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Isolation and phenotypic characterization of coagulase negative staphylococcus isolated from mastitic Cows in and around Zway town, Ethiopia

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Abstract
A total of 230 Holstein freesia, Jersey and cross- breed lactating cows from farms and smallholders were examined during the study period and 97 (42.2%) cows were positive for mastitis by CMT screening and clinical examination of udder. out of these cows 7.0% (16/230) and 35.2% (81/230) showed clinical and subclinical mastitis respectively. Blind teat was examined in 12(1.3%) out of 920 quarters. Multivariable logistic regression showed that there was a statistically significant difference ($P< 0.05$) observed between the prevalence of mastitis/CNS and potential risk factors such as age, parity, breed, milk hygiene, lactation stage and floor type. However antibiotic used was not statistically significant ($p>0.05$). The association of the different potential risk factors and the occurrence of mastitis in and around Zway/Batu farms and smallholder dairy cows of Zway area. Accordingly, the risk factors such as breed (p=0.004, X^2 11.23), lactation stage (p=0.000, X^2= 26.63), floor type (p=17.48, X^2=0.000), milk hygiene (p=21.73, X^2=0.000), parity (p=29.07, x^2=0.000), age (p=29.07, x^2=0.000), showed statistically significan t with the occurrence of mastitis. Whereas antibiotic used (p=7.59, X^2=0.06) which is statistically insignificant.
Antibiotic susceptibility to Amoxicillin, Naldic acid, Oxytetracycline, Cefoxitin, Streptomycin, Compound sulphonamide, Kanamycin and Ceftriaxone was determined by agar disc diffusion (Kirby-Bauer method). All the current isolates were sensitive to kanamycin, Amoxicillin and Ceftriaxone; moderately sensitive to streptomycin and Oxytetracycline. However, the isolates were resistant to compound sulphonamide, Naldic acid and cefoxitin which is similar to different researches made on similar isolates. Since mastitis is an economically important disease, hygienic milking practice, use of effective antibiotics, proper extension packages to dairy farm owners and strategic mastitis control programs should be of paramount importance.

Keywords: Antimicrobial sensitivity test, Biofilm, CNS, Mastitis, Zway/Batu

1. Introduction
Mastitis is inflammation of the parenchyma of the mammary gland regardless of the cause. Mastitis is therefore characterized by a range of physical and chemical changes in the milk and pathological changes in the glandular tissue. The most important changes in the milk include discoloration, the presence of clots and the presence of large numbers of leukocytes. There is swelling, heat, pain and edema in the mammary gland in many clinical cases. However, a large proportion of mastitic glands are not readily detectable by manual palpation nor by visual examination of the milk using a strip cup; these quarters represent subclinical infections. Because of the large numbers of subclinical cases, the diagnosis of mastitis depends largely on indirect tests, which depend, in turn, on the somatic cell concentration (SCC) or electrolyte (sodium or chloride) concentration of milk. It seems practicable and reasonable to define mastitis as a disease characterized by the presence of a significantly increased SCC in milk from affected glands. The increased SCC is, in almost all cases, due to an increased neutrophils concentration, represents a reaction of glandular tissue to injury and is preceded by changes in the milk that are the direct result of damage to glandular tissue. However, the exact clinical and laboratory changes that occur in the udder as a result of infection can also be caused by other factors in the absence of infection (Radostits, 2006) [46].
Identification of cows infected with mastitis is necessary to make decisions regarding treatment, culling or isolation of infected animals. Common methods used to identify infected cows include: milk microbiology ("cultures"), the California Mastitis Test (CMT), individual

cow SCC values and electrical conductivity. Individual quarter cultures. Microbiologic examination of milk samples may be used for control programs (such as segregation plans) or for detection of new pathogens. Culturing is also used to determine antibiotic susceptibility of mastitis pathogens. Microbiologic examination of milk samples is often considered to be the gold standard for identification of infected quarters (Makovec and Ruegg, 2003) [33].

One of the groups of bacteria that cause mastitis is called coagulase-negative staphylococci (CNS). These bacteria are of great interest because they are currently the most commonly isolated microorganisms in cows and heifers in herds, and are currently considered emerging pathogens of bovine mastitis (Pyorala *et al*., 2009) [52]. CNS are normally found on the healthy skin of the nipple and the hands of the milker. They are often called "opportunistic microorganisms" because they live in areas where it is easy to colonize the teat canal and penetrate the secretory tissue. Implementing mastitis control programmes over the past 30 years has led to a reduction in the overall incidence of clinical mastitis in most herds. In some cases, the decrease has been 90%. Whereas the clinical disease caused by major pathogens such as Staphylococcus aureus and Streptococcus agalactiae decreased significantly, less important pathogens such as CNS have been increasingly taking on greater importance.

