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Original Research Article

MecA Gene Prevalence in *Staphylococcus aureus* Isolates from Dairy Cows in Turkey

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ABSTRACT

This cross-sectional study was performed between October 2010 and July 2012 from six different municipalities in Turkey to determine the prevelance of *mec*A gene in *Staphylococcus aureus* isolates from milk samples of mastitic cattle by PCR. Milk samples (n:1600) from 50 different Holstein Fresian herds were evaluated by California Mastitis Test and 480 clinical mastitis cases were diagnosed and taken for further investigation. Out of 480 samples, 151 have been identified to harbor *Staphylococcus aureus* by API-*Staph*® (Biomereux) identification panel and the results have been evaluated by API-*web* system. Kirby Bauer Disc Diffusion Test was used for determination of Cephoxitin® (Oxoid) susceptibility. By PCR, 24 isolates (15.89 %) were found to carry *mec*A gene and yielded an amplification product of 154 bp. This report represents that a significant number of MRSA was found among *Staphylococcus aureus* isolates in mastitis cases.

Key Words: *Staphylococcus aureus*, cattle, mastitis, *mec*A, MRSA

Türkiye'de Sütçü Sığırlardan Elde Edilen *Staphylococcus aureus* İzolatlarında *mec*A Geninin Prevelansı

ÖZET

Bu kesitsel çalışma, Ekim 2010-Haziran 2012 tarihleri arasında Türkiye'de 6 farklı belediyeden, PCR yöntemi ile mastitisli sığır sütlerinden elde edilen *Staphylococcus aureus* izolatlarındaki *mec*A gen prevelansını belirlemek amacı ile yapılmıştır. Elli farklı Holstein Fresian sürüsünden elde edilen süt örnekleri (n:1600) California Mastitis Testi ile değerlendirildi ve 480 klinik ve subklinik mastitisli örnek saptanarak ileri incelemeye alındı. 480 adet süt örneğinin 151 adedi API-*Staph*® (Biomereux) tanımlama paneli ile *Staphylococcus aures* olarak değerlendirildi ve sonuçlar API-*web* sistemi tarafından yorumlandı. Cephoxitin®(Oxoid) duyarlılığının tanımlanması için Kirby Bauer Disk Difüzyon Testi yapıldı. PCR ile 24 izolatın (%15.89) *mec*A geni taşıdığı ve 154 bp'lik amplifikasyon ürünü oluşturduğu saptandı. Bu rapor mastitis olgularındaki S*taphylococcus aureus* izolatlarında önemli oranda MRSA bulunduğunu göstermiştir.

Anahtar Kelimeler: *Staphylococcus aureus*, mastitis, *mec*A, MRSA, sığır

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the most epidemic nosocomial pathogen in humans (Barton *et al.*2006, Biedenbach *et al*, 2004)as well as the primary causative agent of mastitis in cattle (Quinn *et al.*2000**;** Songer and Post 2005; Moon *et al*., 2007; Vanderhaeghen *et al.,* 2010). *S.aureus* is considered as a significant pathogen with related virulence factors such as slime factor (biofilms), PVL (Panton Valentine Leucocidine) and some enzymes (proteases, lipases, and elastase), which enable it to destroy host tissues and metastase to other sites (Gordon and Lowry 2008).

Methicillin-resistant *S. aureus* (MRSA) includes *S.aureus* that have acquired a gene, called *mec*A, giving them resistance to methicillin and essentially to all other beta-lactam antibiotics. MRSA was first reported as a nosocomial pathogen in 1961, soon after methicillin was introduced into human medicine to treat penicillin-resistant staphylococci (Anonymus, 2011). Recently MRSA clones have particularly been detected in animal populations (Feβler *et al*. 2010, Lee, 2003). AlthoughMRSA is mostly associated with the acquiring *mec*A gene, the role of inappropriate antibiotics use should also not be under estimated in formation of bacterial resistance

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and multidrug resistant strains (Chambers, 1997). MRSA infection from mastitis cases is partly related with failure in dosage therapy and choice of antimicrobial substance (Juhász-Kaszanyitzky*et al*., 2007, Feßler *et al*., 2010).

Although MRSA has sporadically been isolated from animals since 1972 (Devriese *et al.*, 1972) emergence of animals has recently been more evident in animal populations of livestock dairy herds, swine farms, kennels, and in animals brought to veterinary hospitals (Seguin *et al*., 1999; Van Duijkeren *et al*., 2004; Farzana *et al*., 2004, Voss *et al*., 2005; Moon *et al*., 2007, Juhasz-Kaszanyitzky *et al*., 2007, Buyukcangaz *et al*, 2013). There are many other MRSA cases detected in companion animals (Walther *et al.*, 2008, Van der Eede *et al.*,2009) and raw chicken, turkey, pork, beef, veal, lamb meats (Lee, 2003, Van Loo *et al.*, 2007, De Boer *et al*., 2009).

