BIO-PROSPECTING FOR BROAD SPECTRUM ANTIBIOTIC PRODUCING ACTINOMYCETES ISOLATED FROM VIRGIN SOILS IN KERICHO COUNTY

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Bio-Prospecting for Broad Spectrum Antibiotic Producing Actinomycetes Isolated from Virgin Soils in Kericho County

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

I dedicate this thesis to my dear parents Mr. David Rotich and Mrs. Alice Rotich for their moral and financial support and words of encouragement throughout my studies. Thank you for the many sacrifices that you have made for me to get to this point, may God bless you abundantly.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS

DNA	Deoxy-ribonucleic acid
ESBL	Extended Spectrum β lactamase
MDRO	Multi Drug Resistant Organism
MDR-TB	Multi Drug Resistant *Mycobacterium tuberculosis*
MHA	Mueller Hinton agar
MRSA	Methicillin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
RT	Room Temperature
SCA	Starch Casein agar
VRE	Vancomycin Resistant *Enterococcus*
ATCC	American Type Culture Collection
rRNA	Ribosomal ribonucleic acid
ANOVA	Analysis of Variance
KEMRI	Kenya Medical Research Institute
SCC*mec*	Staphylococcal Chromosomal Cassette *mec*
ADMET	Absorption, Distribution Metabolism, Excretion and Toxicity

ABSTRACT

Antimicrobial drug resistance is a rising concern in the treatment of infectious diseases and necessitates the need for discovery of novel, potent antimicrobial compounds. Since the natural environment remains a potential source of novel antimicrobial products, this study was performed to test the potential of soils from Kericho County for antibiotic-producing Actinomycetes. Soil samples (214) were randomly collected from virgin soils of Kipkelion East, Kipkelion West, Belgut, Ainamoi, Sigowet and Bureti sub-counties in Kericho County from a depth of between 11cm-16cm from the surface of the soil profile. The Actinomycetes were isolated using serial dilution and antimicrobial activity screening done using modified agar disc diffusion method. DNA was then extracted from the isolates that showed activity and the 16S rRNA gene amplified using primers specific for Actinomycetes. The amplified gene was sequenced and phylogeny analysis done. A total of 107 Actinomycetes were isolated and from these only 39 (36.4%) showed antimicrobial activity against five of the six test isolates. These included reference strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 90028) and three clinical strains *Trichophyton mentagrophyte, Microsporum gypseum* and Methicillin Resistant *Staphylococcus aureus* (MRSA). Two of the isolates showed activity against MRSA and four isolates showed a higher potency than the standard drug Chloramphenicol (30µg/disc) against *S. aureus*. Most of the isolates 84.6% also showed good antimicrobial activity against *T. mentagrophyte* though significantly lower than the control drug Itraconazole (2 µg/ml). The 16S rRNA gene was amplified in only 15 isolates. Sequencing showed that 93.3% were of the genus *Streptomyces* while 6.7% were of the genus *Rhodococcus*. From the results, the soils from this region harbour Actinomycetes which may have good potential of producing novel antibiotics against gram positive bacteria and dermatophytes. The metabolites from the isolates that showed good activity should be extracted and purified and their components analysed and compared to the drugs in the market to find out whether there are any novel compounds.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Natural products have always been the most promising resource for discovery of novel bioactive metabolite hence potential drug leads (Cragg & Newman, 2013; Dias *et al*., 2012; Devi *et al*., 2012). Many soil-inhabiting bacteria are known to produce secondary metabolites that suppress microorganisms competing for the same resources (Velayudham & Murugan, 2012).In the medical field, antibiotics are of great importance as they have been used to save lives against many infectious diseases. However, due to poor regulations on the use of antibiotics, resistance is on the rise both in developed and developing countries (Carlet & Pittet, 2013). With slow discovery of new antimicrobial agents, few options are left for the clinicians in fight against infectious diseases (Carlet, 2012; Aly & Balkhy, 2012). Antibiotics first came into play when Alexander Fleming discovered penicillin and during his Nobel peace prize acceptance speech, he warned that it was not hard to make microbes resistant to penicillin by exposing them to concentrations that are not sufficient to kill them (Huttner *et al*., 2013). Three years after the warning, 38% of *Staphylococcus aureus* in a London hospital were found to be resistant to penicillin (Barber & Dowzenko, 1948).

The most common causes of resistance include: poor usage of antibiotics including non-compliance to the dosage prescription, selection pressure due to over use of these antibiotics and transfer of resistant bacteria from one patient to another or from a health

worker to a patient (Aly & Balkhy, 2012; Huttner *et al*., 2013). This is mainly steered by lack of proper guidelines on the usage of antibiotics and lack of proper hygiene and infection control. Bacteria can develop resistance through different mechanisms including; production of hydrolyzing enzymes like Extended Spectrum β Lactamase (ESBL) and carbapenemases as well as by mutations and alterations of the outer membrane proteins (Aly & Balkhy, 2012).

Most gram negative bacteria have developed resistance against most antibiotics. Some of these bacteria are able to produce Extended Spectrum Beta-Lactamase enzymes which are known to hydrolyze all penicillins, monobactams, oxyimino-cephalosporins and early cephalosporins (Drawz & Bonomo, 2010; Bradford, 2001). The ESBL producing bacteria include *Escherichia coli* and *Klebsiella pneumonia* and are known to cause outbreaks especially in hospitals and these bacteria have also been isolated from healthy individuals (Rao *et al*., 2014).

The other bacteria that have been shown to have developed resistance include the methicillin-resistant *Staphylococcus aureus* (MRSA) which is mostly community acquired. This bacteria is normally associated with outpatients especially those with wound infections. This may be due to clinicians prescribing antibiotics before culture results are out (Schweizer *et al*., 2013). The community acquired MRSA carry a small staphylococcal chromosomal cassette *mec* (SCC*mec*) element belonging to type III and IV (David and Daum, 2010). SCC*mec* is a mobile genetic element that carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mec*A gene

(IWG-SCC, 2009). Methicillin resistant staphylococcal lineages come as a result of the acquisition and insertion of the SCCmec element into the chromosome of susceptible strains. These elements are presumably more mobile than the ones in health care-associated MRSA. They are resistant to fewer non-β-lactam classes of antimicrobials and frequently carry genes for the Panton-Valentine eucocidin (PVL).

Actinomycetes are the most valuable prokaryotes economically and biotechnologically (Deepa *et al*., 2014; Mohanraj & Sekar, 2013; Muthu *et al*., 2013; Sharma *et al*., 2011; Lam, 2006). They have been known to secrete secondary metabolites which have different valuable capacities including; production of antibiotics, nutritional materials, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune modulators, and vitamins (Cragg & Newman, 2013; Jeya *et al*., 2013; Muthu *et al*., 2013). They therefore provide many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. (Deepa, Kanimozhi & Panneerselvam, 2014; Gopinath *et al*., 2013; Velayudham & Murugan, 2012). Actinomycetes are the most widely distributed microbes inhabiting the soil environment. They comprise about 50% of the uncultivable soil microbes and therefore form the most dominant and significant group among the soil microbial community. They are the microorganisms responsible for the characteristic earthy smell (Basilio *et al.,* 2003; Chaudhary *et al.,* 2013) and also play a role in degradation of relatively complex, recalcitrant polymers occurring naturally in plant litter and soil and recycling of organic matter (Khasabuli & Kibera, 2014; Shakthi & Murugan, 2012; Goodfellow & Williams, 1983).

Approximately two thirds of the naturally occurring antibiotics have been isolated from Actinomycetes (Okami & Hotta, 1988), where 75% are used in medicine while 60% are used in agriculture (Chaudhary *et al*., 2013). Most of them are from the genera *Streptom*yces and *Micromonospora* (Pandey *et al.,* 2011). Focus on Actinomycetes started in 1940 when Waksman isolated Actinomycin and Streptomycin from these Actinomycetes. Other antibiotics that have been isolated from Actinomycetes include gentamycin, rifampicin and erythromycin which are both used in pharmaceutical industries and in agricultural practice to inhibit phyto-pathogens (Jeffrey, 2008).

The rate of discovery of new compounds from terrestrial Actinomycetes has however decreased since researchers have started employing the use of target based bacterial genomics, combinatorial chemistry, and high-throughput screening. However this approach has not yielded much and this could be due to the Absorption, Distribution Metabolism, Excretion and Toxicity (ADMET) processes that have to be done and most compounds have proved to be toxic (Gurung *et al.,* 2009). Therefore the tropical nature still remains the richest source for new antibiotics (Bredholt *et al.,* 2008).

Many studies have been done where Actinomycetes have been isolated from different places including soil, mountains, swamps and the marine environment (Guo *et al*., 2015; Kumar *et al*., 2013; Rao *et al*., 2012; Shakthi & Murugan, 2012; Olano *et al*., 2009; Gurung *et al*., 2009; Jeffrey, 2008). This has yielded good results where antibiotics against different gram positive and gram negative bacteria have been extracted. These Actinomycetes have also produced good antifungals and also some compounds with anti-tumour activities have been extracted from them. More research is going on with a

view of coming up with new antibiotics. Therefore this study was designed to bio-prospect for novel antibiotic producing Actinomycetes. Kericho County has different types of soil and also has exotic forests which may have novel Actinomycetes and may not have been explored. Hence this study aimed at exploring the virgin soil of Kericho County with the view of identifying novel antibiotic producing Actinomycetes.

1.2 Statement of the Problem

Antimicrobial resistance is a problem that is on the rise and failures on treatment are being reported. This has resulted mainly due to increase in Multi-drug resistant organisms (MDRO) which include Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococci* (VRE) and Extended Spectrum β-Lactamase (ESBL) producing strains like *Klebsiella pneumoniae* and *Escherichia coli.* Options for treating these resistant pathogens are becoming limited since some organisms may result in complete resistance to all the available antibiotics. This therefore has resulted in increased cost of health care since patients have to stay longer in hospitals and may even result to death. This further endangers the life of health workers and the entire population at large. There is need therefore to come up with other novel antibiotics. Most antibiotics currently being used have been isolated from Actinomycetes more specifically the *Streptomycete sp.*, therefore there is need to explore other potential habitats for novel antibiotic producing Actinomycetes. This study focused on bio-prospecting for potential antibiotic producing Actinomycetes from virgin soils in Kericho County.

1.3 Justification

Actinomycetes, which are mostly found in the terrestrial environment, have been found to produce bioactive molecules against other microorganisms. Most of the antibiotics that are currently in use are derived from Actinomycetes. However, these drugs are faced by challenges of resistance hence leading to high rates of treatment failures. The emergence of multi drug resistance tuberculosis (MDR-TB) has further worsened the situation. MDR-TB is resistant to two of the available antibiotics that is, isoniazid and rifampicin which are the most potent first line drugs. There are also cases that have been reported of strains of *Mycobacterium tuberculosis* that are resistant to both first line and second line antibiotics. Therefore, there is need to explore other new habitats for undiscovered novel alternatives. Kericho County has got both acidic and alkaline soils. Most studies have been done on alkaline soils as this is the dominant habitat of Actinomycetes. Few studies have been done on acidic soils, and these soils have proved to have diverse genera of Actinomycetes hence potential of novel antibiotic producing strains. This study therefore aimed at exploring the soils of Kericho County with prospects of finding antibiotic producing Actinomycetes that can help solve the problem of MDRO.

1.4 HYPOTHESIS

Novel antibiotic producing Actinomycetes cannot be isolated from soil samples of uncultivated land in Kericho County.

1.5 OBJECTIVES

1.5.1 Broad objective

To isolate and determine the diversity of Actinomycetes from virgin soils of Kericho County and bio prospect for antimicrobial activity against selected pathogens.

1.5.2 Specific objectives

1. To isolate pure cultures of Actinomycetes from virgin soils of Kericho County.
2. To screen for antimicrobial activity of the isolated Actinomycetes against selected fungal and bacterial pathogens.
3. To characterize and identify the active Actinomycetes isolated from the virgin soils of Kericho County and determine their diversity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of Actinomycetes

A number of methods have been used to classify Actinomycetes and they include; morphological, biochemical and genomic methods. Morphological observations were initially used during characterization. However this alone is not adequate especially when differentiating many genera. Molecular tools together with phylogenetic approaches have greatly helped in classification (Adegboye & Babalola, 2012).

Taxonomy is an important aspect in science and is also important when screening for novel microorganisms. Identification of isolates up to the species level often gives a clue as to whether the metabolite is novel (Labeda, 1987). Taxonomic characterization of antibiotic producing Actinomycetes is therefore an important step in screening for novel antibiotics. Actinomycetes are gram positive filamentous bacteria that belong to the order Actinomycetales, Superkingdom: Bacteria, Phylum: Firmicutes, Class:Actinobacteria, Subclass: Actinobacteridae. The order Actinomycetales is diverse in terms of morphology, phylogeny and chemotaxonomy (Kekuda *et al.*, 2010). They have a high G+C (>55%) content in their DNA (Gurung *et al.*, 2009). Due to the presence of branching filamentous forms, these microorganisms were for many years wrongly classified as fungi (Adegboye & Babalola 2012). Having both characteristics of fungi and bacteria, the word "Actinomycetes" was derived from two Greek words that is, "atkis" meaning a ray and "mykes" meaning fungus (Chaudhary *et al*., 2013; Das *et*

al., 2008). However, they possess more characteristics of bacteria hence are grouped in the Kingdom bacteria (Adegboye & Babalola, 2012; Chaudhary *et al*., 2013). They are characterized as bacteria that have the ability to form branching hyphae at some stage of their development. This group covers a genera consisting of a wide range of morphology from the coccus like *Micrococcus* and rod-coccus which include *Arthrobacter*, through the fragmenting hyphal forms for example *Rothia* and *Nocardia* to the genera with permanent and differentiated branched mycelium like *Streptomyces* and *Micromonospora* (Goodfellow & Williams, 1983).

The order Actinomycetales is divided into four families namely: Streptomycetaceae, Actinomycetaceae, Actinoplanaceae, and Mycobacteriaceae. The cell wall composition in Actinomycetes is of major taxonomic value and differs among the suborders. There are four major types of cell walls based on the composition of the peptidoglycan and its structure (Willey *et al.,* 2008) as shown in the table below.

Table 0.1: Constituents of Actinomycetes' cell wall

Cell wall type	Sugar pattern	Examples of Genera
I	No characteristic sugar pattern	*Streptomyces, Streptoverticillicum*.
II	Araginose, Xylose (Monosaccharide)	*Actinoplanes, Micromonospora*
III	No sugar	*Dermatophilus, Planomonospora*
IV	Galactose, Arabinose	*Mycobacterium, Nocardia*

Source: (Chaudhary *et al*., 2013)

2.2 Occurrence and distribution of Actinomycetes

Majority of Actinomycetes are free living, saprophytic bacteria found in water, soil and colonizing plants (Saha *et al.*, 2012). However, some are human pathogens and the most common is *Actinomyces israelii*. The free living Actinomycetes are widely distributed in various habitats and represent an abundant group of microbes widely distributed in natural ecosystems around the world (Chaudhary *et al*., 2013).

2.2.1 Terrestrial Actinomycetes

Actinomycetes are known to be mostly soil dwelling organisms and play a major role in the management of microbial stability through production of antibiotic substances (Gopinath *et al*., 2013; Kandasamy *et al*., 2012). They play an important role in the degrading of complex polymers including cellulose, chitin, pectin, lignin (Chavan *et al*., 2013; Goodfellow & Williams, 1983; El-gammal *et al*., 2014) . The characteristic earthy smell is normally produced by the Actinomycetes. *Streptomyces* species are known to be the most abundant in soil and *Streptomyces rochei* was first noted as an important group of soil bacteria. Seventy percent (70%) of the world's naturally occurring antibiotics are produced by these *Streptomyces* species. The antibiotics produced by this abundant genus of Actinomycetes include Streptomycin which was the first treatment for tuberculosis. Streptomycin was discovered in 1944 by Waksman which earned him a Noble prize in physiology and medicine in 1952. Other antibiotics include tetracycline, erythromycin, penicillin among others (Rakesh *et al*., 2013; Gopinath *et al*., 2013; Zoe & Amsaveni, 2012).

