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Characterisation of coagulase positive Staphylococcus species isolated from bovine mastitis using protein and plasmid patterns

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Abstract: The objective of this work was to study the protein patterns, plasmid profiles, and antibiotic susceptibility of *Staphylococcus intermedius* and *Staphylococcus aureus* isolates originating from mastitic mammary glands of dairy cattle in different parts of Konya province. A total of 114 Staphylococcus species were isolated and identified by conventional bacteriological methods from bovine mastitis. Of the total isolates 77 were identified as *S. aureus* and 37 as *S. intermedius*. Intra- and inter-species diversities in the coagulase-positive staphylococci were investigated by analysis of whole-cell protein profiles using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Plasmid profiling also demonstrated that 75 *S. aureus* isolates and 36 *S. intermedius* isolates contained plasmid. In addition, 88.3% of *S. aureus* and 59.4% of *S. intermedius* isolates were resistant to penicillin. Sixty-six of the 77 *S. aureus* isolates were also resistant to amoxicillin+clavulanic acid (85.7%). The corresponding number for *S. intermedius* was 17 (45.9%). Only 1 *S. aureus* isolate was resistant to danofloxacin. One of each of the Staphylococcus isolates was resistant to methicillin. Results from the study showed that the susceptibility of *S. intermedius* isolates to antibiotics used widely in mastitis therapy is a matter of concern.

Key words: Mastitis, plasmid, protein, *S. intermedius*, *S. aureus*

Mastitisli ineklerden izole edilen koagülaz pozitif Stafilokok türlerinin protein ve plazmid profilleri ile karakterizasyonu

Özet: Bu çalışmada Konya yöresinin farklı bölgelerindeki ineklerin meme bezlerinde bulunan *Staphylococcus intermedius* ve *Staphylococcus aureus* suşlarının protein patternleri, plasmid profilleri ve antibiyotik dirençliliklerini çalışmak amaçlanmıştır. Mastitisli ineklerden toplam 114 *Staphylococcus* suşu geleneksel bakteriyolojik yöntemlerle izole edilip tanımlanmıştır. Toplam izolatın 77 adedi *S. aureus*, 37 adedi *S. intermedius* olarak tespit edilmiştir. Koagülaz pozitif stafilokokların tür içi ve türler arası genetik çeşitliliği, toplam hücre protein profillerinin sodyum dodesil sülfat poliakrilamid jel elektroforezi analizi (SDS-PAGE) kullanılarak belirlenmiştir. Plazmit analizinde ise, 75 adet *S. aureus* suşunun ve 36 adet *S. intermedius* suşunun plazmit taşıdıkları gösterilmiştir. Buna ek olarak *S. aureus* izolatlarının % 88,3'ü, *S. intermedius* izolatlarının ise % 59,4'ü penisiline dirençli bulunmuştur. 77 adet *S. aureus* izolatının 66 (% 85,7)'sı da amoksisilin klavulanik aside dirençli bulunmuştur. *S. intermedius* için ise bu sayı 17 (% 45,9) olarak bulunmuştur. Yalnızca bir adet *S. aureus* suşunun danofloksasine dirençli olduğu belirlenmiştir. Her bir stafilokok izolatı metisiline dirençli bulunmuştur. Çalışma sonuçları, *S. intermedius* suşlarının mastitis tedavisinde yaygın olarak kullanılan antibiyotiklere olan duyarlılığının endişe verici olduğunu göstermiştir.

Anahtar sözcükler: Mastitis, plazmit, protein, *S. intermedius*, *S. aureus*

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Introduction

Mastitis is one of the major causes of financial losses for dairy cattle farmers (1). Staphylococci are the most prevalent pathogens causing mastitis in ruminants (2). Staphylococcal species associated with bovine mastitis have been classified as coagulase-positive or coagulase-negative. Coagulase-positive *Staphylococcus aureus* is considered a major cause of bovine mastitis (3). Bovine mastitis due to *S. aureus* is generally chronic, not easily cured by antibiotic treatment (4), and it represents a serious economic problem for milk producers and dairy industries (5). In addition, the presence of *S. aureus* in milk could also represent a serious public health problem because some strains have the ability to produce a variety of toxins (6). Rapid and accurate identification of the disease-causing agent is therefore a prerequisite for disease control and epidemiological surveillance (7). In recent years, many different techniques have become available for studying *S. aureus* strains, such as antibiogram analysis, bacteriophage typing, plasmid analysis, and protein electrophoresis. However, limited data are available on the whole cell protein characteristics of Staphylococcus. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), usually combined with a dendrogram derived from the numerical analysis of the whole-cell protein patterns of the strains, has been used extensively to study the differences among different bacterial genera, species, and strains (8). The genotypic methods include plasmid analysis, random amplified polymorphic DNA (RAPD), and ribotyping, which have been used for typing of bacterial strains with high discriminatory potential and good reproducibility (9). *S. aureus* and *S. intermedius* are species of Staphylococcus associated with food intoxication outbreaks that present very similar phenotypic characteristics, which makes their identification and differentiation through traditional culture techniques difficult (10). Despite methods based on the polymerase chain reaction (PCR) to select pathogens among other bacterial species being recommended, very limited research using molecular methods to differentiate *S. aureus* and *S. intermedius* has been reported (10).

