Isolation, identifications and antimicrobial susceptibility pattern of coagulase positive *Staphylococcus* from subclinical mastitic dairy cattle in and around Haramaya University

Firaol Tafa[1], Yitagele Terefe[1], Nateneal Tamerat[1], Endrias Zewdu[2]*

[1]Haramaya University, College of Veterinary Medicine, P.O.BOX 138, Dire Dawa, Ethiopia
[2]Ambo University, College of Agriculture and Veterinary Sciences, Department of Veterinary Laboratory Technology, P.O.Box 19, Ambo, Ethiopia
**Corresponding author ; E-mail:yitagele@yahoo.com/yterefe@haramaya.edu.et*

Abstract

A cross-sectional study was conducted from November 2013 to April 2014 to isolate coagulase positive Staphylococcus (CPS) from subclinical mastitic (SCM) lactating cows, to establishing prevalence, to identify risk factors and to determine the antimicrobial susceptibility pattern of CPS isolates in and around Haramaya University, Eastern Ethiopia. A semi-structured questionnaire survey, California mastitis test (CMT), bacterial isolation and identification and *in vitro* antimicrobial susceptibility tests were conducted during the study. A total of 210 dairy cattle from seven farms were screened for SCM by CMT. The prevalence of SCM in the study areas was 71.4% (150/210). Milk samples were collected from 562 quarters of 150 cows free of clinical mastitis but positive in CMT. Among these cows 38 of them had blind quarters (6.76%). The prevalence of CPS was found to be 66.0% (99/150) and 28.46% (160/562) on animal and quarter basis, respectively. Among the risk factors studied, SCM due to CPS was significantly higher ($P > 0.05$) in older cows (100%) than younger (70.0%), in cows during late lactation (96.3%), in cows which gave more than 5 births (100%), in cross-breed cows (71.2%) than local breed cows (54.3%), in cows with udder / teat injuries (96.3%) and in cows managed under poor house hygiene (82.5%). Antimicrobial susceptibility testing of 9 antibiotics carried out using Kirby-Bauer disc diffusion method and revealed high multidrug resistance of 71 (87.6 %). High level of resistance to common drugs like ampicillin (90.1%), penicillin (67.9%), and tetracycline (54.3%) was registered. In contrast, the CPS isolates were susceptibility for chloramphenicol, gentamycin and kanamycin at 63%, 46.9%, and 41.97%, respectively. In conclusion, the study confirms the importance of CPS as a mastitis causing multidrug resistant bacterium. Therefore, improved management and early treatment of SCM cases with drug of choice is imperative to tackle CPS mastitis.

Keywords: Antimicrobial susceptibility, Coagulase positive Staphylococcus, and risk factors.

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Introduction

Mastitis is an inflammation of the parenchyma of mammary gland that causes physical and chemical changes in milk and leads to pathological condition of the glandular tissue (Ranjan *et al.*, 2011). The majority cases of mastitis are caused by bacteria (Radostits *et al.*, 2007). Mastitis is an important disease of dairy animals and the huge bottleneck, impacting the milk production of Ethiopian diary sector (Demelash Biffa *et al.*, 2005). It is generally associated with poor hygienic husbandry practices, bruising of mammary tissue or other wound predisposes the cow to mastitis while the primary reservoir of contagious pathogens is the mammary gland itself (Hunderra Sori *et al.*, 2005).

In sub clinical mastitis, there is no visible appearance of changes in the milk or udder, but milk production decreases and composition is altered due to bacteria presence. SCM is 3 to 4 times more common than the clinical mastitis and it is not often detected by most of the dairy farmers; hence it is the major factor affecting the productivity of dairy cattle (Mungube *et al.*, 2005). Reduction in milk production and quality, treatment cost, premature culling of cows and loss of genetic potential will follow leading to heavy economic losses (Leitner *et al.*, 2011). Furthermore, it has zoonotic importance since the affected cows have bacteria contaminated milk (Sharif *et al.*, 2009).

Among the various bacterial pathogens, staphylococcus has been reported as the major cause SCM (Kassaye Getahun *et al.*, 2008). The most widely used and generally accepted criterion for identification of pathogenic staphylococci is usually by their ability to produce coagulase due to the fact that coagulase tests correlates well with staphylococcus species pathogenicity (Lamprell *et al.*, 2004). On the other hand, antimicrobial therapy plays a great role in the control of bovine mastitis caused by staphylococcus species. In fact, Microbial identification and susceptibility reports as a means to guide veterinarians for initial therapeutic decisions have paramount importance. Unfortunately, antimicrobial resistance has become a growing concern worldwide. Thus, generation of antimicrobial resistance data is essential for interventions and monitoring of antimicrobial resistance (Basappa *et al.*, 2011).

