Addis Ababa University

College of Health Science, School of Allied Health Science

Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

A Thesis Submitted to School of Graduate Studies, Addis Ababa University in Partial Fulfilment of the Requirments for the Degree of Masters of Science in Clinical Laboratory Science (Diagnostic and Public Health Microbiology Speciality)

By Selfu Girma

June 2016

Addis Ababa, Ethiopia

Addis Ababa University

College of Health Science, School of Allied Health Science

Diagnostic Potential of Auramine O staining for the Diagnosis of Leprosy

By: Selfu Girma

Advisors:

Kassu Desta , Assistant Professor, BSC, MSc. PhD Candiate

Addis Ababa University School of Allied Health Science Department of Medical Laboratory Science , Addis Ababa , Ethiopia

Kidist Bobosha: BSc, MSc, PhD .

Armauer Hansen Research Institute , Addis Ababa, Ethiopia.

Collaborators:

Abraham Aseffa: Senior Scientist, MD, PhD.

Yohannes Tsegaye: Consultant Pathologist, MD, Speciality in Pathology.

Tesgaye Hailu: Data Manager, BSc, MSC.

Armauer Hansen Research Institute

Shimelis Nigusse: Consultant Dermatovenrologist, MD, Speciality in Dematovenerology

All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre

June 2016

Addis Ababa, Ethiopia.

Addis Ababa University

College of Health Science, School of Allied Health Science, Department of Medical Laboratory Science

Diagnostic Potential of Auramine O staining for the Diagnosis of Leprosy

By: Selfu Girma

Approved by the Examining Board

_____	_____
Cahirman, Dep. Graduate Committee	Signature
_____	_____
External Examiner	Signature
_____	_____
Internal Examiner Examiner	Signature
_____	_____
Advisor	Signature

Abstract

Introduction: *Mycobacterium leprae* (*M. leprae*) is the causative agent of leprosy which is a chronic infectious disease. The disease mainly involves the skin and peripheral nerves. The 2015 WHO statistics showed that there were 213,899 new cases with the detection rate of 3.81 per 100,000 population globally in the year 2014. In Ethiopia, the trend of new case detection for the last ten consecutive years was in average 4200 per year. Implementation of more sensitive laboratory tests at medium and lower health facilities is crucial to reduce the disease transmission and leprosy associated disability. The most important aim this study is to avail Auramine O staining for diagnose of leprosy and improve the sensitivity of leprosy laboratory diagnosis on both SSS and skin biopsy section using Light Emitting Diode Florescent Microscope.

Objective: To evaluate diagnostic potential of Auramine O staining for the diagnosis of leprosy using LED FM microscsope.

Methodology: A cross sectional study was conducted on 142 leprosy and 28non-leprosy participants usingAuramine O, Zehil Neelson, Fite-Faraco, H&E and PCR tests on slit skin smear (SSS) and skin biopsy done at AHRI pathology laboratory.Tissue sections prepared from FFPE tissue was used for the stainings and alcohol fixed tissue was used to extract DNA for PCR. Sensititvity, specifity, positive (PPV) and negative predictive values(NPV), and Kappa values were calculated.

Results: The Sensitivity and Specificity of ZN staining on SSS was 58.8% (95% CI:49.2% - 67.9%) and 100% (95% CI: 85.2% -100%); on the other hand Sensisivity and Specificity of Auramine O staining on SSS were 66.7% (95% CI:57.2% - 75.2%) and 100% (95% CI: 85.2% - 100), respectively. Fite-Faraco, Auramine O on tissue and H&E showedsensitivity of 78.1% , 79% and 86.8%, respectively. The sensititvity and specificity of PCRwas 89.5% (95% CI: 82.3% - 94.4%) and 89.5% (95% CI: 82.3% - 94.4%) respectively.

Conclusion and recommendation: Auramine O staining on both SSS and tissue section was better compared with the previous routine diagnostic tests. The sensititvty of PCR was much

higher and we give our emphasis to recommend Auramine O staining for the diagnosis of leprosy inlower health institutions and that of PCR diagnosis at a referral level.

Aknowledgements

I would like to aknlowledge my External and Internal advisors, Dr Abraham Aseffa and Mrs Kidist Bobosha and Mr kassu Desta for their great support without which I would not have proceeded a single step.

I would aslo like to appreciate the great support of ALERT Red Medical Clinic, Minor Surgery Unit and Dermatology Department staff members for their great support during the recruitment of participants and sample collection. I would also like to appreciate the support of all AHRI staff memebers, particularly Sr Genet Amare and Sr Haregewion Yetesha for their wonderful handling and very professional punch biopsy collection.

I would like to acknowledge the support of the staff members of the laboratory of Professor Stewart Cole at Global Helath Institue, EPFL, Switzerland in this study. I want to express my appreciation to Mr Philippe Busso, Mrs Charlotte Avanzi and Prof Stewart Cole for their generous support during my stay for training on molecular techniques of *M.leprae* diagnosis in their lab.

Contents

List of Tables

List of Figures

Acronyms

AAERC: AHRI/ALERT Ethical Review Committee

ABC: Avidine-Biotine complex

AFB: Acid Fast Bacili

AHRI: Armauer Hansen Research Institute

ALERT: All African Leprosy Rehabilitation and Training Center

BB: Borderline

BI: Bacterial Index

BL: Borderline Lepromatus

BT: Borderline Tuberculoid

DNA: Deoxyribonucleic acid

DPX: Di-n-butyl phthalate in Xylene

FFPE: Formalin Fixed Paraffin Embedded

FF: Fite-Faraco

FLS: Fluorescence Staining

GCP: Good Clinical Practice

GCLP: Good Clinical Laboratory Practice

G2D: Grade 2 Disability

H&E: Hematoxlin and Eosin

IL: Indeterminate leprosy

K: Kappa Value

LED: Light-Emitting Diode

LL: Lepromatous Leprosy

MB: Multibacillary

MDT: Multidrug therapy

M. leprae: *Mycobacterium leprae*

NPV: Negatve predictive value

PB: Paucibacillary

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

PGL-1: Phenolic Glycolipid 1

PPV: positive predictive value

PNL: primary neurotic lesion

SSS: Slit Skin Smear

TT: Tuberculoid

WHO: World Health Organization

ZN: Ziehl Neelsen

Operational Definition

- **Clinically diagnosed leprosy Cases**: those patients who are clinically diagnosed and confirmed as leprosy patient and decided to put them under Multi Drug Treatment irrespective of their laboratory result.

- **Alternative Gold Standard:** is a combination of test panels designed for this study due to absence of universally recommended sensitive gold standard test for leprosy. The main part of this test panel is clinical confirmation of the cases as leprosy then this comfirmation should be supported with positive laboratory tests of either SSS ZN, Fite-Faraco or H&E stainings.

- **Negative Control:** Negative control participants are patients who presented with any kind of skin related diseases other than leprosy and who came to ALERT for surgical treatment.

1. Background

1.1 Introduction

Mycobacterium leprae (*M. leprae)*is the causative agent of leprosy or Hansen's disease which is a chronic infectious disease. The disease mainly affects the skin and peripheral nerves and is transmitting through respiratory route and body contact between cases of leprosy and healthy persons(1, 2).The pathogen was identified by the famous Norwegian physician Dr. Gerhard Armauer Hansen in 1873. The 2015 World Health Organization (WHO) weekly epidemiological record report on leprosy shows about 213,899 globally reported new cases and among those about 13,500were with grade-2 (G2D) disability in the year 2014; with the detection rate about 2.5 per 100,000 population. Among thoses globaly registered new cases, 18,597 (8.7%) were from Africa. It is also reported that the rate per 100,000 populations for G2D for Africa (0.36) was highest next to south-east Asia(0.45) and the lowest wasfor the Western Pacific Region (0.02). Ethiopia contributed about 3,757 new cases in the same year, 2014, which was about 1.8% of globally and 20.2% of continentaly reported new cases(3). In Ethiopia, the trend of new cases report for the last ten consecutive years is 4,200 per year in average.

Leprosy manifests itself in various forms and its diagnosis mainly relies on clinical examination; skin lesions, nerve enlargement and loss of sensation. The clinical examination is supplemented with AFB staining on Slit Skin Smear (SSS). According to Ridley and Jopling, leprosy is classified as Indeterminate leprosy (IL),tuberculoid (TT), borderline tuberculoid (BT),borderline borderline (BB), borderline lepromatus (BL) and lepromatus leprosy (LL). This classification is mainly based on the immunological profiles and bacterial load in patients (4). In order to avoid complications in management, WHO in 1982 also recommended use of PB and MB to classify leprosy patients for treatment purpose. Patients with 5 and less skin lesions and BI=0 are considered as PB and those with 6 or more lesions and positive BI are considered as MB. The appearance of the skin lesions such as macules, plaques, papules, nodules, diffuse infiltration, secondary lesions like burns, blisters, fissures, ulcers and scars are also considered during the classification (5-7).

Many individuals will continue to be infected and develop disease regardless of the intense efforts to eliminate leprosy as a public health problem and the widely applied multidrug therapy (MDT). One of the basic reasons for the consistent report of new leprosy cases in different leprosy endemic countries is the on going transmission in the population. Lack of reliable diagnostic tools specially at the early stage of the disease is one of the main reasons for the on going transmission. For the reason that delay in diagnosis or misdiagnosing leprosy itself will increase disabilities among patients, early diagnosis of the disease is important in reducing disabilities and disease transmission, too (8). Hence, several efforts are being undertaken and WHO has also set early detection of leprosy as priority in leprosy control strategy (9, 10). Beside clinical examination, laboratory tests like ZN and FF staining on SSS, nasal swab and formalin fixed paraffin embedded (FFPE) tissue sectionswhich can be performed at medium and lower health service stations are very relevant to increase the efficiency of leprosy diagnosis. Therefore, along with implementation of more sensitive molecular tests, increasing the sensitivity of these routine laboratory tests is crucial to control the transmission of the disease in the population and reduction of disabilities in leprosy patients.

Though clinical diagnosis is the main tool of leprosy diagnosis based on three essential signs which are hypopigmented or erythematous macules with sensory loss and thickened peripheral nerves which the physician mainly used for the diagnosis, acid fast staining(ZN), modified Fite-Faraco (FF) and histopathologic diagnosis are the main laboratory diagnosis which supports clinical examination. According to Ethiopian Tuberculosis, Leprosy and TB/HIV Prevention and Control program, without careful examination leprosy can easily be misdiagnosed for a number of skin diseases like Tinea versicolor, Vitiligo, Psoriasis, Onchocerciasis, Cutaneous leishmaniasis and Kaposi's sarcoma indicating that the demand for a differential diagnosis. Previous reports on validation of leprosy diagnosis have also showed the need for differential diagnosis to reduce misdiagnosis (11, 12).

1.2 Statement of the Problem

Leprosy diagnosis is based on three important signs of the disease; skin lesions with loss of sensation and enlarged peripheral nerve. Basically, clinical findings supported by positive Acid Fast Bacili(AFB)result or in combination with histopathological findings are the standard

practice for leprosy diagnosis. However, since it is easy to perform and interpret compared with histopathology tests which demands a pathologist, ZN staining on SSS is the widely used conventional method of leprosy laboratory diagnosis especially in middle and low settings(13, 14).Although advanced diagnostic techniques like PCR and Immunohistochemisrty tests are more reliable, their applicability at lower health facilities is minimal due to high cost and lack of trained personnel. Based on this fact, ZN staining on SSS remains as a routine conventional method of laboratory diagnosis of leprosy especially at the primary health care centers (15, 16).

Eventhough it is the only option at lower health institutions, the diagnostic potential of ZN staining to detect *M. leprae* particularly in PB patients is poor. This is mainly due to low bacillary load in PB patients and requires time to patiently observe the wide area of the smear to confirm the presence of the bacilli. Therefore, in the absence of lab results or with BI 0 lab reports, the clinician relies only on the clinical examination to confirm the patient is a PB leprosy patient. This requires enriched experience in the field and will remain challenging in primary health care facilities where no dermatologists are available.

Therefore, this study was designed to determine the diagnostic utility of other staining options and compare with ZN to select better sensitive and specific staining, and in addition a staining that can easily be performed with the readily available lab settings in primary health care facilities. In this study, based on the developed alternative Gold Standared which is acombination of clinical diagnosis with three laboratory diagnostic tests, we have compared Auramine O, Fite-Faraco , ZN, H&E and PCR techniques on SSS and skin biopsy samples with great emphasis on Auramine O staining.

2. Literature Review

In leprosy diagnosis, the main source of error which leads to misdiagnosis of the disease is the reliability of examination of patients by uninitiated health workers, where the availability of dermatologist is scarce(17). Even though all the skin lesions show inflammatory involvement of cutaneous nerves, clinical diagnosis may also face some cases of leprosy with neurological involvement and absence of cutaneous lesions; and due to this reason it will be mandatory to rely on the report of neurologist, neurophysiologist and pathologists to reach at the correct diagnosis and avoid the misdiagnosis arisen from this complexity of the disease(18, 19). Moreover, though skin diseases which have pigmentation of skin patch similar with that of leprosy may took their part to lead to this misdiagnosis, the complicated outcome manifested on leprosy patients like arthritis may also lead the diagnosis to rheumatic disease, unless differential diagnosis used exhaustively. Furthermore, in LL cases; occular, sinovial, bone, mucosal and visceral involvement may occur (20-22).

Among many skin disorders which have similar signs with leprosy, tinea versicolor has been studied in different countries with or without leprosy burden for its frequent misdiagnosis with leprosy. A case study at University of Massachusetts Medical School has been performed on a 24 years old Brazilian showed that the hypopigmentation of macules were symmetrically distributed over the upper trunk and fused in some areas and formed large patches. The presentation of clinical impression was suggestive of tinea versicolor and the patient was treated accordingly, which should rather be treated for leprosy. This kind of misdiagnosis may take several months, years or even unable to get the patient again for the appropriate treatment and may lead the dissemination of the disease in the community which interfere with the disease control program(23).

Although it requires at least 10,000 organisms in a gram of specimen to show the presence of the bacilli,and physicians mostly use clinical sign and symptoms for the routine diagnosis of the disease due to this poor sensitivity, ZN staining is still a useful tool to confirm the diagnosis of the disease in combination with the clinical signs and symptoms. Torres P *et al*. performed a

study on sixty participants of both MB and PB caseswho had finished their treatment(24). They further divided the participants with positive and negative BI based on previous clinical and Ridley–Jopling histopathological result. Among 20 MB BI + participants , conventional ZN staining on SSS from skin lesion, ear lobe and nasal swab smear yielded 75%, 55% and 40% positive AFB result, respectively. Only one participant from 30 MB BI- cases (3%) showed positive for AFB in SSS from skin lesion , whereas both ear lobe and nasal swabs remained negative for this case group. In all PB BI– group (n=10) no AFB positive result was detected from all type of stained samples. In this study, the ZN staining from skin lesion and ear lobe together with nasal swab showed high AFB detection rate with less invasive procedure which can still be used for diagnosis and monitoring of treatment progress.

Irrespective of considering the cases being paucibacillary or multibacillary, similar studies in India conducted by Sarker UK , Bhushan P and Jaswal TS showed that sensitivity of SSS AFB were 30.5% , 40.3% and 50% respectively though the sensititvity of SSS AFB results were lower and inconstant due to the type of cases recruited in the studies. Besides their low snsitivity report, all studies showed very high, i.e 100% diagnostic accuracy of SSS AFB (25-27). Following similar approach, studies in India showed that the sensitivity of ZN staining to detect *M.leprae* in FFPE tissue section was 26.7% and 59.9%, which shows a remarkable variation. Despite the reason that the quality of staining and competency of laboratory personnel affects the yield, it is known that AFB staining technique is less sensitive to detect the bacterium in clinically suspected samples specially collected from PB cases which indicates the requirements of the effort to increase the sensitivity of the test or develop additional sensitive diagnostic tools. For this thought, the result obtained from a study by Deepa AS *et al.* for Auramine-Rodamine flourescent staining compared with FF,which is 100% comparable result of sensitivity can be used as a suggestive input (28, 29).

Leprosy diagnosis is not always an easy task even at higher health institution with experienced dermatologist particularly at the early stage of the disease. Based on its availability and simplicity to be performed at different level health institutions, FF staining is being utilized and accepted with good sensititvity and high specificity in most cases for leprosy diagnosis. In a previous study, among 165 analyzed sample; FF detected 99 (60%) positives whereas H&E, ZN

and PCR showed 96 (58.2%), 84 (50.9%) and 111 (67.3%) positivity, respectively. The study also showed the combination of FF and PCR tests yielded 47.1% positive result of the total PB cases and 91.5% of the total MB cases, which is higher as compared with H&E staining with PCR; 42.5% and 89.4% for PB and MB respectively and ZN staining with PCR 41.4% and 87.3% forPB and MB respectively(28). Far from this resut, the study in Brazil showed 26.6% sensitivity of ZN staining on Slit Skin Smear, which is significantly less than the above study in Indiawhich showed 50.9% sensitivity (30).

