Staphylococcus aureus Genotypes of Subclinical Bovine Mastitis Milk in the Middle Western Anatolia

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Abstract: *Background*: *Staphylococcus aureus* is the most common etiological pathogen of bovine mastitis. Subclinical mastitis is characterised by a non-alteration of the milk but can cause food poisoning by production of enterotoxins in milk. Knowledge about the genetic variability within different *S. aureus* populations would help in the design of efficient treatments to prevent subclinical mastitis and provide useful data for epidemiological studies. The aim of this study was to characterize the genetic nature of the *S. aureus* cultured from subclinical bovine mastitis occurring in 16 farms in the middle western Anatolia.

Methods: Two hundred sixty eight milk samples positive with California Mastitis Test (CMT) suggesting the subclinical mastitis of lactating cows in 16 different farms in the Middle Western Anatolia were collected and *S. aureus* were isolated. Identification was carried out by traditional tests and ribotyping confirmed the identification. Staphylococcal Enterotoxins (SE) were detected and typed by Staphylococcal Enterotoxin Test Reversed Passive Latex Agglutination (SET-RPLA) test kit. Genetic characterisation of the isolates was carried out by both ribotyping and pulsed field gel electrophoresis (PFGE).

Results: A total of 77 isolates of *S. aureus* were purified and analysed by both biochemical identification and genotyping. Only 4 isolates (5.19 %) of *S. aureus* were recorded as enterotoxin positive. Genetic characterisation of the isolates was carried out by ribotyping revealed eight ribotypes while pulsed field gel electrophoresis (PFGE) was more discriminative representing 19 pulsotypes.

Conclusion: This study shows no significant association between enterotoxin production, ribogroup and pulsotype profile of the *S. aureus* isolates collected from the Middle Western Anatolia.

Keywords: Bovine subclinical mastitis, *Staphylococcus aureus*, enterotoxin, genotype.

BACKGROUND

Mastitis in dairy cows is a worldwide disease and can be caused by infections with bacteria, yeast and fungi [1-4]. However, the most causative microorganisms are bacteria with intramammary infection and there are different bacterial species responsible depending on the geographical location and management. *Staphylococcus aureus* is a major bacterial pathogen in dairy cattle causing clinical and subclinical mastitis [5-7] and its prevalence ranges from 5 to 50% in different countries. *S. aureus* strains can cause acute clinical, and long lasting subclinical mastitis. No alteration is observed in the milk with subclinical mastitis but high somatic cell count is obtained. If the cell count is too high, the milk is inappropriate for the consumers. Halasa *et al*. [8] reported that this type of mastitis is often chronic and account for up to 30% of all bovine cases which represents an important economic problem for dairy producers with reduction in milk quantity and quality, prolonged costly antibiotic treatments and premature culling. Philpot *et al*. [9] reported that the reduction in milk production attributed to sub-clinical mastitis may account for 70%–80% of the total losses .

Some *S. aureus* strains have the ability of producing heat stable enterotoxins that cause staphylococcal food poisoning (SFP) [10]. SFP symptoms eg. sickness, abdominal cramps, diarrhoeae and a characteristic projectile emesis [11] appear within a few hours (i.e., 1–6 h) after ingestion of contaminated food, depending on individual susceptibility and toxic dose ingested. The knowledge about the genetic variability within different *S. aureus* populations would help in the design of efficient treatments to prevent subclinical mastitis and provide useful data for epidemiological studies.

The occurrence of mastitis in Turkey has been investigated in many studies and although there are some higher percentages in different regions, mastitis prevalence was reported as 30 % in Turkey [12, 13]. Many studies focusing on the phenotypic and genotyping characterization of *S. aureus* isolated from subclinical bovine mastitis in different regions of Turkey were carried out by coagulase gene polymorphisms

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ISSN: 1927-5196 / E-ISSN: 1927-520X/18

detected by PCR [14], determination of classical enterotoxigenic characteristics [15], detection of superantigenic toxin genes [16]), comparing the antibiotic resistance profiles. Ünal and İstanbulluoğlu [17] revealed clonal relations of only a small number of *S. areus* by using Pulsed field gel electrophoresis (PFGE) and a clone of *the bacterium* was broadly detected in dairy farms in Kırıkkale province. However, there is no study by comparison of different methods about the characterization of strains of *S. aureus* related to subclinical mastitis occurring in farms in the Middle Western Anatolia.

