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Detection of some virulence factors in Staphylococcus epidermidis isolated from clinical specimens in Iraq

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Abstract :

120 samples were collected from different clinical samples including swabs from wounds, noses, and pimples, blood, CSF, and medical equipment (catheters for dialysis and heart valves), among different age groups and sexes, from Gazzi Al-Hariri Hospital and Baghdad Teaching Hospital, during the period from Dec. 2022 to Aug. 2023. The ages from 10 to 65 years old were the range of the study. According to the results, 73 (60.83%) of the samples tested positive for Staphylococcus epidermidis, and the 47 females (64.38%) were more infected than the 26 males (35.61%). The age group of (36–50 years) showed a considerable increase in the infection ratio, followed by (25-35 years), then the age group of (51-65 years) and (10-24 years). The current study was performed in Baghdad Teaching Hospital/Department of Bacteriology. Differential and specific culture media were used to confirm the isolated clinical specimens. Virulence factors and biochemical tests were performed. The diagnosis was verified with the Vitek 2 system. Antibiotic sensitivity testing was achieved by applying the disk diffusion method. Ten S. epidermidis isolates were chosen, and their DNA was extracted and amplified using PCR to detect and validate the presence of the (mecAand ermA) genes. The current study used male mice as a model to perform the lethal dose (LD50). The mice weighed between 22 and 30 g and were between the ages of 2 and 3 months. And in the College of Veterinary Medicine, the mice were sheltered in the animal house. The mice were treated with the bacterial suspension that was adjusted to 0.5 McFerland standard, while, the control group was treated with phosphate buffer saline (PBS), and the mice were daily monitored. The mice exhibited transient behavioral abnormalities, decreased movement, and lethargy when compared with the control group. The skin showed pictures of severe dermatitis. When the mice died, their affected skin, liver, intestine, and heart were taken and preserved in the formalin to send them to the laboratory of histopathology. The histopathology techniques were performed in the histopathology laboratory-College of Veterinary Medicine. Normal cytoarchitecture and appearance were seen in the histopathology figures of the control group. While, the figures of the histopathology of the injury group showed severe hepatitis, severe myocardial infarction, severe enteritis, and the skin appeared with severe dermatitis.

Keywords: Virulence factors; Differential culture media; Antibiotic sensitivity test; Lethal dose 50 (LD50); Molecular study.

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الكشف عن بعض عوامل الضراوة للمكورات العنقودية البشروية المعزولة من عينات سريرية في العراق

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الخلاصة :

هدفت هذه الدراسة الكشف عن عوامل الضراوة للمكورات العنقودية البشروية المعزولة من عينات مختلفة والتي تشمل مسحات الجروح وحب الشباب و مسحات الانف, وكذلك مسحات من الأجهزة الطبية مثل (انابيب قسطرة القلب وغسل الكلى), الدم, سائل النخاع الشوكي. لقد تم جمع 120 عينة من مختلف الجنسين والفئات العمرية من مستشفى بغداد التعليمي ومستشفى غازي الحريري خلال الفترة من كانون الأول/ 2022 إلى آب /2023. وكانت الفئة العمرية للدراسة تتراوح بين 10 سنوات الى 65 سنة. ووفقا للنتائج التي تم التوصل إليها، تم اختبار 73 (60.83%) من العينات على أنها إيجابية بالنسبة إلى *S. epidermidis*. وكانت الإصابة في الإناث 47 (64.38%) أكثر من الذكور 26 (35.61%). واظهرت الدراسة ارتفاع نسبة الإصابة بدرجة كبيرة في الفئات العمرية (50–36 سنة)، (35–25 سنة)، ثم الفئات العمرية (65–51 سنة)، تليها الفئات العمرية (10–24 سنة). وقد أجريت الدراسة الحالية في مستشفى بغداد التعليمي/مختبرالتشخيص البكتيري. حيث تم استخدام أوساط زرعية تفريقية وتشخيصية لتأكيد تشخيص العينات السريرية المعزولة. وقد أجريت فحوصات عوامل الضراوة، والاختبارات الكيميائية الحيوية، وتم استخدم جهاز الفايتيك للتأكد من التشخيص. وتم اجراء اختبار الحساسية للادوية المضادة باستخدام طريقة انتشار الأقراص. وقد اختيرت (10) عزلات من *S. epidermidis* لاستخراج ال DNA و تفاعل البلمرة (PCR) لتحديد وتأكيد وجود الجينين (*mecA*, *ermA*). كما استخدمت الدراسة الحالية ذكور الفئران كنموذج لأداء الجرعة المميتة (LD50). وقد اختيرت الفئران بأعمار تتراوح بين شهرين وثلاثة أشهر و22 إلى 30 غراماً من الوزن، وتم حض الفئران في بيت الحيوان في كلية الطب البيطري. وتم مراقبة الفئران يومياً. حيث ظهرت على الفئران تغيرات سلوكية مؤقتة مثل عدم الراحة، قلة الحركة عند مقارنتها بمجموعة السيطرة. كما ان هناك التهاب جلدي حاد. وبعد موت الفئران تم اخذ الأعضاء المصابة كالجلد والكبد والأمعاء والقلب وحفظهم في الفورمالين لإرسالها إلى مختبر الهستوباثولوجي التابع لكلية الطب البيطري. حيث اظهرت نتائج مجموعة السيطرة صور طبيعية للانسجة, بينما أظهرت بقية النتائج التهاب كبدي حاد، والالتهاب القلبي الحاد، والتهاب معوي حاد، وكذلك هناك التهاباً جلدياً حاداً.

