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Full Length Research Paper

Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia

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A cross-sectional study was carried out from November 2008 - April 2009 to estimate the prevalence of mastitis caused by *Staphylococcus aureus*, to assess the associated risk factors and to determine the antimicrobial resistance pattern in Adama town, East Shoa, Ethiopia. From 102 markets oriented small holder dairy farms, a total of 300 lactating cows were tested for mastitis using the California Mastitis Test (CMT). One hundred and forty of the cows (46.7%) had mastitis, of which 10.0% (30/300) and 36.7% (110/300) showed clinical and sub clinical mastitis, respectively. The quarter level prevalence was 29.0% (348/1200); from which the clinical form was 5.4% (65/1200) and the subclinical was 25.6% (283/1200). Of the 65 quarters with clinical cases, 18 had blind teats while 47 had active mastitis. A total of 140 (30 from clinical and 110 from subclinical cases) milk samples were collected and cultured for *S. aureus* of which 59 resulted in growth of the bacterium (10 from clinical and 49 from subclinical cases). Mastitis prevalence showed significant variation among cows of different age groups (p = 0.005), different housing systems (p = 0.000) and at different lactation stages (p= 0.016). Thus, bovine mastitis was more likely to occur in cows above 6 years of age (OR = 3.4, 95% CI = 0.9, 13.7), that were kept in muddy houses (OR = 5.3, 95% CI = 3.2, 8.9) and were at a lactation stage of above 6 months (OR = 3.6, 95% CI = 1.44, 9.03). The results of antimicrobial susceptibility testing revealed that *S. aureus* was highly susceptible to chloramphenicol (100%) followed by gentamycin (91.7%), kanamycin (88.9%) and streptomycin (86.1%). In contrast, isolates were highly resistant to penicillin (94.4%), trimethoprim-sulfamethoxazole (58.3%) and amoxicillin (36.1%). In conclusion, this study confirms the importance of *S. aureus* as a mastitis causing bacterium and identifies risk factors associated with the disease in the Ethiopian setting.

Key words: Mastitis, prevalence, risk factors, *Staphylococcus aureus*, antimicrobial susceptibility test.

INTRODUCTION

Milk is a very nutritional food that is rich in carbohydrate, proteins, fats, vitamins and minerals. However, health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Bradely, 2002). The quality of milk may be lowered by a numbers of factors such as adulteration, contamination during and after milking and the presence of udder infections (Esron et al., 2005). Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2002). Mastitis, inflammation of the mammary gland, is a highly prevalent problem in dairy cattle and is one of the most important threats affecting the world's dairy industry (Wallenberg et al., 2002). *Staphylococcal* mastitis is the commonest and economically the greatest concern wherever dairy farming is practiced. The chief reservoir of this bacterium is an infected udder. The organism is well adapted to survive in

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Abbreviations: CMT, California mastitis test; **NMC,** National Mastitis Council; **NCCLS,** National Committee for Clinical Laboratory Standards.

the udder and usually establishes mild sub clinical infection of long duration. Bacteria are shed into milk from infected quarters (Tsegaye, 1988).

Transmission occurs mainly at milking time through contaminated milking machines, clothes and hands of milkers or machine operators (Radostitis et al., 1994). Clinical signs vary with the severity of the disease and generally include pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase (Miffin, 2004). Bovine mastitis can be clinical with local (in some cases general) clinical signs and milk abnormalities or sub clinical with production losses and lowered milk quality.

In present day of Ethiopia, there is a national drive to alleviate the existing food deficit by devising different agricultural strategies including improvements of the productivity of livestock sector by controlling some of the major infectious disease through regular monitoring. Mastitis, as a disease, has received little attention in Ethiopia, especially the sub clinical form which is mainly caused by *S. aureus* (Mekonnen et al., 2005; Hundera et al., 2005). Efforts have only been concentrated on the treatment of clinical cases. Owing to the heavy financial implications involved and the inevitable existence of latent infection, mastitis is obviously an important factor that limits dairy production. The disease should be studied as it causes financial loss as a result of reduced milk yield, discarded milk following antibiotic therapy, veterinary expense and culling mastitic cows (Hillerton, 1987).

