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Prevalence of subclinical mastitis, Isolation and antimicrobial susceptibility pattern of Staphylococcus aureus from subclinical mastitic dairy farms at Sebeta, Central Ethiopia.

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Abstract

Mastitis is the repeated, inflammatory reaction of the udder tissue either due to microorganism's infections or physical trauma. The mammary gland infection is the most common disease in dairy cattle worldwide.Mastitis had been continuing and challenging for treatment and control.To estimate Prevalence of subclini cal mastitis, to isolate and to determine antimicrobial susceptibility pattern of *Staphylococcus aureus* a cross sectional study was conducted in dairy farms at Sebeta Town from June 2016 to November 2016. A total of 64 quarters free of clinical mastitis were screened randomly from 45 lactating cows using the California mastitis tests. Following strict hygienic procedures milk sample were collected from CMT positive quarters. Isolates, Prevalence of subclinical mastitis and antimicrobial susceptibility pattern of S. aurous were summarized using descriptive statistics. Totally, 35.6% (64/180) of the quarters were found positive for CMT and 105 (58.3%) were CMT negative & 4(2.2%) teats were detected as blind. After a series of bacteriological examinations 46.9% (30/64) S. aurous isolate were identified. 14.1% (9/64) the genus streptococcus and 10.9 % (7/64) *S.intermedius* were recognized while the rest 15.6% (10/64) were other known mastitis microorganisms. Antimicrobial susceptibility test reveals that all *S.aureus* isolates were (100%) susceptible to Erythromycin, Co-Trimoxazole, Vancomycin, Oxacillin and Cefoxitin. However, 22/30 (73.3%), 13/30 (43.3%), 20/30 (66.7%), and 1/30 (3.3%) of *S.aureus* isolates were resistant to Penicillin, Tetracycline, Ampicillin, and Gentamycin respectively. In other hands 5/30 (16.7%), 15/30 (50%), and 7/30 (23.3%) of *S.aureus* isolates were resistant for one drug, two drugs, and three drugs respectively as multidrug resistance**.** Prolonged environmental survival, capability of invasiveness of the bacteria and its ability of producing exo-proteins, and biofilms formation that helps to develop resistant to a number of antimicrobial could make *S. aureus* remain prevalent in most dairy farms. Lack of early detection of the sub clinical causes could also be the other case. Therefore Consistent follow up, avoiding predisposing factors to lesions, and good husbandry practices are very important. Antimicrobials use should be given an attention.

Keywords: *Subclinical mastitis, S. aureus, Bovine, antimicrobial susceptibility, Sebeta*

Introduction

Although milk is considered a complete food (Drewnowski, A, 2010; Ellen, Muehlhoff., et al 2013), low productivity,the cost for mastitis treatment, market distortions, and other factors make a large gap between the demand and supplies in developing countries like Ethiopia (David J. et al., 2015; Shapiro et al. 2015). Mastitis is the continual, inflammatory reaction of the udder tissue either due to microorganism's infections or physical trauma. The mammary gland infection is the most common disease in dairy cattle worldwide (DAS, 2010; Eri ka M et al., 2012). In past century's mastitis had been continuing and challenging for treatment and control.

The Coliforms from environmental agents, Staphylococcus & Streptococcus from the contagious causative agents of bovine mastitis are the dominant pathogens of economic and epidemiological importance (Alemu et al., 2013; Ararsa et al., 2014). Sub-clinical mastitis has increased equivalent with the improvement of high milk-producing breeds contributing to the rising spread of the disease due to lack of detection and awareness (Sharma et al., 2012). Due to its capability to biofilm formation and ability to produce a range of exo-proteins, Staphylococcus aureus has developed resistance to several antimicrobials (Margariti et al., 2014 Fernandes et al. 2011; Atulya et al. 2014).The objective of this study was to assess the prevalence of subclinical mastitis and S.aureus and to determine the antimicrobial susceptibility pattern of the pathogen from subclinical mastitis in dairy cows.

