ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF ALLIED HEALTH SCIENCE

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

SPECTRUM OF FUNGAL ETIOLOGIES IN SPUTUM SAMPLES FROM PULMONARY TUBERCULOSIS SUSPECTED PATIENTS

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A Thesis Submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

October, 2018

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by TAMAGN MULUNEH, entitled:

"Spectrum of fungal pathogens in sputum samples from pulmonary tuberculosis suspected patients at Kotebe primary hospital Addis Ababa Ethiopia" and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and public health microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

External Examiner_________________ Signature ___________Date ____________

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Acknowledgment

First of all I am so thank full for the almighty God who spare my life and help conduct this research. I would like to pass my heartfelt gratitude to Dr. Adane Bitew for his keen interest and motivating behavior which always inspire me to know and dig more in the realm of medical microbiology and it's really an invaluable privilege for me to work this research with one of our countries high caliber experts of medical mycology. I also want to thank my wife Tsion Menbere for her priceless support and inspirational encouragement throughout the process of this research, Mr. Wondwesen Tsegaye and my fellow classmates for supporting and commenting this research.

I would also thank department Medical Laboratory Science, College of Health Science and School of Allied Health Sciences of Addis Ababa University for giving me this chance to take part in such a scientific expedition. Last but not least I'm grateful for the study subjects participated in this study for their keen interest to take part in the study.

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Abbreviations

AAU	Addis Ababa University
AFB	Acid Fast Bacilli
AHB	Addis Ababa Health Bureau
BHI	Brain Heart Infusion Agar
CDC	Center for Disease Control and Prevention
CPA	Chronic Pulmonary Aspergillosis
HIV	Human Immune Deficiency Virus
KOH	potassium hydroxide
LPCB	Lactophenol cotton blue
MDR-TB	Multi Drug Resistant TB
MTB	Mycobacterium Tuberculosis
MTBC	Mycobacterium Tuberculosis Complex
NAA	Nucleic Acid Amplification
PCP	Pneumocystis Pneumonia
PTB	Pulmonary Tuberculosis
QC	Quality Control
RIF	Rifampicine
RTI	Respiratory Tract Infection
SDA	Sabouraud Dextrose Agar
TB	Tuberculosis
WHO	World Health organization

Abstract

Background: pulmonary mycosis is a systemic fungal infection that occurs when fungi cause's diseases of the lungs. *Mycobacterium tuberculosis* (MTB), the causative agent of pulmonary tuberculosis is one of the major micro organisms that infect this site. Clinical and radiological characteristics of pulmonary mycosis are very similar to that of pulmonary tuberculosis thereby making the disease easily misdiagnosed and mistreated as tuberculosis.

Objective: To assess spectrum of fungal etiologies in sputum samples from pulmonary tuberculosis suspected patients at Kotebe primary hospital Addis Ababa, Ethiopia.

Methods: A cross sectional descriptive study was conducted at Kotebe primary hospital on 423 sputum samples from presumptive TB patients. Sputum samples were collected aseptically and Presence of pulmonary Tuberculosis was screened by Xpert MTB-Rif/assay. Microscopically fungal elements were screened using 10%KOH. Cultural identification of the different pathogenic fungi was performed using SDA and BHI medium incubated at $25c^0$ and $37c^0$ for 4-6weeks respectively for each sputum samples. Identification was performed by noting; the growth form, and rate of growth, surface and reversed coloration on SDA and BHI agar plates. Yeasts were identified by conventional biochemical tests and assimilation characteristics. Data analysis was carried out using SPSS version 23 software.

Results: 423 presumptive TB patients were enrolled in this study of which 50.6% were males. MTB was detected in 7.3% of the patients. Mycotic agents were isolated from 179(42.3%) patients of which 106(59.2%) were males. Co- infection of pulmonary Tuberculosis and a fungal agent was seen in 51.6% of MTB positive subjects. 62.7% of HIV positive patients included showed positive fungal culture. Out of the total isolates in this study, the genuses *Aspergillus* and *Candida* were the predominant fungal agents 8.7% and 23.9% respectively. *C. cruzi* was the predominant fungi in MTB positive subjects. Other fungal etiologies isolated in our study include; *Penicillium*, *S. apiospermum*, *Fusarium, Mucor*, *Acremonium*, *Rhodotorulla, curvularia, Exserohilum species*

Conclusion; High incidence of fungal isolation (42.3%) was obtained in patients with respiratory symptoms. We recommend for policy makers to conduct further studies and consider the need of fungal screening in these patients

Keywords: Respiratory fungal infection, presumptive TB patients, HIV infection

1. Introduction

1.1 Background

Mycosis is a collective name of fungal infection of different tissues of animals including Humans. Depending on tissues involved it can be classified as superficial mycoses, cutaneous mycoses, subcutaneous and systemic mycosis (1). Respiratory mycosis is a systemic fungal infection that occurs when fungi cause diseases of the lungs through direct infection of the pulmonary tissues, by invading pulmonary airspaces/lung cavities, or through their ability to trigger an immunological reaction when fungal material is inhaled (2). It can be caused by either endemic or opportunistic fungi or a combination of both (3).

The opportunistic fungal pathogens include *Candida species, Aspergillus species., Penicillium marneffei, Pseudosporium apiospermum the Zygomycetes, Trichosporon beigelii, and Fusarium species., Cryptococcus neoformans.* The primary or endemic systemic fungal pathogens include *Coccidiodes immitis, Histoplasma capsulatum Blastomyces dermatitidis, and paracoccidiodes brasiliensis.* Most cases of primary mycoses are asymptomatic or clinically mild infections occurring in normal patients living or traveling in endemic areas. However, patients exposed to high inoculums of organisms or those with altered host defenses may suffer life-threatening progression or reactivation of latent foci of infection (4). Opportunistic respiratory mycoses have a cosmopolitan distribution. They comprise a large group of fungal diseases, the etiologic agents of which are usually potential pathogens in the immune-compromised or debilitated patients (5).

Mycobacterium tuberculosis (MTB), the causative agent of pulmonary tuberculosis is one of the major micro organisms that infect this site. It is a small aerobic non-motile bacillus which infects one third of the world's population. The proportion of people who become sick with tuberculosis each year is stable or falling worldwide but, because of population growth, the absolute number of new cases is still increasing (5).

Clinical and radiological characteristics of pulmonary mycosis are very similar to that of pulmonary tuberculosis thereby making the disease easily misdiagnosed and mistreated as

tuberculosis. Thus, patients may suffer from avoidable complications of unwarranted chemotherapy (3).

According to 2015 WHO report Ethiopia remains listed in the 22 high TB burden countries in the world with prevalence rate of 191 per 100,000 populations (6). As it is prominent by the absence of adequate publication of respiratory mycosis in relation to suspected TB patients in Ethiopia, one can say that it is a neglected disease which requires our attention. Therefore the objectives of this study are to determine the prevalence rate of fungal infections in suspected TB patients and demographic characteristics in relation to systemic mycoses. The study was conducted on patients visiting Kotebe primary Hospital, one of the busiest health facilities in Addis Abba.

1.2 Statement of the Problem

Global burden of RTI is responsible for one-third of infectious disease associated mortality, with an estimated 4.3 million annual deaths. Among these, fungal infections of the respiratory tract are largely unrecognized or misdiagnosed, and the true burden is difficult to describe (7). It has been reported that the estimated overall incidence of systemic fungal infection is up to 11.3% of which respiratory mycoses that involve the bronchi and lungs comprises 60% (8).

Fungal lung infections rose significantly over the last two decades, which is largely attributed to the extensive use of broad-spectrum antibiotics, long-term use of immunosuppressive agents, and the increasing population of terminally ill, debilitated and immunocompromised patients (2). Case mortality in pulmonary mycosis can be as high as 90% in immune-compromised patients though immune-competent patients generally respond well to antifungal therapy (3). Due to the lack of Specific clinical manifestations and imaging feature, the diagnosis of pulmonary mycosis is difficult and often easily misdiagnosed and mistreated which also contribute to associated high mortality rate (3, 8). In most developing countries like Ethiopia, the problem is further amplified by the preponderance of pulmonary tuberculosis and paucity of diagnostic mycology laboratories. Consequently, patients with respiratory mycoses may be misdiagnosed as tuberculosis, other bacterial diseases, flu-like infections, or any disease of obscure etiology (9).

The epidemiology of respiratory fungal infections in Ethiopia and many other developing countries has remained largely unexplored and neglected. This has led to a widespread biased impression about their true public health significance. Since it imposes major public health threat equally like other respiratory infections, this neglect needs to come to end and has to be seen as a major call for concern (5).

1.3 Significance of the Study

The results obtained in this study can be beneficial to different groups. First of all patient suffering from over masked respiratory mycoses may get the chance of early diagnosis and treatment who otherwise be subjected to the inevitable consequences of undiagnosed or misdiagnosed respiratory mycosis complication and unwanted usage of antibiotics. Clinician will also be alerted to suspect the disease in parallel with pulmonary tuberculosis. This study can also be used as a baseline data for epidemiological studies of respiratory fungal infection in the country, and may indicate the need of fungal diagnosis as part of routine RTI diagnosis.

2. Literature Review

The incidence of systemic fungal infections remains far beyond hoped and despite treatment, most invasive fungal infections are associated with high mortality rates of >50% (7,10). To this effect the incidence and associated risk factors of fungal agents in RTI are studied in different parts of the globe.

A meta-analysis of studies of pulmonary fungal infection by *Shamim et al* indicated that the incidence of fungal pneumonia can be as high as 12–56% in risk groups. *Candida*, *Aspergillus* or rarely *Zygomycetes* (*Mucorales*) are the main fungi isolated in respiratory secretion in critically ill patients. *Aspergillus* is the main fungus that we should bother as a cause of fungal pneumonia in these patients. This review also indicates that *Aspergillus flavus* or *Aspergillus terreus* and other filamentous fungi such as *Mucorales*, *Fusarium*, *Scedosporium* are also isolated in respiratory secretions of these patients. In fact they are by far less frequent than *Aspergillus fumigatus*. According to this review Isolation of *Candida* is by far common in respiratory secretions of up to 57% of bacterial pneumonia cases. *Candida albicans* is the frequent most species isolated (approximately 50%) followed by *C. parapsilosis*, *C. tropicalis* and *C. glabrata*(11).

According to a review by *Rosemary A,* Aspergillosis is most frequently associated with classically immunosuppressed patients undergoing transplantation or treatment for haematological malignancy where prolonged neutropenia remains a significant risk factor. However, it is now emerging as a potentially undiagnosed problem in much larger groups of critically ill patients, notably those with chronic respiratory disease or liver failure (12).

