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RESEARCH ARTICLE

Prevalence of subclinical mastitis in cows, isolation of agents and determination of their antibiotic susceptibility

Aliye Gülmez Sağlam[1]*, Salih Otlu[1], Mustafa Reha Çoşkun[1], Elif Çelik[1], Fatih Büyük[1], Mitat Şahin[1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, University of Kafkas, Kars, Turkey,
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* alis_6223@hotmail.com

İneklerde subklinik mastitis prevalansı, etken izolasyonu ve antibiyotik duyarlılıklarının belirlenmesi

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Öz

Amaç: Bu çalışmada, Kars ilinde yetiştirilen sığırlarda subklinik mastitise neden olan bakteriyel etkenlerin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Toplam 500 süt ineği CMT ile tarandı ve 120'si (% 24) subklinik mastitis olarak tespit edildi. CMT pozitif süt ineklerinden süt örnekleri alındı. Süt örnekleri, bakteri izolasyonu için, Kanlı agar (7% koyun kanlı) ve Eosin Methylene Blue agar üzerine yayıldı. Ayrıca, *Mycoplasma* spp. izolasyonu için süt örneklerinden PPLO broth ve PPLO agara ekim yapıldı. Fenotipik identifikasyon için kültürler, koloni morfolojisi, Gram boyama özellikleri, hemoliz oluşturma özelliklerine göre değerlendirildi ve daha sonra biyokimyasal testlere tabi tutuldu. İzolatların antibiyotik duyarlılıkları Kirby Bauer Disk Difüzyon Testi ile belirlendi.

Bulgular: Toplam 120 ineğe ait süt örneklerinden 229 *Staphylococcus* spp., 7 *Corynebacterium* spp., 16 *Bacillus* spp., 5 *Acinetobacter* spp., 2 *Escherichia coli* ve 2 *Mycoplasma* spp. izole edildi. Antibiyogram sonucunda, izolatların çoğunluğu cefoxitin (30 µg) ve imipenem'e (10 µg) duyarlı bulunmuş ve izolatlar arasındaki en yüksek direnç penicillin'e (10 µg) karşı belirlenmiştir.

Öneri:
Süt ineği yetiştiriciliği yapılan işletmelerde belirli aralıklarla subklinik mastitin taranması, etkenin izolasyon ve identifikasyonu ile karakterizasyonu çiftçiliğinin kârlılığı ve sürdürülebilirliği için önemlidir.

Anahtar kelimeler: Subklinik mastitis, süt ineği, prevalans, bakteri, antibiyotik duyarlılığı

Abstract

Aim: To investigate bacterial agents causing subclinical mastitis in cow husbandry in Kars province, Turkey.

Materials and Methods: Subclinical mastitis was identified in 120 (24%) of 500 dairy cows screened by CMT. Within the scope of the study, it was taken milk samples from 120 dairy cows and evaluated in the laboratory. For bacterial isolation, the milk samples were mixed with vortex and 100 µl of milk were streaked on Blood Agar (with 7% sheep blood) and Eosin Methylene Blue Agar. For *Mycoplasma* spp. isolation, milk samples were transferred to PPLO broth and PPLO agar. For phenotypic identification, cultures were evaluated according to the colony morphology, hemolysis on BA and Gram staining properties, and then were subjected to biochemical tests. Antibiotic susceptibilities of isolates were determined by Kirby Bauer Disc Diffusion Test.

Results: As a result of bacteriological studies, 229 *Staphylococcus* spp., 7 *Corynebacterium* spp., 16 *Bacillus* spp., 5 *Acinetobacter* spp., 2 *Escherichia coli*, and 2 *Mycoplasma* spp. were isolated from milk samples of 120 cows. As a result of antibiogram, the majority of isolates have found sensitive to cefoxitin (30 µg) and imipenem (10 µg) and the highest resistance among the isolates was determined against to penicillin (10 µg).

Conclusion: The screening of subclinical mastitis at certain periods in enterprises, the proper characterization to be determined by isolation and identification of agent are crucial for the profitability and sustainability of dairy farming.

