol, Nutrient, Blood Medium, Mueller Agar, TSI) and cultured aerobically for 24 hours at 37°C. Biochemical tests including catalase and coacervase were performed on Gram-positive bacteria, and IMVC tests were performed on Gram-negative Enterobacteriaceae. After that, the susceptibility of each bacterial species was tested to determine their resistance to antibiotics. Then the highest number of species and the most resistant to antibiotics were taken and a biofilm test was performed to determine the extent of their formation, after which they were diagnosed at the molecular level and a sequencing was performed.

Antibiotic sensitivity test:

Twelve antibiotics disks manufactured as in the table 1-1, Liofilchem (Itali) were tested- for efficacy against *E.coli and Staphylococcus aureus* , Pseudomonas aeruginosa, Enterobacter aerogenes, Serratia marcescens Klebsiella pneumoniae, depending on disk diffusion method by Kirby Bauer(Mahal et al., 2023).The inhibition zone diameters was measured compared with CLSI 2024. (Lewis, 2024).

Table 1-1. Antibiotic discs were used in the study.

No.	Antibiotic (Symbol – concentration)	Manufacture (Origin)
1	Erythromycin (ER- 15 µg)	Bioanalyse (Turkey)
2	Clindamycin(CL-2 µg)	Bioanalyse (Turkey)
3	Ceftriaxone (CTF - 10 µg)	Bioanalyse (Turkey)
4	Lavofloxacin (LEV - 5 µg)	Bioanalyse (Turkey)
5	Gentamycin (GEN - 10 µg)	Bioanalyse (Turkey)

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6	Cefixime (CM-5 15 µg)	Bioanalyse (Turkey)
7	Meropenem (MRP - 10 µg)	Bioanalyse (Turkey)
8	Methicillin (MET - 10 µg)	Bioanalyse (Turkey)
9	Aztreonam (AZ-30 15 µg)	Bioanalyse (Turkey)
10	Piperacillin (PIT - 100 µg)	Bioanalyse (Turkey)
11	Cefepime (CEP-30 15 µg)	Bioanalyse (Turkey)
12	Tetracycline (TE – 10 µg)	Bioanalyse (Turkey)

Biofilm production test.

Biofilm testing was performed for the most antibiotic-resistant bacteria. Biofilm formation was examined according to (Coffey & Anderson, 2014) by the microtiter plate method (MtP), which is described as a quantitative method for biofilm production.

Molecular study.

Molecular identification of the most numerous bacterial species among the total samples, the most resistant to antibiotics and thus the most effective in forming biofilms was carried out.

Genomic DNA extraction

The bacterial isolates were cultured overnight in 5 ml of nutrient broth and incubated for 24 h at 37 °C. The genomic DNA extraction was performed according to the Presto™ Mini gDNA Bacteria Kit Quick Protocol (Geneaid) (Brown et al., 2020).

DNA concentration and purity estimation.

The DNA concentration and purity were measured via a NanoDrop OneC microvolume UV–vis spectrophotometer (Thermo Fisher Scientific, USA)

PCR preparation.

المجلة العراقية للبحوث الانسانية والاجتماعية والعلمية
Iraqi Journal of Humanitarian, Social and Scientific Research
Print ISSN 2710-0952 Electronic ISSN2790-1254

The total volume of the conventional PCR mixture was 25 µl, which was composed mainly of master mix (OneTaq® Quick-Load® 2X), free nuclease water, forward and reverse primer solutions, and a DNA template, as shown in Table 3-7. They were added together within PCR Eppendorf tubes and mixed completely by vortexing and microspinning.

Table 1-2. The volumes of conventional PCR components.

OneTaq® Quick-Load® 2X Master Mix	F-primer 10 pmol/µl	R-primer 10 pmol/µl	DNA template	Free nuclease water
12.5 µl	1 µl	1 µl	2 µl	8.5 µl

Molecular detection of *Staphylococcus aureus,Pseudomonas aeruginosa, Klebsiella pneumoniae* genes

In this study, The *Staphylococcus aureus, Pseudomonas aeruginosa* , , *Klebsiella pneumoniae* genes were detected via conventional polymerase chain reaction (PCR). By Housekeeping genes (*16S rRNA*)

The conventional PCR mixture was prepared as described in Table 3-8, and the thermal cycler program was set as follows, with a specific annealing temperature for each gene, as detailed in Table 1-3.