In Taiwan, pulmonary fungal infection of community-acquired origins is becoming a serious problem. Kuan-Yu and his colligues recommended that it should be taken into consideration for differential diagnosis of community-acquired pneumonia. This study conducted on a total of 140 patients indicated that Ninety-four cases of pulmonary fungal infection (67%) were community acquired. The most frequently encountered fungi were *Aspergillus species* (57%), followed by Cryptococcus *species* (21%) and *Candida species* (14%). There were 72 patients with acute invasive fungal infection, with a mortality rate of 67% (13).

A study in Belgium indicated that, a total number of 675 invasive aspergillosis cases and ≥169 deaths attributed to this infection were calculated in yearly bases. Chronic pulmonary aspergillosis is estimated to be prevalent in 662 cases. Allergic bronchopulmonary aspergillosis

cases were estimated to be 23,119 applying a 2.5% and 15% rate in adult asthma and cystic fibrosis patients respectively. Severe asthma with fungal sensitization cases was estimated to be 30,402 (14). Similar studies in Spain also showed that incidence of invasive aspergillosis is 2.75 cases per 100,000 and mucormycosis is 0.04 cases per 100 000 (15). A Danish study similarly found out estimates of invasive aspergillosis and chronic pulmonary aspergillosis with rates of 4.4 per 100 000 and 3.1 per 100 000 inhabitants, respectively (16).

The incidence of pulmonary mycosis among clinically suspected Tuberculosis patients have also been studied in different countries. A 2014 study in Sikkim, India indicated that out of 200 sputum samples from clinically suspected pulmonary tuberculosis cases, fourteen (7%) patients were positive only for AFB, while fungus as a primary etiological agent was detected in 16 (8%) patients. Fungus as a secondary etiological agent was detected in 4 (2%) of the patients (3). Similarly another study in Visakhapatnam, India on 100 sputum samples of MDR-TB suspects showed 62% infection with opportunistic fungi. Out of which 34 (54.8%) were yeasts and 28 (45.2%) were filamentous fungi (17). Even though this two studies have different figures of respiratory mycosis incidence due to the different methods utilized, both studies indicate that Pulmonary mycosis can be a primary infection in non- tuberculosis cases or co-infection in pulmonary tuberculosis cases. Investigation for fungal cause in clinically suspected cases of pulmonary tuberculosis will prevent misdiagnosis and mistreatment of cases.

Increasing presence of overlapping opportunistic fungal infections in tuberculosis patients were also recorded by different studies. A study done at Washim District, Central India found out the percentage of mycotic infections in 1357 pulmonary tuberculosis patients was 46%. Mainly four types of fungi, i.e. *Aspergillus niger, A. fumigatus, Histoplasma capsulatum* and *Cryptococcus neoformans* were recorded (18). A study done at Aligarh, India on 160 patients with known HIV positive status and having lower respiratory tract infection found out, 14 (8.7%) out of total 160 patients with lower respiratory tract involvement of fungal agents, which included pulmonary *Aspergillosis* in 4, *Candida pneumonia* in 4, pulmonary *Cryptococcus* in 3 and *pneumocystis jiroveci* pneumonia (PCP) in 3 patients. Another Indian study found out that prevalence of opportunistic fungal pneumonia to be *Candida species* 55%, Cryptococcus neoformans 4%, *Aspergillus species* 3% (19).

In Nigeria review of literatures indicated that, there were 78,032 cases of pulmonary TB in 2010. And 19,000 new cases of chronic Pulmonary Aspergillosis have been documented. The 5 year period prevalence of chronic pulmonary Aspergillosis is 60,377 cases (20). Similarly Tanzanian study revealed, Over 3% of Tanzanians suffer from serious fungal infections annually. There were 10,437 estimated post tuberculosis CPA cases, in 2012 (21).

Another study at Kano, Nigeria showed that, out of Two hundred sputum samples from patients with pulmonary symptoms collected and investigated for mycotic infections, one hundred and eleven samples (55.5%) were positive for the different kinds of yeasts and mycelial fungal pathogens (2). Another study by Yahaya, H et al in this place also found 111 (37%) yeasts mainly belonging to the genus c*andida* among 300 sputum samples suspected of pulmonary tuberculosis and from which only 28 (9.3%) were positive to AFB microscopy (22).
A study at Fako- division Cameroon on Respiratory Tract Aspergillosis in 200 Sputum sample of patients suspected of Tuberculosis revealed that, *Aspergillus species* were isolated from 30 (15%) patients, *A. fumigatus* was isolated in 10 (5%) patients while *A. niger*, A. *flavus*, and A. terreus were isolated from 9 (4.5%), 6 (3%) and 5 (2.5%) patients respectively. M. tuberculosis was found in 27 (13.5%) and a co-infection of 9 (4.5%) was documented. The study also recorded other fungal *species*; *Penicillium species* 3 (1.5%) and Histoplasma *species* 1 (0.5%) and yeasts, *Candida species* 55 (27.5%) Others included Cryptococcus *species* 6 (3%) and Torulopsis *species* 1 (0.5%) have also been isolated (5). similar study in Nigeria found that among the 200 sputum samples of patients with pulmonary disorders examined, eighty subjects (40.00%) were found to be positive for fungal pathogens of which 41 (51.25%) were positive with *Aspergillus species* where as 39 (48.75%) were found to be positive with *Candida species* (23). These two articles have similar figures concerning the two major respiratory fungal pathogens.

In Kenya co-infection of pulmonary fungal pathogens and Mycobacterium tuberculosis was identified in 76 (44.18%) of the 172 patients. Yeasts accounted for 46(26.7%), with 33(19%) being *Candida albicans*, 3(1.7%) were identified as *Candida dubliniensis,* 1(0.6%) was *Candida guilliermondii*, while 3(1.7%) were *Candida tropicalis. Cryptococcus laurentii* was isolated in 2(1.2%). Colonization of *Mycobacterium tuberculosis* with moulds was as follows: 2(1.2%) *Aspergillus flavus*, 3(1.7%) *Aspergillus fumigatus* 4(2.3%) *Aspergillus niger*, 2(1.2%)

Scytalidium hyalinum and 4(2.3%) *Trichosporon asahii. Pneumocystis jirovencii oocysts* were positive in 19(11.0%) (25).

Another prospective study at Imo state Nigeria indicated that, Out of the seventy three sputum sample from HIV positive individuals, 50 (68.5%) showed positive growth while twenty three (31.5%) did not show any growth. Males with opportunistic fungal infections had a lower frequency (40%) than females (60%). 40/50 (80%) of the grown organisms were *Candida* organism and 23/40 (57.5%) of them were *albicans*. *Candida albican* had the highest incidence 23/50 (46%) and seen more in the age bracket (25-34). *Cryptococcus neoformans* was isolated in 5/50 (10%) and *Aspergillus flavus and A. fumigatus* were isolated with incidences of 2.0% each (26).

A 2017 review of literature on fungal infections in Ethiopia shows that there is scarcity of evidences on this problem and existing ones focused mainly on superficial and mucosal mycosis while there is almost none on an invasive or systemic infection. The study revealed that the existing literature on any fungi in Ethiopia over a span of two decades revealed only 91 publications, out of which only 45 original articles were on human fungal infections. Out of the 45 publications, 17 were on superficial infections mainly on dermatophyte infections, 14 on mucosal candidiasis and candiduria, 9 on Cryptococcal meningitis, 4 on Pneumocystis pneumonia, and one on fungal keratitis. On the other hand, there was no study on invasive fungal infections such as blood stream infections with *Candida* or chronic pulmonary Aspergillosis (27).

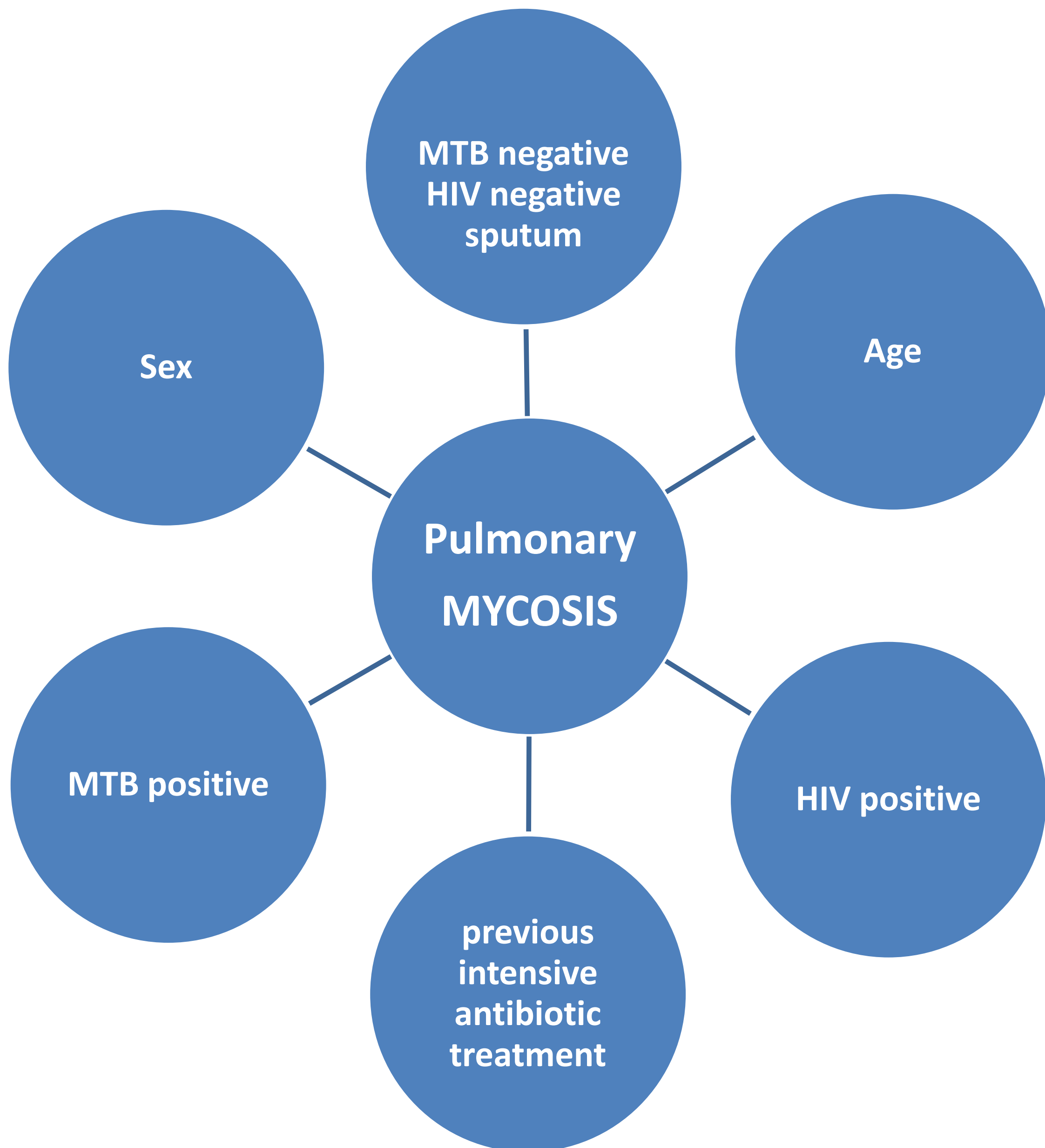


Figure 2: Diagram of conceptual frame work for this study Addis Ababa, Ethiopia June 2018

3. Objective

3.1 General Objective

To assess the rate of fungal etiologies in sputum samples from pulmonary tuberculosis suspected patients

3.2 Specific Objectives

- To determine the prevalence of mycelial fungal agents causing respiratory mycosis among pulmonary TB suspects.
- To determine the prevalence of yeasts causing respiratory mycosis among pulmonary TB suspects.
- To investigate co-infection of pulmonary tuberculosis and respiratory mycosis.