Mastitis has little attention in Ethiopia, (Mekonenet al., 2005). Hard works have only been concentrated on the treatment of clinical cases. Owing to the heavy financial implications involved, wide-ranging use of antimicrobials in animal husbandry potentially leads to maintenance of selective pressure and higher frequency of resistance for wide variety of antimicrobial agents (Silbergeld et al., 2008), and the inevitable existence of latent infection.

Materials and Methods

1. Study Animals and study Areas

The study was conducted in three dairy farms on forty five dairy cows kept under intensive system that were both cross and local lactating breeds in Sebeta town 25km southwest of the capital Addis Ababa with an altitude ranging from 1800 to 3380 meters above sea level. The area has short and medium rainy seasons from March to May and Jun to September respectively with rainfall 680 to 1200mm and temperature of 11.30c to 280c (Abebe, 2006; Sabata Hawas District Rural and Agricultural Office, unpublished data of 2001).

2. Study design and sample collection

A cross sectional study was conducted from June 2016 to November 2016 to isolate pathogenic Staphylococcus aureus and to assess its epidemiological distributionin subclinical bovine mastitis. Milk samples were collected before milking from 45 lactating cows with different stages of lactation following strict hygienic procedures. The first streams of milk were removed from each teat. Starting from the far sides of the udder while dirties were removed by brushing, the flanks and the udder/teats were dipped with 3% iodine and dried thoroughly with clean towel. Orifices of teats scrubbed with gauze moistened with 70% alcohol using separate swabs until they become completely clean. After removing some squirt of milk an equal volume of milk sample taken and test reagent were mixed, then gently agitated in a four-well clean CMT plastic paddle which was marked as 1, 2, 3, and 4 to recognize the individual quarter from which the milk was obtained.Finally about10ml of milk sample was collected separately from CMT positive quarters using a universal sterile screw-capped bottle and stored at -200c until processed in the laboratory at Animal Health Institute.

3. California Mastitis Tests

California Mastitis Tests is a qualitative screening test used as an indirect cell counting method using a detergent sodium lauryl sulfate that dissolves cell membrane and nucleus then DNA is released and forms a transient gel with the detergent. It is a simple but very useful technique for detecting subclinical and acute clinical mastitis providing an immediate result (Blowey & Edmondson, 2010; McGuffey et al., 2011). The presence and severity of mastitis were indicated by the degree of gelling and the color changes which show the pH variation of the milk and therefore, the level of inflammation (Sharma et al., 2011; Paul andCassandra, 2012)

4. Bacteriological examination

Milk samples obtained from different stages of lactation were centrifuged at 2000rpm for 5minutes to concentrate microorganisms and to en hance the sensitivity of standard bacteriological c ulture. The sediments were inoculated on Blood and Nutrient agars simultaneously, incubated for 48hrs at 370c. Sub-culture was made in parallel on blood and MacCkey agar plates. Pure colonies obtained on blood agar transferred to Nutrient agar medium and incubated aerobically at 370c for 48hrs, then kept at +40c and used for a series of bacteriological examinations.Isolation and identification of S.aureus was made as per Quinn et al. (2011) protocol. Hemolysis, colonymorphology,pigmentation and cellular morphology were characterized on Blood agar and on grams reactions (Table1).

Table1.Growth features and colony morphology of the isolate on blood agar, and gram reactions using gram staining techniques.

Colony morphology, pigmentation a nd hemolysis on Blood agar	Gram's reaction	Cellular morp hology under Microscope	Presumpti ve isolates	Reference
Colonies with round shape, Shiny g olden yellow pigmentation, clear zo ne of beta hemolysis, growth with s	Gram positive (stained purple)	Coccid shaped in cluster like grape bunches	Staphyloc occus	Simões et al., 2013, Tong et al., 2015
Colonies with whitish gray pigmenta tion alpha, and weak beta hemolysis with smooth consistency	Gram positive (stained purple)	Coccid shaped i n pair others forming chain	Streptoco ccus	Hossain. (2014)

Presumptive identification of the staphylococci was done based on Oxidative-fermentative, Motility, catalase and oxidase tests (Table2).

