ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

SCHOOL OF GRADUATE STUDIES

DEVELOPMENT AND EVALUATION OF MULTIPLEX PCR FOR SIMULTANEOUS DETECTION OF MAJOR BOVINE MASTITIS CAUSING PATHOGENS

A Thesis Submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

BY

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ABBREVIATIONS

BM	Bovine mastitis
CFU	Colony forming units
CM	Clinical mastitis
EDTA	Ethylene diamine tetra acetic acid
IDF	International dairy federation
NCBI	National center for biotechnology information
m-PCR	Multiplex Polymerase Chain Reaction
SCM	Subclinical mastitis
Taq	Termus aquaticus

ABSTRACT

Mastitis is the most frequently occurring and economically one of the most important infectious disease in dairy cows. In most clinical laboratories, identification methods are based on microbiological culturing of milk and biochemical tests. However, there are many limitations associated with microbiological culture. Therefore, alternative, non-culture-based diagnostic methods are needed. This study was conducted with the objective of developing and assessing the use of multiplex PCR as a tool to identify bacterial species commonly associated with mastitis viz; *Staphylococcus aureus, Escherchia coli*, *Streptococcus agalactiae* and *Streptococcus dysagalactiae*. Conventional techniques based on biochemical and physiological characteristics of bacteria were utilized to identify the bacterial species. Then multiplex PCR assays were optimized using species specific primers for simultaneous detection of bacterial species. For rapid and accurate diagnosis, DNA was isolated from milk samples and subjected to multiplex PCR. Based on multiplex PCR results, *S. aureus* (60%) was found to be the predominant bacteria detected from milk followed by *S. agalactiae* (40 %) and *E. coli* (25%). The detection levels of *S. aureus* and *S. agalactiae* were higher when using mPCR compared to the culture-based method. However, *S. dysagalactiae* was not detected by multiplex PCR. Diagnostic sensitivity and specificity of the optimized multiplex PCR were calculated as 80% and 99.9% respectively. Furthermore, there was a substantial agreement between both diagnostic techniques (kappa result (K= 0.7)). Overall the results suggest that the multiplex PCR assay employed in this study could be used as an alternative routine diagnostic method for rapid, sensitive and specific simultaneous detection of major mastitis agents.

Key-words: ***Bacteria, biochemical test, culture-based methods, Mastitis, Multiplex- PCR***

1. INTRODUCTION

1.1. Background

There are several types of diseases which potentially infect and affect the wellbeing of livestock population among which mastitis is one of the most common (Nicole, 2015). Mastitis is defined as an inflammatory reaction of the mammary gland (Wu *et al*., 2008).

It is induced when pathogenic microorganisms enter the udder through the teat canal, overcome the cow's defense mechanisms, begin to multiply in the udder, and produce toxins that are harmful to the mammary gland (Nielsen, 2009).

Economically, it is one the most important infectious disease in dairy cattle. Besides health disorders of mammary gland, mastitis causes significant losses in milk yield (due to physical and chemical changes of milk), degradation of its nutritive and technological properties, fertility disorders and even systemic disease (Cervinkova *et al* ., 2013).

Mastitis is considered as the most complex disease because of its multifactorial causation (Gaddi *et al*., 2016). Its incidence depends on exposure to pathogens, effectiveness of udder defense mechanisms, and presence of environmental risk factors, as well as interactions between these factors (Oviedo-Boyso *et al*., 2007).

Numerous microorganisms have been described as causative agents of bovine mastitis. Even though majority of the causes are pathogenic bacteria, more than 130 microorganisms such as virus, protozoa and other chemical and mechanical factors are responsible for the occurrence of bovine mastitis (Vianna *et al*., 2008).

According to their epidemiology, mastitis pathogens can be divided broadly into two groups, contagious and environmental. The environmental group includes *Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Enterococcus* sp. and *coagulase-negative staphylococci*; whereas the contagious causes include *Mycoplasma bovis, Staphylococcus aureus* and *Streptococcus agalactiae* (Hameed *et al*., 2008). Although a wide spectrum of bacterial species have been identified as causative agents of mastitis, only a few species including *staphylococci, streptococci* and *coliforms* are responsible for the

majority of infections making them economically and epidemiologically important (Gopinath *et al.*, 2012).

Mastitis occurs mainly in two main forms clinical and subclinical. The clinical mastitis is easily visible and diagnosed by farmers because of the characteristic symptoms on affected udder/quarter and associated changes in milk composition with clots and flakes formation. The subclinical mastitis is hard to be diagnosed visually but characterized mainly by reduction of milk production and alteration of milk constitutes (Gaddi *et al*., 2016).

Mastitis is a complex disease, and thus there is no simple solution for its control, so understanding its occurrence, the related risk factors, and the mastitogenic pathogens involved, are fundamental elements in developing a control program (FAO, 2014).

Despite many years of research mastitis remains the most economically damaging disease for the dairy industry worldwide and it has also public health importance by serving as a vehicle in the spread of diseases like tuberculosis, staphylococcal food poisoning, brucellosis, sore-throat, Q- fever and Leptospirosis (Radostits *et al*., 2007; Sharif *et al*., 2009).

In most clinical laboratories, identification methods are based on microbiological culturing of milk and biochemical tests for further identifications. However, there are many disadvantages associated with microbiological culture. Culture of milk may not yield any bacteria from sub clinically infected glands due to presence of very low number of pathogens when samples are collected. Bacteria may not also be isolated from mastitic milk due to the presence of residual therapeutic antibiotics in the sampled milk that may inhibit bacterial growth. These techniques are also labor-intensive and time-consuming. The whole procedure takes about one week.

For the limitations of cultural and other conventional methods, molecular techniques such as, polymerase chain reaction (PCR) have been developed to identify various mastitis pathogens. The development of PCR-based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial pathogens can be made in hours, rather than the days required for conventional cultural methods. PCR

can also improve the level of detection due to its high sensitivity. Furthermore, PCR can also detect bacteria in the presence of preservatives or residual therapeutic antibodies in milk, thus avoiding the false negative result caused by lack of bacterial growth which is the main limitation of conventional methods.

Different PCR-based methods have been developed for specific and sensitive detection of mastitis pathogens in milk. Due to the fact that mastitis is caused by numerous organisms, the use of multiplex PCR as a diagnostic technique could be effective for specific, rapid and simultaneous detection of major mastitis causing pathogens.

Therefore, this study was designed with the following objectives:

General objective

- To develop and evaluate the use of a multiplex PCR for simultaneous detection of major bovine mastitis causing pathogens in milk.

Specific objectives

- To develop a multiplex PCR assay for detecting the most predominant bovine mastitis casing pathogens viz; *E. coli, S. aureus, S. agalactiae and S. dysa galactiae* using species-specific primers.
- To optimize PCR conditions for *E. coli*, *S. aureus*, *S. agalactiae* and *S. dysagalactiae* using species-specific primers.
- To compare the specificity and sensitivity of cultural methods and multiplex PCR based techniques for bovine mastitis diagnosis.

2. LITERATURE REVIEW

2.1. Definition and general aspects

According to International Dairy Federation (IDF) (1987), mastitis is defined as an inflammation of the mammary gland. Although it may have a traumatic or toxic etiology, it is generally a result of microbiological infection. Mastitis is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder (Sharma, 2007).

It is also defined as inflammation of mammary gland parenchyma, which is caused by bacteria and its toxins (Sharma *et al*., 2006). The bacterial contamination of milk from affected cows render it unfit for human consumption and provide a mechanism of spread of diseases like tuberculosis, sore-throat, Q-fever, Brucellosis, Leptospirosis and has zoonotic importance (Sharif *et al*., 2009).

Mastitis is categorized as acute or chronic on the basis of duration and it is also divided into clinical mastitis and subclinical mastitis on the basis of symptoms. Clinical mastitis is classified by the types of symptoms: mild (clotting of milk), moderate (changes in milk and visible signs of inflammation of the udder), or severe (changes in milk and udder, and systemic signs (IDF, 1987).

2.2. Major causative agents of mastitis

Intramammary infections are the most common causes of mastitis. These are defined as infections of the mammary gland secretory tissue and/or of the ducts and tubules by pathogens. Mastitis is caused by several species of common bacteria, fungi, mycoplasmas and algae (International Dairy Federation, 2011).

Although about 20 to 35% of clinical mastitis cases are of unknown etiology (Wellenberg *et al*., 2002), it is widely accepted that bovine mastitis is mainly bacterial in origin; especially *Staphylococc*i, *Streptococci* and *Coliforms* are the most common pathogens associated with mastitis (FAO, 2014).

Mastitis pathogens are categorized as contagious or environmental (Table-1). Contagious pathogens live and multiply on and in the cow's mammary gland and are spread from cow

to cow, primarily during milking. Contagious pathogens include: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma spp*. and *Corynebacterium bovis* (Radostis *et al*., 2000).

Environmental mastitis can be defined broadly as those intra-mammary infections (IMI) caused by pathogens whose primary reservoir is the environment in which the cow lives (Hogan *et al*., 1999).

Table 1: Etiological agents, natural habitat of agents and clinical types of bovine mastitis **(Source: Quinin *et al*., 1994)**

Etiological agent	Natural habitat	Clinical type of mastitis
Staphylococcus aureus	Udder lesions, skin and mucus membrane	Sub-clinical, chronic, acute and per acute. Including gangrenous mastitis. High % of subclinical carriers can occur in a herd
Streptococcus agalactie	Intramammary in the milk duct	Acute or chronic with recurring clinical cases. Infection can occur in maiden heifers.
Streptococcus dysagalactie	Buccal cavity and genitalia of cattle	Acute
E.coli ***Klebsiella pneumoniae***	Feaces, saw ducts and other bedding	'coliformmastitis', peracute(toxemia) and usually occurs just after calving in cows with low somatic cell count. Life threatening
Actinomyce pyogens	Skin and mucus membrane	Peracute suppurative mastitis

The most frequently isolated environmental pathogens are *Streptococci*, other than *S. agalactiae,* commonly referred to as environmental *streptococci* (usually *S. uberis* and *S. disgalactiae*) and gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. (Hogan *et al*., 1999). Environmental pathogens require moisture, favorable pH and organic material for survival and they enter the gland through the teat canal (Messele, 2012).