The present study evaluated protein patterns, plasmid profiles, and antibiotic susceptibility of *S. intermedius* and *S. aureus* isolates originating from the mammary glands of dairy cattle in different parts of Konya province, Turkey, for the identification of *S. aureus* and *S. intermedius* and to contribute to the understanding of *S. aureus* epidemiology.

Materials and methods

Bacterial isolates

A total of 300 samples of bovine mammary secretions were collected from cattle with subclinical mastitis in 8 herds from different regions of Konya province during 2000 and 2001. Prior to sampling, the California Mastitis Test (CMT) was carried out. Isolation and identification of the strains were performed as described elsewhere (11).

Total protein analysis

The total protein samples were extracted as described by Kishore et al. (12). Total protein analysis was carried out by the method described by Laemmli (13). The gels were stained overnight with Coomassie Brilliant Blue G-250 according to Demiralp et al. (14). Different fragments on the gel were numbered sequentially and the presence and absence of fragments in each sample were scored (present 1, absent 0) and compared with each other using Sintax statistical software.

Plasmid DNA analysis

Plasmid DNA was prepared according to the method described by Anderson and McKay (15). DNA was electrophoresed in 0.8% agarose gel (16).

Antibiotic susceptibility test

The susceptibility test was performed using the disc diffusion technique on Mueller-Hinton Agar (Difco) as described by Bauer et al. (17).

Results

A total of 114 isolates were identified as Staphylococcus. Out of 114 *Stapylococci*, 77 were identified as *S. aureus* and 37 as *S. intermedius* by conventional methods. Plasmids were detected in 75 (97.4%) *S. aureus* isolates and 36 (97.2%) *S. intermedius* isolates. Table 1 summarises the plasmid profiles of *S. aureus* and *S. intermedius* from cows with

Table 1. Antibiotic resistance rates of the *S. aureus* and *S. intermedius* isolates based on plasmid content.

Species	Resistant against	No plasmid (%)	1 Plasmid (%)	> 1 plasmid (%)
S. aureus (77)		2 (2.6)	59 (76.6)	16 (20.8)
	No antibiotic*	2 (2.6)	2 (2.6)	1 (1.3)
	1 antibiotic	0 (0)	5 (6.5)	3 (3.9)
	2 antibiotic	0 (0)	38 (49.3)	9 (11.7)
	> 2 antibiotic	0 (0)	14 (18.2)	3 (3.9)
S. intermedius (37)		1 (2.7)	24 (64.8)	12 (32.4)
	No antibiotic*	0 (0)	11 (29.7)	4 (10.8)
	1 antibiotic	0 (0)	1 (2.7)	1 (2.7)
	2 antibiotic	1 (2.7)	9 (24.3)	2 (5.4)
	> 2 antibiotic	0 (0)	3 (8.1)	5 (13.5)

* Staphylococcus strains are sensitive to all of the antibiotics

subclinically infected udders. Molecular weight of plasmids varied from 20 kb to 1.1 kb. Twenty-six different plasmid profiles for each isolate were identified. Most of the isolates showed only 1 plasmid band with a size of 19 kb (69.8%) while the rest of the isolates had 2 to 4 plasmids ranging from 20 kb to 1.1 kb. Moreover, 2.6% of the isolates had no plasmids and 24.6% isolates had more than 1. Therefore, the numbers of plasmids found in a particular isolate would not necessarily indicate the level of multiple resistance of the isolate.

The highest resistance to the chemotherapeutics used was to penicillin (Table 2). Approximately 88.3% of *S. aureus* and 59.4% of *S. intermedius* isolates were resistant to penicillin. Sixty-six of the 77 *S. aureus* isolates were also resistant to amoxicillin+clavulanic acid (85.7%). The corresponding number for *S. intermedius* was 17 (45.9%). Resistance to this antibacterial agent was the second highest in both species of staphylococci. Only 1 *S. aureus* isolate was resistant to danofloxacin. Only 1 of each Staphylococcus isolates was resistant to methicillin (Table 2).

