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Livestock Diseases

Genomic characterization, antimicrobial resistance profiles, enterotoxin, and biofilm production of methicillin-resistant *Staphylococcus aureus* isolated from clinical and animal products origins in Eastern Turkey[1]

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ABSTRACT.- Baran A., Oz C., Cengiz S. & Adiguzel M.C. 2022**. Genomic characterization, antimicrobial resistance profiles, enterotoxin, and biofilm production of methicillin-resistant *Staphylococcus aureus* isolated from clinical and animal products origins in Eastern Turkey.** Pes*quisa Veterinária Brasileira 42:e06991, 2022*. Department of Microbiology, Faculty of Veterinary Medicine, Ataturk University, Erzurum, 25240, Turkey. E-mail: mcemal.adiguzel@atauni.edu.tr

Staphylococcus aureus is an opportunistic and ubiquitous pathogen found in the skin, nares, and mucosal membranes of mammals. Increasing resistance to antimicrobials including methicillin has become an important public concern. One hundred and eight (108) *S. aureus* strains isolated from a total of 572 clinical and animal products samples, were investigated for their biofilm capability, methicillin resistance, enterotoxin genes, and genetic diversity. Although only one strain isolated from raw retail was found as a strong biofilm producer, the percentage of antimicrobial resistance pattern was relatively higher. 17.59% of *S. aureus* strains tested in this study were resistant to cefoxitin and identified as methicillin-resistant *S. aureus* (MRSA) isolates*. mecA* and *mecC* harboring *S. aureus* strains were detected at a rate of 2.79% and 0.93%, respectively. In addition, staphylococcal enterotoxin genes including *Sea*, *Seb*, *Sec*, and *Sed* genes were found to be 18.5%, 32.4%, 6.5% and 3.7%, respectively. The phylogenetic relationship among the isolates showed relationship between joint calf and cow milk isolates. Multi locus sequence typing (MLST) revealed three different sequence types (STs) including ST84, ST829, and ST6238. These findings highlight the development and spread of MRSA strains with zoonotic potential in animals and the food chain throughout the world.

INDEX TERMS: Genome, antimicrobial resistance, enterotoxin, biofilm, methicillin resistance, *Staphylococcus aureus*, animal products, Turkey, enterobacterial repetitive intergenic consensus, ERIC-PCR, multi locus sequence typing, MLST.

RESUMO.- [**Caracterização genômica perfis de resistência antimicrobiana, produção de enterotoxinas e biofilme *Staphylococcus aureus* resistente à meticilina isolado de origem clínica e de produtos de origem animal no leste da Turquia.**] *Staphylococcus aureus* é um patógeno dúctil e ubíquo encontrado na pele, narinas e membranas mucosas de mamíferos. O aumento da resistência aos antimicrobianos, incluindo a meticilina, tornou-se uma importante preocupação pública. Cento e oito (108) cepas de *S. aureus* isoladas de um total de 572 amostras clínicas e de produtos animais foram investigadas por sua capacidade de biofilme, resistência à meticilina, genes de enterotoxinas e diversidade genética. Embora apenas uma cepa isolada do cru tenha sido encontrada como forte produtora de biofilme, a porcentagem do padrão de resistência antimicrobiana foi relativamente maior. Parte das cepas (17,59%) de *S. aureus* testadas neste estudo eram resistentes à cefoxitina e identificadas como isolados de MRSA. *mecA* e *mecC* abrigando cepas de *S. aureus* foram detectados a uma taxa de 2,79% e 0,93%, respectivamente. Além disso, verificou-se que os genes da enterotoxina estafilocócica,

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incluindo os genes *Sea*, *Seb*, *Sec* e *Sed*, eram 18,5%, 32,4%, 6,5% e 3,7%, respectivamente. A relação filogenética entre os isolados mostrou relação entre os isolados de bezerro e leite de vaca. A tipagem de sequência multiloco (MLST) revelou três tipos de sequência diferentes (STs), incluindo ST84, ST829 e ST6238. Essas descobertas destacam o desenvolvimento e a disseminação de cepas de MRSA com potencial zoonótico em animais e na cadeia alimentar em todo o mundo.

