

Isolation and Identification of a Biofilm-Forming *Staphylococcus epidermidis* Isolated from Clinical Sources

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ABSTRACT

Background: *Staphylococcus epidermidis*, a commensal bacterium found on human skin and mucosa, is a major opportunistic pathogen that causes nosocomial infections related to indwelling devices. **Objective:** Find the link between the multidrug-resistant *S. epidermidis* and the development of biofilm. **Methodology:** A total of 125 clinical samples were collected from various clinical sources from Ghazi Al-Hariri Hospital and Baghdad Teaching Hospital during the study period from October 2023 until January 2024. *S. epidermidis* was identified according to conventional cultural, microscopical characteristics, and biochemical tests. In addition, the identification is confirmed using the VITEK 2 System. Susceptibility testing of all twenty clinical isolates of *S. epidermidis* was performed against 10 antibiotics from several antibiotic classes. DNA was extracted from all isolates, and the presence of the *mecA* gene was investigated using PCR. The biofilm-producing ability was tested for all isolates, and an optimization study was conducted for the higher-producing isolate. **Results:** The results showed that 20 (16%) isolates exhibited identical morphological characteristics and biochemical profiles consistent with *S. epidermidis*. They also showed that most *S. epidermidis* isolates (85%) were multidrug-resistant (MDR), with the highest resistance rate (90%) observed against cefoxitin, whereas the lowest resistance rate (10%) was observed against tetracycline. To test the ability of biofilm formation, the results indicated that all bacterial isolates were biofilm producers at different levels; strong biofilm was detected in 4 (20%) of the tested isolates, 13 isolates (65%) were able to form moderate biofilms, and 3 isolates (15%) were able to form weak biofilms. **Conclusion:** Most of the *S. epidermidis* isolates were (MDR), all of them were able to form biofilm and contained *mecA* gene.

Keywords: *Staphylococcus epidermidis*, Antibiotic susceptibility, Biofilm assay, *mecA* gene.

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INTRODUCTION

The commensal bacterium *Staphylococcus epidermidis* can be detected on human skin and mucous membranes in the intestinal and pulmonary tracts, as the most frequent cause of infections linked to implants. *S. epidermidis* is a classic example of an opportunistic biofilm-forming bacterium. More recent research indicates that some *S. epidermidis* strains may have a role in the etiology of prevalent skin conditions (1,2). However, a significant contributor to nosocomial infections is associated with implanted medical devices (3,4).

Microbial biofilms have emerged as a serious concern for the modern healthcare system in recent years, with their frequency and the threat of antibiotic resistance increasing daily among the general public. The biofilm has a significant influence on infectious disorders, particularly infections linked with indwelling devices such as catheters, implants, artificial heart valves, and prosthetic joints (5).

Furthermore, it has been documented that the biofilm-producing *S. epidermidis* weakens host defenses, including bacterial phagocytosis. Consequently, the only practical way to eliminate the infection is to remove the medical device. This has an impact on morbidity and places a significant financial burden on public health systems (6). Antimicrobial resistance is extremely common in *S. epidermidis*. Methicillin (β -lactam) resistance in *S. epidermidis* is mediated by the *mecA* gene, with a frequency of up to 90% (7). The *mecA* gene, which encodes the penicillin-binding protein PBP2a, is associated with methicillin resistance and reduced affinity for β -lactam antibiotics (8). It is found on a mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (9). The current study aimed to investigate the association between multidrug resistance in *S. epidermidis* and biofilm development.

Methodology

Sample collection

In this study, the samples were collected from October 2023 to January 2024. A total of one hundred and twenty-five clinical samples were collected from cerebrospinal fluid, knee fluid, tissues, blood, wounds, and pus cavities from patients (both genders and different ages) from hospitals in Baghdad (Ghazi Al-Hariri Hospital and Baghdad Teaching Hospital).

