# CHARACTERIZATION OF E. COLI AND *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL AND SUBCLINICAL CASES OF BOVINE MASTITIS IN THE LIMPOPO DAIRY FARM (LIMPOPO, SOUTH AFRICA)

by

BADUGELA NDIVHUWO

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UNIVERSITY OF VENDA

PRIVATE BAG X5050

THOHOYANDOU

Supervisor: DR E Musie

Co- supervisor: Dr M.T Sigidi

Co- supervisor: Prof A.N Traore

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## DECLARATION

I, Badugela Ndivhuwo (student number: 11615918), declare that this dissertation for the award of BSc. Masters Degree in Microbiology of the University of Venda has not previously been submitted for a degree at this or any other institution and that all reference materials contained herein have been duly acknowledged.

Signature……NBadugela…… Date……07/08/2020……

## DEDICATION

I dedicate this work to the most high God my Lord and Savior Jesus Christ for the marvelous grace upon me, giving the potential to acquire all things, marvelously graced me with unending favor of life, my family, colleagues and friends for showing me their endless support day in day out; to my supervisors for giving an opportunity to work with them throughout this project and to myself for not giving up through it all even when facing many challenges.

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## LIST OF ABBREAVIATIONS

| | |
|---|---|
| % | Percentage |
| ≈ | Approximately |
| °C | Degree celcius |
| µg | Microgram |
| µl | Microliter |
| APC | Aerobic Plate Count/ Antigen Processing cells |
| API20E | Analytical Profile Index for 20 Enterobacteria |
| *aur* | Aureolysin gene |
| B-Lymphocytes | Blood Lymphocytes |
| bp | Base pairs |
| BPA | Baird-Parker agar |
| C3 | Complement factor 3 |
| CDC | Center for Disease Control and Prevention |
| CFU | Colony forming units |
| CFU/ML | Colony forming units per milliliter |
| Clf B | Clumping factor B |
| Clf A | Clumping factor A |
| CLSI | Clinical Laboratory Standards Institute |
| CMT | California mastitis test |
| DC | Dendritic Cells |

| | |
|---|---|
| DNA | Deoxyribonucleic Acid |
| DNAse | Deoxynuclease |
| dNTP | Deoxyribose Nucleotide Triphosphate |
| *E. coli* | *Escherichia coli* |
| E.g | For example |
| *eaeA* | *Enteroaggregative enterotoxin* A |
| *EAEC* | Entero-aggregative *E. coli* |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| *EHEC* | *Entero-haemorrhagic E. coli* |
| *EIEC* | *Entero-invasive E. coli* |
| ELISA | Enzyme-linked Immunisorbent Assay |
| *EPEC* | Entero-pathogenic *E. coli* |
| ERY | Erythromycin |
| EspA | *Escherichia coli* secreted preotein A |
| ETA | *Staphylococcal* enterotoxins A |
| Etc | And others |
| ETB | *Staphylococcal* enterotoxins B |
| *ETEC* | *Entero-toxigenic E. coli* |
| F primer | Forward primer |
| F primer | Forward primer |
| FC | Fusidic acid |
| FDA | Food Drug Administration |
| g/ml | Gram per millilitre |
| *gapdh* | Glyceraldehyde 3-phosphate dehydrogenase |
| GEN | Gentamicin |
| GuSCN | Guanidinium thiocyanate |

| | |
|---|---|
| HTST | Higher Temperature Short Time |
| I | Intermediate |
| ICR | Inducible Clindamycin resistance |
| ID/AST testing | Identification and/or Antibiotic susceptibility testing |
| IDF | International Dairy Federation |
| Kb | Kilobytes |
| Lt | Heat labile toxin |
| MAC | MacConkey agar |
| MALDI-TOF-MS mass spectrometry | Matrix assisted laser desorption/Ionization time of flight |
| Mamps | Pathogen-associated molecular patterns |
| *Mdh* | Malate dehydrogenase gene |
| Mec A | Methicillin A |
| mg | milligram |
| MHA | Muller–Hinton agar |
| MHC | Major Histocompatibility Complex |
| MIC | Minimum Inhibitory Concentrations |
| ml | Milliliter |
| MLEE | Multilocus enzyme electrophoresis |
| MLST | Multi-locus sequence typing |
| mmol | Millimole |
| m-PCR | Multiplex PCR |
| m-PCR | Multiplex- Polymerase Chain Reaction |
| MPO | Milk Producers' Organisation |
| MRSA | Methicillin Resistant *Staphylococcus aureus* |
| MSA | Mannitol salt agar |

| | |
|---|---|
| MUP | Mupirocin |
| NaCl | Sodium hydroxide |
| NDA | National Dairy Authority |
| NHL | National Health Laboratory |
| nm | Nanometer |
| NMC | National Mastitis Council |
| Nuc | Thermonuclease gene |
| OX | Oxacillin |
| PBPa | Penicillin-Binding Protein a |
| PCR | Polymerase Chain Reaction |
| PEN | Penicillin |
| PFGE | Pulse Field Gel Electrophoresis |
| PMN | Polymorphonuclear neutrophilic leukocytes |
| PTSAgs | Pyrogenic toxin superantigens |
| Pvl | Panton-Valentine leukocydin |
| R | Resistant |
| R primer | Reverse primer |
| RNA | Ribonucleic acid |
| S | Susceptible |
| *S. aureus* | *Staphylococcus aureus* |
| SA | South Africa |
| SA | South Africa |
| SCC | Somatic cell count |
| SpA | *Staphylococcal* protein A |
| SSSS | *Staphylococcal* scalded skin syndrome |
| *Staphylococcus Species* | *Staphylococcus* species |

| | |
|---|---|
| Stats SA | Statistics South Africa |
| *STs* | *Heat stable toxin* |
| *Stx 1* | *Shiga toxin 1* |
| *Stx 2* | *Shiga toxin* |
| *Stx* | *Shigella like toxin* |
| T-cells | Thymus cells |
| TEMP | Temperature |
| T-Lymphocytes | Thymus lymphocytes |
| TSST-1 | Toxic shock syndrome toxin-1 |
| V | Voltage |
| VRSA | Vancomycin-resistant Staphylococcus aureus |
| *VTEC* | *Verotoxigenic Escherichia coli* |
| WHO | World Health Organization |

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## ABSTRACT

**Background:** *Staphylococcus species* and *Eschericia Coli* has been predominantly found to cause mastitis in dairy farms. Milk harbor various pathogenic microorganisms that causes foodborne and intramammary infections. The aim of this study was to characterize *Staphylococcus spp. and Eschericia coli spp.* isolated from clinical and subclinical cases of bovine mastitis in the Limpopo dairy farm.

**Methods:** Semi structured questionnaire was used prior milk sampling to acquire farm management strategies. A total of 253 milk samples were collected from the dairy farm between 2018 and 2019. California mastitis test was done to screen for mastitis and culture methods were used for the isolation and identification of *E. coli* and *Staphylococcus species*. Further identification and biochemical confirmation for bacterial isolates were performed using API test kit and automated VITEK® 2 system. *Eschericia coli* isolates were characterized using a multiplex PCR. Automated VITEK® 2 system and Kirby Bauer disc diffusion method were also used to determine antibiotic susceptibility of the isolates.

**Results:** The study reported fair farm practices and management system with low mastitis burden. California mastitis test revealed an overall mastitis on 94/250 (37%) of the samples. Of 94 samples cultured, a total of 32 (34%) were positive for *E. coli* strains and 48 (51%) were positive for *Staphylococcus spp.* [*Staphylococcus sciuri* 19 (40%) and *Staphylococcus xylosus* 10 (21%)]. Out of 32 *Escherichia coli* isolates 27 (93%) and 19 (66%) were detected with *astA* gene and *sta* which encodes for enteroaggregative *E. coli* respectively. Most *Staphylococcus species* isolates were highly resistant to Erythromycin (93%); Nalixidic acid (86%). The presence of pathogenic *E. coli and Staphylococcus* species in milk may pose health risks or problem and improving sanitary conditions may reduce the burden of mastitis. For future studies, further analysis of both *E. coli and Staphylococcus* species to determine virulence and resistant genotyping in order to investigate possible mutations is recommended.


**Keywords**: Microbiological quality, Safety, Raw milk, Characterization, PCR, Mastitis, Occurrence.

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 BACKGROUND

South Africa (SA) is a developing country with an estimated population of 58,78 million **(Statistics South Africa, 2016)**. The average annual consumption of unprocessed milk in 2018, was 4,8% which makes approximately 0.5% of the world milk production **(Milk Producers' Organisation, 2019)**. The recorded capita is estimated to be far below the World Health Organization's (WHO) recommendation of 200 liters per capita annually **(Lassen, 2012)**. The reduction in milk production is caused by conditions such as mastitis which has a known negative impact in milk production. Milk from cows with mastitis harbors pathogenic microorganisms of various types that may cause foodborne infections **(Law et al., 2015; Oliver et al., 2005).**

Mastitis, regarded as inflammation of the mammary gland, bacterial infection, trauma, or injury to the udder has been found to be the cause, and leads to decreased productivity of the cow as well as the quality of milk causing. This consequently leads to enormous losses for breeders and the economy of the country. Studies have shown that there is change in the prevalence of mastitis pathogens in SA **(Petzer, 2009)**, it was also noted that the incidence of such environmental pathogens such as *Escherichia* coli *(E. coli*) and *Staphylococcus spp*. (*Staphylococcus spp.*) has increased **(Blignaut et al., 2018)**. The increased prevalence may possibly be attributed to the persistence of environmental pathogens in the udders, and difficulties in decreasing their population in the reservoirs **(Milk South Africa, 2013)**.

The most common mastitis causing organisms are *Staphylococcus spp., Streptococcus spp. E. coli, Klebsiella, Salmonella, Mycoplasma* and *Corynebacterium* **(Benic et al., 2012)**. These microorganisms are normal skin, nasal and gut microflora inhabitants, thus harmful and opportunistic when conditions become favorable. Among the *Staphylococcus spp*., *Staphylococcus aureus* (*S.*

*aureus)* predominantly cause mastitis in milk herds worldwide **(Pekana et al., 2015; Petzer et al., 2009; Wang et al.,2008; Allore et al., 1997)**. The *Staphylococcal* pathogens may infect cows, during lactation or dry season **(Petzer et al., 2009)**. Several dairy farms are still experiencing high levels of *Staphylococcal* mastitis infested with biofilm formations and exotoxin production **(Lee et al., 2014; Takeuchi et al., 2001; Aguilar et al., 2001)**. Mastitogenic zoonoses and toxin transmission is a potential public health hazard **(Blum et al., 2008)**. Specific conditions such as scalded skin syndrome, food toxicity and toxic shock syndrome may be caused by these products **(Becker et al., 2014)**.

Mastitis commonly develops in dairy cows due to invasion of the mammary gland tissue. These mammary glands seem to be a good reservoir of *Staphylococcus spp.* **(Samad, 2008)**. The invasion of these glands allows multiplication and dissemination of the pathogen and increases toxin production **(Samad, 2008)**. These pathogens have long survival periods in cow surroundings including bedding and milking machinery. The consequential effects of mastitis on the economic and public health are detrimental **(Sharma et al., 2013)**. India which is a leader in milk production, and SA which depends on the agricultural sector as a greatest employer is concerned due to losses as a result of milk disposal, reduced milk production, costs for treatment and loss of jobs **(Mohanty et al., 2013; MPO, 2009)**.

Management of mastitis, in many commercial dairy farms, plays a major role in determining raw milk products quality and other derivatives **(Gonzalez and Wilson, 2003)**. A number of factors, that include the quality of raw milk, economic viability of the farmer, reductions in antimicrobial use and animal welfare, also influence the necessity of controlling mastitis **(Asfaw and Negash, 2017)**. Farms characterized by low levels of sanitation, poor teat dip application, inadequate dry cow therapy, poor milking techniques or poor maintenance of machines experience higher levels of mastitis **(Schroeder, 2012)**. Raw milk serves as an ideal medium in the growth of various pathogenic microorganisms **(Kadariya et al., 2014; Zecconi and Hahn, 1999)**.

Furthermore, milk is also an important source of income for commercial farmers and household livestock farms **(Shete and Rutten, 2015)**. South African nutritionists are calling on people to increase their intake of milk products to achieve a balanced

healthy diet **(Wenhold et al., 2016)**. However, milk production industry rarely meets the standard requirements because of various factors such as mastitis **(Seid et al., 2015)**. When the udder is colonized by pathogenic *Staphylococcus species,* it may cause primary clinical signs such as swelling, redness and floccules in the milk, abscesses and fibrosis of the udder **(Branch-Elliman et al., 2013).** Treating *Staphylococcus* mastitis is difficult because of secretion of B-hemolysin potentially leading to fatal gangrene mastitis (**Mellenberger and Kirk, 2001**).

*Staphylococcus spp.* is known as commensal and opportunistic zoonotic pathogens **(Fitzgerald, 2012)**. *Staphylococcal* infections are problematic due to their virulence mechanisms, ease of transmission, persistence, and the ability to colonize the skin or mucosal epithelia as well as antibiotic resistance to conventional treatments **(Rainard et al., 2018)**. *Staphylococcus spp.* and *E. coli* are regarded as the priority or critical pathogens that require a thorough research for the development of new antibiotics because they have exhibited antibiotic resistance to a vast array of antibiotics that are used for their control **(WHO, 2017; WHO, 2015)**.

## 1.2 STUDY RATIONALE

The dairy industry has been greatly devastated by bovine mastitis that results in the decrease of milk production consequently leading to great economic loss for the industry worldwide **(Xi et al., 2017)**. In countries where dairy industry is still in developmental phase, mastitis may be caused by transmissible pathogens. Environmental mastitis caused by *Staphylococcus* and *E. coli* may also become prevalent due to lack of knowledge and routine control measures application **(Sharif and Muhammad, 2009)**. Various studies have greatly contributed knowledge on epidemiological characteristics of these aetiological agents **(Argaw, 2016 Blignaut, 2015; Joshi and Devkota, 2014)**. Due to their impact in economy, food security and issues related with antibiotic use, there is a need to develop the tools available to monitor environmental mastitis **(Rainard et al., 2018 Sordillo et al., 1997)**.

Mastitis not only influences the quality of milk but the yield as well and causes culling of animals until the undesirable characteristics have been satisfactorily treated. It is for these reasons that the processing and value of processed dairy foods or milk derivatives get affected since the valuable components in milk (lactose, casein and

fats) are reduced and defective milk constituents like ions and enzymes are inflated **(Mekibib, 2010)**. There is a lot of documented information on the pathogenesis of *Staphylococcal* mastitis, however, reasons why these pathogens remain a threat in the invasion of the mammary glands have not been clearly identified **(Rainard et al., 2018)**.

In contrast, *Escherichia coli* became a major pathogen causing acute bovine mastitis which usually recovers fast. The rate of recovery is rapid due to the exchange of genetic material between strains through horizontal transfer **(Schmidt et al., 2015)**. The horizontal transfer could give rise to virulent and resistant strains and/or stealthy and contagious strains that could greatly effect agriculture through creation of new variants **(Dyszel et al., 2010)**. This exchange of Mobile Genetic Elements (MGE) encoding virulence and resistance between human and bovine strains is a global issue **(Sung, 2008)**. It is thus possible that the zoonotic risks linked to *Staphylococcus spp.* and *E. coli* (environmental) mastitis will be a future problem **(Rainard, 2018)**.

*Staphylococcus aureus* can harbour vast number of putative virulence genes that may play a role in clinical or subclinical manifestation of the infection in both humans and animals **(Åvall-Jääskeläinen et al., 2018)**. Since variations of strains is associated with virulence potential, to characterize *S. aureus* by phenotype alone can no longer be a reliable control measure for mastitis caused by this organism **(Pilla et al., 2013)**. There are no previous studies that have ever reported about these virulence factors amongst *S. aureus* isolated in dairy farms within the Vhembe District. Data from a previous study conducted in the Limpopo dairy farm showed high prevalence of *Staphylococcus spp*. **(Badugela et al., 2018 unpublished)**. Therefore, the present study aims to phenotypically and genotypically characterize the *Staphylococcus spp.* isolates in bovine mastitis cases by evaluating their virulence genes and antibiotic susceptibility profile.

## 1.3 OBJECTIVES OF THE STUDY

### 1.3.1 PRIMARY OBJECTIVE

To characterize *Staphylococcus spp. and E. coli spp.* isolated from clinical and subclinical cases of bovine mastitis at the Limpopo dairy farm.

### 1.3.2 SECONDARY OBJECTIVES

- To evaluate farm management strategies and determine mastitis prevalence in the Limpopo dairy farm using questionnaire
- To isolate and identify *Staphylococcus spp. and E. coli* from clinical and subclinical cases of bovine mastitis using culture methods and biochemical tests.
- To amplify and identify the specific virulence genes using conventional multiplex polymerase chain reaction (m-PCR).
- To determine the antibiotic susceptibility patterns of the isolates using Kirby Bauer disk diffusion method and automated VITEK® 2 system.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 INTRODUCTION

Mastitis represents the biggest economic losses for dairy farms in many countries ar ound the world **(Petroviski et al., 2006)**. It is generally accepted that inflammatory reaction is caused by microorganisms that infiltrate the teat canal and mammary tissues. The organisms multiply and proliferate to manifest as the different types of mastitis affecting the cow's udder. The infection results in a negative relationship between somatic cell count (SCC) and the yield of milk **(Khan and Khan, 2006)**. Milk from healthy quarters generally contain between 100, 000 - 200,000 somatic cells per millilitre, a value that exceeds 300,000 is abnormal and indicates inflammation in the udder and secretory disturbance **(Viguier et al., 2009)**.

## 2.2 CLASSIFICATION OF MASTITIS

Mastitis is classified based on the extent of the inflammation with predisposing factors like age, breed, nutrition, shed management and stage of lactation. Classification as 'contagious' or 'environmental' may be used following their primary source of infection and route of transmission **(Blowey and Edmondson, 1995; Gomes et al., 2016)**. All the classes categorised as contagious are caused by the presence of bacteria in the teat canal and udder **(Schukken et al., 2004)**. They are capable of establishing sub-clinical infections **(Radostits et al., 1994)**. The main reservoir of environmental mastitis pathogens is the cows inhabit (**Gomes et al., 2016)**. The 3 sub-classes are clinical, subclinical and chronic mastitis (**Figure 2.1**). Clinical mastitis is characterized by inflammation and cuts on the cow's teats, this class is more common in housed cattle because they stay in close proximity **(de Vliegher et al., 2012)**.

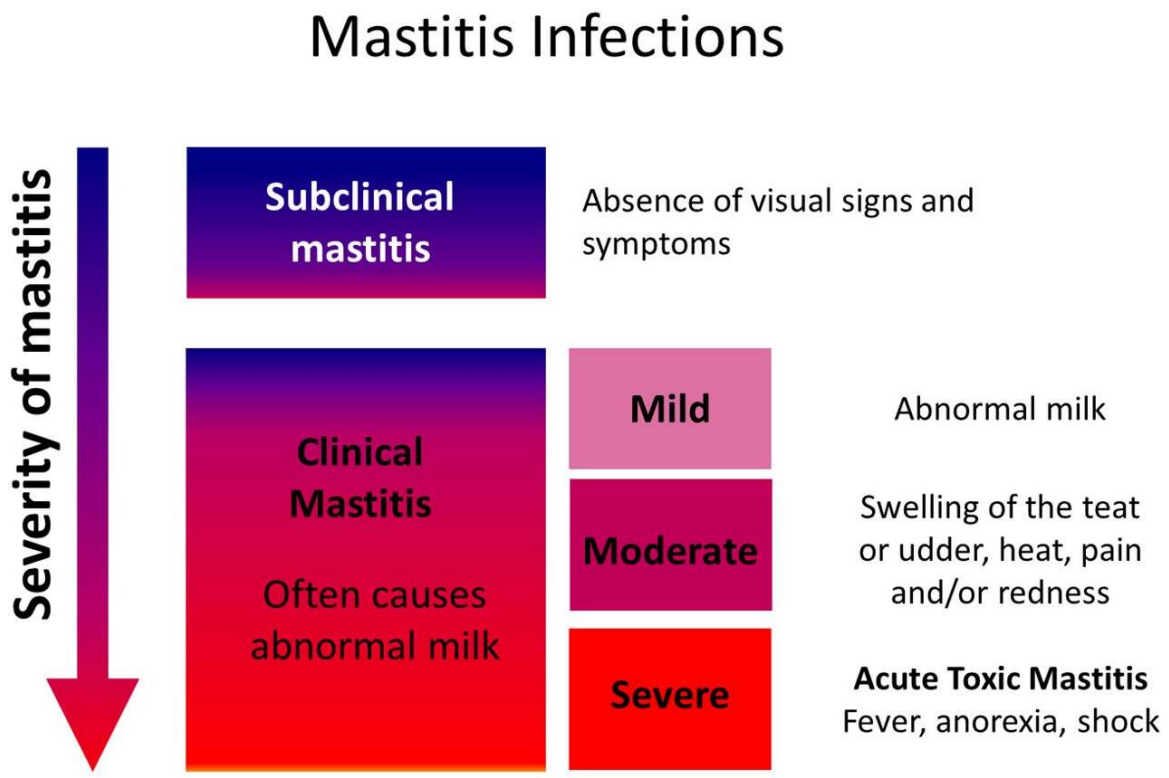



**Figure 2.1:** Classification of Mastitis severity (Farm Health Online, 2018)

Hyperthermia, anorexia, rapid cardiac rate and profound depression are characteristic for para-acute mastitis which is usually sudden in onset. Patches of blue decolouration from ischaemic gangrene, preferably at the base of the udder and around, appear in the most serious cases. Sub clinical mastitis is more threatening because it does not show any apparent signs on the host but it changes the physical and chemical composition of milk. Chronic mastitis manifests in the host for a longer period of time, usually can last from one lactating period to another **(Hughes and Watson, 2018)**. Clinical mastitis is generally more severe in cows than in small ruminants (goat and sheep) **(Contreras et al., 2007)**. Most of the infections are chronic and vary in bacterial shedding (concentration of viable bacteria) in milk, and frequent during continuous lactation.

## 2.3 MICROORGANISMS CAPABLE OF CAUSING MASTITIS

Bacterial organisms are the most common cause of mastitis frequently isolated from dairy cows **(Dieser et al., 2014)**. It has been indicated in ancients' reports that more than 137 microbes are mastitogenic **(Singh et al., 2016; Watts, 1988)**, mainly divided into different groups**.** These groups are contagious, environmental,

opportunistic pathogens, etc **(De Souza, 2018; Schmidt et al., 2015)**. These pathogens are further subdivided into major and minor pathogens based on the source of transmission.

The organisms that cause frequent infections fall within the contagious type of bacteria including *S. aureus* (coagulase positive *staphylococci*), *Streptococcus agalactiae* and the less common infections are caused by *Corynebacterium bovis and Mycoplasma bovis ( M. bovis)* **(Kulkarni and Kaliwal., 2013)**. The microorganisms are primarily found on the teat surface causing udder infection on healthy teats. Environmental mastitis, caused by coliforms (*E. coli* strains) is opportunistic infection which is directly proportional to sanitation and hygiene practice ( Azerverdo et al. 2015)

## 2.4 CONTAGIOUS PATHOGENS

The two mains contagious mastitogens are major and minor contagious pathogens. The major pathogens are mainly found in infected udder quarters. They are called contagious because they spread from infected quarters to healthy quarters (cow to cow transmission). These are the kinds of pathogens that can be found in recurring infections. The major contagious pathogens mainly cause clinical mastitis. These organisms are *S. aureus, Streptococcus and M. bovis* **(Jones and Beiley, 2009)**.

### 2.4.1 *STAPHYLOCOCCUS GENUS*

***Morphological characteristics, classification and structure of Staphylococcus***

*Staphylococcus* belongs to the family *Micrococcaceae*, organisms that are often found as normal human skin and nasal cavity microbiota (**Gomes et al., 2016)** They exist as non-motile, non-spore forming with about 90% found encapsulated **(Harris et al., 2002)**. These bacterial species are facultative anaerobes, with diameter of 0.5-1.5 µm and appear as grape-like clusters of gram-positive spherically shaped organisms when magnified.

The genus comprises over 50 species separated into two groups based on their coagulase activity **(Costa et al., 2013)**. Amongst the *Staphylococcus spp.* , *S. aureus* ranks top in causing diseases and food poisoning possibly because of its virulence factors (**Figure 2.2** and **Table 2.1**) **(Costa et al., 2013; Montville and**

**Mathews, 2008)**. **Pereira et al. (2011)** reported that *S. aureus* cells are able to invade epithelial cells of the mammary glands where they cause chronic infection. A large number of animal species, such as horses, pigs, dogs, cats, rabbits and poultry may be infected by *S. aureus* **(Fitzgerald and Holden, 2016)**.

The cellular structure of *Staphylococcus* is composed of surface proteins that are expressed for the attachment to host proteins. These proteins forms the extracellular matrix of the epithelia and the endothelial surfaces. A study done by **Thakker et al. (1998)** reported that over 90% of strains isolated in clinical studies possess capsular polysacharides. The encapsulated strains of *Staphylococus spp.* are more virulent than non-encapsulated forms. This could be because bacterial capsulation has been reported to help avoid phagocytosis and also facilitates adhesion to host surface **(Figure 2.2).**

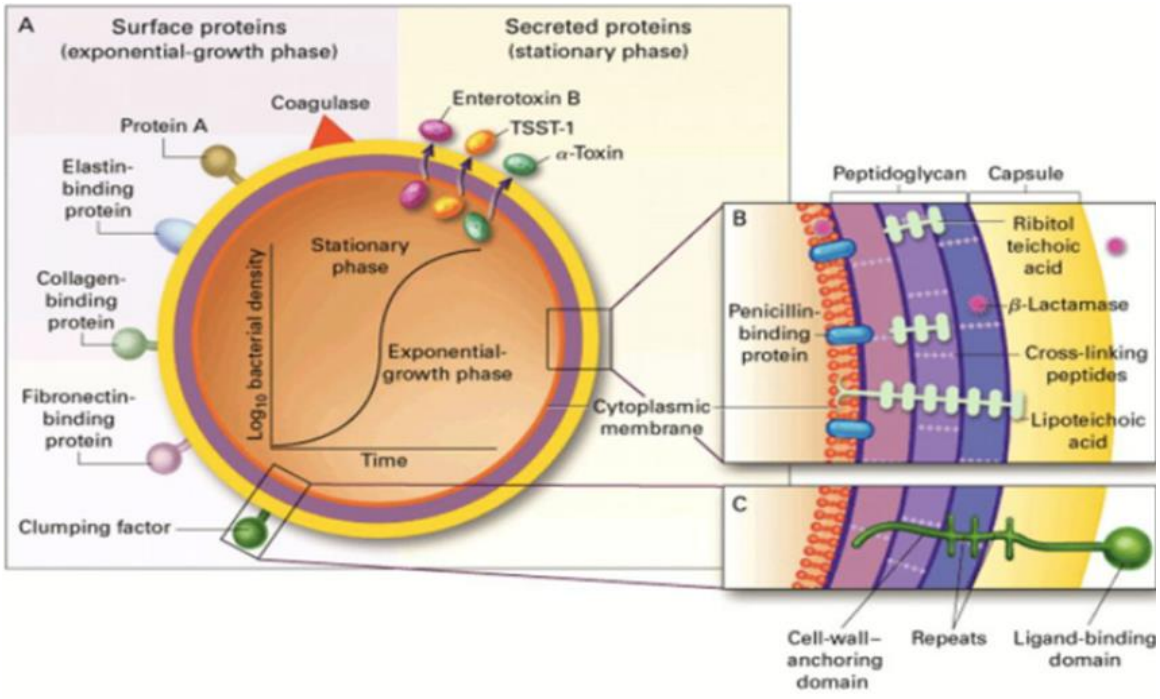



**Figure 2.2:** Schematic representation of *Staphylococcus structure* showing various virulence proteins **(Skipworth, 2009).**

***Virulence factors of Staphylococcus species***

Broad range of *Staphylococcal* infections is associated with a vast number of virulence factors that aid in adherence to surfaces, invade the host immune system and produce harmful toxic effects. Some of the species develop resistance to antibiotics **(Lowy, 1998)**.

