Molecular Detection of *Mycobacterium leprae* in Stained Slit Skin Smear from leprosy patients and comparison of Molecular and Histopathological Findings with Clinical Data

Addis Ababa University, College of Health Sciences, School of Medicine Department of pathology

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List of Abbreviation

AFB…….Acid Fast Bacilli

AHRI……..Armauer Hansen Research Institute

ALERT……...All African Leprosy Rehabilitation and Training Center

BB……...Borderline

BL……..Borderline Lepromatus

BT……..Borderline Tuberculoid

DNA……. Deoxyribonucleic acid

ENL…….Erythema Nodosum Leprosum

FF…….. Fite-Faraco

HB……...Health Bearu

H&E……..Hematoxlin and Eosin

HHC……..House Hold Contacts

IL…….. Indeterminate leprosy

LL……..Lepromatous Leprosy

MB……..Multibacillary

M. leprae…… *Mycobacterium leprae*

MDT……Multi-Drug Therapy

NPV……. Negative predictive value

PCR…….. Polymerase Chain Reaction

PB……..... Paucibacillary

PPV…… Positive predictive value

RR……Reveres Reaction

RELP……..Restriction Element fragment Length Polymorphism

SSS……..Slit Skin Smear

SPSS……Statistical Package for the Social Sciences

TT…….. Tuberculoid

WHO……. World Health Organization

ZN…….....Ziehl Neelsen

Operational definition

Pathology laboratory: - a laboratory that renders diagnostic service based on the examination of tissues and cells from a patient obtained by biopsy, surgical resection and Fine Needle Aspiration Cytology (FNAC) or body fluid specimen.

Histopathology: Microscopic (histological) examination of surgically removed tissues or organs to detect structural abnormalities that can be seen in disease processes

Staining:-Artificial coloration of a substance to facilitate examination of tissues, microorganisms, or other cells under the microscope

Clinically confirmed leprosy Cases:-The patients who are clinically diagnosed and confirmed as leprosy patient and decided them to give medication

A primer - is a short strand of nucleic acid (oligonucleotide) that serves as a starting point for DNA or RNA synthesis

Slit skin smear:- is a sample taken from the earlobe and active lesion of potential leprosy cases by new surgical blade and make a smear in new microscopic slide.

Fite-Faraco stain- is the modifiedAFB stain method to demonstrate *Mycobacterium leprae* in tissue sections.

Bacterial index (BI) - is a method for monitoring the patients' responses to chemotherapy by giving an estimation of the number of acid-fast bacilli present in skin smears of lesions and other specific sites of the skin of leprosy patients.

Abstract

Background:*Mycobacterium leprae* is a causative agent of leprosy which is a chronic infectious disease. The disease mainly involves the skin and peripheral nerves. Leprosy is endemic in tropical countries especially in developing countries.Globally theprevalence of leprosy at the end of 2015 was 210,758 cases (3.2 cases per 100, 000 populations)and the number of new cases reported globally in the same year was 199,992. The Aim of this study was to improve the sensitivity of leprosy laboratory diagnosisusing PCR on Archival ZN Stained Slide of Skin Smear and to compare with other tests.

Objective: To evaluate the diagnostic performance of PCR to detect *M. leprae* on stained Slit Skin Smear collected from clinically confirmed leprosy patients.

Methodology:Retrospective cross sectional study was conducted on 60 clinically confirmed leprosy patients of 42MB and 18PB leprosy cases and Archival sample ZN stained slides on SSS and archival data of H&E and FF on biopsy stained slides of the sixty leprosy patients were collected from the AHRI histopathology laboratory.

Results: The PCR on SSS was positive in 6 (10.00%) PB patients and in 23 (38.67%) MB patients. Among the 23 MB cases detected by PCR 3, and among PB cases detected 2 were from AFB negative slides. Histopathological findings were graded according to Ridley and Jopling scale where most common histological type was BT seen in 14(23.33%) cases followed by BL 8(13.33%) cases, LL 8(13.33%), BB 7 (11.67%), TT 5 (8.33%), and IL 3 (5%). Majority (71.67%) of the cases was MB type and the rest (28.33%) were PB. Faite-Faraco staining was positive in 37 (61.7%) cases.

Conclusion: The PCR on SSS detected a total of 29 out of 60 samples indicating its potential for diagnosis. Although PCR on SSS showed low detection as compared to H&E and FF staining (staining on punch biopsy samples), it detected more positive samples than ZN staining. Therefore, by improving some technical procedures in sample collection and handling, it can be used for diagnosticswhere PCR machines are available.

1. Introduction

1.1. Back ground

Mycobacterium leprae (*M. leprae*) is the causative agent of leprosy or Hansen's disease which is a chronic but curable human diseaseaffecting the skin, peripheral nerves, eyes and mucosalsurfaces of the upper respiratory tract.*M. leprae* is an obligate intracellular pathogen with a tropism for macrophages and Schwann cells of peripheral nerves. This bacterium prefers the cooler regions of the body and, for diagnostic purposes, slit skin smears are often taken from the earlobe and active lesion of potential cases (1).

Leprosy affects all age groups and both sexes, with the most affected being the 15–45 years age-group. In the majority of persons infected with leprosy bacteria, the body's natural immunity is able to kill the bacteria. Only about 5% of individuals infected will develop the disease during their lifetime because the bacteria grow very slowly in the body. Leprosy is associated with poverty and lower socio-economic status and there is no relation between the frequency of leprosy and that of other infectious diseases including HIV (2).

Etiologic Agent

M. leprae is classified under classSchizomycetes, order Actinomycetales, family Mycobacteriaceae, and genus Mycobacterium. *M. leprae* is a straight or slightly curved rod, with rounded ends, measuring 1.5-8 microns in length by 0.2-0.5 micron in diameter. It is red stained with carbolfuchsin using the Ziehl-Neelsen (ZN) stain, and because of its high lipid content, it does not get discolored when washed with acid alcohol. *M. leprae*is different from other mycobacteria in terms of arrangement, since it is arranged in parallel chains, just like cigarettes in a pack, bound together forming the globi (3). The etiologic agent of leprosy was discovered by Norwegian Scientist Gerhard Henrik Armauer Hansen in 1873(4).

Transmission of leprosy

Transmission of leprosy from an infected person to a healthy person is the most accepted mode of transmission. Untreated multibacillary cases, especially lepromatous patients are the most

important source of infection compared to the other types in the spectrum. This is because lepromatous patients harbour significantly higher numbers of bacilli which occurs when bacilli are spread usually by airborne droplets from nasal and/or mouth. Various investigators have also reported that household contacts are at a greater risk of developing leprosy compared to the general population(5). Other routes of transmission such as inoculation through skin or from environmental sources are not proved yet and need further investigation.

Signs and Symptoms of leprosy

Leprosy affects the skin, nerves, and mucous membranes (the soft, moist areas just inside the body's openings).Hypopigmented or erythematous patches/plaques are often the first clinical signs of the disease inmany newly diagnosed leprosy patients. Since many other conditions produce similar lesions, it must beaccompanied by definite sensory loss to be specific for leprosy (6). It also involves nerves where nerve enlargement is also one of the cardinal signs.The nerve damage due to leprosy leads to disability and deformity such as clawing of the hand.

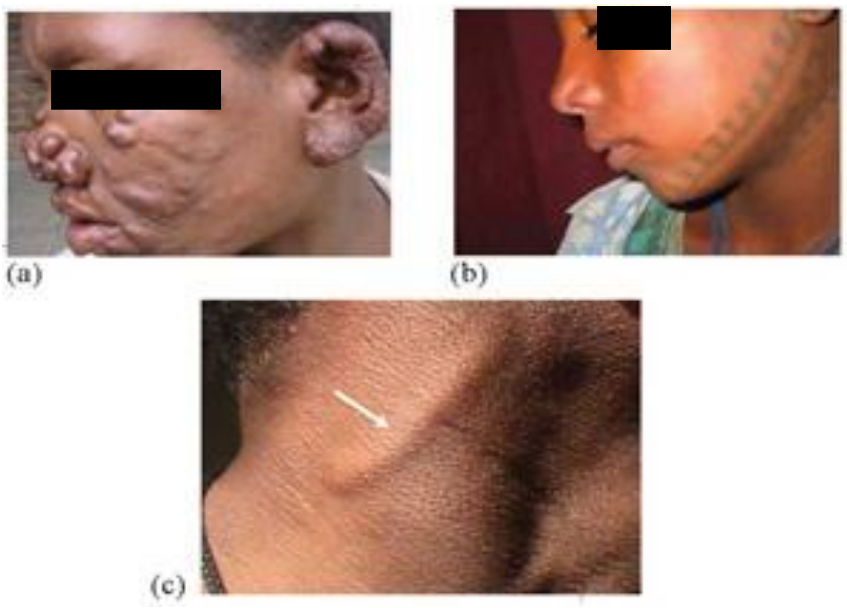

Figure1. Signs and Symptoms of Leprosy
A. Nodules on the face. B skin patch on the face,Can enlarged nerve in the neck
Source: Communicable Diseases Module 18. Leprosy Diagnosis.

