GENOTYPIC CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *ACTINOMYCETES* FROM SPUTUM OF TUBERCULOSIS SMEAR NEGATIVE PATIENTS FROM SELECTED REFERRAL FACILITIES, KENYA

FRIDA MUKWAMUNENE NJERU

MASTER OF SCIENCE

(Molecular Medicine)

JOMO KENYATTA UNIVERSITY

OF

AGRICULTURE AND TECHNOLOGY

2023

Genotypic Characterization and Antimicrobial Susceptibility Testing of *Actinomycetes* from Sputum of Tuberculosis Smear Negative Patients from Selected Referral Facilities, Kenya

Frida Mukwamunene Njeru

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Molecular Medicine of the Jomo Kenyatta University of Agriculture And Technology

2023

DECLARATION

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

Signature: …… **Date:** …….

Frida Mukwamunene Njeru

This thesis has been submitted for examination with our approval as University Supervisors

Signature: …… Date: …….

Dr. Perpetual Ndung'u, PhD
JKUAT, Kenya

Signature: …… Date: …….

Dr. Christine Bii, PhD
KEMRI, Kenya

DEDICATION

I dedicate this thesis to my family. Most sincerely to my beloved husband for his care, support and understanding in all possible ways throughout the process of doing this work. To my lovely daughters Ravine, Zarine and Zakine for their patience and for giving me a humble time in the entire period of this work.

ACKNOWLEDGEMENT

First and foremost, I thank the almighty God for enabling me to reach this far and for His favour and blessings throughout this journey.

My gratitude goes to my supervisors; Dr. Perpetual Ndung'u and Dr. Christine Bii for their academic and technical advice that brought this research work to maturity.

I also thank the Kenya Medical Research Institute through Dr. Christine Bii for allowing me to use their laboratory.

I would also like to acknowledge my colleagues and lab staff of Mycology and bacteriology lab KEMRI who offered their guidance in my lab work.

TABLE OF CONTENT

DECLARATION ..... **ii**

DEDICATION ..... **iii**

ACKNOWLEDGEMENT ..... **iv**

TABLE OF CONTENT ..... **v**

LIST OF TABLES ..... **viii**

LIST OF FIGURES ..... **ix**

LIST OF APPENDICES ..... **x**

ABBREVIATIONS AND ACRONYMS ..... **xi**

ABSTRACT ..... **xiii**

CHAPTER ONE **1**

INTRODUCTION **1**

1.1 Background information 1

1.2 Problem statement 2

1.3 Justification 3

1.4 Research questions 4

1.5 Objectives 4

1.5.1 Main objective 4

CHAPTER TWO 6

LITERATURE REVIEW 6

2.1 *Actinomycetes* and clinical relevance 6

2.2 Tuberculosis co-infection with *Actinomycetes* 8

2.3.1 Phenotypic characterization 9

2.3.2 Molecular analysis 10

CHAPTER THREE 13

MATERIALS AND METHODS 13

3.1 Study site 13

3.2 Study population 13

3.2.1 Target population 13

3.2.2 Inclusion criteria 14

3.2.3 Exclusion criteria 14

3.3 Study design 14

3.4 Sample size 14

3.5 Sampling method 15

3.6 Laboratory procedures 15

3.6.1 Phenotypic characterization of *Actinomycetes* 15

3.6.2 Antimicrobial susceptibility testing 18

3.6.3 Molecular characterization 19

3.7 Data analysis 20

3.8 Ethical consideration 21

CHAPTER FOUR 22

RESULTS 22

4.1. Demographic Parameters of the Actinomycetes positive Patients 22

4.2. Prevalence of Actinomycetes isolated from the four study sites 23

4.3. Isolated *Actinomycetes* 24

4.4. Biochemical test 25

4.5. Antimicrobial susceptibility testing 26

4.6. Polymerase chain reaction and sequencing 29

CHAPTER FIVE 34

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 34

5.1 Discussion 34

5.2 Limitations 37

5.3 Conclusions 37

5.4 Recommendations 38

REFERENCES 39

APPENDICES 50

LIST OF TABLES

Table 4.1: Demographics of the *Actinomycetes* positive cases (n =52) 23

Table 4.2: Biochemical characteristics of representative isolates..... 26

Table 4.3: Antibiotic susceptibility of the isolated *Actinomycetes (n=52).*..... 27

Table 4.4: Antimicrobial activity of different antibiotics against 52 Actinomycetes isolates (Mean ± SD).. 28

Table 4.5: Distribution of the sequenced cases (n =9) 33

LIST OF FIGURES

Figure 4.1: percentages of positive cases per..... 24

Figure 4.2: Colonies of Actinomycetes on Mueller hinton agar. A shows white wrinkeled colonies while B and C shows greyish whitish chalky colonies. 25

Figure 4.3: A shows intertwined filaments while B shows beaded branching filaments of Gram stained Actinomycetes at power × 25

Figure 4.4: Differences in antimicrobial activity of antibiotics against Actinomycetes isolate. 29

Figure 4.5: Representative gel images of amplified 16S rDNA of isolated Actinomycetes. N is the negative control, M is hyperladder 1 kb Molecular Marker. 30

Figure 4.6: Neighbor-joining tree showing the phylogenetic positions of Streptomyces species. The scale bar indicates 5% estimated sequence divergence. 31

Figure 4.7: Neighbor joining tree showing the phylogenetic positions of Nocardiopsis species. 32

Figure 4.8: Aligned sequences of the 9 Actinomycetes species. 32

LIST OF APPENDICES

Appendix I: Consent to participate in a Research Study 50

Appendix II: Consent to Participate in a Research Study (KISWAHILI)..... 54

Appendix III: Assent to Participate in a Research Study 58

Appendix IV: Assent to Participate in a Research Study (KISWAHILI 60

Appendix V: Approval letters 63

Appendix VI: Publication 66

Appendix VII: Minutes of Seminar Presentations..... 67

Appendix VIII: plagiarism report..... 70

ABBREVIATIONS AND ACRONYMS

AMC	Amoxicillin-Clavulanate
AMK	Amikacin
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BMD	Broth Microdilution
CIP	Ciprofloxacin
CLA	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
CMR	Centre for Microbiology Research
CN	Gentamycin
CNS	Central Nervous System
CPGH	Coast Provincial General Hospital
CRO	Ceftriaxone
DNA	Deoxyribonucleic acid
DOXY	Doxycycline
HIV	Human Immunodeficiency Virus
JKUAT	Jomo Kenyatta University of Agriculture and Technology
JOOTRH	Jaramogi Oginga Odinga Teaching and Referral Hospital
KEMRI	Kenya Medical Research Institute
LZD	Linezolid
MDR	Multidrug Resistance
MH	Muller Hinton

MHB	Muller Hinton Broth
MIC	Minimum Inhibition Concentration
MLSA	Multilocus Sequence Analysis
MTB	Mycobacterium Tuberculosis
MTRH	Moi Teaching and Referral Hospital
NCBI	National Center for Biotechnology Information
NCLSI	National Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PPE	Proper protective equipments
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDA	Sabouraud dextrose agar
SERU	Scientific ethical review unit
SXT	Trimethoprim-Sulfamethoxazole
TB	Tuberculosis
U.K	United Kingdom
U.S	United States

ABSTRACT

Actinomycetes are organisms belonging to the order *Actinomycetale*. Most of them are opportunistic pathogens causing infections in immunocompromised patients. In most cases, respiratory infections caused by *Actinomycetes* display symptoms that mimic tuberculosis. This can lead to misdiagnosis resulting to delayed or inappropriate treatment. Therefore, this study aimed at isolating, characterizing and determining drug susceptibility of *Actinomycetes* from the sputum of TB smear negative patients from selected referral facilities in Kenya. This employed a cross-sectional study design where a total of three hundred and eight-five (385) sputum samples from TB smear negative patients were collected and screened for the presence of *Actinomycetes*. Phenotypic identification was done by Gram staining, culture and biochemical test. Genotypic identification was done using rDNA primers to amplify 16S rRNA gene as well as Phylogenetic analysis for confirmation. Antimicrobial susceptibility testing was done by minimum inhibition concentration method (MIC) using 9 antimicrobial agents; amikacin (64 µg/ml), amoxicillin-clavulanate (64/32 µg/ml), ceftriaxone (256 µg/ml), ciprofloxacin (64 µg/ml), clarithromycin (64 µg/ml), gentamycin (64 µg/ml), co-trimoxazole (trimethoprim-sulfamethoxazole) (8/152 µg/ml), doxycycline (64 µg/ml) and linezolid (64 µg/ml). Data was analyzed by statistical package, R Windows using the Kruskal-Wallis test, a *P* value of less than 0.05 was considered significant. Fifty two (13.5%) of 385, were identified as *Actinomycetes* using the phenotypic methods. Of the 52 *Actinomycetes* isolates, 32 (8.3%) were amplified for16S rRNA gene. Nine of the amplified genes were sequenced and compared with those in National Center for Biotechnology Information (NCBI). Phylogenetic analysis confirmed the 9 sequenced genes were members of *Actinomycetes.* Eight of them were members of the genus *Streptomyces* while only one was a *Nocardiopsis* species. Antimicrobial susceptibility testing was done on the 52 isolates. The isolates showed highest susceptibility to Gentamycin and Ciprofloxacin at 100% and 98% respectively. Highest resistance was observed in Ceftriazone and Clarithromycin at 11.5% and 26.9% respectively. Multidrug resistance was observed in four isolates. *Staphylococcus aureus* ATCC 25923 was used for quality control. There is *Actinomycetes* infections in TB smear negative patients hence it is important to investigate suspected pulmonary pathologies for potential *Actinomycetes* infection for proper diagnosis and interventions.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Actinomycetes are a group of aerobic and anaerobic bacteria belonging to the order *Actinomycetales*. The organisms in this group are similar in their morphology containing filamentous branching structures which fragment into bacillary or coccoid forms (Sullivan & Chapman, 2010). They are Gram positive, filamentous, partially acid-fast and relatively slow-growing branched bacteria (Brown, 2003). Their filamentous appearance make them look like fungi though they are grouped as bacteria due to their cell wall components, particularly, their cell envelope lipid and peptidoglycan compositions. They have a worldwide distribution and they exist as saprophytes in the soil and other natural habitats. They are ubiquitous in the environment and they have been isolated worldwide from soil and organic matter. Soil is their main habitat though they can also be found in water bodies, in the air as well as in the plant remains (Oskay *et al*., 2004). They can be transmitted through the wind in the dust and dirt into the lungs or skin and cause health problems (Aghamirian and Ghiasian, 2009). They have many microbiologic characteristics in common with members of the genera *Mycobacterium*.

According to the global tuberculosis report 2014, tuberculosis (TB) is found in all regions of the world and it is one of the world's biggest microbial threats (Zumla *et al.*, 2015). Tuberculosis infections can occur with other bacteria (co-infection) leading to delayed diagnosis and insufficient treatment. Pulmonary TB is a serious cause of morbidity and mortality and it can exist with other pulmonary diseases caused by opportunistic organisms (Ekrami *et al*., 2014, Arora *et al*., 2015).

Among other bacteria, aerobic *Actinomycetes* are common causes of co-infection with pulmonary tuberculosis. Aerobic *Actinomycetes* that cause pulmonary infections have

the same clinical and radiographic features as those of pulmonary tuberculosis. *Nocardia, Rhodococcus, Streptomyces, Gordonia and Tsukamurella* are examples of these aerobic *Actinomycetes* (Franco-Paredes 2014). These organisms can co-infect with pulmonary TB or can be the actual cause of the infection.

In Kenya, the current incidence of aerobic *Actinomycetes* infections is unknown. This could be because these infections can be difficult to diagnose or recognize which consequently underestimate their incidence. Identification of clinically significant *Actinomycetes* to the species level is very important since it can help in defining the spectrum of disease caused by each species as well as establishing the pathogenicity of the species.

Therefore the aim of this study is to detect, characterize and test the antimicrobial susceptibility of *Actinomycetes* in TB smear negative and retreatment patients from selected referral facilities in Kenya.

1.2 Problem statement

Most of the *Actinomycetes* are opportunistic pathogens with the majority of infections occurring in patients with immunosuppressive conditions. However, low percentage of immunocompetent patients also get infected. In immunosuppressed patients, cutaneous disease may disseminate to the lung and central nervous system (CNS). The *Actinomycetes* that affect the respiratory system mostly display symptoms that mimic Tuberculosis (Valenzuela-Tovar *et al*., 2005; Silva *et al*., 2012), hence in most cases are misdiagnosed as tuberculosis or even regarded as a contaminant or commensal organism. Due to this problem, patients have ended up not being treated or being treated with wrong medication hence denying the patients appropriate clinical management and subsequently leading to increase in morbidity and mortality as well as increased drug resistance. Understanding the significance of Actinomycetes infections and their current

level of resistance in smear negative TB cases is essential for reducing mortality and morbidity associated with misdiagnosis.

There is limited data on *Actinomycetes* infections from clinical samples as this is not routinely carried out in Kenya, yet such data is necessary to inform of their significance, antimicrobial susceptibility profiles and their management.

Therefore the aim of this study was to determine the significance of *Actinomycetes* infections in sputum of TB smear negative and retreatment patients as well as to determine the antimicrobial susceptibility profiles of the isolates.

1.3 Justification

The high TB/HIV co-infection burden in Kenya is a significant predisposition to opportunistic pathogens due to their severe immune debilitating nature. The main aim of national and international tuberculosis (TB) control programs is the detection and management of smear-positive pulmonary disease. However, TB-smear negative (ie. patients with clinical and radiological evidence of pulmonary TB but repeatedly negative sputum investigations) is a common clinical problem. Unfortunately, patients with worsening clinical symptoms but negative on MTB diagnostic algorithm are considered MTB relapse/retreated and any fungal-like growth on TB cultures are usually considered culture contaminants without any consideration as possible pathogens (Mwaura *et al.,* 2013). Data generated from this study will provide knowledge to researchers on the significance of *Actinonycetes* infections in TB smear negative and retreatment patients. This study will also generate data on the genotyping of *Actinomycetes* which will provide necessary information on *Actinomycetes* species associated with infections in TB smear negative and retreatment cases.

The cost implications of wrong treatment, morbidity and mortality associated with missed, delayed or misdiagnosis is unwarranted. Emerging resistance to antibiotics is a

global threat ascribed to irrational use of antibiotics. This is a major public health threat, Hence, routine screening as well as Antimicrobial Susceptibility Testing is necessary to protect the public and minimize exposure to non-compliant drugs that could be fuelling resistance. Data obtained from this study will be necessary to inform policy on the effectiveness of the available antibiotics of choice for *Actinomycetes* infections.

1.4 Research questions

1. What is the prevalence of *Actinomycetes* from sputum of TB smear negative patients from selected referral facilities in Kenya?
2. What are the antimicrobial susceptibility profiles of *Actinomycetes* isolated from sputum of smear negative TB patients?
3. What are the genotypic characteristics of *Actinomycetes* from sputum of smear negative TB cases from selected referral facilities in Kenya?

1.5 Objectives

1.5.1 Main objective

To determine the genotypic characteristics and antimicrobial susceptibility profiles of *Actinomycetes* from sputum of TB smear negative patients from selected referral facilities in Kenya.

1.5.2 Specific objectives

1. To determine the prevalence of *Actinomycetes* from sputum of TB smear negative patients from selected referral facilities in Kenya.

