EPIDEMIOLOGY OF BOVINE MASTITIS IN SMALLHOLDER DAIRY FARMS IN UNGUJA ISLAND OF ZANZIBAR, TANZANIA

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EXTENDED ABSTRACT

Bovine mastitis remains to be the most important disease facing smallholder dairy industry in Zanzibar. Direct and indirect cost incurred during the control of clinical cases of mastitis together with prolonged subclinical mastitis reduces quantity and quality of milk produced, and hence, decrease smallholder dairy farmers' income. This study was conducted to assess the characteristics and farm management practices of smallholder dairy cattle farming system and their relation with milk production, to quantify the occurrence of subclinical and clinical bovine mastitis and to identify their potential risk factors associated with both forms of the disease. In addition, pathogens causing clinical and subclinical bovine mastitis were isolated and tested for antimicrobial resistance. The study was carried out in three districts of Unguja island of Zanzibar in the United Republic of Tanzania. The selected districts were North B, Central and West districts. Two hundred and three farmers with 425 lactating dairy cows were included in a cross-sectional study that used questionnaire interviews and direct observation to assess characteristics of dairy farming. To quantify the prevalence of subclinical mastitis, questionnaire interviews, direct observations, California mastitis test and laboratory analysis were used. Longitudinal study was used to quantify the occurrence of clinical mastitis. It was found that a dairy farmer possesses an average of seven dairy cattle including two lactating cows. Farmers in West district had bigger average number of lactating cows (3) compared to Central (2) and North B (2), the difference was statistically significant ($p<0.05$). More than two thirds (72%) of the farmers interviewed kept crossbreed dairy cattle that were managed mostly by zero grazing system (65%). About 61% of the respondents had constructed shed for their dairy animals. About 86% of the farmers provided maize bran, pollard or a mixture of the two as supplement feeds for their lactating cows ($p<0.05$). Average daily milk production was 7.6 ± 3.6 litres per cow per day. It was found that,

average milk production in West district (8.0 litres/day) was higher than average production in Central (7.2 litres/day) and North B (6.9 litres/day), the difference between districts was statistically significant (p<0.05). Two hundred and one farms, 416 dairy cows and 1,648 lactating quarters were tested for the prevalence of bacterial and mycotic subclinical mastitis (SCM). Based on California Mastitis Test (CMT) results, the overall prevalence of SCM was 28.6, 48.8 and 64.7% at quarter, cow and farm level, respectively. Based on bacterial isolation the prevalence was recorded at 42.9, 70.9 and 78.6% at quarter, cow and farm level, respectively. Out of 831 isolates from subclinical cases of bovine mastitis, *Staphylococcus aureus* composed of 36.8%, *Pseudomonas aeruginosa* formed 17.8%, *Staph epidermidis* formed 16.1%, *Klebsiella* spp. formed 9.5%, *Micrococcus* spp. formed 6.3% and *Escherichia coli* formed 4.9%. In addition, it was found that some drugs such as penicillin was no longer appropriate for the treatment of mastitis since 87.6% of isolates were resistant to this drug. The overall prevalence of mycotic subclinical mastitis as defined by mycotic pathogens isolation were 19%, 33.9% and 49.3% at quarter, cow and farm level, respectively. The quarter level prevalence was significantly higher in Central district (26.1%) compared to North B (20.1%) and West (14.4%). The majority of isolated mycotic pathogens were *Aspergillus spp* (48.2% of isolates), *Candida spp* (41.1%), and *Saccharomyces spp* (7.4%). Other minor mycotic pathogens comprised of *mucor spp* (1.2%), *Microsporum spp* (0.6%), *Prototheca spp* (0.6%), *Sporobolomyces spp* (0.6%) and *Trichophyton spp* (0.3%). Cow level incidence rate per 100 cow-year at risk, cumulative incidence and cumulative hazard function as reported by farmers were 34.0, 18.3 and 0.27 respectively while those defined by pathogen isolation were 31.8, 17.1 and 0.25, respectively. Quarter level incidence rate per 100 quarters-year at risk, cumulative incidence and cumulative hazard function at quarter level as reported by farmers were 14.5, 7.8 and 0.11 respectively, and those defined by pathogen isolation were 12.9, 7.0 and 0.10, respectively. Out of 212

pathogens isolated from clinical cases of mastitis, major isolates were *Staphylococcus aureus* (35.4%), *Staphylococcus epidermidis* (13.7%), *Candida spp* (8.5%), *Pseudomonas aeruginosa* (6.6%), *Klebsiella spp* (6.1%) and *Aspergillus spp* (4.7%). Risk factors associated with the increased occurrence of subclinical mastitis as defined by CMT screening included herd size and lactation stage. Risk factors for subclinical mastitis as defined by bacteria isolation were dirty drainage, uses of towel to dry udder and number of parity while as defined by mycotic isolation were type of housing and milking technique. The protective factors associated with decreased occurrence of subclinical mastitis as defined by CMT screening included availability of water and uses of calf sucking before milking. The risk factors associated with bacteria isolations were number of milking per day, milking technique, hand wash before milking and amount of milk produced. Risk factors associated with mycotic isolation included number of milking per day and hand wash before milking. Risk factors associated with increased incidence of clinical mastitis as reported by farmers and pathogens isolation included cows' age and breed, number of parity, lactation stage, milk production, cow hygiene, floor hygiene, study district and calf suckling before milking. Washing hands before milking decreased the occurrence of clinical mastitis. Findings of this study have shown that dairy farming in Unguja Island operates under poor farm management, poor hygiene and improper milking techniques leading to low milk production and increased likelihood of lactating cows being infected with mastitis. Furthermore, the findings revealed high prevalence of subclinical and incidence of clinical mastitis at farms, cows and quarters levels with both contagious and environmental bacterial as well as mycotic pathogens being involved. Findings of this study provide information required to set up strategies to control mastitis in Zanzibar.

DECLARATION

I, Talib Saleh Suleiman, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that it has neither been submitted nor concurrently being submitted for a degree award in any other institution.

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DEDICATION

This work is dedicated to my late lovely mother Asma Ally and my late father Saleh Suleiman. It is also dedicated to my wife Wahida Kassim Soud.

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LIST OF ABBREVIATIONS

ASDP-L	Agriculture Service Support Programme
ASSP	Agriculture Sector Development Programme – Livestock
BA	Blood Agar
BSC	Body Condition Score
CDC	Centre for Disease Control
CI	Confidence limit
CICM	Cumulative Incidence of Clinical Mastitis
CM	Clinical Mastitis
CMT	California Mastitis Test
CNS	Coagulase Negative Staphylococci
D	Design effect which
e^2	e^2 is precision
ESBL	extended Spectrum Beta-Lactamase
G-	Gram negative
G+	Gram positive
GDP	Gross Domestic Products
h	Hour
HHs	Households
IRCM	Incidence Rate of Clinical Mastitis
IU	International units
LF	Left Front
LR	Left Rear
MCA	MacConkey Agar

MRSA	Methicillin-resistant *Staphylococcus aureus*
NBS	National bureau of statistics
^{o}C	Degree Celsius
OCGS	Office of Chief Government Statistician
OR	Odds Ratio
r^2	Correlation Coefficient.
RF	Right Front
roh	Rate of homogeneity
RR	Right Rear
SCC	Somatic Cell Counts
SCM	Subclinical mastitis
SD	standard deviation
SDA	Sabouraud Dextrose Agar
SE	Standard Error
SFMT	Surf Field Mastitis Test
SMS	Short Massages Service
spp	Species
T	Trace
VIC	Veterinary Investigation Centre
WST	White Side Test
X^2	Chi Square test
β	Regression coefficient
μg	Microliters

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Dairy Farming within Zanzibar Agriculture Sector

Agriculture sector remains a major component of Zanzibar economy with high contribution to the Zanzibar Gross Domestic Products (GDP). It ensures the livelihood, wellbeing and improved food security of people living in rural areas. Over 70% of Zanzibar population depends on agriculture. Contribution of agriculture sector to the Zanzibar GDP rose from 23.4% in 2005 to 30.8% in 2009 (OCGS, 2010) but dropped to 27.9% in 2014 (OCGS, 2015). The livestock sub-sector provides employment opportunities to more than 45,684 households and its contribution to the Zanzibar GDP rose from 4.1 in 2005 to 4.5% in 2009. According to 2007/2008 National sample census of agriculture, Zanzibar had a total of 155 624 heads of cattle, out of which 148 744 (95.6%) were indigenous and 6 880 were improved dairy cattle (OCGS, 2012). Unguja island has a total cattle population of 77 313 kept by 15 722 households; out of which 72 680 are indigenous and 4 633 are improved dairy cattle (OCGS, 2012). The dairy cattle kept included pure exotic breeds (*Bos taurus*) of Jersey, Ayrshire and Friesian and their crosses with Tanzania short horn zebu (*Bos indicus*).

Dairy farming in Zanzibar is divided into three categories: smallholder, medium scale and large-scale dairy farming. Smallholder dairy farming started in early 1980s. This serves as an important source of milk production after the collapse of Government owned farms of Bambi and Kizimbani in early1990s (Biwi, 1992; Mshangama and Ali, 1995). In this category, a farmer owns between 1-10 dairy cattle and according to OCGS (2012), 95% of dairy farmers in Zanzibar belong to this category. Medium scale dairy keeping, in which a farmer owns between 11 to 15 dairy cattle comprises about 3% of dairy farmers. Large scale dairy keeping, in which a farmer owns more than 15 dairy cattle make about 2% of

the dairy farming in Zanzibar (OCGS, 2012). Milk production from pure exotic and cross breed cattle in Zanzibar remains below the expected potential and no studies have been carried out within Zanzibar to explain possible factors that contribute to the poor productivity of dairy cattle.

1.2 Bovine Mastitis

The term mastitis is derived from two Greek words; "*masto*" which means mammary gland and "*itis*" which means inflammation (Radostits *et al*., 2006). Bovine mastitis is defined as inflammation of cows' mammary gland caused by intruding pathogenic microorganisms through teat canal (Bradley, 2002; Sharma *et al*., 2011). It is characterized by physical change of infected udder, chemical and microbiological changes of udder secretion (Ijaz *et al*., 2014). Primarily, mastitis results from interaction between the host, causative agents and environmental factors leading to localized inflammation within the udder (Gebru and Getu, 2015; Gomes and Henriques, 2016). It may also lead to raise in body temperature. Microorganisms including bacteria, fungi, algae and viruses may have the potential to cause mastitis once find their way inside the udder (Sharma *et al*., 2012). After intruding the udder, pathogens multiply and produce toxins that damage milk cell resulting to the occurrence of inflammation as body defence mechanism and this leads to increased number of somatic cells in the milk (Qadri *et al*., 2015). In addition to microorganisms, mechanical or thermal trauma and chemical injury can predispose mammary gland to infection leading to mastitis (Bogni *et al*., 2011; Litwinczuk *et al*., 2015).

1.3 Types of Bovine Mastitis

Classification of bovine mastitis depends on pathogens involved, visibility of clinical signs and degrees of severity, udder tissue damage and amount of inflammation generated (Argaw, 2016).

1.3.1 Types of bovine mastitis based on causative agents

Based on the causative agents, mastitis can be divided into three types; contagious, opportunistic and environmental mastitis (Hamadani *et al*., 2013; Kulkarni and Kaliwal, 2013).

1.3.1.1 Contagious mastitis

This type of mastitis is caused by pathogens living in the host mammary gland and cause damage to the milk tissue. Such pathogens cause both, subclinical and clinical mastitis (Bradley, 2002). Contagious mastitis can spread from one quarter to another or from one cow to another through milking. Major bacteria species causing this type of mastitis are *Staphylococcus aureus* and *Streptococcus agalactiae* (Fox and Gay, 1993; Magro *et al*., 2017).

1.3.1.2 Environmental mastitis

It is caused by environmental pathogens living in the host surroundings with potential to invade the mammary gland through teat canal orifice, multiply within the udder, stimulate host immune system and causes inflammation. Poor environmental hygiene and improper milking technique play vital role in the spread and persistence of this type of mastitis. Teat orifices remain open during or soon after milking and make possible for these environmental pathogens to invade the udder. *Strep uberis*, *Klebsiella spp*, *E. coli* and *Enterobacter spp*. make good example of bacteria causing this type of mastitis (Kromker *et al*., 2014; Argaw, 2016; Badiuzzaman *et al*., 2016).

1.3.1.3 Opportunistic mastitis

This type of mastitis is caused by opportunistic pathogens that exist on the teat skin. Normal flora, considered non-pathogenic can invade the udder, stimulate host immune

system and cause subclinical or mild clinical mastitis (Kulkarni and Kaliwal, 2013). Coagulase negative staphylococci such as *S. epidermidis*, *S. saprophyticus*, *S. simulans* and *S. chromogenes* are good examples of pathogens causing this type of mastitis (Pyörälä and Taponen, 2009; El-Jakee *et al*., 2013; Hosseinzadeh and Dastmalchi Saei, 2014).

1.3.2 Types of bovine mastitis based on clinical manifestation

1.3.2.1 Subclinical mastitis

This is a hidden form of mastitis with no observable signs of illness or any visible changes to the milk produced. Subclinical mastitis (SCM) is very challenging form of mastitis in most of the developing countries where farmers sell their milk without considering the level of somatic cell counts (SCC). Normally, farmers are unaware about SCM in their farms neither have technical know-how to diagnose the disease. Occurrence of SCM may be 40 times more compare to clinical mastitis (CM) in a farm (Shaheen *et al*., 2016). This form of mastitis has huge influence in decreasing quality and quantity of milk produced (Batavani *et al*., 2007; Ogola *et al*., 2007). Wide range of pathogens have been isolated from apparently health milking cows (Dieser *et al*., 2014).

1.3.2.2 Clinical mastitis

Clinical mastitis can be recognized by its clear observable signs such as swelling, udder redness, pain, induration, hotness, sudden reduced milk production and alteration of milk secretion that may become watery with flakes, clots or bloody milk (Qadri *et al*., 2015). Clinical bovine mastitis can be categorized in different form depending on the severity of clinical symptoms (Hossain *et al*., 2017). Peracute: characterized by high inflammation with systematic signs of fever, depression, shivering and loss of appetite followed by reduction in milk production and changes in milk composition. Acute form: characterized by udder inflammation with mild systematic signs like mild fever and depression.

Subacute form: characterized by mild udder inflammation without systematic signs (Hamadani *et al*., 2013).

1.3.2.3 Chronic mastitis

This type of mastitis is characterized by prolonged period of infection time which can be extended to months or year. Usually mastitis persists in the infected animal for months or can persist from one lactation to another. Chronic mastitis can persist as subclinical that erupt into clinical form from time to time. Changes in udder formation and milk composition can be observed for longer period of time. Many types of bacteria can invade and persist within the udder of an infected cow (Dogan *et al*., 2006).

1.4 Pathogens Causing Mastitis

Aetiological agents of bovine mastitis vary from bacteria (Belayneh *et al*., 2014; Belachew, 2016; Zeryehun and Abera, 2017), to mycoplasma (Al-Abdullah and Fadl, 2006; Masic *et al*., 2017), fungi (Zhou *et al*., 2013; Ghodasara and Gajbhiye, 2015) and algae (Marques *et al*., 2008; Bozzo *et al*., 2014) or virus (Wellenberg *et al*., 2002). More than 137 pathogens' species and subspecies have been identified to cause bovine mastitis (Watts, 1988; Du Preez, 2000). However, most cases of clinical mastitis are caused by bacteria (Blowey and Edmondson, 2010). This can be divided into major and minor bacteria causative agents (Hamadani *et al*., 2013).

