Original article

Antibiotic-resistant *Staphylococcus epidermidis* isolated from patients and healthy students comparing with antibiotic-resistant bacteria isolated from pasteurized milk

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ABSTRACT

Antibiotic-resistant *Staphylococci* are a global issue affecting humans, animals, and numerous natural environments. Antibiotic-resistant *Staphylococcus epidermidis* is an opportunistic pathogen frequently isolated from patients and healthy individuals. This study aimed to examine the antibiotic resistance of *S. epidermidis* isolated from patients, healthy students and compare the results with antibiotic-resistant bacteria isolated from pasteurized milk. Clinical strain isolation was performed in several hospitals in the Riyadh. Skin swabs from 100 healthy undergraduate candidate students were obtained at King Saud University. The pasteurized milk samples were obtained from local market (company, X). After isolation, identification and susceptibility tests were performed using an automated system. A multiplex *tuf* gene-based PCR assay was used to confirm identification. Biofilm production and biofilm-related gene expression were studied. *S. epidermidis* represented 17% of clinical bacterial isolates, and 1.7% of isolates obtained from healthy students were multiantibiotic-resistant. All patient strains were teicoplanin- and vancomycin-susceptible, while all student strains were gentamicin-, levofloxacin-, moxifloxacin-, and trimethoprim/sulfamethoxazole-susceptible. All the bacteria isolated from pasteurized milk were benzylpenicillin and oxacillin-resistant strains. Of the *S. epidermidis* strains, 91% could produce biofilms, and *mecA, icaADBR, ica-ADB, ica-AD, ica-A* only*, and ica-C* only were expressed in 83, 17.1, 25.7, 37.1, 20, and 0% of the strains, respectively. This work demonstrates that *S. epidermidis* can be accurately identified using a multiplex *tuf*-based assay, and that multiantibiotic-resistant *S. epidermidis* strains are widespread amongst patients and healthy students.

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1. Introduction

Staphylococcus spp. are common commensal bacteria that colonize human skin and have been isolated from diverse sources such as meat, milk, cheese, soil, sand, seawater, freshwater, dust, and air (Kloos et al., 1991). *S. epidermidis* is frequently isolated from the axillae, head, nares, and epithelial tissues (Kloos and Musselwhite, 1975), and it has been suggested that *S. epidermidis* may prevent the colonization of several pathogenic microorganisms such as *Staphylococcus aureus* (Duguid et al., 1992). Gram-positive, non-spore forming, nonmotile, facultative anaerobic, and catalase-positive *staphylococci* bacteria can be classified into coagulase-negative and positive *staphylococci*. *S. epidermidis* is the most clinically significant of the coagulase-negative staphylococci (Namvar et al., 2014; Bowden et al., 2005), which causes infections in the presence of broken skin, especially in immunocompromised patients (Schoenfelder et al., 2010). It contains high concentrations of peptidoglycans and cell wall-anchored proteins, which interact with targets in the host. In hospitalized patients, intact medical

devices are considered a major vector of *S. epidermidis* (Ziebuhr et al., 2006). In general, *Staphylococcus* spp. are considered a major cause of nosocomial infection. *S. epidermidis* results in approximately 13% of prosthetic valve endocarditis infections, with a high rate of intracardiac abscess formation (38%) and mortality (24%) (Chu et al., 2009). *S. epidermidis* grows rapidly on blood agar, resulting in white, raised, cohesive colonies 1–2 mm in diameter that are nonhemolytic (Salyers and Dixie, 2002). In aerobic conditions, it can produce acid from fructose, maltose, sucrose, and glycerol. Its sensitivity to novobiocin is used to distinguish it from *Staphylococcus saprophyticus* (Schaefler, 1971).

