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Molecular identification, virulence, and antibiotic-resistant genes characteristics of *Staphylococcus* spp., isolated from milk samples

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Abstract

Background: Food safety and food-borne infections are major subjects of global interest. Dairy products are considered as important source for these infections.

Aim: The present study was conducted to identify the occurrence and to genotype isolates of *Staphylococcus* spp. recovered from milk samples in Al-Diwaniyah City, Iraq.

Methods: The current study included the collection of 50 milk samples purchased from local stores in the current city. These samples were subjected to bacterial cultivation and biochemical tests. Later, the growth was used to extract the genomic DNA that was exposed to PCR and partial gene sequencing both targeted the *16S rRNA* gene at a specific genetic piece.

Results: The PCR results demonstrated the amplification of the genetic fragment of five genetic clusters for each of *Staphylococcus aureus* (SAD11, SAD12, SAD13, SAD14, and SAD15), *Staphylococcus epidermidis* (SED1, SED2, SED3, SED4, and SED5), and *Staphylococcus intermedius* (SID1, SID2, SID3, SID4, and SID5). The PCR products were sent out to sequencing and reported that the current isolates were similar in their genetic content with global isolates at 95.34% to 97.59%, 96.21% to 97.57%, and 96.09% to 97.88%, respectively, of identity.

Conclusion: The present findings show high genetic variations among isolates of *S. aureus*, *S. epidermidis*, and *S. intermedius* recovered from milk samples, and these genotypes are found in different infection settings related to humans and animals, which may pose high risks to humans and animals.

Keywords: Food hygiene, Food-borne diseases, Food safety.

Introduction

Staphylococcus aureus is a Gram-positive, coagulase-positive, spherical pathogenic bacterial species belonging to the family *Staphylococcaceae*. It is a commensal inhabitant on many parts of the human body and when present without symptoms it is found on the skin, especially of the anterior nostrils, perineum, groin, and axilla (Lakhundi and Zhang, 2018).

Due to its adaptability to hosts and environment, it causes many infections of varying severity, ranging from mild to lobar pneumonia and septicemia. *Staphylococcus aureus* infections are also considered a major cause of both hospital and community-originated infections. The organism induces disease involving the circulation, cutaneous infection, pneumonia, and foodborne diseases, as well as cause infections by medical devices, such as endocarditis (Diekema *et al*., 2001; Lindsay and Holden, 2004; Schito, 2006).

The bacterium secretes unique illness-inducing virulence factors, such as enterotoxins causing diseases, such as foodborne diseases. *Staphylococcus aureus* is in a group of bacteria that can build up resistance to a wide range of antimicrobial drugs. The methicillin utilization resulted in the methicillin-resistant *S. aureus* (MRSA) occurrence, which significantly increased morbidity and mortality. The occurrence of MRSA infections is being increasingly documented globally. There are different MRSA strains, but those commonly seen produce a modified penicillin-binding protein, PBP (2a or 2′) (Lakhundi and Zhang, 2018).

This altered PBP has a weak binding affinity to penicillin that is mediated by a 34-amino-acid segment located near the primary binding site. The altered PBP is encoded by the *mecA* gene, which is clustered on a mec (SCCmec), a chromosomal element (Katayama *et al.*, 2000).

Recently, *mecC*, a less common carrier, was detected in isolates from MRSA, which are resistant most likely to all penicillins, except for some medicines such as ceftobiprole. Moreover, Vancomycin is routinely used

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mostly because it is more effective in vitro than penicillin. Vancomycin is weighed as the last resort for some severe MRSA infections. However, due to its escalated use, moderate to highly resistant isolates to vancomycin have emerged in countries around the globe. Early identification of MRSA infections, using phenotypic and molecular techniques, and rapid sending of these results to the clinician, could enhance patient care. This might reduce the use of inappropriate antimicrobials, antimicrobial resistance, and cost (Buonomini *et al*., 2020).

Since these bacteria have a wide range of distribution and sources in different environments, such as milk, the present study was conducted to identify the occurrence and genotype isolates of *Staphylococcus* spp. recovered from milk samples in Al-Diwaniyah City, Iraq.

Materials and Methods

Samples

Here, 50 samples of raw milk from different local outlet stores in Al-Diwaniyah City in Iraq have been collected, filled in sterile bottles, and then immediately delivered to the microbiology laboratory in the College of Veterinary Medicine, University of Al-Qadisiyah, in the same city and then stored at refrigerator at 4°C until culturing bacteria.

Bacterial cultivation

These specimens were put in Brain Heart Infusion Broth overnight at 37°C for first enrichment, then inoculated into mannitol salt agar, and incubated at 37°C overnight the next day for optimization as to culture just *Staphylococcus* spp.

Molecular identification

Five isolates of each species (the most virulent isolates) were selected for molecular identification by PCR.

DNA extraction

The protocol and its Wizard Genomic DNA Purification Kit (Promega) were employed for the extraction of the DNA. From the bacterial broth, 1 ml was used as starting material for the extraction. A NanoDrop was recruited to measure the DNA according to its quality and quantity.

PCR

The PCR targeted the *16S rRNA* gene by utilizing the sets of the primer; F: AGAGTTTGATCATGGCTCAG and R: GGTTACCTTGTTACGACTT. The reaction mixture contains 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 2 µl of any of dNTPs, 0.4 µM for the respective primer and 2.5 U DNA polymerase in a volume of 50 µl. The addition of about 10 to 100 ng DNA to the reaction mixture gives to total volume of 50 µl. The PCR was performed using an early denaturation step of 2 minutes at 94°C then 35 cycles of 1 minute at 94°C, 1.5 minutes at 55°C and 1 minute at 72°C, of main denaturation, annealing, and main extension steps, respectively, which finally followed by a final extension step at 72°C for 3 minutes. The expected PCR amplification product has a size of 1500 base pairs. The agarose gel at 1% mixed with ethidium bromide was employed at 80–90 Volt for 60 minutess.