1.4.1 Bacterial pathogens

Staphylococcus aureus is a predominant bacterium isolated from both clinical and subclinical cases of bovine mastitis (Zecconi, 2010; Arga *el al*., 2012; Thaker *et al*., 2013). *Staphylococcus aureus* is considered very contagious pathogen among mastitis causing agents (Delgado *et al*., 2011). The pathogen can be transmitted from one infected quarter

to another or from one infected cow to another during milking (Gitau *et al*., 2014). Studies conducted around the world to isolate pathogens of bovine mastitis, reported *Staphylococcus aureus* as one on the top of the table with percentage ranging from 26 to 44.5% (Abera *et al*., 2010; Kwanashie *et al*., 2012; Lakshmi and Jayavardhanan, 2016). Coagulase Negative Staphylococci (CNS) has been linked with the occurrence of both CM and SCM in dairy cattle. A study conducted in Sweden by Thorberg *et al*. (2009) reported isolation of eleven species of CNS from milk collected from subclinical cases of bovine mastitis that include *S. epidermidis*, *S. simulans*, *S. chromogenes*, *S. xylosus*, and *S. haemolyticus*. The CNS may cause chronic form of SCM and are more resistant to antibiotic treatment (Taponen and Pyörälä, 2009).

Streptococcus agalactiae, is a highly contagious pathogen isolated from bovine mastitis cases around the world (Keefe, 1997; Biressaw and Tesfaye, 2015). Many studies described *Strep agalactiae* as second pathogenic microbe isolated from cases of bovine mastitis after *Staph aureus* (Saidi *et al*., 2013; Yohannis and Molla, 2013; Shiferaw and Telila, 2016). Despite the fact that *Streptococcus spp* are susceptible to diverse type of antibiotics, they have remained to be predominant among isolated pathogens of bovine mastitis. They normally cause decrease in the quality and quantity of milk produced (Keefe, 1997). *Streptococcus uberis* is another bacterial pathogen within Streptococcus genus that is involved in the occurrence of clinical and subclinical mastitis (Davies *et al*., 2016).

Most of Gram negative bacteria causing bovine mastitis are considered as of environmental origins. The main sources being bedding material, soil, manure and other organic or inorganic materials around the lactating cows (Hogan and Smith, 2002). *Escherichia coli* is another bacterial pathogen isolated in both clinical and subclinical

bovine mastitis (DaRong *et al*., 2010; Tanzin *et al*., 2016). Isolation of *E. coli* in previous studies comprised between 22% and 30% among isolated pathogens (Mahenthiran and Michael, 2016; Nasef and Dawod, 2016). *Klebsiella spp*. is another Gram negative environmental pathogen that has been found to cause bovine mastitis especially subclinical form (Gogoi *et al*., 2017). Other common bacteria species isolated from bovine mastitis cases included *Bacillus spp*, *Pasteurella spp*, *Corynebacterium spp*, *Proteus spp* and *Pseudomonas spp* (Akram *et al.*, 2013). A number of anaerobic bacteria have been reported to cause bovine mastitis as well. *Clostridium Perfringens* has also been reported in clinical cases of bovine mastitis in cows and buffalos (Osman *at al*., 2009; Odongo *et al*., 2012).

1.4.2 Fungal pathogens

More efforts have been directed to isolation of bacteria as causative agents of bovine mastitis, little attention has been paid to the isolation of other pathogenic microorganisms. The involvement of fungi as causative agents in subclinical and clinical bovine mastitis has increased in recent years (Pachauri *et al*., 2013; Bakr *et al*., 2015). Pachauri *et al*. (2013) described the isolation of different types of fungi from clinical and subclinical cases of bovine mastitis in a study conducted in India. The isolates identified were mainly *C. albicans*, *A. fumigatus* and *A. niger*. Another study conducted in Egypt reported isolation of *C. albicans*, *A. fumigatus*, *A. niger* and *C. neoformans* (Bakr *et al*., 2015). Enany *et al*. (2007) also isolated *C. albicans*, *C. spp*, *A. niger* and *Rhodotorula spp* in Egypt. Identification of fungi as causative pathogen of bovine mastitis has not only reported in underdeveloped countries, but also in developing and developed countries. A group of researchers in Argentina reported the isolation of *A. fumigatus* and *A. niger* (Pellegrino *et al*., 2013). In Tanzania, Mdegela *et al*. (2009) reported the isolation of yeast and Cryptococci from Mvomero and Njombe districts.

Fungi mastitis can be divided into two types, primary fungi mastitis that occurs directly without previous infection of bacterial pathogens and secondary fungi mastitis that occurs with or after previous case of mastitis (Akdouche *et al*., 2014). Primary fungi mastitis can be caused by environmental opportunistic fungi that residing at cows' surroundings. Whenever these pathogens enter the udder, they trigger host immunity to initiate inflammation process and cause mastitis. Secondary fungi mastitis can follow after mastitis infection caused by bacterial pathogens. Fungi may invade the udder during administration of intramammary infusion if unhygienic precautions are not taken into consideration. Uses of contaminated surgical equipment during treatment of udder infection or presence of contaminated injuries or wound in the teats or udder can provide suitable source of fungi infection to the udder (Stanojevic and Krnjajic, 2002).

1.4.3 Algae causative agents

Demonstration of achlorophylic algae as causative pathogens for both CM and SCM has been reported in different places around the world (Ognean *et al*., 2011; Zaini *et al*., 2012). The most isolated species of algae are *Prototheca zopfii* and *Prototheca blaschkeae* (Janosi *et al*., 2001; Marques *et al*., 2008).

1.5 Pathogenesis of Mastitis

Under normal circumstances, teat orifice acts as the first line of defence mechanism of the udder. When a cow is not milked, the sphincter muscle is closed very tight to prevent introduction of any type of microorganism inside the udder (Bogni *et al*., 2011). During milking or soon after milking, the sphincter muscles become dilated and leave the teat orifice open for one to two hours. Whenever proper post milking care was not taken into consideration, pathogenic micro-organisms may take this opportunity to pass the teat canal into the udder. Once in the udder those pathogens attached to the milk cells can multiply

into cluster of cells called biofilm that facilitate the pathogen persistence in the udder (Gomes *et al*., 2016). Inflammation begins when these pathogens start to produce virulence factors that damage milk producing cells evidenced by swelling and death. The death of milk producing cells can be caused by toxins produced by invading pathogens or some pathogens that invade and multiply within the milk cells (Zhao and Lacasse, 2008). When pathogens enter inside the udder the basic body defence mechanism is phagocytosis killing by polymorphonuclear neutrophils. The effectiveness of phagocytosis depends on pathogens' virulence and host immune efficiency (Kerro Dego *et al*., 2002). At this stage, macrophages and damaged epithelial cells release chemical attractants that increase vessel permeability and activate the migration of leukocytes from blood vessels into the udder. The leukocytes act as another defence mechanism that engulf and destroy the invading microorganisms. If the leukocytes manage to destroy and eliminate the invading pathogens, the infection will be cleared and milk production and composition become normal. In case the elimination is not successful, the pathogens persist and proceed to attack smaller milk ducts and milk alveolar. More leukocytes will be imported from blood vessels to the udder and inflammatory reaction continues and more milk producing cells will be completely destroyed leading to decreased amount of milk produced and increased number of somatic cells count in the milk.

1.6 Diagnosis of Mastitis

Proper and accurate diagnosis is very critical to ensure successful control and treatment of bovine mastitis. Farmers can easily identify occurrence of clinical cases of mastitis based on clinical manifestations and notify the nearby veterinarian. For subclinical mastitis, due to the absence of clinical signs, a farmer remains unaware of the disease in his dairy farm. Any decision that will be taken, either to treat the infected cow or otherwise, should be based on the accurate diagnostic results. Different methods of diagnosis can be used,

starting from visual inspection to the measurement of increased number of somatic cells and isolation of causative microorganisms (Sanotharan *et al*., 2016; Hossain *et al*., 2017).

1.6.1 Visual inspection

Any observable physical change in the milk, quarter, udder or animal as a whole should be taken as indication of mastitis (Lam *et al*., 2009). Change in the milk can include clotting, watery milk, bloody milk, visible pus or any other abnormal discharge. Change in the udder or a quarter of the udder such as pain, induration, change of colour, swelling and hotness should highly be noticed. Sudden decrease in milk production, fever, in-appetite and decreased time of resting (lying down) in a milking cow should be considered as indicative sign of clinical case of mastitis.

1.6.2 Somatic Cell Count (SCC)

Occurrence of both clinical and subclinical bovine mastitis results into increased number of somatic cells in the milk. Somatic cells are combined leukocytes and epithelial cells that have been shed from interior parts of mammary gland. Leukocytes which make about 75% of the somatic cells includes macrophages, neutrophils, lymphocytes and erythrocytes while epithelial cells make about 25% of the somatic cells (Sharma *et al*., 2011). Under normal condition the amount of somatic cell in one millilitre of milk range between 0 and 1.5×10^6. The number of neutrophils increase in the udder as body defence mechanism responding to the presence of infection. The measurement of somatic cell in the milk is known as Somatic Cells Count (SCC). The number of SCC in the normal milk should be less than 1.0×10^5 per millilitre of milk but during infection the somatic cells counts can become more than 1.0×10^6 (Guha *et al*., 2012; Deb *at al*., 2013). Measurement of SCC in the milk can be counted directly by using Coulter counter which counts the number of electric impulses resulting from particles passing between two electrodes or by using

fossomatic which counts the number of cell nuclei that become fluorescent due to ethidium bromide (Djabri *et al*., 2002). Both techniques can measure the number of somatic cells directly but require specialized technical personnel and equipment and cannot be practical method under field situation in most part of Zanzibar. In addition, there are numbers of indirect methods of measuring increased number of SCC in milk. such methods include California Mastitis Test (CMT), White Side Test (WST) and Surf Field Mastitis Test (SFMT) (Badiuzzaman *et al*., 2016). California Mastitis Test (CMT) has been found to be the best indirect method to measure SCC among those three techniques (AL-Edany *et al*., 2012).

1.6.3 California Mastitis Test (CMT)

California mastitis test (CMT) is an indirect method of measuring the somatic cell count in the milk (Iqbal *et al*., 2006). It was first described by Schalm and Noorlander (1957) as a quick, simple that can be used in the field to qualitatively predict SCCs from individual milk or composite milk sample (El-Hewairy *et al*., 2015). The CMT reagent is composed of Alkyl Aryl Sulfonate (3%), Sodium hydroxide (1.5%) and Bromo cresol purple (1: 10 000) as an indicator (Barua *et al*., 2014; Kamal *et al*., 2014). The test can be performed by mixing about 2-3 ml of milk with equal amount of CMT solution using commercial available CMT pad. The mixture is then hand rotated and the result observed is based on jelly formation as described earlier by Barnum and Newbould (1961). The results of the jelly formation can be summarized as shown in Table 1.1.

Dingwell *et al*. (2003) described the CMT as appropriate cow side test with high sensitivity (82.4%) and specificity (80.6%). The method, which is cost effective, easy to use and instantly provide results of individual cow at farm level. The CMT is

recommended as the most appropriate method to diagnose subclinical mastitis under field condition (Sharma *et al*., 2010; Kasikci *et al*., 2012).

Table 1.1: CMT indicative result summary and interpretation

Result abbreviation	Result	Mixture consistency
-	Negative	No change in consistency
T	Trace	No change in consistency but when paddle is tipped a slime is seen in the bottom
1+	Weak positive	A thick slime is formed but when the paddle is swirled the solution does not move to the centre
2+	Distinct positive	A thick lumpy gel formed, when swirled quickly moves toward the centre
3+	Strong positive	A distinct gel formation which tends to adhere to the bottom of the paddle, when swirled, distinct central peak is formed

1.6.4 Isolation of causative pathogens

Due to varieties of pathogens causing the disease, identification of specific pathogens for each mastitis cases is crucial. Isolation and identification of involved pathogens is crucial for proper treatment. For this purpose, milk samples should be collected in hygienic manner to avoid any type of contamination. Teats should be washed using fresh and clean water then dried by single use paper towel, and scrubbed using a piece of cotton gauze soaked in 70% methylated alcohol. The first one or two strip of milk should be discarded before collecting milk sample directly to the sterile and unique labelled universal bottle as described by Quinn *et al*. (1994). Soon after collection, milk samples should be transported in a cool box containing ice pack at 4°C to a laboratory for pathogen isolation. For optimal results, milk samples should be cultured on the same day of collection or deep frozen at -20°C until culture within 72 hours.

For isolation of bacterial pathogens, milk samples can be cultured using MacConkey agar (MCA) and blood agar (BA). Bacterial growth should be observed after 24 and 48 hours and if no growth is observed, the quarter from where the milk sample was collected is considered negative. For positive growth, colony morphology, pigmentation and haemolytic reaction should be observed. Gram stain is used to distinguish G+ and G- bacteria and to study the microscopical features of the isolates. Different biochemical tests as described by Vashist *et al*. (2013a) are used to identify the bacteria at genus and species level. Oxidase test, modified oxidase test, catalase, coagulase and urease tests can be used to characterize Gram positive bacteria (Pati and Mukherjee, 2016). For Gram negative bacteria, lactose fermentation, indole production, the methyl red test, the Voges-Proskauer test and the citrate utilization test can be used for characterization (De los Santos *et al*., 2013).

Samples for fungi and algae isolation are cultured on Sabouraud dextrose agar (SDA), incubated at 37°C and observed daily for two weeks. If no growth is observed, the quarter is considered negative for fungi infection. Gram stain and lacto-phenol cotton blue can be used to identify yeast and yeast like fungi followed by sugar fermentation as biochemical test (Pachauri *et al*., 2013). For identification of moulds, morphological growths that include texture, pigmentation of both sides of growth can be used. Transparent adhesive tape technique can be used to observe the filament and microfilament formed under microscope (Sukumar and James, 2012).

1.6.5 Testing for antimicrobial resistance

Most of the time, treatment of mastitis begins before knowing the causative agent involved or without proper antibiotic testing. This may lead to the use of antibiotics which are not effective or the use of antibiotics in cases where bacteria are not involved as causative

agents. Improper use of antibiotics may increase the emergence of resistance among bacteria in both animal and human populations (Padol *et al*., 2015). Testing for appropriate antimicrobial should be emphasised (Awandkar *et al*., 2013). Kirby-Bauer disc diffusion technique as described by Vashist *et al*. (2013b) and can be used to test antimicrobial susceptibility and resistance pattern. A portion of pure colony of bacterial isolates is transferred in a tube with 5 ml of nutrient broth and incubated at $37^{\circ}C$ for 24 hours. The mixture is then transferred into a Muller Hinton agar plate and spread using sterile glass tube. The antibiotic discs are then placed on the plate using sterile forceps and incubated for 24 hours at $37^{\circ}C$. The diameter with no growth around each antibiotic disc is measured using transparent ruler and the measurement was recorded in millimetres. Based on the length of diameter of no growth zone around antibiotic disc, the results can be then interpreted into sensitive, intermediate or resistant to the specific antibiotic in the disc.