Table 2. Antibiogram of *S. aureus* and *S. intermedius* isolated from mastitic udders with subclinical mastitis. Resistance to amoxicillin (A), penicillin (P), danofloxacin (D), oxacillin (O), Amoxicilline+Clavulanic acid (A+C), ampicillin+sulbactam (Amp+S), methicillin (M), cloxacillin (Cl).

Staphylococcus sp.	Number of resistant *S. aureus* (%)	Number of resistant *S. intermedius* (%)	Total number of resistant Staphylococcus sp. (%)
P	68 (88.3)	22 (59.4)	90 (78.9)
A+C	66 (85.7)	17 (45.9)	83 (72.8)
Cl	2 (2.5)	1 (2.7)	3 (2.6)
O	1 (1.2)	1 (2.7)	2 (1.7)
A	16 (20.7)	10 (27)	26 (22.8)
Amp+S	12 (15.5)	4 (10.8)	16 (14)
D	1 (1.2)	0 (0)	1 (0.8)
M	1 (1.2)	1 (2.7)	2 (1.7)

Seventy-seven *S. aureus* isolates and 37 isolates of *S. intermedius* were typed by analysis of whole cell protein profiles by SDS-PAGE. Protein profiles from some of the *S. aureus* and *S. intermedius* strains are shown in Figure 1a. The cluster dendrogram produced by numerical analysis of whole-cell protein profiles is shown in Figure 1b. Genetic distances were between 0% and 96%. SDS-PAGE of whole-cell protein extracts of *S. aureus* and *S. intermedius* isolates produced patterns containing 17 to 49 discrete bands with molecular weights of < 14.4- > 116 kDa. Analysis of protein profiles of the *S. aureus* isolates resulted in a dendrogram (Figure 1b) showing that average distances within isolates were 0%-73% for *S. aureus* and 0%-96% for *S. intermedius*. Some isolates showed 0% genetic distance (Table 3). This close relationship can be explained by the fact that isolates (46-47 and 48-49; Figure not shown) were isolated from 2 living quarters of 2 different cows, suggesting that transmission from one location to another could occur. However, isolates 50 and 54 from 2 different cattle in the same herd suggested that transmission from cow to cow was also possible (Figure not shown). Percentages of distances lower than 28% were

