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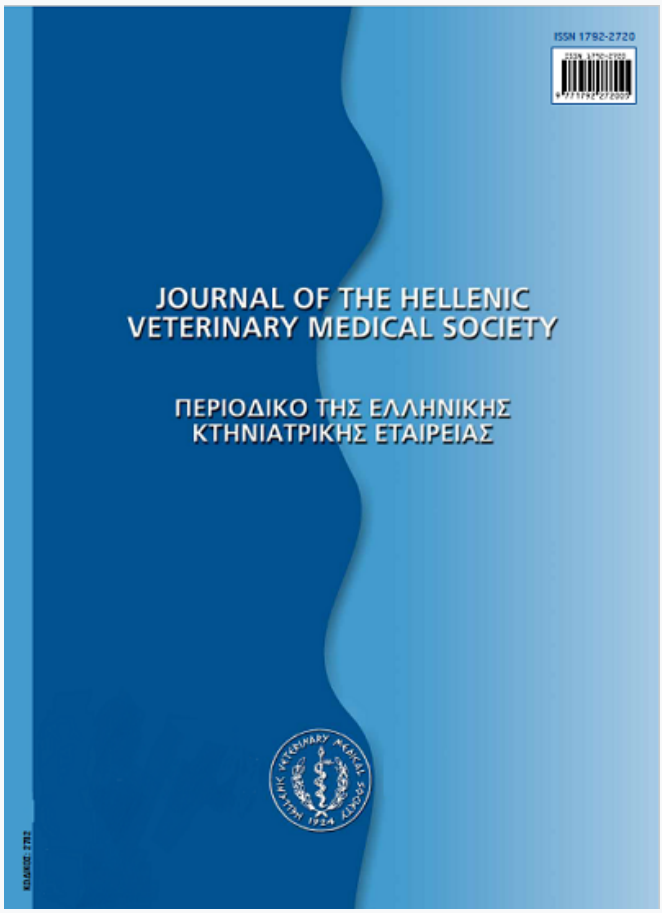

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Research article
Ερευνητικό άρθρο

The Prevalence and Antibiotic Resistance of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Milk and Dairy Products in Balikesir, Turkey

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ABSTRACT. Methicillin-Resistant *Staphylococcus aureus* is an important pathogen that causes severe infections in humans and animals. The aim of this study was to determine the prevalence and the antimicrobial profile of methicillin-resistant *S. aureus* (MRSA) in cow bulk tank milk and dairy products in the region of Balikesir in Turkey. Of 175 milk and dairy products' samples, 26 were found to be positive for *coagulase-positive staphylococci* and 3 (2 samples from cow bulk tank milk and 1 sample from tulum cheese) were MRSA phenotypically being resistant against both oxacillin and cefoxitin. Among these, 17 were confirmed as *S. aureus* by the detection of *nuc* gene and one as MRSA carrying the *mec*A gene. All MRSA isolates were found to be also resistant against ampicillin, penicillin and sulfamethoxazole-trimethoprim. Consequently, even though the prevalence of MRSA in cow bulk tank milk and dairy products was relatively low (1.70%), it may pose serious risks in terms of food safety and public health. In order to prevent the prevalence of MRSA in dairy products, hygienic measures, especially in terms of personal hygiene and disinfection of equipment in all stages of dairy production, should be taken, and HACCP and GMP regulations should be implemented.

Keywords: antibiotic resistance, cow bulk tank milk, MRSA, dairy products

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INTRODUCTION

Staphylococcus aureus, which is part of the normal flora of the skin and the mucous membranes of humans and animals, is an oportunistic pathogen equiped with numerous virulence factors causing serious infections (A alar *et al.*, 2012). Antibiotic resistance is one of these factors involved in nosocomial and community infections (Suda ıdan *et al.*, 2008). The improper use of antibiotics both in human and veterinary medicine, in agriculture, and animal husbandry for more than 50 years has led to the development of antibiotic-resistant strains of *S. aureus*, as well as of other pathogens (Hardy *et al.*, 2004). Methicillin resistance among *S. aureus* strains has especially increased rapidly in last decades (Ito *et al*., 2003). Methicillin (2,6-dimethoxyphenyl penicillin) was first developed in 1959 by George Rawlinson and Ralph Batchelor in England (Rolinson *et al.*, 1960; Dutfield, 2009) and released under the name "Celbenin" for the treatment of penicillin-resistant *S. aureus* infections (Knox, 1961). Shortly thereafter in 1961, the first isolation of MRSA was reported in England (Jevons, 1961; Enright *et al.*, 2002). An altered new form of penicillin-binding protein PBP was synthesized by MRSA strains called "PBP2a" (Enright *et al*., 2002); PBP2 mediates methicillin resistance in staphylococci, coded by the *mec*A gene (Yasuda *et al*., 2000).

