Original Research Article

Isolation, Identification and Antimicrobial Susceptibility Test of the Most Important Bacterial Pathogens from Subclinical Mastitis in Western Tigray, Ethiopia

Abstract

A cross sectional study was carried out in Western Zone of Tigray, North West Ethiopia with the objectives of identifying the etiologies of mastitis and their antimicrobial drug susceptibility test. From 122 CMT positive quarter samples examined, 114(93.44%) were found culture positive for one or more bacterial species. From the species of bacteria isolated Staphylococcus aureus with a prevalence of 57(42.43%) and Staphylococcus epidermidis with a relative prevalence of 47(33.07%) were the first and second predominate isolated bacterial species found in this study respectively. The third predominant bacterial species isolated in this study is Enterobacter aerogen with relative prevalence of 10(7.46%) whereas proteus Spp. with 7(5.22%) prevalence Escherichia coli 5(3.73%), Klebsiella pneumonae 4(2.99%) and Psedomonas aerogenosa 4(2.99%) were with the lowest prevalence of bacterial agent identified. Of total isolated bacterial species, 38 isolate of three bacterial species were tested for susceptibility to six antimicrobial discs. Higher resistance to penicillin (100%) and 50% susceptibility to Tetracycline was observed in case of Staphylococcus aureus. In Staphylococcus epidermidis, it was observed a 100% resistance to penicillin and 50% susceptibility to Chloroamphencole. Moreover, 100% resistance to Penicillin and Vancomycin was found in Escherichia coli. Lack of strategic control measures and improper attention to the health of the mammary glands contribute to the higher infestation rate and drug insusceptibility. Based on the findings of the present study, avoiding indiscriminate use of antimicrobial therapies and regular antimicrobial susceptibility test of bacterial pathogen should be conducted.

Keywords: California Mastitis Test; etiologies, drug susceptibility, teats

1. Introduction

Ethiopia is believed to have the largest livestock population in Africa [8]. Data from the estimation of [9] indicates that the country is a home for about 60.39 million cattle, 31.3 million sheep and 32.74 million goats, 11.2 million equines, 1.42 million camel, 56.1 million poultry with immense bee and fishery resources and Tigray region accounts for 4.82 million cattle, 2.47 million sheep, 4.3 million goats, 0.91 million equines, 0.43 million camel and 6.19 million poultry of the country. Dairy production is one of the sectors of livestock production in many parts of Ethiopia [34] and makes a major contribution to national and household economies. Despite Ethiopia have large dairy cows' population, the national and regional milk supply is low compared to its potential due to lower genetic potential, lack of market, inefficient animal nutrition and different disease of animals in which mastitis is the disease among the fore mentioned disease in dairy cow production [34 and 1]. Mastitis is the most prevalent infectious disease of adult dairy cows and the infection is possibly developed when the cow is lactating or dries off [3]. Mastitis cause a great deal of reduction in productivity, influence the quality and quantity of milk yield, cause culling of animals at an unacceptable age, distort animal welfare and cause death of animals.

Although mastitis caused by more than twenty different groups infectious agents including bacteria, viruses, yeast, fungi and rickettsia, bacterial pathogens have the greatest share of that organism causing mastitis [6]. The bacteria that cause mastitis in dairy cows are classified in to contagious and environmental bacteria. The most common contagious bacteria mentioned in the literature are *S. aureus, Stre.agalactiae,* and *Str. dysgalactiae* and environmental bacteria are coli forms (e.g. *E.coli* and Klebsiella spp.), *Str.uberis,* and Enterococci spp. Other bacteria of importance are including *S.epidermidis, Actinomyces pyogenes*, *P.aeruginosa*, *Nocardia asteroides, Clostridium perfringens,* Mycobacterium, Mycoplasma, Pasturella and Prototheca species and yeasts are included [23, 28 and 30].

Mastitis was prioritized as one of major disease of dairy cows worldwide [4]. The incidence and distribution of the disease causing organisms has not been studied systematically in the study area. Therefore, the objective of the present study was to identify the etiological agents and conduct antimicrobial drug susceptibility test of the identified important bacteria.