4. Hypothesis

There is no difference in the rate of fungal infection among PTB positive and negative patients

5. Methods and Materials

3.1 Study Area

The study was conducted in Addis Ababa at Kotebe primary hospital. Addis Ababa is the capital city of Ethiopia which has an estimated population of 3.6 million in the city proper and a metro population of more than 4.6 million (28). Kotebe primary hospital is one of the busiest health facilities in Addis Ababa with 155 health professionals giving service to more than 43,140 peoples dwelling around it. The facility has an average new outpatient turnover of 150-200 per day. The laboratory now acting as a referral laboratory for MTB screening for five health centers was awarded four star according the last assessment made by ENAO, the national accrediting organization. Even though there is no mycology laboratory in the facility, the facility has well organized TB clinic and TB laboratory with an average turnover of 10-15 new TB suspects per day.

5.2 Study Design and Period

A descriptive cross sectional study was conducted from March 2018 to August, 2018

5.3 Population

5.3.1 Source Population

The source populations were patients attending TB clinic of Kotebe primary hospital and its referring sites.

5.3.2 Study Population

Patients suspected of pulmonary tuberculosis and referred to the laboratory of Kotebe primary hospital for TB diagnosis and those who fulfill the inclusion criteria.

5.4 Inclusion and Exclusion Criteria

5.4.1 Inclusion Criteria

All sputum samples from patients clinically suspected of pulmonary Tuberculosis those who were sent to Kotebe primary hospital laboratory for Genexpert MTB Rif assay tests were included in the specified period of the study.

5.4.2 Exclusion Criteria

- Insufficient sputum samples
- sputum from patients lacking full clinical data was excluded
- Confirmed TB patients on follow up

5.5 Study Variables

5.5.1 Dependent Variables

- Prevalence of fungal etiologies on culture
- Prevalence of pulmonary Tuberculosis

5.5.2 Independent Variables

- Age
- Sex
- HIV sero status
- Previous anti TB treatment

5.6 Measurement and Data Collection

5.6.1 Sample Size Calculation

The sample size of the study was determined using a single population proportion formula. In Ethiopia we lack published studies on prevalence of respiratory mycosis. Assuming prevalence of respiratory mycosis is 50%, 95% CI and 5% margin of error and 10% for the non-response rate in determining my sample size for this study. Therefore the sample size is given as follows:

n= $(Z\alpha/2).2\mathbf{P}\ (\mathbf{1}-\mathbf{P})./\boldsymbol{d}\mathbf{2}$

Where:

n = the sample size

$(Z\alpha/2).2$ = at 95% confidence interval Z value ($\alpha = 0.05$). = 1.96

P = the proportion of occurrence of respiratory mycosis 50% (0.5).

d = margin of error at 5% (0.05).

n= (1.96).2 (0.5). (1-0.5). / (0.05).2 = 384

The minimum Sample size is therefore=384

10% non response rate= (422). (10). /100= 38.4

The Sample size is therefore = 422+39=423

The Sample size is therefore is **423**

5.6.2 Sampling Method:

Convenient sampling method was utilized to achieve the estimated sample size. All pulmonary tuberculosis suspected patients visiting Kotebe primary hospital laboratory within the specified time of the study

5.6.3 Data Collection Procedure

5.6.3.1 Demographic data

As soon as ethical approval from AAU and AHB was issued data collection was started. Age, sex and HIV sero status of each study subject was obtained from laboratory request form brought by the patient. Data was collected with professional laboratory technician. Before the actual data collection, a pre-test on calculated sample size for pre test was conducted using demographic and clinical data collection formats and log books

5.6.3.2 Sample collection and processing

Two sputum samples were collected for TB diagnosis and mycological analysis with the assistance of experienced Medical laboratory scientists. Sputum samples were expectorated from lower respiratory tract and collected in sterile screw capped containers to avoid contamination from external sources in the following order as described by Brooks *et al (*23).

Collection Procedure

Patients were asked to produce the samples in an open air space away from other people to avoid aerosol spread. The patients were instructed to inhale deeply 3 to 4 times before coughing out from the chest. The sputum produced was carefully spit into the container without contaminating the outside of the container. The lid of the container was screwed tightly before being processed, with utmost care not wrapping the container with the laboratory request form.

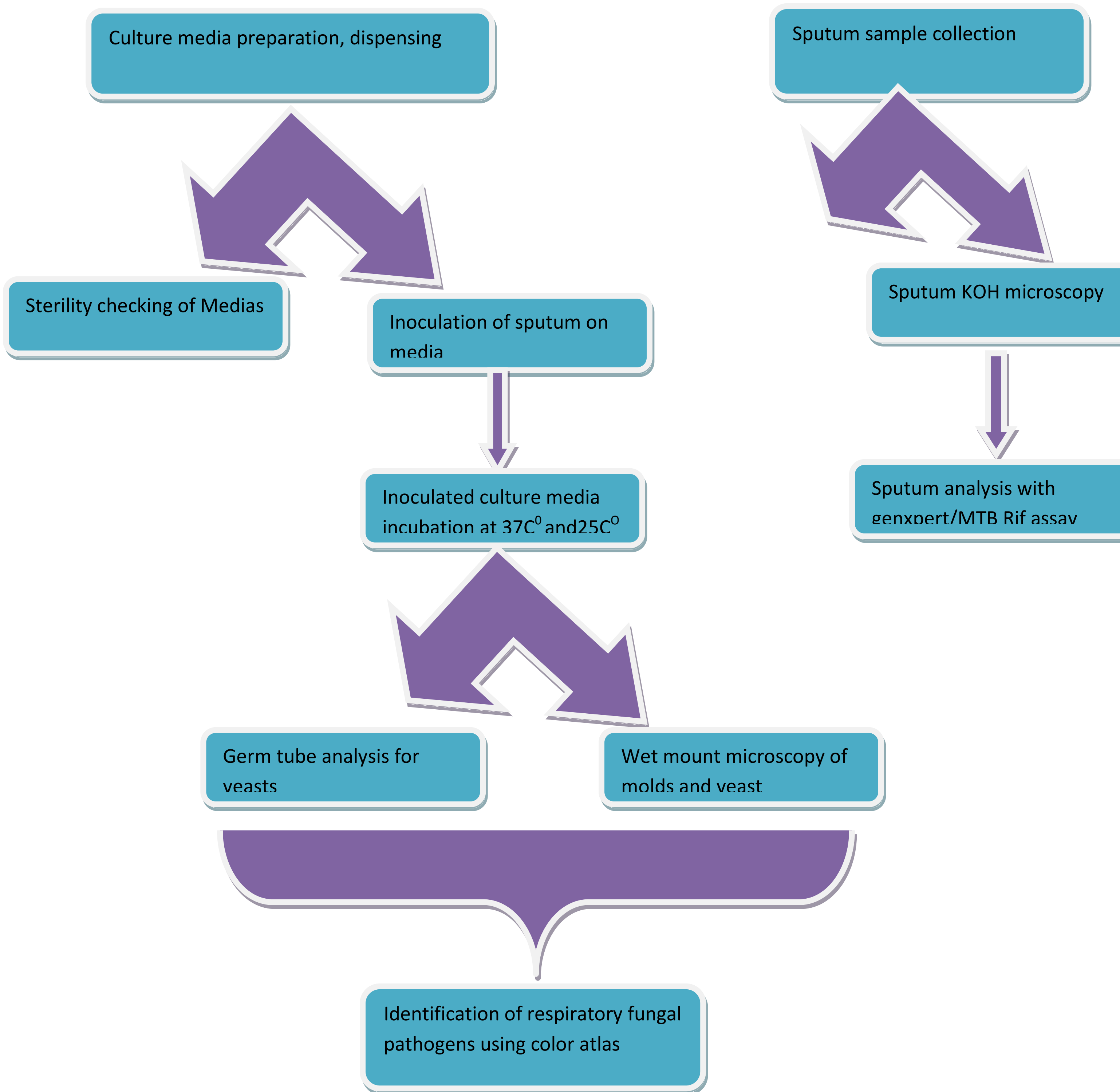
Sample transportation; sputum sample for mycological analysis was transported to mycology laboratory of Addis Ababa university school of Laboratory science within 6 hours of collection by following the appropriate sputum sample transportation method (**29).**

5.6.4 Laboratory Analysis

5.6.4.1 MTB Screening Using Genxpert

Presence of pulmonary Tuberculosis was screened on the same day of sample collection using the country's current guideline, GeneXpert MTB-Rif/assay. GeneXpert MTB/RIF assay is a rapid diagnosis test of Tuberculosis (TB) and drug resistance. It is based on a principle of nucleic acid amplification (NAA) test which simultaneously detects DNA of Mycobacterium tuberculosis complex (MTBC) and resistance to Rifampicin (RIF). (i.e. mutation of the rpoB gene) in less than 2 hours. The primers in the XpertMTB/RIF assay amplify a portion of the rpoB gene containing the 81 base pair "core" region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with Rifampicin resistance. The Centers for Disease Control and Prevention (CDC) recommends that NAA testing be performed on at least one respiratory specimen from patients who have a moderate or high suspicion of having pulmonary TB (30).