Therefore, the aim of the present study was to detect staphylococcal enterotoxins and determine the genetic profiles of *S. aureus* strains isolated from milk of cows suffering from subclinical mastitis in the Middle Western Anatolia by using PFGE and automated ribotyping. To our knowledge, ribotyping is the first study revealing riboprofiles of subclinical mastitis causing *S. aureus* in Turkey.

METHODS

Isolates

Two hundred sixty eight milk samples positive with California Mastitis Test (CMT) indicating the subclinical mastitis of lactating cows in 16 different farms in the Middle Western Anatolia were collected for microbial evaluation in 2010-2011. Each milk sample was aseptically collected into sterile bottles just before milking and they were transported in a cool box at 4ºC to the laboratory. The CMT and bacteriological

Table 1: Characterisation of *S. aureus* Isolates

Isolate No.	Farm	SET-RPLA	Ribo-group	Pulso-type	Isolate No.	Farm	SET-RPLA	Ribo-group	Pulso-type	Isolate No.	Farm	SET-RPLA	Ribo-group	Pulso-type
6	E1	-	1	G1	120	C1	-	3	G11	224	A3	-	7	G19
9	E1	-	1	G19	121	C1	-	7	G11	225	D1	-	3	G11
11	E1	-	2	G1	122	C1	C	5	G12	228	D1	-	4	G11
15	E1	-	3	G2	128	C2	-	7	G11	231	D1	-	7	G11
18	E1	-	2	G3	131	C2	B	3	G13	232	D1	-	5	G11
22	E1	-	1	G1	132	C2	-	7	G11	233	D1	-	3	G11
27	E1	-	5	G4	138	C2	-	8	G11	234	D1	-	5	G11
44	E1	-	5	G15	142	A1	-	7	G11	240	D1	-	2	G11
49	E1	-	7	G7	143	A1	-	2	G11	243	D1	-	7	G11
50	E1	-	1	G5	144	A1	-	6	G11	244	D1	-	5	G11
54	E2	-	7	G5	146	A1	-	8	G14	249	D2	-	1	G19
55	E2	-	3	G5	156	A1	-	3	G11	253	D2	-	7	G11
56	E2	-	4	G6	163	A1	-	2	G16	262	D2	-	1	G19
58	E2	-	3	G11	169	A1	-	1	G17	264	D3	-	1	G19
60	E2	-	4	G19	181	A1	-	1	G19	272	D3	-	7	G11
84	E2	-	7	G7	185	A1	-	3	G11	273	D3	-	1	G19
85	E3	-	5	G11	190	A2	-	3	G11	274	D3	-	7	G11
92	B1	-	7	G11	191	A2	-	3	G11	276	S1	-	1	G19
94	B1	-	7	G11	193	A2	-	3	G11	279	S1	-	3	G19
96	B1	-	3	G11	194	A2	-	3	G11	283	S1	-	3	G19
97	B1	-	2	G11	195	A3	-	3	G11	287	S2	-	7	G11
98	B2	-	3	G19	205	A3	-	1	G19	288	S2	-	1	G19
101	B3	B	8	G8	208	A3	-	2	G18	289	S2	-	1	G19
102	B3	-	6	G9	210	A3	-	1	G19	292	S2	-	1	G19
115	C1	B	4	G10	211	A3	-	2	G11	299	S2	-	3	G19
118	C1	-	7	G11	220	A3	-	3	G11					

analyses were started within 24 h after sampling. For isolation of *S. aureus*, 10 ml of milk sample were centrifuged at 6000 rpm for ten minutes and after discarding the supernatant, the sediment were inoculated on Mannitol Salt Agar (MSA) with incubation at 37°C for 24 to 48 h for primary isolation. Suspected colonies were selected and subcultured on blood agar medium for obtaining pure culture. *S. aureus* was identified at the species level using standard biochemical methods including Gram stain, catalase, tube coagulase, oxidase, DNase activities, Growth on Baird Parker medium and fermentation of mannitol [18] and API Staph test (BioMerieux, France). Isolates identified as *S. aureus* by these classical tests were then evaluated for enterotoxin production and genotyping by both automated ribotyping and PFGE (Table **1**).

Detection of Staphylococcal Enterotoxins (SE)

Strains were grown in 10 mL of tryptone soya broth (CM0219B, Oxoid) for 16–18 h at 37 ˚C by shaking aerobically. After centrifugation, the supernatant was tested for the presence and typing of SEs by Staphylococcal Enterotoxin Test Reversed Passive Latex Agglutination (SET-RPLA) (TD900, Oxoid).

