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, X²=11.23), lactation stage (p=0.000, X²= 26.63), floor type (p=17.48, X²=0.000), milk hygiene (p=21.73, X²=0.000), parity (p=29.07, x²=0.000), age (p=29.07, x²=0.000), showed statistically significant with the occurrence of mastitis. Whereas antibiotic used (p=7.59, X²=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). [40]

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 antimicrobial 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 (Schukken 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 Zway 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 ºC. 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 box and transported to the Addis Ababa University College of veterinary medicine and agriculture laboratory. The sample were either cultured or stored at 4 °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 °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 °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% H2O2 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.

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, cefoxitin, 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 sulphonamamide (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 ($\chi^2$). P values were calculated and $p<0.05$ was considered as statistically 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 test 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).

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 ($p= 0.000$, $x^2=0.000$), milk hygiene ($p=21.73$, $x^2=0.000$), parity ($p= 0.000$, $x^2=0.000$), age ($p= 29.07$, $x^2=0.000$), showed statistically significant with the occurrence of mastitis. Whereas antibiotic used ($p=7.59$, $x^2=0.06$) which is statistically in significant.

Table 1: Prevalence of mastitis at quarter level

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

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

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

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>No of cows examined</th>
<th>No of positive n (%)</th>
<th>$X^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactation Stage</td>
<td>Early 1-3</td>
<td>62</td>
<td>9(14.52%)</td>
<td>26.63</td>
</tr>
<tr>
<td></td>
<td>Mid 4-6</td>
<td>93</td>
<td>49(52.69%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Late &gt;6</td>
<td>75</td>
<td>39(52%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Young (&lt;4)</td>
<td>99</td>
<td>22(22.2%)</td>
<td>29.07</td>
</tr>
<tr>
<td></td>
<td>Adult(4-6)</td>
<td>60</td>
<td>32(53.33%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Old(&gt;7)</td>
<td>71</td>
<td>43(60.53%)</td>
<td></td>
</tr>
<tr>
<td>Milk Hygiene</td>
<td>Good</td>
<td>77</td>
<td>16(21.33%)</td>
<td>21.73</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>153</td>
<td>81(52.26%)</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>1-2</td>
<td>99</td>
<td>22(22.22%)</td>
<td>29.07</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td>59</td>
<td>59(52.54%)</td>
<td></td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Biofilm production on MCRA</th>
<th>Number of isolated bacteria</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>32</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>

MCRA: 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).

| Antibiotic used | 4 72 44(61.1%) | Yes 60 | 19(31.66%) | 7.59 | 0.06 |
| No             | 170            | 78(45.88%) |

<table>
<thead>
<tr>
<th>Floor type</th>
<th>4.3. Biofilm production status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concrete</td>
<td>136 42(30.38%) 17.48 0.000</td>
</tr>
<tr>
<td>Mud</td>
<td>94 54(58.51%)</td>
</tr>
</tbody>
</table>

Fig 2: Number of isolates from clinical and subclinical mastitis

![Image of isotates on Modified Congo red agar](image_url)

Fig 3: Growth on Modified Congo red agar

Table 3: CNS isolates and Biofilm production status

![Table 3: CNS isolates and Biofilm production status](table_url)
Table 4: Summary of antimicrobial sensitivity test for CNS isolate (ng of isolate=32)

<table>
<thead>
<tr>
<th>Antibiotic disc</th>
<th>Susceptible n (%)</th>
<th>Intermediate n (%)</th>
<th>Resistance n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (25 µg)</td>
<td>21 (65.6%)</td>
<td>4 (12.5%)</td>
<td>7 (21.9%)</td>
</tr>
<tr>
<td>Sulphonamide (300 µg)</td>
<td>6 (18.8%)</td>
<td>-</td>
<td>26 (81.3%)</td>
</tr>
<tr>
<td>Oxytetracycline(30 µg)</td>
<td>23 (71.9%)</td>
<td>-</td>
<td>9 (28.1%)</td>
</tr>
<tr>
<td>Ceftriaxone (30 µg)</td>
<td>27 (84.4%)</td>
<td>2 (6.3%)</td>
<td>3 (9.4%)</td>
</tr>
<tr>
<td>Kanamycine (30 µg)</td>
<td>30 (93.8%)</td>
<td>-</td>
<td>2 (6.3%)</td>
</tr>
<tr>
<td>Amoxacillin (30 µg)</td>
<td>28 (87.5%)</td>
<td>-</td>
<td>4 (12.5%)</td>
</tr>
<tr>
<td>Naldixic Acid (30 µg)</td>
<td>13 (40.6%)</td>
<td>9 (28.1%)</td>
<td>10 (31.3%)</td>
</tr>
<tr>
<td>Cefoxitin (30 µg)</td>
<td>5 (15.6%)</td>
<td>14 (43.8%)</td>
<td>13 (40.6%)</td>
</tr>
</tbody>
</table>

4.5. Antimicrobial resistance pattern

From the total CNS isolates as shown in figure (4) 85% were resistant 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=Naldixic Acid, S3=compound sulphonamide, FOX=Cefoxitin
CRO=Ceftriaxone, S=Streptomycin, OT =Oxytetracycline, k=Kanamycine 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) and 44.1% Girma (2010). 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), 49.7% in tgray by Enquebahir et al., 2008, [3] Mekbib et al., 2010, [36] Lake et al., 2009, [39] Sori et al., 2005 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). 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 received 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 US$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), Workineh et al. (2002), Bishi (1998) [8], and Fekadu (1995) who reported 40.4% in southern Ethiopia, 38.2% in Addam-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 Mekbib et al. (2010), Lakew et al. (2009) and Sori et al. (2005) 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
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) 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, 28] 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) [10] and Kerro and Tareke (2003) [28] 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) [10], 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 managerial 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. CNS the major cause of subclinical mastitis are found sensitive to kanamyin (93.8%),Amoxacillin (87.5%),Ceftriaxone(84.4%), and Amoxicillin(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 Amoxicillin (75%). In this study CNS is highly susceptible to Ceftriaxone which is (84.4%) and this closely agrees with Basappa et al. 2011 (83.8%) 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) [52].

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 Staphylococcus 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 1. All the current isolates were sensitive to kanamyin, Amoxicillin and Ceftriazone; 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.

7. References
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