**I) *Staphylococcus* Adherence factors (Adhesins)**

Most of surface proteins act as adhesins and in damaged tissues as fibrinogens, fibronectins, collagen and extracellular matrix **(Merriman, 2015)**. These factors are functional in disease establishment that is usually lethal since *S. aureus* clump in the presence of antibodies against cell associated factors. To date, there have been about 22 *Staphylococcal* adhesins that have been identified and characterized. *Staphylococcal* protein A (SpA) and clumping factor (Clf A and B ) are also a typical member of MSCRAMM **(Foster and Hook, 1998)**.

**II) *Staphylococcal* exoproteins**

All pathogenic strains of *Staphylococcus spp*. secretes exotoxins and enzymes such as nucleases, proteases, lipases, hyaluronidase and collagenases. The proteins are functional for the degradation of the host tissues and convert them into nutrients necessary for growth of bacteria **(Dinges et al., 2000)**. These exoproteins belong to a group of toxins known as pyrogenic toxin superantigens (PTSAgs) **(Lina et al., 2004)**. The widely studied characteristic of this group is the super antigenecity, which refers to its ability to stimulate proliferation of lymphocytes. The most important superantigens are known as *Staphylococcal* enterotoxins A and B (ETA and ETB) as well as toxic shock syndrome toxin-1 (TSST-1). These toxins have been implicated in disease including menstrual toxic shock syndrome, endorcaditis, sepsis and food poisoning **(Spaulding et al., 2013)**. ETA and ETB have been implicated in *Staphylococcal* scalded skin syndrome (SSSS) **(Handler and Schwartz, 2014)**.

The effect that some of the exoprotein have on host cell is that the cytolytic activity they posses perforates the plasma membrane and cause cytolytic cell leakage leading to cell lysis **(Foster, 2005)**. The exoproteins that posses such activity include Panton-Valentine leukocydin as well as hemolysins **(Kaneko and Kamio, 2004)**.

Alpha hemolysin is permeable through the eukaryotic cell membrane and once passed through, it oligomerize into a b-barrel that perforates the membrane and causes osmotic cytolysis. The cytolysis particullarly occurs on the human platelet and monocytic cells **(Craven et al., 2009)**.The functions of various virulent exoproteins are summarized in **Table 2.1**.

**Table 2.1:** The virulence factors and their function (**Costa et al., 2013**)

| Virulence factors | Putative function |
|---|---|
| Cell surface factors: | |
| Staphylococcal protein (SpA) | Bind to IgG, interfering with opsonization and phagocytosis |
| Collagen binding protein | Adherence to collagenous tissues and cartilage |
| Clumping factor protein (Clfp A and Clfp B) | Mediate clumping and adherance to fibrinogenin the presence of fibronectin |
| Secreted factors: | |
| Staphylococcal enterotoxins (SEA, A, B, C, D, E, G) | Massive activation of T cells and antigen presenting cells |
| Toxic shock syndrome | Induce lysis on leukocytes |

***Epidemiology of Staphylococcus spp.***

There is a wide distribution of *Staphylococcus spp.* in nature that is present in about 25-30% of normal individuals in the anterior nares and skin, of which 50% are intermittent carriers **(Grundmann et al., 2006)**. Bacterial colonization is a risk factor for subsequent infection caused by the colonizing clone **(Von Eiff et al., 2001)**. In various parts of the world reports, *Staphylococcus spp.* is isolated from domestic animals (pets), wild animals and livestock. In some parts of Europe, the zoonotic risk associated with the emerging burden of livestock-associated MRSA is high (**Köck et al., 2010**). This trend has been recognised in Africa in different age groups **(Butaye et al., 2016)**.

**Coagulase negative *Staphylococcus spp*.**

The importance of Coagulase negative *Staphylococcus* group is that they are indicators for intramammary infections in cows. In adequately mastitis controlled herd, minor organism can still be found causing opportunistic mastitis. They are increasingly found incriminated for more than 30% of subclinical and 20% of clinical cases **(Radostits et al., 2007)**. Coagulase negative *Staphylococci* are composed of over 30 typical opportunistic species, representing one of the major nosocomial pathogens. These organisms have a substantial impact on human life and health.

Coagulase-negative *Staphylococci* prevalence have increased in many countries and are now predominant emerging mastitis pathogens over *S. aureus* in most countries **(Gomes and Henriques, 2016; Tremblay et al., 2013)**. *Staphylococcus epidermidis* is the most crucial species among the coagulase negative species. It has been implicated in infections associated with prosthetic devices and catheters. Coagulase negative *Staphylococci* possess fewer virulence properties as compared to *S. aureus*, thus presents different disease spectrum **(Cunha et al., 2004)**.

**Coagulase positive *Staphylococci***

Coagulase-positive *Staphylococci* (CPS) are common commensal microorganisms and opportunistic pathogens in humans and animals. Several reports have described zoonotic transmission of methicillin Resistant *Staphylococcus aureus* (MRSA) strains between human and animals they work closely with **(Schmidt et al., 2015; Torres et al., 2010)**. The α and β hemolysins are the important factors that are commonly linked to the pathogenesis of *Staphylococcus* **(Linehan et al., 2003)**. In *S. aureus*,

the accessory gene regulator also has a down-regulating function that yields determinants of cell- associated virulence in a density-dependent pattern **(Lyon et al., 2000)**.

## 2.5 ENVIRONMENTAL PATHOGENS

Environmental pathogens are sourced from the environment and transferred to the cow not from the infected cow to another **(Radostis et al., 2000)**. They are the most ubiquitous pathogens that even the well-controlled herd may still encounter in high incidences of clinical mastitis caused by environmental pathogens. Poorly designed over-crowded unhygienic bedding with zero grazing systems is one of the most important factors that favours the growth of these pathogens. The preventive strategies such as teat dipping after milking and dry cow therapy are unable to control environmental pathogens (**Pekana et al., 2016**).

### 2.5.1 *ESCHERICHIA COLI*

#### Morphological characteristics, classification and structure of *E. coli*

*Escherichia coli* belong to gram-negative rod that ranges about 2.0 µm long and 0.25–1.0 µm in diameter. *E. coli* is a common humans and animals gut flora. Species that are flagellated are usually mobile and those that are non-flagellated are not. **Figure 2.3** shows typical *E. coli* with characteristic features.

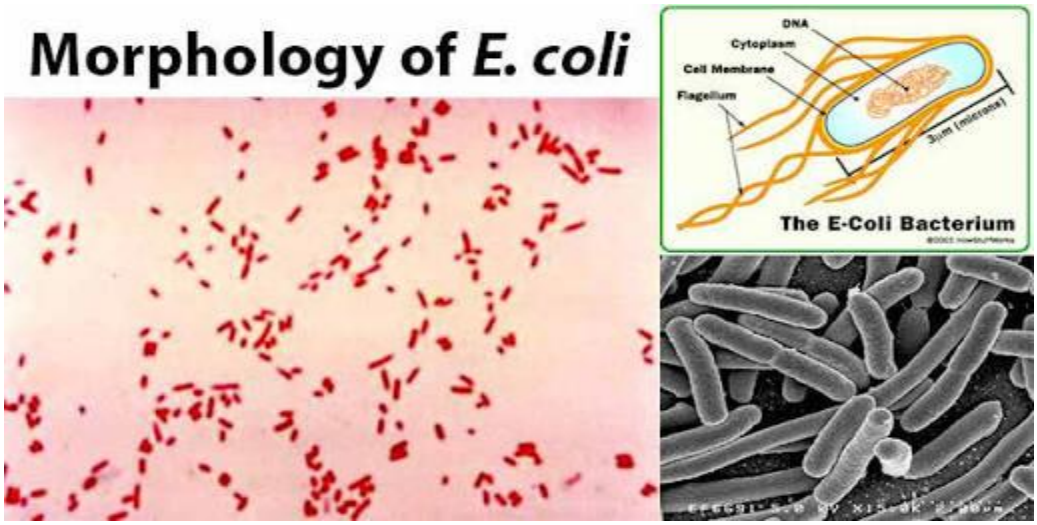



**Figure 2.3:** Morphology of *E. coli* (online source)

## Epidemiology of *E. coli*

Coliforms are the major environmental pathogens. They belong to the family *Enterobactericiae*, consisting of gram-negative rod-like, lactose fermenting bacteria. Coliforms are natural inhabitants of the colon flora. Approximately 20% of clinical mastitis cases caused by *E. coli* **(Sandholm et al. 1995)**. The course of the infection depends on the host response rather than the virulence factors of the pathogenic strains **(Bramley, 1991)**. Isolation of the *E. coli* strains is often not possible in mastitis cases since they release endotoxins once they induce cells to undergo apoptosis or necrosis.

## *E. coli pathogenic pathotypes classified based on virulence*

*Escherichia coli* pathogenic strains are able to cause various diseases in organisms. Some strains exist with the ability of colonizing ruminants that produce food without showing any signs and symptoms, and the may be recognised as a public threat within the farm community and general public. **Nataro and Kaper (1998)** categorized E. *coli* pathogens into five major pathotypes based on their virulence factors. The phathothotypes are: *Enteroaggregative E. coli* (*EAEC*), *Enterohaemorrhagic E. coli* (*EHEC*), *Enteroinvasive E. Coli* (EIEC), *Enterotoxigenic E. coli* (*ETEC*), *Enteropathogenic E. coli* (*EPEC*) and the fifth pathotype is the *Diffuse Adhering E. coli* (*DAEC*) which is proposed recently and not significantly established. Some studies report *Verotoxigenic E. coli (VTEC*) among the pathogenic pathotypes which include a small proportion of O157: H7 serotype (**Nguyen and Spendario, 2012; Msolo et al., 2016**).

### VI. *Enterotoxigenic E. coli* (*ETEC)*

The most commonly known diarhoeagenic *E. coli* is the *Enterotoxigenic E. coli (ETEC)*. Its virulence is associated with secretion of heat labile (LT) and heat stable (HS) enterotoxins which triggers the gastrointestinal mucosa causing secretion of fluid responsible for diarrhea (**Qadri et al., 2005**). The transmission of ETEC is mostly through ingestion food and water of contaminated with fecal (**Centers for Disease Control and Prevention, 2005**). It has been observed that this pathotype rarely cause recurrent infection since the host develop immunity towards successive encounters (**Walker and Black, 2010**).

### VII. *Vero toxigenic producing E. coli (VTEC) / Shiga producing E. coli (STEC)*

The *Verotoxigenic E. coli (VTEC)*, commonly known as Shiga toxin-producing *E. coli* (*STEC*) is the major cause of foodborne infections transmitted to humans primarily through consumption of contaminated foods such as raw milk, raw or undercooked ground meat profducts, and contaminated raw vegetables (**WHO, 2015**). The most prevalent animal associated serotype is the serotype 0157: H7. It has been implicated in numerous outbreak cases reported worldwide. The affected populaces were highly concerned with such outbreaks (**Nguyen and Sperandio, 2012**). The STEC or enterohaemorrhagic term came about because of its ability to induce fatal human infections known as the hemorrhagic colitis and the hemolytic uremic syndrome (**Orden et al., 2008**).

Literature revealed the discovery of *VTEC* dating back to the late 1970s. Since the discovery of VTEC in the 1970s, various studies have reported been reporting different serotypes isolated from humans and animals and specifically around 380 isolates in study conducted by **Nguyen and Sperandio (2012)** have been reported with serotype 0157: H7. Symptoms of STEC infections can vary, but mostly incude severe diarrhoea (often bloody and accompanied with little or no fever), stomach cramps and vomiting.

### VIII. *Enteropathogenic E. coli (EPEC)*

*Enteropathogenic E. coli* strain has been associated with infants' diarrhea in the developing countries (**Olesen, 2005**). The strain is identified by the gene *eaeA* and *bfpA* which are intimin and bundle-forming pilli inducer and promoter genes functional for adhesion and wiping out the intestinal microvilli lesions (**Alizade et al., 2014**). Strain isolated from animals differs from strain recovered from animals in that it lacks bundle-forming pilli (**Cortés et al., 2005**). Most cases related with *EPEC* are confused with *E. coli* infections because the infection onset is not distinct. The symptoms include watery diarrhea, bloody, vomiting and rarely fever (**Lee et al., 2012**).

### IX. *Enteroaggregative E. coli (EAEC)*

*Enteroaggregative E. coli* (*EAEC*) strain is associated with severely persistent diarrheal infections in children from developing countries (**Okhuysen and Dupont,**

**2010**). Most commonly reported symptoms of foodborne illness associated with this pathotype are watery diarrhea with or without blood, vomiting and low grade fever. The literature does not well describe the role and virulence factors of *EAEC* strain as well as the *EIEC* pathotypes, however studies report mainly on **(Puño-Sarmiento et al., 2013).**

### X. *Enteroinvasive E. coli (EIEC)*

*Enteroinvasive E. coli (EIEC)* has similar biochemical characteristics with *Shigella.* The two are usually confused because they both cause mild diarrhea or dysentery and it have been suggested that they share common ancestors (**Liu et al., 2013**; **Aribam et al., 2013**). Infection is initiated by producing several outer membrane proteins that service the pathogen for binding and invading the intestinal wall and causing diarrhea that often resemble that caused by ETEC (**Prats and Llovet, 1995**).

## 2.6 PATHOGENESIS

Animals are regarded as reservoir of virulent *E. coli* pathogens and serves as carriers of diseases to humans. Sometimes transmission may be via foods derived from farm animals due to fecal contamination and this is phenomenal in developing countries (**Alpers et al., 2009**). The larger global population contact pathogen via global distribution of food and once an outbreak occurs, it is impossible to trace and control the foodborne pathogen (**Werber et al., 2012**).

Pathogenesis of S. *aureus* in intramammary infection was scrutinized in detail when *Staphylococcus* comes in direct contact with the teat. The pathogen passes through the teat channel and establishes intramammary infections **(Ndyamukama, 2016)**. Both strain-virulence and host condition are determined by the severity of the condition. Experiments with experimentally induced infections demonstrated that very little colony forming units (CFU) is necessary to induce an infection. Healthy mammary glands are highly sensitive to *S. aureus* infection **(Rainard et al., 2018)**.

The early stage of infection can contribute to infection by other strains adhering to the intact epithelia and spread across the cisterns of the canals **(Rainard et al., 2018)**. Only an intramammary epithelium, which causes an inflammatory response to

the tissue and lumen, has thus far been characterized when they reach a threshold concentration. The direct interaction of bacteria with the epithelium, also released and secreted bacterial products such as pathogen-associated molecular patterns (MAMPs) that enhances bacterium detection by the immune system **(Lebeer, 2010)**. The host and infecting strain are likely to be dependent on the incubation period, epithelium of cisterns and ducts and then alveoli will be damaged by growing *Staphylococci* **(Rainard et al., 2018)**. The epithelium lining is wounded by haemolysins and enzymes. *Staphylococci* may then use their numerous adhesins to adhere to the basal membrane and the extracellular matrix **(Chavakis, 2002)**.

There can be a number of reactions at the initial clinical stage of an acute clinical phase in which host body temperature and anorexia are elevated. **Figure 2.4** shows manifestation of bacteria invading the teat canal and the mammary glands. The bacteria adhering to the interior tissue lining of the mammary gland is prevented from being eroded during milking (I A-C). Bacteria then secrete virulence factors that inflame and damage the mammary glands **(Kulkarni and Kaliwal, 2013)**. The produced toxins increase permeability of the blood vessels promoting the adherence of polymorphonuclear neutrophilic leukocytes (PMN) to the infection site.

Polymorphonuclear neutrophilic leukocytes may phagocytose the bacteria or may be destroyed by the invading organism **(Sharma and Jeong., 2013)**. Both scenarios result in secretion of other substances that induce vasodilation of blood vessel, thereby increasing the number of PMN that allows blood clotting factors to settle into the infection area. The influx of these substances constitutes the inflammatory response **(National Mastitis Council, 1996)**. The secretary glands function and potential milk production decrease once mammary stomal and parenchymal tissues begin, and the effect is irreversible **(Sharma and Jeong, 2013)**.

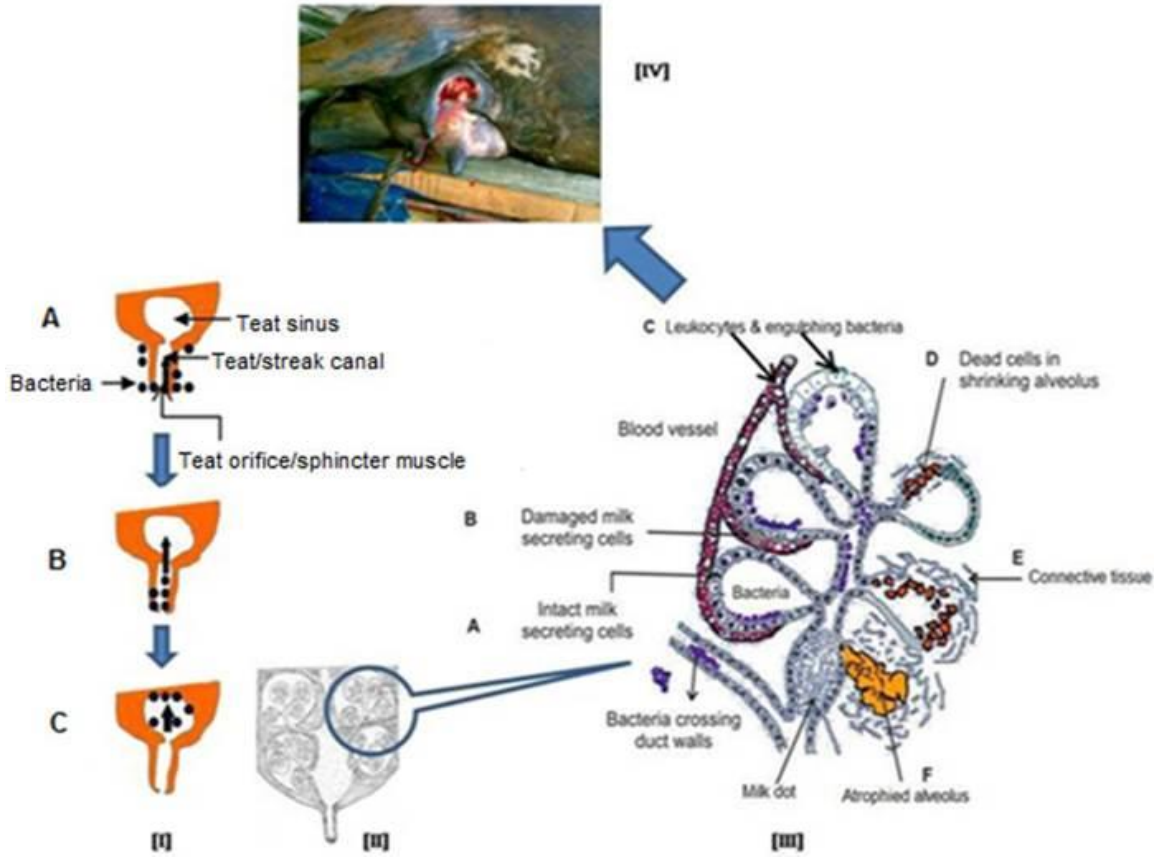



**Figure 2.4:** Schematic representation of the process of mastitis and subsequent damage to the mammary gland (**Sharma and Jeong, 2013**). [I] Bacterial evade the surface of mammary gland (udder, A) gain entrance and adhere onto the teat canal (B). Onset of the disease when environment is conducive within the mammary gland (C), [II] A diagram of normal mammary gland without any inframammary infection, [III] After disease onset, cellular defence mechanism is induced and PMN cells proliferate and influx the into the infected site as well as release toxins that cause fibrosis and necrosis of the mammary tissues (A to F), [IV] mastitis affected teats**.**

## 2.7 HOST IMMUNE RESPONSE TO *STAPHYLOCOCCUS AND E. COLI* PATHOGEN

Neutrophils protects the host against *S. aureus* infection by sensing pathogen entry and replication and destroy tissues that secrete inflammatory signals (chemoattractants and cytokines). Immune cells interact with *Staphylococcal* products via Toll-like receptors and G-protein combined receptors, while cytokines activate immune receptors **(Thammavongsa et al., 2015 Spaan et al., 2013)**. *Staphylococcus aureus* secretes several proteins that interrupts the deposition of complement on the surface of the bacteria. Complement factor 3 (C3) is cleaved by aureolysin (Zn-dependent metalloprotease) to generate functionally active C3a and

C3b. Complement factors I (fI) and H (fH) degrade or bind C3b, preventing its accretion on the cell surface. Different *S. aureus* lineages are associated with this polymorphic aureolysin gene (*aur*) **(Laarman, 2011)**.

## 2.8 LABORATORY DIAGNOSIS OF MASTITIS

Supreme mastitis infections cases (new) are experienced during the first 10 days and last 2 weeks of the dry period. Culture method has remained the golden standard in diagnosis of illnesses (**Reither et al., 2007**). The only challenge in culture methods is selecting pathogenic strain from non-pathogenic strain. PCR assays may be conducted to detect *E. coli* based on amplification of specific genes (**Bekal et al., 2008**).

Majority of clinical mastitis cases appears immediately after calving **(Baillargeon and LeBlanc, 2010)**. Subclinical mastitis detection is not apparent because the milk appears normal but contain elevated somatic cell count and potential presence of pathogen in milk **(Sharma et al., 2010)**.

The methods used to analyse and count *Staphylococcus* depend on the cause for testing the food and also on the history of the test material **(Cowan and steel, 2004)**. Culture method may be done for diagnosis where Baird-parker or Mannitol salt agar is used for isolation to provide suggestive evidence by fermentation of Mannitol **(Eley, 1992)**.

Baird-Parker agar, lysostaphin sensitivity, coagulase, thermo-nuclease production, glucose and mannitol fermentation can be conducted on enterotoxigenic and non-enterotoxigenic strains of *S. aureus* to study their colonial morphology **(Argaw and Addis, 2015)**. Definitive identification using biochemical and enzyme based tests can be used for samples sent to the laboratory. Furthermore, for differentiation at species level, catalase, coagulase, deoxynuclease (DNAse) as well as phosphatase tests can be done **(Mathanraj, 2009)**. In some cases, phage typing can also be done **(Roberts and Chambers, 2005)**.

### 2.8.1 CULTURE BASED AND BIOCHEMICAL TESTS

The traditional identification of bacteria in the clinical microbiology laboratory is carried out by isolating microorganism on agar and analysed for phenotypic characteristics **(De Souza, 2018)**. For selective growth of the *Staphylococcus* genus, culture media such as Baird-Parker agar (BPA), Mannitol salt agar (MSA) and blood agar can be used. Baird parker agar comprises of lithium chloride, potassium tellurite and egg yolk that selects *Staphylococcus* by reducing tellurite that result to formation of black colonies **(De Souza, 2018)**.

On blood agar, it shows haemolytic characteristic and grows as yellow colonies on nutrient agar **(Mathanraj, 2009)**. When *S. aureus* is cultivated on mannitol salt agar, it ferments the mannitol and produces yellow colonies **(Kateete et al., 2010)**. This particular yellow pigment is called staphyloxanthin and is a carotenoid that is regarded as a virulence factor. The synthesis of this carotenoid helps the bacterial cell to evade the destruction by the host immune reactive oxygen **(Chambers and Deleo, 2009)**.

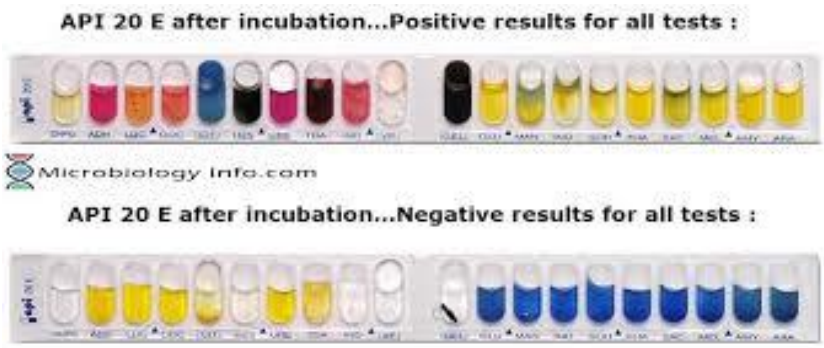
The identification of *Staphylococcus spp.* in milk samples has been shown to increase by freezing, thawing and incubation before plating or centrifugation and cultivation of sediments **(Artursson et al., 2010)**. To differentiate the strains, coagulase positive *Staphylococcal* strain produce lipase and lecithinase which break down lipids and lecithin in the egg yolk the resultant is a formation of dual halo **(De Souza, 2018)**. Mannitol salt agar selects *Staphylococcus* due to the presence of 7.5% sodium hydroxide (NaCl) and other bacteria are inhibited **(De Souza, 2018)**.

To determine the cell purity, typical *Staphylococcus* colonies are subjected to gram staining for observation of their morphology and specific stain. When the morphological characteristics are confirmed catalase and coagulase tests can be done to identify *Staphylococcus aureus (S. aureus)* and other coagulase positive species **(De Souza, 2018)**. *Staphylococcus aureus* is catalase positive, oxidative negative also hydrolyses urea and reduces nitrates to nitrites.

### 2.8.2 AUTOMATED COMMERCIAL KITS

Automated tests and commercial kits based on miniature biochemical experiments are also perfected for the detection and isolation in research laboratories to identify

and isolate *Staphylococcus spp*. **(De Souza, 2018)**. The commercial API staph system kit (BioMérieux, Marcy-l'Etoile, France) consist of 19 dehydrated miniature tests (**Figure 2.5**) that are inoculated for microbial resuspension. The incubation for biochemical tests takes 18- 24 hour at 37ºC **(De Souza, 2018)**. The microorganisms are identified from the database by generating a seven-digit profile number following manufacture instructions.




| Triad | I | | | II | | | III | | | IV | | | V | | | VI | | | VII | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | oxidase |
| Reaction | + | + | + | - | - | - | + | + | - | + | - | - | - | + | + | - | - | + | + | - | + |
| Point | 1 | 2 | 4 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 0 | 0 | 2 | 4 | 0 | 0 | 4 | 1 | 0 | 4 |
| Add | 7 | | | 0 | | | 3 | | | 1 | | | 6 | | | 4 | | | 5 | | |
| 7-digital Code | 7031645 | | | | | | | | | | | | | | | | | | | | |

**Figure 2.5:** API 20E test system (Adapted online) (Beckman Coulter, California, United States) panels contains 27 miniature biochemical tests for conventional identification (**Figure 2.6**) of which 18 are used for identification of *Staphylococcus spp*. The panels are read visually or automatically after incubation for 24- 48 hours. A six-digit code number generated by microscan walk/ away system is used to identify the microorganism in the database. **(De Souza, 2018)**.

