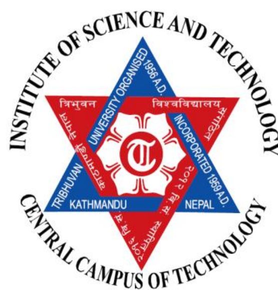
EVALUATION OF GENEXPERT MTB/RIF ASSAY OVER FLUORESCENCE MICROSCOPY FROM TUBERCULOSIS SUSPECTED PATIENTS OF CENTRAL REGION NEPAL



A

Dissertation Submitted to the **Department of Microbiology**

Central Campus of Technology

Tribhuvan University, Dharan, Nepal

In Partial Fulfillment of the Requirements for the Award of Degree

of Masters of Science in Microbiology

(Medical)

By:

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RECOMMENDATION

This is to certify that **Ms. Binita Adhikari** has completed this dissertation work entitled "**Performance Evaluation of GeneXpert MTB/RIF assay over Fluorescence Microscopy from Tuberculosis Suspected Patients of Central Region Nepal"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Medical). To our knowledge this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Hemanta Khanal** this dissertation work of **Ms. Binita Adhikari** entitled **"Performance Evaluation of GeneXpert MTB/RIF assay over Fluorescence Microscopy from Tuberculosis Suspected Patients of Central Region Nepal"** has been approved for the examination and is submitted to the Tribhuvan University in Partial fulfillment of the requirements for M. Sc. degree in Microbiology (Medical).

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ABSTRACT

Tuberculosis is one of the major public health problems worldwide and especially in developing countries like Nepal, and occurrence of drug-resistant is more challenging. The rapid detection of tuberculosis bacteria and drugs resistance permits the establishment of an effective treatment regimen, minimizes the risk of further resistance development and limits the spread of drug-resistant *Mycobacterium tuberculosis* strains. The Xpert MTB/RIF test is a fully automated nucleic acid amplification test for the detection of MTB DNA and rifampicin resistance associated mutation in *rpoB* gene that provides the result within 2 hours. The present study was aimed to evaluate the performance of Xpert MTB/RIF assay over fluorescence microscopy from tuberculosis suspected patients visiting GENETUP, Kathmandu.

The clinical samples, both pulmonary and extrapulmonary, were collected, processed and examined in the laboratory of GENETUP. The collected samples were examined by Fluorescence microscopy (FM) method and by Xpert MTB/RIF assay for the presence of acid-fast bacilli *Mycobacterium tuberculosis* and its resistance to rifampicin, one of the effective anti-TB drugs. Out of 451 suspected samples 177 (39.25%) were detected with the disease and among them 10 cases were found to be rifampicin-resistant TB (RR-TB). Higher proportion of males (60.5%) was detected with TB and which was found to be more in the mid age groups 20-40 years. The sensitivity of Xpert MTB/RIF assay was found to be 95.1% and it detected 79 more MTB cases in smear negative samples than detected by FM; while specificity of Xpert MTB/RIF was found to be 77.3%. Likewise, the sensitivity and specificity of Xpert MTB/RIF assay for PTB samples were 96% and 79.4%, and that for EPTB samples were 80% and 51.9% respectively. The failure of Probe E hybridization denoted the occurrence of mutation and presence of RR-TB. Xpert MTB/RIF assay can be effectively used for the rapid detection of TB and mutation in the gene responsible for rifampicin-resistance.

Keywords: TB, RR-TB, Fluorescence microscopy (FM), Xpert MTB/RIF assay

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LIST OF ABBREVIATION

AFB	: Acid fast bacilli
BCG	: Bacillus Calmette-Guerin
CDC	: Center for Disease Control and Prevention
DST	: Drug susceptibility test
DR-TB	: Drug resistant TB
EDTA	: Ethylene diaminetetraacetic acid
EPTB	: Extrapulmonary tuberculosis
FM	: Fluorescence microscopy
GENETUP	:German Nepal Tuberculosis Project
LJ	: Lowenstein-Jensen
LPA	: Line Probe Assay
MDR-TB	: Multi-drug resistant tuberculosis
MGIT	: Mycobacteria Growth Indicator Tube
MTB/RIF	: *Mycobacterium tuberculosis*/Rifampicin
NPV	: Negative predictive value
NTM	: Non-tuberculous Mycobacteria
NTP	: National Tuberculosis Program
PCR	: Polymerase Chain Reaction
PPV	: Positive predictive value
RR-TB	: Rifampicin-resistant tuberculosis
SLI	: Second line injectables
SPSS	: Statistical Package for the Social Sciences
TB	: Tuberculosis
WHO	: World Health Organization
XDR-TB	: Extended-drug resistant tuberculosis
ZN	: Ziehl-Neelsen

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CHAPTER-I

INTRODUCTION

1.1 Background

Tuberculosis (TB) is an ancient infectious human disease caused by the bacterium *Mycobacterium tuberculosis* and has affected mankind for more than 4,000 years (Zaman 2010). It is one of the chronic diseases and spreads from person to person through air which involves the inhalation of aerosol droplets containing tubercle bacilli (Balasubramanian et al 1994, Chadha 2009). The disease mainly affects the lungs making pulmonary disease the most common presentation (Adigun and Bhimji 2017). In 15-20% of active cases, the disease being multi-systemic, infection spreads outside the lungs causing other kinds of TB collectively known as extra pulmonary tuberculosis (EPTB) (Halezeroğlu and Okur 2014, Jindal 2017). The systems mainly affected by EPTB are pleura (in tuberculous pleurisy), the central nervous system (in tuberculous meningitis), the genitourinary system (in urogenital tuberculosis), the lymphatic system (in scrofula of neck), and the bones and joints (Pott's disease) (Jindal 2017). The other form of widespread and potentially more serious TB is called disseminated or military TB (a case where pulmonary TB and EPTB is found in same patient) which is significantly contributing to TB- related morbidity and mortality (CDC 2015).

The genus *Mycobacterium* lays within the order Actinomycetales which it shares with other bacteria such as *Corynebacterium, Nocardia* and *Rhodococcus.* Mycobacteria are nonmotile, non-sporulating, weakly gram-positive acid-fast bacilli that appear as straight or slightly curved rods, 1-4 µm long and 0.3-0.6 µm wide microscopically (Barry et al 1998). *Mycobacterium tuberculosis* is one of the members of Mycobacterial species which also includes *Mycobacterium bovis*, *Mycobacterium africannum*, *Mycobacterium canetti*, *Mycobacterium microti*, etc (Harshey and Ramakrishnan 1977, Stead et al 1995). The unique mycolic acids expressed by these bacteria in the cell

envelope play a important role in the structure and function of cell wall (Barry et al 1998).

TB remains the major human threat in spite of the updated health-care systems and worldwide implementation of TB control programs (WHO Report 2016). It's a global concern for all developed and developing countries and tends to be more complicated due to the persistence in ageing population and emerging drug-resistant strains (Park et al 2013, WHO 2014). In 2015, the World Health Organization (WHO) estimated 10.4 million individuals had TB but only 6 million cases had been reported to the WHO. This indicates that half of the TB cases are unrecognized to the WHO (WHO Report 2016). In 2016, there was an estimation of 490,000 people developing multi-drug resistant (MDR) TB worldwide and an additional estimation of 110,000 cases of rifampicin-resistant (RR) TB eligible for MDR-TB treatment. The countries having largest numbers of MDR/RR-TB cases (47% of the global total) were China, India and the Russian federation. 6.2% of these cases were estimated to be extensively drug-resistant (XDR) TB and it was reported by 123 WHO states by the end of 2016 (WHO MDR 2018, WHO Report 2018). In 2016, 1.3 million TB deaths among HIV-negative and an additional 374,000 deaths among HIV-positive people was estimated. An estimated 10.4 million people had TB and among which 90% were adults, 65% were male, 10% were people living with HIV (74% in Africa) and 56% were in five countries: India, Indonesia, China, the Philippines and Pakistan (WHO Report 2018).

The incidence of TB disease accounts for 4% of the global burden in South-East Asia region. In 2014 there was an estimated 4 million incidence of TB and about 460,000 people died in this region among which India and Indonesia had the largest numbers of cases making 23% and 10% of the global total respectively. An estimation of 99,000 MDR-TB cases was made among the pulmonary TB cases which accounted for 30% of the world's MDR-TB cases in 2014. Bangladesh, Democratic People's Republic of Korea, India, Indonesia, Myanmar and Thailand are six of the thirty high MDR-TB-burden countries that are in South-East Asia region (WHO 2015).

Tuberculosis remains a major public health concern in Nepal with huge socio-economic burden to the country. The prevalence rate of 211 per 100,000 populations has been estimated by the WHO. In 2013, 59,000 people with active TB were identified and this rate seems to be declining slowly since 1990 and is mostly static since 2000. The case notification rate of TB in 2011/2012 in terai region, hills and mountains were found to be 144, 110 and 66 per 100,000 populations respectively. The drug resistant surveys conducted between 1996/1997 to 2010/2011 revealed that 1.1-3.7% of new TB patients while 12.5-17.2% of retreatment TB patients have MDR-TB. In 2012, GENETUP conducted an XDR-TB survey which showed that among MDR-TB patients, 28% had pre-XDR TB and 8% had XDR TB. It was also estimated that about 2% of the registered MDR-TB patients had resistance against the second line injectable anti-TB medication (NSP Report 2018).

The proper and rapid diagnosis and treatment of TB is a necessity and can be difficult as well as challenging in a developing country like Nepal. The infection or disease is suspected from a number of investigations, clinical signs and symptoms which includes the diagnostic procedure such as radiography, acid-fast bacilli (AFB) smear microscopy and culture, molecular methods like line probe assay (LPA), Xpert MTB/RIF and other nucleic acid amplification (NAA) tests (WHO 2013). According to the annual report of 2016, a total of 4,321 TB treatment centers, 96 urban health centers, 581 microscopy centers and 27 GeneXpert centers have been providing the TB services in Nepal. 14 drug resistant TB treatment centers and 81 sub-centers are providing services for drug-resistant TB cases. At the central level, TB reference laboratories at the National TB center (NTC), Bhaktapur, and GENETUP/NATA, Kathmandu are providing the culture and drug susceptibility testing services (NSP Report 2018).

The use of the rapid test Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) has expanded substantially since 2010, when WHO first recommended its use (WHO Factsheet 2017). In Nepal Xpert MTB/RIF was first implemented in 2011/2012 for the rapid diagnosis of TB (NTC 2016). It is a semi-quantitative nested real-time Polymerase Chain Reaction (PCR) in-vitro

diagnostic test for the detection of MTB DNA in sputum samples and also the detection of rifampicin resistance associated mutations of the *rpoB* gene (XpertMTB/RIF 2015). It is considered as one of the valuable, highly specific and sensitive new tool for the early detection of TB along with RR-TB. The cartridges provided are pre-loaded with all the necessary reagents for sample processing, DNA extraction, amplification and detection of amplified *rpoB* gene target. 5% of rifampicin resistant strains are mono-resistance to rifampicin while a high proportion (about 95%) of it is found to be co-existing resistance to isoniazid, hence helps in the detection of MDR-TB with high level of accuracy (NIHCE 2011, WHO Xpert 2011, Weyer et al 2012, WHO Xpert 2016). Not only sputum samples, this system also evaluate the result from other samples like pus/fluid or tissues from lymph nodes, cerebrospinal fluid, gastric aspirate and pleural fluids (Lawn et al 2013, Maynard-Smith et al 2014, Nhu et al 2014).