TERMOS DE INDEXAÇÃO: Genoma, resistência antimicrobiana, enterotoxina, biofilme, *Staphylococcus aureus*, resistência à meticilina, produtos de origem animal, Turquia, consenso intergênico repetitivo enterobacteriano, ERIC-PCR, tipagem de sequência multiloco.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen that is part of the microbiota of the skin, nares, and mucosal membranes of mammalians. It is responsible for various infections, such as osteomyelitis, wound infections, pneumonia, endocarditis, meningitis, brain abscesses, and impetigo (Papadopoulos et al. 2018, Haag et al. 2019, Adame-Gómez et al. 2020). Mammary gland infection of cows named mastitis is the important issue in dairy operation (Vautor et al. 2003). In addition, the enterotoxin-producing strains are responsible for food (milk, cheese, raw meat products, etc.) poisoning, which is crucial for public health (Schelin et al. 2011). Besides the prevalence of reports of staphylococcal infections in humans, the animal hosts are important because of the zoonotic potential for this important pathogen (Peton & Le Loir 2014).

Invasion and colonization of *S. aureus* strains are associated with the presence of various virulence factors (Adame-Gómez et al. 2020). Among these factors, adhesion proteins and the ability to form biofilms on biotic and abiotic surfaces occupy an important position. Biofilm formation has disadvantages such as facilitating the placement and release of different toxins in the pathogenesis of human and animal infections, limiting the effectiveness of antimicrobial therapy, and increasing the resistance to disinfectant substances on surfaces in the food industry (Aguilar et al. 2001, Vergara et al. 2017). In human medicine, biofilm-related infections have been reported due to infected medical instruments and equipment, resulting in increased morbidity and mortality (Moormeier & Bayles 2017). On the other hand, biofilm formation on surfaces used in the food industry is an important problem for food matrices and food handlers (Achek et al. 2020). Biofilm formation can also affect the severity and clinical presentation of mastitis that can occur in livestock (Karahan et al. 2009). In this respect, the ability to create biofilms is a valuable parameter used in monitoring *S. aureus*.

Treatment of *S. aureus* infections from mild skin infection to invasive disease, especially in humans, is becoming more difficult due to increased methicillin resistance (Gurusamy et al. 2013). It has been reported that as an extremely versatile pathogen, Methicillin resistant *S. aureus* (MRSA) is colonized especially in livestock and spreads in the society (Aires-de-Sousa 2017). It can also be transmitted to humans through meat products due to close contact with animals and cross contamination of animal products (Silva et al. 2020). Hence, careful and continuous monitoring of the spread of MRSA in non-hospital settings such as livestock is required (Caggiano et al. 2016).

Staphylococcal enterotoxins (SE), another problem caused by *S. aureus*, is responsible for severe gastroenteritis along with vomiting, nausea, and diarrhea if swallowed with food. The five main classical SE types (SEA, SEB, SEC, SED and SEE) identified to date are held responsible for these intoxications (Balaban & Rasooly 2000). Food poisoning caused by *S. aureus* has been reported due to raw milk or dairy products (Grispoldi et al. 2019). The presence of species capable of producing SE in milk obtained from livestock animals with subclinical mastitis is a major concern, indicating the necessity to monitor milk and dairy products remarkably.

Animal isolates of *S. aureus* have been reported to exhibit different phenotypic characteristics that vary depending on the host origin. Six biotypes have been identified so far and named human, beta-haemolytic human, bovine, goat species, bird-abattoir, and non-host specific. These biotypes have been obtained by applying sophisticated characterization methods (Peton & Le Loir 2014). The fundamental questions about the population biology of *S. aureus* can be obviously answered when the strains were characterized using a new perspective on methods. Hence, DNA sequence-based approaches such as Multi locus sequence typing (MLST) are widely used to analyze population structures. The high reproducibility of the data obtained by this method is an important advantage (Larsen et al. 2012).

The aim of this study was to screen *S. aureus* isolates originating from animal and animal product samples in eastern Turkey for the existence of phenotypic and genotypic methicillin resistance. Further characterization of the strains was also carried out using enterotoxin production, biofilm-forming capability, ERIC-PCR, and MLST.

