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# Study of Antimicrobial Resistance Bacterial Genes in Infective Endocarditis

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**ABSTRACT**

**Background:**Endocarditis is a dangerous inflammation of the heart's inner lining, known as the endocardium, which encompasses the chambers and valves. Endocarditis is typically induced by an infection. Bacteria, fungus, or other pathogens enter the bloodstream and adhere to compromised regions of the heart. In the absence of prompt intervention, endocarditis can impair or obliterate the heart valves.

**Method:** 230 Blood sample were collected from Infective Endocarditis patients, blood culture used for bacterial growth and identification of bacterial species were completed by microscopic examination, culture characteristics, and biochemical tests, and the use of the Vitek 2 diagnostic system for final identification of the isolated bacteria. Biofilm were detected by microtiter plate and Congo agar methods and Ethidium bromide used for efflux pumps dertection, icaAand norA genes for Staphylococcus spp. and mexR gene for Pseudomonas aeruginosa were detected by PCR.

**Results:** 51 positive culture for bacterial growth, 18 were staph. aureus bacteria and 7 were P. aeruginosa. Staphylococci were the most biofilm forming (9) positive by the CRA method and (8) moderately forming by the MTP method, while P. aeruginosa were the least biofilm forming by both methods (1),(1). Staphylococcus showed positive results at various concentrations of the dye, while the bacteria Pseudomonas aeruginosa were positive for the efflux pumps only at concentrations (1,2) of the dye. icaA gene were found in 21.4% and 25% of S. aureus and S. epidermidis respectively, while norA gene found in 7.1% of S. aureus and mexR gene in 25% of P.aeruginosa.

**Conclusion:** Staph. aureus and P. aeruginosa have many virulence factors genes that contributed in the infective Endocarditis infection and they were resistant to many antibiotics due to these genes.

**Keyword:** Biofilm, Endocarditis, PCR, Efflux pump.

## INTRODUCTION

Endocarditis is a perilous inflammation of the heart's inner lining, known as the endocardium, which encompasses the chambers and valves. Endocarditis is typically induced by an infection. Bacteria, fungus, or other pathogens infiltrate the bloodstream and adhere to compromised regions of the heart. In the absence of prompt intervention, endocarditis may impair or obliterate the heart valves (1).

The majority of cases are attributed to viridans streptococci, Streptococcus gallolyticus, Staphylococcus aureus, coagulase-negative staphylococci, HACEK organisms (Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella), Pseudomonas aeruginosa, and enterococci. Less common organisms encompass pneumococci, Candida, gram-negative bacilli, and polymicrobial entities (2).

Endothelial damage permits either direct infection by pathogenic organisms or the formation of an uninfected platelet-fibrin thrombus, which serves as a reservoir for transitory bacteremia, with the exception of S. aureus, which can infect intact endothelium. These organisms infiltrate the bloodstream via the skin, mucosal surfaces, or previously infected areas and cling to nonbacterial thrombi resulting from valvular injury or turbulent blood flow. In the absence of host defenses, this bacterium proliferates, creating tiny colonies and disseminating into the bloodstream. Left-sided infections are far more prevalent than right-sided infections, with the exception of intravenous drug users (3).

Antibiotic resistance poses a substantial risk to contemporary healthcare delivery. The ESKAPEE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp., and Escherichia coli) are particularly difficult to treat owing to their inherent and acquired capacity to swiftly evolve resistance mechanisms in reaction to environmental challenges. The formation of biofilm is a crucial element in the development of antimicrobial resistance and tolerance. Numerous investigations have indicated the role of efflux pumps in antibiotic resistance, both directly through drug extrusion and indirectly through biofilm development (4).

icaA is a gene that facilitates biofilm formation in S. aureus and S. epidermidis, contributing to the adhesion of bacterial cells to membranes. This gene is regarded as a principal virulence factor, as it confers protection against antibiotics, enhances the bacteria's resistance to the host's immune defenses, and increases their capacity to adhere to and colonize surfaces. The norA gene is an integral component of the key chromosomal genes in S. aureus, responsible for encoding efflux pumps and facilitating elevated multidrug resistance, hence significantly contributing to the antibiotic resistance of S. aureus(5). The norA gene is regarded as the most extensively researched efflux mechanism for S. aureus, first found and documented in 1986 in a Japanese hospital in fluoroquinolone-resistant bacteria. Drug efflux pumps confer broad-spectrum antibiotic resistance to microorganisms by expelling foreign compounds from their cells. P. aeruginosa possesses many efflux systems, comprising at least ten pumps, with the most well-characterized system being the MexR-OprMoperon(6,7).