As a group, CNS are the most prevalent bacteria found in bovine milk samples in many areas around the world (Schukken *et al*., 2009) [49]. They are cultured from milk from cows with and without elevated SCC and have been associated with mild clinical mastitis (CM) cases (Thorberg *et al*., 2009) [54]. Also, they are abundantly present on teat apices and other body sites and in the cows' environment (Taponen *et al*., 2008) [53]. Still, their clinical/pathogenic relevance when cultured from milk remains a point of discussion. Some consider them as true mastitis pathogens with important virulence factors (Zhang and Maddox, 2000) [58], a high level of an-timicrobial resistance (Rajala-Schultz *et al*., 2009) [47], and the ability to cause chronic infections (Gillespie *et al*., 2009) [18]. Others regard them as minor pathogens (Schuk-ken *et al*., 2009) [49].

On the other hand, they have as well been considered as potentially interesting microorganisms that can protect quarters against IMI with more harmful pathogens, either when causing IMI (Lam *et al*., 1997) or when colonizing bovine teats (De Vliegher et *al*., 2004) [10]. In some studies (Zadoks *et al*., 2001) or for some major pathogens (Lam *et al*., 1997), the presence of CNS was not protective against IMI with other species. An association between the presence of CNS and increased risk of mastitis with major pathogens has also been documented (Hogan *et al*., 1988) [21].

The economic impacts of bovine mastitis and intrammary infection have lead to the development of various therapeutic strategies to control them. Many drugs belonging to various therapeutic classes have been assessed (Koivula *et al*., 2005) [27]. The production of high quality milk is realistic and a meaningful goal for all aspects of dairy industry and the primary motivator for establishing mastitis control program in dairy herd. Herds that have undergone successful comprehensive mastitis control program also need to develop strategies to control infection with environmental organism and need to use an effective monitoring system for new infection (Smith *et al*., 1985) [50].

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 the study area and to isolate CNS and conduct *in vitro* antimicrobial susceptibility and biofilm production test on the isolates.

2. Study Methods and Materials

2.1. Study Area

The study was conducted in and around Zway from December 2014 to May 2015. Zway is located in the East shoa Zone of the Oromia National state about 163 kilometres away from Addis Ababa. This town has a latitude of 7°9′N and 38°7′E longitude, with an elevation of 1650masl. The 2007 national census reported a total population for Ziway of 43,660, of whom 22,956 were men and 20,704 were the majority of the inhabitants said they practiced Ethiopian Orthodox Christianity, with 51.04% of the population reporting they observed this belief, while 24.69% of the population were Muslim, 0.42% practiced traditional beliefs, and 22.07% of the population were Protestanten. The rainfall is bimodal unevenly distributed with an average annual rain fall of 761 mm. It extends from February to September with a dry period in may-June. In winter there is much less rainfall than in summer. It has a tropical lowland climate. Monthly temperature variation is highly depend on rainfall, due to its location close to the equator and the seasons are only distinguished by the intensity of rain, which is the most in August and the least in December. The average annual temperature in Zway is 21 °C. The soil is fine sandy, loam with sand, silt clay in proportion of 34:48:18% respectively. Average PH of soil is 7.88 (CSA, 2007).

2.2. The study population

A total of 230 Holstein freesia, Jersey and cross- breed lactating cows from farms and smallholders were examined during the study period. The study populations were lactating cows of different age, lactation stage, parity and milk production. Dairy farms of the town were selected using systematic random sampling methods. The study was also conducted at cow and quarter level. The milk was collected from the lactating cows which were suspected with subclinical and clinical mastitis.

2.3. Study design

A cross-sectional study was conducted in which 230 lactating dairy cattle were tested for the presence of clinical and sub clinical mastitis to isolate and identify MRSA and their biofilm production status From December 2014 to May 2015 in selected dairy farms located in and around Batu town.

2.4. Sampling method and sample size determination

A total of 230 dairy cattle were considered from both small holder and large scale dairy farms having an average of 2 to 8 and 23 to 55 lactating cows respectively. Purposive sampling technique was applied on all available dairy cattle in the study area. Batu, Adami tulu and Bulbula were the targeting site for the sample collection. Questionnaires and direct observations of the farms were used to collect information regarding the risk factors used in the analyses.