Methods used for bacterial population analysis were presented by some reports (Cuny *et.al*. 2010, Enright *et al*., 2000). Such as a European-wide study of MRSA blood culture isolates concluded that MRSA CC398 accounted for less than 1% of all MRSA cases from humans (Grundmann *et al*., 2010, Köck *et al*., 2011).New variants of spa-type t011 of MRSA are reported as a serious problem in dairy farms, which require urgent attention (Spohr *et al*., 2011).

There are over 30 species of staphylococci including *S. aureus*,whichare the most pathogenic for both humans and animals, and can be differentiated from other Staphylococcal species (e.g. *S.pseudointermedius, S. hyicus, S. warnerii, S. saprophyticus, S.chromogenes*) by a positive coagulase reaction in the diagnostic laboratory (Quinn *et al*. 2000, Songer and Post, 2005). Commercial identification panels such as Vitec, Sensititre, BACTEC, API-*Staph* identification systems have been used to identify *Staphylococcus* to sub-species inboth human and animal isolates for many years (Brown *et al*. 2005). *Mec*A detection by PCR is accepted as the gold standard method for identifiying MRSA (Anonymous, 2011). There are also some FDA-approved commercial Polymerase Chain Reaction (PCR) assays for MRSA detection, namely, BD GeneOhm MRSA ACP (BD Diagnostics) ®, BD GeneOhmStaphSR (BD Diagnostics) ®, Xpert MRSA (Cepheid) ®, Xpert MRSA/SA SSTI (Cepheid) ®, Xpert MRSA/SA BC (Cepheid) ® and LightCycler MRSA (Roche) ® (Marlowe and Bankowski, 2011). Most FDA-approved PCR methods are essentially equivalent in performance, with sensitivities ranging from 82 to 100% and specificities ranging from 64 to 99% (Harbartha *et al*., 2011).

The purpose of this study was to investigate the presence of *mec*A gene in *S.aureus* isolates from mastitis cases in Holstein Freisean dairy cows.

MATERIALS AND METHODS

Sample processing

This study was performed between October 2010 and July 2012. Milk samples (n: 480) were taken from Holstein Fresian cattle (n: 1600) from 50 different herds in six different municipalities and their provinces of Turkey; i.eBursa (Nilufer, Karacabey, Yenisehir, Buyukorhan, Orhaneli), Edirne, Luleburgaz, Canakkale, Afyonkarahisar and Balikesir with a capacity of ≥100cattle. All the cities had high dairy cow densities, and are located in the northwest region of Turkey except Afyonkarahisar. In all farms, regular monitoring procedures for mastitis were implemented for lactating cows twice a day; such as pre and post milking teat disinfection, using an iodine teat dip (0.75 % active iodine), counting somatic cells from all lactating quarters by California Mastitis Test (CMT). CMT was scored between I-IV based on the characteristics of the thickening observed, III and IV (300.000 cells/ml of milk) was determined as subclinical mastitis. CMT positive samples were taken under sterile conditions for further bacteriological analysis.

Culture

An enrichment procedure was implemented according to De Boer *et al.*(2009) without second step enrichment procedure for the detection of *S.aureus*. For this purpose, sterile sample tubes containing milk were centrifuged at 3000 rpm (664 × g) for 5 min, and a sterile, ultrafine, cotton-tipped swab was dipped and rotated into the precipitate. Swabs were then placed into a Mueller Hinton Broth (Oxoid® CM0405) containing 6.5 %NaCl

(Merck-K37303004 -721) individually and incubated aerobically at 37°C for 24 h.A loopful of this culture was then streaked onto Baired Parker Agar (Oxoid® CM 961) containing 5 %Egg Yolk Tellurite Emulsion (Oxoid®-SR0054C) and incubated for 48 h in aerobic conditions. One grey-blackcoloured *Staphylococus* spp. suspect colony was streaked onto Colombia Agar (Biomerioux®-1001499590) to obtain pure isolate for use in identification. API-Staph® (Biomerieux, Lyon, France) commercial identification panel was used for classifying candidate *Staphylococcus* isolates according to the manufacturer's guideline. The results were evaluated by API-Web® system. Kirby Bauer Disk diffusion test for Cephoxitin (Oxoid ®-CT0 119B) was applied to all *S.aureus* isolates and the results were evaluated according to directives of Eucast 2012 (Anonymous, 2012).

