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Simplex, Duplex and Multiplex PCR for detection of up to six Enterococcus virulence genes in Staphylococcus spp. isolated from burn patients

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Abstract:

The high incidence of Gram-positive cocci, particularly Staphylococcus aureus, poses a severe threat in burn patients, causing a wide variety of diseases ranging from minor skin infection to life-threatening sepsis cases, leading to infection treatment difficulties. This study aimed to investigate a genotypic approach to detect up to six virulence genes in Staphylococcus spp. that may be passed from Enterococcus spp. isolated from the same niches of burn patients. The study analyzed 200 clinical samples (wound, urine, stool, and blood) from 75 burn patients. The six chosen virulence genes, including asa1, gelE, cylA, esp, hyl, and efa, were detected using simplex, duplex, and multiplex PCR alongside phenotypic testing in Staphylococcus spp., in addition to antibiotic resistance genes vanA and aph(3'). Initial identification used selective media and biochemical tests, followed by 16S rRNA sequencing; of these samples, 38 (19%) were confirmed as Staphylococcus species. The distribution included S. epidermidis (31.5%), S. aureus (28.5%), S. haemolyticus (23.6%), S. hominis (13%), and S. saprophyticus (2.6%). Notably, all 38 isolates grew on HiChrome media typically specific for Enterococcus faecium. The asa1 gene was the most prevalent; it was recorded in up to 100% of isolates, with significant differences $p<0.0001$. Other genes like gelE and hyl also showed high frequency, while efa, esp, and cylA levels fluctuated across different PCR methods. A key finding the coexistence of these two bacterial species in the same niche raises concerns about the potential horizontal transfer of virulence-associated traits between them, highlighting the importance of rapid and accurate diagnosis to minimize this potential risk.

Key words: Burn infections, Staphylococcus spp., Virulence factor genes, VRS, antimicrobial genes.

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Introduction

Microorganisms responsible for burn wound colonization can originate from the patient's endogenous flora. In addition, transmission can occur through contaminated hospital environmental surfaces, water, fomites, air, as well as through contact with the contaminated hands of healthcare personnel [1]. In post-burn infections, the dominant isolated bacteria are Gram-negative bacteria, whereas after fourteen days and continuing, the microbial profile may shift toward the predominance of Gram-positive bacteria, particularly in patients with prolonged hospital stays and more extensive or deeper burn injuries; this can result in attenuate the immune response, as a consequence of an elevation in risk factors [2]. *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Enterococcus faecalis* have been considered the five bacterial species predominant among burn patients [3]. *Staphylococcus* spp. are considered important causes of early burn wound infection due to their ability to possess and express a variety of virulence-associated genes, as well as acquire additional genetic determinants through horizontal gene transfer from other bacterial strains. Moreover, they have the capacity to develop diverse mechanisms of antimicrobial resistance, which can complicate treatment and increase severity of infection [4]. Globally, a high proportion of burn-associated infections, exceeding 50% in some reports, has been linked to Methicillin-resistant *S. aureus* (MRSA), which is capable of causing invasive infections in burn patients [5,6].

The severity of *Staphylococcus* is associated with a variety of bacterial surface components and extracellular proteins, including hemolysin, metalloproteinases such as gelatinase, collagenases, and hyaluronidase. These virulence determinants facilitate tissue invasion and contribute to the development of severe complications such as pneumonia, sepsis, and other manifestations with invasive burn wound infections [6]. It is known that three types of hemolysis are produced by bacteria which including: α-Hemolysin promotes ADAM10 metalloproteinase activity, resulting in E-cadherin shattering and contributing to pore formation. This degradation can decrease neutrophil activity and enhance cytokine release. β-Hemolysin breaks down sphingomyelin in the plasma membrane to produce phosphocholine and ceramide; these processes are essential for hemolytic activity. However, δ-Hemolysin are needed in bulk to disrupt cell membrane integrity through pore formation, shifting membrane

bending, and hydrolyzing them. The severity of *S. aureus* having the previous factors is increased by disrupting host cell membrane integrity, thereby enhancing bacterial survival and evasion of host immune response [5].

Hyaluronidase, encoded by the chromosomal *hyl* gene, contributes to bacterial dissemination by degrading hyaluronic acid, a main component of connective tissue and cartilage; this degradation facilitates the spread of bacteria to adjacent cells [7]. Another important virulence factor is the extracellular serine protease encoded by the *esp* gene. Although it is primarily associated with *Staphylococcus epidermidis*, it also plays a critical role in biofilm formation by *Staphylococcus aureus*. Several studies have reported the presence of the *esp* gene in diverse *S. aureus* isolates, indicating genetic variability that may influence both virulence and antibiotic resistance [8].

Infective endocarditis can occur when *Staphylococcus aureus* attaches to damaged or inflamed heart valves through adhesion factors such as von Willebrand factor and fibrin. Although the *efaA* gene is mainly associated with *Enterococcus faecalis*, similar genes in *S. aureus*, including *ClfA* and *eap*, contribute to endocarditis pathogenicity [9].

The attachment of bacteria to collagen, which is the most abundant protein in humans, represents a critical initial step in the establishment and progression of several bacterial infections, including those caused by *Staphylococcus* spp. The aggregation substances encoded by the *asa1* gene are important virulence determinants that enhance bacterial pathogenicity and may facilitate the spread of bacterial genetic material through the conjugation process [10]. All these virulence gene factors mentioned above were investigated using primers designed to target their presence in *Staphylococcus* spp.

Antimicrobial-resistant bacterial strains pose a serious threat to human health. *Staphylococcus aureus* has developed resistance to many commonly used antibiotics, which is very concerning for human health, leaving only limited treatment options. Understanding the cellular characteristics of resistant bacteria, in addition to their resistance mechanisms, may improve treatment strategies and help identify new therapeutic targets. Furthermore, exposure to environmental stressors such as sub-inhibitory antibiotic concentrations and bacteriophages may induce genetic mutations and contribute to the development of antibiotic resistance, thereby posing significant challenges to effective medical treatment. [11,12].

Methicillin-resistant *Staphylococcus aureus* strains are frequently isolated from burn wounds and bloodstream infections, with prevalence differing according to local epidemiology and specimen type; for instance, Irshad et al. [13] reported that 81% of burn patients were colonized by *Staphylococcus aureus* within 24 hours of admission, indicating rapid early colonization.

Wang et al. [14] reported that multiplex PCR enables the efficient and simultaneous screening of multiple targets. In a previous study, Gram-positive cocci other than Enterococci were recovered from Azide blood agar and Enterococcus faecium HiChrome agar, both of which are highly selective for Enterococcus spp. [15]. Furthermore, experimental evidence demonstrated the transfer of some virulence genes between pathogenic *Enterococcus* and non-pathogenic *Staphylococcus*, and vice versa (manuscript accepted and under publication), suggesting genetic similarity and the potential for gene exchange between these closely related Gram-positive cocci. Therefore, the current study aims to detect six virulence factor-associated genes—*asa1, gelE, cylA, esp, hyl,* and *efa*—three of which were previously evaluated phenotypically, using specific primers originally designed for their amplification in *Enterococcus* spp. Their presence in Staphylococcus recovered from the same ecological niche will be investigated using simplex, duplex, and multiplex PCR techniques. In addition, the antibiotic resistance of these isolates will be evaluated, including detection of the Vancomycin *(vanA)* and Gentamycin *(aph(3'))* resistance genes.

Materials and methods

Samples collection and bacterial identification

The present investigation included all suspected Staphylococcal isolates collected in a study by Al-Maliki *et al*. [15] on isolation of enterococci from burn patients. A total of 200 clinical specimens from 75 patients with burn injuries who were admitted to the Burn unit at Al-Fayhaa hospital in Basrah, Iraq. Seventy-five of the samples were from wound swabs, and blood, 38 from urine, and the last 12 were from stool samples. For many burn cases, more than one sample was collected from the same patient when available (with concern for the patient's acceptance and the physician's) to study the spreading of pathogens between these sites. All samples were initially inoculated directly into Brain Heart Infusion broth (BHIB) (Himedia, India) and incubated at 37 ºC for 24 h. Following enrichment, the culture was subsequently streaked on Azide blood agar plates (Himedia, India) supplemented with

0.04% sodium azide (Merck, Germany) and incubated at 37 ºC for 24-48 h. Subsequently, the isolates were cultured on Mannitol Salt Agar (MSA) under the same incubation conditions. After incubation, selected colonies were subcultured on HiCrome *E. faecium* Agar Base (Himedia, Labs) and incubated for 24- 48 h at 37 ºC. Nutrient agar was used for preservation of the isolates and for performing several biochemical assays, including Gram stain (Biotech, India), Oxidase and Catalase production. In addition, the ability of isolates to hydrolyze aesculin was evaluated on bile-esculin agar (Himedia, India) [16]; hemolysis, gelatin liquefaction, and biofilm formation were performed also. This was followed by molecular identification of the developed bacterial isolates.