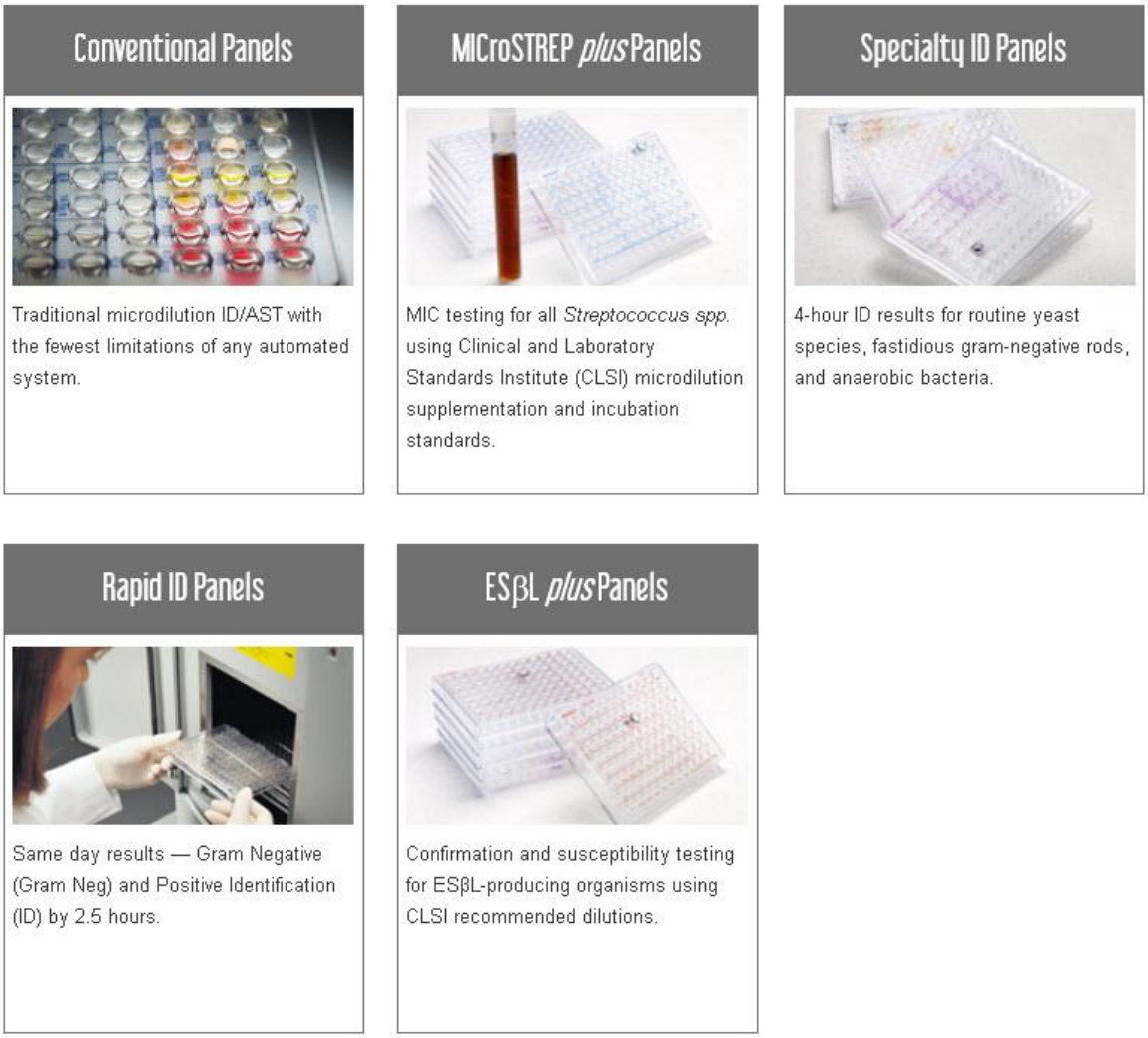



**Figure 2.6:** MicroScan ID/AST panels (Accessed online)

Another automated system-VITEK® 1 system consist of 30 microcavities of which 28 are the actual/ experimental test and the other 2 are the control tests. The cavities contain references for identification of *Staphylococcus spp*. The identification is achieved by insertion of a card filled with a suspension of the microorganism prepared in saline and sealed with the VITEK® filling/sealing module **(De Souza, 2018)**. Analysis is done after 10-13 hours. Limitation that has been noticed to VITEK 1 is that it failed to identify coagulase negative species which led to evolution of the system to VITEK® 2 which automatically performs all bacterial identification **(Ferreira et al., 2012)**. The VITEK® 2 automated microbiology system uses growth-based technology. The system is compacted into three formats (VITEK® 2 compact, VITEK 2, and VITEK® 2 XL) has different automation. The VITEK® 2 compact system is shown on **Figure 2.7a**. All three systems have the same colimetric reagent cards, which are automatically incubated and interpreted. The identification card that the

VITEK® 2 system uses are called reagent card and they are four identification (**Figure 2.7b**). Each card contains 64 wells evaluating different metabolic activities, including acidity, alkanity, hydrolysis and growth in the presence of inhibitors. (**Murray et al., 2007**).

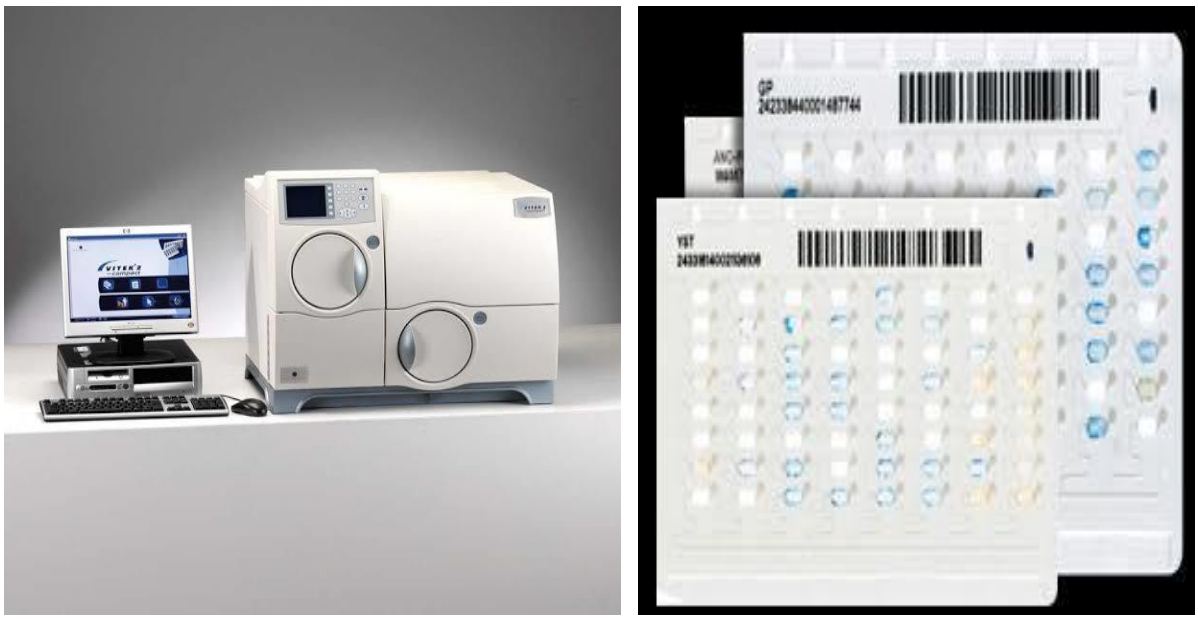


**Figure 2.7**: **a)** VITEK 2 Compact Instrument and Workstation **b)** VITEK 2 GN Colorimetric Identification Card (**Pincus, 2006**).

## 2.8.3 MATRIX ASSISTED LASER DESORPTION/ IONIZATION TIME OF FLIGHT SPECTROMETER (MALDI-TOF)

Recently microorganisms are identified using techniques of protein profile analysis. Matrix assisted laser desorption/Ionization time of flight mass spectrometry (MALDI-TOF MS) promise to be a new technology for bacterial identification **(De Souza, 2018)**. Results produced by MALDI-TOF MS have shown high rates of agreement between the reference identification techniques such as PCR and sequencing **(Dubois et al., 2010)**.

The method is based on pouring the sample onto a conducive metal plate with a matrix **(De Souza, 2018)**. The desorbed and ionized molecules are accelerated through an electric field and enter a metal tube submitted to the vacuum (the travel tube through which the molecule pass) before they arrive to the detector **(De Souza,**

**2018)**. Small ions (m/z) migrate rapidly through the flight tube than bigger ionsDepending on the sample, the time of arrival of the detector varies, producing va rious peaks and mass spectra according to their m / z ratio. The results are transferred to a graph, which gives multiple peaks and obtains a particular graph for each bacterial species **(De Souza, 2018)**.

Results are translated fast in a computerized database to compare sample spectrum with database. **(De Souza, 2018)**.The available commercial systems are MALDI Biotyper (Bruker Daltonics) and VITEK MS (BioMérieux, Marcy-l'Etoile, France) (**Figure 2.8**). Rapidity of the systems is an advantage but the cost limits its use. It has been predicted that since the use of MALDI-TOF MS has more economical advantages (rapid results, labour, uncomplicated, requires less training, and the results are more easily interpreted) in comparison to traditional methods, the technology might replace traditional methods of bacterial identification **(De Souza, 2018)**.


**Figure 2.8**: The MALDI-TOF MS systems (adapted online)

## 2.9 PREVENTION AND CONTROL OF MASTITIS

To manage mastitis, it is essential to decrease exposure of the teat to potential pathogens or by increasing resistance of dairy animals to the infection (**Kulkarni and Kaliwal, 2013).** It is impossible to entirely eliminate mastitis from a herd, but it can be minimised through comprehensive husbandry practices and sanitation, post-milking teat dipping, treatment during non-lactating periods, and culling of chronically infected cows **(Kulkarni and Kaliwal, 2013; Kurjogi and Kaliwal, 2011; Khan and Khan, 2006)**. The hygienic approach of washing hands with soap and water, washing teats and udder in sanitizing solution was also suggested as a control measure **(Jones, 2006)**.

The washing step is followed by thorough drying of the teats and udder with individual paper towels then dipping teats in an effective germicidal teat dip **(Kulkarni and Kaliwal, 2013)**. About 30 seconds of contact time is allowed before wiping off the teat dip with an clean towels and thoroughly scrubbing the teat end with a cotton swab soaked in alcohol. In cases where in all four quarters are being treated, cleaning starts from the farthest teat toward the closest **(Gooder, 2014)**. Commercial antibiotic products are used in single dose containers formulated for intramammary infusion and lastly the teats are dipped in an effective germicidal teat dip after treatment (**Kulkarni and Kaliwal 2013).**

## 2.10 TREATMENT OF MASTITIS

In South Africa, animal producers have unrestricted access to 12 of 22 prescriptions free FDA approved registered medicines, whereas the remaining 10 intramammary drugs are restricted to veterinary clinics usage **(Karzis et al., 2016)**. The prescription free antibiotics can be improperly used and may contribute to increase in antibiotic resistant strain emergence and/or persistence in cows, humans or both **(Henton et al., 2011; Burgos et al., 2005)**. Research studies have indicated that the resistance of *Staphylococcus spp.* to antibiotic may be improved through diet (increasing vitamin E, selenium, vitamin A and β-carotene), genetics, and to a lesser extent, vaccination (**Mathew et al., 2007)**.

The economic effect of mastitis as a chronic disease in dairy farming needs further study into the development of new antimicrobial therapy technologies. Increasing concern for human health, mainly due to the emergence of bacteria with resistance, also necessitates the production of alternative anti-effective agents **(Pieterse and Todorov, 2010)**. Bacteriocins can be seen as an alternative and provide some advantages over traditional antibiotic therapy **(Kulkarni and Kaliwal, 2013; Dos Santos et al., 2005)**.

Penicillin can be used for the treatment of *Staphylococcal* infection if the strain has not developed antibiotic resistance mechanism. An alternative may be ampicillin, but the choice of antibiotic depends on the type and severity of the infection **(Stewart and Costerton, 2001)**. Drug-resistant pattern can also determine the antibiotics to be used: Cefazolin, Cefuroxime, Vancomycin, Clindamycin and Rifampin have been used. Some strains such as MRSA are resistant to methicillin and several β-lactam antibiotics including Penicillin due to the production of Penicillinase which inactivates the antibiotic **(Boyce et al., 2005)**. The gene that is responsible for MRSA resistance is the *mecA* gene that encodes Penicillin binding protein 2 (PBP2a) **(Ehlert, 1999)**.

## 2.11 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The most commonly used methods of antimicrobial detection include broth microdulutio or rapid automated instrument methods that use commercially available products and equipment, as well as methods of disk diffusion **(Reller et al., 2009)**.

The reference methods recommended by the Clinical Laboratory Institute (CLSI) for detecting resistance in *Staphylococcus spp.* include the determination of minimum inhibitory concentrations (MICs) by method of Agar or broth dilution and by the disc diffusion method (**Wayne, 2015**).

There are also several automated systems available for the *Staphylococcus spp.* antimicrobial susceptibility test. The largest market is shared by two products: Vitek (BioMérieux, Marcy-l'Etoile, France) and Microscan (Beckman Coulter, California, United States). VITEK® 2 technology represents a smarter way to automate ID/AST testing. It provides rapid, automatic, standardised validation of every test result with next generation expert software. VITEK® 2 is a unique system that uses a

phenotypic expert system instead of commonly used rules-based expert systems which are incapable of recognizing unusual results (i.e. mixed cultures) and new resistance phenotypes for which no rules exist **(Winstanley et al., 2014)**.

## 2.11.1 BROTH DILUTION TESTS

The earliest tool for measuring antimicrobial resistance was the microbroth or tube di lution test (**Figure 2.9**) described on the study done by **Ericson and Sherris (1971)**. The procedure encompasses preparing the two-fold dilutions of antibiotics in a broth then dispensed in test tubes inoculated with standardized bacterial suspension of $1x10^5$ CFU/ml. The incubation occurs at 35°C and the tubes are visualised for potential bacterial growth shown by turbidity. The minimum inhibitory concentration (MIC) is represented by the lowest concentration of antibiotic that inhibited bacterial growth. The MIC results are quantitative, which serves as an advantage to the technique. But the technique also has its limitations in that it mostly involves manual preparations of antibiotics for each individual test which is work intensive. As an addition, errors may possibly occur during the preparations of antibiotic solutions **(Murray et al., 2007)**.

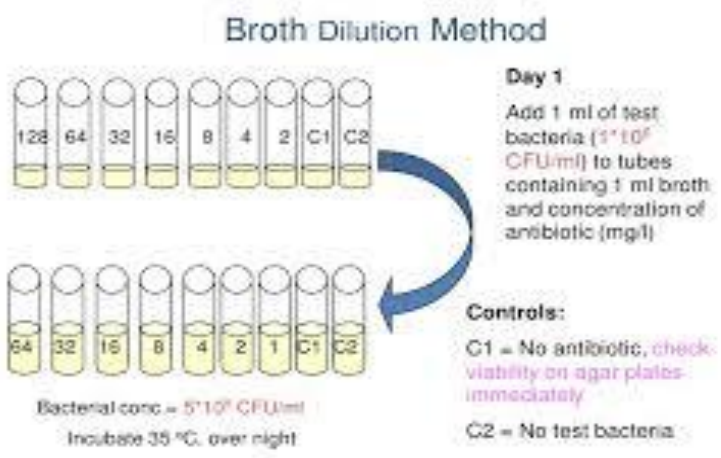



**Figure 2.9**: The Broth Dilution Method (online web)

## 2.11.2 ANTIMICROBIAL GRADIENT METHOD

The antimicrobial gradient method blends dilution methods theory with diffusion methods principle in order to determine the Minimum Inhibitory Concentrations (MIC)

value **(Varley et al., 2009)**. It is based on the possibility of creating the antimicrobial agent concetration gradient measured in the agar medium. The E-test (BioMérieux, Marcy-l'Etoile, France) is a commercial version of this technique. Briefly, a strip impregnated with an increasing concentration gradient of the antimicrobial agent from one end to the other is placed on the agar surface, previously inoculated with the microorganism to be tested. E-tests (**Figure 2.10**) are calibrated to give performance substantially equivalent to the US CLSI reference methods and the MIC. The BSAC comparison methods vary from CLSI, in particular with the test media and the MIC breakpoints used to measure susceptibility. It is more practical to use similar gradient test conditions as used by BSAC methods in routine tests **(Mushtaq et al., 2010)**.

**Figure 2.10**: Etest®/Biomerieux test (Adapted online)

## 3 DISK DIFFUSION TEST

Diffusion techniques were discovered in the same year the broth dilution was discovered. In the year 1959 Kirby–Bauer method was introduced. This method uses paper discs impregnated with various defined concentrations of different antibiotics to determine drug resistance. The impregnated discs are placed onto the surface of the agar. After incubation (16–24 h at 35 °C) zones of growth inhibition around each of the antibiotic discs are measured to the nearest millimetre (**Figure 2.11**). A clear circular zone of no growth in the immediate vicinity of a disc indicates susceptibility

to that antimicrobial. The size of the zone can be compared to the MIC using reference tables, and results can be reported as the organism is susceptible (S), intermediate (I) or resistant (R) **(Vijayakumar et al., 2016)**. This method has relatively low cost. In contrast its limitation is that it is not suitable for slow and anaerobically growing microorganisms. To add more, as this test relies on proper diffusion, the molecular weight of drug molecules is an important factor. Also, false results due to imperfections and unevenness of the agar plates if diffusion is possibly affected. The fact that the test only provides qualitative results and no quantitative MIC values, is a major drawback (**Mushtaq et al., 2010)**

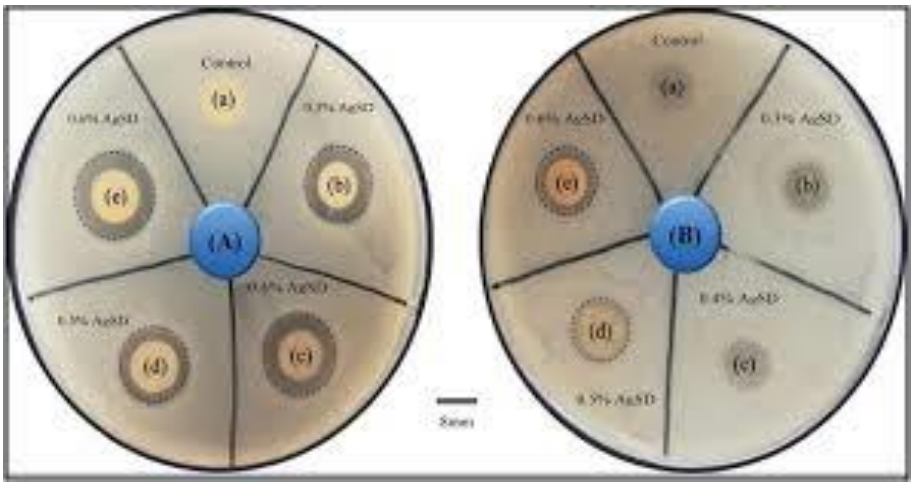



**Figure 2.11**: Disc Diffusion Method (Accessed from online web)

## 2.12 POLYMERASE CHAIN REACTION (PCR) METHOD

### 2.12.1 MULTIPLEX PCR

Multiplex PCR (m-PCR) is the simultaneous detection of multiple genome targets in a single reaction using different primers specific for each target. A m-PCR protocol was designed by **Rocchetti (2014)** for direct detection of a wide range *of Staphylococcus spp.* on blood cultures. The method was found to be sensitive, specific, and fast, and showed good agreement with the phenotypic results **(De Souza, 2018)**. The principle involves the primer mediated DNA amplification based on the ability of DNA polymerase to synthesize a new strand of DNA complementary to the template strand **(Demidov, 2002)**. Amplification cannot occur without primers because they are required for DNA polymerase to add a nucleotide

to their pre-existing 3' OH group. More nucleotides are added when DNA polymerase elongate its 3' end to generate an extended region of the double stranded DNA **(Demidov, 2002)**.

### 2.12.2 REAL-TIME PCR

Real-time PCR has been used to classify fastidious pathogens which were historically examined by conventional PCR. As the technique is now more widely available and more user friendly, it has been applied to investigating commonly found bacteria like *S. aureus (***De Souza, 2018).** Studies have showed that real-time PCR is an effective and rapid tool for differentiating *S. aureus* from other coagulase positive *Staphylococci* and for identifying negative coagulase *Staphylococci* **(Skow et al., 2005)**.

### 2.12.3 SPA LOCUS TYPING

The spa typing approach is based on the sequencing of the protein A gene (spa) polymorphic X region present in all strains of *S. aureus* **(Hallin et al., 2009)**. This technique integrates sequence knowledge from several household genes to compare strains close to the multilocus enzyme electrophoresis (MLEE) **(Shopsin et al., 1999).**

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 ETHICAL CLEARANCE

This study was approved by University of Venda Research and Ethics Committee. Ethical clearance was obtained **(SMNS/18/MBY/32/0712)** and permission to conduct the study within the farm premises was sought from the Farm managers/owners and a written consent form was granted **(Appendices A).**

## 3.2 STUDY SITE AND POPULATION

The study was conducted in the Limpopo dairy processing company in Madombidzha, a few kilometres from Makhado. It is the biggest enterprise in the Limpopo Province that has tons of dairy products supplied across the Province as well as to the neighbouring countries like Zimbabwe. The farm is situated about 80 km from the University of Venda in Thohoyandou (**Figure 3.1**). There are 2 operational divisions, manufacturing and production of dairy products as well as fruit beverages with a production capacity of approximately 50 000 litres of products per day.


**Figure 3.1:** Geographical location of Vhembe district showing the area of the study (**Vhembe District profile, 2017**)

The Limpopo dairy's herd consist of more than 1000 dairy cattle which are milked daily divided in 3 shifts (morning shift, afternoon shift and evening shift). Two different breeds are milked, 3\4 Holstein and 1\4 Jersey breed. The study population is the lactating cows with or without observable signs and symptoms of mastitis.

## 3.3 SURVEY DATA COLLECTION

Data regarding different potential risk were obtained from the farm records and captured on to the questionnaires **(Appendices B)**. The purpose of the questionnaire was to generate basic information on herd management system, nutrition, hygienic practices, disease detection, housing, and prevention, and control actions.

## 3.4 PHYSICAL EXAMINATION

For physical detection of mastitis, signs observed were clots and blood in milk, as well as the cow's temperature. The cow udder's were examined by visual examination and palpation to detect possible fibrosis, cardinal signs of inflammation, visible injury, tick infestation and swelling of the supramammary lymph nodes. During examination, attention was given to inflammatory signs, the size and consistency of the udder quarters as described by **Radostits et al. (1994)**. Examination of milk for somatic cells was done as described by **Philpot and Nickerson (1999).** The cows that were diagnosed with mastitis were given antibiotic treatment if were on state severe state.

## 3.5 SAMPLE COLLECTION

Sample collection was done on lactating cows, except those having received antibiotics in the 3 days before sampling. The sample collection was done as per **National Mastitis Council (NMC) instructions (2006)** prior to routine milking. To reduce contamination of the teat ends during sample collection, the teats close to the personnel were sampled. Approximately 10 ml of milk was collected into a sterile screw capped tubes after udder preparation by farm personnel.

The samples were then transported in an iced cooler to the microbiology laboratory at University of Venda where they were stored at 4° C for a maximum of 24 hours until inoculation on a standard bacteriological media.

## 3.6 SAMPLE SCREENING BY CARLIFONIA MASTITIS TEST

Screening for clinical and sub-clinical mastitis was done using California mastitis test (CMT) as described by **NMC (1990), Quinn et al. (2002)** and **Zeryehun and Abera (2017).** Briefly, the udder was washed with water and antiseptics and dried with clean paper towel. Two millilitre (2 ml) of milk was drawn into the beaker and an estimated equal volume of California mastitis test reagent (4% Sodium hydroxide (NaOH) in distilled water and 1% bromothymol blue) was put in the 4 cups of the CMT paddle. Equal amount of milk from the respective teats of the cow was added and gently mixed by rotating the paddle in a horizontal plane for 10 seconds. The reaction developed almost immediately with milk containing a high concentration of somatic cells. The peak of reaction was obtained within 10 seconds and test results were scored. The results were read as per manufactures recommendation and were scored based on the amount of thickness of gel formed as described by **Hoque et al. (2015)**. The CMT results were scored as 0 (negative), 1 trace, 2 (weak positive) and 3 (strong positive) based on gel formation. The score of 1 and 2 were considered indicators of subclinical mastitis and 3 for clinical mastitis. Cows were considered positive for CMT, when at least one-quarter turned out positive. A herd was considered positive for CMT, when at least one cow in a herd is tested positive with CMT. The total number of blind teats as well as those with clinical infection was subtracted from the total number of teats and the difference was used to calculate the prevalence of subclinical mastitis

## 3.7 MICROBIAL ANALYSIS

### 3.7.1 BACTERIAL ISOLATION

The collected milk samples, upon arrival in the laboratory were shaken and inoculated directly onto the different media without enrichment. Bacterial isolation was done as described by **Zeryehun and Abera (2017)**. About 200 µl of milk sample

was plated on Mannitol salt agar (MSA) and MacConkey agar (MAC) (Davies Diagnostics (Pty) Ltd, United Kingdom) and incubated at 37°C. The plates were checked for growth after 24 hours, 48 hours, and 72 hours to monitor the slow growing bacteria. The plates were examined for growth, morphological features, such as colony size, shape, and colour. Bacterial identification of bacteria on primary culture was made based on growth characteristics. Colonies that appeared yellow with zones on the media were presumed as *S. aureus* (mannitol fermenters) and coagulase negative *Staphylococcus spp.* produce small pink or red colonies with no colour change to the MSA medium. Isolates that appeared pink on MAC and colourless with halo were considered as *E. coli*. *E. coli* isolates were further confirmed by API biochemical tests and catalase and coagulase biochemical tests were used to confirm *S. aureus* isolates. Pure cultures were prepared through sub culturing and incubation on nutrient agar base (Davies Diagnostics (Pty) Ltd, United Kingdom) for further identification.

### 3.7.2 IDENTIFICATION OF *STAPHYLOCOCCUS SPP.* ISOLATES BY VITEK® 2 SYSTEM

For secondary identification of *Staphylococcus spp.* isolates, purified presumptive isolates were confirmed using VITEK® 2 Systems, software version 08.01 (BioMérieux, Marcy-l' Étoile, France) as described by **Layer et al. (2006)** with slight modification. The modification was that all strains stored at −80°C were subcultured overnight on Mueller hinton agar before blind testing. The procedures recommended by the manufacturer were strictly followed. Strains were taken out of the freezer, grown on Colombia agar with 5% sheep red blood cells for 16 to 24 h at 37°C, re-plated, and grown again for 16 to 24 hours at 37 37°C just before testing. Bacterial suspensions were prepared for both indentification cards by emulsifying bacterial isolates in 0.45% saline equal to a 0.5 McFarland turbidity standard with a VITEK® 2 instrument (DensiChek; BioMérieux, Marcy-l'Etoile, France) ( software version 4.01).

