Phenotypic and molecular determination of biofilm formation and antibiotic resistance of coagulase negative staphylococci isolated from food samples of animal origin in Turkey

Türkiye'de hayvansal kaynaklı gıda örneklerinden izole edilen koagülaz negatif stafilokokların biyofilm oluşturmaları ve antibiyotik dirençliliklerinin fenotipik ve moleküler olarak belirlenmesi

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Abstract

Aim: This study aimed to investigate the antibiotic resistance and biofilm formation characteristics of the coagulase-negative Staphylococcus (CNS) isolated from various animal food samples (raw milk, cheese, chicken, meat).

Methods: A total of 60 Staphylococcus isolates collected from various animal food samples (raw milk, cheese, chicken, meat) were obtained from the frozen culture collection of the Microbiology Laboratory of the Gazi University. The isolates were assessed regarding antibiotic resistance, biofilm and s-layer formation, and biofilm-associated genes.

Results: In total, 22 CNS isolates were identified. The CNS isolates were highly resistant to fosfomycin (68.2%; 15/22), fusidic acid (63.6%, n=14/22), and tetracycline (59.1%, n=13/22). There was an intermediate resistance to other antibiotics as well. There was very strong slime production (54.5%, n=12/22) and S-layer production (40.9%, n=9/22). The biofilm formation of CNS isolates was better at 24 hours. Regarding the biofilm-producing genes, 10 (45%) of 22 CNS isolates were positive for the icaA gene, and 4 (18%) isolates were positive for the icaD gene, while all of the isolates were negative for the bap gene.

Conclusion: The CNS existing in foods from animals may lead to public health problems with clinical implications as a result of being one of the important factors associated with antibiotic resistance. The bacteria that can gain antibiotic resistance and biofilm formation capabilities in the stages of production and consumption may be a critical healthcare issue for humans and animals.

Keywords: Antibiotic resistance; biofilms; coagulase; PCR; staphylococcus

Öz

Amaç: Bu çalışmanın amacı, çeşitli hayvansal gıda örneklerinden (çiğ süt, peynir, tavuk, et) izole edilen koagülaz-negatif Stafilokokların (KNS) antibiyotik direnci ve biyofilm oluşturma özelliklerini araştırmaktır.

Yöntemler: Çeşitli hayvansal gıda örneklerinden (çiğ süt, peynir, tavuk, et) izole edilen toplam 60 Staphylococcus izolatı, Üniversitenin Mikrobiyoloji Laboratuvarı'nın dondurulmuş kültür koleksiyonundan elde edilmiştir. İzolatlar antibiyotik direnci, biyofilm ve S tabakası oluşturma ve biyofilmle ilişkili genler açısından değerlendirilmiştir.

Bulgular: Test edilen izolatların 22'si KNS olarak tanımlanmıştır. KNS izolatları fosfomisin (%68,2; 15/22), fusidik asit (%63,6, n=14/22) ve tetrasikline (%59,1, n=13/22) yüksek oranda dirençli bulunmuştur. Diğer antibiyotiklere karşı orta düzeyde direnç tespit edilmiştir. Slime üretimi (%54.5, n=12/22) ve S-tabakası üretiminin (%40.9, n=9/22) oldukça yüksek oranda olduğu belirlenmiştir. KNS izolatlarının biyofilm oluşumunun 24 saatlik deneyde daha güçlü olduğu görülmüştür. Biyofilm oluşumu ile ilgili gen araştırmasında, 22 KNS izolatının 10'unun (%45) icaA geni için ve 4'ünün (%18) icaD geni için pozitif olduğu, izolatların tümünün bap geni için negatif olduğu belirlenmiştir.

Sonuç: Hayvanlardan elde edilen gıdalarda bulunan KNS'ler, antibiyotik direnci ile ilişkili önemli faktörlerden biri olması sebebiyle klinik etkileri olan halk sağlığı sorunlarına yol açabilmektedirler. Üretim ve tüketim aşamalarında antibiyotik direnci ve biyofilm oluşturma yeteneği kazanabilen bakteriler, insanlar ve hayvanlar için kritik bir sağlık sorunu oluşturabilmektedirler.

Anahtar Sözcükler: Antibiyotik direnci; biyofilm; koagülaz; PCR; stafilokok

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INTRODUCTION

Coagulase-negative staphylococci (CNS) have been considered commensal or opportunistic microorganisms that could impact the health of humans and animals. The CNS seems important regarding food safety, as they possess the potential to contaminate food products derived from animals, including minced meat, cheese, raw chicken meat, milk, and sausage (1-3).