2.5. Study methodology

2.5.1. Questioner survey

Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions, previous history of mastitis, milking hygiene, tick infestation presence of teat lesion, floor type, antibiotic use and milk abnormalities like clotting and abnormal secretion) were collected for 216 lactating cows from farm records when available and by interviewing the farm owner when not.

2.5.2. Physical Examination and CMT Screening

Physical Examination of the Udder: Mammary glands were examined by inspection and palpation. Swelling and pain reaction up on palpation, changes in the consistency of the udder, changes in the colour of milk and the presence of flakes in the milk were considered as indicators of clinical mastitis.

CMT Screening: The California Mastitis Test (CMT) was carried out according to the method described by Quinn *et al*. (1999) [43]. A squirt of milk, about 2 ml from each quarter was placed in each of four shallow cups in the CMT paddle. An equal amount of the 3% commercial reagent was added to each cup. A gentle circular motion was applied to the mixtures, in a horizontal plane for 15 seconds. Reactions were graded as 0 and Trace for negative, +1, +2 and +3 for positive.

2.5.3. Milk sample collection

Milk samples were taken into the sterile tube after cleaning of the teat ends using the alcohol soaked cotton. The first 3-4 streams of milk were discarded. Teats towards sample collection were sampled first and then the far ones. The collecting vial was held as near as possible and by turning the teat to a near horizontal as possible and by turning the teat to a near horizontal position, approximately 10ml of milk was collected into a universal sample collection bottle. After collection, the sample was placed in ice pox and transported to the Addis Ababa University College of veterinary medicine and agriculture laboratory. The sample were either cultured or stored at 4 ^{o}C until cultured within few days (Quinn *et al*., 1999) [43].

2.5.4. Bacteriological isolation and identification technique

Culture procedure: - Samples which were CMT positive were streaked aseptically on the sterile blood agar enriched with 5% sheep blood and incubated at 37 ^{o}C for 24-48 hr. Then examine the plate for the presence of staphylococcus colonies. Isolates of staph identified based on morphological aspects (creamy, grayish, white or yellow colonies) and hemolytic pattern on blood agar. Sub-culture the colonies with nutrient blood agar and incubated at 37 ^{o}C for 24-48 hr to get pure culture (Hogan *et al*., 1999) [20].

Gram's staining: All suspected cultures of *Staphylococcus* species were subjected to gram's stain and observed under a light microscope for gram's reaction, size, shape and cell arrangements. The gram-stained smears from typical colonies that showed gram- positive cocci occurring in bunched, grapelike irregular clusters were taken as presumptive *Staphylococcus* species (Quin *et al*., 2002).

Catalase test: Pure culture of the isolates were picked using a sterile loop from the agar slant and mixed with a drop of 3% H_2O_2 on a clean glass slide. If the organism was positive, bubbles of oxygen were liberated within a few seconds and the catalase negative isolates did not produce bubbles. The catalase positive cocci were considered as staphylococci (Quin *et al*., 2002).

Mannitol salt agar: The colonies that were identified by gram-staining and catalase test as Staphylococcus were streaked on MSA plates and incubated at 37 °C and examined after 24-48 h for growth and change in the colour of the medium. The presence of growth and change of pH in the media (red to yellow colour) were regarded as confirmative identification of the salt tolerant staphylococci. Phenol red pH indicator detected the acidic metabolic product of mannitol.in this study color change was not observed.

O-F Basal Medium (Himedium, India): To first 100 ml of sterile medium aseptically add 10 ml sterile 10% dextrose solution. To second add 10 ml sterile 10% lactose solution. To third 100 ml add 10 ml sterile 10% sacoharose solution. Mix and dispense in 5ml amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation solution. In this study fermentation was seen in both in oil coated and none coated tubes.

Coagulase test: The tube coagulase test was performed in sterile tubes by adding 0.5 ml of selected isolates of Staphylococcus grown on Tryptone Soya Broth (TSB) at 37 °C for 24 h to 0.5 ml of citrated rabbit plasma. 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 min intervals for the first 4 h of the test and then after 24 h incubation. The reaction was considered positive if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative. In this case no clotting was observed (Hogan *et al*., 1999) [20].

2.6. Antibiotic sensitivity test

Susceptibility of bacteria to the commonly used antimicrobials was conducted using Kirby-Bauer method (Quinn *et al*., 2002) [42]. About eight antimicrobials such as, compound Sulphonamide, Naldic acid, cefoxstin, Streptomycin, Kanamycine, Oxytetracycline and Ceftriaxone were selected from main class of antimicrobials and investigated for sensitivity testing. The antibiotic disks were applied on the surface of the inoculated agar plates using aseptic technique. Each disk was pressed down to ensure complete contact with the agar surface. After measuring the zone of inhibition, it was classified as sensitive, intermediate and resistant according to National Committee for Clinical Laboratory Standard (NCCLS) break point to interpret the inhibition zone (Quinn *et al.,* 2002) [42].