MATERIALS AND METHODS

Isolation and identification of *Staphylococcus aureus*. A total of 572 samples (379 clinical samples - abscess, ear, joint, oral, tissue, and urine - 50 raw retail milk, 93 cow milk, 50 cheese) were included in this study. Twenty-five grams of raw retail milk and cheese samples were homogenized in 225mL of 1/4 strength Ringer's solution (Merck, Darmstadt, Germany) using a stomacher (Neutec Group, Inc., NY) for 2 min. The homogenized samples were incubated at 37°C for 24 h. After incubation, a loopful of homogenate was streaked onto Baird-Parker agar (HiMedia, Mumbai, India) supplemented with egg yolk tellurite emulsion (HiMedia). A typical colony of *S. aureus* showing a white margin and bigger than 2mm was subcultured onto Mueller Hinton Agar (Oxoid, Basingstoke, United Kingdom). Clinical samples were collected using sterile swabs and placed into tubes containing Amies medium (Biomedics, Madrid, Spain). Milk samples were taken into sterile tubes (Isolab, Wertheim, Germany) under aseptic conditions. One loopful from each tube was streaked on blood agar (Oxoid, Basingstoke, United Kingdom) plates containing 5-7% sheep blood and incubated for 24 h at 37°C aerobically. Suspect *S. aureus* isolates choosing by Gram staining, catalase, oxidase, and tube coagulase test, and DNase test agar, were subpassaged (one colony per sample) onto MH agar to obtain pure cultures. The isolates were stored in cryogenic vials containing Mueller-Hinton (MH) broth (Oxoid, Basingstoke, United Kingdom) with 20% glycerol at -80°C. Frozen culture stocks were passaged on MH agar (Oxoid) and incubated aerobically for 16-24 h at 37°C for subsequent analyses.

Colonies found to be Gram-positive, catalase, coagulase, and DNAse positive were confirmed to be *S. aureus* by PCR amplification of *nuc*

gene. In this sense, genomic DNA was extracted using the boiling method. Briefly, a few colonies were suspended in 40µL single-cell lysis buffer (SCLB, including TE Buffer, Tris–HCl, and disodium EDTA), which was then heated to 80°C for 10 min and cooled down to 55°C for 10 min in a thermocycler to lyse bacterial cells. The suspension was then diluted 1:2 in distilled water and centrifuged at 4500 × *g* for 30 s to remove cellular debris (Sahin et al. 2017).

PCR reaction for confirmation of suspected *S. aureus* isolates was performed using a Dream Taq Green PCR master mix (Thermo Scientific, Waltham/MA, USA) (25µl) including *nuc*F and *nuc*R primers (Table 1) (Baran et al. 2017), with following protocol; initial denaturation at 95°C for 15 min, 35 cycles of amplification (denaturation at 91°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min), and final extension at 72°C for 10 min in a thermal cycler. PCR products were visualized on a 1% agarose gel containing ethidium bromide for 90 min at 75V and 120mAmp. Gels were photographed in a gel documented system using Bio-Rad Gel Doc XR System (Bio-Rad Laboratories, Hercules/CA, USA).

Biofilm formation assay. The selection of the biofilm-forming *S. aureus* isolates was carried out as previously reported (Lade et al. 2019). A 96-well microplate was used. Briefly, *S. aureus* isolates were incubated aerobically on MH agar at 37°C overnight. A colony was then removed and incubated overnight at 37°C in MH broth and a bacterial suspension equivalent to 0.5 McFarland standard turbidity was prepared. The test medium used in the study was 0.5% or 1.0% d - (+) - glucose, 1.0% or 2.0% NaCl, or TSB supplemented with both 1.0% glucose and 1.0% NaCl. Final sugar or salt concentrations in TSB 7.5g/L or 12.5g/L d - (+) - glucose, 15.0g/L or 25.0g/L NaCl, or both 12.5g/L glucose and 15.0g NaCl. The culture medium was inoculated to a final bacteria concentration of 10^6 CFU/mL and dispensed into the wells of the microtiter plates (200µL/well). The well left empty was used as a negative control. Biofilms were grown aerobically at 37°C for 24 hours under constant conditions. Following incubation, bacteria were cultured from each microtiter plate and the wells were washed twice with 200µL of phosphate buffered saline (PBS, pH 7.4) to remove non-adherent bacteria, adherent bacteria were fixed by heating at 65°C for 1 hour. Then it was stained 150µL of 0.1% (w/v) crystal violet for 5 min. The excess crystal purple stain was then discarded and the plates were washed twice with PBS (200µL) to remove residual dye and then allowed to dry for 30 minutes at room temperature. The stain-adhering biofilm was dissolved in 150µL of 33% glacial acetic acid per well for 30 min. The resulting biofilm formation was evaluated by measuring the absorbance at 595nm with a 96-well ELISA reader (Multiscan FC, Thermo Fisher Scientific, Waltham/MA, USA). Each experiment was performed in duplicate. The negative control value was omitted and the data were displayed as mean absorbance ± standard deviation (SD). When the ABS595 value was three times the mean absorbance SD of the negative control, *S. aureus* strains were considered to form biofilms.