Hematoxilin and Eosin staining has a great role in the disease diagnosis, prognosis and for further classification of the disease in to different classes to assist in the medical treatment (6). Despite its inconsistency, different studies showed that the sensititvity of histopatology using H&E staining to diagnose different forms of leprosy is valuable.Reja AH , Ray R and Teixeira AC et al.performedseprate studies in India and Brazil showed that the sensitivity of histpathologic diagnosis to agree with clinical diagnosis was 58.2%, 78% and 67.6%, respectively (28, 31, 32).

As mycolic acid is the component of *M.leprae* cell wall which can be stained with flourescence dyes and examined under flourescent microscope, using flouresence staining to facilitate the lab diagnosis of leprosy is an alternative. Different studies recommended Auramine O staining for the diagnosis of leprosy for its better yield of positive results. Following modified deparaffinization method of tissue sections with xylene- peanut oil mixture, which has a great impact to protect the more delicate waxy coat of the organisms and prevent shrinkage, the sensitivity of Auramine O staining yields as high as 90% detection rate of the organism irrespective of the disease type. Moreover, sensitivity of this fluorescent stain in IL, TT, BT and BB leprosy was 100% using FF staining as a Gold standard. Based on the obtained results florescence staining techniques was recommended for alternative method with high sensitivity(33). However, detection of acid fast bacilli under florescent microscope using Auramine O staining can increase sensitivity and decrease the time required for smear examination, storage of Auramine O stained slides for quality control purpose or further confirmations is not as such practical as storing ZN, FFor H&E stained slides for longer time. This is due to the fading of the fluorochrome based Auramine O stain over time. As indicated in

some literatures, the maximum storage period of time for slides stained with Auramine O with 3+ concentration of bacilli to be still positive with scanty concentration is 5 months, even stored in the dark at 22°C(34).

Although confirmation of clinically diagnosed leprosy cases is done through detection of acid-fast bacilli or typical histopathologic features of skin and nerves, the sensitivity of acid fast staining to find bacilli in paucibacillary cases is very low due to less concentration of bacilli. Moreover, finding of typical histopathology features like inflammatory cells infiltrationin paucibacillary cases may not be present; which in turn leads the yield of the result inconclusive and makes the diagnosis more challenging. Requirement of additional diagnostic techniques like PCR is mandatory for the diagnosis of such inconclusive cases to ensure early diagnosis and make the patients receive the treatments ontime(35). The characteristic of *M.leprea* unable to grow on culture medium and its extremely slow generation time of 12–14 days in selected live animals demands advanced molecular diagnostic techniques for the application even at lower level health institutions (36).

This major threat, failure to cultivate the organism on artificial culture media, also urges researchers in the world to focus on developing some immunological and molecular diagnostic techniques. Though serological and immunological tools to detect *M. leprae* are mostly bases detection of major unique components like phenolic glycolipid-I(PGL-I) or T cell immune response like measuring interferon-gamma (IFN-γ) production, the very divergent of the strength of the cellular and humoral responses affects the development process of good diagnostic tools in the area. To overcome this limitation, use of PCR amplification of *M. leprae* DNA in difficult-to-diagnose cases favors the correct diagnosis and increase possibility of early identification. There are a number of DNA targets like Proline-rich antigen(pra-36 KDa), RLEP,Ag85B and 16S rRNA which can be used in different PCR methods like PCR-Southern Hybridization,Multiplex-PCR, TaqMan real- time PCR and SyBr green real-time PCR. The PCR techniques is contributing a lot not only in diagnosis of difficult cases like primary neural leprosy (PNL) and PB but also in treatment monitoring, transmission and household contact surveillance (37).

In china, it is highly suspected that there is significant number of delayed diagnosis of leprosy based on the reason that more than 85% of the reports are showing only MB cases. To overcome this delayed diagnosis, some potential diagnostic tools like antigen specific antibody detection assays and antigen-specific whole blood assays were evaluated for their performance in diagnosis and monitoring of the disease. A total of 31, multibacillary (MB; n=20) and paucibacillary (PB; n=11) patients were recruited. As it is well established thatMB patients typically have high antibody titers against *M. leprae* antigens while PB patients do not, 90% (18/20) of MB and 45.5% (5/11) of PB sera demonstrated positive responses against PGL-I by ELISA. Despite the relative absence of antibody responses, PB patients do typically demonstrate cellular responses to *M. leprae* antigens. Positive response by secreting IFN-γ upon incubation with the antigen LID-1 in WBA was 40.0% and 72.7% for MB and PB leprosy cases, respectively. The use of better diagnostic tools like measuring IFN-γ secretion in PB cases enables to reduce delay in diagnosis and may have greatinputs to facilitate the diagnosis and also in further reduction of physical damage and disease transmission (38).

Though Immunological diagnostic tools that employ humoral immune responses such as evaluating anti-PGL-I antibodies and quantifying IFN-γ are being used, PB cases mostly do not exhibit antibodies against the bacilli and in MB cases IFN-γ production is diminished unless there is continuous exposure to an infective source. Molecular diagnostic tests prevail in defining leprosy diagnosis of suspected patients with clinically suggestive or atypical lesions presenting with negative bacilloscopy or inconclusive histopathology. This is mostly applicable for PNL patients which can be missed easily as they do not exhibit cutaneous lesions alternatively using nerve biopsy(36). Detection of *M. leprae* DNA in paraffin-embedded skin biopsy specimens was performed on 51 PB cases using TaqMan real-time PCR assay byYan W et al. in China. The assay was sensitive enough to detect up to 8 fg DNA which is equivalent to 240 bacilli and good specificity with no cross-reactivity compared with 21 other bacterial species used as control (39).

To evaluate the sensitivity of PCR on different gene targets, comparative study on RLEP, 16S rRNA, rpoT and Sod A gene targets perfomed on clinical and environmental samples in India. Skin Slit Smear and blood samples were used as clinical samples and the sensitivity obtained

from amplifying RLEP gene in Skin Slit Smear was83% which is higher than other amplified regions(40). Another study in India performed by Banerjee S et al using multiplex PCR on samples from nasal swab recruiting a total of 156 MB and PB cases reported 86% sensitivity (41). Though the aim of the study was to better understand the route of leprosy transmission, the study by Job CK *et al.* reported that the sensititvity of PCR to diagnose leprosy even in a sample collected from unbroken skin wash was 80% which confirms the high sensitivity of the assay (42).

Inorder to emphasize their recommendation of using PCR test targeting 130 bp RLEP for confirmatory diagnosis instead of using 372 bp, a group of researchers in Brazil performed a study on skin biopsies of leprosy lesions collected from 110 non-treated patients. The sensitivity of using 130 and 372 bp target RLEP were 73.6% and 52.7% respectively, showing siginificant variation. Advantage of using 130 bp target PCR has further been elaborated through evaluating it sensitivity in different classifications of leprosy. The sensititvity of the test in diagnosing TT and BT cases which their basiloscopy mostly is very low or null was 40% and 55.5%, respectively. Eventhough it is not assertive for its admirable result for all classifications , the sensititvity of the test to diagnose BB, BL and LL which are mostly with high bacilloscopy was 100 % as expected (43). In similar study conducted by Kamal R *et al*. the sensitivity of in-situ PCR in different classification of leprosy was 62.5% in cases of INT,TT,BT and BB category collectively and 88.8% of BL and LL category as well (44)

The most important aim of this study on Auramine O staining is to enable the laboratory diagnosis of leprosy easy and to make the diagnosis on SSS and skin biopsy section more sensitive using LED florescent microscope, which is available at most of the lower health centers of Ethiopia distributed by the ministry of health which is primarily intended for the increment of quality diagnosis of M. tuberculosis.

3. Hypothesis and Objective

3.1 Hypothesis

Auramine O staining has a comparable degree of sensitivity with Fite-Farco staining on tissue section and Skin Slit Smear to detect *M. leprae* in clinically diagnosed leprosy patients.

- **H_1:** Auramine O staining has equal sensitivity with that of Fite-Faraco staining to detect *M. leprae.*

3.2 General Objective

To evaluate diagnostic potential of Auramine O staining for the diagnosis of leprosyusing Light Emitting Diode Fluorescence Microscope (LED-FM).

3.2.1 Specific objectives

- To determine the sensititvity of Auramine O on both SSS and tissue sections.
- To determine the sensitivity of Auramine O in different histopathologic classification of the disease.
- To compare Auramin O staining with other staining techniques
- To determine the sensitivity of PCR for the diagnosis of leprosy.

4. Material and methods

4.1 Study Design

A cross sectional study was conducted at Armauer Hansen Research Institute and ALERT Hospital from January 1, 2015 to April 30, 2016. All clinically diagnosed leprosy cases examined by dermatologists at the out patient department of dermatology unit of the hospital has been sent to Red Medica clinic for further investigation by senior dermatologist to put the patients on MDT. Clinically confirmed new and relapse leprosy cases were recruited in the study before the introduction of their medical treatment. Furthermore, voluntary non-leprosy participants who came to minor surgery department of the hospital for their routine surgical care were enrolled in the study and used as controls.

4.2 Study Setting

This study was conducted at All African Leprosy and Tuberculosis Rehabilitation and Training Center-Hospital (ALERT),which is a governmental hospital under Federal Ministry of Health which has about 300 beds for admitted patients, including Dermatology, Ophthalmology, Medical, Surgery, Trauma and other departments. The hospital also has Red Medical clinical which only serves leprosy associated cases. The patient registration log book of the clinic shows that in average eight new leprosy patients per week visit the clinic. The hospital also provides pathology diagnostic service including histopathology, which is fully supported by Armauer Hansen Research Institute (AHRI). All units and departments including dermatology unit send clients to the pathology unit for demanding the pathology diagnosis. The pathology unit is also engaged in different types of active researche related with different diseases like Leishmaniasis, TB and Cancer. The Unit is equipped with different types of instruments such as Microtome, Tissue Embedding Console, Cryotome, Tissue Processor, Incubator, Refrigerators and Safety-hood. Currently the pathology lab has engaged one pathologist, one dermatopathologist, one laboratory technologist and two laboratory technicians.

4.3 Study Population

Study participants were new (non-treated) leprosy cases with any form of the disease, with or without reactions and with the age group of 15 yrears and above who came to ALERT leprosy

clinic. Non leprosy cases of the same age group were also enrolled for skin biopsy samples from discarded skin specimens after their routine surgical treatment was performed and were used as negative controls.

4.3.1 Inclusion Criteria of Leprosy Cases

Clinically diagnosed new and relapse leprosycases within the agegroup of 15 years old and above who are willing to give informed consent/assent were included in the study.

4.3.2 Exclusion Criteria of Leprosy Cases

Patients who are critically ill, children with the age group under fifteen years old , pregnant women , patients who are under anticoagulation therapy and also patients under anti-leprosy treatment were exluded from this study.

4.3.3 Inclusion Criteria of Non leprosy cases

Skin samples were colleted from leftover specimens of non leprosy patients after their routine surgical treatment only after having their consent/assent to take part in the study.

4.4 Sample size Determination and Sampling Method.

We have followed conventional sampling method where we recruited leprosy patients who consecutively came to the leprosy clinic as long as they are clinicaly confirmed and willing to participate till we reached the reguired sample size for the study. Sample size was determined by the formula recommended by WHO Tropical Disease Research (TDR) diagnostics evaluation expert panel and also used by Karimollah H. Tilaki for sensitivity and specificity sample size calculation to obtain the required optimum amount of sample size to evaluate diagnostic tests (45, 46).

$$n = \frac{(Z_{\alpha/2})^2 \, P\,(1-P)}{(d)^2}$$

Where:

n = required sample size based on sensitivity

p = anticipated sensitivity measured as a proportion

d= margin of error

Thought;

Anticipated sensitivity of Auramin O staining obtained from previous study (90%) = 0.9 (33) .

We measured the sensitivity within 5% margin of error,then $d$was 0.05 expressed in proportion.

To measure the sensitivity under 95% confidence interval;

$$\text{Therefore, } n = \frac{(1.96)2 \times 0.9 \times (1-0.9)}{(0.05)2}$$

$$= \frac{3.8416 \times 0.09}{0.0025}$$

$$\approx 139$$

The estimated sample size including 10% non-respondents was **153**.

We have followed conventional sampling method to recruit all participants who continuously came to the hospital as long as they are clinically diagnosed as leprosy and till we accomplished collecting samples of the reguired sample size.

4.4.1 Non-leprosy Control Groups

Skin samples from minor surgical ward was colleted from patients who came to ALERT hospital for surgical treatments for skin diseases other than leprosy and who gave consent to take

part in our study.Specimen wascollected from discarded skin after the patients' routine surgical treatment was performed. Skin biopsy samples were collected only from twenty eight non leprosy patients.

4.5 Data Collection Procedures

Senior dermatologist at ALERT hospital clinically screened the leprosy cases to confirm from other suspected skin disorders. After obtaining informed consent/assent from willing patients, a structured questionnaire used to collect the patient's socio-demographic characteristics and clinical data was filled by clinical nurses at Red Medical clinic. Laboratory request forms together with the patients who are clinically diagnosed for leprosy were sent to ALERT hospital central laboratory for SSS collection and routine laboratory diagnosis and to the AHRI pathology unit for skin punch biopsy collection.

4.6 Specimen Collection and Processing

4.6.1 Slit Skin Smear Collection

While collecting SSS for routine ZN diagnosis, the laboratory technologist prepared one additional slide from the same site for Auramine O staining following all the standard and safety measures. Afterfixing the collected smear with gentle flaming, the slide was sent to AHRI laboratory where the Auramine O staining was performed following protocols used by Siddiqui *et al* (47).

4.6.2 Skin Punch Biopsy collection

Under the supervision of the dermatologist, skin biopsy samples were collected using 6 mm skin puncher for the adult and 5mm puncher for child participants by well trained nurses who have experience in skin punch biopsy following the standard procedure. Punch biopsy collection has not been performed on cosmetic and sensitive body parts like face and scrotal area. During and after biopsy collection, all the required ethical and safety procedures has been taken to keep the participants away from any risk and make them comfortable. There were no unusal occurrence during sample collection and all the procedure was performed safely. After collection, the punch

biopsy divided in to two parts using surgical blade; one part immersed in the pre-labeled bottle containing 10% buffered formaldehyde solution for the stainings and the other part kept in nunc tube which contains 70% ethanol for DNA extraction (29, 48).

4.6.3 Leftover Non-leprosy skin sample collection

Nuses working in minor surgery department of ALERT hospital collected about 10 mm skin sample from discarded leftover specimen after the routine surgical procedure and cut it in two parts for 10% formalin and 70% ethanol fixation. They sent the samples to AHRI pathology laboratory after the end of the their routine duty.Similar procedures were perfomed on these non leprosy skin samples like those samples collected from participants affected by leprosy.

4.6.4 Tissue Processing, Embedding and Sectioning

Biopsysamples collected in formaldehyde was preserved inside the bottle for two up to three days. Tissue processing performed using TISSUE-TEK®VIP automatedtissue processor based on the standard tissue processing procedure of AHRI pathology laboratory. The next day the tissue embedded using the tissue embedding station using real-paraffin wax as an embedding medium which has also been used in the infiltration part of tissue processing .

Series of tissue sections prepared at 4μm thickness using a rotary microtome and fixed on one end frosted microscopic slide coated with 50% egg-albumin which islocally prepared adhesive. A total of three slides, each of them containing four consecutive ribbons of section form the same tissue has been prepared for Auramine O, modified FF, and HE staining (49, 50).

4.6.5 DNA Extraction

DNA extraction from70% ethanol fixed skin biopsy samples collected from both clinically confirmed leprosy and non leprosy cases was conducted in AHRI pathlogy lab using Qiamp microbiome extraction kitDNA extraction kit supplied by QigenGmbH, Hilden, Germany. To increase the concentration of extracted pathogen DNA, the final volume of elution buffer was 100 micro litter which is less than that of 200 micro litter specified by the company (51).

4.7 Laboratory Staining Techniques

4.7.1 AFB staining of SSS

One of the slides containing SSS was pre-labeled with the participant's code number. The slide dried for 15 minutes at room temperature and fixed passing slowly through the flame of a spirit burner for 3 times, with the smears upwards position; then covered with 1% carbolfuchsin solution , heated by burning spirit lamp under the slide until vapor begins to rise from the carbolfuchsin and left for 5 minutes. After five minutes the slide washed with running tap water and decolorized with 1% acid-alcohol for 10-20 seconds, rinsed with water gently, counter stained with 0.2% methylene blue for 1minute, washed with tap water and air dried. Finally it was examined with 100X objective of conventional light microscope(52, 53).

4.7.2 Auramine O staining of SSS

The other slide containing the smear was flooded with 0.1% Auramine O solution for 20 minutes on staining rack , then rinsed with sterile water; decolorized with 0.5% acid-alcohol for 2 minutes, then rinsed with sterile water; counterstained with 0.5% Potassium permanganate for 4 minutes, then rinsed with sterile water, air dried and examination under LED fluorescence microscope using 40X objective(33).

4.7.3 Modified FF Staining of Tissue Section

After warming the slide which contain tissue sections inside an oven at 60^0c for few minutes, the slides deparaffinzed in two changes of jars containing two parts of xylene and one part of vegetable oil for 15 minutes. Then the slides blotted well with filter paper to remove the xylene-oil remnant and hydrated with distilled water. Filtered 1%calbolfuchine added onto the slides and kept for 20 minutes with out applying any heat. After washing with tap water the slidedecolorized with 10% H_2SO_4 for 2 minutes. Similarly, after washing the decolorizer with tap water it stained with methylene blue solution for 20 seconds. Just after the specific 20 seconds the slide washed with water, blotted, cleared with xylene, mounted with DPX mounting medium and examined under 100X objective of the microscope (28, 54).