## METHODS

### Study design

This study included 230 patients suffering from heart disease and bacteremia (115 males and 115 females), whose ages ranged between 20-85 years and were admitted to the resuscitation unit, cardiac catheterization unit, internal medicine unit, and dialysis unit in Kirkuk Teaching Hospital and Azadi Teaching Hospital, in addition To 30 groups as a negative control in the time period from October 2023 to March 2024.

230 blood samples were collected from patients with heart disease and bacteremia who suffered from fever, chills, shortness of breath, high blood pressure, and tachycardia. Ten ml of blood was collected by drawing blood from the patient's vein using a vacutainer syringe. Ten ml of blood was injected into two 5 ml blood culture bottles sterilely (5 ml for aerobic culture and the other for anaerobic culture) and the bacteria causing the bloodstream infection were isolated. Take blood samples directly into two aerobic and anaerobic blood culture bottles containing Brain Heart Infusion Broth and the anticoagulant Sodium-Polyanethole Sulfonate SPS.

### Laboratory Diagnosis

### Primary culture on brain heart infusion

Blood culture bottles were examined daily for a duration of up to 7 days for evident indicators of bacterial proliferation, including medium turbidity, colony formation on the surface of red cells (cotton balls), hemolysis, gas production, and clot formation.

### Subculture

Suspected secondary cultures from blood cultures were done directly on blood agar, MacConkey agar, Cetramideagarand chocolate agar on days 1, 3, and 7. All isolates were incubated aerobically and anaerobically at a temperature of (35-37°C) for 24 hours, then suspicious colonies were selected for final microscopic examination, culture characteristics, and biochemical tests, and the use of the Vitek 2 diagnostic system for final identification of the isolated bacteria.

### Biofilm detection

The microtiter plate method and Congo agar method were employed for the detection of bacterial biofilms.

### Efflux pumps detection

Ethidium bromide stain was used to detect efflux pumps in various bacterial isolates obtained from patients with infective endocarditis.

### Molecular diagnosis

### DNA Extraction

The kit was used to extract genomic DNA from Bacteria was DNA Presto™Mini.

### Examination of the purity of the extracted DNA

The extracted DNA was detected by using a Nanodrop spectrophotometer, which is a special device for detecting and measuring the concentration of nucleic acid. DNA is detected by determining the concentration of nucleic acid (ng\ml) (DNA) and measuring the purity of the nucleic acid by reading Absorbance at a wavelength between 260/280 nm.

### Preparation of polymerase chain reaction components

The polymerase chain reaction mixture was prepared using the PCR PreMix kit prepared by the Korean company Bioneer, according to the company’s instructions.

**Table 1:** Components of Monoplex PCR master mix and their volumes

| Component | 20 µlreaction volume |
|---|---|
| Template DNA | Variable (3 µl) |
| Forword primer (10 pmole,ul) | 1.0 µl |
| Reverse primer (10pmole,ul) | 1.0 µl |
| Nuclease Free Water | 15 µl |
| Total volume | 20 µl |

**Table 2:** Conditions used in the PCR thermocycler condition

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Pre-denaturation | 95 ºC | 5min | 1 cycle |
| Denaturation | 95 ºC | 30 sec | 30cycles |
| Annealing | 58 ºC | 30sec | 30cycles |
| Extension | 72 ºC | 30sec | 30cycles |
| Final extension | 72 ºC | 5min | 1 cycle |

**Agarose gel electrophoresis**

This method was used to separate DNA molecules of different sizes, and electrophoresis was performed as stated in (Mohammed et al., 2014).

**Primers used for gene detection**

**Table 3:** Primers used to detect genes

| Primers | Sequence of necliotides 3’ | Gene size target bp |
|---|---|---|
| icaA | GCACAATGAAAACGAAAAGG | 259 |
| icaA | ATGAGTCCAGCCATATTCTTTTTC | |
| norA | TTCATATGATCAATCCCCTTT | 391 |
| norA | ACCACCAAACGGCGATATAA | |
| mexR | AACTACCCCGTGAATCTCGAC | 360 |
| mexR | GGCAAACAATCTCGTCATGC | |

**Results**

The result of present study showed 51(22.1%) were positive for cultural growth while 179(177.8%) were negative, as showed in table (4).

