

# **APPLICATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) IN MICROBIOLOGICAL WATER QUALITY ANALYSIS**

By

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## DECLARATION

I declare that this thesis is my own work, and has not been presented for award of degree in any other institution of learning to the best of my knowledge

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## ABSTRACT

Water is life thus access to safe drinking water is essential to health and a basic human right. Kenya is considered a water scarce country whose water sources are becoming more contaminated through changes in land use and poor solid waste management. This has in turn affected the quantity and quality of portable water available. There are nearly 1.7 billion cases of waterborne diarrheal disease every year with over 760 000 deaths of children under five years. Traditional methods of identification of waterborne pathogens are less specific, sensitive, time-consuming and laborious, so there is a need for the development of innovative methods for their rapid identification. Recent advances in molecular cloning and recombinant DNA techniques have revolutionized the detection of pathogens in foods. In this study the application of a LAMP based technique for the rapid identification of the *Escherichia coli*, *Enterococcus faecalis* and *Clostridia perfringens* DNAs was undertaken. Suitable primers were designed based on specific gene *MalB* of *Escherichia coli*, *tuf* gene of *Enterococcus faecalis* and *CPE* gene of *Clostridia perfringens* for amplification. Selective media for the various indicator microbes were used and specific primers for both LAMP and PCR designed. The study reveals that bottled water from Majesty and Starpop companies were contaminated by coliforms while the ground water from Kikuyu Springs, rainwater from Lang'ata and tapped water from Naivasha's Fishermans campsite were contaminated by the three target bacteria. This was possible via culture work as LAMP and PCR assays were not fully optimized to enable the detection of *Escherichia coli*, *Enterococcus faecalis* and *Clostridium perfringens* DNAs. The study shows LAMP as a viable tool in microbial water quality analysis from the little amplification achieved with the positive controls that had high DNA concentrations

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## TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENT.....	iii
LIST OF FIGURES .....	vii
LIST OF TABLES.....	viii
CHAPTER ONE .....	1
1.0 INTRODUCTION .....	1
1.1 RATIONALE AND JUSTIFICATION .....	4
1.2 OBJECTIVES AND HYPOTHESIS .....	5
1.2.1 General objective .....	5
1.2.2 Specific objectives .....	5
1.2.3 HYPOTHESIS .....	5
CHAPTER TWO .....	6
2.0 LITERATURE REVIEW .....	6
Introduction.....	6
2.1 Sources of Water Pollution .....	7
2.2 Unsafe Water as a Vehicle of Diseases.....	7
2.3 Determining the water quality.....	9
2.4 Importance of using indicator microbes.....	10
2.5 Common Indicator Microbes tested for water contamination.....	10
2.6 Methods of Microbial Analysis.....	13
2.7 Advantages of molecular techniques over the culture based techniques .....	14
2.8 Principle of LAMP.....	14
2.9 Selection of genes for detection of bacterial water contamination .....	18
CHAPTER THREE .....	20

3.0 MATERIALS AND METHODS.....	20
3.1 Sampling .....	20
3.2 Lab processing .....	20
3.3 Molecular Analysis .....	22
CHAPTER FOUR.....	27
4.0 RESULTS .....	27
4.1 Positive controls Confirmation .....	27
4.2 Optimizations for PCR and LAMP methods .....	29
4.2.1 LAMP Assays .....	30
4.2.2 PCR Assays.....	32
4.2.2.1 Optimization of conventional PCR assays for detection of <i>Enterococcus faecalis</i> DNA.....	32
4.2.2.2 Optimization of Touch-down PCR for detection of <i>Clostridium perfringens</i> DNA.....	33
CHAPTER FIVE .....	37
5.0 DISCUSSION .....	37
5.1 Various water sources .....	37
5.2 Culture results .....	38
5.2.1 Gram Staining .....	38
5.2.2 Detection of coliforms and <i>Escherichia coli</i> .....	39
5.2.3 Detection of enterococci and fecal <i>Streptococci</i> group.....	39
5.2.4 Detection of the sulphite reducing <i>Clostridia</i> group.....	40
5.3. Molecular techniques .....	41
5.4. Summary .....	41
CHAPTER SIX.....	43
6.0 CONCLUSION AND RECOMMENDATIONS.....	43
6.1 CONCLUSION.....	43

APPENDICES .....	51
Appendix 1: List of common pathogens found in water .....	51
Appendix 2: Standards and guidelines for various water parameters in drinking .....	52
Appendix 3: Details of water samples collected, their costs and classification by water source.....	53

## LIST OF FIGURES

Figure 1: Diagram illustrating the principle of LAMP

Figure 2: *Clostridium perfringens* at magnification X100

Figure 3: *Escherichia coli* at magnification of X40

Figure 4: *Enterococcus faecalis* at magnification X40

Figure 5: Genomic DNA of *Escherichia coli* and *Enterococcus faecalis* isolates extracted using FastDNA™ SPIN Kit for Soil

Figure 6: A photo of 1.5% agarose gel showing optimization of LAMP assay with designed primers at temperature range of 60-65 °C

Figure 7: A 1.5% agarose gel image showing the optimization of *Enterococcus faecalis* at temperature range of 60 - 65 °C

Figure 8: A 1% agarose gel image showing the gradient PCR done on *Enterococcus faecalis*

Figure 9: A 1% agarose gel image showing touchdown PCR on *Clostridium perfringens*



## LIST OF TABLES

Table 1; Primers used for PCR assays for *Escherichia coli*, *Enterococcus feacalis* and *Clostridium perfringens* detection

Table 2 Primers used for LAMP assays for *Escherichia coli*, *Enterococcus feacalis* and *Clostridium perfringens* DNA detection

Table 3: DNA concentration from Water samples extracted using FastDNA™ SPIN Kit for Soil

Table 4: Water Samples tested by culturing on selective media and pH readings

## LIST OF ABBREVIATIONS

<b>BIP</b>	Backward Inner Primer
<b>BPA</b>	Bisphenol A
<b>Bsn</b>	<i>Bacillus subtilis</i> BSn5
<b>Bst</b>	<i>Bacillus stearothermophilus</i>
<b>DNA</b>	Deoxyribonucleic acid
<b>dsDNA</b>	Double Stranded Deoxyribonucleic Acid
<b>FIP</b>	Forward Inner primer
<b>ISA</b>	Iron Sulphate Agar
<b>KAAA</b>	Kanamycin AesculineAzide Agar
<b>KEBS</b>	Kenya Bureau of Standards
<b>KNBS</b>	Kenya National Bureau of Statistics
<b>LAMP</b>	Loop Mediated Isothermal Amplification
<b>LB</b>	Loop primer backward
<b>LF</b>	Loop primer forward
<b>LP</b>	Loop primers
<b>PET</b>	Polyethylene terephthalate
<b>ssDNA</b>	Single Stranded Deoxyribonucleic Acid
<b>VBNC</b>	Viable but non-cultivable
<b>VRB</b>	Violet Red Bile agar
<b>WHO</b>	World Health Organization

## CHAPTER ONE

### 1.0 INTRODUCTION

Access to safe drinking-water is a component of effective policy for health protection. There are approximately one billion people lacking access to this precious commodity and over 2.5 billion without adequate sanitation services (WHO & UNICEF, 2006) The statistics constitute half of the developing world without water and more than 35% of the world's population (UN, 2013).

The health effects of drinking contaminated water can range from no physical impact to severe illness or even death. Some of these effects can be immediate, while others may not be noticed for many years. There are several factors that affect the possible impact on health such as the age and general health status of the person, the type of contaminant, the amount, and how long the person has been drinking the contaminated water. These health effects can include gastrointestinal, respiratory infections, trachoma, helminthes and skin infections and stomach illnesses like nausea, vomiting, cramps, and diarrhea.

Diarrhea occurs world-wide and causes 4% of all deaths and 5% of health loss to disability. It is most commonly caused by gastrointestinal infections which kill around 2.2 million people globally each year, mostly children in developing countries (WHO, 2000). The use of clean water in hygiene is an important preventive measure but contaminated water is also an important cause of diarrhea as it spreads a host of bacterial, viral and parasitic organisms.

Simple hygiene behaviors, especially hand washing with soap, improved sanitation, as well as safe food and water handling practices are key. They help prevent contamination of treated, safely stored drinking water and reduce the risk of waterborne, foodborne, and person-to-person transmission of diarrheal diseases. They have been suggested to significantly reduce the above mentioned illnesses in poor settings like Karachi (Fung & Cairncross, 2009) and in Kenya (Schmidt *et al.*, 2009); (Luby *et al.*, 2005)

Water quality is affected by changes in nutrients, sedimentation, temperature, pH, heavy metals, non-metallic toxins, persistent organics and pesticides, as well as the biological factors (Palaniappan *et al.*, 2010). Fecal pollution causes alarm in relation to water bodies used for drinking water supply, recreational activities and harvesting seafood due to likely exposure to a wide array of pathogenic bacteria, protozoa and viruses as shown in the Appendices (Ahmed *et*

*al.*, 2009).

Various sources which are affected by both human and natural activities such as sewage treatment plants, agricultural run-off, wild animals, defective on-site wastewater treatment systems and industrial wastewater outlets are known to be potential sources of water pollution. The microbial pathogens found in water and wastewater are difficult, time consuming and expensive to detect, isolate and identify when done regularly. To deal with this problem microbiological quality of water is generally assessed by enumerating fecal indicator bacteria such as *Escherichia coli* and *Enterococci spp.* which are commonly found in the feces of warm-blooded animals including humans (USEPA 2000a). Indicator microorganisms, commonly faecal coliforms, are used to determine the relative risk of the presence of pathogenic microorganisms in a sample. Lack of detection of the common indicator organisms used in water quality analysis does not imply the water is necessarily safe from Protozoa and viruses. This has been demonstrated by outbreaks of cryptosporidiosis in Clark County, Nevada, Canada in water previously determined free of all the indicator microorganisms (Hellard, 1997).

The presence of these indicators in water bodies generally points to fecal pollution and potential public health risks, however, their identification is important in the implementation of appropriate mitigation strategies (Scott *et al.*, 2003). This is normally done using culturing procedures.

Culturing procedures are laborious and involve the use of enrichment and selective media in an attempt to isolate pathogens from background bacteria. It is often difficult to achieve appropriate enrichment, which makes the work even more tedious. Moreover, concentrations may be too low for cultural detection but are high enough to cause infection. Therefore, a molecular detection method is needed for detection, identification, and characterization, since such methods are highly specific and sensitive.

Some of the molecular techniques employed include Nucleic Acid Sequence-Based Amplification (NASBA), polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) with PCR being the most preferred technique (Keer and Birch 2003). Most of these methods consist of concentration of the organism of interest from the water sample, extraction of the nucleic acid (RNA or DNA) from the target organism, amplification of the genomic segment(s) chosen and quantification of the amplified genomic segment(s) (Girones *et al.*, 2010). New advances have led to the use of methods that amplify the nucleic acid material at isothermal conditions and this include techniques like Loop mediated Isothermal Amplification (LAMP), Helicase -dependent amplification (HDA), rolling circle amplification (RCA) (Michaela *et al.*,

2012), multiple displacement amplification (MDA) and recombinase polymerase amplification (RPA) (Laura and Giuseppe, 2013).

Kenyans use water from varied sources that are likely to provide water suitable for drinking and are identified as improved sources. These include a piped source within the dwelling or plot, public tap, tube well or borehole, protected well or spring, and rainwater. Lack of ready access to a water source may limit the quantity of suitable drinking water that is available to a household as contamination may occur during transportation. Home water treatment can be effective in improving the quality of household drinking water.