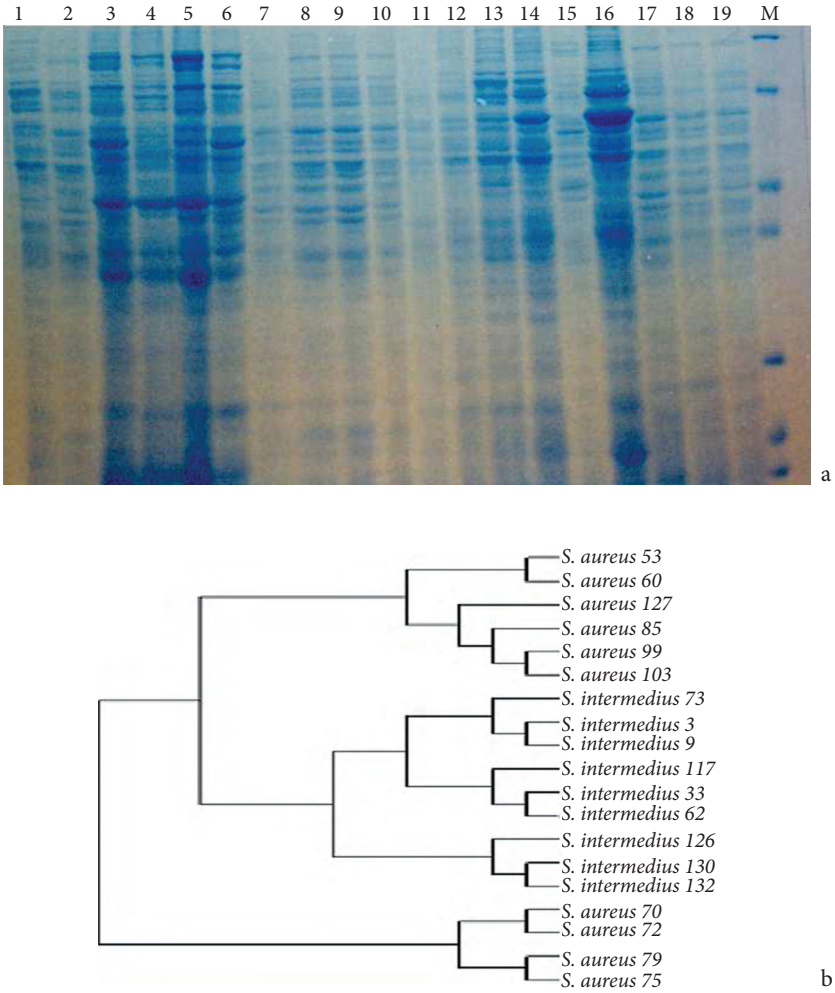

Figure 1. a: Electrophoretic pattern of protein profiles of *Staphylococcus aureus* isolates. From left to right, 1-10: *S. aureus* isolates 53, 60, 70, 72, 79, 75, 85, 99, 103 and 127; 11-19: *S. intermedius* isolate 3, 9, 33, 62, 73, 117, 126, 130 and 132. M: Marker (β galactosidase 116.0 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, RE Bsp 981 25.0 kDa, β lactoglobulin 18.4 kDa, Lysozyme 14.4 kDa).
b: Dendrogram of the *S. aureus* and *S. intermedius* isolates based on protein profiles.

Table 3. Nei's genetic distances values based on total cell proteins of *S. aureus* and *S. intermedius* isolates, 1-10: *S. aureus* isolates 53, 60, 70, 72, 79, 75, 85, 99, 103 and 127; 11-19: *S. intermedius* isolate 3, 9, 33, 62, 73, 117, 126, 130, and 132.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	pop ID
1	***																		
2	0.00	***																	
3	0.86	0.86	***																
4	0.86	0.86	0.06	***															
5	1.03	1.03	0.06	0.13	***														
6	0.94	0.94	0.10	0.17	0.03	***													
7	0.29	0.29	0.72	0.72	0.72	0.66	***												
8	0.34	0.34	0.66	0.66	0.66	0.60	0.03	***											
9	0.34	0.34	0.66	0.66	0.66	0.60	0.03	0.00	***										
10	0.38	0.38	0.60	0.60	0.60	0.54	0.06	0.03	0.03	***									
11	0.60	0.60	0.60	0.60	0.60	0.66	0.48	0.54	0.54	0.48	***								
12	0.48	0.48	0.72	0.72	0.72	0.79	0.48	0.54	0.54	0.48	0.06	***							
13	0.54	0.54	0.94	0.94	0.79	0.86	0.54	0.60	0.60	0.66	0.25	0.17	***						
14	0.48	0.48	1.03	1.03	0.86	0.94	0.60	0.66	0.66	0.72	0.29	0.21	0.03	***					
15	0.38	0.38	0.72	0.72	0.72	0.79	0.38	0.43	0.43	0.48	0.13	0.13	0.25	0.29	***				
16	0.43	0.43	0.94	0.94	0.94	1.03	0.54	0.60	0.60	0.66	0.25	0.17	0.06	0.03	0.25	***			
17	0.66	0.66	0.54	0.54	0.54	0.60	0.43	0.38	0.38	0.43	0.34	0.34	0.38	0.43	0.25	0.38	***		
18	0.60	0.60	0.72	0.72	0.72	0.79	0.38	0.43	0.43	0.48	0.29	0.29	0.34	0.38	0.21	0.34	0.10	***	
19	0.60	0.60	0.72	0.72	0.72	0.79	0.38	0.43	0.43	0.48	0.29	0.29	0.34	0.38	0.21	0.34	0.10	0.00	***

found when protein profiles of some *S. aureus* isolates were compared to those of isolates from *S. intermedius*. By this analysis, in the dendrogram 2 clusters were detected; the first cluster is subdivided into 2 subgroups. The first subgroup contains isolates of *S. aureus*. The second subgroup contains all isolates of *S. intermedius*. The second cluster contains 4 of the *S. aureus* isolates.

Discussion

Based on plasmid and antibiotic resistance, several isolates from both species were identical. For example, isolates of *S. aureus* 101, 102, 106, 108, 109, 110, and 113 have the same plasmid profiles (19 kb plasmid) and antibiotic susceptibility (resistance to P, A+C, A, and Amp+S). Isolate of *S. aureus* 99 and isolates of *S. intermedius* 111 and 112 have the same plasmid (19 kb plasmid) and antibiotic susceptibility patterns (resistance to P and A). Numbers of P and A+C resistance isolates with 19 kb plasmid are 41, P, A+C, A, and Amp+S resistance isolates with 19 kb plasmid are 8, P resistance with 19 kb plasmid are 4, and P, A+C, and A with 19 kb plasmid are 4. Younis et al. (1) studied phenotypic characteristics of 319 *S. aureus* strains isolated from bovine mastitis in 15 Israeli dairy herds and reported differences in protein patterns between 50 and 36 kDa.