Pathogenesis of *M. leprae*

*M. leprae*primarily invades Schwann cells in the peripheral nerves leading to nerve damage and development of disabilities, the incubation period of the disease ranges from three to six years in the tuberculoid form of disease and three to ten years in the multi bacillary form (7), pathogenesis of *M. leprae* replicates intracellularly, typically with in skin histiocytes, endothelial cells, and the Schwann cells of nerves, it may also affect mucosa (mouth, nose and pharynx), testes, kidney, voluntary/smooth muscles and vascular endothelium. Neural tropism of the leprosy bacillus is through its binding and entry into Schwann cells causing demyelination, which results in decreased sensory motor function (8, 9). The cell mediated immunity plays the major part in determining the response of the host to the infection. Most of the world's population is naturally immune to leprosy and the immune response of the patient and the density of the bacteria in the lesion (Bacterial Index: BI) determine the clinical manifestation and the severity of the disease (10). Although leprosy affects both sexes, in most parts of the world males are affected more frequently than females often in the ratio of 2:1.women are more likely to delay the disease and to present with impairmentwomen experience greater social impact than males (11). Leprosy is a slowly progressive, spectral disease. At one end of the spectrum is tuberculoid leprosy wherein patients mount a strong cell-mediated immune response against M. leprae, resulting in the reduction and eventual clearance of the infecting bacteria. At the other end of the spectrum is the lepromatous condition, in which patients display disseminated infection with high bacillary load, high levels of anti-*M. leprae* antibodies, and a weak cell-mediated immune response towards M. leprae antigens. In between these two polar forms, unstable borderline cases with specific clinical, immunological and pathological characteristics exist (12).

Figure 2 Damage to the eyes, face and hands of leprosy patients.
Source: Communicable Diseases Module 18. Leprosy Diagnosis

The clinical presentation is correlated with the quality of the immune response, Tuberculoid leprosy is the result of high cell mediated immunity with a strong Th1 type immune response limiting the disease progression to skin lesions and well defined nerve damage (no humoral response). Lepromatous leprosy is characterized by low cell- mediated immunity with a predominant humoral Th2 response (high production of IgG or IgM antibodies), leading to an inadequate immune response to an intracellular bacterium and to the uncontrolled multiplication of the bacilli (13).

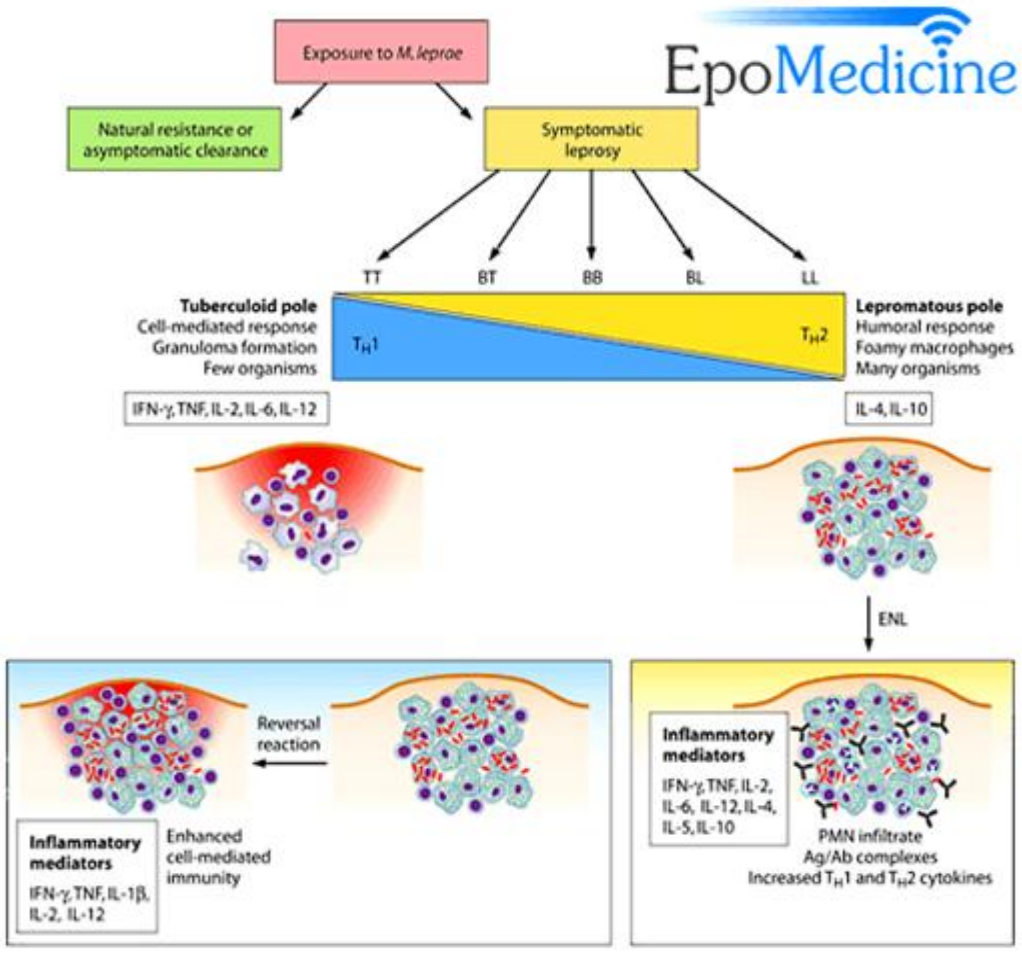

Figure 3 Pathogenesis of *M. leprae.*

Epidemiology of leprosy

Leprosy is endemic in tropical countries, especially in developing countries. Its prevalence has decreased markedly since the introduction of MDT in the beginning of the 1980s. However, many endemic countries, specifically locatedin Southeast Asia, in the Americas, Africa, Eastern Pacific and Western Mediterranean, still concentrate a large number of cases. Leprosy has a long history in Ethiopia; literature indicated that leprosy had been recognized as major public health problem for more than half a century.In the past 11 years, epidemiological trends of leprosy in Ethiopia reported more than 14% disability rate between the years 2000 and 2011, and around 5000 new cases of leprosy per year on average are reported despite the efforts ofstakeholders(14).

Leprosy is a chronic and curable infectious diseaseaffecting more than 200,000 people every year globally according to WHO report of 2015. The 2016 WHO reportshow that the global registered prevalence of leprosy at the end of 2015 was 210, 758 cases (3.2 cases per 100, 000 population)Number of leprosy cases with grade-2 disabilities detected among new cases in the same year were 14,059 (2.5 per1000 000 population), where in Africa it was 2 887 (4.1 per 1000 000 people), In Ethiopia the number of new cases detected in the same year was 3 970. The number of new cases indicates the degree of continued transmission of infection. Global statistics show that 199, 992 (94%) of new leprosy cases were reported from 14 countries reporting more than 1000 new cases eachyear(15).

In 2016 WHO has launched a new global strategy, The Global Leprosy Strategy 2016–2020: Accelerating towards a leprosy-free worldwhich aims to reinvigorate efforts for leprosy control and to avoid disabilities, especially among children affected by the disease in endemic countries, and also our work in the laboratory methods of sensitivity has a major input for this target(16).

Disease classification

The bases for the Ridley-Jopling classification system are clinical, histological features and bacteriological index. According to this classification scheme, there are two poles of spectrum and three borderline forms of leprosy. The two poles of the spectrum are lepromatous leprosy and tuberculoid leprosy. The borderlines are designated as borderline tuberculoid (BT), mid

borderline (BB) and borderline lepromatous (BL)(17). A sixth classification, indeterminate leprosy (IL), is also commonly used.

