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RESEARCH ARTICLE

Molecular Identification of Bacteria Isolated from Dairy Herds with Mastitis

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Summary

The purpose of the present study was to identify environmental and contagious aerobic pathogen agents causing bovine clinical and subclinical mastitis using sequencing. A total of 244 cows were studied for the presence of mastitis using California Mastitis Test (CMT), clinical observations and microbiological isolations. Milk samples were obtained from 226 quarters of 123 cows which were diagnosed having clinical (11.4%) and subclinical (88.6%) mastitis. From these milk samples, 38 (16.8%) had no bacterial growth and from remaining 188 samples (83.2%) microorganisms were isolated. A total of 42 species were identified by sequencing amplified 16S rRNA fragments. The most common species were *Staphylococcus aureus* (22.9%) followed by *Escherichia coli* (10.1%), *S. chromogenes* (8.5%), *S. haemoliticus* (7.5%) and *Hafnia alvei* (6.4%). Of 188 isolates 43 were found contagious (22.9%), and 145 (77.1%) environmental agents. Use of sequencing offer identification of aetiological agent species level useful and practical that can help to chose a suitable therapeutic agent.

Keywords: *Mastitis, Sequencing, Microorganism, Identification*

Mastitisli Sığır Sütlerinden İzole Edilen Bakterilerin Moleküler İdentifikasyonu

Özet

Bu çalışmanın amacı, klinik ve subklinik mastitislere neden olan bulaşıcı ve çevresel aerobik patojen bakteriyel etkenlerin sekans analizi kullanılarak identifikasyonlarının yapılmasıdır. Toplam 244 sığır Kalifornia Mastitis Test (CMT), klinik gözlemler ve mikrobiyolojik identifikasyonlar yapılarak mastitis yönünden incelenmiştir. Yüzyirmiüç sığırın 226 meme lobundan alınan sütlere klinik (%11.4) ve subklinik (%88.6) mastitis teşhisi konulmuştur. Bunların 38 (%16.8)'inde herhangi bir bakteri üremez iken; 188 (%83.2) sütten izolasyon yapılmıştır. Kırk iki tür 16S rRNA fragmentleri çoğaltılarak sekans analizi ile identifiye edilmiştir. *Staphylococcus aureus* (%22.9) en çok izole edilen tür olmakla birlikte bunu *Escherichia coli* (%10.1), *S. chromogenes* (%8.5), *S. haemoliticus* (%7.5) ve *Hafnia alvei* (%6.4)'nin izlediği belirlenmiştir. Yüzseksensekiz izolatın 43 (%22.9)'ü bulaşıcı ve 145 (%77.1)'i çevresel etkenler olduğu tespit edilmiştir. İzolatların tür düzeyinde doğru bir şekilde identifikasyonlarının yapılmasında sekans analizinin kullanılmasının faydalı ve pratik olduğu; ayrıca uygun antibiyotik seçimine katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler: *Mastitis, Sekans analizi, Mikroorganizma, İdentifikasyon*

INTRODUCTION

Bovine mastitis is a frequent cause of economic loss in dairy herds in Turkey [1-3] as well as throughout the world [4-6]. The economic losses caused by bovine mastitis in Turkey reach to about 28 million dollars [7]. The aetiology of bovine mastitis is characterized by the inflammation of the mammary gland which is mostly

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caused by infectious agents. Among infectious agents that cause mastitis, bacteria, yeasts and algae can be cited [8,9]. Many microorganisms were determined as causative agents of mastitis and the most common cause of mastitis in Turkey were reported as *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Actinomyces pyogenes, Escherichia coli, Corynebacterium bovis, Pasteurella multocida, Bacillus subtilis, Bacillus cereus,* and *Micrococcus* spp. [1-3,10-12].

Bacteria involved in bovine mastitis are classified as either contagious or environmental pathogens based on their epidemiological association with the disease. A major group of mastitis pathogens which include *S. aureus, S. agalactiae* and *Mycoplasma* spp. are classified as contagious. Milking procedure helps the spread of these bacteria from one cow to another. A second group of mastitis agents are called environmental pathogens. These are the opportunistic microorganisms that can be found in the vicinity of where cows live, including in soil, water, manure, as well as bacteria from its own flora. The major pathogens that can cause environmental mastitis are *S. uberis, S. dysgalactiae, Enterococcus* spp., *E. coli, Klebsiella* spp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Arcanobacterium pyogenes*, *Serratia* spp., *Nocardia* spp. [8,13].