4.7.4 Auramine O Staining of Tissue Section

Similar to FF staining, the slide which containing the sections was warmed inside an oven at 60^0c for few minutes and deparaffinzed in two changes of jar which contain two parts of xylene and one part of vegetable oil, for 15 minutes. After blotting to remove all the xylene-oil remnant and hydrated in distilled water, the slide was flooded with 0.1% Auramine O solution for 20 minutes, then rinsed with sterile water; decolorized with 0.5% acid-alcohol for 2 minutes, again rinsed with sterile water, counterstained with 0.5% Potassium permanganate for 4 minutes, then rinsed with sterile water , blotted , cleared and mounted with DPX mounting medium to make it ready for microscopic examination using LED florescence microscopy at 40X objective. In this particular study, to overcome fading of stored slides; microscopic digital images of all positive slides which show the presence of the bacilli were storedfor quality control purpose (33).

4.7.5 H&E Staining

One of the slides which contain tissue section for H&E staining was deparafinized and rehydrated using dry oven at 60^0c for 30 minutes and in two changes of xylene for 10 minutes and in series of decreasing concentration of alcohol and finally in tape water. The slidestained using Harris hematoxilin reagent for 8 minutes, decolorized in 0.5% acid-alcohol for 3 seconds and differentiated in running tap water . It was also counter stained with 0.5% Eosin for 1 minute. After dehydrated with alcohol and cleared with xylene, the slide was mounted with DPX mounting medium for histopathologic microscopic examination by the pathologist (38).

4.8 Polymerase Chain Reaction (PCR)

Conventional PCR was performed using specific primers previously designed at Global Health Institute, Ecole polytechnique Federale de Laussane,Switzerland to detectRLEP *M. leprae*specific repetitive region. The primers used were 2μM RLEP 7 (5'-TGA GGC TTC GTG TGC TTT GC-3') and 2μM RLEP 8 (5'-ATC TGC GCT AGA AGG TTG CC -3') per sample. We used 1% agarose gel for electrophoresis to run the amplified PCR product. A ladder which was able to show 100-1000 bp product within 100 bp variation has been used. After reading the gel which contain the PCR product using UV radiation producing gel reader, both the soft copy and the hard copy of the gel readings were kept accordingly.

Flowchart of the Study Procedures

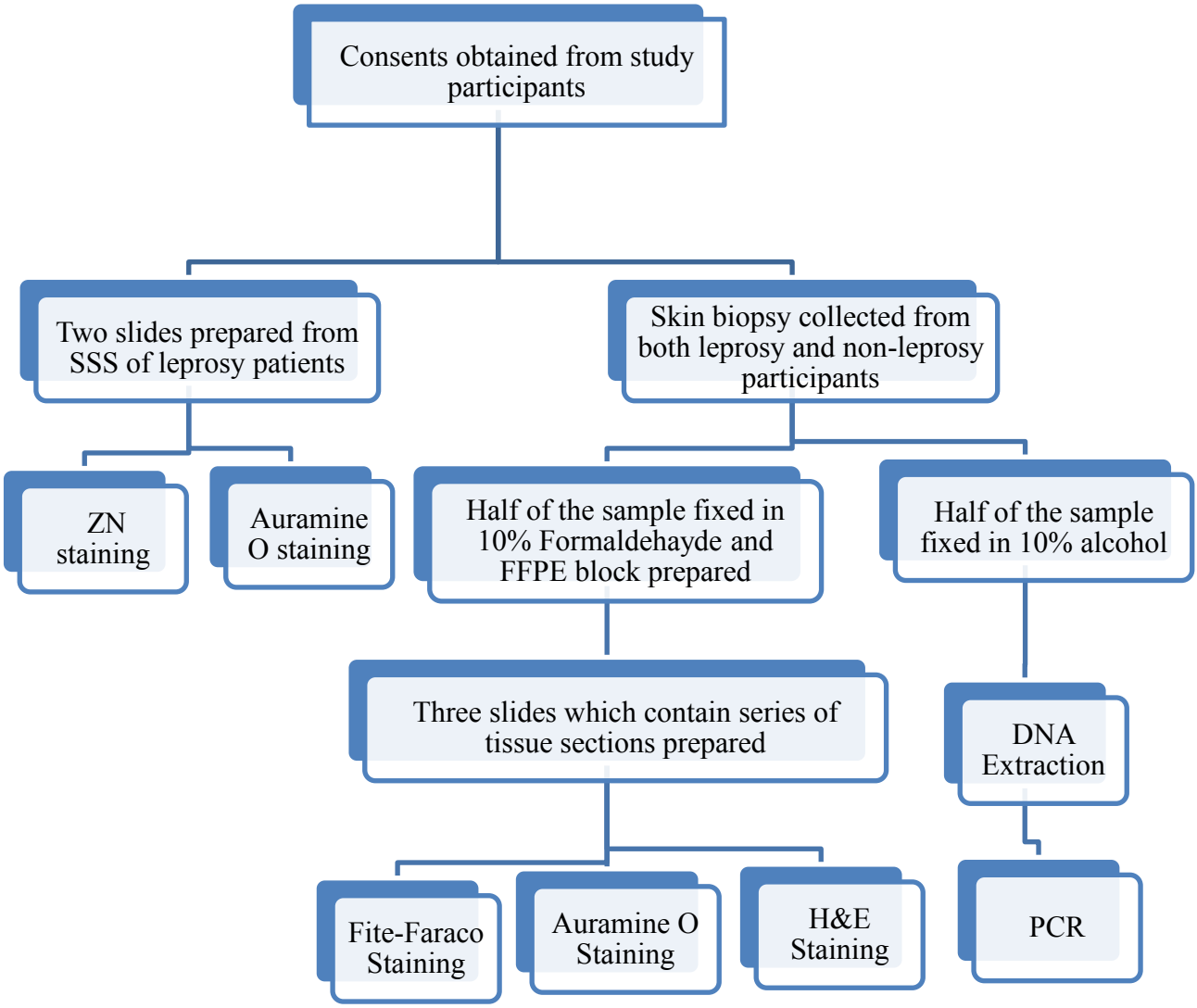


Figure 1.Flow Chartofstudy procedures

4.9 Quality Control

All sample collection procedures conducted based on the standard GCP and GCLP procedures. During the staining procedures and PCR, known positive skin samples for *M.leprae* and known negative skin sample for *M. leprae* were been used as positive and negative control, respectively to run parallel with the participants' sample.All the slide readings were checked by other laboratory technologists blindly.

4.10 Data Management

Socio-demographic data, clinical information and laboratory results were rearranged at AHRI data management unit for software data analysis.The rearranged data were entered in the analyzing software for analysis. The hard copy of all information was kept secured in the data management unit.

4.11 Data Analyses

Since there is no universally recommended gold standard for leprosy laboratory diagnosis, we have established a combination of tests to be used as a gold standard for this specific study. Clicical diagnosis was the necessary part of this combination and it should be supported with atleast onepositive result of the three stainings. These three laboratory testsselectedto supportthe clinical diagnosis are H&E, SSS ZN and FF stainings due to their routine application to diagnose leprosy worldwide.

All obtained Socio-demographic data, clinical information and laboratory results were double eneterd to Stata SE version 11 for statistical analysis. Data which did not require to be recoded like age, number of lesion, nerve involvement and bacterial load entered in the software directly. Catagorical data like microscopy and PCR results, disability, type of reaction and disease classification recoded to numerics before entered to the software. Sensitivity, Specificity, PositivePredictive Value and Negative Predictive Value were calculated including 95% confidence intervals (CI) using the designed alternative gold standard. Kappa value was also calculated to measure the agreement between laboratory technologists blind readings of slides stained with Auramine O.

4.12 Ethical Considerations

Ethical clearance was obtained from AHRI/ALERT Ethical Review Committee (AAERC) and Addis Ababa University College of Health Science , Department of Medical Laboratory Science Ethicsand Research Review Committee (DRERC).The purpose and importance of the study was clearly explained for participants to obtain their full agreement to be involved the study. A written informed consent form for adult participants was signed before recruiting them in the study. Signature of agreement on the consent form for parents and guardian representing their children was also signed to recruit any child in the study. SSS was collected by well-trained laboratory technologist and punch biopsy collection was done by experienced clinical nurses who have been well trained and has been practicing the procedure for many years in AHRI pathology unit for similar research works. Though there was no study participant who withdrew from the study, withdrawal at any stage of the study was possible for participants without any preconditions.

4.13 Dissemination of Results

The findings of this study will be presented to Addis Ababa University Allied Health Science department and Armauer Hansen Research Institute. The findings will also be presented in seminars and symposia. The collective findings of the study will be published in international Journals. Morover, the output of the study will be presented to Ministry of health to improve the laboratory diagnosis of leprosy.

5. Results

5.1. Socio Demographic Characteristics

A total 170 participants were involved in the study during January1, 2015 up to April 30, 2016.Among all particpants 142 were clinically confirmed leprosy cases and 28 were non-leprosy control group. All clinically diagnosedleprosy participants were recruited from Red Medical Center of ALERT hospital. The non-leprosy control participants were recruited from Minor Surgery department, who came for surgical treatment due to different reasons including carcinosis.Five samples collected from leprosy participants were excluded from the analysis due to the absence of SSS ZN result or unable to get good tissue section after tissue processing. The sex proportion was dominanted by male participats contributing 108(63.9%)and the total number of female participants were 61 (36.1%). The mean age of participats was 35. 8(+/-14.6 SD)yrs. ranging beteween 15 and 75 yrs.

Table 1. Age and sex distribution of study participants (n=170)

Variable	Frequency (%)
Age Group	
15-25	46 (27.1)
26-35	61 (35.9)
36-45	24 (14.1)
≥ 46	39 (22.9)
Gender	
Male	108 (63.9)
Female	61 (36.1)

5.2 ClinicalFeatures

Among these clinically confirmed leprosy cases, 19.72% (28/142) of them showed five or less skin lesion and about 80.28 % (114/142) showed 6 or more skin lesions. The smallest duration of hospital visit after experiencing the symptom was one month while one patient explained he had been experiencing the symptom for 14 years. The mean time for patients to visit the health institution after recognizing the syptoms of leprosy was two years.There were 59.57% of leprosy patients who have visited the health institution with observable physical damage. Five (3.5%)of the participants who completed their multi drug treatment were categorized as relapse based on clinical criteria. There were a total of 32 (22.5%) participants presentedwith leprosy reaction classified as Nurites15.6% (5/32), Reversal reaction 68.8% (22/32)and Erythema Nodusum Leprosum 15.6% (5/32). Regarding family history, 36 (25.53%) particicpants were living together with leprosy patient who is or was suffering from leprosy.

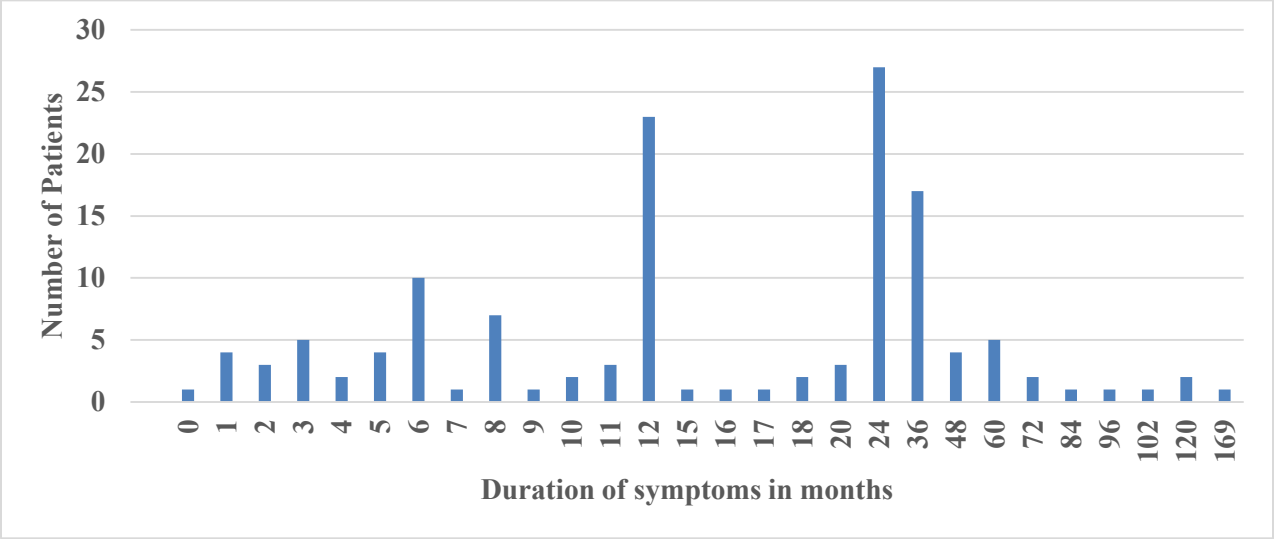


Figure 2. Distribution of duration of symptoms before hospital visit in months.

Among 28 skin sample collected from non-leprosy control group,the dominant number of cases which was reasoned for surgical treatmenet was Carcinosis contributing 32.1% (9/28) . The remaining 67.9% (19/28) samples were collected from participants who came for sugical treatment of different cases including correction amputation.

5.3Auramine O and ZN staining on Skin Slit Smear

Slit Skin samples (SSS) collection were performed on three different body parts of the leprosy patients to increase the probability of detecting acid fast bacili. The right and left earlobe were the common sample collection body sites for all participants and as a third site either one of eyebrows, forhead or one of the the arms were chosen. The routine ZN staining was perfomed for 142 leprosy participants and 5 (3.5%) of them were incomplete dueto different reasons and were excluded from analysis. A total of 137(96.5%) SSS AFB results were anlaysed and the Sensitivity, Specifity, PPV and NPV were 58.8% (95% CI:49.2% - 67.9%) , 100% (95% CI: 85.2% -100%), 100% (95% CI:94.6% -100%) and 32.9% (95% CI:22.1% - 45.1%), respectively.

In the same manner , results from Auramine O stainings performed on all SSS collected from 137 were analysed . The Auramine O staining was also examined by two laboratory technologists blindly. The Sensitivity,Specificity, PPV and NPV were 66.7% (95% CI:57.2% - 75.2%), 100% (95% CI:85.2% - 100%),100% (95% CI:95.3% -100%) and 37.7% (95% CI:25.6% -51%) respectively.The kappa value calculated to show interpersonal agreement between two laboratory technologists who read the slides blindly yielded 0.869 and P value was 0.0000 which is statisticaly significant.

5.4 Histopathologic Examination

Slides stained with H & E staining was reported by an experienced pathologist who maily works on demartopathologic examination of skin samples with different dermatologic disorders. Beyond histopathologic comfirmation of the disease, different histopatholoic features of leprosy like granuloma, epetheloid cells, foamy macrophages ,type of cell infiltration, giant cells and type of inflammation were used to diagnose and classify the disease in to its different forms. The Sensitivity andSpecificity were 86.8% (95% CI:79.2% -92.4%) and 100% (95% CI: 92.9% - 100%), respectively. The NPV and PPV of histopathologic report showed 76.9% (95% CI: 64.8% - 86.5%) and 100%(95% CI: 96.3% - 100%) respectively. The dominant histopathologic disease classification was BT contributing 26.3%(26/99) of histopathologically reported positive samples followed by LL,BL,BB,TT and INT which accounts 24 (24.2%), 20(20.2%), 14 (14.1%), 9(9.1%) and 6(6.1%) respectively.

5.5 Auramine O and Fite-Faraco stainings on Tissue Section

Light Emitting Diode Florocense Microscope was used to evaluate Auramine O staining to look for bacilli in tissue section. All the slides reported by the first laboratory technologist were reexamined blindly by the other one. Any visible clear solid bacilli were reported as a positive. The Sensitivity and Specificity of the staining revealed 78.9%(95% CI: 70.3% - 86%) and 90.2%(95% CI: 78.6% - 96.7%) respectively. While PPV and NPV accounted 94.7%(95% CI: 88.1% - 98.3%) and 65.7% (95% CI: 53.4% - 76.7%) respectively, the kappa and P values were 0.9506 and 0.0000 respectively showing very strong agreement between the readings by the two lab technologists.

Similarly with Auramine O staining, tissue sections stained with Fite-Faraco were examined by two laboratory technologists independently under 100X objective of ordinary lightmicrosope. The agreement between the readings was very strong (k = 0.9630) with P value of 0.0000,whereas the Sensitivity and Specificity were 78.1% (95% CI: 70.8% - 86.5%) and 100% (95% CI: 93% - 100%). The other statistical paramertes PPV and NPV were 100% (95% CI: 95.9% - 100%) and 67.1% (95% CI: 57.1% -79.2%) respectively.