الكلمات المفتاحية: أوساط تفريقية; فحص الحساسية للأدوية; عوامل الضراوة; الفحص الجيني; الجرعة المميتة (LD50).

Introduction

One of the most common human microflora that colonizes the mucosal membrane and skin is the coagulase negative staphylococci (CoNS) [1]. Systemic and local infections are belonging to this species [2]. *Staphylococcus epidermidis* is an example of CoNS that is considered a significant nosocomial infection of humans and animals [3]. *Staphylococcus epidermidis* is a common source of nosocomial infection due to its genetic characteristics and ubiquity, which enhances its resistance to severe environmental conditions. [4]. This agreed with a local study that revealed *Staphylococcus epidermidis* was considered an opportunistic pathogen [5]. MIRZAEI and YOUSEF said in their studies that " Methicillin-resistant *Staphylococcus epidermidis* (MRSE) bacteria are being recognized as true pathogens as they are able to resist methicillin and commonly form biofilms. *Staphylococcus epidermidis* is also considered a reservoir of drug resistance genes " [6]. Staphylococcal

chromosomal cassette mec (SCCmec) facilitates the acquisition of the *mecA* gene, which makes *S. epidermidis* resistant to methicillin. [7]. Some isolates tested as methicillin-susceptible phenotypically, despite having the *mecA* gene in their genomes [8]. The formation of biofilm is the reason for the pathogenicity of CoNS and is considered one of their virulence factors, in addition to lipase, gelatinase, and hemolysin [9]. *Staphylococcus epidermidis* possessed low virulence factors that were suggested by previous studies, while recent studies confirmed that *Staphylococcus epidermidis* can acquire virulence from *Staphylococcus aureus* with increased resistance to methicillin [10]. *Staphylococcus epidermidis* showed its ability to colonize animals such as mice and cause entero-pathogenic effects on the internal organelles and skin of mice. *S. epidermidis* are considered life-threatening pathogens to humans and animals [11].

Materials and methods

1. Sample collection

120 different clinical samples were collected from different age groups and sexes. These clinical specimens include blood, cerebrospinal fluid (C.S.F), swabs from nasal, wound, and acne, and medical devices (dialysis catheters and heart valves). The research period was from Dec. 2022 to Aug. 2023. The clinical specimens were collected from Ghazi Al-Hariri Hospital and Baghdad Teaching Hospital.

2. Bacterial isolation and identification

The collected specimens were inoculated on the blood agar and nutrient agar and then incubated at 37ºC for 24 hours. The isolated colonies were examined for their shape, size, color, pigments, and haemolytic activity. Then, selective and differential media were used to cultivate the resulting colonies. The plates were incubated at 37ºC for 24 hrs. The Identification of S. epidermidis isolates were detected by morphological characteristics on culture media, biochemical tests, antibiotic sensitivity tests, vitek-2 system, virulence factors tests, molecular study, and detection of the pathogenicity of the bacteria in mice has been done.

2.1 Virulence factors

Specific culture media were used to detect virulence factors, such as egg yolk agar for lipase (12). Gelatinase agar for the detection of gelatinase, DNase agar for detecting of enzyme deoxyribonuclease, and skim milk agar for protease (13).

2.2 Biochemical tests

Biochemical tests were performed for further investigation, these tests include slide coagulase, Gram staining, Voges-Proskauer (VP), and urease [14]. Catalase, hydrogen sulfide production test, hemolysis, and motility were performed for the isolated colonies [15].

2.3 Antibiotic sensitivity test

Antibiotic susceptibility testing was achieved on Mullar-Hinton agar, by disk diffusion method, using eight antibiotic disks: methicillin, novobiocin, gentamicin, erythromycin, penicillin, trimethoprim, tetracycline and ciprofloxacin [16].

2.4 VITEK 2 system

The VITEK-2 system confirms the isolated bacteria. The range of positive isolation probabilities was 92-99%. [17].

3. Biofilm assay

The biofilm assay was achieved using the microplates with U-bottom and 96 wells. The 1% glucose was added to the brain heart infusion (BHI) broth, and 2 ml of this broth was used to sub-culture *Staphylococcus epidermidis*. 200µL of the prepared bacterial suspension that was adjusted to the 0.5 McFarland standard was added to the wells of the plates, and incubated at a temperature of 37 °C for 24 hours. The wells were drained gently, and phosphate-buffered saline (PBS) was used to wash the wells three times. After air drying, the produced biofilm was fixed with 200µl of absolute methanol for 15 min. Wells drained and air dried, the wells were stained with 200µL of (0.05%) crystal violet for 5 min., then, drained, and washed with PBS three times, and air dried. 200µL of ethanol (absolute) was added to the wells. The enzyme-linked immunosorbent assay (ELIZA) reader was used to read the optical density at 630 nm [18].