Xpert MTB/RIF assay when compared to sputum microscopy, culture and drug susceptibility test provides a more reliable, sensitive and specific result. The test also exhibits high sensitivity and specificity for detecting pulmonary tuberculosis in immunocompromised patient. Hence this study is carried out to evaluate the performance of GeneXpert MTB/RIF assay over fluorescence microscopy from tuberculosis suspected patients in GENETUP, Kathmandu, Nepal. This study not only covers the cases of Central Region but also represents the condition of whole country and the scenario of drug-resistant TB. It provides the current information on prevalence of pulmonary tuberculosis in Nepal and the role of GeneXpert MTB/RIF Assay in detection of *Mycobacterium tuberculosis* along with Rifampicin resistant *Mycobacterium tuberculosis* (RR-MTB) in short period of time. By this study, we can expand the knowledge and importance of this rapid technique among common people and the necessity to set-up this test in every TB centers and sub-centers.

1.2Objectives

1.2.1 General Objective

To evaluate the performance of GeneXpert MTB/RIF assay over fluorescence microscopy from tuberculosis suspected patients of central region Nepal.

1.2.2 Specific Objectives

a. To compare Xpert MTB/RIF assay with microscopic technique.
b. To detect rifampicin resistance *Mycobacterium tuberculosis*.
c. To study the pattern of probe hybridization among positive cases.

CHAPTER-II

LITERATURE REVIEW

2.1 Mycobacterium tuberculosis

Mycobacterium tuberculosis (MTB) is one of the species of genus *Mycobacterium* that are within the order Actinomycetales. The presence of unique mycolic acids in the cell envelope of this genus is an important aspect for the structure and function of its cell wall (Barry et al 1998). The cell wall represents some distinguishing features of this genus such as its acid-fastness nature; utmost hydrophobicity; resistance to drying, acidity/alkalinity and several antibiotics; also shows its exclusive immune-stimulatory properties (Daffé and Draper 1997).

MTB are slow growing having 12 to 24 hours of division rate and a lengthened culture period on agar taking up to 21 days. Some proposed mechanisms have described about this slow growing behavior as the limitation in nutrient uptake and waste product excretion through its highly impermeable cell wall and also due to slow RNA synthesis rate (Harshey and Ramakrishnan 1977). Previously there was an assumption that MTB evolved from *Mycobacterium bovis* during cattle domestication (Stead et al 1995) but later several genome sequencing projects carried out for both the species proved it wrong. It was found that successive deletions of DNA of regions of difference (RD) were the reason for the branching off of members of MTB complex (Brosch et al 2002).

2.1.1 Structure of Mycobacterium tuberculosis

It is a slender or curved rod under microscope and represents a typical cell wall structure composed of mycolic acids, complex waxes and unique glycosides. The mycolic acids contain extremely long (C_{60} to C_{90}) side chains which are joined to muramic acid moiety of the peptidoglycan with the help of phosphodiester bridgs and to arabinogalactan by esterified glycolipid linkages. Trehalosedimycolate (cord-factor) and mycobacterial sulfolipids are other

components of cell wall that may play a virulent role. Another unique constituent is lipoarabinomannan (LAM) which is thought to contribute in pathogenesis. They grow very slowly with doubling time 18-24 hours. This sluggish growth in-vitro and even in-vivo shows two consequences of clinical significance; one of which is an insidious and chronic infection process taking several weeks to months to become clinically patent, and the other one is very slow appearance of identifiable colonies on solid media taking 4 to 6 weeks. The colonies on agar media are observed as irregular, waxy and buff colored, and the bacteria piled up into clumps or ridges (McMurray 1996).

2.1.2 Pathogenesis and lesion development

The infection of human MTB initiates with the inhalation of tubercle bacilli containing aerosol droplets directly from an infected/smear positive person. The infectious dose is found to be between 1 to 200 bacilli (Rich 1951, Balasubramanian et al 1994). The bacilli make a way to alveoli where they are engulfed by alveolar macrophages, after which more bacteria are phagocytosed by neutrophils and monocytes secreting more cytokines and chemokines. This step aids in the initiation of organizing the early granuloma. Then the bacteria are also phagocytosed by dendritic cells which then migrate to regional lymph nodes where the mycobacterial antigens are presented to the lymphocytes. It leads to the formation of well-organized granuloma which is surrounded by epithelioid macrophages, foam cells and multinucleated giant (Langhans) cells (McCune et al 1966). This lesion (tubercle) maybe enveloped by fibroblast and its center progresses to caseous necrosis. The liquification of caseous material and erosion of tubercle that occurs in an adjacent airway may lead to cavitation and the release of enormous number of bacilli into sputum, while the tubercle is found to become calcified gradually in the resistant host (McMurray 1996).

The presence of high oxygen pressure favors the growth of bacilli in the upper region of lungs lobe. Dissemination within the lungs and other organs may also take place through the blood in the early state of disease (Park et al 1992). Only about 10% of the infected person is thought to develop the primary progressive disease (Comstock 1982).A person goes in the state of latency in

the absence of primary progressive disease which is checked by the host immune system. This ability of establishing chronic asymptomatic infection tends to a development of pathogenic MTB when reactivated and transmitted to other person (Verver et al 2005).

Primary infection are usually asymptomatic but the symptoms like hemoptysis, coughing, pleuritic pain, fever, night sweats, anorexia, cachexia, etc are seen in secondary disease. In about 50% of the cases of immunocompetent people, the secondary TB can lead to dissemination and death, while in 25%-30% cases, it results in chronicity. In 20%-25% of the cases, possibility of recovery is seen if the host re-establishes immune control over the disease (Bates 1980).

2.2 Tuberculosis

Tuberculosis is caused by *Mycobacterium tuberculosis* and mainly causes pulmonary disease but in average 15% of the cases lead to infection in other body parts (WHO 2013).It is said that the bacillus emerged as a human pathogen around 70,000 years ago in Africa and then disseminated to other continents (Gutierrez et al 2005, Hershberg et al 2008) with the increase in human population, agricultural migration and other factors (Wirth et al 2008, Comas et al 2013). The morbidity and mortality rate of TB was declining in 20th century due to better health practices, use of Bacillus Calmette-Guerin (BCG) vaccine, and the introduction of antibiotics in 1950s, which was again disturbed due to the increasing number of new cases in mid-1980s. The reason for this was raising poverty, increasing homeless people, and emerging AIDS disease declining their cell-mediated immune response (Frieden et al 1995).

The other risk factors responsible for tuberculosis are fluctuating economic status, increasing human population density, health-care systems and services, status of HIV infection, poverty, poor nutritional consumption, pollution and TB control programs and activities (NSP Report 2018).

2.2.1 Recent scenario of tuberculosis in Nepal

TB is one of the major public health problems in Nepal. According to National Tuberculosis Program (NTP) Nepal report of 2017, 31764 cases of TB were registered at NTP in 2016/2017 of which 71% were pulmonary cases and out of which 77% were bacteriologically confirmed. 30% of the bacteriologically confirmed cases were confirmed using Xpert MTB/RIF test. 41.2% which is about half of all the TB cases were reported only from Central Region; out of which 35% TB cases were from Kathmandu valley and 25% cases were from Kathmandu district only. 57%, (more than half of the cases) were reported from terai belt, 40% and 3% from hilly and mountain region respectively. 47% of the cases were reported in middle age group from 15 to 44 years, whereas the children contribute to 5.6% cases and males were found nearly 1.8 times more than females with reported TB cases. According to Global TB report, 6000 to 7000 people are dying in Nepal due to TB disease (NTP 2017).

2.3 Drug resistant tuberculosis (DR-TB)

Drug resistant bacteria develop resistant to the antimicrobial drugs and is considered as a biological phenomenon noticed in *Mycobacterium tuberculosis*, since the discovery of streptomycin, the first anti-TB drug (Pyle 1947).The diagnosis of DR-TB is performed by drug susceptibility testing which can be done by phenotypic or culture-based methods and by genotypic or molecular methods. Some of the commercially available molecular diagnostic systems are GeneXpert system (Xpert MTB/RIF assay), Genotype MTBDRplus and MTBDRsl assay, and INNO-LiPARif.TB line probe assay, etc. (WHO DR-TB 2011).

2.3.1 Multi-drug resistant tuberculosis (MDR-TB)

Tuberculosis that does not respond to two most effective anti-TB drugs, isoniazid and rifampicin are termed as MDR-TB. The reasons behind the emergence of MDR-TB are mainly due to the mismanaged treatment process and due to the person-to-person transmission. The diagnosis is performed by special laboratory tests, either by culture-based tests or molecular tests such as Xpert MTB/RIF assay. Culture-based tests are prolonged one, while recent

molecular techniques provide the result within few hours and are also easy to set-up in resource limited settings.

An estimation of 490,000 people developing MDR-TB was given in 2016, plus 110,000 people with RR-TB were also up to MDR-TB treatment. 47% of the global total cases of MDR/RR-TB were seen in India, China and Russian federation out of which 6.2% were estimated as XDR-TB (WHO MDR 2018). According to annual report by NTP Nepal, 2017, high proportion of drug resistant pattern among second line drugs (SLD) used for MDR patients was observed during routine surveillance. 39.3%, 3% and 4% cases were resistant to fluoroquinolones (FQ), second-line injectables (SLI) and FQ and SLI both respectively. This indicates that 42.3% of MDR-patients need pre-XDR treatment and 4% of them may need XDR treatment (NTP 2017).

2.3.2 Extensively drug-resistant tuberculosis (XDR-TB)

It is a form of TB which is resistant to at least four of the important anti-TB drugs. It involves resistant to the most powerful isoniazid and rifampicin plus resistant to any of the fluoroquinolones (such as levofloxacin or moxifloxacin) and to at least one of the three SLI drugs (amikacin, capreomycin or kanamycin). Like MDR-TB, a person can develop XDR-TB due to mismanaged use of drugs or directly through the infected person; and like normal TB cases, he/she can be infected with bacteria but may not develop the active disease.

XDR-TB cases have been reported by 123 states by the end of 2016, and it is estimated that about 6.2% of MDR-TB cases have XDR-TB throughout the world. Due to limited facilities and resource settings, many developing and under-developed countries lack the complete diagnostic processes to test resistance and detect XDR-TB (WHO XDR 2018).