Table2. Summary of Presumptively identification tests and isolated bacterial pathogens

primary tests	Presumptive isolates		References
	Staphylococcus	Streptococcus	
O-F	F	F	Chakraborty et al., 2011
Motility	non-motile	non-motile	Pollitt et al., 2015
Catalase	Positive	Negative	Eason and Fan, 2014; Karen Reiner 2010
Oxidase	Negative	Negative	Gebrewahid et al.,2012
MacConkey Agar plate	Negative	Negative	Anderson,Cindy (2013)

Keys: O-F = Oxidative fermentative

The catalase test differentiates staphylococci from Streptococcus and the coagulase test identifiesStaphylococcus aureus from the CPS (Eason and Fan , 2014; Santos et al., 2015). Presumed S. aureus using tube Coagulase tests were sub-cultured on Mannitol salt agar (MSA) to increase the capability of detectionthe pathogenand finally cultured on Purple agar base (Kateete et al., 2010; Costa et al., 2011; Peetermanset al., 2015)

5. Antimicrobial susceptibility test for S. aureus

Antimicrobial susceptibility test was done by disc diffusion method, according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2016). Well isolated Staphylococcus aureus colonies were tested for their antimicrobial susceptibility profile. Three to five well isolated colonies were transferred and emulsified into 5ml sterile 0.85% saline water and its turbidity was compared to 0.5 McFarland standard solutions visually. The turbidity of the suspension was adjusted to obtain turbidity optically comparable to 0.5 McFarland standard solutions. After adjusting the turbidity, sterile cotton swab was dipped into adjusted suspension and then inoculated onto Mueller Hinton agar plate by rotating 600. Antimicrobials disc were applied on the media using forceps and then incubated for 24 hrs in upside position. Measurement of zone of inhibition was done by using digital caliper (CLSI, 2016). Nine (9) antimicrobial discs were used: Ampicillin-30µg, Cefoxitin-30µg, Erythromycin-15µg, Vancomycin-30µg, Penicillin-G-10 IU, Tetracycline-30µg, Co-trimoxazole-25µg, Oxacillin-1µg, and Gentamycin-10µg.

Results

All the180 quarters were checked for mastitis using CMT consequently, the prevalence of sub-clinical mastitis was found 35.6% (64/180) where 58.3 % (105/180) teats were CMT negative. 3.9 % (7/180) of the quarters had various types of lesions with alteration of consistency and color of the milk sample, positive for clinical mastitis, and 2.2 %(4/180) teats were found blind. From the total of samples collected from sub-clinical mastitic infections, 46.9% (30/64) was S. aureus. Tests and isolates of subclinical mastitis were summarized in table 3 and table 4 below.

Table3. Summary of tests for the Staphylococcus species

Tests	Isolate (Staphylococc	Remarks
Gram reaction	Gram Positive coccid	
Catalase test	Positive	
Coagulase test	Positive	
Mannitol salt agar	Positive	
Purple agar base (1% maltose)	Positive	

Table4. Bacterial isolates from subclinical mastitis quarter milk samples.

Bacterial isolates	No	%
Staphylococcus aureus	30	46.9
S. intermedius	7	10.9
Genus Streptococcus	9	14.1
Other mixed infections	10	15.6

The antimicrobial susceptibility testing result showed that all Staphylococcus aureus isolates were (100%) susceptible to Erythromycin, Co-Trimoxazole, Vancomycin, Oxacillin and Cefoxitin. However, 22/30 (73.3%), 13/30 (43.3%), 20/30 (66.7%), and 1/30 (3.3%) of Staphylococcus aureus isolates were resistant to Penicillin, Tetracycline, Ampicillin, and Gentamycin respectively. In other hands 5/30 (16.7%), 15/30 (50%), and 7/30 (23.3%) of Staphylococcus aureus isolates were resistant for one drug, two drugs, and three drugs respectively as multidrug resistance in this study (Table 5).