Antimicrobial susceptibility testing. Antimicrobial susceptibility profiles of *S. aureus* isolates to eight antimicrobial agents was determined using the standard disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2021). Commercial discs (Oxoid, Basingstoke, United Kingdom) used included cefoxitin, amoxicillin-clavulanic acid, ampicillin-sulbactam, ceftiofur, oxacillin, gentamicin, tetracycline, oxytetracycline, and penicillin. EUCAST clinical breakpoints were used for interpretation and reporting of antimicrobial susceptibility of isolates (EUCAST 2021). Multidrug resistance was considered as resistance at least three different antimicrobial classes (Sigirci et al. 2020).

Methicillin resistance *S. aureus*. Cefoxitin (30µg; Oxoid) disk diffusion assay was performed following EUCAST guidelines v. 11.0, valid from 2021-01-01 for interpretation of the results and using *S. aureus* ATCC 29213 as a negative control (EUCAST 2021). Isolates with phenotypic resistance to cefoxitin were investigated for detection of the *mec* genes (*mecA*, *mecB*, *mecC*, and *mecD*) by PCR (Horie et al. 2009, Stegger et al. 2012, Schwendener et al. 2017, Nagasawa et al. 2020). To amplify the *mec* genes of the isolates by PCR, primer sequences and annealing temperature were summarized in Table 1. PCR reaction was performed using a Dream Taq Green PCR master mix (Thermo Scientific), forward and reverse primers (10pmol/µl) accordingly *mec* genes, template DNA, and deionized water.

Enterotoxin gene detection. The *sea*, *seb*, *sec*, and *sed* loci were determined among the extracted DNA of *S. aureus* isolates by multiplex polymerase chain reaction using specific primers (Table 1). A volume of 25µl of PCR solution contained Dream Taq

Table 1. PCR primers used in this study for amplification

Target gene	Primer sequence (5'-3')	Temperature (°C)	Size (bp)	Reference
nuc	CCTGAAGCAAGTGCATTTACGA	60	166	Nagasawa et al. (2020)
	CTTTAGCCAAGCCTTGACGAACT			
mecA	AAACTACGGTAACATTGATCGCAAC	62	313	Horie et al. (2009)
	CTTGTACCCAATTTTGATCCATTTG			
mecB	TTAACATATACACCCGCTTG	57	279	Becker et al. (2018)
	TAAAGTTCATTAGGCACCTCC			
mecC	GAAAAAAAGGCTTAGAACGCCTC	59	718	Wu et al. (2016)
	GAAGATCTTTTCCGTTTTCAGC			
mecD	TCCTTTAGCGATAGATGGTGAA	59	867	Schwendener et al. (2017)
	CTCCCATCTTTTCTCCATCCT			
SeA	GGTTATCAATGTGCGGGTGG	60	102	Sharma et al. (2017)
	CGGCACTTTTTTCTCTTCGG			
SeB	ATGTAATTTTGATATTCGCAGTG	60	683	
	TGCAGGCATCATATCATACCA			
SeC	CTTGTATGTATGGAGGAATAACAA	60	283	
	TGCAGGCATCATATCATACCA			
SeD	CTAGTTTGGTAATATCTCCT	60	317	
	TAATGCTATATCTTATAGGG			

Green PCR master mix (Thermo Scientific), forward and reverse primers (10pmol/µl), template DNA, and deionized water. PCR was performed as follows: initial denaturation at 95°C for 15 min, 35 cycles of amplification (denaturation at 91°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min), and final extension at 72°C for 10 min in a thermal cycler. PCR products were visualized on a 1% agarose gel containing ethidium bromide for 90 min at 75V and 120mAmp. PCR products was analyzed by loading 10µl of PCR mixture onto agarose 1% in the presence of 100bp DNA ladder. After gel electrophoresis, the gel was exposed to U.V by using Bio-Rad Gel Doc XR System (Bio-Rad Laboratories, Hercules/CA, USA).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR. Genetic relationship between isolates was performed by ERIC-PCR. DNA was extracted and quantified as previously described. ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) was used with each isolate (Van Belkum et al. 1995). PCR mixture consisted as previously described in section of isolation and identification of *S. aureus* in this study. PCR amplification consisted of an initial denaturation for 4 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, annealing for 1 min at 25°C and extension for 2 min at 72°C and final extension for 7 min at 72°C. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide. A DNA ladder of 100-3000bp was included in all gels. The presence and absence of band pattern were exported to Microsoft Excel and used to generate a data matrix (Albufera et al. 2009). The unweighted pair group method with arithmetic mean (UPGMA) and complete linkage algorithms were used to analyze the percentage of similarity and matrix data (Garcia-Vallvé et al. 1999). Relationships between the various band patterns were visualized by the online ITOL[4].