2.4 Diagnosis of tuberculosis

Immediate diagnosis of pulmonary tuberculosis is a must for TB control, for early and proper treatment and also for the reduction of dissemination of infection in the community. In clinical practices it is difficult to diagnose TB

rapidly and efficiently with only a test (WHO 2011). We cannot depend on only chest x-ray which is useful but it can give false positive result in which similar symptoms and clinical findings may be due to the community-acquired pneumonia (Woodring et al 1986, Krysl et al 1994). Simultaneously acid-fast bacilli smear test and culture tests are performed for the cases compatible or suspected with TB. The diagnosis of TB does not depend only on a test but a series of diagnostic procedures are required even in the presence of new and recent rapid diagnostic tools (WHO 2013).

2.4.1 Radiologic study

Chest x-ray is the primary method of diagnosing TB for both suspected patients and those infected with pulmonary TB but is not found to be specific (Woodring et al 1986, Krysl et al 1994). The findings from chest x-ray reveal the cavitary opacity in the posterior apices in the upper lobes and in the superior segment of lower lobes in case of post-primary TB. Cavitation is considered as the hallmark of the post-primary TB which can be recognized in about half of the patients (Woodring et al 1986, Lee et al 1993, Krysl et al 1994, Geng et al 2005).

Chest computed tomography (CT) is usually required that detects the fine lesions which is often missed by chest-ray. It ascertains the unclear lesions and evaluates the complications. The important radiologic findings in chest CT is the tree-in-bud pattern which shows multiple branches of linear structure that indicates the bronchogenic dissemination of the disease, and also reveals the caseous necrosis in the respiratory and terminal bronchioles (Woodring et al 1986, Lee et al 1993).

2.4.2 AFB smear microscopy

Sputum is the most essential sample for laboratory diagnosis of pulmonary tuberculosis. The mostly used method is direct sputum smear microscopy, which is a simple, rapid and effective test available in most of the health-care centers and can be set up easily (Hopewell et al 2006, WHO 2011). Two types of microscopy are widely used for diagnosing TB: conventional light microscopy and fluorescence microscopy. Conventional light microscopy or

Ziehl-Neelsen (ZN) microscopy is found to be more specific but have variable sensitivity (20%-80%). Fluorescence microscopy is found to be 10% more sensitive than light microscopy and also the test can be performed in less time (Steingart et al 2006). In AFB microscopy, both viable and non-viable bacilli are stained and counted. Mycobacteria along with other genera of the same family are acid-fast in nature, so microscopy does not help to determine the individual species, including *Mycobacterium tuberculosis*. A positive smear is found to be 10^4 bacilli per ml or more (MLM 2014).

Fluorescence microscopy was acquainted in 1930s for the improvement and advancement of the outcomes of smear microscopy in which fluorochrome dyes are used in staining the smear (Hopewell et al 2006). In one of the study, sensitivity of conventional light microscopy and fluorescence microscopy was found to be 64% and 72% respectively; while specificity was found to be 96% and 81% respectively (Cattamanchi et al 2009). In other study, sensitivity of fluorescence microscopy for diagnostic and follow-up samples was found to be 94.3% and 60.7% respectively (Selvakumar et al 2012).

2.4.3 Culture

Although laboratory diagnosis of TB relies on microscopic examination, clinicians are advised to obtain culture confirmation due to variable sensitivity of microscopic technique and also to gain information about the drug susceptibility (NIHCE 2011, Hopewell et al 2006, WHO 2011). The sensitivity of mycobacterial culture method is higher but the conventional solid medium culture method requires longer period of time (4-8 weeks). Culture is mainly performed on solid media, Lowenstein-Jensen (LJ) media, or in broth media (Kanchana et al 2000). Commercially different automatized incubators and tools that can automatically detect MTB growth are available such as the Bactec "Mycobacterial Growth Indicator Tube 960" (MGIT960, Becton-Dickinson, Sparks, MD, USA) and the MB/Bact Alert 10 3D (Biomerienx, Durham, NC, USA). But these liquid culture techniques are expensive and unable to provide rapid mycobacterial identification, also cannot identify contaminated or mixed cultures. In contrast, solid media

cultures provide all the information only with the observation of colonies (NIHCE 2011).

Slants of LJ media are inoculated with decontaminated or concentrated sputum samples. The advantage of this egg-based medium is that it is easy and economical to prepare, limits the contamination rate and characteristic morphology of the colony can be easily observed. The tubes are inoculated with the sample and spreaded entirely on the surface medium. Then the tubes are kept in slanted position for about a week with the cap of tube slightly loosened. Later the caps are tightened and incubated at 37℃ in upright position and examined weekly for 8 intervals. The colony of MTB are usually buff-colored, dry and very distinctive. Liquid culture is mainly done in Mycobacteria Growth Indicator Tube (MGIT) which helps in making the semi-quantitative evaluation of bacterial load by determining the time taken by the tubes to signal positive in the BACTEC MGIT 960 system (MLM 2014).

2.4.4 Molecular methods

2.4.4.1 Nucleic acid amplification (NAA) testing

NAA tests have high specificity but variable sensitivity especially in the case of smear negative or paucibacillary where the clinical symptoms are doubtful (Greco et al 2006, Ling et al 2008). When compared to smear microscopy, it has more positive predictive value (PPV) with smear-positive samples; also can rapidly identify MTB in 50%-80% of smear-negative, culture-positive samples. When compared to culture, this test can detect the MTB bacteria in less time then culture for 80%-90% of TB suspected patients whose TB is finally confirmed by culture (Greco et al 2006, Ling et al 2008, CDC 2009). NAA tests cannot detect non-viable bacteria giving false-positive result; but are able to differentiate MTB from non-tuberculous Mycobacteria (NTM) (CDC 2009).

2.4.4.2 Line Probe assay (LPA)

The conventional process of DST is a slow and troublesome process, so rapid DST of isoniazid and rifampicin or only rifampicin is recommended in smear-

positive and culture-positive cases (WHO MDR 2008). LPA is one of the types of molecular assay that has specific gene markers to detect rifampicin resistance alone or in combination with isoniazid. Isoniazid resistance usually occurs due to mutation in *katG*, followed by mutations at *InhA* active site (20%-35%) and in the promoter region of *ahpC*. 96% of rifampicin resistance occur in *rpoB* region (Ramaswamy and Musser 1998, Zhang and Yew 2009).

The steps involved in this assay are extraction of DNA from clinical specimen or Mycobacterial isolates, PCR amplification of resistance determining region of the gene, hybridization of labeled PCR products with oligonucleotide probes immobilized on a strip, and colorimetric development which allows for the lines to be seen where the probes are kept (WHO MDR 2008).

2.4.4.3 Xpert MTB/RIF assay

It is a rapid, automated NAA based assay that detects MTB along with the rifampicin resistant TB directly from the specimen within 2 hours (WHO Xpert 2013).It is provided with GeneXpert cartridges which are already loaded with all the requiring reagents for sample processing, extraction of DNA, amplification, and laser detection of amplified *rpoB* gene target. This test is highly sensitive and specific for determining TB and rifampicin resistance. It is found that only 5% of rifampicin resistance is mono-resistance, while 95% of it is concurrent resistance to isoniazid. Hence, it can be said that rifampicin-resistance is a marker for MDR-TB (NIHCE 2011, WHO Xpert 2011, Weyer et al 2012). Mutation in the *rpoB* gene that encodes ß-subunit of RNA polymerase is the cause of emergence of rifampicin-resistant tuberculosis (Telenti et al 1993). The mutation mostly occurs in the 81 bp region (codons 507-533) of the *rpoB* gene, also called as rifampicin resistance-determining region. In most of the studies, it is found that mutation occurs in codons 516, 526 and 531 (Somoskovi et al 2001, Caws et al 2006). Rifampicin is a derivate of rifamycin and cross-resistance between other rifamycins can also be found to occur. The condition also appears when bacteria is resistant to rifampicin but susceptible to other rifamycins such as rifabutin or rifalazil, especially when mutation occurs in some codons like 518 or 529 (Yang et al 1998, Cavusoglu et al 2004).

In one of the study, higher sensitivity of 90% was shown by Xpert MTB/RIF assay in patients with positive smear, 87% in pulmonary TB samples, 82% in adults and 81% in HIV-positive patients; while an overall pooled sensitivity and specificity of 85% and 98% was shown compared to culture (Li et al 2017). In another study, sensitivity of this assay in smear-positive cases was found to be 85.4% and specificity in smear negative cases was found to be 81% (Shrestha et al 2018). The other study carried out in EPTB cases revealed its overall sensitivity 59% and specificity 92%, with sensitivities of 91% for pus, 80% for lymph nodes aspirate and 51% for fluids, using culture as the reference (Scott et al 2014).

2.5 GeneXpert Technology

GeneXpert system includes thermal and optical system modules, self contained disposable cartridge (having 11 chambers), software and barcode scanner, printer and UPS (uninterruptible power source). The technology employs random access fluidic systems that bridge micro fluidics with macro fluidics and impose proven smartcycler technology. It uses multiplex, rapid real-time PCR technique (6 colors). The design is so made that there will be no wet interface between cartridge and instrument; has integrated ultrasonic horn for rapid lysis of spores; and has total internal control that means no external positive or negative controls are required. The smart fluidics includes encoded software driven motors for valve movement and integral hydraulic drives. It provides automated data reduction and result interpretation.

Probe check, specimen processing control (SPC) and GeneXpert real-time PCR assay are the three elements of molecular design that checks all the key failure modes that could result in false negative result. Probe check verifies bead-rehydration, PCR tube filling, probe integrity and dye or quencher instability. SPC contains intact organisms, DNA or RNA, detection probe, and primers present in primer-probe bead. SPC co-processes with target organisms throughout the prep process and also co-amplifies with target. It helps to check-out the effectiveness of sample processing steps, detects degradation of enzymes and other components, and detects sample inhibition. GeneXpert

real-time PCR assay possess DNA positive control (PC) and internal control (IC) (XpertMTB/RIF 2015).

The cartridges are pre-loaded with lyophilized beads and reagent. Among the 5 beads, 2 beads contain polymerase, dNTPs, probe and bovine serum albumin (BSA); 2 other beads contain primers, probes and BSA; while 1 bead contain SPC (about 6,000 non-infectious *Bacilli globigii* spores). The reagents contain Tris buffer, surfactants and EDTA (Ethylenediaminetetraacetic acid). A bottle of sample reagent is present along with the cartridge which is made of sodium hydroxide and Isopropanol. When the sample loaded cartridge is put in the instrument, the sample combines with SPC. The sample and SPC is captured by a filter, and then the cells lysed ultrasonically release nucleic acid. The DNA then mixes with bead reagent, after which PCR amplification and detection occurs simultaneously (XpertMTB/RIF 2015).

2.5.1 Principle of Xpert MTB/RIF assay

The multichambered cartridge is preloaded with necessary buffers and reagent beads required to run the test. The sample reagent and sputum sample or other samples like gastric aspirate, broncho-alveolar lavage, pus, is added to the cartridge in the ratio 2:1. The cartridge is then incubated and transferred to the GeneXpert instrument. The spores of *Bacillus globigii* which serves as an internal sample processing and PCR control are automatically resuspended and processed, and the resulting *B. globigii* DNA is amplified during the PCR step. The presence or absence of MTB, rifampicin resistance and semi-quantitative estimation of MTB concentration (high, medium, low, very low) is indicated by standard user interface. If the result is displayed negative for both MTB and *B. globigii* internal control, it is reported as invalid.

