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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

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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 Actinomycete*s* 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

need for proper screening of TB smear-negative cases to detect infections by Actinomycetes and also conduct the antimicrobial susceptibility test to determine which antibiotic is effective.

Keywords

Actinomycetes, *Tuberculosis*, Antibiotics, Resistance, Kenya

1. Introduction

Actinomycetes are a group of aerobic and anaerobic bacteria belonging to the order Actinomycetales. Most of the aerobic Actinomycetes are Gram positive, filamentous, partially acid-fast, and relatively slow-growing branched bacteria. They have many microbiologic characteristics in common with members of the genera Mycobacterium. They have a worldwide distribution and they exist as saprophytes in the soil and other natural habitats. They can be transmitted through the wind in the dust and dirt into the lungs or skin and cause infections [1]. Several species of aerobic Actinomycetes that cause infections in human 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* [2].

Most of the Actinomycetes are opportunistic pathogens with the majority of infections occurring in patients with immunosuppressive conditions. However, a low percentage of immunocompetent patients also get infected. The Actinomycetes that affect the respiratory mostly display symptoms that mimic TB; hence in most cases, they are misdiagnosed or regarded as contaminant or commensal organism. Therefore, patients end up not being treated or being treated with the wrong medication. Missed or delayed treatment leads to increased morbidity and mortality and promotes drug resistance. The mandate of the National and International Tuberculosis (TB) Control Programs is the detection and management of pulmonary TB. However, patients with clinical and radiological evidence of pulmonary TB but are repeatedly sputum smear-negative is a common clinical dilemma. Therefore, understanding the significance of Actinomycetes in TB-smear negative and retreatment cases is essential for reducing the morbidity, mortality and drug resistance associated with a missed diagnosis.

Therefore, the aim of this study was to isolate and determine the antimicrobial susceptibility profiles of Actinomycetes from TB smear negative and retreatment cases referred to TB reference facilities in Kenya.

2. Materials and Methods

2.1. Study Site

The samples were collected from the TB reference facilities from TB patients who were considered retreatment, relapse or smear negative from different parts

of the Country. The Samples were analyzed at Kenya Medical Research Institute—Centre for Microbiology Research, Mycology Laboratory-Nairobi.

2.2. Sampling

Patients were purposively selected on criteria that they were retreatment cases, relapse and gene expert negative. Informed consent was obtained after which expectorated sputum was obtained from 385 assented patients.

2.3. Phenotypic Characterization of Actinomycetes

The sputum specimens were Gram stained and examined under X100 magnification for microscopic characteristics of Actinomycetes. The sputum samples were cultured on Mueller Hinton Agar and Sabouraud's dextrose agar (SDA) and incubated aerobically at 35˚C ambient air. The cultures were observed periodically for growth for four weeks. Suspected colonies were identified by their chalky, firm and leathery texture [3] suspicious colonies were sub-cultured on fresh Mueller Hinton Agar plates and incubated at 35˚C until sufficient growth was obtained. Pure colonies were then inoculated into 1.0 mL of 15% sterile glycerol stocking solution and stored at −80˚C for further analysis.

2.4. Biochemical Characterization

The suspected isolates with microscopic and cultural characteristics of Actinomycetes were subjected to the following biochemical tests; Casein hydrolysis, Simon citrate, urea hydrolysis and catalase test.

2.5. Genomic DNA Extraction and PCR Amplification

The stocked pure isolates were revived by culturing on Mueller Hinton agar plates and incubated at 35˚C for 72 hours. A loop full of a single colony was picked for use in the extraction of the total genomic DNA. The extraction process was based on the Qiagen DNA Extraction kit protocol. The extracted DNA was stored at −20˚C for further analysis. The DNA from each isolate was used as a template for amplification of the 16S rDNA gene which is a conserved gene in the bacteria [4]. The 16S rDNA sequence was amplified using the following group specific pair of primers; F-Act 243 (5'-GGATGAGCCCGCGGCCTA-3') and R-Act A3 (5'-CCAGCCCCACCTTCGAC-3') [5]. The following profile was used to carry out the Amplifications: 10 min at 95˚C and 35 cycles of 94˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min, 45 s, followed by 15 min at 72˚C. Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide and visualized under fluorescence light.

2.6. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done using broth microdilution (BMD) method according to the CLSI guidelines for rapidly growing mycobacteria and aerobic Actinomycetes [6].