Table 1. Primers of the PCR for the virulence gene detection of *Staphylococcus* spp. isolated from milk samples.

Gene name	Primer	Primer sequence (5′ to 3′)	Anneal temp (°C)	Product size (bp)	References
Enterotoxin A (*sea*)	sea A-F	GCAGGGAACAGCTTTAGGC	55	521	Løvseth *et al*. (2004)
	sea A-R	GTTCTGTAGAAGTATGAAACACG			
Biofilm-associated protein (*bap*)	bap-F	CCCTATATCGAAGGTGTAGAATTG	56.5	971	Booth *et al*. (1995)
	bap-R	GCTGTTGAAGTTAATACTGTACCTGC			
Thermonuclease (*Nuc*)	Nuc-F	CGATTGATGGTGATACGGTT	55	279	Simojoki *et al*. (2012)
	Nuc-R	ACGCAAGCCTTGACGAACTAAAGC			
Elongation factor Tu (*tuf*)	tuf-F	CCAATGCCACAAACTCGT	56	412	Mugabi (2018)
	tuf-R	CCTGAACCAACAGTACGT			
Hemolysin A (*hlaA*)	hlaA-F	GGTTTAGCCTGGCCTTC	55	550	Løvseth *et al*. (2004)
	hlaA-R	CATCACGAACTCGTTCG			
Coagulase (*coa*)	Coa-F	ATAGAGATGCTGGTACAGG	55	710	Booth *et al*. (1995)
	Coa-R	GCTTCCGATTGTTCGATGC			
cytotoxin (*pvl*)	pvl-F	GTGCCAGACAATGAATTACCC	60	255	Fazal *et al*. (2023)
	pvl-R	TTCATGAGTTTTCCAGTTCACTTC			

Sequencing of 16S rRNA gene (partial)

The PCR-amplified DNA sequences were sent for sequencing by ABI3730XL system (Macrogen Inc. Korea), and phylogenic analysis and tree were performed using NCBI-Websites and MEGA X software.

Detection of virulence genes

All isolated bacteria (68 isolates = 32 isolates from human specimen and 36 isolates from chicken samples) were screened for the presence of virulence genes. Polymerase chain reaction (PCR) assays were applied to target the virulence determinants enterotoxin gene (*stn*), host recognition and invasion gene (*inv*A), changes in the interaction of host immune system gene (*spv*C), fimbrial proteins gene (*fim*A), iron acquisition gene (*iro*B), and survival within macrophage gene (*msg*A) (Chaudhary *et al*., 2015; Borges *et al*., 2017; Soubeiga *et al*., 2022). The details of the primers are displayed in Table 1. Before the experiment, we performed the trial run for each primer for optimization MgCl and annealing temperature, which were displayed in Table 2. 25 µl Vol of reaction was made by PCR master mix (a 2x EconoTaq® PULS GREEN 2X Master Mix, DA5110, Lucigen).

Detection of antibiotic resistance genes

Streptomycin (*aad*A1), aminoglycoside (*aac(3)11a*), β-lactam (bla_{TEM}), Cloramphenicol (*cat*A), Sulfonamides (*sul*1), Tetracycline (*tet*A), Macrolides resistance (*erm*B), and Quinolones (*qnr*B) were employed (Chuanchuen *et al*., 2008) and listed in Table 3. For each primer, the PCR conditions are shown in Table 4.

Ethical approval

Not needed for this study.

Results

Bacterial isolation and identification

Out of 60 milk samples, 53 positive samples (81.66%) were detected. Including 21 *S. aureus*, 17 *Staphylococcus intermedius,* and 15 *Staphylococcus epidermidis*. The PCR results demonstrated the amplification of the genetic fragment of five genetic clusters for each of *S. aureus* (SAD11, SAD12, SAD13, SAD14, and SAD15), *S. epidermidis* (SED1, SED2, SED3, SED4, and SED5), and *S. intermedius* (SID1, SID2, SID3, SID4, and SID5) (Fig. 1). The PCR products were sent out to sequencing and reported that the current isolates were similar in their genetic content with global isolates at 95.34% to 97.59%, 96.21% to 97.57%, and 96.09% to 97.88%, respectively, of identity (Table 5 and Fig. 2). Antibiotics have been developed to treat *S. aureus* infections, leading to the selection of more virulent strains with mutations that confer resistance to antibiotics. In recent decades, many *S. aureus* strains have acquired resistance to multiple antibiotics, posing a serious problem in modern clinical practice. As a result, the genotyping of strains has become an urgent task to guide epidemiological study and clinical practice. The present study recognized the following genotypes; SAD12, SAD14, SAD15, SED1, SED2, SED4, SID4, SID5, and SID1.