A 192-bp segment of the MTB gene is amplified by the PCR assay in a hemi-nested real-time PCR. Five overlapping molecular beacon probes (Probes A to E) are present in the cartridge which is complementary to entire 81-bp rifampicin resistance-determining core region of the wild-type *rpoB* gene which helps in the detection of RR-MTB. If mutation has occurred in the *rpoB* gene target, it inhibits the hybridization of one or more *rpoB*-specific

molecular beacon, thus reducing the signal from corresponding probes. MTB is identified when at least 2 of the 5 molecular beacons give a positive signal with cycle threshold (C_T) values ≤38 and which differ by no more than two cycles. Similarly, *B. globigii* is detected when the single *B. globigii* molecular beacon produces a C_T of ≤ cycles. Rifampicin resistance detection and *rpoB* mutation can be determined by the difference in C_T between the first (early C_T) and last (later C_T) MTB-specific molecular beacons (ΔC_TMax). Rifampicin resistance is identifies if the ΔC_TMax is >3.5 cycles, while it is said to be rifampicin susceptible if the ΔC_TMax is ≤3.5 cycles. It is considered rifampicin intermediate if the last probe returns a C_T of >38 and the first probe has C_T>34.5 because the assay is terminated at cycle 38 and a ΔC_TMax of >3.5 cannot be measured (Piatek et al 1998, El-Hajj et al 2001, Helb et al 2010).

2.6 Treatment of tuberculosis

Tuberculosis can be treated and cured with proper guidance and supervision of health workers, regular intake of drugs and appropriate treatment. According to WHO, it was estimated that about 53 million lives were saved with proper diagnosis and treatment between 2010 to 2016 (WHO Report 2018).According to WHO guidelines for treatment of tuberculosis updated in 2017, for drug susceptible TB, 6 months rifampicin-based regimen is still recommended, which involves 2 months intensive phase with first-line drugs isoniazid (H), rifampicin (R), pyrazinamide (P), and ethambutol (E); and continuation phase of 4 months with drugs isoniazid and rifampicin (2HRZE/4HR). It is recommended that daily dose of medicines is effective than thrice a week dose. Antiretroviral treatment is suggested in all the TB patients living with HIV (WHO TB-Treatment 2017).

In case of drug resistant tuberculosis, five effective second-line drugs is recommended in intensive phase for patients with RR-TB and MDR-TB, followed by continuation phase with high-dose isoniazid and/or ethambutol. The five drugs include pyrazinamide and other four core second-line drugs, among which one drug is chosen from group A, one from group B, and at least two from group C. In case, above combination of drugs seems ineffective, a drug from group D2 and other from group D3 may be added to make five.

Group A includes fluoroquinolones such as levofloxacin, moxifloxacin, gatifloxacin; group B includes amikacin, capreomycin, kanamycin, streptomycin; Group C includes ethionamide, cycloserine, linezolid, clofazimine; Group D2 includes bedaquiline, delamanid; and group D3 includes p-aminosalicylic acid, imipenem-cilastatin, meropenem, amoxicillin-clavulanate (WHO DR-TB 2016).

Patients with RR-TB are also given MDR-TB like treatment even if there is no confirmation of isoniazid-resistant. The drug regimen of second-line drugs lasts for 9-12 months. This regimen is not applicable for those with XDR-TB or those resistant to second-line drugs; instead they need longer MDR-TB regimen in which one of the new drugs (bedaquiline, delamanid) may be added (WHO DR-TB 2016).

2.6.1 Mode of action of anti-TB drugs

Rifampicin is considered as one the most powerful anti-TB drug and in combination with isoniazid, also called as isonicotinic acid hydrazide creates an effective treatment regimen. Rifampicin binds with the ß-subunit of RNA polymerase thus inhibiting the elongation of messenger RNA, whereas not alike rifampicin, isoniazid acts only against active bacilli and works by inhibiting mycolic acid synthesis through NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by inhA (Zhang et al 1992, Blanchard 1996, Rawat et al 2003). Ethambutol is found to be bacteristatic that interferes with the biosynthesis of arabinogalactan in cell wall (Takayama and Kilburn 1989). Pyrazinamide is one of the first-line drugs that helped in shortening the treatment period to 6 months. It is proposed that it acts by converting to pyrazinoic acid that destroys the bacterial membrane inhibiting membrane transport. Other study also proposed that pyrazinoic acid and its n-propyl ester inhibit the fatty acid synthase type I in replicating bacilli (Zimhony et al 2000, Zimhony et al 2007). Streptomycin is found to act by inhibiting the initiation of translation phase thus hampering in synthesis of protein (Moazed and Noller 1987).

Among second-line drugs, fluoroquinolones suppress the two important enzymes topoisomerase II (DNA gyrase) and topoisomerase IV that makes bacteria viable (Fàbrega et al 2009). Kanamycin and amikacin works by hindering protein synthesis by altering 16 SrRNA (Alangaden et al 1998, Suzuki et al 1998), whereas capreomycin and viomycin which has similar structure bind at the same site in ribosome and thus inhibiting protein synthesis (Stanley et al 2010). Similarly, ethionamide which has similar structure to isoniazid acts by interfering with mycolic acid synthesis same as done by isoniazid (Carette et al 2011). Linezolid acts by inhibiting protein syntheses in early stage by binding to the 50 S subunit of ribosome (Zhang 2005).

2.7 Prevention and control measures of TB

TB can be prevented by following several management rules such as early identification of certain population group which is at high risk of TB infection, and screening the active TB disease as well as the latent infection in each individuals living in those high risk areas. Surveillance should be carried out for identifying the outbreaks and availability of proper treatment facilities need to be provided. Control measures program should be carried out especially for those living in highly crowded areas like hospitals, prisons and slum area (WHO STOP-TB 2008). BCG vaccine may be effective against lung disease and help in prevention of TB in children and adults. Similarly proper management and facilities of health-care systems helps in control and prevention of TB (WHO DR-TB 2014).

TB infected person are encouraged to stay in well-ventilated and separate room, not to spend more time in crowded place, and follow protective measures. Both drug-susceptible and drug-resistant TB becomes active if a person has weak immune system, so a he/she need to be taught the importance of healthy lifestyle, proper hygiene, balanced diet, negative impact of smoke and alcohol, and other preventive measures (WHO DR-TB 2014). The control measures also need to be strictly followed in health-care settings as both the staffs and visitors are at high risk for being infected. CDC has recommended 3

level of control measures in health-care settings which includes: administrative measures, that focus on availability of recommended laboratory settings, regular cleaning and sterilization of equipments, implementation of work practices effectively, proper training and guidance to both staffs and patients, etc.; environmental controls that focus on use of ventilation and controlling air flow to remove contaminated air; and lastly respiratory protective equipment that encourages in use of protective equipments and carryout respiratory protective programs (CDC 2016).

CHAPTER-III

MATERIALS AND METHODS

A comparative cross sectional study was carried out in National TB Reference Laboratory, German Nepal Tuberculosis Project (GENETUP), Kathmandu, Nepal from April to June 2018. The clinical samples were collected from the tuberculosis suspected patients who visited GENETUP. Sample collection, processing, microscopic examination and Xpert MTB/RIF assay were all performed in the reference laboratory of GENETUP. In this study, a total of 451 samples were collected which included both pulmonary and non-pulmonary samples, and among which 410 sputum samples, 16 pus, 9 broncho-alveolar lavage (BAL), 7 FNAC (Fine Needle Aspiration Cytology), 5 gastric aspirates (GA), 4 pleural fluid were collected. All types of patients including new, and those treated previously (category I and category II) were included in this study.

3.1 Sample collection

Sample collection was done keeping in mind the type and amount of sample required for the different tests to be performed. For sputum sample collection, each patient was provided with two sterile containers, one for early morning secretion, and other for the sputum expectorated 2 hours later or on the spot. The inadequate samples or samples with saliva or food particles were rejected and asked to bring the following day. Both the samples were collected and processed for microscopic examination, while only one proper and adequate sample was processed for Xpert MTB/RIF assay. Only one sample was collected for samples other than sputum, which was expectorated by the referring hospitals or clinics and sent to GENETUP, or collected itself in the GENETUP hospital. The samples were processed and examined the same day of collection, or if not possible, were stored in the refrigerator at 2-8ºC.

3.2 Sample decontamination

Sputum samples were not decontaminated and examined directly. Samples other than sputum require decontamination, so for this purpose, conventional N-acetyl-L-cysteine (NALC)-NaOH method was used. In a biological safety cabinet-2, the sample and about equal volume of NALC-NaOH solution was added in a 50 ml centrifuge tube. The tube was tightly capped and mixed well on a vortex-type mixer until the sample liquefied. The tube was allowed to stand on room temperature for 15 minutes. Then, phosphate buffer (pH 6.8) was added to the mixture up to 50 ml mark of the tube and mixed. The tube was then centrifuged at 3000xg for 20 minutes, after which the supernatant fluid was decanted carefully and 1 ml of phosphate buffer was added to it. This suspension was used for smear preparation and Xpert MTB/RIF assay. In presence of adequate sample, Xpert MTB/RIF assay was directly carried out without the decontamination process.

3.3 Fluorescence staining method

Fluorescence staining method was carried out for the detection of acid-fast bacilli. For this purpose, smear was prepared on clean, sterile glass slides and heat-fixed. On this heat-fixed slide, auramine-o solution was flooded and left for 20 minutes. The stain was then rinsed off with distilled water, and again flooded with 0.5% acid-alcohol as a decolorizing agent and let stand for 2 minutes. The slides were then rinsed off with distilled water, followed by flooding with 0.1% Methylene blue (or 0.5% potassium permanganate) as counter stain and rinsed with distilled water after 1 minute. The slides were then placed on slide rack to let dry and were examined under fluorescence microscope using 40X objective.

3.4 Xpert MTB/RIF assay (GeneXpert MTB/RIF manual)

3.4.1 Sample preparation

All the following processes were performed in biological safety cabinet level-2. The sample collection container was uncapped carefully and with the help of disposable pipette or sterile stick, about 1 ml of the sample was transferred to a screw-capped conical tube. The conical tube was labeled with sample ID same as labeled in corresponding sample container. Then, sample reagent provided with Xpert MTB/RIF assay kit was added to the sputum sample in the ration 2:1 (v/v), and to the decontaminated sample in the ratio 3:1 (v/v). The cap of the tube was put tightly to avoid the possible leakages and was vortexed for about 10 seconds. The tube was then incubated at room temperature for 15 minutes in a tube rack. During this period, the tube was again vortexed after 10 minutes of incubation and let stand for remaining time.