Actinomycetes isolates from soil have also proved to have activity against plant pathogens. A study done by Jeffrey, 2008 revealed that Actinomycetes isolated from Agriculture soils showed antagonistic activities against selected phytopathogens which included; *Fusarium palmivora*, *Bacillus subtilis*, *Pantoae dipersa* and *Ralstonia solanacearum*. In Turkey, two Actinomycetes strains with different antimicrobial activities were isolated (Oskay *et al*., 2004). One was active against *Erwinia amylovora* which is a bacteria that cause fireblight in apple and the other was active against *Agrobacterium tumefaciens* a causal agent of Crown Gall disease. In Kenya, Actinomycetes strain active against *Pyricularia grisea* that cause blast in finger millets was isolated (Opande *et al*., 2013)

2.2.2 Aquatic Actinomycetes

It has been noted that Actinomycetes isolated from marine sediments could be valuable for isolation of novel strains for production of novel antibiotics (Deepa *et al*., 2013). *Micromonosporae*, *Rhodococci*, and *Streptomycetes* are common species of Actinomycetes in marine environment such as streams, rivers, lake mud, river sediments, beach sands, sponge and marine sediments though other Actinomycetes may also be found (Goodfellow & Williams, 1983). There has been evidence that Actinomycetes only form a small fraction of bacteria found in marine habitats and it has been argued that this small percentage may be due to washouts from the terrestrial habitat. Isolates affiliated with *Rhodococcus*, *Dietzia*, *Micrxobacterium* and *Nocardia* have been isolated from Lake Magadi which is a soda lake in Kenya (Ronoh *et al*., 2013). They can also occur in lakes and rivers, however low numbers of Actinomycetes

occur in the water column but rather in sediments. It has been noted that Micromonospora are common in fresh water lake. Erikson (1941), further claimed that they had a role in turnover of cellulose, chitin and lignin. Other studies have also confirmed the presence of Micromonospora in streams, rivers and river sediments and are considered to be an important part of the aquatic microflora (Goodfellow & Williams, 1983).

Actinomycetes producing secondary metabolites active against cancerous tumours and plasmodium have been isolated from marine environments (Olano *et al*., 2009; Prudhomme *et al*., 2008). There is usually a challenge in isolation of Actinomycetes from the aquatic environment. This is attributed to specific necessities like sea salt while in some cases these microorganisms are obligate halophiles (Tsueng *et al*., 2008).

2.3 General characteristics

Actinomycetes are filamentous Gram positive bacteria characterized by the formation of branching threads or rods. The hyphae are usually non septate but under certain conditions, septa may be observed in some forms. The sporulating mycelium on the other hand may be branching or non-branching and they may be either spiral shaped or straight while the spores are cylindrical, straight or oval (Chaudhary *et al*., 2013).

On culture media, they have a characteristic dry, powdery appearance with different colors depending on the media used (Devi *et al*., 2012). Therefore the macroscopic and microscopic examination on agar plates is useful in the identification of their genus. Macroscopic characteristics include shape, size, colour, consistency on different media,

the absence or presence of aerial mycelium and extent of spore formation (Muthu *et al*., 2013). Microscopic examination on the other hand helps in bringing out some features like fragmentation or non-fragmentation of substrate and aerial mycelium, presence of sclerotia, spore chain morphology and spore surface ornamentation (Khanna *et al*., 2011; Muthu *et al*., 2013; Parungao *et al*., 2007). On media, the Actinomycetes form a network of branching hyphae both on the surface and at the bottom part of the agar. The hyphae on the surface are referred to as aerial hyphae while the hyphae at the bottom are referred to as substrate hyphae. Most Actinomycetes are non-motile but when motility is present, it is confined to flagellated spores (Sharma, 2014).

Recording of the colour of aerial mycelium is done in a simple way that is white, green, red, grey, blue and violet (Sharma, 2014). Incase two colours are observed, both colours should be recorded. When melanoid pigments are produced (i.e. greenish brown, brownish black or distinct brown), the strains will be grouped as melanoid pigment produce hence (+) or melanoid pigment not produced hence (–). The reverse pigments are recorded based on whether the colony has a distinctive colour on the reverse side of the plate. Therefore this is recorded as distinctive (+) or none (-). In the case where the colour is pale such as pale yellow or yellowish brown, it will be graded as (-). If a strain produces soluble pigments apart from melanin, the colour will be noted i.e red, orange, green, yellow, blue and violet. If the pigment is not produced then it will be in the ‘not produced’ group hence (-) (Shirling & Gottlieb, 1966; Sivakumar, 2001; Sharma, 2014).

2.4 Life cycle of Actinomycetes

The life cycle of Actinomycetes has been studied well in *Streptomycetes*. On the surface of the agar, the growth cycle of the colony is initiated when a spore germinates into branched filaments that consist of a network of one or more multinucleoid filaments that elongate and branch repeatedly to form substrate mycelium also called vegetative mycelium(Miguélez *et al*., 1999). Several days after, growth of the substrate mycelium produces specialized aerial hyphae, known as the aerial mycelium that extends from the substrate mycelium into the air. These two mycelia are multinucleated. There after the aerial hyphae undergoes septation to create uninucleoid compartments. These compartments then differentiate to form spore chains (Manteca & Sanchez, 2010; Miguélez *et al*., 2000). The life cycle of *Streptomycetes* is shown in Figure 2.1

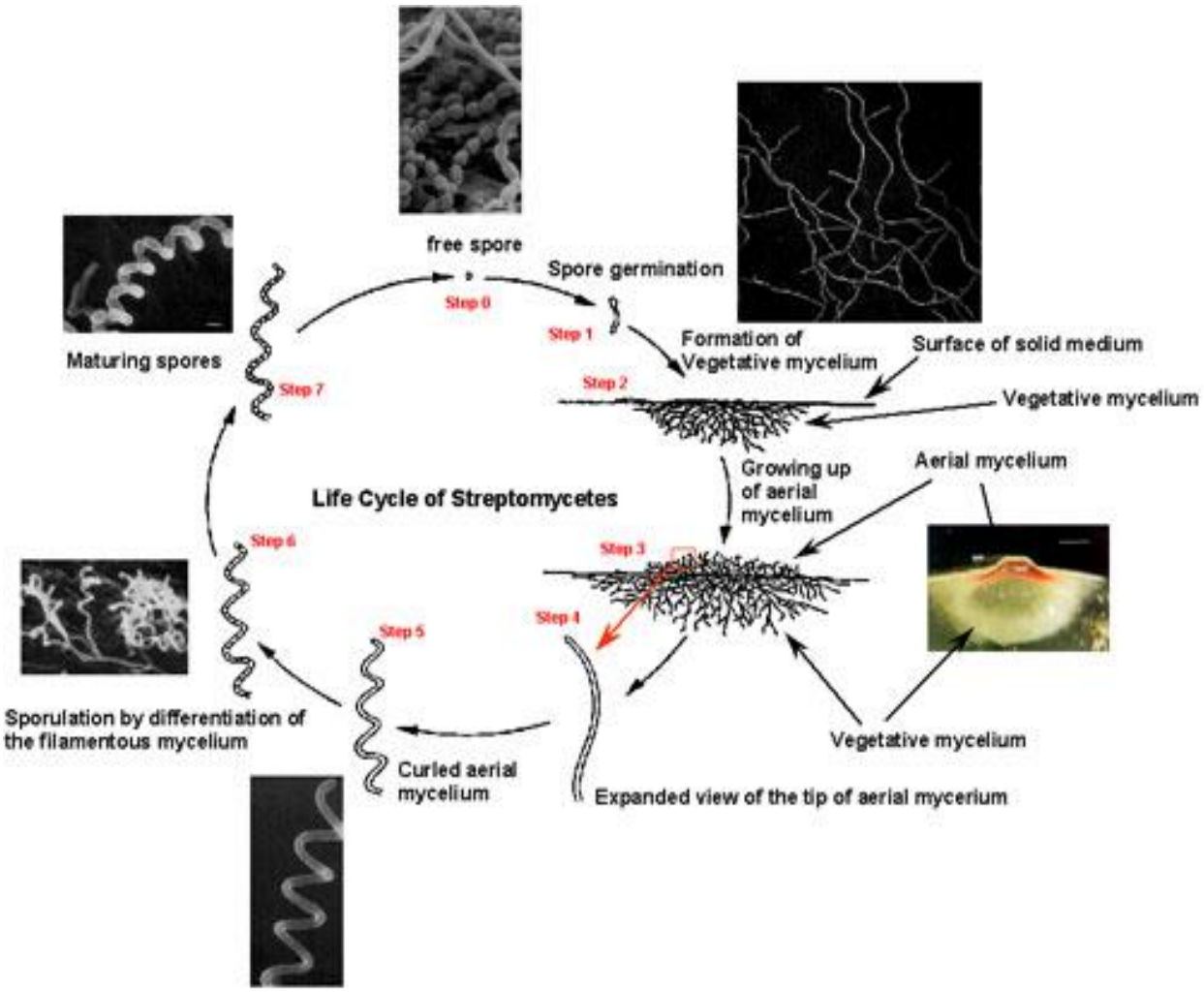

Figure 2.1: Life cycle of Streptomycete, source: Miguélez *et al*., 2000

Differentiation of Streptomyces in liquid media has not been studied extensively and this may be due to the fact that *Streptomycetes* do not sporulate under these conditions (Manteca & Sanchez, 2010). However, most industrial processes interested in secondary metabolite production use liquid cultures since the metabolites are produced at the end of the proliferation stage by the substrate mycelium (Manteca & Sanchez, 2010; Fernandez & Tomasini, 2003). The production of secondary metabolites is not normally directly involved with growth or reproduction of the organism. However they are

produced as a means of defense from predators. These metabolites may be toxic to the organism producing it and therefore genes which code for these metabolites are clustered with genes that confer resistance to that particular compound hence this prevents the organism from killing itself (Martin *et al.*, 2005).

2.5 Production of Secondary Metabolites

Secondary metabolites are compounds produced by strains of certain microbial species and also some plants (Barrios-Gonzalez *et al.*, 2003). These metabolites do not play a physiological role during exponential phase of growth as opposed to primary metabolites (like amino acids, nucleotides, lipids and carbohydrates), that are essential for growth. A characteristic of secondary metabolites is that they are not normally produced during the phase of rapid growth but rather are produced when growth is limited by the exhaustion of one key nutrient source: car- bon, nitrogen or phosphate (Barrios-Gonzalez *et al.*, 2003; Drew & Demain, 1977). These compounds are known to be structurally diverse and most are biologically active, antibiotics being the best known secondary metabolites (Berdy, 2005).

They usually are composed of various chemical moieties, such as polyketide backbones, amino acid derivatives and sugars (Adegboye & Babalola, 2012). The synthesis of these metabolites is catalyzed by a number of enzymes, usually encoded by genes. These genes occur adjacent to one another in clusters which contain all the necessary genes for the synthesis of a particular secondary metabolite. This includes: the genes that encode

the biosynthetic enzymes, regulatory proteins, genes for resistance to the toxic action of secondary metabolites and genes for secretion of the metabolites.

Type-I polyketide synthases (PKS-I) and non-ribosomal peptide synthetases (NRPS) are multimodular enzymes that are involved in the synthesis of secondary metabolites (Nikolouli & Mossialos, 2012). The synthesis of other constitutive compounds such as sugars are done by other enzymes often encoded by genes adjacent to the PKS and NRPS genes (Ichikawa *et al*., 2013). Through further processes such as elongation, synthesis, glycosylation, alkylation and oxidation, structurally diverse and complex metabolites are produced. Transcriptional regulators and transporters strictly regulate the whole process of production and transportation of secondary metabolites (Ichikawa *et al*., 2013; Park *et al.,* 2010). The genes encoding for tailoring enzymes, transcriptional regulators and transporters are also often located adjacent to PKS and NRPS genes. The gene cluster responsible for the synthesis of each secondary metabolite is usually between the size of 10 -100 kb (Adegboye & Babalola, 2012).

2.6 Economic Importance of Actinomycetes

Actinomycetes have been known to be very valuable prokaryotes both economically and biotechnologically. They produce secondary metabolites which are used to produce different important products which range from antibiotics to important enzymes, vitamins, anticancer agents and also the metabolites are of importance in the agricultural sector where they are known to produce pesticides and herbicides (Deepa *et al*., 2013; Manivasagan *et al.,* 2013; Oskay *et al*., 2004; Parungao *et al*., 2007).

2.6.1 Agricultural Importance

Actinomycetes constitute a significant component of most soils (Barakate *et al.*, 2002), and being saprophytic in nature, they are important colonizers of the soil's organic material which mostly consists of insoluble polymers. These bacteria therefore have the ability to solubilize these polymers whether of plant or animal origin. This ability to penetrate and solubilize these polymers allows the Actinomycetes to persist in the Microbial succession (Kandasamy *et al*., 2012). They also exhibit a unique life cycle hence play a role in the cycling of organic matter in the soil ecosystem and also have a role in the biological buffering of soils, biological control of soil environments by nitrogen fixation and degradation of high molecular weight compounds like hydrocarbons in the polluted soil (Chaudhary *et al*., 2013).

Many studies have been done to prove that some of the Actinomycetes can degrade a variety of compounds, for instance lignocelluloses and many other polymers occurring in soil and litter and a range of xenobiotic compounds. The key enzymes of the degradation of lignin substructures have been identified (Zimmermann, 1990). *Thermomonospora mesophila*, *Streptomyces badius* and *Streptomyces viridosporus* have all been proved to degrade lignin under different environmental conditions where the end products of biochemical reactions have been analysed (Borgmeyer & Crawford, 1985; Giroux *et al*., 1988; Godden *et al*., 1992). *Streptomyces setonii* has also demonstrated catabolism of vanillic acid and there is further evidence that lignin-related

compounds can be degraded by *Norcardi*a and *Rhodococcus* (Goodfellow & Williams, 1983)

Currently, pollution of the environment by different chemicals is rampant. Some Actinomycetes have shown some importance as they can act on the different unnatural compounds introduced to soil. Previous studies have shown *Nocardia steroids* and *Rhodococcus sp* being common microorganisms in soils polluted with petroleum and Actinobacteria like Arthrobacter have often been stimulated by accumulation of hydrocarbons in soil (Goodfellow & Williams, 1983). A study has also been done to investigate the ability of Actinomycetes (*Nocardia sp., Gordonia sp , Micromonospora sp.* And *Rhodococcus sp*.) and other bacteria (*Micrococcus* and *Sporosarcina sp.*) to degrade motor cycle spent oil (Idemudia *et al.,* 2014). The percentage of hydrocarbon degradation by Actinomycetes (66.6 %) was relatively higher than that of the other bacteria (59.4%). Mesophilic Actinomycetes on the other hand occur in sewage sludge. Previous evidence showed that Coryneb*acterium, Microbacterium*, and *Rhodococcus* strains were present in activated sludge from a dairy (Goodfellow & Williams, 1983). Removal of phthalate esters from activated sludge and prevention of deflocculation has been achieved with *Rhodococcus erythropolis*. *Nocardia amarae* on the other hand can grow in large quantities in the foam of activated sludge and it has been shown to have a strong demulsification ability.

Actinomycetes have also been found to be responsible for the characteristic earthy smell due to production of a compound known as geosmin (Olano *et al*., 2009; Sharma, 2014;

Rakesh *et al*., 2013). They are also important rhizosphere inhabitants of many plants. However, there is little evidence of the roles they play apart from the possible fact that they are responsible for protecting plant roots from attack by phytopathogens and also enhance the growth of plants (Dileep *et al.,* 2013). Previous studies have shown that antibiotics isolated from different regions have the ability to inhibit plant pathogens for instance; Actinomycetes isolated from Turkey's farming soil have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *Agrobacterium tumefaciens* a causal agent of Crown Gall disease (Jeffrey, 2008). Also a research done in Kenya showed that the isolated Actinomycete had the ability to inhibit *Phytophthora infestans* which causes tomato late blight disease (Mutitu *et al.*, 2008).