As WHO states, mainly for treatment purpose, leprosy is broadly classified as paucibacillary and multibacillary. In the multi-Bacillary (MB) type, bacillary load is high and this form includes lepromatous leprosy (LL), borderline lepromatous (BL) and mid-borderline leprosy (BB), in which the skin smear is positive and/or the number of skin lesions is more than five. In the Pauci-Bacillary (PB) type, the bacillary load is low and it includes tuberculoid (TT), borderline tuberculoid (BT), indeterminate (I) and pure neuritic (PN) form, in which the skin smear is negative and/or the number of skin lesions is 1up to 5 (18).

Leprosy reactions are classified in to two main types: type 1 Reversal reactions (RR) and type 2 Erythema Nodosum Leprosum (ENL) is immune-mediated inflammatory complications of the disease which can occur before, during or after successful completion of multi-drug treatment. They are a major cause of nerve damage and morbidity in a significant proportion of leprosy patients. RR occurs in 30% of patients and involves a sudden activation of an inflammatory response to *M. leprae* antigens and it's the main cause of nerve damage in leprosy affected patients present with swollen hands and feet, exacerbation of cutaneous lesions and neural involvement. ENL affects patients with poor cellular immune responses and present in MB patients with high levels of anti- *M. leprae* immunoglobulins, antigen antibody complex deposition. ENL is characterized by an abrupt onset of erythematous and painful nodules accompanied by systemic such as fever, lymph node infraction, bone tenders and hepatosplenomegaly(19), both RR and ENL contribute immenselyto the burden of leprosy and need to be diagnosedand treated early to prevent nerve function impairmentand permanent disability(20).

If we further look in to the five characteristics of these forms, in lepromatous leprosy (LL) macrophage and foam cell collections present with numerous bacilli interspersed with sparse number of lymphocytes. In Borderline lepromatous (BL) leprosy, there are macrophage granulomas, numerous lymphocytes and moderate numbers of bacilli. In Borderline tuberculoid (BT) leprosy, lymphoepithelioid granuloma presents with occasional Langerhans giant cells and in Tuberculoid leprosy (TT), immature epithelioid cells are present together with Langerhans giant cells and numerous lymphocytes. In Indeterminate leprosy some nerve inflammation is

seen with rare acid fast bacilli and an absence of clear epithelioid or macrophage granulomas , BB shows almost equal admixture of epitheloid cells and macrophages without forming a distinct granuloma(21)

Diagnosis of leprosy

M. leprae remains a diagnostic challenge in developing countries for many clinicians because of the low sensitivity of conventional methods in detecting M. leprae bacilli in clinical specimens of SSS and biopsy. The diagnosis of leprosy is based on observation of clinical symptoms, eventually supported by bacteriologic and histopathological features. at present, the diagnosis of leprosy is based on the 3 cardinal signs of the disease, which are Hypo pigmented or reddish patches with definite loss of sensation; enlarged peripheral nerve and AFB positive slit-skin smear or biopsy (22).The Clinical classification gives information confined to only gross appearances of the lesions. A great variation has been observed in the interpretation of the histopathological examination and reports(23).Hence, the high sensitivity methods includingPCRhave to be introducedto confirm the diagnosis of leprosy (24). Currently the main focus is on promoting early detection of leprosy cases to reduce the disease burden and especially the disabilities it causes.

Among many skin disorders which have similar signs with leprosy, cutaneous sarcodiosis and leismaniaand other have been studied in different countries with or without leprosy burden for its frequent misdiagnosis with leprosy.Diagnosing solely on the clinical basis will lead to error in cases of Indeterminate, borderlines leprosy and can occur difficulties in classification and treatment. Due to its clinical diversity as well as its ability to mimic other diseases, leprosy is sometimes difficult to diagnose clinically. Therefore, histopathological and molecular correlation offers better examination and a helpful diagnostic tool to confirm the diagnosis (25).

I. Ziehl Neelsen (ZN) staining

The primary laboratory method for the diagnosis of *M. leprae* is Ziehl Neelsen staining in SSS. ZN staining on SSS is the widely used conventional method of leprosy diagnosis and can aid in the confirmation of a diagnosis in a suspected case. The reproducibility of SSS result depends on

individual and as 10,000 bacilli per ml of sample is required for reliable detection in stained smears(26). A negative ZN staining only indicates that the concentration of bacilli is below 10, 000 bacilli/ml and this does not necessarily mean that the person is not infected. This issue is especially problematic for individuals with pure neural leprosy(PNL), indeterminate (IL) and paucibacillary (PB) disease, which harbor a low burden of bacteria.

Although confirmation of clinically diagnosed leprosy cases is done through detection of acid-fast bacilli or typical histopathologic features of skin and nerve punch biopsy samples, 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 essential for the diagnosis of such inconclusive cases to ensure early diagnosis and make the patients receive the treatments on time (27).

II. Histopathology

Histopathologic examination is one of the most important tools for detecting *M. leprae*. The histopathological analysis and bacilloscopy are useful in prompt diagnosis and exact typing of the disease and are complementary diagnostic proceduressupporting the clinical diagnosis of leprosy patients. The bacilloscopy of histological sections (BI/H) and bacilloscopy of slit skin smear (BI/S) have an important role for patient diagnosis and follow up, and for choosing the adequate chemotherapeutic regimen(28). The Histopathological examination of skin can provide information regarding cellular morphologypresence of AFB and to accurately classify the lesion in the leprosy spectrum.

Due to the potential neural damage and consequent disabilities and the stigma of leprosy for humans, the correct histopathological diagnosis is mandatory to assist the doctor regarding the spectral form of the patient's disease and its prognosis, favoring a therapeutic outcome during follow-up and confirmation of the leprosy diagnosis to determine the disease load in a given population and the correct clinical classification to determine the risk of patients developing incapacities are important motives for performing the histopathological exam. The pathologist is expected to provide a definitive diagnosis(29).

Studies have shown thatHistopathological examination detectsdefinitive features of leprosy only in 35% of early cases and in the remaining cases histology show only chronic inflammatory signs which are common to many dermatoses(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(31). The finding of typical histopathology features in paucibacillary cases may be missed and reported as non specific, which in turn leads to inconclusive result and makes the diagnosis challenging so it requires additional diagnostic techniques like molecular techniques to ensure early diagnosis and to provide the patients receive the treatments on time.

III. Molecular detection of *M. leprae*

Definitive identification of *M. leprae* has been possible through the development of methods for the extraction, amplification, and identification of *M. leprae* DNA in clinical specimens using PCR. This technique has been applied not only to skin biopsy samples, but also to several different types of specimens such as skin smears, nerves, urine, oral or nasal swabs, blood, and ocular lesions (32). PCR allows detecting slow growth or uncultivable microorganisms and showing significantly better results compared to common microscopic examinations. It is based on the amplification of specific sequences of *M. leprae*genome and in the identification of the fragment of amplified DNA. PCR assay has been applied to support monitoring disease status in leprosy patientsor in those who present with nerve damage in the absence of skin lesions.

In aleprosy study conducted on SSS of Childhood and Adolescent, ZN stain was positive in 17/73 (23.28%) cases and *M leprae* specific RLEP PCR in non stained skin smears was positive in 56/73 (76.71%) cases, therefore the PCR detection in skin smear is more sensitive than AFB and can serve as a good, minimally invasive diagnostic tool for diagnosis of leprosy. *M. leprae*65kDa, 18kDa, 36kDa, due to their large size, undergo damage/fragmentation during the procedure andtherefore they are not widely used. However, this does not occur with RLEP amplicon, which is specific for *M. leprae*. Moreover, it is a repetitive sequence with 28 repetitions in the *M leprae* chromosome and more sensitive than other amplicons which have been used for PCR(33). A study in India showed extraction and detection of *M. leprae* DNA from ZN stained skin smear negative slides and they detected *M. leprae* DNA in 32.6% (34).

This indicates that PCR amplification of *M. leprae* DNA isimportant in diagnosis of leprosy due to its sensitive, specific, and rapid detection of microorganisms in clinical specimens especially in the PB form of leprosy.

Another study in India showed that PCR and reverse transcription-PCR-based techniques having a specificity of 100% and a sensitivity of 34 to 80 % in paucibacillary patients and in greater than 90%of multibacillary patients. Therefore, PCR can provide an excellent adjunct to clinical and histopathological diagnosis of leprosy(35).

Among its many utilities, PCR may allow support of the conventional methods for the analysis of clinical samples in difficult to diagnose cases, such as pure neural leprosy, indeterminate and paucibacillary leprosy. With microscopic visualization, all mycobacteria are phenotypically indistinguishable demonstrating subclinical infection in contacts; monitoring treatment; determining patients' cure or resistance to MDT drugs; and help understand the mechanisms of *M. leprae*transmission (36).