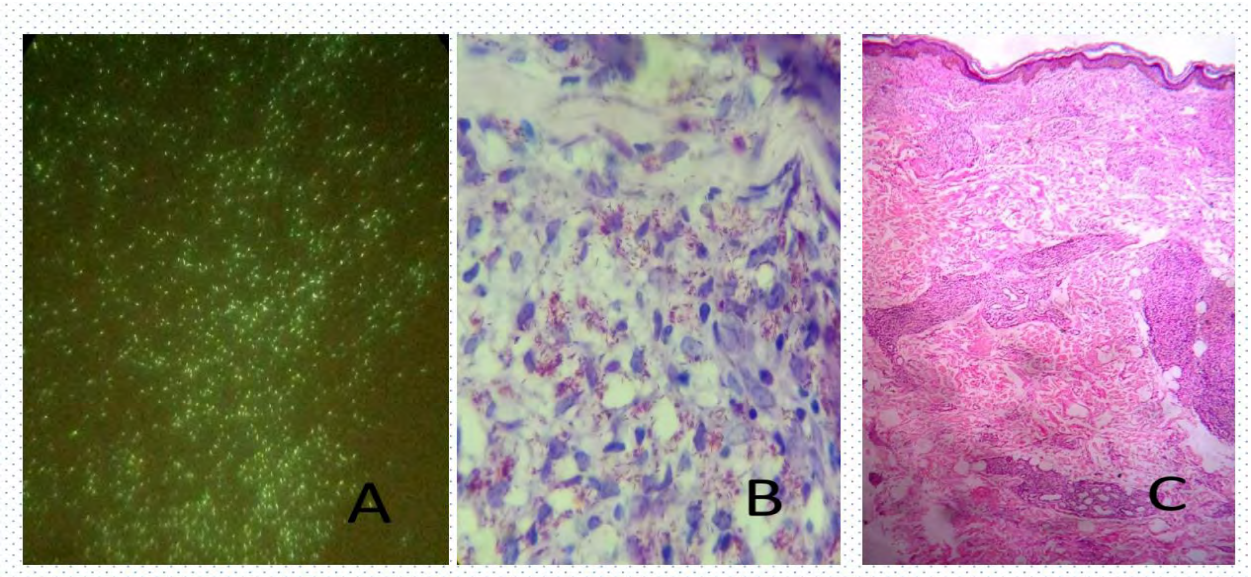


Figure 3.Auramine O (40X), Fite-Faraco (100X) and H&E staining (40X). A: Solid AFB stained with Auramine Ostaining. B: Positive Fite-Faraco staining. C: H&E staining showing typical

histopathologic feature of leprosy (A picture of digital camera mounted on LEDFM and Oridinary Light Microscope).

5.6 *M.leprae* Detection using Polymerase Chain Reaction

All 170 extracted DNA samples were amplified using PCR program specifically developed for *M. leprae* DNA amplification using theremocycler . Among 142 DNA samples from clinically confirmed leprosy cases, 5of them were exluded from data analysis due to their incomplete results. The sensitivity of PCR was 89.5% (95% CI: 82.3% - 94.4%) without any cross reactive positive result on 28 non-leprosy control skin samples with 80.4% (95% CI: 66.9% - 90.2%) specificity.The PPV and NPV were 91.1% (95% CI: 84.2% - 95.6%)and 77.4% (95% CI: 63.8% - 87.7%) respectively.

The sensitivity of PCR to diagnose different forms of leprosy i.e. Neg, INT, TT, BT, BB and BL was 57.9% (22/38), 66.7% (4/6), 66.7% (6/9), 88.5% (23/26), 92.9% (13/14), 100% (20/20) and LL 100% (24/24) respectively. Despite the fact that the extremely low probability of *M.lepromatosis* to be found in Ethiopia, all *M.leprae* PCR negative samples were amplified for the presence of *M.lepromatosis* DNA using specific primers and expectedly all of the sample were negative for the strain.

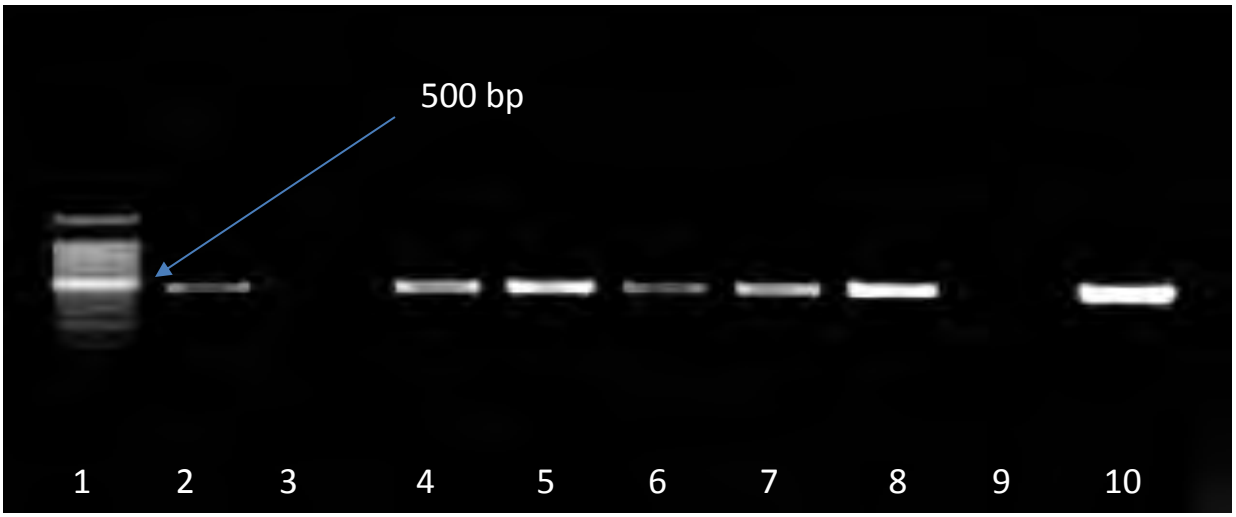
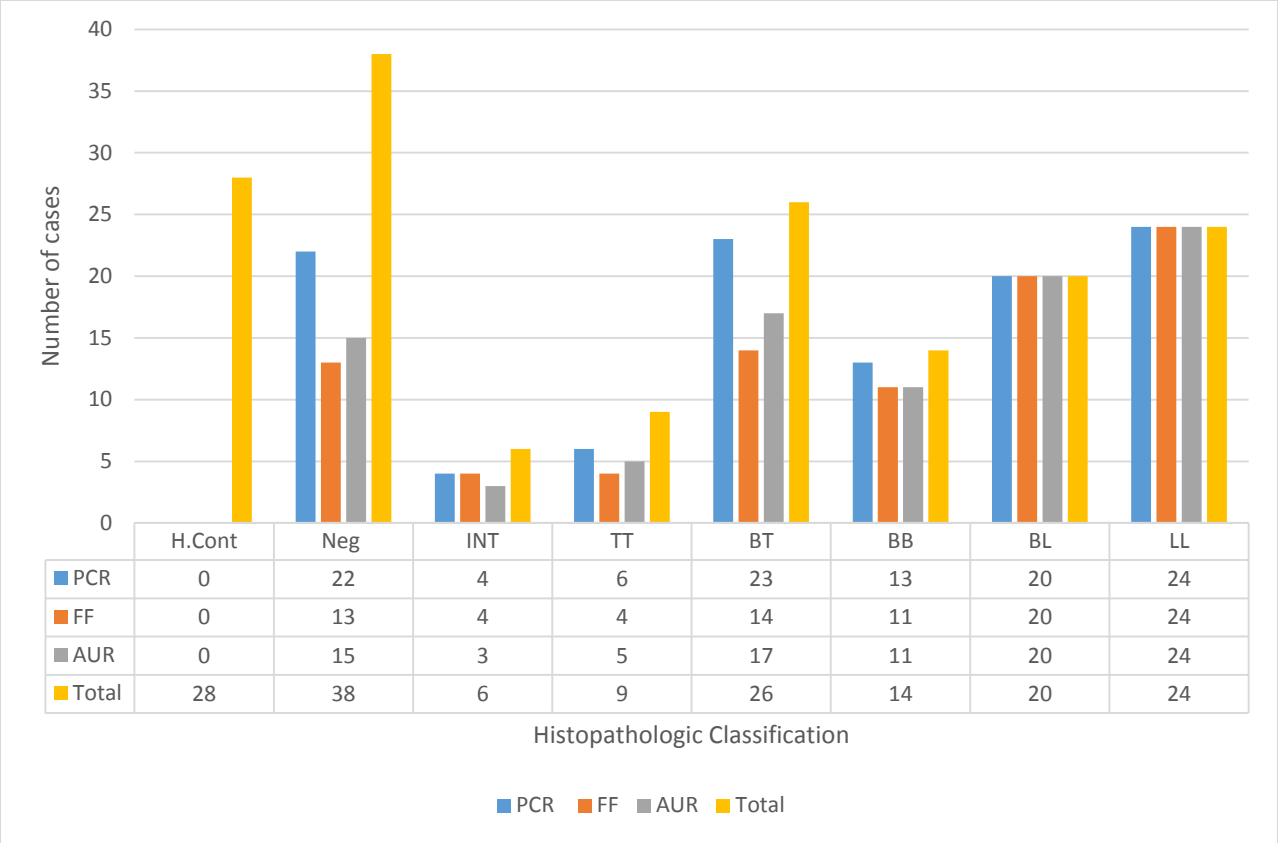


Figure 4.Shows 500 bp bands on Agarose gel showing positive resuts for M. leprae (1. 100bp ladder, 2,4,5,6,7,8. Positive samples, 3. Negative Sample, 9.Negative control, 10. Positive control)

5.7 Performance of Laboratory tests on Histopathologically confirmed Leprosy cases

Based on histopathologic diagnositic classification , the performance of different laboratory tests was compared. Among 137 clinically confirmed cases, a total of 58 (42.3%) samples were histopathologicaly confirmed and classfied under BB, BL or LL,which were expected to contain high bacterial concentration. Almost all of the other laboratory tests had similar reults in this group indicating the samples were positive for*M.leprae.*On the other hand a total of 79 (57.7%)samples wereclassified as BT, TT, INT or Neg which were expected to have low or null basiloscopy count.

Amongst histopathologicaly confirmed tissue samples which were expected to have low or null basiloscopy count, Fite-Faraco, tissue Auramine O and PCR were able to detect 44% (35/79), 51% (40/79) and 70% (55/79) positive results respectively.Thirty eight histopathologically reported as negative samples were clinically leprosy confirmed and the positivity of PCR, Auramine O and Fite-Faraco were 57.9% (22) , 39.5% (15) and 34.2% (13).



	H.Cont	Neg	INT	TT	BT	BB	BL	LL
PCR	0	22	4	6	23	13	20	24
FF	0	13	4	4	14	11	20	24
AUR	0	15	3	5	17	11	20	24
Total	28	38	6	9	26	14	20	24

Figure 5. Graph showing Histopathologic classification of Leprosy with diagnostic potential of different laboratory tests. The graph shows the high diagnostic performance of PCR and Auramine O staining especially in Histopathologically Negative and Pauci Bacilary (INT,TT and BT) classifications which are expected to have low bacterial concentration.

5.8 Discussion

Leprosy diagnosis is mainly dependent on the clinical diagnosis of the disease because of lack of a reliable laboratory diagnostic tests, especially for all types of disease presentation with less concentration of bacilli or inconclusive histopathologic features of the disease. As an alternative laboratory diagnostic method, the potential of Auramine O staining using Light Emitting Diode Flourescense microscope has to our knowledge not been evaluated in Ethiopia. The present study evaluated and confirmed the diagnostic potencial of Auramine O and the other laboratory diagnostic tests.

As ALERT hospital is specialized in dermatology having a number of senior dermatologists, most of the clinically diagnosed leprosy cases in this study were positive for atleast one of the laboratory diagnostic results.This evidence shows how special training or experience can favor the quality of clinical diagnosis. Even for highly trained clinicians, experience and exposure for the diagnosis of different dermatologic disorders has worthful impact to differentiate clinically similar dermatologic disorders with leprosy and due to this fact about 90% of clinically confirmed cases were positive atleast for one laboratory diagnostic tests. About 84 (59.57%) participants of this specific study who came for the sake of medical care after recognizing the symptoms had developed disability which emphasizes the importance of early diagnosis and medical treatment to avoid or reduce disease related physical damage (17).

Though Slit Skin Smear is frequently collected sample from leprosy suspected patients specially in resourse limited areas for AFB staining to look for the presence of bacilli,there are a number of reasons for poor and inconsistent senstitvity of the test including quality of collected sample, quality of the staining, experience of examining technician, concentration of bacilli in the collected sample which is related to the classification of the disease and observers fatigue. As described elsewhere , the sensitivity of skin slit AFB is not more than 60%. A study in India reported the Sensititvity, PPVand NPV of ZN staining were 59.9%, 69%, and 45.7%, respectively(28)with comparable sensitivity, 58.8% of this study . Whereas similar study done in Brazil and India showed that sensitivity of ZN staining on slit skin smear was 26.7% ,30.5%, 40.3% and 50% respectively with 100% specificity. Though the specificity is consistnent with our study, most studies showed relatively lower sensitivitycompared with our study. The main possible reason for this variation of sensitivity is involvmenet of considerable number of

participants in the studies who are PB cases which mostly give negative SSS AFB result (25-27, 30).

Evaluation of diagnostic performance of Auramine O on SSS has showen a better yield of positivity than that of routne ZN staining in this study. Though there are no published data on the senstitvity of Auramine O staining using LEDFM, the obtained sensitivity in this study of 66.7% was with very good agreement between the two readings of two blinded laboratory technolgists supported by 0.869 kappa value. The better sensitivity of Auramine O staining on the same SSS was due to the ease to detect bacilli stained with fluorescent dye and it was very easy to cover the whole stained field within a short period of time without fatigue using 40X objective of the LEDFM.

Beyond its main application in disease diagnosis and prognosis, H&E staining is also used in leprosy classification to assist the decision of treatment duration accordining to the classification of the disease as either PB or MB. Emphasizing the importance of histopathologic examination specially in doubtful cases of leprosy, different studies showed valuable sensitivity of histopathologic examination for the diagnosis of the disease(6). The sensitivity of histopathology diagnosis in this study (86.8%) is relatively very high when compared with the sensitivities 58.2%, 67.6% and 78% of different studies conducted in India and Brazil(28, 31, 32) .The main reason for this high sensititvity obtained in this study could be the high proportion of MB participants and experience of dermatologists at ALERT hospital to identify and send very distinctive leprosy patients to the pathology laboratory. Further more in this study we have developed an alternative gold standard which includes clinical diagnosis, Histopathology, FF and SSS ZN results rather than taking only clinical diagnosis as a gold standard.

Together with its appreciated high specificity, Fite-Faraco staining is a widely accepted laboratory diagnostic test for leprosy on tissue sections with commendable sensititvity. Its combinations with different laboratory tests like PCR and H&E will also have high yield of positivity to support the clinical diagnosis as explained in the study in India by Reja AH et al (28). Though its specificity is very high, the sensitivity of Fite-Faraco staining will also be affected by the type of the disease to be diagnosed like most of the other laboratory diagnostic tests of leprosy. Nayak SV and Reja AH from India reported in their study that the sensitivity of

Fite-Faraco staining was 44.6% and 60% respectively. Partcipants involved in the study conducted by Nayak SV were dominantly PB cases and can be considered as the reason forlower sensitivity than our current sudy, which is 78.9%. Though the sensitivity in our study seems closer with that of the study by Reja AH, still the type of leprosy could be the basic reason for this considerable variation since about 80% of participants in our study were operationally classified as MB (28, 55).

As mentioned in different studies, diagnosis of leprosy in any forms of the disease still requires better sensitive diagnostic tools to increase the positivity of the results. Fluorescence staining for the diagnosis of leprosy using conventional flourescent microscope has been eveauated to check its alternative use in the diagnosis.Considering difficulties of detecting bacilli in IL,TT and BT forms of the disease, a study by Nagarajappa A in India showed the sensitivity of Auramine-Rhodamine flouresent stainng to detect *M.leprae* from clinically confirmed particpants was 90% which was not consistent with 72.3% of detection rate from clinicaly confirmed participants. In the same study Sesitivity (100%,), Specificity (25.4%), NPV (100%) and PPV (12.9%) of flouresent staining compaired with Fite-Faraco staining was not that much consistent with our study's Sensitivity (78.9%), Specificity (100%), NPV (100%) and PPV(66.7%) were, and respectively, The significant variation of reporting positive results by Fite-Farco 22.9% (16/70) and Auramine- Rhodamine 90% (63/70) from clinically suspected participants in the study of of Nagarajappa A *et al* by itself was very inconsistent. This may be due to either underreport of Fite-Faraco or over report of Auramine- Rhodamine positive results (33).

Recommended as a potential sensitive diagnostic tool for leprosy laboratory examination, PCR test was proven for its highst sensitivity of all laboratory diagnostic tests with additional recommendation for diagnosis of different types of leprosy in different studies. Sensitivity reported by Turankar RP, Job CK and Banerjee S *et al*. performing PCR on SSS,unbroken skin wash and nasal swab were 83%, 80% and 86% respectively (40-42). Regardless of the different sample types (SSS, nasal swab or unbroken skin wash) used in these studies, the reported sensitivities were comparable with our study which is 89.5%. The possible reason for slightly higher sensitivity (89.5%) could be dueto using DNA extracted from skin biopsy.

