



Study on Bovine Mastitis with Isolation, Identification and Antimicrobial Resistance Patterns of Streptococci Species from Raw Milk in Bishoftu Town, Ethiopia

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Abstract

Mastitis is the most complex and costly disease of dairy cows occurring throughout the world including Ethiopia. A cross-sectional study was conducted from December 2017 to April 2018 with the objectives to isolate, identify and determine antimicrobial susceptibility of Streptococci species in milk from dairy cows with diagnosis of subclinical mastitis, in Bishoftu town, Oromia, Central Ethiopia. A total of 55 milk samples were collected from California Mastitis Tests positive dairy cows. Out of 55 samples taken, 15 samples were positive for bacteriological examination of streptococcal species with the isolation rate of 2(13.3%) *Streptococcus agalactiae*, 6(40%) *Streptococcus uberis* and 7 (46.7%) *Streptococcus dysgalactiae*. Furthermore, the results of antimicrobial susceptibility testing revealed that isolated Streptococci species were highly susceptible to Norfloxacin (86.7%) and Nalidixic acid (40%). However, the isolates were resistant to penicillin (66.7%) and streptomycin (73.3%). Thus, it could be concluded that bovine mastitis due to the Streptococci species, is a major challenge to the dairy producers in Bishoftu town. Moreover, appropriate control and preventative measures must be instituted and dairy farmers and workers must be trained on proper milking and hygiene practices in order to reduce the prevalence of mastitis in the study area. The penicillin and streptomycin-resistant Streptococci species could be a source of serious infection in humans and hence comprehensive studies including molecular characteristics of the drug resistance gene of Streptococci species should be conducted since dairy cattle might serve as a reservoir of infection for humans.

Keywords: Antimicrobial Susceptibility; CMT; Dairy; Isolation; Mastitis; Streptococci species

Introduction

Ethiopia is believed to have the largest livestock population in Africa. The livestock sector has been contributing a considerable portion to the economy of the country and still promising to rally round the economic development of the country [1]. However, due to various reasons, the country does not benefit from this sector in terms of products such as milk, meat, and etcetera [2].

Mastitis is a complex inflammation of the mammary gland that generally interaction between management practices. In addition, mastitis has different causes, different degree of intensity and variation in duration with different sequelae [3]. Mastitis occurs worldwide among dairy animals and it has been described to have an extreme economic impact. According to

different research outputs, dairy cattle mastitis contributes up to 70% of reduced milk production, 9% of discarded milk after treatment, 7% of the cost of veterinary services and 14% of premature culling. The disease can be classified as clinical or subclinical [4,2].

Generally, bovine mastitis often follows a number of major factors involving the cow, the pathogen and the environment. The general predisposing factors influencing the occurrence of mastitis are hygiene, milking equipment, and techniques, housing conditions, breed, level of production, the shape of the udder and teats, season and bacteria present in the environment [5]. The disease is caused by a multitude of etiological agents that includes bacteria, virus, fungi, and algae [6,7]. The most common bacterial pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, other Streptococcus species and coliforms [8]. Other organisms may also include *Arcanobacterium pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens*, Mycobacterium, Mycoplasma, Pasteurella and Prototheca species and yeasts [5].

The disease in dairy herd is of great economic importance due to a reduction in the milk yield, the change in milk quality, the possibility of permanent change to one or more quarter, or even to the entire udder and death of the cow as well as loss due to increased premature culling rate and cost of treatment [9]. Mastitis has also an effect on the health and wellbeing of the calves. Infectious agents particularly various species of bacteria, viruses [7], and fungi [10] are the most important etiologic agents of mastitis [11].

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Streptococci are among the major mastitis pathogens which have a considerable impact on cow health, milk quality and productivity [12]. *Streptococcus agalactiae* is causing contagious mastitis, an obligated pathogen of the mammary gland, which is transmitted directly among cows during milking [13]. It infects the gland cistern and ducts of the mammary gland causing irritation; swelling and subclinical mastitis [14]. *Streptococcus dysgalactiae* is described as alpha-hemolytic and associated only with Intramammary Infection (IMI) among the environmental streptococci. Besides, *Streptococcus dysgalactiae* is one of the most prevalent that may infect mammary glands as favorable conditions arise [15]. *Streptococcus uberis* is also another important udder pathogen in the modern dairy industry [16].

According to one meta-analysis report of Getaneh and Gebremedhin, bovine mastitis was a serious problem for the dairy sector in Ethiopia. Besides, based on their evaluation on 39 different studies, the overall prevalence of bovine mastitis was 47.0% and higher proportion of subclinical mastitis (37%) was the most predominant challenge for the dairy farms as compared to the clinical mastitis (8.3%) [17].