Seven different categories of results express specificity in the VITEK® 2 system: excellent identification, very good identification, good identification, appropriate identification. Each of these four groups only shows one identifying result) low discrimination (More than one identification results is obtained, whereupon the program suggest carrying out additional tests such as oxidase, hemolysis,

pigmentation indole and motility tests to obtain the correct identification), inconclusive identification and unidentified identification.

## 3.8 MOLECULAR CHARACTERIZATION OF THE ISOLATES

### 3.8.1 CHARACTERIZATION OF *STAPHYLOCCUS SPP*.

#### *I. DNA extraction of Staphylococcus spp.*

DNA samples were extracted from *Staphylococcus* isolates using boiling method as reported by **Englen and Kelley (2000)** with some modifications. About 6 colonies were picked from plates and emulsified into 2 ml brain heart infusion and left for incubation overnight at 37°C. About 500 µl of overnight culture was transferred to a new 2 ml tube and centrifuge at 13 000 rpm for 5 minutes. The supernatant was decanted from the tubes and the pellets re-suspended in 500 µl sterile distilled water, vortexed for 2 seconds and heated for 15 minutes at 100°C.

Subsequent cooling followed at -20 °C for 10 minutes followed by centrifugation at 13 0000 rpm for 5 minutes. The supernatant was transferred into a new micro centrifuge tube and stored at -20 °C till further use.

#### *DNA Amplification*

Isolated DNA was checked for purity and quantified by Nano drop 1000 UV-Vis spectrophotometer (Thermo Fischer Scientific, Waltham, Massachussets, US). Polymerase chain reaction (PCR) was done as described by **Asfour and Darwish (2011).** Briefly, a quadruple PCR assay targeting pairs of specific oligonucleotide primers as shown in **Table 3.1** was used in this study. The PCR reaction mixture (25 µl) comprised of 1 µl of F and R primers, 1 µl DNA sample, 12.5 µl (1x) of PCR master mix and 4.5 µl of nuclease-free water (Qiagen, Hilden, Germany) in a PCR tube. The amplification was carried out in a PT-100 Thermocycler. Reactin conditions. The reaction conditions were optimized at 94˚C for 4 minutes as initial denaturation, followed by 40 cycles (denaturation 94˚C for 60 seconds, annealing at 56˚C for 60 seconds and elongation at 72˚C for 60 seconds). Final extension step was set at 72˚C for 10 minutes. DNA isolated from *S. epidermidis* was used as positive control while nuclease free water was used as negative control. The amplicons were separated on a 1.5% agarose gel for 45 minutes at 80 V. The gel was then visualized under Floro transimullinator (UVITEC Limited, Cambridge,

United Kingdom). The sizes of amplicons were determined by comparison to a 100 bp molecular weight marker (Fermentas, Waltham, Massachusetts, United States).

**Table 3.1:** Primers that were used for amplification of genes encoding *Staphylococcus spp.* virulence factors.

| | Primer sequence | Amplicon Size (bp) | References |
|---|---|---|---|
| *Nuc* | nuc-1: 5'-GCGATTGATGGTGATACGGTT-3'<br>nuc-2 5'-AGCCAAGCCTTGACGAACTAAAGC-3' | 280 | **Johnson et al. (1991)** |
| *Mec A* | MecA1 5′-GTAGAAATGACTGAACGTCCGATAA-3′<br>MecA2 5′-CCAATTCCACATTGTTTCGGTCTAA-3′ | 310 | **McClure et al. (2006)** |
| *Pvl* | pvl-1 5′-ATGTCTGGACATGATCCAA-3′<br>pvl-2 5′-AACTATCTCTGCCATATGGT-3′ | 970 | **Ma et al. (2008)** |
| *Eta* | ETA-1 5′- CTA GTG CAT TTG TTA TTC AA-3′<br>ETA-2 5′-TGC ATT GAC ACC ATA GTA CT-3′ | 119 | **Kalorey (2007)** |

### 3.8.2 CHARACTERIZATION OF *E. COLI* STRAINS

#### *DNA extraction of E. coli isolates*

An in-house silica/guanidium thiocyanate method originally described by **Boom et al (2010)** and optimized by **Delair et al. (2017)** was used for DNA extraction. The reagents were purchased from Qiagen (Hilden, Germany**).** The modifications were (1) addition of 250 ml 100% ethanol to the lysis buffer to enhance binding of the DNA to the extraction matrix (celite), (2) addition of celite to the mixture and shaking in the rocking platform before washing steps for complete mixing and binding of DNA to the celite, (3) elution of DNA from the extraction matrix with 150 µl Qiagen water. The sample were stored at 4˚Cfor further analysis.

About 29 *E. coli* positive isolates were selected and inoculated into nutrient broth in eppendorf tubes and incubated overnight. From the overnight culture, 2 ml was aliquoted into 2 ml sterile Eppendorf tubes and centrifuged for 2 minutes at 13,000 rpm to pellet the cells and decant the supernatant. DNA was extracted following summarized steps in **Figure 3.2A and 3.2B**. The spin columns that were used were also prepared in-house following method done by **(Borodina et al., 2003; Delair et al., 2017)**.

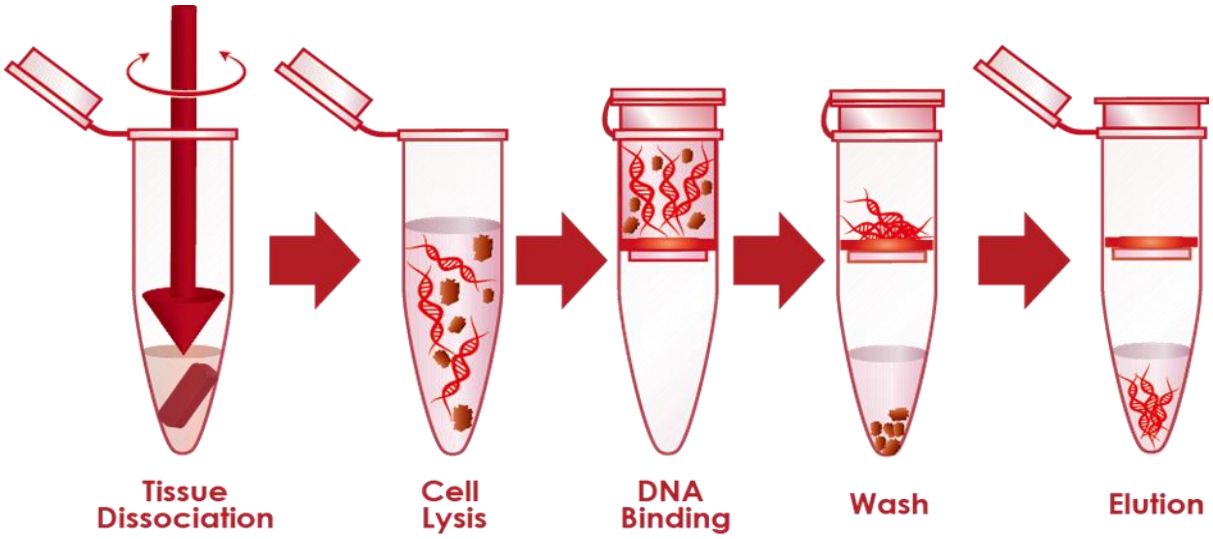



**Figure 3.2A:** Summary of DNA extraction (Taken online).

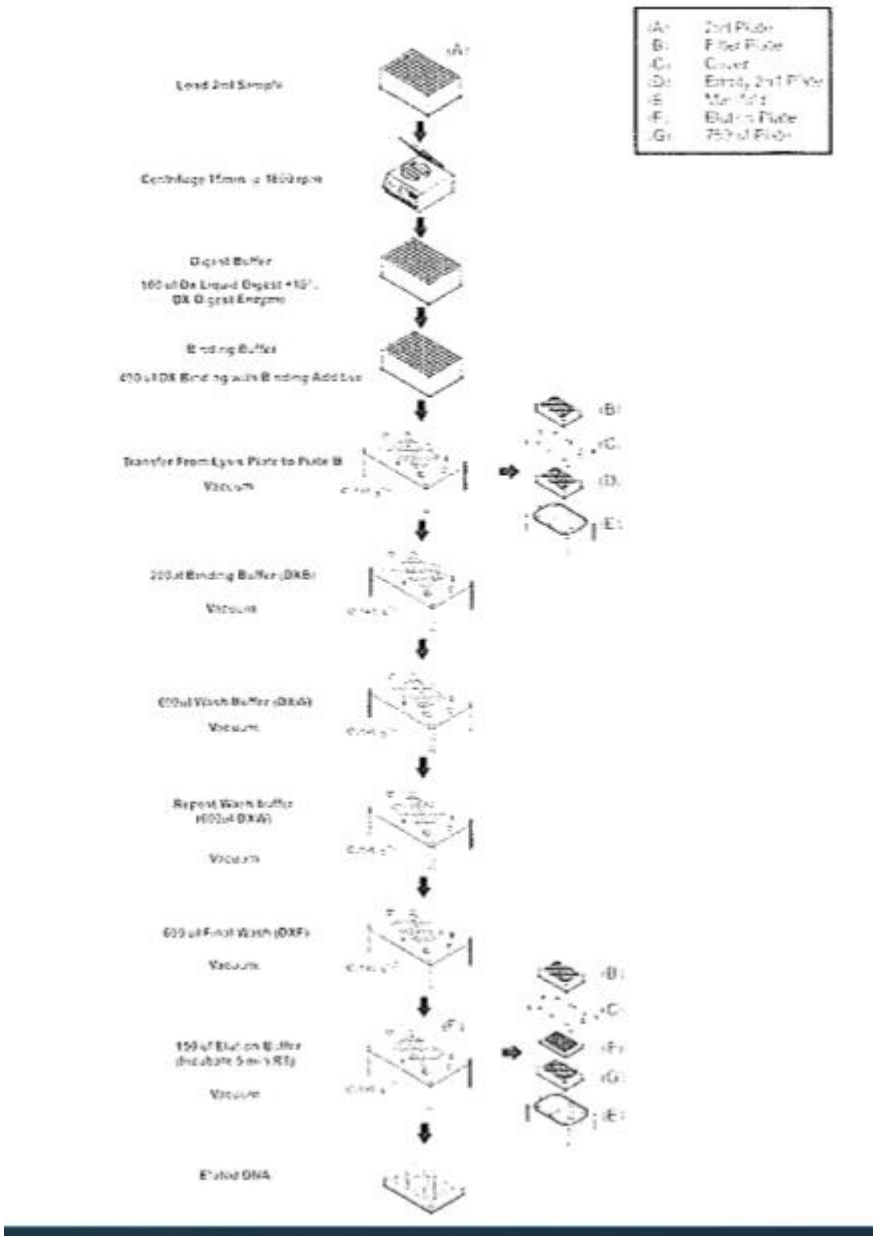


**Figure 3.2A: The 96 well plate modified Boom extraction protocol (Delair, 2017)**

### *Multiplex PCR*

The single step 11 gene multiplex polymerase chain reaction (m-PCR) was used as described by **Omar and Barnard 2014 (2010)**. The amplification targeted the 6 pathotypes of *E. coli* (**Table 3.2**). The house keeping *mdh* gene was used as an internal control for identification of the *E. coli* strains and an external control DNA derived from human glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) gene was also used as external control to determine if the PCR worked with no false positives. housekeeping gene as some environmental *E. coli* do not express the *mdh* gene. Each reaction consisted of 1x Qiagen PCR multiplex mix (containing HotstartTaq,

DNA polymerase, multiplex PCR buffer and dNTP mix), 2 µl primer mix for different *E. coli* pathotypes (Table 3.2), 2 µl sample DNA, 5 µl PCR grade water. Steps for different conditions were followed as described by **Omar and Barnard (2014).** The amplicons was analysed on a horizontal agarose slab gel [2.5 % (w/v)] with ethidium bromide (0.5 mg/mℓ) in Tris-acetate- Ethylene-diamine-tetraacetic acid (TAE) buffer (40 mM Tris acetate; 2 mM Ethylene-diamine-tetraacetic acid (EDTA), pH 8.3). Electrophoresis was performed for 1 to 2 h in electric field strength of 80 V; PCR products were visualised with UV light (UVITEC Limited, Cambridge, United Kingdom). This procedure was followed for all the experiments and the relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with that of the standards run with the samples on each gel using 100 bp markers (Fermentas, Waltham, Massachusetts, United States).

**Table 3.2:** Primers used in the m-PCR reaction to determine *E. coli* pathotypes (Omar and Barnard, 2014).

| Pathogen | Primer | Sequence (5´-3´) | Size (bp) | Conc (1 M) | Reference |
|---|---|---|---|---|---|
| *E. coli* | *Mdh (F)*<br>*Mdh (R)* | GGTATGGATCGTTCCGACCT<br>GGCAGAATGGTAACACCAGAGT | 304 | 0.1 | Tarr et al. (2002) |
| EIEC | *Ial (F)*<br>*Ial (R)* | GGTATGATGATGATGAGTCCA<br>GGAGGCCAACAATTATTTCC | 650 | 0,2 | Lopez-Saucedo et al. (2003) |
| EHEC/ Atypical EPEC | *EaeA (F)*<br>*eaeA (R)* | CTGAACGGCGATTACGCGA<br>CCAGACGATACGACGCAG | 917 | 0.3 | Aranda et al. (2004) |
| EHEC | *Stxl1 (F)*<br>*(R)*<br>*Stxl2 (F)*<br>*(R)* | ACACTGGATCTCAGTGG<br>CTGAATCCCCCTCCATTATG<br>CCATGACAACGGACAGCAGTT<br>CCTGTCAACTGAGCACTTTG | 779 | 0.5<br>0.3 | Moses et al. (2006) |
| EPEC | *Bfp (F)*<br>*(R)* | AATGGTGCTTGCGCTTGCTGC<br>TATTAACACCGTAGCCTTTCGCTGAAGTACCT | 410 | 0.3 | Aranda et al. (2004) |
| EAEC | *Eagg(F)*<br>*(R)* | AGACTCTGGCGAAAGACTGTATC<br>ATGGCTGTCTGTAATAGATGAGAAC | 194 | 0.2 | Pass et al. (2000) |

| | | | | | |
|---|---|---|---|---|---|
| ETEC | *Lt1 (F)*<br>*(R)*<br>*Sta (F)*<br>*(R)* | GGCGACAGATTATACCGTGC<br>CGGTCTCTATATTCCCTGTT<br>TTTCCCCTCTTTTAGTCAGTCAACTG<br>GCCAGGATTACAACAAAGTTCACA | 360 | 0.1<br>0.5 | Pass et al. (2000) |
| *E. coli* toxin | *AstA (F)*<br>*(R)* | GCCATCAACACAGTATATCC<br>GAGTGACGGCTTTGTAGTC | 106 | 0.3 | Kimata et al. (2005) |
| External control | *Gapdh (F)*<br>*R)* | GAGTCAACGGATTTGGTCGT<br>TTGATTTTGGAGGGATCTCG | 238 | 0.3 | Mbene et al. (2006) |

**Note:** *mdh-Malate dehydrogenase, ial-invasion associated protein, astA-Arginine N-succinyltransferase, Bfp-bundle forming pili, gapdh-glyceraldehyde 3-phophate dehydrogenase*

## 3.9 ANTIBIOTIC SUSCEPTIBILITY TESTING

### 3.9.1 ANTIBIOTIC SUSCEPTIBILITY TESTING FOR IDENTIFIED AND CONFIRMED E. COLI ISOLATES

Antibiotic susceptibility profile of the isolated bacteria was tested against 6 selected antibiotics (**Table 3.3**). The antimicrobial agents tested were selected based on two factors: 1) the recommendation of NCLS and 2) the actual veterinary practice. The testing was done using the Kirby Bauer disk diffusion method as described by **Carey et al. (2010)** with some modification. Briefly, Fresh 18-24 hours old cultures was suspended into 5 ml of distilled water in a test tube and adjusted to meet 0.5 McFarland turbidity standards. Thereafter, bacterial suspension was evenly swabbed onto Mueller-Hinton agar plate (Merck, Kenilworth, New Jersey, United States) using a sterile swab. The plates were allowed to dry before placing the antibiotic discs. The discs were placed on the plates with the aid of sterile forceps leaving some space in between for zone of inhibition interpretation. The plates were then incubated at 37˚C for 18-24 hour. The plates were removed from the incubator and a ruler was used to measure the diameter of the zone of inhibition around the disks rounded to the nearest millimetre. Then results were classified as susceptible or resistant and intermediate results interpretation were taken as resistant.

**Table 3.3** Antibiotic selected for *E. coli* antibiotic susceptibility testing

| Antimicrobial class | Antimicrobial agent | S Diameter Zone | I Diameter Zone | R Diameter Zone |
|---|---|---|---|---|
| Penicillins | Ampicillin (10µg) | ≥14 | 12-13 | ≤11 |
| Nitrobenzenes | Chloramphenicol (10µg) | ≥16 | 13-15 | ≤12 |
| Macrolides | Erythromycin (10µg) | ≥21 | 15-20 | ≤14 |
| Sulphonamides | Trimethoprim-sulfamethoxazole (10µg) | ≥16 | 11-15 | ≤10 |
| Fluoroquinolones | Ciproflaxin (5 µg) | ≤21 | 16-20 | ≥15 |
| Synthetic quinolone | Nalixidic acid (30µg) | ≥19 | 14-18 | ≥13 |

**S-Suscdeptible I-intermediate, R-Resistant**

### 3.9.2 ANTIBIOTIC SUSCEPTIBILITY TESTING OF *STAPHYLOCOCCUS SPP*.

Antibiotic susceptibility test for *Staphylococcus spp.* were done using the VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) using software version 08.01 and AST-GP71 (*Staphylococci*) cards. Cefoxitin screening for Oxacillin resistance and susceptibility of the coagulase negative *Staphylococci* isolates were determined using the VITEK® GP67 card. The AST-GP71 cards contained two wells for Inducible Clindamycin resistance (ICR), one with 0.5 g/ml of Clindamycin and the other with a combination of 0.25 g/ml of Clindamycin and 0.5 g/ml of Erythromycin. The VITEK® 2 system was used as described according the manufacturer's instructions. Briefly, Identification cards were inoculated with microorganism suspensions prepared in a test tube and transfer into a special rack (cassette). The identification card was placed in the neighbouring slot. The filled cassette was placed into a vacuum chamber station and, the organism suspension forced through the transfer tube into micro-channels that fill all the test wells. Prior to loading into the machine for incubation at 36.5ºC, the inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card. Data (not provided) for each incubated card were collected at 15-minute intervals during the entire incubation period and recorded on Microsoft Excel for Windows 2010.

## 3.10 STATISTICAL ANALYSIS

Survey data were organized summarized and analysed using simple descriptive analysis from where Microsoft Excel for Windows 2010 were used and results were expressed as mean ± Standard error (SE). The mean between experimental conditions was compared by One-Way Analysis of Variance (ANOVA) while Pearson correlation was used for correlation analysis. All data analyses were performed using SPSS version 23.0.

# CHAPTER 4

# RESULTS AND DISCUSSION

Mastitis being a constant global challenge affecting dairy industry **(Reshi et al., 2015)**, is usually found to be caused by contagious *Staphylococcus spp*. and environmental *E. coli* strains **(Suojala et al., 2010; Ali et al., 2017)**. Given the rising incidence and public concerns about the infections caused by these etiological agents, especially those that harbour resistance genes, it is empirical to understand their prevalence, virulence and antibiotic resistance in order to provide viable control of their breakout. The main aim of this study was to characterize *Staphylococcus spp.* and *E. coli* from cases of bovine clinical and subclinical mastitis in a Limpopo dairy processing company (Limpopo province, South Africa). The results of the study showed that the farm practiced good farm management system with low mastitis burden. The microbiological results also showed that *E. coli* and *Staphylococcus spp.* were obtained from clinical and subclinical mastitis cases.

## 4.1 SURVEY DATA

Survey data was obtained through questionnaire generating basic information on herd management system, nutrition, hygienic practices, disease detection, housing, prevention and control measures.

### 4.1.1 HOUSING AND FEEDING PRACTICE

The results of the study showed that house design for cattle was concrete floor free stall and the passageway can be cleaned by flushing water after each milking session. It was observed that the bedding used for all the groups of cattle within the herd was sandy. All the cattle received their feeds of a total mixed ration produced within the site and monitored by an appointed veterinarian. The feeds contained balanced concentrations of raw materials, minerals and all required feed stuffs. The results of the survey showed that farm practice, feeding and management system were followed and contributed to low mastitis burden in the dairy farm.

Survey data relies mostly on the information provided by the respondents where the information provided may be limited or exaggerated. The respondent in this survey indicated that to ensure biosecurity, adult cows and heifers were not allowed to be bought from the outside source or breeders. They use pure exotic breeds (Holstein) and carefully select them in case of cross-breeding in the farm. This study reports on fair farm practices and management system with low disease burden. The low disease burden in our study could be supported by the fact that this particular farm in the study keeps Holstein exotic breed in a concrete free stall where the distribution of their total mixed ration (produced on site) is carried out. The livestock feeding system is a contributing factor of the herd health as well as the quality of the produce. Livestock in the study are fed local farm grown total mixed ration feed to ensure good and quality health that is monitored by an appointed veterinarian.

Furthermore, several studies have reported on higher incidence of clinical mastitis in tiestall than in free stall housing (**Kalmus et al., 2006**, **Gordon et al., 2013**). In tiestall farms, the main risk factors for clinical mastitis are reported to be teat injuries, short stalls and shortage of bedding material **(Kalmus et al., 2006).** Also, an increased frequency of lying down and rising may lead to increased risk of teat tramping, leading to increased clinical mastitis incidence **(Oltenacu et al., 1990).**

### 4.1.2 BIOSECURITY

The study observed that procurement of adult cows and cow heifers' from outside sources were not permitted and pure exotic dairy breeds (Holstein) were. If by any chance, there was consideration of buying adult cows from outside sources; sellers were asked about their somatic cell count and all historical records. The animals were verified for their health status following the herd health plan that included mastitis and other diseases. The health plan was compiled with the input of veterinary officer. For management of mastitis cases that occur within the farm, dry farm management was practiced, and the cows were quarantined. All disease cases and vaccination programmes (Bovine somatotropin (RBSI), Scougard and *E. coli*) that happen within the farm were kept on records. This study reports on fair farm practices and management system with low disease burden. The results of our study is contrary to a survey conducted by **Welay et al. (2018**) in Ethiopia with remarkably

poor livestock management and high burden of disease. A survey done by **Katsande et al. (2013)** in Zimbabwe reported that farmers predominantly use cross breed in dairy farming which are most likely to be positive for mastitis compared to the local indigenous breeds.

## 4.2 PREVALENCE OF MASTITIS USING CARLIFONIA MASTITIS TEST

A total of 253 clinical and subclinical samples were collected from March to October 2019 and the prevalence of mastitis was determined using California mastitis test. The results are presented in **Table 4.1**. The overall prevalence of mastitis was found to be 94/253 (37.6%) and it was higher during winter 58/100 (58%) compared to summer 24/153 (24%).

**Table 4.1:** Table showing prevalence of overall mastitis using California mastitis test (n-253)

| Samples | Overall mastitis |
|---|---|
| **Summer seasons (n=153)** | n=36 (24%) |
| **Winter seasons (n=100)** | n=58 (58%) |
| **Total (n=253)** | n=94 (37%) |

**Note: n=number of samples**

The results of the prevalence of mastitis were found to be 37% and these results are in agreement with a study done by **Koivula et al. (2007)**. The results of this study were higher to the 7.4% recorded in 2018 (Limpopo Dairy farm records). These results are high because the study analysed both subclinical and clinical mastitic milk samples whereas farm records showed only clinical mastitis cases. The possibility of high prevalence has been attributed to the inadequate post-milking teat dipping with disinfectant, antibiotic treatment or dry cow therapy **(Iraguha et al., 2015)**. The high mastitis prevalence was observed from both clinical and subclinical mastitis in cold season than hot season. This is in agreement with another study conducted by **Iraguha et al. (2015)** where high prevalence was also observed in dry season (cold season).

The prevalence of cow-level mastitis reported in many studies across the African continent ranges from 8 to 64% **(Abebe et al., 2016)**. The results of our study are contrary to reported data from a study conducted by **Mdegela et al. (2009)** showing a 51.6% prevalence in Tanzania and a study conducted by **Abebe et al. (2016)** in Ethiopia which recorded 62.6% in prevalence. However, the results of our study were different to results reported by **Plozza et al. (2011); Tripura et al. (2014)** and **Gianneechini et al. (2002)** who respectively reported on prevalence of 49.5% in South Wales, 51.8% in Bangladesh and 52.4% in Uruguay. In addition, a higher pooled prevalence rate of 68% (sub-clinical and clinical mastitis) was recorded in India **(Krishnamoorthy et al., 2017)**. The low prevalence in our study could be accounted by the breed kept within the farm and the farm management practice.

## 4.3 MICROBIAL ANALYSIS

### 4.3.1 Prevalence of pathogens by culture methods

Samples that tested positive for mastitis using California mastitis test were cultured on selective media (MacConkey and Mannitol salt agar) for isolation of *E. coli* and *Staphylococcus spp.* Out of 94 samples, 48 (51%) tested positive for *Staphylococcus spp.* and 32 (34%) were positive for *E. coli.* Results are shown in **Table 4.2.**

**Table 4.2:** Prevalence of pathogens using culture method (n=94)

| Sample | *E. coli (%)* | *Staphylococcus (%)* |
|---|---|---|
| **Clinical (n=22)** | 13 (59) | 9 (41) |
| **Subclinical (n=72)** | 19 (26) | 39 (52) |
| **Total (n=94)** | 32 (34) | 48 (51) |

Note: Co-infection not recorded

The results of the study showed that *E. coli* or/and *Staphylococcus spp.* were detected and therefore may be responsible for mastitis. *E. coli* is the major pathogen causing environmental mastitis and it is one of the most important pathogens that has received more attention due to its high incidence relatively to other mastitis pathogens **(Castañeda et al., 2013)**. *E. coli* has been regarded as an indicator of faecal contamination in environmental samples. However, in the milk industry; it is

regarded as poor hygiene indicator and shows insufficient sanitary practices during milking **(Disassa et al., 2017)**. This study demonstrated the presence of *E. coli* in subclinical and clinical mastitic milk or cases. Our results were in agreement with a previous study conducted by **Elmonir et al. (2018)** in Egypt which reported 13.2%. Our results were also in agreement with studies done by **El-Razik et al. (2011)** and **Enany et al. (2012)** which reported the presence of *E. coli* in milk from mastitis cases.