Many studies have reported that *S. epidermidis* has genetic mechanisms to overcome harsh environmental conditions, such as extreme salt concentrations and osmotic pressures (Rogers et al., 2009). *S. epidermidis* strains show a high degree of diversity according to sequence types (STs; allelic profiles) (Miragaia et al., 2007). Strains receive resistance determinants through gene acquisition and genetic recombination (Miragaia et al., 2008). In 2011, a study reported that >70% of *S. epidermidis* strains are resistant to methicillin (oxacillin) (Farrell et al., 2011). Although it is an opportunistic pathogen, it plays an important role in balancing the skin microflora and serves as a source of resistance genes (Otto, 2009).

In the United States, at least two million people are infected with antibiotic-resistant bacteria each year, and approximately 23,000 of them die, according to the Center for Disease Control and Prevention. In 2016, the World Health Organization published a fact sheet demonstrating that antibiotic resistance is a considerable threat to global health; it can affect anyone, of any age, in any country. Misuse of antimicrobial agents is a significant cause of antibiotic resistance, which leads to increased medical expenses and mortality (World Health Organization, 2016). Methods of microbial gene transfer include transformation, transfection, transduction, and lipofection. Regarding *Staphylococcus* spp., there are many reports confirming that genetic transfer is mediated by phage transduction and conjugation. Resistance to multiple antibiotics (such as methicillin and vancomycin) is a major obstacle in the treatment of infections caused by *Staphylococcus* spp. (Lacey, 1980; Chen and Novick, 2009; Chan et al., 2011). There are several sources of antibiotic-resistant *S. epidermidis*, including environment surfaces, personal clothing, medical devices, and the skin of health care workers and patients (Brumfitt and Hamilton-Miller, 1989). In Saudi Arabia, although many studies have investigated methicillin-resistant *S. aureus*, there have been very few comparable studies of antibiotic-resistant *S. epidermidis*. In this study, we investigated the antibiotic resistance of *S. epidermidis* strains isolated from patients and healthy students in Riyadh, Saudi Arabia.

2. Materials and methods

2.1. Collection of specimens

Sample collection was performed during two academic semesters (2016–2017). Skin swab samples were collected from healthy male undergraduate students registered in the Botany and Microbiology Department in the College of Science at King Saud University (KSU), Riyadh City. During the same period of time, samples were obtained from patients with clinical symptoms of bacterial infection. All subjects provided written informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the Ethics Committee of the Deanship of Scientific Research and Research Center for the College of Science approved the protocol (KSU-SE-17-2). Clinical isolates were collected from several hospitals in the Riyadh region, including Iman General Hospital, Al-Yamamah General Hospital, and Buraidah Central Hospital.

The clinical specimens included blood, urine, wound swabs, and endotracheal tube secretion (ETTSc). The pasteurized milk samples were obtained from local market (company, X).

2.2. Isolation and primary identification

Samples were cultivated on nutrient agar (Oxoid, United Kingdom) at 37 °C for 24 h. Negative control samples were incubated for another 24 h to ensure no growth. Purification was performed by serial sub cultivation on nutrient agar to obtain single colonies in pure culture. All single cultures were preserved in a sterile glycerol solution (30%) at −80 °C until needed.

The macroscopic and microscopic characteristics were studied using mannitol salt agar (Oxoid) and Baird-Parker egg yolk agar (Oxoid) and a light microscope (Motic, Taiwan). Catalase, slide coagulase activity, motility, blood agar hemolysis, nitrate reductase, urease, and lysozyme and lysostaphin resistance tests were performed as in (Freney et al., 1999).