Therefore, the objectives of this study were to estimate the prevalence and identify associated risk factors of bovine mastitis in Adama town and to isolate and identify *S. aureus* from mastitic milk and to conduct *in vitro* antimicrobial susceptibility test on the isolates.

MATERIALS AND METHODS

Study area

The study was conducted in Adama town, East Shoa, Ethiopia, which is located about 100 km south east of Addis Ababa at an altitude of 1650 meter above sea level. Its annual temperature ranges from 13.9^{o}C - 29^{o}C. The mean annual rainfall of the area is 1024 mm. The livestock population of the area in 2004/2002 estimated to be 70,622 cattle, 36,142 sheep, 42,968 goats and 2,193 equine (CSA, 2004).

Study animals

The study animals included 300 lactating cross breed cows from 102 market oriented small holder dairy farms.

Study type

The study was a cross-sectional study in which 300 lactating cows were tested for the presence of clinical and sub clinical mastitis.

Sample size determination

The sample size was determined from the cluster of 102 small holder dairy farms which are found in and around Adama. The sampling frame from the study site indicated that the farms were small holder dairy farms having an average of two to three lactating cows each. Therefore, all the lactating cows from the 102 dairy farms were considered for this study which consisted of a total of 300 lactating cows.

Study methodology

Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions and previous history of mastitis) were collected for 300 lactating cows from farm records when available and by interviewing the farm owner when not. Clinical examination of the udder, screening using the California mastitis test (CMT) and bacteriological examination were also carried out.

Clinical inspection of the udder

Udders of the cows were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. In addition, milk from each quarter was withdrawn and checked for any change in colour and consistency.

California mastitis test (CMT)

The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it was carried out according to procedures given by Quinn et al. (1994). A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples show gel formation within a few seconds. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from +1 -+3. If at least one quarter was positive by the CMT then the cow was considered positive. Therefore, a cow was considered mastitic if one or more quarters were CMT positive with or without isolation of microorganisms.

Milk sample collection

Milk samples were collected according to the National Mastitis Council NMC (1990). After a quarter had been washed with tap water and dried (in cases when there was a considerable amount of dirt to be removed) the teat end was swabbed with cotton soaked in 70% ethyl alcohol. Approximately 10 ml of milk was then collected aseptically from clinical and subclinical (CMT positive) mastitic cows into sterile universal bottles after discarding the first three milking streams. Samples from each quarter were transported on ice to microbiology laboratory of the Faculty of Veterinary Medicine, Addis Ababa University, where they were immediately cultured or stored at 4°C for a maximum of 24 h until cultured on standard bacteriological media.

Bacteriological examination of milk samples

Bacteriological examination was done according to the NMC (1990), Quinn et al. (1994) and National Committee for Clinical Laboratory Standards (NCCLS) (1997). A loopful of milk sample was streaked on tryptose blood agar base enriched with 7%

Table 1. Number and percentage of *S. aureus* isolated from clinical cases and CMT positive subclinical cows.

Forms of mastitis	No examined	No of *S. aureus* isolated	Prevalence (%)	95% CI
Clinical	30	10	33.3	(17.7, 52.5)
Subclinical	110	49	44.5	(35.6, 54.8)
Total	140	59	42.1	(33.8, 50.6)

defibrinated sheep blood (Oxoid, UK) using the quadrant streaking method for each quarter. Blood agar plates were incubated aerobically at 37°C for 24 - 48 h. The plates were examined for gross colony morphology, pigmentation and haemolytic characteristics at 24 - 48 h. Presumptive colonies of *S. aureus* were selected and sub cultured on nutrient agar (Oxoid, UK) and incubated aerobically at 37°C for 24 - 48 h. After this incubation on nutrient agar, bacteria were identified according to their Gram reaction, morphology and the catalase test. *S. aureus* were identified by the tube coagulase test (4 h), haemolysis, pigment production (golden yellow), mannitol and maltose fermentation. Samples were considered positive for *S. aureus* when at least one colony was identified as *S. aureus*.