The increase of infections caused by antibiotic-resistant bacteria continues to be a major problem worldwide (Spellberg *et al*., 2008; Arefi *et al.,* 2014). MRSA can cause serious invasive infections such as pneumonia, septicemia, osteomyelitis and endocarditis in humans (Mattner *et al.,* 2010). MRSA is the most frequently identified antibiotic-resistant pathogen in many parts of the world, and especially in Europe, America and Middle Eastern countries (Grundmann *et al.,* 2006). The MRSA strains have been divided into three groups: hospital-associated (HA-MRSA), community-associated (CA-MRSA) and livestock-associated (LA-MRSA) (Karpiškova *et al.,* 2009; Huber *et al.,* 2010). Initially, MRSA was known to be associated with hospital-acquired infections, and then it began to appear frequently in healthy individuals in the community (Witte *et al.,* 2008).

In recent years, MRSA has been identified as an emerging pathogen in livestock (pigs, cattle and poultry) and companion animals (Antoci *et al.,* 2013; Cuny *et. al.*, 2013). The presence of LA-MRSA in food-producing animals has raised questions regarding the presence of MRSA in food of animal origin (Feßler *et al.,* 2011). The European Food Safety Authority (2008) underlines that products of animal origin represent a potential source of MRSA, and food contaminated by MRSA could be dangerous. The main risk for the development of food poisoning is the presence of *S. aureus* in foods (Kluytmans, 2010), and MRSA may be involved in food-poisoning outbreaks (Jones *et al.,* 2002).

The aim of the present study was to determine the prevalence of MRSA, and their antimicrobial pattern in cow bulk tank milk collected from farms and dairy products from retail markets in Balikesir Province, Turkey.

MATERIALS AND METHODS

Sample collection

In total 50 cow bulk tank milk samples and 125 dairy products' samples (15 yoghurt, 40 white cheese, 10 kashar cheese, 15 tulum cheese, 12 mihalic cheese, 13 curd cheese, 10 sepet cheese, and 10 butter) were collected from farms and retail markets respectively in Balikesir Province and transferred to the laboratory under refrigeration and analyzed in the same day.

Isolation and identification of MRSA

From each sample, 25 ml or g were aseptically weighted and added into stomacher bags containing 225 ml Mueller-Hinton Broth (Oxoid CM0405, England) with 6.50% NaCl and homogenised in a stomacher (IUL, blender, Spain) for 2 minutes. The homogenates were incubated at 35±2 °C for 16–20 hours for enrichment and then aliquots of 10 µl were spread onto the CHROMagar MRSA (CHROMagar MR502, Paris, France) medium plates and incubated at 35±2 °C for 18-24 hours. At the end of the incubation, according to the manufacturer's recommendations, MRSA suspected colonies showing a pink to mauve color were further examined for coagulase production. Suspected colonies were transferred onto Trypcase Soy Agar (TSA; bioMérieux, 43011, France) before coagulase test. Petri dishes without colonies showing a pink to mauve color were left for an additional 24 hours incubation and then were re-evaluated (EFSA, 2009). Coagulase test was performed by Staphytect Plus (Oxoid-DR0850, Basingstoke, UK). In addition, the MRSA Slidex latex agglutination test (bioMérieux, Marcy l'Etoile, France) was performed for the detection of protein A, clumping factor and capsular polysaccharides. At the completion of the latex tests, coagulase-positive staphylococci were incubated in Brain Heart Infusion Broth (BHI; Merck, Germany) at 35±2 °C for 24 hours and then were stored in BHI broth with glycerol 20% w/v at –80 °C until further analysis.

Detection of the nuc gene in S. aureus isolates and the mecA gene in the MRSA isolates

DNA extraction was performed using a commercial MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Germany), according to the manufacturer's instructions. A PCR cycling program consisted of an initial denaturation step at 94 °C for 10 min, followed by 23 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, and extension at 72°C for 2 min, and a final extension step of 72°C for 5 min.

The *nuc* gene was amplified using nuc 1 (5'-GCGATTGATGGTGATACGGTT-3') and nuc 2 (5'-AGCCAAGCCTTGACGAACTAAAGC-3') primers. The *mec*A gene was amplified using *mec*A 1 (5'-AAAATCGATGGTAAAGGTTGGC-3') and *mec*A 2 (5'-AGTTCTGCAGTACCGGATTTGC-3') primers (Maes *et al.,* 2002). Electrophoresis was performed using 1.50% agarose gel at 90 V for 75 minutes. Samples at 279 bp were considered positive for *S. aureus,* and samples at 533 bp were considered MRSA.

