YIELD OF AUTOMATED REAL TIME PCR IN THE DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS (EPTB) IN PAKISTAN

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Pulmonary tuberculosis

Tuberculosis - Diagnosis

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FINAL APPROVAL

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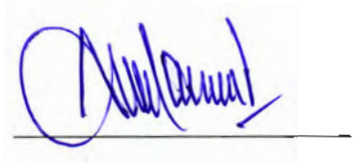

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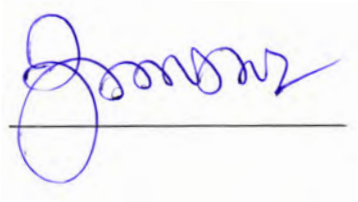

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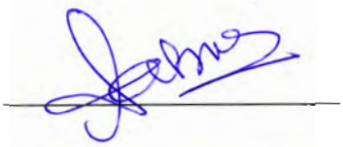

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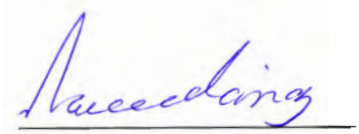

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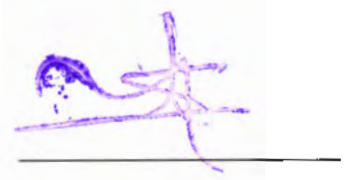

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DEDICATION

I dedicate this thesis to my parents, for without their support in more ways than can be mentioned, encouragement, advice and catharsis I wouldn't have been able to fare as I have. It would be remiss of me to not mention my siblings and supportive friends, the mere presence of whom marks a great difference. You all have shared my burden in an unimaginable way and for that I'll be ever in your debt.

Anum Khan

DECLARATION

It is hereby declared that the work present in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

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CONTENTS

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LIST OF ABBREVIATION

AFB	Acid fast bacilli
AIDS	Acquired immunity deficiency syndrome
AMTDT	Amplified mycobacterium tuberculosis direct test
ATT	Antitubercular treatment
CA	Cobas amplicor
CD4	Cluster of differentiation 4
CFU	Colony forming unit
CND	Culture not done
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTB	Cutaneous tuberculosis
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EMB	Ethambutol
EPTB	Extrapulmonary tuberculosis
FM	Fluorescence microscopy
FNA	Fine needle aspirate
GC	Growth control
GI	Gastro intestinal
GU	Genitourinary
HIV	Human immunodeficiency virus
ICT	Immunochromatographic test
IgG	Immunoglobulin G

IGRAs	Interferon γ release assays
INH	Isoniazid
LPAs	Line-probe assays
LJ	Lowenstein-jensen
MDR	Multi drug resistance
MGIT	Mycobacteria growth indicator tube
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NAAT	Nucleic acid amplification test
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
$Na_3C_6H_5O_7$	Tri sodium citrate
NRL	National reference lab
NTM	Nontuberculous mycobacteria
OFX	Ofloxacin
PANTA	Polymyxin B , amphotericin B , nalidixic acid , trimethoprim , azlocillin
PCR	Polymerase chain reaction
PPD	Purified proteins derivative
PZA	Pyrazinamide
Rcf	Relative centrifugal force
RBCs	Red blood cells
RIF	Rifampicin
rRNA	Ribosomal RNA
RNA	Ribonucleic acid

LIST OF ABBREVIATIONS

rpo	RNA polymerase
RMP	Rifampicin
RR	Rifampicin resistance
SIRE	Streptomycin, isoniazid, rifampicin, ethambutol
SM	Streptomycin
SM	Smear
TB	Tuberculosis
TBM	Tuberculosis meningitis
WBCs	White blood cells
WHO	World health organization
XDR	Extensively drug resistance
ZN	Ziehl neelsen

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ABSTRACT

Extrapulmonary tuberculosis (EPTB) represents 19% of total tuberculosis (TB) cases in Pakistan. Since routine procedures like culture and microscopy have restrictions, rapid and absolute diagnosis of extrapulmonary tuberculosis is challenging. Latest diagnostic released have upgrade this prospective yet beyond the means of many high burden resource limited settings these new diagnostic approaches demand highly sophisticated set up. Automated Xpert MTB/RIF assay with its influential utility for identification of MTB and rifampicin resistance assures to overcome these operational difficulties to great extent. A total of 597 samples submitted to the National Reference Laboratory (NRL) Pakistan for EPTB diagnosis from January 2015 to September 2015 were comparatively investigated with the new molecular-based GeneXpert MTB/RIF (Xpert) assay system, Fluorescence microscopy and conventional culture methods. Valid results were obtained for 512 specimens. Sensitivity and Specificity of Gene Xpert when compared with Fluorescence microscopy was found to be 96.8% and 92.8% respectively while calculated sensitivity and specificity in reference with culture method was 74% and 85%. Good diagnostic potential of Gene Xpert was found for non sterile specimen like pus (65.7%) and lymph node (56.2%) and comparatively low sensitivity for sterile specimens like CSF (8.2%) when compared with reference methods. The assay correctly detected rifampicin resistance in all cases found resistance on phenotypic DST. There was not any case resistant on Gene Xpert and susceptible on phenotypic DST and vice versa. MDR TB was reported in 13.6% cases. Ofloxacin mono resistance was 9.2% in general and 40% among MDR isolates. In conclusion, In resource-constrained settings this platform is exceptionally an optimistic approach for close to patient TB diagnosis.