Mastitis either occurs with clinical symptoms (clinical mastitis) or without them (subclinical mastitis). The reduction in milk production attributed to subclinical mastitis may account for 70%-80% of the total losses [14]. Somatic cell counts (SCC) in milk may be used to identify the presence of subclinical mastitis. California Mastitis Test (CMT), a qualitative measurement of the SCC in milk, is a screening test for mastitis that can be used easily. Although CMT and SCC are used for the determination of mastitis, the definitive test for the diagnosis of mastitis is bacteriological isolation and identification [15,16].

Approximately 150 agents were reported to cause mastitis [8,9]. The correct identification of pathogen that causes infection facilitates the choice of antibiotics for therapy. The use of bacterial identification based on 16S rRNA sequencing is also helpful in veterinary microbiology especially for identification of coagulase negative staphylococci (CNS) [17,18]. For molecular identification a pure culture, DNA extraction and 16S rRNA amplification is necessary [19]. The purpose of the present study was to identify environmental and contagious aerobic pathogen agents causing bovine clinical and subclinical mastitis using sequencing.

MATERIAL and METHODS

Materials

A total of 244 dairy cattle at 13 dairy farms were investigated from January 2008 to December 2008. Among these cattle 123 were with clinical or subclinical mastitis and 266 milk samples were obtained from 123 dairy cattle with (15-20 samples per herd). Milk samples were taken by a veterinary practitioner in all these herds

Diagnosis of Mastitis

Clinical Mastitis: Clinical findings like abnormalities of secretions, abnormalities of size, consistency and temperature of mammary gland were examined by visual inspection and palpation. Pain reaction upon palpation, changes in the milk (blood tinged milk, watery secretions, clots, pus), and changes in consistency of udder were considered as indications of the presence of clinical mastitis.

Subclinical Mastitis: Cows, which did not have clinical mastitis, were subjected to further investigation for subclinical mastitis by using California Mastitis Test (CMT). The procedures and interpretations were performed according to Quinn et al.[20].

Sampling

Milk samples were collected aseptically with the following procedure: Before sampling, teat ends were disinfected with cotton swabs soaked in 70% alcohol and allowed to dry and the first streams of milk were discarded. Sterile tubes were filled with samples about 5 ml [21] by the veterinarian and transported in icebox to the Laboratory of Microbiology, Veterinary Faculty of Adnan Menderes University for further studies.

Methods

Isolation and Identification of Microorganisms

All positive samples (clinic and subclinic) were analyzed microbiologically as described previously [20]. For this, 0.01 ml milk was plated onto 7% sheep blood agar, as well as on Mac Conkey agar. The plates were incubated at 37°C for 72 h under aerobic conditions. The classical characteristics (colony morphology, haemolysis, Gram stain, catalase, coagulase, potassium hydroxide (KOH 3%) and oxidase test) were incestigated of isolated microorganisms.

DNA Extraction

Following isolation, DNA extraction was performed isolated strains.

From Gram Negative Bacteria: Frozen bacteria were subcultered on blood agar and DNA was extracted from colonies by simple boiling method. Shortly, few colonies were removed and suspended in 100 µl of sterile distilled water in 0.2 ml tube and boiled 15 min at 94°C in thermalcycler (Eppendorf AG, Hamburg, Germany). After centrifugation at 16.000 rpm for 5 min, 2 µl of supernatant was used for the PCR.

From Gram Positive Bacteria: For DNA extraction, a single bacterial colony was obtained from a fresh culture and suspended in 30 µl of lysis solution (250 U/ml lysozyme and 25 U/ml lysostaphin 10 mM Tris-HCl, 5 mM EDTA). The suspension was incubated at 30 min at 37°C followed by 10 min at 95°C. After phenol-chloroform extraction and ethanol precipitation, DNA was re-suspended in 50 µl distilled water and 2 µl of bacterial DNA was used as a template for PCR amplification (http://saureus.mlst.net/misc/info.asp). All extracted DNA was stored at -20°C until use. For all experiments, quality control strains, *E. coli* strain (ATCC 25922) and *S. aureus* (ATCC 29213) were used.