The increase of soil pollution by use of pesticides and herbicides has seen several microorganisms being used as biological control agents. Actinomycetes have been shown to play an important role in the biological control of insects through production of compounds that are active on insects. Insecticidally active compounds against the house fly *Musca domestica* has been demonstrated (Hussain *et al.,* 2002). Actinomycetes isolated from Bangladesh also showed insecticidal activity. The *Streptomyces sp*. Produced metabolites that had insecticidal activity against the second instar larvae of *Sitophilus oryzae* which is a rice weevil that attacks several crops (Haque *et al.,* 2013).

A number of plant diseases have also been reported to be caused by Actinomycetes especially the genus *Corynebacterium*. Streptomycetes are known to cause scab of

potatoes and sugar beet (Doumbou *et al.,* 1998; Hopwood *et al.,* 2007). The disease, which occurs in many potato-growing regions of the world, is generally associated with Streptomyces scabies. This incidence of common scab is greatest in dry, neutral to alkaline soils.

2.6.2 Medical Importance

Actinomycetes are the foundation of the antibiotics and therefore hold an important role in human health (Jose & Jha, 2016; Chaudhary *et al*., 2013). Approximately two thirds of the naturally occurring antibiotics have been isolated from Actinomycetes mostly from the genera *Streptomyces* and *Micromonospora* and therefore these natural products are a source of lead structures in the development of new drugs (Magarvey *et al.*, 2004; Pandey *et al*., 2011; Rao *et al.*, 2012). Search for new antibiotics effective on drug resistant organisms is currently an important area in medical research. The species belonging to the genus *Streptomyces* are the most dominant Actinomycetes in the soil (Dileep *et al*., 2013). The capacity of the members of the genus *Streptomyces* to produce commercially significant compounds, especially antibiotics, remains unsurpassed. This could be attributed to the extra-large DNA complement of these bacteria (Kurtböke, 2012).

Other bioactive secondary metabolites produced by Actinomycetes which are important in the medical sector include antifungal agents, antitumor agents, immunosuppressive agents and enzymes (Chaudhary *et al*., 2013). Most of the known and valuable antibiotics in use such as streptomycin, gentamicin, rifampicin and erythromycin are

products of Actinomycetes. For instance, streptomycin is produced by *Streptomyces griseus*, kanamycin by *S. kanamyceticus*, neomycin and phosphomycin by *S. fradiae*, and thienamycin by *S. catleya* (Manteca & Sanchez, 2010).

These antibiotics have different mechanisms among pathogenic organisms. Some target bacterial ribosomes and hence are used in the treatment of respiratory infections for example both tetracycline and erythromycin act against Legionnaires' disease (Sharma, 2014). Others attack the cell walls like vancomycin while some target the bacterial RNA polymerase like Rifamycins which is used to treat both tuberculosis and leprosy. Amphotericin is one of the minority antibiotics that attack fungal membranes. In addition to these, metabolites like Adriamycin which are isolated from Actinomycetes prevent DNA replications hence are used in treating cancer. Most of these antibiotics are harmless to human cells hence have no side effects.

Metabolites produced by Actinomycetes have also been reported to act against insects that causes diseases. For instance, Actinomycetes isolated from desert soil in Egypt were shown to produce metabolites that exhibited 100% lethal effect on 3rd instar larvae of mosquitoes *Culex pipiens* (El-Khawagh *et al.*, 2011). This mosquito is the main vector of Rift Valley Fever virus (Baba *et al.*, 2016). The strains of Actinomycetes that produced these metabolites were as follows; *Streptomyces fungicidicus, Streptomyces griseus, Streptomyces albus, Streptomyces rochei, Streptomyces violaceus, Streptomyces alboflavus* and *Streptomyces griseofuscus*.

Actinomycetes have also proved to be the causal agents of many human and animal infections. These diseases include actinomycosis caused by *Actinomyces bovis* and nocardiosis caused by *Nocardia steroids* (Peabody & Seabury, 1957). The natural habitat of *A. bovis* is the human mouth and oropharynx, while that of *N. steroids* is the soil. These medically important Actinomyces organisms cause infections characterized by chronic progression, abscess formation with fistulous tracts and draining sinuses (Sullivan & Chapman, 2010). The diagnosis of actinomycosis and nocardiosis is often delayed.

2.7 Identification Techniques of Actinomycetes

2.7.1 Classical approach

This incorporates morphological, physiological and biochemical characteristics.

2.7.1.1 Morphological techniques

Culture has always been the gold standard method used in the identification of Actinomycetes. Its morphology on agar can provide a clue to its identity. Hence morphological characteristics are widely used for characterizing its genera based on features like presence or absence of spores on the substrate mycelium or the formation of zoospores in specialized spore vesicles or sporangia (Atta *et al.,* 2011). Other characteristics on culture media include powdery appearance and colour that range from white to grey to pinkish to yellow and are normally hard to pick from the culture media as they are tough (Opande *et al*., 2013). Some Actinomycete strains also produce melanoid pigments which range from light-green brown, brown black or distinct brown

pigment depending on the medium used. Others produce soluble pigments apart from melanin which range from orange, red, green, violet, blue and yellow while some strains have the capability of producing a characteristic pigment on the reverse side of the colony (Sharma 2014).

2.7.1.2 Biochemical Techniques

Biochemical tests are incorporated so as to further identify the organism comparing it with the known strains. Some of the biochemical tests that are routinely carried out include catalase test, oxidase test, casein hydrolysis, starch hydrolysis, urea hydrolysis, esculin hydrolysis, citrate utilization, nitrate reduction, acid production from sugar, NaCl resistance, temperature tolerance and thereafter with the morphological characteristics and biochemical tests results, one can be able to identify the isolates by referring to Bergey's Manual of Determinative Bacteriology (Pandey *et al*., 2011). This method of identification through biochemical tests is however time consuming and uses a lot of chemicals. With the advancement of technology in molecular study, other methods have come in which are fast and accurate (Jeffrey, 2008; Kumari *et al.*, 2013).

2.7.2 Molecular Approach

Modern molecular tools have been applied in characterization of Actinomycetes including; the 16S rRNA sequences which have proved to be valuable in the Actinomycetes taxonomy (Adegboye & Babalola, 2012).The study of nucleic acids in characterization of strains is the most potent approach and through this, one can easily know whether one strain is related to the other as these nucleic acids make up gene

products or are genes themselves (Clarridge, 2004). The aspect of molecular science includes both classification and identification and this originated from the early studies of nucleic acid hybridization. RNA is important in analysis of organisms for taxonomic purposes. This analysis usually focuses on three different molecules of ribosomal RNA (rRNA) that is, 5S (~ 120 bp), 16S (~1540 bp) and 23S (~2400 bp) (Giovannoni & Cary, 1993) . These molecules are the essential elements in protein synthesis hence are present in all living organisms and this is why they are important indicators of relatedness of organisms. Other factors that make these rRNA ideal for evolutionary relationships analysis include the fact that the lateral transfer of rRNAs between different organisms is extremely rare and the longer rRNAs (16S, 18S and 23S) contain regions of highly conserved, moderately variable and highly variable sequences. The conserved regions are essential as they provide primer directed sites for PCR as well as convenient hybridization targets for the cloning of rRNA genes (Gentry *et al.*, 2006; Letowski *et al.*, 2004). Therefore the introduction of nucleic acid sequencing techniques has taken the identification to another level.

Phylogenetic studies based on 16S ribosomal DNA sequences are increasingly being used in the study of bacteria including Actinomycetes (Clarridge, 2004). The 16S rRNA gene, consisting of 1542 bp, is highly conserved among microorganisms and is therefore an excellent tool for studying phylogenetic relationships (Sacchi *et al.*, 2002). The 16S rRNA genes of many phylogenetic groups have characteristic nucleotide sequences called oligonucleotide signatures.

Oligonucleotide signatures are sequences which occur in most or all members of a particular phylogenetic group (Woese, 1987) and can be used when designing primers which are genus or species-specific (Bavykin *et al*., 2001).

The features of the 16SrRNA gene that make it a useful phylogenetic tool also make it useful for bacterial detection and identification in the clinical laboratory Patel, (2001). These features include the fact that its present in all bacteria hence is a universal target in bacterial identification, the function of 16S rRNA has remained constant suggesting that random sequence changes are a more accurate measure of time (evolution) and lastly the 16S rRNA gene is large enough (approximately 1,500 bp) to contain statistically relevant sequence information, but more importantly the molecule consists of approximately 50 functional domains (Janda & Abbott, 2007; Patel, 2001). These domains are important because any introduction of selected changes in one domain does not really affect sequences in other domains. These characteristics are among the reasons Woese, (1987) referred to rRNA as the "ultimate molecular chronometer."

Analysis of 16S rDNA begins by extracting DNA (Macrae, 2000). Thereafter amplification of the gene coding for 16S rRNA is done by polymerase chain reaction. With the advancement in technology, primers have been designed to specifically target the 16S rRNA sequence of Actinomycetes (Jeffrey, 2008; Sivakumar, 2001). The amplified products are then purified and sequenced using a DNA sequencer which determines the order in which the bases are arranged within the length of the sample (Xu, Li, & Jiang, 1996). Using phylogenetic analysis procedures, the generated 16S

rRNA sequences are aligned and compared to the sequences in the GenBank database in the National Centre for Biotechnology Information website. These type of simple comparisons of sequence positions will provide an estimate of how closely related the organisms are. This analysis however allows for identification up to the genus level only (Jeffrey, 2008; Tamura *et al.*, 2013) and this is because the 16S rRNA gene is too conserved to provide good resolution at the species and subspecies levels (Cho & Tiedje, 2001). Therefore analysis of the 16S rRNA gene offers a time saving alternative to the classical methods of identification (Alfaresi & Elkosh, 2006) and also allows for the assessment of a broader range of diversity than that obtained by physiological studies (Brambilla *et al.*, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site.

Kericho County is located within the highlands west of the Rift Valley. It has an area of 2,111 km^2 and lies between longitude 35º 02’ and 35º 40’ East and between the equator and latitude 0 23’ South. It’s located in a region with high altitude and therefore its climate is characterized by rains. The soil pH ranges from strongly acidic (4.78) to slightly alkaline (7.15) (Andae, 2014). It is mainly divided into two regions that is; the upper highlands where tea is mostly grown hence the acidic soils and the lower highlands where pyrethrum and maize are grown hence the slightly alkaline soils (Ministry of Agriculture, 1987). These two types of soils enabled isolation of Actinomycetes favoured by both alkaline and acidic soils. The sampling sites included; Kipkelion East, Kipkelion West, Ainamoi, Belgut, Sigowet and Bureti as shown in Figure 3.2

3.2 Experimental Design

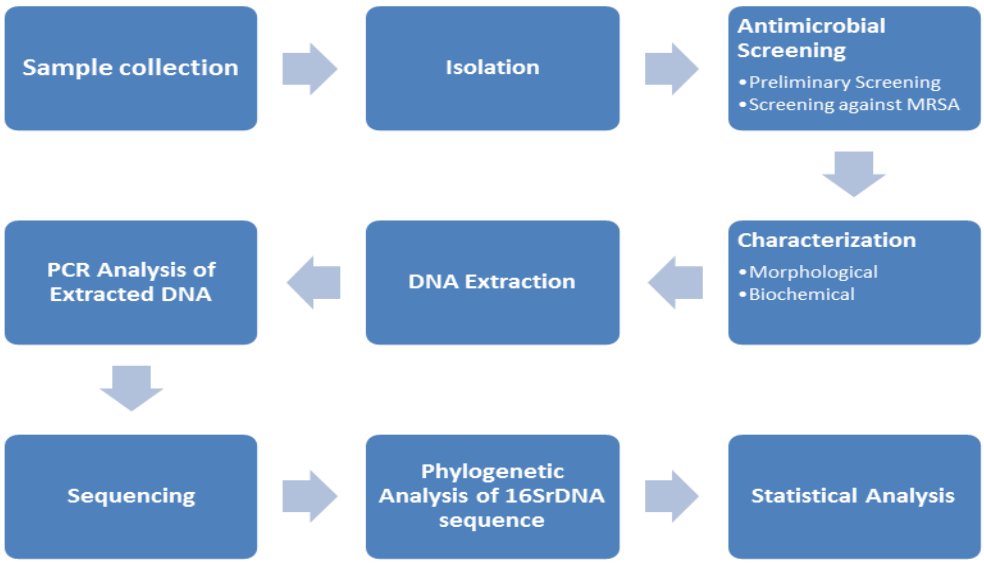

Figure 3.1: Work flow of the study

3.3 Sample collection

Sampling was done by random sampling. An area was identified randomly in a sampling location and then the soil was collected. A radius of 2 kilometers was measured from one sampling point to the other. The soil samples were only collected from virgin land that is, land that has not been cultivated. A depth of approximately 11-16 cm was dug from the soil surface. 11-16cm is the depth where Actinomycetes are abundant and its population gradually reduces with depth (Chaudhary *et al*., 2013). The soil depth was measured using a ruler and soil was scooped using a sterile spoon and the coordinates of the sampling point noted. The collected soil was put in sterile zip lock bags and labeled. The coordinates of each sampling point was taken and this was used to generate the map shown in Figure 3.2 using Google maps. Later, the soils were air dried on a sterile brown

paper in a room at room temperature for a week to reduce the population of gram negative bacteria before transporting them to the laboratory for processing (Jeffrey, 2008). After air drying, the soil samples were repacked into sterile labeled zip lock bags and transported to KEMRI, Mycology Laboratory at the Centre for Microbiology Research for analysis.