Molecular identification

DNA was extracted from each suspected *Staphylococcus spp.* by picking a single colony, cultivating them in sterile tubes containing 5 mL brain heart broth, and incubating at 37 ºC for 24 h. DNA extraction was performed according to the manufacturer's instructions of Presto™ Mini g DNA Bacteria Kit (Geneaid Company, South Korea). The extracted DNA was then stored at -20 °C until further use after being visualized using a 1% agarose gel.

Amplification of the *16S rRNA* gene for bacterial identification was done by applying Polymerase chain reaction (PCR) recruiting the universal *16S rRNA* gene primers shown in Table 1). The components for amplification were used in a total of 50 µl, including: 25 µl Taq Promega green master mix (Promega USA), 2 µl DNA template (50 ng/µl), 2 µl of each of the forward and reverse primers (10 picomole\µl). The 50 µl mixture was prepared with Nuclease free water (NFW); the mixture was vortexed and centrifuged for a short time. The PCR reaction conditions were following [17]; the size of the *16S rRNA* gene product was approximately 1500 bp, which was visualized using 2 % agarose gel electrophoresis [17].

Preparation of amplified PCR product for sequencing and identification:

The amplified *16S rRNA* gene was sent to Macrogen Company/ South Korea for sequencing. The upcoming sequencing outcomes were firstly treated using the Chromas https://chromaspro.software.informer.com/ program. The program was applied automatically to remove low-quality sequences to improve sequencing results, leaving high-quality data to be read by the author; then the bacterial species were identified at the genus and species levels

with Basic Local Alignment Search Tool (BLAST). The high similarity percentage between the corrected subject sequences under investigation and the reference sequence in National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov [18].

Phylogenetic tree: Multiple Sequence Alignment (MSA) was used in the current study to compare the identified bacterial strains and draw the phylogenetic tree. Thirty-eight *16S rRNA* concatenated sequences belonging to the 5 identified *Staphylococcus* spp. obtained in this study were aligned with *16S rRNA* sequences of their reference strains applying MSA. The Molecular Evolutionary Genetics Analysis (MEGAX) http://www.megasoftware.net was applied to draw the constructed phylogenetic tree [19]. This program achieved multiple sequence alignments and used the neighbor-joining (NJ) method for phylogenetic tree construction.

Detection of virulence factors phenotypically

Production of hemolysin

The production of hemolysin by the identified staphylococci spp. was investigated on Blood agar plates supplemented with 5 % blood. The inoculated Blood agar plates with Staphylococci were incubated for 24 hours at 37 ºC. The development of a clear zone around the colonies was recorded as complete hemolysis (β- hemolysis); partial hemolysis (α- hemolysis) was marked when a greenish zone around the colonies appeared. However, no zone of inhibition was recorded as non-hemolysis (γ- hemolysis) [20].

Production of gelatinase

To detect gelatinase synthesis, a type of metalloproteinase, the Staphylococci isolates were stabbed with a sterile needle into gelatin (12 %) agar tubes. The tubes were incubated at 37°C for 24 h; development of liquefied gelatin after cooling at 4 °C for 4 represented positive gelatinase activity [20].

Detection of biofilm formation on Congo Red Agar (CRA):

The production of biofilm was investigated phenotypically on CRA plates prepared following [21]. The bacterial isolate under investigation was tested by streaking them on the CRA medium; the CRA plates were incubated for 24 h at 37 °C. Developed colonies in black color with dry crystals were consider as +ve (*i.e.*, slime layer producers), while a negative result (*i.e.*, no slime layer producer) was recorded by appearing pink-white colonies.

Genotypic detection of virulence factors

In the current study, the presence of six virulence-related genes among the identified *Staphylococcus* spp., namely *asa1,* encoding aggregation substances; *esp,* encoding extracellular surface protein; *efaA*, associated with endocarditis-specific antigen A; *hyl*, encoding hyaluronidase; *gelE*, encoding gelatinase; *cylA*, associated with cytolysin production. The primer sequences employed for molecular detection are presented in Table 1. Furthermore, two antimicrobial resistance genes, *vanA*, and (*aph 3'),* related to vancomycin and gentamicin resistance, were examined. Thus, a total of eight target genes were screened in all Staphylococci isolates included in the study. These primers were commercially synthesized by Macrogen (South Korea). Conventional PCR was performed using three amplification approaches — simplex, duplex, and multiplex PCR to detect the selected virulence and antimicrobial resistance genes.

Table 1: Primer sequences for detection of *16S rRNA*, virulence associated and antimicrobial resistance genes (*van A, aph (3')*) using PCR analysis

Primer Name	DNA Sequences (5'-3')	Product size bp	References
16S rRNA	F-AGAGTTTGATCCTGGCTCAG	1500	[17]
	R-GGTTACCTTGTTACGACTT		
asa1	F-GCACGCTATTACGAACTATGA	375	
	R-TAAGAAAGAACATCACCACGA		
gelE	F-TATGACAATGCTTTTTGGGAT	213	
	R-AGATGCACCCGAAATAATATA		
cylA	F-ACTCGGGGATTGATAGGC	688	[22]
	R-GCTGCTAAAGCTGCGCTT		
Esp	F-AGATTTCATCTTTGATTCTTGG	510	
	R-AATTGATTCTTTAGCATCTGG		
Hyl	F- ACAGAAGAGCTGCAGGAAATG	276	
	R-GACTGACGTCCAAGTTTCCAA		
Efa	F-CGTGAGAAAGAAATGGAGGA	499	[23]
	R-CTACTAACACGTCACGAATG		
van A	F-GGGAAAACGACAATTGC	732	[24]
	R-GTACAATGCGGCCGTTA		
aph (3')	F-GGCTAAAATGAGAATATCACCGG	523	[25]
	R-CTTTAAAAAATCATACAGCTCGCG		

a) Gradient PCR [22]: Gradient PCR was used for each primer of the 8 genes (virulence and resistance gene primers) in gradient PCR at different annealing temperatures ranging from 53-60 °C to find the best conditions for amplification, and 56.6°C was determined to be appropriate for the virulence factor genes, and 56 for the antibiotic's resistance genes.

b) Simplex PCR: The components for the PCR reaction tube for amplification of the six tested genes were prepared in a total of 25 µl using Go Taq Promega green master mix; the amount of the components and the PCR conditions for every single primer were according to reference of the primer (Table 1), except the annealing temperature was optimized in the present study at 56.6 °C for 35 sec, applying gradient PCR for all virulence-associated genes. The product sizes of the eight genes, including antimicrobial resistance genes, were separated and visualized by 2 % agarose gel electrophoresis.

c) Duplex PCR:

Four reactions were applied using four virulence gene primer pairs according to Kiruthiga *et al*. [26]; three of the reaction tubes mixture compromised two virulence genes each as follows: (1: *asa1* and *gelE*; 2: *cylA* and *esp*; 3: *hyl* and *efaA*; and the fourth reaction contains: *vanA* and *aph (3')*). The reaction reagents for gene amplification and the PCR conditions were run following Kiruthiga *et al*. [26] in a total of 50 µl, and using the optimized annealing temperature 56.6ºC for the virulence-associated genes and 56ºC for antimicrobial resistance genes. The product sizes of the eight genes, including antimicrobial resistance genes, were separated and visualized by 2 % agarose gel electrophoresis.

d) Multiplex PCR: The multiplex PCR technique was recruited in the current study to amplify all the virulence factor genes in one tube according to Vankerckhoven *et al*. [22]. One PCR reaction tube (100µl) was standardized, containing all six virulence associated genes (*asa1*, *gelE*, *cylA*, *esp*, *hyl,* and *efaA*). The amplification program was performed using the previously described PCR conditions under the same optimized annealing temperature. The size corresponding to the six genes was visualized applying 2 % agarose gel electrophoresis.