Less than half of Kenyan households (45%) treat their drinking water. The main method of treatment is boiling (29% of households), while 18 % of households add bleach or chlorine to make the water portable. Appropriate water treatment methods are more common among urban households (57%) than among rural households (40%) (Kenya National Bureau of Statistics and ICF Macro, 2010). Thus the need for a rapid, cheap, sensitive, and selective diagnostic technique remains an essential requirement for water quality analysis (Rompre *et al.*, 2002).

In this study the use of convectional technique in microbial water quality analysis i.e. growth of target organism on selective culture media was compared to use of PCR and Loop Mediated Isothermal Amplification (LAMP) assays. This was important as the latter techniques took a shorter time to yield results.

## 1.1 RATIONALE AND JUSTIFICATION

The currently used methods of bacterial detection rely on culturing techniques that either measure a metabolic endpoint or determine growth of a microorganism after an extended incubation period. These are restrictive as there is inability to identify organisms that have a viable but non-cultivable state (VBNC) as it is with *Enterococcus faecalis*. The method's also time consuming, and the media used are only selective for particular microbes. This makes the determination and enumeration of contaminants in water supplies a daunting task and in most cases contamination is detected long after the water has been consumed.

Microbial water quality often varies rapidly and over a wide range. Short-term peaks in pathogen concentration may increase disease risks considerably and may trigger outbreaks of waterborne diseases. Furthermore, by the time microbial contamination is detected, many people may have already been exposed. It is for this reason that rapid and specific techniques in suspected cases of contamination have to be put in place to reduce chances of further infection to water consumers.

New molecular methods that allow direct measurement of cellular properties without incubation are becoming available and have the potential to reduce the measurement period to about one hour. These new technologies also allow for the expansion of the number and types of microbiological indicators that can be measured. The methods are not only sensitive but can differentiate different strains of the same microbe.

In addition to simplicity of the technique used, the sensitivity of the method should be high enough to identify more than the traditional indicator microbes used even in their limited numbers in the water samples. Therefore there is need for exploring a DNA detection strategy based on LAMP assay that is simpler and quicker than the conventional PCR methods, and is as sensitive and reliable as PCR (Hiroka et al., 2008).

LAMP reaction occurs under isothermal conditions that don't require expensive programmable thermocyclers, which are used in the other conventional PCR methods. In this regard the cost of machinery is reduced as only a heating block or a water bath is required.

## **1.2 OBJECTIVES AND HYPOTHESIS**

### **1.2.1 General objective**

To apply Loop Mediated Isothermal amplification assay as a rapid molecular method and compare it with the other conventional methods for analysis of microbial water quality analysis

### **1.2.2 Specific objectives**

- (i) To design and optimize a LAMP assay based for microbiological water quality analysis
- (ii) Determine the presence of *Escherichia coli*, *Enterococcus faecalis* and *Clostridia perfringens* in drinking water using LAMP.
- (iii) Determine the sensitivity and specificity of LAMP for microbial analysis for indicator species, in comparison with culture technique as well as the PCR method.

### **1.2.3 HYPOTHESIS**

LAMP based assay can detect *Escherichia coli*, *Enterococcus faecalis* and *Clostridium perfringens* that contaminate drinking water

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### Introduction

Water is essential to life. An adequate, safe and accessible supply must be available to all. Today different people utilize water from varied sources. Rainfall holds a vital role in the hydrological cycle and is identified as an important source of water that sustains life on earth. However there are challenges in achieving the appropriate quality and adequate quantity. Other water sources supplied by the rain water include the surface water (lakes, rivers and oceans) and underground water that results from the seeping of rainwater through the soil layers. These water sources are however polluted and there are efforts by many people to access safe drinking water. A clean and treated water supply to each house may be the norm in developed nations, but in developing countries, access to both clean water and sanitation to all, is yet to be achieved, and waterborne infections are common.

Uncollected garbage, overflowing latrines and non-functional residential and municipal drainage pipes plague poor people in urban areas while children are found playing in “latrine streams” filled with disease causing agents. Lack of access to safe water and toilets severely impacts on the lives of young girls and women who frequently travel many miles in search of water for their families (The World Watch Institute, 2007). It is estimated that more than a third of Africans practice open defecation. The effects of open defecation are serious; with urgent concerns of ground water resource pollution, contamination of agricultural produce. Consequently open defecation is a major contributing factor to a multiplicity of water and sanitation related diseases, such as diarrhea, cholera and typhoid. Not only is open defecation (and lack of improved sanitation facilities) detrimental to human health but also to economic and social development, e.g., the productive activities of impoverished populations, such as schooling, are severely restricted by ill health from contaminated water. Defecation into plastic bags and throwing into the bush, gutters and backyards is rampant in the slum dwellings. It has double negative impact on the environment i.e. pollution with plastic bags and human waste.



## **2.1 Sources of Water Pollution**

Contamination of drinking water could result from the agricultural sector, urban run-off/storm water, and municipal point sources which constitute the discharges from publicly owned waste water treatment plants. Contaminants from these sources include pesticides, metals, nitrates, solvents, with over 200 of such chemical components being documented in ground water alone (Palaniappan, *et. al.*, 2010).

In addition to this, water contamination by sewage can occur from raw sewage overflow, septic tanks, leaking sewer lines, land application of sludge and partially treated waste water. Sewage itself is a complex mixture and can contain many types of contaminants. Of all these, the greatest threats posed to water resources arise from contamination by bacteria, nitrates, metals, trace quantities of toxic materials, and salts. Overflow into drinking water sources can cause disease from the ingestion of microorganisms such as pathogenic *Escherichia coli*, *Giardia lamblia*, *Cryptosporidium parvum* Hepatitis A virus and helminthes (Health Canada, 2012).

## **2.2 Unsafe Water as a Vehicle of Diseases**

Safe drinking water is free of injurious chemicals or microbial contamination. Waterborne pathogens represent a serious and growing hazard, and infectious diseases continue to affect populations throughout the world. Other problems such as aging of water treatment infrastructures, and the increasing occurrence of organisms resistant to conventional disinfection treatments also increases the indices of these infections. Diarrheal illnesses remain the second most common cause of death among children under five globally, following closely behind pneumonia, the leading killer of young children. Together, pneumonia and diarrhea account for an estimated 40 per cent of all child deaths around the world each year. Nearly one in five child deaths is due to diarrhoea, a loss of about 1.5 million lives each year. The toll is greater than that caused by AIDS, malaria and measles combined. Africa and South Asia are home to more than 80 per cent of child deaths due to diarrhea. Fifteen countries account for almost three quarters of all deaths from diarrhea among children less than five years of age annually. (UNICEF/WHO, 2009)

Diarrhea kills more than three million people each year with the major diseases transmitted by water including cholera, typhoid, bacillary dysentery, infectious hepatitis, and Giardia (The Right to Water, 2003). On the other hand, major diseases caused by lack of water include scabies, skin sepsis and ulcers, yaws, leprosy, trachoma, dysenteries (WHO, “World Health Report” 2002). Unfortunately the water and sanitation crisis hits hardest the most vulnerable populations of the world’s poorest. It is estimated that half of the world population lives in abject poverty on less than \$2 per day. Water-related preventable diseases are killing these people and arresting development in their communities

### **2.2.1 Water Contaminants and Waterborne Diseases**

The level of pollution is so high that many of the drinking water sources including municipal water systems, wells, lakes, rivers are contaminated. Some brands of bottled water have even been found to contain high levels of contaminants in addition to plastics’ chemical leaching from the bottle i.e. Bisphenol A (BPA) and polyethylene terephthalate (PET) (Gleick, 2010). Scientific studies show that exposures to low doses of BPA are associated with a wide range of adverse health effects in later life. Most experts agree that the amount of BPA that could leach into food and drinks through normal handling is probably very small, but there are concerns about the cumulative effect of small doses. BPA has been linked to breast and uterine cancer, an increased risk of miscarriage, and decreased testosterone levels (Bosquiazzo *et. al.*, 2010; Sugiura *et. al.*, 2005). BPA can also wreak havoc on children’s developing systems (United Nations, 2009).

Of interest here however are the microbial contaminants. The greatest microbial risks are associated with ingestion of water that is contaminated with human or animal (including birds’) feces. This can occur due to lack of water treatment or even inadequate treatment resulting to waterborne diseases like gastroenteritis, cholera, hepatitis, typhoid fever and giardiasis. The causative agents of these diseases are bacterial and viral pathogens as well as protozoan parasites.

Contaminated water remains a concern and has been associated with the sporadic outbreak of diseases in Kenya and others in the world. Some reported cases of outbreaks here in Kenya include (a) case of dysentery caused by *Shigella dysenteriae* type 1 in rural western Kenya in the mid-1990s (Malakooti *et. al.*, 1997). (b) A cholera outbreak spreading across the country that mainly affected inmates from the Kamiti Maximum Prison resulting to 11 deaths and over fifty

admissions to hospitals in 2009. (c) More recent outbreaks of measles and waterborne diseases in the horn of Africa affecting Kenya and Ethiopia were reported by (Nordqvist, 2011). Other developing countries have also recorded cases of waterborne diseases outbreaks. In 1994, deaths of thousands of refugees from Rwanda occurred at the border limits between Rwanda and the Democratic Republic of Congo due to the spread of cholera which was caused by lack of clean water (Harry, 1998). After the 2010 earthquake in Haiti, an outbreak of cholera emerged that resulted in >385,000 infections and 5,800 deaths as of July 7, 2011 (Reimer *et al.*, 2011). Those at greatest danger of waterborne disease are children below 5 years of age, people who are incapacitated or living under polluted conditions and the elderly.

### **2.3 Determining the water quality**

There are several parameters looked into when determining status of water and these include; alkalinity, color of water, pH, taste and odor, dissolved metals and salts (i.e. sodium, chloride, potassium, calcium, manganese, magnesium etc). Others include microorganisms such as fecal coliform bacteria (*Escherichia coli*), *Cryptosporidium*, and *Giardia lamblia*, and metalloids (such as lead, mercury, arsenic, etc.), as well as dissolved organics and heavy metals (Health Canada, 2012).

There are several challenges when testing for water pathogens thus making the procedure impractical as a routine process for instance, the procedures may require concentration of a large water sample ( between 10-20 liters), the complexity of the task and the overall time consumed. This has resulted to the testing for the presence of an indicator bacterial species that would signal fecal contamination (Sharma *et. al.*, 2003).

The indicators are characterized by simply isolating them on selective media, and comparing them with the known pathogenic microbes. The indicator microbes are quite unique due to their ability to have resistance against disinfectants as compared to the pathogens, their inability to multiply in water, and are generally absent from other bacteria containing sources that get into contact with water e.g. vegetable matter, soils etc. Thus, the presence of the indicator organism will signal fecal contamination and the possible presence of pathogens, while its absence in both pre- and post-treatment water will suggest that the water is probably free of pathogens. Most of the non-pathogenic microbes found in water however don't exactly fit in all of the above criteria

but the coliform, *Escherichia coli*, are closely followed by other organisms such as the fecal *Streptococci*, and *Bacteroides* (Ashbolt *et. al.*, 2001).

Coliforms have routinely been used as indicators of fecal contamination since they are normal inhabitants of the mammalian gastrointestinal system. Previous studies such as those done by (Pierre and Franco, 1993) have shown that both somatic coliphages and *Clostridia perfringens* can be used as indicators for assessing the virological and parasitological qualities of treated drinking waters due to their survival in harsh conditions that results from the formation of spores. On the other hand, *Enterococcus faecalis* is frequently isolated from environmental waters and has recently been used as a human fecal indicator for microbial source tracking (Wheeler *et al.*, 2002).

