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Prevalence and Antimicrobial Susceptibility of Coagulase Negative *Staphylococci* Isolated from Haramaya University Dairy Farm

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Abstract: A cross-sectional study was conducted from November 2013 to April 2014 at Haramaya University Dairy farm to isolate coagulase negative *Staphylococcus* and to evaluate the antibiotic susceptibility profiles of the isolates. Coagulase negative *Staphylococcus* is gram-positive, catalase-positive, cocci-shaped and coagulase-negative bacteria. Coagulase negative *Staphylococcus* has emerged to be pathogens causing intra-mammary infections in dairy cows and it is opportunistic pathogens that reside on the teat skin which can be distinguished from coagulase positive *Staphylococcus* species by using coagulase tube test. In this study, milk samples from 144 udder quarters of 40 lactating cows were collected and cultured. Out of 160 quarters examined, 16 (10%) were found to be blind teats from which bacterial isolation was not attempted and 144 (90%) quarters had normal teats. Infection rates of coagulase negative *Staphylococcus* at cow level was found to be 36 (90%) from 40 cows and 54 (33.75%) out of 160 quarters at teat level were positive for coagulase negative *Staphylococcus*. Mastitis due to coagulase negative *Staphylococcus* was more likely to occur in cows above six years of age (61.1%). Lactating animals those who have more than 5 months of lactation period showed the highest number (44.4%) of isolates for coagulase negative *Staphylococcus* and regarding parity, infection rate of coagulase negative *Staphylococcus* were found from those cows that gave birth for 3rd -5th parity (51.9%). And the chi-square values showed that there was significant association between age, lactation stages and parity with coagulase negative *Staphylococcus* occurrence ($P<0.05$). Antimicrobial susceptibility of coagulase negative *Staphylococcus* against six antimicrobial agents was tested by using Kirby's Bauer disk diffusion method. The overall activity of antimicrobials on 30 isolates tested, the highest numbers of coagulase negative *Staphylococcus* were susceptible to Chloramphenicol (80%) followed by Gentamicin (73.30%), Tetracyclin (20%), Amoxicillin (16.70%) and the highest resistance was found in Penicillin (83.30%) and Ampicillin (76.70%). Culling of chronically infected animals and old aged cows from the farm should be very important.

Key words: Antimicrobial Susceptibility Test · CNS · Lactating Cows · Mastitis

INTRODUCTION

Mastitis is inflammation of mammary gland, primarily resulting from invasion of the mammary gland by pathogenic microorganisms through the teat canal resulting in physical, chemical, pathological and bacteriological changes in glandular tissues and milk [1, 2]. Mastitis is a multi-factorial disease, requiring exposure to a combination of environmental and pathogenic factors and with variable responses between animals. Many risk factors have been identified for clinical and subclinical mastitis in dairy animals such as breed, age, parity, stage of lactation [3]. To date, more than 50 *Staphylococcus* species and subspecies have been characterized to cause Staphylococcal mastitis. Based on their ability to produce the enzyme coagulase, *Staphylococcal* species associated with bovine mastitis have been classified as coagulase positive or coagulase negative [4]. More than ten different Coagulase Negative *Staphylococcus* species have been isolated from mastitic bovine milk samples. The most common species found are *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus epidermidis* and *Staphylococcus hemolyticus* [5]. CNS has traditionally been considered to be minor pathogens. Recently, however, the importance has increased and they

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have become the predominant pathogens isolated from subclinical mastitis in several countries [6, 7]. One of the important reasons for failure of treatment is assumed to be indiscriminate use of antibiotics without testing in vitro sensitivity of causal organisms. This practice at one hand increases economic losses and on other results in development of resistance to commonly used antimicrobials. For suitable antibiotic therapy, bacterial isolation and antibiotic sensitivity studies are always essentials. Mastitis is considered as one of the major reasons for use of antibiotics in dairy animals. Antimicrobial susceptibility tests help to guide the veterinarian in selecting the most appropriate antimicrobial agent for treatment of IMI caused by *Staphylococcus* species [8]. Therefore, the objectives of the study were to isolate and identify the coagulase negative staphylococcus from Haramaya University dairy farm and to evaluate the susceptibility of the isolates for different antimicrobial agents.