Accordingly, research in mastitis to determine the etiology and testing for antimicrobial susceptibility is the core to treatment, prevention, and control strategies, as well as to save economic loss from milk, milk product and culling [18]. Therefore, the objective of this research was to isolate, identify and to determine antimicrobial susceptibility of streptococci species from Mastitic dairy cows in selected dairy farms of Bishoftu town in Ethiopia

Methods and Materials

Study area

The study was conducted in selected dairy farms of Bishoftu town based on the abundance of dairy farms in the area from December 2017 to April 2018. Bishoftu is located 45 km southeast of Addis Ababa. The area is located at 9°N latitude and 40°E longitude at an altitude of 1850 meters above sea level (masl) with an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 4°C, respectively, with a mean relative humidity of 61.3% [19]. The domestic animals raised in the area are 91,040 cattle population which takes the first rank followed by 39,055 goats, 39,048 sheep, 22,676 donkeys, 6,136 horses, and 2,015 mules [20].

Study animal: Animals included in the study were lactating dairy cows of crossbreed (Holstein-Friesian × Boran) from selected dairy farms of Bishoftu town, Oromia, central Ethiopia.

Study design and Sample size determination: A cross-sectional study was conducted from December 2017 to April 2018. A total of 55 dairy farms from different management systems were selected and assessed based on willingness and cooperation. In addition, 55 milk samples were collected from the individual lactating cows through systematic random sampling. The sample size was determined based on the formula given by [21].

Hence, the expected proportion of apparently healthy dairy cow shedding Streptococci species is set at 50% (P₁) while

the proportion of subclinical mastitic dairy cows expected is estimated at 80% (P₂). The power of the study is set at 80%, 95% confidence interval and a significance level of P<0.05 was used.

$$\text{i.e. } N \geq \frac{(Z_1 - \alpha/2 + Z_1 - \beta)^2 [P_1(1 - P_1) + (P_2(1 - P_2))]}{(P_1 - P_2)^2}$$

Where $Z_1 - \alpha = 50\%$ significance level

$Z_1 - \beta = 80\%$ power level

P₁ = Expected proportion in apparently healthy dairy cow

P₂ = Expected proportion in sub clinical mastitic dairy cows

$$N_1 \geq \frac{7.89 \times [0.5(1 - 0.5) + 0.8(1 - 0.8)]}{(0.5 - 0.8)^2}$$

N₁ ≥ 36, the total sample required is at least 36 dairy cows. However, during the study period, a total of 55 milk samples were taken to increase the recovery rate of the isolate.

Study methodology

Clinical examination of the udder: The udders were examined visually and then palpated to detect any possible fibrosis, inflammatory swelling, and atrophy of the tissue by a veterinary practitioner. The size and consistency of the mammary quarter were inspected for the presence of any abnormalities such as disproportional symmetry, swelling, firmness, and blindness of the teat canal. In addition, two streaks of milk from each quarter in a strip cup were inspected visually for the presence of any flakes, clots, pus, watery appearance, blood and color change [5].

Sample collection, handling, and storages: Milk samples were collected following the standard procedures by the national mastitis council [22]. After a quarter had been washed with tap water and dried (in cases when there was a considerable amount of dirt), the teat end was swabbed with cotton soaked in 70% ethyl alcohol. Approximately 10 ml of milk sample was then collected aseptically from clinically and sub-clinically (CMT positive) mastitic cows into sterile universal bottles after discarding the first milking streams (squirt). The bottles were labeled with a permanent marker and transported on packed icebox to the microbiology laboratory of the college of veterinary medicine, where they were immediately cultured or stored at 4°C until processed for bacterial isolation.

California Mastitis Test (CMT): After the physical examination, milk samples were tested by the California mastitis test. Briefly, a squirt of milk from each quarter of the udder was placed in each of four 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 California Mastitis Test (CMT) is a simple cow-side indicator of the somatic cell count of milk [23,24]. The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it operates by disrupting the cell membrane of any cells present in the milk sample, allowing the DNA in those cells to react with the test reagent, forming a gel and this occurs after mixing CMT reagent and milk in equal proportions (1:1).



Accordingly, the results were scored as 0 (negative), 1 (weakly positive), 2 (distinct positive) and 3 (strongly positive). Milk samples with a test result of CMT 1 to 3, were classified as evidence of subclinical mastitis [5]. If at least one quarter was positive by the CMT then the cow was considered positive for mastitis.

Bacterial isolation and identification: All aseptically collected milk samples were inoculated on sheep blood agar (OXOID) and incubated aerobically at 37°C and cultured plates were examined after 24-48hr of incubation for any visible growth. Firstly, identification of the bacteria was performed by appreciating the growth on blood agar plate. Then followed by colony morphology gross examinations (colony size, shape, and color), hemolysis (presence or absence and type of hemolysis), gram stain staining (Gram-positive or negative, bacterial shape, structure, arrangement), then gram-positive culture on nutrient agar which then were incubated aerobically at 37°C for 24 to 48 hours for growth of bacteria. Then, the colonies were sub-cultured in selective media such as Edward's media to identify Streptococcal species. Moreover, the colonies were identified using primary and secondary biochemical tests at least to determine the genus of the suspected isolate [25] Table 1.