## **2.4 Importance of using indicator microbes**

Despite the vast developments in modern microbiological techniques, routine detection of pathogenic bacteria, viruses, and protozoa in drinking water is not practical because of the varied pathogens in existence, their uneven distribution in water, and the overall time and expense associated with routine monitoring of all pathogens due to their culturing not being straightforward (Health Canada, 2012; Ashbolt *et. al.*, 2001). However, safe water demands that water is free from pathogenic bacteria. It is therefore easier to use a variety of indicators that are less difficult, less expensive, and less time consuming to monitor. This allows for use of a larger sample size which gives a better representation of the water quality and, therefore, better protection of public health.

## **2.5 Common Indicator Microbes tested for water contamination**

### **2.5.1 Coliforms**

These have been defined by World Health Organization as any rod-shaped, non-spore-forming, gram-negative bacteria capable of growth in the presence of bile salts or other surface-active agents. These groups of bacteria are cytochrome-oxidase negative and able to ferment lactose at either 35 or 37 °C with the production of acid, gas, and aldehyde within 24 to 48 hours. Further identification has been done by genus and species levels based on studies performed on  $\beta$ -galactosidase-positive Enterobacteriaceae (Ashbolt *et al.*, 2001). They are further subdivided to

Total coliforms and fecal coliforms. The fecal coliforms like *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* and *Citrobacter* are thought to have co-evolved with mammals, and have “learned” throughout evolution to utilize lactose and be resistant to bile acids. Microbiological media that contains these chemical is used to detect the presence of fecal coliforms in a water sample. (Teplitski & Butler, 2014)

### **2.5.2 *Escherichia coli***

*Escherichia coli* is a facultative anaerobic, Gram negative, non-spore-forming and rod shaped bacterium that belongs to the family Enterobacteriaceae. It is a member of the coliform group of bacteria found to naturally occur in the intestines of humans and warm-blooded animals. It has also been found to survive on plants or in soil and water (Nataro and Kaper, 1998). Interestingly, fecal concentrations of the typical non-pathogenic *E. coli*, used to indicate recent faecal contamination, will always be greater than those of the pathogenic strains, even during outbreaks (Health Canada, 2012).

The vast majority of waterborne *E. coli* isolates have been found to be capable of producing the enzyme  $\beta$ -glucuronidase (Fricker *et al.*, 2008a; 2008b), and it is this characteristic that currently facilitates their detection and identification. This facilitates the difference in color when *E. coli* is grown on Violet red bile agar to a pink coloration as compared to the other coliforms which appear white in color. To further confirm *E. coli* an indole test is performed.

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme. Detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye rosindole-the red ring observed.

The major drawback of relying on fecal coliforms or *E. coli* as indicators is that many of the same organisms have been found to be associated with plants or aquatic invertebrates. For example the common isolation of *Klebsiella pneumoniae* 342, a well-characterized beneficial bacterium that serves as a biological fertilizer for wheat and other plants. Nonetheless, positive coliform results should be regarded as an indicator of possible fecal contamination. (Teplitski,

2014)

### **2.5.3 *Enterococcus faecalis***

*Enterococcus faecalis* was formerly classified as part of the Group D Streptococcus system, a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. These organisms are considered one of the primary causes of nosocomial and environmental infections due to their ability to survive in the environment and to their intrinsic resistance to antimicrobials. Enterococci such as *Enterococcus faecalis* and *E. faecium* are considered as the most suitable indicators of fecal pollution in an aquatic environment. The two bacteria are associated with faecal pollution of water, linked to swimmer-associated gastroenteritis and demonstrate a wide range of antibiotic resistance. The organism has been the conventional indicators of fecal pollution in an aquatic environment because of their long-term survival and low growth rate in such an environment. Enterococci are also easy to analyze during the determination and characterization of pathogen indicators in an aquatic environment. (Manero *et al.*, 2002; Koide *et al.*, 2007)

### **2.5.4 *Clostridium perfringens***

*Clostridium perfringens* was initially referred to as *Bacillus perfringens* and then *C. welchii*. It falls under the genus *Clostridium* and is a member of the sulphite-reducing clostridia. It is a Gram-positive, anaerobic spore-forming rod that reduce sulphite to sulphide, and is capable of fermenting lactose, reducing nitrate and liquefying gelatin.

The bacteria, like most of the members in the *Clostridium* genus, flourishes in the absence of oxygen. They are found inhabiting soil, water as well as decomposing plant and animal material since they are saprophytes. *Clostridium perfringens* is commonly found in human and animal faeces. It produces environmentally resistant spores that survive in water and in the environment for longer periods than the vegetative cells of *E. coli* and other faecal indicators. Its removal from water is achieved by coagulation and filtration, but the spores of these bacteria can be resistant to chlorine at concentrations normally used in water treatment (Ashbolt *et al.*, 2001).

Since this bacteria is generally present in faeces in much lower numbers than *Escherichia coli*

and *Enterococci*, it is less sensitive as an indicator of faecal contamination (Environment Agency, 2010). Low numbers may occasionally occur in water supplies, but they do not represent a risk to health. These bacteria will not grow to significant numbers, or produce toxins, in water supplies, as conditions are usually unsuitable.

The genus *Clostridium spp*, whilst consisting mainly of saprophytes, contains some species which are regarded as opportunistic pathogenic bacteria that produce toxins that causes illness e.g. *Clostridium botulinum* and *Clostridium perfringens* have also been associated with food poisoning, and some strains of *Clostridium perfringens* can produce severe but self-limiting diarrhea in humans and animals if ingested in large numbers (Environment Agency, 2002)

### **2.5.5 Others Indicator Organisms**

Apart from the above mentioned bacteria others used in water monitoring include *Pseudomonas aeruginosa*, often occurring in human feces in much lower numbers than coliform organisms, frequently are found in untreated water in the presence of coliform, *Staphylococcus aureus*, *Shigella* (Kenya Standards 1996) coliphages which are bacteriophages that infect coliform bacteria. Phages are valuable prototypes for enteric viruses because they share many underlying properties and features notably composition, morphology, structure, size and site of replication. In addition, their resistances against environmental factors make coliphages more applicable than faecal bacteria for indicating faecal contamination of water (Grabow 2001; Lin & Ganesh, 2013).

### **2.6 Methods of Microbial Analysis**

Traditional approach has relied on culturing the bacteria which is a laborious procedure that involves enriched and selective media in an effort to isolate the organisms from background bacteria and also to determine concentration of bacteria in the water sample. Achieving appropriate enrichment is often difficult thus making the work even more tedious. There has been an emergence in the use of molecular techniques especially when testing for water pathogens. This is because pathogen concentrations sometimes may be too low for cultural detection but still high enough to cause infection (Girones *et al.*, 2010).

Molecular techniques that involve direct DNA detection do have the advantages of giving an absolute indication of the presence or absence of target organism and are now being used in water analysis. They are being adopted also for their sensitivity, rapidity and quantitative analytical characteristics for studying specific pathogens, including new emergent strains and indicators. They can be used to evaluate the microbial quality of water, the efficiency of pathogen removal in drinking water and wastewater treatment plants. Examples of these techniques include immunological assays like ELISA while nucleic acid based amplifications methods comprise of PCR and the in situ hybridization (ISH) methods (Annie *et. al.*, 2002).

## **2.7 Advantages of molecular techniques over the culture based techniques**

- 1) Through molecular techniques, non-cultivable microbes can be identified as the cultivation techniques only allow growth of microbes selected for by that specific growth medium.
- 2) The time required to get results is much less when the molecular techniques are used since it takes a few minutes to a few hours unlike some cultural techniques that take days to give results
- 3) Specificity is much higher in the use of molecular techniques especially those relying on amplification of DNA as the primers used are highly specific.

These molecular methods have a few limitations when it's direct nucleic acid (DNA or RNA) detection. No isolate is recovered as one can only detect short stretches of DNA, thus the amount of genetic information obtained is limited.

## **2.8 Principle of LAMP**

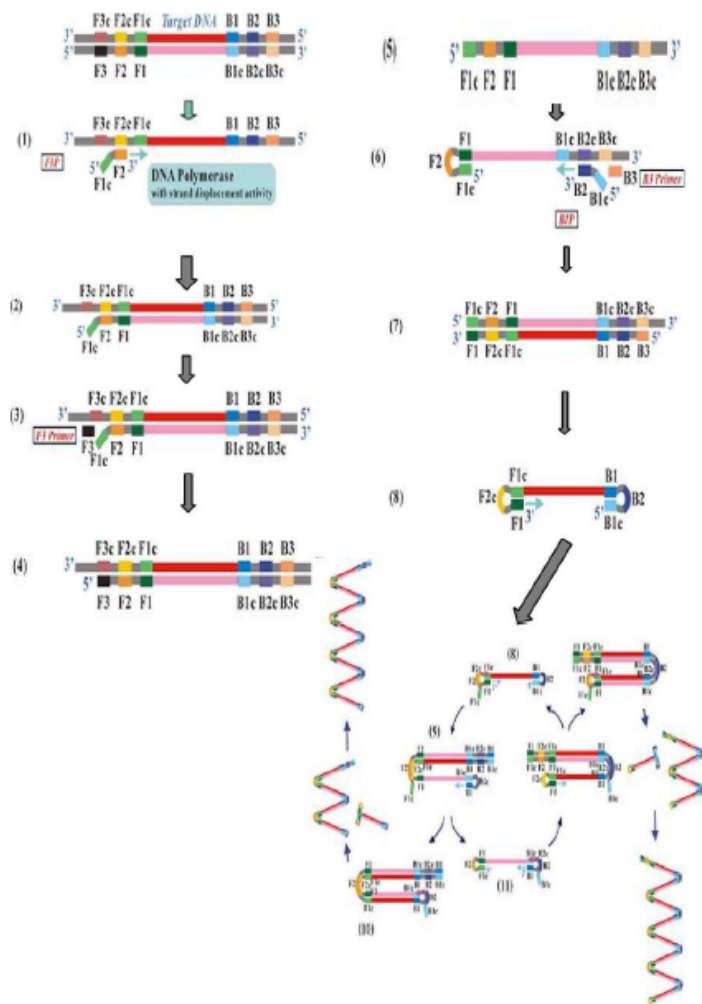
"LAMP" which stands for **L**oop-mediated Isothermal **A**mplification is a simple, rapid, specific and cost-effective nucleic acid amplification method developed by Eiken Chemical Co., Ltd. It is characterized by the use of four different primers specifically designed to recognize six distinct regions on the target gene with the process being performed at a constant temperature (ranging between 60 - 65 °C) using a strand displacement DNA polymerase.

When the DNA template and the reagents are incubated at isothermal conditions, the six target sites of the gene of interest, from the 5' end are amplified after being recognized by the four



primers included in the reaction. The reaction employs the use of *Bst*/*Bsn* DNA polymerase to amplify the nucleic acid. As the amplification time progresses, there are dome shaped products that are formed as a result of the loop formation and the displacement activity of the polymerase. The products are detectable as from 15-40 min from the start of the reaction (Norihiro *et. al.*, 2008).

### LAMP PRINCIPLE



**Figure 1: Diagram illustrating the principle of LAMP** The Forward Inner Primer (FIP) contains F1c and F2 binding sites which bind complementary to the target gene and are elongated by the *Bsn/Bst* DNA polymerase. The Loop primer, F3 binds to its complementary site causing a loop to be created on the 3' end, the Backward Inner Primer (BIP), which contains B1 and B2 binding sites, are also elongated by the enzyme. The other loop primer, B3 then binds to its complimentary site, creating a loop and this is then displaced forming a doom shaped product (8) and the cycle continues and results to structures (9) (10) and (11). The diagram is courtesy of Tomita *et al.*, 2008.