MATERIALS AND METHODS

Description of Study Area: The study was conducted in Haramaya University dairy farm, which is located in eastern Hararghe zone of Oromia Regional State, Eastern part of Ethiopia. Haramaya University is approximately 511 km far from Addis Ababa. The vegetation in the University constitutes the available pasture land which is predominantly native grasses and legumes interspersed with open acacia shrub land. The elevation of the University is about 2000m above sea level and geographically located at $41°59^{1}58^{11}$ latitude and $09°10^{1}24^{11}$ longitudes. The mean annual temperature and relative humidity are 18°C and 65%, respectively. Haramaya University receives an average rain fall of approximately 900mm and climatically found at highland areas. There are four season; a short rain season (from mid March to mid May), a short dry season (from end May to end of June), a long wet season (early July to mid October) and a long dry season (end of October to end of February. Main pasture production is expected after the short rain season, continuing until the end of the long wet season [9].

Study Animals: The study was conducted at Haramaya University dairy farm on milking cows. The farm has a total of 200 dairy cows comprising local (80) and cross breeds (120). Out of 200 cows found in the farm 80 were lactating cows.

Study Design: A cross-sectional study was conducted from November 2013- April 2014 to isolates Coagulase negative *Staphylococcus* from Haramaya University Dairy Farm in intensively reared cows depending on lactation period, age and parity.

Data Collection: Data on each cow was collected in a format designed for this purpose. Data collected include age, parity, lactation stage, teat conditions (blind or normal), bacteriological culture and antimicrobial susceptibility tests were recorded. To determine the association of risk factors, data including age, parity and lactation stage were collected from the record sheet of the farms. Age determination for cattle was done according to De Luhunta and Habel [10].

Sample Size: Selection of lactating cows was made using a deliberate unbiased process. Out of 80 lactating cows 40 cows were selected purposively without taking into account that the cows recently treated, those with physiological status modified and with chronic mastitis. Individual teats were numbered and marked as left front, left rear, right front and right rear respectively.

Teat Canal Examination and Milk Sample Collection: Physical examination was performed to detect for the presence or absence of blind teats. The milk samples were collected before milking early in the morning and strict aseptic procedures were followed when collecting milk samples in order to prevent contamination. Each teat was cleaned with clean tap water, dried and wiped thoroughly with 70% of ethyl alcohol. About 10 ml of milk was collected from normal teats using sterile collecting bottle and the first stream of milk from each quarter was discarded. Viscosity and appearance of milk secretion from each quarter was examined for the presence of clots, flakes, blood and watery secretions. After collection, the samples were transported immediately to the Veterinary Microbiology laboratory of the College of Veterinary Medicine, Haramaya University. Upon arrival, the samples were stored in a refrigerator at 4°C until processing [11].

Isolation and Identification of CNS: Bacterial isolation and identification have been conducted at Veterinary Microbiology laboratory of the College of Veterinary Medicine, Haramaya University. Isolation and identification of CNS was done by culturing the milk

sample on 5% blood agar aerobically for 48 hrs at 37°C and bacterial colonies were identified based on morphological characteristics on 5% Blood agar, Gram staining, catalase test and coagulase test. All suspected culture of *Staphylococcus* were subjected to Gram stain and observed under light microscope for Gram reaction, size and shape and cell arrangements. The Gram stained smears from typical colonies that show Gram positive cocci occurring in grape like irregular cluster were considered as presumptive staphylococcus. Pure culture of the presumptive isolate were picked using sterile loop from the nutrient agar and mixed with a drop of 3% H_2O_2 on a clean glass slide. Those Gram positive cocci librating bubbles of oxygen within a few seconds were considered as catalase positive. And finally tube coagulase test was performed in sterile test tubes by adding 0.5 ml of selected isolates of *Staphylococcus* grown on Tryptone Soya Broth at 37°C for 24 hr to 0.5 ml of rabbit plasma that was obtained from National Veterinary Institutes. After mixing by gentle rotation, the tubes were incubated at 37°C along with a negative control tube containing a mixture of 0.5 ml of sterile TSB and 0.5 ml of rabbit plasma. Clotting was evaluated at 30 minute intervals for the first 4 hr of the test and then after 24 hr incubation. Thus, the isolates that were Gram positive cocci, catalase positive and that did not show clotting on rabbit plasma were considered as coagulase negative *Staphylococci* [2]. It is necessary to identify CNS to the species level [12].