Antibiotic susceptibility assay

a) Antibiotic discs assay: Six antibiotics, including vancomycin (30 µg), meropenem (10 µg), levofloxacin (5 µg), ceftriaxone (30 µg), gentamicin (10 µg), and piperacillin (100 µg), were chosen to be tested during the present study relies on the Clinical and Laboratory Standards Institute

[27] and Basrah's hospitals protocol in Iraq to treat burn patients' infections. The disc agar diffusion procedure was applied for antimicrobial susceptibility testing for each isolate following Nichollas, [28]: a colony from each grew staphylococci for 24 hours on nutrient agar medium was taken with a sterile loop, placed in a glass tube containing 3 ml of normal saline to make bacterial suspension and compared with a turbidity of the prepared McFarland concentration of 0.5 ($1.5x10^8$ CFU/ml). 100 µl of bacterial suspension was taken and spread onto the Mueller-Hinton agar medium. The dishes were left to dry, and then the antibiotic disc was placed on the dish. The dishes were incubated for 24 hours at 37°C. After incubation, the inhibition diameters were determined as sensitive, intermediate, and resistant bacteria and recorded depending on the inhibition doses recommended by CLSI [27] for each antibiotic.

b) Vancomycin and gentamicin resistance gene detection: PCR was performed using specific primers targeting vancomycin (*vanA)* and gentamicin (*aph* (3')) resistance genes as listed in Table (1). The annealing temperature of the primers was optimized using gradient PCR; the amplification components were prepared in a final volume of 50 µl according to the protocol described by Dutka-Malen *et al*. [24] and Vakulenko *et al*. [25], using the same quantities and concentrations of reaction components described previously. The PCR program also depends on [24,25], using the optimized annealing temperature at 56 °C for 35 sec. Duplex PCR was conducted to simultaneously detect the *vanA* and *aph* (3') genes, applying the same optimized annealing temperature at 56 and the same program. The resulting product sizes of both genes (732 bp and 523 bp, respectively) were visualized by 2 % agarose gel electrophoresis [29].

Statistical analysis

The results were analyzed statistically using SPSS Statistics Package Ver. 24.0.0.1. Cochran's Q test was applied to evaluate the occurrence of virulence-associated and antimicrobial resistance genes phenotypically and genotypically, significantly at $p < 0.0001$ level.

Results

Bacterial identification

Following the methodology described by MacFaddin [30], the bacterial isolates were subjected to microbiological and biochemical examinations for preliminary identification. Of the 200 clinical samples examined, 122 (61%) exhibited turbidity following incubation in brain heart infusion broth (BHIB). These positive samples consisted of 75 (37.5 %)

swabs, 31 (15.5 %) urine, 12 (6 %) stool, and 4 (2 %) blood. Subsequently, 110 out of 122 positive samples demonstrated bacterial growth on Azide blood agar. These include 70 (63.6 %) wounds, 26 (23.6 %) urine, 12 (10.9%) stool, and 2 (1.8 %) blood samples obtained from burn patients. The bacterial colonies observed on cultured medium were circular, slightly convex circular and characterized by a smooth white-to-creamy appearance with a raised edge. Among the 46 isolates displaying white colony morphology, 38 were subsequently identified as *Staphylococcus* spp. Upon culturing these isolates on bile-esculin agar, 10 isolates obtained from burn wounds produced black or brown colonies, indicatinga positivee result. The preliminary biochemical characterization of these isolates revealed Gram-positive cocci that were oxidase-negative and catalase positive.

Molecular identification

The thirty-eight biochemically tested isolates, oxidase-negative and catalase-positive, catalase positive were identified later on using the *16S rRNA* gene, as five different *Staphylococcus* spp. were collected from four types: blood, urine, stool, and wound swabs. Twelve 12 (32%) as *Staphylococcus epidermidis* (accession numbers PV943977 to PV943988), were isolated from the wound swabs. Eleven (29%) as *Staphylococcus aureus* (accession numbers PV943989- PV943999), 10 were isolated from wound swabs, and 1 from a urine sample. Five isolates (13 %) as *S. hominis* were identified (accession numbers PV944009- PV944013); 4 were isolated from wounds and 1 from a urine sample. Nine isolates were *S. haemolyticus* (accession numbers PV944000-PV944008); 4 were isolated from urine samples, 4 from wounds, and 1 from blood. One as *S. saprophyticus* (accession number PV944014) was isolated from urine samples. Multiple isolates of the same *Staphylococcus* were found in the same patient; for example, one *S. hominis* and one *S. haemolyticus* were isolated from wound and urine, respectively, from the same patient. Also, from a urine sample of a patient, one isolated bacterium each of *S. aureus* and *S. haemolyticus* were identified. However, regarding the source of isolation, the majority of identified *Staphylococcus* isolates were recovered from wound samples, accounting for 31isolates (81.6 %). These include 12 isolates of *S. epidermidis*, 10 of *S. aureus*, 5 of *S. hominis*, and 4 of *S. haemolyticus*. Urine samples yielded six isolates (15.8 %) compromising 4 *S. haemolyticus* and one isolate each *S. aureus* and *S. saprophyticus*; only one isolate (2.6 %) identified as *S. haemolyticus* was obtained from the blood

sample. A phylogenetic analysis was performed using *16S rRNA* sequences of all identified *Staphylococcus* isolates, and the resulting tree is illustrated in Fig. 1. When compared with their reference strains (ATCC), the phylogenetic tree confirmed the identification and highlight the genetic evolutionary clustering of the five *Staphylococcus spp.* recovered from burn patients.