The emergence of antimicrobial resistance in pathogenic bacteria presents a significant issue attributable to the indiscriminate administration of antibiotics for both the treatment and prophylaxis of infections in animals, coupled with their utilization as growth promoters in animal husbandry and medical therapies. The prevalence of antibiotic resistance among bacterial populations has escalated concomitantly with the heightened application of antibiotics within both community settings and the food production sector (4).

The slime factor is a glycocalyx material with an amorphous capsule structure consisting of 40% carbohydrates and 27% proteins. The slime is an extracellular polysaccharide that provides adhesion to plastic and metal surfaces and gives the bacteria an antiphagocytic property while preventing the entry of antimicrobials into the bacteria. The slime production of coagulase-negative and positive Staphylococci is associated with its virulence as it protects the bacteria from the host defense while contributing to biofilm formation (5).

The S-layer consists of a (glyco) protein known as the S-protein with a molecular mass between 40 and 200 kDa. The surface layer proteins crystallize into a two-dimensional layer outside the bacterial cell wall. The S-layer is a protective sheath against harmful environmental agents and determines the cell shape of archaea. Pathogens are capable of utilizing variations in S-layer structures to acclimate to diverse stressors, including the immune responses of the host and significant environmental fluctuations, whereas non-pathogenic organisms may employ S-layer variations to express alternative genes coding for S-layer proteins to adapt to unfavorable ecological conditions. It has been suggested that S-layer proteins are involved in cell protection and surface recognition and may be potential mediators in the early stages of auto-aggregation and adhesion (6). Therefore, they may be related to biofilm formation as a virulence factor.

In the food industry, CNS can adhere to the surfaces of various tools and equipment in the factories and pipelines during the process, transfer, and storage of foods. The bacteria can grow in a three-dimensional scaffolding structure with extracellular polymers in the form of biofilms in trapped wastes. The biofilm is an exopolysaccharide structure consisting of polysaccharide-glycocalyx, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances (7).

A variety of bacterial and external factors can influence the adhesion and accumulation. The *Staphylococci* specifically produce an extracellular polysaccharide adhesin, which is an intercellular polysaccharide adhesin (PIA) or polymeric N-acetylglucosamine (PNAG) as mediated by the enzymes encoded by the icaADBC operon. The genes in this operon are icaA and icaD (8). Recently, a protein called bap (biofilm associated protein), which was identified in *S. aureus,* has been reported to be associated with skin biofilm formation in animals. The bap gene encodes a large protein of 2,276 amino acids (aa) and is known to promote both primary binding to inert surfaces and intercellular adhesion (9).

The food of animal origin may be associated with antibiotic resistance and cross-contamination of CNS, as well as biofilm formation in the chain from source to consumer due to misuse of antibiotics, inadequate processing, and non-compliance with hygiene rules. At this point, it is critical to investigate the impact of CNS in the food of animal origin (10).

This study aimed to investigate the prevalence of antibiotic resistance, S-layer, slime production, and biofilm formation characteristics of the CNS isolated from various animal food samples (raw milk, cheese, chicken, meat) collected from Ankara, the capital of Turkey.

MATERIAL AND METHODS

Bacterial isolates

A total of 60 *Staphylococcus* isolates were obtained from the frozen culture collection of the Microbiology Laboratory of the Faculty of Science, Gazi University. These isolates were collected from various food samples (raw milk, cheese, chicken, meat) between June 2021 and June 2022. The isolates were stored at -20^{0}C

in a glycerol Brain Heart Infusion medium (LabM, UK) and recovered in Triptic Soya Broth (Merck, Germany) at 37^{0}C overnight. Ethics committee approval is not required since bacterial isolations were made from food samples sold in markets, and our research had no human studies or clinical isolates.

Identification of staphylococcus isolates and determination of antibiotic resistance

The isolates were obtained as pure cultures using Mannitol Salt Agar (LabM, UK). The isolates were identified at the Microbiology Laboratory of Gazi University Hospital with MALDI-TOF MS. The determination of antibiotic resistance status as MIC value with VITEK 2 Compact (BioMérieux) device, according to the MIC values in the EUCAST 2023 (11) guideline.