In Eastern Ethiopia, specifically around Haramaya University small holder dairy development activities are expanding in non-organized manner due to the increasing demand for milk. However, there is a growing production inefficiency posed by health constraints including drug resistances. This is

partly due to the fact that very limited research was employed to address the issue in the area. Therefore, the present work was undertaken with the objectives of isolation and determination of *in vitro* antimicrobial susceptibility pattern of CPS, in addition to assessment of potential risk factors relation to CPS prevalence in the study area.

Material and Methods

Study area

The study was conducted in dairy farms in and around Haramaya district including Haramaya University and Harar city, Eastern Ethiopia. The elevation of study areas is about 2000 m above sea level. It has mean annual temperature and relative humidity of 18°C and 65%, respectively. It receives an average rain fall of approximately 900 mm. Animal production is undergone with agricultural activities side by side (HADB, 2009).

Study design and Study animal

In the cross-sectional study lactating cows were considered for the presence of sub clinical mastitis. The study animals are both cross and local breed lactating cows. The local dairy cows are managed mainly under traditional extensive husbandry system; while most of the cross bred cows are often managed under a small-scale semi-intensive management system.

Sampling Method

The study was carried out in seven farms with different herd sizes ranging from 12 to 60 animals. The sampling of the farm was employed purposively based on accessibility and willingness of the owners. Totally 210 lactating cows were randomly selected for Clinical examination and the subsequent CMT screening from the seven farms after proportional allocation of various sample of cows based on the size of farms.

Study Methodology

Different potential risk factors are considered in the study including breed, age, body condition, udder and teat condition i.e. injuries or blindness, lactation stage, parity, housing conditions, hygienic practice i.e. milker, udder and barn hygiene, and history of antimicrobial treatment in which all information were collected through questionnaire survey, observation and farm records. Before

CMT screening for SCM examination of udder and teat was employed. As result, among 210 sampled cows' 60 of them were excluded from the study due to clinical mastitis. Then those CMT positive cows were considered for further bacteriological examination to check the presence of CPS.

Clinical examination of the udder and teats

The udder was first examined visually and then through palpation to detect possible fibrosis, inflammatory swellings, visible injury, atrophied tissue and any other abnormality. The size and consistency of mammary quarters were inspected for the presence of any unusual findings, such as disproportional symmetry, swelling, firmness and blindness. Viscosity and appearance of milk secretion from each mammary quarter were examined for the presence of clots, pus, flakes, blood, and watery secretions (Kivaria *et al*, 2004). Thus, cows with clinical mastitis and blind teats were not included for further examination.

California mastitis test

CMT was employed on 150 cows to diagnose the presence of SCM. A squirt of milk from each quarter of the udder was placed in each of the four shallow cups in the CMT paddle and an equal amount of the CMT reagent was mixed to it. Evaluation of the degree of gel formation was done by gently rotation of the CMT paddle and the results were determined based on the degree of gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from +1 to +3 (Quinn *et al.*, 2002). If at least one quarter was positive, then the cow was considered as SCM patient.

Collection of milk sample

Milk samples were collected according to Quinn *et al.*, (2002). Milk samples for culture were collected before milking. Hands of the sample collector and quarters of the udder were washed thoroughly and dried. Teats were disinfected with cotton soaked in a 70% alcohol solution before sampling. Approximately 10 ml of milk was collected aseptically with sterile universal tubes after discarding the first three milking streams. Then sample from each quarter was recorded accordingly and transported in icebox with ice packs to the Microbiology Laboratory of College of Veterinary Medicine, Haramaya University. Up on arrival to the laboratory, the samples were either immediately cultured or stored at + 4°C for a maximum of 24 h until culturing on standard bacteriological media.