2. To determine the antimicrobial susceptibility pattern of the *Actinomycetes* isolated from sputum of TB smear negative patients from selected referral facilities in Kenya..

3. To determine the genotypes of *Actinomycetes* isolated from sputum of TB smear negative patients from selected referral facilities in Kenya.

1.6 Scope

This study covered only aerobic *Actinomycetes* in smear negative TB patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Actinomycetes* and clinical relevance

There are several genera of *Actinomycetes* that are associated with human infections. For instance, the species of aerobic *Actinomycetes* that can cause disease in humans and animals include *Nocardia*, *Gordona*, *Tsukamurella*, *Streptomyces*, *Rhodococcus*, *Actinomadura*, and *Corynebacteria*; while the species of anaerobic *Actinomycetes* that are pathogenic to man and animals include *Actinomyces*, *Arachnia*, *Rothia*, and *Bifidobacterium*. (Sullivan & Chapman, 2010).

Among the aerobic *Actinomycetes, Nocardia* is the most common pathogen with over 100 species (Ercibengoa *et al*., 2016) some of which are medically important. The medically significant *Nocardia* species include *Nocardia abscessus, N. asteroides, N. nova, N. farcinica, N. brasiliensis, N. pseudobrasiliensis, N. otitidiscaviarum,* and *N. transvalensis*. *Nocardia africana*, *N. paucivorans,* and *N. veteran* have been found to be significant agents of pulmonary disease (Cloud *et al.*, 2004). *Nocardia* species have a worldwide distribution and the incidence rate of isolation of *Nocardia* species is on the rise with approximately 500-1,000 new cases in the U.S. (Ishikawa *et al.*, 2004). Dust, sand, soil and swimming pools are the common places where pathogenic species are found. Pulmonary infections occur by inhaling contaminated dust (Helal *et al*., 2008). The pathogenesis of Nocardia species involves inhibition of the fusion of phagosomes with the lysosomes in the macrophages. The neutrophils and monocytes are also not able to kill these organisms.

The infections caused by these species range from minor cutaneous lesions to severe pulmonary disease or central nervous system disease, (Hashemi-Shahraki *et al*., 2015). Most of the *Nocardia* infections occur in immunocompromised people as well as those with long-term corticosteroid exposure, malignancy, HIV infection, and a history of transplantation (Peleg *et al*., 2007). The human pathogens affect both immunosuppressed and immunocompetent patients (Minero *et al*., 2009). *Nocardial* infection can be challenging to identify as they grow slowly in routine microbiological media consequently underestimating its incidence, (Yildiz and Doganay, 2006). Pulmonary *Nocardiosis* mimics pulmonary tuberculosis in both clinical symptoms, being chronic in nature and radiological characteristics making it difficult to differentiate from *M. tuberculosis*, (Valenzuela-Tovar *et al*., 2005). However, there have been cases of pulmonary tuberculosis co-infection with *Nocardia*. The most common symptoms of *Nocardiasis* include chronic cough, chest pain, dyspnoea and haemoptysis (Subhash *et al*., 2001).

Other aerobic *Actinomycetes* that are known to cause respiratory infection include *Streptomyces*, *Rhodococcus, Gordonia*, *Tsukamurella* etc (Poonwan *et al*. 2005).

Streptomyces species releases toxins which damage mitochondria and kills human natural killer cells (Aghamirian and Ghiasian, 2009). They are mostly associated with infections of the cutaneous and subcutaneous tissues. These infections are referred to as actinomycetoma (Hamid, 2011; Kirby *et al*., 2012). They have also been reported to cause respiratory disorders of individuals living in moldy houses (Aghamirian and Ghiasian, 2009).

Rhodococcus has about 50 species (Euzéby, 2013) and according to a report by UK Standards for Microbiology Investigations (2016), *Rhodococcus equi* is the species associated with pulmonary and cutaneous infections in immunocompromised patients. It has been identified as an opportunistic pathogen in humans and can cause disease and

even death mostly in HIV patients (Silva *et al*, 2010). It causes an infection called *rhodococcosis which* affects the lungs leading to an infection that presents like pulmonary tuberculosis (Silva *et al*., 2012).

Tsukamurella species are opportunistic pathogens and can also cause pulmonary and cutaneous infections in immunocompromised people (Poonwan *et al*., 2005). The infections caused by these organisms are mostly spread through clinical instruments (Safaei *et al.,* 2018).

On the other hand, anaerobic *Actinomycetes* associated with human disease is the genus *Actinomyces* which comprises of about 42 species (Euzéby, 2009). Among these species, *Actinomyces israelii* is the most common cause of disease in human. It causes a disease known as *Actinomycosis* which affects all age groups, mid-life being the most affected age group. Mostly *Actinomyces* species are normal flora of the mucous membrane. *Actinomycosis* affects different parts of the body including cervical, facial, abdominalpelvic, and thoracic (Sullivan & Chapman, 2010). Thoracic infection is established in the lung and its symptoms simulate those of pulmonary tuberculosis (Yildiz and Doganay 2006).

2.2 Tuberculosis co-infection with *Actinomycetes*

Pulmonary tuberculosis is one of the biggest microbiological hazards in the world and it continues to be a major cause of morbidity and mortality worldwide (Zumla *et al.*, 2015). There is a growing understanding that some pathogens may have the acid-fastness feature to some extent and that these bacteria may clinically resemble pulmonary TB. In fact, some respiratory microbes including some species of aerobic *Actinomycetes* share this feature with Mycobacterium tuberculosis and the nontuberculous mycobacteria (Franco-Paredes & Ray 2012). It has been clear during the clinical evaluation of TB cases that certain individuals initially considered to have pulmonary TB from a clinical and radiographic evaluation were really infected with

aerobic actinomycetes (Franco-Paredes, 2014). Moreover, lung infections caused by these organisms can result in cavitary illness, manifesting with productive cough for many weeks, hemoptysis, fever, night sweats, weight loss and malaise, which are a clinical picture of pulmonary tuberculosis (Savini *et al*., 2012). Therefore, Aerobic actinomycetes may either co-infect or be the actual pathogen among those in whom pulmonary TB was initially suspected. Hence, clinicians should be mindful of the possibility of misdiagnosing these organisms as tubercle bacilli due also to the similarities found among these pathogens in their clinical presentation and radiographic imaging.

2.3 Detection of *Actinomycetes*

Identification of *Actinomycetes* is based on phenotypic characterization and genotypic characterization.

2.3.1 Phenotypic characterization

Microscopic characteristics of these organisms are determined using Gram stain. They are Gram positive and partially acid fast. Culture has been used for many years as the gold standard method in the identification of *Actinomycetes.* Morphology of the colonies is determined by culturing on blood agar plates or other non-selective culture media. Appearance of colonies on agar plates can provide a guide to the identity. Some of these characteristics include; formation of spores on the substrate mycelium, zoospores in specialized spore vesicles or sporangia (Atta *et al*., 2011). The colour of different *Actinomycetes* range from white to grey to pinkish to yellow, light-green brown, brown black or distinct brown pigment depending on the culture medium used (Sharma *et al*., 2014). Some have powdery appearance and some are tough and hard to pick from the culture media (Opande *et al*., 2013).

Biochemical tests are used to help in further identification of these organisms up to the species level. On biochemical tests, citrate utilization, urea hydrolysis and resistance to lysozyme are determined (Lowman & Aithma, 2010). Other biochemical tests used include catalase test, oxidase test, casein hydrolysis, starch hydrolysis, esculin hydrolysis, nitrate reduction, acid production from sugar, NaCl resistance.

A combination of morphological characteristics and biochemical tests can help in identifying these organisms by referring to Bergey's Manual of Determinative Bacteriology (Pandey *et al*., 2011). However, biochemical method of identification is time consuming tedious and uses a lot of chemicals. With the advancement of technology in the molecular study, fast and accurate methods have come in (Kumari *et al*., 2013; Jeffrey, 2008).

2.3.2 Molecular analysis

The aspect of Molecular science includes both classification and identification and this originated from the early studies of nucleic acid hybridization. Molecular analysis involves the analysis of gene sequences which helps in the realization of phylogenetic relationship of organisms and also helps in the detection of new species. Molecular biology techniques have become very popular and important in discovering bacterial diversity. Molecular tools have a great potential to assist in the identification of bacteria and to further their investigation. For taxonomic purposes, RNA is very important in the analysis of organisms. This analysis usually focuses on three different molecules of ribosomal RNA (rRNA) that is, 5S (~ 120 nucleotides), 16S (~1540 nucleotides) and 23S (~2400 nucleotides) (Giovannoni & Cary 1993). These molecules are important indicators of relatedness of organisms since they are present in all living organisms. The 16S rDNA sequencing is very useful in recognition of uncommon bacteria, slow growing bacteria and bacteria with unique phenotypic profiles (Woo *et al*., 2008). 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 bases, is highly conserved among microorganisms and is therefore an excellent tool for studying phylogenetic relationships (Sacchi *et al* 2002). A report by UK Standards for Microbiology Investigations (2016) gives examples of molecular methods used for typing of *Actinomycetes* which include 16S rRNA gene sequencing, Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Analysis (MLSA). The 16S rRNA analysis method is time-saving as compared to the classical methods of identification (Alfaresi & Elkosh 2006). This method also allows for the assessment of a broader range of diversity than that obtained by physiological studies (Brambilla *et al.* 2001).

Sequence-based identification is a method of identifying clinical isolates that are either slowly growing or difficult to identify by biochemical profiling. Several reports have described the advantage of sequencing a portion of the 16S rRNA gene for identifying clinical isolates of *Mycobacterium* spp. and other bacteria (Patel, 2001). These methods have the advantage over phenotypic methods in that they are independent of the physiological state of anorganism; they are not influenced by the composition of the growth medium or by the organism's phase of growth.

2.3.3 Antimicrobial susceptibility

Antimicrobial resistance has become a global threat and a major public health concern that threatens the effective treatment of a range of infections caused by microbes. Most microorganisms are no longer susceptible to the common drugs used to treat them. Bacteria causing common or severe infections have developed resistance to antibiotic coming to market (Prestinaci et al., 2015). The major contribution to the antimicrobial resistance is the irrational and unregulated use of antibiotics in human.

Antimicrobial_susceptibility testing of aerobic *Actinomycetes* isolated from clinical specimens is very important as it helps to guide physicians in choosing antibiotics to

treat the infections caused by these organisms. It is considered a challenge to understand the antimicrobial susceptibility of pathogenic *Actinomycetes* due to their slow growing and filamentous growth characteristics which limits sufficient standardization of AST methodology (Hamid, 2011). The most commonly used methods include broth microdilution method, agar dilution method, e-test and disc diffusion method. However, CLSI approves the broth microdilution method as a standard method for antimicrobial susceptibility testing of *Actinomycetes* (NCLSI, 2003).

Due to misidentification of *Actinomycetes* as well as characterization to genus level only, there has been a problem in the treatment of diseases caused by these organisms. Trimethoprim-sulphamethoxazole is a commonly used antibiotic for aerobic *Actinomycetes* (van de Sande *et al*., 2014; Uhde *et al.*, 2010; Hamid, 2011); Zampella *et al*., 2017). However, some other antibiotics like linezolid have also been found to be sensitive to these organisms. Moreover, other options have been suggested, for example, combinations such as vancomycin-imipeneme-amikacin (Savini *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The sputum samples used to conduct this study were obtained from selected referral facilities in Kenya. The study sites were chosen based on the National TB prevalence and they included TB clinics in Nairobi County (Mbagathi county Hospital), Mombasa County (Coast Provincial General Hospital), Kisumu County (Jaramogi Oginga Odinga Teaching and Referral Hospital) and Uasin Gishu County (Moi Teaching and referral Hospital). These facilities were also considered because they are regional referral facilities with high TB prevalence. Sample processing and analysis was done at Kenya Medical Research Institute - Centre for Microbiology Research (KEMRI-CMR) mycology laboratory. The KEMRI-CMR Mycology laboratory is located at the Kenyatta National Hospital compound behind the Government Chemist laboratories.

3.2 Study population

The study population was TB patients at the study sites either as an in- patient or out-patient who are smear negative, relapse or retreatment. Eligible participants were identified after assessment by the clinical officers on duty.

3.2.1 Target population

The target population was the sputum samples of TB patients who were confirmed to be TB smear negative and/or retreatment cases.

3.2.2 Inclusion criteria

Sputum samples from newly diagnosed smear negative TB patient in the selected sites.

Sputum samples from relapse patients with recurrent symptoms.

Sputum samples from patients who has given informed consent.

3.2.3 Exclusion criteria

Samples from patients with extra pulmonary TB.

Sputum samples from patients who declined to sign informed consent.

3.3 Study design

This was a cross sectional laboratory-based study, where the purposive sampling method was used, to determine the prevalence of *Actinomycetes* infections in TB smear negative and retreatment cases from the selected referral facilities in Kenya.

3.4 Sample size

The sample size was determined using fisher's formula. A prevalence of 50% was used to calculate the minimum number of samples required to carry out the study. The 50% prevalence was used because there is no data showing the prevalence of *Actinomycetes* from clinical samples in Kenya.

$$n = \frac{z^2 \times p(1-p)}{d^2}$$

Where

n=anticipated sample size

P=prevalence of 50%

= 0.5 Probability of pathogen isolation.

z=1.96(confidence interval at 95%)

d=the margin of error at 5%

$$n = \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2}$$

Hence; **n=385**

Therefore three hundred eighty five samples were collected.

3.5 Sampling method

This study utilized purposive sampling. Illegible participants were identified after assessment by the clinical officers on duty. The purpose of the study was explained in detail and subsequently, consent/assent was sought. The participants who met the inclusion criteria were purposively sought and sputum samples were collected. The sputum samples from each study site were transported to KEMRI Mycology laboratory in a cold chain system as soon as possible. Patient details (metadata) were collected using a structured questionnaire.

3.6 Laboratory procedures

3.6.1 Phenotypic characterization of *Actinomycetes*

Phenotypic identification was carried out by microscopic characteristics, colonial morphologies and biochemical tests.

3.6.1.1 Microscopic characterization

Gram staining was done to check for microscopic characteristics of *Actinomycetes*. Direct smear of sputum samples were prepared and stained using Gram staining method following the protocol described by Cappuccino & Sherman (2002)**.** The smears were then examined under microscope using × 100 oil immersion magnification.

3.6.1.2 Culture/colonial morphology

The sputum samples that showed microscopic characteristics of *Actinomycetes* on gram stain were cultured on Sabouraud's dextrose agar medium and incubated at 35°C for up to 3 weeks. This was done in a level 2 biosafety cabinet. Plates were observed periodically for growth of *Actinomycetes*. Colonies suspected to be *Actinomycetes* on the plates were identified as chalky, firm with leathery texture (Rao *et al*. 2012). The colonies were purified by sub culturing on Mueller Hinton agar. Pure colonies were then suspended into 1ml of 15% sterile glycerol triptic soy broth and then stored at -80^{o}C until further analysis.

3.6.1.3 Biochemical Characterization

Different biochemical tests were carried out using fresh colonies as follows:

3.6.1.3.1 Catalase test

Catalase is an enzyme that breaks down hydrogen peroxide to water and oxygen. This helps microorganisms to survive since hydrogen peroxide is lethal to cells. This test was performed by picking a pure colony using a sterile loop and placing it on sterile glass slide containing a drop of hydrogen peroxide. A positive result means that there was the production of free oxygen which was indicated by the production of bubbles (Cappuccino & Sherman 2002).