Determination of biofilm-forming properties of CNS isolates

Congo red agar method: This study was conducted according to the method previously described by Freeman et al., 1989 (12). The isolates were inoculated on Congo red agar, and black colonies formed as a result of growth were considered biofilm positive, while red or pink colored colonies were considered biofilm negative. Biofilm-producing *S. aureus* ATCC 25923 was used as a positive control, and non-producing S. epidermidis ATTCC 12228 was used as a negative control.

Determination of the S layer: The surface layer (S-layer) of *Staphylococcus* isolates was determined by evaluating the growth on TSA (Tryptic Soy Agar, LabM, UK) medium supplemented with 0.1 mg/mL Coomassie Brilliant Blue R 250. The presence of the S-layer was indicated by the observation of a dark blue color in the middle of the colonies with a light gray halo around it as a result of the breakdown of proteins in the medium (13).

Microplate Method: The isolates were cultivated overnight in 5 mL Luria Bertani broth (LB broth) without NaCl at 37°C under shaking conditions. Active cultures were diluted to 0.5 McFarland with LB broth supplemented with 1% glucose, and 130 µl of these suspensions were transferred to 96-well microtiter plates. At the end of the incubation period, after the wells were washed twice with sterile distilled water and the plates were dried at room temperature, 130 µl of 1% crystal violet was transferred to the wells and kept at room temperature for 30 minutes. The plates were then washed twice with sterile distilled water, and 130 µl of ethanol (96%) was transferred to the wells. After incubating for 30 minutes at room temperature, the dye bound to the biofilm layer was dissolved. At the end of the incubation, the dissolved crystal violet dye at OD 570 and OD 595 was measured on an Elisa reader. This experiment was performed in 3 parallel and 3 replicates for all isolates. The result of the biofilm measurement was calculated by subtracting the mean OD values of the control wells (wells containing only LB broth without NaCl) from the mean OD values of the three parallel wells according to the biofilm grading criteria in Table 1 (14).

Detection of biofilm-associated genes of CNS isolates: Genomic DNA isolations of *Staphylococcus* isolates were performed in our laboratory using the Thermo Scientific GeneJet DNA Purification Kit. The protocol of the commercial kit for DNA isolation was optimized for CNS isolates, and since the cell wall of *Staphylococcus* is resistant to lysozyme, lysostaphin (Merck, Germany) was obtained as a cell-wall-degrading enzyme. The purity and amount of bacterial DNA samples were determined based on absorbance measurement using a microvolume spectrophotometer (Biotek, Epoch, USA), according to the OD values (A260/A280) given by the DNA samples. DNAs with good purity were stored in a deep freezer at -20^{0}C to be used for PCR. PCR was used to detect the presence of icaA, icaD, and bap genes in CNS isolates from various food samples. The primer sequences of these genes used in PCR are given in the table below (Table 2) (15-17).

PCR amplification was performed using GeneMark, Hot Start Master Mix II 5X. After the PCR mixture was prepared, the appropriate Tm was selected for each gene, and the amplification of the genes was performed with a thermal cycling device (Applied Biosystems). In the cycle, temperatures and times of 94 °C 4 min for initial denaturation, 30 cycles of 94 °C 45 s denaturation, 52 °C 30 s binding, 72 °C 1 min extension, and 72 °C 7 min for final extension were used. For imaging, 1% agarose gel, 1 kb DNA ladder was used as a marker, 1 µL Runsafe dye (Cleaver) and 5 µL amplicon were mixed and loaded into the wells respectively, run at 80 V for 45 minutes and visualized with Epi Blue on an imaging device (Azure Biosystems C200).

RESULTS

Identification of staphylococcus isolates and determination of antibiotic resistance profile

Of 60 *Staphylococcus* isolates, 22 were identified as CNS species by MALDI-TOF MS. Of these 22 isolates, 8 were obtained from cheese, 4 from milk, 6 from raw chicken, and 4 from raw meat. There were 6 different species of *Staphylococci* among 22 isolates. These were *S. epidermidis* (40.9%, n=9/22) as the most common species, which was followed by *S. saprophyticus* (22.7%, n=5/22), *S. pasteuri* (18.2%, n=4/22), *S. warneri* (9.1%, n=2/22), *S. haemolyticus* (4.5%, n=1/22), and *S. cohnii* ssp. *urealyticus* (4.5%, n=1/22, Table 3).