The detection of *M. leprae* by PCR techniques is specific and as few as 10 to 100 bacilli can be detected (37). RLEP primers assay was specific for *M. leprae* and able to detect 10 fg of purified *M. leprae* DNA, or approximately 300 bacteria in infected tissues(38). There are a number ofdifferent sequences*M. leprae* DNA targetsfor PCR amplification like Proline-rich antigen(pra-36 KDa),18-kDa antigen,65-kDa antigen, RLEP and 16S rRNA which can be used in different PCR methods (39).

GeneXpert test is a molecular test which detects the DNA in TB bacteria. It uses a sputum sample and can give a result in less than 2 hours. It can also detect the genetic mutations associated with resistance to the drug Rifampicin. The GeneXpert system simplifies molecular testing by fully integrating and automating sample preparation, amplification and detection by PCR, could be developed for leprosy. No data exist so far concerning the relative performance of different laboratories and methods for *M. leprae* DNA detection using GeneXpert (38),(40).

Treatment of leprosy

Leprosy is treated with multidrug therapy (MDT), and standard therapy regimens are applied according to the operational classification established by the WHO. Multidrug therapy, usually consisting of Rifampicin, Clofazamine, and Dapsone, is used to treat all cases of leprosy to reduce the development of drug resistance. To treat PB leprosy casesconsistingtwo drugs Rifampicin and Dapsone for 6 months and to treatMB leprosy casesconsistingthree drugs Rifampicin, Clofazimine and Dapsone for 12 months.In patients receiving MDT, a high proportion of bacilli are dead within days, suggesting that many symptoms of leprosy must be due in part to dead cells. Thus, development of a drug to rid the body of dead organisms is essential(41).

Prevention

Early detection of the disease is important, since physical and neurological damagesare irreversible even if cured. Medications can decrease the risk of those living and in contact with untreated leprosy patients from acquiring the disease (42). BCG vaccine is recommended and widely used in endemic countries, with consistent evidence of its protection against leprosy.

1.2. Statement of the problem

The clinical and microscopic examinationsare not enough for detection of *M. leprae* because both lack the power to detect PB patients. The inability of the bacteria to grow in artificial media and its extremely slow generation time of 12–14 days in selected live animals are the major challenges. Therefore, there is need for a more sensitive and fast diagnostic tool for early diagnosis of leprosy cases to prevent the deformities and disabilities. Serological tests based on specific *M. leprae* antigens also do not detect all clinical cases because most of the patients at thePB stage of infection do not develop significant levels of antibody response(43).New case detection rates per year are still high-indicating active transmission. Additionally, in tuberculoid patients, in which bacilli are rare or nonexistent, there is always a limitation in using bacilloscopy as an auxiliary exam, once negative results do not rule out the disease(44).Delay in diagnosis can lead to increased risk of nerve damage, at present; the diagnosis of leprosy in Ethiopia is mainly based on clinical evaluation and microscopic detection of acid-fast bacilli in tissue smears. Microscopic examination is not enough for detection of *M. leprae* because at least 10,000 organisms per gram of tissue are needed for the detection of *M. leprae* by the AFB staining procedure.

1.3. Significance of the study

Even though Leprosy is a problem in developing countries like Ethiopia, there are limited studies on the laboratory diagnostic methods. The conventional method, ZN staining has low sensitivity. Therefore, this study showed the potential of PCR for detection of *M. leprae* on slit skinsmear samples. It provides baseline information for further investigations. The study forwards important recommendations that can be used during designing prevention andcontrol strategies of the diseases(45).

3. Hypothesis and Objective

3.1. Hypothesis

PCR on slit skin smear detects more clinically confirmed leprosy cases than ZN AFB staining.

3.2. General Objective

To evaluate the diagnostic performance of PCR to detect *M. leprae*on stained slit skin smear collected from clinically confirmed leprosy cases.

3.3. Specific Objectives

- To assess the sensitivity and specificity of PCR in ZN stained slides of SSS from leprosy cases.
- Tocompare the laboratory tests with clinical classification.
- To compare PCR and other laboratory tests with leprosy spectrum

4. Materials and Methods

4.1. Study area

The samples were previously collected from ALERT hospital from new untreated patients. The samples collection was done by a lab technician at the Central lab of ALERT.Slit Skin Smear were collected from three different body parts of the leprosy patients to increase the probability of detecting acid fast bacilli. The right and left earlobes were the common sample collection sites and as a third site either one of eyebrows, forehead or one of the arms were chosen for diagnosis and the second slit skin smear was collected for the research purpose. ZN staining was performed and done and slides were stored at AHRI Histopathology laboratory.

4.2 Study Design and Period

Retrospective cross sectional study was conducted from February to May, 2017.

4.3Sample size and sampling technique

One hundred thirty seven clinically diagnosed PB and MB leprosy cases of archival SSS ZN stained slideswhich were examined and stored at AHRI histopathology laboratory from 2015 to 2016. Out of 137 we selected 60 archival SSS ZN stained slides for this study. The sampling technique was purposive sampling where all forms of leprosy were represented.

4.4Inclusion and Exclusion Criteria

4.4.1 Inclusion Criteria

Archival SSS ZN stained slidesof clinically diagnosed leprosy cases and stored at AHRI histopathology laboratoryfrom 2015 to 2016.

4.4.2 Exclusion Criteria

Archival ZN stained SSS samples with incomplete data are excluded for this specific study.

4.5Sample and Data Collection

4.5.1 Data collection instrument

Client request format, laboratory registration and pathology laboratory test results (secondary data).

4.5.2 Sample collection

Systematically collected archived ZN stained slidesof skin smear based on thedata sheet which includes all information on demographic characteristics like age, gender, clinical diagnosis and performed laboratory diagnosis regarding each 60samples ofpatient from AHRI Histopathology laboratory.

The Armauer Hansen Research Institute (AHRI) is a biomedical research institute which was established by the Swedish and Norwegian Save the Children organizations in collaboration with the University of Bergen, with the principal objective of pursuing basic research in leprosy. AHRI is located in the same compound with ALERT. While AHRI was engaged in leprosy research, the ALERT hospital was fully involved in leprosy treatment and management until the portfolio of the two institutions expanded to include other infectious diseases. Both ALERT and AHRI are managed by the Ethiopian Federal Ministry of Health.

4.5.3 Data collection

All data including the clinical data and Histopathological data of the PB & MB leprosy casesof 60 archived samplesfrom 2015 to 2016 were collected and organized using the structured check list and Patients ageranged 15 to 70 years in AHRI Histopathology laboratory.

4.5.4 Quality control

The appropriateness of the reagents was rechecked with a known positive and negative sample for internal quality control and data quality control measures was taken to maintain the quality of data by crosschecking the test result and lab registration book. During the PCR procedure,

known positive and negative DNA samples for *M. leprae* were used as positive and negative control, respectively and optmization.

4.6 Specimen processing

4.6.1 DNA extraction

We extract DNA from ZN stained slides of SSS by DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA (Azevedo MC, et al, 2016) We removed the oil by xylene from ZN stained slide and air dry completely, then we added molecular grade water 100µl to facilitate sample releaseand scrap the smear by new surgical blade. The scrapped sample was transferredto a new eppendorf (1.5 ml micro centrifuge tube). Then briefly centrifuged at 6,000 rpm for 15sec then180µl of lysis buffer ATL & 20µl Proteinase k was added, and then incubated at 56ºc for 1hr. At the end of incubation time 200µl buffer AL and 200µl ethanol were alsoadded to the lysate and vortex. The tube containing lysate was centrifuged 30 sec at 6,000 rpm. This mixture was transferred to a spin column and centrifuged at 6000xg for 1 min discarding the flow through and transferred to a new collection tube. Then 500 µl of buffer AW1 was added and centrifuged at 6000xg for 1 min. Theflow through was again discarded and the column transferred to a new collection tube. Then 500 µl of buffer AW2 was added andcentrifuged at 6000xg for 1 min; discarding the flow through the column was transferred to new collection tube and centrifuged at 20,000xg for 3min. Finally, the column was transferred to a sterile 1.5 ml tube and 50 µl ofthe elution buffer was added and centrifuged at 20,000xg for 1 min.