3.6.1.3.2 Citrate utilization

Some microorganisms produce enzyme citrase which utilizes citrate as the sole carbon source (Harold, 2002). This test was performed by picking a pure colony of the isolate and streaking it on the slant of Simon's citrate agar. The slants were then incubated at 30 °C for 72 hours. 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 (Cappuccino & Sherman 2002). Therefore, a positive test is indicated by blue colour on the inoculated slant after incubation.

3.6.1.3.3 Urea hydrolysis

Urea 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. Production of ammonia creates an alkaline environment that causes phenol to turn to a deep pink. This test was done by inoculating the isolate into sterile urea agar containing the pH indicator phenol red and incubated at 30 °C for 72 hours (Cappuccino & Sherman 2002). The presence of deep pink colour indicated a positive reaction hence presence of urease. Failure of this colour to develop indicated the absence of urease hence a negative result.

3.6.1.3.4 Casein Hydrolysis

Casein is a major protein found in milk. Casein hydrolysis tests the ability of a microorganism to degrade the casein by producing proteolytic enzyme, called proteinase (caseinase) which breaks the peptide bond. This test was done by streaking the isolates on skimmed milk agar medium and incubating at 30 °C for 7hours. Clear zones around the colonies demonstrated Hydrolysis (Ten *et al* 2005).

3.6.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done using broth microdilution (BMD) method as described below, according to the CLSI (formerly known as NCLSI) guidelines for rapidly growing mycobacteria and aerobic *actinomycetes* (NCLSI, 2003).

3.6.2.1 Inoculum preparation.

The revived fresh cultures were used for antimicrobial susceptibility testing. To achieve an optical density of a 0.5 McFarland standard, fresh, pure colonies of the isolates were picked from a Mueller Hinton agar plate, using a sterile loop, and transferred to 2 ml sterile water. The inoculum was then vortexed for 15 to 20 s, to assist in breaking up clumps.

3.6.2.2 Broth Microdilution

The antimicrobial agents tested included amikacin (64 µg/ml), amoxicillin-clavulanate (64/32 µg/ml), ceftriaxone (256 µg/ml), ciprofloxacin (64 µg/ml), clarithromycin (64 µg/ml), gentamycin (64 µg/ml), co-trimoxazole (trimethoprim-sulfamethoxazole) (8/152 µg/ml), doxycycline (64 µg/ml) and linezolid (64 µg/ml). Staphylococcus aureus ATCC 25923 was used for quality control. Mueller hinton broth was prepared, sterilized and 4.9ml of sterile mueller hinton broth dispensed into sterile tubes. Three mililiters of the antimicrobial agents was added to 3ml of respective solvents (sterile water, DMSO, ethanol) and serial diluted to make a twofold serial dilutions. Into the 4.9 ml mueller hinton broth, 0.1 ml of the respective antimicrobial agents' dilutions was added to make a tenfold dilution. Two hundred microliters of broth/antibiotics dilutions was then dispensed into wells of microtiter plates using a multichannel pipette. Ten microliters of the aforementioned inoculum was added to each well containing 200 µl of Mueller-Hinton broth (MHB)/antibiotic dilution. Microtiter plates were sealed using parafilm and incubated at 35°C in ambient air. The MICs were read at 48 h. If required due to

poor growth, plates were reincubated for a further 24 h and a final MIC reading was done on day 3 (72 h). The MIC was defined as the lowest concentration of antimicrobial agent to inhibit visible growth.

3.6.3 Molecular characterization

Molecular analysis was done on the isolates to confirm the identification. DNA extraction was the first step in the Analysis of 16S rDNA (Macrae, 2000). The extracted DNA was then subjected to Polymerase chain reaction in order to amplify the gene coding for 16S rRNA using primers that are specifically designed to target the 16S rRNA sequence (Sivakumar, 2001; Jeffrey, 2008). The PCR product was then sequenced.

3.6.3.1 DNA extraction

The *Actinomycetes* isolates were revived by culturing on Mueller Hinton agar and incubated at 35°C for 72 hours or until sufficient growth was achieved. DNA was extracted using the boiling method. A loopful of pure colonies were put in 1ml of PCR water (DNase/RNase-free water) and emulsified to obtain a turbid suspension. This suspension was placed in a heating block and heated at 95^0c for 10 minutes. The tubes were cooled and then centrifuged at 1400 rpm for 5 minutes. Two hundred microliters of the supernatant which contains the DNA was transferred to sterile tubes. The extracted DNA was stored at -20°C for further use (Dashti *et al*., 2009, Junior *et al*., 2016).

3.6.3.2 PCR amplification

The DNA extracted from the positive isolates was used as a template for amplification of the 16S rDNA. The 16S rRNA gene is a conserved gene in bacteria (Kumar *et al*., 2010). The 16S rRNA gene was amplified using the following group-specific pair of primers; F-Act 243 (5´-GGATGAGCCCGCGGCCTA-3´) and R-Act A3 (5´-

CCAGCCCCACCTTCGAC-3´) as described by (Monciardini *et al.,* 2002)**.** A final reaction volume of 25 µl was prepared containing; 1µl 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 each primer. The following program was used to carry out the amplifications: initial denaturation at 95°C for 10 min followed by 35 cycles of; denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min.

3.6.3.3 Detection of amplified PCR Products

Agarose gel electrophoresis was used to detect PCR products. Ten microliter volumes of PCR products were separated through a 1% agarose gel by electrophoresis at 125 volts. The gel was stained with ethidium bromide (0.5 ug/ml), and visualized by ultraviolet light against a standard molecular base pair (1kb) ladder and photographed.

3.6.3.4 16S rRNA gene sequencing

PCR product of selected isolates were sequenced using DNA sequencer to determine the order in which the bases are arranged within the length of the sample. This was done in Japan using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 3130ABI Genetic Analyzer (Applied Biosystems), according to the manufacturer's instruction. The generated 16S rRNA sequences were aligned and compared to the sequences in the GenBank database in the National Centre for Biotechnology Information website. The comparisons of sequence positions provided an estimate of how closely related the organisms are as well as to confirm the identification and for the species identification.

3.7 Data analysis

Statistical analysis was performed using a statistical package, R Windows using the Kruskal-Wallis test to compare any difference in susceptibility between different antimicrobial agents used on *Actinomycetes* isolates. A *P* value of less than 0.05 was considered significant.

3.8 Ethical consideration

The study was appoved by the Scientific Ethical Review Unit (KEMRI - SERU) and given the ethical clearance letter (SERU Number 3340). Additional approval was given by JKUAT Board of Post Graduate studies.

CHAPTER FOUR

RESULTS

4.1. Demographic Parameters of the Actinomycetes positive Patients

A total of 385 samples were collected and out of them 52 were positive for *Actinomycetes* by phenotypic methods. The 52 positive cases comprised of 33 (63%) males and 19 (37%) females with an odds ratio of 1.7. Distribution of the positive cases among <20, 20-29, 30-39, 40-49 and <50 age categories (in years) was as follows; 4(8%), 16(31%), 11(21%), 11(21%) and 10(19%) respectively. Distribution among the sites where samples were collected namely; Mbagathi Hospital (MB), Coast Provincial Genaral Hospital (CPGH), Moi Teaching and Referral Hospital (MTRH) and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) was 14(27%), 10 (19%) 11(21%) and 17(33%) respectively. Retreatment cases were 39(75%) while new cases were 13(25%) with an odds ratio of 3.0. (Table 4.1)

Table 4.1. Demographics of the *Actinomycetes* positive cases (n =52)

Variable		Frequency (n)	Percentage (%)
Gender	Male	33	63%
	Female	19	37%
Age Category (years)	<20	4	8%
	20-29	16	31%
	30-39	11	21%
	40-49	11	21%
	>50	10	19%
Site/Region	MB	14	27%
	CPGH	10	19%
	MTRH	11	21%
	JOOTRH	17	33%
History of treatment	Retreatment	39	75%
	New cases	13	25%

4.2. Prevalence of Actinomycetes isolated from the four study sites

Out of the 385 participants recruited in the study, the prevalence of *Actinomycetes* was 13.5% (52/385). JOOTRH had the highest number of cases, 17 (4.4%), followed by Mbagathi county hospital with 14 (3.6%), then MTRH with 11 (2.9%), while CPGH had the lowest number of cases, 10 (2.6%). (Figure 4.1).

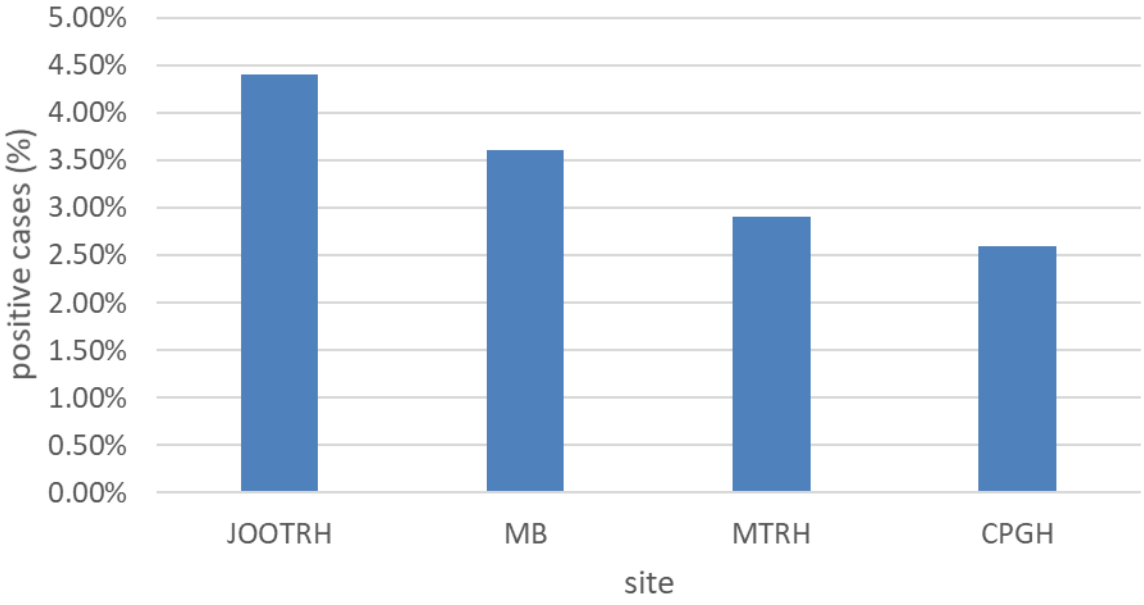


Figure 4.1: percentages of positive cases per site.

4.3. Isolated *Actinomycetes*

The textures of the isolated Actinomycetes were hard to scrap from culture media while a few were easier to pick, Figure 4.2. All the isolates were Gram-positive and their microscopic morphologies ranged from some having highly intertwined filamentous hyphae to branching hyphae, Figure 4.3.

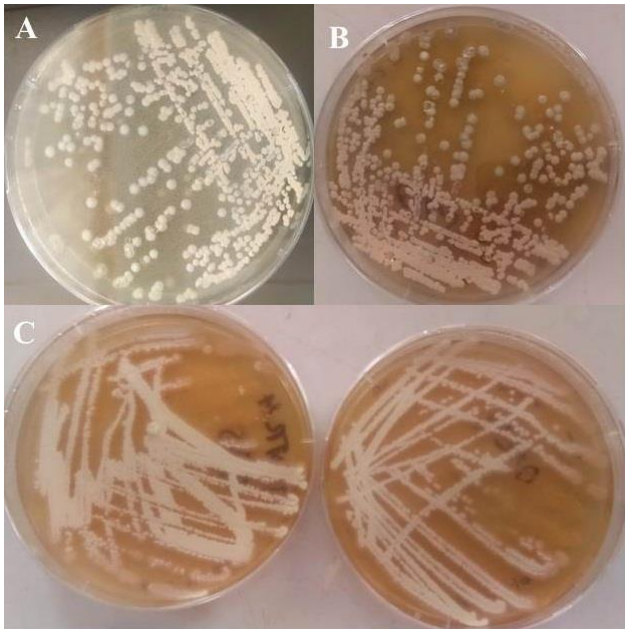


Figure 4.2: Colonies of Actinomycetes on Mueller hinton agar. A shows white wrinkeled colonies while B and C shows greyish whitish chalky colonies.

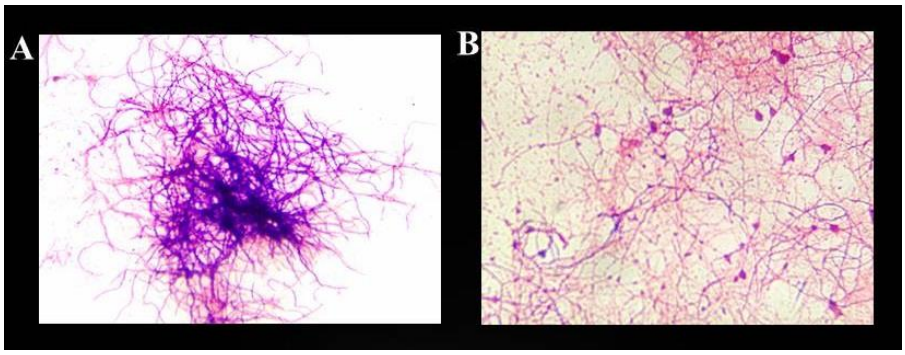


Figure 4.3: A shows intertwined filaments while B shows beaded branching filaments of Gram stained Actinomycetes at power ×100

4.4. Biochemical test

All isolates showed the ability to utilize citrate as the sole carbon source and were also all catalase positive hence the ability to break down hydrogen peroxide to oxygen and

water. Casein hydrolysis test was positive on 20 of the isolates. The biochemical characteristics of representative *Actinomycetes* isolated are as shown in table 4.2 below.

Table 4.2. Biochemical characteristics of representative isolates

Isolate	Casein hydrolysis	Urease	Citrate utilization	Catalase
L228	+	+	+	+
L232	-	+	+	+
L226	+	+	+	+
L224	+	+	+	+
L220	+	+	+	+
L188	-	+	+	+
L131	-	+	+	+
L103	-	+	+	+
L86	+	+	+	+
L101	+	+	+	+

4.5. Antimicrobial susceptibility testing

Antimicrobial Susceptibility Testing was done using nine antimicrobial agents and interpretation of MICs was done according to CLSI guidelines. All the *Actinomycetes* isolated were susceptible to Gentamycin 52 (100%). They also showed high susceptibility to Ciprofloxacin 51 (98%), co-trimoxazole (Trimethoprim – sulphamethoxazole) 49 (94%) and amikacin 47 (90%). On the other hand, the isolates were less susceptible to clarithromycin, linezolid, amoxicillin-clavulanate, and ceftriaxone with the highest resistance being observed in Clarithromycin 14 (26.9).

Multidrug resistance (MDR) was defined as an isolate being resistance to more than three classes of antibiotics (Condas *et al.*, 2015). MDR was observed in 7.7% (4/52) of the isolates. The percentages of antibiotic susceptibility are shown in Table 4.3.

Table 4.3: Antibiotic susceptibility of the isolated *Actinomycetes (n=52)*.