MLST analysis. MLST analysis was performed using the primer pairs and PCR conditions previously reported by a group of researchers Enright et al. (2000). Seven housekeeping genes including *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), and *yqi* (acetyle coenzyme A acetyltransferase) were used. The PCR products were used to Sanger sequencing and the results were analyzed in PubMLST *S. aureus* database[5]. The sequence typing (ST) of strains was assigned for each strain based on which alleles are present from the database.

Statistical analysis. The statistical analysis was performed using SPSS statistics 20 (Statistical Package for the Social Science; SPSS Inc., Chicago/IL, USA). To compare the data in this study, Pearson probability value (*P* value) was calculated using the Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Due to its potential for transmissibility and the presence of infected animals, *Staphylococcus aureus* has long been associated with livestock. Although whole genome sequencing and deep genomic analyses have shown that livestock-associated strains differ from anthropogenic strains, the existence of a strain variation between reservoirs raises concerns about the presence of this infectious agent. In addition, these factors of animal and human origin share virulence factors, however, the exchange of genes of different virulence factors that seem to be important in host adaptation may expand the host range at the same time and thus threaten public health (Fluit 2012, Abd El-Hamid et al. 2019). In this context, regular monitoring of the presence and virulence factors of *S. aureus* should be revealed in terms of both animal and human health. Although there are different reports on the presence of *S. aureus* and the characterization of virulence factors in animal and animal product samples in Turkey (Karahan et al. 2009, Ektik et al. 2017, Keyvan et al. 2020, Issa & Aydin 2021), to our best knowledge, there is no comprehensive data on the genomic characterization and methicillin resistant *S. aureus* (MRSA), especially in eastern Turkey. Thus, in the present study, *S. aureus* was screened and compared in a total of 572 different clinical samples (abscess, ear, joint, oral, tissue, milk, and urine) and animal products (retail milk and cheese) from the eastern region of Turkey.

Phenotypic biofilm production of *S. aureus* isolates was analyzed by the microtiter plate test, which has been reported to be highly subjective (Avila-Novoa et al. 2018) and used extensively as the gold standard. The results were obtained from the 108 *S. aureus* isolates (were from clinical milk samples (n=86), raw retail milk (n=8), cheese (n=4), joint (n=4), abscess (n=1), ear (n=1), oral (n=1), tissue (n=2), and urine (n=1)) tested for phenotypic biofilm formation, the biofilm producer of 1 (0.93%) and 107 (99.07%) showing not biofilm producer. In addition, the strain named 107-Co-R.Mlk isolated from raw retail milk was found as a "strong" biofilm producer. In contrast to our findings, *S. aureus* isolates from food origin in Mexico (Avila-Novoa et al. 2018) and India (Sharma et al. 2017) were reported a higher rate of biofilm producer, which was thought to be caused by the low number of food-origin *S. aureus* isolates included in this study.