Identification of bacterial isolates

All clinical samples were cultured in Brain Heart Infusion Broth at 37°C overnight, followed by culturing on Mannitol Salt Agar. Each plate was incubated at 37°C overnight, and then a single isolated colony was transferred into Hichrome Agar. Then, a single colony was cultured on Blood Agar. Numerous morphological, microscopic, and biochemical tests (Coagulase, Catalase, Oxidase, Citrate utilization, Urease, and Motility test) were used to identify each bacterial isolate. The VITEK 2 System was used to identify each bacterial isolate (99%).

Antibiotic susceptibility test

The disc diffusion technique was used to test ten different antibiotics (Cefoxitin 30 μ g, Gentamicin 10 μ g, Azithromycin 15 μ g, Erythromycin 15 μ g, Tetracycline 30 μ g, Ciprofloxacin 5 μ g, Levofloxacin 5 μ g, Clindamycin 2 μ g, Trimethoprim 5 μ g, and Rifampin 5 μ g) on a Mueller-Hinton agar according to (10).

Detection of biofilm development

The microtiter plate assay, 96-wells, based on (11) was used to determine the potential of bacterial isolates to produce biofilm as follows:

In a 96-well polystyrene microtiter plate, 100 μ l of *S. epidermidis* broth with a turbidity equal to 0.5 McFarland and an equal volume of 2 \times Luria Bertani (LB) broth supplemented with 20% glucose were added to each well, and plates were incubated at 37°C overnight, while the control well contained 200 μ l of LB broth supplemented with 20% glucose. Then, the cultures were gently withdrawn, and the wells were cleansed three times with phosphate-buffered saline. Cells that adhered were fixed with 100% methanol for 10 min, followed by 15 min staining with 0.4% crystal violet. The wells were left to air-dry after being cleaned three times with sterile distilled water. Next, 250 μ l of 33% acetic acid was added to each well for 15 min. The plate was then placed in the plate reader, and the optical density (OD) of the stained adhering bacteria was measured at 595 nm. These OD values were interpreted as indicators of bacterial adhesion to the surface and biofilm production. Biofilm formation was quantified using the formulas in Table (1).

Table (1): Classification of bacterial isolates according to OD of NC (negative control) in the microtiter plate assay method

Mean OD value of NC	Adherence Biofilm Formation
$OD_s \leq OD_c$	non-biofilm
$OD_c < OD_s \leq 2 \times OD_c$	weak biofilm
$2 \times OD_c < OD_s \leq 4 \times OD_c$	moderate biofilm
$4 \times OD_c < OD_s$	strong biofilm

OD_c= the mean of three optical densities of a negative control; OD_s= the mean of three optical densities of each *S. epidermidis* isolate.

DNA extraction

Genomic DNA was extracted from *S. epidermidis* isolates according to the protocol of the Wizard Genomic DNA Purification Kit (Promega, USA). Extracted DNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Fischer Scientific, USA). The genomic DNA was stored at -20°C.

Detection of the *mecA* gene by PCR

The presence of the *mecA* gene DNA was detected by polymerase chain reaction using specific forward and reverse primers for *mecA* (571-bp) designed in this study using the Primer3 Plus program (<https://www.primer3plus.com/>) and checked for specificity using the Primer-Blast program (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The forward primer had the following sequence: 5'-TTGGCCAATACAGGAACAGCA, and 5'-GGTGGATAGCAGTACCTGAGC was used as a reverse primer.

The reaction mixture was brought to a total volume of 20 µl and amplified in a thermal cycler (Techne, UK). The parameters of thermal cycling were set as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of three steps, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with a single cycle at 72°C for 5 min for extension.

RESULTS

Collection of samples

Results revealed that among 125 samples, only 20 isolates (16%) exhibited identical morphological characteristics and biochemical profiles consistent with *S. epidermidis*. On the other hand, 105 isolates were identified as other types of bacteria, and *S. aureus* ranked first, as shown in Figure (1).