1.7 Epidemiology of Bovine Mastitis

1.7.1 Prevalence of bovine mastitis

Number of studies have been conducted to demonstrate the prevalence of bovine mastitis in eastern part of the African continent. In Ethiopia, the farm level prevalence of bovine mastitis has been reported to be 74%, cow level prevalence has been shown to be 42-71% while quarter level prevalence ranged between 28 to 44% (Mekibib *et al*., 2010; Biressaw and Tesfaye, 2015; Abebe *et al*., 2016; Mekonnin *et al*., 2016). In Kenya, cow level prevalence was between 56-82% while quarter level prevalence was estimated as high as 87.4% (Ondiek *et al*., 2013; Gitau *et al*., 2014; Nkoroi and Maitho, 2014). Epidemiological survey on bovine mastitis conducted in Tanzania mainland revealed high prevalence of both clinical and subclinical mastitis (Mdegela *et al*., 2005; Karimuribo *et al*., 2005; Karimuribo *et al*., 2006; Kivaria *et al*., 2007). In Zanzibar, very few studies

were previously conducted to demonstrate the prevalence of bovine mastitis. In Unguja Island, Gitau *et al*. (2003) observed cow level prevalence of subclinical bovine mastitis of 68% while in Pemba island, Suleiman *et al*. (2013) found high prevalence of both clinical and subclinical mastitis. Cow level prevalence of clinical mastitis was 31.1% while subclinical mastitis prevalence at cow level was 55.8% and at quarter level was 34.4%

1.7.2 Incidence and incidence rate of bovine mastitis

Studying the Incidence Rate of Clinical Mastitis (IRCM) in dairy cattle is rare due to cost implication and time taken to follow-up. Most of the studies estimated the point incidence during field visit or are based on farmer recalling the number of incidence during defined previous period of time. High incidence rate of clinical bovine mastitis range between 20 and 26 per 100 cows at risk per year as reported before. Previously studies also indicated cow level incidence of clinical mastitis range between 20 and 53% (Abd El-Razik *et al*., 2011; Almaw *et al*., 2012; Arga *et al*., 2012). This high level of incidence rate shows how important this disease is in dairy industries (Shpigel *et al*., 1998; Cristina *et al*., 2016; Kumar *et al*., 2016).

1.8 Impact of Bovine Mastitis

Bovine mastitis remains as the most important disease in dairy sector around the world. Economic importance, animal welfare and public health hazard cannot be underestimated together with the social impact caused by huge burden of controlling the disease.

1.8.1 Economic importance

Always it is very difficult to calculate economic losses due to mastitis because productivity and production efficacy vary among cows, farms, cities or country (Bradley, 2002; Hogeveen, 2005). When subclinical mastitis is involved, the economic loss can be

estimated from the decreased milk production below cows' potential production level while in clinical bovine mastitis, farmers can easily realize the cost implication from treatment, discarded milk during treatment and decreased milk yield soon after treatment (Singh *et al*., 2014). Economic loss can be estimated from milk production losses, milk discarded, diagnostic cost, treatment cost, veterinary consultancy, premature culling of chronic cases, death and cow replacement cost (Hellerton and Berry, 2005; Halasa *et al*., 2007; Gebru and Getu, 2015). The cost implication of clinical bovine mastitis can be divided into direct costs which is equivalent to 29% of the total cost and indirect cost which is equivalent to 71% of the total cost (Rollin *et al*., 2015). Direct cost includes disease diagnostic measure, therapeutic measures, unsaleable milk, cost of veterinary services, labour work and animal death while indirect cost include prolonged milk production loss, premature culling, replacement loss and reproductive loss. Indirect losses include decreased milk production, decreased milk quality due to the increased somatic cells counts, change of milk composition and lowering the shelf life of the infected milk (Petrovski *et al*., 2006). Many studies have been conducted around the world to estimate the cost implication caused by both clinical and subclinical bovine mastitis. The parameters that have been shown to have negative economic impact include declined milk production, discarded milk, veterinary examination, high cost of drugs, treatment and veterinary services, premature culling, penalty for farmers with higher milk somatic cell counts (Sharifi *et al*., 2014; El-Tarabany and Ali, 2015; Kvapilik *et al*., 2015). In addition to this direct and indirect cost implication caused by mastitis at farm level and the effects can be extended to the processing and products produced from milk with higher somatic cell counts (Geary *et al*., 2013). Cost of controlling the disease increase the economic importance of bovine mastitis by reducing total revenue gained by dairy cattle farmers (Mwabonimana *et al*., 2015).

1.8.2 Public health hazard

1.8.2.1 Biological hazard

Public health hazard implication of mastitis come from the range of zoonotic pathogens causing disease to human population whenever infected cow milk is used without pasteurization (Bradley, 2002; Morwal *et al*., 2017). Shaheen *et al.* (2016) in his review of bovine mastitis, mentioned the occurrence of an outbreak of *Campylobacter jejuni* as a result of consumption of untreated milk. Furthermore, he explained the possible transmission of *Prototheca zopfii*, *Mycobacterium bovis*, *Pseudomonas Spp*., *Listeria monocytogenes*, *Salmonella Spp*., *Campylobacter Spp*., *E. coli* and *Cyrptococcus neoformans* to human if untreated milk is consumed. Presence of *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *E. coli* in the milk may pose threat to human because the same pathogens have the potential to cause diverse human diseases (Hameed *et al*., 2006). LeJeune and Rajala-Schult (2009) also mentioned the outbreaks of salmonellosis, campylobacteriosis, and *E. coli* (O157: H7) infection caused by consumption of raw milk. Zoonotic nature of *Candida albicans* can also put human population at risk of contracting the disease when fresh milk is consumed (Sartori *et al*., 2014; Mousa *et al*., 2016). Previously isolated algae from infected milk included *Prototheca zopfii, Prototheca wickerhamii*, *Prototheca blaschkeae* and *Prototheca cutis*, all of which are pathogenic to both animals and human population (Bakula *et al*., 2014). Girma *et al*. (2014) classified milk borne public health hazards into biological, chemical and physical hazards. Mastitis can induce biological hazards such as pathogens and chemical hazards such as different types of drugs used in veterinary medicine. In addition, milk can comprise of materials such as foreign objects, insects, hair, plastics, metal and fabric, which may cause physical injury to the consumer (Aguiar *et al*., 2018).

1.8.2.2 Chemical hazard

Extensive use of antibiotics to treat or control bovine mastitis raise public health concerns in two ways; the first one emerges from the antibiotic residues that enter into the human food chain which may result into the direct or indirect toxicity of allergic reaction. The second way is the possibility of developing antibiotic resistance by variety of bacterial strains and leading to failure of therapy to the common clinical diseases (Vishnuraj *et al*., 2016). Khaniki (2007) in his review of chemical contaminants in milk, described the presence of antibiotics in the milk as the main chemical contaminants. Antibiotics such as tetracyclines, penicillin and sulphonamides may result into the allergic reaction in sensitive persons whenever contaminated milk is consumed. Transfer of antibiotic-resistant genes from non-pathogenic bacteria to the human pathogenic bacteria is another huge concern of antibiotic residue in the milk (Khaniki, 2007). In a study conducted in Addis Ababa, Ethiopia, 20.8% of the interviewed dairy farmers were found not observing drug withdrawal period after bovine mastitis treatment (Tilahun and Aylate, 2015). The threat of antibiotic residue in milk intended for human consumption can impose two aspects of threats; the first being allergic reaction and the second aspect is development of antibiotic resistance in the gut bacteria of human (Chowdhury *et al*., 2009).

1.8.3 Animal welfare due to mastitis

One among five freedoms as defined by animal welfare is freedom from discomfort. Cell damage in the udder can cause pain and discomfort to the animal. Change of behaviour, like depression, abnormal gesture, in appetite and kicking are among signs of pain during clinical cases of mastitis. Although it is difficult to assess the pain in dairy cattle suffering from mastitis, sensory emotions reveal the availability of pain and discomfort during clinical mastitis (Leslie and Petersson-Wolfe, 2012). Sign of discomfort and change of behaviour can begin some days before the onset of clinical mastitis when infected cow

shows signs of sickness and reduce feed intake (Sepulveda-Varas *et al*., 2014). Behaviour change during clinical cases of mastitis has been discussed previously by different authors. these behaviour changes can persist during clinical mastitis and can continue to a period of between three and ten days after treatment (Medrano-Galarza *et al*., 2012; Fogsgaard *et al*., 2015). Clinical case of bovine mastitis, especially acute form may lead to the generalized fever and depression and if left untreated may lead to the weakening of cow condition and death (Wellenberg *et al*., 2002).

1.9 Factors Influencing the Occurrence of Bovine Mastitis

Occurrence of bovine mastitis depends on the interaction of three elements. A host animal, in this context, a cow at risk of contracting the disease, virulent pathogen capable of invading the mammary gland and cause a disease together with favourable environment and farm management practice (Schroeder, 2012; Kurjogi and Kaliwal, 2014; Reshi *et al*., 2015; Hajie and Teka, 2017).

1.9.1 Host based factors

Previous studies mentioned diverse cow based risk factors that significantly increase or decrease the occurrence of bovine mastitis. Risk factors such as cows' breed, stage of lactation, number of parity, amount of milk produced, udder formation, location and direction of teats can put a cow at risk of contracting the disease. Older cows with more number of parity and prolonged lactation stage tend to have weak sphincter muscles in the teats orifice compared to younger cows. This makes easier for contagious and environmental pathogen to invade and colonized the udder and hence increase disease occurrence (Byarugaba *et al*., 2008; Karimuribo *et al*., 2008; Moges *et al*., 2012; Elbably *et al*., 2013; Abebe *at al*., 2016; Asmare and Kassa, 2017; Naseemunnisa *et al*., 2017). Dairy breeds Jersey, Friesian and Ayrshire which have high milk production records have

higher risk of contracting mastitis compared to their crosses with indigenous zebu (Tuke *et al*., 2017). Udder morphology, flatten teat ends, direction and distance of teat location from ground also have an effect to the occurrence of bovine mastitis (Nakov and Trajcev, 2012; Nakov *et al*., 2014).

1.9.2 Management and environmental factors

Previously identified environmental and farm management risk factors that have significant role in the occurrence of bovine mastitis included increased herd size and number of lactating cows, intensive or semi intensive rearing system, cow hygiene, poor farm hygiene and inappropriate milking techniques (Suleiman *et al*., 2013; Iraguha *et al*., 2015; Sarba and Tola, 2017). Poor hygiene condition of the barn and cows increased the chances of environmental pathogens to invade the udder and cause mastitis. Improved dairy farm management in terms of floor cleanliness, udder and hand wash before milking have been found to have significant influence on decreased cases of clinical mastitis (Rahman *et al*., 2009; Shittu *et al*., 2012; Nakov *et al*., 2014; Iraguha *et al*., 2015). Seasonal variation seems to have significant influence on occurrence of both clinical and subclinical mastitis. The occurrence has been found to be higher during long and short rains compared to dry seasons (Biffa *et al*., 2005). Allowing the calf to suckle sucking before and after milking may influence complete withdrawal of milk from the udder, eliminate number of pathogens and hence decrease occurrence of bovine mastitis (Sedano *et al*., 2010). Some researchers have revealed negative impact of this practice since it may transmit contagious pathogens from one infected quarter to another (Kivaria *et al*., 2004; Shittu *et al*., 2012).

1.9.3 Pathogen factors

Virulence of causative pathogen could play a vital role in the development of subclinical or clinical mastitis. Most contagious pathogens possess virulence genes that capacitate

them to be attached to the milk cell in the udder and avoid host body immune system. It has been observed that contagious pathogen such as *S. aureus* increase the risk of developing mastitis compared to none contagious pathogens. Some bacteria have selective advantage of biofilm formation that enable them to persist in the udder and increase resistance to antimicrobial treatment (Fadlelmoula *et al*., 2007; Gomes *et al*., 2016; Magro *et al*., 2017). Presence of gene encoding Shiga toxin and heat-stable enterotoxin play an important role for *E. coli* virulence toward bovine mastitis (Guler and Gunduz, 2007).

1.10 Antimicrobial Resistance Profile

Multi-drug resistance is a threat to the control and treatment of mastitis since it reduces the choice of drugs to be used (Awandkar *et al*., 2013). Most farmers in the study area use penicillin, streptomycin and tetracycline to treat their animals and a combination of penicillin and neomycin or kanamycin for intra-mammary infusion. Drug resistance differ from one country to another around the world (Sumathi *et al*., 2008; Alekish *et al*., 2013; Kasozi *et al*., 2014). Most previous studies have demonstrated the susceptibility of *S. aureus* to enrofloxacin, tetradelta combinations of amoxicillin plus clavulanic acid and cefalexin plus kanamycin but resistance to amoxicillin, streptomycin, lincomycin, bacitracin and penicillin. Coagulase Negative Staphylococci (CNS) have been found to be susceptible to rifaximin, tetradelta, amoxicillin plus clavulanic acid, cefalexin plus kanamycin, ceftiofur, cloxcillin, enrofloxacin, lincomycin but resistant to streptomycin, penicillin and amoxicillin. *Streptococcus spp* are susceptible to many antibiotics, except lincomycin and streptomycin. *Escherichia coli* is susceptible to ceftiofur, enrofloxacin, tetradelta enrofloxacin, cefalexin + kanamycin, amoxicillin + clavulanic acid but resistant to cloxacilin, lincomycin, penicillin and amoxicillin (Reza *et al*., 2011; Ikiz *et al*., 2013; Idriss *et al*., 2014). Multi drug resistance in veterinary medicine may raise a huge public health concern since the same drugs are used to treat infections in human population. The

threat may be through introduction of antibiotic residue into the human food chain that leads to the direct or indirect toxicity or allergic reaction; or by failure therapy to the common clinical diseases (Vishnuraj *et al*., 2016). Most farmers in Zanzibar use drugs to treat their animals without proper advice from qualified veterinarians and do not abide to the withdrawal period indicated by drug manufacturer (Tilahun and Aylate, 2015).

1.11 Problem Statement and Justification of the Study

1.11.1 Problem statement

Zanzibar smallholders' dairy cattle production system is fragmented without proper channel of milk collection, processing and marketing. Each individual farmer finds his/her own way of selling fresh unprocessed milk direct to consumers. The same situation persists when extension and veterinary services are considered; each farmer finds his own service provider for extension and treatment of his animals. This, leads to poor milk production in terms of quantity and quality and rejection of milk by Zanzibar milk processing plant or tourist market. Bovine mastitis remains to be the most important disease which reduces quality and quantity of milk produced by smallholder dairy cattle farmers. Previous studies conducted in Unguja Island indicated high prevalence of subclinical bovine mastitis (Gitau *et al*., 2003). Most cases of bovine mastitis in the study area treated by farmers themselves or para-veterinarian that focus on treatment of clinical cases rather than investigation of causative pathogens or strengthening the control measures. There is no proper strategy of controlling bovine mastitis prepared under Zanzibar situation. There is no previous study conducted in Unguja Island that characterised dairy farming system; or that estimated the prevalence of both clinical and subclinical mastitis. Baseline information about the factors that increase the occurrence of both clinical and sub clinical bovine mastitis in Unguja island of Zanzibar is missing. Previously study conducted in the study area concentrated only on the isolation of aerobic

bacterial pathogens while neglecting anaerobic, fungal and algae. Existing information gaps with regard to the real characteristics of dairy farming, current status of clinical and subclinical bovine mastitis, pathogens profile causing CM and SCM, status of antimicrobial drug profile specific for Zanzibar should be addressed.

1.11.2 Justification of the study

Prolonged subclinical bovine mastitis drastically reduced the amount of milk produced and, hence, decreases dairy farmer income. In addition, direct and indirect costs incurred during clinical cases of mastitis increase the importance of this disease by decreasing daily farmer's income. Complete knowledge about the epidemiological characteristics of bovine mastitis will help decision maker to prepare strategies for controlling the disease in the study area. If proper control measure against bovine mastitis is put in place and dairy farmers are forced to follow them, it may reduce the prevalence of both clinical and subclinical mastitis, and hence, increased smallholder dairy farmers income.

1.12 Objective of the Study

1.12.1 Overall objective

To investigate characteristics of smallholder dairy farming and epidemiology of bovine mastitis in Unguja Island of Zanzibar, Tanzania.