The percentage of virulence genes (*bap, coa, hla*A, *nuc, pvl, sea,* and *tuf*) of *Staphylococcus* spp. from this study are presented in Table 6. The detection of virulence genes in *Staphylococcus spp.* isolates by PCR technique is shown in Figure 3. Result analysis of total *Staphylococcus spp.* isolates that used in this test showed that *hlaA* gene has the highest percentage (87.18%) followed by *bap, nuc,* and *coa* gene which had 77.14% and 62.14%. The least virulent gene from all tested *Staphylococcus spp.* isolates were *sea, pvl,* and *tuf* genes which had 27.58%, 22.7%, and 11.27%. Under *S. aureus* isolates category, the highest virulent gene was *hla*A which had 100%, followed by *bap* which had 90.47%, *coa* with 80.95%, *tlh* with 37.5%, and the *nuc* gene was found to be 71.42% and the least under

Table 2. PCR therocycler conditions for the detection of virulance genes of *Staphylococcus* spp. isolated from milk samples.

Gene name	PCR conditions
Enterotoxin A (*sea*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minutes at 72°C; 7 minutes at 72°C
Biofilm-associated protein (*bap*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56.5°C and 1 minutes at 72°C; 7 minutes at 72°C
Thermonuclease (*Nuc*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C; 7 minutes at 72°C
Elongation factor Tu (*tuf*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 1 minutes at 72°C; 7 minutes at 72°C
Hemolysin A (*hlaA*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minutes at 72°C; 7 minutes at 72°C
Coagulase (*coa*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minutes at 72°C; 7 minutes at 72°C
cytotoxin (*pvl*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minutes at 72°C; 7 minutes at 72°C

Table 3. Primers of the PCR for the antibiotic resistance gene detection of *Staphylococcus* spp. isolated from milk samples.

Gene name	Primer name	Primer sequence (5′ to 3′)	Anneal temp (°C)	Product size (bp)	References
Stryptomycin gene (aadD)	aadA-F	CAAACTGCTAAATCGGTAGAAGCC	296	60	Klingenberg *et al*. (2004)
	aadA-R	GGAAAGTTGACCAGACATTACGAACT			
Glycopeptide gene (vanA)	vanA-F	GGGAAAACGACAATTGC	732	54	Simeoni *et al*. (2008)
	vanA-R	GTACAATGCGGCCGTTA			
Glycopeptide gene (vanB)	vanB-F	ATGGGAAGCCGATAGTC	635	55	Simeoni *et al*. (2008)
	vanB-R	GATTTCGTTCCTCGACC			
Aminoglycoside gene aacA-aphD	aacA-aphD- F	TAA TCC AAG AGC AAT AAG GGC	227	56	Adwan *et al*. (2014)
	aacA-aphD-R	GCC ACA CTA TCA TAA CCA CTA			
β-lactam gene (blaZ)	BlaZ-F	ACTTCAACACCTGCTGCTTTC	240	57	Martineau *et al*. (2000)
	BlaZ-R	TGACCACTTTTATCAGCAACC			
Cloramphenicol gene (catA)	catA-F	GCG AAC GAA AAA CAA TTG CA	748	62	Schwarz *et al*. (1991)
	catA-R	TGA AGC TGT AAG GCA ACT GG			
Tetracycline gene (tetM)	tetM-F	AGT GGA GCG ATT ACA GAA	158	54	Adwan *et al*. (2014)
	tetM-R	CAT ATG TCC TGG CGT GTC TA			
Macrolides resistance gene (ermC)	ermC-F	AAT CGT CAA TTC CTG CAT GT	299	56	Adwan *et al*. (2014)
	ermC-R	TAA TCG TGG AAT ACG GGT TTG			
Quinolones gene (qnrB)	qnrB-F	ATGACGCCATTACTGTATAA	408	57	Abdu and Mirabeau (2019)
	qnrB-R	GATCGCAATGTGTGAAGTTT			

this category was found to be of *sea*, *pvl* and *tuf* genes which had 33.33%, 23.8%, and 9.52%, respectively. Under *S. intermedius* isolates category, the gene with the highest percentage were *bap* and *nuc* genes which were present in all tested isolates (100%), followed by *hlaA, coa,* and *sea* which found to be (88.23%, 58.82%, and 29.41%), respectively. The least under this category were *tuf* and *pvl* genes with 17.64%. Under *S. epidermidis* isolates category, the highest virulent gene was *hla*A which had 73.33%, followed by *nuc* and *bap,* which both had 60%, the intermediate percentages were of *coa* and *pvl* with 46.66% and 26.66%, respectively. The least under this category was *tuf* genes with 6.66%. The virulence gene patterns of *Staphylococcus spp.* isolated in this study is presented in Table 7. *Staphylococcus spp.* isolates have shown 13 different virulence gene patterns. There were 6 isolates (SAD01, SAD02, SED06, SED08, SED09, and SID13) carrying all tested genes, and the tested isolates were carrying at least one of seven tested virulence genes. The most frequently repeated pattern was V9 (bap, hlaA, nuc) which appeared in nine isolates.

The percentages of nine resistance genes of 53 *Staphylococcus spp.* isolates (21 *S. aureus*, 17 *S. intermedius,* and 15 *S. epidermidis*) are shown in Table 8. The detection of antibiotic resistance genes in *Staphylococcus spp.* isolates by PCR technique is shown in Figure 4. The data analysis of the total tested isolate showed a variety of percentages from high, moderate to low. The highest percentage was of *bla*Z with 94.22% followed by *tet*M and *erm*Cgenes which had 92.26% and 90.68%, respectively. The moderate percentages of resistant genes were of *aacA-aph*D and *cat*A which had 79.51% and 61.56%, respectively. The lowest percentage of total tested isolates was of *van*B, *qnr*B, and *van*A gene which had 17.31%, 13.12%, and 13.12%, respectively.