Bacteriological isolation and identification

Bacteriological examination was done according to the protocols of the (ISO, 2003) and (Quinn *et al.,* 2002). The milk sample was vortexed and a loop full of milk was used for the quadrant streaking method for each quarter on tryptose blood agar base enriched with 7% sheep blood (Oxoid, UK). Blood agar plates were incubated aerobically at 37°C for 24-48 hr. The plates were examined for gross colony morphology, pigmentation and hemolytic characteristics. Presumptive pure colonies were selected which appear to be circular, raised, smooth, glistening with a color ranging from grey-white to orange and sub-cultured on mannitol salt agar media (Oxoid, UK) and incubated aerobically at 37 °C for 24–48 h. The cultured colonies were sub-cultured on nutrient agar (Oxoid, UK) at 37 °C for 24-48 h. Characterization of isolated mastitis causing pathogens was done based on the patterns of haemolysis, morphologic features, Gram stain, biochemical test like catalase test and coagulase test. A sample was considered positive for CPS when at least one colony was identified as coagulase positive by the coagulase test.

Antimicrobial Susceptibility test

It was done by culturing the selected CPS isolates on Mueller Hinton agar media. The isolates were tested for 9 antimicrobials using the Kirby-Bauer disk diffusion method. The antimicrobial disks have various concentrations. Antibiotic discs were applied onto the inoculated MHA using disc dispenser and gently pressed to ensure intimate contact with the surface. The plates were incubated aerobically at 37 °C for 18-24 h. The zones of inhibition were measured using a ruler and Oxford mathematical set divider. The results were reported as the diameter of the zone surrounding the individual disk in which bacterial growth was absent (CLSI, 2007). Based on this, the isolates were defined as resistant, intermediate and susceptible.

Data analysis

The data of questionnaire, observation and laboratory results were recorded and analyzed using SPSS version 20. Descriptive statistics was employed to summarize the data including frequency and percentile. The Chi-square test was also undertaken to demonstrate the statistical association between SCM positive cases and potential risk factors. Furthermore, multi-collinearity between the independent variables was checked by linear regression collinearity diagnostics. The association between variables was considered as statistically significant at $P \leq 0.05$. The confidence interval was set at 95%.

Result

Prevalence

From the 210 lactating cows examined 60 (28.57%) and 150 cows (71.4%) had clinical and subclinical mastitis, respectively. SCM due to coagulase positive *Staphylococcus* accounted for 66% (99/150) and 28.5% (160/562) prevalence at cow and quarter level, respectively (Table 1). Among the total of 600 teats examined, 38 (6.3%) were blind, of these blind teats 13 were from the left front (LF), 13 were from left rear (LR), 5 were from right front (RF) and 7 were from right rear (RR) quarters.

Table 1: Rate of infection due to CPS at quarter level.

Tested quarters	Total No. examined	CPS infected quarters	Prevalence (%)	X^2	P- value	CI
Left Front (LF)	137	39	24.38	1.023	0.796	95 %
Left Rear (LR)	137	42	26.25			
Right Front (RF)	145	36	22.5			
Right Rear (RR)	143	43	26.88			
Totally	562	160	28.5% (160 /562)			

Questionnaire survey and observation

In the present study parity, stage of lactation, udder injuries, age, breeds and house hygiene had significant association ($P < 0.05$) with the prevalence of subclinical mastitis due to CPS (Table 2). Cross breed cows are more affected than local ($P < 0.05$). Lactating cows with above 8 years age is highly infected with CPS, while those at the age interval of 5-8 years is less susceptible to CPS ($P < 0.05$), likewise prevalence of CPS was significantly high in cows with parity number more than 5 and less in those with parity1 up to 3 ($P < 0.05$). Unlike early stage lactation, cows in late stage lactation Cows suffered most from CPS ($P < 0.05$). Infection rate is high in cows with injured udder/teat ($P < 0.05$). Cows which are managed in poor house condition showed significantly high CPS prevalence ($P < 0.05$). However, milkers hand hygiene, udder hygiene and common towel used for drying teat did not show statistically significant ($P > 0.05$) variations (Table 2).

Table 2: Prevalence of subclinical mastitis due to CPS with different potential risk factors.