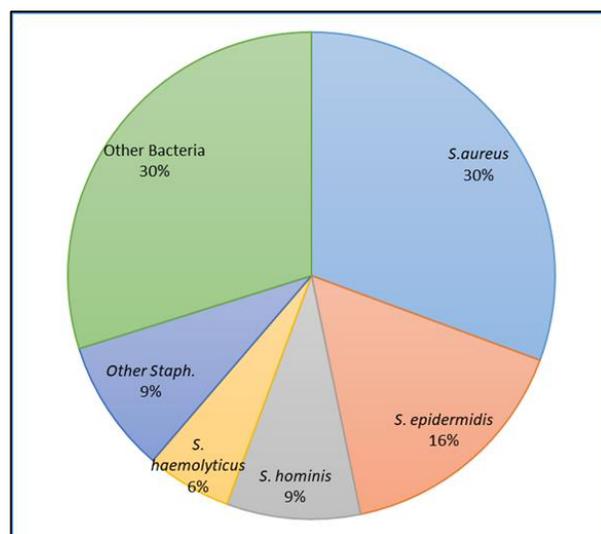


Figure (1): Distribution of the isolated bacterial species.

Isolation and identification of *S. epidermidis*

Cultural appearance on different cultural media

Morphological identification of *S. epidermidis* isolates was primarily achieved by investigating the appearance of colonies on different culture media, as can be seen in Table and Figure (2).

Table (2): Colony Morphology of Bacterial Isolates on Different Culture Media

Media	Colony's structure and/or appearance	Reference
Mannitol Salt Agar	Colonies are small, pink or white, creamy, moist, and rounded in shape	(12)
Blood agar	Colonies appear as small, white-to gray	(13)
Hicrome Agar	Colonies are blue colored	(14)
Nutrient agar	Colonies are small, pale-cream	(15)
Chocolate agar	Colonies are white and raised	(16)

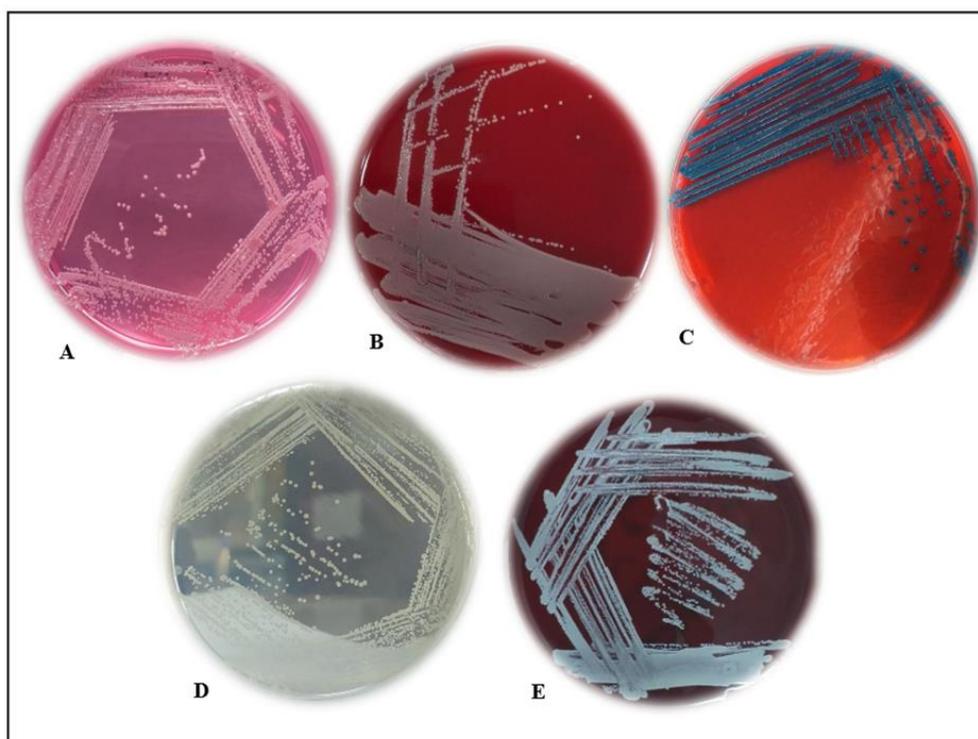


Figure (2): Growth of *S. epidermidis* colonies on different culture media: A- MSA, B- Blood Agar, C- Hicrome agar, D- Nutrient Agar, and E- Chocolate Agar after aerobic incubation of 18-24 h at 37°C.