### 2.8.1 Main Advantages of LAMP Technique

These include;

1. The technique utilizes cheap infrastructure in the sense that only a heating block or a water bath that can maintain the temperatures required constant is required.
2. High specificity is achieved through the use of the four primers that recognize six distinct sites on target DNA in the initial steps of LAMP and two primers (recognition of four distinct sequences) during the subsequent steps ensures high specificity for target amplification. Moreover, in LAMP four primers (six distinct recognition sequences) are simultaneously used to initiate DNA synthesis from the original unamplified DNA to generate a stem-loop DNA for subsequent LAMP cycling, during which the target is recognized by four sequences. Therefore, target selectivity is expected to be higher than those obtained in PCR and SDA (Notomi *et al.*, 2000).
3. The technique is also known for its high amplification efficiency at constant temperatures without a significant influence of the co- presence of non-target DNA. Its detection limit is a few copies, being comparable to that of PCR.
4. The products are a mixture of stem-loop DNAs with various sizes of stem and cauliflower- like structures with multiple loops induced by annealing between alternately inverted repeats of the target sequence in the same strand, the latter of which would enable their simple, easy and selective detection, such as via mechanisms similar to multivalent antigen-antibody interactions.
5. LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase and a regular laboratory water bath or heat block for reaction.

Applications of the LAMP technique are already being used in fields such as medicine, agriculture, food, animal husbandry, and in environmental protection. LAMP technology has been developed into commercially available detection kits for a variety of pathogens including bacteria and viruses. *Plasmodium falciparum* (Lucchi *et. al.*, 2010), sleeping sickness (Njiru *et al.*, 2008) as well as to detect catfish bacterial pathogen *Edwardsiella ictalurifish* (Yeh *et. al.*,

2005).

Analysis of water and environmental samples by LAMP has been used for identification of *Cryptosporidium* oocysts (Koloren *et. al.*, 2011) and other indicator organisms of fecal origin than pollute rain water and other water sources (Ahmed, 2008).

The current focus on LAMP methodology is as a diagnostic system to be employed in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic. The combination of LAMP and novel microfluidic technologies such as Lab-on-a-chip may facilitate the realization of genetic point-of-care testing systems to be used by both developed and developing countries in the near future (Stedtfeld *et. al.*, 2012)

## **2.9 Selection of genes for detection of bacterial water contamination**

For use of LAMP technique in detection of bacterial DNA, a specific gene target region was selected. For the identification of each of the three indicator organisms a marker is to be selected. The selection was based on either genes commonly used for identification of the target indicator bacteria or genes that expressed proteins unique to the organism of choice that helped identify the bacteria. *Tuf* gene present in *Enterococcus faecalis*, *MalB* gene in *Escherichia coli* and the *CPE* gene from *C. perfringens* were chosen. These genes served as sites for specific primer design that were used in both PCR and LAMP assays.

### **2.9.1 *Tuf* gene**

The *tuf*-gene codes for the elongation factor Tu (EF-Tu). EF-Tu is a GTP-binding protein plays a central role in the protein synthesis. It mediates the recognition and transport of aminoacyl-tRNAs and their positioning to the A site of the ribosome. The highly conserved function and ubiquitous distribution render the elongation factor a valuable phylogenetic marker among eubacteria and even throughout the archaeobacterial and eukaryotic kingdoms. In the bacterial genome, there is one up to three *tuf*-genes present in various copy numbers, when only one is present in the majority of gram-positive bacteria with the low GC content (Ke *et. al.*, 2000). There exist 2 variants of *tuf*-genes (*tufA* and *tufB*) (Cupáková *et. al.*, 2005)

### **2.9.2 *Clostridium perfringens* Enterotoxin (CPE)**

The gram-positive bacterium *Clostridium perfringens* is a human and veterinary pathogen that produces at least 14 different protein toxins. Alpha toxin, produced by all toxinotypes of *C. perfringens*, has hemolytic and lethal activities. It is the primary mediator of gas gangrene caused by *C. perfringen*. Toxinotypes B and C produce pore-forming beta toxin while Types B and D express epsilon toxin, which is also a pore forming toxin. Type E of *C. perfringens* produces iota toxin with ADP-ribosyltransferase activity (Fernandez *et. al.*, 2007).

*CPE* is a single polypeptide with 319 amino acids and a molecular mass of 35 kDa. Intoxication by CPE causes, firstly, inhibition of absorption of ions and fluid by intestinal epithelial cells and, secondly, death of these cells leading to secretion of fluid into the intestinal lumen. The gene that encodes for the Enterotoxin E and is produced only by sporulating cells and accumulates in a large inclusion body inside the mother cell, from which it is released after lysis at the end of sporulation. (Mueller-Spitz *et. al.*, 2010a)

### **2.9.3 *MalB* gene**

The *malB* gene codes for a maltose transport protein a region that includes the *lamB* gene which encodes a surface protein recognized by an *E. coli* specific bacteriophage (Rompré *et. al.*, 2002). Amplification of the *E. coli* lamB region by using a primer annealing temperature of 50°C was found to selectively detected *E. coli* and *Salmonella* and *Shigella spp* (Bej *et. al.*, 1990).

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Sampling

Distribution of the sampling sites were based on the Kenya Demographic and Health Survey 2008-09 that classified water sources for Kenyans as piped water within the dwelling areas, public taps, and tube well or borehole, protected well or spring, and rainwater. There were 11 water samples collected 1 rainwater sample from Lang'ata, 5 different brands of bottled water samples (Spa, Majesty, Starpop, Blue label and Aqual Ice ) 1 groundwater sample from Kikuyu Springs and 4 tap water samples 3 from Nairobi City Water and Sewerage Company distribution lines and 1 from Naivasha's Fisherman Campsite.

The water samples were aseptically collected and put in glass bottles for tap waters, rainwater and the groundwater. For the tap water samples collected the bottles used in their sampling contained sterile sodium thiosulphate. They were then transported to the laboratory in a cooler box at  $4\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Analyses were done with six hours from time of collection.

A summary of the samples collected is as shown in the **Appendix 3**. For the choice of bottled water brands it was informed by the very low costs used to peddled in the streets and at a mall despite

#### 3.2 Lab processing

Samples once received in the laboratory had their pH determined after which filtration was carried using a  $0.45\text{ }\mu\text{m}$ -pore-sized (47 mm diameter) nitrocellulose membranes (Pall Gelman Sciences, Sigma Aldrich). For positive controls, cultured strains of *Escherichia coli*, *Enterococcus faecalis*, and *Clostridium perfringens* were obtained from the Government Chemist Laboratory (Kenya).

##### 3.2.1 Detection of the coli form organism and *Escherichia coli* (fecal coli form)

Two hundred and fifty millimeters of the water per sample, was concentrated by filtration through a

0.45µm membrane that was then placed on Violet Red Bile agar (VRB). This media has lactose as a fermentable carbohydrate that is selective for coliforms from the non-lactose fermenting organism. The plates were then incubated at 37 °C and observed after 24 hours for growth and enumeration. Confirmation of *E. coli*, a fecal coliform, was done by testing for Indole production.

### **3.2.1.1 Growth in MacConkey Broth**

The media containing peptic digest of 20 g/L of animal tissue, 10 g/L lactose, 5 g/L Sodium chloride, 5 g/L Sodium taurocholate and 0.01 g/L Bromo cresol purple was prepared and 9 mL dispensed into tubes containing sterile Durham tubes after which sterilization at 121 °C for 15 minutes followed. Upon cooling, four loopfuls of a VRB 24 hour cultures were then put and the tubes incubated at 37°C for 24 hours. A positive test for coliform organisms was identified by the presence of gas in the Durham's tube as well as medium color change from purple to yellow due to acid production. (American Public Health Association, 1992)

### **3.2.1.2 Indole test**

Indole production medium containing 1 g of tryptone and 3 g of beef extract in 1000 mL distilled water was prepared and dispensed into tubes as volumes of 9 mL and autoclaved at 121 °C for 15 minutes. The medium was then inoculated using four loopfuls of a 24 hour culture and incubated in a water bath at 44 °C. Four drops of Kovac's reagent were added as a confirmatory test with a positive reaction indicated by formation a red colored ring. (American Public Health Association, 1992)

### **3.2.2 Detection of fecal streptococci group**

Two hundred and fifty millimeters of the water per sample, was concentrated by filtration through a 0.45µm membrane that was then placed on Kanamycin Aesculine Azide Agar (KAAA) which constitutes of 20 gm/l tryptone, 5 gm/l yeast extract, 5 gm/l NaCl<sub>2</sub>, 1 gm/l sodium citrate, 1 gm/l Aesculine, 0.5 gm/l Ferric ammonium citrate, 0.15 gm/l sodium azide and 10 gm/l agar. The plates were then incubated at 37 °C for 48 hours. The confirmatory test

conducted was observation of continued growth at  $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  after 24 hours of incubation. *Streptococci faecalis* was used as the control for the test.

### **3.2.3 Detection of the sulphite reducing clostridia group**

Two hundred and fifty millimeters of the water per sample, was concentrated by filtration through a  $0.45\mu\text{m}$  membrane that was then placed in a broth of Iron Sulphite Agar (ISA) which constitutes of 10 gm/l tryptone, 0.5 gm/l sodium sulphite, 0.5 gm/l iron II citrate and 12 gm/l agar. The media is used for the detection of thermophilic anaerobic organisms. The tubes were incubated at  $37\text{ }^{\circ}\text{C}$  for 24hours when observations were made. *Clostridium perfringens* was used as the control for the test

## **3.3 Molecular Analysis**

### **3.3.1 Primer Design**

The primer sequences for LAMP amplification were designed based on the Enterococcus faecalis *Tuf* gene Accession number GenBank: AF124221.1 *Escheriachia coli's* *MalB* gene accession number GenBank: M96870.1 and *Clostridia perfringens* *CPE* gene Accession numbers GenBank: GQ225716.1 from NCBI. The primers were designed using the Primer Explorer Version 4 software available on <http://primerexplorer.jp/elamp4.0.0/index.html>. The software was used to design one set of primers (five to six primers per target organism). The same sequences from NCBI were used for the design of PCR primers by the Primer 3 software available on <http://bioinfo.ut.ee/primer3-0.4.0/>



**Table 1; Primers used for PCR assays for *Escherichia coli*, *Enterococcus faecalis* and *Clostridium perfringens* detection**

The software's helped design the list of primers indicated below

<b>Primer Name</b>	<b>Primer sequence</b>	<b>Target Gene</b>
<b>EC_Fw</b>	ATTTCTGGTCCTGGTGCCGG	MalB gene
<b>EC_Rv</b>	AAATCAACCCGGGCGGCACATTA	
<b>Strp_Fw</b>	TATCGCAGGTTCTGCTTTGA	Tuf gene
<b>Strp_Rv</b>	TTTCAACTTCGTCACCAACG	
<b>Clos_Fw</b>	TGGTTGGATATTAGGGGAACC	CPE gene
<b>Clos_Rv</b>	TTCATTTGGACCAGCAGTTG	

**Table 2: Primers used for LAMP assays for *Escherichia coli*, *Enterococcus faecalis* and *Clostridium perfringens* DNA detection**

Primer Name	Primer sequence	Target Gene
<i>Escherichia coli</i>		MalB gene
Ecoli_F3	ATATTTCTGGTCCTGGTGCC	
Ecoli_B3	CCCAGTTCTAATGTGCCGC	
Ecoli_FIP	CCACCAGCTTCAGAGGAGCG-AACATCGATGTTGGCTTCGG	
Ecoli_BIP	ATACCAACGAAACCGCGAACGA-CCGGGTTGATTTCCATCTGC	
Ecoli_LB	CGTTTTTCGATGTGCGTTTAGC	
<i>Enterococcus faecalis</i>		Tuf gene
Strpfeac_F3	TGACAAACCATTCATGATGC	
Strpfeac_B3	AATTTACGGAACATTTCAACAC	
Strpfeac_FIP	CGTTCAACACGTCCTGTAGCA-CAGTCGAAGACGTATTCTCA	
Strpfeac_BIP	GGTGAAGTTCGCGTTGGTGA-GTAACGGTTGTTTTAGATGTTTC	
Strpfeac_LF	ACAGTACCACGTCCAGTGAT	
<i>Clostridia perfringens</i>		CPE gene
Cloperf_F3	TCTGCAGATAGCTTAGGAAAT	
Cloperf_B3	GTGTAAATTAAGCTTTTGAGTCC	
Cloperf_FIP	GCAGCAGCTAAATCAAGGATTTCTT-GTTCATTAATTGAAACTGGTGAA	
Cloperf_BIP	TGATGCATTAAACTCAAATCCAGCT-AAGGGTATGAGTTAGAAGAACG	
Cloperf_LF	GTGTTTTAACAGTTCCATCTACAG	

### **3.3.2 DNA Extraction**

Two hundred and fifty millimeters of the sample was first concentrated by filtration and DNA was then extracted using the Fast DNA Spin Kit (MP Biomedicals, LLC) following the manufacturer's instructions. The extracted DNA samples were then stored at – 20 °C for long term storage and for amplification by LAMP and PCR.