Chapter 1
Introduction

1. INTRODUCTION

Tuberculosis is a communicable malady triggered by *Mycobacterium tuberculosis*, a human pathogen (Glickman *et al.*,2001) first explicate in ' 1882 by Robert Koch (Gengenbacher *et al.*,2012). Tuberculosis has burdened humanity from the indefinite time period even Chinese and prehistoric Babylonian writings referred to this disease. It has been divulged through molecular genetic studies that *Mycobacterium tuberculosis* is the universal cause of Tuberculosis in humans, originated 3 million years ago (Ahmad, 2010).

1.1 Global overview

In spite of early serious events to control Tuberculosis, with more than eight million cases and two million deaths yearly, it remains a global health crisis (Glickman *et al.*, 2001;Yazaret *et al.*, 2014). In reference to WHO report 2015, 1.5 million individuals get killed due to this lethal disease including 890 000 males, 480 000 females and 140 000 children. Among them 0.4 million were HIV-positive whereas 1.1 million were HIV-negative.In 2014, 9.6 million individuals were figured out to have fallen sick with TB worldwide including 5.4 million males, 3.2 million females and 1.0 million children. South East Asia and Western Pacific areas carry 58% of this burden. Africa accounts for 28% of the world's TB cases during the year 2014. India, Indonesia and China had largest of the TB cases representing 23%, 10% and 10% of the worldwide aggregate, separately. Expectedly MDR-TB kill 190 000 people during 2014. By 2015 105 countries had reported XDR-TB. Approximately 9.7% of individuals with MDR-TB have been found to have XDR-TB (WHO, 2015).

1.2 Tuberculosis in Pakistan

Tuberculosis (TB) is a noteworthy issue in Pakistan as it has been widespread and was one of the overlooked health problem in past. TB contributes 5.1 percent of the entire disease load in Pakistan (Vermund *et al.*, 2009). Pakistan rank fourth among 22 countries with highest number of TB cases (WHO, 2015). Estimated TB prevalence in Pakistan is 342 for every 100 000 populace with frequency of 275/100 000 and 58% case detection rate for different types of TB. By 2014 298 446 cases were detected (Fatima *et al.*, 2015). 65% of tuberculosis cases in the eastern Mediterranean region were contributed by this region (NTP, 2013).

1.3 About MTB Bacilli

Mycobacteria a member of order Actinomycetales are weak Gram positive, immotile, non spore forming, acid fast bacilli which when observe under the microscope become visible as straight or curved rods. The unique mycolic cell wall impart following characteristic; uttermost hydrophobicity, distinguishing immune-stimulatory properties, acidfastness, resistance to drying, and drug resistance (Sakamoto *et al.*, 2012).A solitary MTB has a generation time 18–24 h at 37 °C under ideal accessibility of oxygen and nutrients, and on agar within 3-4 weeks appears as white to light-yellow colony (Fig 1.1) (Gengenbacher *et al.*, 2012).*Mycobacterium tuberculosis* and the closely related species *M. bovis, M. africanum, M.microti, M. pinnipedii, M. caprae,* and *M. canetti* form the M. tuberculosis complex (MTBC)(Ahmad, 2010). The genome of MTB is made up of 4,411,529 base pairs, containing 4,000 genes. After E.coli genome of MTB present the second largest bacterial genome sequence accessible right now (Cole *et al.*, 1998).

Fig 1.1 Colonies of Mycobacterium tuberculosis on Lowenstein-Jensen media

(Subramani *et al.*, 2014)

1.4 Transmission of Mycotuberculosis Infection

Tuberculosis is a contagious ailment and patients with pulmonary TB are the significant source of infection (Ahmad, 2010).In humans usually the infection initiate by directly inhaling liquid particles called aerosols carrying infectious bacilli from the patients with pulmonary disease.It has been reported that individual aerosol droplet can carry 1 to 400 mycobacterium tuberculosis bacteria, whereas the infectious dose range between 1 to 200 bacilli (Sakamoto, 2012).As the droplet nuclei are small, so they remain hanged in air for quite a few minutes to hours. The risk of acquiring infection depends on various elements such as the number of inhaled organisms, immune status of host, contact distance and how infectious a source is (Ahmad, 2010).

1.5 Latent infection

Latent tuberculosis is a condition in which individuals immune system effectively respond to the inhaled MTB resulting in growth retardation of bacilli, contributing to bacterial dormancy. Immuno compromised latent individuals may get infected with asymptomatic MTB but disease cannot be transmitted to other individuals. Almost one third of the whole world's population is infected with MTB latently among them 5-10% are capable of developing active TB. Individuals with HIV and other immuno deficient people, for example those with tumor or taking immunosuppressive drugs are more prone to active form of the disease (Fogel, 2015).

1.6 Disease Progression (Active infection)

Disease progression occur as follow (Fig 1.2)

1.6.1 First Stage

During first stage i.e. from 3 to 8 weeks, bacteria in the lungs alveoli disseminate to lymph nodes through lymphatic circulation after bacterial inhalation through aerosols, which results in formation of primary or Ghon complex leading to reactivation of tuberculin.

1.6.2 Second Stage

During second stage bacteria are carried to many organs including other parts of lungs through blood, resulting in occurrence of severe and sometimes fatal disease such as tuberculosis meningitis or military tuberculosis. This stage lasts for about 3 months.

1.6.3 Third Stage

During this stage pleurisy occurs, resulting in severe chest pain. This condition is a result of either hematogenous dissemination or bacterial release into pleural space from sub pleural bacterial concentrations in lung. It is supposed that free bacteria and their elements communicate with CD4 T lymphocytes which are first attracted then multiply and finally release inflammatory cytokines. This stage usually last for 3 to 7 months but some time can be delayed up to 2 year.