Closer inspection of *S. aureus* isolates showed that the highest percentage was of *bla*Z and *tet*M gene which both had 95.23%, followed by ermC, aacA-aphD, and

Table 4. PCR therocycler conditions for the detection ofantibiotic resistance genes of *Staphylococcus* spp. isolated from milk samples.

Gene name	PCR conditions
Stryptomycin gene (*aadD*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minutes at 72°C; 7 minutes at 72°C
Glycopeptide gene (*vanA*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 1 minutes at 72°C; 7 minutes at 72°C
Glycopeptide gene (*vanB*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minutes at 72°C; 7 minutes at 72°C
Aminoglycoside gene aacA-aphD	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 1 minutes at 72°C; 7 minutes at 72°C
β-lactam gene (*blaZ*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 1 minutes at 72°C; 7 minutes at 72°C
Cloramphenicol gene (*catA*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 62°C and 1 minutes at 72°C; 7 minutes at 72°C
Tetracycline gene (*tetM*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 1 minutes at 72°C; 7 minutes at 72°C
Macrolides resistance gene (*ermC*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 1 minutes at 72°C; 7 minutes at 72°C
Quinolones gene (*qnrB*)	5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 1 minutes at 72°C; 7 minutes at 72°C

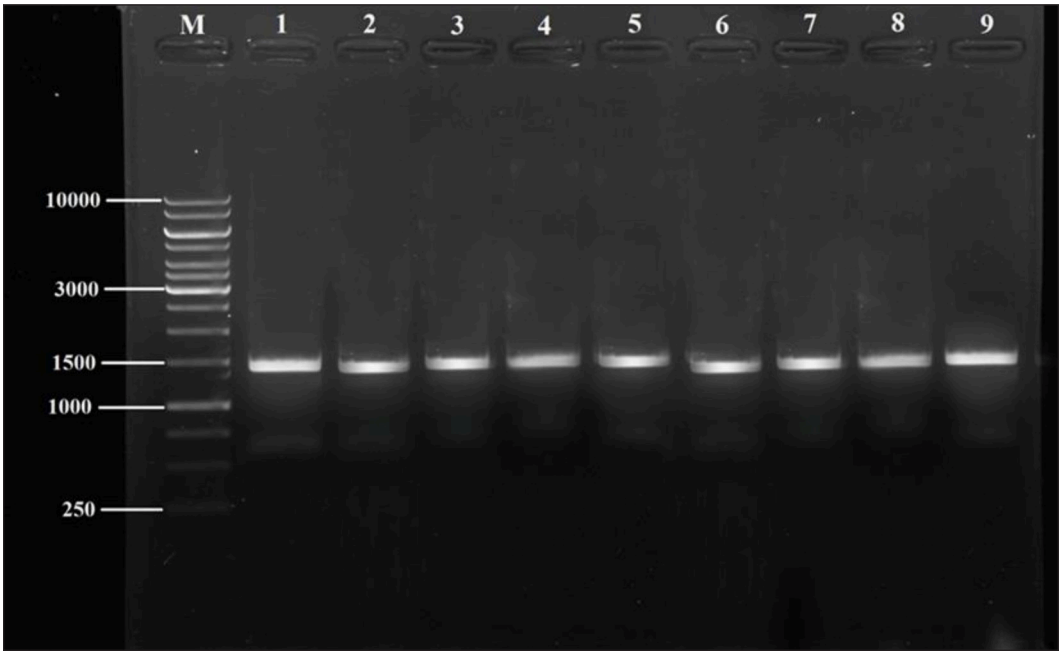

Fig. 1. Image of the *16S rRNA* related amplification on 1% agarose gel of nine isolates of *Staphylococcus* spp from milk samples. M: DNA ladder and Lanes of 1 to 9: SAD12, SAD14, SAD15, SED1, SED2, SED4, SID4, SID5, SID1.

catA genes which had 90.47%, 76.19%, and 66.66%, respectively. *qnr*B, *van*B and *van*A genes were having the same percentage (14.28%). The lowest antibiotic resistance gene percentage of *S. aureus* isolates was of *aad*D gene with 9.52%.

Under the category of *S. intermedius* isolates, there was no gene with 100% and the highest percentage was of *blaZ* gene which had 94.11% and followed by *tet*M, *erm*C, *aacA-aph*D and *cat*A genes with 88.23%, 88.23%, 82.35%, and 64.7%, respectively. It is apparent

Table 5. Identity rates of current study of *16S rRNA* related sequencing alignment of nine isolates of *Staphylococcus* spp from milk samples.

Strain code	Accession Number	Similarity%
SAD11	PP321323	97.59%
SAD12	PP321324	96.78%
SAD13	PP321325	97.34%
SAD14	PP321326	97.50%
SAD15	PP321327	95.34%
SED1	PP321328	96.79%
SED2	PP321329	95.03%
SED3	PP321330	97.57%
SED4	PP321331	96.73%
SED5	PP321332	96.21%
SID1	PP321333	97.88%
SID2	PP321334	96.83%
SID3	PP321335	97.03%
SID4	PP321336	96.09%
SID5	PP321337	96.71%

that the lowest percentage of seawater, sediment, and total tested isolates was of *aad*D, *qnr*B, and *van*A gene, which had 11.76%.

Under the category of *S. epidermidis* isolates, the highest percentage was of *bla*Z, *tet*M, and *erm*C gene which had 93.33% and followed by *aacA-aph*D, *catA,* and *van*B genes with 80%, 53.33%, and 20%, respectively. The lowest percentage of seawater, sediment, and total tested isolates was of *aad*D gene, which had 6.66%.