### **3.3.3 Polymerase Chain Reaction**

DNA amplifications using specifically designed primers by conventional PCR and touch-down PCR before running a gel electrophoresis was carried out.

Conventional PCR was carried out in a total volume of 30 µL containing 10 pmoles of each primer, 10X GenScript PCR Buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1.25 mM MgCl<sub>2</sub>, 1 unit *Taq* DNA polymerase (GenScript Corporation) and 3 µL of DNA template. The PCR was done in a programmed thermal cycler (PTC-100 Programmable Thermal Controller (96-well), MJ Research, Gaithersburg).

Where conventional PCR didn't yield any result a Touchdown PCR was carried out in order to increase specificity of the reactions.

### **3.3.4 LAMP reactions**

Detection of the three microbes was based on the amplification of specific genes targeted by specifically designed primers that would have loop properties to form dumbbell shaped products. The reaction consisted of *Bst* DNA polymerase that had displacement activity and the reaction was isothermal. The LAMP products were analyzed under U.V. light on agarose gels.

The LAMP reaction was carried out in a total volume of 25 µL reaction. The mixture consisted of the following reagents; 1X reaction buffer with 6 mM MgSO<sub>4</sub> (New England Biolabs, Beverly, MA), 0.8 M betaine (USB Corporation, Cleveland, OH), 1.0 mM dNTP's (USB Corporation, Cleveland, OH), 0.2 µM each of F3 and B3 primers, 1.6 µM each of FIP and BIP, 0.32 U/µL *Bst* DNA polymerase (New England Biolabs, Beverly, MA) and appropriate amount of the template genomic DNA. The reaction was carried out at 65 °C for 1 h and inactivated at 80

°C for 10 min. The amplified products were resolved through a 1.5% agarose gel stained with ethidium bromide. The characteristic marker line pattern was expected on all samples that were positive.

### **3.3.5 Agarose gel electrophoresis**

A 1% agarose gel was prepared by dissolving 1 g of agarose powder into 100 mL of 1X TAE buffer, the mixture stirred and boiled in a microwave, then ethidium bromide (10 mg/μl) was added and the mixture allowed to cool before being poured into the casting trays in order to polymerize. The PCR products (7 μL), were then mixed with the 6X loading dye and samples loaded into the gel, alongside a molecular weight DNA marker. Electrophoresis was set at 70 volts for 1 hour, followed by visualization of the DNA under Ultra Violet illumination. The gel photo was analyzed and documented using the software; KODAK Gel Logic 200 Imaging System (RaytestGmbH, Straubenhardt).

For LAMP assay monitoring a 1.5 agarose gel was prepared and run at 75 volts for one hour Under UV illumination, the gel showed many bands of different sizes, forming ladder-like structures which are the various length stem-loop products of the positive LAMP reaction.

## CHAPTER FOUR

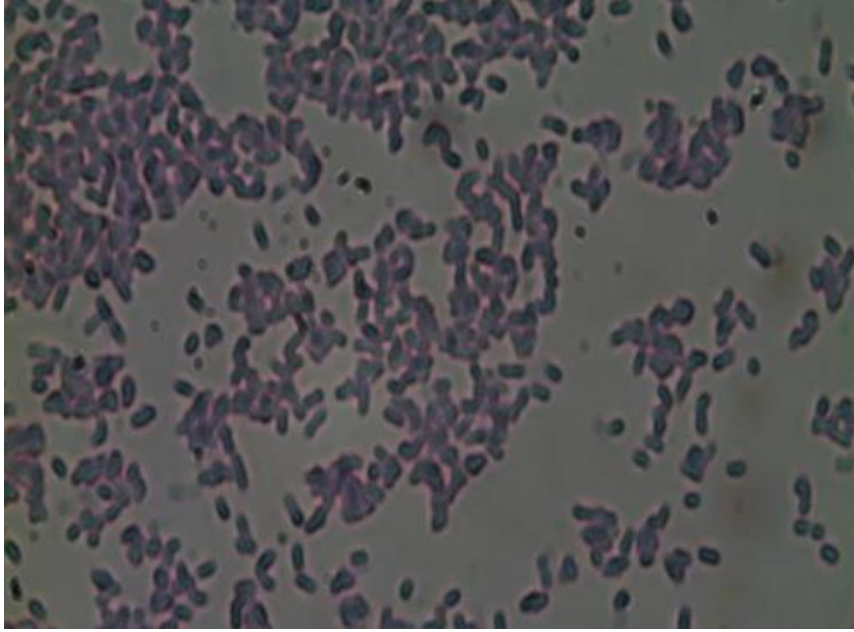
### 4.0 RESULTS

#### 4.1 Positive controls Confirmation

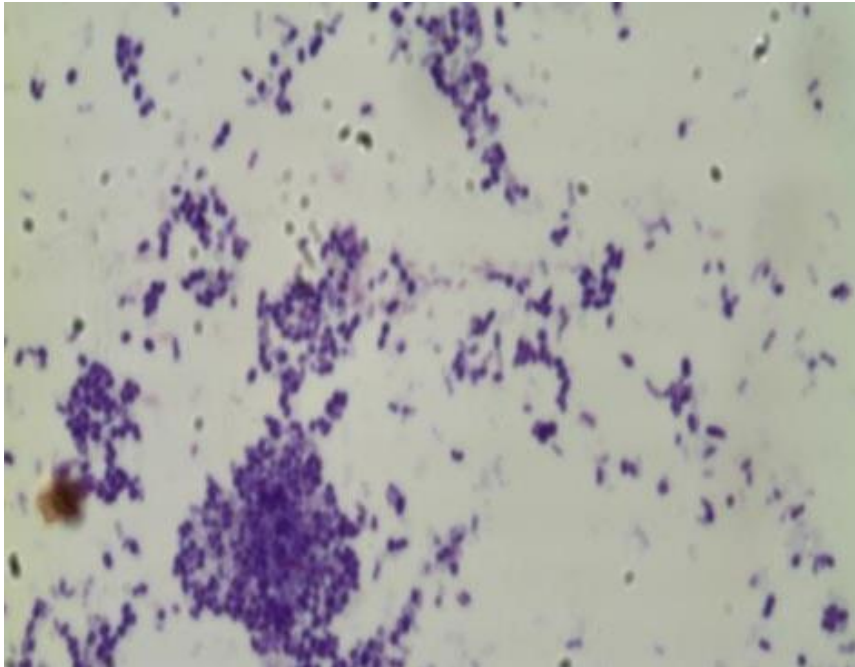
After culturing *Clostridia perfrigen*, *Escherichia coli* and *Enterococcus feacalis*, the bacteria were confirmed by Gram staining and microscopic observation. As shown in Plate 1 *Clostridium perfringens* was observed as gram positive bacilli, Plate 2 showed *Escherichia coli* was Gram negative short rods. *Enterococcus feacalis* was seen as gram positive cocci in Plate 3. Therefore, the procedure confirmed the identities of the bacteria.



**Figure 2:** *Clostridium perfringens* at magnification X100



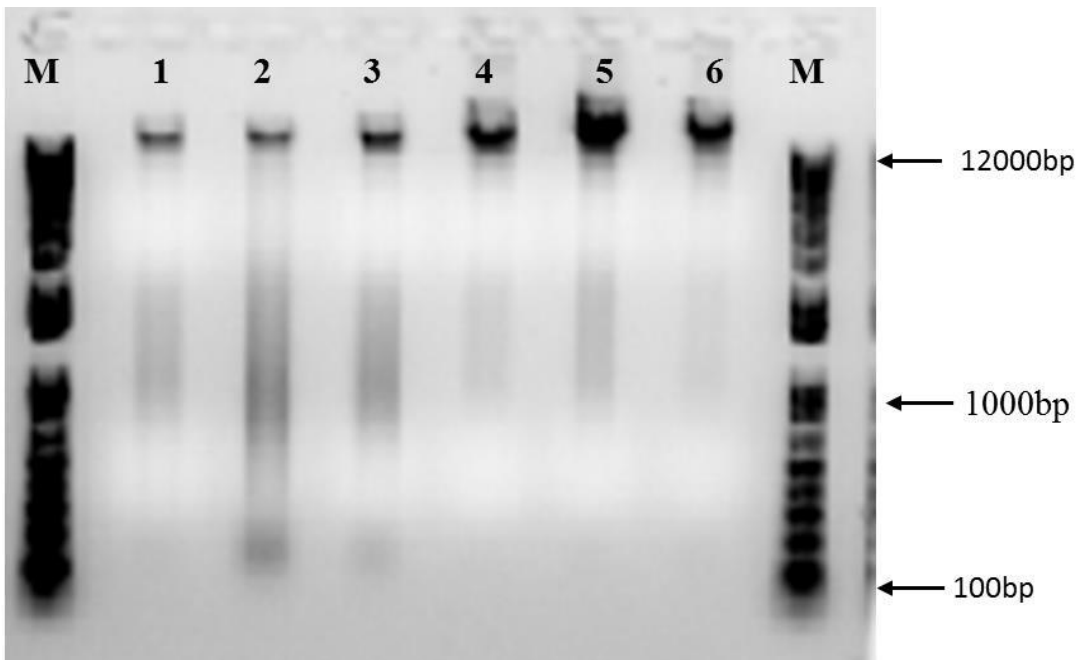
**Figure 3:** *Escherichia coli* at magnification of X40



**Figure 4:** *Enterococcus faecalis* at magnification

## 4.2 Optimizations for PCR and LAMP methods

Most DNA extracts are not reasonably pure and therefore estimates of concentration using spectrophotometric measurements of UV absorption may not be adequate. In this case, quantification of the DNA was achieved by running the DNA samples on 1 % agarose gel stained with ethidium bromide (0.5µg/ml) High molecular weight DNA appeared as a well-resolved band whilst the smearing below the band indicates mechanical or chemical degradation.