Antimicrobial Sensitivity Test: Antimicrobial sensitivity test patterns of CNS isolates were determined using Kirby-Bauer-disk diffusion method [2]. Pure colonies were inoculated into 5 ml of the peptone water and incubated at 37ºC for 24 hours until turbidity is seen and compared to the 0.5 McFarland standards. The isolates were streaked using sterile swab onto Muller-Hinton agar plates and then the antibiotic impregnated disks were applied on the surface of inoculated plates by using antibiotic dispenser and finally the plates were inverted and incubated at 37°C for 24 hours. The diameters of growth-inhibition were measured in millimeters and reported as: Susceptible, Intermediate or Resistant [13]. The antimicrobial discs were selected based on their availability and commonly used antimicrobials for treatment by Haramaya University dairy farm. Antimicrobials used in this study were Amoxicillin, Ampicillin, Penicillin, Chloramphenicol, Gentamicin and Tetracycline. National Committee for Clinical Laboratory Standard (NCCLS) break point were used to interpret the inhibition zone [2] and the manufacturers' manual were used to interpret the inhibition zone.

Statistical Analysis: All the research findings were stored in Microsoft Excel spreadsheet and analyzed using SPSS 16.0 version. The prevalence of status of teat (normal vs. blind), CNS, age groups, stage of lactation and parity, antimicrobial sensitivity test were expressed using percentage. The association between CNS, age groups, stage of lactation and parity were assessed by Chi-squared (χ^2) test and differences were regarded statistically significant at P-value less than 0.05.

RESULTS

Teat Condition and CNS Isolation and Identification at Teat and Cow Level: From 40 lactating cows that have 160 teats, 144 (90%) teat were apparently normal teats. From 160 teats 54 (33.75%), 90 (56.25%), 16 (10%) were positive, negative and not isolated for CNS were isolates respectively at teat level. 36 (90%) CNS was isolated at cow level considering that at least 1 CNS is isolated among the normal teats of a single cow were taken as representative (Table 1).

Risk Factors: Out of the 54 CNS isolated from among the groups were above 6 years old which accounts 61.1 %, of the 54 CNS isolated from the different stages of lactating animals those who have more than 5 months of lactation period showed the highest number (24) of isolates for CNS and regarding with parity out of the 54 CNS isolated 28 (51.9%) were found from those cows that gave birth from 3-5 calves. And the chi- square values showed that there were significant association between the age group, stage of lactation and parity with CNS occurrence (Table 2).

Antimicrobial Susceptibility Test: A total of 30 CNS isolates were tested for susceptibility to six antibiotics. These antibiotics were Amoxicillin, Ampicillin, Penicillin, Chloramphenicol, Gentamicin and Tetracycline (Table 3). The susceptibility pattern of CNS to antimicrobial agents varied among isolates. In this study, of the 30 isolates of CNS were susceptible to the antibiotics Chloramphenicol and Gentamicin. Resistance to penicillin, Ampicillin and Tetracycline was observed while Amoxicillin gave the intermediate sensitivity to some isolates of CNS.

Table 1: Frequency of teat condition, CNS isolates at teat and at cow level

Teat condition	Frequency (%)	CNS isolates at teat level	Frequency (%)	CNS isolates at cow level	Frequency (%)
Blind	16 (10%)	Not isolated	16 (10%)		
Normal	144 (90%)	Negative	90 (56.25%)	Negative	4(10%)
		Positive	54 (33.75%)	Positive	36 (90%)
Total	160 (100%)		160 (100%)		40 (100%)

Table 2: Result of risk factor

Risk factors	Number of examined	CNS positive	χ^2	P- value
Age (years)				
2-3	8	14.8%		
4-6	13	24.1%	14.886	0.005
> 6	33	61.1%		
Total	54	100%		
Lactation (months)				
Early (1-2)	16	29.6%		
Mid (3-5)	14	25.9%	19.105	0.001
Late (>5)	24	44.4%		
Total	54	100%		
Parity				
Few (1-2)	8	14.8%		
Moderate (3-5)	28	51.9%	11.96	0.018
Many (>5)	18	33.3%		
Total	54	100%		

Table 3: The susceptibility pattern of CNS isolates to antimicrobial agents

	Coagulase Negative Staphylococci		
Antimicrobials	Susceptible no. of isolates (%)	Intermediate no. of isolates (%)	Resistance no. of isolates (%)
Penicillin (10u)	0 (0.00)	5 (16.70)	25 (83.30)
Gentamicin (10 µg)	22 (73.30)	8 (26.70)	0 (0.00)
Chloramphenicol (30µg)	24 (80.00)	6(20.00)	0 (0.00)
Ampicillin (10 µg)	0 (0.00)	7 (23.30)	23 (76.70)
Amoxicillin (25 µg)	5 (16.70)	18 (60.00)	7 (23.30)
Tetracycline (30 µg)	6 (20.00)	4 (13.30)	20 (66.70)