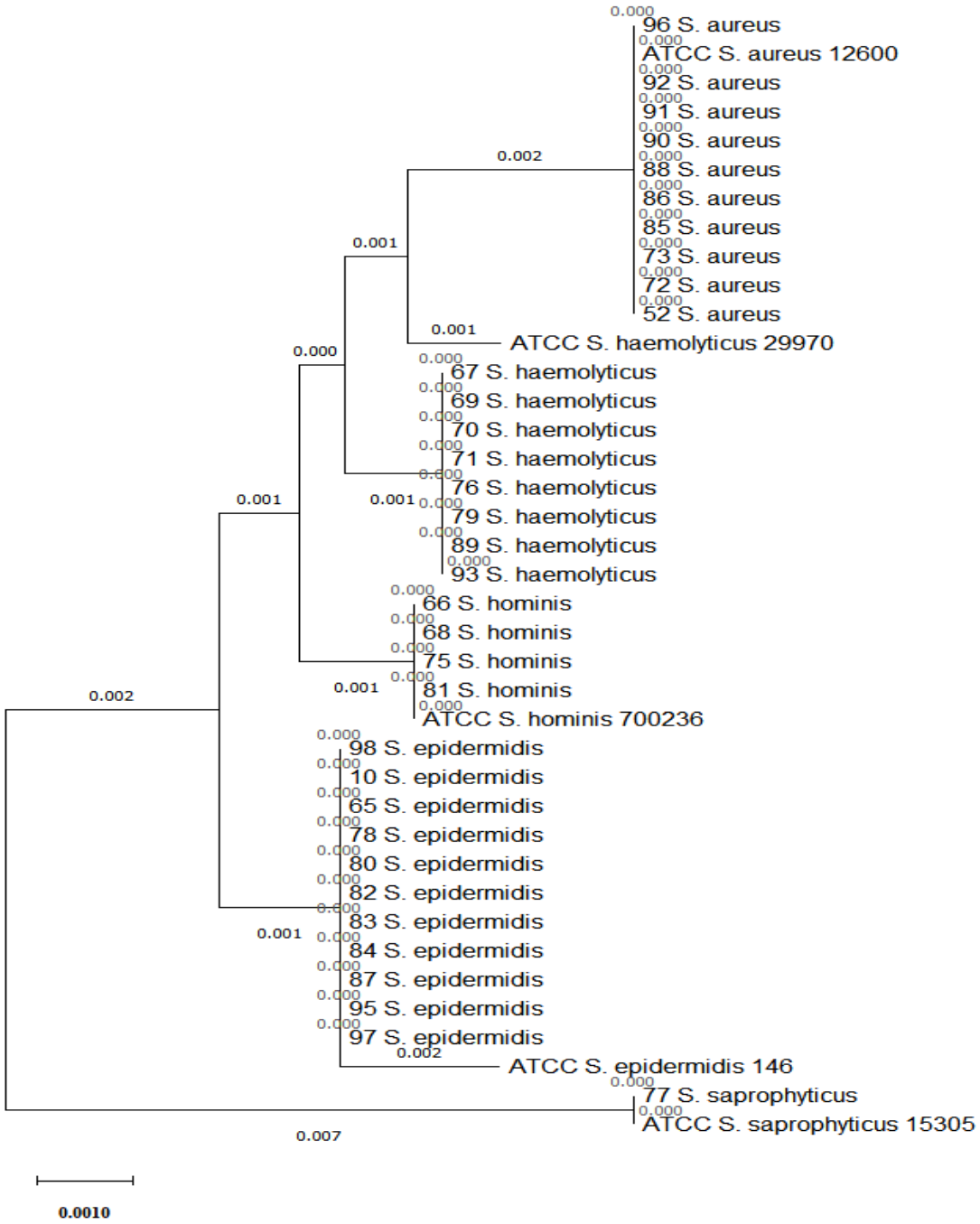

Fig.1: Constructed Neighbor-joining tree revealing the distribution and phylogenetic relationships of thirty-eight identified *Staphylococcus* spp. compared with their reference strains (ATCC). The lengths of the horizontal branches were scaled. The value of Bootstrap after 1000 repetitions are pointed.

Phenotypic detection of virulence factors

Hemolysin production: The five identified *Staphylococcus* species exhibited distinct hemolytic patterns; β-hemolysis, characterized by the presence of a clear zone surrounding the bacterial growth, was observed in 19 isolates of the 38 (50 %). These isolates comprised 9 (23.6 %) *S. epidermidis,* 8 (21 %) *S. haemolyticus,* and 2 (5.2 %) *S. hominis*. In contrast, γ-hemolysis was detected in 15 isolates, including 9 (23.6 %) *S. aureus,* 2 (5.2 %) of each of *S. epidermidis* and *S. hominis,* and one isolate (2.6 %) each of *S. haemolyticus* and *S. saprophyticus*. Additionally, α-hemolysis was observed in four isolates (10.5%), compromising 2 (5.2 %) *S. aureus* isolates and one isolate (2.6 %) each of *S. epidermidis* and *S. hominis*.

Gelatinase production: Twenty-seven of 38 *Staphylococcus* isolates (71 %) exhibited gelatinase hydrolysis, compromising Eleven isolates of each of *S. epidermidis* (28.9 %) and *S. aureus*, three isolates of *S. haemolyticus* (7.8 %), and one of each of *S. hominis* and *S. saprophyticus* (2.6 %).

Biofilm formation: Twenty isolates (52.6 %) out of 38 Staphylococci were marked as biofilm producer on CRA plates, that involving 10 (26.3 %) belonging to *S. epidermidis*, 4 (10.5 %) from each *S. aureus* and *S. haemolyticus*, and 2 (5.2 %) from *S. hominis*.

Genotypic detection of virulence factors

The agarose gel electrophoresis outcome of amplified PCR products of the virulence-associated genes (shown in Fig. 2) was 688 bp, 510 bp, 499 bp, 375 bp, 276 bp, and 213 bp ,corresponding to *gelE, hyl, asa*, *efaA, esp,* and *cylA* genes, respectively, as appeared in Fig. 2a, b, c, d, e, and f, respectively, as a model of the simplex PCR**.** The recorded prevalence of virulence genes using simplex PCR was included; *gelE* in 31 (81 %), *hyl* in 32 (84 %), whereas *asa1* was detected in all isolates 38 (100 %), *efaA* in 25 (65 %), *esp* in 29 (76 %), and finally *cylA* in15 (39 %).

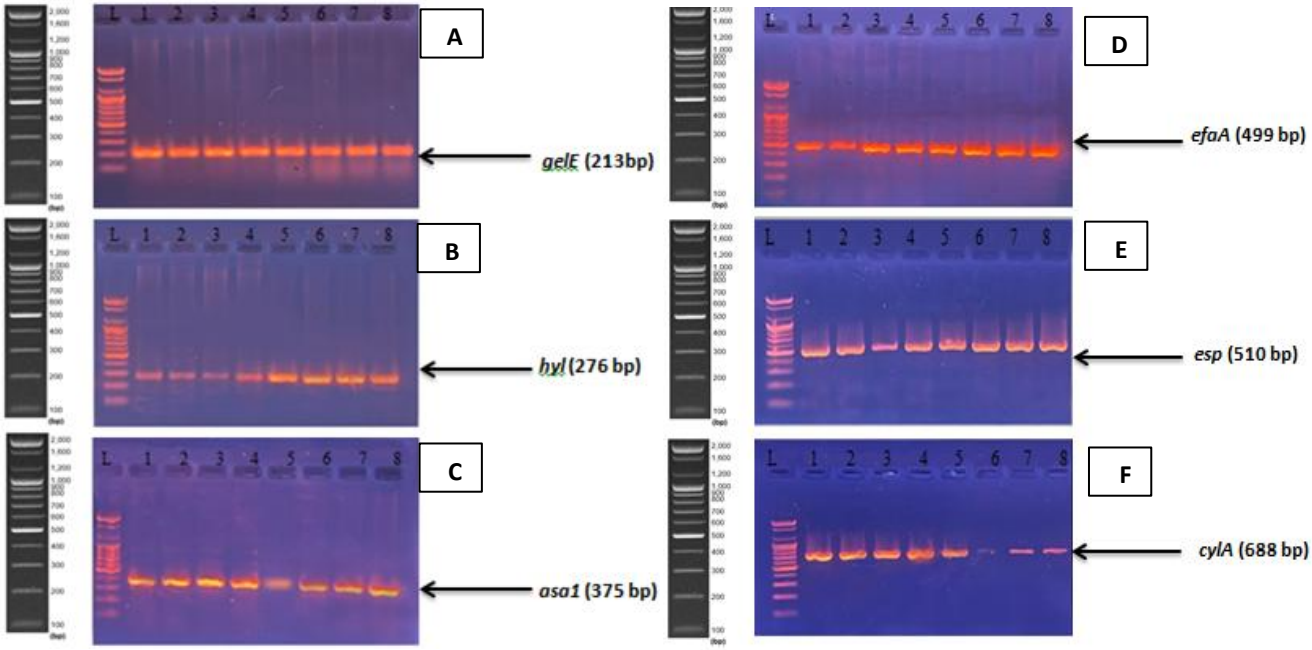

Figure (2): A model of agarose gel electrophoresis of simplex PCR for the six virulence factors genes were as follows: (*gelE* 213bp (A), *hyl* 276 bp (B), *asa1* 375 bp (C), *efaA* 499 bp (D), *esp* 510 bp (E), and *cylA* 688 bp (F). Lane L: 100 bp DNA ladder, Lane 1-8 a model of identified bacterial isolates, 4 belonging to *S. epidermidis* and 4 to *S. aureus*, isolated from patient' wound.

Duplex PCR: The virulence genes *asa1*, *gelE*, *esp*, *cylA, hyl*, *efaA* were observed in 30 isolates (78.9 %), 28 isolates (73.7 %), 14 isolates (36.8 %), five isolates (13.2 %), 21 isolates (55.26 %), and 12 isolates (31.57 %), respectively. The band patterns corresponding to each gene were as shown in Figure 3, since Fig.3a showed the combination of *asa1* and *gelE* depending on the band size differences 375bp and 213bp respectively. While Fig.3b explains the bands of *efaA* and *hyl* with 499bp and 276bp respectively, and finally the genes *cylA* and *esp* with 688bp and 510bp band sizes, respectively, as shown in Fig.3c.