Environmental pathogens reside in soil, bedding materials, manure and other organic matter. Therefore, efforts at prevention or control of environmental mastitis should focus on cleanliness of a cow's workplace and cleanliness of a cow. Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced (Schukken *et al*., 2005).

2.3. Pathogenesis of mastitis

The teat itself is the first line of defense against the penetration of bacteria into the udder. Normally, the sphincter muscle closes the teat canal tightly when the cow is not being milked, preventing the entry of pathogens. This sphincter is lined with a waxy material derived from stratified squamous epithelium called keratin. This keratin contains antimicrobial agents, such as long-chain fatty acids, that obstructs the migration of bacteria and assists in combating the infection (Paulrud, 2005).

Most of the time, invasion of the teat occurs during milking. The sphincter requires 2 hrs to return back to the contracted position. This helps organisms from the environment (manure, bedding, etc.) or those found on injured skin at the tip of the teat to easily enter through the opened or partially opened teat canal (Viguier *et al*., 2008).

When using of milking machine, there is admission of undesired air in the milking unit (Organisms present in the milk or at the teat end are propelled into the teat canal and cistern slipping or squawking of the unit or removal of teat cup without first shutting off the vacuum). Additionally, fluid accumulates within the mammary gland as parturition approaches, resulting in increased intramammary pressure and mammary gland vulnerability caused by the dilation of the teat canal and leakage of keratin and other mammary secretions (Rainard and Riollet, 2006). Once inside the teat, the bacteria may encounter leukocytes (white blood cells) present naturally in small numbers in the milk. These cells are the cow's second line of defense because they can engulf and destroy bacteria (Figure-1). During this process, the leukocytes release substances that cause the movement of additional leukocytes from the blood into the milk (Sordillo and Daley, 1995).

If bacteria are not entirely destroyed, they continue to multiply and begin to invade smaller ducts and alveolar areas. They liberate toxins and induce leukocytes and epithelial cells to release chemo attractants, including cytokines such as tumour necrosis factor-α (TNFα), interleukin (IL)-8, IL-1 (Sordillo and Daley, 1995) eicosanoids like prostaglandin F2a (PGF2α) oxygen radicals and acute phase proteins (APPs) (e.g. haptoglobin [Hp], serum amyloid A [SAA])]) (Giri *et al*., 1984).

Milk-secreting cells damaged by these toxins and other irritants release substances that lead to increased permeability of blood vessels. Additionally, leukocytes mainly polymorpho-nuclear neutrophils (PMNs) will be attracted to the site of infection (Paape *et al*., 2003).

PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) and neutral and acidic proteases (such as elastase, cathepsin G, cathepsin B and cathepsin D (Owen and Campbell, 1999). The released oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-b-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH). Destruction of most of the PMNs takes place by apoptosis once their task is fulfilled. Subsequently, macrophages engulf and ingest the remaining PMNs (Paape *et al*., 2002). The dead and sloughed off mammary epithelial cells, in addition to the dead leukocytes, are secreted into the milk, resulting in high milk Somatic cell count.

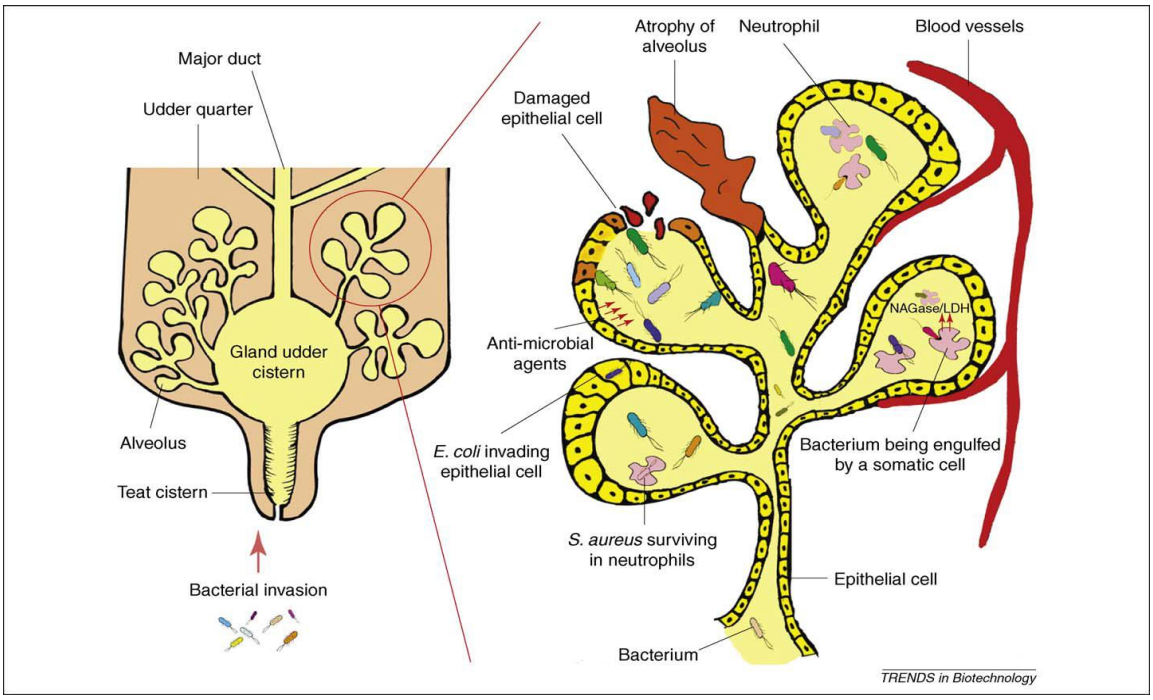

Figure 1: Schematic representation of mastitis development in an infected udder**.**
Source (Viguier *et al*., 2009).

If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity **(**Figure-1). The blood–milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk in addition, with the persistence of infection and clogged duct, the entrapped milk causes the secretory cells to revert to a resting (non-producing) state and the alveoli begin to shrink. Substances released by leukocytes lead to the complete destruction of alveolar structures, which are replaced by connective and scar tissues. The destruction of milk secretory tissue is, in effect, the cow's third line of defense to bring the infection under control (Zhao and Lacasse, 2008).

2.4. Types of mastitis

Mastitis can be either clinical or subclinical depending on how carefully the cow is observed during the time of diagnosis is made (IDF, 1987).

2.4.1. Clinical mastitis

This is a type of mastitis with a visible symptom such as clots or discolorations of the milk. In case of mild clinical mastitis (CM) only flakes or clots appear in the milk, whereas severe cases are associated with heat, swelling and discoloration of the udder, as well as abnormal secretion. Severe CM can also exhibit systemic reactions, such as fever and loss of appetite (IDF, 1987).

2.4.2. Subclinical mastitis

Mastitis can exist in the absence of visible signs of infection, and is then referred to as subclinical mastitis (SCM). SCM is the most prevalent form of mastitis (Akers, 2011). Subclinical mastitis is characterized by changes in milk composition e.g. somatic cell count (SCC); leukocytes and epithelial cells), and changes in milk pH and ion concentration, without clinical signs of inflammation (Guidry, 2007).

SCM can be diagnosed by presence of pathogens in bacteriological cultures of milk, but bacteriological sampling is not practically feasible as a routine test. The current standard method of detecting SCM is to measure SCC. In the healthy lactating mammary gland, the milk SCC is often < 100,000 cells/ml of milk, while the SCC can increase to > 1,000,000 cells/ml of milk during subclinical mastitis. The major factor affecting the SCC at the herd and individual level is the presence of intra mammary infections (IMM) (Radostits *et al*., 2007).

Other inflammatory parameters, such as electrical conductivity, lactose, lactate dehydrogenase, acute phase proteins, etc. have been proposed as indicators of SCM, and some have the potential of being adapted to in-line use (Hamann, 2005; Pyorala, 2003).

Based on the duration of infection mastitis can be further classified as acute or chronic, where a sudden onset defines acute cases and chronic mastitis is characterized by an

inflammatory process that lasts for months and results in progressive development of fibrous tissue (IDF, 1987).

2.5. Tests for pathogen identification

Essentially, bovine mastitis should be regarded as herd problem and the methods of investigation and diagnosis should reflect this fact. According to Quinn et al. (1994), the diagnostic methods include:

- Total and leukocyte cell counts for both herds and individual cows.
- Indirect chemical tests.
- Microbiological investigation to determine: a) the major pathogens causing mastitis in a herd, b) the percentage of subclinical carrier cows, c) the antibiotic susceptibility patterns of the major pathogens so that effective treatment can be administered.
- Clinical examination and detection of abnormal milk using a strip cup.

2.5.1. Qualitative examination of milk

Changes in color of milk can be caused by the presence of blood (red or brownish) or pus (yellow). The consistency may be increased, resulting in thicker, "sticky" milk, or it may be more than usually watery. Flakes and clots are always abnormal. The smell of the secretion may also be altered as a result of mastitis (Quinn *et al*., 1994).