In this study, commercially available (Microbiologics®) *S. aureus*ATCC 25923 and MRSA ATCC 33591, ATCC 43300 and ATCC 700699 were used in all steps as reference strains.

PCR

A PCR method described by DelVecchio *et al.*(1995) with some modifications was implemented to detect *mec*A gene from the MRSA isolates. Primer pairs, MRS1 (5'-TAGAAATGACTGAACGTCCG-3') and MRS2 (5'-TTGCGATCAATGTTACCGTAG-3') were used to amplify a 154 bp product. Pure *S.aureus* subcultures on TSA (Tryptic Soya Agar) were diluted in 50 µL DNase/RNase-free deionized sterile water (Lonza®, Accugene-BE51200). A commercial DNA isolation kit (GenJet Genomic DNA purification kit K0722, Fermentas, Thermo Scientific ®) was used for the extraction of DNA according to the manufacturer's instructions. Amplifications were carried out with 25 µl reaction volumes consisting of 2 µl of the sample DNA, 0,2 mM concentration of each of four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0,2 mM each of the primers MRS1 and MRS2, 0.5 U of *Taq* polymerase and 0,625 mM $MgCl_2$(Fast-Start taq DNA polymerase, dNTP pack 04738381001, Roche-Diagnostics®, Indianapolis, USA). Samples were amplified with an initial denaturation at 94°C for 5 min, followed by 35 cycles with each cycle consisting of 40 s of denaturation at 94°C, 40 s of annealing at 55°C, and 50 s of extension at 72°C in a gradient thermal cycler (Techne TC-3000TX-G®, Bibby Scientific, UK). Ten microliters of the PCR amplicon was loaded into a 2 % (wt/vol) agarose gel (SeaKem – Lonza®) in 1X TAE buffer using EzVision One loading dye (Amresco), and run concurrently. 100 bp DNA Ladder SM0241, 50 µg Thermo Scientific®) and the PCR product obtained from the positive control ATCC 43300 were included in each run. Bands were visualized in an UV-Tech Fire Reader **(**Progen Scientific®**,** Merton, London, SW19 3UU).

RESULTS

A total of 151(31.45%) *S.aureus* isolates were identified by API-Staph® detection kit out of 480 subclinical mastitis cases.Sixtytwo (41.05%) isolates were determined as resistant to Cephoxitin (30 µg) according to EUCAST Clinical Breakpoint Table Version 2.0, 2012 (Anonymous, 2012). Twenty four of the 151 isolates were found to harbor *mec*A gene. Figure 1 show distinct PCR product bands corresponding to respective molecular size stained with Ez-Vision Dye in agarose gel. The locations of mastitic dairy cow isolates, where *mec*A was detected from are shown in Table 1. Figure 2 demonstrates the distribution of *mec*A carrying *S. aureus* isolates and their locations in Turkey.