1.12.2 Specific objectives

i. To determine characteristics of smallholder dairy farming system and their relation to milk production,

ii. To establish prevalence of bovine subclinical mastitis,

iii. To establish incidence of bovine clinical mastitis,

iv.   To identify potential risk factors associated with the occurrence of clinical and subclinical bovine mastitis,

v.   To evaluate antimicrobial resistance patterns in common bovine mastitis causing bacteria.

1.13 Organization of the Thesis

This thesis is organised in seven chapters preceded by an extended abstract which summarizes the objectives, materials and methods, key research findings and conclusion. Chapter one consists of introduction, problem statement, justification of the study, study area and objectives. Chapter two summarize general methodology used to undertake the study. Chapters three to six present the results obtained from each specific objective which are synthesised into either published paper (papers I and II) or publishable manuscripts (papers III and IV) submitted for publication consideration in peer reviewed scientific journals. The format and writing style of the individual papers is according to the targeted instruction of the journal. Chapter seven provides the conclusions and recommendations based on the findings of this study.

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CHAPTER TWO

2.0 GENERAL METHODOLOGY

2.1 Study Design

This research work was divided into four studies. In the first three studies as presented in papers one to three, cross-sectional study design was applied. Longitudinal study design was applied in the fourth study as presented in paper four. Cross-sectional study design (Putt *et al*., 1987) was used to describe characteristics of dairy farming and its effect on milk production, to quantify the prevalence of bovine subclinical mastitis caused by bacterial pathogen and to quantify the occurrence and determinants of intra-mammary infection caused by mycotic pathogens. Longitudinal study design (Caruana *et al*., 2015) was used to quantify the Incidence rate of clinical mastitis.

2.2 Study Area

This study was carried out in three districts of Unguja Island (Fig. 2.1). The Island is one of the two islands that form Zanzibar which is part of the United Republic of Tanzania. Unguja lies between latitude 05º 72'' and 06º 48'' south and between longitude 39º 30'' and 39º 51'' East. The total land area of Unguja is 1 666 square kilometres. The study districts were North B, Central and West districts. The island has a tropical climate with temperature ranging from 24 to 32°C with higher temperature recorded during January and February and lowest temperature during June and July. The island experiences two rainy seasons with long rainfall from March to May and September to November is for short rains. Unguja island experiences two dry seasons, from December to March and June to September. On average, annual percentage humidity in Unguja island is 73% with highest being in April and lowest in October. These climatic conditions are appropriate for environmental pathogens to persist.
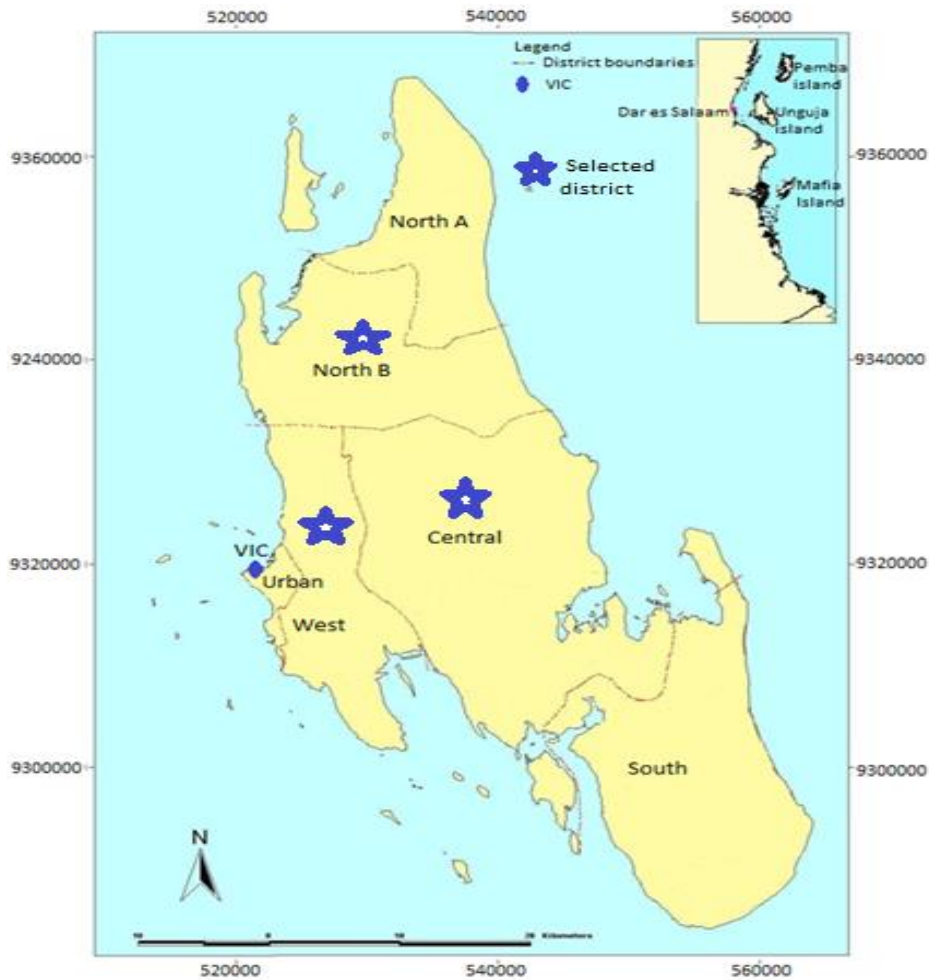
Figure 2.1: Map of Unguja Island

2.3 Sampling Size

Sample size was determined using the formula for estimation of disease proportion using cluster sampling as described by Bennett *et al.* (1991) as shown in formula (1).

$$n = \frac{p(1-p)Dz^2}{e^2 b} \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots (1)$$

Where n is a sample size (number of households); p = estimated prevalence of SCM for previous studies at farm level in previous study which was 0.67 (Suleiman *et al.,* 2013)]; e^2 is precision which is equal to absolute estimated error at 5% (0.05); b is number of lactating dairy cows to be sampled from each household that is two (Suleiman *et al.,* 2016); z is a confidence level at 95% (1.96); D = Design effect which can be calculated using the following formula D = 1 + (b-) roh = 1.2; roh is a rate of homogeneity which

was estimated at 0.2.

Using that formula, a minimum of 200 dairy farmers were required for this study. However, due to proportional sampling of farmers among selected Shehias, a total of 203 farmers were selected during the first study to identify characteristics of dairy farming and its effect on milk production. In the second study, to quantify the prevalence of bovine subclinical mastitis caused by bacterial pathogen, and third study, to quantify the occurrence and determinants of intra-mammary infection caused by mycotic pathogens, a total of 201 dairy farmers continued with those two studies. This was caused by two drop out of farmers due to the absence of lactating cows during cross-sectional study. In the fourth study, two more farmers dropped from the study and hence the study continued with the remaining 198 dairy farmers in study number four.

2.4 Sampling Technique and Sample Frame

This study used multi stage sampling procedure. The first stage was for selection of study district. One district from each region of Unguja was purposively selected based on the number of cattle. Selected districts were North B, West and Central. Second stage was for selection of participating Shehias. A simple random sampling was used to select 28 Shehias from those three districts. The list of all Shehia with smallholder cattle rearing was obtained from the Department of Livestock Development. Third stage involved selection of households (HHs). From each selected Shehia a list of all HHs with dairy cattle was prepared by field assistants using pre-prepared sample frame (Appendix 1). From the sampling frame, a total of 203 HHs were randomly selected based on proportion of dairy cattle in the selected Shehia. Each of selected HH was regarded as cluster and all lactating dairy cows from those HHs were subjects for this study.

2.5 Data Collection Procedure

Five data collection procedures were used for this study. Those included questionnaire interview, field observations, physical examination, cow side screening using California mastitis test kits (CMT) and laboratory based culture isolation, identification and characterisation of causative agent.

2.5.1 Questionnaire interview

At the beginning of this study, each participating household was interviewed by using a simple structured questionnaire (Appendix 2). The aim of this interview was to collect information about the farmers' awareness and practices, and compare them with the outcome variables. List of all independent variables collected were shown in Table 2.1 while clarification of outcome variables from each study is detailed in subsection 2.7. Six field assistants were trained as enumerators who assisted during the enter period of data collection.

2.5.2 Field observation

Field observation was used to assess environmental condition, management procedures (Appendix 2) and individual cow assessment in terms of hygiene and physical soundness (Appendix 3). Data from this field observation were compared with the outcome variables based on the specific study.

2.5.3 Physical examination

Physical examination of all lactating cows from each participating household was conducted in study two, three and four. This included examination of general condition of the cow, udder and individual teat. All information observed were recorded in special form (Appendix 3). The aim of this examination was to assess udders and teats condition, presence of injury, blind teats or clinical signs of mastitis.

2.5.4 Cow side test

California Mastitis Test (CMT) was used to screen lactating cows for subclinical mastitis (SCM). In this test one or two strips of milk (about 2 millilitres) from each teat was milked directly into the CMT pad after discarding the first two strips. Each milk sample in the CMT pad was mixed with equal amount of CMT reagent and the results were recorded according to the standard procedure (Varatanovic *et al*., 2010) depending on the reaction formed. The results were recorded as N for negative, T for trace and +1, +2 and +3 for positive (Barnum and Newbould, 1961).

2.5.5 Laboratory investigation

In second, third and fourth studies, quarter milk samples from both CMT positive and negative quarters were aseptically collected directly from each teat into a 20-mls sterile and unique labelled universal bottle according to the procedure described by Quinn *et al*. (1994). In study number four, only milk samples from infected quarters were collected for laboratory analysis. The milk samples were collected after discarding the first two strips to avoid contamination. Soon after collection, the samples were stored in a cool box containing ice pack with a temperature of 4°C and transported to the Maruhubi Veterinary Investigation Centre (VIC) for culture isolation, identification and characterization of the pathogen.

In the laboratory, milk samples were divided into two portions and used for culture of bacteria and fungi. All samples arrived at laboratory were cultured on the same day or deep frozen at $-20\,°C$ waiting for culture within 72 hours.

2.5.6 Isolation and identification of bacterial pathogens

For isolation and identification of bacterial pathogens, each sample was cultured in duplicate; two plates of MacConkey and two plates of blood agar. One set was incubated

aerobically and another an-aerobically. Bacterial growth was observed after 24 and 48 hours. If no growth observed, the quarter was considered negative. For positive growth, colony morphology, pigmentation and haemolytic reaction were observed. Gram stain was used to distinguish between Gram positive and negative bacteria and to study the microscopical features of the isolated bacteria. Different biochemical tests as described by Vashist *et al.* (2013a) were used to identify genus and species of isolated bacteria. Modified oxidase test was used to differentiate between Gram positive cocci bacteria. Those with positive results were identified as *Micrococcus spp.* while those with negative results were subjected to catalase and coagulase tests. Isolates with positive results in both tests were identified as *S. aureus*. Isolates that tested positive to catalase and negative to coagulase were subjected to oxidase test and those with negative results were confirmed to be S. *epidermid*is (non-haemolytic) or *S. haemolyticus* (haemolytic). Rod shape Gram positive bacteria were exposed to catalase and oxidase tests. Negative results for both tests confirmed the presence of *Trueperella pyogenes* while isolates that were positive to catalase and urease were identified as *Corynebacterium spp.*

Based on culture characteristics on MacConkey agar, Gram negative bacteria were categorised into lactose fermenters with pink colonies and non-lactose fermenters (colourless colonies). Lactose fermenters were subjected to catalase, urease, indole tests and citrate fermentation, isolates with positive results for catalase and indole with negative results for urease and citrate fermentation were identified as *E. coli* and those with citrate fermentation, catalase and urease positive and indole negative were identified as *Klebsiella spp*. Non-lactose fermenters were subjected to catalase and oxidase tests. Isolates with positive results to both tests were identified as *Pseudomonas aeruginosa* while those with catalase positive and oxidase negative were identified as *Proteus spp*.

2.5.7 Testing for antimicrobial sensitivity

Testing for antimicrobial sensitivity was done using Kirby-Bauer disc diffusion technique as described by Vashist *et al.* (2013b). A total of eight antibiotics commonly used in Zanzibar to treat bovine mastitis were used in this test. The selected antimicrobial included Amoxicillin (30μg), Cephalexin (30μg), Gentamycin (10μg), Kanamycin (30μg), Neomycin (30μg), Tetracycline (30μg), Penicillin G (2IU) and Streptomycin (10μg). A total of seven species of bacterial pathogens were tested for antimicrobial sensitivity. The tested bacteria isolates included *E. coli* (13 isolates), *Klebsiella spp.* (57 isolates), *Micrococcus spp.* (35 isolates), *P. aeruginosa* (82 isolates), *S. aureus* (217 isolates), *S. epidermidis* (58 isolates) and *T. pyogenes* (14 isolates). A portion of pure colony of bacteria isolated was transferred in a tube with 5 ml of nutrient broth and incubated at 37°C for 24 hours. The mixture was then transferred into a Muller Hinton agar plate and spread using sterile glass tube. The antibiotic discs were then placed on the plate using sterile forceps and incubated for 24 hours at 37°C. The diameter with no growth around each antibiotic disc was then measured using transparent ruler and the measurement was recorded in millimetres.

2.5.8 Isolation and identification of mycotic pathogens

Isolation and identification of fungi and algae pathogens were performed by culturing each sample in duplicate on Sabouraud dextrose agar (SDA). One petri-dish was incubated at 37°C and the second was incubated at room temperature (24°C). The growth of both fungi and algae was observed after every 24 hours for a period of seven days. If no growth observed, the sample was considered negative. For positive growth samples, microscopic and biochemical tests were conducted to identify the species of fungi and algae isolated. Characterization and identification of yeast and yeast like fungi was conducted microscopically by staining with Gram and lacto-phenol cotton blue stain while

biochemical characteristics was done by sugar fermentation through inoculation of the isolated fungi to 1% of each five sugar as described by Pachauri *et al*. (2013). Identification of moulds was conducted through observation of morphological growths that included texture and pigmentation of both sides of growth. Microscopic examination was conducted using transparent adhesive tape technique to observe the filament and microfilament formed as described earlier (Sukumar and James, 2012).

2.6 Daily Monitoring of CM

During longitudinal study each cow was followed for the period of 24 months. All farmers were previously trained about clinical mastitis and it manifestations. Farmers were responsible to notify the researcher whenever clinical mastitis is observed. Each clinical case of mastitis was attended by researcher or field veterinary assistants. Physical examination was performed and milk sample from infected quarter was collected for laboratory diagnosis. Proper and free treatment for all reported cases was provided after sample collection.

2.7 Data Processing and Analysis

Data from the first three studies were stored and analysed using Epinfo® version 7 for window where descriptive statistic, chi square test (χ^2) and linear or logistic regression analysis were performed. Data from the fourth study were stored in Microsoft Excel 2007 where basic descriptive calculation was done and then, transferred to Epinfo® version 7 for window where chi square test (χ^2) and logistic regression analysis were performed. Graphic presentation was prepared in Microsoft Excel 2010.

The outcome variable in the first study was recorded as litres of milk produced per cow per day. This was compared with different cows' and farms' level factors that may

contributed to the low quantity of milk production. The outcome variable in the second and third study was the prevalence of SCM as defined by CMT, bacteria culture positive or mycotic culture positive. The outcome variables were calculated at three levels of quarter, cow and farm levels based on the following formula as derived from Stevenson, 2005 as shown in formula (2).

$$Prevalence\ of\ SCM = \frac{Number\ positive*}{Total\ number\ tested\dagger}\ x100 \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\ (2)$$

* based on CMT, bacteria culture or mycotic culture positive

† number tested either at quarter, cow or farm levels

During those studies, a quarter was considered positive for CMT screening if the score was positive one and above while a cow was regarded positive if at least one quarter was CMT positive and a farm was considered positive if at least one cow from that farm was CMT positive. Based on bacteria culture results, a quarter was considered culture positive if bacteria were isolated from the sample collected while a cow was considered culture positive if bacteria were isolated from at least one quarter of that cow and a farm was regarded positive if at least one cow from that farm was positive. Based on mycotic culture results, a quarter was considered culture positive if fungi or/and algae were isolated from the sample collected while a cow was considered positive if fungi or/and algae were isolated from at least one quarter of that cow and a farm was regarded positive if at least one cow from that farm was positive for fungi or/and algae isolation.