2.6.1. Inoculum Preparation

The revived fresh cultures were used for antimicrobial susceptibility testing. To achieve an optical density of a 0.5 McFarland standard, pure colonies of the isolates were picked from a Mueller Hinton agar plate after 72 h, using a sterile loop, and transferred to 2 ml sterile water. The inoculum was then vortexed for 15 to 20 s for uniformity

2.6.2. 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. Briefly, Mueller Hinton broth was prepared and sterilized. Into sterile tubes, 4.9 ml of sterile Mueller Hinton broth was dispensed. Three milliliters of the antimicrobial agents were added to 3 ml of respective solvents (sterile water, DMSO, ethanol) and serially diluted to make a two-fold serial dilution. Into the 4.9 ml Mueller Hinton broth, 0.1 ml of the respective antimicrobial agents' dilutions was added to make a ten-fold dilution. Two hundred microliters of broth/antibiotics dilutions were then dispensed into wells of microtiter plates using a multichannel pipette. Ten microliters of the aforementioned inoculum were 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 re-incubated for a further 24 h and a final MIC reading was done after 72 hours. The MIC was defined as the lowest concentration of antimicrobial agent to inhibit visible growth.

2.7. Statistical Analysis

Statistical analysis was performed using a statistical package, R Windows version 3.5.2 by applying mean values using one-way ANOVA with post-hoc least square differences (LSD) method. A P value of less than 0.05 was considered significant.

3. Results

3.1. Demographic Parameters of the Patients

Out of 385 samples collected, 52 were positive for Actinomycetes by culture. The 52 cases were from different age groups with the youngest being 17 years and the oldest being 80 years old. Male were the most affected 33/52 (63%) and female were 19/52 (37%). Thirty nine (75%) of the cases had a history of TB treatment (either on treatment, finished treatment, retreatment) while 13 (25%) had no history of TB treatment. Table 1 below shows the cases based on age groups verses gender and history of treatment.

Table 1. Demographics of the actinomycetes positive cases.

Age	No. of cases	Gender/Clinical presentation				History of TB treatment	No history of TB treatment
		Male		Female			
		With History of TB treatment	No history of TB treatment	With History of TB treatment	No history of TB treatment		
Less than 20 yrs.	4	0	2	1	1	39 (75%)	13 (25%)
20 to 29 yrs.	16	6	2	5	3		
30 to 39 yrs.	11	8	1	2	0		
40 to 49 yrs.	11	7	0	4	0		
50 yrs. and above	10	4	3	2	1		
Total	**52**	**33 (63%)**		**19 (37%)**			

3.2. Isolated Actinomycetes

The different phenotypic features of the isolated Actinomycetes on Mueller Hinton Agar are shown in Figure 1. All the isolates were gram-positive characteristic of Actinomycetes. The microscopic characteristics of representative Actinomycetes on gram stain are shown in Figure 2.

3.3. Biochemical Test of Suspected Actinomycetes Isolates

All isolates showed the ability to utilize citrate as the sole carbon source and were also all catalase positive hence the ability to breakdown hydrogen peroxide to oxygen and water. Most of them were able to produce urease enzyme while only a few of them were able to hydrolyze casein. Figure 3 and Figure 4 show casein and urease test respectively.

3.4. Molecular Confirmation of the Isolates

Confirmation of the isolated Actinomycetes was done by Polymerase Chain Reaction. The DNA extraction was done then PCR. The Gel image of amplified DNA of the representative isolates is shown in Figure 5.

3.5. Antimicrobial Susceptibility Testing

Antimicrobial Susceptibility Testing was done using nine antimicrobial agents which included broad-spectrum cephalosporin, first line and second line antibiotics. Minimum Inhibition Concentration was determined using broth micro dilution method. The antibiotic susceptibility is shown in Table 2 and representative MICs are indicated in Table 3. Most of the isolates were susceptible to most antibiotics. However, four isolates showed multidrug resistance. Ceftriaxone

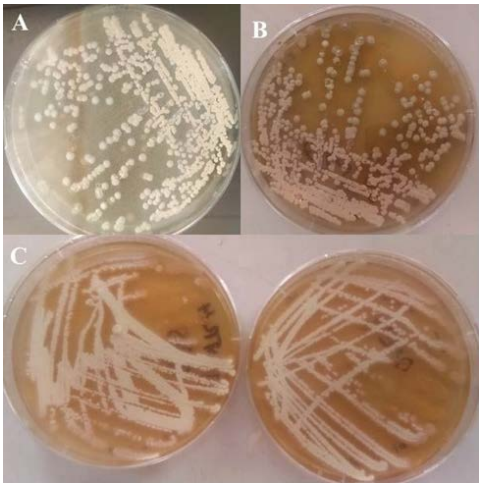

Figure 1. Culture. Typical colonies of Actinomycetes on Mueller Hinton agar. Plate (A) shows white wrinkled colonies while (B) and (C) show greyish whitish chalky colonies.