2.5.2. Direct microscopy

The milk sample can be centrifuged and a stained smear made from the pellet. A Gram-stain is used routinely to detect gram- positive pathogens such as *staphylococci*, *streptococci* and this will also reveal yeast, such as *Candida albicans*, that stain deeply by crystal violet. An MZN (Modified Ziehl-Neelsen)-stained smear can be made if *Norcardia asteroid* is suspected and a ZN **(**Ziehl-Neelsen)-stained smear for the rare cases when bacteria such as *Mycobacterium fortuitum* or *M. bovis* are present (Quinn *et al*., 1994).

2.5.3. California Mastitis Test (CMT)

This practical test was developed in the 1950's during a California testing program; it gives a measure of the SCC of the sampled milk. The reagent (3% sodium lauryl sulphate is often

used) is a detergent which ruptures somatic cells in the milk, thereby releasing DNA. This forms a precipitate with other serum components, fat particles and the CMT reagent, causing visible gelling of the milk. A pH-indicator (for example bromocresol purple) may be added to the reagent. The test procedure is simple and straightforward: after the stripping milk is discarded, a few streams of (fore) milk from each quarter are milked into four plastic dishes set on a paddle. The paddle is then tipped nearly vertically to drain excess milk. An equal volume of the reagent is added from a plastic squeeze bottle and the two components mixed by swirling (Quinn *et al*., 2002).

2.5.4. Flow cytometry (FC)

Flow cytometry (FC) is a method by which physical and chemical characteristics of cells or particles can be measured as they travel in suspension past a sensing point. This method has been developed recently to quantify Somatic Cell Counts in milk, and is particularly good for detecting subclinical mastitis (Tian *et al*., 2005; Holm *et al*., 2004).

2.5.5. Bacterial Culture as gold standard test

The traditional diagnostic test for identifying pathogens in milk is bacterial culture, with the presence of bacteria indicated by their growth on an appropriate growth medium following a period of incubation (Hogan *et al*., 1999).

Bacterial species are identified based on phenotypic characteristics including, colony morphology, serotyping and analysis of enzymatic profile (Hogan *et al*., 1999; Oliver *et al*., 2004). Bacterial culture is currently regarded as the gold standard for identifying mastitis pathogens (Hogan *et al*., 1999). Some of the current SCC and alternative methods for detection of mastitis are summarized below in table 2.

Table 2: Summary on current and alternative methods for detection of mastitis Source (Viguier *et al*., 2009).

Name of Assay	**Principles**	**Advantage**	**Disadvantage**
California mastitis test (CMT)	This assay indirectly measures the SCC in milk samples. A bromocresol purple containing detergent is used to break down the Cell membraneof somatic cells and the subsequent release and aggregation of nucleic acid forms a gel like matrix with a viscosity that is proportional to the leukocyte numbers.	Cost effective (US $12 for 350 test), rapid, user friendly and can be used 'on-site' or in the laboratory.	Can be difficult to interpret and less sensitive
Fossomatic SCC	This counter operates on the principle of optical fluorescence. Ethidium bromide penetrates and intercalates with nuclear DNA, and the fluorescent signal generated is used to estimate the SCC in milk.	Rapid and automated.	The device is expensive (US$7 000) and complex to Use.
Delaval cell counter	This counter operates on the principle of optical fluorescence, whereby propidium iodide is used to stain nuclear DNA to estimate the SCC in milk	Rapid and the device is easily transportable.	Relatively expensive.

Electrical conductivity (EC) test	This test measures the increase in conductance in milk can used by the elevation in levels of ions such as sodium, potassium, calcium, magnesium and chloride during inflammation.	Can be used 'on Site'	Non- mastitis related variations in EC can present problems in diagnosis.
pH test	The rise in milk pH, due to mastitis, is detected using bromothymol blue.	User friendly, cost effective and rapid	Not as sensitive as other tests.
Enzymes	Assays are used to detect enzymes, such as NAGase and LDH.	Assays are rapid.	Assays might be laboratory-based.

2.5.6. PCR as diagnostic test

An alternative test for pathogen identification is the molecular technology, polymerase chain reaction. PCR detects DNA sequences that are unique to specific species or group of bacteria. Thus, confirming the presence or absence of bacterial DNA from both viable and non-viable organisms. The need for a rapid and accurate test led the development of PCR assay to detect mastitis causing pathogens (Riffon *et al*., 2001).

The type of PCR used determines the number of pathogens that can be detected in a test. Simplex PCR identifies one target per PCR reaction, whereas most mastitis PCR assays now use multiplex PCR, enabling the simultaneous detection of multiple pathogens in a single assay (Koskinen *et al*., 2009).

Multiplex PCR requires several primer sets in each reaction, each designed to amplify DNA associated with one species or species group (Gillespie and Oliver, 2006). Multiplex PCR may be linked with a reduction in analytical sensitivity (10-100 fold lower) when compared with simplex PCR, using the same primers for each target (Pheuktes *et al*., 2001). This could be a result of competition between individual reactions for reagents (Medico *et al*., 2000). However, multiplex PCR enables reduction in cost per sample as more pathogens can be detected with in one reaction (Steele, 2015).

The identification of bacteria at the species level is based on amplification of a target gene, which is highly conserved within the species, but variable between species. The rRNA genes are highly conserved in bacteria. In addition to the 16S rRNA gene, which is well established as a standard target for the identification of species of bacteria the 16S to 23S rRNA intergenic spacer of the rRNA operon has proven useful for identification of bacteria at the species level (pheuktes *et al*., 2001).

When comparing diagnostic tests, it is important to use the best and most accurate gold standard as the reference test. However, there is no perfect method that can definitively determine the 'true' bacteriological status of the mammary gland (Dohoo *et al*., 2011).

Culture procedures are generally accepted as the only method to reliably detect the cause of intramammary infections (Dohoo *et al*., 2011), and the current gold standard for identifying mastitis pathogens (Oliver *et al*., 2004).

Comparative evaluation of bacterial culture and PCR, in terms of their ability to identify mastitis pathogens based on different criteria is summarized (Table 3)

Table 3: Comparison of bacterial culture and PCR as mastitis diagnostic test

Criteria	**Culture method**	**PCR**	**Reference**
Target of test	Many potential infectious agents can be detected by their growth condition	Target of PCR test is much more limited i.e. bacteria that are targeted by the primers.	Hogan *et al*., 1999
Viability of bacteria	Bacteria must be viable so that it can be identifie d by its growth condition	Not dependent on the viability or biological functio nality of bacteria	Cressier and Bissonnate, 2011
Test chara cteristics	Large amount of sample needed as starting materi al compared to PCR.	PCR involves replication of Target DNA by Doubling number of copies of the target sequence. So only small amount of target DNA is required.	Koskinon *et al*., 2009

Repeatability	Reproducibility of results b/n laboratories may vary due to human factors, variation in tests used and quality of samples	It focuses on the presence or absence of target nucleic a cid. So, problem of reproducibility of culture i.e. depend o n phenotypic characterization is lower.	Gillespie and Oliver, 2005.
Rapidity	Takes a minimum of 48 hours to confirm results	Much more rapid than culture. With this method pathogen can be identified in as little as 4 hours once sample received.	Hogan *et al*., 1999 Koskinen *et al*., 2010
Cost	Cheaper than PCR methods	Expensive. As PCR require instruments and computer for analysis and more equipment depend on the method of DNA extraction	Paradis *et al*., 2012

2.6. Importance of bovine mastitis

Mastitis is of great economic importance to milk producers, because the disease has negative impact on several important aspects of cow and herd performance. Incurred costs are of both direct and indirect nature (Kossaibati & Esslemont, 1997). Direct costs include veterinary costs, increased labor requirement, discarded milk (during the course of treatment), and reduced milk yield and quality. Indirect costs are those that are not always obvious to the milk producer, and are therefore referred to as hidden costs. They include increased risk of subsequent disorders, reduced fertility (extra services per conception and, as a result of this, an extended calving interval), increased risk of culling, and, occasionally, mortality (Christel Nielsen, 2009). The losses due to mastitis can be a combination of some or all of these direct or indirect effects.

2.6.1. Milk production losses

Intra mammary infection, even if restricted to sub-clinical levels, has been reported to affect milk production negatively. The reduction in milk production is largely due to physical damage to the mammary parenchyma of the affected mammary gland (Zhao and Lacasse, 2008).

In both clinical and sub-clinical mastitis, there is a loss in milk production. Moreover, the loss in milk production does not only occur during the case itself, even after the mastitis case is cured, the milk production level of the cow stays lower. The economic damage of a lower milk production per cow depends on the structure of the farming business (Hogeveen, 2005).

2.6.2. Discarded milk

Because of treatment of a clinical case, milk has to be discarded during the treatment days and waiting time. In general, it is assumed that milk had to be discarded for 6 days: 3 days treatment and 3 days withholding period (Huijps *et al*., 2008).

2.6.3. Treatment costs

There are two elements of the treatment cost: veterinarian fees and the cost of drugs. Obviously these two costs vary between countries. Besides delivering drugs (in many countries), the veterinarian might have to spend time on diagnosis of a (clinical) mastitis case or supportive therapy (FAO, 2014).

2.6.4. Effect on Milk quality

Mastitis influences the quality of milk. Some of these changes cause a less efficient processing of milk and might result in products with less favorable properties. Examples are an unstable and rancid taste of milk, a lower cheese yield and a decreased shelf life (Ma *et al*., 2000).

2.6.5. Public health impacts of mastitis

The principal bacterial infection associated with ingestion of milk and milk products are caused by different bacterial genera. The bacteria that are transmitted through milk and cause disease problems in man are bacteria causing mastitis in cattle and transmissible to

human when man uses raw milk from infected udder. Example of such type of bacteria includes *Mycobacterium*, *Brucella*, and *Staphylococcus* and *Streptococcus* species. Antibiotic residues following treatment of mastitis can be a potential hazard to humans in allergic reaction and possible transfer of resistance to other organisms. Drugs are intended to be toxic to various forms of microorganisms as such may have inherent toxic, mutagenic, teratogenic, drug resistance and carcinogenic effect to humans (Gashaw, 2015).