Determination of Antibiotic Resistance

The antibiotic suspectibility of the 3 phenotypically MRSA isolates to 10 antibiotics/antibiotic combinations was determined by the disk diffusion method (Baur *et al.* 1961). Disks containing the following antibiotics were spotted onto the TSA with a 24 mm interval: penicillin G 10 µg, gentamicin 10 µg, erythromycin 5 µg, ampicillin 10 µg, sulfamethoxazole-trimethoprim 25 µg, ciprofloxacin 5 µg, tetracycline 30 µg, chloramphenicol 30 µg, cefoxitin 30 µg, and oxacillin 1 µg. The results were interpreted according to Clinical and Laboratory Standard Institute (2012; 2014) criteria. Antibiotic discs were purchased from Thermo Scientific (Oxoid, England).

Reference Strains

S. aureus (ATCC 33592 *nuc* gene and ATCC 43300 *nuc* gene and *mec*A gene positive) reference strains were purchased from Microbiologics Inc. (St. Cloud, Minnesota USA).

RESULTS

Coagulase-positive staphylococci were isolated from 26 samples. Of these isolates, 16 were isolated from cow bulk tank milk; two from white cheeses; three from kashar cheeses; two from tulum cheeses; two from mihalic cheeses; and one from sepet cheese.

By Slidex MRSA latex agglutination test, three of these isolates found to be MRSA. Of these, two were isolated from bulk tank milk and one from tulum cheese.

The *nuc* gene was detected in 17 of 26 phenotypically coagulase-positive staphylococci isolates (65.38%), while the remaining 9 isolates were *nuc* gene-negative (Fig 1; Table 1). The *mec*A gene was detected in only one out of 17 *S. aureus* isolates (5.88%) (Fig. 2).

DISCUSSION

In this study, *S. aureus* was isolated from 14 of 50 (28%) cow's bulk tank milk samples and two of them (14.28%) were MRSA showing resistance against both oxacillin and cefoxitin (Table 1). The *nuc* gene was detected in 17 of 26 coagulase-positive staphylococci isolates and 9 isolates were *nuc* gene-negative (Fig 1). Consequently, using *nuc*-specific PCR as the sole molecular method for diagnosing *S. aureus* might result in the misidentification of *S. aureus* and MRSA as coagulase-negative staphylococci (Hoegh *et al*., 2014).

A few studies have evaluated the prevalence of MRSA in cow bulk tank milk samples in Turkey and worldwide. Daka et *al.* (2012) reported that 78 of the 160 raw milk samples (48.70%) were *S. aureus*

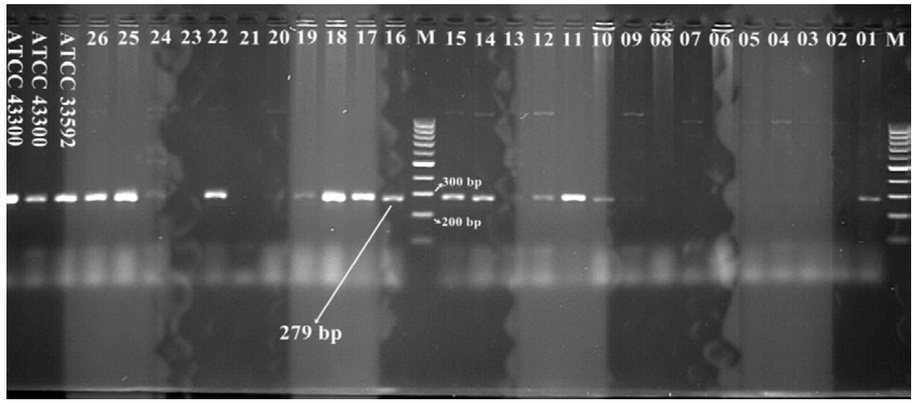

Figure 1. Electrophoresis image of *nuc* positive *S. aureus* isolates.
(M: Marker, ATCC 33592 and ATCC 43300: Positive