4. Molecular study

PCR amplification and DNA extraction were used to detect and validate the existence of the (*mecA*, *ermA*) genes, using specific primers. Electrophoresis on agarose gel was performed in a comparison with DNA ladder marker 100 pb.

5. Lethal dose 50 (LD50)

LD50 (the median lethal dose leading to the death of 50% of the animals). The College of Veterinary Medicine provided the lab mice housing in its animal house. The age of the mice was from 2 to 3 months and the weight of 22-30 g. Male mice (15 total mice) were classified into three groups: control group, intraperitoneally injection group, and skin group (and there were 5 mice in each group). The control group was treated with phosphate-buffered saline. In the second group, the mice were injected intraperitoneally with a bacterial suspension that adjusted to the 0.5 McFerland standard, which contains 1.5 x 108 cells/ml. In the third group, the superficial layers of the skin were scratched and contaminated by the bacterial suspension. Then, the clinical condition of the mice was monitored; after two weeks, symptoms of lethargy and infection started to appear. When the mice died, they were dissected and preserved in formalin to send the selected internal organs and skin to the laboratory for histopathology[19]. The Histopathology techniques were performed in the histopathology laboratory- College of Veterinary Medicine.

6. Statistical analysis

The statistical analysis was performed using SPSS version 26. Mathematical methods (Mean and Standard Deviation) were used, and the statistical significance was determined by the Chi-square test.

Result and Discussion

The current study was performed on 120 specimens of different sexes and age groups. The results showed that 73 samples were positive for Staphylococcus epidermidis. These clinical specimens were collected from blood, C.S.F, acne, wound, nasal, swabs, and medical devices (dialysis catheters and heart valves), during the period from December 2022 until August 2023, and they were collected from Ghazi Al-Hariri-Hospital and Baghdad Teaching Hospital. The study population's age range was 10 to 65 years old. Age was distributed among 73 positive cases 26 (35.61%) males and 47 (64.38%) females, and this showed that the females were affected more than males. Furthermore, there was no significant difference (P-value > 0.05) between the type of samples and the sex, as shown in Table 1.

Table 1: Distribution the type of specimens among sexes of the isolated S. epidermidis

Type of sample	No.	Male	%	Female	%	P-value (Chi-square test)
Blood	29	8	27.85	21	72.41	0.213*
Wound swab	8	3	37.5	5	62.5	0.238*
Nasal swab	7	3	42.85	4	57.1	0.223*
Acne swab	7	3	42.85	4	57.1	0.157*
C.S.F.	7	2	28.57	5	71.42	0.223*
Heart valve	8	5	62.5	3	37.5	0.223*
Dialysis Catheter	7	2	28.57	5	71.42	0.157*

Total	73	26	35.61	47	64.38	
*** P-value > 0.05 (Non-Significance)**						
Mean		3.71		6.71		
SD		2.138		6.343		

The majority of the respondent cases were in the age group of (36 to 50) years, followed by the age group of (25 to 35) years, and (51 to 65) years, and then the age group of (10 to 24) years, as shown in table 2.

Table 2: Distribution the type of samples among age groups of the isolated S. epidermidis

Type of sample	Age groups								Total	%	P-value (Chi-square)
	(10-24)	%	(25-35)	%	(36-50)	%	(51-65)	%			
Blood	2	6.89	10	34.48	15	51.72	2	6.89	29	39.72	0.238*
Wound swabs	1	12.5	3	37.5	2	25	2	25	8	10.95	0.238*
Nasal swabs	2	40	3	60	2	40	-	-	5	6.84	0.223*
Acne swabs	4	57.1	3	42.85	-	-	-	-	7	9.59	0.157*
C.S.F.	2	28.57	-	-	3	42.85	2	28.57	7	9.59	0.223*
Heart valve	-	-	3	37.5	3	37.5	2	25	8	10.95	0.223*
Dialysis catheter	-	-	-	-	3	42.85	4	57.1	7	9.59	0.157*
*** P-value > 0.05 (Non-Significance)**											

The current study revealed that the high percentage of positive cases reported from blood samples (29, 39.72%) among the total isolates of 73 samples appeared much higher than the other patient specimens collected from different cases. A previous study in the Afzalipour Teaching Hospital in Kerman, Iran also agreed with our study and showed that the clinical isolates obtained from blood were 77% [20].

1. Identification of *Staphylococcus epidermidis*

The *S. epidermidis* was collected from different clinical specimens and the diagnosis is done by specific tests depending on routine laboratory techniques. Firstly, the samples were cultured on different culture media, and the results are as follows:

1.1 Identification of Nutrient Agar

The colonies appeared white, 2-3 mm in diameter, raised with a round shape and complete edges, the resulting colonies were sub-cultured on other differential and selective media, and other investigations were performed.