2.6.6. Pre-mature Culling and replacement

Cows with mastitis have a higher risk of being culled. The cost due to premature replacement of animals due to mastitis is probably one of the largest areas of economic loss However; it is also a hidden cost. When a cow is culled, direct costs are the costs of rearing or buying a replacement animal (mostly heifers). Indirect costs are a decreased efficiency of milk production by the replacement animal, since the milk yield of multiparous cows is higher than that of primiparous cows. Moreover, the milk production of a heifer might be disappointing (heifers have a relatively high culling rate). On the other hand, there are also possible returns from culling a cow, mostly the price of meat. The costs of involuntary culling of a cow differ over time, depending on milk production, parity, lactation stage and reproductive status. The direct costs are the cost of rearing or buying a replacement animal. At the same time there are returns from culling a cow, mostly the price of the meat. Indirect costs could be decreased efficiency of milk production by the replacement animal, as usually a multiparous cow is more productive than a primiparous one (Halasa *et al*., 2007).

2.6.7. Subsequent Disorders

Mastitis is associated with increased risk of lameness (Peeler *et al*., 1994) and clinical mastitis has been reported to be associated with concurrent or subsequent diagnosis of ketosis, displaced abomasum and non-parturient paresis (Gröhn *et al*., 1989). Clinical mastitis is not a risk factor for reproductive disorders (Gröhn *et al*., 1990), but both CM and SCM are known to adversely affect reproductive performance (Petersson *et al*., 2006).

3. MATERIALS AND METHODS

All the laboratory activities and experiments were conducted at Animal biotechnology and Molecular Biology Laboratories, National Agricultural Biotechnology Research center, Holeta.

3.1. Milk samples

The milk samples required for this research were collected from dairy farm found in Holeta research center. A total of 20 milk samples were collected from bulk tanks. Samplings of milk were performed according to Watts (1990). Milk containers were agitated for about 2-3 min using sterile agitator. Then about 50 ml milk sample were collected from the top of each container using a sterile dipper and 10ml of sample were Poured into a screw-cap bottle. All collected samples were immediately kept in an insulated container with ice packs and transferred to the laboratory without delay and processed using bacterial culturing and PCR assays.

3.2. Microbiological culture

The organisms used in this study include *E. coli, S. aureus, S. agalactiae* and *S. dysagalactiae*. For identification of these bacterial spp, all milk samples collected were diluted to dilution factors of 10^{-1} and 10^{-2}; then, 0.1 ml of each of diluted sample was inoculated into blood agar base (Himedia, India) supplemented with 7% sheep blood and incubated at 37 ^{0}C for 24hr. Single colony of different colony types of the positive cultures was subcultured on blood agar plates so as to obtain a pure culture for further analysis. The bacterial Species were identified according to Quinn *et al*. (1999). Briefly, bacterial colony morphology was characterized; then, primary genus identification was made using Gram staining, oxidase and catalase tests. The bacterial species were further identified using recommended secondary biochemical tests. *Staphylococci* and *Streptococci* were differentiated by their respective positive and negative response to the catalase test. Furthermore, *Staphylococcus* species were identified by their reaction on coagulase tests, catalase tests and growth on Mannitol salt agar. *S. aureus* and *CNS* were further distinguished by their coagulase activity using tube coagulase test. Biochemical tests for distinguishing *Streptococci* include CAMP test, Aesculin hydrolysis on Edwards medium and Growth on MacConkey Agar. Biochemical tests for distinguishing *E. coli* from other

Enterobacteriaceae included citrate utilization test, motility test, indole production test, oxidase test, methyl red test, triple sugar iron test and colony characteristics on MacConkey Agar.

3.3. Extraction of bacterial genomic DNA

3.3.1. Bacterial genomic DNA Extraction from culture

Bacterial strains were grown overnight in nutrient agar (Himedia, India) at 37°C. Bacterial DNA was extracted by boiling a bacterial suspension in water. A loop full of a colony was added to 100 µl of sterile water. After boiling the suspension for 13 minutes, the suspension was frozen for 5 minutes in ice and centrifuged at 14,000 rpm for 15 min to pellet the cell debris (Reischl *et al*., 2002). The supernatant from the centrifuged tubes was transferred to new 1.5 ml clean plastic tube and used as a template for PCR amplification. The extracted DNA samples were detected by electrophoresis in 1.5 % agarose gel and also quantified using Nanodrop Spectrophotometer to determine the quality and concentration and then stored at -20°C for further use.

3.3.2. Bacterial genomic DNA Extraction from milk

DNA was extracted directly from milk samples using the method described by Jiusheng *et al*. (2008). About 1.5 ml of liquid milk sample was transferred into an eppendorf tube and centrifuged at 5,000 rpm for 10 min. The pellets were resuspended in 500 µl of enzyme incubation buffer (20 mM Tris-HCl, pH 8.0, 1.2% Triton x-100, 20 mg/ml of lysozyme, 2 mM EDTA) followed by incubation for 30 min at 37°C. Then, 25 µl of proteinase K (20 mg/ml) was added and incubated at 56°C for 2 h. After that, the DNA was extracted with phenol-chloroform-isoamylol (25:24:1) and precipitated by ethanol. The DNA was dissolved in 500 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration and purity was determined by the absorbance at 260 nm with a nanodrop spectrophotometer (Biofisher, Italy) and then stored at -20°C for later usage.

3.4. PCR Primer design and standardization

An extensive study of potential molecular targets of bacterial genome was carried out by Clustal W and other sequence alignment tools for aligning the sequences of the target

software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

All PCR primers were designed to have similar melting temperatures as needed for multiplex PCR. Other primer designing parameters such as, product size, primer size and primer GC% were adjusted for designing of specific primers for each bacterial species.

3.5. Optimization of Individual PCR Assays

PCR reactions for *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* were initially optimized separately. The PCR was carried out in 0.5-ml tubes in a reaction volume of 25 µl. All PCR reactions contained 200 µM each of dATP, dTTP, dCTP, dGTP and 1X Taq buffer and different concentrations of the extracted DNA. Preliminary trials with different magnesium concentrations (1, 1.5, 2 and 2.5 mM), Taq DNA polymerase concentrations (0.5, 0.6, 1 and 1.25 U per reaction) and primer concentrations (0.12, 0.25 and 0.4 µM) were performed to define the optimal PCR conditions for each individual PCR assay. Amplification was performed in an automated thermocycler; the annealing temperature and numbers of cycles were subsequently optimized for each individual assay.

3.6. Optimization of Multiplex PCR

To optimize the multiplex PCR, different concentrations of Taq polymerase, MgCl2 and combination of variable concentrations of primers were used. Accordingly, the optimal conditions for the assay were determined by testing different magnesium concentrations (2, 2.5, and 3 mM), Taq DNA polymerase concentrations (1, 2, 2.5 and 3 U per reaction) and different combinations of primer concentrations (0.3, 0.4, 0.5 and 0.6 µM) as well as different concentrations of extracted DNA were used. The annealing temperature and numbers of cycles were used from the previously optimized reactions of individual PCR conditions as a starting point.

3.7. Visualization and documentation of amplified products

To separate the amplified fragments, 7µL of PCR product was added to 2µL of loading dye stained with gel red. The PCR products were then separated by electrophoresis on a 2 % agarose gel at 70V for 60 minutes and visualized using image analyser (GelDoc-IT™ Imaging System - UVP, USA) at 300 nM. As a marker, 4µL of 100bp DNA ladder (Bioneer, South Korea) was loaded in the gel in parallel with PCR products to determine the size

3.8. Sensitivity evaluation of the multiplex PCR assay

To evaluate the detection limit of the developed PCR, all bacterial species were cultured overnight. The bacterial suspension of each species was serially diluted from 10^8 to 10^1 CFU/ml, in ultra–heat-treated (UHT) or pasteurized milk purchased from a local grocery store. Bacterial DNA was extracted directly from the diluted suspensions with the method previously mentioned in subheading 3.3.2., and used as the template for determining the detection limit (sensitivity) of the PCR assays (Lee *et al*., 2008).

3.9. Determining the comparative efficiency of detection

To determine the comparative efficiency, all collected milk samples were processed simultaneously for culture based bacterial isolation and DNA extraction from milk. In culture method, the species level identification of selected colonies recovered was performed using biochemical tests. In multiplex PCR method, detection of target pathogens was carried out using the DNA extracted from milk.

3.10. Statistical analysis

All collected data were entered into Microsoft Excel 2007. *S. aureus, S. agalactiae, S. dysagalactiae* and *E. coli* total isolates and percentage frequencies were evaluated according to microbiological culture and mPCR results. The specificity and sensitivity and its 95% confidence intervals of the PCR method was calculated in comparison with in vitro culture method according to Mackinnon (2000). Unweighted Kappa test was performed to test the fitness of PCR to bacterial culture as gold standard test.

4. RESULTS

4.1. PCR primers

A total of four sets of primers for the highly conserved regions of target pathogenic bacterial genomes were used in this study. All sets of primers were designed based on conserved nucleotide sequence of 16S rRNA for amplifying of *S. agalactiae, S. dysagalactiae, E. coli and S. aureus* using previously published sequence entries available in the NCBI – GenBank database generating 204 bp, 766 bp, 700 bp and 556 bp product sizes respectively. All oligonucleotide primers used in this study were synthesized by Himedia (India) Ltd. and procured as lyophilized powders. The working concentration of 10µM was prepared with sterile filtered distilled water and stored at -20ºC. Sequences of the four PCR primer pairs for multiplex PCR, their corresponding gene targets and size of expected amplification products are shown below in table 4.