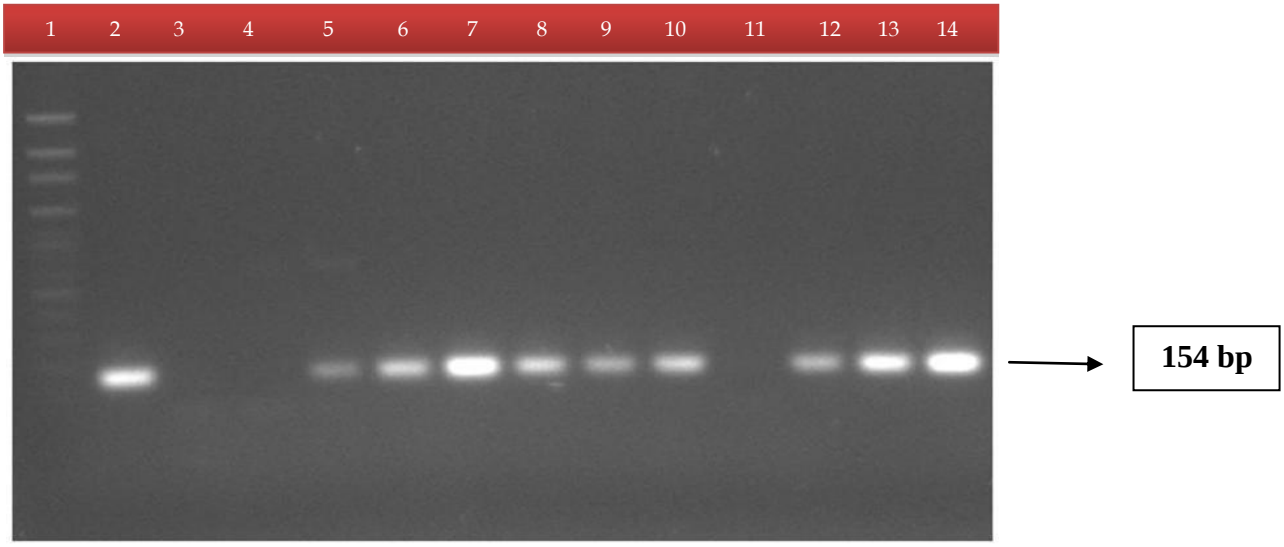

Figure 1: PCR results in agarose gel of reference *S.aureus* strains and some isolates in this study. 1) 1-100 bp Marker, 2) 2- *S.aureus* ATCC 33591, 3) 3-*S.aureus* ATCC 25922, 4) 4- *E.coli* ATCC 25922, 5) 5-10 *mec*A positive *S. aureus* isolate, 6) 11- *mec*A negative *S. aureus* isolate, 7) 12-14 *mec*A positive *S. aureus* isolate

Table 1. Distribution of *mec*A positive *Staphylococcus aureus* isolates based on geographical regions

Number	Isolate Number	Cephoxitin Susceptibility	*mec* A gene carriage	Region
1	**B10**	R	+	Bandirma-Balikesir
2	**73**	R	+	Karacabey-Bursa
3	**77**	R	+	Karacabey-Bursa
4	**91-A**	R	+	Karacabey-Bursa
5	**91-B**	R	+	Karacabey-Bursa
6	**118**	R	+	Afyon
7	**126**	I	+	Karacabey-Bursa
8	**149**	S	+	Canakkale
9	**165-B**	S	+	Canakkale
10	**168**	S	+	Canakkale
11	**185**	S	+	Yenisehir-Bursa
12	**187**	S	+	Yenisehir-Bursa
13	**199**	R	+	Yenisehir-Bursa
14	**207**	S	+	Yenisehir-Bursa
15	**209**	I	+	Yenisehir-Bursa
16	**215**	R	+	Yenisehir-Bursa
17	**216**	R	+	Yenisehir-Bursa
18	**217**	I	+	Mustafakemalpasa-Bursa
19	**218**	S	+	Mustafakemalpasa-Bursa
20	**368**	S	+	Afyon
21	**372**	R	+	Afyon
22	**466-G**	R	+	Karacabey-Bursa
23	**466-S**	R	+	Karacabey-Bursa
24	**480-K**	R	+	Karacabey-Bursa

S: Susceptible, I: Intermediate, R: Resistant

Figure 2. Map of *mec*A distribution of *Staphylococcus aureus* isolates derived from dairy cattle, NorthwesternTurkey.

DISCUSSION

Mastitis due to *S.aureus* infection in cattle is a problem worldwide. Recently, MRSA clones (*mec*A positive) from mastitis cases in dairy cows have been detected from different countries (Lee, 2003; Farzana *et al*, 2004; Moon, J.S. *et al.*,2007; Juhasz-Kaszanyitzky *et al.*, 2007). A study from Serbia (Zutic *et al*, 2012) indicated that 5 (5.9%) MRSA were found in 84 (39.6%) *S.aureus* isolates from 212 (20.7%) cows with subclinical mastitis. In another study (Vanderhaeghen *et al*., 2010), 11 (9.3 %) out of 118 *S.aureus* isolates were found to harbor *mec*A gene in isolates from Belgian cows. Spohr *et al*.(2011) detected 5.1–16.7 % MRSA in milk samples of dairy cows in the first level of investigation of three different farms, and the respective proportions in the second herd level investigation were 1.4–10.0 %.The presence of MRSA in cattle has recently been reported in Turkey (Turkyilmaz *et al*, 2010) and in our work. Also, Ucan and Aslan (2002) from Konya region determined MRSA prevalence as 1.33 % in milk samples from mastitic cattle while Kirecci and Colak (2002) reported that MRSA prevelance was 8.7% in milk samples from masitic cattle. In another report by Kaynarca and Turkyilmaz (2010) a total of 16 (10.6 %) MRSA was isolated, while Turkyilmaz *et al*. (2010) reported to detect %17.2 MRSA from masitic milk samples. Contrary to these, Tel *et al.* (2012) could not detect MRSA in bovine originated *S.aureus* strains in south eastern region of Turkey. None of the studies performed in Turkey mentioned above did not cover the northwestern region of Turkey and we could not compare our results that we found percentage of MRSA as 15.89, with them. Vast differences in MRSA prevalence rates reported in these studies could be primarily related to differences in regions, isolation and identification procedures including PCR and possibility in horizontal gene transmission between *Staphylococcus* species.The high percentage of *mec*Agene carriage in our isolates, on the other hand, may be the due to horizontal transmission (Chambers, 1997) of this gene between the strains found together in one sample or environment that we observed that most of the milk samples had more than one *Staphylococcus* species including Coagulase Negative *Staphylococcus* spp. (CoNs) and Coagulase Positive *Staphylococcus* spp. (CoPs).