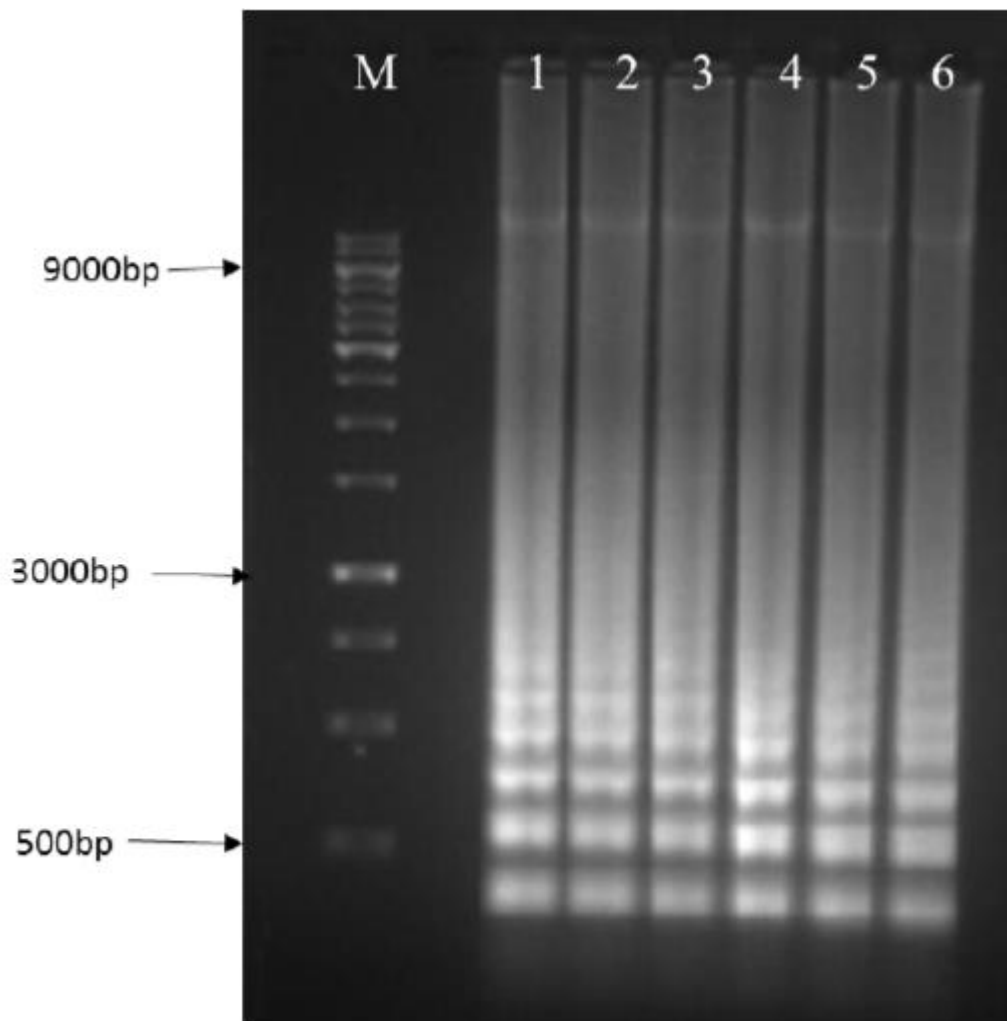
**Figure 5: Genomic DNA of *Escherichia coli* and *Enterococcus faecalis* isolates extracted using FastDNA™ SPIN Kit for Soil. M: 1 Kb DNA ladder (Invitrogen, Burlington, Ontario, Canada); Lanes 1, 2 and 3: *Escherichia coli* isolates; Lane 4, 5 and 6: *Enterococcus faecalis* isolates**

The genomic DNAs extracted from the three bacteria isolates were then used for the optimization of both LAMP and conventional PCR assays

## 4.2.1 LAMP Assays

### 4.2.1.1 Optimization for LAMP Assays for detection of *E. coli* DNA

After designing oligonucleotide primers from target DNA of *E. coli*, the LAMP assay was optimized by varying amplification temperature to obtain an optimal temperature for the assay. As shown in Figure 4 the DNA template obtained from the target gene of *E. coli* was amplified at all the temperature conditions tested

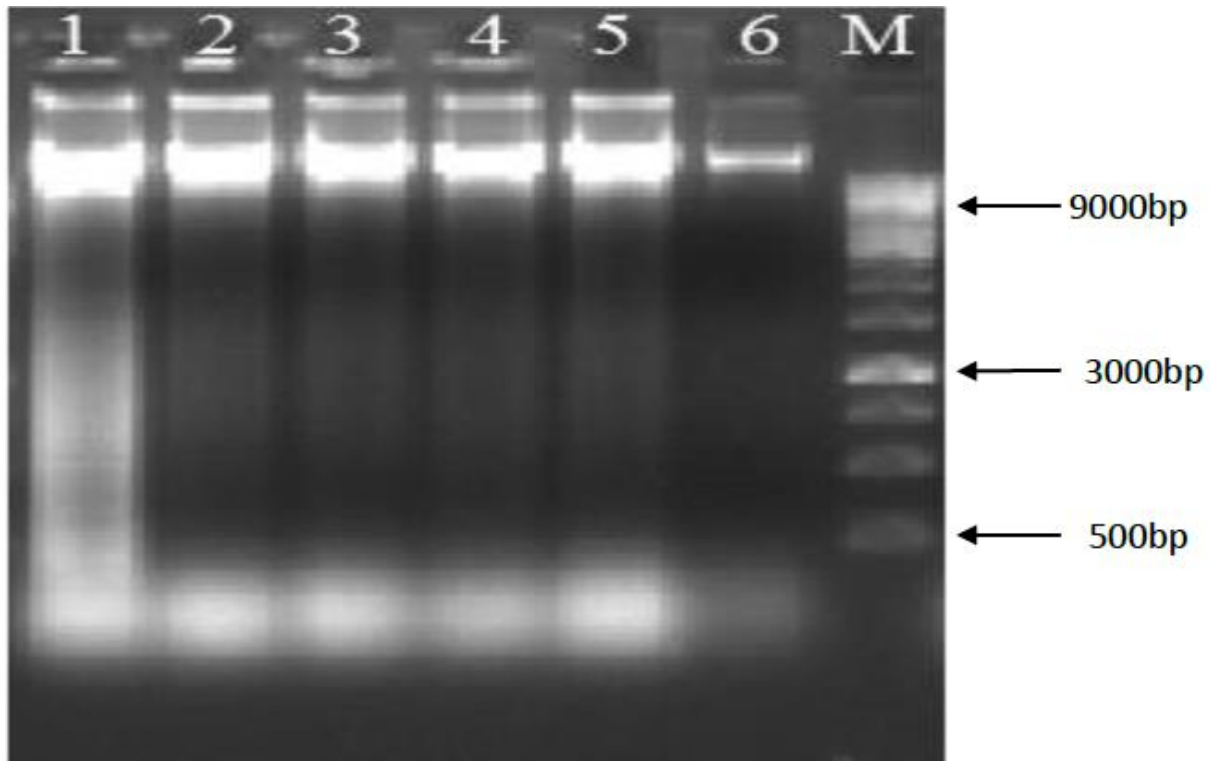


**Figure 6:** A photo of 1.5% agarose gel showing optimization of LAMP assay with designed primers at temperature range of 60-65 °C; M: 1kb ladder (New England Biolabs); Lane 1-6 60-65 °C temperature range used.



#### 4.2.1.2 Optimization for LAMP Assays for detection of *Enterococcus faecalis* DNA

After designing oligonucleotide primers from target DNA of *Enterococcus faecalis*, the LAMP assay was optimized by varying amplification temperature to obtain an optimal temperature for the assay. There was also addition of LB primer from *Escherichia coli*. As shown in Figure 5 the DNA template obtained from the target gene of *Enterococcus faecalis* was amplified at 60 °C but without the many bands of different sizes, forming ladder-like structure.



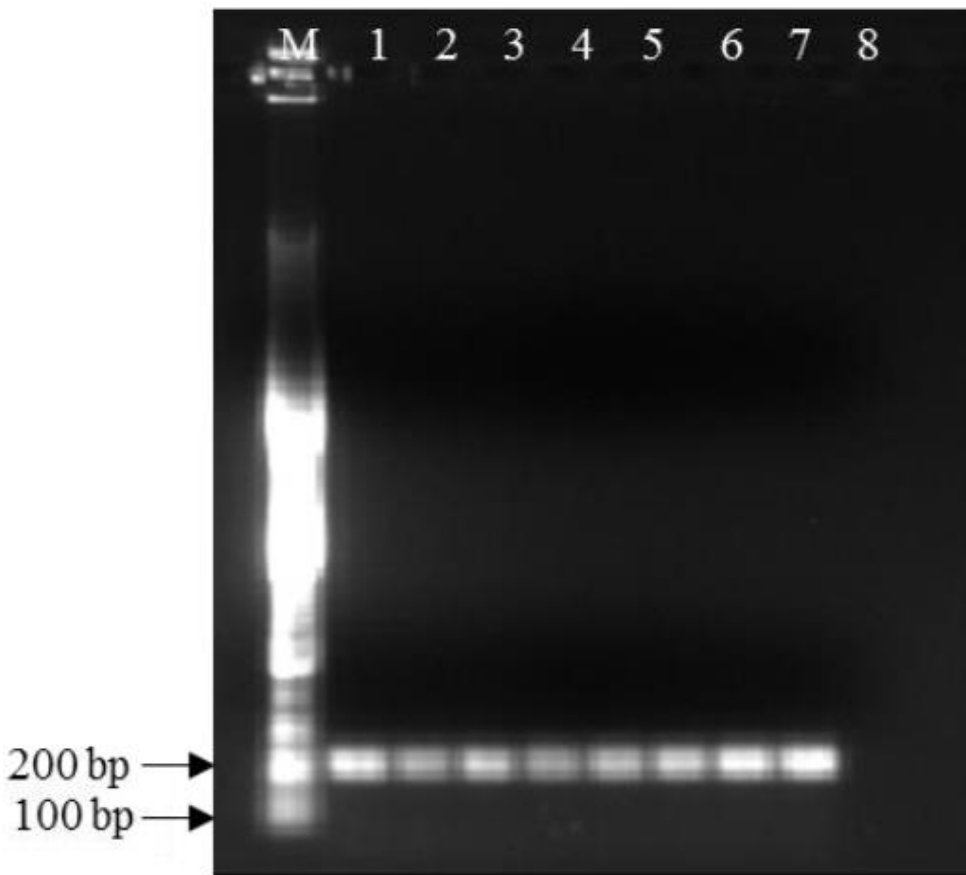
**Figure 7:** A 1.5% agarose gel image showing the optimization of *E. faecalis* at temperature range of 60 - 65 °C. **M:** 1 Kb ladder **Lane s1-6:** Temperature range of 60 – 65 °C after incubation for 60 minutes.

Amplification as observed in Lanes 1-5 was only achieved with the inclusion of LB primer from *Escherichia coli*.

## 4.2.2 PCR Assays

### 4.2.2.1 Optimization of conventional PCR assays for detection of *Enterococcus faecalis* DNA

The conventional PCR Assays was optimized using primers designed from the target DNA of *Enterococcus faecalis*, by varying the annealing temperatures, using the principle of a gradient PCR. The objective here was to achieve an optimal annealing temperature. As shown in Figure 6, a specific band corresponding to 200bp was observed for all annealing temperatures tested. The observation indicates that these annealing temperatures could be successfully used in conventional PCR assay.