Risk factor	Total No. Examined	No of Positive Prevalence (%)	X^2	P-value	Confidence Interval (CI)
Breed					
Cross	104	74 (71.2 %)	4.014a	0.045	95 %
Local	46	25 (54.3%)			
Age					
3-5 years	50	35 (70%)	18.773 a	0.000	
6 – 8 years	76	40 (52.6%)			
> 8 years	24	24 (100%)			
Lactation stage					
1-3 months	58	31 (53.4%)	15.171 a	0.001	
4-5 months	65	42 (64.6%)			
> 5 months	27	26 (96.3%)			
Parity					
1-3 births	85	48 (56.5%)	10.098 a	0.006	
4-5 births	56	42 (75%)			
Over 5 births	9	9 (100%)			
Udder/teat condition					
Not injured	123	73 (59.3%)	13.468 a	0.000	
Injured	27	26 (96.3%)			
Udder hygiene					
Poor hygiene	75	54 (72%)	2.046 a	0.121	
Good hygiene	75	45(60%)			
Hand hygiene before milking					
Washed	72	42 (58.3%)	3.627 a	0.057	
Not washed	78	57 (73.1%)			
Usage of towel					
Common towel	39	28 (71.8%)	0.789 a	0.375	
No use of towel	111	71(64 %)			
House hygiene					
Good	70	33 (47.1%)	20.798 a	0.000	
Poor	80	66 (82.5%)			

Antimicrobial susceptibility pattern

From the total 160 CPS isolates, antimicrobial susceptibility tests were conducted on 81 randomly selected isolates. CPS isolates were found to be relatively susceptible to chloramphenicol (63.0%) followed by gentamycin (46.9%) and kanamycin (42.0%) (Table 4). However, there was high multi-drug resistance of 71 (87.6 %), among the CPS isolates (Table 3). In addition, high drug resistance encountered against some commonly used drugs including ampicillin (90.1%) penicillin (67.9%) and Tetracycline (54.3%) (Table 4).

Table 3: Multidrug Resistance pattern of CPS isolates.

Level of Resistance	No of CPS isolate	Percent (%)	Cumulative Multidrug Resistance, No (%)
No resistance	1	1.2%	-
Resistance for 1 drug	9	11.1%	-
Resistance for 2 drugs	21	25.9%	21 (25.9%)
Resistance for 3 drugs	11	13.6%	32 (39.5 %)
Resistance for 4 drugs	24	29.6%	56 (69.1 %)
Resistance for 5 drugs	10	12.3%	66 (81.4 %)
Resistance for 6 drugs	5	6.2%	71 (87.6 %)
Total	81	100 %	71 (87.6 %)

Table 4: Antibiotic susceptibility pattern of CPS isolate from subclinical mastitic cows.

Antimicrobials	Resistant No (%)	Intermediate No (%)	Susceptible No (%)
Amoxicillin	24 (29.6)	51(63.0)	6 (7.4)
Gentamycin	1 (1.2)	42 (51.9)	38 (46.9)
Streptomycin	29 (35.8)	49 (60.5)	3 (3.7)
Penicillin	55 (67.9)	25 (30.9)	1 (1.2)
Chloramphenicol	0 (0.0)	30 (37.0)	51 (63.0)
Kanamycin	3 (3.7)	44 (54.3)	34 (42.0)
Ampicillin	73 (90.1)	4 (4.9)	4 (4.9)
Tetracycline	44 (54.3)	34 (42.0)	3 (3.7)
Erythromycin	32 (39.5)	46 (56.8)	3(3.7)

Discussion

The 71.4 % subclinical mastitis prevalence of the present study is close to the work of Ararsa Duguma *et al* (2014). However, it is greater than the reports of Mesele Abera *et al*, 2013 and Rigbe Haftu *et al.*, 2012 who reported 36.7 % and 33.8 % of SCM, respectively. This study revealed the prevalence of SCM due to CPS at cows' level (66%) and quarter's level (28.46%), similarly Oudessa and Tareke (2003), report relatively lower prevalence of CPS as a cause of SCM in the country. The high prevalence of CPS might be associated with its ability to colonize teats frequently, its existence in intracellular and localize within micro abscesses in the udder and hence resistant to antibiotic treatment (MacDonald, 1997). Variation in the rate of CPS infection among different areas across the country could be due to the difference in management practices, hygienic status and the breed composition.

Prevalence of SCM due to CPS was significantly higher ($P < 0.05$) in cross breed cows than the local breeds which is also true in the work of Mulugeta Yohannis and Wassie Molla (2013) who reported high prevalence of mastitis in Jersey breed than local zebu cattle. This could be due to anatomically large pendulous udder of cross breed cows which can be easily exposed to environmental injuries which in turn predispose cows to suffer from mastitis. Age wise the present study indicate significantly ($P < 0.05$) higher CPS infection at advanced age which is in agreement with the finding of Rigbe Haftu *et al.*, (2012). This is probably due to prolonged period of exposure to the infecting organisms and predisposing factors like stress of lactation which favor dilation of teat canal due to repeat milking thereby facilitating the entry of pathogens into the teat canal to cause subclinical intramammary infection (Shittu *et al.*, 2012).

