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RESEARCH ARTICLE

Phenotypic and Genotypic Determination of Antibiotic Resistant and Biofilm Forming *Staphylococcus aureus* Isolated in Erzincan Tulum Cheese [1]

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Summary

In this study, biofilm formation and antibiotic resistance of *Staphylococcus aureus* strains isolated from Erzincan tulum cheeses were phenotypically and genotypically investigated. Samples collected from 100 different Erzincan tulum cheese were inoculated into Baird-Parker agar to isolate *S. aureus*. Identification of *S. aureus* was performed with Gram staining, hemolysis or coagulase tests. Determination of the *nuc, mecA, vanA,* and *vanB* genes in isolates was performed by Polymerase Chain Reaction (PCR). Congo red agar was used for the biofilm formation of *S. aureus*. Antibiotic resistance was determined by antibiotic discs of oxacillin (1 µg), cefoxitin (30 µg), vancomycin (30 µg), amoxicillin-clavulanic acid (20 µg), and penicillin (10 units). A total of 72 of 100 (72%) samples were positive for *Staphylococcus* spp. Of 72 samples, 61 (84.7%) were phenotypically and genotypically identified as *S. aureus*. Of 61 isolates, 37 (60.6%) formed a biofilm. Of 61 isolates, 49 were determined to resistant to antibiotics of oxacillin (methicillin) (9), cefoxitin (8), amoxicillin-clavulanic acid (4), and, penicillin (28). Vancomycin-resistance was not detected. Only the *nuc* and *mecA* genes were detected in 10 of 61 (16.3%) strains of *S. aureus*. In this study, the rate of *S. aureus* determined in Erzincan tulum cheeses was high. Considering the high rate of contamination and antibiotic resistance due to poor hygienic conditions, it was concluded that Erzincan tulum cheese, now a PDO cheese, should be considered to be great risk for public health.

Keywords: *Antibiotic resistance, Biofilm, Staphylococcus aureus, Polymerase Chain Reaction, Tulum cheese*

Erzincan Tulum Peynirinden İzole Edilen *Staphylococcus aureus* İzolatlarında Antibiyotik Direncinin ve Biyofilm Oluşturma Özelliğinin Fenotipik ve Genotipik Olarak Belirlenmesi

Özet

Bu çalışmada Erzincan tulum peynirinden izole edilen *Staphylococcus aureus*'ların biyofilm oluşturabilme yetenekleri ve antibiyotik dirençlilikleri fenotipik ve genotipik yöntemlerle araştırıldı. Araştırmada 100 adet Erzincan tulum peyniri numunesi toplandı. Peynir örneklerinden *Staphylococcus aureus* izolasyonu için Baird-Parker agar'a ekim yapıldı. İzolatlar Gram boyama, hemoliz ve koagülaz testleriyle *S. aureus* olarak identifiye edildi. Polimeraz Zincir Reaksiyonu ile izolatlar *nuc, mecA, vanA* ve *vanB* genleri yönünden incelendi. *S. aureus*'ların biyofilm oluşturma yeteneği için Kongo Red agar ve oksasilin (1 µg), sefoksitin (30 µg), vankomisin (30 µg), amoksisilin-klavulonik asit (20 µg) ve penisilin (10 unit) diskleri antibiyotik dirençliliğin saptanması amacıyla kullanıldı. Test edilen örneklerin 72 (%72)'si *Staphylococcus* spp. pozitif bulundu. Bunlardan 61 (%84.7) örnek fenotipik ve genotipik olarak *S. aureus* olarak identifiye edildi. İzolatların 37 (%60.6)'sinin biyofilm oluşturduğu saptandı. Disk difüzyon yönteminde 61 izolattan 9 (%14.7)'unda oksasilin (metisilin), 8 (%13.1)'inde sefoksitin, 4 (%6.5)'ünde amoksisilin-klavulonik asit, 28 (%45.9)'inde ise penisilin dirençliliği tespit edilirken vankomisin dirençli izolata rastlanmadı. Polimeraz Zincir Reaksiyonu sonucunda 61 örnekten 10 (%16.3)'unda *nuc* ve *mecA* genleri saptanırken *vanA* ve *vanB* genlerine rastlanmadı. Bu araştırmada Erzincan tulum peynirinde *S. aureus*'un yüksek bir oranda bulunduğu görülmüştür. Kontaminasyon oranının yüksek olması ve izolatların antibiyotik dirençliliği göz önünde bulundurulduğunda hijyenik şartlarda üretilmeyen bu peynirlerin halk sağlığı açısından büyük risk oluşturabileceği kanaatine varılmıştır.