1.2 Identification of blood agar

The colonies of bacteria appeared white, raised, cohesive, and about (1–2) mm in diameter after overnight incubation at 37ºC, and it was not hemolytic bacteria (gamma hemolysis) on blood agar.

1.3 Identification of mannitol salt agar

The colonies appeared pink after 24 hours, at 37ºC, on mannitol salt agar, and without producing a change in the color of the medium, because *S.epidermidis* did not ferment the mannitol.

1.4 Identification of chrome agar

The colonies appeared blue in color because the Staphylococcus epidermidis did

not ferment the D-mannitol contained in the medium, this medium was a selective chromogenic medium.

2. Antibiotic sensitivity test

Different patterns of antibiotic susceptibility were shown by S. epidermidis, as shown in Table 3. The highest rate of resistance (100%) was seen with erythromycin, penicillin, tetracycline, trimethoprim, methicillin (95.89%), novobiocin (84.93%), followed by the lowest resistance rate of ciprofloxacin (71.23%), gentamicin (41.09%). An intermediate resistance was seen with gentamycin (24.65%). Sensitivity patterns appeared with gentamycin (34.24%) ciprofloxacin (28.76%), novobiocin (15.06%), and methicillin (4.11%). A local study showed agreement with current one in resistant patterns of *Staphylococcus epidermidis* towards antibiotics [21].

Table 3: Antimicrobial susceptibility test of the isolated S. epidermidis

NO.	Antibiotics	Resistance	%	Intermediate	%	Sensitive	%
1	**Novobiocin**	62	84.93	-	-	11	15.06
2	**Methicillin**	70	95.89	-	-	3	4.11
3	**Erythromycn**	73	100	-	-	-	-
4	**Gentamicin**	30	41.09	18	24.65	25	34.24
5	**Penicillin**	73	100	-	-	-	-
6	**Tetracycline**	73	100	-	-	-	-
7	**Ciprofloxacin**	52	71.23	-	-	21	28.76
8	**Trimethoprm**	73	100	-	-	-	-

3. Tests for some virulence factors and biochemical tests

The virulence factors of *Staphylococcus epidermidis* were detected by using selective and differential culture media, such as skim milk agar for protease, DNase for DNA enzyme, egg yolk agar for lipase and nutrient gelatin agar for gelatin, and the results revealed that *S.epidermidis* had protease and lipase enzymes and lacked DNase and gelatinase enzymes. The biochemical tests revealed that *S. epidermidis* was coagulase-negative, non-motile and Voges-Proskauer VP, hydrogen sulfide (H_2S), and urease were positive and showed gamma hemolysis. The current study agreed with a local one that performed biochemical tests like the coagulase test that was used to differentiate Coagulase-Positive Staphylococci from other Coagulase-Negative Staphylococci [22].

4. Biofilm formation

The 36 (49.31%) of the S. epidermidis isolates were strong biofilm producers, 27 (36.98%) moderate biofilm producers, and 10 (13.70%) weak biofilm producers. as in Table 4, and according to the micro-titer plate assay, as shown in Figure 1.

Table 4: Biofilm formation of the isolated S. epidermidis

Biofilm	Strong	Moderate	Weak
Type of sample	36	27	10
Total %	49.32%	36.99%	13.70%

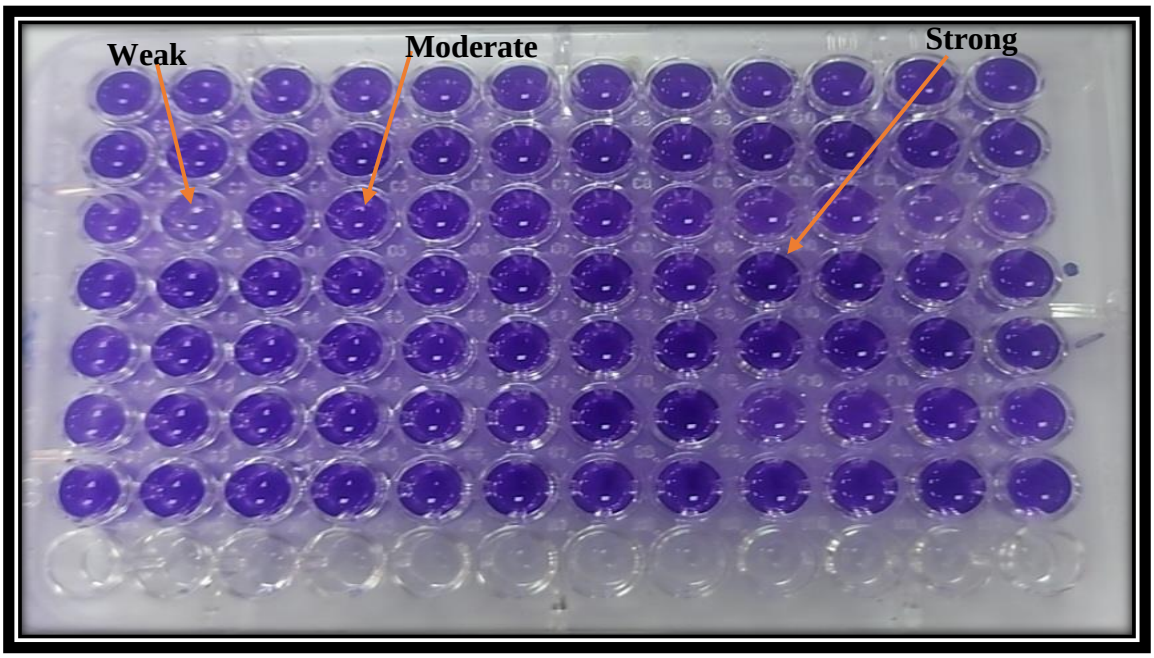

Figure 1: A micro-titer plate showed biofilm stained with crystal violet of the isolated S. epidermidis