Figure 2. Diagram of work flow



4.6.4.2 Mycological Analysis

Direct microscopy:

With the use of sterile plastic pipette few drops of 10% potassium hydroxide was placed on the centre of a clean glass slide and using a sterile wire loop this was mixed with a portion of the sputum. The preparation was flattened under a cover slip and examined with magnification x40 objective for the presence of hyphal fragments and yeast cells.

Principle: The principle of this test is base on that the alkali is to digest the keratin surrounding the fungi so that the hyphae and conidia (spores) can be seen (29).

Methylin Blue Preparation

A large drop of methylin blue was placed on a clean grease free glass slide with the help of Pasteur pipette. A small quantity of the sample was picked up with the tip of wire loop and stirred gently. In case of a colony, small portion was teased with sterile teasing needles and spread uniformly onto the glass slide. The set up was cover sliped gently in such a way that air bubble is avoided, and microscopy follows. Methylene blue used long with KOH imparts colored background and the fungal elements when present shows prominent refractive objects (WHO, 2009) (31).

Culture:

Irrespective of the outcome of the direct sputum microscopy, given that the full characterization of mycotic agents is achieved through culture all samples were cultured by streaking on two plates of Sabouraud's dextrose agar (SDA) and brain heart infusion agar (BHI) with chloramphenicol which was prepared according to the manufacture's instruction. All inoculated plates of SDA and BHI were incubated at inverted position for 4-6 weeks at 25^0C and 37^0C aerobically respectively. Incubated plates were examined every three days for any fungal growth.

Principle: BHI: This medium contains brain heart infusion, peptone, and dextrose which provide nitrogen, carbon, sulfur, vitamins, and carbohydrate source. Chloramphenicole is a broad spectrum antibiotic against a range of gram positive and gram negative organisms. The SDA media is comprised of enzymatic digest of casein and animal tissues which provide a nutritious

source of amino acids and nitrogenous compounds for the growth of fungi and yeasts. Dextrose is the fermentable carbohydrate incorporated in high concentration as a carbon and energy source. Agar is the solidifying agent. Addition of antibiotics like Chloramphenicol acts as broad spectrum antimicrobials to inhibit the growth of a wide range of gram-positive and gram-negative bacteria (32).

Cultural Identification: After appropriate incubation, the growth form, and rate of growth-surface and reversed coloration on SDA and BHI agar plates was noted. Pure isolates were obtained by sub-culturing on new plates and colonies growing out of the inoculation area were regarded as contaminants.

Microscopy: The different fungal pathogens were identified with the various morphological features associated with the characteristic sporing head according to **Collier *et al* (33).** *A* Color Atlas of Pathogenic Fungi was used for microscopic and macroscopic identification of fungal isolates. Yeasts were identified by conventional biochemical tests and assimilation characteristics. In addition, Yeasts will also be identified by their smell, pseudomycellia and budding cells. Further investigation include **Chromagar for identification of *candida species***

5.7 Quality assurance

5.7.1 Data Quality Assurance

The quality of sputum samples and appropriate labeling of specimens was checked before analysis. Expiration date of 10% KOH solution, Genexpert kit, SDA, chromagar and BHI Medias were inspected; internal inbuilt QC run and daily preventive maintenance procedure for genexpert analyzer was also inspected prior to testing. Prepared SDA, chromagar and BHI Medias were checked for sterility by incubating at 25-30^{0}C for weeks. Data was cleaned and checked for completion before analysis and pre-test was done before regular data collection start.

5.7.2 Quality Control

5.7.2.1 Pre- analytical

A sterile sputum collection cup was issued to patients. Patients are asked to produce sputum from deep by deep breathing three times and coughing so as to expectorate purulent sputum. Collected sputum samples were inspected for eligibility. Reagents used for Xpert MTB/RIF assay, SDA,

chromagar BHI media preparation and KOH was checked for expiry date and any abnormal color change. Preventive maintenance of equipments was inspected.

5.7.2.2 Analytical:

- Test procedures for each test were strictly followed according to standard operation system of the tests.
- Media preparation was performed according to manufacturers' manual
- Prepared SDA, chromagar and BHI Medias were checked for growth support by incubating at 25-30^{0}C for weeks
- Sterility of each batch of prepared media was checked by incubating uninoculated media at 25-30^{0}C for weeks.

5.7.2.3Post analytical

- Results generated were appropriately documented electronically
- To prevent loss of data backup data was also documented on result registration log book.
- Appropriate disposal system by incineration was utilized after disinfection of Specimens and cultures of organisms by autoclaving.

5.8 Data Analysis and Interpretation:

Descriptive analysis was utilized for this study. Socio-demographic characteristics of study subjects, clinical manifestations and laboratory results was compiled and entered into SPSS version 23

5.9Ethical Consideration

This study was conducted with the ethical approval of the Addis Ababa University, AHB and the authorites of Kotebe primary hospital. Personal information about the participants was treated confidentially. Each participant was informed briefly about the study, outcomes and confidentiality. Those showing interest to be included and willing to give consent were only included in the study. Information sheet, consent and assent forms were utilized for this agreement. Patients with positive 10% KOH fungal microscopy were informed with their respective physicians and dealt accordingly.

5.10 Dissemination of Results:

The final outcome of the study will be submitted and presented to Department of Medical Laboratory Science, School of Allied Health Science and College of Health Sciences of Addis Ababa University. The result will also be reported to the host of the study, Kotebe primary hospital and also to AHB. And finally the study will be sent to some medical journals for publication purpose.

5.11 Operational Definition

Presumptive TB patient; Tuberculosis suspected patient. (34)

Co-infection: The presence of more than two infections on the same host. (4)

Opportunistic fungi: Fungi that cause disease in immune compromised host. (1)

Endemic fungi: Fungi that cause disease in any host regardless of its immune status. (1)

6 Results

6.1 Demographic Analysis

A total of four hundred twenty three (N=423) study participants were enrolled in the present study, of which 214(50.6%) were males and 209(49.4%) were females as shown in table 1. The ages of the study subjects ranged from 7 year to 80 year with a mean age of 35 years. Approximately, about 50% of the participants were between the age brackets of (25-44). There were 67 HIV positive subjects and 19 patients who were previously treated with anti TB drugs.

Age Group		Sex		Total
		F	M	
	0-14	7	11	18
	15-24	43	37	80
	25-44	98	111	209
	45-64	52	46	98
	>64	9	9	18
Total		209	214	423

PTB Pulmonary TB Age = WHO age classification for health, 2007.

Table 1: Age and sex distribution of PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa, Ethiopia, 2018 (n=423)

6.2 Detection and Isolation Rates of MTB and Fungi in sputum

6.2.1 MTB detection

A total of 423 fresh sputum samples were collected and examined for the presence of mycotic agents and *M. tuberculosis*, out of which 31(7.3%) patients were positive for *M.tuberculosis* by genexpert MTB Rif assay. As indicated on table 2, male subjects presented more positive samples as compared to their female counterparts 20(64.5%) and 11(35.5%) respectively.

Sex	Genxpert MTB Rif assay result		Total
	Negative	Positive	
F	198	11	209
	94.7%	5.3%	100.0%
M	194	20	214
	90.7%	9.3%	100.0%
Total	392	31	423
	92.7%	7.3%	100.0%

Table 2: Occurrence of MTB based on Sex among PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa,Ethiopia,2018,(n=423)

6.2.2 Fungal Pathogens Isolated on culture

Eleven different fungal species were isolated from 179(42.3%) patients among of which 106(59.2%) were males and 73(40.8%) were female subjects as shown on Table 3. Here again there are more males than females in the positives subjects showing statistically significant association between gender and fungal isolation from sputum χ^2 (1, N = 423) = 9.239, p = .002). Among of the 423 patients; culture of 18 subjects presented more than one fungal isolate. Out of the 179 study subjects whom sputum was positive for fungal culture, fungal species were detected by KOH direct microscopy in 132(73.7%) of the sputum samples.

Table3: Distribution of fungi in relation to sex among fungal culture positive PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa,Ethiopia,2018,

Isolate	Female (%)	Male (%)	Total
Aspergillus flavus	1(50.0%)	1(50.0%)	2(0.5%)
Aspergillus fumigatus	5(20.8%)	19(79.2%	24(5.7%)
Aspergillus niger	5(45.5%)	6(54.5%)	11(2.6%)
Acremonium species	1(50.0%)	1(50.0%)	2(0.5%)
Airobasidium pullulans	1(100.0%)	0(0.0%)	1(0.2%)
Alternaria species	0(0.0%)	1(100.0%)	1(0.2%)
Candida.albicans	18(39.1%)	28(60.9%)	46(10.9%
Candida.cruzi	13(39.2%)	21(61.8%)	34(8%)
Candida.parapsilosis	1(50.0%)	1(50.0%)	2(0.5%)
Candida.tropicalis	7(36.8%)	12(63.2%)	19(4.5%)
Curvularia	1(100.0%)	0(0.0%)	1(0.2%)
Exserohilum	1(100.0%)	0(0.0%)	1(0.2%)
Fusarium species	4(40.0%)	6(60.0%)	10(2.4%),
Mucor species	3(50.0%)	3(50.0%)	6(1.4%)
Paecyclomycea	0(0.0%)	1(100.0%)	1(0.2%)
Penicillium species	11(50.0%)	11(50.0%)	22(5.2%)
Rhodotorulla species	1(33.3%)	2(66.7%)	3(0.7%)
Scedosporium apiospermum	6(60.0%)	4(40.0%)	10(2.4%),
Total	79(40.1%)	118(59.9)	196

Many of the positive sputum fungal cultures were dominated by the genus *Aspergillus* and *Candida*. *A. fumigatus* was isolated in 24 (5.7%) of the patients. *A. niger* and *A.flavus* was isolated in 11(2.6%) and 2(0.5%) patients respectively. Other fungal agents isolated include in descending order; *C.albicans*, *C.cruzi*, *Penicillium species*, *Penicillium species* *Candida.tropicalis*, *Scedosporium apiospurmum*, *Fusarium species*, *Mucor species*, *Acremonium species*, C.*parapsilosis*. The following groups of fungal isolates has a prevalence of 1(0.2%), *Airobasidium pullulans*, *Alternaria species*, *Curvularia* species, *Exserohilum species*.