Table 4: Primer sequences, lengths and expected product size used in mPCR.

Target	Sequence	Length	Start	Stop	GC%	Self-complementarity	Self-3' complementarity
S.aureus	**F: TCAACCGTGGAGGGTCATTG** **R: GTTGTCACCGGCAGTCAACG** **Expected size: 556bp**	**20** **20**	**599** **1154**	**615** **1135**	**55** **55**	**5** **4**	**3** **3**
E.coli	**F: AAGAAGCACCGGCTAACTCC** **R: GGCGCTTCTCTTTGTATGCG** **Expected size: 700bp**	**20** **20**	**422** **1157**	**441** **1168**	**55** **55**	**4** **4**	**0.00** **2**
S. dysagalactiae	**F: TGGTGAGGTAACGGCTCAC** **R: CCTGGTAAGGTTCTTCGCG** **Expected size: 766bp**	**20** **20**	**152** **889**	**171** **870**	**55** **57.8**	**5** **4**	**3** **4**
S. agalactiae	**F: GCCTCATAGCGGGGGATAAC** **R: GTGTCTCAGTCCCAGTGTGG** **Expected size: 204bp**	**20**	**83**	**102**	**60**	**2**	**2**

4.2. Specificity of primers

The Specific primers described above (Table 4) were proven to be specific since on agarose gel only one band was observed. the nucleotide sequence data comparison (BLASTN) showed that the four primers are specific for each bacterial species.

Specificity was also examined by using DNA template of one species and primers from the other Species (e.g. using of *E. coli* DNA in the presence of *S. aureus* primers) which did not show any amplification indicating the primers are specific. There was no amplified product seen in case of No Template Control (NTC) indicating the amplified product is specific. The test absence of PCR products in the negative control also shows that there were not any contaminations during the PCR assays and avoids any possible false positives.

4.3. Simplex PCR assays

Each Simplex PCR assay for amplification of each organism was performed using 25µl PCR reaction volume containing 2.5 µl (1X) of PCR buffer, 1.5 µl (1.5 mM) of $MgCl_2$, 2 µl (200 µM) of each of dNTP, 0.125 µl (0.65U) of Taq DNA polymerase (Himedia, India), 1.0 µl (0.25µM) of each of forward and reverse primers, 1 µl of the DNA template and 16 µl of nuclease free water to make 25 µl reaction volume. PCR assays were done in a Thermocycler (Edvocycler version 6.2) using the following cycling conditions: after an initial denaturation cycle of three minutes at 95 °C, the reaction mixes were subjected to 30 amplification cycles of 1 minute at 95 °C and 1 minute at 63 °C and two minutes at 72 °C and final extension of 10 minutes at 72 °C. All PCR products were at their expected size i.e. 700bp for *E. coli* (figure-2), 204 bp for *S. agalactiae* (figure-3), 566 bp for *S.aureus* (figure-4) and 766 bp for *S. dysagalactiae* (figure-5).

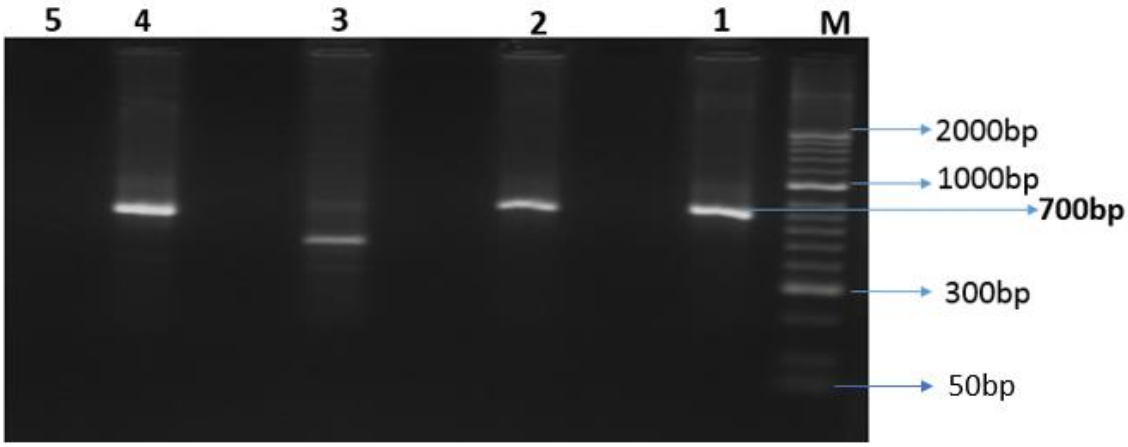

Figure 2: PCR amplification of 700 bp *E. coli* analyzed by electrophoresis on a 2% agarose gel, Lane M: 100 bp DNA ladder, Lanes 1, 2, 3 and 4 PCR amplified 700 bp product of *E. coli.* Lane -5 : Negative control. (NTC).

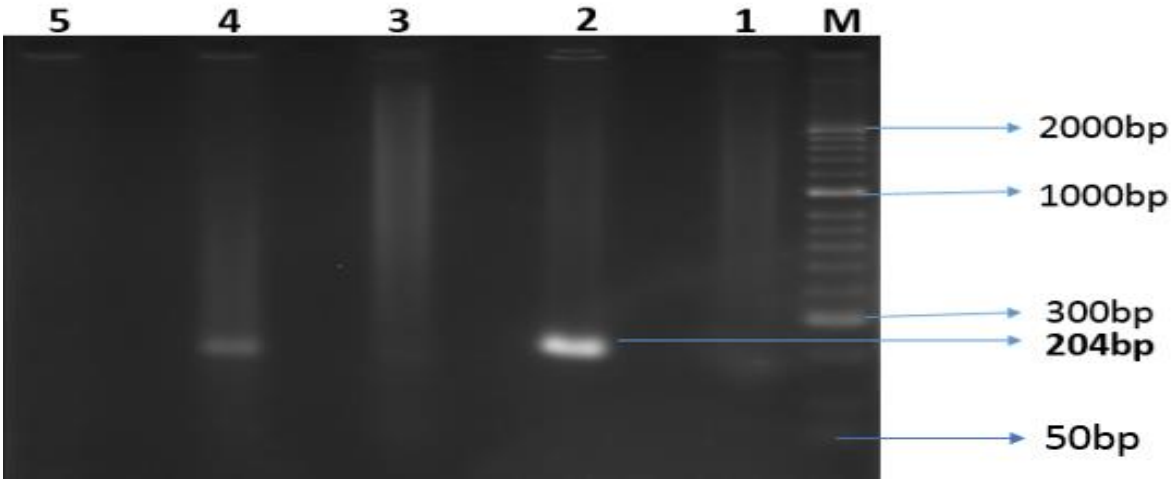

Figure 3: PCR amplification of 204 bp *S. agalactiae* analyzed by electrophoresis on a 2% agarose gel, Lane M: 100 bp DNA ladder, Lanes 2 and 4: PCR amplified 204 bp product of *S. agalactiae*. Lanes 1 and 3 none amplified samples. Lane -5: Negative control (NTC).

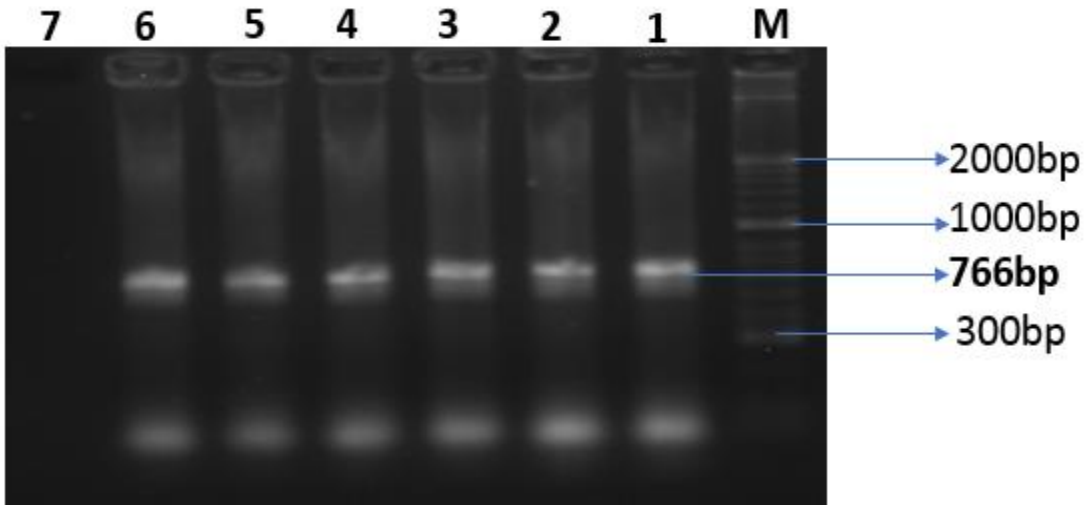

Figure 4: PCR amplification of 766 bp *S. dysagalactiae* analyzed by electrophoresis on a 2% agarose gel. Lane M: 100 bp DNA ladder, Lanes 1-6: PCR amplified 766 bp product of *S. dysagalactiae* and lane 7: Negative control.