2.3. Identification and susceptibility tests

Identification and susceptibility test were performed using the automated VITEK 2 system (BioMérieux, Marcy-l'Étoile, France) using GP to identify *enterococci*, *streptococci*, *staphylococci*, and selective groups of gram-positive bacteria. AST-ST01 was used to determine the susceptibility of *Streptococcus pneumoniae*, beta-hemolytic *streptococci*, and *viridans streptococci*. Identifications were confirmed using a multiplex *tuf* gene-based PCR method according to (Delgado et al., 2009). Briefly, chromosomal DNA was extracted using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, Missouri, USA). Lysozyme (30 μg/mL; Sigma-Aldrich) was added at the cell lysis step. PCR was performed using 1 μL of purified DNA as a template, a 2× QIAGEN Multiplex PCR kit (QIAGEN, Hilden, Germany), and 12 μM of each of the three primers: *tuf-g* (5′-GGTGTACCAGCATTAGT-3′), *tuf-a* (5′-TTCAG TATGTGGTGTAA-3′), and *tuf-e* (5′-TTCGTGCATACCGATGA-3′). Oligonucleotide primers were purchased from Eurofines Genomic (Luxembourg, Germany). PCR conditions were 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 5 min. Amplicons were analyzed by gel electrophoresis in a 1.5% agarose gel and visualized under ultraviolet light with ethidium bromide.

2.4. Detection of mecA

The *mecA* gene was detected by PCR using the primers *mecA* forward (5′-GGTCCCATTAACTCTGAAG-3′) and *mecA* reverse (5′-AGTT CTGCAGTACCGGATTTTGC-3′), as in (Cafiso et al., 2001). Oligonucleotide primers were purchased from Eurofines Genomic (Luxembourg, Germany).

2.5. Biofilm production test

Biofilm production was tested by a microtiter method, as described in (Christensen et al., 1985). *S. epidermidis* isolates were aerobically cultivated on tryptic soy broth (TSB; Oxoid) supplemented with 0.25% glucose (Sigma-Aldrich) at 37 °C for 12 h with shaking at 150 rpm. Samples were diluted 1:100 in TSB, then 200 μL were added into each well of a 96-well polystyrene microtiter plate. The plate was aerobically incubated at 37 °C for 12 h, then washed and stained with crystal violet dye (0.1%).

A microplate reader Multiskan™ FC (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to measure the optical density (OD) at 490 nm. The results were considered positive for biofilm production when the OD was >0.12. The *icaA, B, C, D,* and *R* genes were detected by a multiplex biofilm-related

Table 1
Primers used in the multiplex biofilm-related gene-based PCR assay.

Primer	Sequence 5′ → 3′		Amplicon size (bp)
	Forward	Reverse	
icaA	ACAGTCGCTACGAAAAGAAA	GGAAATGCCATAATGACAAC	103
icaB	CTGATCAAGAATTTAAATCACAAA	AAAGTCCCATAAGCCTGTTT	302
icaC	TAACTTTAGGCGCATATGTTTT	TTCCAGTTAGGCTGGTATTG	400
icaD	ATGGTCAAGCCCAGACAGAG	CGTGTTTTCAACATTTAATGCAA	198
icaR	TAATCCCGAATTTTTGTGAA	AACGCAATAACCTTATTTTCC	453

gene-based PCR method according to (de Silva et al., 2002) and (Zhou et al., 2013). The primers used in this assay are listed in the Table 1.

2.6. Experimental design and analysis

A completely randomized design was used in this study, and the data are represented as percentages. The antibiotic resistance
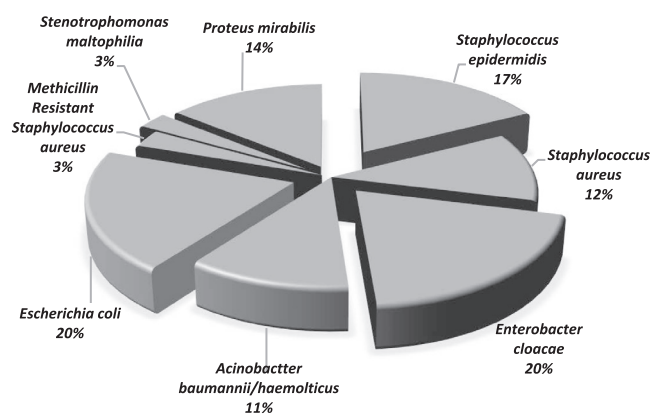
Fig. 1. Percentages of different species in clinical isolates collected from patients in several hospitals in the Riyadh region (n = 200).

pattern was analyzed by Ward linkage using SPSS Statistics 24 (IBM, USA).