The Brazilian research group study (43) was similar with our study due to similar gene target which they amplified and the type of sample used extracting DNA. Eventhough theyrecruited a relatively high number of PB cases , the sensitivity and specificity found was73.6% and 100% resepctively. In our study, the total senstitvity of PCR was 89.5% with 80.4% specificity.The possible reason for this variation of sensitivity icould be probably recruitment of high number of MB cases in our study which has direct relationship with bacterial concentration. Moreover, in our study we used a combination of tests as gold standard and this certainly has high sensititvity than using only AFB staining as a gold standard like in the Brazilian study . The relatively high sensitivity observed in our study using a gold standard with better sensititvity has made the specificity lower than the Brazilian study.

The same study in Brazil (43) reported that PCR has sensitivity of 40% for TT, 55.5% for BT, and 100% for all BB, BL and LL cases. This was very good evaluation to support that PCR can improve the diagnostic challenge of PB cases which mostly have negative bacterial index. In our study, the sensititvity of PCR was 66% for TT, 88% for BT and 93% for BB and 100% for both BL and LL cases. The result for BB,BL and LL case was very comparable with that of the Brazilian study but the sensitivity which we found for PB cases was higher than the Brazilian study. The possible reason for this variation could be the higher sensititvity of the primer pair we used in this study and it could be recommended for the routine diagnostic purpose.

In this specific study, we alternatively compared the sensitivity of PCR with that of the established gold standard taking clinical diagnosis as a reference for comparison. The sensitvity of PCR and the designed gold standard which incorporated three laboratory tests were 81.8% and 83.2% respectively. This comparable result of sensitivity shows the ideal diagnostic potential of a single run of PCR for diagnosis of the disease and support the clinical diagnosis by detecting cases within a short period of time. Moreover its 70% (55/79) sensitivity among leprosy classes expected to have low or null bacilloscopy and sensititviy of 57.9% (22/38) among histopathologically reported negative samples was highly encouraging.

5. 9 Conclusion and Recommendation

5.9.1 Conclusion

This particular study evaluated the diagnostic potential of Auramine O staining using Light Emitting Diode Florocese Microscope and it is possibly the first study evaluating LED-FM for the diagnosis of leprosy. Auramine O staining has been confirmed for a better sensitivity,66.7% than the routine AFB staining,58.5% on Slit Skin Smear and Fite-Faraco staining on tissue sections. Beyond its better sensitivity in general, its diagnostic support to the clinical diagnosis of PB type leprosy is very helpful based on its comfortable and easiness to perform the diagnosis using LED-FM.

The other interesting outcome of this study is the the remarkably good sensitivity of PCR for the diagnosis of all type of leprosy in general and PB type in specific. In addition to the usual clear interpretation of PCR result which bases on specific base pair bands visible on agarose gel, the fast and easy DNA extraction does not need special expertisies of laboratory perssonel beyond average training. In PB type of leprosy, which mostly confuses the clinicians with other type of skin diseases, PCR may play a great role as a differential diagnosis to reach to the correct diagnosis of leprosy.

5.9.2 Recommendation

- In this study laboratory diagnosis of leprosy using LED-FM and Auramine O staining showed better sensitivity than the routine AFB staining. Therefore, Auramine O staining could replace the routine AFB staining even at health center level if enough training is provided for the laboratory personnel.

- PCR should be implemented as a routine laboratory diagnostic tool at least at refereal level where the PCR facility is available. And in the future emphasis should be given to implement PCR diagnostic facility at selected hospitals to provide better service nearest to leprosy affected and suspected individuals.

- Fite-Faraco staining should always be performed along with H&E staining for histopathologic diagnosis to support the clinical diagnosis as significant number of cases were positive by Fite-Faraco in this study which are missed by H&E.

- As there is a siginificant number of relapse cases detected in this study and due to strong association of relapse cases with drug resistance, diagnosis of drug resisitance pattern of *M.lepra* should be investigated specially on relapse cases and on patients who did not respond to MDT. Drug resistance tests should be integrated with the routine leprosy diagnosis for timely prevention of the occurrence and dissemination of future MDR strain in the country.

6. Limitation and Strength of the Study

6.1 Limitation

Knowing the samples collected from clinically diagnosed leprosy patients has an impact on the laboratory technologist that he could not analyse laboratory tests blindly.

6.2 Strength

To our knowledge there was no report on the diagnosis of leprosy using Light Emitting Diode Florosence microscope (LEDFM) and will add its contribution in low income leprosy endemic countries for early diagnosis.

7. References

1. Barbieri RR, Sales AM, Illarramendi X, Moraes MO, Nery JA, Moreira SJ, et al. Diagnostic challenges of single plaque-like lesion paucibacillary leprosy. Memorias do Instituto Oswaldo Cruz. 2014;109(7):944-7.

2. Singh A, Weng X, Nath I. Skin Biopsy in Leprosy. National Institute of Pathology (ICMR), Safdajung Hospital Campus, New Delhi,. November, 2011;5:73-86.

3. WHO. World Health Organization Weekly epidemiological record. 2015.

4. Martinez AN, Ribeiro-Alves M, Sarno EN, Moraes MO. Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens. PLoS neglected tropical diseases. 2011;5(10):e1354.

5. Pardillo FE, Fajardo TT, Abalos RM, Scollard D, Gelber RH. Methods for the classification of leprosy for treatment purposes. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2007;44(8):1096-9.

6.Thakkar S, Patel SV. Clinical profile of leprosy patients: a prospective study. Indian journal of dermatology. 2014;59(2):158-62.

7. Gupta R, Kar HK, Bharadwaj M. Revalidation of various clinical criteria for the classification of leprosy--a clinic-pathological study. Lepr Rev. 2012;83(4):354-62.

8. Buhrer-Sekula S, Smits HL, Gussenhoven GC, van Leeuwen J, Amador S, Fujiwara T, et al. Simple and Fast Lateral Flow Test for Classification of Leprosy Patients and Identification of Contacts with High Risk of Developing Leprosy. Journal of Clinical Microbiology. 2003;41(5):1991-5.

9. WHO. Global Strategy for Further Reducing the Leprosy Burden and Sustaining Leprosy Control Activities. 2005.

10. Geluk A, Bobosha K, van der Ploeg-van Schip JJ, Spencer JS, Banu S, Martins MB, et al. New Biomarkers with Relevance to Leprosy Diagnosis Applicable in Areas Hyperendemic for Leprosy. Journal of Immunology (Baltimore, Md : 1950). 2012;188(10):4782-91.

11. Mandal BC, Bandyopadhyay G. Leprosy mimicry of lupus vulgaris and misdiagnosis of leprosy--a case report. Indian journal of leprosy. 2012;84(1):23-5.

12. Federal Mistry of Ethiopia, Tuberculosis, Leprosy and TB/HIV Prevention and Control Programme manua, fourth edition. 2008.

13. Shahab UA, Mostaque A, Rahman A. Role of Florourchrome stain in the diagnosis of Lyprosy. JCMCTA. 2008;19(1):15-6.

14.Salvatore N, Pieter AM, Bernard N. The Diagnosis of Lyprosy. Lyprosy mailing list. 2011.

15. Banerjee S, Biswas N, Kanti Das N, Sil A, Ghosh P, Hasanoor Raja AH, et al. Diagnosing leprosy: revisiting the role of the slit-skin smear with critical analysis of the applicability of polymerase chain reaction in diagnosis. International journal of dermatology. 2011;50(12):1522-7.

16. Bhamidi S, Scherman MS, Jones V, Crick DC, Belisle JT, Brennan PJ, et al. Detailed structural and quantitative analysis reveals the spatial organization of the cell walls of in vivo grown Mycobacterium leprae and in vitro grown Mycobacterium tuberculosis. The Journal of biological chemistry. 2011;286(26):23168-77.

17. Kumar B, Dogra S. Leprosy: A disease with diagnostic and management challenges! Indian journal of dermatology, venereology and leprology. 2009;75(2):111.

18. Garbino JA, Ura S, Belone AdFF, Marciano LHSC, Fleury RN. Clinical and diagnostic aspects of the primarily neural leprosy. Hansen int. 2005;29(2):130-6.

19. Mahajan R, Dogra S, Kaur I, Yadav S, Saikia UN, Budania A. Type II reaction without erythema nodosum leprosum masquerading as lymphoma. Lepr Rev. 2012;83(4):378-83.

20. Rath D, Bhargava S, Kundu BK. Leprosy mimicking common rheumatologic entities: a trial for the clinician in the era of biologics. Case reports in rheumatology. 2014;2014:429698.

21. Salvi S, Chopra A. Leprosy in a rheumatology setting: a challenging mimic to expose. Clinical rheumatology. 2013;32(10):1557-63.

22. Sheetal S, Arvind C. Lest we forget Hansen's disease (leprosy): an unusual presentation with an acute onset of inflammatory polyarthritis and the rheumatology experience. International journal of rheumatic diseases. 2009;12(1):64-9.

23. Yang S, Makredes M, O'Donnell P, Levin NA. A case of Hansen Disease presenting as tinea versicolor. Dermatology online journal. 2013;19(4).

24. Torres P, CamarinA J, Gomeze R, Olmos A, Navarro C, Olmos A. Comparision of PCR mediated amplification of DNA nad the classical method for detection of mycobacterium leprae in different types of clinical samples in Lyprosy patients and contacts. Lepr Rev. 2003;74:18-30.

25. Bhushan P, Sardana K, Koranne RV, Choudhary M, Manjul P. Diagnosing multibacillary leprosy: a comparative evaluation of diagnostic accuracy of slit-skin smear, bacterial index of granuloma and WHO operational classification. Indian journal of dermatology, venereology and leprology. 2008;74(4):322-6.

26. Sarker UK, Mohammad QD, Uddin MJ, Chowdhury RN, Bhattacharjee M, Mondol G, et al. Socio-demographic characteristics, types and Slit Skin Smear (SSS) of the leprosy patients: a hospital based study. Mymensingh medical journal : MMJ. 2014;23(3):435-40.

27. Jaswal TS, Jain VK, Jain V, Singh M, Kishore K, Singh S. Evaluation of leprosy lesions by skin smear cytology in comparison to histopathology. Indian journal of pathology & microbiology. 2001;44(3):277-81.

28. Reja AH, Biswas N, Biswas S, Dasgupta S, Chowdhury IH, Banerjee S, et al. Fite-Faraco staining in combination with multiplex polymerase chain reaction: a new approach to leprosy diagnosis. Indian journal of dermatology, venereology and leprology. 2013;79(5):693-700.

29. Deepa AS. A comparative study of Zehil-Nillson, Modified Fite-Faraco with Auramine-Rodamine staining in detection of mycobacterium leprae in tissue sectionShri BM Medical Collgee Hospital and Research Center, Bijapur 2010.

30. Rosa FB, Souza VC, Almeida TA, Nascimento VA, Vasquez FG, Cunha Mda G, et al. Detection of Mycobacterium leprae in saliva and the evaluation of oral sensitivity in patients with leprosy. Memorias do Instituto Oswaldo Cruz. 2013;108(5):572-7.

31. Ray R, Mondal RK, Pathak S. Benefits and limitations of fine needle aspiration cytology in the diagnosis and classification of leprosy in primary and secondary healthcare settings. Cytopathology : official journal of the British Society for Clinical Cytology. 2015;26(4):238-43.

32. Teixeira AC, Cruvinel DL, Roma FR, Luppino LF, Resende LH, Sousa T, et al. Evaluation of the agreement between clinical and laboratorial exams in the diagnosis of leprosy. Revista da Sociedade Brasileira de Medicina Tropical. 2008;41 Suppl 2:48-55.

33. Nagarajappa A, Prabhu D. Sensitivity Of Fluorescent Microscopy In Detecting Mycobacterium Leprae In Tissue Sections. IJPA. 2010;11(2).

34. Sarita S, Muhammed K, Najeeba R, Rajan GN, Anza K, Binitha MP, et al. A study on histological features of lepra reactions in patients attending the Dermatology Department of the Government Medical College, Calicut, Kerala, India. Lepr Rev. 2013;84(1):51-64.

35. Lavania M, Jadhav RS, Turankar RP, Chaitanya VS, Singh M, Sengupta U. Single nucleotide polymorphisms typing of Mycobacterium leprae reveals focal transmission of leprosy in high endemic regions of India. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2013;19(11):1058-62.

36. Mendum TA, Schuenemann VJ, Roffey S, Taylor GM, Wu H, Singh P, et al. Mycobacterium leprae genomes from a British medieval leprosy hospital: towards understanding an ancient epidemic. BMC genomics. 2014;15:270.

37. Martinez AN, Talhari C, Moraes MO, Talhari S. PCR-based techniques for leprosy diagnosis: from the laboratory to the clinic. PLoS neglected tropical diseases. 2014;8(4):e2655.

38. Wen Y, You YG, Yuan LC, Yuan YH, Zhang Y, Duthie MS, et al. Evaluation of novel tools to facilitate the detection and characterization of leprosy patients in China. BioMed research international. 2014;2014:371828.

39. Yan W, Xing Y, Yuan LC, De Yang R, Tan FY, Zhang Y, et al. Application of RLEP real-time PCR for detection of M. leprae DNA in paraffin-embedded skin biopsy specimens for diagnosis of paucibacillary leprosy. The American journal of tropical medicine and hygiene. 2014;90(3):524-9.

40. Turankar RP, Pandey S, Lavania M, Singh I, Nigam A, Darlong J, et al. Comparative evaluation of PCR amplification of RLEP, 16S rRNA, rpoT and Sod A gene targets for detection of M. leprae DNA from clinical and environmental samples. International journal of mycobacteriology. 2015;4(1):54-9.

41. Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, Guha S, et al. Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts--a pilot study from India. BMC infectious diseases. 2010;10:252.

42. Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: a study of skin and nasal secretions of household contacts of leprosy patients using PCR. The American journal of tropical medicine and hygiene. 2008;78(3):518-21.

43. Goulart IM, Cardoso AM, Santos MS, Goncalves MA, Pereira JE, Goulart LR. Detection of Mycobacterium leprae DNA in skin lesions of leprosy patients by PCR may be affected by amplicon size. Archives of dermatological research. 2007;299(5-6):267-71.

44. Kamal R, Natrajan M, Katoch K, Katoch VM. Evaluation of diagnostic role of in situ PCR on slit-skin smears in pediatric leprosy. Indian journal of leprosy. 2010;82(4):195-200.

45. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, et al. Evaluation of diagnostic tests for infectious diseases: general principles. Nature reviews Microbiology. 2010;8(12 Suppl):S17-29.

46. Vora RV, Pilani AP, Mehta MJ, Chaudhari A, Patel N. De-novo histoid hansen cases. Journal of global infectious diseases. 2014;6(1):19-22.

47. Siddiqui MR, Velidi NR, Pati S, Rath N, Kanungo AK, Bhanjadeo AK, et al. Integration of leprosy elimination into primary health care in orissa, India. PLoS One. 2009;4(12):e8351.

48. Kumar A, Girdhar A, Girdhar BK. Six monthsfixed duration multidrug therapy in paucibacillary leprosy: risk of relapse and disability in Agra PB cohort study. BMJ open. 2012.

49. Barbieri RR, Sales AM, Illarramendi X, Moraes MO, Nery JAdC, Moreira SJM, et al. Diagnostic challenges of single plaque-like lesion paucibacillary leprosy. Memorias do Instituto Oswaldo Cruz. 2014;109(7):944-7.

50. Ray R, Mondal RK, Pathak S. Benefits and limitations of fine needle aspiration cytology in the diagnosis and classification of leprosy in primary and secondary healthcare settings. Cytopathology : official journal of the British Society for Clinical Cytology. 2014.

51. Huijsmans CJ, Damen J, van der Linden JC, Savelkoul PH, Hermans MH. Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effect on downstream molecular applications. BMC research notes. 2010;3:239.

52. Paula Vaz Cardoso L, Dias RF, Freitas AA, Hungria EM, Oliveira RM, Collovati M, et al. Development of a quantitative rapid diagnostic test for multibacillary leprosy using smart phone technology. BMC infectious diseases. 2013;13:497.

53. Guerrero MI, Colorado CL, Torres JF, León CI. ¿Es la resistencia de Mycobacterium leprae a los medicamentos un verdadero motivo de preocupación? Primera aproximación a la vigilancia molecular de pacientes colombianos multibacilares con tratamiento previo para lepra y sin él. Biomédica. 2013;34(0):137.

54. Geluk A, Klein MR, Franken KL, van Meijgaarden KE, Wieles B, Pereira KC, et al. Postgenomic approach to identify novel Mycobacterium leprae antigens with potential to improve immunodiagnosis of infection. Infection and immunity. 2005;73(9):5636-44.

55. Nayak SV, Shivarudrappa AS, Mukkamil AS. Role of fluorescent microscopy in detecting Mycobacterium leprae in tissue sections. Annals of diagnostic pathology. 2003;7(2):78-81.