**Table 4:** Results of blood cultures for endocarditis patients and the control group

| Result | IE patients | Controls |
|---|---|---|
| Positive | 51 (22.1%) | 0 |
| Negative | 179 (177.8%) | 30 (100%) |
| Total | 230 (100%) | 30 (100%) |

The result of bacterial culture revealed 34 (14.78%) were gram positive bacteria while 17 (7.39%) of isolates were gram negative bacteria, figure (1)

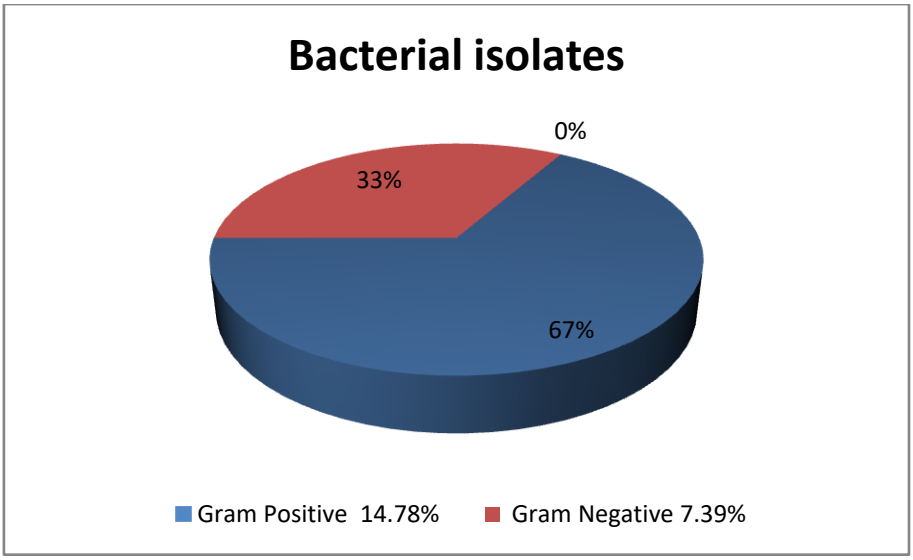


**Figure 1:** Distribution of bacterial isolates that caused IE

Isolation results showed that out of 51 positive samples, (18) samples were S. aureus 7.83%, (5) samples were S. epidermidis 2.17%, and (3) samples were Micrococcus luteus 1.30% and the rest of the isolates are as shown in Table (5), where the isolates were identified based on the phenotypic characteristics of the colonies growing on the culture media, biochemical tests, and molecular tests.

**Table 5:** Types of bacterial isolates that cause endocardial infections and bacteremia

| **Isolates** | **No.** | **Precentage(%)** |
|---|---|---|
| Gram positive | 34 | 14.78 |
| S. aureus | 18 | 7.83 |
| S. epidermidis | 5 | 2.17 |
| S. haemolyticus | 2 | 0.87 |
| S. hominis | 2 | 0.87 |
| E. faecalis | 2 | 0.87 |
| Micrococcus luteus | 3 | 1.30 |
| Micrococcus lylae | 2 | 0.87 |
| Gram negative | 17 | 7.39 |
| E. coli | 5 | 2.17 |
| K. pneumoniae | 5 | 2.17 |
| P. aeruginosa | 7 | 3.04 |
| Total | 51 | 100 |

Staph aureuswere identified based on their phenotypic characteristics after growing them on blood agar medium under aerobic conditions. The results showed large, smooth, raised, low-convex, opaque colonies. Most of the colonies were dyed in a creamy yellow color, as in Figure (2), surrounded by clear areas. (Areas of beta hemolysis). As for its growth on mannitol salt agar medium, it produces small colonies and the medium turns yellow because the bacteria grow in high concentrations of salinity and have the ability to ferment mannitol sugar and produce acid, which works to change the color of the medium's reagent from red to yellow.