Keywords: Subclinical mastitis, dairy cow, prevalence, bacteria, antibiotic susceptibility

Introduction

Mastitis is an inflammation of mammary gland characterized by pathological changes of udder tissue that cause physical and chemical changes in milk (Baştan 2013). Mastitis is one of the major disease, that cause economic losses worldwide due to the affects on milk quality and quantity (Behiry et al 2012, Sharma et al 2011), occurs as clinical and subclinical form with acute and chronic course (Çokal and Konuş 2012). Clinical mastitis emerges with important disease symptoms in milk and udder tissue. It was reported that diagnosis of subclinical mastitis which does not present any clinical findings is difficult and more important than clinical mastitis in terms of huge economic losses as a rate of 3-26% milk drop (Şimşek and Aksakal 2005).

According to the origin of the pathogens, mastitis is divided into two groups as contagious and environmental mastitis. Contagious mastitis includes contagious pathogens such as *Staphylococcus* aureus, *Mycoplasma bovis* and S*treptococcus agalactiae*. Environmental mastitis is caused by coliform group agents consist of *Escherichia coli*, *Streptococcus* uberis, *Str. Dysgalactiae*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. (Preethirani et al 2015, Bhatt et al 2011).

It was emphasized that more than 130 etiological agents were isolated from mastitis cases. Among these, bacterial agents take place on the top and with a rate of 95% of bovine mastitis agents were defined as *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis* and *E. coli* (Hillerton and Berry 2005, Macun et al 2011). However, agents such as *Corynebacterium bovis*, *Bacillus subtilis*, *B. cereus, Micrococcus* spp., *Nocardia* spp. *Cryptococcus* spp, have been reported less frequently (Kuyucuoğlu and Uçar 2001). Some yeast and molds are reported rarely as a causative agent of mastitis (Çokal and Konuş 2012).

The diagnosis of mastitis is based on the chemical and microbiological examination together with the clinical manifestation. In the case of subclinical mastitis diagnosis it is widely used of various chemical and microbiological tests. Subclinical mastitis can be considerably demonstrated with the California Mastitis Test (CMT), which is based on determining the number of somatic cells in milk. Isolation of infectious agents plays an important role in antibiotic selection to case treatment and definition of infection agent (Blowey and Edmondson 1995, Rişvanlı and Kalkan 2002).

In this study, it was aimed to estimate the prevalence of subclinical mastitis in cows in Kars region and to isolate the causative agents and determine the susceptibility of agents against to various antibiotics.

Material and methods

Population size sampled

In this study, 500 dairy cows raising in family type enterprises (10-20 heads) in the villages connected to the center of Kars were examined with CMT in terms of subclinical mastitis. The experiment was carried out with the approval of the Local Ethical Committee in Kars (KAÜ-HADYEK/2015-024).

Diagnosis of subclinical mastitis by CMT

CMT was used for diagnosis of subclinical mastitis. In brief, 2 ml of milk sample was taken into the CMT paddle and the same amount of CMT kit reagent was added onto each cup, the paddle was rotated for few seconds and the results were recorded (Schalm et al 1971).

Milk samples

Within the scope of the study, it was taken approximately 40 ml of milk into sterilized falcon tube from each udder lobe of animals whic were determined to be subclinical mastitis by CMT. Right after the nipples were cleaned with 70% ethyl alcohol the first milking discharge were collected under aseptic conditions. Milk samples were transported to Kafkas University, Faculty of Veterinary Medicine, Microbiology Department Laboratories in cold chain and short time. Totally 318 milk samples from different udder lobes of 120 cows, which were found to be positive by CMT, were taken for microbiological evaluations.

I*solation and identification studies*

For bacteriological isolation, at first the milk samples were homogenized by vortexing. Then 100 µl of milk were streaked on Blood agar enriched with 7% sheep blood (Oxoid, CM0271) and Eosin Methylene Blue agar (Oxoid, CM0069) plates. Plates were incubated at 37 oC for 24-48 h in aerobic condition. For *Mycoplasma* spp. isolation, milk samples were incubated at 37 °C for 72 h in Mycoplasma broth medium (supplemented with horse sera, thallium acetate and penicilin, Oxoid, CM0403)) in microaerobic condition (Merck, Anaerocult C, 1.16275). After preenrichment step in Mycoplasma broth medium, liquid cultures were transferred to Mycoplasma agar (supplemented with horse sera, thallium acetate and penicilin, Oxoid CM0401) and incubated for two weeks at 37 °C under the same conditions.