Table 9 shows the antibiotic resistance gene patterns of *Staphylococcus spp.* isolated from seawater and sediment samples. Seven gens were investigated and *Staphylococcus spp.* isolates have shown 12 different antibiotic resistance gene patterns. The presence of antibiotic-resistance genes was varied from three to nine genes out of nine tested genes. There were five isolates (SAD02, SAD04, SED07, SED10, and SID12) carrying all tested genes and belong to patterns A1 pattern.

The most frequently repeated pattern was A10 pattern (aacA-aphD, ermC, vanA, vanB) which was reported in 14 isolates, followed by A7 pattern (aacA-aphD, aadD, ermC, vanA, vanB) which were reported in 12 isolates. All pattern was reported in 8 isolates, A12 and A1 patterns were reported in five isolates each. The other

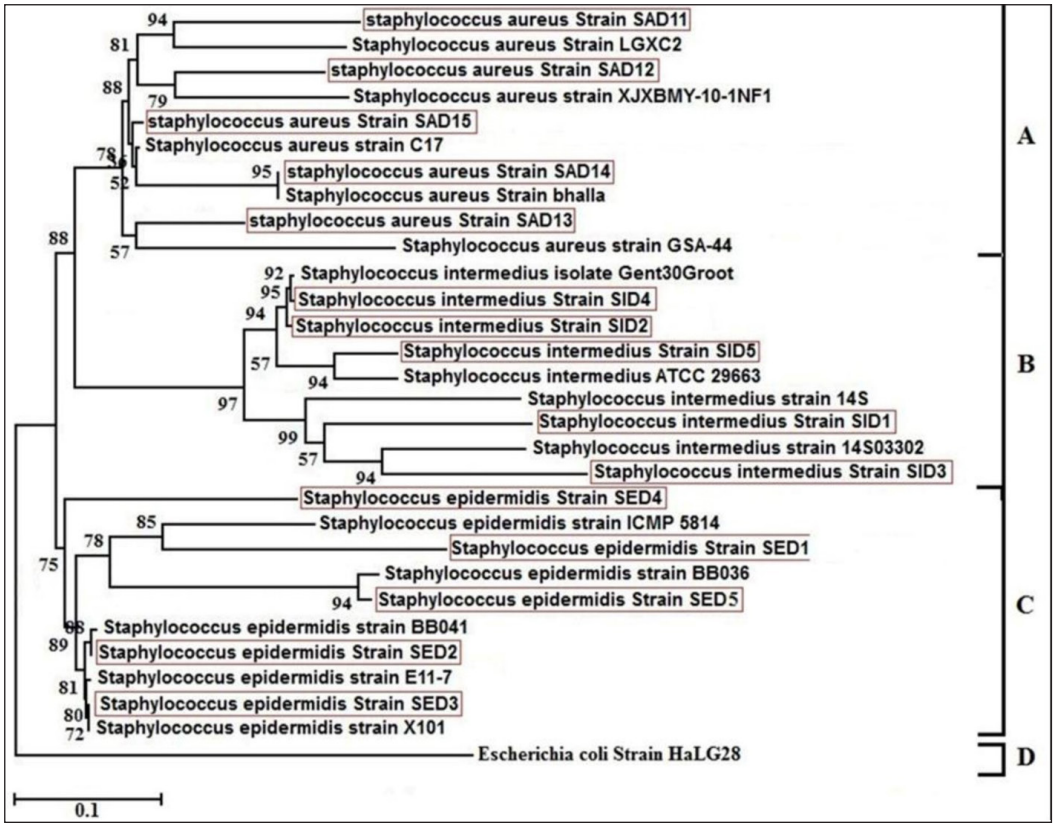

Fig. 2. Phylogenetic tree of of *16S rRNA* related sequencing alignment of nine isolates of *Staphylococcus* spp from milk samples.

Table 6. Percentage of virulence genesof *Staphylococcus* spp. isolated from milk samples.

Gene name	*S. aureus* *n* = 21 (%)	*S. intermedius* *n* = 17 (%)	*S. epidermidis* *n* = 11 (%)	Total (%)
Biofilm-associated protein (*bap*)	19(90.47)	17(100)	9(60)	83.49
Coagulase (*coa*)	17(80.95)	10(58.82)	7(46.66)	62.14
Hemolysin A (*hlaA*)	21(100)	15(88.23)	11(73.33)	87.18
Thermonuclease (*Nuc*)	15(71.42)	17(100)	9(60)	77.14
cytotoxin (*pvl*)	5(23.8)	3(17.64)	4(26.66)	22.7
Enterotoxin A (*sea*)	7(33.33)	5(29.41)	3(20)	27.58
Elongation factor Tu (*tuf*)	2(9.52)	3(17.64)	1(6.66)	11.27