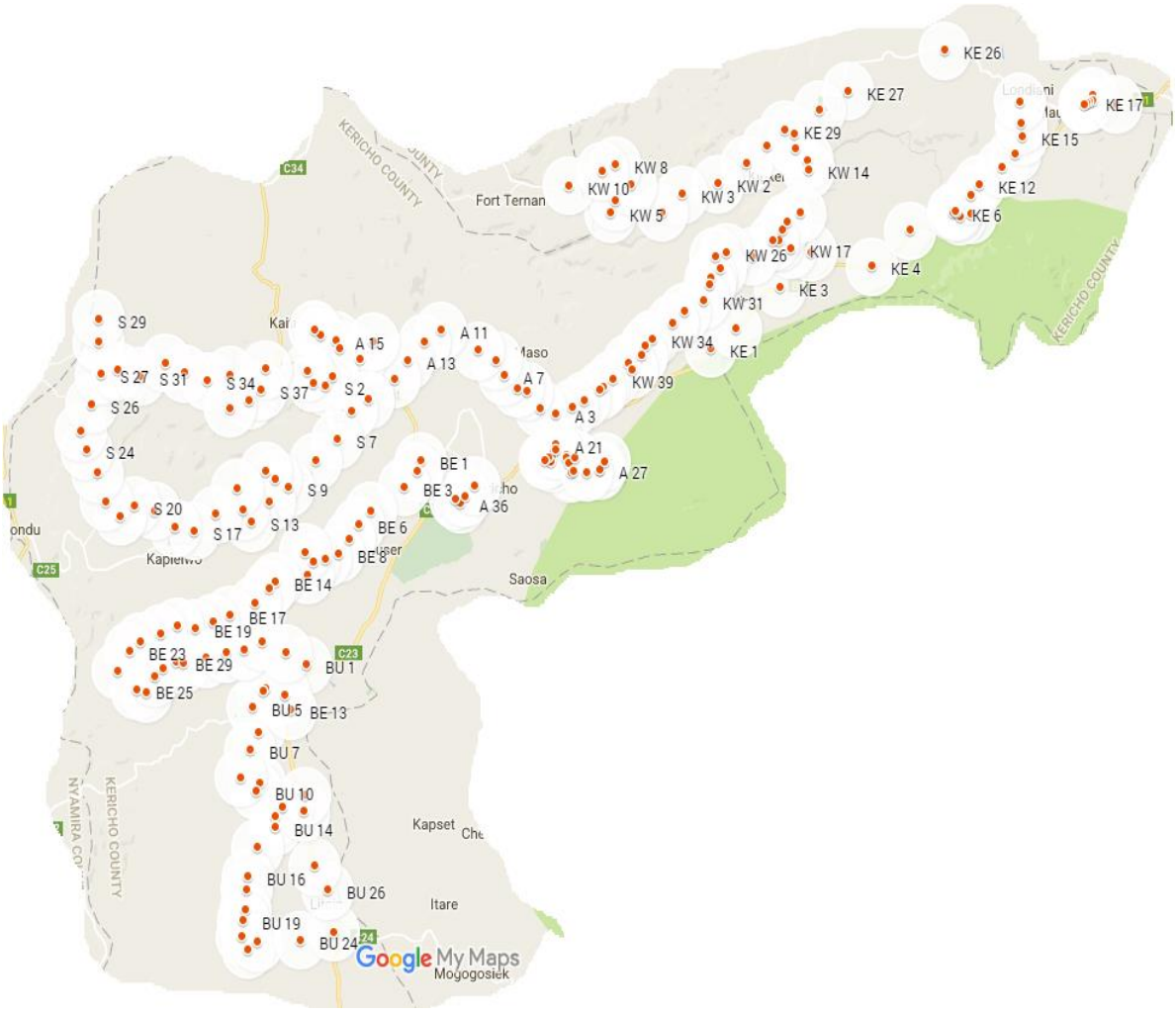

Figure 3.2: Map Showing Sampling Points in Kericho County

Note: (KE-Kipkelion East, KW-Kipkelion West, BU-Bureti, BE-Belgut, S-Sigowet, A-Ainamoi)

3.4 Determination of Sample size

The following simple formula (Cochran, 1963) was used to calculate the sample size:

$$n = \frac{Z^2 P(1-P)}{e^2}$$

Where n = sample size,

Z = Z statistic for a level of confidence which in this case is 95% confidence level hence 1.96

P = expected prevalence or proportion in this case is 50% hence 0.5

e = precision which in this case is 5% hence 0.05

$$\text{Therefore sample size} = \frac{(1.96^2)\ (0.5)\ (0.5)}{0.05^2}$$

$$= 384 \text{ samples}$$

A total number of two hundred and fourteen soil samples were collected as opposed to the expected three hundred and eighty four samples. This was due to the area being vast and financial constraints. The number of soil samples collected from every site is shown in the Table 3.1 below.

Table 0.1: Soil samples collected from each location

Sampling location	Number of soil samples collected
Kipkelion East	30
Kipkelion West	40
Ainamoi	41
Sigowet	38
Belgut	35
Bureti	30

3.5 Isolation and Cultivation of Actinomycetes

Actinomycetes were isolated by serial dilution method (Gebreyohannes *et al.,* 2013) from the 214 soil samples, where stock solution was prepared by diluting 1 gram of soil in 9 mL of sterile distilled water and vortexed for 3 minutes. From the stock solution, 1 mL was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} by serial dilution method. Thereafter 0.1 mL of the suspension from 10^{-1}, 10^{-3}, and 10^{-5} were used to spread aseptically on starch-casein agar (Oxoid USA) plates supplemented with Rifampicin 5µg/ml and Fluconazole 25µg/ml to inhibit bacterial and fungal contamination respectively (Gurung *et al.,* 2009; Rao *et al*., 2012). The starch casein agar is which is recommended for detection of Actinomycetes. The plates were then incubated aerobically at 30^{o}C for 10 days. Plates were observed periodically for growth of Actinomycetes. Suspected colonies were identified by their chalky, firm and leathery texture (Rao *et al*., 2012). The colonies were picked and sub cultured for purity by

streaking on Starch Casein agar. Pure colonies were then inoculated into 1.5mL of 15% sterile glycerol stocking solution. They were then stored at -35^{o}C until further analysis

3.6 Antimicrobial screening

3.6.1 Preliminary Antimicrobial Screening

Antimicrobial screening was done using modified agar disc diffusion method as described by Kirby Bauer (1979). The test pathogens used were both reference isolates that is the American Type Culture Collection (ATCC) and clinical isolates which were provided at KEMRI and were as follows: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 90028), *Trichophyton mentagrophyte* (clinical isolate) and *Microsporum gypseum* (clinical isolate). The stocked Actinomycetes isolates were revived by sub-culturing on Nutrient Agar plates (Oxoid, USA). The colony was then picked and inoculated into 5ml Mueller Hinton broth (Oxoid, USA) and incubated at 30^{o}C for 10 days. The test isolates were revived on Nutrient Agar (Oxoid, USA) plates for bacteria and Potato Dextrose Agar (Oxoid, USA) plates for fungi. Thereafter 0.5 McFarland of the test isolates were made using sterile distilled water. A sterile swab was dipped into the suspension and excess liquid was removed by gently rotating the cotton swab against the inner surface of the test tube. To obtain even growth, the entire Mueller Hinton agar plate surface was swabbed uniformly with the cotton swab (Gebreyohannes *et al*., 2013).

Paper discs were prepared from Whatman No 1 filter papers and sterilized by autoclaving at 121^{o}C, 15psi for 15 minutes (Ngeny *et al.*, 2013). The disc (6 mm in

diameter) was impregnated with 15µLof the 10 day old culture broth and placed on Mueller Hinton Agar inoculated with the test isolates. They were then incubated at 37^{o}C for 24 hours for bacteria and at 30^{o}C for 48 hours for yeast and for 96 hours for the molds. The isolates with antimicrobial activities were identified by measuring the inhibition zone in millimeters (mm) using a ruler. The absence of growth or a less dense growth of test bacteria near the disc was considered as positive for production and secretion of antibacterial metabolite by the isolates (Kekuda *et al*., 2010). Conventional antibiotics were used as positive controls namely: Chloramphenicol (30µg/disc) for bacteria and Itraconazole (2 ug/ml) for fungi while a disc impregnated with normal saline was used as a negative control (Han *et al.*, 2013). The Actinomycetes isolates that showed antimicrobial activity were further tested for activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

3.6.2 Screening for activity against Methicillin Resistant *Staphylococcus aureus*

The Actinomycetes isolates that showed antimicrobial activity against the above pathogens were further tested against Methicillin-resistant *Staphylococcus aureus* (clinical isolate) obtained from KEMRI archives. Kirby Bauer disc diffusion method was used as described in section 3.6.1.

3.7 Morphological and Biochemical Characterization

3.7.1 Macroscopic Characterization

The isolates that showed activity against the test pathogens were cultured on Starch Casein Agar plates and incubated at 30^{o}C for 7 to 10 days. The colour of aerial mycelium, reverse colour, nature of the colony was observed and recorded.

3.7.2 Microscopic Characterization

This was done using two methods as follows:

3.7.2.1 Modified Slide culture technique

The Actinomycete isolates that showed activity were streaked on corn meal agar plate and a sterile cover slip was aseptically placed on top then it was incubated at 30^{o}C for 4-7 days depending on the growth rate of the isolate. Thereafter the cover slip was aseptically removed using sterile forceps and placed on a grease free glass slide that contained a drop of sterile water. This was then observed under X 40 magnification and then a fixed mount was done and observed under high power using a light microscope. Identification was done using macroscopic and microscopic morphological characteristics (Kandasamy *et al*., 2012)

3.7.2.2 Gram Stain

A thin smear of the 7 day old Actinomycetes cultures were inoculated into grease free slides. Thereafter they were heat fixed and placed in a staining rack. The slides were then flooded with Crystal violet for one minute and then rinsed with distilled water gently. Gram's iodine was then gently flooded on the smears and allowed to stand for

one minute before gently rinsing with distilled water. This was then decolourised using 95% ethyl alcohol for 5 seconds and immediately rinsed with water to avoid overdecolorization. Finally safranin was gently flooded on the smears and let to stand for 45 seconds before rinsing with distilled water. The slides were then blot dried using absorbent paper then viewed using a light-microscope under oil-immersion (x1000) (Cappuccino & Sherman, 2002).

3.7.3 Biochemical Characterization

Different biochemical tests were carried out as follows:

3.7.3.1 Casein Hydrolysis test

Casein is a major protein found in milk. This tests the ability of a microorganism to degrade the casein by producing proteolytic enzyme, called proteinase (caseinase) which breaks the peptide bond. Isolates were streaked on skimmed milk agar medium and incubated at 30 °C for 7 days.

Hydrolysis was demonstrated by clear zones around the colonies (Ten *et al.*, 2005).

3.7.3.2 Citrate utilization

Citrate test is used to show the ability of a microorganism to utilize citrate as the sole carbon source (Harold, 2002). The utilization of citrate depends on the presence of an enzyme citrase produced by the organism. Isolates were also streaked on Simon's citrate slant agar and incubated at 30 °C for 7 days. Bromothymol blue is used as an indicator and therefore when the citric acid is metabolized, the CO_2 generated combines with

sodium and water to form sodium carbonate an alkaline product, which changes the colour of the indicator from green to blue and this constitutes a positive test (Cappuccino & Sherman, 2002).

3.7.3.3 Urea hydrolysis

Urea hydrolysis was carried out as described by Cappuccino and Sherman, (2002). This test is done to test the presence of urease. Urease is a hydrolytic enzyme that attacks the nitrogen and

carbon bond in amide compounds such as urea to form alkaline end product ammonia. The test isolate was inoculated into sterile urea agar slants containing the pH indicator phenol red and incubated at 30 °C for 7 days. Production of ammonia creates an alkaline environment that causes phenol to turn to a deep pink. This is a positive reaction for the presence of urease. The absence of urease indicates a negative result.

3.7.3.4 Catalase test

Catalase enzyme present in some microorganisms breaks down hydrogen peroxide to water and oxygen and this helps them in survival since hydrogen peroxide is lethal to cells. A modified version of the method described by Cappuccino and Sherman, (2002) was used in which isolates were grown on starch casein agar plates at 30^{o}C for 7 days and thereafter a colony was picked with a sterile stick and placed on a sterile glass slide containing a drop of hydrogen peroxide. Production of bubbles was indicative of positive results hence the production of free oxygen.

3.8 Molecular Characterization

3.8.1 Extraction of Bacterial Genomic DNA

A loop full colony of 5-day subcultures grown on Nutrient agar plates (Oxoid, USA), of the 39 Actinomycetes that showed antimicrobial activity were used for the DNA extraction using a Qiagen DNA Extraction Kit with strict adherence to the manufacture's protocol. The Actinomycetes were recovered by centrifuging the cultures at 20,000 x g for 5 minutes before resuspending them in 180 µl enzymatic lysis buffer followed by an incubation at 37^{o}C for 30min. 25 µl of Proteinase K and 200 µl of Buffer AL (without ethanol) were then added to the samples and vorted before incubating at 56^{o}C for 30 min. After the incubation, 200 µl of 100% ethanol was added into the samples and contents vortexed thoroughly before pipetting them into the provided DNeasy Mini spin columns and centrifuged at 10,000 x g for 1 minute (ensuring the membrane was completely dry and free from any residual ethanol). The flow-through was discarded and the spin column placed in a new collection tube. 500 µl of Buffer AW1 was added and then centrifuged at 10,000 x g for 1 minute. The flow-through was discarded and the spin column placed in a new collection tube. 500 µl of AW2 Buffer was then added and contents centrifuged at 20,000 x g for 3 minute to wash and dry the membrane. The flow-through was discarded again and the spin column placed in a sterile microfuge tube. 200 µl of AE Buffer was then pipetted directly onto the membrane and contents incubated at room temperature for 1 minute before centrifuging at 10,000 x g to recover the DNA. The presence of the extracted DNA was then verified on 0.8% agarose gel in 1 x TAE buffer and visualised under UV light after staining with

Ethidium bromide (Nonoh *et al.,* 2010).The DNA samples were then stored at -20^{o}C until further use.

3.8.2 PCR Analysis of Extracted DNA

Total DNA from each isolate was used as a template for amplification of the 16S rDNA. The 16S rRNA gene is a conserved gene in the bacteria (Kumar *et al.,* 2010). The full length of the 16S rDNA sequence was amplified using the following group specific pair of primers synthesized at International Livestock Research Institute, Kenya; F-Act 243 (5´-GGATGAGCCCGCGGCCTA-3´) and R-Act A3 (5´-CCAGCCCCACCTTCGAC-3´) described by (Monciardini *et al*., 2002). A final reaction volume of 50 µl was prepared containing; 1 µl of genomic DNA, 5 µl of 10x Genescript *Taq* Buffer, 3 µl of 2.5 mM dNTPs, 0.4 µl of 20 mg/ml BSA, 0.5 µl of 5U Genescript *Taq* polymerase and 1 µl of 20 pmole of each primer. Amplifications were then carried out according to the following cycling conditions: 10 min at 95°C and 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, 45 s, followed by 10 min at 72°C. Amplification products were analyzed by electro-phoresis in 2% (w/v) agarose gels stained with ethidium bromide.

3.8.2.1 Purification of samples for sequencing

The PCR products were then purified using the QIAquick® PCR purification Kit from Qiagen according to the manufacturer's instructions and the samples were sent to Macrogen Netherlands for sequencing. The primers used in amplification were the ones used in sequencing of both forward and reverse strands.

3.9 Phylogenetic analysis of the 16S rDNA Sequence.

A contig, which is a consensus sequence of both the reverse and forward sequence was generated using the BioEdit software aligner (Hall, 1999). The 16S rDNA sequences generated were compared to sequences in the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Phylogenetic and molecular evolutionary analysis was then conducted using MEGA version 6 (Tamura *et al.*, 2013). The sequences were aligned using the MUSCLE program against the nearest neighbours and the evolutionary history was inferred using the Neighbor-Joining method (Saitou, 1987). The evolutionary distances were computed using Maximum Composite Likelihood model (Tamura *et al.*, 2004). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed.

3.10 Statistical Analysis

The antimicrobial results obtained were analyzed using one way ANOVA to compare the level of significance between the isolates' antimicrobial activities and the positive control by using SPSS version 20.

CHAPTER FOUR

RESULTS

4.1 Isolated Actinomycetes

A total of 107 Actinomycetes were isolated from a total of 214 soil samples based on their typical morphology of chalky, firm and leathery texture. Most of the soil samples that had no Actinomycetes had fungal contamination despite the fact that an antifungal had been added to the isolation media. After incubation of the pure cultures for 10 days, the morphological characteristics were observed and recorded. It was noted that colonies had different colours ranging from red, yellow, grey and white as shown by the representative isolates in Plate 4.1. It was further observed that most colonies had textures which were hard and difficult to scrap off the agar as these are the characteristics of Actinomycetes (Salam & Rana, 2014). The textures of 90% of the isolates were hard to scrap being one of its characteristics of Actinomycetes while 10% were easier to pick. The 39 isolates that showed antimicrobial activity had different range of macroscopic morphologies. Most of them (11) were greyish with whitish edges, powdery, dry colonies while five of them were creamish, greyish, dry, raised round colonies. Some colonies were seen to change colour on further incubation for example, IS75 appeared to have yellow aerial mycelium but on further incubation, it became greyish-whitish chalky colony.