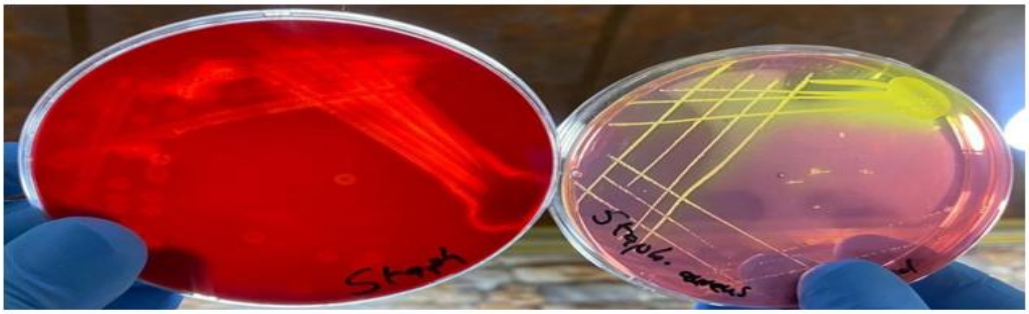


**Figure 2:** Staph aureus isolate on Blood agar and mannitol agar

P. aeruginosa grow on MacConkey agar medium and produce circular, smooth, pale-colored colonies because they do not ferment the sugar lactose. They have an odor similar to the smell of sweet or fermented grapes, and often produce the bluish-green pigment pyocyanin in cteramide agar, as in Figure (3).

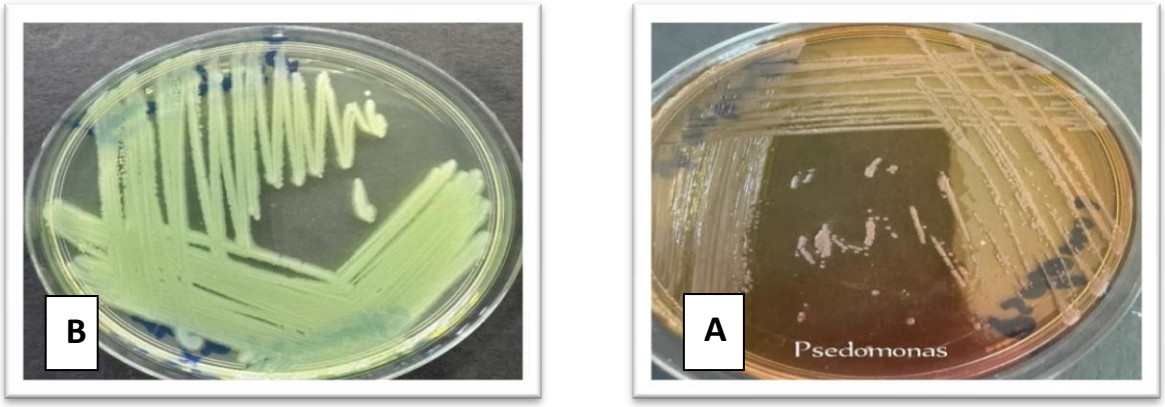


**Figure 3:** A-P. aeruginosa on MacConkey agar B-P. aeruginosa on cetramide agar

**Biofilm formation**

The biofilm formation of bacteria was detected by two methods: microtiter plate and Congo red agar. The results showed that staphylococci were the most biofilm forming (9) positive by the CRA method and (8) moderately forming by the MTP method, while P. aeruginosa were the least biofilm forming by both methods (1),(1) as shown in Table (5).

**Table 5:** Biofilm detection in bacterial spp.

| Bacteria | CRA | | | MTP | | |
|---|---|---|---|---|---|---|
| | Positive | Moderate | Negative | Positive | Moderate | Negative |
| S. aureus | 9 | 7 | 2 | 2 | 8 | 3 |
| P. aeruginosa | 1 | 1 | 4 | 1 | 1 | 4 |

**Detection of efflux pumps**

Ethidium bromide dye was used to detect efflux pumps in various bacterial isolates obtained from patients with infective endocarditis. The results of the current study showed that most isolates of Staphylococcus showed positive results at various concentrations of the dye, while the bacteria Pseudomonas aeruginosa were positive for the efflux pumps only at concentrations (1,2) of the dye used as shown in Table (6) and Figure (4).

**Table 6:** efflux pumps detection in bacterial spp.

| Bacteria | Ethidium bromide(mg/ml) | | |
|---|---|---|---|
| | 0 | 1 | 2 |
| S. aureus | + | + | + |
| P. aeruginosa | - | + | + |

**Figure 4:** Efflux pumps appearance in different concentration of Ethidium bromide

**Detection of the icaA gene**

The results of our study showed that the detection rate of the icaA gene in Staphylococcus aureus and S. epidermidis bacteria using PCR technology was positive for (3) out of 4 S. aureus isolates, at a rate of 75% as shown in Figure (5).