5. *Staphylococcus epidermidis* identification using a molecular technique (convolutional)

The PCR amplification and DNA extraction were performed to detect and validate the existence of the (mecA, ermA) genes with specific primers. After the process of extraction, the electrophoresis was carried out on an agarose gel in comparison with a DNA ladder marker of 100 pb., to identify DNA fragments and identify the outcome of the PCR interaction in the presence of standard DNA, as well as to differentiate the bundle size of the PCR interaction on the Agarose gel, as shown in Figure 2**.**

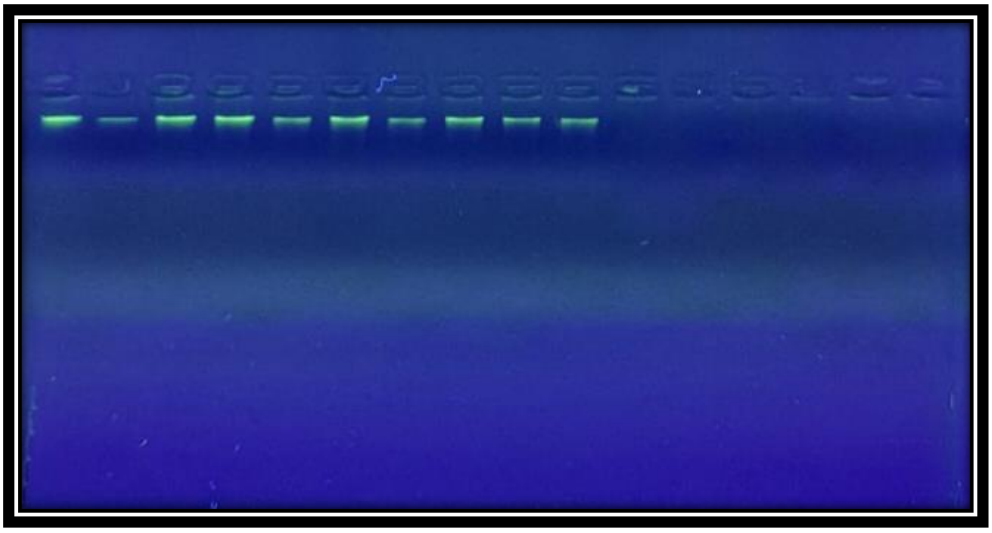

Figure 2: Gel electrophoresis of genomic DNA extraction from (10) Staphylococcus epidermidis, 1% agarose gel at 5vol /cm for 30 min.

According to the findings, the *mecA* gene is present in the genomes of all 10 isolates of *S. epidermidis* as shown in Figure 3.