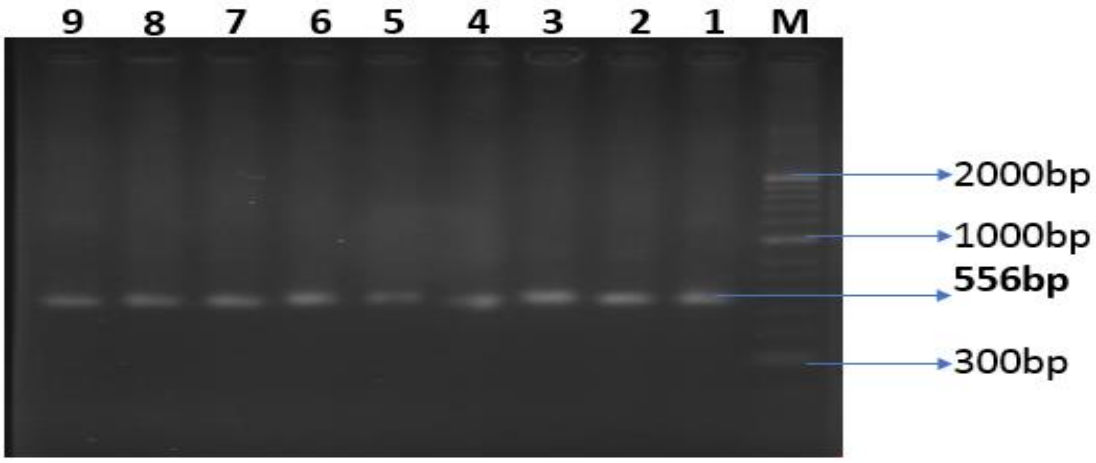

Figure 5: PCR amplification of 556 bp *S.aureus* analyzed by electrophoresis on a 2% agarose gel. Lane M: 100 bp DNA ladder, Lanes 1-9: PCR amplified 556 bp product of *S.aureus.*

4.4. Multiplex PCR assay

Multiplex PCR was optimized to allow for the species-specific primers to be used together in a single reaction mix to differentially amplify DNA from each of the four-bacterial species. Standardization of Multiplex PCR was initially started using the previously optimized condition for each bacterial target and combination of variable concentrations of primers and after several attempts. Accordingly, each mPCR reaction was performed using 2.5µL buffer (10X), 1.0 µL of dNTP (0.2mM), 2.0µL (0.5 µM) of each primers of

S. agalactiae and 2.5 µl (0.65 µM) of each of *S. aureus*, *S. dysagalactiae* and *E. coli* and 0.5µL (0.2 U) of Taq DNA polymerase, 4µl (2 mM) of $Mgcl_2$ and 1µL genomic DNA of each bacterium (during optimization) and 3µl total genomic DNA extracted from milk. Thermocycler conditions were: 96°C for 5 min; 35 cycles of 96°C for 1 min, 63°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 10 min.

4.5. Multiplex PCR and primers

When used in conjunction with all four primers in the optimization of mPCR the species-specific primers yielded amplicons of 700, 566 and 204 bp from *E. coli*, *S. aureus* and *S. agalactiae* respectively with the exception of *S. dysagalactiae* (766 bp) which was absent in the mPCR product (figure-6). Once optimized at the stated conditions, these primers consistently gave a single amplicon of the specified size from each species. Even though an extra non-specific band was observed in the PCR product occasionally, this band was always lighter than the specific band and did not occur at the same position as that from one of the other primers. It, therefore, never interfered with interpretation of the results.

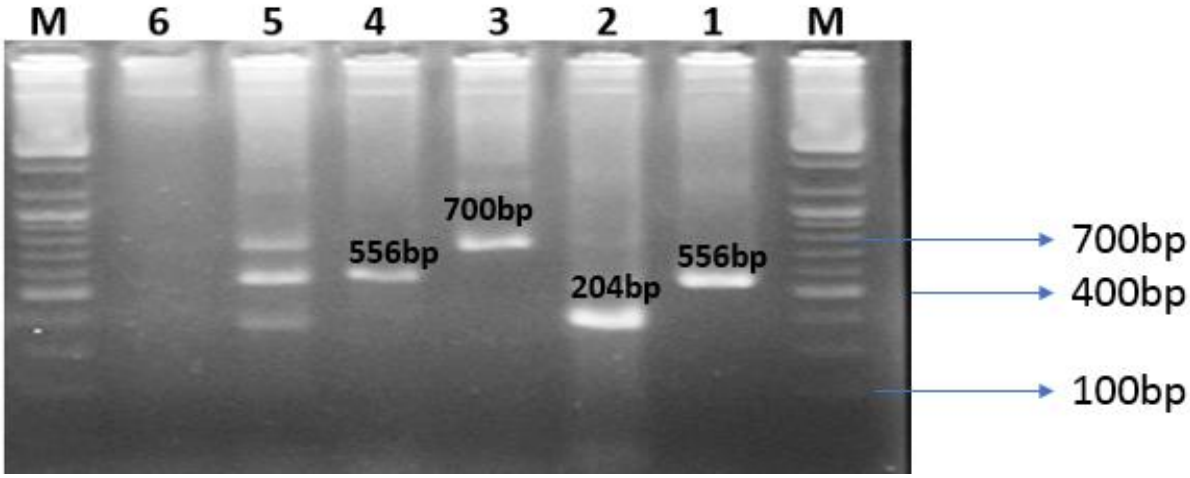

Figure 6: Amplification products of the different primer combinations were analyzed by electrophoresis on a 2% agarose gel. Lane M: 100-bp DNA ladder; Lane 1: *S. aureus*, Lane 2: *S.agalactiae*, lane 3: *E.coli*, Lane 4: *S. aureus* and Lane 5: Mixture of these 4 primers pairs amplified specific genes from the DNAs from the 4 organism; lane 6: Negative control.

4.6. Detection limit of PCR for each organism

The lowest detectable concentration of bacteria was determined for each bacterial species. The detection limit for *S. aureus* (figure- 7, lane 5) and *E. coli* (data not shown) was 10^3 CFU/ ml. For *S. agalactiae* the detection limit was 10^4 CFU/ml (figure-8). Agarose gel

electrophoresis demonstrated that all PCR results have a unique and expected amplification product. With increasing dilution, the intensity of DNA bands decreased and completely disappeared at 10^2 CFU/ ml (in case of *S. aureus* and *E. coli*) and at 10^3 CFU/ ml in case of *S. agalactiae.*

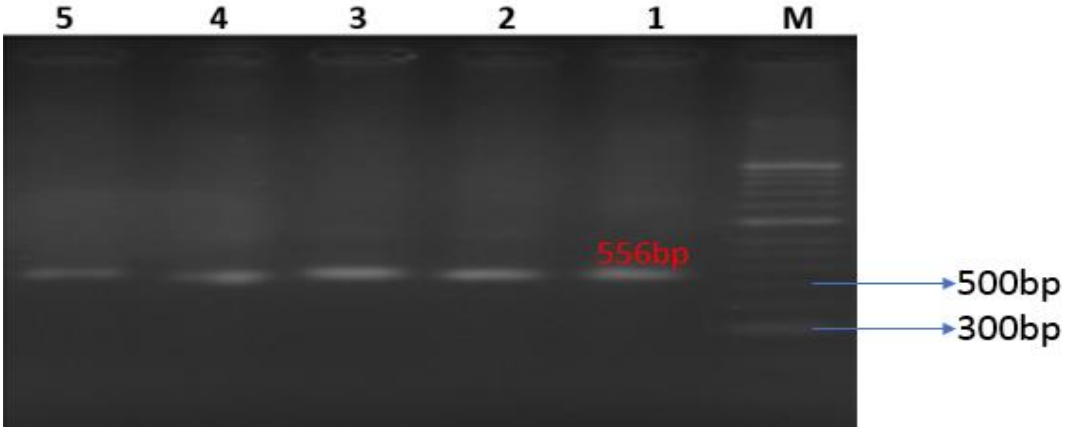

Figure 7: Sensitivity of *S. aureus* on Agarose gel electrophoresis illustrating the sensitivity of mPCR test using the combination of primers for each bacterium. Lane M: 100 bp ladder, Lane 1: sample with 10^7 cfu/ml of bacteria, lane 2: sample with 10^6 cfu/ml of bacteria, lane 3: sample with 10^5 cfu/ml of bacteria, lane 4: sample with 10^4 cfu/ml of bacteria, lane 5: sample with 10^3 cfu/ml of bacteria.

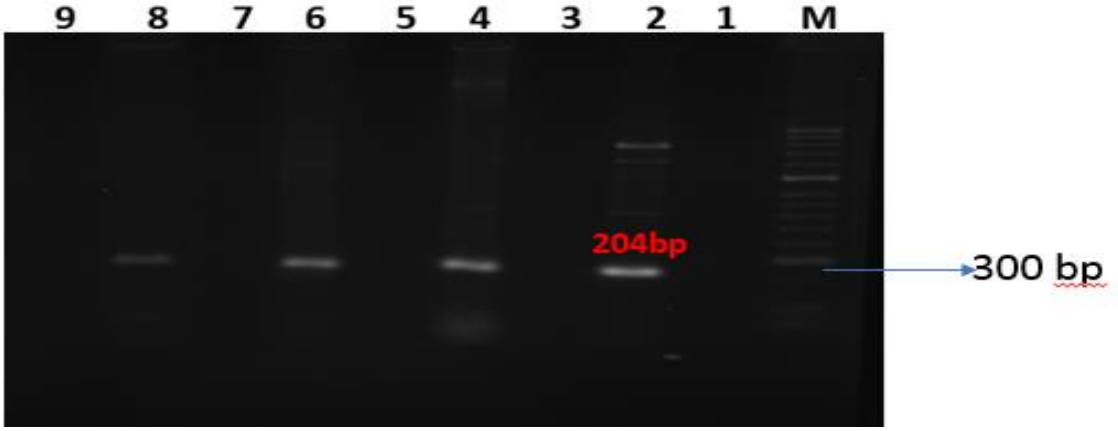

Figure 8: Sensitivity of *S. agalactiae* on Agarose gel electrophoresis illustrating the sensitivity of mPCR test using the combination of all primers for each bacterium. Lane M: 100 bp ladder, Lane 2: sample with 10^7 cfu/ml of bacteria, lane 4: sample with 10^6 cfu/ml of bacteria, lane 6: sample with 10^5 cfu/ml of bacteria, lane 8: sample with 10^4 cfu/ml of bacteria, lane 9: sample with 10^3 cfu/ml of bacteria, lanes 1, 3, 5, 7 and 9 negative controls.