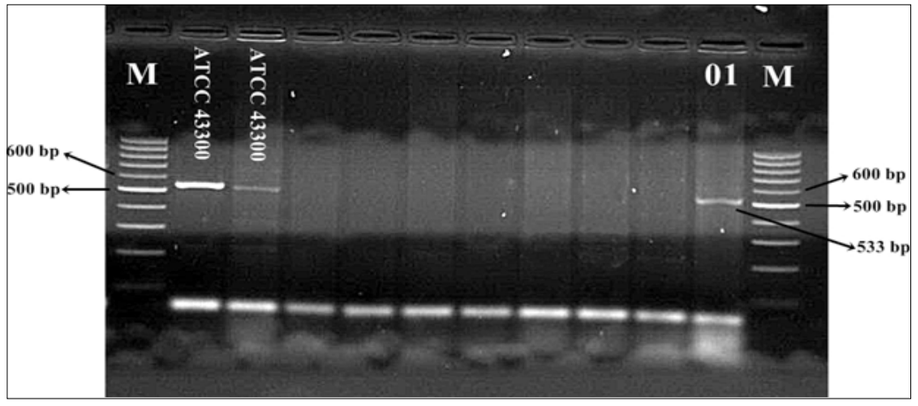

Figure 2. Electrophoresis image of *mec*A positive isolates and reference strain.
(M: Marker, ATCC 43300: Positive Control Strain, 1:*mec*A positive 533 bp)

and 60.30% of them were phenotypically MRSA. Vyletelova *et al.* (2011) isolated *S. aureus* from 326 of 703 tank milk samples (46.30%) and 20 isolates of them (6.10%) were MRSA carrying the *mec*A gene. Paterson *et al.* (2012) found MRSA strains in seven (0.46%) of the 1500 bulk tank milk sample as they carry the *mec*A gene. Kanaan and Al-Shammary (2013) found MRSA phenotypically in 4 (13.40%) of the 15 raw milk. Riva et *al.* (2015) MRSA was detected in seven (1.83%) of 383 raw milk samples. Cortimiglia *et al.* (2016) found MRSA genotypically in 32 (3.80%) of the 844 bulk tank milk. This study's results showed a lower prevalence than the results from several studies in world (Daka et *al.*, 2012) and higher from other (Vyletelova *et al.,* 2011, Paterson *et al.*, 2012, Kanaan and Al-Shammary 2013, Riva *et al.*, 2015, Cortimiglia *et al.*, 2016). The reports on the prevalence of MRSA in milk and dairy products vary significantly between different studies and different countries or even among regions within the same country where they carried out and even among herds within the same region. Furthermore, during the milking process, contamination of milk from staff, equipment, and the environment may affect the

Table 1. Prevalence of genotypically identified *S. aureus* and MRSA isolates in cow bulk tank milk and dairy products

Samples	Number of *S.aureus* positive samples(%)	Number of MRSA positive samples (%)
Cow Bulk Tank Milk	14 (87.50)	-
Yoghurts	-	-
White Cheeses	1(50.00)	-
Kashar Cheeses	-	-
Tulum Cheeses	1(50.00)	1(100)
Mihalic Cheeses	-	-
Sepet Cheeses	1(100)	-
Curd Cheeses	-	-
Butter	-	-
Total	17 (65.30)	1 (33.30)

results (Kalsoom *et al*., 2004).

Türkyılmaz *et al.* (2010) reported that 16 of the 93 (17.20%) *S. aureus* strains isolated from the mastitis cow milk samples were phenotypically MRSA and all of them were carrying *mec*A gene. Pehlivano lu and Yardımcı (2012) reported that 65 of the 306 milk samples with mastitis (21.20%) were *S. aureus* and 20 (30.70%) of them were phenotypically MRSA *mec*A gene was found in 37 isolates (56.90%). Vyletelova *et al.* (2011) isolated *S. aureus* from 326 of 703 tank milk samples (46.30%) and 20 isolates of them (6.10%) were MRSA carrying the *mec*A gene. Vanderhaeghen *et al.* (2010) detected the *mec*A gene in 11 of the 118 *S. aureus* strains (9.30%) obtained from clinical and subclinical mastitis milks. Two strains (18.10%) were isolated from clinical mastitis milk and nine of them (81.80%) from subclinical mastitis milk. Erdem and Türkyılmaz (2013) detected genotypically MRSA in two (1.30%) of 145 mastitis milk samples. Ünal (2013) reported that *mec*A gene was found in two isolates (3.30%) from 60 *S. aureus* isolates which were isolated from milk with subclinical mastitis. These variations may be due to the differences in animal-husbandry systems, in national antimicrobial policies and regulations which may contribute to the variety of prevalence estimates (Grave *et al.*, 2010). Mirzaei *et al.* (2012) examined 100 raw milk samples and reported that 14 (14%) isolates carried the *mec*A gene. Therefore, the results of our study is showed similarity with the results of Mirzaei *et al.* (2012).