Anahtar sözcükler: *Antibiyotik direnci, Biyofilm, Staphylococcus aureus, Polimeraz Zincir Reaksiyonu, Tulum peyniri*

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INTRODUCTION

Tulum cheese is produced commonly in Turkey. However, there is no standard production methods including ripening time. Therefore, quality of tulum cheese varies. Tulum cheese is traditionally made of sheep's milk. Following milking, raw milk is filtered without any heat treatment or pasteurization, and then fermentation process is started. Fermentation temperature of milk varies from 28°C to 30°C. Fermentation process is completed between 1.5 and 4 hours depending on the strength and amount of yeast [1].

Staphylococcus aureus, a health-threatening pathogen associated with food can cause many diseases including mild skin infections, pneumonia or septicemia in people and animals. The most common carriers of these pathogenic bacteria are human being. Approximately 50% of people are commensal carriers of *S. aureus*. Antibiotic-resistant pathogens can be contaminated by milk and milk products, and meat and meat products. *Staphylococcus aureus* strains may have an adhesive protein known as biofilm made of polysaccharide. It was indicated that strains with biofilm were more virulent compared to those without biofilm; therefore, strains with biofilm become resistant to antibiotics [2]. Food-borne contamination can play an important role in transporting methicillin-resistant *S. aureus* (MRSA) which has been frequently encountered in recent years [3]. Methicillin-resistant strains may become multiresistant; therefore, treatment of these strains can be very difficult. Vancomycin has been used successfully in the treatment of methicillin-resistant strains of *S. aureus*; however, in recent years, only medium-level resistance was determined. Therefore, determination of vancomycin-resistant strains is of critical importance [4].

The purpose of this study was to investigate phenotypically and genotypically biofilm formation and antibiotic resistance of *Staphylococcus aureus* isolated from Erzincan tulum cheese with high consumption rate in Turkey.

MATERIAL and METHODS

Study materials consisted of 100 samples of Erzincan tulum cheese in Erzincan province. Samples as 200 g in weight were collected from markets via aseptic conditions in the sterile bags and then were brought to the laboratory in the cold chain.

Ten grams of each sample was placed in sterile stomacher bags and then 90 ml 0.1% sterile peptone water was added. Stomacher bags were mixed for homogenization at least 3 minutes. One ml of homogenates was transferred in 9 ml peptone water tubes. Decimal dilutions were performed to determine the exact structure of the colonies due to unknown bacterial density in the sample. Dilution in 0.1 ml was incubated for aerobic culture on Baird-Parker agar at 37°C for 24-48 h. After incubation growth typical and atypical colonies were isolated for analyzing [5].

Phenotypic Methods

Gram staining was performed for growth colonies on Baird-Parker [6]. Hemolysis and coagulase tests were performed on isolates identified as Gram-positive [5].

Investigation of Resistance to Antibiotics by the Disc Diffusion Method

Bacterial suspension was prepared in accordance with the recommendations of the Clinical Laboratory Standards Institute (CLSI) [7]. Briefly, suspension from the 24-h culture of bacteria in 0.9% NaCl solution which is equal to the 0.5 McFarland turbidity standards (1×10^8 CFU/ml) was prepared by using the direct colony suspension method. Suspension was spread onto the surface of Mueller-Hinton agar (Oxoid CM337) plates using sterile swabs. After the medium surface dried oxacillin, cefoxitin, vancomycin, amoxicillin-clavulanic acid and penicillin discs were placed on the plates. The zone diameters of the discs were measured after incubation at 35°C for 24 h. The results were evaluated on the basis of standards set, in accordance with recommendations of the Clinical Laboratory Standards Institute [7]. Because oxacillin (1 µg) resistance also determines methicillin resistance, an oxacillin antibiotic disc (1 µg) was used in the resistance investigation of methicillin.

Genotypic Methods

DNA extraction was performed with the phenol/chloroform extraction method [8].