8. Annex

I. Information sheets(English version)

Information sheets for Leprosy patients

Tittle of the study -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

General Information

Mycobacterium leprae (*M. leprae*) is the causative agent of Leprosy which is a chronic infectious disease. The disease mainly involves the skin and peripheral nerves and it is mainly transmitted by contact between cases of leprosy and healthy persons and also by respiratory route. In Ethiopia, the trend of new case detection for the last eight consecutive years was in average 4200 per year. In low and middle income countries like Ethiopia, the routine laboratory diagnosis has less performance and some studies are required to increase the diagnostic performance of these tests and this will help to detected leprosy cases and get treated before it causes severe damage on patients.

Therefore,you are invited to participate in this study for which we would like to use the Skin Slit Smear (SSS) and Skin Punch Biopsy. SSS sample means a sample which the laboratory technologist will take from you using sterile surgical blade for your routine diagnostic purpose. We are also going to use skin punch biopsy for a research purpose which is going to be used by highly trained nurses using standard skin puncher. So we kindly request to give some of your time to read and understand the prepared explanation and ask any kind of question which is not clear for you. You have a full right not to be involved in this study or even to terminate the participation in the middle of the study and this will never affect your routine diagnosis and treatment.

Purpose:

The purpose of this study is to evaluate the diagnostic performance of laboratory diagnostic methods of leprosy. The output of this study will help to improve the laboratory diagnosis of leprosy. By making the detection of the disease early, the patient will start his/her treatment soon and it is possible to prevent the probability of occurrence of disability due to the delayed diagnosis and treatment.

Procedures to be carried on

You are invited to participate in the study, after giving your consent you will give 6 or 5 millimeters (≈ the size of the punched 6 mm paper) of skin punch biopsy samples based on your age and two to three small drops of skin slit juice for the preparation SSS. We will do an investigation for Mycobacterium leprae at Armauer Hansen Research Institute Histopathology laboratory. Half of the skin sample will be sent abroad Ethiopia for molecular analysis. The obtained clinical and demographic data will be used to investigate which type of leprosy will found at which type part of the country and this will help to take early preventive and curative measures by the concerned bodies.

The informed consent will be given to senior clinical nurse who is working at Red Medical clinic to serve leprosy patients for long period of time. The assigned experienced nurse will take the consent and required data in separate room which found in the clinic to assure your privacy. She/he also take demographic and clinical data from you because this data has great value in the study as it will help to correlate the clinical and demographic data with the lab diagnostics methods and it will also help to know some factor which will lead the organism to resist the treatment. After you gave the consent the runner who is assigned for this duty will take you to the sample collection area. The skin punch biopsy will be collected by very experience nurse assigned for this specific duty and the skin slit smear will be collected by the laboratory technologist in ALERT hospital who is practicing the procedure day to day. All the laboratory diagnosis will be performed at AHRI laboratory. Some of the skin sample will be sent abroad for molecular diagnosis. You have full right not to give biopsy sample.

Risks associated with the study:

There is a probability of risk associated with the skin punch biopsy and SSS collection. Most side effects are short term which include slight bleeding or bruising, tenderness, some people may experience pain during the procedure, infection and scarring. The dermatologist together with the well experienced clinical nurses in the sample collection area is able to control all these rarely happening risks, immediately. If the participant senses any sign of infection at the site from which the sample collected, he/she should communicate the focal person with the attached telephone number for appropriate treatment.

Benefits of the study:

There will be no financial or other direct benefit to you. But the result of the study will play a role in Leprosy control program, in the country.

Compensations:

You will get thirty birr (30.00 birr) as a compensation for thetimeyou spent in the study. If there is any related risk with the sample collection you will get free medical care and transportation cost will be covered.

Confidentiality of your information:

The results of the lab findings will be kept confidential and could only be accessed by the researcher and the responsible physician. There will be no personal information to be attached to your data. Separate registration form will be prepared to give a code number for individual participants and this registration code will kept very confidentially. To keep the confidentiality of the participants we will use the necessary codes given to the participants while analyzing results. All collected raw data will be stored at AHRI data management unit in secured manner.

The collected sample for this study will be used only for this particular study. Your samples will not be sold or used directly to produce commercial products. The left over will stored in the institute based the rule and regulations of Ethics. Research studies that want to use the left over samples will be reviewed by the Institutional Review Committee andtheNational Ethical Research Committee.

Termination of the study:

We will respect your decision if you later on changed your mind. Your withdrawal of consent will not affect your right to receive medication. The collected sample will be used only for your routine diagnosis and treatment. We are not using your sample for our study if you have decided to withdraw yourself from the study.

If you have any question please contact

Selfu Girma (principal investigator)

Cell phone: +251-913-261720

AAERC Secretary

Phone: +251-011-8962183

Information sheet for control group (non-leprosy cases)

Tittle of the study -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

General Information

Mycobacterium leprae (*M. leprae*) is the causative agent of Leprosy which is a chronic infectious disease. The disease mainly involves the skin and peripheral nerves and it is mainly transmitted by contact between cases of leprosy and healthy persons and also by respiratory route. In Ethiopia, the trend of new case detection for the last eight consecutive years was in average 4200 per year. In low and middle income countries like Ethiopia, the routine laboratory diagnosis has less performance and some studies are required to increase the diagnostic performance of these tests and this will help to detected leprosy cases and get treated before it causes severe damage on patients.

Therefore, you are invited to participate in this study for which we would like to use Skin Biopsy which is discarded after your routine surgical treatment. Nurses who are involved in your routine treatment are going to collect about 10 mm of skin biopsy for the research purpose from the discarded skin sample after your routine treatment. So we kindly request to give some of your time to read and understand the prepared explanation and ask any kind of question which is not clear for you. You have a full right not to be involved in this study or even to terminate the participation in the middle of the study and this will never affect your routine diagnosis and treatment.

Purpose:

The purpose of this study is to evaluate the diagnostic performance of laboratory diagnostic methods of leprosy. The output of this study will help to improve the laboratory diagnosis of leprosy. By making the detection of the disease early, the patient will start his/her treatment soon and it is possible to prevent the probability of occurrence of disability due to the delayed diagnosis and treatment.

Procedures to be carried on

After you routine surgical procedure for your medical care, about 10 millimeters sample will be collected from the leftover skin. This sample will be divided in to two parts and used for histopathology and molecular diagnosis to be used as negative control.

Risks associated with the study:

As long as the sample is going to be collected from the discarded skin, there is no probability of associated risk.

Benefits of the study:

There will be no financial or other direct benefit to you. But the result of the study will play a role in Leprosy control program, in the country.

Compensations:

You will get thirty birr (30.00 birr) as a compensation for the time you spent in the study.

Confidentiality of your information:

The results of the lab findings will be kept confidential and could only be accessed by the researcher and the responsible physician. There will be no personal information to be attached to your data. Separate registration form will be prepared to give a code number for individual participants and this registration code will kept very confidentially. To keep the confidentiality of the participants we will use the necessary codes given to the participants while analyzing results. All collected raw data will be stored at AHRI data management unit in secured manner.

The collected sample for this study will be used only for this particular study. Your samples will not be sold or used directly to produce commercial products. The left over sample will be stored in the institute based on the rule and regulations of Ethics. Research studies that want to use the left over samples will be reviewed by the Institutional Review Committee and the National Ethical Research Committee.

Termination of the study:

We will respect your decision if you later on changed your mind. Your withdrawal of consent will not affect your right to receive medication. The collected sample will be used only for your routine diagnosis and treatment. We are not using your sample for our study if you have decided to withdraw yourself from the study.

If you have any question please contact

Selfu Girma (principal investigator)

Cell phone: +251-913-261720

AAERC Secretary

Phone: +251-118-962183

II. Consent and Assent forms.

Consent form form for leprosy patients (Age ≥ 18 years) (English version)

Tittle of the study -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

1. I, the undersigned person, understand that this study is going to be conducted on Comparing laboratory diagnosticmethods of Leprosy.
2. I understand that if I am enrolled in this study, 6 mm of skin punch biopsy and three drops of Skin Slit Juice form the affected site of the skin. Half of the skin sample will be sent abroad Ethiopia for molecular analysis.
3. I understand that collection of punch biopsy may cause short term side effects andit may include slight bleeding or bruising, even though collected experienced professionals. The dermatologist will control all these rarely happening risks, immediately.
4. If my result is positive I will be treated according to the national guideline.
5. I understand that all the results will be explained to me and all the data obtained will be kept strictly confidential by using only code numbers.
6. I am clear for all explanation and agree to participate in the study. I put my signature for my agreement.

Participant Name…… Name of Witness ……..

Signature ……. Signature …….

Date …….. Date ……..

Consent form fornon-leprosy cases (Age ≥ 18 years) (English version)

Tittle of the study -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

1 I, the undersigned person, understand that this study is going to be conducted on Comparing laboratory diagnosticmethods of Leprosy.
2 I understand that if I am enrolled in this study, 10 mm of skin biopsy will be collected from the left over tissue.
3 If my result is positive I will contacted for the dermatologist for further investigation and care, if required.
4 I understand that all the results will be explained to me and all the data obtained will be kept strictly confidential by using only code numbers.
5 I am clear for all explanation and agree to participate in the study. I put my signature for my agreement.

Participant Name…… Name of Witness ……..

Signature ----------Signature …….

Date …… Date ……..

Assent form for leprosy patients (Age15 to 17 years) (English version)

Tittle -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

I, the undersigned person, understand that this study is going to be conducted on Comparing laboratory diagnostic methods of Leprosy.

1. I understand that if my child is enrolled in this study, 5 mm of skin punch biopsy and three drops of Skin Slit Juice form the affected site of the skin.Half of the skin sample will be sent abroad Ethiopia for molecular analysis.
2. I understand that collection of punch biopsy may cause short term side effects and it may include slight bleeding or bruising, even though collected experienced professionals. The dermatologist will control all these rarely happening risks, immediately
3. If result of my child is positive he/she will be treated according to the national guideline.
4. I understand that all the results will be explained to me and my child ,all the data obtained will be kept strictly confidential by using only code numbers.
5. I am clear for all explanation and agree for participation of my child in the study.I put my signature for my agreement.

Participant Name…… Name of parent/guardian……

Signature ----------Signature ----------

Date ……Date……

Name of witness ……..

Signature …….

Date…….

Assent form for non-leprosy patients (Age15- 17 years) (English version)

Tittle of the study -Evaluation of the Diagnostic Potential of Auramine O Staining for the Diagnosis of Leprosy

1 I, the undersigned person, understand that this study is going to be conducted on Comparing laboratory diagnosticmethods of Leprosy.
2 I understand that if I am enrolled in this study, 10mm of skin biopsy will be collected from the left over tissue.
3 If my result is positive I will contacted for the dermatologist for further investigation and care, if required.
4 I understand that all the results will be explained to me and all the data obtained will be kept strictly confidential by using only code numbers.
5 I am clear for all explanation and agree for participation of my child in the study.I put my signature for my agreement.

Participant Name…… Name of parent/guardian……

Signature ----------Signature ----------

Date ……Date……

Name of witness ……..

Signature ……. Date…….

III. Questionnaires

For Participants (leprosy patients)

Date___/___/_____

1. ☐New case ☐ Relapse

2. Current Leprosy Classification ☐ MB ☐ PB

3. Patient information's

- ✓ Hospital No._____
- ✓ AHRI No. _____
- ✓ Study Number _____
- ✓ Sex ☐ M ☐ F
- ✓ Age (Yrs.) _____
- ✓ Address: Region _____ Woreda _____

Kebele _____ House No. _____

- ✓ Telephone number - _____

4. Is there other member of the family suffered from leprosy ☐ Yes ☐ No

5. If no, is there any family member with the same symptoms ☐ Yes ☐ No

Comments: _____

6. Leprosy symptoms

- ✓ Number of lesions: _____
- ✓ Number of nerves involved _____
- ✓ Disability: ☐ Yes ☐ No
- ✓ If Yes: 1. Eye _____, Right_____ , Left_____
  2. Hand_____, Right_____, Left_____
  3. Foot_____, Right_____, Left_____
- ✓ Duration of symptom (in months) _____

7. Leprosy reaction

- ✓ Any developed reaction, ☐ Yes ☐ No
- ✓ If Yes, ☐ ENL ☐ RR

8. Classification ☐ TT ☐ BT ☐ BB ☐ BL ☐ LL

9. In case of relapse only

- ✓ When was the previous Leprosy diagnostic (number of months or years) _____
- ✓ What was the previous Leprosy classification ☐ MB ☐ PB
- ✓ Did you take the treatment (antibiotics), ☐ Yes ☐ No
- ✓ If yes, what was the antibiotic? ☐ MDT: Dapsone / Rifampicin / Clofazimine

  ☐ MDT: Dapsone / Rifampicin

  ☐ Other (please specify)_____

- ✓ Did you complete the whole dose, ☐ Yes ☐ No
- ✓ For how long you took your medication (in months) _____

Examining Physician: _____ Signature _____

---

9. Sites of SSS sample collected 1._____ 2._____3._____

Bacterial Index_____ Morphologic Index_____

10. Part of the body where the biopsy sample collected

- ✓ Upper limb_____ 1.1 right_____ 1.2 Left_____
- ✓ Lower limb_____ 2.2 Right_____ 2.2 Left_____
- ✓ Trunk _____ 3.1 Front_____3.2 Back_____
- ✓ Other (Specify) _____

For participants (non-leprosy cases)

Date___/___/_____

1. Patient information's

- ✓ Hospital No._____
- ✓ AHRI No. _____
- ✓ Study Number _____
- ✓ Sex ☐ M ☐ F
- ✓ Age (Yrs.) _____
- ✓ Address: Region _____ Woreda _____

Kebele _____ House No. _____

- ✓ Telephone number - _____

2. Have you ever suffer from lyprosy ☐ Yes ☐ No

4. Is there other member of the family suffered from leprosy ☐ Yes ☐ No

5. Resason for sugery :

_____

_____

_____

10. Part of the body where the biopsy sample collected

- ✓ Upper limb_____ 1.1 right_____ 1.2 Left_____

- ✓ Lower limb_____ 2.2 Right_____ 2.2 Left_____
- ✓ Trunk _____ 3.1 Front_____3.2 Back_____
- ✓ Other (Specify) _____

Examining Physician: _____ Signature _____

IV. Information sheets, concent and assent forms in Amharic version

የስጋ ደዌ ተተቂ ለሆኑ የጥናቱ ተሳታፊዎች የመረጃ ቅጽ

የጥናቱርእስ፡-የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

አጠቃላይመረጃ

የስጋ ደዌ በሽታ ማይኮባክቴሪየም ሊፕሬ ተብሎ በሚጠራ ጀርምአማካይነት የሚከሰት ሲሆን በሽታዉ በአብዛኛዉ ከሚያጠቃቸዉ የሰዉነታችን ክፍሎች ዉስጥ ቆዳና ነርቭ ዋነኞቹ ናቸዉ፡፡ በሀገራችን ኢትዮጵያ ላለፉት ስምንት ተከታታይ አመታት በበሽታዉ የሚያዙ አዳዲስ ህመምተኞች ቁጥር በአማካይ በአመት እስከ አራትሺህ ሁለት መቶ ደርሷል፡፡ እንደኢትየጵያ ባሉ አነስተኛ ናመካከለኛ ገቢ ባላቸዉሀገሮች በሽታዉን ለመመርመር የሚዉሉ መሰረታዊ የላብራቶሪ የምርመራ አይነቶች በሽታዉን በብቃት የመመርመር ብቃታቸዉን ለመገምገም ጥናትማኪ ያሄድያስፈልጋል፡፡ ይህም በሽታዉ ወደተባባሰ ደረጃ ሳይደርስ በጊዜ ለማወቅና በሰዉነት ላይ የከፋ ጉዳት ሳያደርስ ህክምናዉን በጊዜ ለመጀመር ያስችላል፡፡

ስላዚህ እርሶ በዚህ ጥናት እንዲሳተፉ ተጋብዘዋል:: ለዚህ ጥናት የሚሆነዉን ናሙና የምንጠቀመዉ ከቆዳዎት ላይከሚወሰድ ጠብታፈሳሽና በተጨማሪም ከታመመዉ የቆዳዎ ክፍል ላይ በጥንቃቄ ትንሽ የቆዳ ናሙና ተወስዶ ነዉ፡፡ ከቆዳ ላይ የሚወሰደዉ ጠብታ ናሙና አለርት ሆስፒታል ባሉ ልምድ ባላቸዉ ላብራቶሪ ቴክኖሎጂስቶች ሲሆን ናሙናዉን ለሙሰድም ለዚሁ ተግባር በተዘጋጀ ንጹህ ምላጭ ይጠቀማሉ፡፡ ከታመመዉ የቆዳዎ ክፍል ላይ በጥንቃቄ የሚወሰደዉን ትንሽ የቆዳ ናሙና ደግሞ በሙያዉ ለረጅም አመታት በሰሩ ልምድ ያላቸዉ ነርሶችና ለዚሁ ተግባር በተዘጋጀ ንጹህ የምላጭ አይነት በመጠቀም ይወሰዳል፡፡ በመሆኑም ጥቂት ጊዜ ወስደው ስለጥናቱ የተዘጋጀውን ይህን የማብሪረያ ጽሁፍ እንዲያነቡና ግልጽ ያልሆነለዎትን ማንኛውንም ጥያቄ በመጠየቅ ስለጥናቱ በቂ ግንዛቤ እንዲኖሮዎት ያስፈልጋል:: በዚህ ጥናት ለመሳተፍ ካልፈለጉ አይገደዱም እንዲሁም መሳተፍ ከጀመሩ በኋላ በማንኛውም ግዜ ከጥናቱ አቋርጠው መውጣት ይችላሉ:: ይህንን በማድረግዎ በማህከሉ የሚደረግልዎትን ህክምና በምንም መልኩ አያስተጓጉልም::