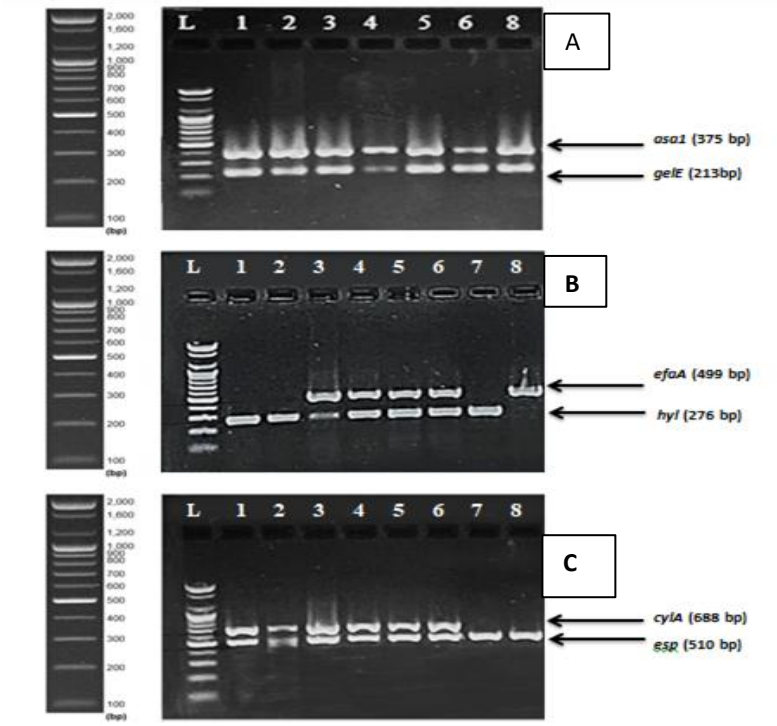

Fig. 3: An agarose gel electrophoresis of three duplex PCR for the six virulence genes (*asa1* 375 bp and *gelE* 213 bp), (*efaA* 499bp and *hyl* 276bp) and (*cylA* 688 bp and *esp* 510bp), as shown in A, B, and C Respectively. Lane L: 100 bp DNA ladder, Lane 1-8: Model of bacterial isolate belonged to four staphylococci species; *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. hominis* isolated from patient' wound.

Multiplex PCR: the gene frequencies using multiplex PCR were recorded as follows: 38 (100 %) for *asa1*, 15 (39.47 %) for *gelE*, 11 (28.94 %) for *esp*, 3 (7.89 %) for *cylA,* 15 (39.47 %) for *hyl*, and finally 11 (28.94 %) for *efaA*. A model for multiplex PCR bands is shown in Fig. 4. The percentage frequency of all the genes frequency according to the type of PCR (simplex, duplex, or multiplex) was explained in more detail in Table (2.

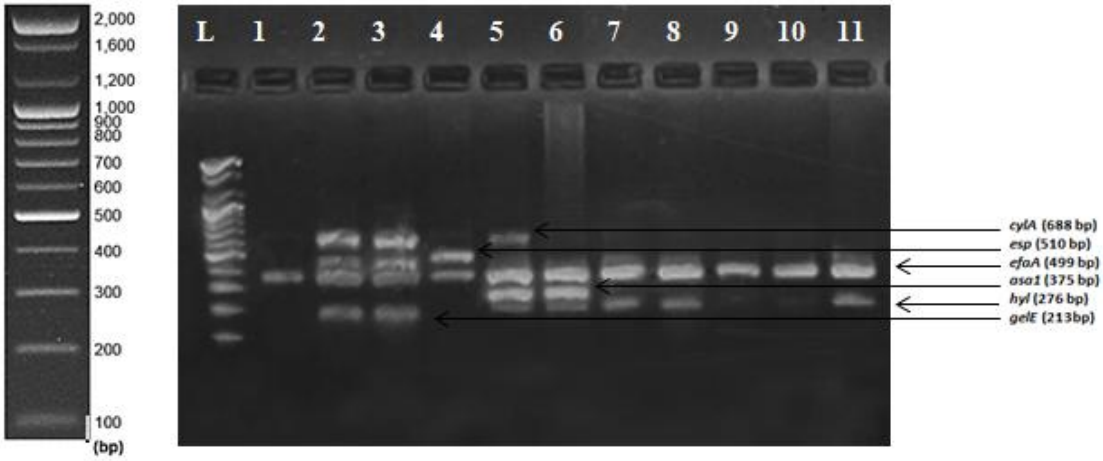

Fig. 4: An agarose gel electrophoresis representing a model of Multiplex PCR product for *cylA* with a size of 688 bp, *esp* with size 510 bp, *efaA* size 499 bp, *asa1* correspond to 375 bp, *hyl* with size of 276 bp, and *gelE* with size 213bp. Lane L: 100 bp DNA ladder, Lane 1-11: Selected identified staphylococci belonging to the three species; *S. epidermidis*, *S. aureus*, and *S. haemolyticus* isolated from wound swabs taken from several patients, which repeatedly showed higher virulence factors.

Table 2: The percentage and distribution of six virulence genes in clinical *Staphylococcus* isolates applying three PCR protocols.

PCR protocols	Isolates	Virulence genes					
		asa1	***gelE***	***hyl***	***efaA***	***esp***	***cylA***
Simplex PCR	*S. epidermidis* (12)	12 (31.57%)	9 (24%)	11 (28.94%)	7 (18.42%)	7 (18.42%)	4 (10.52%)
	S. aureus (11)	11 (28.94%)	8 (21.05%)	6 (15.78%)	6 (15.78%)	9 (23.68%)	6 (15.78%)
	S. haemolyticus (9)	9 (23.68%)	8 (21.05%)	9 (23.68%)	7 (18.42%)	7 (18.42%)	2 (5.26%)
	S. hominis (5)	5 (13.15%)	5 (13.15%)	5 (13.15%)	4 (10.52%)	5 (13.15%)	3 (7.89%)
	S. saprophyticus (1)	1 (2.63%)	1 (2.63%)	1 (2.63%)	1 (2.63%)	1 (2.63%)	0 (0%)
Total	(38) isolates	38	31	32	25	29	15
Duplex PCR	*S. epidermidis* (12)	9 (23.68%)	11 (28.94%)	8 (21.05%)	4 (10.52)	6 (15.78)	1 (2.63%)
	S. aureus (11)	10 (26.31)	9 (24%)	4 (10.52%)	2 (5.26%)	4 (10.52%)	2 (5.26%)
	S. haemolyticus (9)	7	6	7	3	2	1

		(18.42)	(15.78)	(18.42%)	(7.89%)	(5.26%)	(2.63%)
	S. hominis (5)	3 (7.89)	2 (5.26%)	2 (5.26%)	2 (5.26%)	1 (2.63%)	1 (2.63%)
	S. saprophyticus	1 (2.63%)	1 (2.63%)	0 (0%)	1 (2.63%)	1 (2.63%)	0 (0%)
Total	38 isolates	30	29	21	12	14	4
Multiplex PCR	*S. epidermidis* (12)	12 (31.57%)	5 (13.15%)	4 (10.52%)	3 (7.89%)	4 (10.52%)	1 (2.63%)
	S. aureus (11)	11 (28.94%)	3 (7.89%)	5 (13.15%)	2 (5.26%)	3 (7.89%)	1 (2.63%)
	S. haemolyticus (9)	9 (23.68%)	4 (10.52%)	4 (10.52%)	2 (5.26%)	2 (5.26%)	1 (2.63%)
	S. hominis (5)	5 (13.15%)	3 (7.89%)	2 (5.26%)	3 (7.89%)	2 (5.26%)	0 (0%)
	S. saprophyticus (1)	1 (2.63%)	0 (0%)	0 (0%)	1 (2.63%)	0 (0%)	0 (0%)
Total	(38) isolates	38	15	15	11	11	3

Correlation between phenotypic and genotypic results

Despite 27 *Staphylococci spp.* isolates revealed the ability to produce gelatinase on the medium, 31 isolates have been shown to have the corresponding gene (*gelE*), which encodes for gelatinase, in simplex PCR, 29 isolates in duplex PCR, and 15 in multiplex PCR. The presence of the *gelE* gene in *S. epidermidis* bacteria (all of which were isolated from wound swabs) was genetically and phenotypically consistent in 9 isolates. One isolate was both genetically and phenotypically lacking this character, whereas the other two were only phenotypically documented. Eleven isolates of *S. aureus* displayed it phenotypically, whereas eight isolates displayed the gene genetically; the remaining three isolates that did not exhibit it genetically were recovered from wound swabs. The gene was present in all five *S. hominis* isolates; however, one strain, which was obtained from a wound swab, exhibited consistently with phenotypic. All *S. haemolyticus* isolates had the *gelE* genotype; however, only three isolates obtained from urine exhibited it phenotypically. The incidence of the *gelE* gene was recorded at 81.60%, 76.3%, and 39.50% using Simplex, duplex, and Multiplex respectively, with a significant

difference using Cochron Q test at $p<0.0001$ between simplex and duplex compared to multiplex PCR.