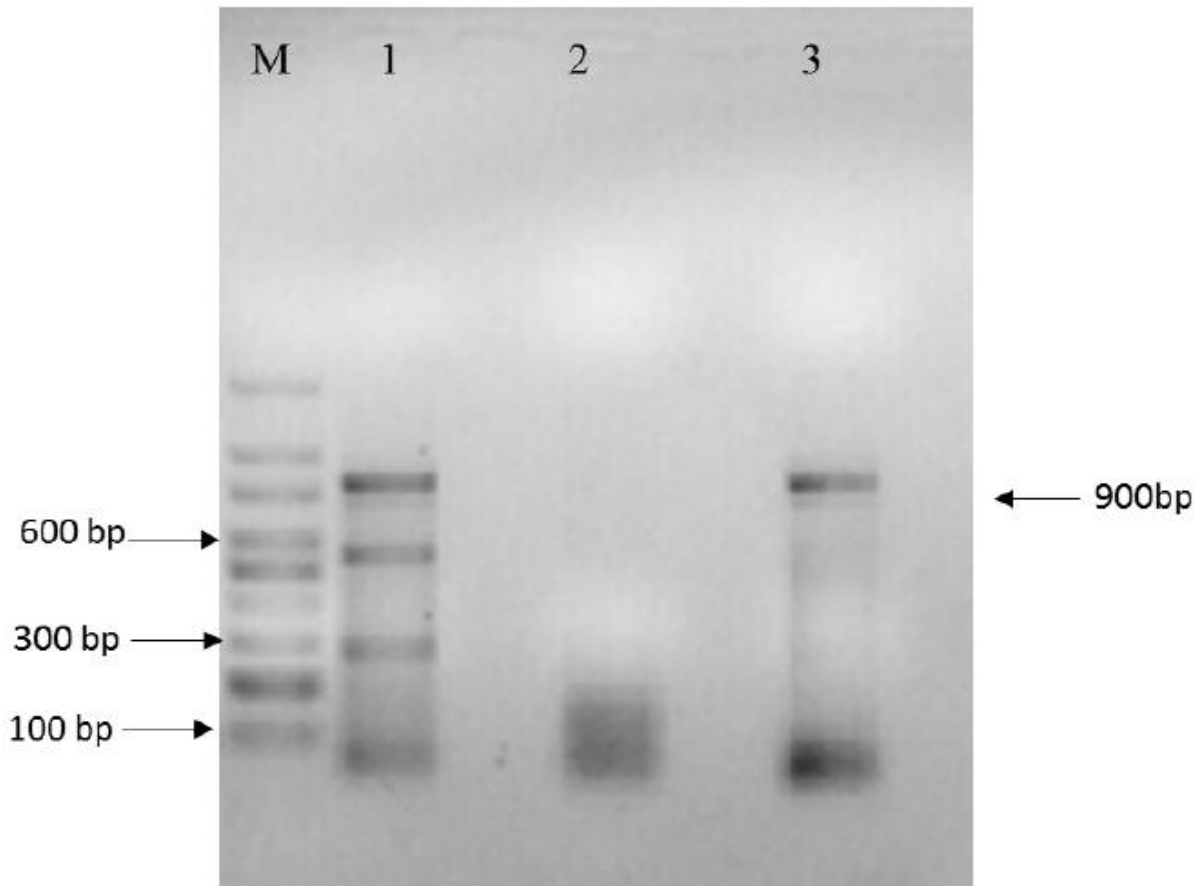


**Figure 8:** A 1% agarose gel image showing the gradient PCR done on *Enterococcus faecalis*. M: 100 bp DNA Ladder (New England Biolabs) Lanes 1-8: Amplification at temperatures of 53, 54, 55, 56, 57, 58, 59 and 60° C, respectively.

The target bandwidth of 200bp from the isolates was observed at all the temperature range used.

#### 4.2.2.2 Optimization of Touch-down PCR for detection of *Clostridium perfringens* DNA

In the optimization of conditions most suitable for the detection of *Clostridium perfringens* DNA, conventional PCR did not yield any results thus using touch-down PCR was used as an alternative



**Figure 9:** A 1% agarose gel image showing touchdown PCR on *Clostridium perfringens*. M 100 bp DNA ladder **Lanes 1-3:** Various isolates of *C. perfringens* extracted at different times. A Touchdown PCR focuses on maximizing target product yield by only ensuring amplification of fragments only containing the sequence of interest. The target bandwidth of primer was about 235bp and from the amplification no such length was achieved. The optimal conditions for the *Clostridium perfringens* detection was however not achieved despite several other trials

**Table 3 DNA concentration from Water samples extracted using FastDNA™ SPIN Kit for Soil**

Water Type	Sample ID	Concentration.(ng/μl)	260/280
<b>Rain water</b>	Rainwater	8.6	2.46
<b>Bottled water</b>	Blue label	6.6	3.48
	Aqual ice	5.2	2.1
	Majesty	7.9	3.16
	SPA	16.5	1.74
	Starpop	6.2	2.23
<b>Tap water</b>	Naivasha	13	2.18
	Muthaiga	15.2	1.77
	Dog pound	15.3	2.13
	Kikuyu treated	14.3	2.1
<b>Ground water</b>	Kikuyu Raw	17.3	2.29

After DNA extraction, the highest concentration of total DNA obtained was 17.3 ng/μl from the raw waters of Kikuyu Springs while water from Aqual ice had the lowest at 5.2 ng/μl. The purity levels were relatively high with all the samples having an OD260:OD280 ratio of  $1.8 \pm 1$ . The samples were then analyzed by PCR and LAMP assays to detect *Escherichia coli* and

*Enterococcus faecalis* DNAs as the conditions were optimized. The gel images obtained did not reveal the presence of target DNAs of the organisms.

**Table 4: Water Samples tested by culturing on selective media and pH readings**

No.	Sample ID	<i>E. coli</i>	<i>E. faecalis</i>	<i>C. perfringens</i>	pH
1	Lang'ata Rainwater	+	+	+	6.39
2	Blue label	-	-	-	6.19
3	Aqual ice	-	-	-	5.97
4	Majesty	-	-	-	6.91
5	SPA	-	-	-	
6	Starpop	-	-	-	7.19
7	Naivasha	+	+	+	5.70
8	Muthaiga	-	-	-	7.01
9	Dog pound	-	-	-	6.89
10	Kikuyu treated	+	+	-	6.36
11	Kikuyu Raw	+	+	+	6.73

**Legend:** + means present, - means absence of the organism, respectively.

All tap water samples except from Fisherman's Camp in Naivasha were not contaminated by the three target bacteria being assayed, similarly to the bottled water samples. However, two of the bottled water samples (Starpop and Majesty) showed growth of coliform in the Violet bile agar with the colonies having variety of colors. Raw water from Kikuyu Springs as well as the rain water samples were contaminated by all the three bacteria.

pH which is a measure of hydrogen ions present in test sample was found to range between 5.7 from Naivasha and 7.19 from Starpop Bottling Company. Some sources were found to contain acidic water the Naivasha samples as it was outside the range of drinking water requirements as by the Kenya Standards by KEBS shown in Appendices 3

## CHAPTER FIVE

### 5.0 DISCUSSION

In order to neutralize the chlorine content present in the tap water samples that would otherwise interfere with the culturing steps sterile sodium thiosulphate was added. Transportation of samples to the laboratory in a dark cool box at  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$  was to help minimize changes in the microbial content with exposure to light and high temperatures. As recommended by WHO and UNEP (Gray, 2008)

#### 5.1 Various water sources

The raw groundwater collected from Kikuyu Springs was found to be contaminated by culture techniques employed. By conventional PCR and LAMP assays, the water samples were not contaminated. This could be attributed to the very low DNA quantities extracted (as shown in Table 4) or that the assays were not sensitive enough to detect bacterial DNAs in the water samples. After treatment there was detection of *Enterococcus faecalis* and *Escherichia coli* but no *C. perfringens*. This indicated the inefficiency in the treatment process or possible contamination of the sampling container which had to be dipped into the tank containing the treated water.

Rainwater is generally considered safe to drink and acceptable for household use. The sample collected was very turbid and after bacterial concentration by filtration and analysis by cultures all the indicator bacteria were found present. The presence of the three bacteria could be as a result of fecal matter introduced on the roof or in gutters, originating from birds, monkeys, lizards, or other animals that have access to the roof.

In Nairobi, the Public is supplied with water from the Nairobi City Water and Sewer Company distribution system and are usually subjected to varied treatment processes such as coagulation, filtration, and chlorination. The water sampled from the distribution system was free of the three targeted bacteria as seen via the cultures done, which showed their absence. This was an indication that the disinfection procedures used were effective. The LAMP detection of *Escherichia coli* and *Enterococcus faecalis* DNAs was not achieved despite the optimization of the assay and the system having been successful.

Bottled water samples analyzed by culture method were found to lack any of the three target indicator bacteria. However, water samples obtained from Starpop and Majesty companies had coliforms indicating that the treatment process used is not effective. The two samples contaminated by coliforms were also the cheapest in terms of monetary value and are normally peddled by street vendors.

Water bottling has become a competitive business in the Kenyan market with a market value exceeding Ksh. 12 billion. Due to overcrowding in the industry and the high demand for bottled water, how to beat competition becomes the greatest challenge for companies to stay profitable (Standard Media Group (11th August 2013)). As a result some unscrupulous traders have taken advantage of the increasing demand for bottled water to market products that are not certified. Some bottled water on the streets and shops are found to have a fake Kenya Bureau of Standards seal. For example, the University of Nairobi, department of Chemistry carried a chemical composition analysis of the bottled water in the country. In one of the brands, the results from the sample taken were different from the ones on the label. “The results from the laboratory and on the label though different, complied with standard KS 05-459 as per the Kenya Bureau of Standards,” it indicated. Consequently, this questions the ability of the standards body to enforce the law. In the past, employees of the board have been accused of leaking the standard mark of quality to unscrupulous traders, who go ahead to bottle the water without meeting the required specifications (Standard Media Group, 25th Feb 2014).

## **5.2 Culture results**

### **5.2.1 Gram Staining**

Gram staining is a differential staining technique that groups bacteria into two: gram-positives and gram-negatives. Gram staining depends on the structure and composition of the cell wall of bacteria. Gram positive bacteria have thicker peptidoglycan layer than gram negative bacteria thus retains the Crystal violet iodine complex within the cell wall despite the application of the alcohol that decolorizes the stain. The gram negative organism fails to retain the complex and gets decolorized. On other hand gram positive continues to retain the complex and remain purple. To observe the decolorized cells secondary stains, Safranin, is added which stains the gram negative organisms pink. By Gram staining and observation under the microscope the bacteria cultures used as the positive controls had their identities confirmed and the cell structures well displayed. *Escherichia coli* were determined to be Gram negative short rods, *Enterococcus faecalis* as Gram



positive cocci and *Clostridium perfringens* were determined by the bacillus nature and were Gram positive.

### **5.2.2 Detection of coliforms and *Escherichia coli***

*Escherichia coli* is a widely preferred index of faecal contamination and is also used as an indicator of how effective water treatment is. However, as with the other coliform indicators, it is more sensitive to disinfection than many pathogens (in particular viruses and protozoa). The use of Violet Red bile agar as a selective media in food products, water, milk and dairy products is for the detection of coli-aerogenes content. It allows for determination and enumeration of coliform organism. These organisms utilize lactose rapidly to produce purple- pink colonies surrounded by purple haloes while non-lactose fermenters produce pale colonies with greenish zones (American Public Health Association, 1992). Confirmation of *E. coli* from the purple-pink colonies was achieved by the indole test due to the high tryptophan content. Incubation of the microbes was done at 44 °C in order to be able to detect *E. coli* since it's the only organism present in water capable of producing indole at this temperature (ISO 9308-1: ISO 9308-2).

The presence of *E. coli* in surface and groundwater sources indicates that the water is contaminated by fecal material and is microbiologically unfit for human use without further treatment (Health Canada, 2012). The detection of *E. coli* in treated waters should not be ignored, as the basic assumptions that pathogens may be present and that treatment has been inadequate is a possibility, but its absence does not necessarily indicate that pathogens have been eliminated (Gray, 2008).