Biochemical test

Biochemical tests of *S. epidermidis* isolates are based on the production or absence of enzymes thereof to visualize a biochemical change with a substrate and the colony of bacteria, as can be seen in Table and Figure (3), which was confirmed by the VITEK 2 system with a probability of 99% as *S. epidermidis*.

Table (3): Biochemical Test Results of the Isolate

Test	Result	Reference
Catalase	+	(17)
Oxidase	-	(18) (19)
Coagulase	-	(20)
Urease	+	(21)
Simmon Citrate	-	(22)
Motility	-	(23) (24)

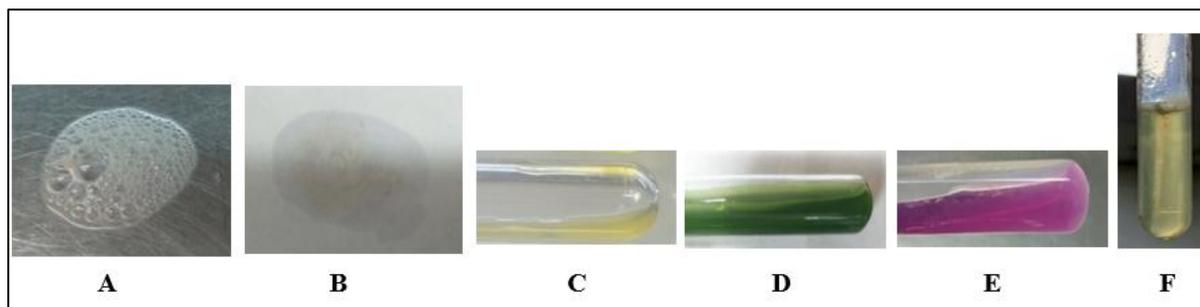


Figure (3): Result of Biochemical test of *S. epidermidis* (A- Catalase +ve, B- Oxidase -ve, C- Coagulase -ve, D- Citrate -ve, E- Urease +ve, F- Motility -ve).

Antibiotic susceptibility test of *Staphylococcus epidermidis*

The results are listed in Table (4), Figure (4), compared with the published data updated by the (10):-

Table (4): Antibiotic sensitivity test of *S. epidermidis* isolates against different antibiotics

Class	Antibiotic Discs (Symbol)	Antibiotic Concentration (µg)	No. of Isolates (%)		
			Resistant	Intermediate	Sensitive
Beta-lactam	Cefoxitin (FOX)	30	18 (90)	0 (0)	2 (10)
Aminoglycoside	Gentamicin (CN)	10	12 (60)	1 (5)	7 (35)
Macrolides	Azithromycin (AZM)	15	15 (75)	2 (10)	3 (15)
	Erythromycin (E)	15	15 (75)	0 (0)	5 (25)
Tetracycline	Tetracycline (TE)	30	2 (10)	4 (20)	14 (70)
Quinolones	Ciprofloxacin (CIP)	5	6 (30)	4 (20)	10 (50)
	Levofloxacin (LEV)	5	7 (35)	4 (20)	9 (45)
Lincosamide	Clindamycin (CD)	2	7 (35)	4 (20)	9 (45)
Antifolates	Trimethoprim (TR)	5	9 (45)	0 (0)	11 (55)
Ansamycins	Rifampin (RIF)	5	3 (15)	0 (0)	17 (85)