Antibiotic susceptibility test: The antibiotic susceptibility tests of the Streptococci isolates were performed according to the National Committee for Clinical Laboratory Standards (NCCLS) method using the Kirby-Bauer disk diffusion test on Muller-Hinton agar. Reliable results can be obtained with disk diffusion tests that use standardized methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behavior of strains among clinically susceptible and resistant categorizations [26]. The performance standards of the eleven selected antibiotics (Penicillin, Nalidixic Acid, Streptomycin, and Norfloxacin) were indicated in Table 2.

Pure colonies on nutrient agar were taken with a wire loop and transferred to a tube containing 5 ml of Saline water and emulsified. The broth culture was incubated at 37°C for 4 hours until it achieved the 0.5 McFarland turbidity standards. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller- Hinton agar plate within a sterile safety cabinet. The plates were held at room temperature for 15 minutes to allow drying. Antibiotic discs with a known concentration of antimicrobials were placed and the plates were incubated at 37°C for 24 hours. The measurement of the zone of inhibition was done by using a digital caliper and was interpreted as susceptible, intermediate and resistant based on the diameter of the zone of inhibition of individual antibiotic agents.

Data analysis

Data collected from field and laboratory investigations were recorded, screened and coded using Microsoft Excel 2013 program and analyzed using STATA version 13.0 software. Descriptive statistics were used to figure out the proportions of Streptococci species isolate. Antibiotic efficacy was determined by comparing the zone of inhibition of each drug with the standard.

Table 1: Summary of Culture characteristic and Biochemical tests used as reference for identification.

Biochemical test	<i>Streptococcus agalactiae</i>	<i>Str. Dysgalactiae</i>	<i>Str. uberis</i>
Hemolysis	Beta	Alpha	Gamma
Catalase test	Negative	Negative	Negative
Oxides	Negative	Negative	Negative
KOH	Positive	Positive	Positive
Edward media	Positive	Positive	Positive
CAMP test	Positive	Negative	Negative
Gram stain	Positive cocci	Positive cocci	Positive cocci
Colony clear	Slightly blue	Green	Grey

KOH: Potassium Oxide; CAMP: Christie Atkins Munch, and Peterson
Source: (Vandepitte et al., 2003)

Table 2: Performance Standards for Antibiotic Susceptibility Testing.

Antibiotic disc	Disk potency (µg)	Resistance (≤)	Intermediate	Susceptible (≥)
Nalidixic Acid	30	13	14-18	19-37
Penicillin	10	19	20-27	15
Streptomycin	10	11	Dec-14	15
Norfloxacin	10	12	13-16	17

Results

Streptococci Species isolation and identification

The results of the present study revealed that out of 55 samples, 15 samples were found to be positive for Streptococci Species. Isolates of *Streptococcus agalactiae* were showing β hemolysis on Blood agar plates (Figure 1A) and showed enlarged the area of β-hemolysis formed by *Streptococcus agalactiae* by CAMP test (Figure 1B). Upon Gram's staining of the isolates under 100x using light microscope, purple-colored, small cocci-shaped organisms arranged in single, pairs or short-chain were identified (Figure 1C).

Proportion of Streptococci Isolates from different farms

In the present study, a total of fifty-five milk samples were collected from Bishoftu, Geneses, and field dairy farms and the milk sample was passed through the CMT screening test to assess the status of subclinical mastitis. Out of the total, a higher proportion (29.2%) of streptococcal species are isolated from Genesis and farm different farms located in Bishoftu followed by field farms (14.3%) Table 3.

According to the present study, different streptococcal species were isolated from different farms with a proportion of Streptococci agalactiae (13.3%), *Streptococci dysgalactiae* (46.7%), and Streptococci uberis (40.0%) Table 4.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the current study revealed that 86.67% and 40% of total isolates were susceptible

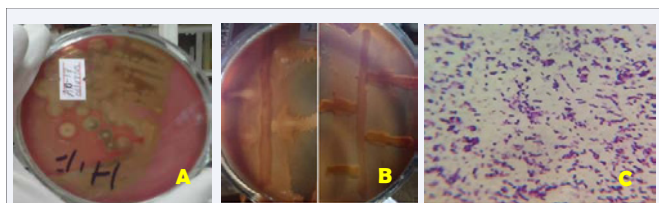


Figure 1 β hemolysis on blood agar (A) and CAMP test (B) and Gram-positive cocci Streptococci species (C).