Antibiotic	Interpretation	Frequency (n)	Percentage (%)
	Sensitive	51	98.1%
CIP	Intermidient	0	0%
	Resistant	1	1.9%
	Sensitive	49	94.2%
SXT	Intermidient	0	0%
	Resistant	3	5.8%
	Sensitive	44	84.6%
LZD	Intermidient	0	0%
	Resistant	8	15.4%
	Sensitive	47	90.4%
AMK	Intermidient	0	0%
	Resistant	5	9.6%
	Sensitive	41	78.8%
AMC	Intermidient	4	7.7%
	Resistant	7	13.5%
	Sensitive	52	100%
CN	Intermidient	0	0%
	Resistant	0	0%
	Sensitive	37	71.2%
CLA	Intermidient	1	1.9%
	Resistant	14	26.9%
	Sensitive	46	88.5%
DOXY	Intermidient	6	11.5%
	Resistant	0	0%
	Sensitive	32	61.5%
CRO	Intermidient	14	26.9%
	Resistant	6	11.5%

Most of the antibiotics used in this study were effective in inhibiting the growth of *Actinomycetes* isolates. However, the antibiotics showed a statistical-significant

difference (P< 0.001) in inhibition concentrations of *Actinomycetes* isolates, Table 4.4 or Figure 4.4

Table 4.4: Antimicrobial activity of different antibiotics against 52 Actinomycetes isolates (Mean ± SD).

Drugs	**Mean MIC of 52 *Actinomycetes* isolates**	**Range of MIC**	**Mean MIC of *S. Aureus* ATCC 25923**
CIP [e]	0.840 ± 4.412	0.062 – 32	0.25
CN [e]	0.389 ± 0.355	0.062 – 2	1.0
DOXY [e]	0.578 ± 0.761	0.062 – 4	0.5
CLA [d]	6.284 ± 11.339	0.062 – 32	1.0
AMK [cd]	3.191 ± 4.651	0.062 – 16	4.0
LZD [bc]	6.567 ± 10.447	0.500 – 32	8.0
AMC [b]	8.002 ± 10.453	0.062 – 32	0.125
CRO [a]	16.644 ± 19.489	0.500 – 64	4.0
SXT [a]	15.392 ± 17.351	1.188 – 76	9.5

e- Have a significant difference at P< 0.001 to all other drugs; **b**-Have a significant difference at p< 0.001 and P< 0.01 to some drugs and P< 0.05 to others while it's not significant to others; **bc**-Have a significant difference at P< 0.001 and P< 0.01 to some drugs and not significant to others; **d-** Have a significant difference at P< 0.001 and P< 0.01 to some drugs and not significant to others; **cd**- Have a significant difference at P<0.001 and P< 0.05 to some drugs and not significant to others; **a-** Have a significant difference at P< 0.001 to most drugs and P< 0.01 to others.

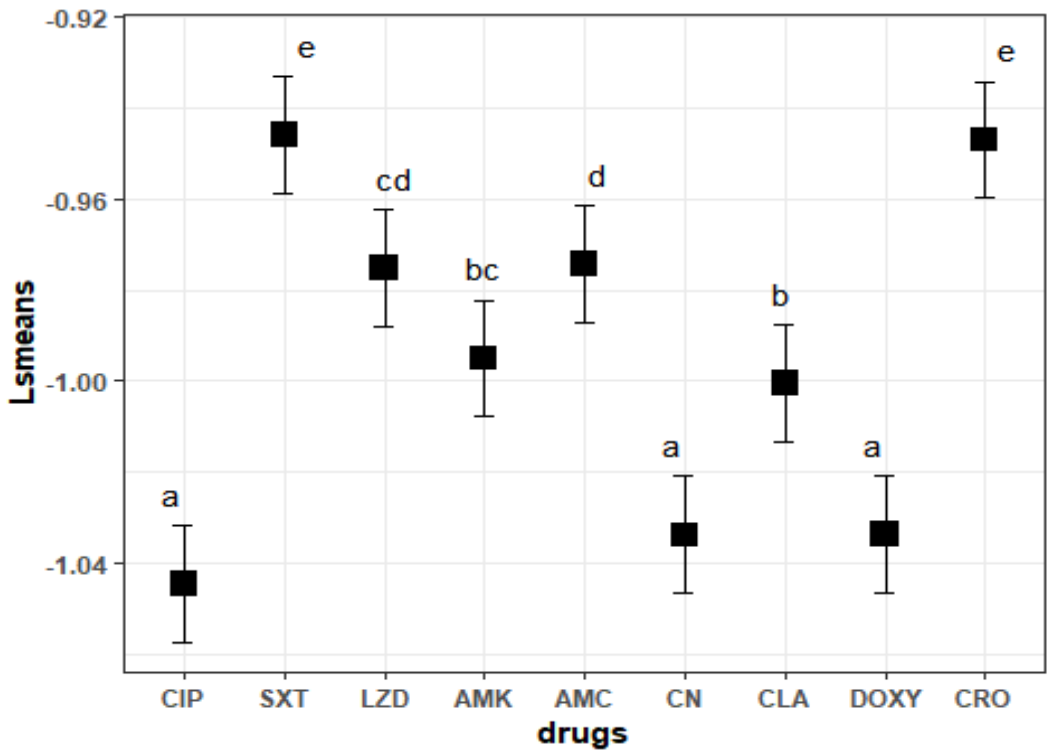


Figure 4.4: Differences in antimicrobial activity of antibiotics against Actinomycetes isolate.

4.6. Polymerase chain reaction and sequencing

Out of the 52 isolates that had phenotypic characteristics of *Actinomycetes*, 32 (61.5%) isolates were able to be amplified by the pair of primers used. These isolates had 16S rRNA gene of up to about 1300 base pairs. Representative gel images of the amplified DNA is shown in Figure 4.5.

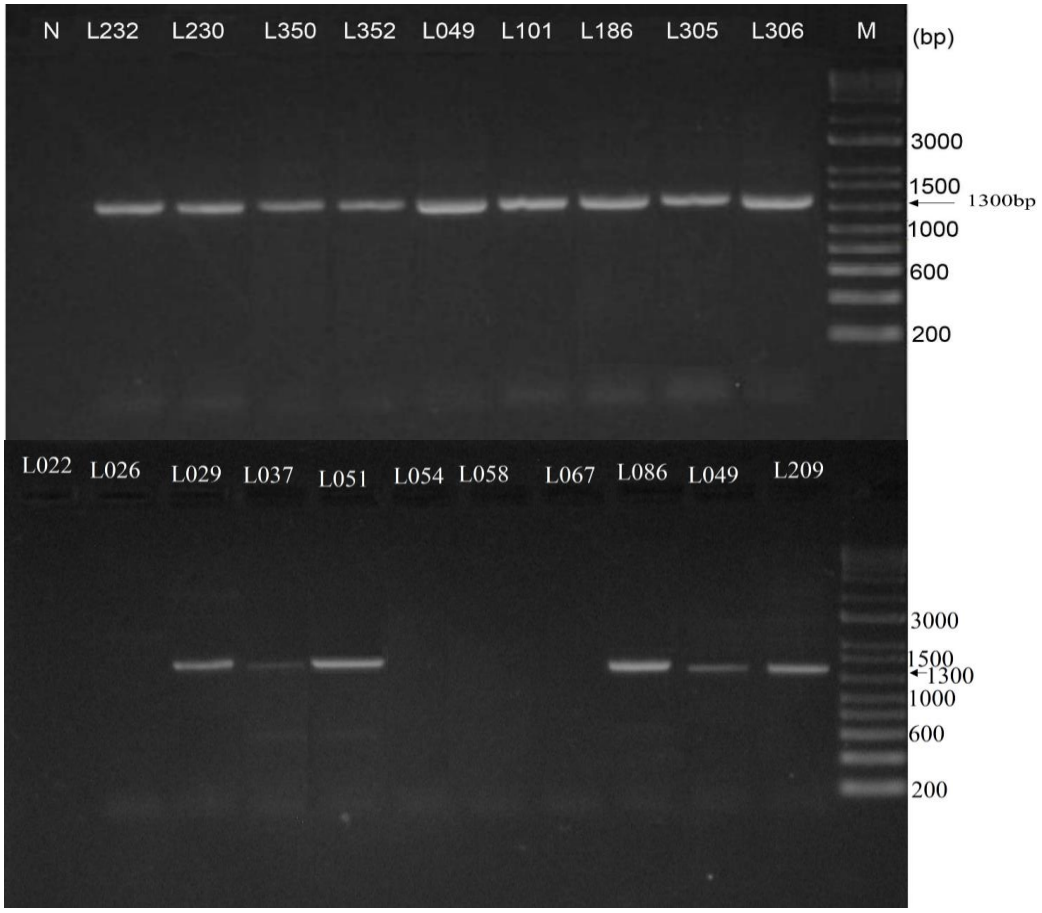


Figure 4.5: Representative gel images of amplified 16S rDNA of isolated Actinomycetes. N is the negative control, M is hyperladder 1 kb Molecular Marker

From the 32 isolates that were amplified, 9 were able to be sequenced and the 16S rRNA sequences compared with those of other *Actinomycetes* whose sequences are already in NCBI database. The trees were constructed using the Neighbour-Joining method based on the alignment of 16S rRNA sequences in 1000 bootstrap replications using MEGA 7 software. GeneBank accession numbers of reference sequences are indicated in parenthesis. According to the phylogenetic analysis of the 16S rRNA gene, the isolates were members of *Actinomycetes.* Eight were members of the genus *Streptomyces* while only one was a *Nocardiopsis* species. The phylogenetic trees of

representative samples (in bold) are drawn in Figures 4.6 and 4.7 while figure 4.8 shows the aligned sequences.

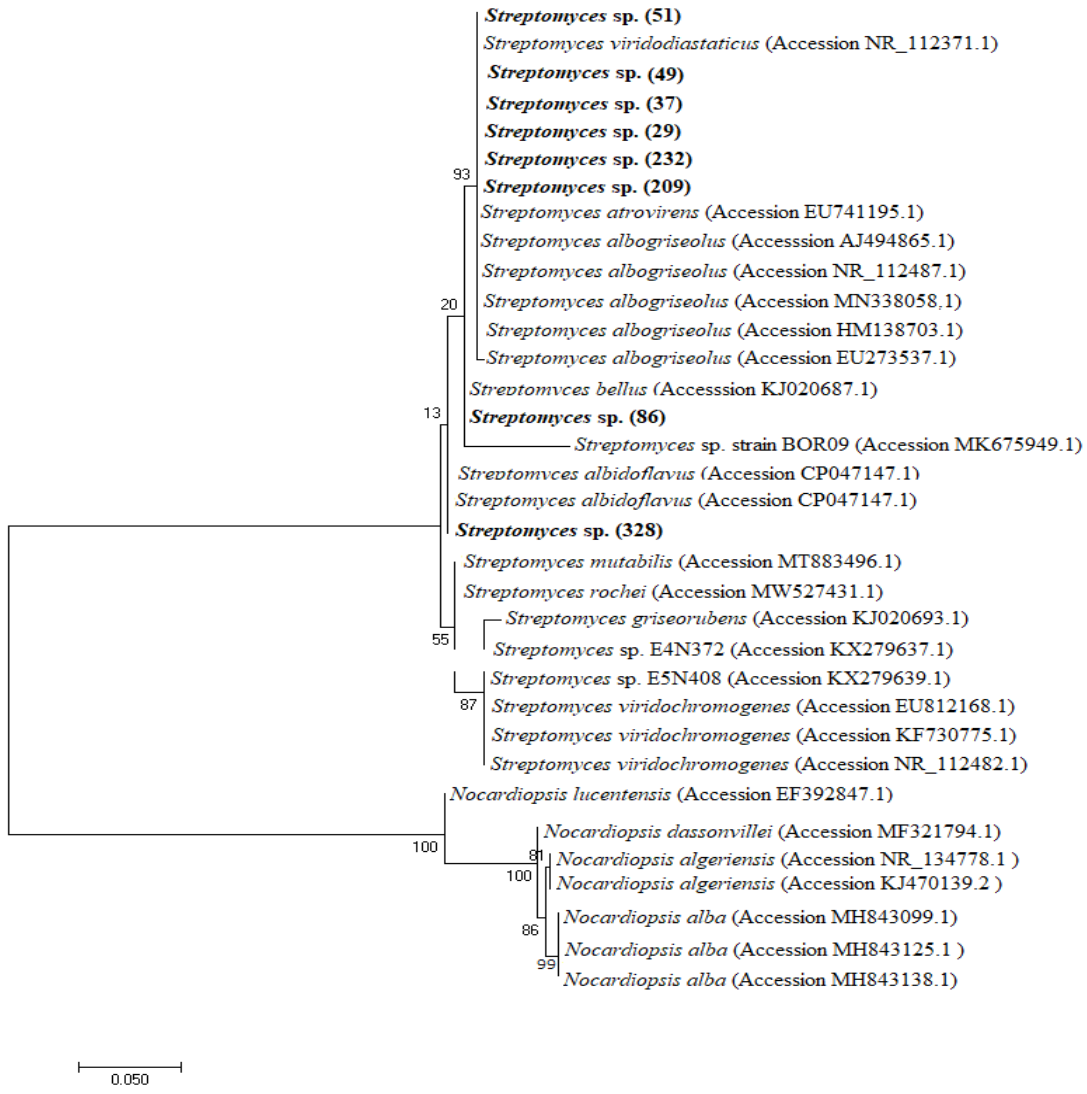


Figure 4.6: Neighbor-joining tree showing the phylogenetic positions of Streptomyces species. The scale bar indicates 5% estimated sequence divergence.

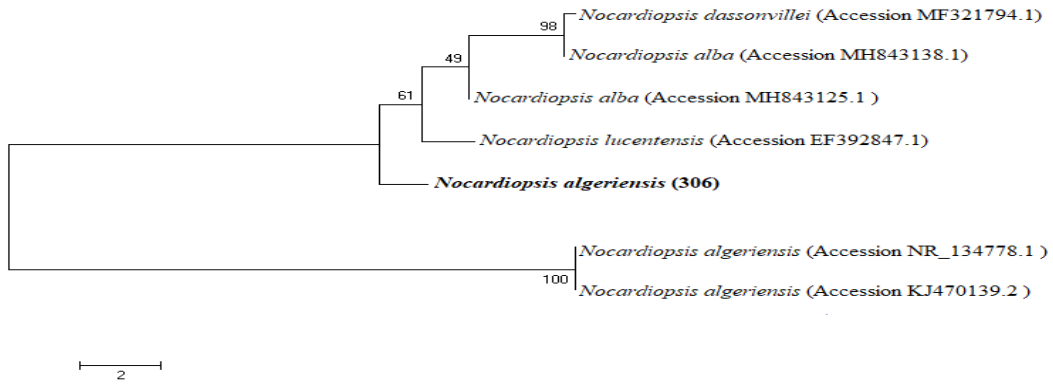


Figure 4.7: Neighbor joining tree showing the phylogenetic positions of Nocardiopsis species.