3.4.2 Cartridge preparation

The cartridge provided with Xpert MTB/RIF assay kit was carefully kept in the safety cabinet avoiding shaking or bumping. It was then labeled with sample ID same as labeled in the corresponding conical tube. With the help of sterile transfer pipette provided with the kit, liquefied sample was drawn in to the pipette until the meniscus was above the minimum mark. The cartridge lid was opened and the sample was transferred to it carefully. The cartridge lid was closed and the pipette was discarded into biohazard bin.

3.4.3 Starting the test

The GeneXpert Dx system software was opened and the test was created by entering the sample ID number labeled on the cartridge manually, while the barcode on the cartridge was entered by scanning with the help of scanner. The cartridge was then loaded into the instrument after the blinking of green light on the instrument module door. The result was displayed in the computer after 1 hour 50 minutes, and after the completion of test, the cartridge was disposed into biohazard waste container.

3.5 Interpretation of results

Rod-shaped or curved bacilli are seen under fluorescence microscope. The organisms fluoresce bright yellow, non-specific debris stains pale yellow, and the background seems almost black. The 40X objective is used to avoid false-positive result due to fluorescing debris. The bacilli may occur singly, in small groups containing few bacilli or in large clumps. The stained smear should be scanned rapidly and systematically from one side to the other in sweeps. The bacilli must be counted in appropriate number of fields for the degree of positivity, and scanning of at least 100 fields must be done to report smear as negative. In 400X (40X objective and 10X eye piece), if no AFB is found in one length, it is reported as no AFB; 1-2 AFB in one length requires confirmation; scanty or exact number is written if 3-24 AFB are seen in one length. If 1-6 AFB seen in one field, it is reported as 1+, 2+ for 7-60 AFB in one field, and 3+ for >60 AFB in one field.

The GeneXpert instrument system generates the results from measured fluorescent signals and embedded calculation algorithms, which can be observed in the View Results window. If mutation in the *rpoB* gene has been detected with SPC as Not Applicable (NA) and probe check as PASS, the results is shown as MTB Detected, Rif Resistance Detected. If mutation in *rpoB* gene is not detected with SPC as NA and probe check as PASS, the result is MTB Detected, Rif Resistance Not Detected. If mutation in *rpoB* gene could not be determined due to insufficient signal detection,, SPC as NA and probe check as PASS, the result is MTB Detected, Rif Resistance Intermediate.

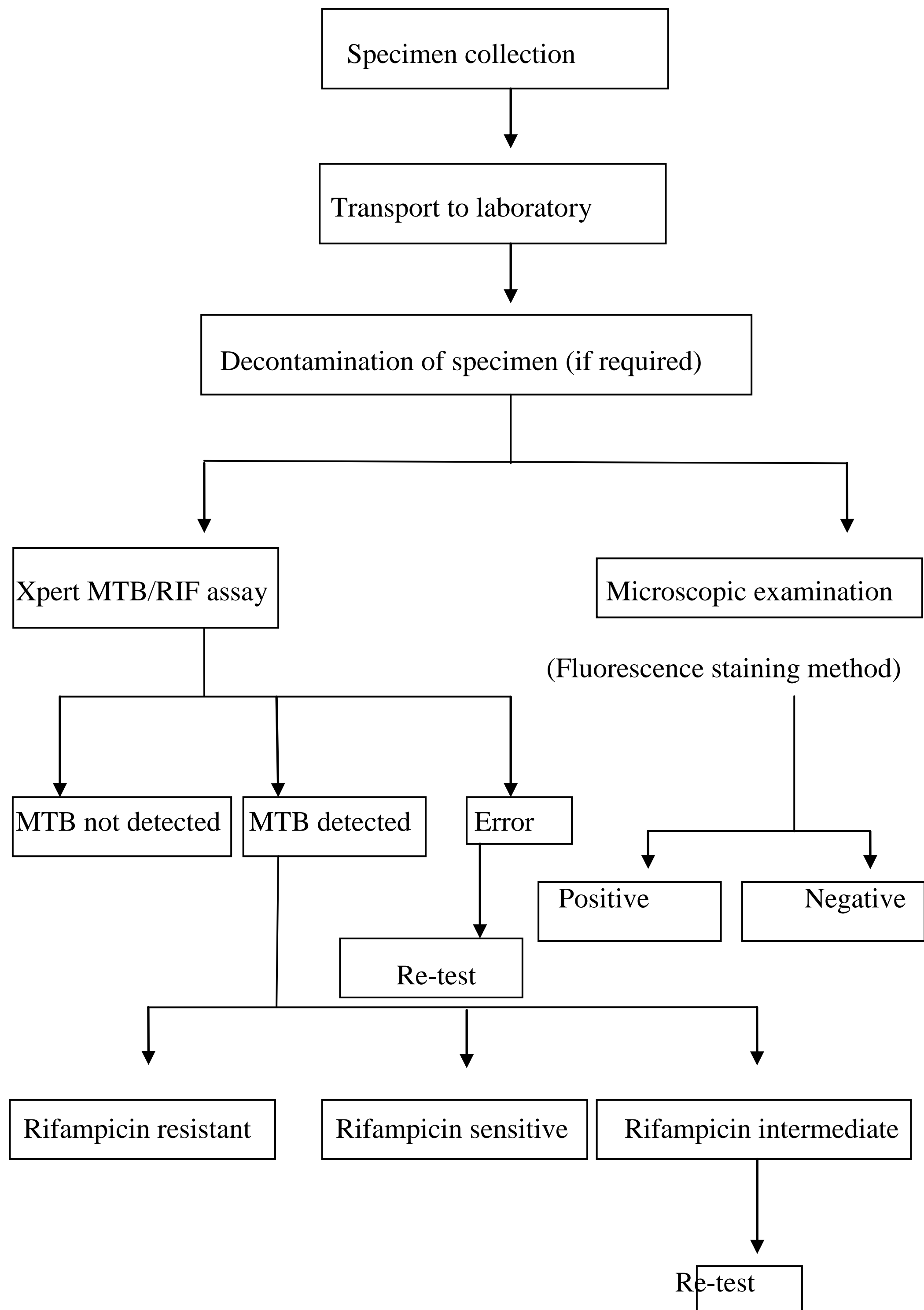
MTB Not Detected is shown if MTB target is not detected within the sample, and both SPC and probe check is shown PASS. Sometimes the result is shown as Invalid, Error or No Result if the presence or absence of MTB cannot be determined due to several reasons like SPC does not meet the acceptance criteria, the sample was not properly processed or if PCR was inhibited. In this case, the test should be repeated using the new cartridge and left-over sample

reagent-treated sample if sufficient (used within 4 hours of sample preparation) or using the left-over sample and preparation as before.

3.6 Data analysis

The data was entered in MS Excel 2007 and the database was created in SPSS 16.0 for Windows. The data was transferred to variables as required and analyzed after correcting the errors. Summary output tables for descriptive statistic crosstabulation between variables were produced and Chi-square test was used to compare categorical variables. The test was considered statistically significant if the p-value obtained was less than 0.05.

Flowchart of the procedures



CHAPTER-IV

RESULTS

A total of 451 samples, including sputum samples and other samples like broncho-alveolar lavage (BAL), pus, fine needle aspiration cytology (FNAC), gastric aspirate (GA) and pleural fluid were collected and analyzed in the study period. Sputum and BAL are considered as PTB samples while remaining are considered as EPTB samples. Among them, 338 were the new cases; while 113 were the previously treated patients, out of which 105 of them were Category I patients and 8 were Category II patients (TB relapse cases).23

In the study, 107 male patients and 70 female patients were detected with MTB. In this way, MTB was detected in 177 of the suspected patients, among which 164 cases were rifampicin sensitive, 10 cases were rifampicin resistant and 3 cases were found to be rifampicin intermediate.

4.1 Sex wise distribution of suspected population

In this study, the number of males suspected with tuberculosis was higher than that of females. Among total population, 289 were reported as males and 162 as females which is illustrated in **Figure 1.**

TB suspected population

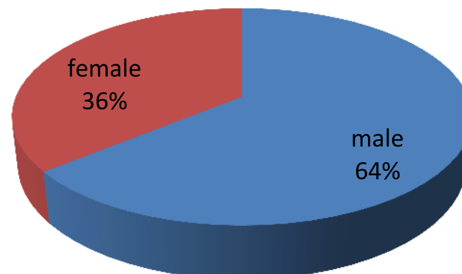


Figure 1: Sex wise distribution of TB suspected population

4.2 Age wise distribution of TB suspected population

Patients of different ages suspected to have tuberculosis were reported in the study which is categorized into several age groups. The numbers of males were significantly higher than females in all age groups. The number of suspected males and females were more in age group 20-30 years as shown in **Figure 2.**

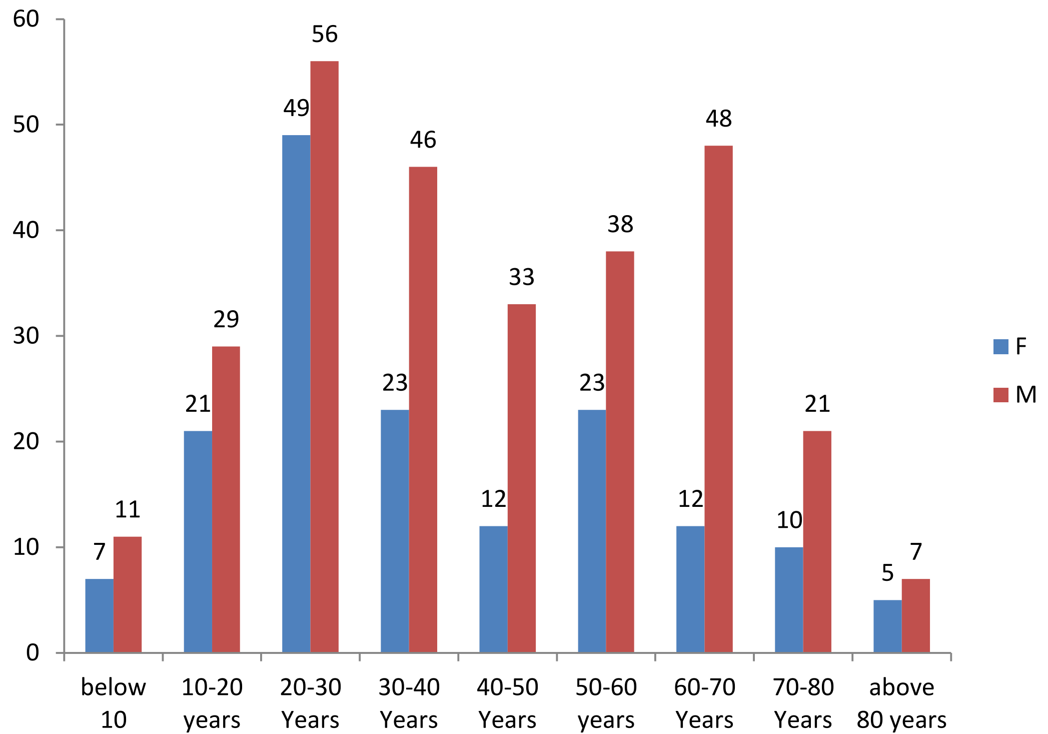


Figure 2: Age wise distribution of TB suspected population

4.3 Sex wise distribution of TB detected population

The percentage of TB detected in male and female patients is similar to that of TB suspected patients. In this study, 60.5% (107) of male and 39.5% (70) of female patients were found to have infected with TB as illustrated in **Figure 3.**