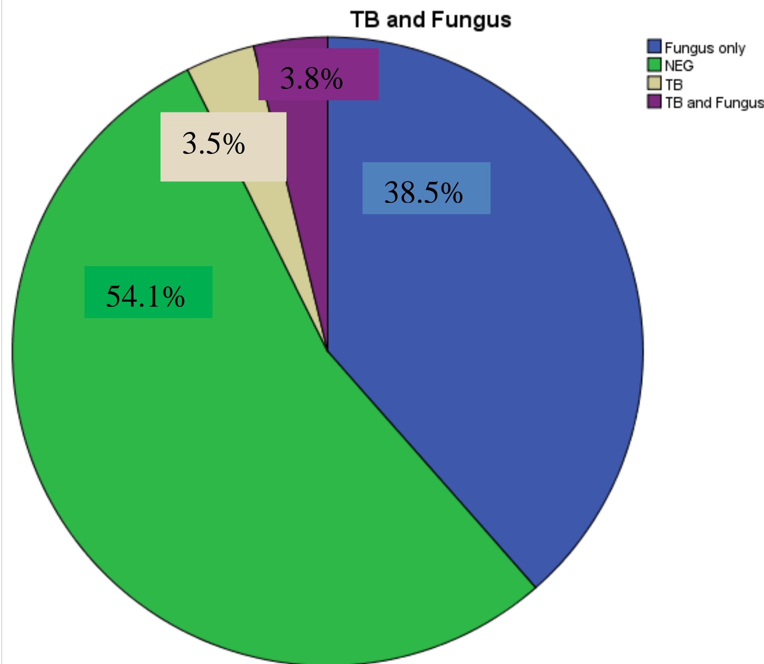
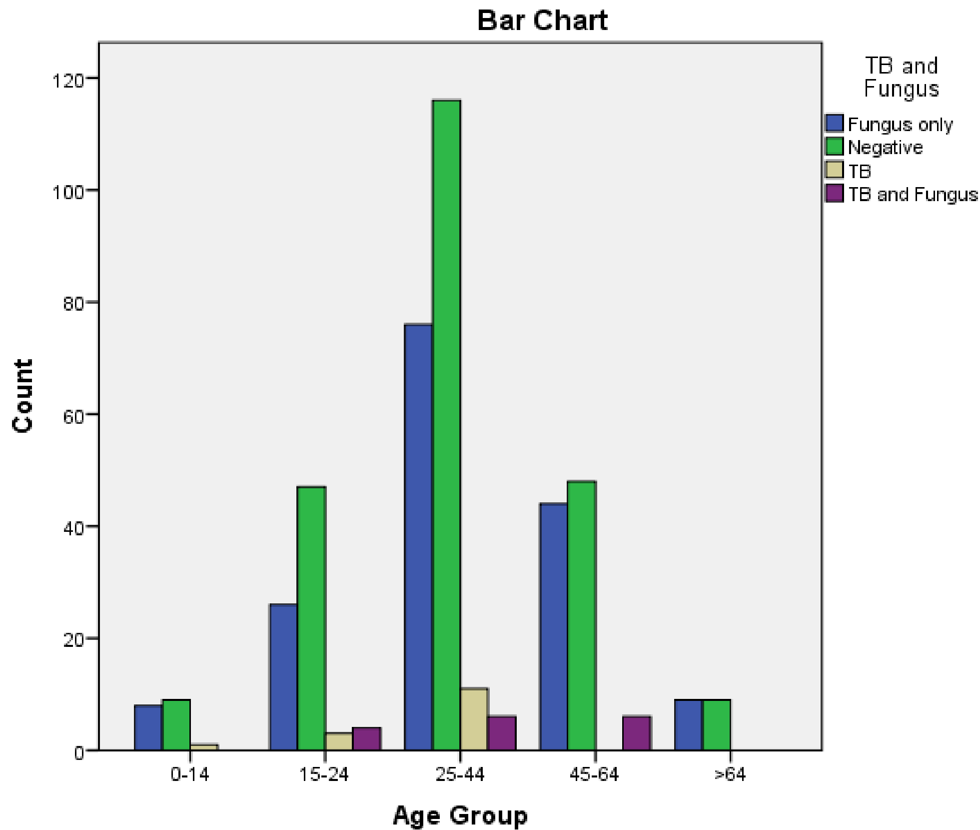


Figure3: Composition of etiologic agents MTB and fungal pathogens among patients suspected of pulmonary Tuberculosis at Kotebe primary hospital, Addis Ababa, Ethiopia, 2018, (n=423).

The above pie chart (figure2) illustrates the proportions of MTB, fungal agents and negative sputum samples with respect to each other. More than half of the studied subjects (55.9%) were positive either for MTB or fungal or both. Fungal positivity by sputum fungal culture was considerably higher than MTB detection by Genxpert MTB Rif assay. *M. tuberculosis* was detected in 31(7.3%) patients of whom approximately half of them were still showed positive fungal culture.

6.2.2 Fungal Pathogens Isolated In Relation To Age

Fungal agents were isolated from the sputum of patients in all age group, the age bracket of (25-44) years of age was the one containing the highest incidence of fungal isolates as indicated on figure 3 and followed by (45-64) age group, (15-24) age group, (>64) age group.



Age = WHO age classification for health, 2007

Figure4 Composition of etiologic agents MTB and fungal pathogens with respect to age among patients suspected of pulmonary Tuberculosis at Kotebe primary hospital, Addis Ababa, Ethiopia, 2018, (n=423).

As indicated on table 4, *C.cruzi* is the predominant isolate in the (0-14) and (15-24) age groups. While *C. albicans* is the predominant one in the (25-44) and (>64) age groups. *A.fumigatus* along with *C.albicans* were the predominant fungi in the age groups (45-64)

Table 4: Distribution of fungi in relation to different age groups among fungal culture positive PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa,Ethiopia, 2018,

Isolate	**Age group**					**Total**
	0-14	15-24	25-44	45-64	>64	
Aspergillus flavus	0	0	1	1	0	2
Aspergillus fumigates	1	1	10	10	2	24
Aspergillus niger	2	1	3	4	1	11
Acremonium species	0	0	0	2	0	2
Airobasidium pullulans	0	0	1	0	0	1
Alternaria species	0	0	1	0	0	1
Candida.albicans	0	6	27	10	3	46
Candida.cruzi	3	6	14	10	1	34
Candida.parapsilosis	0	0	0	2	0	2
Candida.tropicalis	0	4	10	5	0	19
Curvularia	0	1	0	0	0	1
Exserohilum	0	0	0	0	1	1
Fusarium species	0	4	5	0	1	10
Mucor species	1	1	2	2	0	6
Paecyclomycea	0	1	0	0	0	1
Penicillium species	1	3	11	7	0	22
Rhodotorulla species	0	0	2	1	0	3
Scedosporium apiospermum	0	5	1	4	0	10
Total	8	33	88	58	9	196
%	4.1%	16.8%	44.9%	29.6%	4.6%	100%

PTB Pulmonary TB Age = WHO age classification for health, 2007.

6.2.3 Fungal Pathogens Isolated With Respect To Risk Factors

Fungal and TB co – infection was observed in 16(3.8%) patients of whom 12(75%) patients were males. C.*cruzi* was the predominant fungi in MTB positive group, 8(50%). The rest fungal agents co detected with MTB were; *A.fumigatus*, A.*niger*, C.*albicans*, C.*tropicalis*.

6.2.3.1 HIV and fungal infection

There were 67 HIV positive patients included in this study as shown by table 5 below, among these subjects, fungal infection was observed in 42(62.7%) of the patients. This finding shows HIV and fungal infection have significant association $\chi 2$ (1, N = 423) = 13.53, p = .0023).

Table 5 Sputum fungal culture in relation to HIV sero status among PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa, Ethiopia, 2018, (n=423)

Sputum culture		**HIVsero status**		**Total**
		Negative	**Positive**	
Negative		219	25	244
		63.9%	4.5%	57.7%
Positive		137	42	179
		36.1%	95.5%	42.3%
Total		356	67	423
		100.0%	100.0%	100.0%

As the distribution fungal agents in HIV positive patients is illustrated on table 6, *C.albicans* shows the highest incidence in HIV positive patients 16(36.4%) followed by *A.fumigatus* and *Fusarium species* with same incidence of 6(13.6%)

Table 6 Distribution of fungal pathogens in HIV positive subjects among PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa,Ethiopia,2018,

Isolate		**Frequency**
	A.fumigatus	6(14.3%)
	A.*niger*	2(4.8%)
	C.*albicans*	16(38.1%)
	C.*cruzi*	3(7.1%)
	C.*parapsilosis*	1(2.4%)
	C.*tropicalis*	4(9.5%)
	Fusarium species	6(14.3)
	Penicillium species	5(11.9)
	Scedosporium apiospermum *Rhodotorulla species*	2(4.8%) 1(2.4%)
Total		**42**

6.2.3.2 Previous anti TB treatment and fungal infection

As indicated on table 5, fungal pathogens in relation to patients with previous anti TB treatment was also noted. Among 19 previously treated patients by anti TB drugs, 16(84.2%) patients showed positive fungal sputum culture. this shows statistically significant association χ^2 (1, N = 423) = 14.3, p = .002) between fungal infection and previous intensive antibiotic therapy and TB infection.

Table 7 Sputum fungal culture in relation to previous anti TB treatment among PTB suspected patients, at Kotebe primary Hospital laboratory, Addis Ababa,Ethiopia, 2018,(n=423)

Sputum culture		**previous anti TB treatment**		**Total**
		No	**Yes**	
	Negative	241	3	244
		59.7%	15.8%	57.7%
	Positive	163	16	179
		40.3%	84.2%	42.3%
Total		404	19	423
		100.0%	100.0%	100.0%

7. Discussion

The main objective of the present study was to determine the prevalence of mycotic agents in the sputum sample of presumptive pulmonary TB patients.

Out of 423 patients participated in this study, 214(50.6%) were males and 209(49.4%) were females this agrees with a study conducted in Ethiopia by Awoke et al where, 52.3% of the participants were males (35). And the mean age in our study was 35 years old similar to a retrospective study in Addis Ababa Ethiopia by Boja et al; in which the mean age was 35.1 years old (36).

In the present study of the 423 study subjects from which sputum samples were collected, MTB was detected in 31(7.3%) patients. There was 1 Rifampicine resistant TB and 1 rifampicine indeterminate TB. This is consistent with finding of a study done in India by Tshering et al in which they found MTB in (7%) of the patients and closer to a study done in Cameroon by Anna et al which was 13.5% (3,5). MTB positivity rate of our study is slightly lower than studies conducted in different part of the Ethiopia, 14.6% Northwest Ethiopia, and 43.6% in Addis Ababa. (u34, 35) MTB positivity rate of our study among gender is 20(64.5%) male and 11(35.5%) female patients. This agrees with the findings of 2007 WHO Report (36), which put the males as more prone to TB infection than females. This might be attributed to more exposure of males to external environment than females. This also agrees with the findings of a study done in Gondar, Ethiopia by Kefyalew et al where (58%) of the positive patients were males and (42%) were females (38).