Antimicrobial resistance pattern test

Antimicrobial susceptibility test was conducted on randomly selected *S. aureus* isolates (n = 36) were isolated during the study. The isolates were tested for 8 antimicrobials using the Kirby-Bauer disk diffusion method (Quinn et al., 1994; NCCLS, 1997). The following antimicrobial disks (Oxoid, Basing Stoke, UK) with their corresponding concentration were used: Streptomycine (S, 10 ∝g), amoxacillin (Am, 2 ∝g), gentamycin (Gn, 10 ∝g), polymyxin B (PB,300 ∝g), penicillin (P, 10 ∝g), chloramphenicol (C,30 ∝g), trimethoprim-sulfamethoxazole (B, 10 ∝g) and kanamycin (K,30 ∝g). The inhibition zone was reported as the diameter of the zone surrounding the individual disk in which bacterial growth was absent. Based on this, the isolates were defined as resistant, intermediate and susceptible are according to the guide lines of the NCCLS (1997).

Data collection and analysis

All the collected data about age, parity, lactation stage, previous history of mastitis in the housing system were recorded. Depending on clinical inspection and CMT results cases were categorized as either positive or negative; positive cases were further categorized as clinical and subclinical mastitis. The age of the study animals was determined from birth records and categorized as young adults (≥ 3 - 5 years), adults (≥ 6 - ≥ 9 years), and old (> 9 years). Parity was also categorized as few (with 1 - 2 calves), moderate (3 - 4 calves) and many (> 4 calves). Lactation stage was classified as early (< 3 m), medium (3 - 6 m) and late (> 6 m); and the housing was categorized as good (house with concrete floor) and poor (house with mud floor). Data related to previous history of the mammary quarters and causes of blindness were obtained from clinical records of the farm and interviews with the owner of the farms. The data were recorded in Microsoft Excel spread sheet for statistical analysis. Logistic regression was used to see the association of the potential risk factors with occurrence of mastitis using Stata 9 statistical software. The final model was fit using step wise logistic regression. The degree of association between risk factors and the prevalence of mastitis were analyzed using odds ratio (OR). In all the analysis, the level of significance was set at 5%.

RESULTS

Prevalence

Of the total 300 lactating cows examined during the study period 140 (46.7%) had mastitis, of which 10.0% (30/300) and 36.7% (110/300) showed clinical and subclinical mastitis, respectively (Table 1). The quarter level prevalence was 29.0% (348/1200); from which 23.5% (283/1200) and 1.5% (18/1200) were found to be of subclinical form and blind teat, respectively. The remaining 3.9% (47/1200) were of a clinical form revealing active cases of mastitis with visible signs of inflammation on the udder and changes in milk quality.

Bacterial isolation

A total of 140 samples were collected and cultured from all the 30 clinical cows (47 teats) and 110 CMT-positive subclinical cows (283 teats). Growth of *S. aureus* was observed from the 33.3% (10/30) and 44.5% (49/110) cows with clinical and subclinical mastitis (Table 2).

Associated risk factors

(Table 3) shows the association between the occurrence of mastitis (both clinical and subclinical) in the selected cows and different potential risk factors. Accordingly, mastitis prevalence showed significant variation among different age groups (p = 0.005), housing system (p = 0.000) and lactation stages (p=0.046). Thus, bovine mastitis was more likely to occur in cows that were above 6 years of age (OR = 3.8, 95% CI = 1.0, 15.0), were keptin muddy houses (OR = 5.4, 95% CI = 3.2, 9.2) and were at a late lactation stage (OR = 3.2, 95% CI = 1.2, 8.2) in comparison to cows that were younger than 6 years of age, were kept in houses with concrete floors and were at the early lactation stage. No association was observed in this study between mastitis prevalence and parity or a previous history of mastitis.

Antimicrobial susceptibility

From a total of 59 isolates of *S. aureus* obtained from the study antimicrobial susceptibility tests were performed on 36 isolates. Due to the relatively small size, no separate

Table 2. Multivariable logistic regression analysis of the association of different potential risk factors associated with subclinical mastitis (n = 300).