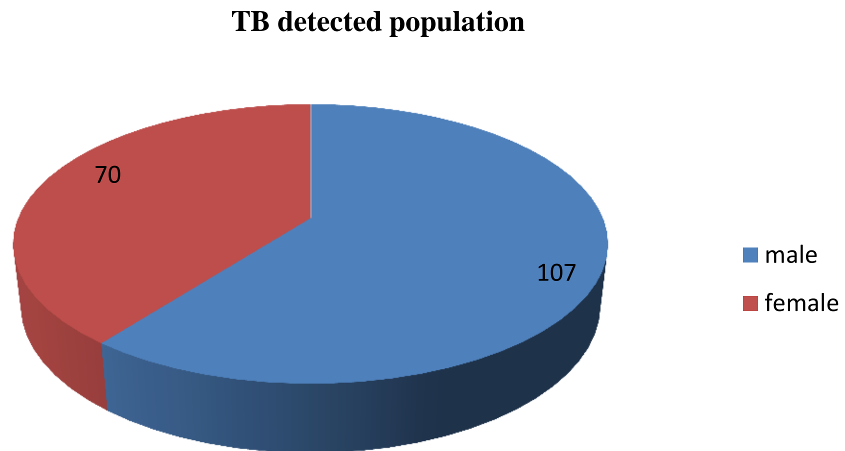


Figure 3: Sex wise distribution of TB detected population

4.4 AFB smears result with respect to category of age

In this study, the age group was divided into nine categories, among which age group 20-30 years showed highest number of smear positive result compared to other age groups, followed by 30-40 years. No smear positive result was seen in age group below 10 years, while least smear positive result was seen in age groups 70-80 years and above 80. Remaining age groups showed similar type of result. The above results are analyzed among total number of cases and not among individual age-group cases. The detail information of relation between category of age and smear result along with degree of positivity is given in **Table 1.**

Table 1: AFB smear results with respect to category of age

Category of age (Years)	AFB Smear Results					Total	P-value	Likelihood Ratio
	1+	2+	3+	Negative	Scanty			
Below 10	-	-	-	18(4.0%)	-	18 (4.0%)		
10-20	9(2.0%)	2(0.4%)	1(0.2%)	38(8.4%)	-	50 (11.1%)		
20-30	22(4.9%)	3(0.7%)	7(1.6%)	70(15.5%)	3(0.7%)	105 (23.3%)		
30-40	11(2.4%)	4(0.9%)	1(0.2%)	51(11.3%)	2(0.4%)	69 (15.3%)		
40-50	5(1.1%)	7(1.6%)	1(0.2%)	32(7.1%)	-	45 (10.0%)	0.028	0.012
50-60	8(1.8%)	-	-	51(11.3%)	2(0.4%)	61 (13.5%)		
60-70	6(1.3%)	2(0.4%)	1(0.2%)	50(11.1%)	1(0.2%)	60 (13.3%)		
70-80	4(0.9%)	-	-	27(6%)	-	31 (6.9%)		
Above 80	1(0.2%)	-	-	11(2.4%)	-	12 (2.7%)		
Total	66(14.6%)	18(4.0%)	11(2.4%)	348(77.2%)	8(1.8%)	451 (100%)		

4.5Xpert MTB/RIF results with respect to category of age

In this study, 177 cases of MTB were detected by Xpert MTB/RIF assay, which was highest among the age group 20-30 years, followed by 30-40 years and 10-20 years. The above results are analyzed among total number of cases and not among individual group. No MTB was detected in patients below 10 years of age. The number of MTB detected and not detected cases are shown in **Table 2**.

Table 2: Xpert MTB/RIF result with respect to category of age

Category of age (Years)	Xpert MTB Result		Total	p-Value
	Detected	Not detected		
Below 10	-	18(4.0%)	18(4.0%)	
10-20	21(4.7%)	29(6.4%)	50(11.1%)	
20-30	60(13.3%)	45(10.0%)	105(23.3%)	
30-40	34(7.5%)	35(7.8%)	69(15.3%)	
40-50	19 (4.2%)	26(5.8%)	45(10.0%)	0.0
50-60	18(4.0%)	43(9.5%)	61(13.5%)	
60-70	14(3.1%)	46(10.2%)	60(13.3%)	
70-80	9(2.0%)	22(4.9%)	31(6.9%)	
Above 80	2(0.4%)	10(2.2%)	12(2.6%)	
Total	177 (39.2%)	274 (60.8%)	451 (100%)	

4.6AFB smears result according to different clinical samples

Among all the PTB and EPTB samples, sputum samples showed higher degree of positivity(1+, 2+ and 3+) and few scanty results, a pus sample was reported as 1+, while other samples except BAL and GA gave scanty results during AFB smear microscopy. The above results are analyzed among total samples and not among individual samples, the detailed report of which is given in **Table 3.**

Table 3: AFB smear result according to different clinical samples

Samples	AFB Stain Results					Total	p-value
	1+	2+	3+	Negative	Scanty		
BAL	-	-	-	9 (2.0%)	-	9 (2.0%)	0.00612
FNAC	-	-	-	6 (1.3%)	1 (0.2%)	7 (1.6%)	
GA	-	-	-	5 (1.1%)	-	5 (1.1%)	
Pleural fluid	-	-	-	3 (0.7%)	1 (0.2%)	4 (0.9%)	
Pus	1 (0.2%)	-	-	13 (2.9%)	2 (0.4%)	16 (3.5%)	
Sputum	65 (14.4%)	18 (4.0%)	11 (2.4%)	312 (69.2%)	4 (0.9%)	410 (91%)	
Total	66 (14.6%)	18 (4.0%)	11 (2.4%)	348 (77.2%)	8 (1.8%)	451 (100%)	

4.7Xpert MTB/RIF result according to different clinical samples

All clinical samples showed positive MTB result except gastric aspirate and pleural fluid. MTB was detected in 157 sputum samples which accounts for 38.29% of the total sputum samples, while it was detected in 13 pus samples which is about 81.25% of the total pus samples collected. Likewise, the disease was detected in 3 BAL and 4 FNAC samples. The percentage written for each value in the table below is calculated with respect to the total samples and not with each sample type as shown in **Table 4.**

Table 4: Xpert MTB/RIF result according to different clinical samples

Samples	Xpert MTB Result		Total	p-Value
	Detected	Not detected		
BAL	3(0.7%)	6(1.3%)	9(2.0%)	0.002
FNAC	4(0.9%)	3(0.7%)	7(1.6%)	
GA	-	5(1.1%)	5(1.1%)	
Pleural fluid	-	4(0.9%)	4(0.9%)	
Pus	13(2.9%)	3(0.7%)	16(3.5%)	
Sputum	157(34.8%)	253(56.1%)	410(91%)	
Total	177(39.2%)	274(60.8%)	451(100%)	

4.8 AFB smear result and Xpert MTB/RIF result according to patient type

Here, the new cases represents the patients suspected with TB and had not received any TB treatment before or have taken anti-TB drugs for less than 1 month. First treatment or Category I patients represents the patients who have completed their first treatment course; while relapse cases or Category II patients represents those who have completed their Tb treatment course the second time, after the first treatment failure.

In first treatment cases or Category I patients, MTB was detected in 27 smear positive cases and in 26 smear negative cases; white the disease was not detected in 2 smear positive cases. In new cases, MTB was detected in all positive cases and in 51 smear negative cases. The disease was also not detected in all smear negative cases. Similarly, in TB relapse cases or Category II patients, MTB was detected in 1 smear positive case and in 2 smear negative cases; while it was not detected in 3 of smear positive cases. The detailed data of AFB smear result along with degree of positivity and Xpert result is given in **Table 5.**

Table 5: AFB smear result and Xpert MTB/RIF result according to patient type

Patient Type	Xpert MTB Result	AFB Smear Results					Total	p-value
		1+	2+	3+	Negative	Scanty		
First Treatment cases	Detected	16	5	3	26	3	53	.000
	Not detected	1	-	-	50	1	52	
New Cases	Detected	46	13	8	51	3	121	.000
	Not detected	-	-	-	217	-	217	
Relapse cases	Detected	1	-	-	2	-	3	.641
	Not detected	2	-	-	2	1	5	
Total	Detected	63	18	11	79	6	177	0.00
	Not detected	3	-	-	269	2	274	
Total		66	18	11	348	8	451	

4.9 Rifampicin resistant according to patient type

In the study, RR-TB was detected in 10 of the total cases, among which it was detected in 6 of the total 105 first treatment cases (Category I patients) and in 4 of the total 338 new cases; while no RR-TB was detected in relapse cases (Category II patients) as shown in **Table 6.**

Table 6: Rifampicin resistant according to patient type

Patient type	Rifampicin				Total	p-Value
	Not detected	I	R	S		
First Treatment cases	52	1	6	46	105	0.047
Relapse cases	5	-	-	3	8	
New cases	217	2	4	115	338	
Total	274	3	10	164	451	

I= Intermediate, R= Resistant, S= Sensitive

4.10 AFB smear result versus Xpert MTB/RIF result

Among 451 samples, 177 cases were MTB positive cases which accounts for 39.25% of the total tuberculosis suspected cases. Among the smear positive cases, 5 of the samples were found to be MTB negative when examined by Xpert MTB/RIF assay, while all other smear positive samples were also MTB positive. Similarly, among the smear negative cases, 79 of the cases were found to be MTB positive, while other smear negative cases were also MTB negative. The data of AFB smear result versus Xpert MTB result is given in **Table 7.**

Table 7: AFB smear result versus Xpert MTB/RIF result

AFB Smear Results	Xpert MTB Result		Total	p-Value
	Detected	Not detected		
1+	63	3	66	.002
2+	18	-	18	
3+	11	-	11	
Negative	79	269	348	
Scanty	6	2	8	
Total	177	274	451	

4.11Xpert MTB/RIF Rifampicin resistant result

Among the total of 177 MTB detected cases from suspected patients, 164 cases were rifampicin sensitive, 10 cases were rifampicin resistant and 3 cases were reported as rifampicin intermediate. The percentage calculated for each case is among the TB detected cases as shown in **Table 8.**

Table 8: Xpert MTB/RIF rifampicin resistant result

Rif Resistance	Xpert MTB Result		Total	P-Value
	Detected	Not detected		
Intermediate	3(1.7%)	-	3(1.7%)	
Resistant	10(5.6%)	-	10(5.6%)	0.00
Sensitive	164(92.7%)	-	164(92.7%)	
Total	177(100%)	-	177(100%)	