The disc agar-diffusion method was applied and performed according to NCCLS guidelines (NCCLS, 2002) in the Mueller-Hinton agar. Antimicrobial susceptibility for the following antibiotics was conducted; cefoxitin (30 µg), streptomycin (25 µg), kanamycine (30 µg), Oxy-tetracycline (30 µg), ceftriaxone (30 µg), amoxicillin (30 µg), compound sulphonamide (30 0 µg) and Naldic acid (30 µg).

2.8. Biofilm production of CNS on Modified Congo red agar (MCRA)

Phenotypic characterization of biofilm production was performed as proposed by Freeman *et al.,* (1989). A pure one isolate of *S. aureus* colony from 18-20 hour growth on nutrient agar were streaked on modified congo red agar (MCRA) plates and incubated aerobically for 24-48 hours. Then it was left at room temperature for more than 4 days, to observe if the bacteria colonies form a delayed black pigmentation. Positive result shows when the Congo red dye interacts with certain

polysaccharides; forming black colonies on MCRA, whereas non biofilm producers form red colonies (Freeman *et al.*, 1989).

3. Data Collection and Analysis

Data collected from each study animal and laboratory work results were coded into appropriate and enter Microsoft spread sheet. Then analysis was performed using spss versin 20. Association of specific variables breed, parity, lactation stage, milk hygiene, floor type, antibiotic were performed by using Pearson Chi square (x^2). P values were calculated and $p \leq 0.05$ was considered asstatistically significant.

4. Results

4.1. Prevalence of mastitis and CNS

A total of 230 Holstein freesia, Jersey and cross breed lactating cows from farms and smallholders were examined December 2014 to may 2015 and 97 (42.2%) cows were positive for mastitis by CMT screening and clinical examination of udder. out of these cows 7.0% (16/230) and 35.2% (81/230) showed clinical and subclinical mastitis respectively. Blind teat was examined in 12(1.3%) out of 920 quarters. Multivariate logistic regression showed that there was a statistically significant difference ($P< 0.05$) observed between the prevalence of mastitis and potential risk factors such as age, parity, breed, milk hygiene, lactation stage and floor type. However antibiotic used was not statistically significant ($p>0.05$) (Figure 1).

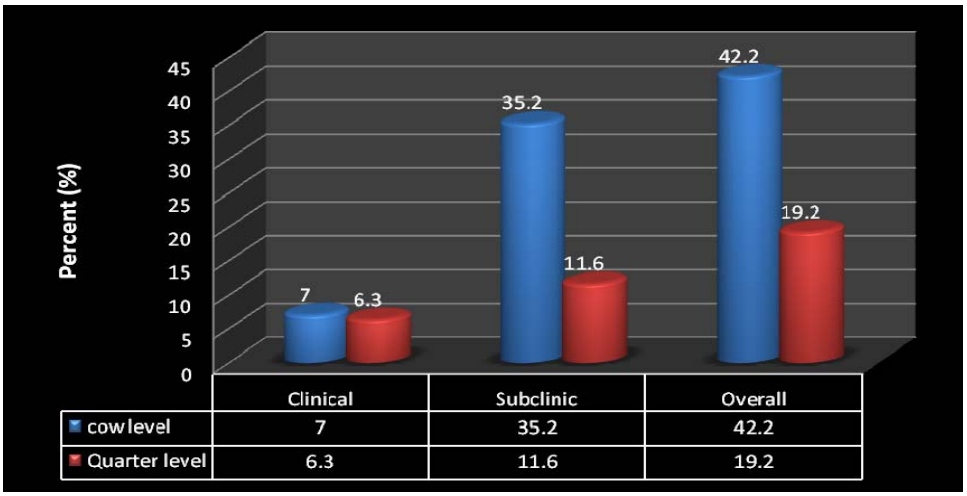

Fig 1: Prevalence of mastitis at cow and quarter level

Table 1: Prevalence of mastitis at quarter level

Quarter	No of cows examined	Positive (%)	Negative (%)	Blind (%)	Chi square	p-value
RF	230	36(15%)	194(84.35%)	1(0.44%)	66.16	0.000
LH	230	52(22.21%)	178(77.39%)	6(2.61%)	192.93	0.000
LF	230	34(14.78%)	196(85.22%)	1(0.44%)	61.95	0.000
RH	230	55(23.91%)	175(76.09%)	4(1.74%)	116.59	0.000
Total	920	177(19.24%)	743(80.76%)	12(1.3%)		