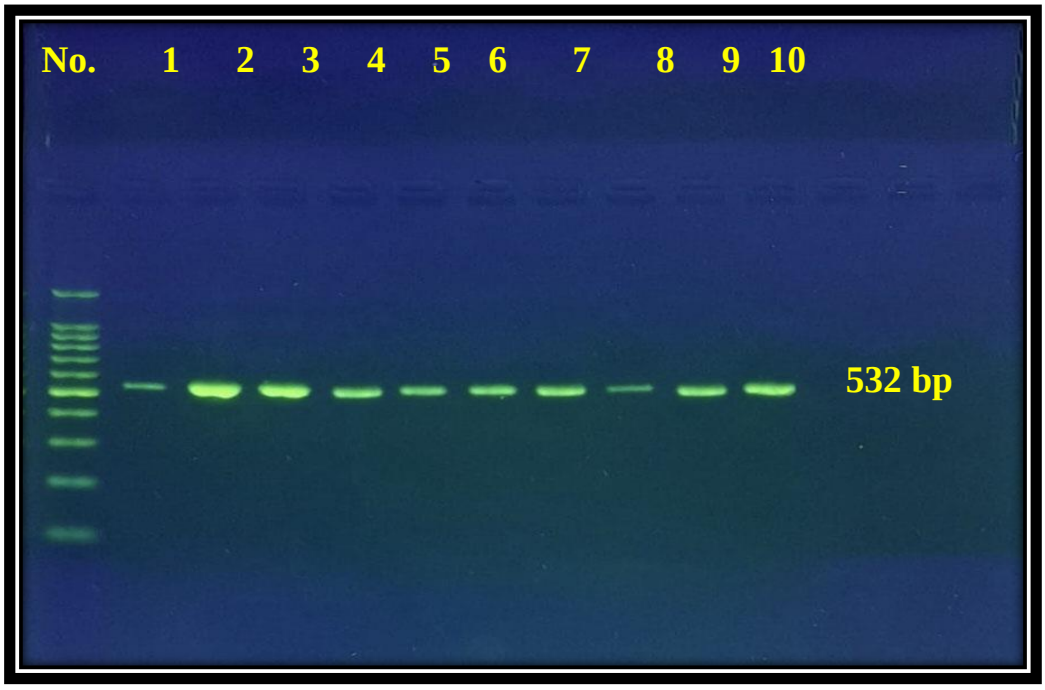

Figure 3: PCR product, *mecA* gene, the band size 532 bp. The product was electrophoresed on 2% agarose at 5 volt/cm^2. 1x TBE buffer for one hour. N: DNA ladder (100).

The PCR method verified that none of the ten *S. epidermidis* isolates had the *ermA* gene in their genomes as shown in Figure 4.

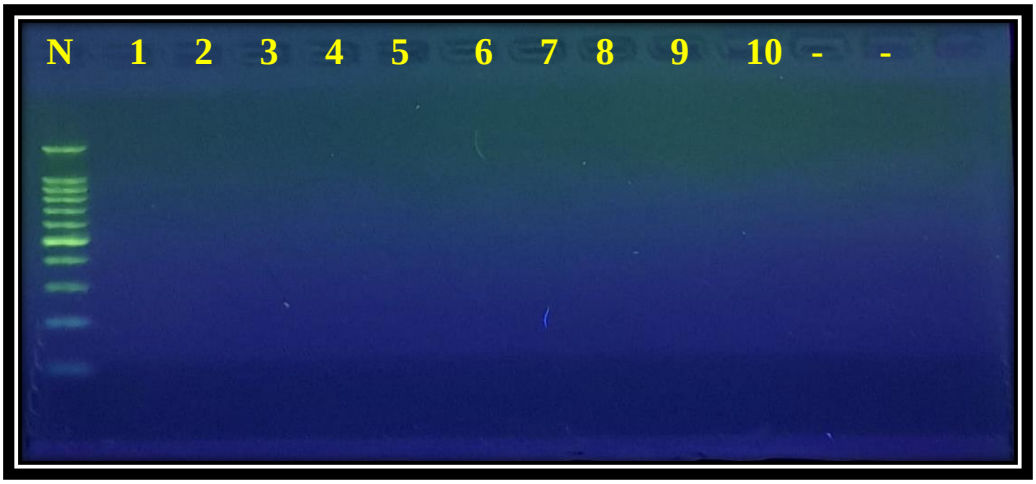

Figure 4: PCR product, *ermA* gene, the band size of *ermA* gene was190 bp, no band appears on the gel. The product was electrophoresed on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1hr. N: DNA ladder (100)

The current study demonstrated that among 73 isolates of Staphylococcus epidermidis, there were 70 (95.89%) isolates resistant to methicillin. Methicillin resistance has been identified in 75–90% of hospital isolates of Staphylococcus epidermidis that exhibit the mecA gene that is carried by phylococcal cassette chromosome mec (SCCmec) [23]. Staphylococcal Cassette Chromosome mec (SCCmec) is a mobile genetic element that includes the mecA genes that exhibited resistance to methicillin and encodes 2a (PBP2a), an alternative binding protein with a low affinity for β-lactam antibiotics [24]. Also, the current study revealed that some S. epidermidis isolates showed sensitivity to methicillin (4.11%) and had mecA gene. This is explained by the study of Bilyk who said "Constitutive expression of mecA does not lead to homogeneous resistance" [25]

6. Lethal dose (LD50) study

The term LD50 describes an estimate of the toxin concentration that, under control conditions, will be a lethal dose to 50% of a large number of test animals of a particular species. The bacterial suspension of *S. epidermidis* was adjusted to 0.5 McFarland standard containing 1.5x10^8 cells/ml.

7. Histopathological examination

This study represented the pathogenicity of commensal Staphylococcusepidermidis in laboratory-test male mice. According to the results of histopathology, the liver, skin, heart, and small intestine of the control group had normal cytoarchitecture and appearance, as shown in Figure 2.