A few national and international studies have been reported on the prevalence of MRSA in cheeses. Arefi (2013) identified seven (14%) and 18 (36%) of 100 cheese samples (50 Feta cheese and 50 traditional white cheese) as MRSA and *S. aureus*, respectively. Eight of these 23 isolates (34.78%) were found to carry the *mec*A gene. Two of them were isolated from Feta cheese and six of them were isolated from traditional white cheese. Huber *et al.* (2010) reported that they could not detect MRSA in 200 cheese made from raw milk. Yücel and Anıl (2011) obtained 16 *S. aureus* isolates from 90 cheese samples. Also methicillin resistance was detected in nine of 79 (11.39%) coagulase positive isolates which were isolated from cheeses. Özpınar (2011) reported that

61 of the 100 Erzincan Tulum cheeses (61%) were *S. aureus* and 10 of them (16.30%) were genotypically MRSA by PCR. In Mirzaei *et al*.'s study (2011) MRSA was genotypically detected in two (4%) of 50 traditional cheese samples by PCR. Can and Çelik (2012) found *S. aureus* in 12 of the 200 unpackaged cheese samples (100 white cheese and 100 Tulum cheese). Two out of 12 *S. aureus* strains (5 from white cheese and seven from Tulum cheese) were found to be MRSA. MRSA positive samples were isolated from Tulum cheese. Kanaan and Al-Shammary (2013) found MRSA phenotypically in 6 (40%) of the 15 soft white raw cheese with whey samples. This study's results are similar to those of Arefi *et al.* (2014) and higher than other researchers' results (Huber *et al.,* 2010; Yücel and Anıl, 2011; Özpınar, 2011; Mirzaei *et al.,* 2011; Can and Çelik, 2012; Kanaan and Al-Shammary, 2013). These differences may be due to variations in cheese-production technology, the number of samples, and the fact that the milk used in production was not pasteurized. They may also be related to different level of hygiene standards performed during their production and of the production, storage and retail sales conditions.

Tulum cheese has a high dry matter and fat content, and production techniques vary from region to region. In the traditional manufacture of Turkish Tulum cheese, the cheese milk is not pasteurised. So, the presence of MRSA in tulum cheese samples is probably linked to the use of raw milk in their production, the use of animal skins as containers during ripening and filling them by hand or with a contaminated thick stick (Tekin en, 2000; Turkish Food Codex, 2015). Furthermore the production under unhygienic conditions, mostly in temporary dairies without the use of starter cultures is a serious contributing factor of contamination as well as the release in the market of cheeses before the completion of the ripening period (Çakır, 2011).

In this study MRSA strains were not detected in butter samples. In accordance with our findings, Mirzaei *et al.* (2011) did not detect the *mec*A gene in staphylocci isolated from 50 butter samples.

S. aureus isolates may show phenotypical resistance against cefoxitin and oxacillin, despite the absence of the *mec*A gene. This resistance may be regulated by newly described *mec*A homologue *mec*C gene or other factors such as overproduction of beta-lactamase and mutations that occur in the structure of PBP (Garcia-Alvarez *et al.,* 2011; Petersen *et al.,* 2013). Lee (2003) found 12 MRSA strains isolated from bovine milk samples that were resistant to oxacillin, penicillin, and ampicillin; six of the 12 MRSA isolates were resistant to cefoxitin; three were resistant to ciprofloxacin; eight to erythromycin; 11 to gentamicin; and five to tetracycline. None of the isolates were resistant to trimethoprim/sulfamethoxazole.

CONCLUSIONS

The prevalence of MRSA in raw milk and dairy products, both from pasteurized and unpasteurized milk represents a potential threat for public health. In order to control the presence of MRSA in milk and dairy products, milk must be obtained strictly from healthy animals. HACCP (Hazard Analysis and Critical Control Points), GMP (Good Manufacturing Practices) and GHP (Good Hygiene Practices) should be implemented for milk and dairy products throughout the whole production chain of dairy products from milking to retail sales. In addition, the use of raw milk in cheese production should be avoided; adequate pasteurization should be applied; and necessary precautions must be taken to prevent contamination after pasteurization. Regarding the treatment of infections in dairy animals, antibiotics should be used in a controlled and conscientious manner. Furthermore, the status of antibiotic resistance should be monitored regularly. Studies on the prevalence and antibiotic resistance of MRSA should be conducted regularly, especially in foods of animal origin and the environment food-production facilities.

DISCLOSURE STATEMENT

No competing financial interests exist.

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