2. Material and Methods

2.1. Description of study area

The study was carried out in Western Zone of Tigray Region, North West Ethiopia. It is surrounded with Tahtay Adibayo, Tselemti and Asgede Tsimbla districts in the East, Sudan in West, Amhara region in South and Eritrea in the North and consists of three agro-ecological zones which comprised of 75% low land, 15.7% mid land and 9.3% high land. It is located at a distance of 580 to 750 km North West of Mekelle the capital city of Tigray and covers an area of 1.5 million hectare [13]. The geographical location of the zone is at altitude 13°42′ to 14°28′ north and 36°23′ to 37°31′ east. The minimum and maximum range of rainfall and temperature are 600 to 1800 mm and 27 0c to 45 0c in the lowland areas respectively whereas 100c to 220c temperature range both in midland and highland areas. The altitude of the zone ranges from 500 to 3008 m.a.s.l. and livestock production is the predominant economic activity with about 95% of the total population engaged directly or indirectly [19]. Main cattle breeds raised are the local Arado (in both high land and mid land) and Begait cattle (in lowland). Semi-intensive production system is practiced in urban kebelle of the zone while extensive production system is dominant in all districts. The main crops cultivated in the lowland areas of the zone are Sesame, Cotton and Sorghum while Teff, Wheat, Barley, Noug, Lentils, Finger millet, Field peas and Faba beans are cultivated crops in both mid land and high land areas of the zone [31].

2.2. Study animals

The study animals that had been sampled were lactating smallholder dairy cows of different herds with different calving history. The study included 355 small holders lactating dairy cows which were indigenous local Arado (n=160), Begait breed (n=170) and cross breed (n=25) managed under the traditional extensive and semi intensive system of production.

2.3. Study design

A cross sectional study was carried out from November 2017 to June 2018. During the beginning of the study community identification and assessment was completed and list of study kebeles were done purposively based transport facility. The number of representative sample animals was proportionally allocated to the selected peasant associations based on the number of dairy cattle population and simple random sampling technique was used to select the study animals.

2.4. Sample size

Since there was no reasonable research in the study area so far, the expected prevalence was assumed to be 50%. The required sample size was determined based on the assumption of expected prevalence of 50% and by the formula given by [33] and the study was considering 95% confidence interval and 5% of absolute precision.

$$\frac{n = 1.96^2 Pexp\ (1 - Pexp)}{d^2}$$

Where, n= sample size

1.96= the value of Z at 95% confidence interval

Pexp= expected prevalence (50%)

d= desired absolute precision (5%)

Therefore, the calculated sample size was 384 samples due to various inconveniences 355 lactating dairy cows were taken.

2.6. Milk sample collection

Milk sample was collected by strict aseptic procedure to prevent contamination with microorganisms. Teat ends were cleaned and disinfected first using 70% ethyl alcohol before sampling. First jets milk was discarded to reduce the number of contamination of teat canal. Sterile universal bottle with tight fitting cups were used. The universal bottle was labeled with permanent marker before sampling. To reduce contamination of teat ends during sample collection, the near teats were sampled first and then followed by the far ones [24]. Then, 10ml milk sample was collected to the sterile universal bottle from CMT positive quarter, placed in racks for ease of handling and transported in ice box to Mekelle Regional Veterinary Laboratory and samples were stored at 4^{o}C until cultured for isolation.

2.7. Bacteriological isolation

Identification of mastitis causing pathogens was carried out following the standard procedures for diagnosis of bovine mastitis described by [25]. According [22] bacteria may be concentrated in the cream layer of refrigerated milk sample and held within clumps of fat globules. Hence milk samples that had been refrigerated were warmed at room temperature (25^{0}c) for about an hour and shake it in order to made dispersion of bacteria and fat and the samples were allowed to

stand for a while for the foam to disperse before inoculation. Loopful of milk sample was streaked on 7% sheep blood agar and plates were incubated aerobically at 37°C and the plates checked for growth after 24, 48 and up to 72 hrs to rule out slow growing bacteria pathogen. The culture was considered negative if no growth occurs after 72 hrs. For the primary identification colony size, shape, color, hemolytic characteristics, pigment production, catalase production and oxidase test was conducted. Staphylococcus species were identified based on growth characteristics on Manitol salt agar, pigment production on blood agar, maltose fermentation and Coagulase test. Gram negative bacterial isolated that had been growth on MacConkey agar were identified to their species level by oxidase reaction, catalase test, Triple Sugar Iron(TSI), Urease test and the ″IMViC″ (indole, methyl red, vagesproskaur, and citrate test) [24].