In the fourth study, the outcome variable was the occurrence of CM as reported by farmer and as defined by isolation of pathogens from milk samples collected for the period of two year. The results were presented as incidence rate per 100 cows year at risk, cumulative

incidence and Cumulative hazard function. For the calculation of incidence rate the following formula derived from CDC (2012) as shown in formula (3).

$$IRCM = \frac{Number\ of\ new\ cases\ of\ CM\ for\ two\ years}{Time\ each\ individual\dagger\ observed\ totaled\ for\ all\ individuals} \dotsc \dotsc \dotsc (3)$$

Hence:

IRCM = Incidence rate of clinical mastitis,

CM = Clinical mastitis, and

†Individual = number of quarters / cows at risk of developing clinical mastitis for the period of two years.

Probability occurrence of clinical case of mastitis was presented as cumulative incidence and was calculated using the following formula as derived from CDC (2012) as shown in formula (4).

$$CICM = \frac{Number\ of\ new\ cases\ of\ CM\ for\ two\ years}{Population\ size\ \dagger at\ risk\ of\ devoloping\ CM\ for\ two\ years} \dotsc \dotsc (4)$$

Hence:

CICM = Cumulative incidence of clinical mastitis,

CM = Clinical mastitis, and

† Population size = number of quarters or cows at risk of developing CM for the period of two years.

Cumulative hazard function was calculated by dividing the conditional probability of individuals (quarter/cow) affected with clinical mastitis during two years' time interval divided by the specific time interval.

Independent variables in all four studies were different cows and farm level risk factors that may contributed to the occurrence of an outcome variable either positively or negatively. List of cows' and farms' level factors are shown in Table 2.1. These factors were compared with the occurrence of outcome variable using linear regression analysis in the first study. In the second to the fourth studies, these factors were compared with the

occurrence of subclinical or clinical mastitis using logistic regression analysis as detailed in the specific paper in each study. To consider the effect of confounding, forward stepwise regression analysis was performed. Only variable with p-value ≤ 0.05 were included in the final model of risk factors that influenced the prevalence of SCM or incidence of CM.

Table 2.1: List of cows' and farm' level factors compared with outcome variables in all four studies

Cow level factors	Farms' level factors	
	Farms' situation	Farmers' practice
Age	Location (district)	Milk per day
Breed	Housing	Milking technique
Number of parity	Type of floor	Calf suckling before milking
Stage of lactation	Floor hygienic	Calf suckling after milking
Milk produced (litres)	Drainage system	Wash udder
Body condition score (BCS)	Type of roof	Towel dry
Cow hygiene	Roof condition	Separate towel per cow
Udder hygiene	Rearing system	Application before milking
Teat condition	Water supply	Application after milking
	Herd size	Hand wash
	Lactating cows	Pasture used
		Concentrates provision

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CHAPTER THREE

PAPER ONE

Characteristics of dairy farming and its effect on milk production: a case study of Unguja island of Zanzibar, Tanzania

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CHAPTER FOUR

PAPER TWO

Prevalence of bovine subclinical mastitis and antibiotic susceptibility patterns of major mastitis pathogens isolated in Unguja island of Zanzibar, Tanzania

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CHAPTER FIVE

PAPER THREE

Occurrence and determinants of intra-mammary infection in dairy cows caused by mycotic pathogens in Unguja Island of Zanzibar, Tanzania

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Occurrence and determinants of intra-mammary infection in dairy cows caused by mycotic pathogens in Unguja Island of Zanzibar, Tanzania

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Abstract

Background: Previous studies described high prevalence of clinical and subclinical bovine mastitis in Unguja island of Zanzibar. Both contagious and environmental bacterial pathogens have been isolated as causative agents during those studies. Investigation of fungi and algae as causative agents of mastitis in Zanzibar Island has never been conducted before. A cross-sectional study was conducted with the objective of estimating the prevalence of bovine mycotic subclinical mastitis and associated risk factors.

Results: The overall prevalence of mycotic subclinical mastitis as defined by mycotic pathogens isolation was 19.0%, 33.9% and 49.3% at quarter, cow and farm level, respectively. The quarter level prevalence was significantly higher ($P < 0.05$) in Central district (26.1%, n = 496) compared to North B (20.1%, n = 288) and West (14.4%, n = 864). Out of 338 isolates, 336 were found to be fungi identified as *Aspergillus spp* (48.2% of isolates), *Candida spp* (41.1%), *Saccharomyces spp* (7.4%), *mucor spp* (1.2%), *Microsporum spp* (0.6%), *Sporobolomyces spp* (0.6%) and *Trichophyton spp* (0.3%). Only two isolates were described as algae and were identified as *Prototheca spp* (0.6%). Type of housing and milking techniques were associated with the increased cases of mycotic

mastitis while number of milking per day and practice of farmer to wash hand before milking decreased the occurrence of mycotic mastitis.

Conclusion: It is concluded that poor dairy farm management practices and unhygienic milking procedures are the main source of infection leading to the high prevalence of mycotic mastitis. Therefore, farmers should observe proper farm husbandry practices and milking procedures so as to improve the quality of milk produced through reduced magnitude of the disease.

Keywords: Subclinical mastitis, prevalence, fungi, algae

Background

Bovine mastitis is considered as the most important and complex disease of dairy industry due to the multi aetiological agents of the disease around the world. These pathogenic microorganisms vary from bacteria, virus, fungi and algae. Generally, these can be divided into two categories, contagious and environmental pathogens [1]. Contagious pathogens are the ones that can cause infection and can be transmitted from one cow to another or from one infected quarter to another during milking. Dairy farm management plays vital role in transmission of these pathogens either because of poor hygiene or improper milking techniques. Environmental pathogens are normal floras which are harmless, living in the cow's surroundings like floor, soil, milking equipment, on the skin of a cow or person who milks the cow or even in the fodders or feeding material. These pathogens invade the udder through teat canal when sphincter muscle is relaxed and keratin which closes the teat orifice is washed out during milking [2]. Due to highly nutritive value of milk together with favourable temperature, these microorganisms multiply and produce toxins and antigens that trigger the inflammatory response of the animal leading to mastitis [3, 4]. Majority of Clinical Mastitis (CM) and Subclinical

Mastitis (SCM) is caused by bacteria pathogens [5,6]. Involvement of mycotic as causative agents of CM and SCM has been reported in previously studies [7, 8].

Mycotic mastitis can be divided into two types, the first is primary mycotic mastitis occurs directly without having previous infection of bacterial mastitis or antibiotic treatment. The second is secondary mycotic mastitis that appears with or after bacterial infection, after surgical procedure or injuries in the teats or can be introduced after the use of intramammary infusions, especially when proper hygiene is not observed [9, 10]. Always, mycotic mastitis is under diagnosed because most veterinarians opt for antibiotic use to treat any case of CM rather than taking milk samples for laboratory. Whenever sick cows do not respond to antibiotics therapy, mycotic mastitis can be suspected [7]. The isolation of different species of fungi from milk was previously demonstrated in both developed and developing countries [11, 12]. The isolation of achlorophylic algae have been demonstrated from both CM and SCM in deferent parts of the world with *Prototheca zopfii being* the most algae isolated specie [13, 14].

Previous study on bovine mastitis in Zanzibar demonstrated high prevalence of both CM and SCM based on California Mastitis Test (CMT) and bacteria isolation [15]. No studies have been conducted so far to investigate the involvement of mycotic pathogen in bovine mastitis. Due to the importance of mycotic mastitis in the occurrence of both CM and SCM and the risk factors associated with, this study was designed to a) investigate the prevalence of SCM caused by mycotic pathogens in Unguja Island and b) identify associated important risk factors.

Material and Methods

Study area

This study was carried out in Unguja Island of Zanzibar, Tanzania. Unguja Island lies between latitudes 05° 72'' and 06° 48'' South of Equator and longitude 39° 30'' and 39° 51'' East of Greenwich about 45 kilometres from the east cost of Tanzania Mainland. The island has a tropical climate with temperature ranges from 24°C to 32°C, experiencing two rainy seasons from March to May and from September to November and two dry seasons from November to March and from May to September. On average, annual percentage of humidity is 73% with highest humidity percentage recorded during April and lowest humidity percentage recorded during October. These climate and weather provide appropriate condition for environmental pathogen to persist within cow environment.

Study design and sample size

This cross-sectional study was conducted between January and July 2014. Sample size was determined using the formula to estimate disease proportion using cluster sampling [16].

$$n = \frac{p(1-p)Dz^2}{e^2 b}$$

Where n is a sample size (number of households); p = estimated prevalence of SCM at farm level in previous study which was 0.67 [15]; e^2 is precision which is equal to absolute estimated error at 5% (0.05); b is number of lactating dairy cows to be sampled from each household that is two [17]; z is a confidence level at 95% (1.96); D = Design effect which can be calculated using the following formula D = 1 + (b-) roh = 1.2; roh is a rate of homogeneity which was estimated at 0.2. Using that formula, a minimum of 200 dairy farmers were required to conduct this study. However, due to proportional sampling of farmers among selected Shehias, a total of 201 dairy farmers were selected.

Sampling strategies

Multi stages sampling procedure was used to obtain dairy cow for this study. The first stage used purposive sampling to select three out of six districts of Unguja Island. The only criterion used to include the district in the study was number of households rearing dairy cattle in that district based on official government statistics [18]. The selected districts were North B, Central and West. Random selection of participating Shehia (smallest administrative unit in Zanzibar) was a second stage of sampling procedure. A total of 28 out of 59 Shehias with dairy keeping activities were selected. In the third stage, a total of 201 were selected based on proportional sampling of dairy households from each Shehia. Each household was considered as cluster and all dairy lactating cows were included in the study.

Data collection procedure

Each participating dairy cattle farmer was interviewed using simple structured questionnaire. The aim of this interview was to collect information about farmers' awareness, knowledge and practices and compare them with the outcome variables of occurrence of SCM in dairy cows as defined by isolation of mycotic pathogens isolation. Field observation was used to assess environmental condition, management procedure together with individual cow assessment in terms of hygiene and physical soundness in relation to the occurrence of SCM. All lactating cows from each participating household were subjected to physical examination where general condition of the cow, udder and individual teats were assessed for observable clinical signs of CM.

Cow side test and laboratory investigation

California Mastitis Test (CMT) was used to screen lactating cows for subclinical mastitis (SCM). Two millilitres of milk were directly into the CMT pad mixed with equal amount

of CMT reagent and the result was read immediately depending on the reaction formed. The results were recorded based on the standard procedure for using CMT reagent [19, 20]. For identification of mycotic pathogens, milk sample from each quarter was aseptically collected according to the procedure described before [21]. To avoid sample contamination, the firsts two drops from each quarter were discarded before collection. Unique sterile and labelled universal bottles were used to collect about 10 millilitres of milk for laboratory analysis. The samples were stored in a cool box containing ice packs with temperature of 4°C and transported to Maruhubi Veterinary Investigation Centre for fungal and algal isolation and identification. In the laboratory, the samples were cultured on the same day or deep frozen at -20°C until culture within 72 hours. Each sample was cultured on Sabouraud dextrose agar (SDA) and incubated in duplicate, one petri-dish incubated at 37°C and the second was incubated at room temperature (24°C). The growth of both fungi and algae was observed after every 24 hours for a period of seven days. If no growth observed, the sample was considered negative. For positive growth samples, microscopic and biochemical tests were conducted to identify the species of fungi and algae isolated. For characterization and identification of yeast and yeast like fungi, observation was conducted microscopically by staining with Gram and lacto-phenol cotton blue stain while biochemical characteristics was done by sugar fermentation through inoculation of the isolated fungi to 1% of each five sugar as described earlier [7]. For identification of moulds, morphological growths that included texture, pigmentation of both sides of growth was observed. Microscopic examination was conducted using transparent adhesive tape technique to observe the filament and microfilament formed [22].

Data analysis and Definition of outcome variables

Data analysis was done using Epinfo® version 7 for window where descriptive statistic and logistic regression analysis were performed. Graphic presentation was prepared in Microsoft Excel 2010. The outcome variable in this study was the prevalence of SCM as defined by isolation of mycotic pathogens from milk samples collected. Independent variables included different cows and farm level risk factors that may contributed to the occurrence of bovine mastitis. The results were analysed at three levels of farm, cow and quarter. A cow's quarter was considered SCM positive if mycotic pathogen were isolated from the sample collected. On the other hand, a cow was considered positive if mycotic pathogens were isolated from at least one quarter and a farm was positive if at least one cow from that farm was positive. Test for significance of proportions as defined by SCM prevalence in different categorical variables was performed using Chi Square (x^2) test. Risk factors were identified by using two steps; the first being simple regression analysis where 31 risk factors were separately compared with the outcome variables and only variables that score p-value equal or less than 0.2 were forwarded to second step of multiple regression analysis [23]. During this step, forward stepwise regression analysis was performed to consider the association of this risk factors to the occurrence of SCM. Only variable with p-value ≤ 0.05 were included in the final model of risk factors that influenced the prevalence of SCM as defined by isolation of mycotic pathogens.

Results

Prevalence of bovine SCM as defined by mycotic pathogens

During this study, a total of 201 dairy farmers with 416 lactating dairy cows were involved. A total of 1,664 quarters were examined, hence 1,648 (99%) were normal and 16 (1%) were blind. The results indicated that out of 1,648 milk samples cultured, 316 (19.0%) samples revealed mycotic pathogens growth, of these, 286 (17.4%) samples

showed one species while 26 (1.6%) samples showed two species. Eighteen samples (1.1%) showed more than two mycotic pathogen species and were considered contaminated. These contaminated samples together with samples with no growth were considered negative. A total of seven types of fungi (99.4%) and one type of algae (0.6%) were isolated from quarter milk samples. where most of the isolates were *Aspergillus spp* (48.2%), *Candida spp* (41.1%) and *Saccharomyces spp* (7.4%), together with other minor isolates (Fig. 5.1).