Polymerase Chain Reaction (PCR): 16S rRNA genes were amplified by PCR using universal 16S primers. To amplify 16S rRNA gene universal primers S16S20 5' AGA GTT TGA TCC TGG CTC AG 3' and 16S1390 5' GAC GGG CGG TGT GTA CAA 3' were used [22,23]. PCR experiments were carried out the following selected conditions: 2.5 U Taq polymerase (Fermentas), 10X Taq buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM $MgCl_2$, 0.4 pmol primers, and 0.2 mM dNTP, 2 µl of template sample DNA in a final volume of 30 µl. Amplification was obtained with an initial denaturation step at 94°C for 10 min followed by 35 cycles at 94°C for 30 s, and 50°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. Five µl of PCR products were separated on a 1% agarose gel and stained in 2 µl/ml ethidium bromide. The DNA fragments were visualized by UV. Samples with expected size (1371 bp) amplicons were further analyzed by sequencing.

Sequence

The amplicons with expected size were sent to Macrogen Korea in 96 well plates for sequence analysis (Macrogen Inc., 1001 World Meridian Venture Center, #60-24, Gasan-dong, Geumchun-gu, Seoul, 153-781, Korea). Sequence analysis was done after purification using ABI Primse sequencing system. Sequences obtained were compared to gene bank using Nucleotide-Nucleotide BLAST program at National Centre of Biotechnology Information web page (http://www.ncbi.nlm.nih.gov*) (Fig. 1).*

RESULTS

Isolation

A total of 244 cows were investigated from 13 dairy farms surveyed for mastitis cases and clinical or sub-clinical mastitis were diagnosed in 123 (54.9%) of these cows.

Of these 123 cows 14 (11.4%) had clinical and 109 (88.6%) had subclinical mastitis. From 123 cows with mastitis 226 milk samples were taken whereas 22 of them had mastitis in only one quarter, 99 had in two quarters and 2 had in three quarters. Among 226 samples 38 (16.8%) had no bacterial growth and from remaining 188 (83.2%) milk samples microorganisms were isolated. The total number of all cows, cows with mastitis, milk samples taken, and no growth and isolation status were given in *Table 1*. Among these 188 mastitis

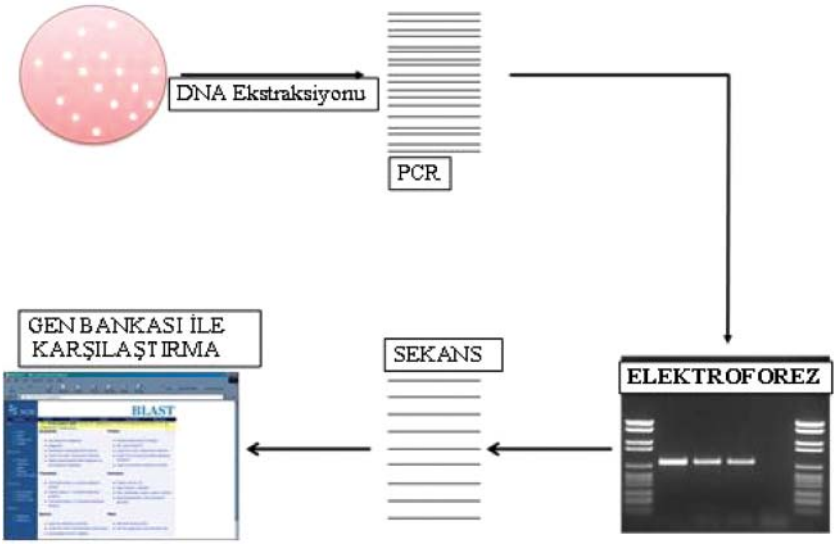


Fig 1. Molecular identification of bacteria based on 16S rRNA sequencing

Şekil 1. Bakterilerin 16S rRNA sekansına dayalı moleküler identifikasyonu

agents 136 were Gram positive (72.3%) and 52 were Gram negative (27.7%) bacteria *(Table 2).*

PCR

PCR was done for all strains to amplify from 16S rRNA gene. A 1371 bp long band was obtained with PCR by using universal 16S primers *(Fig. 2)*.