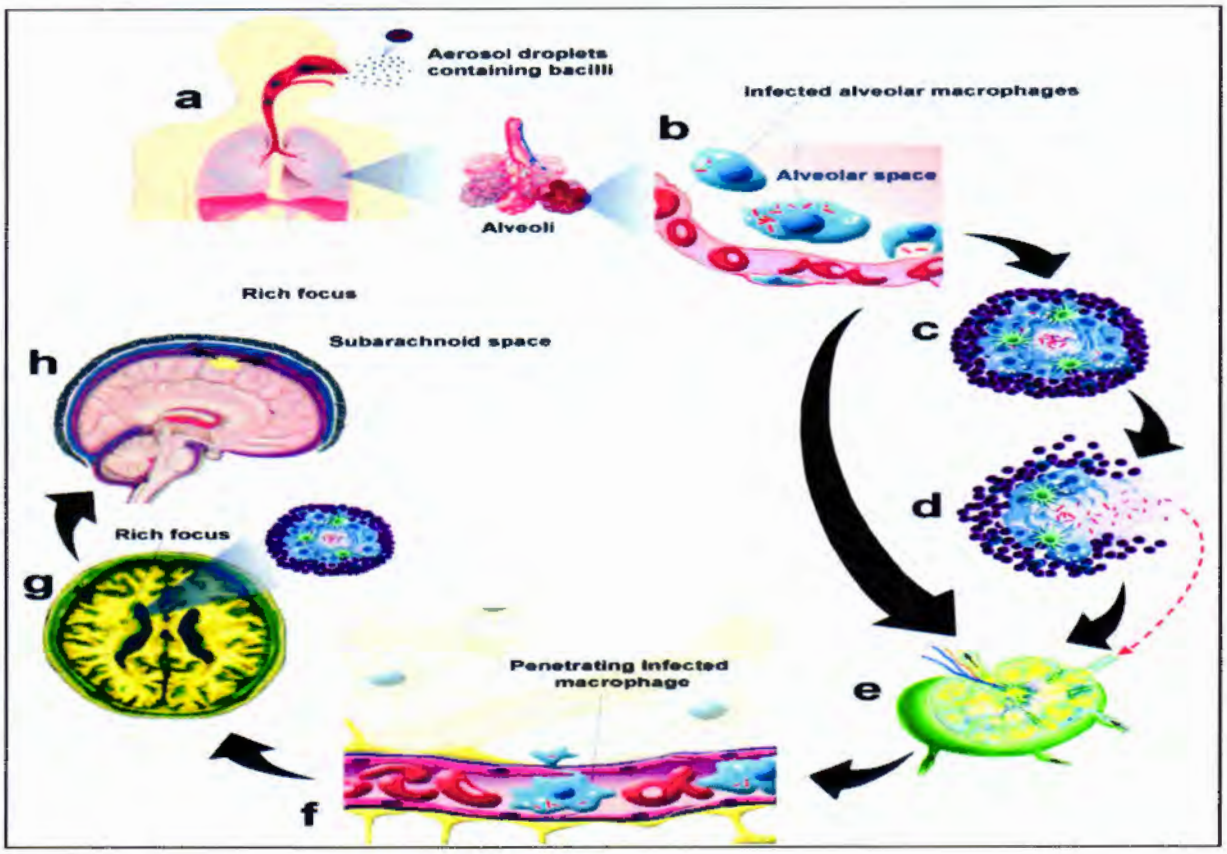


Fig 1.2Pathogenisis of mycobacterium tuberculosis causing TBM (a) Transmission of MTB through aerosole (b) Phagocytosis by macrophages (c) Granuloma formation (d) Bacilli escaping from granuloma (e) Dissemination of bacilli to regional lymphnode (f) (g) Bacilli seed to the meninges through blood forming Rich foci (h) Rupturing of Rich foci and onset of TBM (Faksri *et al.*, 2012)

1.6.4 Last Stage

The last stage may take up to three years for disease advancement. This stage is characterized by development of extrapulmonary lesions e.g. in bones and joints which are responsible for persistent back pain in some patients (Smith, 2003).

1.7 Clinical Manifestation of Tuberculosis

The clinical picture of tuberculosis are inconsistent, rely on host and microbes characteristics as well as on interaction between both (Yang *et al.*, 2004).Clinically tuberculosis is presented as pulmonary or extra pulmonary (Wares *et al.*, 2015).

1.7.1 Pulmonary tuberculosis

According to the definition given by WHO pulmonary tuberculosis referred to involvement of lungs parenchyma or tracheobronchia. Miliary TB is also assorted as pulmonary TB in light of the fact that there are abrasions in the lungs. A patient affected with both types i.e. pulmonary and extrapulmonary TB ought to be named an instance of pulmonary TB (WHO, 2015).

1.7.1.1 Clinical Sign and Symptoms

Patients with pulmonary TB often show unclear signs and symptoms. Almost 5% of active cases of pulmonary TB are asymptomatic (Leung, 1999).Symptoms comprise mild fever, loss of appetite, tiredness, nocturnal sweats, and loss of weight (Zumla *et al.*, 2013).With acute onset of TB Erythema nodosummay occur that is related to the progression of specific immunity.In around 10% of individuals with Tuberculosis show increase in WBC's count and deficiency of RBC's. Persistent Cough is the most typical manifestation of lung infection might be unproductive in early stage but may leads to mucopurulent sputum production afterwards. Hemoptysis might likewise happen (Leung, 1999).