2.8. Antimicrobial sensitivity test

In vitro antibiotic sensitivity test was carried out on the most important mastitis causing bacteria's. Six antimicrobials agent such as Chloroamphinicol, Penicillin, Vancomycin, Tetracycline, Gentamycin and Ciprofloxacin were tested by Kirby-Baur disc diffusion method. A loop full of colony from the growth of isolates was transferred to the nutrient broth in tubes and incubated at 37°C for 5hrs. Mueller-Hinton agar which was used as plating medium was inoculated with broth (bacterial suspension) by using sterile cotton swab. Then antibiotic disks were applied and pressed onto the plate with forceps on the surface of the inoculated agar plates using aseptic technique. Plates were incubated at 37°C for 18hrs. The diameters of zones inhibition was measured and interpreted as sensitive, intermediate and resistant to different antibiotics according to national committee for clinical laboratory standard (NCCLS) [26].

2.9 Data analyses

The collected raw data was entered into Microsoft Excel data sheets and analyzed using STATA 11.1 statistical software. Descriptive statistics and percentages was summarized the result.

3. Results and Discussion

3.1. Bacteriological examination

With regard to the bacteriological isolation of the milk sample, out of 122 CMT positive sample used for bacteriological culture, 114(93.44%) sample were found culture positive for single or

more bacterial species and 8(6.56 %) CMT positive collected milk samples were found culture negative (Table 1). The result of the present study is lower than the report of [5] who reported (9.2%) negative culture, [18] who reported 15(10%) and [32] who reported (9.82%) culture negative milk sample. The failure to isolate bacteria from the CMT positive milk samples could be associated with spontaneous elimination of the infection, low concentration of pathogen in milk, intermittent shedding of pathogen and intracellular location of pathogens and presence of inhibitory substance in milk [29]. It might also be due to some cases of delayed healing of the infection from which organisms may have disappeared or reduced, while infiltration of leukocytes continued until complete healing [32] and death of the bacteria prior to culture due to delay of culture after the sample was collected [36].

Table 1: Proportion of cultured samples to positive culture

CMT positive quarter	Cultured sample	Positive culture	% positive culture
144	122	114	93.44

From the species of bacteria isolated *S. aureus* was the highest isolated which accounted 57(42.43%) of all isolate (Table 2). The finding of high level isolation rat of *S. aureus* in present lower than the report of [15] who found a prevalence of 54.4% *S. aureus* around Areka town, Southern Ethiopia and related with the finding [14] who reported 39.4%, [32] who reported 44.03% in and Around Sebeta, Ethiopia. The high prevalence of this organism may be associated with its frequent colonization of teats as they are commensals of the skin, its ability to intracellular persistence and localize within micro abscesses in the udder and hence resistant to antibiotic treatment [16]. The bacteria usually establish chronic, subclinical infections and are shed in the milk which serves as a source of infection for other healthy cows during the milking process [27 and 21]. Transmission among cows increase whenever there is lack of effective udder washing and drying, post- milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups from infected quarters [27]. However, the incidences of the *S. epidermidis* are second predominate isolated bacterial species in this study with a relative prevalence of 47(33.07%) (Table 2). The result of the present study is higher than the report of [18] who reported 2(1.33%), [32] reported 20(14.93%), [15] reported a prevalence of 21(8.4%) and [11] who reported 8(11.27%) in Adis Abeba City Ethiopia but, lower than the report of [21] who repot of 51.9% in Bahir Dar and its environs Amhara, Region.

The high isolation rate of *S. epidermidis* in this study could be associated with its characteristics of chronicity as all isolated were found from sub-clinical mastitis. The highest isolation rate of *S. epidermidis* from milk sample collected from bovine and other dairy animals which had mastitis, indicated that they could be pathogenic even causes more mastitis than *S. aureus* as reported by [35].The third predominant bacterial species isolated in this study is, *E.aerogen* with relative prevalence of 10(7.46%) (Table 2). The finding of the present result is higher than the report of [32] who reported 2(1.33) relative prevalence of *E. aerogen* in Dairy Farms of Holeta Town, Central Ethiopia. *E.coli* is the other coli form bacterial species isolated with 5(3.73%) prevalence in this study which is in consent with the result of [6,17, and 20] who reported 4.60%, 3.64% and 3.14%, respectively from different parts of Ethiopia and less than the report of [2] who reported 47(39.5%). *K.pneumonae* account 4(2.99%) prevalence which is in agreement with report of [12] who reported 3(2.5%) in West Harerghe zone, Doba district and 3.30% infestation rate of by [20]. *P.aerogenosa* 4(2.99%) prevalence reported in this study also less than [12] who reported relative prevalence of 5(4.1%) whereas proteus Spp. with 7(5.22%) prevalence reported in this study higher than [10 and 12] who reported 2(2.7%) and 1(1.9%) prevalence respectively (Table 2).Characteristics of pathogens, environmental condition and level of management to risk factor which influence the prevalence of mastitis could be the reason for these differences in occurrence [29].