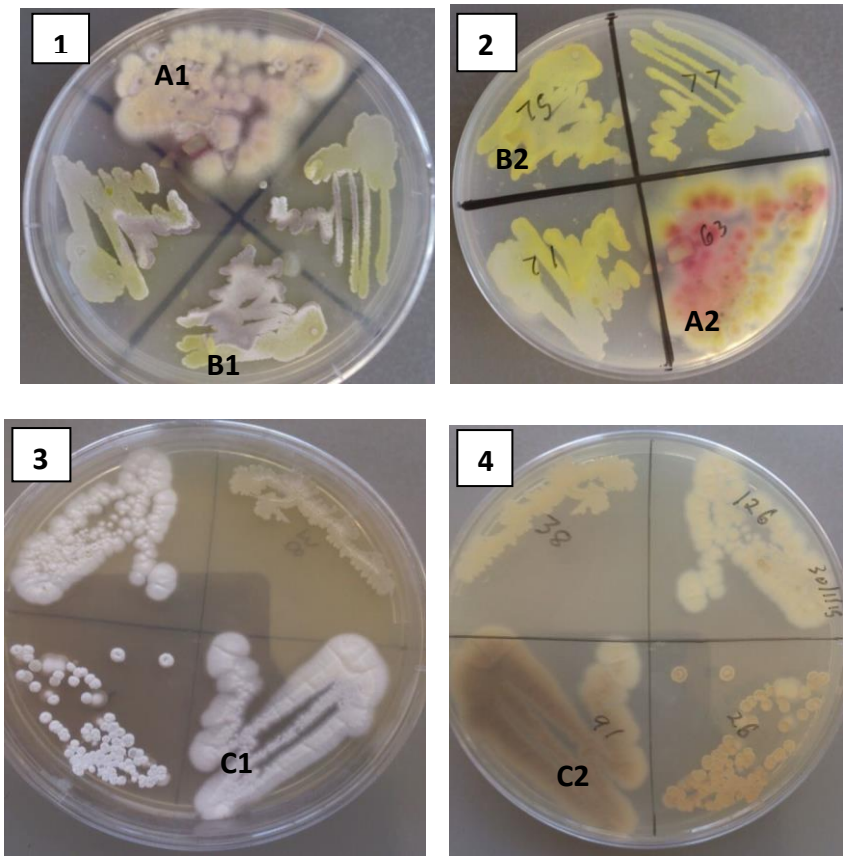

Plate 4.1: Morphologies and colours of representative Isolates (Aerial mycelium's morphology and colour - A1=IS63: light yellowish-red leathery colony, B1=IS75: greyish-whitish chalky colony, C1=IS91: white wrinkled colony. Substrate mycelium's morphology and colour- A2=IS63: yellow which turns red on further incubation, B2=IS75: bright yellow mycelium, C2=IS91: brownish mycelium

The microscopic observations revealed that colonies ranged from some having highly intertwined filamentous hyphae (A) to log-like fragmented hyphae (B) while some had branching hyphae(C). Clustered and single spores were also seen (D). All the isolates

were gram positive as they retained the primary colour (crystal violet) hence appeared blue and this is a characteristic of Actinomycetes as shown in C and D.

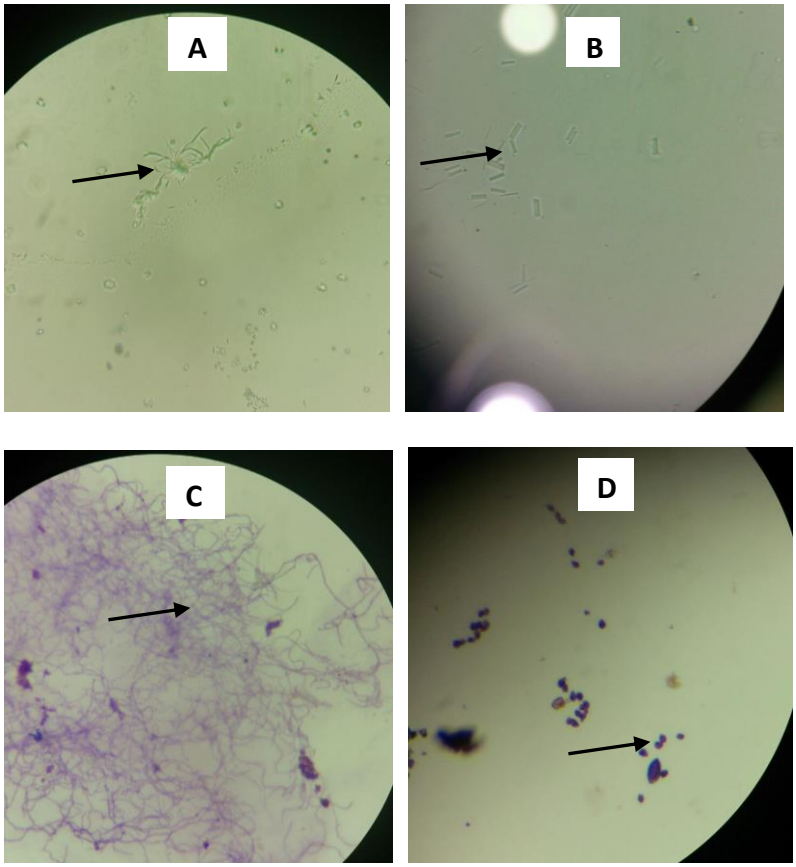

Plate 0.1: Microscopic morphologies (A and B shows morphologies on wet mount while C and D shows morphologies on Gram stain)

4.2 Antimicrobial activity screening of the isolated Actinomycetes

Out of the 107 isolated Actinomycetes, only 39(36.4%) showed antimicrobial activity against the five of the six test isolates with inhibition zones ranging from 6mm to 34mm. Sixteen Actinomycetes isolates showed antimicrobial activity against *S. aureus* (ATCC 25923), two showed activity against *C. albicans* (ATCC 90028), thirty three showed

antifungal activity against *T. mentagrophyte* (clinical isolate), twelve Actinomycetes isolates showed antimicrobial activity against *M. gypseum* (clinical isolate) while two showed activity against Methicillin Resistant *S. aureus* (clinical isolate). This is shown in Figure 4.1 below.

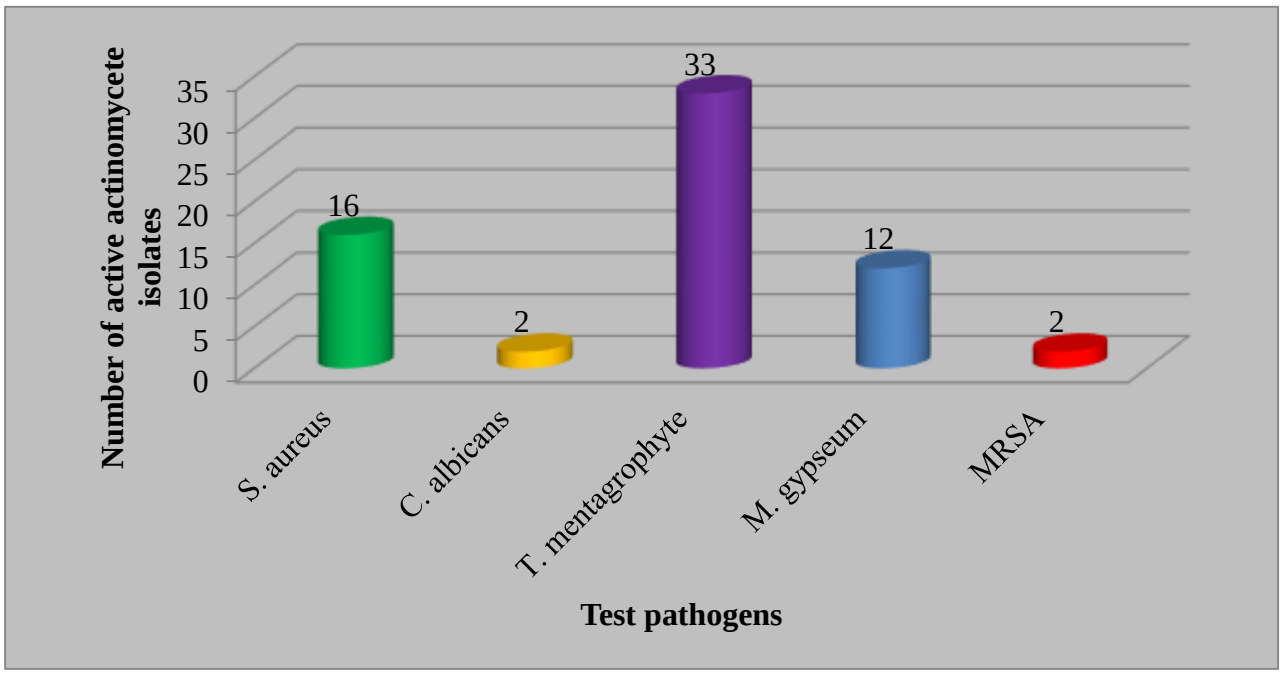

Figure 4.1: Proportion of the isolated Actinomycetes with antibiotic activity

The antimicrobial activity of all the isolates tested against *S. aureus* (SA) were statistically significant (P<0.001) (Table 4.1). The inhibition zones of IS30, IS42, IS71 and IS77 were at the maximum of 30.00mm, 30.33mm, 27.00mm, and 32.67mm respectively against *S. aureus* and were significantly higher than Chloramphenicol (23.67 mm) as shown in Table 4.1. The zones of inhibition for Isolates IS6 and IS40 tested against *C. albicans* (CA) were significantly lower than that of Itraconazole

(29.67mm) with zones of 10.33mm and 8.33mm respectively, however they were statistically significant ($P<0.001$). None of the Isolates was active against *E. coli* (EC).

Most isolates screened (84.6%) were active against *T. mentagrophyte* (TM) and most of these isolates were isolated from red soils. The inhibition zones were significantly lower ($P<0.001$) from those of Itraconazole (39.67mm) which was the control drug. The inhibition zones of the active isolates tested against *M. gypseum* (MG) were significantly lower ($P<0.001$) than that of the control (38.33mm). The two isolates that showed antibiotic activity against the Methicillin resistant *S. aureus* were also statistically lower than the control ($P<0.01$). Isolate IS58 showed good activity (20.33mm) compared to Chloramphenicol (25.33mm) as opposed to IS72 that had an inhibition zone of 10.33mm.

Table 0.1: Antimicrobial activity (mm) exhibited by Isolated Actinomycetes

ISOLATES	Test Pathogens					
	SA	EC	CA	TM	MG	MRSA
IS1	12.33±0.58	_	_	_	_	_
IS3	_	_	_	8.33±1.53	_	_
IS6	_	_	10.33±0.58	_	11.67±1.16	_
IS7	_	_	_	22.67±0.58	_	_
IS20	6.33±0.58	_	_	10.33±0.58	_	_
IS26	7.33±0.58	_	_	_	15.33±0.58	_
IS30	30.00±1.00[a]	_	_	26.33±0.58	18.33±0.58	_
IS34	_	_	_	20.33±0.58	_	_
IS38	_	_	_	30.33±0.58	_	_
IS40	_	_	8.33±0.58	_	_	_
IS42	30.33±0.58[a]	_	_	23.67±0.58	_	_
IS46	10.33±0.58	_	_	11.33±0.58	_	_
IS48	_	_	_	15.00±1.00	_	_
IS52	_	_	_	15.67±0.58	_	_
IS53	_	_	_	21.33±1.16	_	_
IS55	6.33±0.58	_	_	19.67±0.58	_	_
IS58	_	_	_	32.67±0.58	17.67±0.58	20.33±0.33
IS59	9.00±1.00	_	_	_	_	_
IS63	_	_	_	24.67±0.58	11.67±0.58	_
IS67	_	_	_	9.33±0.58	18.00±1.00	_
IS71	27.00±1.00[a]	_	_	27.33±0.58	14.67±0.58	_
IS72	_	_	_	13.67±0.58	20.33±0.58	10.33±0.33
IS75	_	_	_	34.67±0.58	18.00±1.00	_
IS77	32.67±0.58[a]	_	_	25.67±0.58	_	_
IS91	_	_	_	17.67±0.58	15.33±0.58	_
IS92	_	_	_	11.67±0.58	_	_
IS98	7.33±0.58	_	_	7.33±0.58	_	_
IS100	_	_	_	9.67±0.58	16.67±0.58	_
IS101	_	_	_	8.67±0.58	_	_
IS104	_	_	_	15.00±1.00	_	_
IS105	_	_	_	14.33±0.58	_	_
IS118	_	_	_	12.67±0.58	_	_
IS123	6.33±0.58	_	_	7.67±0.58	_	_
IS124	_	_	_	7.67±0.58	_	_
IS125	7.33±0.58	_	_	7.67±0.58	10.33±0.58	_
IS126	_	_	_	8.67±0.58	_	_
IS127	7.33±0.58	_	_	8.33±0.58	_	_
IS128	6.33±0.58	_	_	_	_	_
IS129	10.33±0.58	_	_	8.67±0.58	_	_
Control	**23.67±0.58**	**25±0.58**	**29.67±0.58**	**39.67±0.58**	**38.33±0.58**	**25.33±0.33**

Note: Superscripted figures indicate higher significant value (P<0.001) from the control Inhibition zone diameters mean ±SD of the three replications

(-): no antimicrobial activity, Control drug for bacteria: Chloramphenicol (30µg/disc), Control drug for fungi: Itraconazole (2µg/ml)

Plate 4.3 below shows an inhibition zone in A by IS30 that measures 27mm. IS41 has no inhibition zone hence no antimicrobial activity against *T. mentagrophyte*. B show antimicrobial activity by IS71 against *S. aureus.*

A B

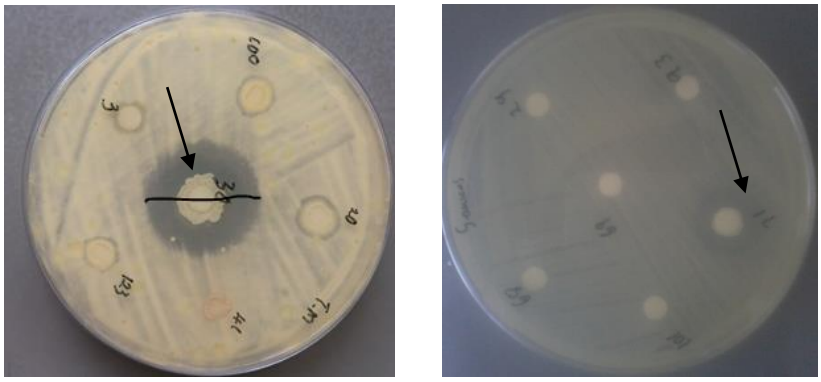

Plate 0.2: Plates showing Antimicrobial Activity of isolated Actinomycetes against A: *T. mentagrophyte* and B: *S. aureus*

Twenty one of the Actinomycetes with antimicrobial activity had activity against two or more test pathogens. Most 9(43%) of these Actinomycetes isolates were active against both *S. aureus* and *T. mentagrophyte*. The two isolates that showed antibiotic activity against MRSA were also both active against *T. mentagrophyte* and *M. gypseum* (Table 4.1). Other 3 isolates also showed antibiotic activity against three test pathogens that is; *S. aureus, T. mentagrophyte* and *M. gypseum*. Five (24%) Actinomycete isolates showed antimicrobial activity against both *T. mentagrophyte* and *M. gypseum* which are both dermatophytes, one (5%) isolate (IS6) showed antimicrobial activity against both *C.*

albicans and *M. gypseum* while also one (IS26) showed activity against both *S. aureus* and *M. gypseum* as shown in figure 4.2.