1.7.2 Extrapulmonary Tuberculosis

Extrapulmonary Tuberculosis is related to presence of MTB infection in any organ except lungs. Tuberculosis bacilli might be carried through blood circulatory or lymphatic system to any part of body, and undergo dormancy for several years before causing infections (Chander *et al.*, 2010).Profoundly vascular areas, for example, spine meninges, lymph nodes, growing ends of the bones and kidney are normally influenced in EPTB. Other infected sites may include liver, GI tract, pleura, genito-urinary tract, skin, peritoneum and pericardium (Wares *et al.*, 2005).Among entire TB cases 15 to 20 % are contributed by extrapulmonary tuberculosis (Sharma & Mohan, 2004).

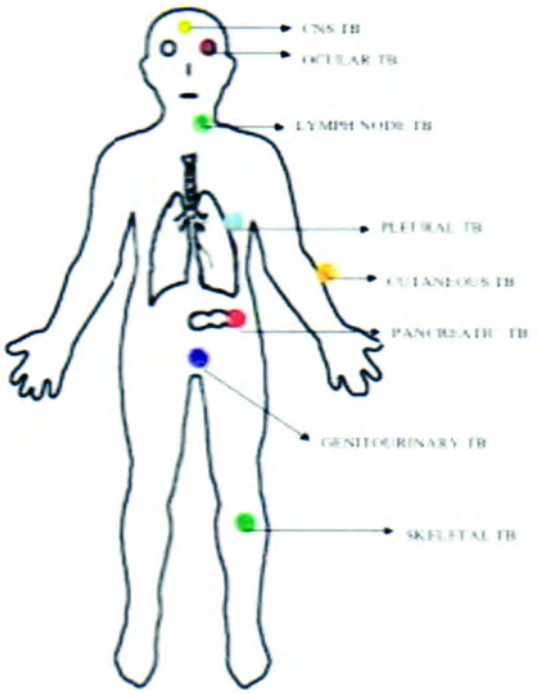


Fig 1.3 Various forms of extrapulmonary tuberculosis

(Kandola & Meena, 2014)

1.8 Extrapulmonary Tuberculosis and HIV Infection

Extrapulmonary TB specifically is a vital reason for morbidity in individuals infected with HIV. According to accessible information tuberculosis in individuals infected with HIV bring about activation of latent mycobacterial infection (Shafer *et al.*, 1991). 50 -70% of patients co-infected with HIV have higher prevalence rate of extrapulmonary TB as compared to non HIV cases (10 -34%) (Chandir *et al.*, 2010).Unique features of AIDS-related tuberculosis are extrapulmonary disease, rapid development, tissue ulcer, negative tuberculin test and lymph node infection. Anti-tuberculosis drugs results in detrimental reactions in AIDS patients. Risk of relapse of this disease is uncertain in HIV infected patients (Golden &Vikram, 2005).

1.9 Clinical Picture of Different Forms of EPTB

1.9.1 Tuberculous Lymphadenitis

One of the most usual form of EPTB is lymph node TB. Cervical areas are mostly targeted however involvement of axillary, groin area, mesenteric, mammary and mediastinal tissues, have also been observed (Golden &Vikram, 2005).One of the important aspects of lymph node tuberculosis is its distribution among various age groups and sex. Females and younger age groups are more vulnerable to this type of EPTB. Targeted age group is 20 to 40 years (Handa *et al.*, 2011).Patient usually presents symptoms such as pain free lump in specific lymphatic areas, but sometimes they experience painful mass with fever. Local abscess is observed in almost 25% of individuals infected with lymph node TB, which in later stages developed into sinus if remain untreated (Chen & Wood, 1998).According to Jones and Campbell findings peripheral tuberculous lymph nodes include following five stages.

- Compact, movable, distinct, enlarged nodes are often observed in stage one.
- Large elastic nodes joined to nearby tissue are noticed in stage two.
- In stage 3 abscess formation leads to central softening.
- Collar-stud abscess development is observed in stage four.
- Sinus formation is found in stage five (Mohapatra & Janmeja, 2009).

1.9.2 Pleural Tuberculosis

The second most prevalent type of extrapulmonary tuberculosis is tuberculosis of pleura (Ruan *et al.*, 2012).Irrespective of close association of pleura and the lungs pleural tuberculosis is still referred to as extrapulmonary (Sharma & Mohan, 2004).Pleural tuberculosis develops as a result of raised protein level in the pleural fluid in response to the delayed hypersensitivity reaction that leads to increased absorbance of protein by pleural capillaries. More fluid production occurs as a result of expanded protein concentration in the pleural fluid. Moreover pleural inflammation hinders the lymphatics in the parietal pleura, which prompts lessen pleural fluid removal from the pleural space. So increased pleural fluid production in combination with decreased pleural fluid clearance results in pleural effusion (Light, 2010).Patients with pleural tuberculosis experienced intense febrile illness described by chest pain, non productive cough however without increase in the peripheral WBC count. Other reported manifestations include night sweat, cold, fatique, breathlessness and weight loss (Vorster *et al.*, 2015). Pleural based nodules and thickening are rarely presented by patients with pleural TB (Light, 2010).

1.9.3 Skeletal Tuberculosis

Skeletal tuberculosis frequently includes the spine trailed by peripheral osteoarticular tuberculosis (Golden & Vikram, 2005).Osteoarticular TB affect 1-2 % patients annually, additionally half of osteoarticular TB influences the spine (Vardhan & Yanamandra, 2011).

1.9.3.1 Tuberculosis Osteomyelitis

Tuberculosis of spine is one of the ancient malady known. The infection is commonly known as Pott's spine. Initially anterior inferior section of vertebral body is affected followed by central part of the body or disk. Anterior, paradiskal and central part of the vertebral body are frequently involved. Clinical symptoms of spinal tuberculosis include local inflammation, gibbus, abscess, rigidity and contraction of the muscle, local pain and a notable spinal distortion. Neurologic deficits are prominent involving cervical and thoracic regions. Early neurologic involvement may advance to paraplegia, if left untreated (Garg & Somvanshi, 2011).