The CNS isolates were highly resistant to trimethoprim-sulfamethoxazole (100%, n=22/22), fosfomycin (68.2%; n=15/22), fusidic acid (63.6%, n=14/22), and tetracycline (59.1%, n=13/22). The majority of them were susceptible but showed increased exposure to levofloxacin (95.5%, n=21/22) and ciprofloxacin (68.2%, n=15/22). The antibiotic resistance of CNS isolates is shown in Table 3.

Determination of biofilm-forming properties of CNS isolates

The biofilm formation characteristics of 22 CNS isolates were evaluated utilizing both phenotypic and molecular methodologies. The results related to the phenotypic characteristics of biofilm formation among the CNS isolates are shown in Table 4.

It was found that the majority of CNS isolates displayed very strong slime production (54.5%, n=12/22). The others had strong (18.2%, n=4/22) and weak (18.2%, n=4/22) slime productions, while the others (9.1%, n=2/22) were non-producers. The S-layer production was very strong (40.9%, n=9/22, strong (18.2%, n=4/22), and weak (4.5%, n=1/22), while the others (36.4%, n=8/22) were non-producers (Table 4).

Biofilm production of the isolates by the microplate method was studied separately at 24 and 48 hours, and the biofilm production of the plates was measured at 570 and 595 nm. It was shown that the biofilm formation of *Staphylococcus* isolates was better at 24 hours, and there was no difference between the measurements at 570 and 595 nm (Table 4).

In the 24-hour microplate study, 3 (13.6%) isolates showed strong adhesion, 6 (27.3%) isolates showed moderate adhesion, 13 (59.1%) isolates showed weak adhesion, 1 (4.5%) isolate showed strong adhesion, 1 (4.5%) isolate showed moderate adhesion, 18 (81.8%) isolates showed weak adhesion. 2 (9.1%) isolates showed no adhesion in the 48-hour study (Table 4).

Detection of biofilm-associated genes of CNS isolates

It was found that 10 (45%) of 22 CNS isolates were positive for the icaA gene, and 4 (18%) isolates were positive for the icaD gene, while all of the isolates were negative for the bap gene. The gene profiles of the isolates are given in Table 5.

Table 1. Biofilm grading criteria

	Adhesion	Biofilm Grade
$OD \leq OD_C$	Non-adhesive	0
$OD_C < OD \leq 2 \times OD_C$	Weak adhesion	I
$2 \times OD_C < OD \leq 4 \times OD_C$	Moderate adhesion	II
$4 \times OD_C < OD$	Strong adhesion	III

OD: Optical density, ODC: Optical density of control well

Table 2. Primer sequences

Gene name	Primer sequences	Amplicon size
icaA	Forward(5'-CCTAACTAACGAAAGGTAG-3') Reverse (5'-AAGATATAGCGATAAGTGC-3')	1315 bp (15)
icaD	Forward (5'- AAACGTAAAGAGGTGG-3') Reverse (5'- GGCAATATGATCAAGATAC-3')	381 bp (16)
bap	Forward (5'-CCCTATATCGAAGGTGTAGAATTGCAC-3') Reverse (5'-GCTGTTGAAGTTAATACTGTACCTGC-3')	971 bp (17)