The nuc Gene Detection by Polymerase Chain Reaction

According to the protocol of Maes et al.[9] primer pairs of the *nuc* gene sequence were used to follow 279 bp DNA fragments. Preparation of the reaction mixture was made using $MgCl_2$ (Sigma) 2 mM dNTPs (Sigma) 250 µM, primers 20 pmol/µl (Sigma), 0.4 µM of each of the primers, *Taq* polymerase (Sigma) 2 U, and DNA 5 µl. The amplification process was performed with 30 cycles in a thermalcycler (Techne, UK). The amplification cycle was performed as initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, connecting at 51°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 2 min. Products were stained with 6 µM ethidium bromide and electrophoresis (Thermo, USA) 120 V for 40 min was performed. The products were examined under an UV-transilluminator (Vilber Lourmat, France). *Escherichia coli* ATCC 11230 was used as the *nuc* (-) control strain [9].

Detection of Methicillin Resistance by Polymerase Chain Reaction

The same procedures of detection of the *nuc* gene were applied to follow the 533 bp DNA fragment. In the test, *S. aureus* ATCC 46300 was used as the *mecA* (+) control and

the *S. aureus* ATCC 1065 strain was used as the *mecA* (-) control [9].

Detection of Vancomycin Resistance by Polymerase Chain Reaction

The protocol indicated by Clark et al.[10] was used to perform PCR by using primer pairs (Sigma) forming the *vanA* and *vanB* gene sequence to follow 1030 bp DNA fragments for *vanA*, and 433 bp DNA fragments for *vanB*. The reaction mixture was consisted of 2.5 U *Taq* polymerase (Sigma), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTPs mix (Sigma), 0.5 µM of each primer (Sigma primers), 1.5 mM $MgCl_2$, and 5 µl DNA. The amplification process was performed with 30 cycles in thermalcycler (Techne, UK). The amplification cycle was performed as initial denaturation at 94°C for 10 min, denaturation at 94°C for 30 sec, connecting at 58°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 10 min. The PCR products were stained in 1.8% agarose gel with 10 ml of ethidium bromide and electrophoresis (Thermo, USA) 110 V for 1 hour was performed. The products were examined under a UV-transilluminator (Vilber Lourmat, France). 1030 bp for *vanA* and 433 bp for *vanB* were determined as positive [10]. *E. faecium* ATCC 51559 (*vanA*) and *E. faecalis* ATCC 700802 (*vanB*) strains were used for positive control [11].

Determination of Biofilm Creation Feature

Congo Red agar (CRA) method developed by Freeman et al was used to determine the slime-posivity [12]. Colonies isolated as *S. aureus* were incubated on CRA at 37°C for 24 h. After incubation black-colored colonies were determined as biofilm positive.

Statistics

The SPSS (Statistical Package for Social Sciences for Windows, 16.0) program was used for the statistical analysis. Frequency distributions and the chi-square statistical methods were applied. Results were evaluated at 95% confidence interval at a level of $P<0.05$.

RESULTS

Staphylococcus spp. was determined in 72 of 100 Tulum cheese samples on the market after incubation on Baird-Parker's agar. Moreover, all 72 isolates were determined as Gram positive and cocci by Gram-staining. Of 72 isolates, 61 (84.7%) were identified as *S. aureus* by hemolysis and coagulase tests.

When 61 isolates of *S. aureus* were tested phenotypically, 9 (14.75%) were oxacillin resistant, 8 (13.11%) were cefoxitin resistant, 4 (6.55%) were amoxicillin-clavulanic acid resistant, and 28 (45.90%) were penicillin resistant.

Although the *nuc* gene was detected in all 61 *S. aureus* isolates the *mecA* gene was only detected in 10 (16.39%) isolates *(Fig. 1)*. On the other hand, neither the *vanA* nor *vanB* genes were not detected.