Table 3: Overall isolation rate of Streptococci Species from selected farms in the study area.

Farm	Total examined animals	Streptococci Positive
	N=55	N (%)
Bishoftu	24	7 (29.2)
Field farm	7	1 (14.3)
Genesis	24	7 (29.2)

to Norfloxacin and Nalidixic acid respectively, which was followed by penicillin and streptomycin with susceptibility rate of 33.33 % and 26.67%, respectively Table 5.

Discussion

In the current study, a total of Fifty five raw milk samples were collected and processed bacteriologically and biochemical tests were performed to detect streptococcal species from mastitic milk samples. All the Streptococcal species isolates were able to show β hemolysis on Blood agar plates, characteristic enlarged the area of β -hemolysis formed by *Streptococcus agalactiae* by CAMP test and purple-colored, small rod-shaped Gram-positive cocci on Gram's staining. Besides, all streptococcal isolates were catalase-negative and KOH positive but the hemolysis and the colony character varies depending on the species which are in agreement with the reports of [27,25].

Besides, in the present study out of 55 samples, Streptococci species were isolated from 15 of the milk samples after screened with CMT test and these shows streptococcal are the cause of sub mastitis in the dairy farms of the study area. A similar finding was reported by [28,29] who reported streptococci infection were cause of sub-clinical mastitis and major problems in the dairy farms

Laboratory results indicated that from 55 milk samples subjected to bacteriological examination 46.67% were isolates of *Streptococcus dysgalactiae*, 40% were isolates of *Streptococci uberis* and 13.33% were isolates of *Streptococcus agalactiae*. A higher proportions of *Streptococcus uberis* in the present study were comparable with [30] who reported 49.98% from Pakistan but disagreed with [31] who reported 12.1% at Holeta district, but much higher than 4.23% by [32], 1.48% by [33] in and around Bahir dar and (6.53%), by [34].

Similarly, a higher finding of *Streptococcus dysgalactiae* in the present study disagreed with [35] who reported 24% in and around Ereka town. The higher proportion of *Streptococcus*

dysgalactiae isolates in current study area might be due to lack of inter-cow hand washing and disinfection in the milking area and contaminations of milkers' hands and this facilitates the spread of mastitis in the present study and this finding was in agreement with the previous study by [35] who reported similar reasons for the higher spread of *Streptococcus dysgalactiae* between cows within dairy herds may occur directly or by way of the milking machine or environment. The lower findings (13.33%) for *Streptococcus agalactiae* in the current study disagreed with [33] who reported a higher isolation rate (27%) for *Streptococcus agalactiae*.

Streptococcus agalactiae survives poorly outside the udder, and established infections are eliminated by frequent use of penicillin and other antibiotics. The current study on antimicrobial sensitivity test revealed that Gentamycin was the first line effective antibiotic. The finding of the present study is in agreement with the report of [36] who reported Gentamycin was the most effective antibiotic of the total isolates found to be susceptible because these drugs were the least frequently used. Moreover, the present findings showed highly susceptible to Nalidixic acid followed by Norfloxacin, however, penicillin and streptomycin were not as good as a drug of choice. In this study, marked susceptibility *Streptococcus agalactiae* to penicillin coincided with [36].

Conclusion and Recommendations

This study showed a high percentage of bovine mastitis mainly due to the subclinical type and is a major health problem of dairy cows in the study farms. Both contagious and environmental pathogens such as *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* were among the isolated bacteria. Of this, *Streptococcus dysgalactiae* were the predominant isolates. Moreover, antimicrobial susceptibility testing of the isolates revealed that Norfloxacin and Nalidixic acid were the most effective antibiotics, and could be the drug of choice whereas penicillin and streptomycin were found to be poor in their efficacy to the major of the isolates. Bovine sub-clinical mastitis is an important problem and a serious threat to the dairy industry in the study area. Thus, proper husbandry practice, regular cleaning of the cows, proper milking practices and milking of infected cows after apparently healthy animals

Table 4: The frequency of streptococcal isolates.

Positive Bacterial Isolates (N=55)			Total
<i>Str. agalactiae</i>	<i>Str. dysgalactiae</i>	<i>Str. uberis</i>	
2(13.3%)	7(46.7%)	6(40%)	15(100%)

Table 5: Frequency of the antimicrobial susceptibility test result.

Antimicrobials tested	Isolates of streptococcal Species (N=10)		
	S	I	R
Nalidixic Acid	6(40%)	5(33.3%)	4(26.7%)
Penicillin	5(33.3%)	-	10(66.7%)
Streptomycin	4(26.7%)	1(6.6%)	10(66.7%)
Norfloxacin	13(86.7%)	2(13.3%)	-



should be practiced. Furthermore, there should be a regular mastitis checkup. Moreover, further serotyping and molecular techniques are needed to identify the isolate to the strain level.

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