Risk factors	Total No	No (%) Positive	Crude OR and 95% CI	Adjusted OR and 95% CI with SWLR	P-value
Age (years)					
3 - 5	101	31 (30.7)	1	1	0.002
6 - 9	179	98 (54.7)	2.7 (1.6, 4.6)	3.3 (1.5, 7.1)	0.051
> 9	20	11 (55.0)	2.8 (1.0, 7.3)	3.8 (1.0, 15.0)	
Parity					
1 - 2	123	48 (39.0)	1	1	0.464
3 – 4	130	67 (51.5)	1.7 (1.0, 2.7)	1.3 (0.6, 2.5)	0.698
>4	47	25 (53.2)	1.8 (0.9, 3.5)	1.2 (0.8, 3.3)	
Lactation stage (m):					
< 3	75	26 (36.7)	1	1	0.046
3 - 6	191	97 (50.8)	1.3 (0.6, 2.6)	1.6 (0.7, 3.5)	0.660
>6	34	20 (58.8)	2.5 (1.0, 6.5)	3.2 (1.2, 8.2)	
Previous mastitis history					
No	288	131 (45.5)	1	1	0.058
Yes	12	9 (75.5)	3.6 (1.0, 13.6)	2.8 (0.6, 10.4)	
Housing					
Concrete	155	45 (29.0)	1	1	0.000
Muddy	145	95 (65.5)	4.7 (2.7, 8.9)	5.4 (3.2, 9.2)	

OR = Odds ratio, CI = confident interval, SWLR = Step wise logistic regression.

Table 3. Resistance of *S. aureus* isolates to different antimicrobials (n = 36).

Antimicrobials	Resistant	Intermediate	Susceptible
	No (%)	No (%)	No (%)
Amoxicillin	13 (36.1)	10 (27.8)	13 (36.1)
Gentamycin	0 (0)	3 (8.3)	33 (91.7)
Streptomycin	2 (5.6)	3 (8.3)	31 (86.1)
Penicillin	34 (94.4)	0 (0)	2 (5.6)
Chloramphenicol	0 (0)	0 (0)	36 (100)
Kanamycin	0 (0)	4 (11.1)	32 (88.9)
Trimethoprim-sulfamethoxazole	21 (58.3)	14 (38.9)	1 (2.8)
Polymyxin B	0 (0)	13 (36.1)	23 (63.9)
Mean	9 (25.0)	6 (16.7)	21 (58.3)

analysis was undertaken for clinical and subclinical isolates of *S. aureus*. In this study *S. aureus* were found to be highly susceptible to chloramphenicol (100%) followed by gentamycin (91.7%), kanamycin (88.9%) and streptomycin (86.1%). However these isolates were highly resistant to penicillin (94.4%) and trimethoprim-sulfamethoxazole (58.3%) followed by amoxicillin (36.1%). The antimicrobial resistance profiles are shown in Table 3.

DISCUSSION

The mastitis prevalence of 46.7% in cows and 29% in quarters reported in this study is in line with some earlier reports of 40% in cows and 19% in quarters by Kerro and Tareke (2003). This report was also in agreement with the assertion by Radostits et al. (2000) that, in most countries and irrespective of the cause, the prevalence of mastitis is about 50% in cows and 25% in quarters. The infection rate in cows was similar to the findings of Abdelrahim et al. (1990), who found a prevalence of 45.8% in Sudan. However, the present findings are lower than the prevalences report in Ethiopia (e.g. 52.8% by Hundera et al. (2005) around Sebeta, 53.35% by Haile (1995) in South Wollo, 53.5% by Tolossa (1987) in Kallu province, 61.11% by Tolla (1996) in South Wollo, 63% by Biru (1989) and 68.1% by Zerihun (1996) in Addis Ababa).