RF= right front, LH= left hind, LF =Left Front, RH= right hind

4.2. Risk factors associated with prevalence of CNS

Association of risk factors with occurrence of mastitis (CNS): The association of the different potential risk factors and the occurrence of mastitis in and around Zway/Batu farms and smallholder dairy cows of Zway area are shown in table2. Accordingly, the risk factors such as breed (p= 0.004, x^2 11.23), lactation stage (p= 0.000, x^2= 26.63), floor type (p= 17.48, x^2=0.000), milk hygiene (p=21.73, x^2=0.000), parity (p=29.07, x^2=0.000), age (p=29.07, x^2=0.000), showed statistically significant with the occurrence of mastitis. Whereas antibiotic used (p=7.59, x2=0.06) which is statistically in significant.

Table 2: The prevalence of bovine mastitis based on lactation, milk hygiene, parity number, floor type, breed, age and antibiotic used.

Risk factors		No of cows examined	No of positive n (%)	X^2	p-value
Lactation Stage	Early 1-3	62	9(14.52%)	26.63	0.000
	Mid 4-6	93	49(52.69%)		
	Late >6	75	39(52%)		
Age	Young (<4)	99	22(22.2%)	29.07	0.00
	Adult(4-6)	60	32(53.33%)		
	Old(>7)	71	43(60.53%)		
Milk Hygiene	Good	77	16(21.33%)	21.73	0.000
	Poor	153	81(52.26%)		
Parity	1-2	99	22(22.22%)	29.07	0.000
	3-4	59	59(52.54%)		

	>4	72	44(61.1%)		
Antibiotic used	Yes	60	19(31.66%)	7.59	0.06
	No	170	78(45.88%)		
Floor type	Concrete	136	42(30.38%)	17.48	0.000
	Mud	94	54(58.51%)		

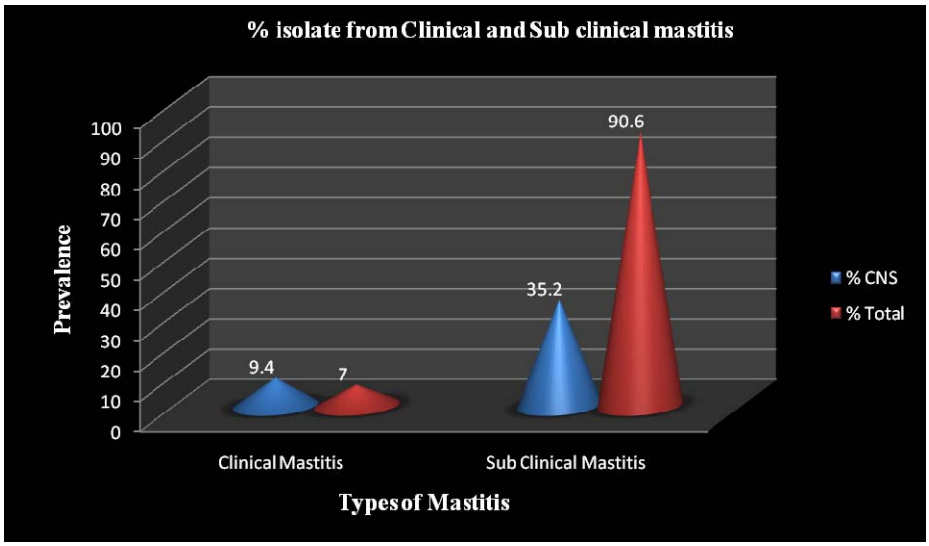

Fig 2: Number of isolates from clinical and subclinical mastitis

4.3. Biofilm production status

A total of 32 pure isolated CNS were subjected for biofilm production on a modified Congo red agar. However none of the isolate showed a black colony with dry crystalline consistency (0%), rather they were found forming a red colony which indicates a bacterium with no biofilm production (Table 3) (Figure 3).