Polymerase Chain Reaction (PCR) for detection of *M. leprae*

All 60 extracted DNA samples were amplified using Conventional PCR and performed using specific primers RELP repetitive region for *M. leprae* previously used in AHRI histopathology (microsynth, the swiss DNA Company, Switzerland) to detect RLEP *M. leprae* specific repetitive region. The primers used were 5µl of 2µM RLEP 7 (5'-TGA GGC TTC GTG TGC TTT GC-3') and 5µl of 2µM RLEP 8 (5'-ATC TGC GCT AGA AGG TTG CC -3'), 25µl hot start master mix, 10 µl molecular grade water and 5µl DNA template was added to amplify 500 bp fragment (Avanzi et al., 2016) in a final volume of 50µl. Molecular grade water and known *M. leprae* DNA was used as negative and positive controls.Amplification was started by 95ºc for 5 minute and followed by 39 cycles consisted of 95ºc of denaturation for 30 second,58ºc of

annealing temperature for 40 seconds and 72 ºc of extension for 30 seconds. The final extension time 10 minutes at 72^0c and hold at 4ºc.Molecular grade water and known *M. leprae* DNA was used as negative and positive controls respectively.

Detection of amplified product

The amplified PCR product was detected using 1% agarose gel electrophoresis. Each 50 µl amplicon was mixed with 5µl loading dye and 10µl of it was loaded on 1% agarose gel prepared in a Tris- Acetate EDTA (TAE) buffer containing 1µl Ethidium bromide. The gel was exposed at120 V for 30 minutes; and finally the presence of amplified DNA was visualized by exposing the gel for UVradiation in transilluminator. The PCR results were interpreted by observing the presence or absence of the amplified 500-bp band in an agarose gel using a reference DNA ladder with 100-1000 within 100 bp variations. After reading the PCR product using UV radiation, both the soft copy and the hard copy of the gel readings were kept accordingly.

4.7 Study Variables

4.7.1 Dependent variables

PCR

H&E and FF staining

Archival SSS ZN stained slides

4.7.2 Independent variable

Clinical characteristics

4.8 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 R software for analysis. The hard copy of all information was kept secured in the data management unit.

4.9 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. The three laboratory testsselectedto supportthe clinical diagnosis are H&E, SSS ZN and FF staining, due to their routine application to diagnose leprosy worldwide. andwe used H&E staining as a gold standard for this specific study (46). Data were statistically described in terms of mean, frequency (Number of cases) and relative frequency (percentages).

All obtained Socio-demographic data, clinical information and laboratory results were entered to SPSS V 21 software for statistical analysis. Data which did not require to be recoded like age and number of lesion was entered in the software directly. Categorical data like microscopy and PCR results, disability, type of reaction and disease classification recoded to numeric 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.

4.10 Dissemination of Results

The findings of this studywill bepresented and submitted to Addis Ababa University College of Health Sciences School of Medicine Department of pathologyandArmauer Hansen Research Institute. The findings can also be presented in seminars and symposia. The results of the whole study was disseminated to the concerning health institutions, government bodies such as MOH, HBand other concerning stack holders. Said to be scientific the work will be published in international journals and accessible for further study.

4.11 Ethical considerations

The study was conducted after obtaining institutional ethical clearance from ethical review committee of Addis Ababa University, College of Health Sciences School of Medicine, department of pathology and Armauer Hansen Research Institute.Findings of the study were confidential and all records return to appropriate place after the data collection safely and timely.

5. Result

5.1 Socio-demographic and clinical characteristics

A total of 60 clinically diagnosed leprosy patients' archival samples which fulfilled the inclusion criteria were included in our study. The study samples were from patients with age range of 15 to 70 years and with the mean age of 37.9. Majority of the leprosy cases were found in the age group of 26 to 35 years.We included samples from clinically diagnosed 42(70%) male and 18(30%) female leprosy cases and the male to female ratio was 2.3:1.

Among the clinically confirmed leprosy cases, 30% (18/60) were paucibacillary (PB)with five or less skin lesion, of this 9/17 (52.9%) showed disability.Multibacillary (MB) cases were 70.0 % (42/60) with6 or more skin lesions, of which 21/43 (48.8%) had disability. A total of 14 MB patients had reaction where 13 (30.23%had reversal reaction (type 1) and only 1(2.33%) had Erythema Nodusum Leprosum (type 2 reaction) [Table 1].

Table 1 Socio-demographic and clinical characteristics of study subjects(n=60)

Variable	**Frequency (%)**
Age Group	
15- 25	10 (16.67%)
26- 35	23 (38.33%)
36- 45	13 (21.67%)
> 46	14 (23.33%)
Gender	
Male	42 (70%)
Female	18 (30%)
Clinical status	
PB	17/60 (28.33%)
MB	43/60 (71.67%)
Reaction	
PB	6 (5-NU, 1- RR) (35.3%)
MB	14(13- RR, 1-ENL) (32.5%)
Disability	
PB	9/17 (52.9%)
MB	21/43 (48.8%)

5.2. PCR on SSS

The amplified PCR products for each sample were run on gel electrophoresis and pictures were acquired using a UV transilluminator. Figure: 1 show some of the PCR positive and negative samples. In general, we found 29 (48.33%) samples being positive by PCR on SSS. Among these positive samples 3 MB and 2 PB samples were from AFB negative slides. The figure shows the 500bp bands in agarose gel for *M. leprae*detection.

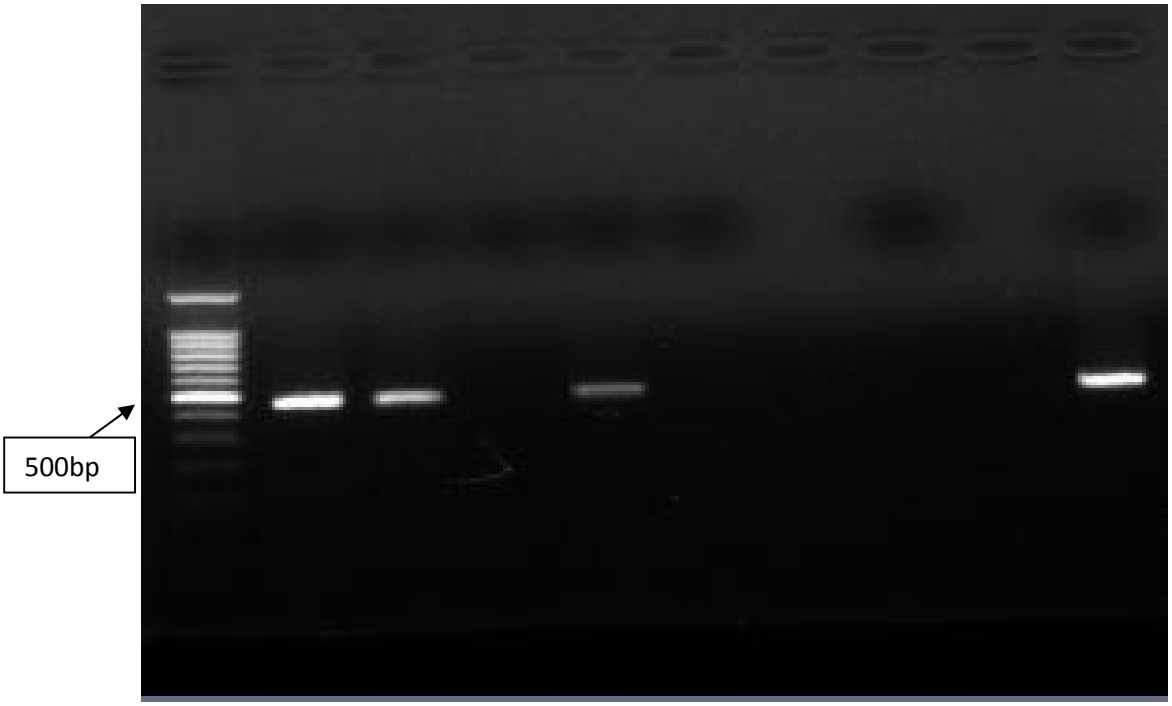

Figure 4 PCR result of SSS samples showing *M. leprae* positive and negative samples.

1- 1kb DNA ladder; 2, 3 and 5 Positive samples of patients; 4 and 6 Negative Sample of patients; 8-Negative control & 10- Positive control.

5.3. Comparison of different laboratory methods with clinical classification

We found that out of the sixty clinically diagnosed leprosy cases, the PCR on SSS detected 23 (38.3%) in MB and 6 (9.9%) in PB forms.The ZN staining picked 21 (35%) in MB cases and 3 (5.000%) in PB cases and confirmed or supported MB cases by H&E were32 (53.3%) and 13 (21.6%) PB cases.As to FF staining,30 (50 %) in MB and7 (11.6%) PB was detected [Table 2].