4.12Probe hybridization among RR-TB cases

The pattern of hybridization of probes was noted from the automated analytic result appearing in the computer screen from where it was found that in case of all the 10 rifampicin resistant cases, all the probes were hybridized except Probe E. It suggested that mutation has occurred in Probe E region of the RRDR of *rpoB* gene as illustrated in **Table 9.**

Table 9: Probe hybridization pattern among RR-TB cases

Probes hybridization failure	Number of cases
Probe A (507-511)	-
Probe B (512-518)	-
Probe C (518-523)	-
Probe D (523-529)	-
Probe E (529-533)	10(100%)
Total	10(100%)

4.13 Overall sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay over fluorescence microscopy (FM)

The sensitivity of Xpert MTB/RIF assay compared to smear positive samples was found to be 95.1% and the specificity compared to smear negative samples was 77.3%. The positive predictive value and negative predictive value was found to be 55.4% and 98.2% respectively as shown in **Table 10.**

Table 10: Overall sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay over FM

Xpert result	AFB smear result		Total	
	Smear positive	Smear negative		
MTB detected	98	79	177	PPV=55.4%
MTB not detected	5	269	274	NPV=98.2%
Total	103	348	451	
	Sensitivity= 95.1%	Specificity=77.3%		

Note: PPV: Positive Predictive Value, NPV: Negative Predictive Value

4.14 Sensitivity and specificity of both pulmonary and extra pulmonary samples

The sensitivity of Xpert MTB/RIF assay compared to smear positive pulmonary sample (sputum and BAL) was found to be 96%, and specificity compared to smear negative pulmonary sample was found to be 79.4% as given in **Table 11.**

Table 11: Sensitivity and specificity of Xpert MTB/RIF assay for PTB samples.

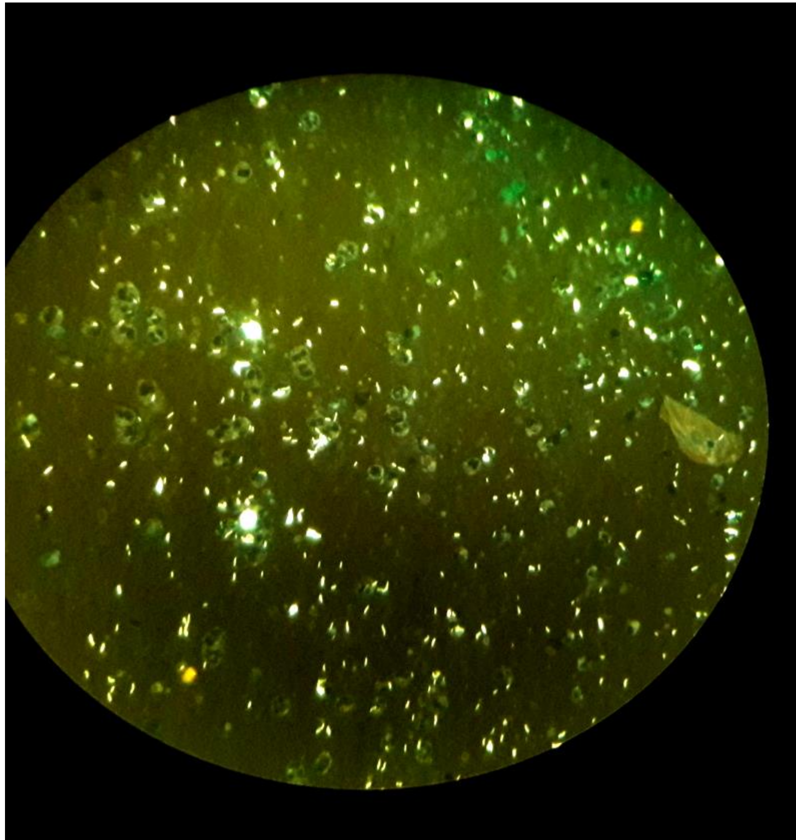
Xpert result	AFB smear result for PTB sample		Total
	Smear positive	Smear negative	
MTB detected	94	66	160
MTB not detected	4	255	259
Total	98	321	419
	Sensitivity=96%	Specificity=79.4%	

The sensitivity of Xpert MTB/RIF assay compared to smear positive EPTB samples was found to be 80%, and specificity compared to smear negative samples was found to be 51.9% as given in **Table 12.**

Table 12: Sensitivity and specificity of Xpert MTB/RIF assay for EPTB samples.

Xpert result	AFB smear result for EPTB samples		Total
	Smear positive	Smear negative	
MTB detected	4	13	17
MTB not detected	1	14	15
Total	5	27	32
	Sensitivity=80%	Specificity=51.9%	

Photograph 1: Staining the smear with auramine-o stain



Photograph 2: Observation of acid-fast bacilli under fluorescence microscope

Photograph 3: GeneXpert Dx system

Photograph 4: Researcher working in Class-II Biological Safety Cabinet

CHAPTER-V

DISCUSSIONS

This study is carried out by testing the clinical samples by fluorescence microscopy and GeneXpert MTB/RIF assay method. Both the methods were performed and analyzed with respect to different factors like gender, age groups, types of samples, and different patient types.

In this study, MTB was detected in 177 (39.25%) of the total 451 samples by Xpert MTB/RIF assay which is similar to the study carried out in Pakistan which detected MTB in 45.3% of total samples (Iram et al 2015). Among the TB detected cases, 60.5% were males and 39.5% were females, which implies that male to female ratio of TB positive cases is 1.5: 1 which is slightly less than 1.8: 1 as shown by Annual report of NTP Nepal (NTP 2017).

The TB suspected population was higher in mid-age groups 20-30 years and 30-40 years, which is similar to TB positive cases. The TB positive patients were found to be higher in age group 20-30 years followed by 30-40 years and 10-20 years in both AFB smear result and Xpert MTB/RIF result. This result agrees with the final annual report which states that highest proportion (47%) of TB cases was reported in middle age group between 15-44 years (NTP 2017). This result is also similar to the study done in Kenya where patients between 18-47 years of age were at high frequency of smear positive result (Muia et al 2017). The relationship between both AFB smear result and Xpert MTB/RIF assay result with age group was found to be statistically significant ($p<0.05$).

In this study, both pulmonary and extrapulmonary samples were collected for the tests to be carried out. Out of 451 samples, 419 (92.9%) were PTB samples (sputum and BAL), while 32 (7.1%) were EPTB samples (pus, FNAC, pleural fluid and gastric aspirate). Among the positive cases, 160 (90.4%) was found to be PTB, while 17 (9.6%) cases were EPTB cases. This result is not relatable to the annual report where 71 % of the total was PTB cases (NTP 2017). In

fluorescence microscopy, 24% (98 out of 410) sputum samples showed positive result with high degree of positivity (1+, 2+ and 3+) compared to other samples, and few scanty result; while 15.6% (5 out of 32) of EPTB samples showed positive result which matches with the result in the other study where 15.8% of EPTB samples were positive (Iram et al 2015). In GeneXpert MTB/RIF assay, 38.29% (157 out of 410) of sputum samples showed positive result which is 14.29% more than that detected by FM. Similarly, 53.1% (17 out of 32) of EPTB samples showed positive result which is 37.5% more than that detected by FM. Likewise, 3 BAL sample was found to be positive which was not detected by FM. This result reveals the higher sensitivity of Xpert MTB/RIF assay compared to smear microscopy in detecting both PTB and EPTB cases. Similar result was obtained in Kenya where Xpert MTB/RIF assay detected 32.25% of positive sputum samples (Muia et al 2017). A study in Pakistan showed 22.5% of positive EPTB samples which is less than our result; while it was similar in case of detecting positive pus sample where our study showed highest yield of positivity (65%) for pus among EPTB positive samples (Iram et al 2015). There was significant difference between clinical samples with both FM and Xpert MTB/RIF assay ($p<0.05$).

In this study, Xpert MTB/RIF assay result was compared with AFB smear result according to different patient types. Firstly, new cases, first treatment cases and relapse cases comprised of 74.9% (338), 23.3% (105) and 1.8% (8) of the total TB suspected cases respectively. Among these samples, MTB was detected in 68.4% (121 out of 177) in new cases, 29.9% (53 out of 177) in first treatment cases and 1.7% (3 out of 177) in relapse cases of the total 177 MTB positive cases by Xpert MTB/RIF assay. This result disagrees with a study where TB was detected in 18.5% of new cases and 67% of previously treated cases (Shrestha et al 2018). In comparison to smear microscopy, Xpert MTB/RIF assay detected MTB in 79 smear negative cases, among which 26 were smear negative in first treatment cases, 51 were smear negative in new cases and 2 were smear negative in relapse cases. Likewise, Xpert MTB/RIF assay did not detect 5 smear positive cases of first treatment and relapse cases.

Both the tests for new and first treatment case was statistically significant (p<0.05), while for relapse cases, there was no significant difference (p<0.05).

Xpert MTB/RIF assay detects MTB along with RR-MTB which is also considered as marker of MDR-TB. In this study RR-TB was detected in 5.6% (10 out of 177) of the MTB positive patients, among which 6 cases was detected among first treatment case and 4 cases was detected among new TB cases. This result somewhat agrees with the study carried out by WHO worldwide in 2015 where 21% previously treated and 3.9% of new cases developed MDR/RR-TB, which shows that more proportion of MDR/RR-TB is detected in previously treated cases than in new cases (Falzon et al 2017). Another study carried out in India also agrees with the result where highest proportion of MDR and XDR TB was seen among previously treated patients (Udaykumar et al 2014). In this study, RR-TB was not detected among relapse cases or Category II patients. This may be due to less number of relapse cases in our study. This lower frequency of relapse cases also suggest that risk of drug resistance (MDR and XDR) is probably low in the study area. This study also reveals that previous history of treatment of TB with anti-TB drugs is also one of the important factors responsible for the development of drug-resistant TB. There was significant association between rifampicin resistance and patient types (p<0.05)

The effectiveness of Xpert MTB/RIF assay can be determined by the overall result compared between FM and Xpert MTB/RIF assay. In this study, FM detected 21.73% (98 out of 451) MTB positive cases; while Xpert MTB/RIF assay detected 39.25% (177 out of 451) MTB positive cases among total TB suspected cases which is 17.5% more than that detected by FM. But it did not detect 5 smear positive cases. These type of cases were considered as MOTTs (Mycobacteria other than tuberculosis), or might be the presence of other bacteria from the order Actinomycetales which shares similar characteristic with Mycobacteria, and the report was made as MTB negative.

From this study, the overall sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay compared to AFB smear result was found to be 95.1%, 77.3%, 55.4% and 98.2% respectively. The result of our study was less than

that of one of the study where sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay was 97.7%, 100%, 100% and 98.9% respectively (Muia et al 2017). In comparison to another study, the overall sensitivity of our study was more but specificity, PPV and NPV was less (Zeka et al 2011).