Table 3: CNS isolates and Biofilm production status

	Biofilm production on MCRA	
Number of isolated bacteria	**Positive**	**Negative**
32	0(0%)	32(100%)

MCRA: Modified Congo red agar

Fig 3: Growth on Modified Congo red agar

4.4. *In vitro* antimicrobial susceptibility test

Antimicrobial sensitivity test was done for all of the bacterial isolates in which CNS showed highly susceptible to kanamycine (93.3%), Amoxicillin (87.5%), Ceftriaxone (84.4%) and moderately to streptomycin (65%) and oxytetracycline (71.9%). However it was resistance to sulphonamide (81.3%), cefoxitin (40.6%), and Naldic acid (31.3%). And also resistance more than two drugs are shown in table below (Table 4).

Table 4: Summery of antimicrobial sensitivity test for CNS isolate (no of isolate=32)

Antibiotic disc	Antibiotic susceptible Coagulase negative staphs		
	Susceptible n (%)	Intermediate n (%)	Resistance n (%)
Streptomycin (25 µg)	21 (65.6%)	4 (12.5%)	7 (21.9%)
Sulphonamide (300 µg)	6 (18.8%)	-	26 (81.3%)
Oxytetracycline(30 µg)	23 (71.9%)	-	9 (28.1%)
Ceftriaxone (30 µg)	27 (84.4%)	2 (6.3%)	3 (9.4%)
Kanamycine (30 µg)	30 (93.8%)	-	2 (6.3%)
Amoxacillin (30 µg)	28 (87.5%)	-	4 (12.5%)
Naldic Acid (30 µg)	13 (40.6%)	9 (28.1%)	10 (31.3%)
Cefoxitin (30 µg)	5 (15.6%)	14 (43.8%)	13 (40.6%)

4.5. Antimicrobial resistance pattern

From the total CNS isolates as shown in figure (4) 85% were resistance to one drug, 30% to two drugs and 25% were resistant to for more than two drugs.

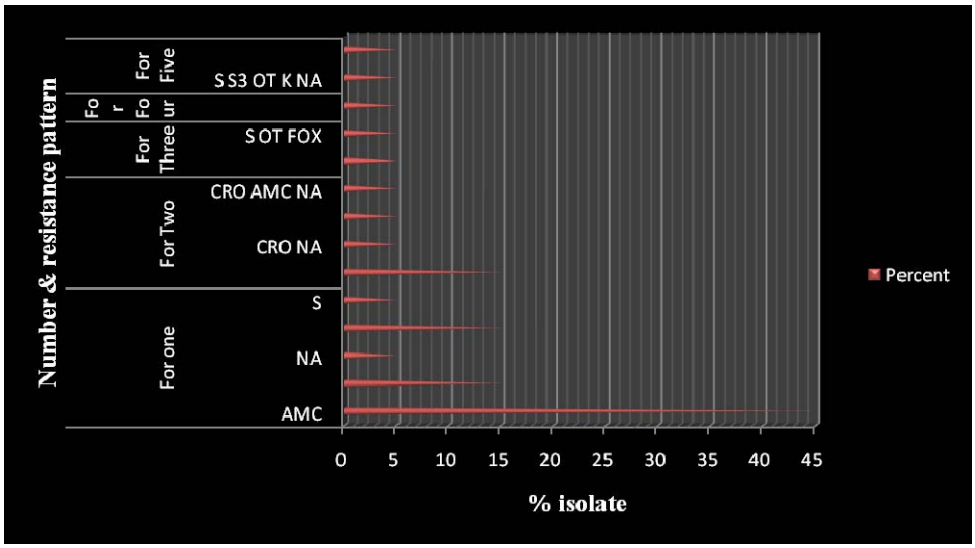

Fig 4: Antimicrobial resistance pattern of CNS isolated from dairy milk in and around Zway
AMC=Amoxicillin NA=Naldic Acid, S3=compound sulphonamide, FOX=Cefoxitin
CRO=Ceftriaxone, S=Streptomycin, OT =Oxytetracycline, k=Kanamycin S3=compound sulphonamide, and Naldixic acid.