Mycotic agents were isolated from 179(42.3%) patients. Similar study was conducted in India and Nigeria and found out 10% and 55.00% of the sputum samples from patients suspected of pulmonary Tuberculosis were positive for fungal culture respectively. (3, 2) In our study the distribution of positive sputum fungal culture with respect to gender indicates that 106(59.2%) were males and 73(40.8%) were female subjects showing significant association between gender and fungal isolation χ^2 (1, N = 423) = 9.239, p = .002). This finding is in agreement with the findings of Yahaya et al in Nigeria who observed that fungal infection was higher in males compared to females as men are more vulnerable to fungal infections than females due to their great exposure to the surrounding (20). This finding disagrees with the findings of Taura et al a

study in Negeria and Anna et al in Cameroon (5, 2) in which they concluded that sex is independent of the distribution of the fungal infections.

In our current study, positivity rate of sputum fungal culture was highest in the age group of 25-44 by 82(19.4%) and lowest in the age group of 0-14 by 8(1.9%) This is consistent with the findings of Anna et al in Cameroon and Yahaya et al in Nigeria. And disagree with the findings of Tshering et al in which the study indicated that pulmonary mycosis is more common in patients of more than 70 years of age compared to patients of less than 70 years of age. (3, 5, 2)

Out of the total isolates in our study the genus *Aspergillus and Candida* were the predominant fungal agents 8.7% and 23.9% respectively. This finding is in agreement with the results of Taura et al in Nigeria who found out 36.9% *Aspergillus* species and 36.04% *Candida* species in their study (2). A study in china by Bai-ling et al also depicted that *Aspergillus* is the main pathogen of pulmonary mycosis. (8). The pattern and isolation rate of mycotic agents obtained in the present study was comparable with those of earlier two studies in Nigeria, Other fungal pathogens isolated in our study include in descending order of frequency; *Penicillium species* 5.2%, *Scedosporium apiospermum* and *Fusarium species* both with equal frequency 2.4%, *Mucor species* (1.4%), *Acremonium species* 2(0.5%), *Rhodotorulla species* (0.7%) The following groups of fungal isolates has a prevalence of 1(0.2%), *Airobasidium pullulans*, *Alternaria species*, *Curvularia species*, *Exserohilum species*. (2, 20)

Fungi those rarely found in sputum of immunocompetent patients have also been isolated in our current study. This includes *Scedosporium apiospermum* and *Fusarium species* both with equal frequency 2.4%, *Rhodotorulla species* (0.7%) Most of these fungi were isolated from HIV positive patients included in the study. Unusual fungi for this site like; *Airobasidium pullulans*, *Alternaria species*, *Curvularia* species, *Exserohilum species* were also isolated in our study. Even though their number is minimal, it needs further exploration.

There were 67 HIV positive patients participated in this study. Among these subjects, Fungal infection was observed in 42(62.7%) of the patients showing statistically significant association χ^2 (1, N = 423) = 13.53, p = .0023). *C.albicans* shows the highest incidence in HIV positive patients 16(36.4%) followed by *A.fumigatus* and *Fusarium species* with same incidence of 6(13.6%), *Penicillium species* 5(11.9%) and *Rhodotorulla species* 1(2.4%) This finding is in agreement with the results of Nwako a study in Nigeria and Andrew in USA where they both indicated that

majority of fungal infections in immune-compromised patients are related to either *Candida* infections or *Aspergillus*(26,39).

There were 19 patients included in our current study those previously treated with anti TB drugs. And 16(84.2%) of this subject's sputum showed positive fungal growth showing significant association χ^2 (1, N = 423) = 14.3, p = .002) between fungal infection and previous intensive antibiotic therapy and TB infection. This result is in agreement with the finding of Taura et al in which they found that fungal infection among antibiotic users is 90.99 %(2).

Among 31 patients positive for pulmonary Tuberculosis a Co- infection of pulmonary Tuberculosis and fungal agents was seen in 16(51.6%) subjects. Higher incidence of fungal co-infection was recorded in the MTB positive group. This finding is consistent with results of Elizabeth et al in Kenya with co –infection of MTB and fungal agents in 44.18% of their study subjects and Sunita et al in India with a coinfection rate of 46%. They concluded that Pathogenic fungi may be significant co-infecting pathogens complicating the management of TB. Clinicians in Kenya should be aware of co-infection of *Mycobacterium tuberculosis* with opportunistic pulmonary fungal and bacterial pathogens. HIV infection is a significant pre-disposition to pulmonary tuberculosis. The two conditions present severe immune suppression. Confounded by prolonged TB treatment, this group represents a high risk for acquiring opportunistic fungal pathogens. (18, 25)

8. Strengths and Limitations of the Study

8.1 Strengths

Since there was no published study previously done on the same area in Ethiopia, the findings of this study may serve as a baseline data for further study; the study have addressed the major and important fungi causing respiratory mycosis both in immune compromised and competent patients.

8.2 Limitations

Due to time and financial constraints

- All risk factors for fungal infection and drug susceptibility test were not performed in.
- Cultural identification for the presence of MTB couldn't be performed

9 Conclusion and Recommendations

Conclusion

High incidence of fungal isolation (42.3%) was obtained in patients with respiratory symptoms. Male subjects were found to show more positive fungal culture which was statistically significant. There was also strong association between both fungal infection and HIV positive patients and patients previously treated with anti TB drugs. Co- infection of fungal agents was also seen in (51.6%) of MTB positive subjects. *C. cruzi* was the predominant fungi in MTB positive subjects. *Aspergillus* and *Candida* were the predominant species among the positive subjects and therefore found to preferentially cause or help in complicating Bronchopulmonary disorders hence, in the absence of specific predictions with regards to pulmonary symptoms, the possibility of fungal colonization needs to be explored.

Recommendations:

- Since the isolation of fungal agents in presumptive TB patients is greater than MTB itself, we recommend fungal screening in presumptive TB patients so that patients get the appropriate diagnosis and treatment.
- As our study has indicated the prevalence of fungal infection seems higher in MTB positive and HIV positive patients, so we recommend fungal screening would also be important for good treatment outcome of these patients
- As our study provides baseline information in terms of indicating the great possibility of fungal infection among presumptive TB patients, hence, we recommend further study to be done.

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11. Annexes

Annex 1: Reagent Preparation (Stains and Media) And Quality Control

A. 10% Potassium hydroxide

Formula;

KOH	10g
Glycerol	20ml Distilled
Water	80 ml

Dissolve the potassium hydroxide in distilled water, and then add glycerol. Mix well. Filter sterilizes. Store in sterile amber bottle and keep for 3 months. Purpose: To digest or clear organic material e.g. tissue cells in a specimen in order to allow fungal structures to be more easily demonstrated.

Principle: Fungi are unaffected by KOH. Glycerol prolongs shelf-life by preventing crystallization and preserves the slides for a few days.

Procedure 1

- Add a drop of 10% KOH to specimen on slide. Coverslip.
- Gentle heating may aid in dissolving debris
- If specimen is thick, it may take 15-30 minutes to dissolve
- Observe under low light microscope

B. Lacto Phenol cotton Blue (LPCB)

Formulae:

Distilled water	20.0 ml
Lactic acid	20.0 ml
Phenol crystals	20.0 g

Cotton blue 0.05 g

Glycerol 40.0 ml

Dissolve phenol in the lactic acid, glycerol, and water by gently heating. Then add aniline blue.

C. Sabouraud Dextrose Agar with Chloramphenicol

Approximate Formula per Liter of Purified Water

Pancreatic Digest of Casein...5.0 g

Peptic Digest of Animal Tissue..5.0 g
Dextrose...40.0 g
Agar..15.0 g
Chloramphenicol...0.05 g

Storage Instructions: store plates in the dark at 2 – 8°C ready for use. For slopes: Dispense 10ml. amounts into bottles.

PRINCIPLES OF THE PROCEDURE

Sabouraud Dextrose Agar is a peptone medium supplemented with dextrose to support the growth of fungi. The peptones are sources of nitrogenous growth factors. Dextrose provides an energy source for the growth of microorganisms. Chloramphenicol is a broad-spectrum antibiotic which is inhibitory to a wide range of gram-negative and gram-positive bacteria. For slopes: Dispense 10 ml. amounts into UGB bottles. Autoclave 121oC/15 minutes.Store at RT. Final pH 7.0 at 250C.

Test procedures

Gene xpert, MTB/rif assay

1. 4ml of sputum sample wasmixed with 8ml of sample reagent (1:2). and thoroughly mixed and left for 10min
2. After 10min the mixture wasmixed by hand and left for 5min
3. 0A0fter 5min 2ml of the prepared sample wastransferred to a cartridge for MTB/rif assay
4. The cartridge wasput in genexpert closed system real-time PCR analyzer
5. After 2hr the result wasready

Quality Control

Pre- analytical

A sterile sputum collection cup was issued to patients. Patients are asked to produce sputum from deep by deep breathing three times and coughing so as to expectorate purulent sputum. Collected sputum samples were inspected for eligibility. Reagents used for Xpert MTB/RIF assay, SDA, BHI media preparation and KOH were checked for expiry date and any abnormal color change. Preventive maintenance of equipments were inspected.

Analytical:

Test procedures for each test were strictly followed according to standard operation system of the tests. Media preparation was performed according to manufacturers' manual Prepared SDA Medias was checked for sterility by incubating at 25-3^{0}C for weeks sterility of each batch of prepared media was checked by incubating uninoculated media at 25-3^{0}C for weeks if there is any kind of microbial growth the prepared batch was discarded. Growth support of each batch of prepared Medias was checked with known representative yeast species C. albicans and mold of Aspergillus species

Post analytical;

- Results generated was appropriately documented electronically
- To prevent loss of data backup data will also be documented on result registration log book.
- Appropriate disposal system by incineration was utilized after disinfection of Specimens and cultures of organisms by autoclaving.

Annex 2: English Version of Participant Information Sheet, Consent/Assent

1. **Participant information sheet** Addis Ababa University College of health sciences school of allied health science department of medical laboratory sciences

Title: mycological findings of sputum samples from pulmonary Tuberculosis suspected patients at Kotebe Health center Addis Ababa Ethiopia.

Introduction First of all we would like to thank you in advance for your cooperation and consent in participation in this study. Please listen when it is read for you about the general information of the study. If you have any question regarding the study please ask freely.

Aim of the study: To assess mycological findings of sputum samples from pulmonary tuberculosis suspected patients at Kotebe Health center Addis Ababa.