Regarding hemolytic activity, 19 out of 38 staphylococci isolates showed either α or β hemolysis; however, 15, 4, and 3 isolates displayed the *cylA* gene bands in simplex, duplex, and multiplex PCR, respectively. Only two of the eight *S. epidermidis* isolates with a beta-hemolytic phenotypic activity had the *cylA* gene. Furthermore, it was noticed that two isolates had the gene but had a gamma-hemolytic phenotype.

Interestingly, none of the six *S. aureus* isolates that had the *cylA* gene displayed beta-hemolysis (Four isolates exhibited gamma hemolysis, whereas two displayed alpha hemolysis). Five of these isolates were obtained from wound swabs and one from urine. A beta-hemolytic phenotypic activity was seen in seven isolates of *S. haemolyticus* bacteria; however, only two of them were consistent with the genotypic findings —one from wounds and the other from urine—that had the *cylA* gene.

The *cylA* gene was present in three *S. hominis* isolates; only one, which was isolated from urine, had phenotypic beta-hemolytic activity. The remaining two isolates were wound isolates and had gamma-hemolytic activity. Despite not having the gene, a third isolate that was also taken from wounds had phenotypic beta-hemolytic activity. The lack of beta-hemolytic capacity in *S. saprophyticus* bacteria is consistent in their genotype and phenotype tests.

Concerning biofilm producing, the outcome of the current study demonstrated that twenty bacterial isolates were biofilm formers on CRA, while the corresponding gene *asa1* was observed as the most recorded gene. *asa1* gene was observed in 38, 35, and 38 isolates out of 38 isolates (total of all *Staphylococcus* spp. under investigation) using simplex, duplex, and multiplex PCR, respectively. Two of *S. epidermidis* and *S. Hominis*, and four of *S. aureus* and *S. hemolyticus*, were among the isolates that showed biofilm formation on Congo red agar plates. All but three of them were separated from patient's wound swabs (one from blood and two from urine samples of the same patient). However, there was a significant difference between *asa1* gene recording using simplex and multiplex compared to duplex at $p<0.0001$ using Cochran's Q test.

Regarding the result obtained for the *hyl* gene which showed 84.20%, 55,30%, and 39.50% using simplex, duplex and multiplex PCR respectively, there was a significant difference at level $p<0.0001$ between simplex and duplex which is also differ significantly compared to multiplex

PCR using Cochran' Q test. However, statistically, the other three genes in the current study, namely *efa*, *esp,* and *cyl* were recorded approximately same result as shown in table (2), since all of them different significantly at p<0.0001 between simplex PCR compared to duplex and multiplex PCR (no difference was recorded between duplex and multiplex for these three genes).

It is worth mentioning that three genes encoding three 3 virulence factors, namely *hyl, efa,* and *es,p* were investigated genotypically only in the current study.

Antibiotic susceptibility

Antibiotic susceptibility testing was investigated on the identified bacterial isolates using the disk agar diffusion method, followed by detection of two antimicrobial resistance genes. As illustrated in Table 3, notably, most of the isolates in the current study were resistant to vancomycin and piperacillin. Less resistance was recorded for Gentamicin, Levofloxacin, and Ceftriaxone, while all isolates of *S. epidermidis, S. aureus, and S. saprophyticus* were sensitive to Meropenem.

According to the obtained outcome of antimicrobial sensitivity, it was observed that the more active antibiotic towards *S. epidermidis, Staph. aureus, Staph. saprophyticus,* and *Staph. hominis* was Meropenem, with a significant difference at $p< 0.0001$ using the Cochran's Q test, followed by Levofloxacin, which is different from the other antibiotics, without significant differences compared to Ceftriaxone, Vancomycin, and finally Piperacillin that showed no activity towards *S. epidermidis*. However, Gentamicin shows a comparable activity to Meropenem toward *Staph. hominis* without significant differences. In detail, *S. aureus* revealed a diverse response against the antimicrobial under investigation, where Meropenem was the best antibiotic detected against *S. aureus* with a significant difference ($p<0.0001$) compared to Ceftriaxone and Levofloxacin; however, Gentamicin showed activity towards only 1 isolate. All 11 tested *S. aureus* were resistant to both Vancomycin and Piperacillin. *S. haemolyticus* showed resistance to most of the 6 investigated antibiotics, with Meropenem that showed an outstanding activity towards these resistant bacteria, with a significant difference (p <0.0001) compared to Gentamicin, Piperacillin, and Ceftriaxone, whereas less activity was recorded for Vancomycin and Levofloxacin toward this bacterial isolate. One out of 5 isolate of *S.hominis* was resistant to each of Meropenem, followed by Gentamicin and Levofloxacin, with a significant difference

at $p<0.05$ compared to Piperacillin, Ceftriaxone, and Vancomycin where 4, 4, and 5 out of 5 isolates were resistant, respectively (Table 3). The last tested bacterial species in the present study was *S. saprophyticus*, which showed equal sensitivity towards Meropenem, Ceftriaxone, Piperacillin, and Levofloxacin, whereas it was resistant to both Vancomycin and Gentamicin.

Table 3: Antimicrobial susceptibility of *Staphylococcus* spp. obtained from clinical isolates

Clinical isolates	Number of antimicrobial resistance isolates					
	VA 30 µg	**GEN 10 µg**	**LEV 5 µg**	**PRL 100 µg**	**MRP 10 µg**	**CRO 30 µg**
S. epidermidis (12)	11	8	2	12	0	10
S. aureus (11)	11	10	6	11	0	6
S. haemolyticus (9)	7	9	5	9	4	9
S. hominis (5)	5	1	2	4	1	4
S. saprophyticus (1)	1	1	0	0	0	0
Total (38)	35	29	15	36	5	29

VA: Vancomycin, GEN: Gentamicin, LEV: Levofloxacin, PRL: Piperacillin, MRP: Meropenem, CRO: Ceftriaxone.

Detection *of vanA)* and *aph (3')*) resistance genes in Staphylococcal isolates

The gentamicin (*aph (3')*) and vancomycin *(vanA)* resistance genes were also uncovered in the current research. The outcome revealed that the *aph (3')* gene was more widespread, since it was recorded in 27 of Staphylococci clinical isolates: nine (23.68% each) of both *S. epidermidis* and *S. aureus*, five of them belonged to *S. haemolyticus* (13.15 %), three of *S. hominis* were positive for this gene (7.89 %), and finally only one (2.63 %) was for *S. saprophyticus*. Interestingly, only five out of the thirty-eight isolates were shown to have the *vanA* gene; mainly, 2 (5.26 %) of them belonged to *S. epidermidis*, and 1 (2.63 %) from each of *S. aureus*, *S. haemolyticus*, and *S. hominis*. The sources of the resistant isolates were two isolates of *S. epidermidis* and one isolate from each of *S. aureus* and *S. hominis* from wounds, and one isolate of *S. haemolyticus* from urine were showed phenotypic and genomic concordance for vancomycin resistance; however, resistance was more frequently observed phenotypically than genotypically. Six *S. epidermidis* isolates from wounds showed gentamicin resistance

in both phenotypic and genomic aspects. However, three isolates displayed the gene but did not exhibit resistance, which has been shown by the other two that don't harbor the encoding gene. Eight *S. aureus* isolates showed genotype-phenotype concordance in gentamicin resistance; seven were isolated from wounds and one from urine, whereas genotype-phenotype concordance was observed in two isolates that exhibited phenotypic resistance; the third isolate exhibited only genotype-specific resistance. There was no genotypic-phenotypic agreement for gentamicin resistance in *S. hominis* isolates, since three isolates had the gene but did not exhibit its phenotypic expression; two isolates were obtained from wounds and one from urine,;however, one isolate from wounds that had the phenotypic resistance did not have the gene. The results of resistance testing to gentamicin showed that all 9 *S. haemolyticus* isolates exhibited the phenotype, while the genotype was recorded in only five isolates, three of which were recovered from urine, one from blood, and one from wounds. The phenotypic and genomic pattern of gentamicin resistance is consistent in the *S. saprophyticus* isolate.