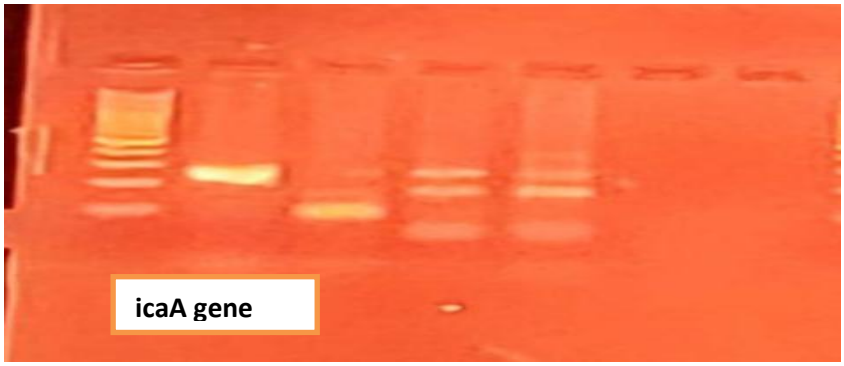


**Figure 5:** showed the product of the PCR reaction with a size of 259 and 378 bp for the genetic detection of the icaA gene of S. aureus and, which was migrated in an agarose gel at a concentration of (1)% at a voltage difference of (70) volts for (60) minute.

**Detection of the norA gene**

The results of study showed that the detection rate of the norA gene in Staph. aureus bacteria using PCR technology. The results were positive for (2) isolates out of 4, at a rate of 50% as shown in Figure (6).

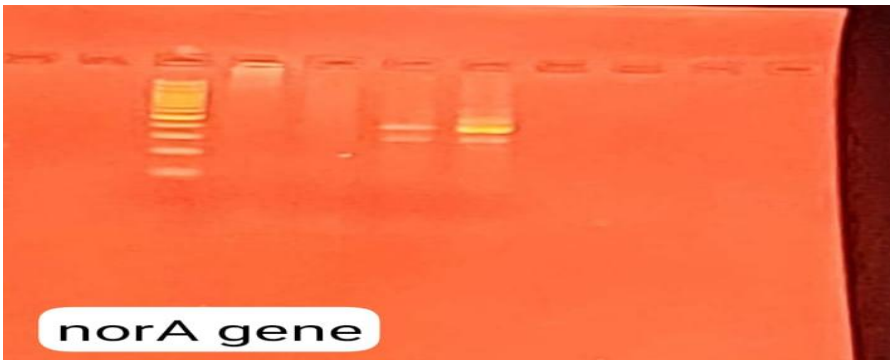


**Figure 6:** showed the product of the PCR reaction with a size of 391 bp for the genetic detection of the norA gene of bacteria S. aureus which was migrated in an agarose gel at a concentration of (1)% at a voltage difference of (70) volts for (60) minute.

**Detection of the mexR gene**

The results of study showed that the detection rate of the mexR gene in P. aeruginosa bacteria using PCR technology. The results were positive for (4) isolates, at a rate of 100% as shown in Figure (7).

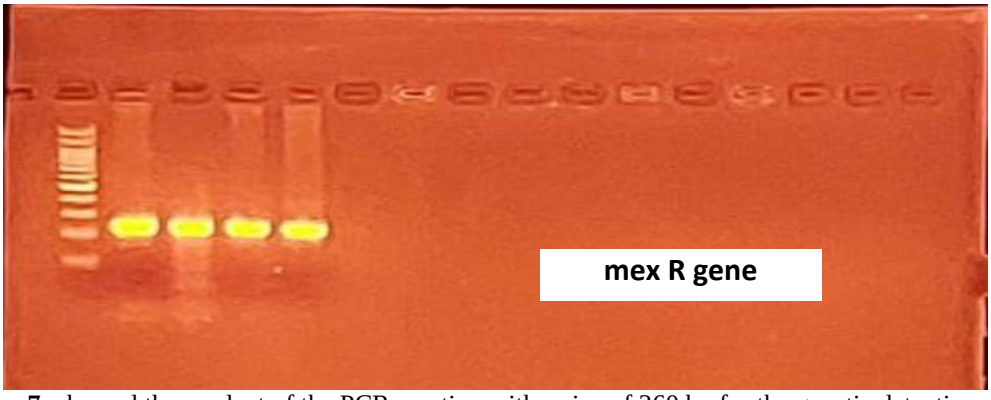


**Figure 7:** showed the product of the PCR reaction with a size of 360 bp for the genetic detection of the mexRgene of bacteria S. aureus which was migrated in an agarose gel at a concentration of (1)% at a voltage difference of (70) volts for (60) minute.