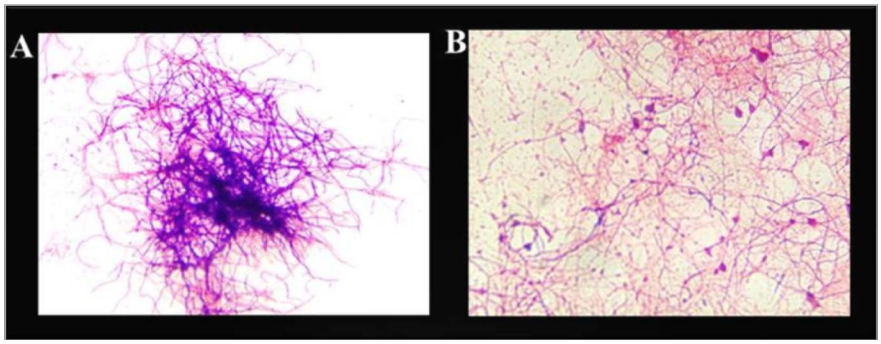

Figure 2. Gram Reactions of some of the Actinomycetes isolated. (A): Gram-positive intertwined filaments; (B): Gram-positive beaded branching filaments at power ×100 magnification.

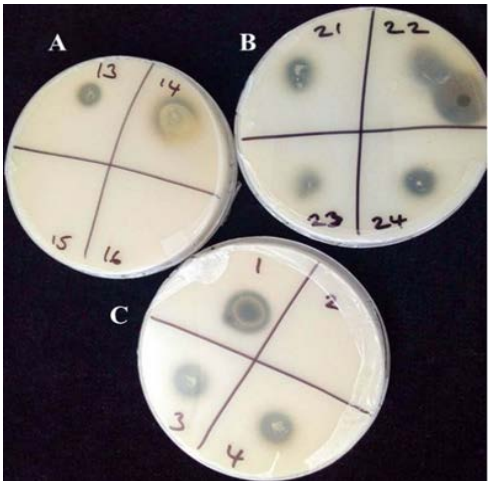

Figure 3. Casein test. Isolate 2, 15 and 16 are negative for casein hydrolysis while the rest are positive.

Figure 4. Urease test of some of the isolated Actinomycete showing positive urease test. The last one is a negative control.

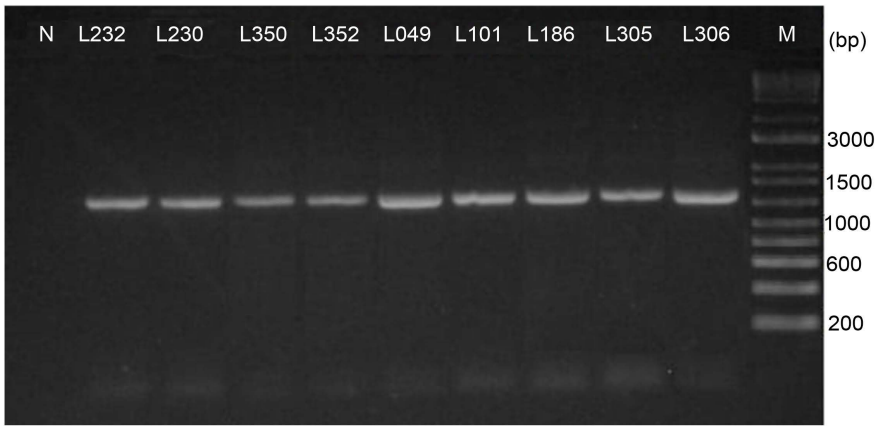

Figure 5. Representative gel image of amplified 16S rDNA of isolated Actinomycetes. N is the negative control, M is hyperladder 1 kb Molecular Marker.

Table 2. Antibiotic susceptibility of the isolated actinomycetes.