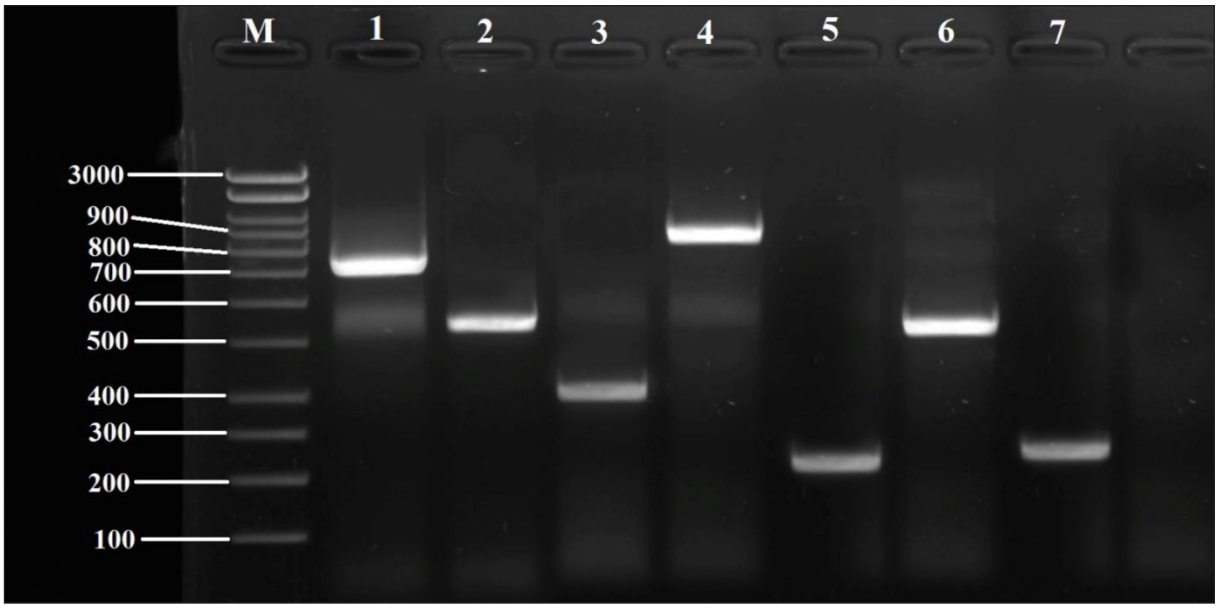

Fig. 3. Detection of virulence genes in *Staphylococcus spp.* isolates by PCR technique, electrophoresed on 1.5% (w/v) agarose gel. Lane M: 100bp DNA ladder; Lane 1: *coa* gene in SAD01; Lane 2: *hla*A gene in SED06; Lane 3: *tuf* gene in SED09; Lane 4: *bap* gene in SAD02; Lane 5: *pvl* gene in SID13; Lane 6: *sea* gene in SID15; Lane 7: *nuc* gene in SAD04.

patterns were repeated in lower frequency in tested *Staphylococcus spp.* isolates.

Discussion

For SAD14 and SAD15 Genotype: A study by Loewen *et al.* (2017) reports that these two genotypes, SAD14 and SAD15 are two very similar types of *S. aureus* strains and that these two genotypes seemed to have a relatively heightened occurrence in community-acquired infections. The research also suggests that in the case of SAD14 and SAD15, a relatively lower antibiotic resistance profile could be observed in contrast to SAD12. The researchers concluded that a potential shift in the epidemiology of *S. aureus* infections could be observed where these two genotypes could gain importance in a community-acquired setting. They argue that the SED1, SED2, and SED4 strains have been demonstrating a greater likelihood to persist long-term, which would imply that they are extremely successful inhabitants, able to thrive in the human host for a long time. Recently it has also been shown that SED4 could play a role in increased antibiotic resistance by finding a high prevalence among emerging antibiotic-resistant strains, of which MRSA was the most popular. In light of this, individual genotyping has been proposed as a potential tool to accurately predict the susceptibility of strains to antibiotics. For SID4, SID5, and SID1 genotype: It was found that the genetic structure of *S. aureus* establishes the existence of specific genetic DNA markers that set them apart from other genotypes. Specifically, they studied the SID4, SID5, and SID1 genotypes involving well-differentiated strains. SID4 was found to have a relatively higher prevalence in HAIs compared to other

Table 7. Virulence genes patternof *Staphylococcus* spp. isolated from milk samples.

	Isolates code	Virulence Genes	Pattern
S. aureus	SAD06, SAD07, SAD08, SAD14, SAD15, SAD16	bap, coa, hlaA	V1
	SAD10, SAD11, SAD19, SAD20	bap, coa, hlaA, nuc	V2
	SAD03, SAD04, SAD05	bap, coa, hlaA, nuc, pvl, sea	V4
	SAD01, SAD02	bap, coa, hlaA, nuc, pvl, sea, tuf	V5
	SAD12, SAD21	bap, hlaA, nuc	V9
	SAD09, SAD17	bap, hlaA, nuc, sea	V10
	SAD13, SAD18	coa, hlaA, nuc	V12
S. epidermidis	SED01, SED02, SED11	bap, coa, hlaA, nuc	V2
	SED06, SED08, SED09	bap, coa, hlaA, nuc, pvl, sea, tuf	V5
	SED07, SED10	bap, coa, hlaA, nuc, sea	V6
	SED05, SED17	bap, coa, nuc	V7
	SED03, SED04, SED12, SED13, SED14, SED15, SED16	bap, hlaA, nuc	V9
S. intermedius	SID12, SID14	bap, coa, hlaA, nuc, pvl	V3
	SID15	bap, coa, hlaA, nuc, pvl, sea	V4
	SID13	bap, coa, hlaA, nuc, pvl, sea, tuf	V5
	SID11	bap, coa, hlaA, nuc, sea	V6
	SID03, SID05, SID07, SID09	bap, hlaA	V8
	SID01, SID10	coa, hlaA	V11
	SID02, SID04, SID06, SID08	nuc	V13

Table 8. Prevalence of antibiotic resistance genesof *Staphylococcus* spp. isolated from milk samples.