Ribotyping

Strains identified as *S. aureus* with conventional biochemical tests were then characterized by automated ribotyping using *Eco*RI in a robotized instrument (Riboprinter™ Microbial Characterization System, Qualicon, DuPont, Wilmington, DE) and the Riboprinter ™ System Data Analysis Program. The procedure used for processing each sample is described in detail by the manufacturer. The identification of each strain was obtained when the corresponding pattern matched one of the pattern of the DuPont Identification library of the Riboprinter® with a similarity %0.86. The characterisation consisted of combining profiles within a similarity range equal or larger than 0.93 to form a dynamic ribogroup that reflected the genetic relatedness of the strains [19]. Each ribogroup was numbered by the system used in the study. A similarity dendogram was generated based on the banding pattern similarities by using SPSS version 21.0.

Pulsed Field Gel Electrophoresis (PFGE) Analyses

The genetic relationships of *S. aureus* isolates collected from cows with subclinical mastitis were compared by the DNA macrorestriction patterns obtained from PFGE following *Sma* I digestion. PFGE typing of strains was performed according to the methods of Vanderlinde [20] and Hennekinne *et al.* [21] with some modifications. The electrophoresis was carried by using a Chef Mapper (Bio-Rad Laboratories, Hercules, CA) and pulse times were ramped from 5 s to 40 s for 19 h. Gels were stained with ethidium bromide, visualized using an ultraviolet transilluminator, and photographed. Strains of *S. aureus* were placed in groups of identical or related strains by comparing the banding patterns produced, using a combination of photographic visiual inspection and computer analysis (SPSS version 21.0, SPSS Inc., Chicago, IL) was carried out to create a similarity dendogram. A pulsotype (PT) was defined as a unique electrophoretic banding pattern. Strains with identical restriction profiles were assigned as the same type.

RESULTS

Isolation and Identification of *S. aureus*

A total of 185 isolates were collected from 463 mastitis positive milk samples and identification was carried out by both conventional biochemical tests and ribotyping. However, only 77 isolates collected from 8 out of 16 different farms were identifed as *S. aureus* by both conventional biochemical tests and ribotyping (Table **1**).

Detection of Staphylococcal Enterotoxins

Using the SET-RPLA test, 73 out of the 77 isolates of the *S. aureus* (94.8%) showed no enterotoxin activity, only 4 isolates (5.19 %) isolated from mastitis positive milk samples were recorded as positive. Three isolates (numbered 101, 115 and 131) synthesized Staphyloccal enterotoxin B however, only one isolate (122) synthesized Staphyloccal enterotoxin C (Table **1**). No correlation was obtained between enterotoxin profiles and pulsotypes of the isolates (Table **1**).

Ribotyping

In this study, automated riboprinting was applied to the 77 isolates of *S. aureus* for both confirmation of the classical identification and ribotyping. All the isolates tested were typeable. Restriction of the total DNA with *Eco*RI yielded about 14 fragments of 1–50 kb in size. A total of eight banding patterns were obtained among the strains (Figure **1**). The most prevalent ribotype was Ribotype 5 represented by 20 isolates (Table **1**).

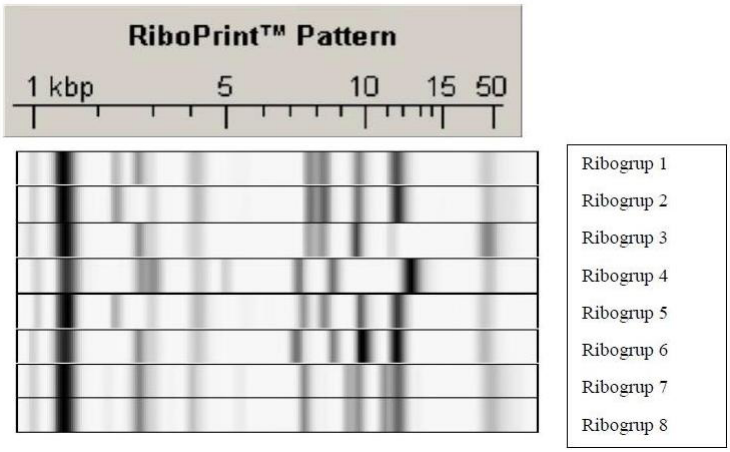

Figure 1: Ribotypes from *S. aureus* isolated from different subclinical mastitis milk samples (ribogroup numbers were given by automatic ribotyping system).