አላማው፡

የዚህ ጥናት ዓላማ የስጋደዌ በሽታን የላብራቶሪ ምርመራ የመመርመር አቅም መገምገም ነዉ፡፡ከጥናቱ የሚገኘዉ ዉጤት የስጋ ደዌ በሽታን የላብራቶሪ ምርመራዎች ለማሻሻል ይለዳል፡፡የስጋ ደዌ በሽታን በተገቢዉ ጊዜና ፍጥነት መርምሮ ማወቅ ህክምናዉን ቶሎ ለመጀመና እንዲሁም በበሽተኞች ላይ ሊደርስ የሚችለዉን የአካል ጉዳት ለመከላከል ይረዳል፡፡

ቅደምተከተል:

በተደረገልዎት አጠቃላይ ምርመራ በዚህ ጥናት ለመሳተፍ ሃኪሙ ብቁ መሆንዎትን ካረጋገጠ ስለጥናቱ አላማ እና ስለሚጠበቀው ውጤት በቂ መረጃ ይሰጠዎትና የስምምነት ቅጽ እንዲፈርሙ ይደረጋል:: በዚህ ጥናት ሲሳተፉ በትክክል የስጋደዌ በሽታ እንዳለቦት ለማወቅ የሚያስችል በጤና ባለሙያ የህክምና ምርመራ ይደረግልዎታል:: ከመደበኛ ለላቦራቶሪ

ምርመራ በተጨማሪ ለምርምር አገልግሎት የሚውል ከተንዳዉ የቆዳ ክፍል ላይ ናሙና ይሰጣሉ፡፡ የተወሰደዉ ናሙና ለምርምሩ ይዉላል፡፡ ለምርመራ የሚወሰደዉ የቆዳ ናሙና መጠን ስድስት ሚሊሜትር ያህላል፡፡ ሀሉም የምርመራ አይነቶች አለርት ሆስቲታል በሚገኘዉ በአርማወር ሃንሰን የምርምር ኢንስቲቲዉት ይኪያሄዳሉ፡፡ የተወሰነዉ የናሙና ክፍል ለሞለኪዉላር ምርመራ ዉጪ ይላካል፡፡ ከቆዳዎት ላይ የሚወሰደዉን ናሙና ያለመስጠት ሙሉ መብት አሎት፡፡

ስጋትናጉዳት:

በዚህ ጥናት ሊሳተፉ ናሙናው የሚወሰደው የሙያውን ስነ-ምግባር በሚያሟላና በቂ እዉቀት ባላዉ ባለሙያ ይከናወናል:: አልፎ አልፎ ናሙናዉ በሚወሰድበት ጊዜ የመድማት ምልክት ሊያሳይ ይችላል፡፡ በተጨማሪም የህምም ስሜት፣ጠባሳ እንዲሁም ኢንፌክሽን ሊያጋጥም ይችላል፡፡ ነገር ግን ናሙናዉን የሚወስደዉ ሀኪምና ነርሶች አስፈላጊዉን ህክምና ያደርጉሎታል፡፡ ናሙናዉን ከሰጡ በኋላ የኢንፌክሽን ምልክት ካዩ ከታች በተጠቀሱት ስልክ ቁጥሮች በመደወል አስፈላጊዉን እርዳታ ያገኛሉ፡፡

ጥቅሞች:

በዚህ ጥናት በመሳተፍዎ የሚያገኙት ቀጥተኛ የሆነ ልዩ ጥቅም የለም:: ነገር ግን የጥናቱ ውጤቶች ወደፊት የስጋ ደዌ በሽታ የላብራቶሪ ምርመራን የተሻለለ ማድረግና በወቅቱም ህክምናዉን ለማስጀመር ለሚደረገው ጥረት ከፍተኛ ጥቅም ይሰጣል::

ማካካሻ፡

በጥናቱ ዉስጥ ለመሳተፍ ሲባል የሚሞሉትን ፎርም ለሙላት የሚወስድቦትን ጊዜ ለማካካስ የሚሆን የትራንስፖርት ክፍያ ሰላሳ ብር (30.00ብር) ያገኛሉ፡፡ ናሙና በሚሰበሰበት ወቀት ማንኛም አይነት ተያያዥ ችግር ካጋጠመ ህክምናዉን በነፃ ያገኛሉ የትራንስፖርት ወጪዎም ይሸፈናል::

ምስጢራዊነት:

ከዚህ ምርምር የምንሰበስበው መረጃ በምስጢ ርይያዛል:: ከእርስዎ የሚገኘው መረጃ የተሳታፊውን ስም በማይጠቅስ መልኩ በቁጥር ወይም በኮድ መልክ ይመዘገባል:: የቱ ቁጥር ወይም ኮድ የየትኛው ተሳታፊ ግለሰብ እንደሆነ በማይታወቅበት ሰነድ በተቆለፈ ቦታ ይቀመጣል:: ይህም ምስጢር ለዋናው ተመራማሪና ለሃኪሙ ብቻ ካልሆነ በስተቀር ለሌላ ለማንም ሰው አይሰጥም::

በጥናቱ ያለመሳተፍ ወይም አቋርጦ የመውጣት መብት:

በዚህ ጥናት ለመሳተፍ ካልፈለጉ አይገደዱም:: እንዲሁም መሳተፍ ከጀመሩ በኋላ በማንኛውም ጊዜ ከጥናቱ አቋርጠው መውጣት ይችላሉ:: ይህንን በማድረግዎ በማህከሉ የሚደረግልዎትን ህክምናበ ምንም መልኩ አያስተጓጉልም::

ጥያቄ ካለዎት አሁን ወይም ሌላ ግዜ ሊጠይቁ ይችላሉ::

ሌላ ጊዜ ለመጠየቅ ቢፈልጉ ከዚህ በታች የተጠቀሱትን ግለሰቦች ማነጋገር ይችላሉ::

ስልፉግርማ (ዋናተመራማሪ)

ስልክ ቁጥር 251-913-261720

አህሪ/አለርት የምርምር ስነምግባር ኮሚቴ

ስልክ ቁጥር 251-011-8962183

አርማወር ሀንሰን የምርምር ተቋም

የስጋ ደዌ ተጠቂ ላልሆኑ የጥናቱ ተሳታፊዎች የመረጃ ቅጽ

የጥናቱ ርእስ፡-የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

አጠቃላይመረጃ

የስጋደዌ በሽታ ማይኮባክቴሪየምሊፕሬ ተብሎ በሚጠራ ጀርም አማካይነት የሚከሰት ሲሆን በሽታዉ በአብዛኛዉ ከሚያጠቃቸዉ የሰዉነታችን ክፍሎች ዉስጥ ቆዳና ነርቭ ዋነኞቹ ናቸዉ፡፡ በሀገራችን ኢትዮጵያ ላለፉት ስምንት ተከታታይ አመታት በበሽታዉ የሚያዙ አዳዲስ ህመምተኞች ቁጥር በአማካይ በአመት እስከ አራት ሺህ ሁለት መቶ ደርሷል፡፡ እንደኢትየጵያ ባሉ አነስተኛና መካከለኛ ገቢ ባላቸዉ ሀገሮች በሽታዉን ለመመርመር የሚዉሉ መሰረታዊ የላብራቶሪ የምርመራ አይነቶች በሽታዉን በብቃት የመመርመር ብቃታቸዉን ለመገምገም ጥናት ማኪያሄድ ያስፈልጋል፡፡ ይህም በሽታዉ ወደ ተባባሰ ደረጃ ሳይደርስ በጊዜ ለማወቅና በሰዉነት ላይ የከፉ ጉዳት ሳያደርስ ህክምናዉን በጊዜ ለመጀመር ያስችላል፡፡ስላዚህ እርሶ በዚህ ጥናት እንዲሳተፉ ተጋብዘዋል:: ለዚህ ጥናት የሚሆነዉን ናሙና የምንጠቀመዉ ከመደበኛ ህክምናዎ ላይ ከሚወገደዉ ቆዳ ላይ አስር ሚሊማትር የሚሆን የቆዳ ናሙና ለምርምሩ እንዲዉል ይወሰዳል፡፡

በመሆኑም ጥቂት ጊዜ ወስደው ስለጥናቱ የተዘጋጀውን ይህን የማብሪረያ ጽሁፍ እንዲያነቡና ግልጽ ያልሆነለዎትን ማንኛውንም ጥያቄ በመጠየቅ ስለጥናቱ በቂግንዛቤ እንዲኖሮዎት ያስፈልጋል:: በዚህ ጥናት ለመሳተፍ ካልፈለጉ አይገደዱም እንዲሁም መሳተፍ ከጀመሩ በኋላ በማንኛውም ግዜ ከጥናቱ አቋርጠው መውጣት ይችላሉ:: ይህንን በማድረግዎ በማህከሉ የሚደረግልዎትን ህክምና በምንም መልኩ አያስተጓጉልም::

አላማው፡

የዚህ ጥናት ዓላማ የስጋደዌ በሽታን የላብራቶሪ ምርመራ የመመርመር አቅም መገምገም ነዉ፡፡ከጥናቱ የሚገኘዉ ዉጤት የስጋ ደዌ በሽታን የላብራቶሪ ምርመራዎች ለማሻሻል ይለዳል፡፡የስጋደዌ በሽታን በተገቢዉ ጊዜና ፍጥነት መርምሮ ማወቅ ህክምናዉንም ቶሎ ለመጀመና እንዲሁም በበሽተኞች ላይ ሊደርስየሚችለዉን የአካልጉዳት ለመከላከል ይረዳል፡፡

ቅደም ተከተል:

መደበኛ ህክምና ከተደረገሎት በኃላ ከሚወገደዉ የቆዳ ክፍል ላይ አስር ሚሊሜትር የሚሆን ናሙና ይወሰዳል፡፡ ይህም ናሙና ከሁለት ተከፍሎ ላ ሂስቶፓቶሎጂና ለሞለኪዉላር ሚርመራ ይዉላል፡፡

ስጋትና ጉዳት:

በዚህጥናትሊሳተፉ ናሙናው የሚወሰደው መደበኛ ህክምናዎትን ካዳርጉ በኃላ ከሚወገደዉ የቆዳ ክፍል ላይ ስለሆነ ምንም ተያያዥናት ያለዉ ጉዳት አይኖረዉም፡፡

ጥቅሞች:

በዚህ ጥናት በመሳተፍዎ የሚያገኙት ቀጥተኛ የሆነ ልዩ ጥቅም የለም:: ነገር ግን የጥናቱ ውጤቶች ወደፊት የስጋደዌ በሽታ የላብራቶሪ ምርመራን የተሻለለ ማድረግና በወቅቱም ህክምናዉን ለማስጀመር ለሚደረገው ጥረት ከፍተኛ ጥቅም ይሰጣል::

ማካካሻ፡

በጥናቱ ዉስጥ ለመሳተፍ ሲባል የሚሞላዉን ፎርም ለሙላት የሚወስድቦትን ጊዜ ለማካካስ የሚሆን የትራንስፖርት ክፍያ ሰላሳ ብር (30.00ብር) ያገኛሉ፡፡

ምስጢራዊነት:

ከዚህ ምርምር የምንሰበስበው መረጃ በምስጢር ይያዛል:: ከእርስዎ የሚገኘው መረጃ የተሳታፊውን ስም በማይጠቅስ መልኩ በቁጥር ወይም በኮድ መልክ ይመዘገባል:: የቱ ቁጥር ወይም ኮድ የየትኛው ተሳታፊ ግለሰብ እንደሆነ በማይታወቅበት መልኩ በተቆለፈ ቦታ ይቀመጣል:: ይህም ምስጢር ለዋናው ተመራማሪና ለሃኪሙ ብቻ ካልሆነ በስተቀር ለሌላ ለማንም ሰው አይሰጥም::

በጥናቱ ያለመሳተፍ ወይም አቋርጦ የመውጣት መብት:

በዚህ ጥናት ለመሳተፍ ካልፈለጉ አይገደዱም:: እንዲሁም መሳተፍ ከጀመሩ በኋላ በማንኛውም ጊዜ ከጥናቱ አቋርጠው መውጣት ይችላሉ:: ይህንን በማድረግዎ በማህከሉ የሚደረግልዎትን ህክምና በምንምመ ልኩ አያስተጓጉልም::

ጥያቄ ካለዎት አሁን ወይም ሌላ ግዜ ሊጠይቁ ይችላሉ::

ሌላጊዜለመጠየቅቢፈልጉከዚህበታችየተጠቀሱትንግለሰቦችማነጋገርይችላሉ::

ሰልፉግርማ (ዋናተመራማሪ)

ስልክ ቁጥር 251-913-261720

አህሪ/አለርት የምርምር ስነምግባር ኮሚቴ

ስልክ ቁጥር 251-011-8962183

አርማወር ሀንሰን የምርምር ተቋም

እድሜአቸዉ ከ18 አመት በላይ የስጋ ደዌ ታማሚ ለሆኑ አዋቂዎች ፈቃደኝነት መጠየቂያ ቅፅ

የጥናቱ ርእስ፡-የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

1. እኔ ስሜ ከዚህ በታች የተገለጸዉ ይህ ጥናት የስጋደዌ መመርመርያ የላብራቶሪ ምርመራዎችን በማወዳደር ያላቸዉን የመመርመር ብቃት ለማጥናት እንደሚካሄድ ተገልፆልኛል፡፡
2. በዚህ ጥናት ውስጥ ለመሳተፍ ፍቃደኛ ከሆንኩ ለጥናቱ የሚያስፈልገውን ከቆዳላይ ተፈቅፍቆ የሚወሰድ (ሶስት ጠብታ) ፈሳሽ እና ከተጎዳዉ ቆዳ ላይስድስት ሚሊሜትር የሚያህል የቆዳ ናሙና እንደሚወሰድ ተነግሮኛል፡፡ግማሹየቆዳናሙናም ለሞለኪዉላር ምርመራ ከኢትዬጵያ ዉC ይላካል*
3. የሰለጠነ ባለሙያ ቢወስደዉ እንኩዋን ከቆዳ ላይ ናሙና በሚወሰድበት ጊዜ ቀለል ያለ መድማትና መቁሰል አልፎ አልፎ መቁሰል ሊያጋጥም ይችላል፡፡ደርማቶሎጂስቶች ሊያጋጥም የሚችለዉን አላስፈላጊ የጎን ዉጤት ከመቆጣጠር በተጨማሪ አስፈላጊዉን ህክምና ሁሉ ያድረጋሉ፡፡
4. የስጋደዌ በሽታ ምርመራ እንደሚደረግልኝና በሽታዉ በርግጥ የስጋደዌ ከሆነ አስፈላጊውን ህክምና እንደማገኝ ተረድቻለሁ፡፡
5. የምርመራውጤቴም እንደሚነገረኝና የኮድ ቁጥር በመጠበቀም በሚስጥርም እንደሚያዝ ተረድቻለሁ፡፡
6. ከላይ የተደረገልኝን ማብራሪያ በሚገባ ተረድቻለዉ፡፡በጥናቱም ዉስጥ እንደምሳተፍ በፊርማዬ አረጋግጣለዉ፡፡

የተሳታፊውስም…… የእማኝስም…….

ፊርማ …… ፊርማ……..

ቀን…… ቀን……

ዕድሜያቸዉ ከ 15 እስከ 17 ለሆኑ ታማሚ ተሳታፊ ታዳጊወጣቶች ናወላጆቻቸዉ/አሳዳጊዎቻቸዉ ፈቃደኝነት መጠየቂያ ቅፅ

የጥናቱ ርእስ፡- የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

እኔ ስሜ ከዚህ በታች የተገለጸዉ ይህ ጥናት የስጋ ደዌ መመርመር ያየላብራቶ ሪምርመራዎችን በማወዳደር ያላቸዉን የመመርመር ብቃት ለማጥናት እንደሚካሄድ ተገልፆልኛል፡፡

1. በዚህ ጥናት ውስጥ ልጄ እንዲሳተፍ/እንድትሳተፍ ፍቃደኛ ከሆንኩ ለጥናቱ የሚያስፈልገውን ከቆዳ ላይ ተፈቅፍቆ የሚወሰድ (ሶስትጠብታ) ፈሳሽእና ከተነዳዉ ቆዳ ላይ ትንሽ የቆዳ ናሙና(አምስት ሚሊሜትር) እንደሚወሰድ ተነግሮኛል፡፡ግማሹየቆዳ ናሙናም ለሞለኪዉላር ምርመራ ከኢትዮጵያ ዉጭ ይላካል፡፡
2. የሰለጠነ ባለሙያ ቢወስደዉ እንኩዋን ከቆዳ ላይ ናሙና በሚወሰድበት ጊዜ ቀለል ያለ መድማትና መቁሰል አልፎ አልፎ መቁሰል ሊያጋጥም ይችላል፡፡ደርማቶሎጂስቶች ሊያጋጥም የሚችለዉን አላስፈላጊ የጎን ዉጤት ከመቆጣጠር በተጨማሪ አስፈላጊዉን ህክምና ሁሉ ያድረጋሉ፡፡
3. ለጄ የስጋደዌ በሽታምርመራ እንደሚደረግለት/ግላትና በሽታዉ በርግጥ የስጋደዌ ከሆነ አስፈላጊውን ህክምና እንደሚያገኝ/ምታገኝ ተረድቻለሁ፡፡
4. የምርመራው ጤቱም ለኔም ለልጄም እንደሚነገረንና የኮድ ቁጥር በመጠበቀም በሚስጥርም እንደሚያዝ ተረድቻለሁ፡፡
5. ከላይ የተደረገልኝን ማብራሪያ በሚግባ ተረድቻለዉ፡፡ ፡፡ ልጄም በጥናቱም ዉስጥ እንዲሳተፍ/ድትሳተፍ በፊርማዬ አረጋግጣለዉ፡፡

የተሳታፊው ስም…… የተሳታፊው ወላጅ/አሳዳጊ ስም……

ፊርማ …… ፊርማ ……

ቀን…….. ቀን……..