Gene name	*S. aureus* *n* = 21(%)	*S. intermedius* *n* = 17(%)	*S. epidermidis* *n* = 11(%)	Total (%)
Aminoglycoside gene (*aacA-aphD*)	16(76.19)	14(82.35)	12(80)	79.51
Cloramphenicol gene (*catA*)	14(66.66)	11(64.7)	8(53.33)	61.56
Glycopeptide gene (*vanA*)	3(14.28)	2(11.76)	2(13.33)	13.12
Glycopeptide gene (*vanB*)	3(14.28)	3(17.64)	3(20)	17.31
Macrolides resistance gene (*ermC*)	19(90.47)	15(88.23)	14(93.33)	90.68
Quinolones gene (*qnrB*)	3(14.28)	2(11.76)	2(13.33)	13.12
stryptomycin gene (*aadD*)	2(9.52)	2(11.76)	1(6.66)	9.31
Tetracycline gene (*tetM*)	20(95.23)	15(88.23)	14(93.33)	92.26
β-lactam gene (*blaZ*)	20(95.23)	16(94.11)	14(93.33)	94.22

genotypes. On the other hand, SID5 and SID1 were reported to be relatively more common among CAIs than other genotypes. Similarly, the research proposes that these three genotypes exhibited specific differences in their antibiotic resistance profiles (Elsayed and Dawoud, 2015).

This result is consistent with what has been found in a previous study of Khasapane *et al*. (2024) how reported

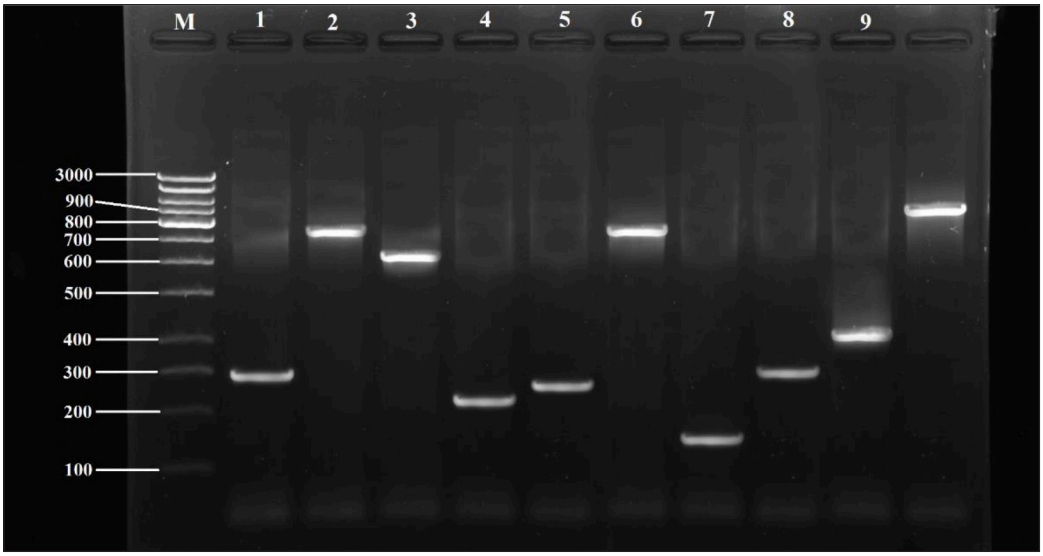

Fig. 4. Detection of virulence genes in Staphylococcus spp. isolates by PCR technique, electrophoresed on 1.5% (w/v) agarose gel. Lane M: 1K DNA ladder; Lane 1: *aad*D gene in SAD01; Lane 2: *van*A gene in SAD01; Lane 3: *van*B gene in SED09; Lane 4: *aacA-aph*D gene in SAD02; Lane 5: *bla*Z gene in SED10; Lane 6: *cat*A gene in SID15; Lane 7: *tet*M gene in SAD04; Lane 8: *erm*C gene in SID15; Lane 9: *qnr*B gene in SAD04.

Table 9. Antibiotic resistance gene patternsof *Staphylococcus* spp. isolated from milk samples.

	Isolates code	Virulence Genes	Pattern
S. aureus	SAD02, SAD04	aacA-aphD, aadD, blaZ, catA, ermC, qnrB, tetM, vanA, vanB	A1
	SAD05	aacA-aphD, aadD, blaZ, catA, ermC, vanA, vanB	A3
	SAD16	aacA-aphD, aadD, ermC	A5
	SAD01	aacA-aphD, aadD, ermC, qnrB, vanA, vanB	A6
	SAD03, SAD08	aacA-aphD, aadD, ermC, vanA, vanB	A7
	SAD06, SAD18	aacA-aphD, aadD, vanA, vanB	A8
	SAD07, SAD09, SAD11, SAD13, SAD15, SAD20, SAD21	aacA-aphD, ermC, vanA, vanB	A10
	SAD10, SAD12, SAD14, SAD17, SAD19	aadD, ermC, vanA, vanB	A11
S. epidermid	SED07, SED10	aacA-aphD, aadD, blaZ, catA, ermC, qnrB, tetM, vanA, vanB	A1
	SED09	aacA-aphD, aadD, catA, ermC, vanA	A4
	SED01, SED03, SED04, SED06, SED08	aacA-aphD, aadD, ermC, vanA, vanB	A7
	SED05, SED11, SED12, SED13	aacA-aphD, ermC, vanA, vanB	A10
	SED15, SED17, SED02	aacA-aphD, ermC, vanB	A11
	SED14, SED16	aadD, vanA, vanB	A12
S. intermedius	SID12	aacA-aphD, aadD, blaZ, catA, ermC, qnrB, tetM, vanA, vanB	A1
	SID15	aacA-aphD, aadD, blaZ, catA, ermC, qnrB, vanA, vanB	A2
	SID03	aacA-aphD, aadD, ermC	A5
	SID06, SID08, SID11, SID13, SID14	aacA-aphD, aadD, ermC, vanA, vanB	A7
	SID04	aacA-aphD, catA, vanA, vanB	A9
	SID01, SID02, SID10	aacA-aphD, ermC, vanA, vanB	A10
	SID05, SID07, SID09	ermC, vanA, vanB	A12