Table 1-3. The thermal cycler program for *S. aureus* gene amplification.

Stages	Steps	Temperature (°C)	Time (min.)	Number of cycles
1	Initial denaturation	94	3	1
2	Denaturation	94	1	33
	Annealing	53	1	
	Extension	72	1	
3	Final extension	72	7	1

Table 1-4. The thermal cycler program for *Ps. aeruginosa* gene amplification.

Stages	Steps	Temperature (°C)	Time (min.)	Number of cycles
1	Initial denaturation	94	2 min	1
2	Denaturation	94	40 sec	30
	Annealing	62	30 sec	
	Extension	72	30 sec	
3	Final extension	72	7 min	1

Table 1-5. The thermal cycler program for *K. pneumoniae* gene amplification.

Stages	Steps	Temperature (°C)	Time (min.)	Number of cycles
1	Initial denaturation	94	3	1
2	Denaturation	94	1	33
	Annealing	53	1	
	Extension	72	1	
3	Final extension	72	7	1

Detection of PCR products by electrophoresis

The agarose gel (1.5%) prepared previously was used to detect the presence and size of the amplified genes. After the gel solidified, the comb was removed, and the tank was filled with 1X TAE buffer. Then, 3 µL of a ladder (100 base pairs) was placed in the first well of the gel, followed by the addition of 2 µL of PCR product from each sample to their respective wells. The electrophoresis lid was placed, and the voltage was set at 90 V for the first 10 min and then reduced to 70 V for approximately 45 to 50 min. The amplified bands were then detected by a gel documentation device with "Image Lab" software.

Sequencing.

genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit, a fragment of the 16sRNA gene was amplified using primers, and PCR reactions were conducted in a total volume of 25 microliters, comprising 12.5 microliters of Master Mix, 1 microliter of each primer, 2 microliters of DNA, and 8.5 microliters of sterile water. The reaction conditions were 94°C for 5 minutes, followed by 35 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, and 72°C for 1 minute), and then 72°C for 10 minutes. The PCR products were then analyzed on a 1.5% agarose gel. purified and sent for Sanger sequencing to Macrogen in South Korea. In order to identify species, sequences were compared using BLAST against the NCBI database, and chromatograms were reviewed using BioEdit.

Results and Discussion.

Prevalence of burn injuries and associated resistant bacteria in patients with burn wounds in Ramadi city. 100 86 bacterial isolates were studied from 100 samples taken from burn wound patients in Ramadi city. It was found that Staphylococcus was the most common pathogen (54%), followed by Pseudomonas aeruginosa (9%), Enterobacter species, led by Klebsiella pneumonia (7%), Escherichia coli (5%), Enterobacter aerogenes (4%), Serratia marcescens (3%), while Staphylococcus haemolyticus, Enterococcus faecalis, Enterobacter cloacae complex, Serratia odorifera, and Klebsiella oxytoca were the only isolates. 14 samples out of 100 did not show growth. Biochemical tests were also performed for bacterial species, including the IMVC test for all enteric bacteria, the coagulase test for Staphylococcus aureus, and the oxidase test for all samples, with gram-negative enteric bacteria being negative for the test and gram-positive bacteria being positive for the test, and the catalase test....

These results confirm what is mentioned in medical literature about the prevalence of S. aureus as a major cause of infection, especially in clinical samples related to wounds and pus (Ghareeb et al., 2023; Shrestha & Rajbhandari, 2018) . However, the rate in this study (54%) is higher than the majority of previous studies, where the rate ranged between 19.9% and 27.9%. This may indicate a specificity in the nature of the samples, the clinicalenv ironment,or weaknesses in infection control procedures.

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Gram-Negative Bacterial Transmission:
Pseudomonas, Klebsiella pneumoniae, and E. coli isolates appeared in amounts comparable to those documented in other investigations. These kinds pose a serious problem since they are becoming more resistant to antibiotics, particularly in hospital (Li et al., 2024a)

According to recent research, Pseudomonas and Klebsiella resistance to antibiotics, particularly carbapenems, is linked to higher mortality rates and more challenging treatment outcomes (Tsai et al., 2025; Zhang et al., 2020)

Rare Isolates: The existence of serratia marcescens, serratia odorifera, and Enterobacter cloacae complex in trace amounts is consistent with the literature characterizing them as opportunistic infection causes that frequently manifest in patients with weakened immune systems or prolonged hospital stays (Li et al., 2024b, 2024c).