Table 3. Identification and antibiotic resistance results of the CNS isolates

Isolates		CN	CIP	LF	E	CLY	LN	DAP	VA	TE	TG	FOS	NF	FA	TRİ/SUL
S. haemolitycus (1)	MIC*	≤0,5	≤0,5	0,25	0,5	0,25	2	0,25	≤0,5	≤1	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	S	R
S. epidermidis (4)	MIC	≤0,5	≤0,5	0,5	1	0,25	2	0,25	≤0,5	≤1	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	S	R
S. warneri (7)	MIC	≤0,5	≤0,5	≤0,12	≥8	≤0,12	2	0,5	≤0,5	≤1	≤0,12	≥128	≤16	16	≤10
	Mean	S	I	I	R	I	S	S	S	S	S	R	S	R	R
S. pasteuri (12-1)	MIC	≤0,5	≤0,5	≤0,12	≤0,25	≤0,12	1	0,25	≤0,5	≥16	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	S	R
S. epidermidis (13A)	MIC	≤0,5	2	1	≤0,25	0,25	1	0,5	1	2	0,25	≤8	≤16	16	≤10
	Mean	S	R	I	S	S	S	S	S	R	S	S	S	R	R
S. pasteuri (14-1)	MIC	≤0,5	≤0,5	≤0,12	≤0,25	≤0,12	1	0,5	≤0,5	≥16	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	S	R
S. epidermidis (25-1)	MIC	≤0,5	2	2	0,5	0,25	1	1	2	2	≤0,12	≤8	≤16	8	≤10
	Mean	S	R	R	S	S	S	S	S	R	S	S	S	R	R
S. epidermidis (28-1)	MIC	≤0,5	2	1	≤0,25	0,25	1	1	2	≥16	≤0,12	≥128	≤16	8	≤10
	Mean	S	R	I	S	S	S	S	S	R	S	R	S	R	R
S. epidermidis (33-1)	MIC	≤0,5	2	1	0,5	0,25	1	0,5	2	2	0,25	≤8	≤16	8	≤10
	Mean	S	R	I	S	S	S	S	S	R	S	S	S	R	R
S. epidermidis (34-1)	MIC	≤0,5	≤0,5	0,25	0,5	0,25	2	0,5	1	≤1	≤0,12	64	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	S	R
S. pasteuri (35-1)	MIC	≤0,5	≤0,5	≤0,12	≤0,25	≤0,12	2	0,5	≤0,5	≥16	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	S	R
S. saprophyticus (37-1)	MIC	≤0,5	≤0,5	0,5	1	0,25	4	≤0,12	≤0,5	≤1	≤0,12	≥128	≤16	8	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	R	R
S. pasteuri (39-1)	MIC	≤0,5	≤0,5	≤0,12	≤0,25	≤0,12	1	0,5	≤0,5	≥16	≤0,12	≥128	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	S	R
S. saprophyticus (41A-1)	MIC	≤0,5	≤0,5	0,5	1	0,25	2	0,25	≤0,5	≥16	0,5	≤8	≤16	2	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	R	R
S. cohnii ssp. urealyticus (42-1)	MIC	≤0,5	≤0,5	1	≥8	≥4	4	≤0,12	1	≥16	0,25	≤8	≤16	16	40
	Mean	S	I	I	R	R	S	S	S	R	S	S	S	R	R
S. epidermidis (43-1)	MIC	≤0,5	2	1	0,5	≤0,12	1	0,5	2	2	≤0,12	≤8	≤16	8	≤10
	Mean	S	R	I	S	S	S	S	S	R	S	S	S	R	R
S. warneri (44A -1)	MIC	≤0,5	≤0,5	0,25	≥8	≤0,12	2	0,5	≤0,5	≤1	≤0,12	≥128	≤16	16	≤10
	Mean	S	I	I	R	S	S	S	S	S	S	R	S	R	R
S. saprophyticus (50-1)	MIC	≤0,5	≤0,5	0,5	0,5	≤0,12	2	≤0,12	≤0,5	≤1	≤0,12	≤8	≤16	8	≤10
	Mean	S	I	I	S	S	S	S	S	S	S	R	S	R	R
S. epidermidis (51-1)	MIC	≤0,5	2	1	≤0,25	0,25	1	0,5	2	≤1	≤0,12	≤8	≤16	8	≤10
	Mean	S	R	I	S	S	S	S	S	S	S	S	S	R	R
S. saprophyticus (53A-1)	MIC	≤0,5	≤0,5	1	1	0,25	4	0,25	≤0,5	≥16	0,25	≤8	≤16	≤0,5	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	R	R
S. epidermidis (59-1)	MIC	≤0,5	2	1	≥8	≥4	2	0,5	2	≥16	≥128	≤16	8	≤10	≤10
	Mean	S	R	I	R	R	S	S	S	R	R	S	R	S	R
S. saprophyticus (60B-1)	MIC	≤0,5	≤0,5	1	1	0,25	4	0,25	≤0,5	≥16	0,25	≤8	≤16	4	≤10
	Mean	S	I	I	S	S	S	S	S	R	S	R	S	R	R

*: mg/L, S: Susceptible, I: Susceptible, increased exposure, R: Resistant, CN: Gentamicin, CIP: Ciprofloxacin, LF: Levofloxacin, E: Erythromycin, CLY: Clindamycin, LN: Linezolid, DAP: Daptomycin, VA: Vankomycin,TE: Tetracycline, TG: Tigecycline, FOS: Fosfomycin, NF: Nitrofurantoin, FA: Fusidic acid, TRİ/SUL: Trimethoprim-sulfamethoxazole