1.9.3.2 Peripheral Osteoarticular Tuberculosis

Peripheral osteoarticular TB also reffered as extraspinal skeletal TB that influence bones or joints. Typically long weight-bearing bones are more affected. Knee and hip involvement is also frequent. Femoral disease is not uncommon. Rarely the disease influence the ribs, sacroiliac and sternoclavicular joints (Pigrau-Serrallach & Rodríguez-Pardo,2013).Disease onset occurs with tbe penetration of synovium with MTB or may be by direct diffusion of a metaphyseal focus with bacilli. An effusion develops that leads to synovial granulation resulting in marginal disintegration due to proliferation of tissue at the joint periphery. Loss of articular cartilage and additional bone break down occur due to spread of granulating tissue from periphery to the centre.This advances to joint demolition, which might be linked with displacement. Eventually joint get stiffened (Spiegel *et al.*, 2005).Characteristic clinical feature ofextraspinal tuberculous osteomyelitis include cold

abscess with nodule formation, mild inflammation and pain and might be wrongly interpreted as tumor (Pigrau-Serrallach & Rodríguez-Pardo, 2013).

1.9.4 Central Nervous System Tuberculosis

Tuberculosis of central nervous system is one of the most dreadful form of EPTB representing 4% of all cases of tuberculosis (Tai, 2013). Developed countries are comparatively less affected than developing nations where the disease is significant source of mortality and morbidity (Girgis *et al.*, 1998).Younger age group, malnutition, alcohol addiction, HIV-coinfection, use of anti rejection medication, maignancies and use of TNFα neutralizing antibody are risk factors for CNS TB (Cherian & Thomas 2011). Disease emerges as a result of rupturing of Rich foci into subarachnoid space (Marx & Chan, 2011). Central nervous system tuberculosis in adults is characterized by manifestations such as headache, fever and neck stiffness in addition with focal neurologic deficits and behavioral changes. Children affected with TBM present symptoms such as seizures, meningismus, fever, nausea and vomiting. Headache is less commonly experienced by children than adult patients. According to the stage of disease clinical manifestation may vary from fatigue, anxiety to coma (Rock *et al.*, 2008).

1.9.5 Abdominal Tuberculosis

Tuberculosis of abdomen representing 12% of EPTB cases and 1-3% of the total cases of TB generally exist in four manifestation described as lymph node tuberculosis, GI tuberculosis, peritoneal tuberculosis and visceral tuberculosis that involve the solid organs (Debi *et al.*, 2014). Delayed diagnosis occurs because the illness can impersonate numerous conditions such as malignancy, inflammatory bowel disease, and other infections (Uzunkoyet *et al.*, 2004). The disease can affect any age group but young adults are more vulnerable to

infection; 66% of patients are 21-40 years of age and the average age of the infected individuals is 30-40 years. Abdominal TB might affect children with different spectrum compared to adults (Kapoor, 1998).

1.9.5.1 Peritoneal Tuberculosis

This form of TB involves the organs like omentum, intestine (small and large), spleen, liver female genital tract including parietal and visceral peritoneum. It represents almost 1-2% of total TB cases (Mimidis & G Kartalis, 2007). The ailment exhibit three different manifestation including wet ascetic, fibrotic fixed and dry plastic form having related clinical symptom except dry plastic form which is not characterized by abdominal distension (Sanai & Bzeizi, 2005). Common clinical findings include weakness, fever, loss of appetite, weight loss, anxiety and abdominal distention (Mimidis & G Kartalis, 2007).

1.9.5.2 Gastrointestinal Tuberculosis

GI tuberculosis of ileocecal region is most common followed by jejunum and colon. Other rarely infected organs include oesophagus, stomach and duodenum (Debi *et al.*, 2014).

1.9.5.2.1 Tuberculosis of the Oesophagus

Tuberculosis of Oesophagus is comparatively uncommon form of EPTB accounts for only 0.2 % of total cases of abdominal TB. Disease mainly occurs by expansion of infection from nearby lymph nodes. Disease is characterized by low fever, odynophagia, ulcer, discomfort in swallowing and most often midoesophageal (Sharma & Bhatia, 2004).

1.9.5.2.2 Gastric TB

Tuberculosis of Stomach and duodenum is rare manifestation of tuberculosis accounts for 1% of cases of abdominal tuberculosis. Rare involvement of stomach is due to its acidic environment, continuous motor activity and the lack of lymphatic follicles within the gastric wall (Bandyopadhyay, 2011).Most influenced area during the infection are the antrum and distal body of the stomach. Patients usually present gastric mass, ulcers and nodular accumulation within the gastric wall. Involvement of duodenum is immensely uncommon (Jadvar *et al.*, 1997).

1.9.5.3 Intestinal TB

1.9.5.3.1 Ileocecal tuberculosis

Ileocecal region being the most affected site of GI tract representing 64% of cases of GI TB. Variable contributing factor such as abundance of lymphoid tissue, high absorption capacity of region, close association of the infectious bacteria with the mucosa made ileum the most influenced site (Debi *et al.*, 2014).Disease is characterized by highly non-specific outcomes such as weakness, weight loss, abdominal pain, fever, loss of apetite, bleeding, diarrhea, ascites and constipation (Suarez *et al.*, 2010).

1.9.5.3.2 Jejunal Tuberculosis

Individual involvement of Jejunum is rarely reported but infection is almost always related with peritonitis. Disease usually presents general symptoms but may be characterized by ulceration, thickening and narrowing of passage (Suarez *et al.*, 2010).