የእማኝ ስም……

ፊርማ……

ቀን……

እድሜአቸዉ ከ18 አመት በላይ የስጋ ደዌ ተተቂ ላልሆኑ የጥናቱ ተሳታፊዎች ፈቃደኝነት መጠየቂ ያቅፅ

የጥናቱ ርእስ፡-የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

1. እኔ ስሜ ከዚህ በታች የተገለጸዉ ይህ ጥናት የስጋደዌ መመርመርያ የላብራቶሪ ምርመራዎችን በማወዳደር ያላቸዉን የመመርመር ብቃት ለማጥናት እንደሚካሄድ ተገልፆልኛል፡፡
2. በዚህ ጥናት ውስጥ ለመሳተፍ ፍቃደኛ ከሆንኩ ለጥናቱ የሚያስፈልገውን ከመደበኛ ሰርጂካል ህክምና የሚወገደዉ ቆዳ ላይ ከ 10 ሚሊሜትር ናሙና እንደሚወሰድ ተነግሮኛል፡፡
3. የስጋደዌ በሽታ ምርመራ ሲደረግ መሽታዉን ምልክት የሚታይ ከሆነ ለበለጠ ክትትልና ምርመራ ከህኪሞች ጋር እነድገናኝ ይደረጋል፡፡
4. የምርመራውጤቴም እንደሚነገረኝና የኮድ ቁጥር በመጠበቀም በሚስጥርም እንደሚያዝ ተረድቻለሁ፡፡
5. ከላይ የተደረገልኝን ማብራሪያ በሚገባ ተረድቻለዉ፡፡በጥናቱም ዉስጥ እንደምሳተፍ በፊርማዬ አረጋግጣለዉ፡፡

የተሳታፊውስም…… የእማ ኝስም…….

ፊርማ …… ፊርማ……..

ቀን…….. ቀን……

ዕድሜያቸዉ ከ15 እስከ 17 ለሆኑ የስጋ ደዌ ተጠቂ ላልሆኑ የጥናቱ ተሳታፊዎች ተሳታፊዎችና ወላጆቻቸዉ/አሳዳጊዎቻቸዉ ፈቃደኝነት መጠየቂያ ቅፅ

የጥናቱ ርእስ፡-የስጋ ደዌ በሽታን በተሻለ ሁኔታ ለመመርመር የሚስችል የላብራቶሪ የምርመራ አይነት መገምገም

እኔ ስሜ ከዚህ በታች የተገለጸዉ ይህ ጥናት የስጋደዌ መመርመርያ የላብራቶሪ ምርመራዎችን በማወዳደር ያላቸዉን የመመርመር ብቃት ለማጥናት እንደሚካሄድ ተገልፆልኛል፡፡

1. በዚህ ጥናት ውስጥ ለመሳተፍ ፍቃደኛ ከሆንኩ ለጥናቱ የሚያስፈልገውን ከመደበኛ ሰርጂካል ህክምና የሚወገደዉ ቆዳ ላይ ከ 10 ሚሊሜትር ናሙና እንደሚወሰድ ተነግሮኛል፡፡
2. የስጋደዌ በሽታ ምርመራ ሲደረግ መሽታዉን ምልክት የሚታይ ከሆነ ለበለጠ ክትትልና ምርመራ ከሀኪሞች ጋር እነድገናኝ ይደረጋል፡፡
3. የምርመራውጤቴም እንደሚነገረኝና የኮድ ቁጥር በመጠበቀም በሚስጥርም እንደሚያዝ ተረድቻለሁ፡፡

4. ከላይ የተደረገልኝን ማብራሪያ በሚግባ ተረድቻለዉ፡፡ ፡፡ ልጇም በጥናቱም ዉስጥ እንዲሳተፍ/ድትሳተፍ በፊርማዬ አረጋግጣለዉ፡፡

የተሳታፊው ስም…… የተሳታፊው ወላጅ/አሳዳጊ ስም……

ፊርማ …… ፊርማ ……

ቀን…….. ቀን……..

የእማኝ ስም……

ፊርማ……

ቀን……

V. Laboratory Procedures

1. Skin Skin Smear (SSS)

1.1 Sample Collection

- Universal precautions should be observed in obtaining skin smears.
- The skin is cleansed with 70% alcohol and air-dried or wiped dry with cotton.
- A fold of skin is made relatively avascular by pinching or mild clamping. If the skin cannot be grasped by pinching, it can be compressed.
- Local anesthesia is generally unnecessary,the compression of the skin by pinching aids in the anesthesia.
- An incision 3-5 mm long and 2-3 mm deep is made with a surgical blade. Mild pressure to maintain relative avascularity is continuously applied to the area until an adequate smear has been obtained.
- A small amount of blood does not interfere with the reading, but large amounts should be avoided and can usually be controlled by the amount of pressure of the pinch.
- If excessive bleeding occurs, it can be wiped away with a cotton swab.
- After the incision is made, and before the blade is withdrawn, the inner surface of the wound is scraped with thenon sharp side of the blade held at a right angle to the incision. Upon scraping, tissue fluid and dermal tissue are obtained.
- The material then is transferred to the cleaned microscope slide. A moderately thick smear, with a visible uniform opacity is made. The smear is made in a circular manner on the slide.
- A single technician takes all smears to insure more uniform and consistent results.
- The smears are then sent to the AHRI pathology lab.

1.2 SSS ZN staining

- Dry the slide with smear at room temperature. Do not heat fix.

- Place slides on a staining rack and fcover the whole slide with filtered 1% carbol fuchsin solution for twenty minutes without applying heat.
- Gently rinse slides well with tap water to remove excess stain.
- Decolorize with 1% acid-alcoho for 2 minute.
- Gently rinse slides thoroughly with tap water.
- Counterstain with 0.2% methylene blue solution for 30 seconds .
- Gently rinse well with tap water and air dry.

1.3 SSS ZN Reporting

- The results are reported on a 0 to 6+ scale using a descriptive phrase or numerical code and this is also an indicator of the total bacillary load of the patient as in dicated in the table below.

S.No	Microscopic Examination	Descripton	Numerical code
1	Over 1000 bacilli per oil immersion field.	Very Numerous	+6
2	100 to 1000 bacilli per oil immersion field.	Numerous	+5
3	10 to 100 bacilli per oil immersion field.	Moderate	+4
4	1 to 10 bacilli per oil immersion field.	Few	+3
5	1 to 10 bacilli per 10 fields.	Very few	+2

6	1 to 10 bacilli per 100 fields.	Rare	+1
7	No AFB seen on entire site.	None found	0

1.4 SSS Auramine O staining

- Dry the slide with smear at room temperature. Do not heat fix.
- Flood the slide with0.1% Auramine Ostain for20 minutes.
- Rinse the slide with water and drain excess water from the slide.
- Flood with 0.5% acid-alcohol for 2 minutes to decoloraztion.
- Rinse the slide with water and drain excess water from the slide.
- Flood the slide with potassium 0.5%potassium permanganate for 5 mintes to couterstain.
- Rinse the slide with water and drain excess water from the slide.
- Air dry, do not blot.
- Examine the smear under 40X objective of LED fluorescent microscope.

1.5 Reporting of Auramine O staining using LEDFM.

- The following table containms the reporting scale of LEDFM microscope reporting recommended by WHO and we also used this scale.

Final Report	Microscopic Readings		
	Bright field(100X Objective)	LEDFM(20-25x Objective)	LEDFM(40X objective)
Negative	Zero AFB/1 length	Zero AFB/1 length	Zero AFB/1 length
Scanty or actual	1-9 AFB/1 length or	1-29 AFB/1 length	1-19 AFB/ 1 length

count	100 HPF		
1+	10-99 AFB/1 length or 100 HPF (=1-9 AFB/10 fields)	30 – 299 AFB/1 length	20- 199 AFB/ 1 length
2+	1-10 AFB/1 HPF on average	10-100 AFB/1 Field on average	5-50 AFB/1 field on average
3+	≥ 10 AFB / 1 HPF on average	≥ 100 AFB / 1 HPF on average	> 50 AFB / 1 field in average

2. Skin Puch biopsy

2.1 Sample collection Procedure

- Arrange all the required dressing materials and equipment for Punch Biopsy collection.
- Choose the appropritae lesion to collect sample from.
- Clean the area using 70% alcohol to disinfect .
- Inject adequate amount of local anesthesia (2% lidocaine) to the skin and subcutaneous tissue .
- Take 6 mm punch biospsy from the numbed area and dress the area
- Divide the collected bunch biopsy in to two parts , half to be fixed in 10% formaldehyde and the other half in 70% ethanol.
- Send the collected sample to AHRI pathology laboratory.

2.2 DNA Extraction

2.2.1 Required materials

- 2 ml Eppendorf tubes

- ATL buffer
- PBS
- Proteinase K 20mg/ml
- Qiamp microbiome extraction kit (Qiagen)

2.2.2 DNA extraction procedure

- Add 200 ml ATL buffer in to 2 ml eppendorf tubes and label the tubes with sample ID.
- Take out the tissue from 70% ethanol and rinse it in PBS.
- Transfer the punch tissue in to eppendorf tube which contains ATL buffer.
- Add 20 µL Proteinase K of 20mg/ml.
- Incubate the tubes at 56^0C in an incubator with shaker till the biopy dissolves.
- Vortex the tubes two or there times with 30 minutes interval to facilitate the reaction.
- Once the tissue dissolved, continue with the DNA purification procedure of Qiamp microbiome extraction kit which also includes the pathogen lysis part.

2.3 Polymerase Chain reaction

- Arrange the required number of micro tubes and lable them with the corresponding sample ID including negative and positive controls.
- Pepare the master mix and primers using the following proportion in the table calculated for one sample.
- Prepare the master mix in a room reserved for master mix preparation to avoid contamination.
- It is better to prepare the final volume of mastermix with 10% more than the required amount to compensate the shortage during pipetting.

For one reaction	
Master Mix	25 µl
2µM Primer- Froward	5 µl

2µM Primer- Reverse	5 µl
Molecular grade H_2O	10 µl
DNA Template	5 µl
Total Volume	**50 µl**

- Mix well the master mix, primers and H_2O using vortexer and take it to the room where the DNA template is kept. Never bring the DNA template to the room reserved for mastermix preparation.
- Add 5 µl respective DNA template to the microtube which contains 45 µl primers and master mix.
- Vortex well and spin briefly to collect all the liquid to the bottom of the microtube.
- Use a PCR program containing the following table.

Prameter	Tempratute in ^0C	Time	Number of cycle
Preheating	950	5 minutes	1
Denature	950	30 seconds	40
Annealing	580	40 seconds	
Elongation	720	30 seconds	
Elongation	720	10 minutes	1
Hold	40	Indefinitely	1

2.4 Reading the amplicon on Agaraose gel.

- Be sure that all the required materials are ready to run the agaros gel.
- Select the appropriate comb to prepare enough number lane for the ladder, all samples and controls (Positive and Negative)

- Prepare 50 ml of 1% agarose gel in 1X TAE buffer and boil it in the microvave to dissolve the agarose completely.
- Put 1µl ethidium bromide in the agarose solution as soon as it is removed from the microwave. N.B. Ethidium bromide is carcinogenic so add it with a great care.
- Aftter dispensing the gel in the box wich contain the comb, wait 30 minutes till the gel gets solidify.
- In the mean time prepare a10 µl ladder which can show a band in 100 bp variation.
- Add 5 µl loading dye in to all samples, controls and the ladder, too.
- After 30 minutes, remove the comb and transfer it to gel running box which contain 1X TAE.
- Add 5 µl ladder to lane 1.
- Then add 10 µl samples consecutivily starting from lane 2.
- Add 10 µl negative and 10 µl positive controls in to the last two lanes, respectively.
- Run the gel runner for 30 minutes.
- Remove the gel from the gel runeer and read the presence of 500 bp bands in the lanes.
- Any visible band in the specific base pair is a positive result for *M. leprae*.

2.5Tissue processing

- Wash the tissue which is fixed in 10% formaldehyde for 48 hours in running tape water to remove the excess remaining formaline in the casset which contain the tissue. Then put the cassete in
    - ✓ 70% alcohol ……...45 minutes
    - ✓ 80% alcohol ……..45 minutes
    - ✓ 90% alcohol …….. 45 hour
    - ✓ 96% alcohol I……..45 hour
    - ✓ 96% alcohol II…….1 hour
    - ✓ Absolute alcohol ……. ..1 hour
    - ✓ Xylene I ……...1 hour
    - ✓ Xylene II……. 1 hour

- ✓ Paraffin I …… 1 hour
- ✓ Paraffin II……...…2 hours or leave the tissue in paraffin II over night.

- The the next day prepare respective blockes for each sample in the cassete.

2.6 Tissue Sectioning

- Prepare three ordinary one end frosted microscopic slides.
- Put a small drop of 50% egg albumin on the them and polish the entire area which the section tissue can lay on.
- Prepare 9 series of tisue sections by adjusting the microtome at 5 μm.
- Put three series of section on each of the three slides.
- Lable and put the lides in an oven adjusted at 70^0C for 30 minutes.
- Proceed to the desired staining techniques on each slides. .

2.7 Fite Faraco staining

- Warm sections and deparaffinize in two changes of a mixture of two parts Xylene/ one part of Vegetable Oil for 15 minutes each.
- Blot the slide with absorbent filter paper and wash in distilled water.
- Stain the slides with filtered Carbol fuchine solution without applying heat.
- Wash in running tap water
- Differentiate/decolorize in 10% $H_2SO_4$for 2 minutes.
- Wash well in running tap water and rinse in distilled water.
- Counter stain with 0.25% methylene blue exactly for 20 seconds.
- Wash and blot dry. Never use Alcohol for dehyderation.
- Clear well in Xylen and mount in a DPX .
- Examine under 100X objective of the microscope.

2.8 Fite-Faraco Reporting

- Use the same reporting system of SSS ZN staining reporting esytem of this study.

2.9 Tissue section Auramine O staining

- Warm sections and deparaffinize in two changes of a mixture of two parts Xylene/ one part of Vegetable Oil for 15 minutes each.
- Blot the slide with absorbent filter paper and wash in distilled water
- Stain the slide with 0.1% Auramine O for 20 minutes.
- Rinse the slide with water and drain excess water from the slide.
- Flood with 0.5% acid-alcohol for 2 minutes to decolorization.
- Rinse the slide with water and drain excess water from the slide.
- Flood the slide with potassium 0.5% potassium permanganate for 5 mintes to Couterstain.
- Rinse the slide with water ,drain excess water from the slide and blot using absorbent filter paper to dry.
- Clear well in Xylen and mount in a DPX
- Examine under 40X objective of LEDFM.

2.10 Tissue Auramine O staining reporting

- Use the reporting system of SSS Auramine O staining .

2.11 H&E attaining

- Deparaffinize sections in 2 changes of xylene, 10 minutes each.
- Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
- 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
- Wash briefly in distilled water.
- Stain in Harris hematoxylin solution for 8 minutes.
- Wash in running tap water for 5 minutes.
- Differentiate in 1% acid alcohol for 30 seconds.
- Wash running tap water for 1 minute.

- Bluing in 0.2% ammonia water 30 seconds to 1 minute.
- Wash in running tap water for 5 minutes.
- Rinse in 95% alcohol, 10 dips.
- Counterstain in eosin Y solution for 30 seconds to 1 minute.
- Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
- Clear in 2 changes of xylene, 5 minutes each.
- Mount with xylene based mounting medium.

❖ **Result**

- ✓ Nuclei ---------- Blue
- ✓ Cytoplasm ----------Pink to red

VI. Assurance of Principal Investigator

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

Name: Selfu Girma

Signature:_____

Date: _____

This thesis has been submitted for examination with my approval as University Advisor.

Name: Kasu Desta, Assitant Professor, BSc, MSc.

Signature:_____

Date:_____

This thesis has been submitted for examination with my approval as External Advisor.

Name: Kidist Bobosha, BSc. MSc, PhD Cand.

Singature:_____

Date:_____