5. Discussion

The current study indicated a prevalence of mastitis 42.2% (35.2% subclinical and 7% clinical) which was comparable to 40.6 and 5.3 subclinical and clinical mastitis studied in the same area respectively (Benta and Habtamu, 2011). The overall prevalence reported in the present study was enclose agreement with the results of various researchers in different corner of the region like, 40% in southern Ethiopia by Kerro and Tareke (2003) [25] and 44.1% Girma (2010) [19], Mungube *et al*. (2004) 46.6% from central highlands of Ethiopia, respectively. Mungube *et al*. (2004) and Abdelrahim *et al*. (1990) who found a prevalence of 45.8% in Sudan. However the prevalence reported in the present study is lower than previous researchers findings in different corners of the country.52.8% Hunderra *et al.* (2005, [22] in Sebeta.49.7% in tgray by Enquebahir *et al*., 2008, [12] Mekibib *et al*., 2010, [36] Lakew *et al*., 2009, [28] Sori *et al*., 2005) [51] and who recorded 71.1% from Holeta, 64.6% from Assela, and 52.8% from Sebeta. The difference in the prevalence of mastitis in present study and other reports could probably be due to differences in farm management practices, breed, geographic location, label of production, study methods and instruments employed by investigators (Radostitis *et al*., 2000) [45]. This study showed higher prevalence of subclinical mastitis compared to clinical form and this is supported by several reports (Abera *et al*., 2010) [2]. Subclinical mastitis has been reported to be higher than clinical mastitis owing to the defense mechanism of udder, which reduces the severity of the disease (Bardley, 2002) [9]. In most developing countries including Ethiopia, subclinical form of mastitis reicived little attention and efforts have been concentrated on the treatment of clinical cases (Hussein *et al*., 1997) [23]. According to Mungbe *et al.* (2005) [37] losses associated with subclinical mastitis in cross breed dairy cows in central highlands of Ethiopia was found to be USS$38 for each cow per lactation. Of 920 quarters examined 12 quarters were blind. The prevalence of blind teat is an indication of serious mastitis problem on the respected farms and of the absence of culling program that can serve as a means to remove a source of this mammary mastitis for other cows.

The overall prevalence of 42.2% at cow level and 17.6% at quarter level is comparable to the findings of Kerro and Tareke (2003) [25], Workineh *et al*. (2002), Bishi (1998) [8], and Fekadu (1995) who reported 40.4% in southern Ethiopia, 38.2% in Adami-Tulu central Ethiopia, 39.8% in and around Addis Ababa and 39.7% in Chaffa valley in north eastern Ethiopia, respectively. However, it is relatively lower than the report of Mekibib *et al.* (2010) [36], Lakew *et al.* (2009) [29], and Sori *et al*. (2005) [51] who recorded 71.1% from Holeta, 64.6% from Assela, and 52.8% from Sebeta respectively. Prevalence of subclinical mastitis (35.2%) was higher than that of clinical mastitis (7%) in the present study, which is in general agreement with several earlier reports from different parts of

Ethiopia (Abera *et al*., 2010; Mekibib *et al*., 2010; Lakew *et al*., 2009; Almaw *et al*., 2008; Getahun *et al*., 2008; Biffa *et al*., 2005; Mungube *et al*., 2004; Kerro and Tareke, 2003; Workineh *et al*., 2002) [2, 36, 29, 3, 17, 6, 25] and elsewhere in Africa (Kivaria *et al*., 2004) [26].

In this study the prevalence of CNS isolate is higher in subclinical mastitis than clinical mastitis 90.6% and 9.4% respectively. This is in line with studies on clinical and subclinical mastitis (Pyorala and Taponen, 2009) [52]. CNS is generally associated with subclinical mastitis and occasional bouts of clinical mastitis. Usually the clinical signs remain mild. CNS can affect the quality of bulk tank milk, as they are a frequent cause of mastitis, although they only slightly increase the SCC. CNS are generally considered as opportunistic pathogens. The prevalence of CNS IMI may vary markedly between regions and countries. In national Swedish surveys (Ericsson Unnerstad *et al*., 2009) on clinical and subclinical mastitis, the CNS prevalence was 6% and 17%, respectively. The control of CNS mastitis is complicated by the heterogeneity of this bacterial group. Moreover, coagulase-negative staphylococci are always present on the udder skin and in teat canals; under favorable conditions they permeate the galactogenic pathway to the quarter. The pathogenic mechanisms of CNS are expressed by two parameters: invasiveness (ability to permeate through protective barriers, to adhere to host cells and to form a biofilm) and toxicity (capacity to produce enzymes and toxins, including haemolysins and proteases) (Bartoszewicz-Potyrala and Przondo-Mordarska, 2002; Bochniarz and Wawron, 2012). Controlling CNS mastitis is difficult because the epidemiology is not clear, and the CNS group consists of about 50 different *Staphylococcus* species (White *et al*., 1989; Matos *et al*., 1991). A variety of CNS species has been isolated from mastitis. *S. chromogenes, S. simulans* and *S. hyicus* are reported most often, but also many other species are frequently mentioned. Identification of CNS species in different studies usually rests on use of commercial identification kits based on biochemical profiles. Recently, diverse identification methods based on genotype have been developed and compared with identification methods based on phenotype. Knowledge about the relative prevalence of specific CNS species in different types of mastitis is, however, scarce.