Table 4. Biofilm forming properties of CNS isolates

No	Isolate number	*Staphylococcus* isolates	Biofilm (slime) production	S-layer production	Biofilm (24 hours)		Biyofilm (48 hours)	
					570 nm	595 nm	570 nm	595 nm
1	1	*S. haemolitycus*	++	+	II	II	0	0
2	4	*S. epidermidis*	+	+++	II	II	I	I
3	7	*S. warneri*	+++	+++	I	I	I	I
4	12-1	*S. pasteuri*	+++	+++	I	I	I	I
5	13A	*S. epidermidis*	+++	-	II	II	I	I
6	14-1	*S. pasteuri*	+++	+++	III	III	I	I
7	25-1	*S. epidermidis*	+++	-	I	I	0	0
8	28-1	*S. epidermidis*	-	-	II	II	I	I
9	33-1	*S. epidermidis*	+++	-	I	I	I	I
10	34-1	*S. epidermidis*	+++	+++	III	III	I	I
11	35-1	*S. pasteuri*	+	+++	I	I	I	I
12	37-1	*S. saprophyticus*	-	+++	I	I	I	I
13	39-1	*S. pasteuri*	++	+++	I	I	II	II
14	41A-1	*S. saprophyticus*	+++	-	II	II	I	I
15	42-1	*S. cohnii ssp. urealyticus*	+++	+++	I	I	I	I
16	43-1	*S. epidermidis*	+++	-	I	I	I	I
17	44A-1	*S. warneri*	+	++	I	I	I	I
18	50-1	*S. saprophyticus*	+	++	I	I	I	I
19	51-1	*S. epidermidis*	++	-	III	III	III	III
20	53A-1	*S. saprophyticus*	+++	++	II	II	I	I
21	59-1	*S. epidermidis*	+++	-	I	I	I	I
22	60B-1	*S. saprophyticus*	++	++	I	I	I	I

+: weak slime and S-layer production, ++: strong slime and S-layer production, +++: very strong slime and S-layer production, 0: no adhesion, I: weak adhesion, II: moderate adhesion, III: strong adhesion

DISCUSSION AND CONCLUSION

The CNS, which are members of the microbiota of the skin and mucous membranes of animals and humans, can also be isolated from milk of animals, foods of animal origin, and environmental samples (18-21). The s*taphylococcus* species could be isolated from the majority of foods of animal origin in our study as well. The majority of CNS species isolated in our study were *S. epidermidis*, which is a member of the normal flora of skin and mucosa in humans and animals (18,21,22).

In this study, numerous antibiotics were evaluated regarding the antibiotic resistance of the bacteria in the food samples, and resistance to some of the antibiotics could be detected. This condition may be associated with the inappropriate use of antibiotics in animals, which can lead to increased antibiotic resistance in bacteria like *Staphylococcus*, and resistant genes may impact the treatment outcomes of humans and animals (19,20,23,24,25).

It was reported in the study of El-Seedy et al. that the CNS isolates obtained from the milk of cows with

Table 5.The biofilm gene profiles of CNS isolates

Number	Isolate number	*Staphylococcus* isolates	icaA (1315bp)	icaD (381 bp)	bap (971 bp)
1	1	*S. haemolitycus*	-	-	-
2	4	*S. epidermidis*	-	-	-
3	7	*S. warneri*	-	-	-
4	12-1	*S. pasteuri*	+	-	-
5	13A	*S. epidermidis*	+	-	-
6	14-1	*S. pasteuri*	+	-	-
7	25-1	*S. epidermidis*	-	-	-
8	28-1	*S. epidermidis*	-	-	-
9	33-1	*S. epidermidis*	+	-	-
10	34-1	*S. epidermidis*	-	-	-
11	35-1	*S. pasteuri*	-	-	-
12	37-1	*S. saprophyticus*	-	+	-
13	39-1	*S. pasteuri*	+	-	-
14	41A-1	*S. saprophyticus*	+	+	-
15	42-1	*S. cohnii ssp. urealyticus*	-	-	-
16	43-1	*S. epidermidis*	+	-	-
17	44A-1	*S. warneri*	-	-	-
18	50-1	*S. saprophyticus*	+	+	-
19	51-1	*S. epidermidis*	-	-	-
20	53A-1	*S. saprophyticus*	-	+	-
21	59-1	*S. epidermidis*	+	-	-
22	60B-1	*S. saprophyticus*	+	-	-
Total			10 (45%)	4 (18%)	0

mastitis were resistant to ampicillin (95.79%), cefoxitin (77.9%), cefuroxime (35.8%), amoxicillin (32.63%) and clindamycin (18.95%). In the same study, the bacteria were susceptible to imipenem (100%), enrofloxacin (96.84%), chloramphenicol (85.26%), and vancomycin (84.21%) (21). Yurdakul et al. obtained the CNS isolates from chicken meat, and the study revealed resistance to ciprofloxacin, erythromycin, tetracycline, and chloramphenicol by 27.2%, 68.1%, 77.2%, and 27.2%, respectively (2). This difference in antibiotic resistance rates may be acceptable because of the variations in the locations and animal food samples of CNS isolates.