DISCUSSION

CNS is normal flora of healthy teat skin and constitutes a constant source of bacteria to colonize end of the teat. This study revealed that the occurrence of CNS in Haramaya University lactating cows were 90% a nd 33.75% at cow and quarter level respectively. In this study, out of 160 quarters examined, 16 (10%) were blind. Whereas, 144 quarters (90%) were found normal. Blind quarters 16 (10 %) reported in this study might be associated with the seriousness of mastitis problem and absence of culling chronically infected animals in this farm. The results of the current study shows that CNS may plays a major role along with other microbes in causing bovine mastitis, since they were isolated in high percentages of 90% indicating that CNS may involve in causing mastitis. High isolation of CNS may be due to poor milking hygiene as this pathogen is mainly spread during milking via milkers' hands and towels. Contamination of end of the teat is a major predisposing factor in the development of CNS [14].

The result of CNS prevalence at teat level (33.75%) in the current study is much higher than the finding of Miline *et al.* [15], Tolossa [16] 10%, 23.2%. respectively and much lower than the findings of Bishi [17], Workineh *et al.* [18] in Ethiopia who reported 54% and 59.7%, respectively. However the finding out of this study was comparable with previous findings, 40.4 % by Kerro and Tareke [19], 34.9 % by Biffa *et al.* [20], 38.4% [21] and 33.6 % by Getahun *et al.*[22] and in urban and per urban smallholder production systems of Ethiopia. The variability in isolation of CNS between reports could be due to difference in farm management practices or due to differences in study methods and instruments employed by the investigators.

The current study shows CNS infection rate increases with age of above 6 years, which is (61.1%). In this study the increase in isolation rate of CNS with age is in accordance with the work of other investigators [23, 24]. The occurrence of CNS infection rate may be because of dilation of teat canal due to repeated exposure during milking, which facilitates the entry of pathogen into the teat canal and causing intra-mammary infection. The result found indicate that parity number starting from three to five (51.9%) and late lactation stages (44.4%) were found to increase occurrence of CNS, this is may be primiparous cows have more effective defense mechanism than multiparous cows [1].

The observed higher prevalence of CNS during late lactation as compared to mid and early lactation stages was in contrast with the report of Kerro and Tareke [19] and in line with studies reported by Getahun *et al.* [22], Almaw *et al.* [23], Abera *et al.* [24] and Haftu *et al.* [25]. The effect of stage of lactation in this study may be due to induced opportunity of infection with time and as well as the prolonged duration of infection. Radiostits *et al.* [26] 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 present studies evaluate the antimicrobial susceptibilities of 30 CNS isolates in Haramaya University dairy farm from 40 lactating cows. The antimicrobial susceptibility tests carried out in this study indicate the existence of susceptibility and resistance of CNS to some of the antimicrobials indicating that relatively higher resistance of CNS, against penicillin (83.30 %) and Ampicillin (76.70%) however, it shows sensitivity to Chloramphenicol (80%) and Gentamicin (70.30%), compared with most of the previous studies from other countries [27]. Reason for higher percentage of antibiotic resistance in CNS isolates could be due to the administration of antibiotics without their susceptibility assessment coupled with their indiscriminate use in the farm. Similar suggestion was given by Jaims *et al.* [28].

Among the 30 CNS isolates tested, the highest rate of susceptibility was generally observed for Chloramphenicol (80%), followed by Gentamicin (73.30%), Whereas, penicillin and Ampicillin showed the lowest activities against CNS in this study, which is in agreement with those of previous reports from Korea and other countries [29]. In this study Chloramphenicol and Gentamicin were the most effective antibiotics where 80% and 73.30% of isolates were susceptible respectively. This might be due to the fact that these drugs were the least frequently used in the study area in treatment of animals so, no resistance was developed. The result of the present study suggests that CNS may play a major role in causing bovine mastitis and the organisms should be considered potential udder pathogens in the routine culturing of milk samples, for which antibiotic susceptibilities should be carried out.

CONCLUSION AND RECOMMENDATIONS

The study attempted to isolate and evaluate antibiotic susceptibility profiles of CNS involved in lactating cows using bacteriology and in vitro susceptibility test. The isolation rate of CNS in this study area was 33.75 at quarter level and 90% at cow level. The result suggests that CNS may play a major role in causing bovine mastitis and antimicrobial resistance problem is comparatively more serious in this study area. Therefore, udder health must be followed up and Culling of chronically infected animals and old aged cows from the farm should be very important. Regular screening test for sub clinical mastitis and use of antibiotics by sensitivity testing is recommended to reduce economic loss. This study did not attempt to identify CNS at species level, so interested parties or researchers can conduct to identify at species level of CNS.

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