Conclusion of Comparison

The results of this study confirm the dominance of S. aureus as a major cause of infection, with a note of high rates compared to previous studies, which calls for a review of infection control policies
The distribution of the rest of the bacterial isolates is consistent with global trends, especially with regard to the spread of antibiotic-resistant bacteria in hospitals .

The results indicate the need to strengthen programs for rationalizing the use of antibiotics and monitoring infections within health institutions to reduce the spread of resistant bacteria.

Antibiotic sensitivity test:

This test is conducted to classify the isolates as MDR. after incubation at 37 ºC for 24 hours. The results showed that all staphylococcal isolates showed high resistance to methicillin at a rate of 100%,According to molecular research, all MRSA isolates possess resistance genes such as mecA and femA, which are essential for antibiotic resistance and influence the severity of the infection (Vittorakis et al., 2024) . Additionally, the community's MRSA frequency is rising, which complicates infection control, There are several risk factors for

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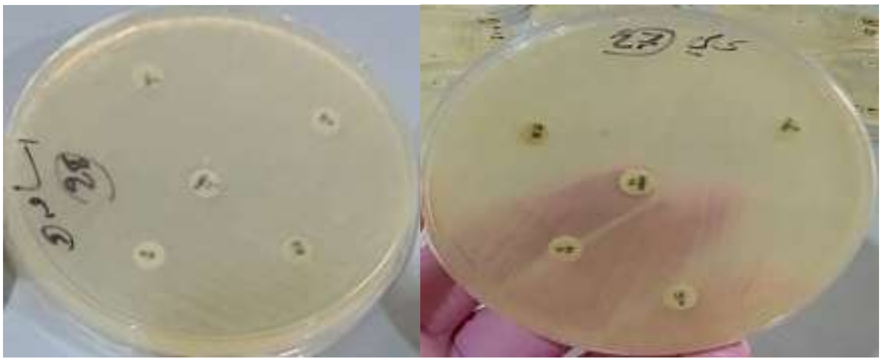
MRSA infection, such as extended hospital admissions, frequent use of antibiotics, internal medical devices or wounds, and close contact with infected individuals or healthcare personnel (Vittorakis et al., 2024).Comparing domestic and foreign research emphasizes how crucial it is to have a molecular and epidemiological understanding of this resistance in order to create successful treatment plans.

It is also becoming more difficult to treat infections caused by Staphylococcus aureus because of its growing resistance to several antibiotics, such as erythromycin (Er), tetracycline (Tet), levofloxacin (Lev), and meropenem (mero), at fairly comparable amounts.



Images showing the resistance of Staphylococcus aureus bacteria to antibiotics.

While the Enterobacteriaceae family in each of the bacteria E.coli, Klebsiella pneumonia, Serratia marcescens, Enterobacter aerogenes showed high resistance to the antibiotics Tet, Lev, Cef, Ceft, and Mero. Several studies have shown increasing resistance of Escherichia coli and Klebsiella pneumoniae to beta-lactam antibiotics, particularly cephalosporins and carbapenems, which are considered last-line antibiotics. For example, strains of K. pneumoniae producing carbapenemase (KPC) enzymes have been isolated in several countries, complicating treatment and limiting available options (Shaikh et al., 2015).

Research on the environment has also revealed that the Enterobacteriaceae family possesses a variety of antibiotic resistances, such as those to cephalosporins and tetracyclines, suggesting the existence of extensive environmental reservoirs that aid in the spread of this resistance (Younus, 2024).