In this study, prevalence of SCM was significantly higher ($P<0.05$) in late lactation as compared to early and mid lactation, like wise Kassaye Getahun *et al.*, (2008) and Rigbe Haftu *et al.*, (2012) states that increased prevalence of mastitis was encountered as lactation stage advance. On the contrary, Oudessa Kerro and Fiseha Tareke (2003) and Mulugeta Yohannis and Wassie Molla (2013) reported that cows at early stage of lactation are more susceptible to mastitis. This study revealed significantly ($P<0.05$) higher prevalence of CPS as parity number increased. Similar findings were previously reported by (Oudessa Kerro and Fiseha Tareke, 2003; Mungube *et al.*, 2005; Demelash Biffa *et al.*, 2005; Kassaye Getahun *et al.*, 2008; Mulugeta Yohannis and Wassie Molla, 2013). The age-multiparous-high prevalence relationship

is explored to be due to decrease in teat patency, degree and frequency of previous exposure in multiparous old cows (Radostits *et al.,* 2007). In addition, teat and udder injuries has shown significantly ($P<0.05$) higher prevalence of CPS (96.3%), which are caused by barbed wire fences, tick infestations and vigorous suckling calves. High level of CPS in injured udder/teat could be due to favorable environment of the wound (injuries) which ultimately leads to secondary bacterial complication.

On the other hands, House hygiene had shown significant ($P<0.05$) effect on the prevalence of CPS. In the case of good house hygiene the prevalence were 47.1%, while cows managed in poor house hygiene had prevalence of 82.5%. In dairy managed under unhygienic houses, manure and wet bedding materials were not frequently removed which in turn favour high occurrence of CPS. This is consistent with the findings of earlier works in Ethiopia that implicated poor barn hygiene to high prevalence of mastitis (Hunderra Sori *et al.,* 2005; Mesele Abera *et al., 2*013, Mulugeta Yohannis and Wassie Molla, 2013).

The present study has demonstrated the presence of high level of multi drug resistance 71 (87.6 %), especially among commonly used drugs like penicillin and tetracycline. The 67.9 % of SCP isolates were resistance to penicillin in this study, likewise Firaol Tamiru *et al*, (2013), Zerfie Taddesse *et al* (2014) and Mesele Abera *et al*, (2013) reported 50 %, 90 % and 94.4 % *Staphylococcus aures* resistance, respectively. There was also 54.3 % resistance among the CPS isolate against tetracycline in this study, likewise Berhanu Andualem, (2009) stated 65.3 % resistance of *Staphylococcus aures* against tetracycline. Chloramphenicol was the most effective drug with 63 % CPS isolates susceptibility in the present study, similarly Rigbe Haftu *et al*, (2012) and Mesele Abera *et al*, 2013 indicate *Staphylococcus aures* susceptibility of 58.8 % and 100% for Chloramphenicol, respectively. According to the present study, Gentamicin is the second effective drug with 48.9 susceptibility of CPS. Similarly, Firaol Tamiru *et al,* (2013) and Zerfie Taddesse *et al* (2014) report 71.4 % and 84.3 % susceptibility of S*taphylococcus aures* to Gentamicin, respectively.

In the current study area there is frequent, prolonged and indiscriminate use of antimicrobial drugs. In addition, there is a growing believe that some of the drugs which have been used in the study area were not introduced in to the country legally after assessment of their quality standard. In fact, it is not uncommon to find these drugs in the hand of farmers which have been bought from illegal outlet which adds up to the conclusion that most of CPS isolates

showed resistance to commonly used antimicrobials due to the mentioned facts. The high prevalence of CPS infection detected in dairy cows has public health concern since CPS bacteria's are capable of producing heat-stable enterotoxins, which might cause staphylococcal food poisoning outbreaks when ingested by humans in sufficient quantities (Lamprell *et al.*, 2004). Therefore, Good hygienic management practices, regular monitoring of antimicrobial resistance and further research to identify species and strains of CPS has paramount significance to reduce and prevent the pathogen effect on dairy industry.

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