3. Results and discussion

In this study, the antibiotic resistance of *S. epidermidis* strains isolated from patients and healthy students was investigated. From patients, 45.71, 28.57, 22.86, and 2.8% of the clinical isolates were obtained from blood, urine, wound swabs, and endotracheal tubes, respectively. The largest percentages of isolates were *Escherichia coli* and *Enterobacter cloacae* (Fig. 1), while 17% of clinical bacteria isolates were *S. epidermidis*, and 12% and 3% were *S. aureus* and methicillin-resistant *S. aureus* (MRSA) respectively.

The normal micro flora isolated from the skin of healthy male students are shown in Fig. 2. Of the samples collected from students, 62.19% displayed no growth on the culture media used in this study. Although 12.82% of the isolates were identified as *Staphylococcus* species, only 1.7% were *S. epidermidis*. Non-staphylococcus bacteria composed 25.01% of the identified isolates.

The percentages of antibiotic-resistant and susceptible *S. epidermidis* isolated from patients and healthy students in Riyadh are shown in Table 2. The scan covered several standard antibiotics and 60 clinical isolates identified as *S. epidermidis*. The results show that 100% of *S. epidermidis* isolated from patients were resistant to daptomycin, and 100% of *S. epidermidis* isolated from students were susceptible to vancomycin and teicoplanin.

Fosfomycin, amoxicillin/clavulanic acid, lmipenem, linezolid, and oxacillin resistance were detected in more than 80% of the iso-
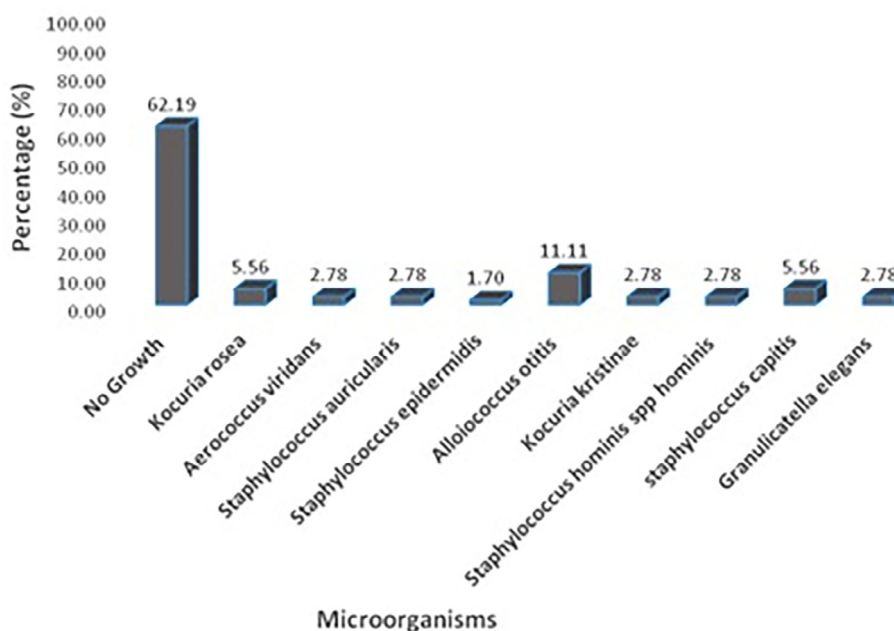
Fig. 2. Percentages of different species in clinical microbial isolates from healthy KSU students (n = 100).

Table 2
Percentages of antibiotic-resistant and susceptible *S. epidermidis* isolated from patients, healthy students and pasteurized milk (n = 60 strains).