Table 2. Comparison of different laboratory methods with clinical classification (n=60).

Laboratory methods	**Category**	**Clinical classification**		Total case
		PB (%)	MB (%)	
Biopsy H&E	Positive	13 (21.666%)	32 (53.333%)	45 (75.000%)
	Negative	5 (8.333%)	10 (16.667%)	15 (25.000%)
	Total	18 (30.000%)	42 (70.000%)	60 (100.000%)
Biopsy FF	Positive	7 (11.667%)	30 (50.000%)	37 (61.667%)
	Negative	11 (18.333%)	12 (20.000%)	23 (38.333%)
	Total	18 (30.000%)	42 (70.000%)	60 (100.000%)
SSS PCR	Positive	6 (10.000%)	23 (38.334%)	29 (48.334%)
	Negative	12 (20.000%)	19 (31.666%)	31 (51.666%)
	Total	18 (30.000%)	42 (70.000%)	60 (100.000%)
SSS ZN	Positive	3 (5.000%)	21 (35.000%)	24 (40.000%)
	Negative	15 (25.000%)	21 (35.000%)	36 (60.000%)
	Total	18 (30.000%)	42 (70.000%)	60 (100.000%)

5.4. Sensitivity, specificity, positive and negative predictive values of the laboratory results

In order to consider a diagnostic method for clinical application, it has to have acceptable sensitivity and specificity. Therefore, we analyzed the sensitivity and specificity of the different methods. The sensitivity and specificity of the ZN staining were 47% and 80% respectively and the PPV and NPV were 88% and 33% respectively. The Sensitivity and Specificity for the

PCRon SSS were 62% and 93% respectively and the PPV & NPV were 96% and 45%. The Sensitivity and Specificity of the FF were found to be 71% and 67% respectively. While the PPV & NPV were 86% and 43% respectively [Table 3].

Table 3 Sensitivity, specificity, positive and negative predictive values of the laboratory results against H&E as gold standard (n=60)

Laboratory tests	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
SSS ZN	47%	80%	88%	33%
SSS PCR	62%	93%	96%	45%
Biopsy FF	71%	67%	86%	43%

5.5. The potential of lab tests in detecting different forms of leprosy

Although leprosy is broadly classified as PB and MB for treatment purpose, based on the Ridley and Jopling classification, leprosy has different spectra. Other than the two polar forms, TT and LL, there are borderline forms which are immunologically unstable mainly BT, BB and BL. Considering the different forms of leprosy, among the PB patients 11.12% are BT confirmed by all tests. PCR detects IL and TT (11.12%)forms but not the other tests. Among the MB cases, 19.45% cases are detected as LL in all tests. In addition, PCR detects 13.89% BB cases. ZN and FF detect5.56% and 11.12% BB respectively (Table 4-A&B). Kappa value was also calculated to measure the agreement between PCR and H&E (0.27) (Table 5).

Table 4-A: Patients categorized by Ridley and Jopling classification

IL	3(5%)
TT	5(8.33%)
BT	14(23.33%)
BB	7(11.67%)
BL	8(13.33%)
LL	8(13.33%)
Total	45(75%)

Table 4-B: Leprosy spectrum percentage of ZN, PCR and FF with PB and MB classification

leprosy spectra	H&E stain N (%)	ZN stain N (%)	PCR N (%)	FF stain N (%)
Paucibacillary n= 9				
IL	1 (11.12%)	0 (0%)	1 (11.12%)	0 (0%)
TT	3 (33.34%)	0 (0%)	1 (11.12%)	0 (0%)
BT	1 (11.12%)	1 (11.12%)	2 (22.24%)	1 (11.12%)
Not specified in spectrum	4 (44.45%)	1(11.12%)	0 (0%)	2(22.23%)
Total	9 (100%)	2(22.24%)	4(44.48%)	3(33.35%)
Multibacillary n = 36				
BB	5 (13.89%)	2 (5.56%)	5 (13.89%)	4 (11.12)
BL	7 (19.45%)	6 (16.67%)	6 (16.67%)	7 (19.45%)
LL	7 (19.45%)	7 (19.45%)	12 (33.34%)	7 (19.45%)
Not specified in spectrum	17(47.23%)	2 (5.56%)	2 (5.56%)	3(8.34%)
Total	36 (100%)	17 (47.23%)	25 (69.46%)	21 (58.36%)

Table: 5 Kappa value of laboratory tests: PCR and H&E

PCR	H&E		Total
	Positive	Negative	
Positive	28(46.667%)	1(1.667%)	29(48.334%)
Negative	17 (28.333%)	14(23.333%)	31(51.666%)
Total	45(75.000%)	45(25.000%)	60(100.000%)
Kappa value	0.27		

5.6 BI and PCR positivity

The BI shows the load of bacilli in patients and also used for treatment follow up. In this specific study, 83.34% had a BI of >3 (20) and those with BI <3 were 16.67% (4). The number of cases confirmed by PCR (29) is more than the AFB detection (24) (Table 6).

Table 6. BI and PCR positivity

BI	Cases confirmed by AFB In SSS	Cases confirmed by PCR
>3	83.33% (20)	68.96% (20)
<3	16.67% (4)	31.04% (9)
Total	100% (24)	100% (29)

6. Discussion

There are major challenges in leprosy control which need continuous and advanced research. The consistent number of new cases in some countries reported in the last decade, the considerable number of new cases in children and the number of new cases coming with grade 2 disabilities are the major ones which indicate the ongoing transmission of the disease. Therefore, the current research on leprosy globally focuses on development of early diagnostic tools to reduce the number of new cases and eventually break the transmission. Along with this improving the existing staining technique or introducing advanced techniques to the routine leprosy diagnosis methods in clinics certainly have a tremendous importance.

ZN Staining of SSS is the main tool available currently for diagnosis of leprosy in the developing countries supporting the clinical diagnosis. However, this conventional technique has its own limitation as it is less sensitive and usually misses PB cases mainly due to their low bacillary load. Therefore, improving this technique or introducing a more sensitive diagnostic method is necessary for early diagnosis of leprosy cases to prevent mainly disabilities. Many studies have reported the advantages of PCR to detect *M. leprae* compared to SSS microcopy (32, 33). Similarly in our study, we run PCR for 60 archived slides of SSSand compared it with theroutine conventional SSS microscopy.Our study demonstrated a positive yield in 45% of all cases of clinically diagnosed leprosy cases by SSS PCR and SSS microscopy was 40%. Although the difference between these methods is insignificant, the result from SSS PCR indicated that it is possible. To detect cases not picked by the ZN staining but needs certain improvements to exploit the potential of this advanced technique.

In the present study, 48.33% of the total cases were SSS PCR positive with 71.43% in BB, 87.5% in BLand 100% in LL subgroups. In our study the sensitivity of PCR in PB case is low as compared with the number of PCR positive casesin MBpatients were as many as 70% of the MB cases were PCR positive, while only30% in the PB cases. This result was expected as MB leprosy has a higher bacterial load than PB leprosy.

However, considering the high prevalence of positive PB case by PCR from SSS in a study conducted in Eastern Nepal(47) and emphasizing on the purpose of introducing such sensitive techniques, there is a need to identify the causes for the low PB detection rate of the SSS PCR in

our study. The possibility of extracting DNA and running PCR from ZN-stained skin smear slides has an added advantage in the screening of smear negative slides in leprosy, which will contribute to accuracy in diagnosis and treatment of leprosy.Leprosy diagnosis is mainly dependent on the clinical diagnosis of the disease because of lack of sensitive laboratory diagnostic tests. There are a number of reasons for poor sensitivity 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.According to standard techniques, skin smears should be taken at least from six different sites for proper evaluation; the skin is cleaned by 70% alcohol and the site is pinched between thumb and index finger, 5 mm long and 2-3 mm deep with a sterile surgical blade to take the specimen. This might not be properly practiced in recent days from lack of experience which has an impact on the result. As PCR is a sensitive method, introducing it in to the diagnosis procedure at least in referral laboratories where GeneXpert is available will contribute in improving the leprosy diagnosis.

The number of positive results by H&E staining is higher than SSS PCR and this is mainly because of the nature of the disease. *M. leprae* is an intracellular bacterium which primarily resides in macrophages (48) These skin macrophages are found in the dermis part of the skin. Skin biopsy sampling includes this part of the skin which makes it easier to find the bacilli. As the *M. leprae*bacilli are residing in macrophages in the dermis and the routine skin slit smear is taken from epidermis, the load of the AFB is low in SSS specimens which might compromise the positivity rate in SSS PCR and SSS AFB.