The prevalence of *Staphylococcus spp*. from subclinical and clinical mastitis cases was found to be 51%. Our findings are highly comparable with findings of a study done in Iran by **Rahman et al. (2016)** which reported the presence of *Staphylococcus spp.* isolated from mastitis cases in sheep. Our results are also in agreement with data recorded by **Liu et al. (2018)** in China. This difference in prevalence may be due to the differences in the geographical distribution of pathogens, environmental and management conditions **(Schaumburg et al., 2014).**

### 4.3.2 IDENTIFICATION OF PRESUMPTIVE ISOLATES

#### *Identification of presumptive Staphylococcus spp.*

A total of 48 presumptive *Staphylococcus* isolates were randomly selected and subjected to automated VITEK® system for the identification of *Staphylococcus spp*. Results are presented in **Table 4.3**. Thirty isolates were confirmed to be *Staphylococcus spp.* [*Staphylococcus sciuri* (40%), *Staphylococcus xylosus (21%))* and *Staphylococcus caprae* (2%)] and 18 isolates were identified as *Enterococcus* and *Enterobacter spp*.

**Table 4.3:** Prevalence of *Staphylococcus spp.* isolated from milk (n=48)

| Pathogen | Positive strains | Percentage (%) |
|---|---|---|
| ***Staphylococcus sciuri*** | 19 | 40 |
| ***Staphylococcus caprae*** | 1 | 2 |
| ***Staphylococcus xylosus*** | 10 | 21 |
| **Other (*Enterococcus faecalis*, *Enterococcus gallinarium*, *Lactococcus garviae*, *Enterobacter cloacae*)** | 18 | 38 |

The results of this study supported the fact that coagulase negative *Staphylococcus spp.* play a prominent role in bovine mastitis and have recently been the global recognizable significant causative agent of bovine subclinical mastitis. Our results are in accordance with previous reports from Algeria where researchers found that Coagulase negative *Staphylococcus spp.* were the most common mastitis causing agents **(Heleili et al. (2012); Awale et al. (2012); Mamache et al. (2014); Pekana et al. (2015) Zaatout et al. (2019)]**. The distribution of coagulase negative species in mastitis cases is different among dairy farms or herds. This was supported by a recent study, in which *Staphylococcus Warneri*, *Staphylococcus epidermidis* and *Staphylococcus hyicus* (*S. warneri*, *S. epidermidis* and *S. hyicus) w*ere identified to be the dominant species among 18 coagulase negative species isolated from California mastitis positive cow milk **(Xu et al., 2015)**.

Although *Staphylococcus. xylosus* (*S. xylosus*) is not known to cause mastitis, it was detected in this study, supporting previous studies that showed that *S. xylosus* is an underestimated pathogenic Coagulase negative *Staphylococcus spp.* in bovine mastitis **(Frey et al., 2013)**. *Staphylococcus xylosus and Staphylococcus sciuri* (*S. sciuri)* affects the composition of milk as suggested by **Vasil et al. (2016)**. *Staphylococcus sciuri* is known as an ancestral species within the genus *Staphylococcus* and it has long being considered as a commensal species **(Nemeghaire et al., 2014)**.

Even though *Staphylococcus caprae (S. caprae)* has never been reported to be a cause of mastitis in cattle, this species has been reported in the current study in at least one sample. *S. caprae* is implicated in causing mastitis in goats (**d'Ersu et al., 2016**). In this study, dairy herd is mixed with goats and sheep's, and might have transferred from goats to cattle (own observation).

Our study also showed that various species of *Lactococcus* were associated with bovine mastitis (e.g., *Lactococcus lactis* and *Lactococcus garvieae*). It is speculated that the presence of *Lactococcus garvieae* in milk might have bactericidal effects against several bacteria including *Staphylococcus aureus (S.aureus) hence S. aureus was not detected* in our study.

### *Identification of E. coli strain using multiplex PCR (m-PCR)*

This study demonstrated the presence of *E. coli* in subclinical and clinical mastitiic milk or cases. The m-PCR assay that targeted 11 genes identifying 6 different *E. coli* virulent pathotypes was used for the detection and amplification of *E. coli* virulent genes. Results indicating the detected targeted genes are shown in **Figure 4.1**.

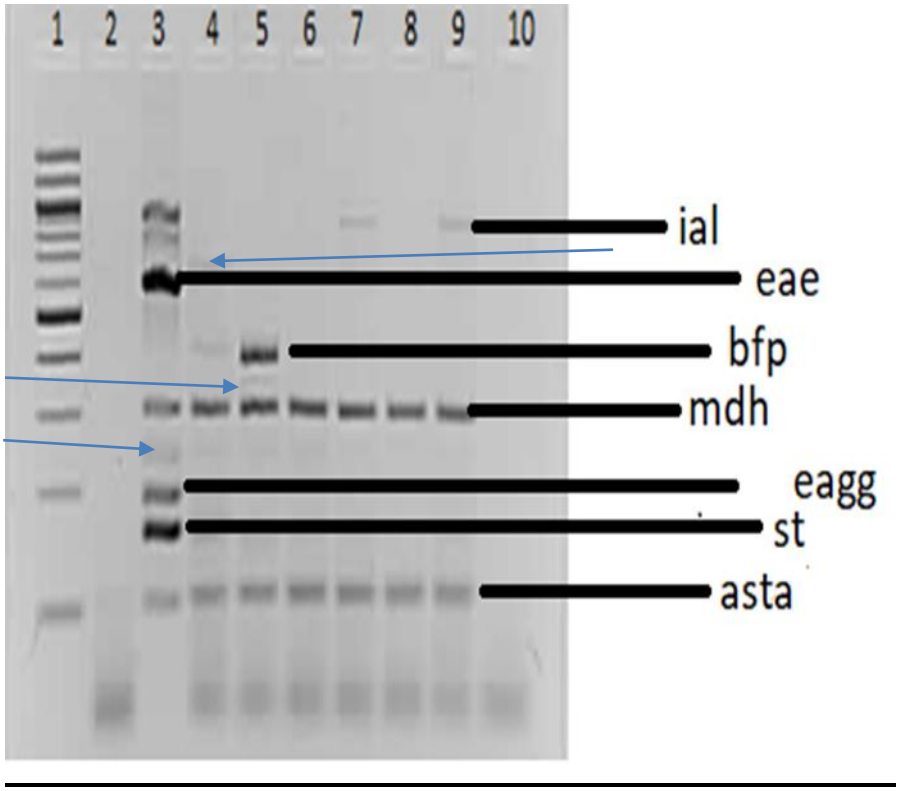



**Figure 4.1**: Agarose gel picture showing the target bands of interest.
Lane1: DNA ladder Lane2: -ve control Lane3: +ve control Lane4-9: Samples; Lane10: Ext -ve

Given the high diversity of *E. coli* species, *E. coli* isolates could include a large diversity of genetic backgrounds and various sets of virulence factors encoding for different traits determining pathogenicity **(Kempf et al., 2016).** Another objective of our study was to amplify and identify the specific virulence genes carried by *E. coli* isolates using conventional m-PCR. Virulence genes tested for in this study were

selected based on their association with *E. coli* strains causing diarrhoeagenic infections.

Out of 32 isolates, 29 were identified and confirmed to be *E. coli* using multiplex PCR. About 27 (93%) and 19 (66%) were positive for *astA* 1 gene (enteroaggregative *E. coli* heat-stable enterotoxin) and *st*a (heat stable toxin) respectively**.** This oi98gene is embedded in a putative transposase (ORF1) and presents polymorphism in diarrheagenic strains. The *Eae g*ene was detected in 35% of the isolates and *stx1* and *stx2*, *Ial* genes were detected in low frequency. Only 3 (10%) *E. coli* isolates were positive for single pathotype (ETEC) and 31% of the isolates carried a combination of atypical EPEC/ enteropathogenic *E. coli; enterotoxigenic E.coli/ enteroaggregative E. coli; and typical enteropathogeenic E.coli/ enterotoxigenic E. coli* (aEPEC/ ETEC; ETEC/EAEC and tEPEC/ ETEC). About 10 (35%) were not identified as virulent pathogens. The results showing the prevalence of virulence genes and pathotypes are shown in **Table 4.4 and 4.5.**

**Table 4.4:** Prevalence of *E. coli* virulence genes from selected presumptive *E. coli* isolates (N=29 isolates).

| Target genes | Number of occurrences (%) |
|---|---|
| *Mdh* (internal control) | 28 (97) |
| *Ial* | 3 (10) |
| *Stxl1* | 1 (3) |
| *Stxl2* | 3 (10) |
| *Bfp* | 6 (21) |
| *Eae* | 10 (35) |
| *Eagg* | 5 (17) |
| *Lt1* | 8 (28) |
| *Sta* | 19 (66) |
| *AstA* | 27 (93) |
| *Gapdh* (external control) | 7 (24) |

**Table 4.5:** Prevalence of pathotypes detected from selected presumptive *E. coli* isolates (n=29)

| Pathotype | Number (%) | Infection | Total (%) |
|---|---|---|---|
| **ETEC** | 3(10) | Single infection | 3(10) |
| **aEPEC/ ETEC** | 7(24) | Co-infections (2 pathotypes) | 9(31) |
| **ETEC/ EAEC** | 1 (4) | | |
| **tEPEC/ ETEC** | 1(4) | | |
| **aEPEC/ETEC/EAEC** | 2(7) | Multiple infections (3 pathotypes) | 5(17) |
| **tEPEC/ EIEC/ETEC** | 1(4) | | |
| **aEPEC/EHEC/ETEC** | 2(7) | | |
| **aEPEC/EHEC/ETEC/EAEC** | 1(4) | Multiple infections (4 pathotypes) | 2(7) |
| **aEPEC/EIEC/ETEC/EAEC** | 1(4) | | |
| ***E. coli* (undefined pathotype)** | 10(35) | | 10(35) |

Approximately all the isolates (90%) were confirmed to be *E. coli* by amplification of *mdh*-housekeeping gene and virulence genes such as Asta, st and eae, were detected together with other pathotypes . The presence/ detection of virulence genes is contradictory to a study that reported a lack of virulence genes in samples (**Kempf et al., 2016**). Another study reported that an approximate 30.3% of isolates did not show amplification of the examined genes which indicate that *E. coli* strains associated with mammary gland infections may use different mechanisms to cause diseases **(Blum et al., 2008)**.

The *asta* gene was highly detected or identified in this study (**Table 4.4).** The *asta* gene (*E. coli* toxin/ *enteroaggregative E. coli* heat-stable enterotoxin) is a toxin gene found in non pathogenic *E. coli* organism. As it is found in various pathotypes, it was also identified in EAEC as a structural gene that code for enterotoxin **(Huang et al., 2006).** In addition, **Soto et al (2009)** reported *asta* as an aggregative heat stable toxin 1 which does not have a clear development of an infection. High prevalence of *E. coli* strains carrying the *astA* gene has also been reported previously from commensal *E. coli* isolates in fresh water **(Masters et al., 2011)**. The presence of *astA* gene is in agreement with previous studies that reported wide distribution of

this gene among diarrheagenic *E. coli* isolates from humans and animals **(Sidhu et al., 2013)**.

The high prevalence of *E. coli* strains carrying *astA* toxin gene is a cause of concern since *E. coli* strains carrying *astA* toxin gene have been shown to cause diarrhoea in developed and developing countries and are carried by commensal *E. coli* strains **(Badugela et al., 2017 (unpublished data); Yatsuyanagi et al., 2003; Savarino et al., 1996).** The *eae* gene, which codes for intimin protein, was detected in 35% of the isolates. This gene is necessary for intimate attachment to host epithelial cells in both the EHEC and EPEC pathotypes which causes haemorrhagic colitis and haemolytic uremic syndrome in humans.

Our results also showed the presence of *Shigatoxin E. coli (*STEC) pathotype. *Shigatoxin E. coli (*STEC) pathotype causes mastitis in bovine and reduce milk quality for human consumption and raw milk from mastitic animals, mostly subclinical mastitis is the main resource for STEC. Many studies showed that the STEC strains are the most prevalent causative agent of milk-poisoning **(Argaw and Addis, 2015; Solomakos et al., 2009; Stephan et al., 2008)**.

The detection of *st* and *lt* gene which encodes for heat-labile and heat stable toxin in our results are not in agreement with data reported by **Caine et al. (2014)**, who reported only 13.5% in dairy farms in the Eastern Cape, (South Africa). The Enterotoxigenic *E. coli* (ETEC) pathotype causes infantile and travellers' diarrhoea in humans regardless of economic state of the countries since contaminated food such as milk and water are the main route of infections. The presence of ETEC in milk is not in compliance with the law in section 15(1), Act, No. 54 of 1972 in South Africa. The pathotype ETEC was the most prevalent pathotype than other pathotypes. In addition, this pathotype was found to be presented with other pathotypes creating multiple infections. Approximately 31% of the isolates carried a combination of atypical *Enteropathogenic Escherichia coli*/ Enteropathogenic E. coli; Enterotoxigenic E. coli/ Enteroaggregative E. coli and typical EPEC (aEPEC/ ETEC; ETEC/EAEC and tEPEC/ ETEC). These results are comparable with work done by **Sidhu et al. (2013)** who found approximately 9% of the isolates carried a combination of EPEC, EIEC, and EAEC virulence genes.

The presence of multiple virulence genes in *E. coli* strain is implicated in the pathogenicity of the organism (**Sarowska et al., 2019**). But the strain has to possess relevant combination of virulence genes that may cause infection by using complex multistep mechanism of pathogenesis involving a number of virulence factors depending upon the pathotype. Some of *E. coli* strains from our study had 3 and 4 multiple genes that are capable of causing HUS and diarrhoea among children and other infections in animals but not showing symptoms in animals such as cattle **(Huasai et al., 2012)**. This observation is of concern, as the presence of multiple genes in pathogens is known to cause more severe diarrhoea in humans.

### 4.3.3 ANTIMICROBIAL SUSCEPTIBILITY PATTERNS

#### *Antimicrobial susceptibility patterns of E. coli Isolates*

Antimicrobial susceptibility testing was done on isolates that were identified and confirmed to be *E. coli* using disc diffusion method. Diameters of the zone of inhibition were used to interpret results as susceptible, or resistant based on Clinical Laboratory Standards Institute (CLSI) standards. The *E. coli* isolates were highly resistant to >55% of selected antibiotics [Erythromycin (93); Nalixidic acid (86%) and Trimethoprim-sulfamethoxazole (86 %)] and susceptible to Chloramphenicol (69%) as well as Ampicillin (66%). The results are shown in **Table 4.6**.

**Table 4.6:** Antimicrobial susceptibility of *E. coli* against 6 selected antibiotics (%) (n=29)

| Antimicrobial class | Antimicrobial agent | S<br>Diameter<br>N (%) | R<br>Diameter<br>N (%) |
|---|---|---|---|
| Penicillins | Ampicillin (10µg) | ≥14<br>19 (66) | ≤11<br>10 (35) |
| Nitrobenzene | Chloramphenicol (10µg) | ≥16<br>20 (69) | ≤12<br>9 (31) |
| Macrolides | Erythromycin (10µg) | ≥21<br>2 (7) | ≤14<br>27 (93) |
| Sulphonamides | Trimethoprim-sulfamethoxazole (10µg) | ≥16<br>4 (14) | ≤10<br>25 (86) |

| | | | |
|---|---|---|---|
| Fluoroquinolones | Ciproflaxin (5 µg) | ≤21 | ≥15 |
| | | 15 (52) | 14 (48) |
| Synthetic quinolones | Nalixidic acid (30µg) | ≥19 | ≥13 |
| | | 4 (14) | 25 (86) |

Antimicrobial agents greatly serve as curative measures against bacterial infections, however, cumbersome detriments arise to animal producers and veterinary when antibiotic resistant bacteria affect therapy (**Bengtsson and Greko, 2014**). Resistance of antimicrobial agents arises from indiscriminate use in animals and human as well as the subsequent transfer of bacteria and resistant genes among animals, humans, animal products, and the environment (**WHO, 2018**). This improper use has contributed to the emergence of resistance, in hospitals, community and livestock settings **(Argudin et al., 2017)**. In our study, the highest resistance of *E. coli* to various antibiotics was observed against the first-line oral antimicrobial agents such as Eryththromycin (93%), Nalixidic acid (83%) and Trimethoprim-sulfamethoxazole (72%) and highly susceptible to Chloramphenicol (69%) and Ampicillin (66%).

Antibiotics reported in this study have also been reported by other researchers and there is evidence of increase in resistance to wide range of antibiotics in *E. coli* isolated from animals **(Jeykumar et al., 2013; Kalmus et al., 2011; Sumathi et al., 2008 and Dhakal et al., 2007)**. Our results demonstrated high resistance to ampicillin and sensitivity to Chloramphenicol and this is in accordance with data reported by **Ranjan et al. (2011)**; **Charaya et al. (2014)** and **Preethirani et al. (2015)** who also reported high susceptibility of *E. coli* isolates to Chloramphenicol, Gentamicin and Ciprofloxcin.

Isolates resistant to more than three antibacterial agents were defined as multidrug resistant. The frequency of multidrug resistant isolates was 8 (28%), 6 (20%) and 5 (17%) for more than 4, 6 and 5 antibiotics tested, respectively and results are shown in **Table 4.7.**

**Table 4.7:** Percentage frequency of occurrence of multidrug resistant *E. coli* from mastitis raw milk (n=29)

| **No. of Antibiotics** | **No. of multidrug resistant strains (%)** |
|---|---|

| | |
|---|---|
| **Two** | 3 (10) |
| **Three** | 6 (20) |
| **Four** | 8 (28) |
| **Five** | 5 (17) |
| **Six** | 2 (7) |
| **Total (29)** | 24 (83) |

No = Number

The multidrug resistance trait of *E. coli* is a cause of concern worldwide **(Kilani et al., 2017)**. Our results showed that isolates were resistant to more than one drug**.** These results are not in accordance with data reported by **Kibret and Abera, (2011)** and **Ibrahim et al. (2012)** who reported lower rate of multidrug resistance**.** The emergence of *E. coli* isolates with different MDR phenotypes has been previously reported and is considered a serious health concern **(Sukumaran et al., 2012)**.

Multidrug resistance is mainly linked to integrons, thus may have integrated multiple genes cassettes in their variable regions, and consequently provide a common promoter **(Kilani et al., 2017)**. These findings represent alarming increased rates in resistant *E. coli* to also Fluoroquinolones, which triggers acquisition of resistance **(Ibrahim et al., 2012; Namboodiri et al., 2011)** and has emerged as a cumbersome problem in both developed and developing countries.

### *Antibiotic resistance of Staphylococcus spp.*

To determine antibiotic susceptibility of *Staphylococcus spp.*, Isolates were subjected to an automated VITEK® 2 System. VITEK® 2 system is a widely used system for determining antimicrobial resistance patterns for clinical isolates such as methicillin resistant and methicillin susceptible *Staphylococcus spp.* **(Bobenchick et al., 2014)**.The MIC of *Staphylococcus spp.* was determined using the automated VITEK® system to evaluate antibiotic resistance and the MIC values of all isolates were recorded and interpreted as resistant or susceptible in **Table 4.8**.

**Table 4.8:** Minimum inhibitory concentration (MIC) of the 15 selected antibiotics for isolated *Staphylococcus spp.* strains on VITEK® 2 system (n= 30)

| Isolates (no) | CEF-S (R>4; S(-)) | | OX R>2;S ≤4 | | GM R>16 ;S≤4 | | CIP R>1;S ≤1 | | MOX R>1;S≤ 0.5 | | ICM R≥16;S ≤10 | | ERY R>2;S ≤1 | | TEL - | | CLI R>0.5;S≤ 0.25 | | LIN R>4;S ≤4 | | DAP R>1;S ≤1 | | TEI R>4;S ≤4 | | VA R>2;S ≤2 | | TET R>2;S ≤1 | | NIT R>64;S ≤64 | | FUA R>1;S ≤1 | | RIF R>0.5;S≤ 0.06 | | TM R>1;S ≤1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | I | MIC | |
| **MSA3b** | NEG | - | >=4 | R | ≤0.5 | S | 4 | R | ≥8 | R | NEG | - | ≥8 | R | 2 | I | 0.5 | S | ≥8 | | ≥8 | | 2 | S | 2 | S | 2 | S | 128 | R | 16 | R | 1 | S | 80 | R |
| **MSA24b`** | NEG | - | ≥8 | R | 2 | S | ≥8 | R | 2 | R | NEG | - | ≤0.25 | S | ≤0.25 | S | >=4 | R | 2 | S | ≥8 | | 4 | S | 2 | S | ≥16 | R | 32 | S | 1 | S | ≤0.5 | S | ≥320 | R |
| **MSA25a** | NEG | - | >=4 | R | ≤0.5 | S | 2 | I | 2 | R | NEG | - | 4 | I | 0.5 | S | >=4 | R | >=8 | | >=8 | | 4 | S | 1 | S | ≤1 | S | 32 | S | 8 | R | ≤0.5 | S | 40 | S |
| **MSA32b** | POS | + | >=4 | R | ≤0.5 | S | 2 | I | 2 | R | NEG | - | 4 | I | 0.5 | S | 0.5 | S | >=8 | | >=8 | | 2 | S | 1 | S | ≤1 | S | 64 | I | 8 | R | ≤0.5 | S | 40 | S |
| **MSA35a** | NEG | - | >=4 | R | 1 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | >=4 | R | >=8 | | >=8 | | 2 | S | 2 | S | 2 | S | 128 | R | >=32 | R | 1 | S | >=320 | R |
| **MSA37** | NEG | - | >=4 | R | ≤0.5 | S | 2 | I | 2 | R | NEG | - | >=8 | R | 2 | I | 0.5 | S | 2 | S | >=8 | | 2 | S | 2 | S | 2 | S | 64 | I | 16 | R | ≤0.5 | S | 80 | R |
| **MSA38a** | NEG | - | 2 | *S | ≤0.5 | S | 2 | I | ≤8 | R | NEG | - | 4 | I | 1 | S | 0.5 | S | 2 | S | 4 | | 2 | S | 2 | S | 2 | S | 64 | I | 8 | R | 1 | S | 40 | S |
| **MSA38b** | NEG | - | >=4 | R | ≤0.5 | S | 1 | S | 2 | R | NEG | - | ≤8 | R | 1 | S | 0.5 | S | >=8 | | >=8 | | 2 | S | 2 | S | >=1 | S | 32 | S | 8 | R | ≤0.5 | S | 20 | S |
| **MSA42b** | NEG | - | >=4 | R | 1 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | 1 | S | 0.5 | S | >=8 | | 1 | S | 1 | S | 1 | S | 2 | S | 64 | I | 8 | R | ≤0.5 | S | 160 | R |
| **MSABTF** | NEG | - | >=4 | R | ≤0.5 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | 2 | I | 0,5 | S | 2 | S | >=8 | | 2 | S | 2 | S | 2 | S | >=64 | I | 16 | R | ≤0,5 | S | 40 | S |
| **MSAB4a** | POS | + | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | >=4 | R | >=8 | R | >=8 | R | 16 | R | 8 | R | >=16 | R | 256 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB4b** | TRM | | >=4 | R | 2 | S | >=8 | R | >=8 | R | TRM | | >=8 | R | >=4 | R | TRM | | >=8 | | >=8 | | TRM | | 16 | | 2 | | >=512 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSA-B5b** | POS | + | >=4 | R | 1 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | 0.5 | S | 0.5 | S | >=8 | | >=8 | | 2 | S | 2 | S | ≤1 | S | 128 | R | 8 | R | ≤0,5 | S | 20 | S |
| **MSA-B6b** | TRM | | >=4 | R | 1 | S | >=8 | R | >=8 | R | TRM | | 4 | I | TRM | | TRM | | >=8 | | >=8 | | TRM | | 16 | | ≤1 | S | 256 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSAB7b** | POS | + | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0,5 | S | >=8 | | >=8 | | 16 | I | 8 | I | >=16 | R | 128 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB7c** | NEG | - | >=4 | R | 2 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | 2 | I | 0.5 | S | >=8 | | >=8 | | 2 | S | 2 | S | 2 | S | 128 | R | 8 | R | 1 | S | 20 | S |
| **MSAB9a** | POS | + | >=4 | R | ≤0.5 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | >=4 | R | >=8 | | >=8 | | 2 | S | 8 | I | >=16 | R | 128 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB11a** | NEG | - | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0,5 | S | >=8 | | >=8 | | 16 | I | 16 | I | >=16 | R | 256 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB12b** | TRM | | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | TRM | | >=4 | R | >=8 | | >=8 | | TRM | | 16 | I | 2 | S | >=512 | R | >=32 | R | 4 | R | 40 | S |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MSAB 13b** | NEG | - | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | TRM | | >=8 | >=8 | TRM | | 2 | S | 2 | S | 128 | R | 16 | R | 1 | S | 40 | S |
| **MSAB 14a** | TRM | | >=4 | R | 2 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | >=4 | R | >=8 | >=8 | 16 | I | 16 | I | 4 | S | 256 | R | >=32 | R | 8 | R | >=320 | R |
| **MSAB 14b** | TRM | | >=4 | R | 1 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | TRM | | TRM | | >=8 | >=8 | TRM | | 16 | I | 2 | S | 128 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSAB 15a** | POS | + | >=4 | R | 1 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0.5 | S | >=8 | >=8 | 16 | I | 8 | I | >=16 | R | 128 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB 16a** | TRM | | 2 | R | ≤0,5 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0.5 | S | >=8 | >=8 | 8 | S | 8 | I | 2 | S | 256 | R | >=32 | R | 1 | R | >=320 | R |
| **MSA17b** | POS | + | >=4 | R | 2 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | 2 | I | >=4 | | >=8 | >=8 | 2 | S | 2 | S | 2 | S | 128 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSAB 18a** | POS | + | >=4 | R | 2 | S | 4 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0.5 | S | >=8 | >=8 | 16 | I | 16 | I | >=16 | R | 128 | R | >=32 | R | ≤0,5 | S | >=320 | R |
| **MSAB 18b** | TRM | | >=4 | R | 2 | S | >=8 | R | >=8 | R | TRM | | >=8 | R | >=4 | R | TRM | | >=8 | >=8 | TRM | | 16 | I | 2 | S | >=512 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSAB 19a** | POS | + | >=4 | R | 1 | S | >=8 | R | >=8 | R | NEG | - | >=8 | R | >=4 | R | 0.5 | S | >=8 | >=8 | 8 | S | 8 | I | >=16 | R | 128 | R | >=32 | R | ≤0,5 | R | >=8 | R |
| **MSAB 19b** | TRM | | >=4 | R | 1 | S | 4 | R | >=8 | R | TRM | | >=8 | R | TRM | | TRM | | >=8 | >=8 | TRM | | 16 | I | 2 | S | >=520 | R | 16 | R | ≤0,5 | S | 20 | S |
| **MSAB 20b** | TRM | | >=4 | R | 1 | S | 4 | R | >=8 | R | TRM | | >=8 | R | >=4 | R | TRM | | >=8 | >=8 | TRM | | 16 | I | 2 | S | 128 | R | 16 | R | >=32 | R | 20 | S |

**Key**: CEF-S- Cefoxitin screen; OX- Oxacillin; GM- Gentamycin; CIP- Ciprofloxacin; MOX- Moxifloxacin; ICM- Inducible clindamycin resistance, ERY- Erythromycin; TEL- Telithromycin; CLI-Clindamycin; LIN- Linezolid, DAP- Daptomycin; TEI- Teicoplanin;VA- Vancomycin ; TET- Tetracycline, NIT- Nitrofurantoin; FUA- Fusidic acid; MUP- Mupirocin; RIF- Rifampicin; TM- Trimethoprim; MIC- minimum inhibitory concentration, R-Resistant; S-Susceptible ; TRM-Reaction terminated

Out of 30 *Staphylococcus* isolates, a total of 9 (30%) were positive for Cefoxitin screen and none were positive for Clindamycin inducible resistance as reported in **Table 4.9**.