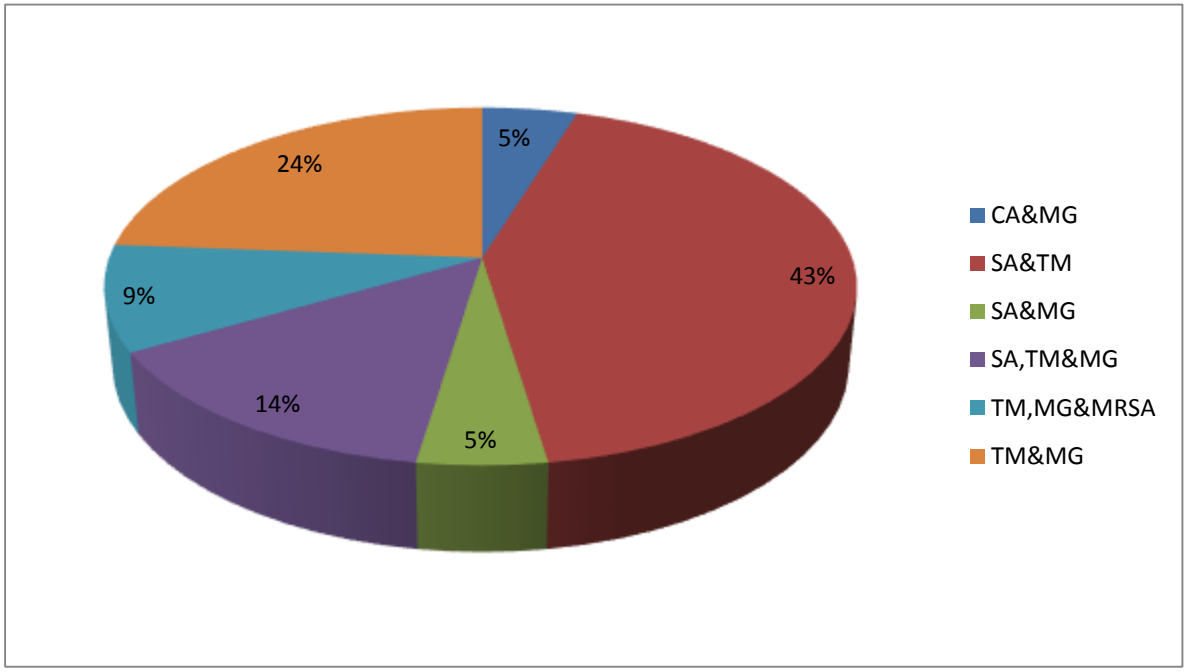

Figure 4.2: Actinomycetes isolates that had antimicrobial activity against more than one test pathogen

Ainamoi had the highest number of isolated Actinomycetes (43.59%) showing antimicrobial activity. This was followed by Belgut with 17.95%, Kipkelion East with 15.38%, Kipkelion West with 12.82%, Sigowet and Bureti both having 5.13% of the active isolates. This is shown in the table 4.2.

Table 0.2: Active Isolates with their Site of Origin

Site	Site Location	Isolate
A2	Ainamoi	IS30,IS34
A4	Ainamoi	IS20
A8	Ainamoi	IS52, IS63
A9	Ainamoi	IS26
A11	Ainamoi	IS42
A12	Ainamoi	IS101
A13	Ainamoi	IS46
A15	Ainamoi	IS48
A19	Ainamoi	IS127,
A29	Ainamoi	IS55, IS58
A32	Ainamoi	IS53
A37	Ainamoi	IS40, IS38
A42	Ainamoi	IS59
KE4	Kipkelion East	IS71, IS72, IS75
KE5	Kipkelion East	IS77
KE21	Kipkelion East	IS67
KE27	Kipkelion East	IS129
KW2	Kipkelion West	IS1, IS3
KW31	Kipkelion West	IS104
KW35	Kipkelion West	IS6, IS7
S14	Sigowet	IS98
S21	Sigowet	IS126
BE4	Belgut	IS91
BE8	Belgut	IS92
BE14	Belgut	IS128
BE20	Belgut	IS118
BE30	Belgut	IS123, IS124, IS125
BU11	Bureti	IS100
BU28	Bureti	IS105

The two isolates that showed antimicrobial activity against MRSA were isolated from Ainamoi (A29) and Kipkelion East (KE4) respectively. The four isolates that showed

significantly higher antimicrobial activity against *S. aureus* compared to the control drug Chloramphenicol were also isolated from Ainamoi (IS30 and IS42) and Kipkelion East(IS71 and IS77). The isolates that showed antifungal properties especially the ones that showed antimicrobial activity against *T. mentagrophyte* were isolated from soil samples from all the six regions.

4.3 Biochemical Analysis

From table 4.3, all isolates showed the ability to utilize citrate as the sole carbon source and also they were all catalase positive hence the ability to breakdown hydrogen peroxide to oxygen and water. Isolate IS42, IS53, IS58 and IS72 had the ability to hydrolyze casein. Most of the morphological characteristics observed were characteristics exhibited by *Streptomyces* species.

The plate below shows IS42 and IS72 showing casein hydrolysis while IS7 and IS75 have no ability to hydrolyze casein.

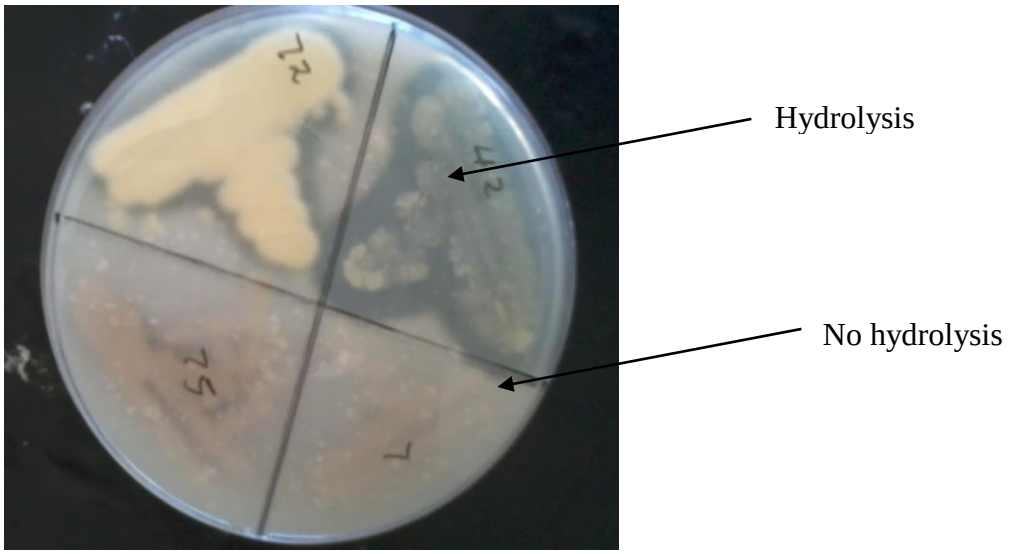

Plate 0.3: Casein Hydrolysis by Actinomycetes isolates

Table 0.3: Macroscopic, Microscopic and Biochemical Characteristics of representative Actinomycetes isolates

Isolates	Aerial mycelium	Substrate mycelium	Pigmentation	Cell form	Casein hydrolysis	Citrate utilization	Urease	Catalase
IS 7	Orange	Creamish	None	Log-like fragmented hyphae	_	+	+	+
IS 34	Greyish	Faded orange	Brown	Long branching hyphae seen	_	+	+	+
IS 40	Cream	Cream	None	Single spores	_	+	+	+
IS 42	Cream	Cream	None	Long branching hyphae	+	+	+	+
IS 48	White creamish	creamish	Brown	Branched hyphae	_	+	+	+
IS 52	Pale orangish	Pale orangish	None	Short fragmented rods observed	_	+	+	+
IS 53	Greyish creamish	Cream	None	Long hyphae with spores	+	+	+	+
IS 58	Mucoid greenish	Light greenish	None	Short rods	+	+	+	+
IS 63	Light yellowish red leathery	Red	None	Spores seen	_	+	+	+
IS 71	Greyish-whitish chalky	Bright yellow	None	Grouped and single spores	_	+	+	+
IS 72	White leathery	White	None	Branched hyphae with spores in twos	+	+	+	+
IS 75	Greyish-whitish chalky	Yellow	None	Long branching hyphae	_	+	+	+
IS 77	Greyish-whitish chalky	Bright yellow	None	Short rods	_	+	+	+
IS 125	Greyish with whitish edges	Brownish	Brown	Intertwined hook-like hyphae	_	+	+	+

Figure 4.3 shows an Actinomycete isolate with a positive result for a urease test hence the pink colour.

Figure 4.3: Isolated Actinomycete showing Positive Urease test

4.4 Molecular Analysis

The DNA from the 39 isolates that showed antimicrobial activity was extracted and amplified. From the 39, only 15(38.5%) isolates with a size of about 1250bp were able to be amplified by the pair of primers used. The two isolates IS58 and IS72 that were active against MRSA were among those that could not amplified using this pair of primers.

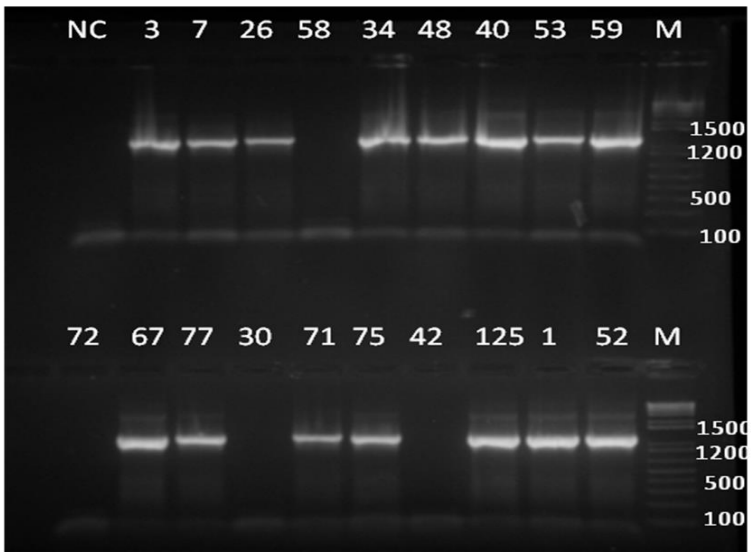

Figure 4.4: Amplifications of 16S rDNA of Actinomycetes isolates

Note: NC is the negative control while M is the molecular ladder. The numbers represent the isolates.

4.5 Phylogenetic Analysis of the 16S rDNA sequences

The 16S rDNA sequences of the 15 isolates were compared with those in NCBI. The phylogenetic analysis of the 16S rRNA gene showed that the isolates were members of the Actinomycetes group and most 14(93.3%) were affiliated to the genus *Streptomyces* while only one (6.7%) showed similarity with the genus *Rhodococcus*. The similarity values were between 98% and 100%. The phylogenetic tree of representative sample amplified is shown in figure 4.5. There were therefore no diverse groups of Actinomycetes found. Though many of the isolates were *Streptomyces* species, they were of different strains as shown in the phylogenetic tree below.

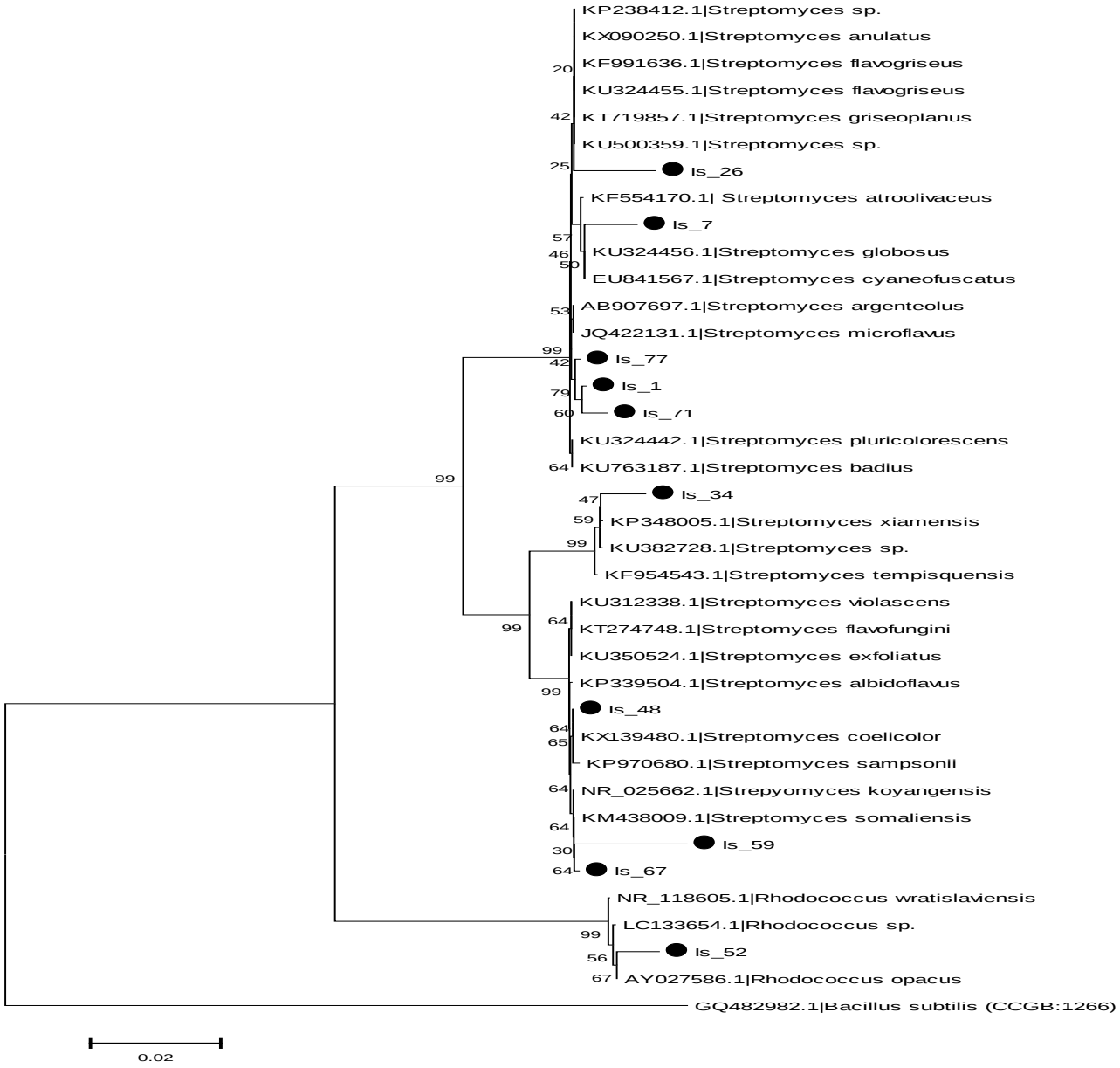

Figure 4.5: Neighbor joining tree showing phylogenetic positions of the partial 16S rDNA sequences of the isolates

The above tree in Figure 4.5 was rooted using 16S rDNA of *Bacillus subtilis* as outgroup. The GenBank accession numbers of the strains are indicated. The scale bar indicates 2% estimated sequence divergence. The percentage of replicate trees in which

the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The 15 Actinomycete isolates that were sequenced were edited using BioEdit version 7.2.5 and sent to NCBI for deposit in the GenBank. The following accession numbers were given to them.

Table 0.4: Accession Numbers of the Sequenced Actinomycetes Isolates

Actinomycete Isolate	**Genus name**	**Accession number**
IS1	*Streptomyces sp.*	KY922837
IS3	*Streptomyces sp.*	KY922838
IS7	*Streptomyces sp.*	KY922839
IS26	*Streptomyces sp.*	KY922840
IS34	*Streptomyces sp.*	KY922841
IS40	*Streptomyces sp.*	KY922842
IS48	*Streptomyces sp.*	KY922843
IS53	*Streptomyces sp.*	KY922844
IS59	*Streptomyces sp.*	KY922845
IS67	*Streptomyces sp.*	KY922846
IS71	*Streptomyces sp.*	KY922847
IS75	*Streptomyces sp.*	KY922848
IS77	*Streptomyces sp.*	KY922849
IS125	*Streptomyces sp.*	KY922850
IS52	*Rhodococcus sp.*	KY922851

CHAPTER FIVE

DISCUSSION

Natural products are the most promising sources for developing future antibiotics (Cragg & Newman, 2013; Sharma *et al*., 2011). About 70% of antibiotics in use have been isolated from Actinomycetes (Gurung *et al.*, 2009) and are known to be the main source of lead compounds for antimicrobial drugs. Kericho County is located in a region with high altitude and the soil pH ranges from 4.78 to 7.15 (Andae, 2014) hence the possibility of finding novel strains of Actinomycetes. Actinomycetes have been known to grow well under neutral or slightly alkaline environments (Basilio *et al*., 2003). However some studies have also proved that novel acidophilic Actinomycetes can be isolated from acidic soils (Guo *et al*., 2015).