PCR inhibitors have been an obstacle to success. All who use PCR are likely to be impacted by inhibitors at some time, this is one of effect in this result that was xylene. PCR inhibitors generally exert their effects through direct interaction with DNA or interference with thermostable DNA polymerases. Direct binding of agents to single- stranded or double-stranded DNA can prevent amplification and facilitate co-purification of inhibitor and DNA. Inhibitors can also interact directly with a DNA polymerase to block enzyme activity. DNA polymerases have cofactor requirements that can be the target of inhibition. Magnesium is a critical cofactor, and agents that reduce Mg2+ availability or interfere with binding of Mg2+ to the DNA polymerase can inhibit PCR (49).

PCR inhibitors are a very heterogeneous group of chemical substances. One certain matrix may contain many different inhibitory substances and the same inhibitors can be found in many different matrices. Organic as well asinorganic substances, which may be dissolved or solid, can appear as PCR inhibitors. Calcium ions are an example for inorganic substances with inhibitory effects on the PCR. However, most of the known inhibitors are organiccompounds, for example, bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulphate (SDS) and it can be found in a variety of biological materials, organs, blood and body fluids (50).

7. Conclusion and Recommendation

7.1. Conclusion

Diagnosing and treating leprosy on clinical basis will continue to be the main diagnostic tools in developing countries however there is a need to introduce simple and advanced diagnostic techniques mainly for early detection of leprosy as such case are difficult for diagnosis. Hence, introducing molecular diagnosis mainly PCR in addition to AFB staining of skin slit smear and histopathology confirmation of suspected leprosy cases is important for definite diagnosis.

The positive PCR result on stained skin slit smear was low in our study when it is compared with other studies. Therefore, there is a need for further improvement of PCR on skin slit smear samples. This will contribute in simplifying the diagnostic process by avoiding invasive procedures. It also contributes to detect patients with low bacillary load as they are difficult to clearly define clinically.

The DNA extraction procedure does not need special expertise of laboratory personnel beyond average training. In AFB negative slides of skin smear which is PB type of leprosy, whichusually confuses the clinicians with other type of skin diseases. Therefore, PCR may play a great role as a differential diagnosis to reach to the correct diagnosis of leprosy.
Recently, the GeneXpert is being used for TB diagnosis with the aim of replacing the routine AFB staining. Similarly, if we collect evidences on the potential of PCR in diagnosing leprosy, there will be a possibility of integrating the diagnosis for TB and leprosy to efficiently use the available resource throughout the country.

7.2. Recommendation

- Introducing PCR for diagnosis of leprosy is important. However further investigations to improve its sensitivity for detection of PB cases are required
- Having different forms of leprosy, the clinical diagnosis, the H&E staining and PCR technique have their own advantages. Therefore, an integrated approach is required to increase the detection rate of leprosy.

- The recent introduction of GeneXpert for TB diagnosis is an advantage for leprosy diagnosis. Therefore, collecting more evidences on the advantages of PCR for leprosy diagnosis will facilitate the use of GeneXpert for both TB and leprosy.

7.3. Limitation of the study

Time was the main limiting factor which obliges us to take small number of archival stained SSS samples for analysis. In the future this has to be done on freshly collected SSS samples. Clinically leprosy negative cases were not included in the study and no Gold standard test for confirmation of leprosy was a limitation.

8. References

1. T. SPaCS. Mycobacterium leprae: genes, pseudogenes and genetic Diversity. Future Microbiol 2011;6(1):57–71
2. WHO (WHO/CTD/LEP/93.3) Report of a meeting on HIV infection in leprosy 1993.
3. Lastória JC AM. Leprosy: review of the epidemiological, clinical, and ethiopathogenic aspects part 1. Anais brasileiros de dermatologia. 2012;89(2):205-18.
4. Rao AL, Prabhakar M, Krupa DS, Manasa N. Leprosy: Disease prevailing from past to present. International Journal of Research in Pharmacy and Chemistry. 2012;2(3):770-8.
5. Lockwood DN SL, Sagili KD, Chaduvula MV, Mohammed I, van Brakel W, et al. Cytokine and protein markers of leprosy reactions in skin and nerves: baseline results for the North Indian INFIR cohort. PLoS Negl Trop Dis. 2011;5(12):e1327.
6. Kumar B, Dogra S. Leprosy: a disease with diagnostic and management challenges! Indian Journal of Dermatology, Venereology, and Leprology. 2009;75(2):111.
7. Pinheiro RO dSSJ, Sarno EN, Sampaio Mycobacterium leprae–host-cell interactions and genetic determinants in leprosy. an overview Future microbiology. . 2011 6 (2):217-30.
8. Bhat RM PC. Leprosy: an overview of pathophysiology. Interdisciplinary perspectives on infectious diseases. Leprosy. 2012;4.
9. Ebenezer GJ, Polydefkis M, Scollard DM. Mechanisms of Nerve Injury in Leprosy.
10. Legendre DP, Muzny CA, Swiatlo E. Hansen's Disease (Leprosy): Current and Future Pharmacotherapy and Treatment of Disease-Related Immunologic Reactions. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2012;32(1):27-37.
11. Lakshmana Rao A PM, Santhi Krupa D and Manasa N. Leprosy: Disease Prevailing From Past To Present. International Journal Of Research In Pharmacy And Chemistry 2012;2(3).

12. Lini N SNPaDK. Quantitative real-time PCR analysis of Mycobacterium leprae DNA and mRNA in human biopsy material from leprosy and reactional cases Medical Microbiology. 2009;58:753–9.

13. Reibel F CE, Aubry A Update on the epidemiology, diagnosis, and treatment of leprosy. Elsevier Masson France. 2015.

14. SileshiBaye. Leprosy in Ethiopia: Epidemiological trends from 2000 to 2011. ADVANCES IN LIFE SCIENCES AND HEALTH 2015;2:1.

15. WHO. Weekly epidemiological record. 2016;91:405-20.

16. Rao PN. Global leprosy strategy 2016–2020: Issues and concerns. Indian Journal of Dermatology, Venereology, and Leprology. 2017;83(1):4.

17. RIDLEY DSJWH. Classification of Leprosy according to immmunity: Five group system. Inter J lepr other Micobacterial Diseases. 1966;34.

18. Alejandra N. Martinez, 1, Constanc ¸a F. P. C. Britto, 2, Jose,´A.C.Nery, et al. Evaluation of Real-Time and Conventional PCR Targeting Complex 85 Genes for Detection of Mycobacterium leprae DNA in Skin Biopsy Samples from Patients Diagnosed with Leprosy. CLINICAL MICROBIOLOGY. 2006:3154–9.

19. Barbosa de lima Fonseca A VsM, Cazzaniga R A, Rodrigues de Moura T, Pacheco de Almeida R, Duuuthie M S, Reed S G, Ribiro de Jesus A. The infulence of innate and adaptative immune responses on the differential clinical outcomes of leprosy. infectious diseases of poverty. 2017.

20. Kahawita I P WSL, J. Lockwood D N. Leprosy type 1 reactions and erythema nodosum leprosum. An Bras Dermatol. 2008;83(1):75-82.

21. U. K. Skin Biopsy-Perspective. InTech. 2011.

22. Noto S SP. Diagnosis of leprosy. Leprosy mailing list archives. 2011.

23. Pokhrel K PS, Shah M, Sushma Subedi S. Clinico-histopathological correlation of leprosy in western region of Nepal-A pioneer pilot study. Indian Journal of Clinical and Experimental Dermatology. July-September 2016;2(3):93-7.

24. S A MOHAMMED SMSMKZ. Demography, clinical presentation and laboratory diagnosis of leprosy by microscopy, histopathologyand PCR from Dhaka city in Bangladesh. 2016.

25. Badhan R KRK, Raj R T, Kumar Bahl R, and Singh Bal M. A Clinico-Pathological Correlation Study of Leprosy in a Tertiary Care Teaching Institute in Northwest Punjab, India. American Journal of Medical Sciences and Medicine. 2014;2(5):99-108.

26. Yan W XY, Yuan LC, De Yang R, Tan FY, Zhang Y, Li HY. 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.

27. Lavania M JR, 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. 2013;19(11):1058-62.

28. Azevedo M C RNM, Vincenzi Fachin L R, Tassa M. Rosa P S, Belone A F F. qPCR detection of Mycobacterium leprae in biopsies and slit skin smear of different leprosy clinical forms. Braz J Infect Dis. 2016.