1.9.5.3.3 Colonic Tuberculosis

Colonic tuberculosis is comprise of 9.2% of total cases of abdominal TB and is refers to the MTB infection affecting colon without involvement of ileoceeal region. Most

commonly encountered clinical symptoms include Pain, anorexia, bleeding, change in bowel habit, decrease in body weight (Sharma & Bhatia, 2004).

1.9.5.3.4 Visceral Tuberculosis

Visceral tuberculosis refers to the tuberculosis of abdominal solid organ and is comparatively rare form of EPTB constituting 15%-20% of total abdominal TB cases. Most commonly involved site among solid organ is GU system followed by other organs including liver, spleen and pancreas. Diagnosis is tough because of non-specific features presented by the patients (Debi *et al.*, 2014).

1.9.5.4 Genitourinary Tuberculosis

The term genitourinary tuberculosis was first introduced by Hans Wildbolz in 1937 (Zajaczkowski, 2012).Genitourinary tuberculosis involves pathogenic occurrence of MTB in the genital and urinary tract (Kandola & Meena 2014). Predominantly the bacilli affect the kidney from where infection expands to other parts of urinary tract. Among genital involvement primary site of infection is epididymis (in male) and fallopian tubes (in women) (Kapoor *et al.*, 2008).Adults (19 year-39 year of age) are more prone to the disease whereas children and older age group are less susceptible (Merchant *et al.*, 2013).Urinary TB is characterized by features like bladder inflammation, urinary frequency, difficulty in urination, renal colic, blood presence in urine, renal insufficiency, pain, and rarely high blood pressure.The common presenting features among male affected with genital TB are related to presence of painless mass in the epididymis and other unclear symptoms such as fever. Women with genital TB usually encountered general presentations such as disturbance of menstrual cycle, fatigue, vaginal discharge, pelvic pain and infertility (Elder, 1992).

1.9.6 Pericardial Tuberculosis

Pericarditis a rare presentation but one of the lethal form of EPTB (Trautner & Darouiche, 2001).The infection occurs because of the diffusion of bacilli into the pericardium by breakdown of tuberculous lesion in the lungs or their spread through blood from adjacent infected organs (Mayosi *et al.*, 2005).Tuberculous pericarditis is portrayed by three fundamental stages referred as effusive, constrictive and effusive constrictive (Evans, 2008).

1.9.6.1 Pericardial Effusion

Tuberculous pericardial effusion ordinarily progress deceptively, presenting unclear systemic manifestations including fever, loss in weight, nocturnal sweating, cough and fatigue (Mayosi *et al.*, 2005).Infrequently patient might experience horseness and dysphagia because of pressure on esophagus. Right upper abdominal pain due to liver blockage has also been illustrated (Mayosi *et al.*, 2005).

1.9.6.2 Constrictive Tuberculous Pericarditis

This complication is observed in 30-60% of cases and emerges at later stage of effusive pericarditis (Evans, 2008). Symptoms may ranges from general characteristics to severe complications. Diagnosis is generally missed on casual clinical examination. The pericardial knock that correspond with early diastolic sound and sudden second heart sound are indefinite (Mayosi *et al.*, 2005).

1.9.6.3 Effusive-Constrictive Pericarditis

Patients experience increased in pericardial pressure. Echocardiography might show pericardial effusion between the pericardial membranes in patients affected with effusive-contrictive pericarditis (Mayosi *et al.*, 2005).

1.9.7 Cutaneous Tuberculosis

CTB refers to the dermatological presentation of TB accounting 2% of total cases of tuberculosis (Frankel *et al.*, 2009). The malady is described by pain-free ulcer. Site of infection is characterized by painless regional lymphadenophaty (Solis *et al.*, 2012). Priorly CTB was classified on the basis of morphology of the lesions but with increasing knowledge it became apparent that despite the fact that clinically lesions appear to be similar but actually they differ in progression, development and prognosis. Tappeiner and Wolff put forward the most broadly acknowledged classification on the basis of route of infection i.e. exogenous and endogenous routes (Frankel *et al.*, 2009).Exogenous route of infection involve direct inoculation of skin as in case of tuberculosis verrucosa, tuberculous chancre and few cases of lupus vulgaris. In case of Endogenous route secondary involvement of skin occur either through blood from distant infectious organ or due to contact with established tuberculosis focus like in most cases of lupus vulgaris, military tuberculosis, orificial tuberculosis and scrofuloderma (Dias *et al.*, 2014).Most frequently encountered variant among all clinical types is scrofuloderma other form such as lupus vulgaris and tuberculosis verrucosa cutis are less common (Singal & Sonthalia, 2010).

1.9.8 Other Forms

There are also reported cases of tuberculosis infecting other body organs. Few are described as under:(Elder, 1992)

- Pathogenic occurrence of *M. tuberculosis* in eye referred to as ocular tuberculosis is predominantly observed in children and young adults. Initial sites of infection are conjunctiva, cornea and sclera. Ocular TB can be named as intraocular TB and extra ocular TB on the basis of the infected area of the eye. Intraocular TB involve infection

of iris and and ciliary body while lid is infected in case of extra ocular TB characterized by the accumulation of pus within the tissue (Kandola & Meena, 2014).

- On rare cases mycobacterium tuberculosis infects the breast. Young, multiparous and lactating women are frequently encountered by the infection. Clinical manifestation includes mass with or without presence of ulcer (Engin, 2000).
- MTB can cause infection of pharynx, oral cavity, salivary gland and thyroid gland. TB of tongue and ear has also been reported. Congenital TB acquired by the infant from diseased mother has also been documented. These forms of TB are quite uncommon even in high burden countries (Elder, 1992).