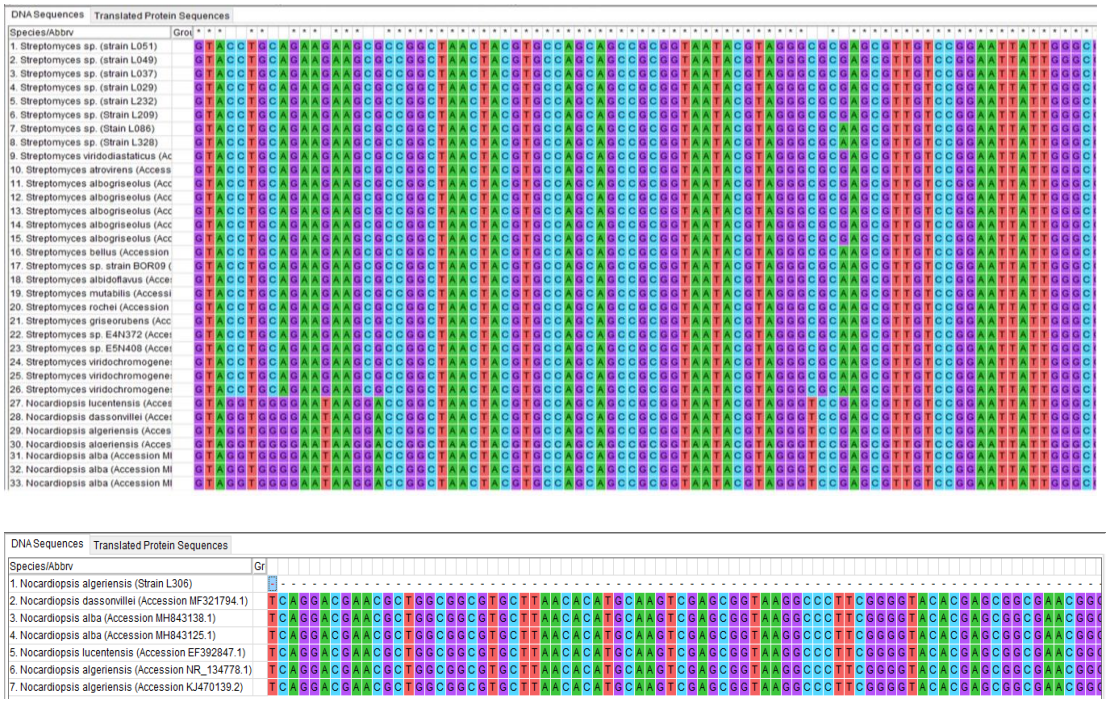


Figure 4.8: Aligned sequences of the 9 Actinomycetes species.

The 9 sequenced cases comprised of 6 (66.7%) males and 3 (33.3%) females. Distribution of the positive cases among <20, 20-29, 30-39, 40-49 and <50 age categories (in years) was as follows; 0(0%), 1(11.1%), 5(55.6%), 1(11.1%) and 2(22.2%) respectively. Distribution among the sites was 2 samples each with JOOTRH having 3 samples to make a total of 9. Retreatment cases were 7(77.8%) while new cases were 2(22.2%), (Table 4.5)

Table 4.5:Distribution of the sequenced cases (n =9)

Variable (%)		**Frequency (n)**	**Percentage**
Gender	Male	6	66.7%
	Female	3	33.3%
Age Category (years)	<20	0	0%
	20-29	1	11.1%
	30-39	5	55.6%
	40-49	1	11.1%
	>50	2	22.2%
Site/Region	MB	2	22.2%
	CPGH	2	22.2%
	MTRH	2	22.2%
	JOOTRH	3	33.3%
History of treatment	Retreatment	7	77.8%
	New cases	2	22.2%

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Actinomycetes are opportunistic microorganisms causing infections in immunocompromised patients particularly, human immunodeficiency virus (HIV)-infected patients. Pulmonary infections caused by these organisms display symptoms that mimic *tuberculosis* and hence can easily be mistaken for *Mycobacteria tuberculosis*. Identification of *Actinomycetes* is based on a variety of phenotypic, chromatographic, and genotypic characteristics. Increased awareness of *actinomycetes* can greatly improve chances for rapid and correct diagnosis. Basic and clinical analysis is necessary for appropriate treatment.

This study examined 385 sputum samples for *Actinomycetes* and reported a prevalence of 13.5% (52/385) by phenotypic characterization. All the 52 isolates were catalase positive and were able to utilize citrate as the sole carbon source. They were able to produce urease enzyme while 38% (20/52) of them were able to hydrolyze casein. These characteristics corresponds with the characteristics described by studies in literature (Sharma *et al*., 2014; Dileep *et al*., 2013). The positive cases were from all age groups with the adults being more affected than children which corresponds with studies done by Järvi *et al.,* 2018 and Toh *et al.,* 2007. A significant difference in isolation of *Actinomycetes* was observed between males and females (Odds Ratio 1.7) which is on contrary to a study done by Toh *et al.,* 2007 which did not find any significant difference in the prevalence by gender of the patient.

Antimicrobial susceptibility testing of aerobic *Actinomycetes* isolated from clinical specimens is very important as it can help in guiding the therapy for infections caused by these organisms. These organisms are slow growing and also most of them form

clumps and aggregates when preparing inoculums hence causing difficulty in carrying out the antimicrobial susceptibility testing (Condas *et al*., 2015; Glupczynski *et al.*, 2006). This study determined the antimicrobial susceptibility patterns of 52 clinical isolates of aerobic *Actinomycetes* which were identified by phenotypic tests. The classes of the antimicrobial agents used in this study are as follows; aminoglycosides (amikacin and gentamycin), penicillins (amoxicillin-clavulanate), cephalosporins (ceftriaxone), quinolones (ciprofloxacin), macrolides (clarithromycin), sulfonamides (co-trimoxazole ie trimethoprim-sulfamethoxazole), tetracyclines (doxycycline) and oxazolidinones (linezolid).

The isolates showed highest susceptibility to Gentamycin 100% (52/52) followed by Ciprofloxacin 98% (51/52), co-trimoxazole (Trimethoprim –sulphamethoxazole) 94% (49/52) and amikacin 90% (47/52). These findings are almost similar to those observed by Hashemi-Shahraki *et al.,* 2015) on a study done on *Nocardia* species. A study done by Hamid, (2011) on S*treptomyces* also showed over 90% susceptibility to Gentamycin. Another study by Silva *et al*., 2010) on *Rhodococcus* also showed 100% susceptibility to Gentamycin.

Trimethoprim-sulphamethoxazole is a commonly used antibiotic for aerobic *Actinomycetes* (van de Sande *et al*., 2014; Uhde *et al.,* 2010; Hamid, (2011); Zampella et al., 2017). This study showed that 94% of the isolates were susceptible to trimethoprim-sulphamethoxazole which corresponds with several other studies done on aerobic *Actinomycetes* (McTaggart *et al.,* 2015); Glupczynski *et al*., 2006); Lowman and Aithma, (2010). However, resistance to trimethoprime-sulphamethoxazole have been observed in different studies including (Uhde *et al.*, 2010; Silva *et al.*, 2010; Hamid, (2011); Condas *et al.,* 2015; Moser *et al.,* 2012; Zampella et al., 2017; Pellegrini et al., 2012) among others. This contradites the results of this study. This resistance could be attributed by overuse of this antibiotic.

On the other hand, the isolates were less susceptible to doxycycline, linezolid, amoxicillin-clavulanate, clarithromycin and ceftriaxone. A study by Hashemi-Shahraki *et a*l., 2015) on *Nocardia* species also showed less susceptibility to ceftriaxone, which is consistent with the results by this study. However, a study by Hashemi-Shahraki *et al.*, (2015) showed high resistance to amoxicillin-clavulanate and ciprofloxacin which is on the contrary to this study. Another study done on *Gordonia* by Moser *et al.*, 2012 showed susceptibility to ceftriazone, linezolid, amikacin, amoxicillin-clavulanate and ciprofloxacin. Resistance to ceftriaxone, ciprofloxacin and clarithromycin was observed on a study done on *Streptomyces* (Pellegrini *et al.,* 2012).

Resistance to three or more classes of antibiotics was considered multidrug-resistant (Condas *et al.,* 2015). Therefore, 7.6% (4/52) isolates showed a multidrug resistance pattern, being resistant to more than 3 classes of antibiotics; penicillin-betalactamise inhibitor (amoxicillin-clavulanate), cephalosporin (ceftriaxone), macrolide (clarithromycin) and oxazolidinone (linezolid). Ceftriazone and Clarithromycin had the highest resistance; 11.5% and 26.9% respectively.

All the antimicrobial agents studied in this study were effective in inhibiting the growth of most *Actinomycetes* isolates. The antimicrobial activity of all the antibiotics tested against the isolates were statistically significant ($P< 0.001$). The MIC of these antibiotics against S *Aureus* ATCC 25923 was also included in the study as a control.

Molecular analysis of *Actinomycetes* is very important as it helps to confirm their identification and to further their investigation. Out of the 52 isolates that had characteristics of *Actinomycetes*, only 32 (61.5%) isolates were able to be amplified by the pair of primers used. These isolates had a size of about 1300 base pairs. Out of the 32 that were amplified, 9 were sequenced and their 16S rRNA sequences were compared with those in NCBI. Phylogenetic analysis showed that they were members of *Actinomycetes.* 8/9 *(*88.9%) of them were members of the genus *Streptomyces* while 1/9

(11.1%) was a *Nocardiopsis* species. This could be because Streptomyces is the most prevalent *Actinomycetes* in the environment (soil) hence can easily be transmitted to human through inhalation (Priyadharsini & Dhanasekaran, 2015; Vijayakumar et al., 2007). Five of the *Streptomyces* species isolated by this study were closely related to *Streptomyces albogriseolus* accession number AJ494865.1 and *Streptomyces atrovirens* accession number EU741195.1 species, Wirth and Ulrich, (2002); (Solano et al., 2009). Other species of *Streptomyces* that are related to the *Streptomyces* species isolated by this study are; *Streptomyces* species strain BOR09 accession number MK675949.1 (Nakaew *et al.,* 2019), *Streptomyces bellus* accession number KJ020687.1 (Aftab & Sajid 2016), *Streptomyces viridodiastaticus* accession number NR_112371.1, *Streptomyces albidoflavus* accession number CP047147.1, and *Streptomyces mutabilis* accession number MT883496.1. The one isolate that was found to be *Nocardiopsis* species aligned with *Nocardiopsis algeriensis* which is closely related to *Nocardiopsis alba,* accession number MH843125.1, that was isolated by Gohel *et al.,* 2018). Although only one *Nocardiopsis* species was isolated in this study, it is significantly important since *Nocardiopsis* species have been observed in indoor environments and also isolated from lung biopsies of farmers suffering from alveolitis (Peltola et al., 2001).

5.2 Limitations

1. The study was not able to sequence all the isolates due to limited resources.
2. Resistance genes was not studied due to limited resources.

5.3 Conclusions

1. This study conclude that *Actinomycetes* are significant pathogens in TB smear-negative and retreatment cases which if not properly screened could go untreated or wrongly treated.
2. The *Actinomycetes* are susceptible to the commonly used antibiotics. However, there is little Multidrug resistance in these organisms.

3. *Actinomycetes* from the genera *Streptomycetes* are the most dominant in TB smear negative and retreatment patients which could be attributed by the fact that *Streptomyces* species are the most prevalent *Actinomycetes* in the environment (soil) hence can easily be transmited to human through inhalation.

5.4 Recommendations

1. There is need for routine screening of all suspected pulmonary pathologies for potential *Actinomycetes* infection.
2. There is need for routine Antimicrobial susceptibility testing of *Actinomycetes* as this will help in guiding on the therapeutic decisions for all clinical *Actinomycetes* isolates.
3. There is a need to explore further on phylogenetic analysis to be able to capture all *Actinomycetes* species of clinical importance. In addition, there is a need for further studies to address one-health aspect due to transmission from the environment to humans and other zoonotic diseases and vice versa.

REFERENCES

Aftab, U., & Sajid, I. (2016). In-vitro antitumor activity and metabolic finger printing of the actinomycetes isolated from various ecological niches in Pakistan. Pakistan Journal of Zoology, 48(5), 1291-1291

Aghamirian, M. R., & Ghiasian, S. A. (2009). Isolation and characterization of medically important aerobic actinomycetes in soil of iran (2006-2007). *The open microbiology journal*, *3*(1), 53-57

Alfaresi, M., & Elkosh, A. (2006). Rapid identification of clinically relevant Nocardia species using real-time PCR with SYBR Green and melting-curve analysis. Journal of medical microbiology, 55(12), 1711-1715.

Arora, A. A., Krishnaswamy, U. M., Moideen, R. P., & Padmaja, M. S. (2015). Tubercular and bacterial coinfection: A case series. *Lung India: official organ of Indian Chest Society*, *32*(2), 172-182

Atta, H. M., El-Sayed, A. S., El-Desoukey, M. A., Mona-Hassan, M., & Manal-El-Gazar, M. (2011). Screening, identification, phylogenetic characterization and optimization of antimicrobial agents biosynthesis production by S. rimosus. World Rural Obs, 3, 40-52.

Bell, M., McNeil, M. M., & Brown, J. M. Nocardia species (Nocardiosis).http://www.antimicrobe.org/new/b117.asp

Brambilla, E., Hippe, H., Hagelstein, A., Tindall, B. J., & Stackebrandt, E. (2001). 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. Extremophiles, 5(1), 23-33.

Brown, J. M. (2003). Nocardia, rhodococcus, gordonia, actinomadura, streptomyces, and other aerobic actinomycetes. *Manual of clinical microbiology*, 502-531.

Cappuccino, J. G., and Sherman, N. (2002). *Microbiology: a Laboratory Manual* 6th edn. San Francisco Benjamin Cummings Pearson Education.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical microbiology reviews, *17*(4), 840-862.

Cloud, J. L., Conville, P. S., Croft, A., Harmsen, D., Witebsky, F. G., & Carroll, K. C. (2004). Evaluation of partial 16S ribosomal DNA sequencing for identification of Nocardia species by using the MicroSeq 500 system with an expanded database. *Journal of clinical microbiology*, *42*(2), 578-584.

Condas, L. A. Z., Ribeiro, M. G., Muro, M. D., VARGAS, A. P. C. D., Matsuzawa, T., Yazawa, K., ... & Ferreira, K. S. (2015). Molecular Identification and antimicrobial resistance pattern of seven clinical isolates of Nocardia spp. in Brazil. Revista do Instituto de Medicina Tropical de São Paulo, 57(3), 251-256.

Dashti, A. A., Jadaon, M. M., Abdulsamad, A. M., & Dashti, H. M. (2009). Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. *Kuwait Med J*, *41*(2), 117-122.

Dileep, N., Junaid, S., Mesta, S. C., & Onkarappa, R. (2013). soil of Thirthahalli, Karnataka, India. *Int. J. Drug Dev. & Res*, *5*(3), 0975-9344

Ekrami, A., Khosravi, A. D., Zadeh, A. R. S., & Hashemzadeh, M. (2014). Nocardia Co-Infection in Patients With Pulmonary Tuberculosis. *Jundishapur journal of microbiology*, *7*(12).

Ercibengoa, M., Bell, M., Vicente, D., & Marimón, J. M. (2016). First isolation of Nocardia grenadensis from a clinical sample. *JMM Case Reports*, *3*(2), e005022.

Euzéby, J. P. (2009). List of prokaryotic names with standing in nomenclature (LPSN). *Formerly list of bacterial names with standing in nomenclature (LBSN)*.

Euzeby, J. P. (2013). List of prokaryotic names with standing in nomenclature-Genus. *Proteus*.

Franco-Paredes, C. (2014). Aerobic actinomycetes that masquerade as pulmonary tuberculosis. *Boletín médico del Hospital Infantil de México*, *71*(1), 37-40.

Franco-Paredes, C. (2014). Aerobic actinomycetes that masquerade as pulmonary tuberculosis. *Boletín médico del Hospital Infantil de México*, *71*(1), 37-40.