Procedure of the sample collection sputum samples was collected in sterile sputum collection tube for MTB diagnosis and mycological diagnosis

Benefits for participants: Study participants will not have any financial incentives or other inducements from participating on this study. However, their results was given and was treated by the prescribing physician based on the KOH mount results and depending on the nature of the disease and the physicians decision; patients may be appointed to await culture results for better treatment.

Risks and complication There is no considerable risk to the study subjects in participating in the study.

Confidentiality: In order to maintain the confidentiality of participants' information, the name will not be given and the samples were coded. Participants will not be prohibited to stop or withdraw at any time from the study. Only interested participants can retrieve their own lab result using their code number. The physicians were responsible for the interpretation of the results and providing treatment. No personal identifier was disclosed to third party or will not appear in any report from this study

Annex 3: English Version Of Consent Form For Participants Older Than 18 Years Old.

The objective and the application of the study were briefly explained to me. I am also informed that my demographic and clinical data wasused for this research purpose from the laboratory request form and they waskept confidential. Moreover, I have been well informed of my right to refuse information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care. It is therefore with full understanding of the situation that I agreed to give the informed consent voluntarily to the researcher to give my specimen for the mentioned study.

Participant name ---------------Signature/fingerprint: ------------- Date ----------- Witness's name----------------- signature: -------------- Date ----------- Investigator's name ------------- signature: ---------- date ---------

Annex 4: Assent Form For The Age 12-17 Years Old

The objective and the application of the study were briefly explained to me. I am also informed that all information contained within the laboratory request is to be kept confidential. Moreover, I have been well informed of my right to refuse information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care. It is therefore with full understanding of the situation that I agreed to give the assent form voluntarily to the researcher to give my specimen for the mentioned study and agreed to use the sample for further study in my signature.

Guardians ‘name--------------------- signature/fingerprint-------date --------- Participant name-------------------- Signature: -------- date --------- Witness’s name--------------------- signature: --------- date --------- Investigator’s name----------------------- signature: ---------- date ---------

Annex 5: Parental/Guardian Consent Form (For Ages Less Than 11 Years Old).

I was informed take whatever time I need to discuss the study with my family and friends, or anyone else I wish to. The decision to let my child join, or not to join, is up to me, and will take him/her about 10 minutes ,it is not painful and my child can stop participating at any time and will not lose any benefits as thereof. As parent or legal guardian, I assure in my signature to become my child a participant in the research study described in this form. Guardian's name-------------------Signature/fingerprint: ---------- Date ----------- Witness's name-------------------- signature: -----------Date ----------- Investigator's name-------------- signature: ---------- date ---------

Annex 6: Amharic Versions Of Patient Information Sheet

እኔ ታማኝ ሙሉነህ በአዲስ አበባ ዩኒቨርሲቲ፣ ጤና ሳይንስ ኮሌጅ፣ የህክምና ላበራቶሪ ሳይንስ ትምህርት ክፍል የሁለተኛ ዲግሪ ተማሪ ስሆን የምርምር ስራየን በመስራት ሊይ እገኛለሁ። እርስዎም በዚህ ጥናት ላይ እንዲሳተፉ ተጋብዘዋል። በጥናቱ ለመሳተፍ ፈቃደኛ ሆነዉ ከተስማሙ መስማማትዎን የሚያሳይ ዶክመንት ላይ እንዲፈርሙ እጠይቃሁ።

መግቢያ

የጥናቱ ርዕስ mycological findings of sputum samples from pulmonary Tuberculosis suspected patients at Kotebe Health center Addis Ababa Ethiopia. ማለትም በመተንፈሻ አካላት ላይ በሽታን የሚያመጡ ፈንገሱች ስጭት በሚል ርዕስ እያጠናሁ እገኛለሁ። ይህ ጥናትም በተሳታፊ ሙለ ፈቃዯኝነት ላይ ተመሠረተ ነዉ።

ከጥናቱ ተሳታፊ የሚጠበቁ

በዚህ ጥናት ለመሳተፍ የሚስማሙ ከሆነ የአክታ ናሙና እንዲወሰድ መስማማት ይጠበቅቦታል። የጤና ባልሙያ ከእርሰዎ ናሙናዉን ይሰበስባል። ከተወሰደዉም ናሙና ላይ የሚገኙ መረጃዎች የእርሶን ማንነት የማይገልጹ ማስረጃዎችን ማለትም ስም፣ አድራሻና የመሳሰለት መረጃዎች ሳይጨምርና ለዚህ ጥናት አገልግሎት ብቻ የሚዉል መለያ ቁጥር በመጠቀም ከዚህ ሆስፒታል ዉጭ ለሚገኙ ለሥራዉ አግባብነት ላላቸዉ ሰዎች ቢነገር የማይቃወሙ መሆኑን መስማማት ይጠበቅቦታል። ናሙና ሰጡ ማለት በሽታው ይገኝብዎታል ማለት አይደለም። በእርሰዎ ናሙና ውስጥ የበሽታ አምጭ ተህዋስ ቢገኝ ከጤና ባለሙያዉ አስፈሊጊውን ህክምና ያገኛሉ።

የመረጃዉ ሚስጥራዊነት

ማንኛዉም የሰጡት መረጃ እና ከተወሰደዉ ናሙና ላይ የተገኘዉ የላቦራቶሪ ዉጤት የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ። ይህን ማህደር ሊያገኙ የሚችሉ የተወሰኑ የጥናቱ ተባባሪ ሠራተኞች ብቻ ናቸዉ። ከዚህም በላይ ስለ እርስዎ ያለዉን ማንኛዉንም መረጃ የይለፍ-ቃል ባለዉ የኮምፒዉተር የመረጃ ማህደር ዉስጥ እንዲቀመጥ ይደረጋል።

ተሳታፊዉ የሚያጠፋዉ ጊዜ

የተዘጋጀዉን የስምምነት ቅጽ ለመፈረምና ናሙና ለመስጠት 3-5 ደቂቃ ያስፈልጋል።

በጥናቱ በመሳተፍ የሚስከትላቸዉ ችግሮች

ናሙና በሚሰበሰብበት ወቅት ምነም አይነት ችግር አያስከትልቦትም።

በጥናቱ በመሳተፍ የሚያስከትላቸዉ ጥቅሞች

ይህ ጥናት የማስተርስ ዲግር መመረቂያ እንደመሆኑ መጠን በመሳተፍዎ የሚያገኙት የገንዘብ ጥቅማጥቅም የለም። ሆኖም በቀኑ በሚደረገዉ የላቦራቶሪ ምረመራ ለሀኪምዎ በመስጠት አስፈላጊዉን ህክምና እንዲያገኙ ይደረጋል እንደ አስፈላጊነቱም የካልቸር ዉጤቶትንም ጠብቆ አስፈላጊዉን ምርመራ ይደረግሎታል። ለወደፊት በተመሳሳይ ሁኔታ ዉስጥ ላሉ በሽተኞች በመረጃ ላይ የተመሰረተ ህክምና ለመስጠት ያግዛል ከፈለጉ የላቦራቶሪ ዉጤቶችን በነፃ ያገኛሉ እንዲሁም ስለ አስፈላጊዉ ህክምና ከሀኪምዎ ጋር ይነጋገራሉ።

የጥናቱ ተሳታፊዎች መብት

ትብብርዎ ሙሉ በሙሉ በፍቃደኝነት ላይ የተመሠረተና ተሳትፎዎን መተውና በማንኛውም ሰዓት ጥናቱን ማቆም ይችላሉ።በጥናቱ ዉስጥ ያሎትን ተሳትፎ በማንኛዉም ጊዜ የማቐረጥ ሙሉ መብትዎ የተጠበቀ ከመሆኑም በላይ ራሰን ከጥናቱ በማግለልዎ ምክንያት የሚቀርብዎት ምንም ዓይነት የሆስፒታል አገልግሎት አይኖርም። ከዚህም በተጨማሪ ጥናቱን በተመለከተ ማንኛዉንም ዓይነት ጥያቄ የመጠቅና ገለፃ የማግነት መብት አለዎት። የላቦራቶሪ ምርመራ ዉጤቱንም በነፃ ማግኘት ይቻላል።

ግንኙነትና ጥያቄ

ይህን ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥዉ ድንገተኛ ችግር ወይም ጥያቄ ካሎት በሚከተለዉ አድራሻ ይጠቀሙ።

ተመራማሪ፣ ታማኝ ሙሉነህ

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አዲስ አበባ ዩኒቨርሲቲ

አማካሪ፣ አዳነ ቢተዉ (ፒ.ኤች. ዲ) ሞባይል +251911039162, ኢ-ሜይል፤ bitewadane@gmail.com

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል! የጤና ሳይንስ ኮሌጅ

አዲስ አበባ ዩኒቨርሲቲ

ለተጨማሪ መረጃ አዲስ አበባ ዩኒቨርሲቲ፤ የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል ይጠይቁ;

ስልክ-+251112755170

ከዚህ በታች የሚገኘው ፊርማዎ ለእርስዎ የተሰጠውን መረጃ ማንበብዎን፣ መስማትዎን እና መገንዘብዎን የሚያሳይ ነው። ከመፈረምዎ በፊት እባክዎትን የጥናቱን ዓላማ፣ የተሳትፎ ጉዳትና ጥቅሙ፣ የመተው፣ የማቋረጥ፣ መብትና ነፃነት እንዳለዎት ይረዱ። ተስማምተዋል? የጥናቱን መግለጫ አንብብያለሁ/ ሰምቻለሁ እናም ተረድቻለሁ። መመሪያው ምን እንደ ሆነና በእኔ ምን ሊከሰት እንደሚችል ተረድቻለሁ። በጥናቱ ላይ ለመሳተፍ፤

እስማማለሁ **-------------------------** አልስማማም **---------------------**

Annex 7: Amharic Versions Of Consent Form For Participants Older Than 18 Years Of Age

የተሳታፊ ስምምነት ቅጽ

ይህ ገጽ “prevalence mycological findings of sputum samples from pulmonary Tuberculosis suspected patients at Kotebe Health center Addis Ababa Ethiopia” ማለትም ማለትም በመተንፈሻ አካላት ላይ በሽታን የሚያመጡ ፈንገሱች ስጭት በ ኮተቤ ጤና ጣብያኢትዮጵያ” በሚል ርዕስ የተሳታፊ ስምምነት ቅጽ ነዉ። በመሆኑም እባክዎን ከዚህ በታች የተዘረዘሩትን ነጥቦች ይረዱና፤ ለመሳተፍ ፈቃደኛ ሆነዉ ከተስማሙ መስማማትዎን የሚያሳይ ፊርማዎን ከታች በተሰጠው ቦታ ሊይ እንዱፈርሙ እጠይቃለሁ።