Sequence

All amplicons obtained by 16S rRNA amplification were sequenced. The results of bovine mastitis aetiology presented in *Table 2* and in *Fig. 3*.

A total of 42 microorganism species were isolated from milk samples. Especially staphylococci are the most frequently isolated mastitis agent.

Contagious and Environmental Microorganisms

Of 188 isolates 43 (22.9%) were contagious (*S. aureus*), and 145 (77.1%) environmental. The most common

Table 1. *The total number of all cows, cows with mastitis, milk samples taken, no growth and isolation status*
Tablo 1. *Tüm sığır, mastitisli sığır, alınan süt örneği, üreme olmayan örnek ve izolat sayıları*

Farm	Number of				
	Total Bovine	Bovine with Mastitis	Milk Sample Taken	No Growth	Isolates
Herd 1	25	7	15	0	15
Herd 2	26	8	16	1	15
Herd 3	14	9	18	2	16
Herd 4	16	10	20	1	19
Herd 5	16	8	15	3	12
Herd 6	13	10	16	1	15
Herd 7	14	10	19	5	14
Herd 8	16	9	19	8	11
Herd 9	15	10	20	5	14
Herd 10	13	8	16	5	11
Herd 11	15	12	19	5	14
Herd 12	35	12	18	2	16
Herd 13	26	10	15	0	15
TOTAL	**244**	**123**	**226**	**38**	**188**

Table 2. *Contagious and environmental agents of bovine mastitis isolated from milk samples*
Tablo 2. *Mastitisli sığır sütlerinden izole edilen bulaşıcı ve çevresel etkenler*

Gram Positives		Gram Negatives	
Microorganism	**Number of Isolated (%)**	**Microorganism**	**Number of Isolated (%)**
Contagious (22.9)		***Contagious (0.0)***	
S. aureus	43 (22.9)	.....	0 (0.0)
Environmental (49.8)		***Environmental (27.3)***	
S. chromogenes	16 (8.5)	*E. coli*	20 (10.1)
S. haemolyticus	14 (7.5)	*H. alvei*	12 (6.4)
B. licheniformis	9 (4.7)	*E. agglomerans*	5 (2.7)
E. faecalis	7 (3.6)	*E. cloacae*	4 (2.1)
L. garvieae	5 (2.7)	*E. hormaechei*	3 (1.6)
S. simulans, S. pseudointermedius	4 (2.0)	*C. freundii, P. stutzeri*	2 (1.0)
S. epidermidis, S. cohnii, B. cereus	3 (1.6)	**	1 (0.6)
S. pasteuri, S. uberis, L. lactis, B. subtilis, B. pumilus	2 (1.0)		
*	1 (06)		

** S. sciuri, S. vitulis, S. equorum, S. xylosus, S. warneri, S. parauberis, S. dysgalactiae, E. durans, A. viridans, A. aneurinilyticus, K. gibsoni, C. mucifaciensis, C. flavescens, M. luteus, A. gandavensis*
*** E. amnigenus, S. somnei, K. planticola, Y. enterocolytica*