Biofilms are multicellular communities held together by a self-generated matrix of extracellular polymeric substance (EPS). The mechanisms used by different bacteria to form biofilms often vary depending on environmental conditions and specific strain characteristics. Biofilm formation is an important microbial survival strategy for a large number of pathogens. The transition from acute infection to chronic infection is often associated with biofilm formation. Biofilms contribute to the virulence of the pathogen and significantly affect the persistence of the pathogen in biological tissues and inanimate surfaces; because biofilm-associated bacteria are tightly bound together, this reduces the effectiveness of detergents, biocides, and antimicrobial agents (26). The effect of biofilms in chronic infections has increased the interest in the characterization of genes involved in biofilm formation (8,17,27). According to our results,

the CNS can usually show strong slime formation, and the rate is strong in almost two-thirds of the isolates. That high rate also applies to S-layer formation in our study. The biofilm formation can occur at the maximum level within 24 hours. The biofilm formation was weaker after 48 hours. This condition may be related to the loss of the adhesion capacity of the bacteria to the plates during the washing stages of the crystal violet method (28).

The icaA, icaD, and bap genes are associated with biofilm formation in CNS. In the *Staphylococcus* isolates obtained from mastitic milk and cheese processing plants, almost 40% were found to be positive for slime formation, all samples were positive for icaA and icaD genes, while bap genes were not detected at all (9). Similarly, in our study, 45% of CNS isolates were positive for icaA gene and 18% were positive for icaD gene, whereas none of the isolates were positive for bap gene. The lack of bap gene positivity may be attributed to low frequency of bap gene positivity in *Staphylococci* species (29,30).

It was reported that antimicrobial resistance and biofilm production could be detected in 103 of 137 *S.aureus* and CNS isolates obtained from sheep and goats with mastitis. None of the *S. aureus* isolates carried the bap gene, and 8 of the 18 CNS isolates harbored this gene. The icaA gene was detected in *S. aureus* and *S. warneri*, and icaD was detected only in *S. aureus*. None of the isolates carrying the bap gene harbored ica genes. None of the biofilm-associated genes were detected in 14 isolates (31).

It has been shown that poultry can be a source of CNS, which is capable of biofilm formation. It was proposed that almost all CNS strains obtained from broilers were capable of biofilm formation, with more than half of the isolates being strong or moderate biofilm producers. The ability to form biofilm limits treatment options and, therefore, increases morbidity and mortality in poultry, and the demonstration of genes involved in biofilm formation will provide a genetic basis for differentiating between symbiotic and potentially invasive CNS strains (18).

It's recognized that the Polymerase Chain Reaction (PCR) approach might not sufficiently evaluate the genetic factors at play in biofilm production, as the existence of a gene does not automatically indicate that the associated protein is operational, nor does it promise that this operation affects biofilm development. Consequently, additional investigations employing sophisticated molecular biology methodologies must be pursued to enhance our comprehension of the genetic factors influencing the adhesive properties of CNS (32).

Staphylococci isolated from milk, cheese, chicken, and beef can lead to antibiotic resistance and biofilm formation at a high rate. Antibiotic resistance can be transferred between bacteria through gene exchange, and the presence of antibiotic-resistant bacteria in foods also poses a significant risk. It is very important to comply with the hygiene and sanitation rules in the chain from the production of animal foods to the consumer tables, and to carry out continuous inspections by paying attention to the HACCP control points.

The CNS existing in the foods of animals may lead to public health problems with clinical implications as a result of being one of the important factors associated with antibiotic resistance. A comprehensive examination of CNS and their antibiotic resistance, in conjunction with their biofilm formation capabilities, which are inherently linked to bacterial persistence and pathogenic behaviors in human contact environments, is essential for effectively regulating and preventing illnesses related to CNS in both humans and animals.

Conflict of interest and financial disclosure

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