A dendogram based on the similarity was obtained. Ribogroups 7 and 8 showed 98% similarity while all the ribogroups were clustered in a group with a 95% similarity (Figure **2**). Automated ribotyping was applied for the first time for evaluation of *S. aureus* isolates collected from subclinical mastitis positive milk samples in Turkey. All the farms contained different ribogroups of *S. aureus* except farms E3, B2 and A2. Farms D2 and D3 contained same ribogrous while farms E3, B2 and A2 contained only one ribogroup of *S. aureus* (Table **1**).

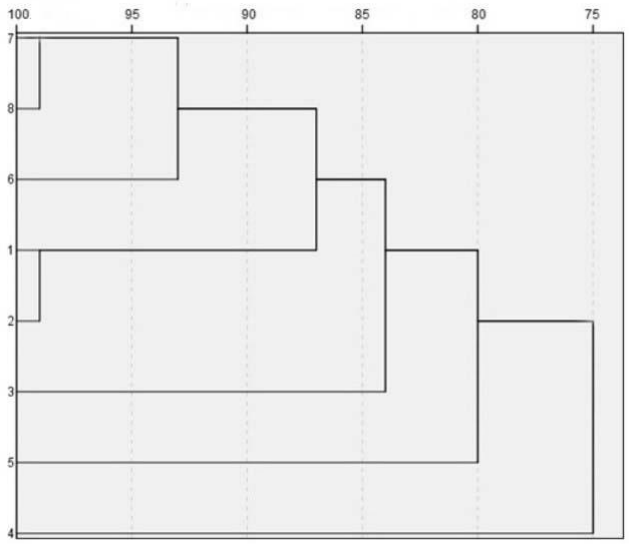

Figure 2: Dendogram constructed on the basis of ribotype patterns of *S. aureus* obtained by *Eco* RI enzyme.

PFGE Analyses

All of the isolates tested were typeable with Pulsed Field Gel Electrophoresis (PFGE). The genetic analyses revealed 19 different PTs varying from 17 to 23 distinct bands in the range from 485 kb to 48.5 kb indicating high genetic diversity among the samples (Figure **3**). Pulsotype G11 contained 38 isolates (49,3%), pulsotype G19 contained 17 isolates (22%), pulsotype G1 contained 3 isolates (3,8%), pulsotype G5 contained 3 isolates (3,8%), pulsotype G7 contained 2 isolates (2,59%). Other pulsotypes were represented by only one isolate. 77 isolates collected from 16 different farms were analyzed with PFGE and farms E3, B1, B2, A2 and D1 contained only one pulsotype either G11 or G19 indicating that the source of mastitis was same strain of *S. aureus*. However other farms were represented by different pulsotypes as indication of different strains of *S. aureus* caused mastitis (Table **2**).

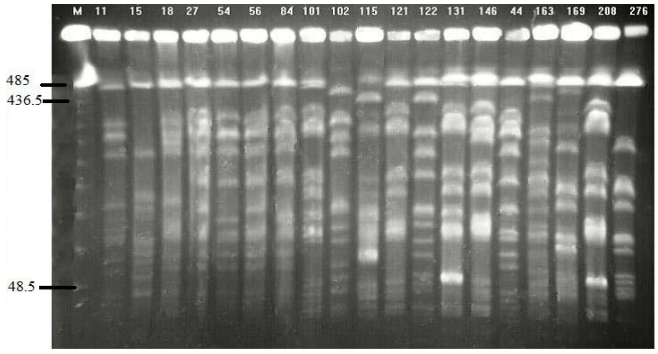

Figure 3: Restriction endonuclease digestion of total genomic DNA of strains representing some of the groups PFGE profiles of *staphylococcus aureus* after restriction digestion with *SmaI* and separation by PFGE. Lanes show some of the pulsotypes represented in Table **1** and M, 48.5–1,000-kB concatamer ladder (Bio-Rad). The PFGE conditions were 1% (w/v) agarose gel in 0.5X TBE, switching pulses of 5 to 40 s at a period of 19 h 6 V/cm.

A dendogram that included all patterns was constructed on the basis of the similarity levels. A high

Table 2: Spread of Pulsotypes in the Farms

Farm	Pulsotype	No. isolates	Farm	Pulsotype	No. isolates
E1	G1,G2,G3,G4,G5,G7,G15,G19	10	A1	G11,G14,G16,G17,G19	9
E2	G5,G6,G7,G11,G19	6	A2	G11	4
E3	G11	1	A3	G11,G18,G19	7
B1	G11	4	D1	G11	9
B2	G19	1	D2	G11,G19	3
B3	G8, G9	2	D3	G11,G19	4
C1	G10,G11,G12	5	S1	G11,G19	3
C2	G11,G13	4	S2	G11,G19	5

degree of similarity was observed between the pulsotypes 4 -7 and 5-6. Secondry level of high degree of similarity was observed between pulsotypes 14-18 and 8-11 (Figure **4**).