After incubation, cultures were evaluated in terms of colony morphology, pigment production, hemolysis on BA and Gram staining features were examined, as well. Suspicious isolates were identified by subjecting to classical biochemical tests (i.e. catalase, oxidase, urease, nitrate reduction tests). Growth characteristics of suspicious Staphylococcus isolates were evaluated on DNase agar (Merck, V873149), Mannitol Salt agar (MSA, Merck VM355104) and Baird Parker agar (BPA, Merck U297806) mediums and coagulase test was performed on these isolates, as well. For *Mycoplasma* spp. identification, the colonies on Mycoplasma agar were examined and evaluated under microscope in terms of typical fried egg shaped morphology.

For fungal isolation, 100 µl of milk samples were inoculated on Sabouraud Dextrose agar (SDA, Merck V371439) and the plates were incubated aerobically at 25°C for 7-10 days. Colonies were firstly evaluated by macroscopically and then stained with lactophenol cotton blue for microscopic examination.

Determination of antibiotic susceptibility of isolates

Antibiotic susceptibilities of the identified microorganisms were determined by Kirby Bauer Disc Diffusion Method (NCCLS 2008) on Mueller-Hinton agar (MHA, BBL 211443). Suspensions of the isolates were prepared in 0.9% physiological saline according to Mc Farland No 0.5 standard. Oxacillin (OX, 1 µg), cefoxitin (FOX, 30 µg), vancomycin (VA, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (DA, 2 µg), enrofloxacin (ENR, 5 µg), gentamicin (CN, 10 µg), imipenem (IPM, 10 µg), cefoperazone (CFP, 75 µg), ampicillin (AM, 10 µg), amoxycillin/clavulanic acid (AMC, 30 µg) ve penicillin (P, 10 µg) discs obtained from Oxoid Company were placed on the MHA and incubated at 37 °C for 24 hours. Antibiogram test results were evaluated according to the criteria reported by NCCLS. Antibiogram test has been not applied for fungal and mycoplasma isolates.

Results

The prevalence of subclinical mastitis

Subclinical mastitis was identified in 120 (24%) of 500 dairy cows screened by CMT and frequently in 2 or 3 breast lobes of cows.

Isolation and identification results

At the result of bacteriological and mycological evaluation out of 318 milk samples taken from 120 cows with subclinical mastitis a total of 297 isolates were obtained from 99 samples while no growth was obtained from the remaining 21 milk samples. The distribution of agents is as follows; 138 (46.46%) *S. aureus*, 67 (25.55%) Coagulase Negative Staphylococci (CoNS), 24 (8.08%) Coagulase Positive Staphylococci (CoPS), 16 (5.38%) *Bacillus* spp., 13 (4.37%) *E. coli*, 7 (2.35%) *Corynebacterium* spp., 6 (2.02%) *Acinetobacter* spp., 2 (0.67%) *Mycoplasma* spp., 9 (3.03%) *Candida* spp., 8 (2.69%) *Aspergillus* spp. and 7 (2.35%) *Penicillum* spp. (Table 1).

Antibiogram test results

While the majority of isolates have found sensitive to cefoxitin (30 µg) and imipenem (10 µg), the highest resistance among the isolates was determined against to penicillin (10 µg) and ampicillin (10 µg) (Table 2).

Discussion

One of the most important problems encountered in dairy cattle farming enterprises is mastitis. Especially subclinical mastitis which can not clinically diagnosed, it is overlooked and therefore spreads rapidly (Viguier et al 2009). Moreover, it causes significant economic and labor loss leading to reduction in milk production, deterioration of milk quality and anavoidable treatment expenses (Baştan 2013). Early diagnosis and treatment of subclinical mastitis is very important in herd management because the incidence is higher than clinical mastitis (Çokal and Konuş 2012).