On the other hand, the report of Biffa (1994) in Wolaita Ethiopia was lower (33.0%) than the present study. This variability in prevalence of mastitis between different reports could be attributed to differences in farm management practices or to differences in study methods and instruments employed by the investigators. The quarter infection rate was higher than the 19% prevalence reported by Kerro and Tareke (2003) in Southern Ethiopia; but lower than the 39% quarter infection rate reported by Abdelrahim et al. (1990). The prevalence of subclinical mastitis in this study was 36.7% which is in agreement with 38.2% prevalence reported by Workneh et al. (2002). In the current study the rate of sub-clinical mastitis (36.7%) was higher than that of the clinical mastitis (10.0%) as was reported by Kerro and Tareke (2003) (62.9 versus 37.0% in Southern Ethiopia), Birru (1989) (39.5 versus 23.9%) and Hundera et al. (2005) (36.67 versus 16.11%) in central, Ethiopia. This variation in prevalence between subclinical and clinical mastitis may be due to the fact that, the defense mechanism of the udder reduces the severity of the disease.

The observed higher prevalence of mastitis during early lactation as compared to mid and late lactation stages was in line with the reports by Kerro and Tareke (2003) who also reported the same findings in Southern Ethiopia and this may be due to an absence of dry period therapy and birth related influences. Radostits et al. (2000) suggested that, the mammary gland is more susceptible to new infection during the early and late dry period, which may be due to the absence of udder washing and teat dipping, which in turn may have increased the presence of potential pathogens on the skin of the teat. The increasing prevalence of mastitis with increasing age is in agreement with the findings by Kerro and Tareke (2003), and by Busato et al. (2000) who found that, the risk of clinical and subclinical mastitis increase significantly with the advancing age of the cow. The finding of a high prevalence of mastitis in houses with muddy floors when compared with concrete floor types ($p < 0.05$) shows the prevalence of mastitis is strongly associated with the housing (bedding) type or condition of the farm. A high proportion of *S. aureus* was also isolated from CMT positive cows kept in poor housing (muddy) conditions. This could be because *S. aureus* is environmentally very robust, surviving wide extremes of temperature and moisture. The organism also readily colonizes teat orifices, damaging roughened epithelium (NMC, 1990). The main source of the infection is the udder of infected cows transferred via milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostitis et al., 1994). From 140 milk samples subjected to bacteriological examination 59 (42.1%) isolates of *S. aureus* were isolated. This finding was in agreement with other studies (Adlan et al., 1980; Vaarst and Envoldsen, 1997; Kerro and Tareke, 2003; Hundera et al., 2005; Mekonnen et al. (2005), in which *S. aureus* was the predominant isolate from clinical and subclinical mastitis. The high prevalence of *S. aureus* can most likely be attributed to the wide distribution of the organism inside mammary glands and on the skin of teats and udders (Jones et al., 1998). *S. aureus* has adapted to survive in the udder and establish chronic and subclinical infections. From there it is shed into the milk, which serves as a source of infection for healthy cows during the milking process (Radostitis et al., 1994). Of the 1200 quarters examined, 18 were blind, which may be an indication of a serious mastitis problem on the respective farms and of the absence of a culling programmed that can serve as a means to remove a source of this mammary pathogens for other cows.

The antimicrobial susceptibility tests carried out in this study indicated the existence of susceptibility and resistance of *S. aureus* to some of the antimicrobials. The average susceptibility (69.4%) of *S. aureus* strains to all antimicrobials tested in this study is in agreement with the existing reports of 62.7% by Mekonnen et al. (2005) in Ethiopia and Myllys et al. (1998) in Finland.

The result of the present study shows that *S. aureus* isolates were resistant to penicillin (94.4%) and Trimethoprim-Sulfamethoxazole (58.3%) followed by amoxicillin (36.1%) and this is comparable with the higher reported resistance of 75 and 83% to ampicillin by Corrales et al. (1995) and Mekonnen et al. (2005), respectively. The resistance of *S. aureus* to penicillin and ampicillin may be attributed to the production of beta-lactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis causing *S. aureus* strains produce beta-lactamase (Green and Bradely, 2004).

The present study has demonstrated the existence of alarming levels of resistance of *S. aureus* to commonly used antimicrobial agents in the study farms and the results are in accordance with reports from earlier studies in other countries (Edward et al., 2002; Gentilini, 2000) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

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