4.7. Testing of the multiplex PCR

To test the applicability of the developed mPCR protocol, a trial of the mPCR was implemented with the use of randomly collected milk samples (n=20). Total genomic DNA was extracted directly from milk using a method described in Subheading 4.3.2. and used as a template in the mPCR.

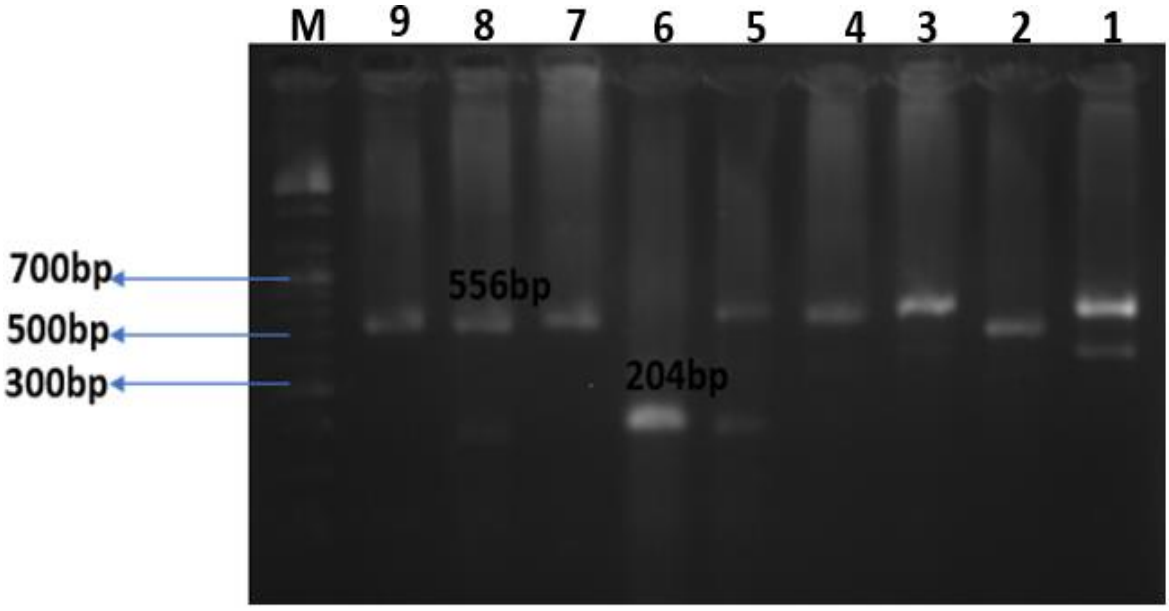

Figure 9: Multiplex PCR products amplified from DNA extracted directly from milk. Lane 1: *E. coli* and *S. aureus*; Lane 2: *S. aureus*; Lane 3: *E. coli*; Lane 4: *S. aureus*, lane 5: *S. aureus* and *S. agalactiae*; Lane 6: *S. agalactiae*; Lane 7: *S. aureus*; Lane 8: *S. aureus* and *St. agalactiae*; Lane 9: *S. aureus*; Lane M: 100-bp DNA ladder.

4.8. Comparison of conventional culture method and mPCR assay

For comparison of mPCR and cultural methods randomly collected milk samples (n=20) were used to identify the bacterial pathogens found in milk by using the two techniques. From the bacteriology, majority of pathogens isolated with respect to the target bacteria were *S. aureus* 10 (17%), *E. coli* 7 (11.8%), *S. agalactiae* 5 (8.5%), *S. dysagalactiae* 3 (5 %). Frequency of bacterial isolates from milk samples (n=20) using microbiological culture are presented in table 6.

The number of positive samples identified by the mPCR were 25, including 12 *S. aureus* (60 %), 8 *S. agalactiae* (40 %), 5 *E. coli* (25%). But *S. dysgalactiae* was not detected with

mPCR. Results regarding the target microorganisms detection by culture and by mPCR are shown in table 6. In general, the detection level of 2 bacterial species (*S. aureus, and S. agalactiae*) was higher in mPCR compared to cultural methods. But cultural method was found to be more sensitive in detection of *E. coli* compared to mPCR. The sensitivity level for *S. dysagalactiae* was much higher in culture method since it was not detected using mPCR at all.

Table 5: Frequency of detection of *S. aureus*, *S. agalactiae*, *E. coli* and *S. dysagalactiae* in milk samples (n=20) using microbiological culture and mPCR.

Microorganism	**Microbiological culture**		**mPCR**	
	Positive	Frequency (%)	Positive	Frequency (%)
S. aureus	10/20	50	12/20	60
S. agalactiae	5/20	25	8/20	40
E. coli	7/20	35	5/20	25
S. dysagalactiae	3/20	15	0	0

According to the result obtained, there are a total of 25 target bacteria showing positive in mPCR test among the total milk samples examined. Twenty target bacteria which were positive in mPCR show the same results as in the culture-based method. Five PCR positive targets show no growth in a microbial culture. Other 5 culture positive target presented negative in PCR tests.

Table 6: Comparison of bacterial culture and PCR based detection

Assay		**Cultural method**		
		Positive	Negative	Total
mPCR method	Positive	**20**	5	25
	Negative	5	**50**	55
	Total	25	55	80

P_0= 0.87, P_e= 0.57, K= 0.7

The diagnosis sensitivity is 80 % and the specificity is 99.9%. Kappa test was performed to compare the agreement of the results from mPCR with the results of bacterial culture. The results revealed that the mPCR diagnosis of mastitis pathogens presented good consistency in comparison with in vitro culture (kappa = 0.7), i.e. mPCR method has a strong discriminating ability to some degree in identifying the major mastitis pathogens from milk as the method of bacterial culturing can do.

Table 7: Sensitivity, Specificity, Positive and negative Predictive Values, Accuracy, Kappa Coefficient and p-value obtained using a multiplex PCR protocol compared to microbiological culture.

STATSTIC	**VALUE**
Sensitivity (%)	80
Specificity (%)	90.9
Positive predictive value (%)	80
Negative predictive value (%)	90.9
Accuracy (%)	87.5
Kappa test	0.7
P-value (%)	31.25

4.9. Comparison based on time required for test results

The mPCR was applied for the assessment of random milk samples, and the results showed that mPCR tool was rapid enough taking 7 hours to assess the samples; in contrast to traditional bacteriological methods that require a minimum of five days (Table 9)

Table 8: Summary on time requirement for both mPCR and cultural methods.

mPCR method		**Cultural method**	
Method	**Time required**	**Method**	**Time required**
DNA extraction	3 hrs.	Media preparation and culturing	2 days
Mastermix preparation	30 min	Sub culturing and primary identification	1 day
PCR amplification	2hr 30 min	Biochemical tests	2 days
Electrophoresis	1 hr.	_____	
Total	7 hours	_____	5 days

5. DISCUSSION

For designing mPCR assay, it was essential that the amplicons, besides being specific, should differ in length by at least 40–50 bp from each other so that they are clearly distinguishable after agarose gel electrophoresis (Shome *et al*., 2011). In this investigation, four pairs of oligonucleotide primers were designed to simultaneously detect four different types of mastitis pathogens by multiplex PCR in a single tube. The primer pairs showed significant affinities only for their target genes. To facilitate PCR product detection, the primers were designed so that the expected size of the products from each target gene would be different to permit size discriminated by gel electrophoresis. The BLAST result of each primer demonstrated the amplification region were 99-100% homology with the query sequence found in the NCBI database. The results revealed that the given primers could only amplify the 16S-rRNA.

All four PCR primers were designed to have similar melting temperatures. In agreement with the findings of Henegariu *et al*. (1997), the relative concentrations of the primers were found to be the most important factor in determining approximately equal yields of amplification products from each of the organisms in a single reaction. One possible explanation of this result might be the different copy numbers of the 16s RNA within the different bacterial species. Little is known about the important factors and common difficulties influencing a multiplex PCR. Other critical factors in multiplex PCR include the concentration of the PCR buffer, the balance between the magnesium chloride and deoxyribonucleotide triphosphate concentrations, and the cycling temperatures (Henegariu *et al*., 1997).

The species-specific primers for *E. coli*, *S. aureus, S. agalactiae* and *S. dysagalactiae* were designed based on a DNA sequence coding for 16S rRNA and yielded amplicons of 700bp for *E. coli*, 556bp for *S. aureus*, 204bp for *S. agalactiae* and 766bp for *S. dysagalactiae*. but these amplicons sizes were not the same with the previous works of Troncarelli *et al*. (2015), who found amplicon sizes of 293bp, 660bp and 1300bp for amplification *S. agalactiae, E. coli* and *S. aureus* respectively, and Shome *et al*. (2011) who found amplicon sizes of 317bp and 572bp for amplification of *S. agalactiae* and *S. dysagaalactiae* respectively. Possible reasons for this different in amplicon size might be due to the

different copy numbers of the 16s RNA within the different bacterial species or influence the GC% on the melting temperature of the primers.

5.2. Detection limit

The newly developed mPCR showed a minimum detection limit of 10^3 CFU/ ml for *S. aureus* which is similar with sensitivity of previously reported assays (Phuektes *et al*., 2001; Lee *et al*., 2008), who reported that level of sensitivity for the detection of *S. aureus* in milk by PCR and by biochip was in the range of 10^3–10^5 CFU/ ml. Detection level of *S. agalactiae* is still lower than previous reports. In another study, the detection limit of multiplex PCR assay described for the detection of mastitis pathogens was reported to be 10^3–10^4 CFU/ml) (Gillespie and Oliver, 2005).