## DISCUSSION

Infective endocarditis is an uncommon yet lethal form of sepsis, exhibiting an overall death rate of 20 to 25% in most studies(8). The results showed that the most common causative agent of infectious endocarditis due to Gram-positive aerobic bacteria was mainly Staphylococcus aureus bacteria, as this bacterium was found to be associated with an increased mortality rate, followed by S. epidermidis, which is the most common species causing this disease, as well as E. faecalis, Micrococcus luteus, S. haemolyticus, and less common microbial causes of IE for aerobic Gram-negative bacteria were E. coli, K. pneumoniae, and P. aeruginosa. This study agreed with a study (9). Staphylococcus aureus, the primary etiological agent of infective endocarditis, has demonstrated significant manipulation of immunothrombosis and flourishes in the fibrin-rich milieu of endocarditis vegetations. The coagulation system, due to its pivotal role in infective endocarditis, presents a compelling therapeutic target for this lethal condition. Nonetheless, a precarious equilibrium exists, and the use of antithrombotic agents in patients with endocarditis frequently entails a significant risk of hemorrhage(10).

One of the most important properties of biofilms is that bacteria can resist antibiotics at high doses. A previous study showed that P. aeruginosa bacteria have the ability to form a biofilm that helps resist antibiotics and spread disease(11). The pathogenicity resulting from Staphylococcus aureus infection is of note of importance as one of the community-associated bacterial infections, due to the virulent ability of these bacteria to produce biofilms. A study conducted in Baghdad showed that biofilm examination showed that 46.15% of the isolates were strong biofilm producers. 46.15% of them had an average ability to produce biofilms, and 7.70% were weak producers(12).

Patel et al. (13) demonstrated that multidrug-resistant efflux pumps facilitate resistance to antimicrobials and biocides in Staphylococcus aureus. Ethidium bromide serves as a substrate for nearly all well-characterized multidrug-resistant (MDR) efflux pumps, except for NorC, prompting an investigation into the efficacy of basic MIC assessments with this molecule for detecting efflux-competent bacteria. Numerous investigations have demonstrated that drug efflux is a significant contributor to the development of resistance to antibiotics and other antimicrobials in S. aureus. Multidrug efflux pumps (MDR EPs) are noteworthy for their ability to expel a diverse array of chemically distinct antimicrobials and are often linked to multidrug phenotypes in bacteria. In S. aureus, around twenty potential multidrug-resistant efflux pumps are encoded in the chromosomes(14).

A recent study by Elmosallamy et al., (15) showed that efflux pumps play an important role in mediating antibiotic resistance in P. aeruginosa isolates. The relationship between the phenotype and genotype, especially the spread of the MexA and MexR genes, confirms the importance of efflux pumps in multidrug resistance in isolated P. aeruginosa strains.

Recent study indicates that S. aureus isolates possess different biofilm-forming abilities, with the presence of these biofilm-associated ica genes clearly showing roles as biofilm-producing markers (16). A study conducted by (17) revealed that 75% of MRSA isolates possessed the icaA gene, with the intercellular adhesion (ica) locus comprising the icaADBC factor, which includes four genes encoding the principal proteins required for PIA production. The initial two genes, icaA and icaD, are pivotal in the biosynthesis of exopolysaccharides. The research aimed to ascertain if norA constitutes a component of the core genome of S. aureus. This subject is pertinent as a significant number of studies on efflux-mediated resistance in this pathogen mostly concentrate on NorA activity, and some research has indicated the absence of the norA gene in certain S. aureus strains(18).

The study conducted by (19) showed that most Pseudomonas aeruginosa isolates possessed the mexR gene, which is considered an important gene for efflux pumps. In a study (20), active efflux pump genes (mex A, mex R) were detected in all multidrug-resistant P. aeruginosa isolates. This is consistent with (21) in Iraq, which reported the presence of a pump gene. Active efflux (mexR) in all MDR isolates and with (22) who found that all isolates (100%) showed the efflux pump (MexA,R).

## CONCLUSION

Staph. aureus and P. aeruginosa have many virulence factors genes that contributed in the infective Endocarditis infection and they were resistant to many antibiotics due to these genes.

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