Interpretations	ANTIBIOTICS								
	CIP	SXT	LZD	AMK	AMC	CN	CLA	DOXY	CRO
Sensitive	98.1%	94.2%	84.6%	90.4%	78.8%	100%	71.2%	88.5%	61.5%
Intermediate	0%	0%	0%	0%	7.7%	0%	1.9%	11.5%	26.9%
Resistance	1.9%	5.8%	15.4%	9.6%	13.5%	0%	26.9%	0%	11.5%

Key: CIP-Ciprofloxacin, SXT-Co-trimoxazole (Trimethoprim-Sulfamethoxazole), LZD-Linezolid, AMK-Amikacin, AMC-Amoxicillin-Clavulanate, CN-Gentamycin, CLA-Clarithromycin, DOXY-Doxycycline, CRO-Ceftriaxone.

and Clarithromycin had the highest resistance; 11.5% and 26.9% respectively while Gentamycin and Ciprofloxacin showed the highest susceptibility; 100% and 98.1% respectively. Interpretation of MICs was done according to CLSI guidelines.

Table 3. The minimum inhibation concetrations of selected actinomycetes.

LAB NO.	ANTIBIOTIC PANEL TESTED AND MIC IN µG/ML								
	CIP	SXT	LZD	AMK	AMC	CN	CLA	DOXY	CRO
ATCC 25923	0.25S	9.5S	8S	4S	0.125S	1S	1S	0.5S	4S
L228	<0.062S	19S	4S	0.25S	<0.0625S	0.5S	<0.0625S	<0.0625S	4S
L232	0.125S	9.5S	8S	<0.0625S	16I	0.25S	1S	<0.0625S	8S
L224	0.25S	19S	2S	4S	0.125S	0.125S	0.125S	0.125S	4S
L222	0.5S	9.5S	2S	16R	4S	0.5S	32R	0.5S	32I
L171	0.125S	1.1875S	1S	1S	<0.0625S	0.25S	0.125S	0.25S	4S
L186	0.0625S	>76R	>32R	16R	32R	1S	>32R	0.5S	64R
L188	0.25S	>76R	2S	16R	>32R	2S	0.5S	0.5S	64R
L131	<0.0625S	9.5S	1S	0.25S	8S	<0.0625S	1S	<0.0625S	8S
L86	0.25S	9.5S	1S	<0.0625S	2S	<0.0625S	<0.0625S	<0.0625S	4S
L51	1S	2.375S	1S	0.25S	32R	<0.0625S	0.5S	1S	64R
L29	1S	19S	2S	1S	32R	0.125S	2S	0.0625S	64R
L230	>32R	>76R	1S	8S	32R	0.5S	32R	2I	16I
L241	0.25S	19S	2S	8S	16I	0.5S	32R	2I	16I
L235	0.125S	4.75S	1S	2S	1S	0.5S	0.25S	1S	4S
L238	<0.0625S	9.5S	>32R	0.5S	2S	0.25S	8R	2I	16I
L341	0.25S	2.375S	>32R	1S	1S	0.125S	0.25S	0.0625S	4S
L333	<0.0625S	4.75S	2S	16R	8S	0.5S	1S	0.25S	64R
L77	0.125S	19S	>32R	2S	4S	0.5S	8R	2I	8S
L314	0.25S	19S	>32R	4S	32R	0.5S	>32R	0.25S	16I

Key: S = Sensitive; I = Intermediate; R = Resistant.

4. Discussion

Actinomycetes are opportunistic microorganisms causing infections in immunocompromised patients particularly, human immunodeficiency virus (HIV)-infected individuals. Pulmonary infections caused by these organisms display symptoms that mimic *tuberculosis* 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.

Three hundred and eighty five samples were collected from patients of different age groups. Out of 385 samples collected, 52 (14%) of them had Actinomycetes. The youngest positive case was 17 years and the oldest being 80 years old. Male were the most affected gender with 33/52 (63%) and female were 19/52 (37%). Thirty nine (75%) of the cases had a history of TB treatment (either on treatment, finished treatment, retreatment) while 13 (25%) had no history of TB treatment.

Direct smears of all the samples were stained using gram stain. Out of these, 88 showed microscopic characteristics of Actinomycetes, *i.e*, gram-positive with filamentous branching structures which fragment into bacillary or coccoid forms [2].

These 88 suspects were further investigated by culture and biochemical tests and 52 were confirmed as Actinomycetes. All 52 grew well in aerobic conditions at 35˚C in SDA and a non-selective medium, MH agar. The colonies exhibited a typical hard to pick from the culture medium with few mucoid. Some exhibited white wrinkled colonies, or greyish whitish chalky colonies typical of Actinomycetes [7] [8] [9].

Actinomycetes have the ability to produce a variety of extracellular hydrolytic enzymes [10], hence, biochemical tests help in the identification of different Actinomycetes. Catalase test, citrate utilization, urease test and casein hydrolysis were the biochemical test done in this study. All the 52 isolates were catalase positive and were able to utilize citrate as the sole carbon source. Most of them were able to produce urease enzyme while only a few of them were able to hydrolyze casein. These characteristics agree with some of the characteristics described by some studies in literature [8].