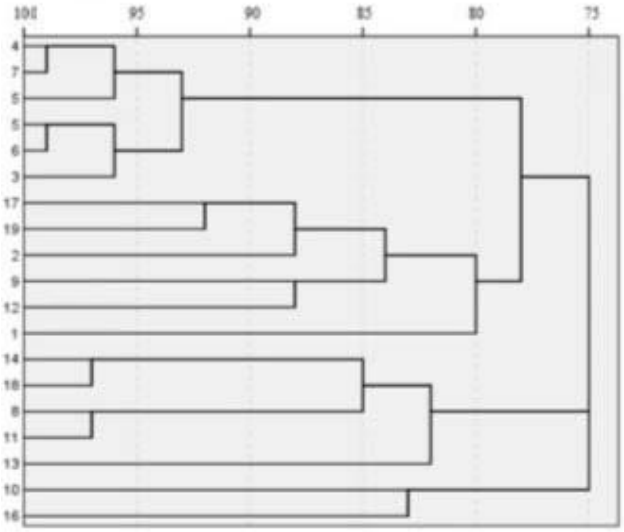

Figure 4: Dendogram constructed on the basis of 19 pulsotype patterns of *S. aureus* strains established with *Sma* I PFGE analysis. Pulsotype numbers are given in Table **1**.

DISCUSSION

Subclinical mastitis causes elevated somatic cell counts (SCC) but no detectable changes in milk or the udder. The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and are contagious. Economic damage due to subclinical mastitis has been mainly attributed to the fact that a subclinical cow is a constant source of infection to otber cows and to milk production loss as reported by Swinkels *et al*. [22]. In this study, only 77 isolates out of 185 were identified as *S. aureus* as a causative organism of subclinical mastitis suggesting that other bacteria were responsible as indicated in Hegde *et al*. [23]. *S. aureus* can access to milk by direct excretion from udders during milking [24] and multiply in milk and produce enterotoxins that cause food poisoning if ingested [25]. Staphylococcal enterotoxins have a remarkable ability to resist heat and denaturation by cooking therefore, even after pasteurization biological activity of toxin remains and can cause food poisoning [26]. A relatively high percentage of classical enterotoxin forming *S. aureus* strains from bovine subclinical mastitis was recorded in many publications [27-29].

Boynukara *et al*., [15] first investigated classical enterotoxigenic properties of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis in eastern part of Turkey with 106 *S. aureus* strains. Twenty seven isolates (25.5%) were found to be enterotoxigenic by reverse passive latex agglutination (RPLA). Of these, 25 (23.6%) were positive for staphylococcal enterotoxin A (SEA), 2 (1.9%) for staphylococcal enterotoxin B. Their study showed that most *S. aureus* strains isolated from bovine subclinical mastitis produced SEA compared to other SEs. However in this study, the prevelance of SE strains were very low (5.19%) being the SEB (3.89%) and SEC (1.29%) among 77 isolates of *S. aureus*.

The present study involved 77 isolates recovered from 16 commercial dairy farmss located in the middle western Anatolia, with different *S. aureus* ribotype and pulsotype prevalence. Although the number of isolates is not too large, it allowed us to develop a small-scale project aimed to assess the relationship between *S. aureus* ribotype and pulsotype pattern and the prevalence of them in dairy farms. Moreover, this study represents, in our knowledge, the first application of ribotyping technique to evaluate the role of *S. aureus* virulence factor patterns in subclinical mastitis prevalence in dairy farms in Turkey.

CONLUSION

The strains producing SEs indicates very low potential of poisoning in milk samples collected from subclinical mastitis infected cows in the Middle Western Anatolia. Also, no significant association between enterotoxin production, ribogroup and pulsotype was observed in the current study. Future studies involving biofilm production ability of the isolates as carried out by Khoramrooz *et al*. [30] may explain a possible correlation between enterotoxin genes with biofilm formation and genotypes.

ACKNOWLEDGEMENTS

We gratefully thank Erdoğan Çakır for his technical assistance in ribotyping. The article being published represent research outcome that was funded by Anadolu University Research Foundation for financial support through Project number 1109F149.

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Received on 15-03-2018 Accepted on 27-03-2018 Published on 13-04-2018

DOI: https://doi.org/10.6000/1927-520X.2018.07.01.1