Results from 33 *S. intermedius* and 45 *S. aureus* showed exactly the same protein profile, although they were characterised physiologically as different species. A similar observation has been reported for other gram-positive cocci, Pediococcus, isolated from human clinical specimens (18). According to their protein profile analysis, a species (ATCC 33314) had a protein profile typical of *P. pentosaceus* instead of *P. acidilactici*. On the basis of the results of conventional physiological tests only in the present work, the identification of these 2 strains remained questionable because the presence or absence of pigmentation would differentiate between the strains. Two key features to differentiate the species from one to another are fermentation of mannitol and DNase reactions, which are known to vary between the strains. In other words, either some *S. intermedius* may have pigmentation or those of *S. aureus* may not.

S. aureus isolated from mastitic udders mostly contains plasmids (19). Plasmid profiles observed in this study suggested no specific plasmid pattern within a species. For the 2 isolates evaluated, 97.3% of isolates were positive for plasmids. Plasmids were generally small, consistent with previous reports (19,20). In agreement with a previous study (21), use of plasmid patterns in conjunction with antibiotic susceptibility profiles in *S. aurues* isolates was of little value in this study. *S. intermedius* isolates also have similar characteristics as evidenced by the present study. Three species of isolates were found to be free of plasmids in this study. In vitro and in vivo studies have shown that variability and instability of plasmid content in staphylococcal isolates could occur. These types of changes have been suggested to be related to the observation that staphylococcal self-transmissible plasmids are capable of mobilising smaller plasmids into recipient isolates (22). This could explain the fact that isolates from the same udder obtained over time may be the same strain, regardless of the plasmid profile. Although some of the *S. aureus* isolates showed resistance to only 1 chemotherapeutic, they were found to contain 3 plasmids (isolate No: 24, 128; data not shown), which suggests that the plasmid observed in the strains could be correlated with chemotherapeutics not used in this study.

Two points regarding 19 kb plasmid need to be emphasised. Firstly, although mastitis cases caused by *S. intermedius* are rare in the literature (23,24), the ratio of the presence of the plasmid (19 kb) by the species (>88.8%) in the study was high. This could indicate that acquisition of the plasmid by *S. intermedius* may be common. However, future studies examining plasmid patterns of this species isolated by different geographic regions will help to clarify this. Furthermore, 20.7% *S. aureus* contained >1 type of plasmid. The corresponding figure for *S. intermedius* was 32.4%. Since virulence plasmids in staphylococci have been reported to be uncommon and plasmids in staphylococci might be correlated with resistance to antibiotics (21), based on the results of this study, it may be speculated that this should be the case for not only *S. aureus* but also for *S. intermedius*.

Differences in the protein pattern, as shown in the SDS-PAGE, clearly demonstrated that different proteins were produced, but further analysis needs to be conducted for the identification of those proteins

and their functions. Since cows were examined in this study (all were subclinically infected with either *S. aureus* or *S. intermedius*), isolation was mainly of virulent strains, while non-virulent strains contaminating the udder and/or the environment, if there were any, were not examined. A comparison between protein patterns of virulent and non-virulent strains will make this clear. On the other hand, the plasmid profile analysis itself does not appear to be an adequate method to differentiate isolates of 2 staphylococci recovered from bovine mastitis.

This study's aim was the isolation and differentiation of 114 isolates of 2 coagulase positive staphylococcal species of *S. aureus* and *S. intermedius* from subclinical mastitis. The classification of the species was performed using conventional culture procedures. The isolates were then further characterised by the determination of plasmid profiles and protein patterns using SDS-PAGE. The results show that the isolates with different protein profiles have different culture properties. Similar results were obtained for plasmid pattern and antibiotic resistance. In conclusion, plasmid profiles or antibiotic resistance pattern do not seem to be very valuable for differentiating between *S. aureus* and *S. intermedius*. Protein profiles of staphylococci showed variation on banding profiles within and between species. However, the isolates from the same species clustered together. Our data indicate that SDS-PAGE has a higher discriminative power than antibiotic resistance and plasmid pattern to distinguish *S. aureus* and *S. intermedius* isolates. Data from other veterinary diagnostic laboratories from the region showed that occurrence of *S. intermedius* mastitis has been an increasing problem not only in this region but also in the rest of Turkey. Additionally, the first report of a brain abscess in a human due to *S. intermedius* appeared recently (25). The isolated strain was not susceptible to methicillin. The patient in the case was reported not to be immunocompromised and no clear origin for the infection was established. Taken together, *S. intermedius* in certain countries or regions might be of concern as an aetiologic agent of subclinical bovine mastitis.

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