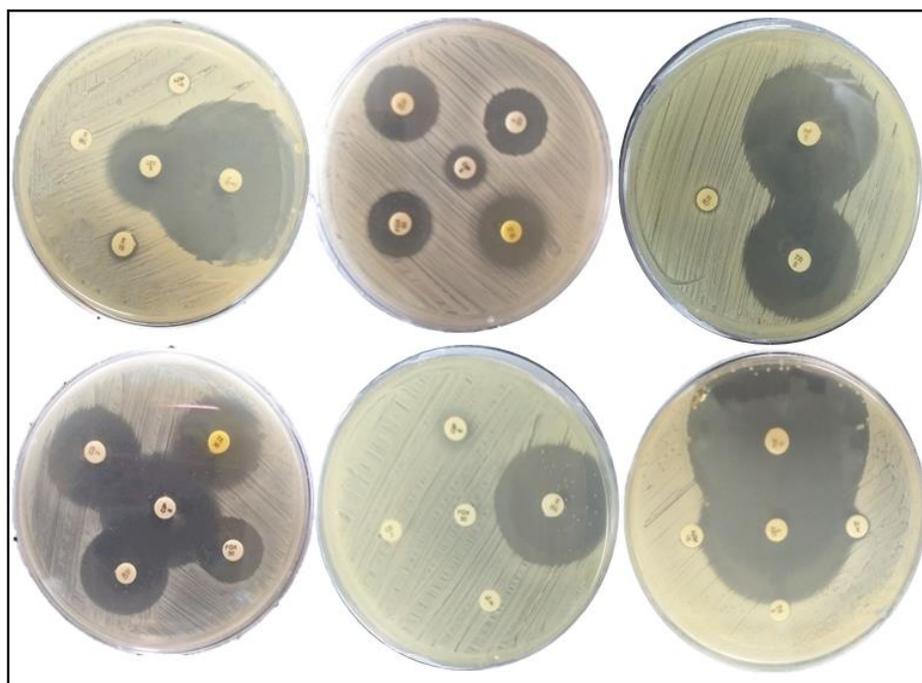


Figure (4): Antibiotic susceptibility test of *S. epidermidis* isolates on Muller-Hinton Agar with different types of antibiotics incubated at 37°C for 24 h.

Biofilm production profile

The obtained results indicated that from a total number of 20 isolates, biofilm formation was observed in all isolates. Strong biofilm formation was observed in 4 (20%) of the tested isolates, whereas 14 (65%) of them were able to form moderate biofilms. On the other hand, only 3 (15%) isolates were recognized as weak biofilm formers, as shown in Table (5).

Table (5): Classification of bacterial isolates according to their biofilm formation in a microtiter plate assay method.

Average OD value	Type of biofilm formation	No. of isolates (%)
$ODs \leq 0.157$	non-biofilm	0 (0%)
$0.157 < ODs \leq 0.314$	weak biofilm	3 (15%)
$0.314 < ODs \leq 0.628$	moderate biofilm	13 (65%)
$0.628 < ODs$	strong biofilm	4 (20%)

Detection of the antibiotic resistance *mecA* gene in *S. epidermidis* isolates

DNA extraction and purification were carried out for all 20 *S. epidermidis* isolates. DNA concentrations for all *S. epidermidis* DNA samples were in the range of 30-427 ng/μl, and the purity of the DNA was 1.9 to 2.1.

By PCR assay, the amplification of the *mecA* gene in the chromosomal DNA of *S. epidermidis* produced an amplicon of size 571 bp in Figure (5). The present results showed that the prevalence of the *mecA* gene was 20(100%) in the DNA of isolates.