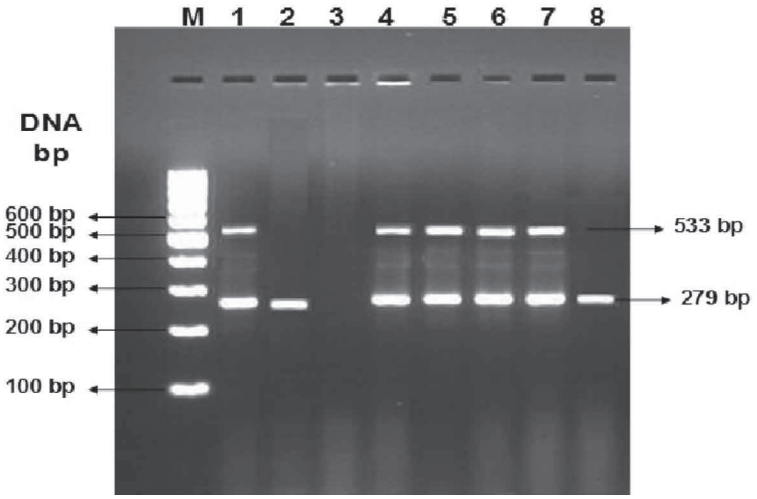

Fig 1. Electrophoresis View of the *nuc* and *mecA* Genes. M: Marker (Gene Ruler Set-Promega, Madison, USA) DNA Ladder Plus (G210A) 100 bp-3000 bp Blue/Orange Loading Dye (G190A), 1: *Staphylococcus aureus* ATCC 46300 (533 bp *mecA* positive and 279 bp *nuc* positive control strains), 2: *Staphylococcus aureus* ATCC 1065 (*mecA* negative and 279 bp *nuc* positive control strains), 3: *E. coli* ATCC 11230 (negative control strain) 4: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 5: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 6: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 7: 533 bp *mecA* positive, 279 bp *nuc* positive study isolates, 8: *mecA* negative, 279 bp *nuc* positive study isolates

Şekil 1. *nuc* ve *mecA* Genlerinin Elektroforez Görünümü. M: Marker (Gene Ruler Seti-Promega, Madison, USA) DNA Ladder Plus (G210A) 100 bp-3000 bp Blue/Orange Loading Dye (G190A), 1: *Staphylococcus aureus* ATCC 46300 (533 bp'da *mecA* pozitif ve 279 bp'da *nuc* pozitif kontrol suşları), 2: *Staphylococcus aureus* ATCC 1065 (*mecA* negatif ve 279 bp'da *nuc* pozitif kontrol suşlar), 3: *E. coli* ATCC 11230 (negatif kontrol suşu) 4: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 5: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 6: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 7: 533 bp'da *mecA* pozitif, 279 bp'da *nuc* pozitif çalışma izolatları, 8: *mecA* negatif, 279 bp'da *nuc* pozitif çalışma izolatları

Table 1. *The results of analysis of antibiotic resistance of biofilm positive and negative S. aureus*
Tablo 1. *Biyofilm pozitif ve negatif S. aureus'larda antibiyotik direnç analiz sonuçları*

Antibiotic	Biofilm Positive 37 (60.65%)		Biofilm Negative 24 (39.25%)		Total 61 (100%)		P*<0.05 Significant
	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	
Oxacillin	9 (24.32%)	28 (75.68%)	0 (0%)	24 (100%)	9 (14.75%)	52 (82.25%)	P*=0.05
Cefoxitin	8 (21.62%)	29 (78.38%)	0 (0%)	24 (100%)	8 (13.11%)	53 (86.89%)	P=0.06
Vancomycin	0 (0%)	37 (100%)	0 (0%)	24 (100%)	0 (0%)	61 (100%)	P=0.19
Amoxicillin-Clavulanic acid	4 (10.81%)	33 (89.19%)	0 (0%)	24 (100%)	4 (6.55%)	57 (93.45%)	P=0.11
Penicillin	25 (67.56%)	12 (32.44%)	3 (12.50%)	21 (87.50%)	28 (45.90%)	33 (54.10%)	P*=0.01

Of 61 *S. aureus* isolates, 37 (60.65%) were positive for biofilm formation. Of 37 biofilm positive isolates, 9 (24.32%) were oxacillin resistant, 8 (21.62%) were cefoxitin resistant, 4 (10.81%) were amoxicillin-clavulanic acid resistant, and 25 (67.56%) were penicillin resistant. On the other hand, vancomycin resistance was not detected *(Table 1)*.