Since the silent course of subclinical mastitis the changes in udder tissue and milk can not detected clinically; hence these changes are determined by various tests. CMT is the most practical and more preferred method among these tests (Midleton et al 2004). As the result of different studies the rates of subclinical mastitis were reported as 29% in South Wales (Plozza et

Table 1. The distribution of microorganisms isolated from milk samples of dairy cows

Microorganisms	The number of microorganism (n)	Isolation rate (%)
S. aureus	138	46.46
CoNS	67	25.55
CoPS	24	8.08
Bacillus spp.	16	5.38
E. coli	13	4.37
Corynebacterium spp.	7	2.35
Acinetobacter spp.	6	2.02
Mycoplasma spp.	2	0.67
Candida spp.	9	3.03
Aspergillus spp.	8	2.69
Penicillum spp.	7	2.35

Table 2. The antibiotic susceptibility profile of defined microorganisms

Antibiotic disc		*S. aureus*	CoNS	CoPS	*Bacillus* spp.	*E. coli*	*Corynebacterium* spp.	*Acinetobacter* Spp.
OX	S(%)	125 (90.6)	64 (95.5)	22 (91.7)	6 (37.5)	9 (69.2)	6 (85.7)	5 (83.3)
	R(%)	13 (9.4)	3 (4.5)	2 (8.3)	10 (62.5)	4 (30.8)	1 (14.3)	1 (16.7)
ENR	S(%)	131 (94.9)	65 (97)	21 (87.5)	13 (81.3)	11 (84.6)	5 (71.4)	4 (66.7)
	R(%)	7 (5.1)	2 (3)	3 (12.5)	3 (18.7)	2 (15.4)	2 (28.6)	2 (33.3)
DA	S(%)	107(77.6)	55 (82.1)	19 (79.2)	15 (93.7)	7 (53.8)	7 (100)	5 (83.3)
	R(%)	31(22.4)	12 (17.9)	5 (20.8)	1 (6.3)	6 (46.2)	0	1 (16.7)
CIP	S(%)	133 (96.3)	64 (95.5)	23 (95.8)	10 (62.5)	8 (61.5)	5 (71.4)	4 (66.7)
	R(%)	5 (3.7)	3 (4.5)	1 (4.2)	6 (37.5)	5 (36.5)	2 (28.6)	2 (33.3)
FOX	S(%)	138(100)	67 (100)	24 (100)	16 (100)	12 (92.3)	7 (100)	6 (100)
	R(%)	0	0	0	0	1 (7.7)	0	0
IPM	S(%)	138 (100)	67 (100)	24 (100)	12 (75)	7 (53.8)	5 (71.4)	4 (66.7)
	R(%)	0	0	0	4 (25)	6 (46.2)	2 (28.6)	2 (33.3)
AM	S(%)	93(67.4)	57 (85.1)	22 (91.7)	8 (50)	10 (76.9)	5 (71.4)	4 (66.7)
	R(%)	45 (32.6)	10 (14.9)	2 (3)	8 (50)	3 (23.1)	2 (28.6)	2 (33.3)
CFP	S(%)	134 (97.1)	64 (95.5)	19 (79.2)	16 (100)	7 (53.8)	6 (85.7)	3 (50)
	R(%)	4 (2.9)	3 (4.5)	5 (20.8)	0	6 (46.2)	1 (14.3)	3 (50)
AMC	S(%)	124 (90)	60 (89.6)	22 (91.7)	11 (68.7)	10 (76.9)	4 (57.1)	4 (66.7)
	R(%)	14 (10)	7 (10.4)	2 (3)	5 (31.3)	3 (23.1)	3 (42.9)	2 (33.3)
P	S(%)	95 (68.8)	57 (85.1)	21 (87.5)	11 (68.7)	8 (61.5)	6 (85.7)	5 (83.3)
	R(%)	43 (31.2)	10 (14.9)	3 (12.5)	5 (31.3)	5 (36.5)	1 (14.3)	1 (16.7)
VA	S(%)	134 (97.1)	62 (92.5)	21 (87.5)	8 (50)	12 (92.3)	5 (71.4)	3 (50)
	R(%)	4 (2.9)	5 (7.5)	3 (12.5)	8 (50)	1 (7.7)	2 (28.6)	3 (50)
CN	S(%)	137 (99.3)	67 (100)	22 (91.7)	9 (56.2)	9 (69.2)	6 (85.7)	5 (83.3)
	R(%)	1 (0.7)	0	2 (3)	7 (43.8)	4 (30.8)	1 (14.3)	1 (16.7)