Patients			Health students and pasteurized milk		
Resistance	Antibiotic		Antibiotic	Health students Resistance	Pasteurized milk Resistance
85.71%	Amox/K Clav		Benzylpenicillin	100%	100%
54.29%	Ciprofloxacin		Oxacillin	40%	100%
37.14%	Clindamycin		Gentamicin	0%	37%
100.00%	Daptomycin		Tobramycin	40%	0%
60.00%	Erythromycin		Levolfoxcin	0%	0%
97.14%	Fosfomycin		Moxifloxacin	0%	0%
14.29%	Fusidic Acid		Erythromycin	60%	16%
54.29%	Gentamicin		Clindamycin	60%	33%
82.86%	Lmipenem		Linezolid	40%	40%
54.29%	Levofloxacin		Teicoplanin	20%	16%
97.14%	Linezolid		Vancomycin	20%	16%
48.57%	Moxifloxacin		Tetracycline	60%	0%
22.86%	Mupirocin		Tigecycline	0%	0%
88.57%	Oxacillin		Fostamycin	100%	80%
14.29%	Rifampin		Nitrofurantoin	20%	0%
5.71%	Synercid		Fusidic Acid	80%	16%
0.00%	Teicoplanin		Rifampicin	40%	80%
34.29%	Tetracycline		Trimethoprim/Sulfamethoxazole	0%	80%
40.00%	Trimethoprim/Sulfamethoxazole				
0.00%	Vancomycin				

lates. In addition, 100% of the *S. epidermidis* strains isolated from healthy students were resistant to benzylpenicillin and fosfomycin, and more than 50% were resistant to erythromycin, clindamycin, tetracycline, and fusidic acid. Conversely, 100% of the *S. epidermidis* strains were susceptible to gentamicin, levofloxacin, moxifloxacin, tigecycline, and trimethoprim/sulfamethoxazole. The strains isolated from pasteurized milk reported that the milk may be one of the bio-resources of antibiotics resistant bacteria such as benzylpenicillin and oxacillin-resistant strains.

Fig. 3 demonstrates the differences in antibiotic resistance between strains isolated from patients and healthy students. For example, although *S. epidermidis* strains isolated from healthy students were not resistant to gentamicin, levofloxacin, moxifloxacin, and trimethoprim/sulfamethoxazole, over 40% of *S. epidermidis* strains isolated from patients were resistant to these antibiotics. Furthermore, strains isolated from patients had no resistance to teicoplanin and vancomycin, whereas 20% of *S. epidermidis* strains isolated from healthy students were teicoplanin- and vancomycin-resistant. Even though more than 60% of *S. epidermidis* isolated from healthy students were resistant to clindamycin, tetracycline, and fusidic acid, less than 40% of *S. epidermidis* isolated from patients showed similar resistance. All *S. epidermidis* strains isolated from healthy students and 97.14% of *S. epidermidis* isolated from patients were resistant to fosfomycin, and 60% of all *S. epidermidis* strains isolated were resistant to erythromycin.

The antibiotic susceptibility patterns of *S. epidermidis* were analyzed using Ward linkage analysis. The pattern included fourth groups. The first group showed resistance to amoxicillin/clavulanic acid, lmipenem, and oxacillin, and susceptibility to tetracyclines, macrolides, oxazolidinones, quinolinones, and lipopeptides. The second group showed resistance to quinolones and susceptibility to tetracyclines, macrolides, glycopeptides, lipopeptides, and oxazolidinones. The third group showed resistance to amoxicillin/-clavulanic acid, azithromycin, oxacillin, lmipenem, fusidic acid, gentamycin, tetracyclines, and macrolides, and susceptibility to quinolones, glycopeptides, ansamycins, and lipopeptides. The fourth group showed resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, cefotaxime, mupirocin, oxacillin, tetracyclines, and macrolides, and susceptibility to glycopeptides, ansamycins, oxazolidinones, quinolones, and lipopeptides.