Molecular analysis of Actinomycetes is very important as it helps to confirm their identification and to further their investigation. This analysis was done by extracting DNA from the isolates that had morphological and biochemical characteristics of Actinomycetes. The extracted DNA was then Amplified using specific primers for 16S rRNA. 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 yielding a band size of about 1300 base pairs.

Antimicrobial susceptibility testing of aerobic Actinomycetes isolated from clinical specimens is very important as it can help in guiding the therapy for infections. Actinomycetes are slow growing and most of them clumps and aggregates hence causing difficulty in carrying out the antimicrobial susceptibility testing [11] [12]. Broth microdilution method was used to perform the AST which is the approved standard method by CLSI for antimicrobial susceptibility testing of Actinomycetes [6]. Interpretation of the MICs was done according to CLSI guidelines.

This study determined the antimicrobial susceptibility patterns of 52 clinical isolates of aerobic Actinomycetes which were identified by phenotypic features.

Most of the isolates were susceptible to most antibiotics. Gentamycin showed the highest susceptibility (100%) followed by Ciprofloxacin (98%), Co-trimoxazole (Trimethoprim-Sulphamethoxazole) (94%) and Amikacin (90%). These findings are almost similar to those observed by Hashemi-Shahraki [13] on a study done on *Nocardia* species. A study done by Hamid [14] on *Streptomyces* species also showed over 90% susceptibility to Gentamycin. Another study by Silva [15] on *Rhodococcus* species also showed 100% susceptibility to Gentamycin.

Trimethoprim-sulphamethoxazole is the commonly used antibiotic for aero-

bic Actinomycetes [14] [16] [17] [18]. Our study showed that most of the isolates (94%) were susceptible to Trimethoprim-sulphamethoxazole which is in agreement with several other studies done on aerobic Actinomycetes [12] [19] [20]. However, resistance to Trimethoprim-sulphamethoxazole has been observed in different studies [11] [14] [15] [17] [18] [21] among others. This is on the contrary to the current study. The resistance could be as a result of overuse of this antibiotic.

On the other hand, most of the isolates were less susceptible to Doxycycline, Linezolid, Amoxicillin-clavulanate, Clarithromycin and Ceftriaxone. A study by Hashemi-Shahraki [13] on *Nocardia* species also showed less susceptibility to Ceftriaxone, which is consistent with the results of our study. However, a study by Hashemi-Shahraki *et al.* [13] showed high resistance to Amoxicillin-clavulanate and Ciprofloxacin which is on the contrary to our study. Another study done on *Gordonia* by Moser *et al.*, [21] showed susceptibility to Ceftriaxone, Clarithromycin, Linezolid, Amikacin, Amoxicillin-Clavulanate and Ciprofloxacin. Resistance to Ceftriaxone was observed on a study done on *Streptomyces* [22] which is on the contrary to the current study.

Resistance to three or more antibiotics was considered multidrug-resistant [11]. Therefore, 4 (7.6%) isolates showed multidrug resistance pattern, being resistance to more than 3 antibiotics; Amoxicillin-Clavulanate, Ceftriaxone, and Linezolid. Ceftriaxone and Clarithromycin had the highest resistance of 11.5% and 26.9% respectively.

All the antimicrobial agents studied in this study were effective in inhibiting the growth of most Actinomycetes tested. The antimicrobial activity of all the antibiotics tested against the isolates was statistically significant with P value less than 0.001 ($P < 0.001$).

5. Conclusion

The findings of this study show that there is a significant Actinomycotic infection in TB smear-negative and retreatment cases. This could be the reasons for persistent symptoms despite treatment. In most TB screening facilities, diagnosis of Actinomycetes is not done due to the lack of technical capability for diagnosis. Misdiagnosis and wrong treatment could be a significant factor in the high morbidity and mortality associated with TB management. Therefore, investigating all suspected pulmonary pathologies for potential Actinomycetes and other infection is recommended. Though most antibiotics tested in this study were susceptible, resistance to a few antibiotics was observed; hence, proper screening and antimicrobial susceptibility testing of Actinomycetes is very important for guiding therapeutic decisions.

6. Limitations of the Study

Due to financial constraints, full genome sequencing of the isolated Actinomycetes could not be done. This would have provided more information especially

on determining the specific species hence better understanding of the species diversity of Actinomycetes in TB-smear negative and retreatment cases.

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Conflicts of Interest

The authors do not have any conflict of interest.

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