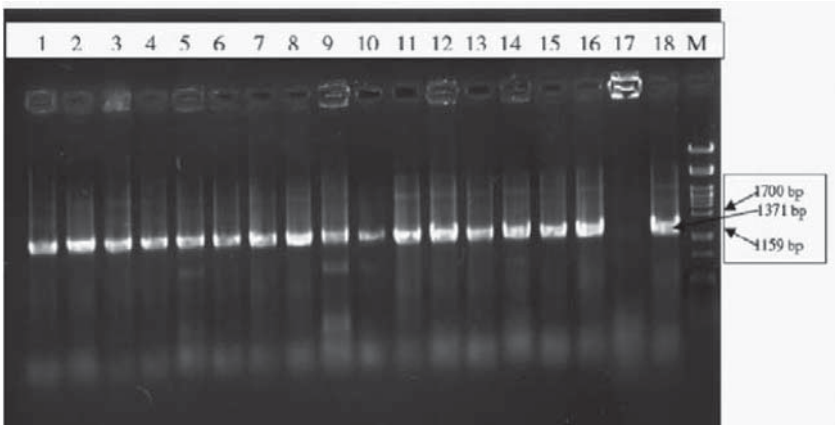
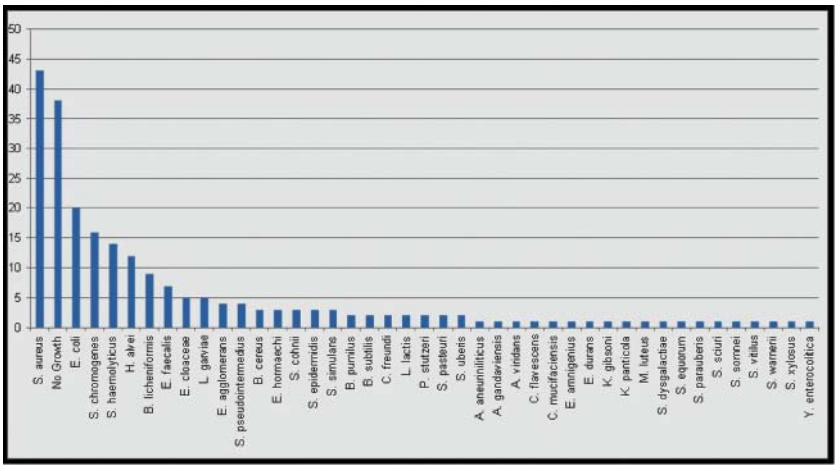


Fig 2. PCR performed by using 16S rRNA universal primers. M: Marker (Lambda phage DNA restricted with PstI enzyme) 1-16: PCR performed by using isolated microorganism's DNA. 17: Negative control (without DNA master mix) 18: Positive control (*S. aureus* ATCC 29213)

Şekil 2. 16S universal primerleri kullanılarak gerçekleştirilen M: Marker (PstI enzimi ile kesilmiş lambda faj DNA'sı). PZR. 1-16: İzole edilen mikroorganizmaların DNA'ları ile kullanılarak yapılan PZR, 17: Negatif Kontrol (DNA'sız master miks), 18: Pozitif Kontrol (*S. aureus* ATCC 29213)

Fig 3. Microorganisms and their numbers isolated from milk samples obtained from cows with mastitis. A total of 42 different species were isolated from 188 isolates and from 38 samples there were no bacterial growth

Şekil 3. Mastitisli sığır süt örneklerinden izole edilen mikroorganizmalar ve sayıları. Otuzsekiz örnekten bakteriyolojik izolasyon yapılamaz iken, 188 izolattan 42 farklı tür mikroorganizma izolasyonu yapıldı



species were *S. aureus* (22.9%) followed by *E. coli* (10.1 % of all isolates), *S. chromogenes* (8.5), *S. haemoliticus* (7.5) and *H. alvei* (6.4). Total number of CNS reached to 51 isolates (27.1%) of 12 species. Among 52 Gram negative isolates (27.7%) 20 were (10.1) *Escherichia coli*, 12 (6.4%) *Hafnia alvei*, 5 (2.7%) *Enterobacter agglomerans*, 4 (2.1%) *E. cloacae,* 3 (1.6%) *E. hormaechei,* 2 (1.0%) *Citrobacter freundii and Pseudomonas stutzeri,* 1 (0.6%) *E. amnigenus, Shigella somnei, Klebsiella planticola*, *Yersinia enterocolytica,* and *Agrobacterium tumefaciens.* Among 136 Gram positives isolates 43 were *S. aureus* (22.9%), 16 (8.5%) *S. chromogenes*, 14 (7.5%) *S. haemolyticus*, 9 (4.7%) *Bacillus licheniformis,* 7 (3.6%) *Enterococcus faecalis,* 5 (2.7%) *Lactococcus garvieae,* 4 (2.0%) *S. pseudointermedius* and *S. simulans*, 3 (1.6%) *S. epidermidis, S. cohnii* and *B. cereus,* 2 (1.0%) *S. pasteuri, S. uberis, L. lactis, B. subtilis* and *B. pumilus,* 1 (0.6%) *S. dysgalactiae, S. sciuri, S. vitulis*, *S. equorum*, *S. xylosus*, *S. warneri, S. parauberis, E. durans, Aerococcus viridans, Aneurinibacillus aneurinilyticus*, *Kurthia gibsoni*, *Corynebacterium mucifaciensis*, *C. flavescens*, and *Micrococcus luteus (Table 3)*.