Table 3 summarizes the identification and detection data, including identification by the VITEK 2 system and the multiplex *tuf* gene-based PCR assay; detection of biofilm production by the microplate method and the multiplex biofilm-related gene-based PCR assay; and detection of the *mecA* gene. The VITEK 2 system identified 100% of the isolates as *S. epidermidis* according to their biochemical characteristics, and 94% were identified by the multiplex *tuf* gene-based PCR assay according their genetic features. The microtiter method indicated that 91% of *S. epidermidis* isolates can produce biofilms, and 83% of the isolates contain the *mecA* gene. Regarding the *ica* genes, 17.1, 25.7, 37.1, and 20% of *S. epidermidis* isolates contained *ica-ADBR, icaADB, icaAD,* and *icaA* respectively.

Our results indicate that *E. coli* and *E. cloacae* bacterial infections were most prevalent in Riyadh. Hamid et al. (2011) reported that *E. coli* and *Staphylococcus* spp. are the major etiological agents in the Assir region of Saudi Arabia, and most published studies from the area have focused on *S. aureus* and MRSA. Our data suggest that *S. epidermidis* merits more attention, as it represented 17% of the identified bacterial isolates. *S. epidermidis* is an important opportunistic pathogen, and the most common source of bacterial infection on intact medical devices (Otto, 2009). The results of this study raise important questions about the use of fosfomycin for the treatment of nosocomial infections due to multidrug-resistant *S. epidermidis*. Fosfomycin can inhibit cell wall synthesis in both gram-positive and gram-negative bacteria. The data suggest that almost all of the strains were resistant to fosfomycin.
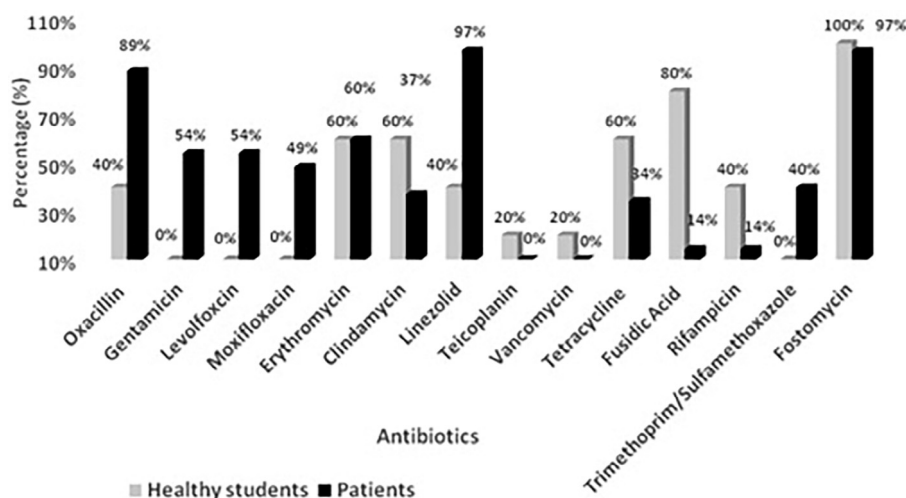
Fig. 3. Comparison of the antibiotic-resistant *S. epidermidis* strains isolated from patients and healthy students (n = 35).

Table 3
Summary of the *S. epidermidis* isolate identification and virulence factor screens.

	Identification by VITEK 2 system	Multiplex *tuf* gene-based PCR assay	Biofilm detection using the microtiter assay	Multiplex biofilm-related gene-based PCR assay				*mecA* gene detection
				Ica-ADBR	*Ica-ADB*	*Ica-AD*	*Ica-A*	
Identified isolates	35	33	32	6	9	13	7	29
Total isolates	35	35	35	35	35	35	35	35
% Identified	100	94	91	17.1	25.7	37.1	20	83

Approximately 89% of *S. epidermidis* isolated from patients were oxacillin-resistant strains, according to the minimal inhibitory concentration test.

Dickinson and Archer (2000) reported that there are difficulties in detecting the oxacillin-resistant phenotype, especially in *S. epidermidis*, as well as phenotypic expression of oxacillin-resistant. *S. epidermidis* in broth can be influenced both by subpopulation resistance expression and by *mecA* transcriptional regulation.