The findings revealed that two isolates from wounds, *S. epidermidis* and *S. aureus*, demonstrated phenotypic and genotypic resistance to gentamicin and vancomycin.

Discussion

The skin serves as the first barrier against microbial invasion; however, damage to this barrier heightens vulnerability to bacterial infections, especially those caused by *Staphylococcus aureus*. Inadequate wound management can exacerbate infection and result in serious complications, including sepsis and systemic infection, potentially leading to fatality [4]. Distinguishing between related cocci that are found frequently in the same environments, such as *Staphylococcus* spp., *Enterococcus* spp., and Streptococci spp., is necessary and desperately following the diagnostic references such as MacFaddin [30].

However, a previous study for Al-Maliki et al., [15], has found that despite the using of an Azide blood agar and Enterococci HiCrome agar that are highly selective medium for the split-up of *Enterococcus* species, 38 isolates, showed up as white colonies on this medium were identified later on in the current study as *Staphylococcus* spp. which reflect a high similarity between these two Gram-positive bacteria. As such, up to 6 Enterococci virulence genes (*asa1, gelE, hyl, efaA, esp,* and *cylA)* that were chosen, depending on different studies, to be

amplified using Enterococcal gene primers by simplex, duplex, and multiplex PCR methods, for their presence in Staphylococci spp., have shown valuable and variable results. In addition, the production of some of the genes is also investigated phenotypically.

The *asa1* frequency in the current study was the highest among the staphylococcal spp., applying simplex, duplex, and multiplex PCR with 100 %, 78.9 %, and 100 %, respectively. Bacterial adhesion to collagen is an essential step in the development and persistence of infections caused by *Staphylococcus aureus* and other *Staphylococcus* spp. The *asa1* gene is associated with bacterial conjugation, adhesion, and pathogenicity, while specific amino acid motifs within *asa1* may promote fibronectin-like binding to eukaryotic cells, enhancing microbial virulence and infection establishment [10]. *The asa1* gene has been recorded at a high percent in *Staphylococcus* spp.. However, the *asa1* gene percentage was recorded in the multiplex higher than the duplex, which is weird or unexpected. This might be due to the use of an equal amount of the two pairs of primers, which in some cases led to the first primer outcompeting the second once owing to its adequacy to adhere to its target and led to failure or weak amplification of the target. The concentration of agarose gel used at that time also affected band separation and appearance; excluding the experimental techniques, mistakes can be overcome in the future.

S. aureus and *Enterococcus faecalis* are widespread opportunistic pathogens accountable for nosocomial infections and biofilm-associated diseases such as endocarditis, urinary tract infections, and chronic wounds [31]. The responsible gene for gelatin hydrolysis, *gelE* is recorded at a high frequency in all examined *Staphylococcus* isolates, as demonstrated in the results section. The generation of biofilm and gelatinase are correlated, which is consistent with Sharma et al. [32]. However, 27 isolates have the ability to produce the gelatinase enzyme phenotypically in the current study compared to genotypic approach, which might be due to inappropriate culture conditions or other factors that stop gene expression, which again reflects the incompatibility between phenotypic and genotypic characterization.

Hyaluronidase in pathogenic models has been shown to be responsible for bacterial colonization, invasion, and biofilm dispersal, especially in immunocompromised individuals [33]. In the current investigation, the *hyl* gene was also high in Staphylococci spp., since 32 out

of 38 isolates harbored this gene (84.21%) using simplex PCR. However, 21(55.25%) and 15 (39.47%) were detected using duplex and multiplex PCR, respectively. The highest frequency for this gene was recorded in *S.epidermidis* and then *S. haemolyticus*. The current investigation is aligned with Hart et al. [34], who recorded a high percentage of this gene in Staphylococci spp.; on another side, the current study is inconsistent with another study by Hart et al. [35], who recorded that 98% of hyaluronidase was produced by *S. aureus*. However, Hart et al. [35] proved in this study that this gene (also named *hysA* in *Staphylococcus*) is present in all clinical isolates of *S. aureus,* unlike other *Staphylococcus* species. However, this gene is usually regulated by other regulatory proteins such as CodYY, which is expressed under different nutritional conditions [33].

The *esp* gene encodes a protein that may also play a crucial role in virulence, since it assists in colonization and stability in urinary tract infections. Abundance studies have demonstrated the structural similarities between *esp* and other proteins associated with biofilm formation produced by Gram (+) bacteria, i.e., the C-α, encoded by the *bca* gene, encodes for β-hemolytic protein in *Streptococcus agalactiae*, R28 in *Streptococcus pyogenes*, and *Bap* in *Staphylococcus aureus* [36]. During the current investigation, *esp* was recorded at the highest percentage using simplex PCR, while only lower in duplex and multiplex PCR for all *Staphylococcus* species. However, this gene was recorded at a high percentage applying simplex PCR in *Staph. aureus*, although the percentage was low using duplex and multiplex PCR. Although this gene was detected as more prevalent in pathogenic *E. faecalis* [15], it has been shown that *esp* may pass from one strain to another via chromosome–chromosome transposition and plasmid conjugation [8,12], which provides the possibility of transfer between Staphylococci and Enterococci. Another surface protein which the accurate role in pathogenicity is not fully understood but might be involved in attachment to biotic and abiotic surfaces and biofilm production, like *esp*, is *E. faecalis* antigen A (*EfaA*) [23], which also investigated in the present study, the results demonstrated that this gene frequency was 65.78%, 31.57%, and 28, 94% using Simplex, Duplex, and Multiplex, respectively. These percent consider not as low as recorded by Mannu et al. [23], who revealed that 19 of 40 diary isolates contain this gene, where no clinical isolate was positive for this gene, which also demonstrated the possibility of transferring this gene between Gram +ve cocci and causing identification interference.

However, the transfer of some virulence genetic materials between closely related Gram +ve cocci has been reported by our group in another study (the study has been accepted and is under journal publication process).

The pathogenic potential of *Staphylococcus* can also be associated with diverse virulence factors, such as hemolysin, which can shift the immune responses or destroy the cell membranes of its host, leading to damage to intercellular connections. This toxin has been shown to affect various types of human cell types such as T-cells, monocytes, macrophage, endothelial, and epithelial cells [5]. In the current investigation, 4, 19, and 15 were recorded to be α, β, and γ hemolysis phenotypically, respectively, while the encoding gene (*cylA*) was observed in only 12, 5, and 3 isolates applying simplex, duplex, and multiplex PCR approach, respectively. Cytolysin is an operon with a multigene consists of Lysin (L) encoded by *cylL1*, *cylL2*, *cylM*, *cylB,* and an activator (A) encoded by *cylA*. This multigene starts at the same point and ends with a transcriptional structure responsible for recognizable function [22, 37]; this might explain that detecting this multigene operon required many primer pairs, which subsequently reflects thelowers sensitivity and low percentage of this genecomparedg to the phenotypic method. Also, Staphylococcal cytolysin genes frequently fail to generate the predicted hemolytic phenotypes due to multilayered regulation, mobile-element RNA effects, metabolic status, and environmental cues that influence transcription, translation, and activity. Additionally, Staphylococcal toxin expression is regulated by quorum sensing and various regulatory pathways, while metabolic status and environmental conditions significantly influence cytolysin production. Consequently, toxin genes may exist without active toxin expression owing to diminished Agr activity, environmental conditions, or host-related factors [37]. However, the recording of α and γ-hemolysis results in *Staph. aureus* or β-hemolysis in *Staph. epidermidis* which was unexpected due to many reasons mentioned above and supported by many studies, since in a study by Ventura et al.,[38], using a type of molecular approach called pulsed-field gel electrophoresis, a strain of *Staph. aureus* called USA300, which is a prevalent strain in the USA, was found to produce thirteen toxin ttypes,including γ-hemolysin AB (HlgAB) and γ-hemolysin CB (HlgCB). Also, Lai et al. [39] reported that different *Staph. aureus* strains have been isolated from different medical cases for patients under hemodialysis, have been shown to be either β- or γ-hemolytic phenotypes. In addition to the possibility of passing them

among these different species of *Staphylococcus,* as suggested by the current study.