Furthermore, Xpert MTB/RIF assay showed sensitivity and specificity of 96% and 79.4% respectively for PTB samples which is incomparable to the study done for PTB where sensitivity and specificity were found to be 82.3% and 100% respectively (Zeka et al 2011). Likewise, the sensitivity and specificity of Xpert MTB/RIF assay for EPTB samples was calculated to be 80% and 51.9% respectively. In contrast, a study showed 59% sensitivity and 92% specificity for EPTB samples using reference as culture (Scott et al 2014). In reference to microscopy, a study found the sensitivity and specificity of 52.1% and 100% respectively which does not match with our result (Zeka et al 2011).

In the overall 177 MTB positive cases, 164 were rifampicin sensitive, 10 were rifampicin resistant and 3 cases were found to be rifampicin intermediate. The rifampicin intermediate cases were put for re-test, while the rifampicin resistant cases were sent to further tests and analysis. Rifampicin resistant occurs when at least one of the molecular beacon probes fail to hybridize. In our study, in all 10 rifampicin resistant cases, Probe E (529-533) did not hybridize with wild type *rpoB* gene. The failure of hybridization of probes denotes the mutation in wild type RRDR of *rpoB* gene resulting in rifampicin resistance. This result is somewhat relatable with one of the study where 64.1% of Probe E failure was noted followed by Probe B (15.2%) and Probe D (14.1%) (Rahman et al 2016). In contrast, the other study showed that more proportion of Probe B failed to hybridize followed by Probe D and Probe E (Kouassi et al 2016).

CHAPTER-VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Tuberculosis is a communicable disease of global concern caused by acid-fast bacilli *Mycobacterium tuberculosis.* Both PTB and EPTB are responsible for TB morbidity and mortality cases. It is also considered as a major public health problem in context of Nepal. The rapid identification and diagnosis of the disease is necessary for its proper treatment, prevention and control. Conventional techniques of diagnosing TB are quite time consuming and some have less sensitivity and specificity rates. WHO recommended Xpert MTB/RIF assay in 2010, which was implemented in Nepal in 2011/2012, as a rapid and fully automated technique which detects MTB DNA along with RR-TB within 2 hours of time and also has higher sensitivity and specificity. Our study was aimed in evaluating the performance of Xpert MTB/RIF assay over FM. Out of 451 samples, 177 (39.25%) samples were detected with MTB among which 10 were RR-TB cases. 60.5% of males and 39.5% of female patients were found to be infected with the disease, higher proportion in age groups 20-30 years and 30-40 years. The sensitivity, specificity, PPV and NPV of Xpert MTB/RIF assay over FM was found to be 95.1%, 77.3%, 55.4% and 98.2% respectively. Xpert MTB/RIF assay detected 79 more MTB positive cases among smear negative samples than that detected by FM. Rifampicin resistance observed in 10 MTB cases was denoted by the failure of Probe E hybridization in all 10 cases. The significant difference was observed between the tests performed by FM and Xpert MTB/RIF assay. Thus, this study helps in expanding the knowledge of risk of TB, and significant role and necessity of implementation of Xpert MTB/RIF assay in every hospital, TB centers and sub-centers, helping in the rapid identification of the disease and developing a proper treatment regimen.

6.2Recommendations

1. AFB microscopy and Xpert MTB/RIF assay should be performed simultaneously for the accurate diagnosis of TB.
2. Xpert MTB/RIF assay must be implemented in every hospitals, and TB centers for rapid identification and detection of the disease.
3. Rapid detection of drug-resistant TB should be done to prevent further dissemination of the disease in the community.
4. RR-TB is considered as MDR-TB, so proper and efficient treatment regimen should be provided to the patients as early as possible.

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APPENDICES

Appendix I

Nepal Anti - Tuberculosis Association
German - Nepal Tuberculosis Project (GENETUP)
Kalimati, Kathmandu

P.B.No. 1494
Phone: 4270483, Fax: 4302454
Email: genetup@wlink.com.np

Ref. No. 2075/2018 Date : 2075.4.14

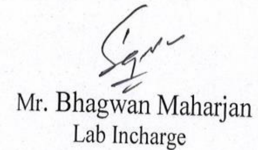
To Whom It May Concern

This is to certify that Ms. Binita Adhikari has been involved as a research student from 16 Baishakh 2075 to 15.Asar 2075 in this National TB Reference Laboratory, GENETUP/NATA.

During this time she has been actively involved in sputum collection, Microscopic examination and GeneXpert MTB/RIF assay.

During the working period she has been shown that she is dedicated, competent and hard working. Ms. Adhikari has maintained good working relationship with other staff in the Laboratory.

I know nothing against her and wish her all best in her future endeavors.



Mr. Bhagwan Maharjan
Lab Incharge

Appendix II

A. Materials used for the study:

1. Equipments:

Class II Biological safety cabinet (BSC)

GeneXpert Dx system equipped with GX2.1 software/computer/printer/barcode wand-reader and operator manual.
Xpert MTB/RIF kit

Fluorescence microscope

Centrifuge

Vortex mixer

Bunsen burner

Refrigerator

2. Chemicals:

Auramine-o stain

0.5% acid alcohol

0.5% potassium permanganate

3. Miscellaneous:

Bunsen burner	One side frosted glass slides
Marker	Gloves
Staining racks	Wooden sticks

APPENDIX III

A. Composition and preparation of staining reagents

Fluorescence staining reagents:

a. Auramine phenol

Auramine	1 gm
Alcohol (denatured ethanol or methanol)	100 ml
Phenol crystals	30 gm
Distilled water	870 ml

Auramine was dissolved in alcohol and phenol was dissolved in water, and both the solutions were mixed. Mixing was done for one hour on a magnetic stirring plate. The bottle was marked as 0.1% auramine, with date and sign with initials and stored in dark. The working solution was filtered in every use.

b. Decolorizing solution

Hydrochloric acid	5 ml
70% ethanol	1000 ml

5 ml of hydrochloric acid was added slowly to 1000 ml 70% ethanol and mixed. The bottle was labeled with 0.5% acid-alcohol and date and sign with initials was marked.

c. Counter stain:

Potassium permanganate

Potassium permanganate	5 gm
Distilled water	1000 ml

Potassium permanganate was added to distilled water and mixed well. The bottle was labeled as 0.5% potassium permanganate, and date and sign with initials was added.

Methylene blue

Methylene blue	1.0 gm
Distilled water	1000 ml

Methylene blue was added to distilled water and mixed well. The bottle was labeled as 0.1% Methylene blue, and the date of preparation and sign with initials was added. The solution was kept in dark or in dark-colored bottles.

Reagents for the NALC-NAOH method of decontamination

1. Sodium hydroxide (NaOH), 4%

Sodium hydroxide pellets	4 gm
Distilled water	100 ml

NaOH was dissolved in distilled water and sterilized by autoclaving at 121ºC for 20 minutes.

2. Trisodium citrate, 2.94%

Trisodiumcitrate.$2H_2O$	2.94 gm
or	
Trisodium citrate anhydrous	2.6 gm
Distilled water	100ml

Trisodium citrate was dissolved in distilled water and sterilized by autoclaving at 121ºC for 20 minutes.

3. NALC-NaOH solution freshly prepared

Equal volumes of solution 1 and 2 were mixed. 0.5 gm of N-acetyl l-cysteine (NALC) was added just before use.

4. Phosphate buffer, 0.067 mol/litre, pH 6.8

- Stock solution A: disodium phosphate, 0.067 mol/litre
  9.47 gm of anhydrous Na_2HPO_4 was dissolved in 1 litre of distilled water.
- Stock solution B: monopotassium phosphate, 0.067 mol/litre
  9.07 gm of KH_2PO_4 was dissolved in 1 litre distilled water.

50 ml of solution A and 50 ml of solution B was mixed. A pH meter was used to confirm that the correct pH has reached and was adjusted using 10% phosphoric acid. The buffer was autoclaved at 121ºC for 15 minutes and stored at room temperature.

APPENDIX IV

Statistical analysis:

AFB smear result versus category of age:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	48.888[a]	32	.028
	52.657	32	
N of Valid Cases	451		

a. 30 cells (66.7%) have expected count less than 5. The minimum expected count is .21.

Xpert MTB/RIF result versus category of age:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	41.690[a]	8	.000
Likelihood Ratio	48.455	8	.000
N of Valid Cases	451		

a. 1 cells (5.6%) have expected count less than 5. The minimum expected count is 4.71.

AFB smear result versus clinical samples:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	39.295[a]	20	.006
Likelihood Ratio	27.749	20	.115
N of Valid Cases	451		

a. 22 cells (73.3%) have expected count less than 5. The minimum expected count is .07.

Xpert MTB/RIF result versus clinical samples:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.882[a]	5	.002
Likelihood Ratio	22.039	5	.001
N of Valid Cases	451		

a. 7 cells (58.3%) have expected count less than 5. The minimum expected count is 1.57.

AFB smear result and Xpert MTB/RIF result according to patient types:

Chi-Square Tests

PATIENT TYPE		Value	Df	Asymp. Sig. (2-sided)
First Treatment	Pearson Chi-Square	29.807[b]	4	.000
	Likelihood Ratio	35.798	4	.000
	N of Valid Cases	105		
New	Pearson Chi-Square	158.327[c]	4	.000
	Likelihood Ratio	180.075	4	.000
	N of Valid Cases	338		
Relapse	Pearson Chi-Square	.889[d]	2	.641
	Likelihood Ratio	1.221	2	.543
	N of Valid Cases	8		
Total	Pearson Chi-Square	176.587[a]	4	.000
	Likelihood Ratio	197.982	4	.000
	N of Valid Cases	451		

a. 3 cells (30.0%) have expected count less than 5. The minimum expected count is 3.14.
b. 6 cells (60.0%) have expected count less than 5. The minimum expected count is 1.49.
c. 4 cells (40.0%) have expected count less than 5. The minimum expected count is 1.07.
d. 6 cells (100.0%) have expected count less than 5. The minimum expected count is .38.

Rifampicin resistant according to patient types:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.766[a]	6	.047
Likelihood Ratio	11.592	6	.072
N of Valid Cases	451		

a. 7 cells (58.3%) have expected count less than 5. The minimum expected count is .05.

AFB smear result versus Xpert MTB/RIF result:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	176.587[a]	4	.000
Likelihood Ratio	197.982	4	.000
N of Valid Cases	451		

a. 3 cells (30.0%) have expected count less than 5. The minimum expected count is 3.14.

Xpert MTB/RIF versus RR-TB:

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	451.000[a]	3	.000
Likelihood Ratio	604.192	3	.000
N of Valid Cases	451		

a. 3 cells (37.5%) have expected count less than 5. The minimum expected count is 1.18.

APPENDIX-V

<u>Formulas:</u>

The sensitivity, specificity, PPV and NPV values were calculated by using the following formula:

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100\%$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100\%$$

$$\text{PPV} = \frac{TP}{TP+FP} \times 100\%$$

$$\text{NPV} = \frac{TN}{TN+FN} \times 100\%$$

PPV= positive predictive value, NPV= negative predictive value

TP= true positive, TN= true negative

FP= false positive, FN= false negative