**Table 4.9:** Methicillin and inducible clindamycin resistance by Cefoxitin screen and inducible clindamycin test (N=30)

| Antibiotic | Positive (%) | Negative (%) | TRM% |
|---|---|---|---|
| **Cefoxitin screen** | 9 (30) | 12 (40) | 9 (30) |
| **Inducible clindamycin** | 0 (0) | 25 (83) | 5 (0) |

TRM-Reaction terminated

Our study reported 30% resistance to Cefoxatin and this finding is not in accordance with a study conducted by **Ansari et al. (2014)** in Nepal who reported 43% resistance. Results of our study was not in alignment with findings reported by **Kumari et al. (2008)** who reported lower resistance of 26%. The MRSA prevalence in our study might have been due to the wide use of B-lactam antibiotics without specific laboratory tests. No Inducible clindamycin resistance was recorded in our study and our results are not contrary to that reported by **Prabhu and Rao, (2011)** and **Ciraj et al. (2009)** who reported prevalence of inducible clindamycin resistance of 13.1%. A total of 30 isolates were resistant to Moxifloxacin, Oxacillin, Ciproflacin, Erythrromycin and Fusidic acid. The isolated strains were also susceptible to Gentamycin, Tetracycline and Rifampicin as shown in **Table 4.10**.

**Table 4.10:** Percentages of resistance of isolates to tested antibiotics using automated VITEK® system (n= 30)

| Antimicrobial | MIC breakpoint (mg/L) | | |
|---|---|---|---|
| | **R** | ***I** | **S** |
| Oxacillin | >2<br>N=29 (97%) | -<br>N= 0 | 4<br>N=1 (3%) |
| Gentamycin | >16<br>N=0 | -<br>N= 0 | ≤4<br>N=30 (100%) |
| Ciprofloxacin | > 1<br>N=25 (83%) | -<br>N=1 (3%) | ≤1<br>N=4 (%) |
| Moxifloxacin | > 1<br>N=30 (100%) | 1<br>0 | ≤0.5<br>N=0 |
| Erythromycin | > 2<br>N=25 (83%) | 2<br>N=4 (13%) | ≤1<br>N=1 (3%) |
| Telithromycin | N=14 (47%) | N= 5 (17 %) | N=5 (17%) |
| Clindamycin | > 0.5<br>N= 7 (23%) | 0.5<br>0 | ≤0.25<br>N=15 (50%) |
| Teicoplanin | > 4<br>N=1 (3%) | -<br>N= 5 (17%) | ≤4<br>N=16 (53%) |
| Vancomycin | > 2<br>N= 1 (3%) | -<br>N=13 (43%) | ≤2<br>N=14 (47%) |
| Tetracycline | > 2<br>N=8 (27%) | 2<br>0 | ≤1<br>N=21 (70%) |
| Nitrofurantoin | > 64<br>N=22 (73%) | -<br>N=5 (17%) | ≤64<br>3 (10%) |
| Fusidic acid | > 1<br>N=29 (97%) | -<br>0 | ≤1<br>N=1 (3%) |
| Rifampicin | > 0.5<br>N=4 (13%) | 0.12 - 0.5<br>- | ≤0.06<br>N=26 (87%) |
| Trimethoprim | > 1<br>N=14 (47%) | -<br>0 | ≤1<br>N= 16 (53%) |

S-Susceptible, * I-Intermediate (regarded as Resistant), R-Resistant

Although antibiotic resistance is commonly linked to clinical studies, recent studies from different ecological niches revealed multidrug resistant bacteria is widespread in the environment but not much is known about the antibiotic resistance of *Staphylococci* isolated from different ecological niches **(Xu et al., 2018)**. In this study, the majority of *Staphylococci spp.* were highly resistant to Moxifloxacin, Oxacillin, Fusidic acid, Ciprofloxacin, Erythromycin and Nitrofurantoin. The current findings are contrary to findings reported by **Aqib et al. (2017),** who reported 100%

efficacy of Moxifloxacin, Ciprofloxicin and other antibiotic to *Staphylococcus aureus* recovered from buffaloes. **Ferreira et al. (2012)** reported high resistance to Oxacillin.

*Staphylococcus Sciuri (S. sciuri)* were more resistant to antibiotics than other *Staphylococcus spp.* recovered in this study. High resistance of 63% was observed on *S. sciuri* to Oxacillin, and Fusidic acid followed by Ciprofloxacin [18 (60%)]. Resistance was also observed for *S. xylosus* to Ciproflaxin, Moxifloxacin and Erythromycin all with 10 (33%). Results are shown in **Table 4.11**.

**Table 4.11:** Distribution frequency of resistance of isolates to tested antibiotics using automated VITEK® system (n= 30)

| **Antimicrobial** | ***S. sciuri*** | | ***S. xylosus*** | | ***S. caprae*** | | **TRM** |
|---|---|---|---|---|---|---|---|
| | R | S | R | S | R | S | |
| **Oxacillin** | 19 (63%) | - | 9 (30%) | 1(3%) | 1(3%) | - | - |
| **Gentamycin** | - | 19(63) | - | 9 (30%) | | 1(3%) | |
| **Ciprofloxacin** | 18 (60%) | 1(3%) | 10 (33%) | - | 1(3%) | - | - |
| **Moxifloxacin** | 19 (63%) | - | 10 (33%) | - | 1(3%) | - | - |
| **Erythromycin** | 19 (63%) | - | 10 (33%) | - | - | 1(3%) | - |
| **Telithromycin** | 10 (33%) | 5 (17%) | 9 (30%) | 1(3%) | - | 1(3%) | 4 (13%) |
| **Clindamycin** | 4 (13%) | 8 (27%) | 2 (7%) | 8 (27%) | 1(3%) | - | 7 (23%) |
| **Linezolid** | - | 3 (10%) | 1(3%) | 1(3%) | - | - | 25 (83%) |
| **Teicoplanin** | 1(3%) | 10 (33%) | 5 (17%) | 5 (17%) | - | 1(3%) | 8 (27%) |
| **Vancomycin** | 6 (20%) | 11 (37%) | 9 (30%) | 1(3%) | - | 1(3%) | 2 (7%) |
| **Tetracycline** | - | 19 (63%) | 8 (27%) | 2 (7%) | 1(3%) | - | - |
| **Nitrofurantoin** | 17 (57%) | 2 (7%) | 9 (30%) | 1(3%) | - | 1(3%) | - |
| **Fusidic acid** | 19 (63%) | - | 9 (30%) | 1(3%) | - | 1(3%) | - |
| **Rifampicin** | 2 (6%) | 17 (57%) | 2 (6%) | 8 (27%) | - | 1 (3%) | - |
| **Trimethoprim** | 4 (13%) | 15 (50%) | 7 (23%) | 3 (10%) | - | 1 (3%) | - |

S-Susceptible,* I-Intermediate (regarded as Resistant), R-Resistant , TRM-Reaction terminated

Multidrug resistant Coagulase negative species in non-healthcare associated environments is a disturbing finding. Our results showed that *S. sciuri* were prevalent and were resistant to Oxacillin and Cefoxatin and these may be due to over-expression of *MecA* gene. Our results are in agreement with the study conducted by **Frey et al. (2013)** and **Ferreira et al. (2003).** A recent study by **Rolo et al. (2017)** has shown that *S. sciuri* has developed Oxacillin resistance using a variety of mechanisms from diversification of the non-binding domain of native PBPs, change in the *mecA* promoter, which led to acquiring the SCC*mec* element and adaptation of the bacterial genetic background. The resistance exhibited by a large percentage of coagulase negative species to these routinely used antibiotics in treatment of *Staphylococcal* infections necessitates the search for newer and more effective antibiotics against this group of organisms **(Fowoyo and Ogunbanwo, 2017)**.

A total number of 22 isolates (73%) were multi-drug resistant and most strains were resistant to six drugs (4; 18%) followed by five (3; 14%) and nine (3; 14%)**.** Results are recorded in **Table 4.12**.

**Table 4.12:** Frequency of Multidrug resistant coagulase negative species from mastitic raw milk (**n=30**)

| No. of antibiotics tested | No. of multidrug resistant strains (%) |
|---|---|
| **Two** | 1 (3) |
| **Three** | 1 (3) |
| **Four** | 1 (3) |
| **Five** | 3 (14) |
| **Six** | 4 (18) |
| **Seven** | 3 (14) |
| **Eight** | 2 (9) |
| **Nine** | 3 (14) |
| **Ten** | 2 (9) |
| **Eleven** | 1(3) |
| **Twelve** | - |
| **Thirteen** | - |
| **Fourteen** | 1(3) |
| **Total=30** | **22(73)** |

High frequency of multidrug resistance can be accounted for by the fact that coagulase-negative *Staphylococci* are known to form biofilms, and this reduces the effect of antimicrobial agents against them **(John and Harvin, 2007)**. The emergence of Teicoplanin resistance among coagulase negative might be the result of selective pressure from the frequent use of vancomycin, however it was interesting to find that vancomycin did not exhibit high resistance like other study reports. Vancomycin can still be the reasonable choice for the treatment of severe infections due to multiple-resistant coagulase negative species **(Ma et al., 2011).**

# CHAPTER 5

# CONCLUSION AND RECOMMENDATIONS

## 5.1 CONCLUSION

The aim of this study was to characterise *Staphylococcus spp.* and *E. coli* isolated from milk obtained from subclinical and clinical mastitis cows. This was done through a combination of techniques including culture, isolation of strains, detection and characterization using a published Multiplex Polymerase Chain (m-PCR) reaction protocol and a semi-automated VITEK® 2 system.

The first objective was to evaluate the farm's management strategies and determine mastitis prevalence in the dairy farm using a questionnaire tool. The results of the survey showed that good farm practices, feeding and management system were followed and contributed to low mastitis burden in the dairy farm.

The second objective was to isolate and identify *staphylococcus spp. and E. coli* from clinical and subclinical cases of bovine mastitis using culture methods, biochemical and molecular biology tests. The results of the study showed that *E. coli* pathotypes and coagulase negative *Staphylococcus spp.* were detected and therefore may be responsible for mastitis.

The third objective was to amplify and identify the specific virulence genes carried by *E. coli* isolates using m-PCR. Virulence genes such as *astA*, *st*a and *eae*A, were detected and isolates carried a combination of aEPEC/ETEC; ETEC/EAEC and tEPEC/ETEC pathotypes.

The last objective was to determine the antibiotic susceptibility patterns of the isolates using Kirby Bauer disk diffusion method and automated VITEK® 2 system. . The *E. coli* isolates were highly resistant to Erythromycin; Nalixidic acid and Trimethoprim-sulfamethoxazole and susceptible to Chloramphenicol as well as Ampicillin. Most *Staphylococci* were highly resistant to Moxifloxacin, Oxacillin, Fusidic acid, Ciprofloxacin, Erythromycin and Nitrofurantoin. This may have future implications on the effective treatments of various infections in dairy cattle.

## 5.2 LIMITATIONS OF THE STUDY

Due to limited funds and lengthy administrative hurdles, this study was conducted in a single farm setting in the Makhado municipality of the Vhembe district. A multi-setting study within the region and beyond could have strengthened the findings. Virulence and antibiotic resistance genes were not screened in *Staphylococcus spp.* due to financial constraints.

## 5.3 RECOMMENDATIONS

The study recommends the following to various stake holders (institutional, provincial and farmers' association):

- ✓ The university should support such studies in order to strengthen the farm productivity and also improve communities within the District
- ✓ Encouraging farm workers to keep on practising good farm management in Limpopo dairy may keep mastitis rate at low and eventually have good control of the infection.
- ✓ The small-scale farmers are also encouraged to get education or awareness of implications of hygiene in their daily routine in order to abstain the public from unnecessary foodborne outbreaks.
- ✓ The presence of *E. coli* may indicate that hygienic practices are not up to standard, most especially during milking process and needs to be improved
- ✓ Improving sanitary conditions may reduce the burden of mastitis caused by diarrhegenic *E. coli* and *Staphylococcus spp.*
- ✓ The presence of pathogenic *E. coli* and *Staphylococcus spp.* in milk may pose health risks or problem. This report should be made available to municipality department dealing with public health to educate communities on the risk of consuming unprocessed contaminated milk
- ✓ The study also recommends routine studies that analyse or reports on management practices and implication of sanitary condition in facilities that produce dairy products
- ✓ For future studies, further analysis of both *E. coli* and *Staphylococcus spp.* to determine virulence and resistant genotyping in order to investigate possible mutations is recommended.

# REFERENCES

Abebe, R., Hatiya, H., Abera, M., Megersa, B. and Asmare, K., 2016. Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. *BioMed Central Veterinary Research*, *12*(1), p.270.

Aguilar, B., Amorena, B. and Iturralde, M., 2001. Effect of slime on adherence of *Staphylococcus aureus* isolated from bovine and ovine mastitis. *Veterinary microbiology*, *78*(2), pp.183-191.

Ajantha, G.S., Kulkarni, R.D., Shetty, J., Shubhada, C. and Jain, P., 2008. Phenotypic detection of inducible clindamycin resistance among Staphylococcus aureus isolates by using the lower limit of recommended inter-disk distance. *Indian Journal of Pathology and Microbiology*, *51*(3), p.376.

Ali, A., Mir, B.A., Bhat, R.R., Muzamil, S., Baba, O.K., Rashid, S.M., Hussain, I., Ahmad, S.B., Mir, M.U.R. and Mir, M.U.R., 2017. Current Advances in Genetic Resistance to Mastitis in Cattle and the development of sensors.

Alizade, H., Ghanbarpour, R. and Aflatoonian, M.R., 2014. Molecular study on diarrheagenic Escherichia coli pathotypes isolated from under 5 years old children in southeast of Iran. *Asian Pacific Journal of Tropical Disease*, *4*, pp.S813-S817.

Allore, H.G., Oltenacu, P.A. and Erb, H.N., 1997. Effects of season, herd size, and geographic region on the composition and quality of milk in the northeast. *Journal of Dairy Science*, *80*(11), pp.3040-3049.

Ammor, S., Tauveron, G., Dufour, E. and Chevallier, I., 2006. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1—Screening and characterization of the antibacterial compounds. *Food control*, *17*(6), pp.454-461.

Aqib, A.I., Ijaz, M., Hussain, R., Durrani, A.Z., Anjum, A.A., Rizwan, A., Sana, S., Farooqi, S.H. and Hussain, K., 2017. Identification of coagulase gene in Staphylococcus aureus isolates recovered from subclinical mastitis in camels. *Pak. Vet. J*, *37*(2), pp.160-164.

Aranda, K.R.S., Fagundes-Neto, U. and Scaletsky, I.C.A., 2004. Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic Escherichia coli and Shigella spp. *Journal of clinical microbiology*, *42*(12), pp.5849-5853.

Argaw, A., 2016. Review on epidemiology of clinical and subclinical mastitis on dairy cows. *Food Science Quallity Management*, *52*, pp.56-65.

Argaw, S. and Addis, M., 2015. A review on staphylococcal food poisoning. *Food Science and Quality Management*, *40*, pp.59-citation_lastpage.

Argudín, M.A., Deplano, A., Meghraoui, A., Dodémont, M., Heinrichs, A., Denis, O., Nonhoff, C. and Roisin, S., 2017. Bacteria from animals as a pool of antimicrobial resistance genes. *Antibiotics*, *6*(2), p.12.

Artursson, K., Nilsson-Öst, M. and Waller, K.P., 2010. An improved method to culture Staphylococcus aureus from bovine milk. *Journal of dairy science*, *93*(4), pp.1534-1538.

Asfaw, M. and Negash, A., 2017. Review on Impact of Bovine Mastitis in Dairy Production. *Advances in Biological Research*, *11*(3), pp.126-131.

Asfour, H.A.E. and Darwish, S.F., 2011. Phenotypic and genotypic detection of both mecA-and blaZ-genes mediated β-lactam resistance in Staphylococcus strains isolated from bovine mastitis. *Global veterinaria*, *6*(1), pp.39-50.

Åvall-Jääskeläinen, S., Taponen, S., Kant, R., Paulin, L., Blom, J., Palva, A. and Koort, J., 2018. Comparative genome analysis of 24 bovine-associated Staphylococcus isolates with special focus on the putative virulence genes. *Peer Journal*, *6*, p.e4560.

Awale, M.M., Dudhatra, G.B., Avinash, K., Chauhan, B.N., Kamani, D.R., Modi, C.M., Patel, H.B. and Mody, S.K., 2012. Bovine mastitis: a threat to economy. *Open Access Scientific Reports*, *1*(5), p.295.

Badugela Ndivhuwo, 2017. The microbiological quality and safety of milk produced by a dairy processing company in the Vhembe district. A mini-dissertation submitted in fulfillment for Bachelor of Science Honors Degree (BSc. Hons) in Microbiology, University of Venda.

Baillargeon, P. and LeBlanc, S.J., 2010. Clinical and economic effects of an internal teat sealant at dry-off on the incidence of clinical mastitis in early lactation. *Bovine Practitioner*, *44*(1), pp.1-11.

Becker, K., Heilmann, C. and Peters, G., 2014. Coagulase-negative *Staphylococci*. *Clinical microbiology reviews*, *27*(4), pp.870-926.

Bengtsson, B. and Greko, C., 2014. Antibiotic resistance—consequences for animal health, welfare, and food production. *Upsala journal of medical sciences*, *119*(2), pp.96-102.

Benić, M., Habrun, B. and Kompes, G., 2012. Clinical and epidemiological aspects of cow mastitis caused by Staphylococcus aureus and its methicillin-resistant

strains. *Rad Hrvatske akademije znanosti i umjetnosti: Medicinske znanosti*, 511(37), pp.113-121.

Bhatia, A. and Zahoor, S., 2007. Staphylococcus aureus enterotoxins: a review. *Journal of Clinical Diagnostic Resource*, *3*(1), pp.188-197.

Blignaut, D.J., 2015. Prevalence of mastitogenic pathogens in pasture and total mixed ration-based dairies during 2008 and 2013 (Doctoral dissertation, University of Pretoria).

Blowey, R.W. and Edmondson, P.W., 1995. Mastitis control in dairy herds. Ipswich.

Blum, S., Heller, E.D., Krifucks, O., Sela, S., Hammer-Muntz, O. and Leitner, G., 2008. Identification of a bovine mastitis Escherichia coli subset. *Veterinary microbiology*, *132*(1-2), pp.135-148.

Bobenchik, A.M., Hindler, J.A., Giltner, C.L., Saeki, S. and Humphries, R.M., 2014. Performance of VITEK® 2 for antimicrobial susceptibility testing of *Staphylococcus spp.* and *Enterococcus spp*. *Journal of clinical microbiology,* 52(2), pp.392-397.

Bogni, C., Odierno, L., Raspanti, C., Giraudo, J., Larriestra, A., Reinoso, E., Lasagno, M., Ferrari, M., Ducrós, E., Frigerio, C. and Bettera, S., 2011. War against mastitis: Current concepts on controlling bovine mastitis pathogens. *Science against microbial pathogens: Communicafing current research and technological advances*, pp.483-494.

Boom, R.C.J.A., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M. and Van der Noordaa, J.P.M.E., 1990. Rapid and simple method for purification of nucleic acids. *Journal of clinical microbiology*, *28*(3), pp.495-503.

Borodina, T.A., Lehrach, H. and Soldatov, A.V. 2003. DNA purification on homemade silica spin-columns. Analytical biochemistry, 321:135-137.

Boyce, J.M., Cookson, B., Christiansen, K., Hori, S., Vuopio-Varkila, J., Kocagöz, S., Öztop, A.Y., Vandenbroucke-Grauls, C.M., Harbarth, S. and Pittet, D. 2005. *Meticillin-resistant Staphylococcus aureus*. *The Lancet infectious diseases*, 5:653-663.

Bramley, A.J., 1991. Scientific summary of the Ghent Meeting 1990 [on mastitis]. *Vlaams Diergeneeskundig Tijdschrift (Belgium)*. Van Messom G., Hill A.W. (Eds.), New insights into the pathogenesis of mastitis*, Flemish Veterinary Journal*, pp. 235–238.

Branch-Elliman, W., Lee, G.M., Golen, T.H., Gold, H.S., Baldini, L.M. and Wright, S.B., 2013. Health and economic burden of post-partum Staphylococcus aureus breast abscess. *PLoS One*, *8*(9).

Burgos, J.M., Ellington, B.A. and Varela, M.F., 2005. Presence of multidrug-resistant enteric bacteria in dairy farm topsoil. *Journal of dairy science*, *88*(4), pp.1391-1398.

Butaye, P., Argudín, M.A. and Smith, T.C., 2016. Livestock-associated MRSA and its current evolution. *Current Clinical Microbiology Reports*, *3*(1), pp.19-31.

Caine, L.A., Nwodo, U.U., Okoh, A.I., Ndip, R.N. and Green, E., 2014. Occurrence of virulence genes associated with diarrheagenic Escherichia coli isolated from raw cow's milk from two commercial dairy farms in the Eastern Cape Province, South Africa. *International journal of environmental research and public health*, *11*(11), pp.11950-11963.

Carey, A.J., Duchon, J., Della-Latta, P. and Saiman, L., 2010. The epidemiology of methicillin-susceptible and methicillin-resistant Staphylococcus aureus in a neonatal intensive care unit, 2000–2007. *Journal of Perinatology*, *30*(2), pp.135-139.

Castañeda Vázquez, H., Jäger, S., Wolter, W., Zschöck, M., Vazquez, C. and El-Sayed, A., 2013. Isolation and identification of main mastitis pathogens in Mexico. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *65*(2), pp.377-382.Feng P, Weagent S, Grant M. (2002-09-01). " Enumeration of Escherichia Coli and the Coliform Bacteria. Bacteriological Analytical Manual (8th ed). FDA/ Center for Food Society & Applied Nutrition.

Cave, R., Misra, R., Chen, J., Wang, S. and Mkrtchyan, H.V., 2019. Whole genome sequencing revealed new molecular characteristics in multidrug resistant staphylococci recovered from high frequency touched surfaces in London. *Scientific reports*, *9*(1), pp.1-13.

Centers for Disease Control and Prevention (2005). *Enterotoxigenic Escherichiacoli (ETEC)*. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/etec_g.html. Accessed on 13 November 2015.

Chambers, H.F. and DeLeo, F.R., 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology*, *7*(9), pp.629-641.

Chandrasekaran, D., 2013. Evaluation of antibiotic resistant mastitis In dairy cows (Doctoral dissertation, Tamil Nadu Veterinary and Animal Sciences University).

Chapman, M.R., Robinson, L.S., Pinkner, J.S., Roth, R., Heuser, J., Hammar, M., Normark, S. and Hultgren, S.J., 2002. Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science*, *295*(5556), pp.851-855.

Charaya, G., Sharma, A., Kumar, A., Singh, M. and Goel, P., 2014. Pathogens isolated from clinical mastitis in Murrah buffaloes and their antibiogram. *Veterinary World*, *7*(11).

Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.I., Herrmann, M. and Preissner, K.T., 2002. Staphylococcus aureus extracellular

adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nature medicine*, *8*(7), pp.687-693.

Ciftci, A., Findik, A., Onuk, E.E. and Savasan, S., 2009. Detection of methicillin resistance and slime factor production of *Staphylococcus aureus* in bovine mastitis. *Brazilian Journal of Microbiology*, 40(2), pp.254-261.

Contreras, A., Sierra, D., Sánchez, A., Corrales, J.C., Marco, J.C., Paape, M.J. and Gonzalo, C., 2007. Mastitis in small ruminants. *Small Ruminant Research*, *68*(1-2), pp.145-153.

Cortés, C., De la Fuente, R., Blanco, J., Blanco, M., Blanco, J.E., Dhabi, G., Mora, A., Justel, P., Contreras, A., Sanchez, A. and Corrales, J.C., 2005. Serotypes, virulence genes and intimin types of verotoxin-producing Escherichia coli and enteropathogenic E. coli isolated from healthy dairy goats in Spain. *Veterinary microbiology*, *110*(1-2), pp.67-76.

Costa, A.R., Batistão, D.W., Ribas, R.M., Sousa, A.M., Pereira, M.O. and Botelho, C.M., 2013. *Staphylococcus aureus* virulence factors and disease. Microbial pathogens and strategies for combating them*: Science, Technology and education*, 1, pp.702-710.

Cowan, S.T. and Steel, K.J., 2004. Cowan and Steel's manual for the identification of medical bacteria. Cambridge university press.

Craven, R.R., Gao, X., Allen, I.C., Gris, D., Wardenburg, J.B., McElvania-TeKippe, E., Ting, J.P. and Duncan, J.A., 2009. Staphylococcus aureus α-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PloS one*, *4*(10).

Croxatto, A., Prod'hom, G. and Greub, G., 2012. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS microbiology reviews*, 36:380-407.

Cunha, M.L.R.S.; Sinzato, Y.K.; and Silveira; L.V.A. 2004. Comparison of methods for the identification of coagulase-negative *Staphylococci. Memórias do Instituto Oswaldo Cruz*, 99: 855-860.

De Souza, M.D.L.R., 2018. Methods for the Identification, Characterization, and Tracking the Spread of Staphylococcus aureus. *In Staphylococcus aureus*.

De Vliegher, S., Fox, L.K., Piepers, S., McDougall, S. and Barkema, H.W., 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. *Journal of dairy science*, *95*(3), pp.1025-1040.

Delair, Z., 2016. *Implementation of molecular methods for the detection and characterization of pathogenic Escherichia coli: industrial and routine monitoring applications* (Doctoral dissertation, University of Johannesburg).

Demidov, V.V., 2002. Rolling-circle amplification in DNA diagnostics: the power of simplicity. *Expert review of molecular diagnostics*, *2*(6), pp.542-548.

Dhakal, I.P., Dhakal, P., Koshihara, T. and Nagahata, H., 2007. Epidemiological and bacteriological survey of buffalo mastitis in Nepal. *Journal of Veterinary Medical Science*, *69*(12), pp.1241-1245.

Dieser, S.A., Vissio, C., Lasagno, M.C., Bogni, C.I., Larriestra, A.J. and Odierno, L.M., 2014. Prevalence of pathogens causing subclinical mastitis in Argentinean dairy herds. *Pak Vet J*, *34*(1), pp.124-126.

Dinges, M.M., Orwin, P.M., and Schlievert, P.M. 2000. Exotoxins of Staphylococcus aureus. Clinical microbiology reviews, 13:16-34.

Disassa, N., Sibhat, B., Mengistu, S., Muktar, Y. and Belina, D., 2017. Prevalence and antimicrobial susceptibility pattern of E. coli O157: H7 isolated from traditionally marketed raw cow milk in and around Asosa town, western Ethiopia. *Veterinary medicine international*, *2017*.

dos Santos Nascimento, J., Fagundes, P.C., de Paiva Brito, M.A.V., Dos Santos, K.R.N. and de Freire Bastos, M.D.C., 2005. Production of bacteriocins by coagulase-negative staphylococci involved in bovine mastitis. *Veterinary microbiology*, *106*(1-2), pp.61-71.

Dubois, D., Leyssene, D., Chacornac, J.P., Kostrzewa, M., Schmit, P.O., Talon, R., Bonnet, R. and Delmas, J., 2010. Identification of a variety of Staphylococcus species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of clinical microbiology*, 48:941-945.