Franco-Paredes, C., & Ray, S. (2012). Causes of persistent acid-fast positive smears in pulmonary tuberculosis. *The American Journal of Medicine*, *125*(12), e3-e4.

Garcia-Bellmunt, L., Sibila, O., Solanes, I., Sanchez-Reus, F., & Plaza, V. (2012). Pulmonary nocardiosis in patients with COPD: characteristics and prognostic factors. *Archivos de Bronconeumología (English Edition)*, *48*(8), 280-285.

George, O., Okumu, W., & Peter, A. (2013). Use of soil streptomycetes from Maseno (Kenya) to inhibit growth of Pyricularia grisea invitro cultures. IOSR J Agric Vet Sci, 6(1), 01-06.

Glupczynski, Y., Berhin, C., Janssens, M., & Wauters, G. (2006). Determination of antimicrobial susceptibility patterns of Nocardia spp. from clinical specimens by Etest. Clinical Microbiology and Infection, 12(9), 905-912.

Gohel, S. D., & Singh, S. P. (2018). Molecular phylogeny and diversity of the salt-tolerant alkaliphilic actinobacteria inhabiting coastal Gujarat, India. Geomicrobiology Journal, 35(9), 775-789.

Hamid, M. E. (2011). Variable antibiotic susceptibility patterns among Streptomyces species causing actinomycetoma in man and animals. Annals of clinical microbiology and antimicrobials, 10(1), 24-35

Harold, B.J., (2002). *Microbiological applications : laboratory manual in general microbiology*, New York: McGraw-Hill.

Hashemi-Shahraki, A., Heidarieh, P., Bostanabad, S. Z., Hashemzadeh, M., Feizabadi, M. M., Schraufnagel, D., & Mirsaeidi, M. (2015). Genetic diversity and antimicrobial susceptibility of Nocardia species among patients with nocardiosis. Scientific reports, 5, 17862.

Helal, Z. H., Khan, M. I., Ashour, M. S. E., & Eissa, S. A. (2008). Detection and characterization of Nocardia from patients diagnosed as tuberculosis in Egypt. *Int J Biomed Sci*, *3*, 179-84.

Ishikawa, J., Yamashita, A., Mikami, Y., Hoshino, Y., Kurita, H., Hotta, K., ...& Hattori, M. (2004). The complete genomic sequence of Nocardiafarcinica IFM 10152. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14925-14930.

Järvi, K., Hyvärinen, A., Täubel, M., Karvonen, A. M., Turunen, M., Jalkanen, K., ... & Pekkanen, J. (2018). Microbial growth in building material samples and occupants' health in severely moisture-damaged homes. *Indoor Air*, *28*(2), 287-297.

Jeffrey, L. S. H. (2008). Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak. *African Journal of Biotechnology*, *7*(20), 3700-3705.

Junior, J. C. R., Tamanini, R., Soares, B. F., de Oliveira, A. M., de Godoi Silva, F., da Silva, F. F., ... & Beloti, V. (2016). Efficiency of boiling and four other methods for genomic DNA extraction of deteriorating spore-forming bacteria from milk. Semina: *Ciências Agrárias, 37*(5), 3069-3078.

Kirby, R., Sangal, V., Tucker, N. P., Zakrzewska-Czerwińska, J., Wierzbicka, K., Herron, P. R., ... & Hoskisson, P. A. (2012). Draft genome sequence of the human pathogen Streptomyces somaliensis, a significant cause of actinomycetoma. *Journal of bacteriology*, *194*(13), 3544-3545.

Kumar, V., Bharti, A., Gusain, O., & Bisht, G. S. (2010). An improved method for isolation of genomic DNA from filamentous actinomycetes. *J Sci Eng Tech Mgt*, *2*, 10-13.

Kumar, V., Bisht, G. S., & Institu, S. B. S. P. G. (2010). An improved method for isolation of genomic DNA from filamentous actinomycetes. *Int. J. Eng. Technol. Manage. Appl. Sci.*, *2*(2). 10-12

Kumari, M., Myagmarjav, B. E., Prasad, B., & Choudhary, M. (2013). Identification and characterization of antibiotic-producing actinomycetes isolates. *American journal of Microbiology*, *4*(1), 24.

Lowman, W., & Aithma, N. (2010). Antimicrobial susceptibility testing and profiling of Nocardia species and other aerobic actinomycetes from South Africa: comparative evaluation of broth microdilution versus the Etest. J*ournal of clinical microbiology*, *48*(12), 4534-4540.

Macrae, A. (2000). The use of 16S rDNA methods in soil microbial ecology. Brazilian *Journal of microbiology,* 31(2), 77-82.

McTaggart, L. R., Doucet, J., Witkowska, M., & Richardson, S. E. (2015). Antimicrobial susceptibility among clinical Nocardia species identified by multilocus sequence analysis. *Antimicrobial agents and chemotherapy*, *59*(1), 269-275.

Minero, M. V., Marín, M., Cercenado, E., Rabadán, P. M., Bouza, E., & Muñoz, P. (2009). Nocardiosis at the turn of the century. *Medicine*, *88*(4), 250-261.

Monciardini, P., Sosio, M., Cavaletti, L., Chiocchini, C., & Donadio, S. (2002). New PCR primers for the selective amplification of 16S rDNA from different groups of actinomycetes. *FEMS Microbiology Ecology*, *42*(3), 419-429.

Moser, B. D., Pellegrini, G. J., Lasker, B. A., & Brown, J. M. (2012). Pattern of antimicrobial susceptibility obtained from blood isolates of a rare but emerging human pathogen, Gordonia polyisoprenivorans. *Antimicrobial agents and chemotherapy, 56(*9), 4991-4993.

Mwaura, E. N., Matiru, V., & Bii, C. (2013). Mycological findings of sputum samples from pulmonary tuberculosis patients attending TB clinic in Nairobi, Kenya. *Virology & Mycology*, *2*(3), 1-6.

Nakaew, N., Ngoenkam, J., Donphraiyon, M., Yot-an, T., Jompaeng, C., Ano-thai, B., & Sungthong, R. (2019). Endophytic actinobacteria of herbal rhizomes & their pharmaceutical potential to form L-asparaginase. *Access Microbiology, 1*(1A), 502.

NCCLS. 2003. Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes. Approved standard. NCCLS document M-24A. NCCLS, Wayne, PA.

Oskay, A. M., Üsame, T., & Cem, A. (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *African journal of Biotechnology*, *3*(9), 441-446.

Pandey, A., Ali, I., Butola, K. S., Chatterji, T., & Singh, V. (2011). Isolation and characterization of Actinomycetes from soil and evaluation of antibacterial activities of Actinomycetes against pathogens. *International Journal of Applied Biology and Pharmaceutical Technology*, *2(*4), 384-392.

Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular diagnosis*, *6*, 313-321.

Peleg, A. Y., Husain, S., Qureshi, Z. A., Silveira, F. P., Sarumi, M., Shutt, K. A., ...& Paterson, D. L. (2007). Risk factors, clinical characteristics, and outcome of Nocardia infection in organ transplant recipients: a matched case-control study. *Clinical Infectious Diseases*, *44*(10), 1307-1314.

Pellegrini, G. J., Graziano, J. C., Ragunathan, L., Bhat, M. A., Hemashettar, B. M., & Brown, J. M. (2012). Scalp abscess due to Streptomyces cacaoi subsp. cacaoi, first report in a human infection. *Journal of clinical microbiology*, *50(*4), 1484-1486.

Peltola, J., Andersson, M. A., Haahtela, T., Mussalo-Rauhamaa, H., Rainey, F. A., Kroppenstedt, R. M., ... & Salkinoja-Salonen, M. S. (2001). Toxic-metabolite-producing bacteria and fungus in an indoor environment. *Applied and Environmental Microbiology, 67*(7), 3269-3274.

Poonwan, N., Mekha, N., Yazawa, K., Thunyaharn, S., Yamanaka, A., & Mikami, Y. (2005). Characterization of clinical isolates of pathogenic Nocardia strains and related actinomycetes in Thailand from 1996 to 2003.*Mycopathologia*, *159*(3), 361-368.

Priyadharsini, P., & Dhanasekaran, D. (2015). Diversity of soil allelopathic Actinobacteria in Tiruchirappalli district, Tamilnadu, India. *Journal of the Saudi Society of Agricultural Sciences, 14*(1), 54-60.

Rana, S., & Salam, M. D. (2014). Antimicrobial potential of actinomycetes isolated from soil samples of Punjab. *India. J Microbiol Exp, 1*(2), 00010.

Rao, K. V. R., Kumar, K. S., Rao, D. B., & Rao, T. R. (2012). Isolation and characterization of antagonistic actinobacteria from mangrove soil. *Journal of Biochemical Technology*, *3*(4), 361-365.

Saadoun, I., Rawashdeh, R., Dayeh, T., Ababneh, Q., & Mahasneh, A. (2007). Isolation, characterization and screening for fiber hydrolytic enzymes-producing streptomycetes of Jordanian forest soils. *Biotechnology*, *6*(1), 120-128.

Sacchi, C. T., Whitney, A. M., Reeves, M. W., Mayer, L. W., & Popovic, T. (2002). Sequence diversity of Neisseria meningitidis 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. *Journal of clinical microbiology*, *40(*12), 4520-4527.

Safaei, S., Fatahi-Bafghi, M., & Pouresmaeil, O. (2018). Role of Tsukamurella species in human infections: first literature review. New microbes and new infections, 22, 6-12.

Saubolle, M. A., & Sussland, D. (2003). Nocardiosis review of clinical and laboratory experience. *Journal of clinical microbiology*, *41*(10), 4497-4501.

Savini, V., Fazii, P., Favaro, M., Astolfi, D., Polilli, E., Pompilio, A., ...& D'Antonio, D. (2012). Tuberculosis-like pneumonias by the aerobic actinomycetes Rhodococcus, Tsukamurella and Gordonia. *Microbes and Infection*, *14*(5), 401-410.

Sharma, M., Dangi, P., & Choudhary, M. (2014). Actinomycetes: source, identification, and their applications. *Int J Curr Microbiol App Sci*, *3*(2), 801-832.

Silva, P. D., Miyata, M., Sato, D. N., Santos, A. C. B., Mendes, N. H., & Leite, C. Q. F. (2010). Rhodococcus equi isolation from sputum of patients with suspected tuberculosis. *Memórias do Instituto Oswaldo Cruz*, *105*(2), 199-202.

Silva, P. D., Santos, A. C. B., Sato, D. N., Silva, J. O., Medeiros, M. I. C., Carneiro, A. M. M., ... & Leite, C. Q. F. (2012). Phenotypic and genotypic characterization of Rhodococcus equi isolated from sputum. *Brazilian Journal of Infectious Diseases*, *16*(5), 409-415.

Sivakumar, K. (2001). Actinomycetes of an Indian mangrove (Pichavaram) environment: An inventory. *Annamalai University, India*, 91-4.

Solano, G., Rojas-Jiménez, K., Jaspars, M., & Tamayo-Castillo, G. (2009). Study of the diversity of culturable actinomycetes in the North Pacific and Caribbean coasts of Costa Rica. *Antonie Van Leeuwenhoek*, *96*(1), 71-78.

Subhash, H. S., Christopher, D. J., Roy, A., & Cherian, A. M. (2001). Pulmonary nocardiosis in human immunodeficiency virus infection: a tuberculosis mimic. *Journal of postgraduate medicine*, *47*(1), 30.

Sullivan, D. C., & Chapman, S. W. (2010). Bacteria that masquerade as fungi: actinomycosis/nocardia. *Proceedings of the American Thoracic Society*, *7*(3), 216-221.

Ten, L. N., Im, W. T., Kim, M. K., & Lee, S. T. (2005). A plate assay for simultaneous screening of polysaccharide-and protein-degrading micro-organisms. *Letters in applied microbiology*, *40*(2), 92-98.

Toh, S. T., Yuen, H. W., & Goh, Y. H. (2007). Actinomycetes colonization of tonsils: a comparative study between patients with and without recurrent tonsillitis. *The Journal of Laryngology & Otology*, *121*(8), 775-778.

Uhde, K. B., Pathak, S., McCullum Jr, I., Jannat-Khah, D. P., Shadomy, S. V., Dykewicz, C. A., ... & Brown, J. M. (2010). Antimicrobial-resistant nocardia isolates, United States, 1995–2004. *Clinical infectious diseases*, *51(*12), 1445-1448.

Valenzuela-Tovar, J. F., Contreras-Pérez, C., Shibayama-Hernández, H., Chávez-González, L., Vázquez-Chacón, C. A., & Olivera-Díaz, H. (2005). Biochemical identification and molecular characterization (PCR-RFLP) of Nocardia isolates from sputum. *Archives of medical research*, *36*(4), 356-361.

Van de Sande, W. W., Fahal, A. H., Goodfellow, M., Welsh, O., & Zijlstra, E. E. (2014). Merits and pitfalls of currently used diagnostic tools in mycetoma. *PLoS neglected tropical diseases*, *8*(7), e2918.

Vijayakumar, R., Muthukumar, C., Thajuddin, N., Panneerselvam, A., & Saravanamuthu, R. (2007). Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycetologica*, 0712050027-0712050027.

Wirth, S., & Ulrich, A. (2002). Cellulose-degrading potentials and phylogenetic classification of carboxymethyl-cellulose decomposing bacteria isolated from soil. *Systematic and applied microbiology*, *25*(4), 584-591.

Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Tse, H., & Yuen, K. Y. (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection*, *14*(10), 908-934.

Yildiz, O., & Doganay, M. (2006). Actinomycoses and Nocardia pulmonary infections. *Current opinion in pulmonary medicine*, *12*(3), 228-234.

Zampella, J. G., Kwatra, S. G., Kazi, N., & Aguh, C. (2017). Madura foot caused by Gordonia terrae misdiagnosed as Nocardia. *Australasian Journal of Dermatology*, *58*(3), e129-e131.

Zumla, A., George, A., Sharma, V., Herbert, R. H. N., Oxley, A., & Oliver, M. (2015). The WHO 2014 global tuberculosis report—further to go. *The Lancet Global Health*, *3*(1), e10-e12.

APPENDICES

Appendix I: Consent to participate in a Research Study

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: Phenotypic, genotypic characterization and antimicrobial susceptibility testing of *actinomycetes* from sputum of TB smear negative patients from selected referral facilities, Kenya.

Name of study site: MTRH, JOOTRH, CPGH and Mbagathi county Hospital

Investigators	**Institution**	**Study role**
Frida M. Njeru	Student at JKUAT (ITROMID)	Principal Investigator
Dr. Christine Bii	KEMRI, Centre for Microbiology Research (Kenya)	Supervisor
Dr.Perpetual Ndung'u	Jomo Kenyatta University of Agriculture And Technology	Supervisor

Purpose of the study

This study is a research study. I am a student at Jomo Kenyatta University of Agriculture and Technology and am conducting a research study to determine the type of germs that cause infections which presents with signs and symptoms of TB but they are not TB. This study will also test if the medicines used to treat these germs are working. This will help in addressing the health challenges in these patients. You are being asked to participate in this study because you are suspected to have TB. I am seeking to obtain with your consent the sputum sample left over after routine laboratory investigations for further investigations. Information obtained from this study will be shared with the facilities to be used to guide treatment and management of patients with this infection.

Who can participate in this study?