Table 2: The relative rate of bacteria species isolated from bovine subclinical mastitis

Bacterial Species	Frequency (%)	Prevalence (%)
Staphylococcus aureus	57	42.43
Staphylococcus epidermidis	47	35.07
Proteu spp.	7	5.22
Escherichia coli	5	3.73
Enterobacter aerogen	10	7.46
Klebsiella pneumonae	4	2.99
Psedomona saerogenosa	4	2.99
Total	134	100

3.2. Antimicrobial sensitivity test

Of total isolated bacterial species, 38 isolate of three bacterial species were tested for susceptibility to six antimicrobial discs. In the current study, *S.aureus* is found susceptibility for Vancomycin (92%); Gentamycin (92%); Ciprofloxacin (83%) and Chloroamphinicol

.Susceptibility to Vancomycin (100%), Gentamycin (80%), Ciprofloxacin (100%), tetracycline (80%) and Chloroamphinicol (50%) was found in case of *S. epidermidis* (Table 3).The report of the present result is similar with report of [7] who reported *S.aureus* and *S.epidermidis* are more sensitive to Gentamycin (100%), Chloroamphencole (100%), and Vancomycin (100%). In the present study, resistance to penicillin (80%) and intermediate to tetracycline (50%) in case of S.*aureus* and (80%) and (50%) resistance to penicillin and Chloroamphencole were obtained in *S.epidermidis*. *E.coli* is major mastitis causing microorganism and ubiquitous in the environment of dairy cows with manure is major source. The antimicrobial susceptibility test of the organism in the present study is susceptible to Chloroamphencole (100%), Ciprofloxacin (100%), Gentamycin (75%) and tetracycline (75%) but, highly resistant to Penicillin (100%) and Vancomycin (100%) (Table 3).The highly resistance to those drug found in the present study is probably due to the indiscriminate and inappropriate doze use of those drugs by animal owners in the study zone, since the farms studied were mainly found in the rural area where veterinary services are not adequate, most farmers use treatment on their own as indicated by animal owners.

Table 3: Antimicrobial sensitivity test

Bacterial		C30		P10		CN10		VA30		CIP5		Te30	
Isolates	N	S	R	S	R	S	R	S	R	S	R	S	R
S.aureus	24	20(83)	4(17)	4(20)	20(80)	22(92)	2(8)	22(92)	2(8)	24(83)	4(17)	12(50)	12(50)
S.epidermidis	10	5(50)	5(50)	2(20)	8(80)	8(80)	2(20)	10(100)	0(0.0)	10(100)	0(0)	8(80)	2(20)
E.coli	04	4(100)	0(0)	0(0)	4(100)	3(75)	1(2)	0(0)	4(100)	4(100)	0(0)	3(75)	1(25)

Total 38

4. Conclusion and Recommendations

The results of the present study indicated a relatively high prevalence of contagious mastitis bacterial pathogen such as *S. aureus* is the major bacterial isolate found followed by *S. epidermidis, E. aerogen*, Proteus spp, *E.coli, K. pneumonae* and *P. saerogen*. In the present study, best drug choice for treatment in the case of *S .aureus* were found to be Vancomycin, Gentamycin, Ciprofloxacin and Chloroamphinicol while Vancomycin, Gentamycin, Ciprofloxacin and tetracycline for S. epidermidis; and Chloroamphencole, Ciprofloxacin, Gentamycin and tetracycline for *E. coli.* . On other hand higher resistance to penicillin (80%) and intermediate to tetracycline (50%) in case of S. *aureus,* penicillin and Chloroamphencole resistance was obtained in *S. epidermidis* whereas *E.coli* 100% resistant to Penicillin and Vancomycin. Based on the findings of the present study, avoiding indiscriminate use of antimicrobial therapies and regular antimicrobial susceptibility test of bacterial pathogen should be conducted.

Data Availability

All relevant data generated and analyzed during this study are available within the article

Consent and Ethical approval

Local ethics committee ruled that no official ethics approval was needed to conduct this research. Before conducting the research, informed consent was obtained from the owners or managers of the dairy farms used in this study

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