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 prevalence of mecA gene in Staphylococcus aureus isolates from milk samples of mastitic cattle by PCR. Milk samples (n=1600) from 50 different Holstein Friesian 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® (Biomerieux) identification panel and the results have been evaluated by API-web system. Kirby Bauer Disc Diffusion Test was used for determination of Cephalosporin® (Oxoid) susceptibility. By PCR, 24 isolates (15.89 %) were found to carry mecA 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, mecA, MRSA

Türkiye’de Sütçü Sığırlardan Elde Edilen Staphylococcus aureus İzolatlarında mecA Geninin Prevelansı

ÖZET

Anahtar Kelimeler: Staphylococcus aureus, mastitis, mecA, MRSA, süt

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; Vanderhaegen 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 metastasize to other sites (Gordon and Lowry 2008).

Methicillin-resistant S. aureus (MRSA) includes S.aureus that have acquired a gene, called mecA, 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 (Anonymous, 2011). Recently MRSA clones have particularly been detected in animal populations (Feßler et al. 2010, Lee, 2003). Although MRSA is mostly associated with the acquiring mecA gene, the role of inappropriate antibiotics use should also not be underestimated 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 (Juhasz-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 (Spoth et al., 2011).

There are over 30 species of staphylococci including S. aureus, which are the most pathogenic for both humans and animals, and can be differentiated from other Staphylococcus 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 in both human and animal isolates for many years (Brown et al. 2005). McfA detection by PCR is accepted as the gold standard method for identifying 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 GeneOhm StaphSR (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 mecA gene in S. aureus isolates from mastitis cases in Holstein Freisian 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 Freisian cattle (n: 1600) from 50 different herds in six different municipalities and their provinces of Turkey; i.e Bursa (Nilufer, Karacabey, Yenisehir, Buyukorhan, Orhaneli), Edirne, Luleburgaz, Canakkale, Afyonkarahisar and Balikesir with a capacity of ≥100 cattle. 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 1-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 Baird Parker Agar (Oxoid® CM 961) containing 5 %Egg Yolk Tellurite Emulsion (Oxoid®-SR0054C) and incubated for 48 h in aerobic conditions. One grey-blackcoloured Staphylococcus spp. suspect colony was streaked onto Columbia Agar (Biomerieux®-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 Staphylococcus aureus isolates and the results were evaluated according to directives of Eucastr 2012 (Anonymous, 2012).

In this study, commercially available (Microbiologics®) S. aureusATCC 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 meca 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 MgCl₂(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 meca 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 meca was detected from are shown in Table 1. Figure 2 demonstrates the distribution of meca carrying S. aureus isolates and their locations in Turkey.
Figure 1: PCR results in agarose gel of reference \textit{S.aureus} strains and some isolates in this study. 1) 1-100 bp Marker, 2) 2- \textit{S.aureus} ATCC 33591, 3) 3-\textit{S.aureus} ATCC 25922, 4) 4- \textit{E.coli} ATCC 25922, 5) 5-10 \textit{mecA} positive \textit{S. aureus} isolate, 6) 11- \textit{mecA} negative \textit{S. aureus} isolate, 7) 12-14 \textit{mecA} positive \textit{S. aureus} isolate

| Table 1. Distribution of \textit{mecA} positive \textit{Staphylococcus aureus} isolates based on geographical regions |
|---|---|---|---|
| Number | Isolate Number | Cephalixin Susceptibility | meca 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 | S | + | 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
Mastitis due to *S. aureus* infection in cattle is a problem worldwide. Recently, MRSA clones (*mecA* 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 *mecA* 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 prevalence was 8.7% in milk samples from mastitic 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 mastic 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 *mecA* gene 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).

Heterogenous 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 *mecA*, can be identified as resistant to meticilllin 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 *mecA* 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 Susceptible (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 Bairded 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 applied for the first time in our 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 antimicrobials such as mannitol salt, oxacillin, and cephoxitin were often combined (De Boer et al, 2009; Wertheim et al, 2001) for differentiating 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 cefoxitoxime (Wertheim et al, 2001) allowed the growth of all reference MRSA strains tested. In our study, no antimicrobial substance was used during the 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 mecA 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 prevalence of MRSA strains were obtained by PCR-based methods proved to be useful tools to monitor the circulation of these strains in these animal husbandry settings. Additional studies 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 implemented by the Ministry of Agriculture of Turkey to 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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