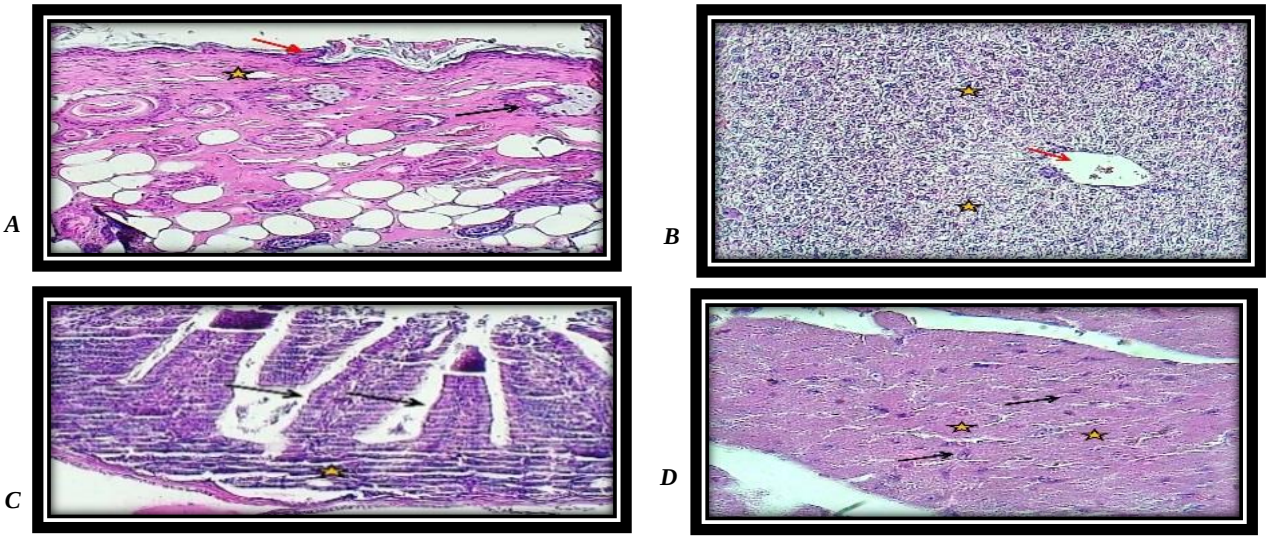

Figure 2: (Control) A- Displayed a normal epidermis; B) Liver section (Control) displaying the appearance and arrangement of the hepatic cords and the central vein (arrow). C-Small intestine (Control) section displays intestinal glands and villi (Arrows) in their typical appearance. D: The cardiac myocardium appears normal.

The histopathological figures of the injury group revealed major vascular congestion at the portal triad and sinusoids with major disorder, damage to the hepatic cords and necrosis, severe hepatitis, and significant hepatocyte atrophy Additionally, there was a notable mono- and polymorphic nuclear leukocytes infiltration, with the formation of giant cells and damage to the central vein cord, as shown in Figure 3.

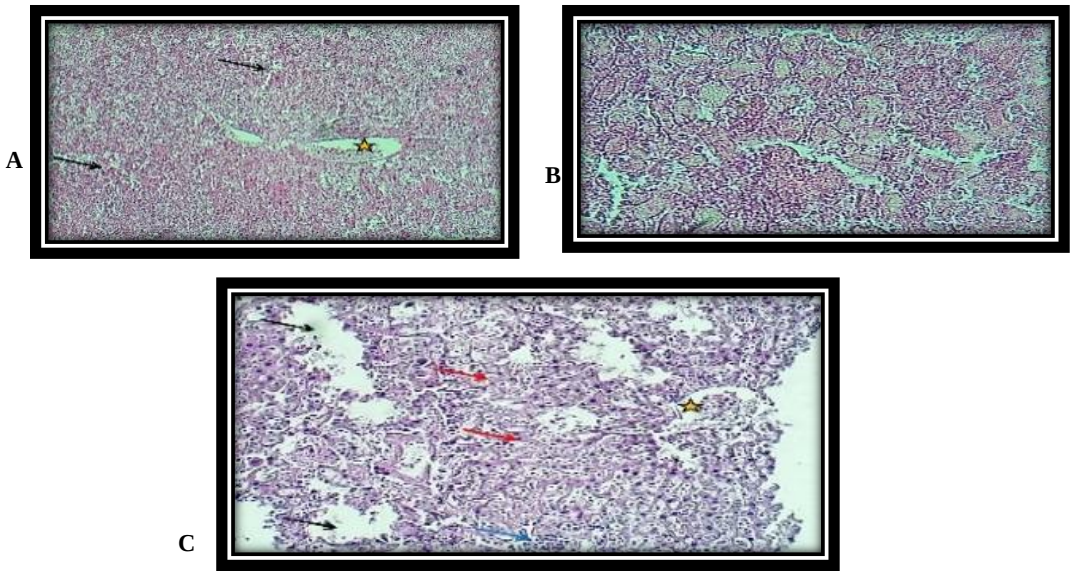

Figure 3: A- A section of the liver has significant necrosis-induced damage of the hepatic cord, with significant vascular congestion at the portal triad. B- Marked damage of hepatic cords by necrosis. C- Shows marked hepatitis, infiltration of leukocytes.

Severe myocardial infarction was observed in the histological figures of the heart. With a marked wavy appearance, edema, atrophy of the afflicted myofibers, and development of ventricular thrombus as shown in Figure 4.