A total of 107 Actinomycetes were isolated based on their typical morphology from the 214 soil samples. Out of the total 107 Actinomycete isolates, 39 (36.4%) showed antimicrobial activity against the test pathogens as shown in Table 4.1. The morphological features of the isolated Actinomycetes showed that 90% had morphological characteristics of *Streptomyces sp.* The characteristics of *Streptomyces* noted were white to grayish on the aerial mycelium with brownish substrate mycelium or white-grayish aerial mycelium with yellow substrate mycelium. It is generally known that morphologically similar strains have a likelihood of producing the same antibiotics and therefore when doing isolation, one is likely to pick one isolate of the similar strains (Mazza *et al*., 2003). However from this study, three morphologically similar strains

with different antimicrobial properties were isolated. IS71, IS75 and IS77 had similar morphologies as shown in Plate 4.1. Their substrate mycelium was bright yellow while the aerial mycelium was yellow which changed to chalky white then grey on further incubation. These three isolates however were noted to have different antimicrobial properties (Table5.1). IS71 had antimicrobial activity against *S. aureus*, *T. mentagrophyte* and *M. gypseum*. IS75 on the other hand had antimicrobial activity against *T. mentagrophyte* and *M. gypseum* while IS77 had activity on *S. aureus* and *T. mentagrophyte*. This is in agreement with Khanna *et al*., (2011), who noted that there are limitations in using traditional morphological methods as this may lead to one missing out on potentially useful strains if one was to eliminate a strain based on identical morphologies.

Out of the 39 Actinomycetes isolates with antimicrobial properties, 41.0% (16 isolates) showed antimicrobial activity against *S. aureus* as shown in Fig 4.1. Four of these isolates had a higher potency than the standard drug Chloramphenicol (30µg/disc). These isolates that showed a higher potency included IS30, IS42, IS71 and IS77 showing inhibition of 30.0mm, 30.33mm, 27.0mm and 32.67mm respectively as compared to the control that had an inhibition zone of 23.67mm (Table 4.1). These isolates therefore showed promising results of having the ability of producing effective antibiotics given that they are still at their crude state. These four could be studied further by analyzing their metabolites to find out whether there are any novel compounds present which could be lead candidates for drugs. *S. aureus* is known to be

the leading cause of nosocomial infections (Lowy, 2003). Previous study showed that *S. aureus* was the prevalent cause of blood stream infections, pneumonia, skin and soft tissue infection (Diekema *et al*., 2001). The IS30 and IS71 also showed good activity against both *T. mentagrophyte* and *M. gypseum* while IS42 and IS77 also showed good activity against *T. mentagrophyte*. Similar results have been reported by Omran and Kadhem, 2012 where an isolate 40A had good activity on *S. aureus, T. mentagrophyte* and *Microsporum canis.*

Consequentially, 2 isolates (5.1%) showed antimicrobial activity against Methicillin resistant *S. aureus.* One of the two isolates (IS58) showed promising activity against MRSA with an inhibition zone of 20.33mm at its crude state as opposed to pure standard drug Chloramphenicol (30µg/disc) which had an inhibition zone of 25.33mm (Table 4.1). IS72 on the other had had an inhibition zone of 10.33mm. Similar results have been reported in a study done at Mt. Everest (Gurung *et al*., 2009) where three isolates, K.6.3, K.14.2 and K.58.5 showed an activity of 18mm, 15mm and 15mm respectively against. The metabolites from this isolate IS58 could be purified further to get its potency at its minimum inhibitory concentration and also find its components to compare with the standard drugs. These two isolates IS58 and IS72 also showed antifungal properties as they had antimicrobial activity against *T. mentagrophyte* and *M. gypseum* with IS58 showing good activity against *T. mentagrophyte* (32.67mm) while IS72 showing good activity against *M. gypseum* (20.33mm). These two isolates therefore not only could be ideal candidates for drug development against MRSA but also dermatophytes. However

from this study, these isolates that had antibacterial activity against MRSA showed no activity against *S. aureus*. Similar results have been reported where two strains ACK 41 and ACK 108 isolated from soil showed antimicrobial activity against MRSA while showing no activity against *S. aureus* (Sawasdee, 2012). It is not clear why this was the case and therefore if these metabolites could be purified and their components isolated it could provide some explanation. However, ACK 108 had very good activity (32.87mm) on *E. coli* as opposed to ACK 41 which had no activity on *E. coli* hence similar to IS58 and IS72.

Trichophyton mentagrophyte and *Microsporum gypseum* on the other hand are dermatophytes which cause infections of keratinized tissues while *C. albicans* mostly cause opportunistic infections. In this study, most of the isolates (84.6%) had activity against *T. mentagrophyte*, 30.8% on *M. gypseum* and 5.1% on *C. albicans*. Though none of the isolates were more potent than the standard drug Itraconazole (2 μg/ml), some isolates showed promising activity like IS38, IS58 and IS75. These had good antimicrobial activity with an inhibition zone of 30.33mm, 32.67mm and 34.67mm respectively against *T. mentagrophyte*. Therefore these isolates with potent activity could be further purified to investigate their relative potency. Most of these isolates active on *T. mentagrophyte* were isolated from the red soils which are known to be acidic. Guo *et al.*, (2015) reported that acidic soils harbor Actinomycetes with antifungal activity.

The search for antifungals active against dermatophytes has faced difficulties in the past (Spadari *et al.*, 2013). In previous studies few isolated Actinomycetes have shown activity against dermatophytes (Augustine *et al.*, 2005, Lakshmipat & Kannabiran, 2009; Deepa *et al*., 2011). A study done in Brazil involving six *Candida sp.* and 5 dermatophytes were tested including *T. mentagrophyte* and *M. gypseum* showed that the antibiotic activity was only on the *Candida sp.* (Spadari *et al*., 2013) . This is in contrast with the current study where there was good activity against the two dermatophytes tested especially against *T. mentagrophyte.* However, a study done in Kenya from soil collected from different national parks had similar results with this study where the Actinomycetes isolated had antibiotic activity against crop fungal pathogen (Nonoh *et al*., 2010). Since most of the Actinomycetes active on the dermatophytes were isolated from red soil which are known to habour fungi and are acidic, the activity shown as opposed to previous studies could be attributed to the fact that the isolated Actinomycetes have developed adaptive mechanisms for survival hence produce metabolites active on fungi (Crawford *et al.*, 1993).

None of the isolated Actinomycetes showed activity against *Escherichia coli* which is a gram negative bacterium. This could be attributed to the outer membrane that contains lipopolysaccharide hence making the cell wall impermeable to lipophilic solutes. Gram positive bacteria on the other hand lack the outer membrane hence is more permeable (Nikaido & Vaara 1985). Several other studies have also shown that Gram positive

isolates are more susceptible to the antibiotics produced by Actinomycetes than Gram negative bacteria (Gebreyohannes *et al*. 2013, Dileep *et al*. 2013).

IS77 was one of the isolates with very good antimicrobial activity. At its crude state, it had the highest inhibition zone of 32.67mm against *S. aureus* and 25.67mm against *T. mentagrophyte.* This strain had 99% similarity against a *Streptomyces sp*. SBT 343 which was reported to have anti- Trypansomal properties (Cheng *et al*., 2015). It also had 99% similarity with *Streptomyces pratensis* (KU973961.1) and *Streptomyces badius* (KY007184.1). *S. badius* has been known to degrade lignin (Goodfellow & Williams 1983; Godden *et al*. 1992) and also has been reported to be among the strains that have a broad spectrum activity against Gram positive bacteria, yeast and fungi (Rifaat *et al.*, 2007) hence with IS77 having activity on Gram positive and a fungi, it can be assumed to belong to the family of *S. badius.*

IS75 had good antimicrobial activity against *T. mentagrophyte* and had the highest inhibition zone of 34.67 in that group. This also had activity against *M. gypseum* and therefore this strain could be assumed to produce antifungals only. It had 1416bp and had 99% similarity with an Actinobacterium isolate (KJ128969.1), *S. badius* (KY007184.1) and *S. lavendulae* (KX698040.1). A strain isolated in soil by Shibata *et al.,* 1980, had similar characteristics as this strain as it had only antifungal properties. This however is in contrast with the strain isolated by (Rifaat *et al.*, 2007) which had activity only on Gram positive bacteria.

IS58 was also another promising candidate for good antibiotics as it had both antifungal and antibacterial properties. It had a good inhibition zone of 32.67mm against *T. mentagrophyte*, 17.67mm against *M. gypseum* and a zone of 20.33mm against MRSA. This strain however was not able to be sequenced by the pair of primers. This strain could be a more rare strain as it also had a different morphology compared to the other isolates. On Starch Casein Agar plate, it appeared as a mucoid colony with a greenish pigment. Some species of Actinomadura are known to be mucoid in appearance according to (Health Protection Agency, 2009) and therefore this isolate could possibly belong to this family.

It was also noted that isolates with good antimicrobial activity were mainly isolated from soils in Ainamoi and Kipkelion East. Ainamoi has red soils which have been known to be acidic and have the potential of habouring novel strains of Actinomycetes (Guo *et al.*, 2015). Kipkelion East on the other hand has blackish soil which has been reported to be slightly alkaline. The promising isolates from this region included IS71, IS72, IS75 and IS77 and were all *Streptomyces sp.* A novel Actinomycete strain N8 isolated from Vidarbha region in India (Deshmukh & Vidhale, 2015) had similar morphological characteristics as IS71, IS75 and IS77. This Actinomycete strain N8 had gray aerial mycelium with yellow substrate mycelium hence similar to the characteristics of the three strains and belonged to the family of *Streptomyces sp*. However, it showed antimicrobial activity against *E. coli* in contrast to the three strains of the current study.

Soil from Vidarbha region was reported to be black and saline which are similar properties of the soil from Kipkelion East.

The biochemical tests showed that 20% of the isolates tested had the ability to hydrolyze casein, however most of the isolates were seen to have the ability to produce urease and catalase enzymes and this could be attributed to similar metabolic pathways (Sharma, 2014). All the isolates showed the ability to use citrate as the main carbon source and were catalase positive. This is similar to the results shown for *Streptomyces sp*. isolated in India (Dileep *et al*., 2013). The ability of Actinomycetes to produce a variety of extracellular hydrolytic enzymes is of great importance and normally the natural source from which it is isolated influences the kind of enzymes produced by the Actinomycete strain (Saadoun *et al*., 2007; Sharmin *et al*., 2005).

Only fifteen isolates (38.5%) were able to be amplified by the actinobacterial-specific primers used. This could be attributed to the fact that the isolates that were not amplified could belong to the rare genera of Actinomycetes and in future more pair of primers should be used including genera specific primers. Also the quality of DNA used in amplification could have been a problem since its quality was not tested due to a limitation of instruments in the lab. The isolates that were not able to be amplified included the two (IS58 and IS72) that were active against MRSA and two (IS30 and IS42) that showed higher potency than the control drug against *S. aureus*. Phylogenetic analysis showed that most of the amplified isolates clustered with known *Streptomyces sp.* and had similarity values of between 98% -100%. Previous study on soil samples

from a reserved area in Kenya have reported these similar results (Nonoh *et al*., 2010) where all the five isolates with antimicrobial activity clustered with known *Streptomyces sp.* The dominance of *Streptomyces* in soil has been documented earlier (Priyadharsini & Dhanasekaran, 2015, Han *et al*., 2013; Basilio *et al*., 2003). *Streptomyces sp.* is also reported to be the most important bacteria in soil as it carries out different ecological functions such as degradation of organic matter and helping in the formation of compost (Adegboye & Babalola, 2012; Crawford *et al*., 1993). It represents almost up to 90% of the Actinomycetes' soil diversity (Xu *et al.*, 1996).

Only one isolate (IS52) clustered with *Rhodococcus opacus* with a bootstrap value of 67, a similarity of 99% and is the first recorded species of *Rhodococcus sp.* on Kenyan soil though *Rhodococcus sp.* was also isolated from Lake Magadi (Ronoh *et al.*, 2013) .This *Rhodococcus sp.* had antimicrobial activity only on *T. mentagrophyte* as opposed to the one isolated in Lake Magadi that showed activity on *E. coli* and *P. aeruginosa.* This therefore suggests that they are of different strains. *Rhodococcus sp.* have been previously described to produce metabolites having high antifungal activity against *C. albicans* and *C. neofarmans* with no antibacterial properties (Chiba et *al*., 1999). This strain of *Rhodococcus* isolated by Chiba *et al*., (1999) was referred to as *Rhodococcus sp*. Mer-N1033 and therefore has similar properties as the species isolated in the current study due to its antifungal properties. *Rhodococcus opacus* on the other hand have been reported to be lipid producers as they have the ability to accumulate triacylglycerol (TAG) and can also degrade hydrocarbons (Castro *et al.*, 2016; Waltermann *et al.*,

2000). Therefore though the isolated *Rhodoccocus* did not have very good activity, it could further be explored for other useful metabolites. The soils in this region therefore did not show diversity of genera as only two genera were seen, that is *Streptomyces sp*. and *Rhodococcus sp.* However the strains were diverse as evidenced by the different antimicrobial properties.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the specific objectives of this study on bio-prospecting of broad spectrum antibiotic producing Actinomycetes isolated from virgin soils in Kericho County, the following conclusions were arrived at;

1. One hundred and seven Actinomycetes were able to be isolated from the virgin soils of Kericho County based on their typical morphologies and pure cultures gotten.
2. The soil from Kericho harbour Actinomycetes which may have good potential of producing novel antibiotics. Thirty nine isolates showed antimicrobial properties against the test isolates however none showed activity against *E. coli*. IS30, IS42, IS71 and IS77 showed antimicrobial activity with an inhibition zone higher than the control drug Chloramphenicol. They also had activity against one or two of the molds tested. This therefore shows that they could be sources of antibiotics that can be used to treat both Gram positive and fungal pathogens. The isolate IS58 showed promising results as it showed good activity in its crude form on Methicillin resistant *Staphylococcus aureus* when compared with the control drug. Majority of the isolates also showed activity against *T. mentagrophyte.* Though they were not more potent than the control drug, the three isolates that had an inhibition zone of more than 30mm could be good candidates for antifungals.

3. Most of these Actinomycetes had morphological characteristics of the genera *Streptomyces*. Phylogenetic analysis showed that the isolates clustered with two genera that is *Streptomyces sp*. and *Rhodococcus sp*. Therefore the Actinomycetes isolated from these soils were not diverse. However the *Streptomyces* strains were diverse and is evident from the different antimicrobial properties exhibited by each.

6.2 Recommendations

1. New techniques should be used for isolation of those taxa that cannot be isolated by conventional methods. Metagenomics can be employed hence those taxa that cannot be cultured can be captured.
2. Actinomycetes isolated showed good antimicrobial activity against Gram positive and fungal pathogens therefore, more studies should be done to bioprospect for novel Actinomycetes with activity against gram positive pathogens and antifungals especially dermatophytes.
3. IS30, IS42, IS71 and IS77 that showed good potency and those against *T. mentagrophyte* that had an inhibition zone of more than 30mm should further be investigated by extracting the metabolites and getting their Minimum Inhibition Concentration. Analysis of the constituents of the metabolites should also be done and compare with the constituents of the available drugs.
4. Metabolites of isolate IS58 which had good activity in its crude form against MRSA should further be extracted and purified and test their activity in their pure state.
5. More test pathogens should be used to test for antimicrobial properties of these isolates so as to get the isolates with broad spectrum activity.