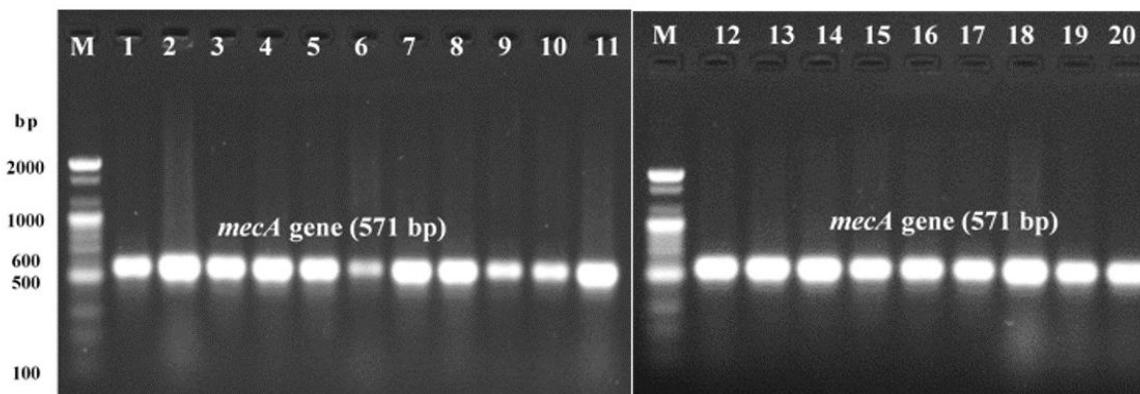


Figure (5): Gel electrophoresis of the amplified *mecA* gene of *S. epidermidis* isolates. Electrophoresis was done on 1% agarose at 75 V for one hr. Lane M: DNA ladder marker (100 bp), Lanes 1-20: amplified *mecA* gene (571 bp) of *S. epidermidis* isolates.

STATISTICAL ANALYSIS

Statistical analysis was performed using Microsoft Excel (Microsoft Office 2021). Descriptive statistics were used to summarize the frequency and percentage distributions of bacterial isolates, antibiotic resistance profiles, and biofilm formation categories. The prevalence of the *mecA* gene among isolates was expressed as a percentage. Optical density (OD) readings for biofilm quantification were analyzed by calculating the mean for each isolate and comparing them with the negative control to classify biofilm production levels according to standard interpretation criteria. All data were presented as frequencies, percentages, and graphical illustrations.

DISCUSSION

Morphological identification of *S. epidermidis* isolates was primarily based on colony morphology: small, pink, or white, creamy, and moist on MSA medium (12). This medium is a differential and selective medium containing sodium chloride in high concentrations (7.5%) to prevent the growth of bacterial species other than *Staphylococci* (13), the Hicrome agar also contains high concentration of sodium chloride, which helps in inhibiting the accompanying microflora and mannitol for differentiation between *S. aureus* that fermented mannitol and give greenish colored colonies and *S. epidermidis* which does not ferment mannitol hence blue colored colonies are observed according to the manufacturer's instruction.

The positive catalase result in the present study is a key feature of the isolated *S. epidermidis* strains, indicating their ability to catalyze the decomposition of hydrogen peroxide into water and oxygen (17). At the same time, they were negative for oxidase due to the absence of cytochrome oxidase (18,19), as well as for coagulase test, so they were unable to convert plasma to clot (20), also the Simmon Citrate (SC) test was negative for *S. epidermidis*, SC is known to detect the ability of an organism to utilize energy from citrate as a sole carbon source, if it can, it will raise the pH so that it changes the color of bromothymol from green to blue (21,22). *S. epidermidis* strains were positive in the Urease test due to the production of urease, which converts urea to ammonia, thereby creating an alkaline pH. A positive result is indicated by a change in the medium color (21,19).

The high resistance of *S. epidermidis* to a wide range of commonly used antibiotics has been considered a major problem that threatens the medical system as well as the community, and reduces antibiotic options. The antibiotic susceptibility profile for the *S. epidermidis* isolates against ten types of antibiotics (Cefoxitin 30 µg, Gentamicin 10 µg, Azithromycin 15 µg, Erythromycin 15 µg, Tetracycline 30 µg, Ciprofloxacin 5 µg, Levofloxacin 5 µg, Clindamycin 2 µg, Trimethoprim 5 µg, and Rifampin 5 µg) that act to inhibit the bacteria through different mechanisms was tested.

Eighteen isolates (90%) appeared to be resistant to Cefoxitin. This result was close to that reported in (25), with a resistance rate of 93.3%. Fifteen isolates showed high resistance to Azithromycin (75%) and Erythromycin (75%). Asante and his group reported the same result, stating that the Azithromycin and Erythromycin resistance rates were 75% (26). Twelve isolates were resistant to Gentamicin (60%). Eladli and his group reported a close result, stating that the Gentamicin resistance rate was 54.29% (27). Resistance by 9 isolates (45%) against Trimethoprim, as well as 7 isolates (35%) against Clindamycin. The result was close to the work of (25), where Clindamycin was resistant (33.3%). While 7 isolates (33.3%) were resistant against Levofloxacin, this result was close to (28), who reported that Levofloxacin resistance was (30.4%). There was low resistance to Ciprofloxacin (30%). These results were similar to those of (29), who reported close results when they stated that the Ciprofloxacin resistance rate was (23.2%). For Tetracycline (10%), and Rifampin (15%) the result was very close to the work of (27) with a rate of (14.29%).