Any person who have been referred to the facility and is suspected of TB and have his/her sputum sample obtained and have agreed to participate and signed the consent form or consent has been given through a relative/guardian.

How many people will take part in this study?

About 385 people who have been referred to the facility, with suspected TB infection have their sputum sample taken will participate in this study.

Potential risks

There is no risk for participation in this study. Sputum sample is routinely obtained for laboratory analysis in patients suspected with TB. Findings obtained from this study will be shared with the facilities for management of this infection.

Potential benefits

There is no benefit that the patients will get from participating in the study. However, findings obtained from this study will be shared with the facilities for management of these infections in future.

Payment for participation

There is no payment or compensation to volunteers for their participation in this study.

Confidentiality

All information and records relating to your participation in the study will remain confidential. Yourname will not be used in any report resulting from this study. The information will not be used for any other purpose other than for this study and will only be accessible to study investigators and authorized person. All computerized records and laboratory specimens for this study will contain only unique study number and not your name. Data will be entered in the computer and will be password protected and only accessible to authorized persons in the study.

Protection of research subjects

KEMRI Scientific and Ethical Review Unit (SERU) is a mandated body concerned with protection of persons participating in medical research; it is also eligible to review research records and information as part of their responsibility to protect human subjects.

Consent to participate

This study is seeking for you/relative/guardian consent/assent for participation. Participation will involve giving consent for use of sputum sample, medical information but not name. There is no follow up or further information needed from you or your relative/guardian. If you consent to participate or act as a legal representative for an adult to participate in the study, i will ask you to sign or append your thumbprint on this form. In case you do not give consent for participation, there will be no penalty. You will receive a signed copy of this consent form to keep.

If you have understood the information in this consent form and what I have explained to you, I am requesting you to confirm and sign up for participation of you/your relative. If there is any portion of this consent form that you do not understand, please ask me before signing it.

Declaration

I have read or have been read to me and understood the information given and willingly consent for me/relative to participate in this study. I have been given the opportunity to ask questions concerning this investigation and all questions has been answered to my full and complete satisfaction.

In case of need for further information about this study please contact **Frida M. Njeru mobile No. 0723-828448 or The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org**

Participants sign…….. Date……..Age……
Sex……

Legal representative Name …….. Sign…….. Date…….

Thumbprint……

Witness Name……. Sign…….. Date…….

Interview /Investigator's Name …… date…….

Appendix II: Consent to Participate in a Research Study (KISWAHILI)

IDHINI YA KUSHIRIKI KATIKA UTAFITI

Jina la Utafiti: Phenotypic, genotypic characterization and antimicrobial susceptibility testing of *actinomycetes* from sputum of TB smear negative patients from selected referral facilities, Kenya.

Jina la hospitali ya utafiti huu: MTRH, JOOTRH, CPGH and Mbagathi county Hospital

Mtafiti	**Taasisi Husika**	**Jukumu la Mtafiti**
Frida M. Njeru	Student at JKUAT (ITROMID)	Principal Investigator
Dr. Bii Christine	KEMRI, Centre for Microbiology Research (Kenya)	Supervisor
Dr.Perpetual Ndung'u	Jomo Kenyatta University of Agriculture And Technology	Supervisor

Madhumuni ya Utafiti

Huu ni utafiti. Mimi ni mwanafunzi katika chuo kikuu cha Jomo Kenyatta university of Agriculture and technology na ninafanya utafiti juu ya viini vinavyosababisha maradhi yanayoonyesha dalili kama za kifua kikuu lakini maradhi yenyewe sio kifua kikuu. Utafiti huu pia utachunguza kama madawa ambayo yanatumiwa kutibu viini hivi yanafanya kazi. Haya yatasaidia katika kutatua changamoto za kiafya kwa wagonjwa hawa. Unahimizwa kushiriki katika utafiti huu kwa sababu unatuhumiwa kuwa na ugonjwa wa kifua kikuu na daktari anayekutibu. Ninakuomba idhini yako ya kuweza kuchukua sampuli ya mate yako uliotowa yatakayobaki baada ya kumalizika kwa uchunguzi ili kufanyia utafiti wa kuwepo kwa viini vingine. Matokeo ya utafiti huu yatapatiwa hosipitali zilizoshiriki ili kutumika kuongoza matibabu na usimamizi kwa wagonjwa wenye maradhi haya.

Ambae anaweza kushirikia katika utafiti

Mtu yeyote ambaye ametumwa katika hosipitali naanatuhumiwa kuwa ana maradhi ya pneumonia isio ya TB na sampuli yake ya mate imechukuliwa kwa uchunguzi, na ametoa idhini kwa kuweka sahihi katika fomu hii ya idhini au idhini kupatikana kupitia kwa mzazi, jamaa au mlezi anaweza kushirika katika utafiti huu.

Ni watu wangapi watashiriki katika utafiti

Watu mia tatu themanini na tano ambao wametumwa katika hosipitali kwa kuwa wanatuhumiwa kuwa ana maradhi ya TB na sampuli yao ya mate imechukuliwa kwa uchunguzi watashiriki katika utafiti huu.

Faida na uwezo wa hatari kwa mshiriki

Hakuna hatari yoyote itapatikana kwa mwenye kushiriki katika utafiti huu. Sampuli ya mate hutolewa kwa kawaida kwa mgonjwa yeyote anayetuhumiwa kuwa na maradhi ya TB. Matokeo yatakayopatikana kutoka kwa utafiti huu yatapatiwa hosipitali zilizoshiriki ili kutumika kuongoza matibabu na usimamizi kwa wagonjwa wenye maradhi yanayo sababishwa na vimelea vyitakavyopatikana.

Malipo kwa ajili ya kushiriki

Hakuna malipo au fidia yoyote itakayotolewa au kupatikana kwa mwenye kushiriki. Kushiriki katika utafiti huu ni kwa hiari.

Kuwekwa siri kwa taarifa na rekodi za mshiriki

Itabakia siri taarifa na rekodi zote zinazohusiana na wewe kuhusu kushiriki katika utafiti huu. Jina lako halitatumika katika ripoti yoyote ya utafiti huu. Taarifa zote zitatumika tu kwa ajili ya utafiti huu na sio kwa matumizi mengine yoyote. Taarifa na rekodi zote za utafiti huu zinawezakutizamwa tu na mtafiti husika. Taarifa zote zitanukuliwa katika tarakilishi na sampuli zote zitakuwa tu na nambari maalum ya utafiti, na sio jina la

mshiriki. Taarifa zote zitawekwa katika tarikilishi iliyo na visawe na kuweza kufunguliwa tu na mtafiti katika utafiti huu au mwenye kupewa ruhusa.

Utetezi wa haki kwa mshiriki

Kamati ya kitaifa ya maadili ya KEMRI, ina mamlaka husika kutetea na kulinda haki za washiriki katika utafiti wa kimatibabu. Pia kamati hii ina mamlaka ya kukagua taarifa na rekodi zote za utafiti kama sehemu ya majukumu yake. Anwani ya kamati hii inapatikana hapo chini katika fomu hii.

Idhini ya Kushiriki

Utafiti huu unakuhimiza kupata kwa idhini yako /jamaa wako ili kushiriki. Kushiriki kwako kutahusisha kutoa idhini kwa matumizi ya sampuli ya mate yako na taarifa ya afya ya kidakitari. Hakutakuwa na kufwatilia au nyongeza zaidi za habari kuitishwa. Ukikubali kushiriki au kutenda kama mwakilishi wa kisheria kwa mtu mzima kushiriki katika utafiti, nitakuhitaji uweke sahihi au ishara ya kidolo chako cha gumba kwenye fomu hii. Iwapo utakosa kutoa idhini ya kushiriki kwako/ jamaa yako, hakutakuwa na adhabu yoyote. Ukikubali kushiriki utapata nakala ya fomu hii ya idhini kujiwekea. Kama umeelewa maelzeo katika fomu hii ya idhini na niliyokueleza, ninakuomba uthibitishe kwa ishara kwa kuweka sahihi au alama ya kidole chako/jamaa wako kwa kushiriki. Kama kuna sehemu yoyote ya fomu hii ya idhini huelewi, tafadhali niulize mimi kabla ya kuweka sahihi au kidole chako.

Azimio la Kushiriki

Nimefahamishwa kusudi na lengo la utafiti huu. Nimesoma/nimesomewa fomu hii ya idhini na nimeelewa vizuri maelezo yote. Nimepewa fursa ya kuuliza maswali kuhusu utafiti huu na maswali yangu yote yamejibiwa kwa ukamilifu na nimeridhika na majibu niliopewa. Ninatoa idhini yangu/jamaa yangu kushiriki katika utafiti huu kwa hiari.

Iwapo utahitaji maelezo zaidi kuhusu utafiti huu, tafadhali wasiliana na mtafiti mkuu au katibu wa kamati ya maadili ya KEMRI kupitia anwani zifuatazo; **Frida M. Njeru mobile number0723-828448 au The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org**

Sahihi ya Mshiriki……Tarehe……..…Umri……Jinsia……

Jina la mwakilishi …….. ……..…….. Sahihi……Tarehe…….

Alama ya kidole cha gumba……..……

Jina la mshahidi ……. Sahihi …… Tarehe …….…….

Jina la mtafiti…… …… Tarehe…….

Appendix III: Assent to Participate in a Research Study

ASSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: Phenotypic, genotypic characterization and antimicrobial susceptibility testing of *actinomycetes* from sputum of TB smear negative patients from selected referral facilities, Kenya.

Name of study site: MTRH, JOOTRH, CPGH and Mbagathi county Hospital

Investigators	**Institution**	**Study role**
Frida M. Njeru	Student at JKUAT (ITROMID)	Principal Investigator
Dr. Christine Bii	KEMRI, Centre for Microbiology Research (Kenya)	Supervisor
Dr.Perpetual Ndung'u	Jomo Kenyatta University of Agriculture And Technology	Supervisor

Declaration

This study is a research study and the purpose of this study has been explained to me satisfactorily. I understand that the study is investigating the germs that cause diseases that show signs and symptoms like those of TB but they are not TB. I understand that the findings of this study will be used to guide in the treatment and management of patients with these infections. I am being requested to participate in this study and my participation is voluntary**.**My participation will involve giving assent for use of my sputum samples. The germs that will be obtained from this study may be exported for further analysis and confirmation where necessary.

I further understand that my participation is voluntary and I may withdraw from the study at any time and without any explanation or loss of right to care and treatment of my condition.

I further understand that there is no compensation to volunteers for their participation.

I have read and/ or have been read to me and understood the information given and willingly consent to participate in the study. I have been given the opportunity to ask questions concerning this investigation and the question has been answered to my full and complete satisfaction. I will receive a signed copy of this consent form to keep.

Incase of need for further information about this study please contact **Frida M. Njeru mobile number 0723828448** or **The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org.**

Participant sign……. Date…….

Thumbprint…… Age……. Sex……..

Witness Name …… sign……. Date…….

Interviewer /Investigator Name……Sign……. date…….

Appendix IV: Assent to Participate in a Research Study (KISWAHILI)

IDHINI YA KUSHIRIKI KATIKA UTAFITI

Jina la Utafiti: Phenotypic, genotypic characterization and antimicrobial susceptibility testing of *actinomycetes* from sputum of TB smear negative patients from selected referral facilities, Kenya.

Jina la hospitali ya utafiti huu: MTRH, JOOTRH, CPGH and Mbagathi county Hospital

Mtafiti	**Taasisi Husika**	**Jukumu la Mtafiti**
Frida M. Njeru	Student at JKUAT (ITROMID)	Principal Investigator
Dr. Bii Christine	KEMRI, Centre for Microbiology Research (Kenya)	Supervisor
Dr.Perpetual Ndung'u	Jomo Kenyatta University of Agriculture And Technology	Supervisor

Azimio la kushiriki

Huu ni utafiti. Nimeelezwa lengo na kusudi la utafiti huu na nimeridhika. Naelewa kuwa utafiti huu unachunguza kuwepo kwaviini vinavyosababisha maradhi yanayoonyesha dalili kama za kifua kikuu lakini maradhi yenyewe sio kifua kikuu. Naelewa kuwa matokeo ya utafiti huu yatatumiwa kwa ajili ya matibabu na usimamizi wa maradhi haya. Ninahimizwa kushiriki katika utafiti huu na naelewa kuwa kushiriki kwangu ni kwa hiari. Naelewa kuwa kushiriki kwangu kutahusisha kutoa idhini kwa matumizi ya sampuli ya mate. Nafahamu kuwa viini vitakavyo patikana kutokana na utafiti huu vinaweza kusafirishwa katika maabara ya nje ya nchi itakapohitajika kufanyiwa uchunguzi zaidi ya kibiologia.

Naelewa kuwa kushiriki kwangu ni kwa hiari na naweza kukataa kushiriki wakati wowote bila kutowa maelezo yeyote. Nimesoma au nimesomewa fomu hii ya idhini na nimeelewa vyema maelezo yote. Ninatoa idhini yangu ya kushiriki katika utafiti huu kwa hiari. Nimepewa fursa ya kuuliza maswali kuhusu utafiti huu na maswali yangu yote yamejibiwa kwa ukamilifu na nimeridhika na majibu niliopewa. Nitapewa nakala iliyotiwa sahihi ya fomu hii ya idhini kujiwekea.