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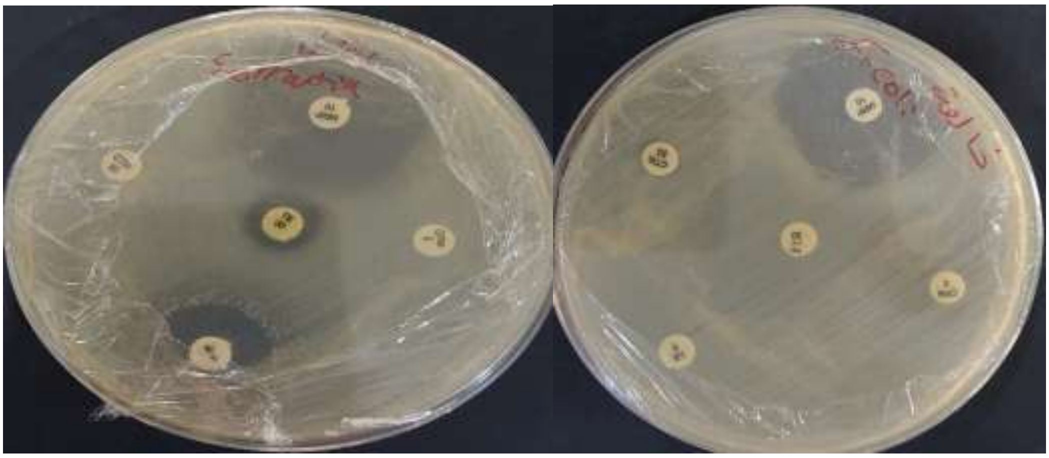
Furthermore, AmpC beta-lactamase enzymes, which are produced by Enterobacter species, confer extensive resistance to beta-lactam antibiotics. Because these enzymes frequently exhibit resistance to inhibitors like clavulanic acid, carbapenems or innovative combinations of beta-lactamase inhibitors are required as viable treatment alternatives (Younus, 2024)



Images showing the resistance of Enterobacteriaceae to antibiotics.

Pseudomonas aeruginosa isolates in the study had a high resistance rate of 76% to the medicines aztreonam and piperacillin, whereas the resistance rate to levofloxacin, meropenem, and cefepime was comparatively lower, reaching about 50%. The complicated nature and developing resistance of these bacteria, which are major sources of infections, particularly in hospital settings, are reflected in these results.

The potential of Pseudomonas aeruginosa to acquire additional resistance genes through plasmids and other genetic pathways, as well as its inherent and intrinsic resistance to numerous antibiotics, are well-known. Among the resistance mechanisms,

the Creation of carbapenemase enzymes, which break down beta-lactam antibiotics like azrinom and piperacillin, making therapy more challenging (Saha et al., 2025).

reduced expression of porin proteins, which lower drug permeability by letting antibiotics penetrate the cell,heightened efflux pump activity, which lowers the effective concentration of antibiotics by removing them from the cell,Resistance to fluoroquinolones like levofloxacin results from mutations in genes like gyrase (Elfadadny et al., 2024)

Images showing the resistance of *Ps. aeruginosa* to antibiotics .

Biofilm formation

***S.aureus**

According to the previously cited study, 53% of the highly resistant bacteria formed strong biofilms and 60% formed moderate ones, and only 6% were weak in biofilm formation. Demonstrating a clear correlation between antibiotic resistance and bacterial biofilm formation. Methicillin-resistant (MRSA) isolates are better at forming a robust biofilm than methicillin-susceptible (MSSA) isolates (Pokhrel et al., 2024).

*** Enterobacteriaceae (K.pneumoniae, E.coli, E.aerogenes)**

The presented study shows a marked variation in biofilm-forming abilities among Enterobacteriaceae genera.E.coli bacteria showed moderate biofilm formation،Klebsiella pneumoniae bacteria were 75% strong and only 25% moderate biofilm-forming This is consistent with what was found by (Karimi et al., 2021), while Enterobacter aerogenes bacteria were 100% moderate biofilm-forming While (Singh et al., 2024) found that strong biofilm formation by Enterobacter aerogenes was associated with delayed wound healing and increased antibiotic resistance.

Molecular Identification.