Table1 describes the prevalence of mycotic mastitis at farm, cow and quarter level in each study district. The overall prevalence at farm, cow and quarter level was 49.3% (n=201), 33.9% (n=416) and 18.9% (n=1638), respectively. Quarter level prevalence was highest in Central districts (26.1%) followed by North B district (20.1%) and West district (14.4%) and the difference was statistically significant (p-value = 0.000). The differences at cow and farm levels were not statistically significant.
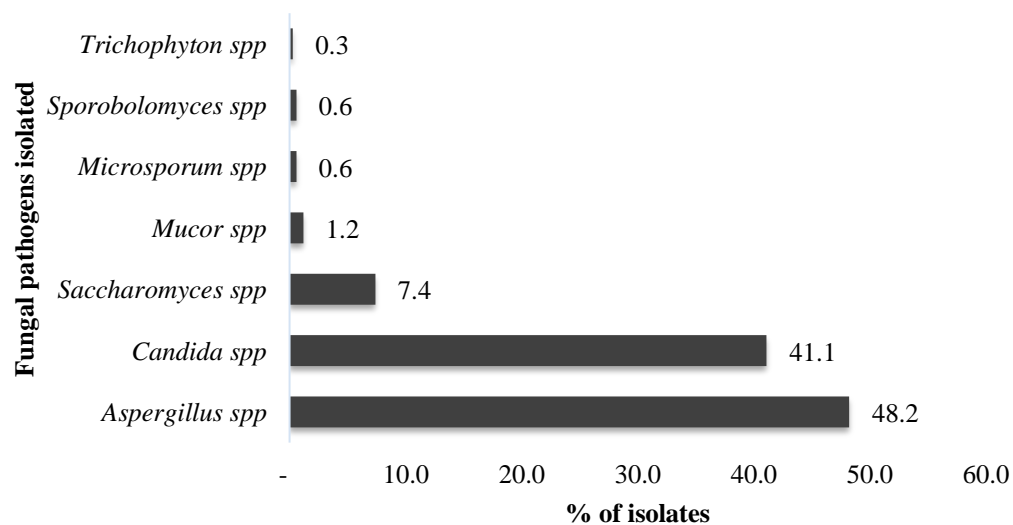
Figure 5.1: Fungal pathogens isolated from quarters milk samples from 338 isolates of fungal and algal isolates

Table 5.1: Prevalence of SCM at farm, cow and quarter level based on mycotic pathogens isolation in each of the study district

District	Farm level (n=201) positive (%)	Cow level (n=416 positive (%)	Quarter level (n=1648) positive (%)
	Prevalence of mycotic mastitis		
North B	15 (34.9)	27 (37.0)	58 (20.1)
Central	37 (55.2)	51 (40.8)	130 (26.2)
West	47 (51.6)	63 (28.9)	124 (14.4)
Total	99 (49.3)	141 (33.9)	312 (18.9)
X^2	4.717	5.400	29.199
p-value	0.094	0.067	0.000

Factors that influenced the prevalence of SCM

comparisons of the results from mycotic pathogens isolation with those obtained from CMT test showed that out of 472 milk samples that tested positive for CMT mycotic pathogens were isolated from 161 samples (34.1%). On the other hand, out of 1176 milk samples that tested negative for CMT, mycotic pathogens were isolated from 151 samples (12.8%) as shown in Figure 4.2. Further analysis indicated that it is more likely to isolate mycotic pathogens from CMT positive samples then from CMT negative samples (OR=4.41). Mycotic pathogens were isolated from two out of 14 milk samples from injured teat (14.3%) compared to 310 (19%) out of 1634 quarter milk samples from normal teat. However, the difference was not significant (p-value = 0.655). Table 4.2 shows other categorical variables that influenced the isolation of mycotic pathogens from quarters' milk sample. Out of 31 risk factors tested, including type of housing and milking technique increased the occurrence of mycotic mastitis while number of milking per day and farmer practice of washing hand before milking decreased the occurrence.
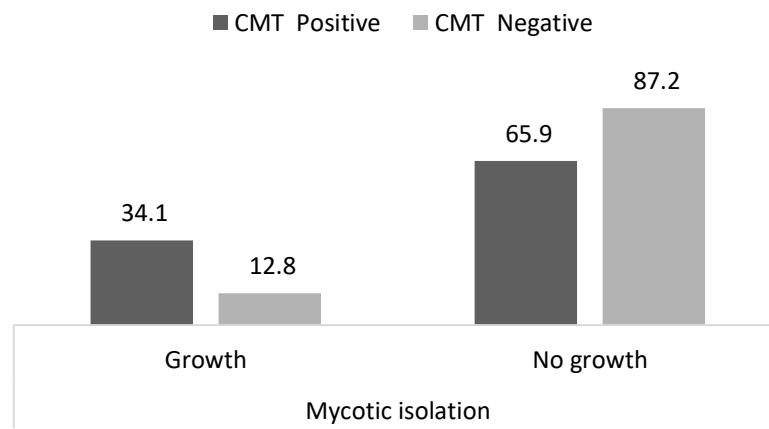
Figure 5.2: The results of isolation of mycotic pathogens as compared to CMT screening test
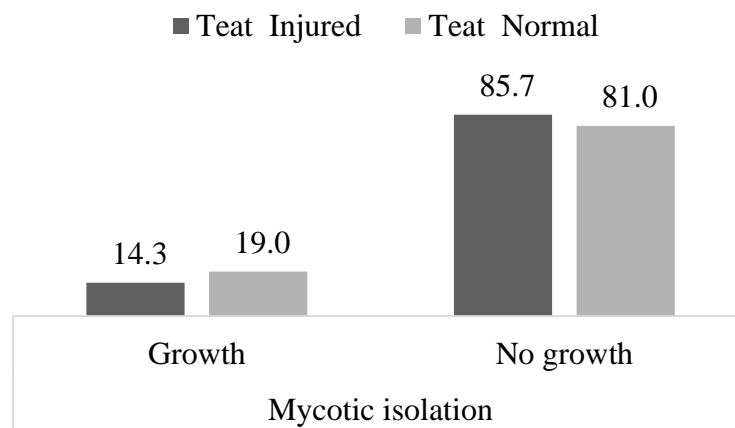
Figure 5.3: The results of isolation of mycotic pathogens as compared to teat condition

Table 5.2: Explanatory variables influencing prevalence of SCM as defined by

mycotic isolation in Unguja island

Variable	OR	95%	C.I.	β	S. E.	Z-Statistic	p-Value
Housing (temporary/permanent)	1.41	1.10	1.81	0.34	0.13	2.70	0.007
Milking Day (Twice/Once)	0.26	0.18	0.40	-1.31	0.20	-6.34	0.000
Milking Technique (Thumb stripped/Hand fist)	1.56	1.04	2.34	0.44	0.20	2.17	0.029
Wash Hand (Yes/No)	0.51	0.33	0.79	-0.66	0.22	-2.99	0.002

OR=Odds Ratio, CI=Confidence limit, β=Regression coefficient, SE=Standard Error

Discussion

Quarter and cow level prevalence of mycotic SCM in dairy cows observed in this study gave an indication of the magnitude and diverse of environmental pathogens that can invade and cause intra-mammary infection. Although the prevalence of mycotic mastitis has been described before in other parts of Tanzania [24], this is the first identification of mycotic pathogens involvement as causative agents of SCM in Zanzibar. The level of infection found in this study does not differ much with the prevalence found elsewhere [11, 25, 26]. Quarter level prevalence was higher in Central district followed by North B and West district. This finding is in line with the level of farm floor hygiene from which the samples were taken hence farms in Central district were dirtier compared to North B (51%) and West district [17]. Dirty environment around the cow may contribute to the increased level of infection because most of mycotic pathogens isolated were of environmental origins which were opportunistic pathogens. High prevalence of mycotic SCM may contribute to the development of chronic cases of clinical mastitis as most of

these pathogens do not respond to antibiotics instead they use some antibiotic as source of energy [7]. Persistent mycotic mastitis may lead to long term infection and contribute to the poor milk production in terms of quantity and quality [3]. The zoonotic nature of these fungi and algae is of high public health concern due to the fact that some of them may resist pasteurization process and eventual be consumed by human [14]. High prevalence of mycotic bovine mastitis has also been reported in other parts of Africa [27].

Prevalence of *Aspergillus spp, candida spp* and yeast like fungi such as *Saccharomyces spp, Microsporum spp, Sporobolomyces spp* and *Trichophyton spp* together with achrophylic algae like *Prototheca spp* described in this study may raise public health concern due to their zoonotic nature. Isolation of fungi from milk samples has been observed in other parts of Tanzania [28] or in African continent [9]. The low rate of isolation of *Prototheca spp* (0.6%) might have been caused by standard procedure of direct streaking of whole quarter milk samples without centrifuging used in this study rather than isolation from milk sediment after centrifuge [27].

Likelihood of isolating mycotic pathogens from CMT positive milk samples was higher compared to the probability of isolating mycotic pathogens from CMT negative samples. Mycotic pathogens did not grow from about 66% of the CMT positive milk samples probable due to presence of other pathogens rather than mycotic; like bacteria and mycoplasma that trigger immunological response of the host and increase amount of Somatic Cell Count (SCC) as observed before [29]. About 13% of CMT negative milk samples developed fungal growth. This may be caused by the mycotic pathogens entering mammary gland but the number of pathogens did not reach the level of intramammary infection that can activate the immunological response of the host animal and, hence, maintained the normal range of SSC [30]. In most cases, mycotic mastitis is predicted

after failure of antibiotic use during treatment. Uses of laboratory facilities to isolate actual pathogens causing mastitis may be valuable tool towards proper treatment.

Most of the studies on the risk factors associated with the prevalence of both CM and SCM conducted within the East African region consider the risk factors based on CMT and/or bacteria isolation but not association with mycotic pathogens isolation separately [31, 32]. The risk factors which were found to be associated with SCM in this study were mainly related to the sanitary measures and milking techniques. Permanent structure with concrete floor smoothen cleaning process, removing dung and reducing amount of fungal contamination from cows' environment and hence minimize the probability of infecting a cow since most of these pathogens were opportunistic living in the cow environment. cows of the farmers who milked their cows twice a day were less infected compared to those of farmers who milked once a day. Time taken from one milking to another may contribute to the amount of opportunistic pathogen invading the teats and grow inside the udder and, hence, increase the chance of isolation during laboratory procedure. Number of samples developed growth was higher in farmers who did not wash their hand after every cow milking. This poor sanitary measure increased the opportunity of these opportunistic pathogens around the cow to invade the mammary gland during or after milking.

Conclusion

Mycotic bovine mastitis observed in this study was substantially important and may contribute to the decreased amount of milk produced under smallholder dairy cattle farming in Zanzibar. Most mycotic pathogens isolated were of environmental origin mainly attributed to farmers' negligence on good sanitary measures around the lactating cows. Poor dairy farm management and unhygienic milking procedures were the main sources of infection. Zoonotic nature of some species of fungi like *Candida spp* or algae

like *Prototheca spp* should not be underestimated in terms of public health hazard because those pathogens known to cause number of disease in human population. To reduce the prevalence of mycotic bovine mastitis in Zanzibar, the dairy cattle farmers should improve farm management, hygiene and sanitary measures during milking so as to minimize the chances of their cows to be infected with these environmental pathogens. Uses of teat dip after milking and dry cow therapy during dry off could prevent cow from this increased occurrence of subclinical mastitis.

Abbreviations

Cl: Confidence limit; CM: Clinical Mastitis; CMT: California Mastitis Test; OR: Odds Ratio; SCM: Subclinical Mastitis; SDA: Sabouraud dextrose agar; SE: Standard Error; spp: species; SSC: somatic cell count; β: Regression coefficient.

Availability of data and material

Raw data will not be made available. We are constrained with our local requirement of confidentiality. We tried to explain all information about data collection and analysis in methods section. More data were presented in the results section in Table 1 and 2 together with attached Fig. 4.1, 4.2 and 4.3. The dataset used in these studies which include all information about dairy farms are available by request from corresponding author.

Authors' contributions

TSS designed the study protocol, carried out sample collection, supervise laboratory analysis, performed statistical analysis, interpreted the results of data analysis and drafted the manuscript. RHM and EDK reviewed, corrected and improved the study concept, participated in study design, improved data presentation, corrected and improved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This research work has been approved by College of Veterinary and Medical Sciences of Sokoine University of Agriculture. Before commencement of the study all participating dairy farmers agreed to be interviewed, examine and taking milk samples from their animals.

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CHAPTER SIX

PAPER FOUR

Occurrence and risk factors influencing the Incidence rate of clinical mastitis in dairy cattle in Zanzibar, Tanzania

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Occurrence and risk factors influencing the Incidence rate of clinical mastitis in dairy cattle in Zanzibar, Tanzania

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Abstract

Background: Clinical bovine mastitis is a disease of economic importance, public health and animal welfare concerns in the dairy sector. The current study was designed to investigate incidence rate of clinical mastitis, identify and quantify important risk factors that influence its occurrence and isolate common pathogenic causative agents from three districts of Unguja island of Zanzibar, Tanzania. This longitudinal study involved 578 dairy cows from 198 randomly selected dairy farms. The cows were followed for a period of two years (2014-2016). Farmers reported each occurrence of clinical case of mastitis to the researcher using mobile phone; either by short message service (SMS) or phone call. Each farmer was previously trained about clinical signs of mastitis. Milk samples for pathogen isolation were collected from each infected quarter and submitted for laboratory analysis.

Results: About 32.8% of participating dairy farms reported at least one case of clinical mastitis. Based on microbiological isolation, 30.8% of the dairy farms were confirmed to have at least one case, with West districts having significantly higher farm level incidence (40.4%). Cow level incidence rate per 100 cow-year at risk, cumulative incidence and cumulative hazard function as reported by farmers were 34.0, 18.3% and 0.27,

respectively, while those confirmed by pathogen isolation were 31.8, 17.1% and 0.25, respectively. Quarter level incidence rate per 100 quarters-year at risk, cumulative incidence and cumulative hazard function as reported by farmers were 14.5, 7.8% and 0.11, respectively, and those confirmed by pathogen isolation were 12.9, 7.0% and 0.10, respectively. Major pathogen isolated in this study includes *Staphylococcus aureus* (35.4%), *Staphylococcus epidermidis* (13.7%), *candida spp* (8.5%), *Pseudomonas aeruginosa* (6.6%), *Klebsiella spp* (6.1%) and *Aspergillus spp* (4.7%). Risk factors that increased the occurrence of clinical mastitis included study location, number of parity, cows' hygiene and dirty floor hygiene.

Conclusion: it is concluded that bovine mastitis is a very important disease of dairy sector in the study area. Both contagious and environmental pathogens were found to be associated with the disease. Improved dairy farm management and sanitary measure may reduce the burden of disease incidence since it reduces number of environmental pathogens that can invade the udder.

Keywords: incidence rate, hazard function, risk factors, pathogens, bacteria, fungi

Introduction

Establishment of smallholder dairy farming in Zanzibar started in late 1980s [1]. However, the development of smallholder dairy farming has not been accompanied with best farm management practices and disease control programme, this has led to poor milk production and occurrence of various animal diseases. Bovine mastitis is one of the major diseases affecting dairy sector in Zanzibar [2]. Bovine Clinical Mastitis (CM) is defined as inflammation of cows' mammary gland caused by invention of pathogenic microorganisms through teat canal [3,4] and it is characterized by physical change of infected udder, chemical and microbiological change of udder secretion [5].

Clinical Mastitis (CM) can be recognized by farmers through its clear observable clinical signs such as swelling, udder redness, pain, induration, hotness, sudden reduced milk production and alteration of milk secretion that may become watery, flakes, clots or bloody milk [6]. clinical mastitis can be categorized in different forms of paracute, acute or subacute depending on the severity of clinical symptoms observed [7]. Generally, pathogens causing CM are divided into three groups known as contagious, opportunistic and environmental pathogens [8,9]. More than 137 pathogens' species and subspecies have been identified to cause bovine mastitis [10]. However, most cases of clinical mastitis are caused by bacteria [11] but mycoplasma, fungi, and algae have been as well reported to cause CM [12,13.14].

Occurrence of any case of CM is a result of an interaction among host animal, causative agents and environmental factors which results into the host body to respond by developing localized inflammation within the udder location. Different cow and farm level risk factors have been associated with the occurrence of CM in dairy cows. Cow level risk factors reported to influence the occurrence of CM include cows breed, age, stage of

lactation, number of parity, amount of milk produced [15,16,17]. Farm management risk factors that influence the occurrence of CM include herd size, barn and cow sanitary measure and milking procedure [18,19,20,21].