1.10 Diagnosis of Extrapulmonary tuberculosis

Early verification of extra-pulmonary form is always tough due to lower bacterial population in specimen compared with the pulmonary one (Katoch, 2004).Moreover, invasive techniques are used for sample collection so obtaining additional sample is always an issue. Confirmation of tuberculosis could be made utilizing direct and indirect approaches (Hillemann *et al.*, 2011).

1.10.1 Indirect Diagnosis of Tuberculosis

Indirect diagnosis is based on recognition of infected target with MTB, yet the tests that are currently accessible do not distinguish among latent and active TB infection. Few method of choice are mentioned as below (Molicotti *et al.*, 2014).

1.10.1.1 Tuberculin skin test

Tuberculin skin test or Mantoux test is preferred technique for recognizing latent tuberculosis infection (Abdel-Samea *et al.*, 2013). Rober Koch developed tuberculin and is compose of blend of around 200 purified proteins derivative (PPD) found in MTB and numerous other mycobacteria. Infected subject develop delayed hypersensitive reaction when

exposed to PPD. The skin reaction might indicate either positive or negative tuberculin test (Molicotti *et al.*, 2014).However, this procedure has a few inconveniences, for example vaccinated individuals show flase-positive results, reading error, patients with recent active TB may show false-negative results, error in technical application of the test and most particularly the low specificity of the test (Kussen *et al.*,2016; Brock *et al.*, 2004).

1.10.1.2 Interferon (IFN) - γ release assays (IGRAs)

IGRAs are recently introduced immune based test used as a substitute to TST for the identification of LTB infection (Diel *et al.*, 2011). The test utilize two specific secretory proteins (ESAT-6,CFP-10) particular for *M.tuberculosis* (Abdel-Samea *et al.*, 2013).QuantiFERON®TB Gold In-Tube and QuantiFERON®TB are two commercially available kits (Kussen *et al.*, 2016).Prior BCG vaccination or exposures to NTM have least effect on the test results because antigens used in assay are specific for *M. tuberculosis* complex with few exceptions (Diel *et al.*, 2011).However the major disadvantage of technique is its incapacity to discriminate latent infection from active one additionally these tests are expensive require advance laboratory setting with highly sophisticated staff. A false negative result is also an issue (Nicol, 2010).

1.10.2 Direct Diagnosis

Direct diagnostic approach is based on mycobacterial isolation, identification on species level and monitoring the drug sensitivity of isolated strain. Microbiological handling of tuberculosis demands particular reagents, non-conventional procedures, additional time, advance equipments and biologically safe environment (Molicotti *et al.*, 2014). Some of the standard techniques used are discussed below:

1.10.2.1 Direct Microscopic Examination

Identification of acid fast bacilli using stained-sputum microscope is still the most convenient, simple, cheap and fast diagnostic procedure (Wright *et al.*, 1998).Acid fast terminology comes from the fact that stain forms complex with mycolic cell wall helping the acid-fast bacilli in retaining the dye even by using acid alcohol. Mycobacterium bacilli are identified using light microscope (100 x oil immersion objective) or a fluorescent microscope (25x or 40x objective) on the basis of dye used (Babady & Wengenack, 2012). Fluorescence and ZN staining share same specificity but sensitivity of Fluorescence staining is found to be 10% more than ZN (Chaidir *et al.*, 2013; Cattamanchi *et al.*, 2009). Low sensitivity and operator performance dependency are the factors that question the reliability of this technique (Molicotti *et al.*, 2014).

1.10.2.2 Culture

When it comes to diagnosis and drug sensitivity testing, culture always serves as a standard diagnostic procedure (Jaiswal *et al.*, 2013).Culture is more efficient than smear microscopy i.e. it can detect as few as 101-102 organisms per ml of the sample (Molicotti *et al.*, 2014).Culture methods based on use of Lowenstein-Jensen (LJ) or 7H11 medium are inexpensive and easy but are time consuming i.e. they take four to six weeks for diagnosis which is the major drawback (Jaiswal *et al.*, 2013).Then there are various commercially accessible partially automated liquid culture system used for detection of both mycobacteria and MTB complex. One of the most extensively used instrument for mycobacterial detection is the broth-based Bactec 460 TB system although the method is costly and is source of radioactive waste moreover, requires sophisticated laboratory setting for proper working(Samra *et al.*, 2000; Welch *et al.*, 1993) .Another culture method called as MGIT system (Mycobacteria growth indicator tube) which is nonradioactive broth-based technique has recently been launched. System use fluorochromes for early growth detection (7-12

days) and drug susceptibility monitoring (Katoch, 2004).Instrument continuously monitors the cultures until positive culture is flagged. Positive growth on either solid or liquid media is further confirmed for non-mycobacterial contamination if any (Babady & Wengenack,2012).

1.10.2.3 Molecular Method

Nucleic acid amplification assays or direct amplification tests based on replication of nucleic acid region particular to MTB complex are the methods of choice for TB diagnosis (Molicotti *et al.*, 2014; Pai *et al.*, 2006).Commercially NAAT are available as Amplicor M. tuberculosis test, Gene Xpert MTB Rif assay, the Amplified *Mycobacterium tuberculosis* Direct test and BDProbe Tec ETtest (Pai *et al.*, 2006; Dorman, 2009).

1.10.2.3.1 Amplicor MTB Test

Amplicor MTB test is endorsed for recognition of TB bacilli in suspected patients with positive smear results. This assay is PCR based which work on mechanism of 16S rRNA gene amplification carrying a sequence particular for MTB complex (Shah & Gupta, 2015) CobasAmplicor MTB test is its automated form. The CA perform following successive events of replication, hybridization and identification of nucleic acid in a single run without any manual interference and can process in parallel for different targets using PCR mixture (Eing *et al.*, 1998).