In the current study, antibiotic sensitivity also was demonstrated for the staphylococci using disc diffusion method, which revealed that 36 out of 38 isolates were resistant to piperacillin, 35 to vancomycin, 29 to ceftriaxone and gentamicin, 15 were resistant to levofloxacin, while only five out of 38 isolates were resistant to meropenem according to CLSI [27]. The bacteria within biofilms are more protected and they can effectively acquire the resistance genes from neighboring cells, including those from different specie,s through horizontal gene transfer facilitated by plasmids and other mobile genetic components [11].

In burn victims, these individuals are at significant risk of severe infections caused by resistant Gram-positive bacteria, notably MRSA, and vancomycin is one of the few first-line viable treatment choices for physicians. Due to altered pharmacokinetics and probable nephrotoxicity in this group, it must be used with caution and a tailored dose [40]. However, a high resistance percentage of Staphylococci spp. was recorded in the current study. Owing to the high percentage recovering of antibiotic-resistant strains, it is essential to detect vancomycin, which is a medication of choice by physicians, as well as the resistance genes of gentamicin in Staphylococci, especially *Staphylococcus aureus*.

Molecular approaches such as PCR have been shown to be used in a numerous study to detect genes associated to vancomycin resistance, such as *vanA, vanB*, and *vanC*. Although high percentage of phenotypically resistant Staphylococci isolates were demonstrated to be resistant to Vancomycin, only 5 *Staphylococcus* spp. out of 38 isolates harbored the *vanA* resistance gene. Geographical differences have been reported in the prevalence of the *vanA* gene; for instance, a study conducted in Sudan found that 10.3% of isolates were identified as vancomycin-resistant *Staphylococcus aureus* (VRSA) carrying the *vanA* gene [41]. As such, looking for vancomycin-resistance genes requires applying more than three pairs of gene primers or designing a primer covering the whole resistance gene locus; furthermore, the conjugative plasmid of vancomycin-resistant *Enterococcus* is considered the original source for this operon, which is carried on the Tn1546 transposon [12]. Although most of the *Staphylococcus* spp. in the current study are resistant to gentamicin phenotypically and genotypically, it has not been recommended

as a suitable choice by physicians due to the consequent complications of this medication, especially for burn patients being gentamicin has two major toxicities that limit its usage in burn patients: renal tubular injury and inner-ear (cochlear and vestibular) impairment. Also, the restricted use of this antibiotic reflects the lower exposuretof most pathogens that might interpret the high resistance of staphylococci to this gene phenotypically and genotypically. Identification of antibiotics resistance is essential for understanding the molecular mechanisms underlying antimicrobial resistance and may facilitate the development of effective therapeutic, preventive, and management strategies against *Staphylococcus* infections. These findings further emphasize the importance of continued research in this area. Although gentamicin resistance is generally less prevalent, the current study's findings revealed that the *aph(3')* gene was detected more frequently than the *vanA* gene. Nevertheless, compared with the well-characterized mechanism of vancomycin resistance, the mechanism responsible for gentamicin resistance in *Staphylococcus* spp. remains incompletely understood.

Interestingly,y and according to the current study's findings, *S. epidermidis,* which is a normal flora of the skin, was the most frequent species, consisting of 32% (12 out of the 38 isolates). Furthermore, isolates of *S. epidermidis* were recorded to have a higher predominance of some virulence factors in the laboratory setting compared to other pathogenic *Staphylococcus* spp. such as *S. aureus*. In addition to record these isolates showed a high percent of β-hemolysis which need more attention in the future.

Despite the variability in detection rates among individual genes, using simplex PCR remains a valuable technique owing to its high sensitivity and specificity in amplifying single target genes without interference from multiple targets [40]. Duplex and multiplex PCR may shorten the time and labor; no sequencing is needed; this might be useful in epidemiological research where time is critical by detecting numerous genes at once in the same reaction tube. However, lower sensitivity for each individual gene was observed due to reagent competition, and low sensitivity and specificity in identifying particular genes. However, the small number of *Staphylococcus* spp. (38 isolates), emerging five other Staphylococci types rather than Staph. aureus, including *S. epidermidus* in a high percentage, recording some results positive phenotypically whereas it was negative genotypically, or vice versa, may be consider as a limitation in generalizing

the conclusion of the current study until a large number of these bacteria were analyzed.

Conclusion

Although more than six virulence genes considered highly specific to *Enterococcus* were investigated in *Staphylococcus aureus* isolated from the same ecological niche, five additional *Staphylococcus* spp. recovered from burn patients were also found to harbor these virulence genes. This finding may indicate possible horizontal gene transfer between these closely related Gram-positive cocci and highlights the importance of further investigating *Enterococcus*-associated virulence genes in *Staphylococcus* and other pathogenic cocci. Furthermore, genes that have not previously been detected in these bacteria could serve as potential targets for future studies and may help distinguish between closely related bacterial species. Despite the high VRS observed in the current study, MRP appeared to be the most effective alternative antibiotic against most pathogenic *Staphylococcus* isolates.

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Ethical approval

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تشخيص ست جينات ضراوة خاصة لبكتريا *Enterococcus* spp. في بكتريا *Staphylococcus* spp المعزولة من الحروق باستخدام التفاعل التسلسلي المفرد، المزدوج والمتعدد

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ان تردد بكتريا المكورات العنقودية Staphylococci spp. خصوصا النوع الممرض *Staphylococcus aureus* يشكل تهديد خطير لمرضى الحروق، وتتسبب بحدوث عدد من الإصابات تتراوح من إصابات جلدية بسيطة الى إصابة معقدة جدا تهدد حياة المريض مثل انتان الدم والذي بدوره يؤدي الى تعقيد سبل المعالجة. هدفت الدراسة الحالية

الى استخدام الطرق المظهرية والجينية لتشخيص عدد من عوامل الضراوة الخاصة ببكتريا *Enterococcus* spp. التي قد تكون انتقلت الى بكتريا المكورات العنقودية المعزولة من مرضى الحروق ذاتهم. شملت الدراسة جمع 200 عينة من الجروح، الادرار، البراز، والدم من 75 مريض بالحروق. تم اختيار ست جينات مسؤولة عن التشفير لعوامل ضراوة في المكورات وهي *asa1*، *gelE*، *cylA* ، *esp*، *hyl* ، و *efa* باستخدام تفاعل البلمرة المتسلسل Polymerase chain (PCR) Reaction المفرد، الثنائي والمتعدد مع استخدام بعض الصفات المظهرية في أنواع بكتريا المكورات العنقودية بلا ضافة الى تحديد جينان المقاومة للمضادين: الفانكومايسين والجنتامايسين *vanA*، *aph(3')* على التوالي. أظهرت نتائج طرق التشخيص الاولية باستخدام الأوساط الزرعية وبعض الاختبارات البايوكيميائية متبوعة بالتشخيص الجيني باستخدام جين *16S rRNA* ان 38 عزلة شخصت على انها مكورات عنقودية تعود الى خمسة أنواع: 31.5% منها كانت *Staphylococcus epidermidis* ، 28.5% كانت *Staphylococcus aureus* ، 23.6% كانت *Staphylococcus haemolyticus* ، 13% *S.hominis* ، و2.6% منها *S.saprophyticus* . جميع هذه العزلات نمت على HiChrome of *Enterococcus faecium* (وهو وسط عالي الخصوصية لتنمية المكورات المعوية). سجل الجين *asa1* أعلى تردد حيث ظهر بنسبة 100% في أنواع المكورات قيد الدراسة *Staphylococcus* spp. وبفارق معنوي عن البقية الجينات عند مستوى احتمالية 0.0001 اما بقية الجينات مثل *gelE* و *hyl* فقد سجلت أيضا نسب ظهور عالية مقارنة مع بقية الجينات التي سجلت بدورها نسب متفاوتة اقل من السابقة. كما اوضحت الدراسة الحالية من خلال النتائج الحالية ان تواجد هذين النوعين من البكتريا في نفس البيئة يشكل عامل خطر لانتقال الجينات المرضية الخطرة بين هذين النوعين المعزولة من نفس المكان المرضي وربما انتقال عوامل أخرى وأكدت الدراسة على ضرورة إيجاد طرق تشخيص سريعة لتقليل الخطر الناجم عنها.

الكلمات المفتاحية: التهابات الحروق ، بكتريا المكورات العنقودية، جينات الضراوة