Economic importance of bovine mastitis includes direct cost such as disease diagnostic measure, therapeutic measures, unsaleable milk, cost of veterinary services, labour work and animal death. In addition, there is indirect costs which are associated with clinical mastitis that include decreased milk quality, prolonged milk production loss, premature culling, replacement loss and reproductive loss [22,23]. Public health hazard implication of mastitis is due to a range of pathogens that can cause disease to the human population [3]. Disease caused by *E. coli, Campylobacter jejuni, Prototheca zopfii and Mycobacterium bovis* can easily be transmitted to humans whenever unpasteurized milk is used [24,25]. In addition to the biological hazard, the chemical effects of different veterinary drugs used to treat sick animals whenever withdrawal period is not observed is also obvious [26].

Recent findings in the study area quantified and reported the prevalence of both clinical and subclinical mastitis [2,27] rather than the incidence and incidence rate of CM due to the high cost of follow-up required for each individual dairy cow for a period of time. Therefore, this study was designed to investigate the incidence rate of CM, identify important risk factors that influence its occurrence and isolate common pathogenic causative agents in three districts of Unguja island of Zanzibar, Tanzania.

Material and methods

A longitudinal study was conducted for a period of two years (2014-2016) to establish the occurrence of clinical mastitis (CM) in dairy cattle kept under smallholder dairy farming

system in Unguja island of Zanzibar in the United Republic of Tanzania. The island experiences four weather seasons per year starting with long rains from March to May and temperature ranges from $23^{\circ}C$ to $32^{\circ}C$. Cool season which is from June to September is always dry with temperature range of between $22^{\circ}C$ and $28^{\circ}C$. Short rain season occurs from October to November and temperature range from $22^{\circ}C$ and $31^{\circ}C$. Hot season starts by the end of November to the beginning of March with temperature range of between $25^{\circ}C$ and $32^{\circ}C$. These range of temperatures and high humidity throughout the year favour the growth of many pathogens surrounding the cows' environment.

Sample size determination and farmer selection

A total of 198 dairy cattle farmers were selected using multi-stage sampling technique to estimate disease proportion [28]. The first stage was purposive sampling of three districts of Unguja island, the second stage was random sampling of 28 Shehias out of 59 with dairy farming activities from those three districts, the third stage was random selection of 198 dairy farmers out of 308 with lactating cows from those 28 Shehias. The selection of dairy farmers was proportional based on the number of dairy farmers in each Shehia. and final stage, each dairy farm was considered a cluster and all lactating cows were included in the study. Brought in and brought out method was adopted during the whole period of the study. By using this method, the animal entered the study when become at risk of contracting bovine mastitis and dropped out of the study when affected with bovine mastitis or cease to be at risk of contracting the disease. In addition, any newly lactating cow; either a cow from within the farm five days after delivery or a newly purchased lactating cow from outside the farm was brought in the study. On the other hand, any dried off, sold or dead cow was excluded from the study. The total number of 578 dairy cows were included in the study.

Data collection procedure

All participating dairy farms were visited at the beginning of the study and by using simple structured questionnaire, all important information about the farm, cows and farm management practices were recorded. Each participating farmer was trained on the clinical signs of bovine mastitis. Then, each farm was visited when clinical mastitis observed in an animal. The farmers were responsible for reporting any occurrence of CM using mobile phone; either by sending SMS or making phone call to the main researcher. All participating dairy farms were regularly visited after every two months for a period of twenty-four months from the starting date. During each visit, information about any new cow brought in or brought out from the study were recorded. Whenever CM was reported, the cow was subjected to physical examination to assess general condition of the cow, clinical signs emerged, udder and teat condition. Milk samples for laboratory analysis before treatment were aseptically collected as described before [29]. All cows infected were treated free of charge. The collected milk samples were transported in a cool box at $4^{o}C$ to Maruhubi Veterinary Investigation Centre for pathogens isolation and identification. Isolation of bacteria was done by culturing each sample in blood and MacConkey agar at $37^{o}C$ and observed after 24 and 48 hours. If no growth occurred after 48 hours the quarter was considered negative for bacteria isolation. Isolated bacteria were subjected to macroscopic, microscopic and biochemical test for identification to the species level as described earlier [27,30]. Isolation of fungi was done by culturing each milk sample in Sabouraud dextrose agar (SDA), incubated at $37^{o}C$ and observed daily for one week. If no growth observed, the quarter was considered negative for fungi isolation. Isolated fungi were subjected to macroscopic and biochemical examination [31] while observation for moulds was based on their morphological growths, pigmentation Microscopic examination, the filament and microfilament formed [32].

Definition of the outcome variables

During this study, a quarter was considered positive if pathogen, bacteria and/or fungi, were isolated from the collected milk sample. A cow was considered positive if at least one quarter was positive and a farm was considered positive if at least one cow from that farm was positive. Cow at risk was a lactating cow with at least five days after delivery while quarter at risk was a healthy and lactating quarter from a cow at risk. Quarters with blind teats were excluded from risk. Brought in cows were those cows started to be milked or purchased by participating farmers while milked during the study period. Brought out cows were those cows that were included in the study but stopped to be at risk either by drying off, being sold or death. Independent variables were different cow and farm level risk factors that were observed during the study.

Data storage and analysis

Data storage was done in Microsoft Excel 2007. The data were then transferred to Epinfo® version 7.2 for windows where descriptive statistic, chi square and logistic regression analyses were performed. Graphic presentation was prepared using Microsoft Excel 2007. Incidence rate, cumulative incidence and cumulative hazard function of clinical mastitis were calculated. The outcome variable in this study was the occurrence of CM as reported by farmer and as defined by isolation of pathogens from milk samples collected. Independent variables included different cows and farm level risk factors that may contributed to the occurrence of clinical cases of bovine mastitis. The results were analysed at cows and quarter levels. A cow's quarter was considered positive if reported by a farmer or pathogens were isolated from the sample collected. On the other hand, a cow was considered positive if at least one quarter from that cow was reported by a farmer of pathogens were isolated from at least one quarter of that cow. Logistic regression analysis was used to screen explanatory variables that significantly influenced the

occurrence of CM as defined by the isolation of pathogens. Variables that had score p-value equal or less than 0.2 during univariable logistic regression were forwarded to multiple logistic regression analysis. To control the effect of cofounding effects, forward stepwise regression analysis was used [33]. Only variable with p-value ≤ 0.05 were included considered as risk factors that influenced the prevalence of CM as defined by pathogens isolation.

Results

During this study, 65 dairy farms (32.8%) out of 198 participating dairy farms reported at least one case of clinical bovine mastitis for the period of two years. West district had higher number of cases reported at farm level (40.4%) compared to Central (29.9%) and North B districts (21.4%), but the difference was not statistically significant (P=0.07). Based on microbiological isolation, 30.8% of the dairy farms were confirmed to have at least one case of clinical bovine mastitis during the study period. The difference among the districts involved in the study were statistically significant (P=0.02). West district had higher number of farms with laboratory confirmed CM (40.4%) compared to Central (23.9%) and North B district (21.4%).

Table 6.1: Cow level Incidence of clinical bovine mastitis in Unguja Island for a

       period of two years (2014-2016)

District	Number observed	Incidence rate per 100 cow-year at risk	Cumulative incidence	cumulative hazard function
Cows reported CM				
North B	96	23.2	13.5	0.19
West	314	39.8	20.7	0.31
Central	168	30.0	16.7	0.24
Overall	578	34.0	18.3	0.27
p-value	0.22			
Confirmed CM by pathogen isolation				
North B	96	23.2	13.5	0.19
West	314	38.0	19.8	0.30
Central	168	26.0	14.3	0.21
Overall	578	31.8	17.1	0.25
p-value	0.18			

Table 6.2: Quarter level Incidence of clinical bovine mastitis in Unguja Island a

       period of two years

District	Number observed	Incidence rate per 100 quarter-year at risk	Cumulative incidence	Cumulative hazard function
Quarters reported CM				
North B	374	12.4	7.2	0.10
West	1243	16.9	8.8	0.13
Central	667	11.7	6.5	0.09
Overall	2284	14.5	7.8	0.11
p-value	0.17			
Confirmed CM by pathogen isolation				
North B	374	12.4	7.2	0.10
West	1243	15.8	8.2	0.12
Central	667	8.2	4.5	0.07
Overall	2284	12.9	7.0	0.10
p-value	0.009			

Out of 578 cows at risk of developing clinical mastitis, the overall incidence rate,

cumulative incidence and cumulative hazard function as reported by farmers were 34.0 per 100 cow-year at risk. 18.3% and 0.27, respectively while based on pathogen isolation were 31.8 per 100 cow-year at risk. 17.1% and 0.25, respectively, for the same parameters (Table 6.1). Further analysis at quarter level indicated the incidence rate, cumulative incidence and cumulative hazard function as reported by farmers as 14.5 per 100 quarters-year at risk, 7.8% and 0.11, respectively while based on pathogen isolation were 12.9, 7.0% and 0.10, respectively, for the same parameters (Table 6.2). The analysis of incidence rate, cumulative incidence and cumulative hazard function at district level at cow and quarter level were as shown in Table 6.1 and 6.2, respectively.

Table 6.3: Percentage distribution of isolated pathogens from each of the participating district

Isolated pathogens	North B n (%)	Central n (%)	West n (%)	Total n (%)
Staphylococcus aureus	11 (28.9)	18 (35.3)	46 (37.4)	75 (35.4)
Staphylococcus epidermidis	7 (18.4)	3 (5.9)	19 (15.4)	29 (13.7)
Candida spp	6 (15.8)	5 (9.8)	7 (5.7)	18 (8.5)
Pseudomonas aeruginosa	3 (7.9)	2 (3.9)	9 (7.3)	14 (6.6)
Klebsiella spp	0 (0.0)	6 (11.8)	7 (5.7)	13 (6.1)
Escherichia coli	2 (5.3)	0 (0.0)	6 (4.9)	8 (3.8)
Aspergillus spp	1 (2.6)	2 (3.9)	7 (5.7)	10 (4.7)
Saccharomyces spp	0 (0.0)	0 (0.0)	3 (2.4)	3 (1.4)
Micrococcus spp	1 (2.6)	0 (0.0)	0 (0.0)	1 (0.5)
Bacillus spp	0 (0.0)	1 (2.0)	0 (0.0)	1 (0.5)
Contaminated	7 (18.4)	1 (2.0)	12 (9.8)	20 (9.4)
No growth	0 (0.0)	13 (25.5)	7 (5.7)	20 (9.4)
Total	**38 (100.0)**	**51 (100.0)**	**123 (100.0)**	**212 (100.0)**

Out of 169 milk samples from quarters with clinical mastitis submitted for pathogens isolation, 79 (46.7%) samples revealed single bacterial pathogens, 19 (11.2%) revealed two bacterial pathogens, 24 (14.2%) revealed a bacterial and fungal pathogen, 7 (4.1%) revealed a single fungal pathogen, 20 (11.8%) revealed more than two pathogens and were

considered contaminated, while 20 (9.4%) did not develop any microbial growth. Contaminated samples and samples with no growth were considered negative bacteria isolation. Out of 169 milk samples collected, 129 (76.3%) were positive for microbial isolation and 40 (23.7%) were negative. Out of 172 pathogens isolated from this study, 82% were bacterial pathogen and 18% were mycotic pathogens. Table 5.3 summarises the number of isolated pathogens from each district. *Staphylococcus aureus* were predominant (35.4%) followed by *Staphylococcus epidermidis* (13.7%), *candida spp* (8.5%), *Pseudomonas aeruginosa* (6.6%), *Klebsiella spp* (6.1%) and *Aspergillus spp* (4.7%).

Cow and farm management characteristics that influence the occurrence of clinical bovine mastitis based on confirmed microbial isolation are indicated in Table 6.4., Lactation stage and cow hygiene were cows' characteristics that increased the occurrence of CM. Study district, floor hygiene and calf suckling before milking were farm management factors that increased the occurrence. Farmers practice to wash hand before milking decrease the occurrence.

Table 6.4: Risk factors influencing quarter level incidence of clinical bovine mastitis in Unguja Island as defined by pathogen isolation

Risk factors	Category	Number observed	Cumulative incidence %	OR	95%	C.I.	p-Value
Study district	North B	374	7.2				
	Central	667	4.5	1.89	1.25	2.89	0.002
	West	1243	8.2				
Lactation stage (Months)	<4	811	7.4				
	4-6	876	6.1	0.66	0.53	0.83	0.0004
	>6	597	7.7				
Cow hygiene	Dirty	1607	8.1				
	Clean	677	4.3	0.64	0.48	0.85	0.0026
Floor hygiene	*Dirty*	1138	8.2				
	Clean	1146	5.8	1.18	1.03	1.36	0.016
Hand wash	Yes	159	3.8				
	No	2125	14.6	4.71	2.00	11.11	0.0004
Calf suckling	Yes	1303	6.1				
	No	981	8.0	0.57	0.36	0.89	0.013

OR=Odds Ratio, CI=Confidence limit

Discussion

High occurrence of clinical mastitis at farm level observed in this study shows how important this disease is at farm level. The observed incidence at farm level was slightly higher compared to what has been reported in other studies conducted in Tanzania [34]. However, the cumulative incidence at farm level observed in this study seem to be lower than what was observed by other studies in developing countries [35] or developed countries [36]. The higher farm level cumulative incidence of CM in the West district compared to the other two districts is in line with the higher quarter level cumulative incidence of CM in that district described in this study (8.2%).

Studies on the Incidence Rate of Clinical Mastitis (IRCM) in dairy cattle are rare due to the cost implication and time taken to follow-up a number of dairy cows for a period of time. Being the first study to estimate IRCM in Zanzibar, there was no similar study from the same area which can be used to compare the observed results. IRCM observed in this study is higher compared to what was observed before in other part of African continent [37]. However, cow level IRCM observed in this study was higher compared to what have been observed in Ethiopia [38], the Netherlands [39] and Canada [40]. IRCM confirmed by isolation of causative pathogen was slightly lower compared to what was reported by farmers since some milk samples did not yield any growth. This may be caused by presence of other pathogens rather than bacteria and fungi that were not taken into consideration during this study. Some pathogens which need specialized isolation media or procedures such as mycoplasma and viruses as previously described [41] and that were not part of this research design. Incidence rate, cumulative incidence and cumulative hazard function in the West district were higher than in Central and North B districts. Increased number cattle and high percentage of lactating cows in the West district as described earlier [42] may have contributed to these findings. Furthermore, high

prevalence of subclinical mastitis in the study area as explained before [27] may act as source of infection that may develop to clinical cases of mastitis.

During the study period, it was found that 81.9% of the isolated pathogens were bacteria and 18.1% were fungi. Most pathogens isolated with exception of *Staphylococcus aureus* were of the environmental origin. Inappropriate farm management, farm hygiene and cow hygiene may be the source of these environmental pathogens incidence of CM. Most of bacterial pathogens observed in this study were also described in previous studies in Tanzania [2,34] and in other parts of the world [39]. *Staphylococcus aureus* was the major pathogenic bacterial species isolated in this study. Similar results have been reported in other studies conducted to identify causative agents of clinical mastitis around the world [43]. In addition to *Staphylococcus aureus,* other major bacterial pathogens observed in this study were *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella spp* and *Escherichia coli*, these are similar to what have been described before as causative agents of CM in other parts of the world [44]. Most fungal pathogens observed in this study belonged to *Candida spp* and *Aspergillus spp*. This is similar to what has been described in other studies conducted in Tanzania [34] and other parts of Africa [45]. A number of studies described *Streptococcus spp.* as one of the major causative agents of CM [46,47]. This study did not identify *Streptococcus spp.* as causative agent of CM. This is in agreement with the finding of study conducted previously in the area to quantify the prevalence of subclinical mastitis [27] and other studies conducted elsewhere [48].