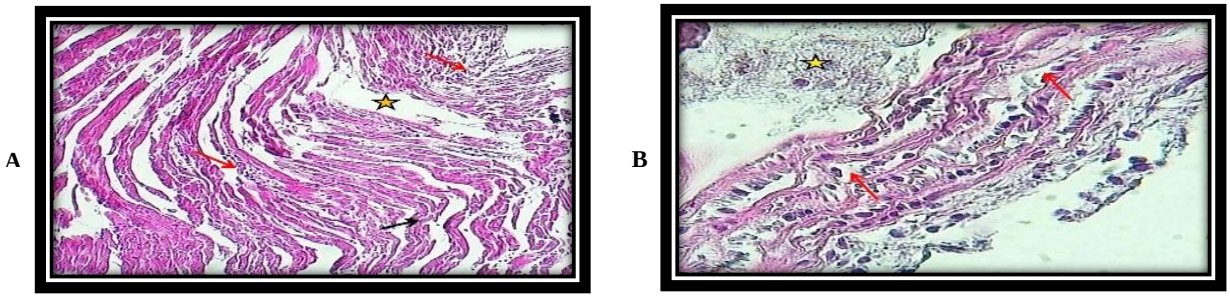

Figure 4: A- severe myocardial infarction seen in the section of the heart. B- Marked thrombus formation

Histopathological figures of the small intestine revealed two types of enteritis: severe destructive enteritis, which was characterized by a complete loss of the cytoarchitecture of the mucosal villi and a notable amount of luminal tissue debris, and severe enteritis, which was characterized by marked damage to the mucosal villi and necrosis of intestinal glands as shown in Figure 5.

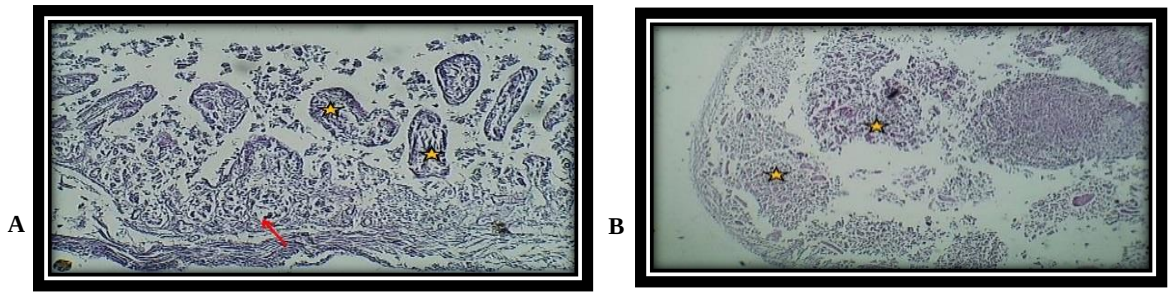

Figure 5: A-A small intestinal section demonstrates severe enteritis, which is characterized by marked mucosal destruction. B: A severe destructive enteritis characterized by marked loss of mucosal villi, a total loss of cytoarchitecture, and marked amount of luminal tissue debris lost.

Severe dermatitis seen in the histopathological figures of the skin was characterized by marked damage with loss of epidermis, and ulcer formation covered by necrotic tissue. The dermis revealed marked degeneration with necrosis of dermal fibrous tissue and infiltration of mononuclear leukocytes as shown in Figure 6.

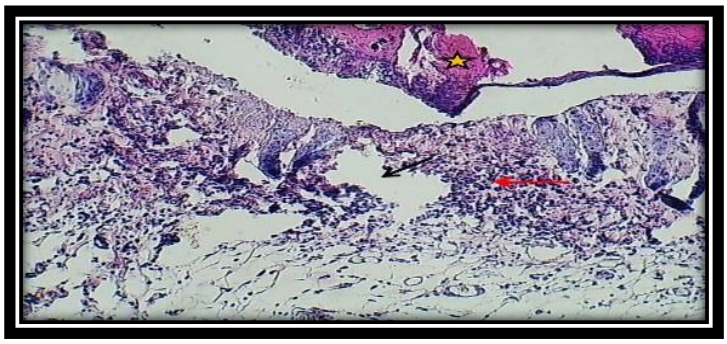

Figure 6: A skin section demonstrates necrotic tissue (asterisk), dermatitis with tissue loss (red arrow), and dermal fibrous tissue necrosis with MNC infiltration.

A local study by Ahmed A. Hammadi and Mohsin A. Essa in the Rafidain journal of science, in 2020 represented the detection of bacterial types belonging to the CoNS group from pathogenic sources and medical devices and the pattern of their resistance to different antibiotics [26]. Another study by asistant Prof. Fakiri S.Alajeeli, Ali S.kadhum, in Al-Salam Journal for Medical Science, 2023, showed the biofilms formation on the plastic devices plays a pivotal role in the pathogenicity of *S. epidermidis* and its connection to acne [27].

Conclusion

The study revealed that 73 cases among 120 samples were diagnosed as positive for S. epidermidis. The 10 to 65 years was the age range of this study. The age group of (36 to 50) years had the higher ratio of the respondent cases. The current study shows that S. epidermidis possessed some virulence factors and was a strong biofilm producer. Furthermore, the mecA gene appeared in all S. epidermidis isolate, but the ermA gene was absent. Antibiotic sensitivity tests revealed that most isolates were resistant to methicilline to the acquisition of mecAgene. Also, S. epidermidis caused pathogenicity in mice.

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