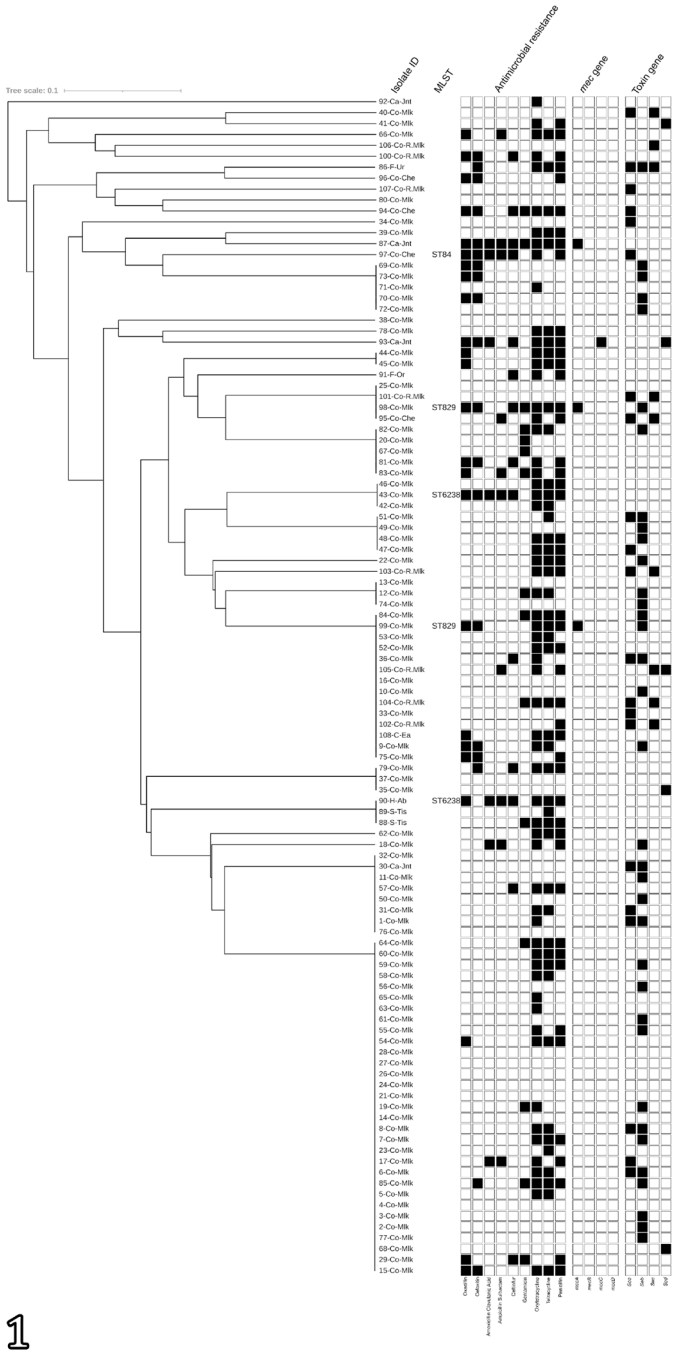
Fifty (54.2%), forty (44.8%), thirty-nine (40.6%), twenty-one (21.9%), sixteen (16.7%), fourteen (14.58%), twelve (12.5%), and seven (7.3%) of *S. aureus* strains (n=96) isolated from clinical and cow milk samples in the current study were resistant to oxytetracycline, tetracycline, penicillin, oxacillin, cefoxitin, ceftiofur, gentamicin, amoxicillin-clavulanic acid, and ampicillin-sulbactam, respectively. On the other hand, among the animal product isolates (n=12), eight (66.7%) were resistance to penicillin, seven (58.3%) were resistance to oxytetracycline, four (33.3%) were resistance to oxacillin, cefoxitin and ceftiofur, three (25.0%) were resistance to ampicillin-sulbactam and tetracycline, two (16.7%) were resistance to gentamicin and only one was resistant to amoxicillin-clavulanic acid (Fig.1). Among the isolates, 34.4% of clinical isolates and 25.0% of animal product isolates were susceptible to all antimicrobials used. On the other hand, 8.3% of animal clinical isolates showed single resistance, 14.6% double resistance and 27.1% multiple resistances. One (8.3%) of the animal product isolates showed single resistance, while four (33.3%) showed multiple resistances (Fig.2). The comparative antimicrobial susceptibility results showed that resistance to penicillin, ceftiofur, ampicillin-sulbactam, cefoxitin, and oxacillin was found to be higher in animal product isolates than in clinical isolates, whereas it was in contrast to tetracycline. At least one isolate was resistant to all antimicrobials tested in this study. In addition, no significant difference was found between antimicrobial resistance and the origin of the isolates ($P<0.05$). The extensive use of antimicrobials in veterinary medicine promotes stabilization of antimicrobial resistance genes that can transfer these genes

[4] Available at <https://itol.embl.de/> Accessed on Jun. 23, 2021.

[5] Available at <https://pubmlst.org/organisms/staphylococcus-aureus/> Accessed on Jun. 23, 2021.