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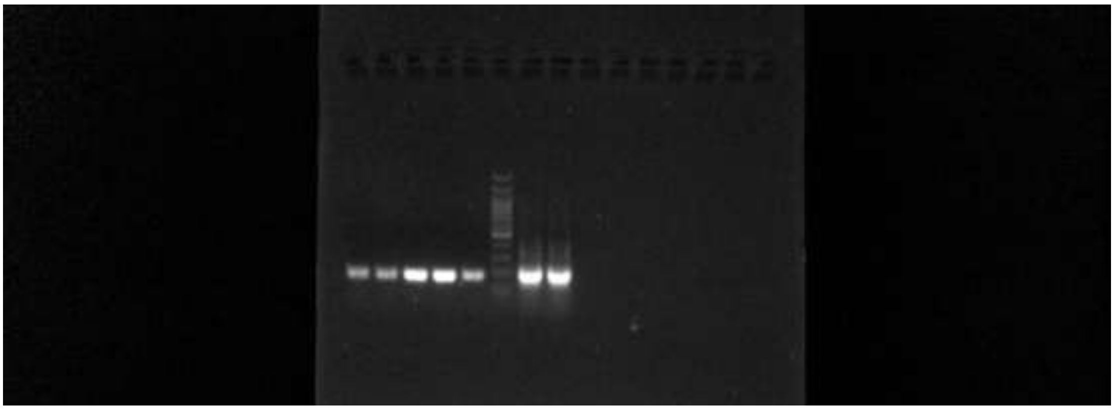
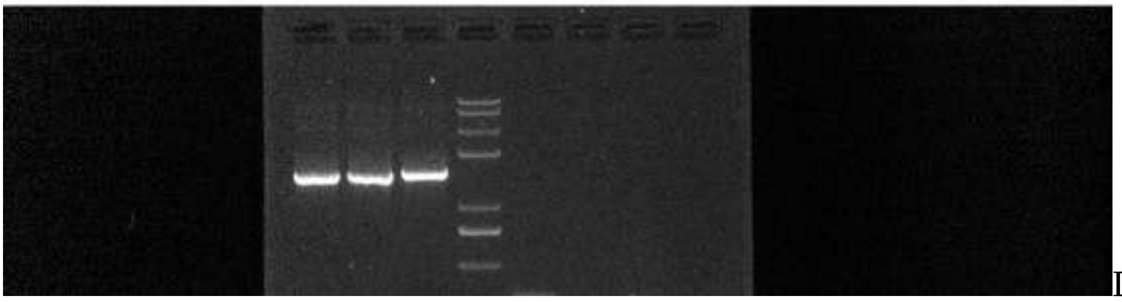
PCR identification.



Diagnostic genes for S.aureus 16sRNA gene are on the left of the image, and diagnostic genes for Ps.aeruginosa are on the right of the image**.**



Diagnostic genes for K. pneumoniae bacteria 16s rRNA gene

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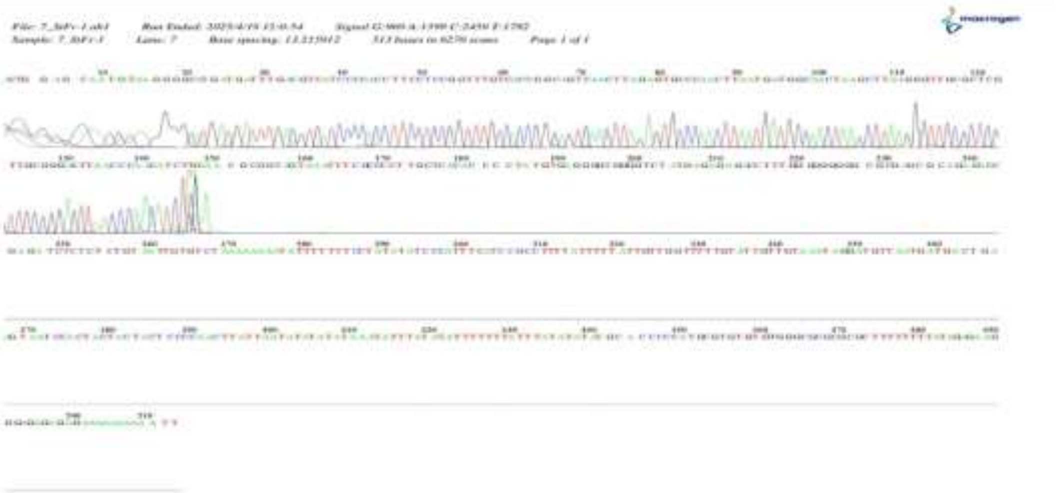
Sequences.



S.aureus forward primar.

k.pneumoniae forward primar.

Conclusion:

The study's conclusion highlights the significance of molecularly identifying the bacteria causing burn infections in order to direct the proper course of treatment, particularly given the high frequency of isolates that are resistant to antibiotics and their propensity to form biofilms, which makes treatment more challenging. In order to lower antibiotic resistance and enhance treatment results in burn units, the study suggests that precise antimicrobial susceptibility testing be performed for every case.This conclusion is supported by comparable results from recent research on burn bacteria in Iraq and the surrounding area, including the detection of virulence genes in isolates of Pseudomonas aeruginosa that are resistant to multiple drugs.

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