29. Teixeira AC CD RF, Luppino LF, Resende LH, Sousa T, et al. Evaluation of the agreement between clinical and laboratorial exams in the diagnosis of leprosy. . Medical Tropical. 2008;41(2):48-55.

30. SS AM. Demography, clinical presentation and laboratory diagnosis of leprosy by microscopy, histopathology and PCR from Dhaka city in Bangladesh. Lepr Rev. 2017;88:122-30.

31. Thakkar S PS. Clinical profile of leprosy patients: a prospective study. Indian journal of dermatology. 2o14;59(2):158-62.

32. Santos AR DMA, Sarno EN, Suffys PN, Degrave WM. Use of PCR-mediated amplification of Mycobacterium leprae DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol. 1993;39:298–304.

33. Kamal R DR GK, Biswas S , Gupta SB , Kumar N , Kumar R , Pengoria R , Chauhan DS , Katoch K , Katoch VM , Singh PK. RLEP PCR as a Definitive Diagnostic Test for Leprosy from Skin Smear Samples in Childhood and Adolescent Leprosy. Indian J Lepr. 2016;88:193-7.

34. Kamble R R SVS MSP, Kamble A A, Ravikumar B P, Jadhav R S. Extraction and detection of Mycobacterium leprae DNA from ZNCF-stained skin smear slides for better identification of negative skin smears. Indian J Med Microbiol. 2010;28:57-9.

35. B. K. World Leprosy Day 2015: Renewing commitment for a leprosy free world. The Indian journal of medical research. 2015;141(1):1.

36. Lastória J C dAMAM. Leprosy: a review of laboratory and therapeutic aspects - Part 2. An Bras Dermatol. 2014;89(3):389-403.

37. Job C K JJ, Williams D L, and Gillis T P. . Role of Polymerase Chain Reaction in theDiagnosis of Early Leprosy. IN1ERNATIONAL JOURNAL IATROSY. 1997.

38. Truman RW AP, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP. Enumeration of Mycobacterium leprae using real-time PCR. PLoS Negl Trop Dis. 2008;2(11):e328.

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

40. Kakoma LN, Mukesi, M. and Moyo, S.R. Effectiveness of GeneXpert Technology in the Diagnosis of Smear-Negative Pulmonary Mycobacterium tuberculosis in HIV Positive Patients in Namibia. Open Journal of Medical Microbiology. 2016;6:133-41.

41. Eichelmann K GSEG, Salas-Alanis J.C, Ocampo-Candiani J. Leprosy. An Update: Definition, Pathogenesis, Classification, Diagnosis, and Treatment. 2012.

42. Rodrigues LC LD. Leprosy now: epidemiology, progress, challenges, and research gaps. . The Lancet infectious diseases. 2011;11(6):464-70.

43. Ruiz-Fuentes JL DA, Entenza AE, Frión Y, Suárez O, Torres P, de Armas Y, Acosta L. Comparison of four DNA extraction methods for the detection of Mycobacterium leprae from Ziehl–Neelsen-stained microscopic slides. . International journal of mycobacteriology 2015;4(4):284-9.

44. Karre S GKK, Gorva A, Veeragandham S, Thungaturthi S R, Malhotra V. Histopathological and Clinical correlation of leprosy in a rural population of South India. Journal of Medical and Dental Science Research. 2015;2(12):14-8.

45. T SAaMP. Leprosy Diagnosis: An Update on the Use of Molecular Tools Lucrecia. Mol Biol 2015;4(4).

46. ANANDARAMA ADIGA D S BHS, RAO G, SAHA D, YELIKAR B R, KARIGOUDAR M. Evaluation of Fluorescent Staining for Diagnosis of Leprosy and its Impact on Grading of the Disease: Comparison with Conventional Staining. Journal of Clinical and Diagnostic Research. 2016;10(10):EC23-EC6.

47. Siwakoti1 S RK, Bhattarai1 N R, Agarwal S, Khanal B. Evaluation of Polymerase Chain Reaction (PCR) with Slit Skin Smear Examination (SSS) to Confirm Clinical Diagnosis of Leprosy in Eastern Nepal. PLOS Neglected Tropical Diseases. 2016;10(12):e0005220.

48. Satapathy J KB, Job C K. Presence of Mycobacterium leprae in epidermal cells of lepromatous skin and its significance. Indian J Dermatol Venereol Leprol.2005;71:267-9.

49. Bessetti J. An Introduction to PCR Inhibitors. Promega Corporation. 2007.

50. C. Schrader AS, L. Ellerbroek and R. Johne. PCR inhibitors – occurrence, properties and removal. Journal of Applied Microbiology 2012;113(5).

8. Annex

8.1. DNA Extraction from ZN Stained Slide of SSS

- Add 100µlof molecular grade water on to the smear on the slide
- Carefully scrub the entire smear in to a new pre labeled 1.5 ml eppindorf microcentrfuge tube using a new surgical blade
- Briefly centrifuge the tube to concentrate the sample
- Resuspend the sample in 180µl buffer ATL and Add 20µl proteinase k and mix by vortex
- Incubate at 56ºc for 1 hour and briefly centrifuge the tube to remove drops inside of lid
- Add 200µl buffer AL to the sample and mix by vortex. Then add 200µl ethanol (96-100%), and mix by vortex
- Briefly centrifuge the tube to remove drop from inside the lid
- Carefully transfer the entire lysate to QIAamp MinElute column in a two ml collection tube without wetting the rim, close the lid and centrifuge at 6000xg for 1 minute.
- Place the QIAamp MinElute in a clean 2 ml collection tube and discard the collection tube containing the flow-through.
- Carefully open QIAamp MinElute column and add 500µl buffer AW1 without wetting the rim, close the lid and centrifuge at 6000xg for 1 minute.
- Place the QIAamp MinElute in a clean 2 ml collection tube and discard the collection tube containing the flow-through.
- Carefully open QIAamp MinElute column and add 500µl buffer AW2 without wetting the rim, close the lid and centrifuge at 6000xg for 1 minute.
- Place the QIAamp MinElute in a clean 2 ml collection tube and discard the collection tube containing the flow-through.
- Centrifuge at full speed for 3 minute to dry the membrane completely.
- Place the QIAamp MinElute in a 1.5ml microcentrfuge tube and discard the collection tube containing the flow-through.
- Carefully open the lid of the QIAamp MinElute column and apply 50µl ATE to the center of the membrane
- Close the lid and incubate at room temperature for 1-5 minute

- Centrifuge at full speed for 1 minute.

8.2. Polymerase chain reaction

- Arrange the required number of micro tubes and label them with the corresponding sample ID including negative and positive controls.
- Prepare 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 master mix 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 H2O	10 µl
DNA Template	5 µl
Total Volume	**50 µl**

1. Mix well the master mix, primers and H2O using vortex and take it to the room where the DNA template is kept.
2. Never bring the DNA template to the room reserved for mastermix preparation.
3. Add 5 µl respective DNA template to the microtube which contains 45 µl primers and master mix.
4. Vortex well and spin briefly to collect all the liquid to the bottom of the microtube.

Cycling condition

step	Temperature	Time	Number of cycle
Initial denaturation	95^{o}c	15 minute	1 cycle
Denaturation	95^{o}c	30 second	39 cycles
Annealing	58^{o}c	40 second	
Extension	72^{o}c	30 second	
Final extension	72^{o}c	10 minute	1 cycle
Hold	4^{o}c	Hold indefinitely	

8.3. Gel Electrophoresis

- We will 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 microwave 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.
- After dispensing the gel in the box which contains the comb, wait 30 minutes till the gel gets solidify.
- In the mean time prepare a10µl ladder which can show a band in 100bp variation.
- Add 5µl 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 5µl ladder to lane 1.
- Then add 10µl samples consecutively starting from lane 2.

- Add 10µl negative and 10µl positive controls in to the last two lanes, respectively.
- Close the lid and connect the wires and set at 120 volt for 30 minutes and run the gel
- Remove the gel from the gel runner and read the presence of 500bp bands in the lanes by using Bio Rad UV illuminator. Any visible band in the specific base pair is a positive result for *M. leprae*.

9. Format for Data collection

Code number	ARLP 001
Age group (yrs) **Sex**	15-24 25-34 35-44 45-55 >=55 Male Female
Clinical Classification	PB MB Lesion Disability Reaction
Archival Data **Archival sample**	H & E, FF, SSS-AFB Stained slides of SSS-AFB