Of the samples collected, 3 out of 13, i.e. rainwater from Nairobi, tap water from Fishermans' Camp Naivasha and sample from the Kikuyu spring were found to be contaminated by *E. coli*. This is an indication that there is a potential health risk from consuming the water and thus need for additional actions to be taken like notifying the responsible authorities, issuing a boil water advisory, investigating the cause of the contamination, and implementing corrective actions

### **5.2.3 Detection of enterococci and fecal *Streptococci* group**

*Enterococcus faecalis* is a low-GC Gram positive bacterium whose primary habitat is the gastrointestinal tract of humans and a wide range of animals. Kanamycin Aesculineazide agar is

able to detect *Enterococci spp* in foodstuffs. Their appearance is characterized by colonies that are round, white or grey in color surrounded by black zones. The media contains selective inhibitory components like Sodium Azide and Kanamycin Sulphate and has an indicator system to detect Aesculine hydrolyzing Streptococci. The black zones formed are as a result of formation of black iron phenolic compounds derived from Aesculine- hydrolysis products and ferrous iron. Confirmation that it was *Enterococci* group was the observation made after growth continued with the incubation of the plates at 44 °C (American Public Health Association, 1992). Most of the *Enterococcus* species are of fecal origin and can generally be regarded as specific indices of human fecal pollution. *Enterococci*, as an index of fecal pollution, can also be used to complement *E. coli* in catchment assessment, in tropical climates (where *E. coli* is less appropriate because of the suspicion of multiplication) and in ground water source evaluation. *Enterococci* can also serve as an additional indicator of treatment efficiency. They are highly resistant to drying and thus may be valuable for routine control after new main lines are laid or distribution systems are repaired, or for detecting pollution of groundwater or surface waters by surface run-off (Gray, 2008). Although some strains of *Enterococci* are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens. From the samples analyzed *Enterococcus faecalis* was detected in the rainwater, and raw groundwater which is significant due to probable recent pollution to these water sources. There is need to treat water from these sources if they are to be portable.

#### **5.2.4 Detection of the sulphite reducing *Clostridia* group**

Use of Iron Sulphite agar allows for the detection of thermophilic anaerobic organisms causing sulphide spoilage in food. The blackening reaction is only presumptive evidence of *Clostridia spp* growth. Black colonies are observed and gas bubbles are indicative of hydrogen sulphide gas production.

Sulphite-reducing clostridia are obligate anaerobic, spore-forming organisms, of which the most characteristic, *Clostridium perfringens*, is normally present in feces, albeit in smaller quantities than *E. coli*. Other than *Clostridium perfringens* these bacteria are not exclusively of faecal origin and can be derived from other environmental sources. The spores can survive in water for

very long periods and are quite resistant to disinfection. Since *C. perfringens* is faecally specific, unlike the other sulphite-reducing clostridia, it is the preferred parameter. Due to its survival, size, and association with particles, this organism has been considered a good surrogate for tracking *Cryptosporidium* oocysts in aquatic systems (Mueller-Spitz, 2010).

Water samples from Naivasha's fisherman Camp, the rain water from Lang'ata and the ground water from Kikuyu springs were found to be contaminated with the *Clostridium perfringens* and therefore could suggest possibility of other pathogens found in water mainly viruses and protozoa. (ISO 6461-1 (1986))It has been proposed that the detection of *C. perfringens* spores in finished water may indicate the potential for protozoan cysts to have passed through the treatment process (WHO & OECD, 2002)

### **5.3. Molecular techniques**

LAMP assays were only able to show amplification of *Escherichia coli* when the DNA concentrations were very high unlike in water samples. There is need to make the assay more sensitive for routine use in the detection of the target bacteria. By conventional PCR more optimization of necessary conditions was required. Thus even with resolving to use Touchdown PCR optimum conditions were not met. Touchdown PCR uses a cycling program where the annealing temperature is gradually reduced (e.g. 1-2°C /every second cycle). The initial annealing temperature is usually several degrees above the estimated T<sub>m</sub> of the primers. The annealing temperature is then gradually decreased until it reaches the calculated annealing temperature of the primers or some degrees below. Amplification is then continued using this annealing temperature. By this method the conditions necessary for amplification of *Clostridium perfringens* was not achieved thus there was need to redesign the primers

### **5.4. Summary**

For a long time water quality has depended on use of cultivation of the indicator microbes. This has its limits especially with the long periods of time used in waiting for results to be generated, and underestimation of the bacterial concentration due to the presence of injured or stressed cells as well as antagonistic organisms' interference (Ahmed *et al.*, 2008).

Over the years there has been an increase in the use of molecular techniques especially in

detection of the viable but non cultivable organisms (VBNC). By using the LAMP technique, primers were designed for target DNA of each target organism and optimization done. However, successful detection was only achieved for *Escherichia coli* where amplification of DNA was observed in the temperature range of 60 – 65 °C.

The samples collected when analyzed with the same conditions as the positive controls, did not have any observable LAMP products despite some of them showing growth in cultures. This could probably have been due to the extremely low DNA concentrations obtained after nucleic acid extraction was done (or that the target organism was not present in the sample being assayed). Optimization of the molecular techniques for detection of *Enterococci faecalis* primers was achieved when one of the loop primers (LB) from the *E. coli* collection was used at 60 °C. Analysis on the collected samples for detection of *E. faecalis* was futile as no amplification was detected. The possibility of lack of detection could be explained as in the case of *E. coli* as the same samples were used.

During primer design for detection of *E. faecalis* the LB sequence was not generated probably because of the short length *tuf* gene sequence used. The lack of the many bands of different sizes, forming ladder-like structure at each lane could have been a necessity requiring further optimization of the conditions for *E. faecalis* detection.

Detection of *Clostridium perfringens* was not realized in the water samples despite several trials during optimization.

## CHAPTER SIX

### 6.0 CONCLUSION AND RECOMMENDATIONS

#### 6.1 CONCLUSION

The LAMP technique is a viable method that could be used for rapid analysis of water quality as shown by the time taken to do the amplification and visualize the results. However there is need for thorough gene analysis when designing the primers, by testing more genes and further optimizing the target genes in both LAMP and PCR assays. This will help in adequately coming up with the specific, unique regions in the target organisms. The analysis can be through more optimization of the target genes in the LAMP assay. Once DNA extraction was done, time required to run a LAMP reaction was 60 min as compared to 3 hrs for PCR and 24-72 hrs for cultures. This is of great significance in case of an outbreak when rapid results are required. Other than the time frame used to obtain results, another significant resource saved was on cost to run a LAMP assay as only a water bath or heating block was required. PCR on the other hand requires a thermocycler which is quite expensive.

Detection of *Escherichia coli*, *Enterococcus faecalis* and *Clostridia faecalis* DNAs from water samples collected was not achieved. This was because the optimum conditions for the LAMP assay were not achieved. This was also the case with the PCR assay. However by culture method the water samples tested, the tap water in the Nairobi City Water and Sewerage Company distribution system was the most portable by virtue of culture results in the selective media used. This is an indication of adequate treatment. However, the absence of the indicator organism doesn't rule out the possibility of finding any of the pathogens. On the other hand, two out of four bottled water samples analyzed were found to contain coliforms but were negative for *E. coli*. The rain water and the ground water from Kikuyu were found to have the three organisms of interest indicating that these sources are contaminated. There is need to have in place measures that ensure their disinfection before use as they could pose a health risk to the users.

The sensitivity and specificity of LAMP technique in detection of *Escherichia coli*, *Enterococcus faecalis* and *Clostridium perfringens* DNA could not be determined thus no results and no conclusion could be drawn.

## 6.2 RECOMMENDATIONS

From this work a number of recommendations were made. This included

- To ensure that the water being consumed is safe, there should be periodic testing of bottled water by the regulatory body KEBS so as to enhance adherence to standards and the prevention of illegal traders. As for the other water sources the regional water service providers like NCWSC should monitor their systems in order to provide safe drinking water. There is need to find cheap and reliable ways to treat the rainwater if it is for portability and not just domestic use.
- From the laboratory analysis concentration of the microbes from the water samples was a big challenge but with improvement using membrane filters or the other techniques such as ultra-filtration, this can be resolved. This is of great importance especially when pathogens such as *Gardia* and *Cryptosporidium* spp are to be detected in the water samples since large volumes, of about 10 - 20 liters, are required to enable their detection.
- There is need to standardize the technique for routine use at the county level by simplifying the method a little further to allow for their use by untrained personnel
- To best determine the threshold level of detection of LAMP, there is need to perform serial dilutions, which will help with the quantification. This is important in indicating the sensitivity and specificity levels of the technique. With such further improvements, the method can be used in pathogen detection from natural waters, due to their low concentration in waters, and to the fact that their minimum infective doses are not determined.
- The need to upscale the study to a national level is important in monitoring the spread of diseases associated with water contamination.
- In conclusion, to further save on time, development of a multiplex system in detection of all the three bacteria or more for water quality analysis is key.

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## APPENDICES

### Appendix 1: List of common pathogens found in water

BACTERIA	VIRUSES	PROTOZOA
Campylobacter	Norwalk- like	Cryptosporidium
<i>Escherichia coli</i>	Polio-myelitis	<i>Giardia lamblia</i>
Salmonella (Non-Typhoid)	Echovirus	<i>EntamoebaHistolytica</i>
Shigella	Coxsackie	Mycrosporidia
Helicobacteroylori	Hepatitis A	<i>Toxoplasma gondii</i>
Salmonella (Typhoid)	Reovirus	<i>Cyclosporacayetanensis</i>
Yersinia	Rotavirus	
Vibrio (Non Cholera)		
Vibrio (Cholera)		
Legionella		
Mycobacterium Avium Complex (MAC)		
Salmonella (Typhoid)		

**Source:** Ahmed, W., Goonetilleke, A., & Powell, D. (2009). Comparison of molecular markers to detect fresh sewage in environmental waters. *Water Research*, 43, 4908–4917

## Appendix 2: Standards and guidelines for various water parameters in drinking

Different regulations bodies in the world have put in place guidelines that are used to determine if water is safe for human use. Illustrated in the table below are the World Health Organization, Kenya Bureau of Standards and European Union regulatory Standards

Water Parameter	Unit	WHO Standards	KEBS Standards	EU Standards
<b>pH</b>		6.5-9.5	6.5-8.5	6.5- 9.5
<b>Coliforms</b>	MPN	0 per 100	Not detectable in 250ml	0 per 100ml
<i>Enterococci spp</i>	MPN	0 per 250ml	Not detectable in 250ml	0 per 250ml
<i>Clostridia spp</i>	MPN	0 per 250ml	Not detectable in 250ml	0 per 250ml

### Sources

**WHO** (1985) guidelines for drinking water quality Vol 1 Recommendations

**KEBS** Kenya Bureau of standards (1996) Kenya Standards. Specification for drinking water

Council Directive 98/83/EC on the quality of water intended for human consumption in the European Union.

**Appendix 3: Details of water samples collected, their costs and classification by water source**

<b>Collection Date</b>	<b>Sample Name</b>	<b>Cost in ksh</b>	<b>Water type</b>
<b>28/3/13</b>	Lang'ata Rain water	Free	Rainwater
<b>29/3/13</b>	Blue label	30 per lt	Bottled from mall
<b>29/3/13</b>	Aqual ice	30 per ½lt	Bottled water from streets
<b>29/3/13</b>	Majesty	30 per ½lt	
<b>29/3/13</b>	SPA water	30 per ½lt	
<b>29/3/13</b>	Starpop	20 per ½lt	
<b>05/04/13</b>	Fishermans Camp	Free	Tap water
<b>24/04/13</b>	Muthaiga Pry sch.	Free	
<b>24/04/13</b>	Dog Pound Unit	Free	
<b>24/04/13</b>	Treated Kikuyu Springs	Free	
<b>24/04/13</b>	Raw Kikuyu spring	Free	Ground water