1. እኔ በመተንፈሻ አካላት ላይ በሽታን የሚያመጡ ፈንገሱች ስጭት በ ኮተቤ ጤና ጣብያኢትዮጵያ” የሚለዉ ጥናት አላማ በደንብ ተገንዝቤአለሁ።

2. ከእኔ የሚወሰደዉ ናሙና ለጥናቱ አላማ ብቻ እንደሚዉል ተረድቻለሁ።

3. ሁለም መረጃዎች እና የናሙና ዉጤቱ ምስጢራዊ መሆኑን ተገንዝቤአለሁ።

4. በጥናቱ ላይ በመሳተፌ ምንም የገንዘብ ክፍያ እንደማላገኝ ተረድቻለሁ።

5. በጥናቱ ያለመሳተፍ እንዱሁም በማንኛዉም ጊዜ የማቃረጥ መብት እንዳለኝ አዉቄአለሁ።

6. ሁለም መረጃዎች በአስተባባሪዉ/ዎች ተገልጾልኝ በደንብ ተረድቻለሁ።

የተሳታፊ ፊርማ፡ --------------------------------

የተሳታፊ አድራሻ፡--------------------------------

ቀን፡--

በስምምነቱ ወቅት የነበሩ ምስክሮች

1.______________________________________

2.____

ይህንን ጥናት በተመለከተ ጥያቄ ቢኖርዎት ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥመዎት ድንገተኛ ችግር በሚከተለዉ

አድራሻ ይጠቀሙ።

ታማኝ ሙሉነህ

ሞባይል፡ +251912130068

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል

የጤና ሳይንስ ኮሌጅ፤

አዲስ አበባ ዩኒቨርሲቲ

ኢ-ሜይሌ፤ tamagnxt@gmail.com

ለተጨማሪ መረጃ፡ አዲስ አበባ ዩኒቨርሲቲ ፤ የሕክምና ላብራቶሪ ሳይንስ ት/ክፍል ይጠይቁ።

ስልክ፡+251112755170

Annex 8: Amharic Versions Of Ascent Form For Participants Less Than 18 Years Of Age

ይህ ገጽ "prevalence mycological findings of sputum samples from pulmonary Tuberculosis suspected patients at Kotebe Health center Addis Ababa Ethiopia" ማለትም ማለትም በመተንፈሻ አካላት ላይ በሽታን የሚያመጡ ፈንገሱች ስጭት በ ኮተቤ ጤና ጣብያኢትዮጵያ" በሚል ርዕስ እድሜያቸው ከ18 ኣመት በታች ለሆናቸው ተሳታፊዎች ስምምነት የመጠየቂያ ቅጽ ነው።

ናሙና የምንወስድበት መሳሪያ ንጽህናው ሙሉ በሙሉ አስተማማኝና ከዚህ በፊት ጥቅም ሊይ ያልዋለ ነው። ናሙና በምንወስድበት ጊዜ የሚኖር ህመም የሚፈጥር ስሜትም ሆነ አደጋ የሚያስከትል ሂደት የለውም። ለጥናቱ የሚወሰደው ናሙና ለጥናቱ አላማ ብቻ ይውላል። የናሙናው ውጤት ምስጥራዊነት የተጠበቀ ሲሆን በናሙናው ውስጥ የበሽታ አምጭ ተህዋስ ቢገኝ ከጤና ባለሙያዉ አስፈላጊውን ህክምና ያገኛሉ። በጥናቱ ላይ በመሳተፍዎ ምንም የገንዘብ ክፍያ አያገኙም። በጥናቱ ለመሳተፍ የመፍቀድም ሆነ ያለመፍቀድ እንዱሁም በማንኛዉም ጊዜ የማቋረጥ መብት አለዎት።

(ስም) በጥናቱ እንዲሳተፍ/ እንድትሳተፍ ይፈቅዳሉ?

ፈቃዮኛ ከሆኑ፤

የተሳታፊ ፊርማ፣-------------------------- የፈቀደው ግለሰብ ፊርማ፣------------------------------

አድራሻ፣------------------------------- ቀን፣--

በስምምነቱ ወቅት የነበሩ ምስክሮች

1.____

2.____

ይህን ጥናት በተመለከተ ጥያቄ ቢኖርዎት ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥመዎት ድንገተኛ ችግር በሚከተለዉ

አድራሻ ይጠቀሙ።

ታማኝ ሙሉነህ

ሞባይል፡ +251912130068

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል

የጤና ሳይንስ ኮሌጅ፤

አዲስ አበባ ዩኒቨርሲቲ

ኢ-ሜይሌ፤ tamagnxt@gmail.com

ለተጨማሪ መረጃ፡ አዲስ አበባ ዩኒቨርሲቲ ፤ የሕክምና ላብራቶሪ ሳይንስ ት/ክፍል ይጠይቁ።

ስልክ፡+251112755170

Annex 9: Demographic and Clinical Data Record Format

Addis ababa University, collage of health sciences, department of medical laboratory science.

Demographic and clinical record format for the prevalence of mycotic agents among pulmonary tuberculosis suspected patients at kotebe primary hospital Addis Ababa Ethiopia.

I. Sample IDo___________Age_____

II. Sex

1	Female	2	Male

III.

	Clinical background
1	HIV sero status
2	Previous anti TB drug treatment

IV. Genxpert MTB Rif assay

1	Negative
2	MTB detected rif resistant not detected
3	MTB detected rif resistant detected
4	MTB detected rif resistant indeterminate

V. Microscope (KOH) lab result

1	Fungal element seen	2	Fungal element seen

VI. Culture result____

VII.

Sample no	Patient name or id	Age Sex	HIV status	Previous anti TB treatment	genexpert	KOH	MYCOLOGY CULTURE (SDA and BHI).					
							Inoculation date					
								Check 1	Check 2	Check 3	Check 4	5

ATTACH BARCODE HERE IF AVAILABLE

LAB CONTROL NO: EPHI NRL /TBL/ F5.4-001

Federal Democratic Republic of Ethiopia, Ministry of Health/NTP/
Tel: 251 (0) 11 551 7011 Fax: 251 (0) 11 551 9366 E-mail: moh@ethionet.et P. O. Box 1234, Addis Ababa, Ethiopia

TB Diagnostic Service Request and Report Form

1. **PATIENT ADDRESS:** Patient Full Name: ______________________ Age: _____ Sex: _____ Region: _________ Zone/Sub city: _________
Woreda: __________ Kebele: _________ H. No: _____ Tel: ________________
Referring Health Facility: ______________ MRN: _____ Co-infection: _______ TB registration No: ______ MDR TB No: ________

2. **TB DISEASE TYPE & TREATMENT HISTORY:**

- Site: i. pulmonary ______________________ ii. Extra pulmonary (specify): _________
- Patient Registration Group: ☐ New ☐ Relapse ☐ Treatment after lost to follow-up ☐ after failure of first treatment ☐ After failure of retreatment, ☐ Pre-XDR , ☐ XDR, ☐ Other, specify, ______________
- Previous TB drug use: ☐ New, ☐ 1st line treatment, ☐ 2nd line treatment, ☐ MDR-TB contact, ☐ Other, _______

3. **REQUEST FOR TESTING AT TB LABORATORY:**

- **Reason:** I. **Diagnosis**: If Diagnosis, ☐ Presumptive TB, ☐ Presumptive DR, II. **Follow up**: If follow up, ☐ at_______ months of 1st line treatment, ☐ MDR Follow up, at _____ months after treatment, ☐ Presumptive XDR,
- **Specimen Type:** ☐ Sputum ☐ Other, Specify: ______________________________
- **Requested tests:** ☐ Microscopy, ☐ GeneXpert MTB/RIF test, ☐ Culture, ☐ Phenotypic DST (First Line _____, Second Line_____) ☐ Line Probe Assay (LPA)

Requested Clinician: Name: _________________Signature ___________Tel:____________ Date of Request _________

....

4. **LABORATORY RESULT (for Laboratory use only) :**

Laboratory Number: ________ ☐ Date of specimen collected (E.C): ___/___/______ Date of sputum received E.C): ___/___/___

4.1 Microscopic examination:

- **Method used:**
  - ✓ Ziehl-Neelsen ☐ Direct Smear ☐ Concentrated Smear
  - ✓ Fluorescence ☐ Direct Smear ☐ Concentrated Smear

Result	Negative	Positive			
		Scanty	1 +	2 +	3+
1st Spot					
2nd Spot					

Name of the Examiner: _______________ Signature: _________ Date: _________

4.2 GeneXpert MTB/RIF Assay Result:

Result	Detected	Not detected	Indeterminate	Invalid	Remark
M. Tuberculosis (MTB)					
Rifampicin Resistance (RR)					

Name of the Examiner: _______________ Signature: _________ Date: __________

4.3 TB Culture result:

Result	Positive for Mycobacterium tuberculosis Complex (MTBC)					Negative	Contaminated
	1-9 colonies Actual Count	10 – 99 colonies (1+)	≥100 colonies (2+)	Confluent growth (3+)	Non tuberculous Mycobacteria (NTM)		

4.4 TB Drug Susceptibility Testing (DST) result:

- **Method used**: **Phenotypic:** ☐ 1st Line, ☐ 2nd Line ☐ **LPA:** ☐ 1st Line, ☐ 2nd Line ☐ **Other, Specify,** ___________

Result	1st line drugs					2nd line drugs							Other	
	H	R	S	E	PZA	Ofx	Lft	Mfx	Am	Cm	Km	Eto		

- **Legend**: **H**=Isoniazid; **R**= Rifampicin **Z**= Pyrazinamide; **E** = Ethambutol, **S**= Streptomycin, **Ofx**= Ofloxacin; **Lft**= Levofloxacin; **Mfx**=Moxifloxacin; **Am**=amikacin; **Cm**=Capreomycin; **Km**= Kanamycin; **Eto**= Ethionamide, **S** = Sensitive; **R** = Resistant; **C** = Contaminated; **ND** = Not done.

Name of the Examiner: _______________ **Sig**_________ **Tel:**_______________ **Date** _________

Date reported: ___/___/___**Reviewed by:** ________________**Date** ___________**Signature**________________

12. Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: TAMAGN MULUNEH (B.Sc.).

Signature:__________________

Date of submission:__________________

This thesis has been submitted with our approval as advisors.

Advisor:

ADANE BITEW (MSc, PhD).

Signature: ____________________

Date: ____________________

Place: Addis Ababa, Ethiopia.