CM was more prevalent in West district compared to North B and Central Districts. Increased number of lactating cows and intensification in the West district together with high prevalence of subclinical mastitis as described before may have contributed to this high incidence of CM [27,42]. Stage of lactation was among the risk factors that was

found to have significant effects on increased cases of clinical mastitis as described before [19,21]. The occurrence tends to increase with the increased stage of lactation. The sphincter muscles in the teats orifice in the cows with prolonged time of milk production tend to be looser after milking. It became easier for environmental pathogen to enter the udder during this period [17,35]. Improved cow hygiene significantly decreased the chances of getting the disease. Safe environment and proper floor hygiene around the cow facilitate the cleanliness of the cow and udder and decrease the amount of environmental pathogen that can invade the udder through the teat canal. Good drainage system and clean floor are example of safe environment. This decreases the possibilities of getting clinical mastitis as previously reported [20]. Improved dairy farm management facilitates the cleanliness of the cow and udder and decreases the number of environmental pathogens and decreased cases of clinical mastitis. Similar results have been previously reported [36]. Farmers practice to let calves suckling before or during milking seem to have negative impact to the increased number of mastitis cases. This may be due to the fact that calves may transmit pathogens from one infected quarter to another during suckling. Hand wash before milking significantly reduced clinical cases of mastitis. This sanitary measure reduced number of pathogens that can enter the udder during or after milking.

Conclusion

High incidence rate, cumulative incidence and cumulative hazard function of clinical mastitis recorded in this study demonstrate the importance of mastitis in the dairy industry. Most of the clinical cases of bovine mastitis were caused by bacterial pathogens but a number of mycotic mastitis in this study raises attention of its importance particularly when chronic cases were involved. Both contagious and environmental bacteria and mycotic remain as very important pathogens involved in bovine mastitis in the study area. Cows' age, number of parity, cow and floor hygiene seem to have an influence on the increased occurrence of bovine mastitis.

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Conflict of interest

The authors declare that they have no conflict of interest and this study is the original research work. Laboratory work of this study was carried out at Maruhubi Veterinary Investigation centre in Unguja Island of Zanzibar, Tanzania. No part of it is submitted anywhere else for publication or conference.

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CHAPTER SEVEN

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

It is well known that smallholder dairy farming is the only reliable source of fresh milk produced in Zanzibar. The findings of this study demonstrate the importance of crossbred cattle of Jersey, Friesian and Ayrshire in milk production. Those crossbred animals are preferred by livestock keepers compared to pure dairy breeds. Land scarcity encourage farmers to practice intensive and semi-intensive methods of dairy keeping which was the most observed during this study. Most of the smallholder dairy farmers do not practice good dairy husbandry practices. Poor and unhygienic environment, improper milking techniques and unorganized dairy cattle husbandry are part of the reasons towards poor milk production.

In Zanzibar, as the case in the rest of the world, bovine mastitis has long been considered to be a disease of economic importance and public health concerns. A high percentage of farmers reporting cases of clinical mastitis, a high prevalence of SCM and elevated incidence rate of CM observed during this study provides evidence of the extent of bovine mastitis in Zanzibar. Higher prevalence of subclinical bovine mastitis as confirmed by CMT, bacteria and mycotic isolation described, provide wider knowledge of the disease, its causative agents and different methods that can be used to diagnose the disease under Zanzibar situation.

During this study it was observed that different dairy farm management practice contributed to the low milk production from smallholder dairy farmers. Rearing system, Pasture used, type of housing, provision of supplements feed and type of supplement,

calves suckling before and after milking, water availability at the farm and farmer practice to wash hand before milking have shown effects on milk production.

The findings of this study demonstrate various farm and cow level risk factors that play a role in the increased or decreased the occurrence of both CM and SCM. Cow level risk factors that increased the occurrence included cow hygiene, lactation stages, number of parity and increased amount of produced milk. Farmers tendency to allow calves suckling during milking, uses of towel to dry the udder, observed to increase the occurrence. On the other hand, farmer practices to wash their hand before milking and milking technique used, number of milking per day are among factors that decreased the occurrence of mastitis. In addition, farm level factors that described to increase the occurrence of mastitis included districts, increased herd size, dirty drainage and floor hygiene.

Furthermore, the findings of this study revealed involvement of both, contagious and environmental pathogens as causative agents of mastitis. This diverse of pathogens demonstrated, increased the challenges in controlling the disease in smallholder dairy farmers. In addition, some pathogens demonstrated in this study are capable of causing diverse of infection in human population. This zoonotic aspect of pathogens causing mastitis increased the importance of controlling mastitis. The observed resistance nature of some bacteria isolated during this study to some antibiotics restricts the choice of drug to treat this condition. Drugs such as penicillin compound is no longer suitable for the treatment of mastitis in Unguja island.

6.2 Recommendations

The reported research findings provide a baseline information to the dairy farmers and decision-making authorities to put in place strategies that will improve milk production

and decrease occurrence of mastitis in Zanzibar. Strategies should focus on increased quantity and quality of milk produced and minimizing the level of contamination of milk produced.

Due to the land scarcity, farmers are encouraged to use intensive dairy cattle keeping using cut and carry as methods of obtaining forage to their animals. To improve quantity of milk produced, farmers should follow good dairy farming practices in terms feeding, farm hygiene and cattle housing. To decrease the occurrence of bovine mastitis, farmers should improve sanitary measure of their cattle house, follow recommended good milking practice and uses of preventive measure to control mastitis. Good milking practice such as hand wash and udder wash before milking should be encouraged. In addition, the uses of separate towel for each cow to dry udder after washing should be emphases. Dairy farmers should improve pre- and post-milking procedures by introducing post milking teat dip uses of dry cow therapy. Farmers practice of using of calves suckling during milking should be used with care. This practice should be avoided if one quarter is infected because it can spread mastitis to the remaining quarters of that cow.

Whenever clinical cases of bovine mastitis occur, farmers should require proper diagnosis of causative agent and treatment. Test for drug sensitivity should be encouraged for suitable choice of drug to be used.

6.3 Relevance

Baseline information obtained from this study will fill the gaps of information about epidemiology of bovine mastitis. This will enable decision-making process of setting up strategies to control mastitis in Zanzibar. Improvement of dairy farming management in terms of feeding, animal husbandry and disease control as recommended will decrease

cases of both clinical and subclinical bovine mastitis, increase amount of milk produced and hence raise farmers' income and improve livelihood of smallholder dairy families.

6.4 Future Studies

i. Future research should focus on the bacteria genetic makeup responsible for the antimicrobial resistance established. mentioning few examples such as conducting study to identify extended Spectrum Beta-Lactamase (ESBL) producing bacteria or Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria or others.

ii. There is a need for conducting an in-depth study on constraint and benefits of dairy farming in Zanzibar with focus on cost and benefits of dairy farming.

iii. There is a need to incorporate modern communication technologies that will improve animal disease reporting system and animal health management. Early reporting will lead to early diagnosis and proper treatment and hence to minimize number of chronic cases.

iv. Further study is needed to establish specific pathogen epidemiology and mapping of common of common pathogens identified during this study.

v. Due to the study design and time constraint, this study failed to isolate other microorganisms known to cause bovine mastitis. further studies should be conducted to identify other mastitis causative pathogens that require specialized isolation procedures.

APPENDICES

Appendix 1: Sample Frame Form

(this form should be used to list all dairy farmers within the selected Shehia for the purpose of creating sample frame which will be used in the proportional random selection of participating smallholder dairy keepers within District)

District _____Shehia_____ Field assistant name _____ Signature _____

SN	Farmer's name	Telephone number	Breed of cattle	In calf Heifers	Pregnant Dry Cows	Lactating cows	Total herd size
	1.1	1.2	1.3	1.4	1.5	1.6	1.7

Cattle
Breeds

Appendix 2: Questionnaire for farmer interview during cross sectional study

Name of Field assistant name _____ Code number of Assistant _____ Signature _____
   1.   Farm identification (the information below is about farm location, owners' identification and number of cattle owned)

SN	Farm ID	Head of household	Telephone number	District	Shehia	For how long you have been keeping cattle? (Years)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

2.   Farm characteristics (the information below is about breed and number of animals owned by the farmer)

SN	Farm ID	Breed of cattle (0=crossbreed, 1=pure breed Dairy cattle, 2=Both)	Herd structure (insert number of animals in each category)						
			Calves (<6monts)	Heifers	Dry Cows	Lactating cows	Breeding bulls	Other	Total herd size
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									

3. Herd management (the following information is about farm management as a whole).

Housing

SN	Farm ID	Where do you keep your animals? (0=communal land, 1=own land field, 2=Backyard, 3=cattle house)	Housing (0=No structure, 1=under the tree, 2=Temporary structure, 3=permanent structure)	Type of floor (0=mud floor, 1=concrete floor, 2=both)	Floor hygienic condition (0=dry, 1=wet, 2=dirty)	Drainage system (0=no drainage, 1=poor, 2=good)	Type of roof (0=none, 1=coconut-mate, 2=iron sheets, 3=both)	Roof condition (0=poor, 1=good)
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Cleaning

SN	Farm ID	How many times do you clean the floor? (0=none, 1=occasionally, 2=once a week, 3=once a day, 4=twice a day)	How do you clean the floor? (0=dung removal, 1=water cleaning, 2=brushing, 3=0+1, 4=0+1+2)	Do you use disinfectant to clean you barn? (0=no, 1=yes)	If Yes, which disinfectant do you use? (specify)	Where do you milk your cows? (0=same housing, 1=separate space for milking)	Do you clean milking area after milking? (0=no, 1=yes)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

Feeding and water supply

SN	Farm ID	What is the rearing system used? (0=Intensive, 1=Semi intensive 2=Extensive,)	Pasture used? (0= communal, 1=established, 2=cut and carry, 3= 1 + 2, 4=0+2)	Do you give concentrates? (0=No, 1=Yes)	If yes to question 3.16, what type of concentrate? (0=Pollard, 1=Maize bran, 2=wheat bran, 3=other specify)	Do you have constant water supply? (0=No, 1=Yes)	What is the source of water? (0=tap water, 1=river, 2=borehole, 3=rain water)	How many times do you supply water to you animals? (0=once, 1=twice, 2=always available)
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

4. Milking procedure

SN	Farm ID	Who is milking? (0=owner, 1=relatives, 2=hired labour, 3= not specific)	How many cows does one-person milk per milking? (Number)	How many times do you milk per day? (0=once, 1=twice, 2=thrice)	What technique used during milking? (0=hand fist, 1=thumb stripped)	Do you use calf for milk let down? (0=no, 1=yes,)	Do you let the calf suck after milking? (0=no, 1=yes,)	Do you wash udder before milking? (0=no, 1=yes,)
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Milking procedure (continue...)

SN	Farm ID	Do your dry udder with towel after washing? (0=no, 1=yes,)	Do you use separate towel for each cow? (0=no, 1=yes,)	Do you apply anything before milking? (0=no, 1=water, 2=milk, 3=Vaseline, 4=cooking oil, 5= milking salve, 6=teat dip)	Do you apply anything after milking? (0=no, 1=water, 2=milk, 3=Vaseline, 4=cooking oil, 5= milking salve, 6=teat dip)	Do you wash your hand during milking? (0=no, 1=yes)	How often do you wash your hands? (0=beginning only, 1=after each cow)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

5. Knowledge and previous record of clinical mastitis (the information below is about farmers' knowledge of mastitis).

SN	Farm ID	Do you know anything about mastitis disease in cattle? (0=No, 1=Yes)	Did clinical mastitis affect your cow for the past one year? (0=No, 1=Yes)	How do you know if your cow has clinical mastitis? (0=Don't know, 1=sign in udder, 2=sign in milk, 3=both 1 and 2)	If yes, which type of treatment did you apply? {0=No treatment, 1=intra-muscular Inj., 2= Intra-mammary, 3=Both 1 and 2, 4=other (specify)}	Do you use any traditional treatment to treat mastitis? (0=no, 1=yes – mention it)	Who treat your animals when sick? (0=Myself, 1=CAHW, 2=Vet Ass. 3=another farmer)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

6. Knowledge of sub-clinical mastitis (the information below is about farmers' knowledge of subclinical mastitis).

SN	Farm ID	Do you know anything about sub clinical mastitis? (0=No, 1=Yes)	How do you know if your cow has sub-clinical mastitis? (0=don't know, 1=milk change, 2=cow side test)	Do you treat your animal with sub clinical mastitis? (0=No, 1=Yes)	If yes in 6.3, which type of treatment did you apply? {0=No treatment, 1=intra-muscular Inj., 2= Intra-mammary, 3=Both 1 and 2, 4=other (specify)}	What do you do with milk during treatment? (0=discard, 1=sell, 2=home use, 3= give other people)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

7. Control methods used for both clinical and sub-clinical mastitis

SN	Farm ID	Which method do you use to control mastitis? {0=none, 1=prophylactic, 2= culling chronic case, 3=other (specify)}	Did you use dry cow therapy during drying period? (0=No, 1=Yes)	Did you use teat application before milking? (0=No, 1=Yes)	What Application	Did you use teat dip after milking? (0=No, 1=Yes)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

Appendix 3: Questionnaire for cow information and Field observations during cross sectional study

Code number of Assistant _____

 Signature _____

Name of Field assistant name _____

1. Cow information (the information below is about each cow under investigation).

SN	Farm ID	Cows' number (Ear tag number)	Cows' name (if any)	What is the breed of the cow? (1=Cross, 2=Exotic)	If the answer in question 8.3 is cross; of which breed? (0=Friesian, 1=Ayrshire, 2=Jersey, 3=others)	If the answer in question 8.3 is exotic, which breed? (0=Friesian, 1=Ayrshire, 2=Jersey, 3=others -specify)	What is the age of the cow under investigation? (Record the age in years)	What is the origin of this cow? (0=same farm 1=same district, 2=Unguja, 3=Pemba, 4=Mainland)

2. Milk, milking and mastitis records (the information below is about milk record of each cow under investigation)

SN	Farm ID	Cows' number (Ear tag number)	What is the number of lactation of this cow? (Parturitions)	For how long has been milked since parturition? (State the length in months)	What is the average milk production per day (litres)	Has this cow had clinical mastitis for the period of one year? (0=No, 1=Yes	If the answer in question 9.4 is yes, which teat was infected? (RF=Right Front, LF=Left Front, RR=Right Rear, LR=Left Rear)	How many times does this cow develop mastitis for period of one year?

3. Physical Examination of cow and udder

SN	Farmer ID	Cow ID	Cow			Udder	
			What is the Body Condition Score (BSC) of the cow under examination? (0=very poor, 1=poor, 2=fair, 3=good, 4=very good) compare with provided picture	Which external parasite can be seen on the cow?	What is the general hygienic status of the cow under examination? (1=very clean, 2=clean, 3=dirty, 4=very dirty) Compare with provided picture	What is the general hygienic status of the udder? (1=very clean, 2=clean, 3=dirty, 4=very dirty)	Which external parasite can be seen on the udder?

4. Physical Examination of teats

SN	Farmer ID	Cow ID	What is the general condition of the teat? N=Normal teat without infection H=Hard teat with signs of infection G=Gangrenous teat, B=Blind teat I=injured teat, A=Abnormal				Which external parasite can be seen attached to the teat? (0=ticks, 1=lice, 2=other....specify)			
			Right Front (RF)	Left Front (LF)	Right Rear (RR)	Left Rear (LR)	Right Front (RF)	Left Front (LF)	Right Rear (RR)	Left Rear (LR)

5. Cow side test results and milk sample collection

SN	Farmer ID	Cow ID	CMT field test				Sample collection for bacterial test			
			Right Front (RF)	Left Front (LF)	Right Rear (RR)	Left Rear (LR)	Right Front (RF)	Left Front (LF)	Right Rear (RR)	Left Rear (LR)