The data obtained in this work could be used as a tool to differentiate between *S. epidermidis* isolated from patients and healthy individuals. All the *S. epidermidis* strains isolated from healthy students were gentamicin-, levofloxacin-, moxifloxacin-, and trimethoprim/sulfamethoxazole-susceptible, whereas all the strains isolated from patients were susceptible to teicoplanin and vancomycin. The gentamicin-resistant strains detected may express enzymes mediating gentamicin resistance, such as aminoglycoside 6′-N-acetyltransferase and gentamicin phosphotransferase.

There is some disagreement between the present results and previous studies. For example, Haque et al. (2009) found that *S. epidermidis* strains isolated from patients showed multiantibiotic resistance as follows: oxacillin, 56%; gentamycin, 44%; erythromycin, 41%; and fusidic acid, 22%. Conversely, strains isolated from healthy controls were susceptible to all antibiotics except 10% of the isolates, which were resistant to penicillin. In our results, we found that 89, 54, 60, and 14.2% of the strains were resistant to oxacillin, gentamycin, erythromycin, and fusidic acid, respectively. Moreover, the strains isolated from healthy students showed multiantibiotic resistance.

Generally, prominent differences were observed in the resistance patterns of *S. epidermidis* isolated from patients and healthy students in the Riyadh region. Phenotypic and genotypic characteristic studies are required to achieve accurate and precise identification of *S. epidermidis*.

The VITEK 2 system is a certified, reliable, automated system used in most medical laboratories. Almost all *S. epidermidis* isolates identified using the VITEK 2 system were confirmed by multiplex *tuf* gene-based PCR assay, except for two isolates, one each from Buraidah Central Hospital and Iman General Hospital.

In accordance with previous studies, identification by the colorimetric VITEK 2 method was verified by genetic methods to ensure accurate identification (Zbinden et al., 2007; Kim et al., 2008). Several multiplex gene-based PCR methods have been suggested for the identification of *S. epidermidis* stains, and nearly all of these methods are based on the detection of the *mecA*, *ica-ABCDR,* and *tuf-GE* genes. These methods can identify the genes that responsible for resistance to methicillin and biofilm formation. For example, Zhou et al. (2013) developed a method to achieve the latter.

The *S. epidermidis* stains isolated from healthy students were negative for the *ica-B, ica-C, ica-R,* and *mecA* genes, and 32.3% of the *S. epidermidis* stains isolated from patients belonged to the fourth group showed resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, cefotaxime, mupirocin, oxacillin, tetracyclines, and macrolides, and susceptibility to glycopeptides, ansamycins, oxazolidinones, quinolones, and lipopeptides according to antibiotic susceptibility pattern analysis.

This study did not provide clear evidence about the genes responsible for the non-production of biofilm strains (BCH-133 BLD, BCH134-TA, and BCH-183 U) because all *S. epidermidis* strains isolated in this work had *ica-A* and not *ica-C*. Additionally, one of the non-producing strains contained *ica-A, ica-D, ica-B,* and *ica-R*. All non-biofilm producing strains were isolated from Buraidah Central Hospital.

4. Conclusions

In conclusion, as all the *S. epidermidis* strains isolated from patients in Riyadh region were susceptible to teicoplanin and vancomycin, these antibacterial agents could be used as alternative

treatments for *S. epidermidis* infections. The results indicate that multidrug-resistant bacteria strains are relatively widespread pathogens in patients, and can be isolated from healthy students and pasteurized milk as well.

It has been found that not all of the strains scanned by the VITEK 2 system were identified as *S. epidermidis* according to the multiplex *tuf* gene-based PCR assay. Also, not all *S. epidermidis* strains had the ability to form biofilms according to microtiter and multiplex biofilm-related gene-based PCR methods.

Acknowledgments

Author contributions

M.G.E. and N.S.A conceived and designed the experiment; M.G.E, S.K, A. S. A. and S. A. A. performed the experiments; J.M.K. analyzed the data and wrote the paper.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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