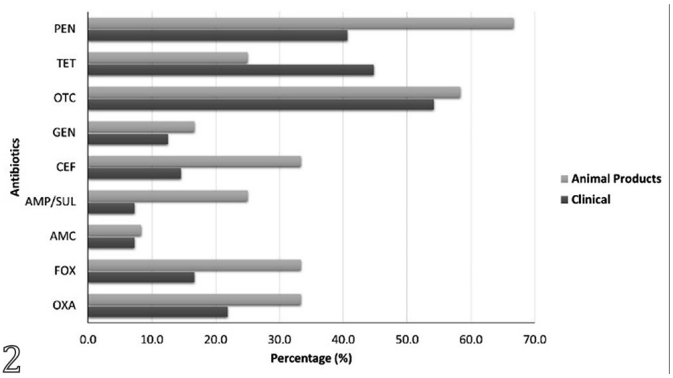
to human-adapted pathogens or bacteria in the human gut microbiota through direct contact, food, or the environment (Argudín et al. 2017). *S. aureus* strains with 25.5% multidrug resistance isolated from animal products in Algeria have been reported by a group of researchers (Achek et al. 2018). Similarly, a quarter of the isolates tested in this study were also harbored MDR. On the other hand, the number of *S. aureus* isolates susceptible to all antimicrobials (33.3%) in the current study was similarly reported by another study (Chen et al. 2020). With regard to isolates of animal origin, most strains showed resistance to penicillin and tetracycline, in agreement with previous studies (Aslantaş & Demir 2016, Gandhale et al. 2017, Achek et al. 2018, Pekana & Green 2018, Yadav et al. 2018, Dan et al. 2019, Vitale et al. 2019, Chen et al. 2020). In addition, there was lower resistance to amoxicillin clavulanic acid and gentamicin as previously reported and supported our findings (Sumathi et al. 2008, Beco et al. 2013), suggesting that those antimicrobials are the most effective drugs against *S. aureus* infections. Of note, resistance to antimicrobial components in bacteria varies as it is related to the use of drugs in a particular area, and therefore the pattern and rate of resistance to certain antimicrobials (Yadav et al. 2018).

Nineteen (19, 17.59%) of 108 *S. aureus* strains tested in this study were resistant to cefoxitin and thus classified as MRSA isolates. In addition, 24 (22.22%) of 108 *S. aureus* strains were resistant to oxacillin. The use of cefoxitin disc test for the detection of MRSA has been reported that is more sensitive and specific than the oxacillin disc test by researchers (Boşgelmez-Tınaz et al. 2006, Alipour et al. 2014). Thus, the cefoxitin disc test was used to detect MRSA strains in this study. The three of nineteen cefoxitin-resistant *S. aureus* isolates were found to be *mecA*-positive (2.78%). Besides, one isolate showing cefoxitin-resistant was detected to be *mecC*-positive (0.93%) by PCR. However, none of the *S. aureus* strains were positive for *mecB* and *mecD* gene as determined by PCR (Fig.1). All *mec* gene carrying *S. aureus* strains were resistant to penicillin, however, two of them were sensitive to enhanced penicillin (amoxicillin-clavulanic acid and ampicillin-sulbactam) tested in this study. This finding indicated that enhanced penicillin may still affect against MRSA strains isolated from clinical samples. On the other hand, two of *mec* gene carrying strains were resistant to enhanced penicillin, indicating careless antimicrobials usage in companion animals leading to a wide resistance spectrum



1

Fig.1. ERIC-PCR band patterns based dendrogram of *Staphylococcus aureus* isolates from different sources. Band profile analyzes were performed using Microsoft Excel to generate the data matrix. The presence (black box) and absence (empty box) of antimicrobial susceptibility patterns, mec genes, and toxin genes are shown. The dendrogram included ST number for five strains. Origin and sources of the isolates are indicated by following abbreviations: canine (C), calf (Ca), cow (Co), feline (F), horse (H), sheep (S), abscess (Ab), cheese (Che), ear (Ea), joint (Jnt), milk (Mlk), oral (Or), raw retail milk (R. Mlk), urine (Ur).



2

Fig.2. Percentage of clinical and animal products-derived isolates resistant to the antimicrobials used in this study. Penicillin (PEN), tetracycline (TET), oxytetracycline (OTC), gentamicin (GEN), cefepime (CEF), ampicillin-sulbactam (AMP/SUL), amoxicillin-clavulanic acid (AMC), Cefoxitin (FOX), oxacillin (OXA).

of MDR. MRSA prevalence has been reported differently by other researchers previously (Li et al. 2017, Liu et al. 2018, Issa & Aydin 2021). It has been reported the detection of MRSA strains (0.14%) isolated from cheese samples in Turkey (Issa & Aydin 2021). In another study reported that MRSA prevalence was 5% in cheese and raw retail milk samples collected in Turkey (Siriken et al. 2018). In contrast to the previous study, the MRSA prevalence of the current study was 17.59% and is thought to be associated with samples' origins and numbers although this association is not certain always.