Risk of mastitis increased with age and parity. This observation is in agreement with the reports of (Abera *et al*., 2010, Biffa *et al*., 2005, Mungube *et al*., 2004 and Kerro and Tareke, 2003) [2, 6, 25] from the country. Mastitis prevalence was high in early and late stage of lactation. This result is consistent with observations of Biffa *et al*. (2005) [6] and Kerro and Tareke (2003) [25] who reported high prevalence of mastitis in the early and late stage of lactation. Breed showed significant influence on the prevalence of mastitis. The observed high prevalence of mastitis in Holstein Friesian compared to local cows is in agreement with the findings of Biffa *et al*. (2005) [6], Girma (2002) and Biru (1989). As stated in Radostits (*et al*., 2007) this may be associated with differences in anatomical and physiological characteristics of the mammary gland, as well as high milk yielding of the cows. Cross breed cows have large udders which can easily be injured and the presence of injury is a predisposing factor for the occurrence of mastitis. It is believed that managemental factors (milking and hygiene) played significant role in the incidence of mastitis (Quin *et al*., 2002) [42].

The association between soil floor and high prevalence of mastitis recorded in this study is consistent with the findings of (Abera *et al*., 2010) [2]. Earlier works implicated poor barn hygiene to high prevalence of mastitis (Sori *et al*., 2005) [51]. Cows with previous history of mastitis were found less likely to be mastitic. This finding suggests that treatment of cows for mastitis may be effective in eliminating the pathogens.

*C*NS the major cause of subclinical mastitis are found sensitive to kanamycin (93.8%),Amoxacillin (87.5%),Ceftriaxone(84.4 %), and Amoxacillin(71.9%) and resistance to sulphonamide (81.5%), cefoxitin(40%), Naldic acid (31.3%) as compared from the isolates. This finding is in close agreement with the findings of Belayneh *et al*. (2014) who reported CNS highly sensitive to Naldic acid (95%) and resistant to Amoxacillin (75%). In this study CNS is highly susceptible to Ceftriaxone which is (84.4%) and this closely agrees with Basappa *et al.* 2011 (83.88%) in India.

Cefoxitin resistant CNS was found to be sensitive to Ceftriaxone which agrees with the reports of Lucianne *et al.* (2013). Because these drugs are the least used in veterinary clinics. Similar suggestions was given by Jaimes *et al.* (2002) [24] in that the development of antibiotic resistance is nearly always as a result of repeated therapeutic use or indiscriminate use of them. Thus cefoxitin resistant isolates of CNS were more resistant to sulphonamide, oxytetracycline. This is in agreement with reports of Machodo *et al*. (2008) [32].

6. Conclusion and Recommendation

The present study revealed that mastitis is still prevalent in smallholder dairy farms in the study area, and further confirms that the subclinical form is the most prevalent. Mastitis prevalence was associated with several risk factors. The study shows that coagulase negative *S*taphylococcus are the most important causes of bovine mastitis, especially subclinical mastitis, in the study area. The current finding indicated an occurrence of low to moderate prevalence of clinical mastitis and moderate to high prevalence of subclinical mastitis at cow, herd and quarter level. Stage of lactation, parity number, age, breed and milk hygiene were the most important risk factor affecting the prevalence of sub clinical mastitis in cow and quarter level l. All the current isolates were sensitive to kanamycin, Amoxicillin and Ceftriaxone; moderately sensitive to streptomycin and Oxytetracycline. However, the isolates were resistant to compound sulphonamide, Naldic acid and cefoxitin which is similar to different researches made on similar isolates. Since mastitis is an economically important disease, hygienic milking practice, use of effective antibiotics, proper extension packages to dairy farm owners and strategic mastitis control programs should be of paramount importance and antibiotic susceptibility of CNS is unpredictable and multiresistant strains are common.

Therefore, based on the above conclusion and recommendation, the following points are forwarded:-

- Antibiotic susceptibility testing should be performed in all cases of clinically and sub clinically significant mastitic infections caused by CNS.
- Culling of old and chronically affected cows, screening of cows and milk for clinical and subclinical mastitis, dry cow therapy, hygiene at milking and cow house hygiene should be considered in attempts to reduce prevalence of mastitis.
- Moreover, extension services and training programs aiming at creation of awareness about the importance and prevention of subclinical mastitis among smallholder dairy farmers is recommended.

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Conflict of Interest
We all authors confirmed that there is no conflict of interest.

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