Dyszel, J.L., Soares, J.A., Swearingen, M.C., Lindsay, A., Smith, J.N. and Ahmer, B.M., 2010. *E. coli* K-12 and EHEC genes regulated by SdiA. *PLoS One*, *5*(1).

Ehlert, K., 1999. Methicillin-resistance in Staphylococcus aureus-molecular basis, novel targets and antibiotic therapy. *Current pharmaceutical design*, *5*(2), pp.45-55.

Eley, A.R., 1992. Laboratory diagnosis. In *Microbial Food Poisoning* (pp. 107-124). Springer, Boston, MA.

Elmonir, W., Abo-Remela, E. and Sobeih, A., 2018. Public health risks of Escherichia coli and Staphylococcus aureus in raw bovine milk sold in informal markets in Egypt. *The Journal of Infection in Developing Countries*, *12*(07), pp.533-541

El-Razik, K.A.A., Abdelrahman, K.A., El-Moez, S.I.A. and Danial, E.N., 2011. New approach in diagnosis and treatment of Bovine Mycotic Mastitis in Egypt. *African Journal of Microbiology Research*, *5*(31), pp.5725-5732.

Enany, M.E., Said, A.M.H. and Aly, S.M., 2012. Molecular characterization of bacterial species isolated from clinical and subclinical mastitis in cattle. *Suez Canal Veterinary Medicine Journal*, *2*, pp.65-73.

Englen, M.D. and Kelley, L.C., 2000. A rapid DNA isolation procedure for the identification of Campylobacter jejuni by the polymerase chain reaction. *Letters in applied microbiology*, *31*(6), pp.421-426.

Ericsson, H.M. and Sherris, J.C., 1971. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta pathologica et microbiologica scandinavica*, (Suppl. 217).

Ferreira, A.M., Bonesso, M.F., Mondelli, A.L. and de Souza, M.D.L.R., 2012. Identification of Staphylococcus saprophyticus isolated from patients with urinary tract infection using a simple set of biochemical tests correlating with 16S–23S interspace region molecular weight patterns. *Journal of microbiological methods*, *91*(3), pp.406-411.

Ferreira, R.B., Iorio, N.L., Malvar, K.L., Nunes, A.P.F., Fonseca, L.S., Bastos, C.C. and Santos, K.R., 2003. Coagulase-negative staphylococci: comparison of phenotypic and genotypic oxacillin susceptibility tests and evaluation of the agar screening test by using different concentrations of oxacillin. Journal of clinical microbiology, 41(8), pp.3609-3614.

Fitzgerald, J.R. and Holden, M.T., 2016. Genomics of natural populations of Staphylococcus aureus. Annual review of microbiology, 70, pp.459-478.

Fitzgerald, J.R., 2012. Livestock-associated Staphylococcus aureus: origin, evolution and public health threat. *Trends in microbiology*, *20*(4), pp.192-198.

Foster, T.J. and Höök, M., 1998. Surface protein adhesins of *Staphylococcus aureus*. Trends in microbiology, 6(12), pp.484-488.

Foster, T.J., 2005. Immune evasion by staphylococci. *Nature reviews microbiology*, *3*(12), pp.948-958.

Fowoyo, P.T. and Ogunbanwo, S.T., 2017. Antimicrobial resistance in coagulase-negative *Staphylococci* from Nigerian traditional fermented foods. *Annals of clinical microbiology and antimicrobials*, *16*(1), p.4.

Fox, L.K. and Gay, J.M., 1993. Contagious mastitis. *Veterinary Clinics of North America: Food Animal Practice*, *9*(3), pp.475-487.

Frey, Y., Rodriguez, J.P., Thomann, A., Schwendener, S. and Perreten, V., 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. Journal of dairy science, 96(4), pp.2247-2257.

Gianneechini, R., Concha, C., Rivero, R., Delucci, I. and López, J.M., 2002. Occurrence of clinical and sub-clinical mastitis in dairy herds in the West Littoral Region in Uruguay. *Acta Veterinaria Scandinavica*, *43*(4), p.221.

Gomes, F. and Henriques, M., 2016. Control of bovine mastitis: old and recent therapeutic approaches. *Current microbiology*, *72*(4), pp.377-382.

Gomes, F., Saavedra, M.J. and Henriques, M., 2016. Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. *Pathogens and disease*, *74*(3).

González, R.N. and Wilson, D.J., 2003. Mycoplasmal mastitis in dairy herds. *Veterinary Clinics: Food Animal Practice*, *19*(1), pp.199-221.

Gooder, R., 2014. A Review of Mastitis Control Practices.

Gordon, P.F., Van den Borne, B.H., Reist, M., Kohler, S. and Doherr, M.G., 2013. Questionnaire-based study to assess the association between management practices and mastitis within tie-stall and free-stall dairy housing systems in Switzerland. *BMC veterinary research*, *9*(1), p.200.

Grundmann, H., Aires-de-Sousa, M., Boyce, J. and Tiemersma, E., 2006. Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. *The Lancet*, *368*(9538), pp.874-885.

Hallin, M., De Mendonça, R., Denis, O., Lefort, A., El Garch, F., Butaye, P., Hermans, K. and Struelens, M.J., 2011. Diversity of accessory genome of human and livestock-associated ST398 methicillin resistant Staphylococcus aureus strains. *Infection, genetics and evolution*, *11*(2), pp.290-299.

Hamadani, H., Khan, A.A., Banday, M.T., Ashraf, I., Handoo, N., Bashir, A. and Hamadani, A., 2013. Bovine mastitis-A disease of serious concern for dairy farmers. *Int J Livest Res*, *3*(1), pp.42-55.

Handler, M.Z. and Schwartz, R.A., 2014. Staphylococcal scalded skin syndrome: diagnosis and management in children and adults. Journal of the European Academy of Dermatology and Venereology, 28(11), pp.1418-1423.

Harris, L.G., Foster, S.J. and Richards, R.G., 2002. An introduction to Staphylococcus aureus, and techniques for identifying and quantifying S. aureus adhesins in relation to adhesion to biomaterials: review. Eur Cell Mater, 4(3), pp.39-60.

Heleili, N., Ayachi, A., Melizi, M., Kassah, A.L. and Mamache, B. (2012) Prevalence of Subclinical Bovine Mastitis and the in Vitro Sensitivity of Bacterial Isolates in Batna Governorate, East of Algeria. *Journal of Animal Science Advances*, 2, 576-582

Henton, M.M., Eagar, H.A., Swan, G.E. and Van Vuuren, M., 2011. Part VI. Antibiotic management and resistance in livestock production. *SAMJ: South African Medical Journal*, *101*(8), pp.583-586.

Hoque, M.N., Das, Z.C., Rahman, A.N.M.A., Haider, M.G. and Islam, M.A., 2018. Molecular characterization of Staphylococcus aureus strains in bovine mastitis milk in Bangladesh. *International journal of veterinary science and medicine*, *6*(1), pp.53-60.

Huang, D.B., Nataro, J.P., DuPont, H.L., Kamat, P.P., Mhatre, A.D., Okhuysen, P.C. and Chiang, T., 2006. Enteroaggregative Escherichia coli is a cause of acute diarrheal illness: a meta-analysis. *Clinical infectious diseases*, *43*(5), pp.556-563.

Huasai, S., Chen, A., Wang, C.J., Li, Y. and Tongrige, B., 2012. Occurrence and characteristics of virulence genes of Escherichia coli strains isolated from healthy dairy cows in Inner Mongolia, China. Brazilian Journal of Microbiology, 43(2), pp.528-534.

Hughes, K. and Watson, C.J., 2018. The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: A One Health focus. *Journal of mammary gland biology and neoplasia*, *23*(1-2), pp.27-41.

Ibrahim, M.E., Bilal, N.E. and Hamid, M.E., 2012. Increased multi-drug resistant Escherichia coli from hospitals in Khartoum state, Sudan. *African health sciences*, *12*(3), pp.368-375.

Iraguha, B., Hamudikuwanda, H. and Mushonga, B., 2015. Bovine mastitis prevalence and associated risk factors in dairy cows in Nyagatare District, Rwanda. *Journal of the South African Veterinary Association*, *86*(1), pp.1-6.

Islam, M.A., Islam, M.Z., Rahman, M.S. and Islam, M.T., 2011. Prevalence of subclinical mastitis in dairy cows in selected areas of Bangladesh. *Bangladesh Journal of Veterinary Medicine*, *9*(1), pp.73-78.

Islam, M.A., Kabir, S.M.L. and Seel, S.K., 2016. Molecular detection and characterization of Escherichia coli isolated from raw milk sold in different markets of Bangladesh. *Bangladesh Journal of Veterinary Medicine*, *14*(2), pp.271-275.

Jeykumar, M., Vinodkumar, G., Bashir, B.P. and Krovvidi, S., 2013. Antibiogram of mastitis pathogens in the milk of crossbred cows in Namakkal district, Tamil Nadu. *Veterinary World*, 6(6), pp.354-356

John, J.F. and Harvin, A.M., 2007. History and evolution of antibiotic resistance in coagulase-negative staphylococci: Susceptibility profiles of new anti-staphylococcal agents. *Therapeutics and clinical risk management*, *3*(6), p.1143.

Johnson, W.M., Tyler, S.D., Ewan, E.P., Ashton, F.E., Pollard, D.R. and Rozee, K.R., 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock

syndrome toxin 1 in Staphylococcus aureus by the polymerase chain reaction. *Journal of Clinical Microbiology*, *29*(3), pp.426-430.

Jones, G. M. 2006. Understanding the basics of mastitis. Virginia Cooperative Extension. *Virginia State University, USA*, 404, pp.1-7.

Jones, G.M. and Bailey, T.L., 2009. Understanding the basics of mastitis.

Joshi, L.R. and Devkota, S.P., 2014. Methicillin-Resistant Staphylococcus Aureus (MRSA) in Cattle: Epidemiology and Zoonotic Implications. *International Journal of Applied Sciences and Biotechnology*, *2*(1), pp.29-33.

Kadariya, J., Smith, T.C. and Thapaliya, D., 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *Biomedical research international*, *2014*.

Kalmus, P., Aasmäe, B., Kärssin, A., Orro, T. and Kask, K., 2011. Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. *Acta Veterinaria Scandinavica Journal*, *53*(1), p.4.

Kalmus, P., Viltrop, A., Aasmäe, B. and Kask, K., 2006. Occurrence of clinical mastitis in primiparous Estonian dairy cows in different housing conditions. *Acta Veterinaria Scandinavica*, *48*(1), p.21.

Kalorey, D.R., Shanmugam, Y., Kurkure, N.V., Chousalkar, K.K. and Barbuddhe, S.B., 2007. PCR-based detection of genes encoding virulence determinants in Staphylococcus aureus from bovine subclinical mastitis cases. *Journal of Veterinary Science*, *8*(2), pp.151-154.

Kaneko, J. and Kamio, Y., 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Bioscience, biotechnology, and biochemistry*, *68*(5), pp.981-1003.

Karzis, J., Petzer, I.M., Donkin, E.F. and Naidoo, V., 2018. Proactive udder health management in South Africa and monitoring of antibiotic resistance of Staphylococcus aureus in dairy herds from 2001 to 2010. *Journal of the South African Veterinary Association*, *89*(1), pp.1-8.

Kateete, D.P., Kimani, C.N., Katabazi, F.A., Okeng, A., Okee, M.S., Nanteza, A., Joloba, M.L. and Najjuka, F.C., 2010. Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of clinical microbiology and antimicrobials*, *9*(1), p.23.

Katsande, S., Matope, G., Ndengu, M. and Pfukenyi, D.M., 2013. Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. *Onderstepoort Journal of Veterinary Research*, *80*(1), pp.00-00.

Kempf, F., Slugocki, C., Blum, S.E., Leitner, G. and Germon, P., 2016. Genomic comparative study of bovine mastitis Escherichia coli. *PLoS One*, *11*(1), p.e0147954.

Khan, M.Z. and Khan, A., 2006. Basic facts of mastitis in dairy animals: a review. *Pakistan veterinary journal*, *26*(4), p.204.

Kibret, M. and Abera, B., 2011. Antimicrobial susceptibility patterns of *E. coli* from clinical sources in northeast Ethiopia. *African health sciences*, 11(3), pp.40-45.

Kilani, H., Abbassi, M.S., Ferjani, S., Salem, R.B., Mansouri, R., Chehida, N.B. and Boubaker, I.B.B., 2017. Diverse Escherichia coli pathovars of phylogroups B2 and D isolated from animals in Tunisia. The Journal of Infection in Developing Countries, 11(07), pp.549-556.

Kimata, K., Shima, T., Shimizu, M., Tanaka, D., Isobe, J., Gyobu, Y., Watahiki, M. and Nagai, Y., 2005. Rapid categorization of pathogenic Escherichia coli by multiplex PCR. *Microbiology and immunology*, *49*(6), pp.485-492.

Kisku, J.J. and Samad, M.A., 2013. Prevalence of sub-clinical mastitis in lactating buffaloes detected by comparative evaluation of indirect tests and bacteriological methods with antibiotic sensitivity profiles in Bangladesh. *Buffalo Bulletin*, *32*(4), pp.293-306.

Köck, R., Becker, K., Cookson, B., van Gemert-Pijnen, J.E., Harbarth, S., Kluytmans, J.A.J.W., Mielke, M., Peters, G., Skov, R.L., Struelens, M.J. and Tacconelli, E., 2010. Methicillin-resistant Staphylococcus aureus (MRSA): burden of disease and control challenges in Europe.

Koivula, M., Pitkälä, A., Pyörälä, S. and Mäntysaari, E.A., 2007. Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. Acta Agriculturae Scand Section A, 57(2), pp.89-96.

Krishnamoorthy, P., Suresh, K.P., Saha, S., Govindaraj, G., Shome, B.R. and Roy, P., 2017. Meta-analysis of prevalence of subclinical and clinical mastitis, major mastitis pathogens in dairy cattle in India.

Kulkarni, A.G. and Kaliwal, B.B., 2013. Bovine mastitis: a review. *International Journal of Recent Scientific Research*, *4*(5), pp.543-548.

Kurjogi, M.M. and Kaliwal, B.B., 2011. Prevalence and antimicrobial susceptibility of bacteria isolated from bovine mastitis. *Advanced Applied Science Research*, 2, pp.229-235.

Laarman, A.J., Ruyken, M., Malone, C.L., van Strijp, J.A., Horswill, A.R. and Rooijakkers, S.H., 2011. Staphylococcus aureus metalloprotease aureolysin cleaves complement C3 to mediate immune evasion. *The Journal of Immunology*, *186*(11), pp.6445-6453.

Lassen, B., 2012, *Dairy production in South Africa-impressions - Country Report 2012/1*, Agri Benchmark Dairy, viewed 07 June 2017, from http://www.agribenchmark.org/fileadmin/Dateiablage/B-Dairy/Country_Report/12_country_information_SA.pdf

Law, J.W.F., Ab Mutalib, N.S., Chan, K.G. and Lee, L.H., 2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in microbiology*, *5*, p.770.

Layer, F., Ghebremedhin, B., Moder, K.A., König, W. and König, B., 2006. Comparative study using various methods for identification of Staphylococcus species in clinical specimens. *Journal of clinical microbiology*, *44*(8), pp.2824-2830.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C., 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nature Reviews Microbiology*, *8*(3), pp.171-184.

Lee, D.W., Gwack, J. and Youn, S.K., 2012. Enteropathogenic Escherichia coli Outbreak and its Incubation Period: Is it Short or Long?. *Osong public health and research perspectives*, *3*(1), pp.43-47.

Lee, S.H.I., Mangolin, B.L.C., Gonçalves, J.L., Neeff, D.V., Silva, M.P., Cruz, A.G. and Oliveira, C.A.F., 2014. Biofilm-producing ability of Staphylococcus aureus isolates from Brazilian dairy farms. *Journal of dairy science*, *97*(3), pp.1812-1816.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. and Mariuzza, R., 2004. Standard nomenclature for the superantigens expressed by Staphylococcus. The Journal of infectious diseases, 189:2334-2336.

Linehan, D., Etienne, J. and Sheehan, D., 2003. Relationship between haemolytic and sphingomyelinase activities in a partially purified β-like toxin from Staphylococcus schleiferi. *FEMS Immunology & Medical Microbiology*, *36*(1-2), pp.95-102.

Liu, G., Ding, L., Han, B., Piepers, S., Naqvi, S.A., Barkema, H.W., Ali, T., De Vliegher, S., Xu, S. and Gao, J., 2018. Characteristics of Escherichia coli Isolated from Bovine Mastitis Exposed to Subminimum Inhibitory Concentrations of Cefalotin or Ceftazidime. *BioMed research international*, *2018*.

Liu, X., Liu, W., Zhang, Q., Tian, F., Wang, G., Zhang, H. and Chen, W., 2013. Screening of lactobacilli with antagonistic activity against enteroinvasive Escherichia coli. *Food Control*, *30*(2), pp.563-568.

López-Saucedo, C., Cerna, J.F., Villegas-Sepulveda, N., Thompson, R., Velazquez, F.R., Torres, J., Tarr, P.I. and Estrada-Garcia, T., 2003. Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic Escherichia coli. *Emerging infectious diseases*, *9*(1), p.127.

Lowy, F.D., 1998. *Staphylococcus aureus* infections. New England journal of medicine, 339(8), pp.520-532.

Lyon, G.J., Mayville, P., Muir, T.W. and Novick, R.P., 2000. Rational design of a global inhibitor of the virulence response in Staphylococcus aureus, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. *Proceedings of the National Academy of Sciences*, *97*(24), pp.13330-13335.

M. Thakker, J.S. Park, V. Carey, J.C. Lee *Staphylococcus aureus* serotype 5 capsular polysaccharide is anti-phagocytic and enhances bacterial virulence in a murine bacteremia model Infect. Immun., 66 (1998), pp. 5183-5189

Ma, X.X., Ito, T., Kondo, Y., Cho, M., Yoshizawa, Y., Kaneko, J., Katai, A., Higashiide, M., Li, S. and Hiramatsu, K., 2008. Two different Panton-Valentine leukocidin phage lineages predominate in Japan. *Journal of clinical microbiology*, *46*(10), pp.3246-3258.

Ma, X.X., Sun, D.D., Wang, S., Wang, M.L., Li, M., Shang, H., Wang, E.H. and Luo, E.J., 2011. Nasal carriage of methicillin-resistant Staphylococcus aureus among preclinical medical students: epidemiologic and molecular characteristics of methicillin-resistant S. aureus clones. *Diagnostic microbiology and infectious disease*, *70*(1), pp.22-30.

Mamache, B., Rabehi, S. and Meziane, T., 2014. Bacteriological study of subclinical mastitis in batna and setif governorates algeria. *J. Vet. Adv*, *4*(2), pp.364-373.

Masters, N., Wiegand, A., Ahmed, W. and Katouli, M., 2011. Escherichia coli virulence genes profile of surface waters as an indicator of water quality. *water research*, *45*(19), pp.6321-6333.

Mathanraj, S., Sujatha, S., Sivasangeetha, K. and Parija, S.C., 2009. Screening for methicillin-resistant Staphylococcus aureus carriers among patients and health care workers of a tertiary care hospital in south India. *Indian Journal of Medical Microbiology*, *27*(1), p.62.

Mathew, A.G., Cissell, R. and Liamthong, S., 2007. Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne pathogens and disease*, *4*(2), pp.115-133.

Mbene, A.B., Houreld, N.N. and Abrahamse, H., 2009. DNA damage after phototherapy in wounded fibroblast cells irradiated with 16 J/cm2. *Journal of Photochemistry and Photobiology B: Biology*, *94*(2), pp.131-137.

McClure, J.A., Conly, J.M., Lau, V., Elsayed, S., Louie, T., Hutchins, W. and Zhang, K., 2006. Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of

methicillin-susceptible from-resistant staphylococci. *Journal of clinical microbiology*, *44*(3), pp.1141-1144.

Mdegela, R.H., Ryoba, R., Karimuribo, E.D., Phiri, E.J., Løken, T., Reksen, O., Mtengeti, E. and Urio, N.A., 2009. Prevalence of clinical and subclinical mastitis and quality of milk on smallholder dairy farms in Tanzania. Journal of the South African Veterinary Association, 80(3), pp.163-168.

Mekibib, B., Furgasa, M., Abunna, F., Megersa, B. and Regassa, A., 2010. Bovine mastitis: Prevalence, risk factors and major pathogens in dairy farms of Holeta Town, Central Ethiopia. *Veterinary World*, *3*(9), pp.397-403.

Mellenberger, R. and Kirk, J.H., 2001. Mastitis Control Program for Staph. aureus Infected Dairy Cows. *Vetmed. Ucdavis. edu*.

Merriman, J.A., 2015. Secreted *Staphylococcus aureus* virulence factors and their role in chronic wound development and persistence.

Mohanty, N.N., Das, P., Pany, S.S., Sarangi, L.N., Ranabijuli, S., Panda, H.K., Univerisity, L. and Krishi Vigyan Kendra, S., 2013. Isolation and antibiogram of *Staphylococcus, Streptococcus and Escherichia coli* isolates from clinical and subclinical cases of bovine mastitis. *Veterinary World*, *6*(10), pp.739-743.

Montville, T.J. and Matthews, K.R., 2008. Staphylococcus aureus. *Food Microbiology: An Introduction*, *2*, pp.189-201.

Moses, A.E., Garbati, M.A., Egwu, A.O. and Ameh, E.J., 2006. Detection of E. coli 0157 and 026 serogroups in human immunodeficiency virus-infected patients with clinical manifestation of diarrhoea in Maiduguri, Nigeria. *Research Journal of Medicine and Medical Sciences*, *1*(4), pp.140-145.

Msolo, L., Igbinosa, E.O. and Okoh, A.I., 2016. Prevalence and antibiogram profiles of Escherichia coli O157: H7 isolates recovered from three selected dairy farms in the Eastern Cape Province, South Africa. *Asian Pacific Journal of Tropical Disease*, *6*(12), pp.990-995.

Murinda, S.E., Nguyen, L.T., Landers, T.L., Draughon, F.A., Mathew, A.G., Hogan, J.S., Smith, K.L., Hancock, D.D. and Oliver, S.P., 2004. Comparison of Escherichia coli isolates from humans, food, and farm and companion animals for presence of Shiga toxin–producing E. coli virulence markers. *Foodbourne Pathogens & Disease*, *1*(3), pp.178-184.

Murray, P.R. Baron, E.J. Jorgensen, J.H. Landry, M.L. Pfaller M.A. 2007 Manual of Clinical Microbiology, *American Society of Microbiology Press,* 1(9), Washington, DC

Mushtaq, S., Warner, M., Cloke, J., Afzal-Shah, M. and Livermore, D.M., 2010. Performance of the Oxoid MIC Evaluator™ Strips compared with the Etest® assay

and BSAC agar dilution. *Journal of antimicrobial chemotherapy*, *65*(8), pp.1702-1711.

Namboodiri, S.S., Opintan, J.A., Lijek, R.S., Newman, M.J. and Okeke, I.N., 2011. Quinolone resistance in Escherichia coli from Accra, Ghana. *BMC microbiology*, *11*(1), p.44.

Nataro, J.P. and Kaper, J.B., 1998. Diarrheagenic escherichia coli. *Clinical microbiology reviews*, *11*(1), pp.142-201.

National Mastitis Council. 2006. Dry Cow Therapy. NMC Fact Sheet. https://nmconline.org/drycow.html.

Ndyamukama, C.F., 2016. *Evaluation of microbial contamination in milk of healthy and mastitic cows in selected Districts in Tanzania* (Doctoral dissertation, Sokoine University of Agriculture).

Nemeghaire, S., Argudín, M.A., Fessler, A.T., Hauschild, T., Schwarz, S. and Butaye, P., 2014. The ecological importance of the Staphylococcus sciuri species group as a reservoir for resistance and virulence genes. *Veterinary microbiology*, *171*(3-4), pp.342-356.

Okhuysen, P.C. and DuPont, H.L., 2010. Enteroaggregative Escherichia coli (EAEC): a cause of acute and persistent diarrhea of worldwide importance.

Olesen, B., Neimann, J., Böttiger, B., Ethelberg, S., Schiellerup, P., Jensen, C., Helms, M., Scheutz, F., Olsen, K.E., Krogfelt, K. and Petersen, E., 2005. Etiology of diarrhea in young children in Denmark: a case-control study. *Journal of clinical microbiology*, *43*(8), pp.3636-3641.

Oliver, S.P., Jayarao, B.M. and Almeida, R.A., 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodbourne Pathogens & Disease*, *2*(2), pp.115-129.

Oltenacu, P.A., Bendixen, P.H., Vilson, B. and Ekesbo, I., 1990. Tramped teats--clinical mastitis disease complex in tied cows. Environmental risk factors and interrelationships with other diseases. *Acta veterinaria scandinavica*, *31*(4), pp.471-478.

Omar, O.K., Barnard, T. G. and Jagals, P. 2010. Development of a competitive PCR assay for the quantification of total Escherichia coli DNA in water. African Journal of Biotechnology, 9:564-572.

Orden, J.A., Cortés, C., Horcajo, P., De la Fuente, R., Blanco, J.E., Mora, A., López, C., Blanco, J., Contreras, A., Sánchez, A. and Corrales, J.C., 2008. A longitudinal study of verotoxin-producing Escherichia coli in two dairy goat herds. *Veterinary microbiology*, *132*(3-4), pp.428-434.

Pass, M.A., Odedra, R. and Batt, R.M., 2000. Multiplex PCRs for identification of Escherichia colivirulence genes. *Journal of clinical microbiology*, *38*(5), pp.2001-2004.

Pekana, A., 2015. Characterization of some virulence and antibiotic resistance genes of staphylococcus aureus isolated from cases of bovine mastitis in Nkonkobe Municipality, Eastern Cape Province, RSA (Doctoral dissertation, University of Fort Hare).

Pereira, U.P., Oliveira, D.G.S., Mesquita, L.R., Costa, G.M. and Pereira, L.J., 2011. Efficacy of Staphylococcus aureus vaccines for bovine mastitis: a systematic review. *Veterinary microbiology*, *148*(2-4), pp.117-124.

Petrovski, K.R., Buneski, G. and Trajcev, M., 2006. A review of the factors affecting the costs of bovine mastitis. *Journal of the South African Veterinary Association*, *77*(2), pp.52-60.

Petzer, I.M., Karzis, J., Watermeyer, J.C., Van der Schans, T.J. and Van Reenen, R., 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association*, *80*(1), pp.17-22.

Pieterse, R. and Todorov, S.D., 2010. Bacteriocins: exploring alternatives to antibiotics in mastitis treatment. *Brazilian Journal of Microbiology*, *41*(3), pp.542-562.

Pilla, R., Snel, G.G., Malvisi, M. and Piccinini, R., 2013. Duplex real-time PCR assay for rapid identification of Staphylococcus aureus isolates from dairy cow milk. *Journal of dairy research*, *80*(2), pp.223-226.

Pincus, D.H., 2006. Microbial identification using the bioMérieux VITEK® 2 system. *Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association*, pp.1-32.

Plozza, K., Lievaart, J.J., Potts, G. and Barkema, H.W., 2011. Subclinical mastitis and associated risk factors on dairy farms in New South Wales. *Australian Veterinary Journal*, *89*(1-2), pp.41-46.