Characterizing the presence of the SE gene in *S. aureus* isolates from different sources in terms of public health and food safety can provide important epidemiological information (Chao et al. 2015). In this context, *Sea*, *Seb*, *Sec*, and *Sed* genes in 108 isolates analyzed in the present study were detected at a rate of 18.5%, 32.4%, 6.5%, and 3.7%, respectively. To best our knowledge, this is the first study to report SE genes from different animal sources in eastern Turkey. It was determined that 53.7% of the isolates in this study had at least one SE gene. Similarly, approximately 50-60% of *S. aureus* isolates harbored SE genes previously reported by other researchers (Figueroa et al. 2002, Morandi et al. 2007, Carfora et al. 2015, Rola et al. 2016, Vasconcelos et al. 2011). Some isolates (11.11%) displayed three genes forming two different profiles that were *Sea-Seb* and *Sea-Sec*. Notably, the prevalence of SE genes in the current study was both relatively abundant and diverse in isolates obtained from animal products (mean, 1.33) and was significantly higher ($P<0.05$) in clinical isolates (mean, 0.56), suggesting that environmental stress conditions (nutrition, temperature, etc.) may result in an apparent loss of mobile genetic elements (i.e. SE genes). It is also concluded that animal product isolates are the main potential source of classical staphylococcal food poisoning.

All *S. aureus* strains (n=108) isolated in this study were characterized by ERIC-PCR to determine the genetic diversity and phylogenetic relationship among the isolates (Fig.1). All the isolates were typeable by ERIC-PCR. The dendrogram displayed two main clades that one of which included only one strain isolated from the calf joint. The tree indicated that a calf joint isolate was genetically close to the cow milk isolates, suggesting thought to be caused by the transmission of pathogens via contaminated feed, milking stuff, or farmer. It has been reported that the large proportion of MRSA colonization in calves may be due to the transmission through farmers (Vandendriessche et al. 2013). However, further analyses such as whole-genome analysis and pulsed-field gel electrophoresis are needed to definitively ascertain the origin of the strains. The three of the cheese strains were genetically distinct from raw retail and clinical milk strains, whereas one cheese strain was more closely related to them. Although the clinical strains isolated from horse, sheep, feline, and canine were formed separate clusters, one canine ear strain (108-C-Ea) was placed in the same cluster with raw retail and clinical milk strains.

Based on the MLST, the isolates belong to sequence type (ST) 84 (n=1), 829 (n=2), and 6238 (n=2) isolated from clinical and animal product samples. ST84, ST829, and ST6238 have been reported infrequently in public MLST databases so far and included only one strain from Colombia in 1998, The Netherlands in 2003, and Switzerland in 2020 in PubMLST database, respectively. The ST84 isolated from cheese samples was MRSA as previously reported from Colombia in the PubMLST database. Two milk isolates carrying *mecA* gene were shared ST 829 (Fig.1). Of note, the strain named 90-H-Ab isolated from horse abscess represented the first MRSA strain member within ST6238. Similarly, this ST number was reported from horse skin samples in the PubMLST database. Interestingly, one cow milk strain was shared the same ST number (ST6238) with horse abscess strain.

CONCLUSIONS

The findings from the current study further point out the everlasting spread of methicillin resistance genes mediated by *mec* genes among *Staphylococcus aureus* isolates of diverse origin with zoonotic potential around the world.

In addition, as previous studies by other investigators have shown, *S. aureus* isolates isolated from the present study were found to harbor staphylococcal enterotoxin genes as well as methicillin resistance gene, further emphasizing the potential for treatment difficulties and severity of the infections.

A large number of staphylococcal enterotoxin-producing strains were detected from milk and cheese isolates, indicating a potential risk for public health. Besides, the higher detection of antimicrobial resistance in clinical isolates may be associated with the fact that heavy use of antimicrobials in food-producing animals.

Future research into the epidemiology of methicillin resistance in animals, people, and the environment will help researchers better understand how to combat this serious One-Health concern.

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