Clinical and Subclinical Mastitis Agents

Of 123 cows 14 (11.4%) had clinical mastitis. Twenty two microorganisms were isolated from these cases. The most frequent pathogens in clinical cases were 10 (5.3%) *S. aureus,* 4 (2.0%) *E. coli,* 2 (1.0%) *E. faecalis,* 2 (1.0%) *S. uberis,* and 1 (0.6%) *S. pseudointermedius, L. garvieae, S. chromogenes, S. chromogenes, S. pseudointermedius* while 2 samples had no bacterial growth. Of 123 cows 109 (88.6%) had subclinical mastitis. From these 166 microorganisms were isolated. The most frequently isolated pathogens were 33 (16.9%) *S. aureus*, followed by 16 (8.5%) *E. coli,* 15 (7.9%) *S.chromogenes*, 14 (7.5%) *S. haemolyticus,* 12 (6.4%) *H. alvei,* 5 (2.7%) *E. faecalis* and *E. agglomerans,* 4 (2.0%) *L. garvieae,* 3 (1.6%) *S. pseudointermedius, S. epidermidis, S. cohnii* and *B. cereus,* 2 (1.0%) *C. freundii, S. pasteuri, L. lactis, B. subtilis* and *B. pumilus,* 1 (0.6%) *P. stutzeri* and others (*E. amnigenus, S. somnei, K. planticola, Y. enterocolytica, S. sciuri, S. vitulis, S. equorum, S. xylosus, S. warneri, S. parauberis, S.dysgalactiae, E. durans, A. viridans, A. aneurinilyticus, K. gibsoni, C. mucifaciensis, C. flavescens, M. luteus, A. gandavensis) (Table 3)*.

DISCUSSION

Determination of aetiological agents of mastitis by continuous survey studies would be helpful for treatment

Table 3. *Distributions of microorganisms isolated from milk samples with clinical and subclinical mastitis*
Tablo 3. *Klinik ve subklinik mastitisli süt örneklerinden izole edilen mikroorganizmaların dağılımı*

Isolates	Number of Isolates	
	Clinical Mastitis (%)	**Subclinical Mastitis (%)**
S. aureus	10 (5.3)	33 (16.9)
E. coli	4 (2.0)	16 (8.5)
E. faecalis	2 (1.0)	5 (2.7)
S. uberis	2 (1.0)	0 (0.0)
P. stutzeri	1 (0.6)	1 (0.6)
L. garvieae	1 (0.6)	4 (2.0)
S. chromogenes	1 (0.6)	15 (7.9)
S. pseudointermedius	1 (0.6)	3 (1.6)
B. licheniformis	-	9 (4.7)
S. haemolyticus	-	14 (7.5)
H. alvei	-	12 (6.4)
E. agglomerans	-	5 (2.7)
S. simulans, E. cloacae	-	4 (2.0)
***	-	3 (1.6)
****	-	2 (1.0)
*****	-	1 (0.6)
Total	**22 (11.7)**	**166 (88.3)**

*** *S. epidermidis, S. cohnii, B. cereus, E. hormaechi*
**** *C. freundii, S. pasteuri, L. lactis, B. subtilis, B. pumilus*
***** *S. sciuri, S. vitulis, S. equorum, S. xylosus, S. warneri, S. parauberis, S. dysgalactiae, E. durans, A. viridans, A. aneurinilyticus, K. gibsoni, C. mucifaciensis, C. flavescens, M. luteus, A. gandavensis, E. amnigenus, S. somnei, K. planticola, Y. enterocolytica*

of these infections. In most of the studies done world-wide as well as in Turkey *S. aureus* was found to be the most frequent agent [3,5,6,24]. In the study, *S. aureus* was the most common (22.9%) species isolated from milk samples which is slightly lower than the rates reported in previous studies done in Turkey that vary between 28.3% and 47.5% [3,11,25] . The main reason for the high frequency of *S. aureus* in intramammary infections may be the transfer of this pathogen from one caw to the other during milking procedures by the herdsman which can be avoided by standard hygienic measures, desinfectation of the milking parlours and milking equipment. In the a recent study it was shown the clonality of isolates from even among different herds [26].