The results demonstrated that all *Staphylococcus epidermidis* isolates formed biofilms, a key factor contributing to bacterial persistence and antibiotic resistance, thereby complicating treatment. Biofilm environments are known to enhance the acquisition of resistance traits, findings consistent with previous studies (30,31,32). Additionally, all isolates harbored the *mecA* gene, which accounts for their resistance to β -lactam antibiotics, including methicillin. This resistance is mediated by the *mecA*-encoded penicillin-binding protein (PBP2a), which has reduced affinity for β -lactam antibiotics, consistent with earlier reports (33, 34).

Conclusion

The present study concluded that most of the *S. epidermidis* isolates (85%) were multi-drug resistant (MDR) and all of them contained antibiotic-associated gene (*mecA*), also all twenty isolates could form biofilm but in different levels for several reasons, such as presence of genes responsible for biofilm formation and the influence of changes in temperature, pH, nutrient content, surface properties and other factors.

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ETHICAL APPROVAL

This study was approved by the ethical committee of the Biotechnology Department College of Science/ Al-Nahrain University Approval letter Rec.Cob./0429/10.

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عزل وتشخيص المكورات العنقودية البشرية المكونة للأغشية الحيوية المعزولة من المصادر السريرية

صفا زهير غازي ، ياسين اسماعيل المعموري

قسم التقنيات الاحيائية الجزيئية والطبية ، كلية التقنيات الاحيائية ، جامعة النهرين ، الجادرية ، بغداد ، العراق

الخلاصة

خلفية عن الموضوع: المكورات العنقودية البشرية، وهي بكتيريا متعايشة موجودة في جلد الإنسان والغشاء المخاطي، هي أحد مسببات الأمراض الانتهازية الرئيسية التي تسبب التهابات المستشفيات المرتبطة بالأجهزة الساكنة. **الهدف:** معرفة العلاقة بين مقاومة الأدوية المتعددة لعزلات المكورات العنقودية البشرية وتطور الاغشية الحيوية. **المواد وطرق العمل:** تم جمع 125 عينة سريرية من مصادر سريرية مختلفة من مستشفى غازي الحريري ومستشفى بغداد التعليمي خلال فترة الدراسة من تشرين الأول 2023 حتى كانون الثاني 2024. تم التعرف على المكورات العنقودية البشرية وفقا للخصائص الزراعية والمجهرية وكذلك الاختبارات الكيميائية الحيوية. علاوة على ذلك، تم تأكيد تشخيص العزلات البكتيرية باستخدام نظام الفايتهك. تم إجراء اختبار الحساسية لجميع العزلات السريرية العشرين لـ البكتيريا ضد 10 مضادات حيوية مختلفة من عدة فئات من المضادات الحيوية ، تم استخراج الحمض النووي من جميع العزلات وتم التحقق من وجود جين *mecA* باستخدام تقنية تفاعل البوليميراز المتسلسل . **النتائج:** أظهرت أن 20 عزلة (16%) أعطت صفات مورفولوجية واختبارات كيميائية حيوية متطابقة لعزلات المكورات العنقودية البشرية. كما أظهرنا أن معظم عزلات البكتيريا (85%) كانت مقاومة للعديد من الادوية (المقاومة المتعددة للمضادات الحيوية) مع أعلى معدل مقاومة (90%) لوحظ ضد السيفوكسيبتين بينما لوحظ أقل معدل مقاومة (10%) ضد التتراسيكلين. **الاستنتاج:** معظم عزلات البكتيريا كانت من نوع (المقاومة المتعددة للمضادات الحيوية)، وكانت جميعها قادرة على تكوين الأغشية الحيوية وتحتوي على الجين المقاوم للميثيسيلين (*mecA*).

الكلمات المفتاحية: المكورات العنقودية البشرية ، حساسية المضادات ، الحيوية فحص الغشاء الحيوي ، جين (*mecA*)