al 2011), 43% in Sri Lanka (Sanotharan et al 2016), 51.8% in Ethiopia (Zeryehun and Abera 2017), 61.9% in Germany (Gundling et al 2015) and 64% in East Kenya (Mureithi and Njuguna 2016). In Turkey, the prevalence of subclinical mastitis was obtained by CMT in some studies and the rate were reported as 15.78% in Kars (Şahin et al 1997), 8.2% in Elazığ (Gülcü and Ertaş 2004), 89.18% and 54.37% in Kırıkkale (Macun et al 2011, Yeşilmen et al 2012). According to the results of CMT applied in current study, subclinical mastitis was determined as 24%. It is clear that these different results can be caused by differences such as farming conditions, milking style (hand or machine), udder care and hygiene.

As the case in many geographical areas in where dairy farming is favoured, the profilies of causative agents is different among subclinical mastitic cows. Saidi et al (2013) determined an isolation rate as 40% for *S. aureus*, 12.5% for *Streptococcus* spp, 2.5% for *Enterobacteriacea*e, 2.5% for *Pseudomonas* spp, 12.5% for *S. aureus + Streptococcus* spp., 7.5% for *Streptococcus* spp., 7.5% for *S. aureus + Mycoplasma* spp., and 5% for *S. aureus + Streptococcus* spp. + *E. coli* in their studies in Algeria. At the result of a study in India reported by Preethirani et al (2015) 64.8% CNS, 18.1% *Streptococcus* spp., 9.8% *E. coli* and 7.3% *S.aureus* were isolated and identified. Tel et al (2009) carried out a study on cow's milk in Şanlıurfa region and reported 258 aerob bacteria including 32.5% *S.aureus*, 71 (27.5%) CoNS, 8.9% *Streptococcus* spp., 6.2% *E. coli*, 5.8% *Trueperella pyogenes*, 3.4% *Bacillus* spp., 3.1% *C. bovis*, 2.7% *Micrococcus* spp., 1.9% *Enterobacter aerogenes*, 1.9% *Candida* spp., 1.5% *Pasteurella multocida*, 1.5% *Klebsiella pneumonia*e, 1.5% *Citrobacter diversus*, 1.1% Pseudomonas aeruginosa. In a similar study conducted by Sevinti and Şahin (2009), out of 79 milk samples of mastitis cows at a rate of 34.3% *S. aureus*, 28.3% CoNS, 16.4% *Streptococcus* spp. and 5.9% *E. coli* were isolated and identified. In another study of bacteriological examination of breast tissue samples taken from cows in Elazığ region, 39.04% *S. aureus*, 17.81% *S. epidermidis*, 14.38% *T. pyogenes* were isolated (Gülcü and Ertaş 2004). Çokal and Konuş (2012) has conducted a study which includes the isolation of aerob bacteria from 117 milk samples that are determined as positive by CMT. In this study the major causative agent is

reported *S. aureus* (41.0%) in mastitis cases and the remaning are as following order; CoNS from 17.1% of cases, 12.0% *E. aerogenes* from, 9.4% *E. coli*, 8.5% *Streptococcus* spp., 6.8% *K. pneumoniae*, 5.1% *Citrobacter* spp. and 2.6% of *Bacillus* spp. In the present study, 500 cows were screened by CMT and 318 milk samples, which include each affected breast lobe of 120 animals that were found as positive, were evaluated and as the result of cultural examinations 46.46% *S. aureus*, 25.55% CoNS, 8.08% CoPS, 5.38% *Bacillus* spp., 4.37% *E. coli*, 2.35% *Corynebacterium* spp., 2.02% *Acinetobacter* spp., 0.67% *Mycoplasma* spp., 3.03% *Candida* spp., 2.69% *Aspergillus* spp. and 2.35% *Penicillum* spp. were identified. When these results are compared with the previous studies (Saidi et al 2013, Tel et al 2009), it is seen that subclinical mastitis is largely constituted by Gram-positive cocci, even *S. aureus*. However, contrary to many other researchers (Gülcü and Ertaş 2004), *Streptococcus* spp. can not be isolated in this study.