In the present study there were no differences in the detection limit between individual PCR and mPCR for detection of all bacteria and this was in agreement with Troncarelli *et al*. (2015) and Riffon *et al*. (2001).

Another study by Phuektes *et al*. (2001), needed enrichment in a multiplex PCR for detection of *S. aureus* and *Streptococcus spp* (*S. agalactiae*, *S. dysgalactiae*, and *S. uberis* to detect levels of 1 CFU/ml, but it is more time-consuming.

In addition to the above findings, Kalin *et al*. (2017) found a minimum detection limit of 10 CFU/mL in milk samples using the commercial DNA isolation kit compared to standard DNA extraction methods which was found to be $6x10^1$ CFU/ml.

According to Wu *et al*. (2008), there are several factors that can cause lower sensitivity of PCR. The characteristics of chemicals used during extraction may cause several problems. Firstly, the DNA molecule can be degraded which may decrease the DNA concentration of extract (PCR template). Secondly, the chemical substances used during extraction can also change the pH value and DNA configuration, thus causing reduction in sensitivity.

The specificity of the PCR reaction is possibly interfered with the milk components and the amount of DNA extracted from somatic cells which greatly increase in mastitis (Henegariu *et al*., 1997).

The sensitivity of PCR for the detection of bacteria in liquid milk can also be affected by protein and calcium ion when the crude DNA extracted from bovine mastitis milk to be used as a template. A high concentration of casein and calcium ion can interfere with the activity of Taq- polymerase in PCR. The sensitivity is still likely increased by modifying extracting protocol and reducing the dilution volume (Wu *et al*., 2008; Riffon *et al.*, 2001).

Approaches used to improve the sensitivity of multiplex PCR assays include methods to improve the sensitivity of the reaction, such as nested PCR or methods to improve the sensitivity of product detection, such as hybridization with radiolabeled probes. However, such approaches increase the expense, labor and time for analysis. Nested PCR assays are also subject to contamination problems due to their high sensitivity and the need to open reaction tubes. It has been suggested (Moe *et al*., 1994) that their use minimized for routine diagnosis.

5.3. Multiplex PCR and primers

When used in conjunction with all four primers in the optimization of mPCR the species-specific primers yielded amplicons of 700, 566 and 204 bp from *E. coli*, *S. aureus* and *S. agalactiae* respectively with the exception of *S. dysagalactiae* (766 bp).

The absence of positive results for *S. dysagalactiae* in milk samples by mPCR might be due to inefficient DNA extraction methods affecting the recovery and detection of bacterial DNA from samples, the concentration of *S. dysagalactiae* might be lower than the detection limit of the PCR assay in milk and incorrect diagnosis of or recording in culture (Steele, 2015). Other possible reasons for the failure might be due to the various PCR Inhibitors that are common in clinical specimens (Kim *et al*., 2001).

The present study is in agreement with Yamagishi *et al*. (2007). That is molecular detection of *S. dysagalactiae* or other species in milk samples was described to be more accurate when simplex PCR is used.

One of the problems often encountered with multiplex PCR is a reduction in sensitivity compared with simplex PCR. This may be due to the competition between individual reactions for dNTPs and Taq polymerase when multiplex primer sets are combined in a

single reaction (Madico *et al*., 2003). In addition, the DNA region to be amplified in *streptococci* spp could be difficult compared to the region in *staphylococci*. If, for example, the GC% of the DNA regions of *S. dysagalactiae* is higher than the other species amplified by the primers, the reagents can be competitively consumed by DNA regions that are easier for amplification since high GC% is usually difficult for PCR amplification.

The GC% of the region amplified in *streptococci* (53%) was higher when compared to the region amplified in *Staphylococci* which was 51%. Therefore, this might be one possible reason for the failure of amplification of *S. dysagalactiae* in mPCR.

This is also in agreement with Pheuktes *et al*. (2001), where it was shown that multiplex PCR may be linked with a reduction in analytical sensitivity (10-100 fold lower) when compared with simplex PCR, using the same primers for each target.

5.4. Comparison of culture and PCR based detection methods

In both conventional and molecular methods higher occurrence of *S. aureus* was found in the present study which might be due to persistent cow to cow spread, possibly by the hands of milkers. This is in agreement with the finding of Su *et al*. (2000), that *S. aureus* and *S. agalactiae* are considered as significant organisms associated with clinical and subclinical bovine mastitis worldwide. In addition to an infected quarter which is the main reservoir, *S. aureus* can also be isolated from the skin of the udder and teats, as well as many other sites in dairy cows, feed and caretakers (Larsen *et al*., 2000). But the present study is distinct from Bradely . (2002); Khaled *et al*. (2010) and Anjali and Kashyap (2017), who have reported higher prevalence of *E. coli*.

E. coli was also encountered in a higher percentage in the examined milk next to *S. aureus*. This agrees with the findings of Dopfer *et al*. (2000), who considered *E. coli* organisms as one of the major etiological agents of clinical mastitis.

The result of the present study showed that the simplex and multiplex PCR were more sensitive than culture-based detection of *Staphylococcus aureus*, and *Streptococcus agalactiae* in milk. Similar results were reported by Riffon *et al*. (2001), Yamagishi *et al*. (2007) and Amin *et al*. (2011). This may be attributed to the fact that PCR detects living

and dead organisms, since it is based on detection of organism DNA, while culture detects only living organisms.

The multiplex PCR assay successfully detected majority of bacteria, as isolated in culture method. Besides, mPCR detected organisms in samples found culture-negative as well as samples showing no growth. Culture-negative milk samples represent a large proportion of samples in conventional bacteriology (Taponen *et al*., 2009).

In conventional culturing, 25–50% of all samples from clinical mastitis harbor no bacterial growth (Makovec and Ruegg, 2003; Bradley*et al*., 2007). Besides, inflammation of the mammary gland, inhibitory metabolic products of bacteria, or unfavorable conditions during sample transportation can also decrease the viability of the mastitis bacteria (Dinsmore *et al*., 1992).

In contrast with Troncarelli *et al*. (2015), who has reported failure of mPCR in detection of *S. aureus* from milk sample, the present study was successful in detection of this bacterium.

Better detection levels for *S. dysagalactiae* were obtained by culture and simplex PCR when compared to the mPCR method for this specific pathogen. These data are different from the study obtained by Phuektes *et al*. (2001), who successfully detects *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* from subclinical mastitis cases using the mPCR. This disparity in results of simplex and multiplex PCR might be due to incompatibility of primers, suboptimal concentration of PCR components or method of DNA extraction.

However, a previous study by Shome *et al*. (2011) was able to detect 10 bacterial strains simultaneously by multiplex PCR.

The sensitivity of molecular method was 80%, which is higher than that obtained by Troncarelli *et al*. (2015). In addition, specificity value by mPCR of the present study was 90.9%, which is in the same line with previous studies, ranging from 96.3% to 100% (Gillespie and Oliver, 2005; Maxwell *et al*., 2008).

According to the Kappa test (K=0.7) there was a substantial agreement between mPCR and cultural diagnostic techniques. A better Kappa test value (K=0.9) was obtained in the similar study by Wu *et al*. (2008). But, the Kappa value of the present study was much higher than the value obtained by Troncarelli *et al*. (2015), that a fair agreement (K=0.3) was observed between the two diagnostic tests.

In general, the present study allows the detection of major mastitis pathogens in a single reaction using smaller amounts of reagents. Another advantage of this assay is that the whole process (DNA extraction, multiplex PCR, and gel electrophoresis) allows the detection and discrimination of major mastitis pathogens in approximately 7 hours in contrast to traditional bacteriological methods that require about five days.

6. CONCLUSION AND RECOMMENDATIONS

As correct species identification is important for mastitis treatment, prevention, control and in epidemiological investigations, as well as in understanding of the significance of infections caused by different bacterial species; this assay would prove to be an adequate tool for the identification of the most common mastitis pathogens, independent of their phenotypic characteristics for diagnosis. In this study, the number of positive samples identified by the mPCR were found to be higher compared to cultural methods. But cultural method was found more sensitive in detection of *E. coli* compared to mPCR. The overall sensitivity and specificity was 80% and 90.9% respectively and this was with a substantial agreement (K=0.7) with cultural methods.

In general, the result of the present study indicates the developed mPRC has the potential to be a rapid, sensitive, and specific diagnostic method for simultaneous identification of *S. aureus, E. coli* and *S. agalactiae* from milk samples, but unable to detect *S. dysagalactiae*. Use of such rapid, sensitive and specific method may be helpful in the field studies of diagnosis and effective treatment, antibiotic selection and ultimately the control of mastitis.

Based on the above findings obtained, the following recommendations are forwarded:

- Large scale farms and clinics should use mPCR alternatively as a rapid and sensitive diagnostic technique of mastitis pathogens for both intervention and surveillance.
- New DNA extraction methods from milk with a performance of successfully removing PCR inhibitors should be developed.
- New mPCR protocols with a performance of amplifying many bacterial and viral mastitis pathogens should be developed.

- Further study should be carried out to improve the detection level of the mPCR.
- Further studies should be carried out to detect *S. dysagalactiae* and other important pathogens using mPCR from milk there by increasing the sensitivity of detection of molecular methods of diagnosis.
- Due to the limit of time and resources, the present study could not exceed to compare mPCR results from clinical and subclinical cases; therefore, a further research should be conducted.

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9. SIGNED DECLARATION SHEET

I, the under signed, declare that, this thesis is my original work, has not been presented for a degree in any university and all the sources of material used for this thesis have been duly acknowledged.

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Date of Submission: January, 2018

This has been submitted for examination with my approval as University advisor.

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