coa gene (42%), and hla gene (38%). The presence of hlaA gene at 38% in Staphylococcus spp. isolates in the present study are in contrast with previous findings in Egypt (34.4% and 43.75%) (Singh *et al*., 2014). Brazil (38% and 58%) (Mühlberg *et al*., 2020), and China (57% and 36%). However, this is lower than those from various studies in China, in which the results of hlaA genes were more than 80% (Wang *et al*., 2016).

In the present study, the identification of the staphylococcal enterotoxin gene sea in the isolates studied was recognized in 27.58% of them. These findings are consistent with previous results obtained by several other authors: German authors (7.10%), Brazilians (10.9%), and Czechs (19.4%). The percentage of isolates that carried the sea gene was significantly lower compared with those obtained in raw meats from the South African market (35.29%), from the market of northern Egypt (52%), and from the supermarkets in Italy (65.60%). The results of *pvl* gene also were aligned with the previous report of Mello *et al*. (2016) who reported that 10.7% of staphylococcal carried *pvl* gene.

Previous study of Karimzadeh and Ghassab (2022), they found nuc gene present in 30% of tested *Staphylococcus* isolates which is interestingly opposite to our finding where nuc gene shown in 77.14%. The coa gene results were in agreement with the previous study of Chmagh and Abd Al-Abbas (2019) who found 77.3% of the tested isolates were carrying *coa* gene. Salina *et al*. (2020) was in agreement with this study, where they found that bap gene in *Staphylococcus* were present in 58% of tested isolates.

The result showed that 90.68% of the isolates of Staphylococcus spp. carrying the erm(C) gene while 9.32% were negative for erm(C) (Table 5). These findings was in contrast to a previous study which reveals that the prevalence of erm(A) gene has been established as the predominant dissemination of the methicillin-resistant strains (Schafer and Phillippi, 2020) but conforms with previous studies which reveals the wide distribution and acceptance of the erm(C) in *S. aureus* isolates. Also, Denmark has an increase occurrence of staphylococcus spp. carrying erm (C) (Qu *et al*., 2010). Some previous studies have revealed that the prevalence of the erm (A) is more than erm (C) gene in the tested isolates. Current study revealed that most of the isolates of Staphylococcus spp. were positive for tetM; in fact 92.26% of the isolates carried tetM, in contrast to previous studies which revealed that the prevalence of erm (A) gene has been established as the predominant dissemination of the methicillin-resistant strains (Fujiwara *et al*., 2017; Schafer and Phillippi, 2020) but a previous study by Bagcigil and his colleagues in Turkey conforms with the results of the current work, revealing a high prevalence of tetM in isolates of *S. aureus* isolates obtained from clinical patients and staphylococcus spp. was revealed to be the leading cause of bovine mastitis (Bagcigil *et al*., 2012).

An opposite result was obtained in the research by Aubry *et al*. (2020), where 30% of strains were found to contain the blaZ gene (Aubry *et al*., 2020). Similar to our study, As of Bagcigil *et al.* (2012) the presence of blaZ gene was determined in *S. aureus* isolates, determined by broth microdilution, originating from bovine mastitis, and all isolates were found to be positive for the blaZ gene.

Other study by Salina *et al*. (2020) showed seventeen (59%) tested isolates contained the cat gene which were in agreement with our results. Amin *et al*. (2021) investigated the presence of qnrB gene, they found 6% of *Staphylococcus spp* were carrying the gene, this result is in consistent with our results where qnrB gene showed 13.12%. in earlier study (Benites *et al*., 2021), the presence of the aacA-aphD gene, which codes for enzymes that act on aminoglycosides, was detected in 28.1% of the *Staphylococcus spp.* isolates, this result contradicts our results that shown higher aacA-aphD gene percentage (79.51%). Monecke and Ehricht (2005) investigate the percentages of aadD gene and they found that 29% of *Staphylococcus spp.* isolates were carrying aadD gene, these findings are not directly in line with our results (9.31%). This result ties well with previous studies (Monecke and Ehricht, 2005) whereinvanA and vanB resistant genes were detected in 34% and 37% of clinical isolates, respectively.

One of the main findings in this study was the wide spread of antimicrobial resistance genes among *Staphylococcus spp*. Resistance genes of *bla*Z, *erm*C, and *tet*M were prevalent. This makes sense as these antibiotics have evolved naturally in the environment and so are primed to select for the spread of the resistance determinants or for selection of locally increasing isolates (that is, resistance evolves due to persistent contact with the antibiotic in the environment, which can increase its use among bacterial populations already present (Rosser and Young, 1999).

Conclusion

The present findings show highly genetic variations among isolates of *S. aureus*, *S. epidermidis*, and *S. intermedius* recovered from milk samples, and these genotypes are found in different infection setting related to humans and animals, which may pose high risks to humans and animals.

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Conflict of interest

The authors declare that there is no conflict of interest for the current study.

Authors' contribution

All authors had participated in samples collection, performing tests, analyzing the data, and preparing and revising the manuscript.

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Data availabilty

The data for the current study are available upon request.

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