6.3 Limitations of the Study

1. One pair of primers was used in amplification of the 16S rDNA.
2. Quantification of DNA was not done

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APPENDICES

Appendix 1: Constituents of Media and Preparation

Starch Casein Agar (Formula is per Litre)

Soluble Starch..... 0.5 g

Dipotassium hydrogen Phosphate..... 0.5g

Sodium proprionate…..... 0.5 g

Casein……..... 0.3 g

Asparagine……..... 0.1 g

Potassium nitrate…..... 0.1g

Ferrous Sulfate..... 1.0 mg

Agar..... 20.0 g

Preparation

63.0 grams was suspended in 1000 ml distilled water. This was then heat to boiling point to completely dissolve the medium. Thereafter it was autoclaved at 15 lbs pressure (121°C) for 15 minutes. Fluconazole and Rifampicin was added and mixed and allowed to cool then 20ul was aseptically dispensed into sterile Petri plates.

Nutrient Agar

Lab-Lemco' powder……..1.0g

Yeast extract…….2.0g

Peptone……5.0g

Sodium Chloride……..5.0g

Agar……15.0g

Preparation

28g was suspended in 1 litre of distilled water. This was then heat to boiling point to completely dissolve the medium. Thereafter it was autoclaved at 121°C for 15 minutes allowed to cool then 20ul was aseptically dispensed into sterile Petri plates.

Mueller Hinton Agar(g/L)

Beef, dehydrated infusion from……30.0g

Casein hydrolysate…….17.5g

Starch…….1.5g

Agar……17.0g

Preparation

38g was suspended in 1 litre of distilled water. This was then heat to boiling point to completely dissolve the medium. Thereafter it was autoclaved at 121°C for 15 minutes allowed to cool then 20ul was aseptically dispensed into sterile Petri plates.

Mueller Hinton Broth (g/L)

Beef, dehydrated infusion from…….30.0g

Casein hydrolysate……..17.5g

Starch……..1.5g

Potato Dextrose Agar

Potato extract……..4.0g

Glucose……20.0g

Agar…… 15.0g

Preparation

39g was suspended in 1 litre of distilled water. This was then heat to boiling point to completely dissolve the medium. Thereafter it was autoclaved at 121°C for 15 minutes allowed to cool then 20ul was aseptically dispensed into sterile Petri plates.

Appendix 2: DNA Extraction Reagents

Enzymatic lysis buffer contains;

- 20 mM Tris•Cl, pH 8.0
- 2 mM sodium EDTA
- 1.2% Triton® X-100
- Immediately before use, 20µl lysozyme was added (20 mg/ml)

Appendix 3: Coordinates of Sampling Points

Kipkelion East Coordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
KE 1	35.41	-0.296	2,270
KE 2	35.425	-0.286	2,304
KE 3	35.45	-0.265	2,138
KE 4	35.503	-0.254	2,224
KE 5	35.525	-0.2354	2,172
KE 6	35.55	-0.226	2,064
KE 7	35.56	-0.227	2,129
KE 8	35.55366	-0.22829	2,161
KE 9	35.5518	-0.22598	2,083
KE 10	35.551	-0.2254	2,120
KE 11	35.5597	-0.2171	2,166
KE 12	35.5648	-0.21151	2,188
KE 13	35.5777	-0.20299	2,254
KE 14	35.5852	-0.19573	2,272
KE 15	35.5895	-0.18698	2,298
KE 16	35.5886	-0.18026	2,317

KE 17	35.6263	-0.16938	2,323
KE 18	35.62813	-0.167808	2,317
KE 19	35.6301	-0.166157	2,338
KE 20	35.62981	-0.16841	2,301
KE 21	35.6283	-0.1693	2,331
KE 22	35.6266	-0.16965	2,318
KE 23	35.6249	-0.17053	2,326
KE 24	35.644	-0.17035	2,319
KE 25	35.588	-0.16924	2,289
KE 26	35.5452	-0.14278	2,304
KE 27	35.489	-0.16351	2,278
KE 28	35.4725	-0.17305	2,159
KE 29	35.45277	-0.183575	2,072
KE 30	35.4426	-0.192149	2,028

Kipkelion West Coordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
KW 1	35.43113	-0.20085	1,992
KW 2	35.4142	-0.21138	1,980
KW 3	35.3939	-0.21687	1,951
KW 4	35.3819	-0.22617	1,866
KW 5	35.3527	-0.22635	1,683
KW 6	35.3554	-0.22011	1,665
KW 7	35.36409	-0.21193	1,620
KW 8	35.3554	-0.201755	1,566
KW 9	3,534,775	-0.20495	1,539
KW 10	35.3284	-0.2124	1,507
KW 11	35.4585	-0.1856	2,086
KW 12	35.45939	-0.19322	2,060
KW 13	35.46596	-0.19926	1,934
KW 14	35.4664	-0.204207	1,904
KW 15	35.46775	-0.24668	2,230
KW 16	35.45642	-0.24511	2,222
KW 17	35.4495	0.2409316	2,172
KW 18	35.45136	-0.23494	2,211
KW 19	35.454342	-0.2310368	2,083
KW 20	35.461675	-0.226468	2,047
KW 21	35.46499	0.2244269	2,020

KW 22	35.446066	-0.24097	2,154
KW 23	35.4341986	-0.248654	2,126
KW 24	35.426519	-0.247252	2,104
KW 25	35.41905	0.2466534	2,071
KW 26	35.41289	-0.24879	2,029
KW 27	35.415644	-0.255361	1,976
KW 28	35.41065	-0.260188	2,018
KW 39	35.40955	-0.2633275	1,994
KW 30	35.4065	-0.271452	2,031
KW 31	35.394957	-0.277105	2,045
KW 32	35.388198	-0.283184	2,008
KW 33	35.3768	-0.29124	2,012
KW 34	35.37233	-0.294947	1,983
KW 35	35.37062	-0.29999	1,954
KW 36	35.3632	-0.30373	1,985
KW 37	35.36483	-0.30698	2,003
KW 38	35.354071	-0.311816	2,037
KW 39	35.34849	-0.31645	2,038
KW 40	35.34662	-0.317778	2,032

Ainamoi Co-ordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
A 1	35.3373	-0.32283	2,023
A 2	35.331	-0.32675	2,054
A 3	35.32125	-0.32994	2,062
A 4	35.311904	-0.326931	2,087
A 5	35.3045	-0.318	2,135
A 6	35.29898	-0.31672	2,142
A 7	35.29133	-0.31032	2,132
A 8	35.2864	-0.302302	2,049
A 9	35.27634	-0.297132	1,918
A 10	35.2677	0.293183	1,864
A 11	35.25524	-0.287009	1,776
A 12	35.24547	-0.2929898	1,852
A 13	35.236008	-0.302572	1,919
A 14	35.22864	-0.311786	1,918
A 15	35.1948	-0.29184	1,951
A 16	35.186118	-0.289463	1,892

A 17	35.18255	-0.28661	1,861
A 18	35.196876	-0.2964694	1,989
A 19	35.20856	-0.301519	1,979
A 20	35.21614	-0.2927023	1,985
A 21	35.32137	-0.345709	2,033
A 22	35.327012	-0.3511895	2,019
A 23	35.327158	-0.352322	2,025
A 24	35.328909	-0.355318	1,989
A 25	35.33028	-0.36023	2,051
A 26	35.33884	-0.359881	2,072
A 27	35.346178	-0.359042	2,094
A 28	35.3466	-0.3586991	2,107
A 29	35.3493988	-0.354364	2,117
A 30	35.331739	-0.3528457	2,035
A 31	35.321	-0.348211	2,027
A 32	35.318839	-0.3523109	2,011
A 33	35.31859	-0.3547299	2,046
A 34	35.3171423	-0.352493	2,024
A 35	35.3149	-0.353777	1,999
A 36	35.2663058	-0.3761373	1,925
A 37	35.263523	-0.373912	1,925
A 38	35.26685	0.373893	1,908
A 39	35.26868	-0.372776	1,948
A 40	35.27456	-0.366965	1,928
A 41	35.319617	-0.3495823	2,021
A 42	35.331029	-0.35967	2,060

Bureti Coordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
BU 1	35.17786	-0.45921	
BU 2	35.16517	-0.47463	1732
BU 3	35.154506	-0.47114	1704
BU 4	35.152748	-0.47265	1687
BU 5	35.14684	-0.48074	1740
BU 6	35.1498	-0.49376	1722
BU 7	35.14503	-0.50308	1716
BU 8	35.14492	-513857	1783

BU 9	35.15065	-0.51983	1812
BU 10	35.148863	-0.52399	1826
BU 11	35.13959	-517290	1792
BU 12	35.16393	-0.53269	1854
BU 13	35.159984	-0.53744	1832
BU 14	35.159938	-0.54262	1834
BU 15	35.14942	-0.55309	1887
BU 16	35.143968	-0.5682	1852
BU 17	35.143459	-0.57484	1874
BU 18	35.142448	-0.58553	1867
BU 19	35.1412	-0.59069	1838
BU 20	35.14064	-0.59926	1851
BU 21	35.14389	-0.60588	1882
BU 22	35.149362	-0.60215	1895
BU 23	35.166877	0.599268	1930
BU 24	35.174108	-0.60091	1931
BU 25	35.1935	-0.59699	1935
BU 26	35.189916	-0.57535	1927
BU 27	35.182414	-0.56279	1912
BU 28	35,182,043	-0.54842	1890
BU 29	35.1762	-0.53488	1866
BU 30	35.17713	-0.52634	1865

Belgut Coordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
BE 1	35.24359	-0.35425	2,018
BE 2	35.24137	-0.35971	1972
BE 3	35.23361	-0.36769	1982
BE 4	35.22476	0.337488	1942
BE 5	35.21494	-0.37995	1920
BE 6	35.20743	-0.38695	1874
BE 7	35.20236	-0.39431	1875
BE 8	35.196	-0.40172	1850
BE 9	35.18877	-0.40453	1833
BE 10	35.18133	-0.40612	1818
BE 11	35.17831	-0.41274	1824
BE 12	35.17652	-0.40107	1745
BE 13	35.16865	-0.48242	1778

BE 14	35.15979	-0.41635	1784
BE 15	35.15644	-0.41975	1789
BE 16	35.14775	-0.4276	1784
BE 17	35.13327	-0.43337	1778
BE 18	35.1238	-0.43732	1725
BE 19	35.11371	-0.44027	1713
BE 20	35.10366	-0.43939	1724
BE 21	35.09408	-0.44296	1694
BE 22	35.08227	-0.44723	1684
BE 23	35.0761	-0.4521	1728
BE 24	35.06917	-0.46212	1762
BE 25	35.08034	-0.47224	1692
BE 26	35.08573	-0.47371	1630
BE 27	35.09021	-0.46544	1729
BE 28	35.09523	-0.46118	1729
BE 29	35.1026	-0.4579	1739
BE 30	35.10673	-0.45833	1715
BE 31	35.11559	0.457794	1716
BE 32	35.12012	-0.45585	1684
BE 33	35.13148	-0.45307	1710
BE 34	35.14165	-0.45173	1698
BE 35	35.152	-0.44724	1739
BE 36	35.16566	-0.45306	1709

Sigowet Coordinates

SITE	LONGITUDE	LATITUDE	ALTITUDE
S 1	35.19244	-0.310632	1966
S 2	35.18846	-0.3156654	1932
S 3	35.181334	-0.31408	1866
S 4	35.17836	-0.3080136	1886
S 5	35.21309	-0.3226037	1981
S 6	35.20362	-0.328366	1987
S 7	35.19532	-0.34301	1960
S 8	35.18289	-0.354102	1954
S 9	35.16742	-0.36777	1913
S 10	35.15964	-0.36324	1855
S 11	35.15386	-0.35944	1805
S 12	35.15612	-0.37531	1880

S 13	35.14569	-0.385565	1852
S 14	35.14081	-0.379542	1858
S 15	35.138	-0.3680497	1806
S 16	35.12503	-0.38166	1825
S 17	35.11265	-0.3903	1806
S 18	35.10234	-0.388602	1772
S 19	35.09056	-0.379898	1748
S 20	35.0787	-0.377103	1714
S 21	35.07069	-0.382801	1760
S 22	35.06204	-0.374954	1728
S 23	35.057595	-0.360417	1712
S 24	35.050899	-0.348695	1664
S 25	35.04797	-0.339163	1569
S 26	35.053771	-0.325081	1419
S 27	35.05977	-0.309514	1289
S 28	35.058025	-0.2930155	1257
S 29	35.058136	-0.281004	1238
S 30	35.0693	-0.307366	1306
S 31	35.081995	-0.3106337	1383
S 32	35.09647	-0.303658	1416
S 33	35.107223	-0.308646	1496
S 34	35.1208	-0.312975	1593
S 35	35.13383	-0.309879	1634
S 36	35.14578	-0.031465	
S 37	35.15167	-0.317786	1737
S 38	35.1444	-0.323059	1695
S 39	35.1334	-0.32699	1697
S 40	35.154498	-0.306752	1755

Appendix 4: Incubated Isolates in Mueller Hinton Broth

Appendix 5: Article on Standard Newspaper on this work

WEDNESDAY, MARCH 29, 2017 The Standar

Medicine. Kenyan researchers unearth key to disease resistance

Hope as soil with healing powers found in Kericho

Virgin soils in Kericho can help cure drug-resistant diseases.

By Gatonye Gathura
newsdesk@standardmedia.co.ke

Kenyan scientists have found a medicinal substance that could play an important role in combating drug-resistant germs.

The researchers from the Jomo Kenyatta University of Agriculture and Technology and the Kenya Medical Research Institute (Kemri) have found the substance in the virgin soils of Kipkelion East and West, Belgut, Ainamoi, Sigowet, and Bureti sub-counties in Kericho County.

Called Actinomycetes, the substance is described on MedicineNet.com as a group of gram-positive bacteria that produce antibiotics, enzymes and vitamins.

Antibiotics are useful in preventing or treating bacterial infections.

The World Health Organisation (WHO) says the emergence of drug-resistant bacteria due to overuse and misuse of antibiotics is the biggest medical crisis facing the world today.

In the January 2017 issue of Advances in Microbiology journal, the researchers say the newly discovered substance could provide new and more potent antibiotics that could be vital in treating such bacteria.

Useful bacteria in soils are not entirely new in the pharmaceutical world. They occur widely, especially in undisturbed soils, and have been the source of most of the current antibiotics in use and even cancer medicines.

But older medicines, explains the all-female team coordinated by Dr Naomi Maina, are no longer effective against most disease-causing germs, hence the unfolding health crisis.

Hit pay-dirt

This is what prompted Mercy Chepkurui Rotich, Esther Magiri and Christine Bii to head back to the forests of Kericho to search for yet-to-be-exploited strains of actinomycetes.

"Kericho County has different types of soil and also exotic forests which may have novel actinomycetes that haven't been explored," says the study.

The research area is located in a region of high altitude and the right soil composition providing a good environment for new bacteria.

The prospectors, as the report indicates, are on their way to hitting pay dirt, having isolated 107 of the organisms, with more than a third - 36 per cent - confirmed at the Kemri labs to be active against several disease-causing germs.

A significant number of the organisms were found to effectively kill certain germs, some notorious for causing hard-to-treat pneumonia and blood and skin infections.

The 2016 Economic Survey reported that pneumonia killed more people in Kenya in 2015 (22,473) than the former leading killer, malaria (20,691).

DISEASE RESISTANCE HEADACHE

- Growing cases of disease resistance threatens the treatment of infections caused by bacteria, parasites, viruses and fungi
- Without effective antibiotics, the success of major surgery and cancer chemotherapy would be compromised.
- Cost of treating patients with resistant infections is high, requiring longer periods, more expensive drugs, and additional tests
- Globally, 480,000 people develop multi-drug resistant TB each year, and drug resistance is starting to complicate the fight against HIV and malaria
- www.rocketscience.co.ke

22,473

The 2016 Economic Survey reported that pneumonia killed more people in Kenya in 2015 (22,473) than the former leading killer, malaria (20,691).

2014

WHO in 2014 listed pneumonia, together with influenza, as the leading killer diseases in Kenya.