Heteronenous and borderline resistance can be develope in some clinical isolates according to the culture conditions and β-lactam antibiotic use. Lee *et al*. (2003) claimed that some susceptibility tests such as Minimal Inhibitory Concentration (MIC), Kirby Bauer Disc Diffusion and Agar Dilusion can over estimate meticillin resistance in such a way that the isolates that do not carry *mec*A, can be identified as resistant to methicillin phenotipically. In this study 62 isolates were found to be resistant to cephoxitin by disc diffusion test, but essentially some of them were not carry *mec*A gene. Among the MRSA isolates, cephoxitin susceptibility

were detected as 13 (54.16 %), 3 (12.5 %) and 8 (33.33 %) of Resistant (R), Intermediate (I) and Susceptable (S), respectively.

In this study, single step enrichment procedure was carried out with Mueller Hinton broth containing 6.5 %NaCl (De Boer *et al*, 2009) without antimicrobial addition followed by plating step in Baired Parker with Egg Yolk PotassiumTellurite implemented for the detection of *S.aureus.* As far as we know, this single step enrichment method for the detection of *S.aureus* from milk samples was appliedfor the first time inour study. Some researchers (Brown *et al*, 2005, Sautter *et al.* 1998) suggested that enrichment of screening swabs is more sensitive than direct plating. Marlowe and Bankowski (2011) reported that broth enrichment prior to plating showed further increase in sensitivity of the selective culture. In this study *S.aureus* and MRSA isolation percentage (31.45 % and 5%, respectively) might be related with enrichment procedure implemented.

Several chemicals and antimicrobialssuch as mannitol salt, oxacillin, and cephoxitinwereoften combined (De Boer *et al*, 2009; Wertheim *et al*, 2001) for differantiating MRSA from Methicillin Susceptible *Staphylococcus aureus* (MSSA). In recent years cephalosporins have been reported to be particularly successful when they are used as an alternative to oxacillin. A phenyl red mannitol broth containing aztreonam and ceftizoxime (Wertheim *et al*, 2001) allowed the growth of all reference MRSA strains tested.In our study, no antimicrobial substance was usedduringthe isolation procedure. Second step enrichment with adding antimicrobials and chemicals might increase and/or decrease the isolation rates of *S.aureus* and MRSA. Previous studies indicate that there is a need for further optimization prior to standardization for MRSA-screening culture methods (Brown *et al*, 2005).

Molecular methods have high sensitivity and rapid turnaround time when comparing other phenotyping methods. PCR assays may also improve clinical outcomes by decreasing the time to identification of coagulase-negative *Staphylococcus* species (CoNS), MSSA, and MRSA and by allowing for earlier, more effective antimicrobial therapy. It was approved that PCR should be considered the 'Gold Standard' for meticillin resistance (Suzuki *et al*., 1992; Tokue*et al*., 1992; Chambers, 1997, Anonymous, 2011). Because of this, in this study, the *mec*A prevalence was detected by a PCR method described by DelVecchio (1995) with some modifications.

European Food Safety Authority (EFSA) (Anonymous, 2009) recommends that further work should be performed on harmonising methods for sampling, detection and quantification of MRSA during carriage in both humans and animals. Epidemiological findings related with MRSA screening studies in animals are very limited worldwide and especially in Turkey. This study represented a considerably high MRSA prevalence in dairy cattle in Turkey. The information concerning the prevelance of MRSA strains were obtained by PCR-based methods proved to be usefull tools to monitor the circulation of these strains in these animal husbandry settings.Additionalstudies needed for control of MRSA prevalence and detection of antimicrobial resistance in MSSA, MRSA and CoNs in lactating cows and animal populations in Turkey. A detailed surveillance should be implementedby the Ministry of Agriculture of Turkeyto monitor the MRSA together with other multiple antibiotic-resistant bacteria especially of major zoonotic importance such as *Salmonella* and *Campylobacter jejuni*.

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