Prabhu, K., Rao, S. and Rao, V., 2011. Inducible clindamycin resistance in Staphylococcus aureus isolated from clinical samples. *Journal of laboratory physicians*, *3*(1), p.25.

Prats, G. and Llovet, T., 1995. Enteroinvasive Escherichia coli. Pathogenesis and epidemiology. *Microbiologia (Madrid, Spain)*, *11*(1), pp.91-96.

Preethirani, P.L., Isloor, S., Sundareshan, S., Nuthanalakshmi, V., Deepthikiran, K., Sinha, A.Y., Rathnamma, D., Prabhu, K.N., Sharada, R., Mukkur, T.K. and Hegde, N.R., 2015. Isolation, biochemical and molecular identification, and in-vitro

antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in South India. *PLoS One*, *10*(11).

Puño-Sarmiento, J., Medeiros, L., Chiconi, C., Martins, F., Pelayo, J., Rocha, S., Blanco, J., Blanco, M., Zanutto, M., Kobayashi, R. and Nakazato, G., 2013. Detection of diarrheagenic Escherichia coli strains isolated from dogs and cats in Brazil. *Veterinary microbiology*, *166*(3-4), pp.676-680.

Qadri, F., Svennerholm, A.M., Faruque, A.S.G. and Sack, R.B., 2005. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clinical microbiology reviews*, *18*(3), pp.465-483.

Radostits, O., C. Gay, W. Hinch cliff and P. constable,2007. Veterinary medicine a text book of the diseaseof cattle Horses, sheep pigs and goats. 10 ed.thLondon: Sounders, pp: 1462-1464

Radostits, O.M., Blood, D.C. and Gay, C.C.,1994. Veterinary Medicine, a Text Book of the Disease of Cattle, Sheep, Goats, Pigs and Horses. 8th Edition, Bailliere, Tindall, London, 1015-1026.

Radostits, O.M., Mayhew, I.G. and Houston, D.M., 2000. *Veterinary clinical examination and diagnosis*. WB Saunders.

Rahman, B., Ownagh, A., Mardani, K. and Ardebili, F.F., 2016. Prevalence and molecular characterization of staphylococci isolated from sheep with subclinical mastitis in West-Azerbaijan province, Iran. In *Veterinary Research Forum* (Vol. 7, No. 2, p. 155). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Rainard, P., Foucras, G., Fitzgerald, J.R., Watts, J.L., Koop, G. and Middleton, J.R., 2018. Knowledge gaps and research priorities in Staphylococcus aureus mastitis control. *Transboundary and emerging diseases*, *65*, pp.149-165.

Ranjan, R., Gupta, M.K. and Singh, K.K., 2011. Study of bovine mastitis in different climatic conditions in Jharkhand, India. *Veterinary World*, *4*(5), pp.205-208.

Regasa, S., Mengistu, S. and Abraha, A., 2019. Milk Safety Assessment, Isolation, and Antimicrobial Susceptibility Profile of Staphylococcus aureus in Selected Dairy Farms of Mukaturi and Sululta Town, Oromia Region, Ethiopia. *Veterinary medicine international*, *2019*.

Reller, L.B., Weinstein, M., Jorgensen, J.H. and Ferraro, M.J., 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical infectious diseases*, *49*(11), pp.1749-1755.

Reshi, A.A., Husain, I., Bhat, S.A., Rehman, M.U., Razak, R., Bilal, S. and Mir, M.R., 2015. Bovine mastitis as an evolving disease and its impact on the dairy industry. Int. J. Curr. Res. Rev, 7(5), pp.48-55.

Roberts, S. and Chambers, S., 2005. Diagnosis and management of Staphylococcus aureus infections of the skin and soft tissue. *Internal medicine journal*, *35*, pp.S97-105.

Rocchetti, T.T., 2014. Detecção do operon ica da produção de biofilme, gene mecA de resistência à oxacilina e identificação de espécies de Staphylococcus spp. diretamente dos frascos de hemoculturas pela técnica de PCR multiplex.

Rolo, J., Worning, P., Nielsen, J.B., Sobral, R., Bowden, R., Bouchami, O., Damborg, P., Guardabassi, L., Perreten, V., Westh, H. and Tomasz, A., 2017. Evidence for the evolutionary steps leading to mecA-mediated β-lactam resistance in staphylococci. PLoS genetics, 13(4), p.e1006674.

Samad, M.A., 2008. Animal husbandry and veterinary science. Volume I; Volume II. Animal husbandry and veterinary science. Volume I; Volume II.

Sandholm, M. and Pyörälä, S., 1995. Dry cow therapy. *The Bovine Udder and Mastitis. Sandholm, M.; Honkanen-Buzalski, T*, pp.209-214.

Sarowska, J., Futoma-Koloch, B., Jama-Kmiecik, A., Frej-Madrzak, M., Ksiazczyk, M., Bugla-Ploskonska, G. and Choroszy-Krol, I., 2019. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic Escherichia coli isolated from different sources: recent reports. *Gut pathogens*, *11*(1), p.10.

Savarino, S.J., McVeigh, A., Watson, J., Cravioto, A., Molina, J., Echeverria, P., Bhan, M.K., Levine, M.M. and Fasano, A., 1996. Enteroaggregative Escherichia coli heat-stable enterotoxin is not restricted to enteroaggregative E. coli. *Journal of infectious diseases*, *173*(4), pp.1019-1022.

Schaumburg, F., Alabi, A.S., Peters, G. and Becker, K., 2014. New epidemiology of Staphylococcus aureus infection in Africa. *Clinical Microbiology and Infection*, *20*(7), pp.589-596.

Schmidt, T., Kock, M.M. and Ehlers, M.M., 2015. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *Journal of dairy science*, *98*(9), pp.6256-6269.

Schreckenberger, P.C., Ilendo, E. and Ristow, K.L., 2004. Incidence of constitutive and inducible clindamycin resistance in Staphylococcus aureus and coagulase-negative staphylococci in a community and a tertiary care hospital. *Journal of clinical microbiology*, *42*(6), pp.2777-2779.

Schroeder, J.W., 2012. Bovine mastitis and milking management. *Drug therapy*, *8*(4).

Schukken, Y.H., Zadoks, R.N., Tikofsky, L., Dogan, B., Klaessig, S., Simpson, K., Wiedmann, M. and Boor, K., 2004. Epidemiology of mastitis: paradigms patterns and parables. *Medecin veterinaire du quebec.*, *34*, pp.48-49.

Seid, U., Zenebe, T., Almaw, G., Edao, A., Disassa, H., Kabeta, T., Gerbi, F. and Kebede, G., 2015. Prevalence, Risk Factors and Major Bacterial Causes of Bovine Mastitis in West Arsi Zone of Oromia Region, Southern Ethiopia. Nature and Science, 13(8), pp.19-27.

Sharif, A. and Muhammad, G., 2009. Mastitis control in dairy animals. *Pakistan Vet. J*, *29*(3), pp.145-148

Sharma N, Jeong DK., 2013 Stem Cell Research: A Novel Boulevard towards Improved Bovine Mastitis Management*. International Journal of Biological Sciences*, 9:818-829.

Sharma, N., Pandey, V. and Sudhan, N.A., 2010. Comparison of some indirect screening tests for detection of subclinical mastitis in dairy cows. *Bulgarian Journal of Veterinary Medicine*, *13*(2).

Shete, M. and Rutten, M., 2015. Impacts of large-scale farming on local communities' food security and income levels–Empirical evidence from Oromia Region, Ethiopia. *Land Use Policy*, *47*, pp.282-292.

Shopsin, B., Gomez, M., Montgomery, S.O., Smith, D.H., Waddington, M., Dodge, D.E., Bost, D.A., Riehman, M., Naidich, S. and Kreiswirth, B.N., 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. *Journal of clinical microbiology*, *37*(11), pp.3556-3563.

Sidhu, J.P., Ahmed, W., Hodgers, L. and Toze, S., 2013. Occurrence of virulence genes associated with diarrheagenic pathotypes in Escherichia coli isolates from surface water. *Appl. Environ. Microbiol.*, *79*(1), pp.328-335.

Singh, N., Singh, P. and Patel, R.K., 2016. Isolation and identification of bacterial organisms from mastitic milk. *J. Livestock Sci*, *7*, pp.46-8.

Skipworth, J., 2009. Structure of *Staphylococcus aureus* illustrating surface and secreted protein in exponential and stationary phase. URL: http://www.flickr.com/pathogenesis+*Staphylococcus*+*aureus*/photos (Accessed April 2018)

Skow, A., Mangold, K.A., Tajuddin, M., Huntington, A., Fritz, B., Thomson, R.B. and Kaul, K.L., 2005. Species-level identification of staphylococcal isolates by real-time PCR and melt curve analysis. *Journal of clinical microbiology*, *43*(6), pp.2876-2880.

Solomakos, N., A. Govaris, A. S. Angelidis et al., 2009. Occurrence, virulence genes and antibiotic resistance of *Escherichia coli O157* isolated from raw bovine, caprine and ovine milk in Greece, *Food Microbiology*, 26(8), pp. 865–871

Sordillo, L.M., Shafer-Weaver, K. and DeRosa, D., 1997. Immunobiology of the mammary gland. *Journal of dairy science*, *80*(8), pp.1851-1865.

Soto, S.M., Guiral, E., Bosch, J. and Vila, J., 2009. Prevalence of the set-1B and astA genes encoding enterotoxins in uropathogenic Escherichia coli clinical isolates. *Microbial pathogenesis*, *47*(6), pp.305-307.

Spaan, A.N., Surewaard, B.G., Nijland, R. and van Strijp, J.A., 2013. Neutrophils versus Staphylococcus aureus: a biological tug of war. *Annual review of microbiology*, *67*, pp.629-650.

Spaulding, A.R., Salgado-Pabón, W., Kohler, P.L., Horswill, A.R., Leung, D.Y. and Schlievert, P.M., 2013. Staphylococcal and streptococcal superantigen exotoxins. *Clinical microbiology reviews*, *26*(3), pp.422-447.

Sperandio, V. and Nguyen, Y., 2012. Enterohemorrhagic E. coli (EHEC) pathogenesis. *Frontiers in cellular and infection microbiology*, *2*, p.90.

Speziale, P., Pietrocola, G., Rindi, S., Provenzano, M., Provenza, G., Di Poto, A., Visai, L. and Arciola, C.R., 2009. Structural and functional role of Staphylococcus aureus surface components recognizing adhesive matrix molecules of the host. Future microbiology, 4(10), pp.1337-1352.

Stats, S.A., 2016. Statistical release: Mid-year population estimates. *Statistics South Africa, Pretoria*, p.3.

Stephan, R., S. Schumacher, S. Corti, G. Krause, J. Danuser, and L. Beutin, 2008. Prevalence and characteristics of *Shiga toxinproducing Escherichia coli* in Swiss raw milk cheeses collected at producer level, *Journal of Dairy Science*, 91( 7), pp.2561–2565.

Stewart, P.S. and Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. *The lancet*, *358*(9276), pp.135-138.

Sukumaran, D.P., Durairaj, S. and Abdulla, M.H., 2012. Antibiotic resistance of Escherichia coli serotypes from Cochin estuary. Interdisciplinary perspectives on infectious diseases, 2012.

Sumathi, S., Chai, S.P. and Mohamed, A.R., 2008. Utilization of oil palm as a source of renewable energy in Malaysia. *Renewable and sustainable energy reviews*, *12*(9), pp.2404-2421.

Sung, J.M.L., Lloyd, D.H. and Lindsay, J.A., 2008. Staphylococcus aureus host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology*, *154*(7), pp.1949-1959.

Suojala, L., 2010. Bovine mastitis caused by Escherichia coli: Clinical, bacteriological and therapeutic aspects.

Takeuchi, S., Maeda, T., Hashimoto, N., Imaizumi, K., Kaidoh, T. and Hayakawa, Y., 2001. Variation of the agr locus in Staphylococcus aureus isolates from cows with mastitis. *Veterinary microbiology*, *79*(3), pp.267-274.

Tarr, C.L., Large, T.M., Moeller, C.L., Lacher, D.W., Tarr, P.I., Acheson, D.W. and Whittam, T.S., 2002. Molecular characterization of a serotype O121: H19 clone, a distinct Shiga toxin-producing clone of pathogenic Escherichia coli. *Infection and immunity*, *70*(12), pp.6853-6859.

Tekle, Y. and Berihe, T., 2016. Bovine mastitis: Prevalence, risk factors and major pathogens in the Sidamo zone snnprs, Ethiopia. *Eur. J. biol. Res*, *4*, pp.27-43.

Thammavongsa, V., Kim, H.K., Missiakas, D. and Schneewind, O., 2015. Staphylococcal manipulation of host immune responses. *Nature Reviews Microbiology*, *13*(9), pp.529-543.

Torres, VJ., Attia, AS., Mason, WJ., Hood, MI., Corbin, BD., Beasley, FC., Anderson, KL., Stauff, DL., McDonald, WH., Zimmerman, LJ., Friedman, DB., Heinrichs, DE., Dunmsn, PM., and Skaar, EP., 2010. Staphylococcus aureus fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. *Infection and Immunity*. 78:1618-1628.

Tremblay, Y.D., Lamarche, D., Chever, P., Haine, D., Messier, S. and Jacques, M., 2013. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *Journal of dairy science*, *96*(1), pp.234-246.

Tulinski, P., Fluit, A.C., Wagenaar, J.A., Mevius, D., van de Vijver, L. and Duim, B., 2012. Methicillin-resistant coagulase-negative staphylococci on pig farms as a reservoir of heterogeneous staphylococcal cassette chromosome mec elements. *Appl. Environ. Microbiol.*, *78*(2), pp.299-304.

Varley, A.J., Sule, J. and Absalom, A.R., 2009. Principles of antibiotic therapy. *Continuing Education in Anaesthesia, Critical Care & Pain*, *9*(6), pp.184-188.

Vasil, M., Pecka-Kiełb, E., Elečko, J., Zachwieja, A., Zawadzki, W., Zigo, F., Illek, J. and Farkašová, Z., 2016. Effects of udder infections with Staphylococcus xylosus and Staphylococcus warneri on the composition and physicochemical changes in cows milk. *Polish journal of veterinary sciences*, *19*(4), pp.841-848.

Verma, H., Rawat, S., Sharma, N., Jaiswal, V. and Singh, R., 2018. Prevalence, bacterial etiology and antibiotic susceptibility pattern of bovine mastitis in Meerut. *Journal of Entomolology and Zoology Studies*, *6*, pp.706-709.

Vhembe district profile.,  2017.

Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R., 2009. Mastitis detection: current trends and future perspectives. *Trends in biotechnology*, *27*(8), pp.486-493.

Vijayakumar, R., Al-Aboody, M.S., AlFonaisan, M.K., Al Turaiki, W., Mickymaray, S., Mariappan, P., Alsagaby, S.A. and Sandle, T., 2016. Determination of minimum inhibitory concentrations of common biocides to multidrug-resistant gram-negative bacteria. *Applied Medical Research*, *2*(3), pp.56-62.

Von Eiff, C., Becker, K., Machka, K., Stammer, H. and Peters, G., 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. *New England Journal of Medicine*, *344*(1), pp.11-16.

Wald, R., Hess, C., Urbantke, V., Wittek, T. and Baumgartner, M., 2019. Characterization of Staphylococcus species isolated from bovine quarter milk samples. Animals, 9(5), p.200.

Walker, C.L.F., Sack, D. and Black, R.E., 2010. Etiology of diarrhea in older children, adolescents and adults: a systematic review. *PLoS neglected tropical diseases*, *4*(8).

Wang, Y., Wu, C.M., Lu, L.M., Ren, G.W.N., Cao, X.Y. and Shen, J.Z., 2008. Macrolide–lincosamide-resistant phenotypes and genotypes of Staphylococcus aureus isolated from bovine clinical mastitis. *Veterinary microbiology*, *130*(1-2), pp.118-125.

Watts, J.L., 1988. Etiological agents of bovine mastitis. *Veterinary microbiology*, *16*(1), pp.41-66.

Wayne, P.A., 2015. Performance Standards for Antimicrobial Susceptibility Testing; 25th Informational Supplement. CLSI M100-S25.

Welay, G.M., Tedla, D.G., Teklu, G.G., Weldearegay, S.K., Shibeshi, M.B., Kidane, H.H., Gebrezgiabher, B.B. and Abraha, T.H., 2018. A preliminary survey of major diseases of ruminants and management practices in Western Tigray province, northern Ethiopia. *BMC veterinary research*, *14*(1), p.293.

Wenhold, F., Muehlhoff, E. and Kruger, H.S., 2016. Nutrition for School-age Children. *Community Nutrition for Developing Countries*, 31, pp.104.

Winstanley, T. and Courvalin, P., 2011. Expert systems in clinical microbiology. *Clinical microbiology reviews*, *24*(3), pp.515-556.

Xi, X., Kwok, L.Y., Wang, Y., Ma, C., Mi, Z. and Zhang, H., 2017. Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry MSE-based untargeted milk metabolomics in dairy cows with subclinical or clinical mastitis. *Journal of dairy science*, *100*(6), pp.4884-4896.

Xu, J., Tan, X., Zhang, X., Xia, X. and Sun, H., 2015. The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. *Microbial pathogenesis*, *88*, pp.29-38.

Xu, Z., Shah, H.N., Misra, R., Chen, J., Zhang, W., Liu, Y., Cutler, R.R. and Mkrtchyan, H.V., 2018. The prevalence, antibiotic resistance and mecA characterization of coagulase negative staphylococci recovered from non-healthcare settings in London, UK. *Antimicrobial Resistance & Infection Control*, *7*(1), p.73.

Yatsuyanagi, Jun, Shioko Saito, Yoshimichi Miyajima, Ken-Ichi Amano, and Katsuhiko Enomoto. "Characterization of atypical enteropathogenic Escherichia coli strains harboring the astA gene that were associated with a waterborne outbreak of diarrhea in Japan." *Journal of clinical microbiology* 41, no. 5 (2003): 2033-2039.

Zaatout, N., Ayachi, A. and Kecha, M., 2020. Epidemiological investigation of subclinical bovine mastitis in Algeria and molecular characterization of biofilm-forming Staphylococcus aureus. *Tropical animal health and production*, *52*(1), pp.283-292.

Zecconi, A. and Hahn, G., 1999. *Staphylococcus aureus* in raw milk and human health risk. *International Dairy Federation*, 345 pp.15-18.

Zeryehun, T. and Abera, G., 2017. Prevalence and bacterial isolates of mastitis in dairy farms in selected districts of eastern Harrarghe Zone, Eastern Ethiopia. *Journal of veterinary medicine*, *2017*.

## APPENDICES

## APPENDIX A

## INFORMED CONSENT FORM FOR DAIRY FARM MANAGERS

This Informed Consent Form is for dairy farm managers and dairy farm personnel's who we are inviting to participate in research on Mastitis. The title of our research is **CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL AND SUBCLINICAL CASES OF BOVINE MASTITIS IN THE LIMPOPO DAIRY FARM**. Study is done by **BADUGELA NDIVHUWO (11615918),** for fulfilment of Master of Science degree to the Department of Microbiology.

**SUPERVISOR: DR E MUSIE** (University of Venda)

**CO-SUPERVISOR: DR MT SIGIDI** (University of Venda)

**CO-SUPERVISOR: PROF AN TRAORE** (University of Venda)

## PART I: INFORMATION SHEET

### INTRODUCTION

I am Badugela Ndivhuwo, a student in the University of Venda under the supervision of Dr E Musie, Dr M.T Sigidi and Prof A.N Traore. We are doing research on Mastitis disease, which is very common in dairy farms and mostly caused by bacteria known as *Staphylococcus aureus* and *E. coli*. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

### PUPROSE OF THE RESEARCH

Mastitis is a disease caused by invasion of the mammary gland tissues. In dairy cows the teats are infected. It has a bad impact in the economy for it reduce the production of dairy products and pose threat on public health globally because of

quality of milk produced. It is most commonly caused by two bacteria that are commonly found in the intestines and the skin of the cows as normal bacteria, but once they find opportunity to invade, they become contagious due to the virulence factors they have. Mastitis can show symptoms and can also not show symptoms in cows but changes the milk composition. The reason we are doing this research is to search for the bacteria and their virulence factors from cows that shows symptoms as well as those that do not show symptoms, and also test the bacteria found to the reaction of commonly used antibiotics in order to be able to know if the found bacteria can be destroyed or can resist the drugs which are currently being used.

**WHAT IS EXPECTED FROM YOU?**

This research will involve collection of milk from the cows (Showing signs and not showing signs of mastitis) within the farm and responding to a few questions from a questionnaire.

**WHY ARE YOU CHOSEN?**

You are in the area of interest.

**DURATION**

The research takes place over 2 seasons, dry and wet seasons. During that time, we will make visits into the farm to perform on farm screening and to collect the milk sample and health records of the cow's appointment prior visit until the research is finished.

**PART II: CERTIFICATE OF CONSENT**

I have read the foregoing information. I have had the opportunity to ask questions about it and any questions that I have asked to have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

**Name of Participant**_____

**Signature of Participant** _____

**Date** _____

**STATEMENT BY THE RESEARCHER/PARTICIPANT**

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. On farm cows screening

2. Cow's milk collection

3. Responding to questionnaires

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

**A COPY OF THIS INFORMED CONSENT FORM HAS BEEN PROVIDED TO THE PARTICIPANT**.

**Name of Researcher** _____

**Signature of Researcher**_____

**Date** _____

**APPENDIX B**

**A questionnaire designed to measure management practice used on Limpopo dairy farm and individual cow general information and health record**

**PART A: GENERAL INFORMATION**

**1) HOUSING**

1.1 How many cows are housed in your farm?

1.2 What is the type of housing for your milking cows?

☐ Tie stall ☐ Free stall ☐ Bedding pack

Other(specify)……

……..

1.3 How are the passage way cleaned?

☐

☐ Scrapped ☐ Flushed with water ☐ Other, Please specify……

1.4 How many times are the passage way cleaned?
.....

1.5 What type of material is the base of your milking cow made of?

☐ Concrete ☐ Sand ☐ Pastu☐mat ☐ Other, please specify
……..

1.6 What type of bedding used ☐

☐ Straw ☐ Saw du☐ ☐ Shav☐gs ☐ Sand ☐ No bedding
Other, specify……

1.7 Bedding management

☐ Less than 2cm dee☐ ☐ greater than 2cm deep

**2. BIOSECURITY**

2.1. Do you buy adult cows and first cows heifers?

☐ Yes ☐ No Yes, if yes what do you do to moving the animals to your farm to make sure that their udder is healthy?

☐ Perform bacterial analysis for each quarter milk sample

☐ Perform bacterial analysis for pooled milk sample

☐ Ask sellers about the somatic cell count of the animals

☐ Perform Carlifornia Mastitis Test

☐ Ask sellers about the somatic cell count of the animals

2.2 Do make any udder health verification prior moving the animals?

☐ Yes ☐ No

2.3 Do you have the herd health health plan?

☐ Yes ☐ No

2.4 Does the health plan include detailed plan for mastitis management and control?

☐ Yes ☐ No

2.5 Was the plan compiled with veterinary input?

☐ Yes ☐ No

2.6 Do you use dry cow management for mastitis cases in your operation?

☐ Yes, for some cows ☐ Yes, for some cows ☐ No cows

2.7 Are dry cows housed with milking cows?

☐ Always ☐ Sometimes ☐ Never

2.8 Which of the following best describe the milking parlour on this operation?

☐ Side opening ☐ parallel ☐ ving ☐ Rotary ☐ Flat barn

Other, please specify……
……

**3. DISEASES**

3.1 Do you keep record of diseases occurring on your farm?

☐ Yes ☐ No

3.2 Do you have vaccination programme against mastitis?

☐ Yes ☐ No

Name the vaccines used

1……
2……

3……..
4……

5……..

3.2 Do you have general vaccination programme for adult cows?

☐ Yes ☐ No

Name the vaccines used

3.3 Are your cows udders dipped or flamed?

☐ Yes, dipped ☐ Yes, flam ☐ No

**4 MASTITIS RELATED RECORD**

4.1 Do you have Mastitic cows in your operation?

☐ Yes ☐ No

4.2 How many cows have been diagnosed with mastitis in your records?

……

4.3 Have you culled any cows because of mastitis?

☐ Yes ☐ No

4.4 Have any cows died from high cell count?

☐ Yes ☐ No

4.5 Which signs do you use to diagnose mastitis

☐ Clots in milk ☐ discoloured milk ☐ blood in milk ☐ hot udder ☐ udder discolouration ☐ change in cow milking order ☐ low temperature ☐ other, please specify……
……..

4.6 What method for detection of clots in milk is used?

☐ Strip cup ☐ Carlifornia mastitis test ☐ inline filter ☐ other, please specify……
……..

4.6 For cows showing clots in milk, what type of treatment is used?

☐ Herbal udder infusion ☐ Antibiotics ☐ other, please specify……
……

If antibiotics are used, what type are used?

☐ B lactams: ……, ……..,
……, ……

☐ Macrolides: ……, ……..,
……, ……

☐ Phenolics: ……., ……,
……., ……

4.7 What consideration do you make when deciding which cows will receive conventional treatment?

……
……

**PART B: INDIVIDUAL COW RECORD AND OBSERVATION**

Cows name/ID: ……

Registration/ Ear tag number:……

Date of birth/Age:……

Breed: …….

Lactation stage: ……..

Vaccination status: ……

Parity level: …….

Previous history of mastitis: ……

Accumulated cases in previous months of lactation: ……..

Pathogens involved: ……..

Cows physical observation

| Physical observation | | none | Mild | severe | Comments |
|---|---|---|---|---|---|
| Cleanliness | Dirty hind limbs | | | | |
| | Dirty udder | | | | |
| Coat condition | Dull coat | | | | |
| | Thick hairy coat | | | | |
| | Hair loss | | | | |
| Teat ends | Sore inflamed teat ends | | | | |
| Adverse reaction to drug administration | | | | | |

**PART C: PERSONAL (DAIRY FARM)**

4.1 How important is a persistent high somatic cell counting your culling decisions?

(Rate from 1 -Not important to 5-Very Important)

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|

1 Not important 2 Partially important 3 Neutral 4 Important 5 very important

4.2 How important is an infection with *Staphylococcus aureus* in your culling decisions?

1 Not important 2 Partially important 3 Neutral 4 Important 5. Very important

4.3 Do you agree with the statements?

i. High somatic cell count (SCC) cows are easy to discover during milking

1 Strongly disagree 2 Agree 3 Neutral 4 Agree 5 strongly agree

ii. To prevent Staphylococcus aureus infection, it is important to look at stall cleanliness intead of milking procedures

1 Strongly disagree 2 Disagree 3 Neutral 4 Agree 5 Strongly agree

iii. Analysis of cows individual SCC is very important

1 Strongly disagree 2 Disagree 3 Neutral 4 Agree 5 Strongly agree

iv. Generally you can not influence causes of sub clinical mastitis

1 Strongly disagree 2 Disagree 3 Neutral 4 Agree 5 strongly agree

v. I know enough about mastitis to keep the herd out of trouble

1 Strongly disagree 2 Disagree 3 Neutral 4 Agree 5 strongly agree

**APPENDIX C**