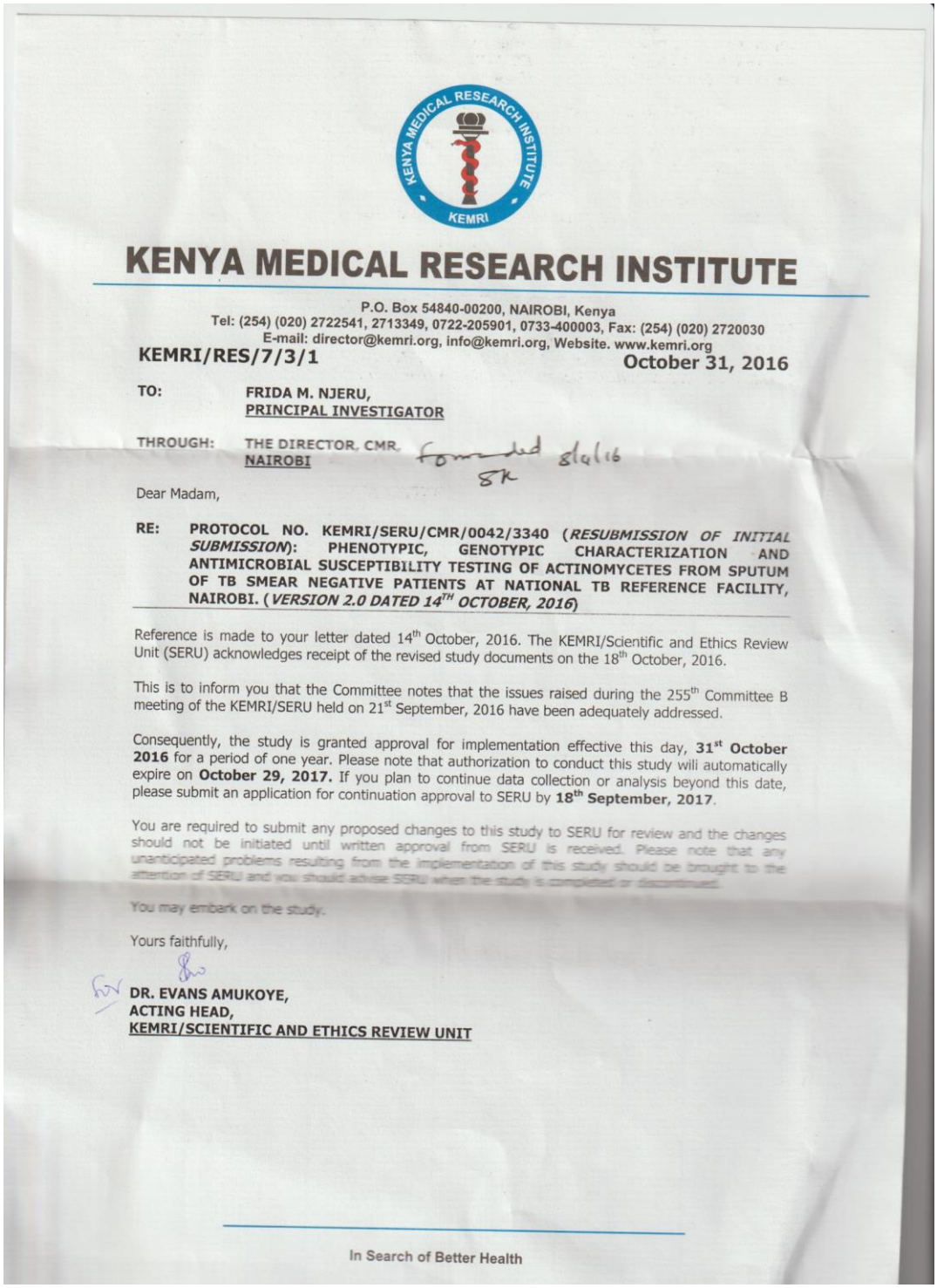
Iwapo utahitaji maelezo zaidi kuhusu utafiti huu, tafadhali wasiliana na mtafiti mkuu au katibu wa kamati ya maadili ya KEMRI kupitia anwani zifuatazo; **Frida M. Njeru mobile number 0723828448 au The Secretary Scientific and Ethics Review Unit P.O.Box 54840-00200 Nairobi. Mobile; +254717719477; Email; seru@kemri.org.**

Sahihi ya Mshiriki……..
Tarehe……..…..Umri…… Jinsia……

Jina la mshahidi ……. Sahihi ……..……..
Tarehe …….…….

Alama ya kidole cha gumba……..……

Appendix V: Approval letters



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **October 31, 2016**

TO: **FRIDA M. NJERU,**
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CMR.
NAIROBI

Dear Madam,

RE: **PROTOCOL NO. KEMRI/SERU/CMR/0042/3340 (*RESUBMISSION OF INITIAL SUBMISSION*): PHENOTYPIC, GENOTYPIC CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING OF ACTINOMYCETES FROM SPUTUM OF TB SMEAR NEGATIVE PATIENTS AT NATIONAL TB REFERENCE FACILITY, NAIROBI. (*VERSION 2.0 DATED 14TH OCTOBER, 2016*)**

Reference is made to your letter dated 14th October, 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the 18th October, 2016.

This is to inform you that the Committee notes that the issues raised during the 255th Committee B meeting of the KEMRI/SERU held on 21st September, 2016 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **31st October 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **October 29, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **18th September, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke
TEL: 254-067-52711/52181-4
FAX: 254-067-52164/52030

REF: JKU/2/11/TM305-2765/2015

31ST OCTOBER, 2018

NJERU, FRIDA MUKWAMUNENE
C/o SOBMS
JKUAT

Dear Ms. Frida,

RE: APPROVAL OF RESEARCH PROPOSAL AND OF SUPERVISORS

Kindly note that your MSc. research proposal entitled: **"PHENOTYPIC, GENOTYPIC CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *ACTINOMYCETES* FROM SPUTUM OF TB SMEAR NEGATIVE PATIENTS AT NATIONAL TB REFERENCE FACILITY, NAIROBI"** has been approved. The following are your approved supervisors:-

1. Dr. Perpetual Ndung'u
2. Dr. Christine Bii

Yours sincerely,

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to: Dean, SOBMS
/cm

0723-828448

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
DEAN
25 FEB
SIGN:.....
SCHOOL OF BIOMEDICAL SC...

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
OFFICE OF THE DIRECTOR, BOARD OF POSTGRADUATE STUDIES
P.O. BOX 62000, 00200 • NAIROBI •KENYA•TEL: (067)-5870000-4 Email: director@bps.jkuat.ac.ke

REF: JKU/2/TM305-2765/2015

11th February, 2022

RECEIVED
02 MAR 2022
KEMRI GRADUATE SCHOOL

Frida M. Njeru
c/o SOBMS
JKUAT

Dear Ms. Njeru

RE: CHANGE OF RESEARCH THESIS TITLE

Reference is made to your letter dated 2nd December, 2021 regarding the change of your research title from "**Phenotypic, Genotypic Characterization and Antimicrobial Susceptibility of Actinomycetes from Sputum of TB Smear Negative Patients at National TB Reference Facility, Nairobi**" to "**Phenotypic, Genotypic Characterization and Antimicrobial Susceptibility Testing of *Actinomycetes* from Selected Referral Facilities, Kenya**".

Kindly note that your request was given due consideration and granted approval. You are therefore expected to use the same on your thesis.

PROF. LOSENGE TUROOP
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Dean, SOBMS
KEMRI Graduate School
Dr. Perpetual Ndung'u
Dr. Christine Bii

Appendix VI: Publication

Scientific Research Publishing

Journal of Biosciences and Medicines, 2019, 7, 1-12
http://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Characterization and Antimicrobial Susceptibility of Actinomycetes from TB Smear Negative and Retreatment Patients in Nairobi, Kenya

Frida M. Njeru[1,2*], Perpetual Ndungu[3], Christine Bii[2]

[1]Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[2]Centre for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya
[3]Department of Biomedical Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
Email: *fnmunene@gmail.com

How to cite this paper: Njeru, F.M., Ndungu, P. and Bii, C. (2019) Characterization and Antimicrobial Susceptibility of Actinomycetes from TB Smear Negative and Retreatment Patients in Nairobi, Kenya. *Journal of Biosciences and Medicines*, 7, 1-12.
https://doi.org/10.4236/jbm.2019.78001

Received: June 21, 2019
Accepted: August 6, 2019
Published: August 9, 2019

Abstract

Actinomycetes are opportunistic pathogens in immunosuppressive patients. Pulmonary actinomycetes infections display symptoms that mimic *Mycobacteria tuberculosis* and can be misdiagnosed and treated as pulmonary TB. Actinomycetes can be co-infection with tuberculosis leading to delayed or inappropriate treatment. This study aimed to identify and determine antimicrobial susceptibility profiles of Actinomycetes from the sputum of TB smear negative and re-treatment patients referred to TB reference facilities in Kenya. Sputum specimens were collected and direct smears stained with Gram's reagents. Culture was done on Mueller Hinton agar and incubated at 35°C for two weeks. Identification was done using phenotypic and biochemical procedures. Confirmation of the isolates was done using Polymerase Chain Reaction. A total of 52/385 (14%) Actinomycetes were isolated and subjected to antimicrobial susceptibility testing using broth microdilution method to determine the Minimum Inhibitory Concentration. Nine antibiotics were tested which included: Amikacin, Amoxicillin/Clavulanic acid, Ceftriaxone, Ciprofloxacin, Clarithromycin, Linezolid, Doxycycline, Trimethoprim-Sulfamethoxazole and Gentamycin. *Staphylococcus aureus* (ATCC 25923) was used as a control. Most of the isolates were susceptible to the test antibiotics. However, four isolates showed multidrug resistance to Ceftriaxone and Clarithromycin with resistance of 11.5% and 26.9% respectively. Gentamycin and Ciprofloxacin showed the highest susceptibility of 100% and 98.1% respectively. The findings of this study confirm that Actinomycetes are significant pathogens in TB smear-negative cases. Although most antibiotics were susceptible, resistance to few antibiotics was observed; hence, there is a

Appendix VII: Minutes of Seminar Presentations

KENYA MEDICAL RESEARCH INSTITUTE

GRADUATE SCHOOL OF HEALTH SCIENCES

P.O. BOX 54840-00200, NAIROBI, KENYA
Tel: (254) (020) 2722541, 0713 112853, 0202711255 or 0713 112854 Fax: (254) (020) 2720030
E-mail: graduateschool@kemri.org Website: www.kemri.org

MINUTE OF VIRTUAL SEMINAR PRESENTATIONS HELD ON THURSDAY 28TH OCTOBER 2021 9AM TO 1PM

FACULTY MEMBERS PRESENT

1. Dr. Raphael Lihana- KEMRI/Chairperson
2. Prof Kenneth Ngure - JKUAT
3. Prof. Mohammed Karama- KEMRI
4. Dr. Christine Bii – KEMRI
5. Dr. Perpetual Ndungu - JKUAT
6. Dr. Joseph Mutai - KEMRI
7. Dr. Willie Sang- KEMRI

STUDENTS PRESENT

1. Georgina Mbeki
2. Cosmas Ndeti
3. Frida Njeru
4. Winnie J. Kemboi
5. Bertha Mwinuka
6. Stella Njagi
7. Thani Suleiman
8. Milker Atieno Simba
9. Silas Lodeke
10. Johana Too
11. Salim Omambia

No.	PRESENTER	Comments	VERDICT
1	Georgine Mbeki TM310-0912-2012 Msc Public Health Predictors Of Client Satisfaction With Family Planning Services Rendered To Women Of Reproductive Age In Two Healthcare Facilities In Nairobi County. Prof.M.Karama KEMRI Dr Mwaniki JKUAT	2nd seminar presentation. 1. Consider refining objectives to make more measurable (eg avoiding use of words like "Assess", "Evaluate" etc. 2. Seems there are only 2 objectives. 3. Analysis- yours is a cross-sectional study; when did this come up	Satisfactory. Proceed
2	Fridah Njeru Msc Molecular Medicine Phenotypic, Genotypic Characterization And Antimicrobial Susceptibility Of Actinomycetes From Sputum Of Tb	1st seminar 1. You needed to give a brief of: What are actinomycetes? 2. You didn't have questionnaires	Satisfactory. Proceed

Raphael Lihana

KENYA MEDICAL RESEARCH INSTITUTE

GRADUATE SCHOOL OF HEALTH SCIENCES
P.O. BOX 54840-00200, NAIROBI, KENYA
Tel: (254) (020) 2722541, 0713 112853, 0202711255 or 0713 112854 Fax: (254) (020) 2720030
E-mail: graduateschool@kemri.org Website: www.kemri.org

VIRTUAL MINUTES FOR THE MSC AND PhD STUDENTS SEMINAR/ PROPOSAL PRESENTATIONS HELD ON 2nd December 2021 9AM (ONLINE)

Faculty Members Present	Students Present
1. Dr. Raphael Lihana- Chairperson 2. Prof Simon Karanja- SoPH JKUAT 3. Prof Kenneth Ngure- JKUAT 4. Dr. Joseph Mutai- KEMRI 5. Dr. Christine Bii- KEMRI 6. Dr. Daniel Nyamongo - JKUAT 7. Dr. Joel Lutomiah- KEMRI 8. Dr.Benjamin Ngugi- KEMRI 9. Prof. Wallace Bulimo - KEMRI	1. Daniel Muvengei 2. Fridah Njeru 3. Cosmas Ndeti 4. Salim Omambia 5. Rose Anyango Oloo 6. Stella Njagi 7. James Mutisya 8. Sarah Atambo 9. Maureen Chepkoech 10. Betty Muriithi 11. Athuman Nyae Chiguzo 12. MESHACK WADEGU 13. Khatra Shariff Said,

No.	Students	Comments	verdict
1	DANIEL M. MUVENGEI TM406-3103/2015 PhD Epidemiology Utilization of a targeted mobile phone intervention in antenatal care and its effect on postnatal outcomes among pregnant women in a pastoralist community in narok county in kenya Prof Simon karanja skaranja@jkuat.ac.ke 0726424669 Dr Peter Wanzala - 0721624374 - wanzap2003@yahoo.com	2nd seminar Title-not clear about the pastoral community, name the community in question Slide are crowded and too wordy. Data collections- the tool is too shallow it needs to capture specific objectives. There is no analysis shown let be it clear. What is the intervention package in the intervention model. The tables are not labelled correctly. Use the appropriate titles. Results- you need to know the youngest age of your respondents. Need to relook at the discussions and results Conclusions and recommendations are not smart relook at them.	To refine and proceed to the next level
2	FRIDAH NJERU TM305/2765/2015 Msc Molecular Medicine Phenotypic, genotypic characterization and Antimicrobial susceptibility of actinomycetes from Sputum of tb smear negative patients from various Refferal facilities, KENYA Dr. Perpetual Ndungu (ndunguperpetual@gmail.com) +254 722 864455 Dr. Christine Bii (biichristinec@gmail.com)	2ND seminar The presentation was clear. Did you identify the origins of the people and how they got the bacteria Recommendations- bring it out clearly. Put observations and recommendations	To refine and proceed to the next level

Raphael Lana

Appendix VIII: plagiarism report

Plagiarism Detector v. 1991 - Originality Report 05/13/2022 12:57:35 PM

Analyzed document: Frida Draft 4 Thesis (2).docx Licensed to: Jomo Kenyatta University of Agriculture and Technology

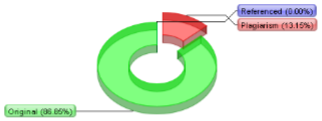
Comparison Preset: Word-to-Word Detected language: En

Check type: Internet Check

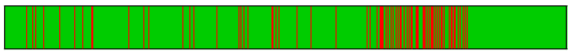
[tee_and_enc_string] [tee_and_enc_value]

Detailed document body analysis:

Relation chart:



Distribution graph:



Top sources of plagiarism: 191

7%	642	1. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/563971/ID_10i2.2.pdf
6%	784	2. https://shsfeapi1.pdc-gate2.com/get_doc.php?id=7851/MTAuMTAwMi85Nzg2NTI3NjcyMTY1.txt
6%	510	3. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4291361/

Processed resources details: 624 - Ok / 202 - Failed

Important notes:

Wikipedia:	Google Books:	Ghostwriting services:	Anti-cheating:
Wiki Detected!	[not detected]	[not detected]	[not detected]

UACE: UniCode Anti-Cheat Engine report:

1. Status: Analyzer [On] Normalizer [On] character similarity set to [100%]
2. Detected UniCode contamination percent: [0.1%] with limit of: 4%]
3. Document not normalized: percent not reached [5%]
4. All suspicious symbols will be marked in purple colorAbcd...
5. Invisible symbols found: [0]

Assessment recommendation:

No special action is required. Document is Ok.

[uace_abc_stats_header]

[uace_abc_stats_html_table]

Active References (Urls Extracted from the Document):

No URLs detected

Excluded Urls:

1. https://www.scirp.org/journal/paperinformation.aspx?paperid=94228
2. https://www.scirp.org/journal/paperinformation.aspx?paperid=94228
3. https://www.researchgate.net/publication/335036054_Characterization_and_Antimicrobial_Susceptibility_of_Actinomycetes_from_TB_Smear_Negative_and_Retreatr
4. https://www.semanticscholar.org/paper/Characterization-and-Antimicrobial-Susceptibility-Njeru-Ndung'u/723944fda9f96ae92f002bc63eea52bc69264847/figure/1

Included Urls:

No URLs detected