DISCUSSION

Staphylococcus aureus threats public health by causing serious hospital infections. Most of the nosocomial infections caused by methicillin-resistant *S. aureus* resulted in substantial losses in the world. Identification of m*ecA* gene in determination of methicillin-resistant is a gold standard. Methicillin-resistant strains are multi-resistant. It is known that genes resistant to vancomycin were transferred from *Enterococcus* to *Staphylococcus*. Wide spread usage of vancomycin resulted in decreased sensitivity of *S. aureus* strains to vancomycin. Therefore, treatment of this infection is difficult. Antibiotic-resistant strains can infect humans via the ingestion of foods contaminated with these resistant strains. Therefore, contaminated foods have great risk potential to human health [13].

There are many studies that presence of *Staphylococcus* spp. and *S. aureus* in cheese has been investigated. Yasar [14] reported that *Staphylococcus* spp. was determined in 47 of 99 cheese samples on the market and in 45 of 72 fresh cheese samples. Of 45 strains, 12 were *S. aureus*. Önganer et al.[15] indicated that *S. aureus* was determined in 30 of 100 fresh curd cheese in Diyarbakır. Öksüztepe et al.[16] indicated that *S. aureus* was determined in 37 of 40 curd cheese on market in Elazığ province. Another study indicated that *S. aureus* was determined in 14 of 181 different cheese samples [17].

In the present study, high contamination rates of *Staphylococcus* spp. and *S. aureus* in cheese are probably associated with raw milk usage, improper use of starter cultures, and lack of sanitary conditions during production and storage, and insufficient ripening period. In addition, different methods and the medium used in the isolation and identification of the organisms may have effects on the results.

Staphylococcus aureus caused many lethal infections before discovery of antibiotics. In addition, staphylococci strains which have gained resistance to penicillin and many other antibiotics became an infection agent causing increased rate of hospital infections in the world. Rosengren et al.[18] indicated that *S. aureus* was determined in 6 of 96 pasteurized milk, and in 38 of 55 non-pasteurized cheese samples; moreover, 39% of these isolates were penicillin resistant. In another study, *S. aureus* was identified in 17 of 24 cheese samples. While these isolates were resistant to penicillin (60%) and oxacillin (5%) none of them were resistant to vancomycin [19]. Spanu et al.[20] reported that *S. aureus* were isolated in 36 cheese samples made of raw sheep's milk. While one third of those strains were penicillin resistant none of them were resistant to neither oxacillin nor vancomycin. In another study, *S. aureus* strains were isolated in 40 of 81 samples consisted of raw milk, cheese and whey. While 50% of them were penicillin resistant only 15% of them were oxacillin resistant; however, none of them were vancomycin resistant [21]. Nohutçu et al.[22] indicated that 79 *S. aureus* isolated in cheese samples were examined for antibiotic resistance; 13.9% were resistant to penicillin and 6.3% were resistant to methicillin. In the present study, high rates of penicillin resistance can be associated with usage of penicillin for the treatment of mastitis and other infections. In addition, antibiotic resistance could be related to usage of milk of animals treated with antibiotics without waiting for the recommended ripening period in humans and calves. Bacteria found in many live and dead surfaces can form a biofilm which is a problem for the pharmaceutical and dairy industries. Biofilm bacteria exhibit resistance to the effect of antibiotics in a variety of ways. Limited diffusion of the antibiotic into the biofilm, different growth rates of bacteria in the biofilm, and the negative effect of micro-environmental changes to the antibiotics are a few example of bacterial resistant. To the best of our knowledge, no information is available in the literature regarding biofilm forming ability of *S. aureus* strains isolated from different varieties of cheese. In this study, of 60.65% *S. aureus* strains formed biofilm; 24.32% were resistant to oxacillin, 21.62% were resistant to cefoxitin, 10.81% were resistant to amoxicillin-clavulanic acid, and 67.56% were resistant to penicillin. Vancomycin resistance was not detected in any of these strains. This

is the first study about biofilm-positive *S. aureus* strains isolated from tulum cheese.

According to these results and statistical data, it was observed that the production of biofilm may be an effective factor for the resistance gaining phenomenon of bacteria against oxacillin and penicillin.

The high rate of *S. aureus* in Erzincan tulum cheese in the present study was concluded as a result of improper hygienic conditions and shortening ripening time. The high rates of antibiotic resistance and the biofilm formation in isolated *S. aureus* indicates that these strains can cause diseases that are difficult to treat. It is thought that Erzincan tulum cheese manufactured with improper hygienic conditions is a threat for public health.

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