Coagulase negative staphylococci (CNS) have long been considered as a relatively inoffensive commensals or contaminants, and now recognized as the cause of severe mastitis cases [27]. A total of 51 (27.1 %) CNS isolates belonging to 12 species were identified in this study. In the previous studies it was stated that CNSs were the most frequently isolated mastitis agents [28,29] which was also confirmed by present study. *S. chromogenes* was the third most common isolate following *S. aureus* and *E. coli*. This shows the importance of sequencing technique which let us to identify CNSs at species basis. Among 12 CNS species identified *S. chromogenes* were followed by *S. haemolyticus*, *S. epidermidis* and *S. simulans*.

Coliform bacteria are also very common environmental pathogen following the CNS species [3,6,10,11]. In our study the most common coliforms were *E. coli, H. alvei* and *E. cloaceae*.

It was reported that *S. uberis* and *S. dysgalactiae* were most important species in environmental *Streptococcus* genus [10,11,28]. However in our study the number of streptococci isolated was lesser than expected (2 *S. uberis*, 1 *S. dysgalactiae*, and 1 *S. parauberis*). As the number of the reports using molecular identification increase exact distribution of causative agents for mastitis will be better understood. Further studies are going on in our laboratory to determine the rates of streptococci as a causative agent for mastitis in our region. All of the streptococcus species isolated in this study were environmental mastitis agents. In this study it was observed that the sequence technique was very useful in the identification of micro-organisms like *S. uberis* and *S. parauberis* which could not have been identified by biochemical methods as stated by Jayarao et al.[30].

The pathogens which cause clinic or subclinic mastitis are not always the same. The diversity of causative agents for subclinical mastitis is very much higher than clinical mastitis. In both clinical and subclinical mastitis *S. aureus* and *E. coli* are the most important agents however their rates are different; *S. aureus* are the 45% of all clinical mastitis but only 19% of subclinical mastitis. *E. coli* rates are twice higher for clinical mastitis than subclinical mastitis (18% *versus* 9.6%). The rates of CNSs are 29.5% among subclinical mastitis but only 9% among clinical mastitis.

In some of the previous studies CNSs were the most frequently isolated bacteria from subclinical cases [1,24,31-33]. However, some authors reported high percentage of clinical cases evoked by CNS [34]. Bayar [1] have reported that all *Staphylococcus* spp. (*S. haemolyticus*, *S. simulans S. auricularis, S. hominis, S. warneri, S. capitis, S. cohnii, S. xylosus, S. epidermidis* and *S. sciuri*) isolated from 221 subclinical mastitis suspected milk samples were CNS. Gianneechini et al.[24] have also reported that 37 (7.4%) and 3 (7.5%) CNS isolations were performed from subclinical and clinical mastitis, respectively. In this study 51 CNS isolations were obtained from which 49 were from subclinical cases. Under the consideration that staphylococci are found in the normal flora of the teat and teat skin, this high ratio (26.2%) explains that mastitis control measurements were insufficient.

As mentioned above, in our survey 16.8% of the bacteriological cultures were negative for all obtained samples. This ratio was varied between 38.0% and 7.3% in other studies [3,11,24,35,36]. In the authors' opinion the possible reasons for this may be the suspected microorganism may be an anaerobic microorganism, a virus, mycoplasma or other microorganisms requiring special mediums for growth.

A successful control program for mastitis can be established with an effective monitoring system for all dairy herds and correct identification of pathogen that cause mastitis. Molecular methods may help for correct identification of agents. Sequencing technique used in identifying bacteria is becoming a frequent method. High cost and insufficient number of specialists were the limiting factors in the expanding use of molecular identification methods in routine veterinary laboratories. But nowadays sequence technique may have greater advantages over traditional phenotypic identification methods by means of reduced cost of sequence and increasing knowledge of the technical personnel.

In conclusion, contagious and environmental agents were isolated at a rate of 22.9% and 77.1% from mastitis suspected milk samples, respectively. Isolation of forty-two species indicates that the choice of antibacterial agents needs a better identification of causative agents to be more effective in curing a wide range of intra-mammary infections.

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