Treatment of mastitis should be targeted towards the causative bacteria whenever possible, but in acute situations, treatment is initiated based on herd data and personal experience. Rapid or on-farm bacteriological diagnosis would facilitate the selection of the most appropriate antimicrobial. Sevinti and Şahin (2009) determined the antibiotic susceptibility of *Staphylococccus* isolates from mastitic milk against β-lactam group antibiotics in Kars region and found that out of 42 *Staphylococcus* strains 25 (59.5%) were resistant to penicillin, while 16 (38.1%) were amoxicillin, 3 (7.1%) were amoxicillin-clavulanic acid, and 2 (4.8%) were cloxacillin-resistant, as well. Furthermore, 5 (11.9%) strains were found to be resistant to enrofloxacin, whereas all strains were found to be susceptible to vancomycin. Güler et al (2005) analysed 235 *Staphylococcus* spp. strains and reported that 63% of strains were resistant to both penicillin and ampicillin, 27.9% to oxytetracycline, 1.8% to trimetoprim-sulfametaxazole. But no resistance was observed to amoxicillin-clavulonic acid, oxacillin, enrofloxacilin and kanamycin-cephalexin. In a study conducted by Yeşilmen et al (2012) it was seen that *Staphylococcus* spp. isolates were found to be susceptible to ampicillin, cefoperazone-sulbactam and cefoxitin antibiotics and all agents were found to be resistant to penicillin. In the present study, it was seen that the most of the isolated strains (24%) were resistant to penicillin, with ampicillin (21,9%) and clindamicin (19,2%) following. All isolates (100%) were found to be susceptible to cefoxitin and 99.6% of them susceptible to gentamicin. It is thought that the high penicillin resistance findings is similar to that reported by the other authors, However, the widespread use of penicillin preparations indiscriminately and especially in these resistant mastitis cases may be the resultant bacteria may have gained enough mutation to develop resistance. The differences in antibiotic susceptibility of agents isolated in this study are thought to be due to the variability in regional strain distribution and unconscious medications.

The development of resistance in bacteria has a great importance for the efficacy of treatment in animals. In recent years except *S. aureus* strains, MecA gene has been identified in CoNS strains, and these were found having resistance to one or more antimicrobials (Büyükcangaz et al 2012). In the Staphylococci, the methicillin resistance emerges as a consequence of the production of penicillin-binding 2a/2' protein (PBP2a/PBP2') encoded by the MecA gene. Although displaying of the MecA gene is accepted as a gold standard in detection of methicillin resistance, in routine practice, tests that are able to determine oxacillin sensitivity are also used as reference methods (Sevgican et al 2009). Kireçci and Çolak (2002)have reported 5 (5.8%) methicillin-resistant *Staphylococcus* spp. strains in a study conducted in Erzurum province. Kırkan et al (2005) has reported 60% oxacillin resistance among *S. aureus* strains studied in Aydın region. 15 (6.6%) of 229 *Staphylococcus* spp. isolates were determined as resistant to methicillin. The most important features of methicillin-resistant *Staphylococcal* strains are that they are resistant to multiple antibiotics (Kaynarca and Türkyılmaz 2010). In this study, all of 15 methicillin resistant strains were found to have also multiple antibiotic resistance. Therefore, it is important to consider multidrug resistance with the methicillin resistance of the disease agents in treatment to be applied in mastitis cases.

One of the most important problems of cattle breeding in Kars is unpredictable economic losses due to the endemic diseases (such as Brucellosis, Anthrax and Leptospirosis) in the region (Otlu et al 2002, Otlu et al 2008).

Conclusion

It can be said that subclinical mastitis has an important role in economic losses due to the reasons such as excessive decline in milk production and short lactations and treatment costs. Because of these reasons, the screening of subclinical mastitis at certain periods in enterprises, the proper characterization to be determined by isolation and identification of agent are crucial for the profitability and sustainability of dairy farming.

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