Table 5. Antimicrobial susceptibility test results

Antimicrobial Agents	Resistance No (%)	Intermediate No (%)
Penicillin G 10 IU	22(73.33)	5 (16.67)
Tetracycline 30 µg	13(43.33)	10 (33.33)
Vancomycin 30 µg	-	-
Erythromycin 15 µg	-	-
Co-Trimoxazole 25 µg	-	-
Ampicillin 30 µg	20(66.67)	7 (23.33)
Oxacillin 1 µg	-	-
Gentamycin 10 µg	1 (3.33)	-
Cefoxitin 30 µg	-	-

Discussion

From 64 CMT positive quarter milk samples 87.5% were positive for the known subclinical mastitis microorganisms. The prevalence of subclinical mastitis in this study35.6%(64/180), comparatively comparable with Haftu et al., 2012 who reported 33.8% in Northern Ethiopia, 36.7%, Abera et al, 2013 in Adama and Girma, 20 10 who reported 33.8% in Holeta nevertheless thi s finding was lower than 75.3% report of Duguma et al., 2014 in central Ethiopia, 71.4% of Tafa, et al., 2015.The result of this report was also lower t han 55.8% & 55.1% prevalence of sub clinical ma stitis reported by Bedada and A. Hiko, 2011 and Z eryehun et al., 2013 respectively. The present stud y reveals 46.9% prevalence of S. aureus from sub clinical mastitis relatively comparable with 43.3% , 48.4% and 43.13% prevalence reported by Duguma,, et al., 2014, Garedew et al., 2015 and Mekibib et al.,2010 respectively but higher than 36 % (Haftu ., et al 2012), 39.44% (Sori et al., 2011)and 36.95% (Dabash et al., 2014). The difference in parity, management type, age, milk yields, ecology, and sample or herd size and study designee in many studies might be the reason for variation in the prevalence of the disease. Subclinical mastitis needs emphasis as most of the time is an undetectable an d neglected disease. It remains costly problem in t he dairy industry, causing a long term reduction in milk quality and production and culling of chronically infected cows (Erika M et al., 2012; David J. et al., 2015).Subclinical form of the disease most of the time caused by S. aureus develops to chronic forms forming micro-abscess, fibrosis and occlusion of ducts which makes diffic ult for control by treatments alone(Molalegne et al ., 2010; Zadoks et al., 2011; Tremblay et al.2013).

The antimicrobial susceptibility tests carried out in this study revealed for the high resistance of Staphylococcus species to Penicillin followed by Ampicillin and Tetracycline. The resistance of S. aureus to Penicillin and Ampicillin may be credited to the production of beta-lactamase, an enzyme that inactivates penicillin and closely related antibiotics. This could be associated with the major use of penicillin for treatment of mastitis cases in the area. Other tested isolates were susceptible for rest antimicrobials.

Conclusion and Recommendations

It was indicated that contagious mastitis is associa ted with milking hygienic practice and Staphyloco ccus aureusi is one of the emerging pathogens that cause chronic subclinical infections in dairy cows . Including this study, prevalence of sub clinical mastitis due to S.aureus was indicated in several previous reports with different rate of incidence. These might be because of lack of early detection and awareness of the sub clinical c ases, environmental survivalof the pathogen, capability of invasiveness, and antimicrobial resistance of S.aureus. There is also multidrug resistance development of the tested isolates. Therefore based on this conclusion set from the study, the following recommendations were forwarded.

Awareness creation and early detection of the dise ase is essential for continual and productive dairy production and to implement controlling measures.

Good husbandry management should be practiced.

Avoiding pre-disposing factors to lesions and/or infestation is very important.

Old cows and cows with chronic mastitis should be culled from the farms.

Attention should be given for currently in used antimicrobial drugs.

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