1.10.2.3.2 Gen-Probe amplified M. tuberculosis direct test (AMTD)

AMTDT is based on principle of rRNA amplification with formation of DNA intermediate following detection of replicated product using DNA probe labeled with acridinium-easter (O'Sullivan *et al.*, 2002; Piersimoni *et al.*, 1998). The test is highly specific but different studies suggest that sensitivity may vary from 65 to 97%. Low bacterial burden in the specimen influences the sensitivity of this test than culture subsequently questioning its effectiveness for screening those specimens that are smear-negative (Gamboa *et al.*, 1998).

1.10.2.3.3 Gene Xpert MTB/RIF Assay

Gene Xpert is WHO approved PCR based automated system. Recently WHO recommended this assay for diagnosis of TB in paucibacillary respiratory specimen because of its sensitivity for these samples. Currently Gene Xpert serves as first line rapid diagnostic test in endemic countries (Tortoli *et al.*, 2012). Assay utilize single reaction to identify Mycobacterium tuberculosis DNA and rpoB gene mutations by using molecular beacons targeting the core region related to Rif resistance. Any alteration in wild type gene (rpoB) is reported as rifampicin resistance (Atehortua *et al.*, 2015; Marlowe *et al.*, 2011; Ioannidis *et al.*, 2011).Assay is characterized by following successive events such as lysis of bacteria, extraction of nucleic acid, replication and finally detection of amplified product. Test is run on Gene Xpert machine by using disposable cartridge containing the entire essential reagent along with internal control in form of spores of *Bacillus globigii* (Hillemann *et al.*, 2011;Moure *et al.*, 2011).Sample is mixed with buffer before addition to the cartridge which is only the manual step (Ioannidis *et al.*, 2011).Sample is either identified as positive or negative after running on a machine which gives result after 90 minutes (Fig 1.4)(Sarwar *et al.*, 2014).Gene Xpert has high sensitivity of 98.3% and specificity of 99% for sputum specimen and for extrapulmonary specimen it varies from 25-100% depending on the site from where sample is taken (Banada *et al.*, 2013).Various distinctive features make Xpert appealing tool for extrapulmonary specimen such as low risk of cross contamination, less technician dependency, require minimal biosafety environment and its sensitivity for smear negative pulmonary specimen especially in HIV infected patients (Vadwai *et al.*, 2011).

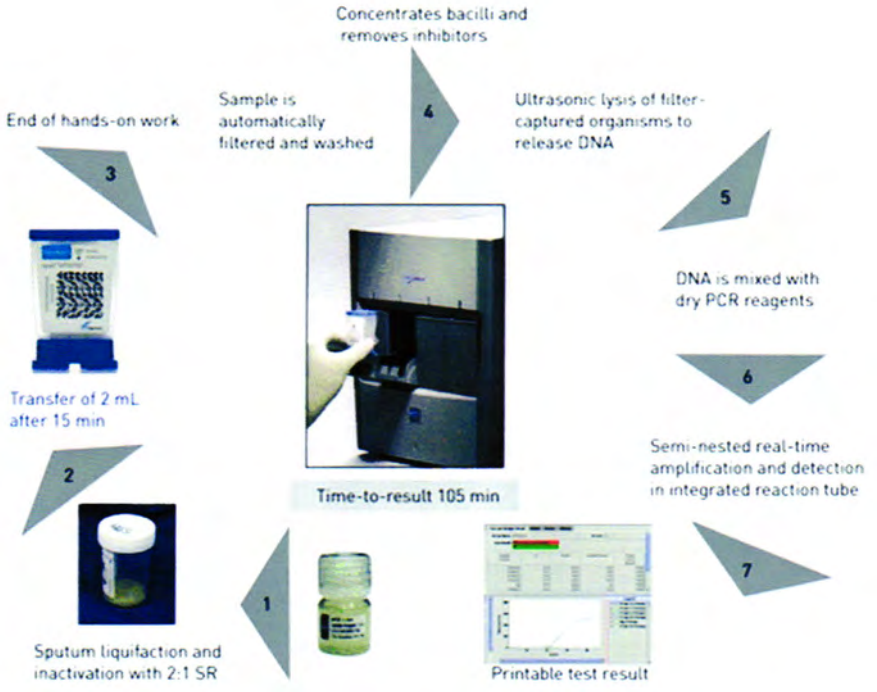


Fig 1.4 Different steps of Gene Xpert MTB/Rif assay(Weyer et al., 2013)

- BD ProbeTec is semi automated strand displacement amplification based test which uses 16S rRNA gene and IS6110 insertion chain as primary amplification target. Other commercially available systems e.g GenoType® Mycobacteria Direct Test and Real-Time PCR are also used for diagnosis of MTB. In short highly sensitive NAA-based tests are always preferred one when it comes to diagnosis of tuberculosis (Sahin, 2013).

1.10.2.4 Immunochromatographic Test

ICT is antibody based test that identify tuberculosis on the basis of the antibodies (IGg) produced in response to five purified MTB antigens. Immobilized MTB antigens produced during active TB is present in a lane on test strips. The test has high specificity for active tuberculosis as actively growing organisms produce the target protein MPB64 (Gounder *et al.*, 2002).Positive result is indicated by of one or more pink line on a strip produced in response to binding of anti human IgG to human IgG (Fig 1.5)(Pottumarthy *et*

al., 2000).The tests do not demand any additional apparatus and give final results within 15 minutes (Grobusch *et al.*, 1998).

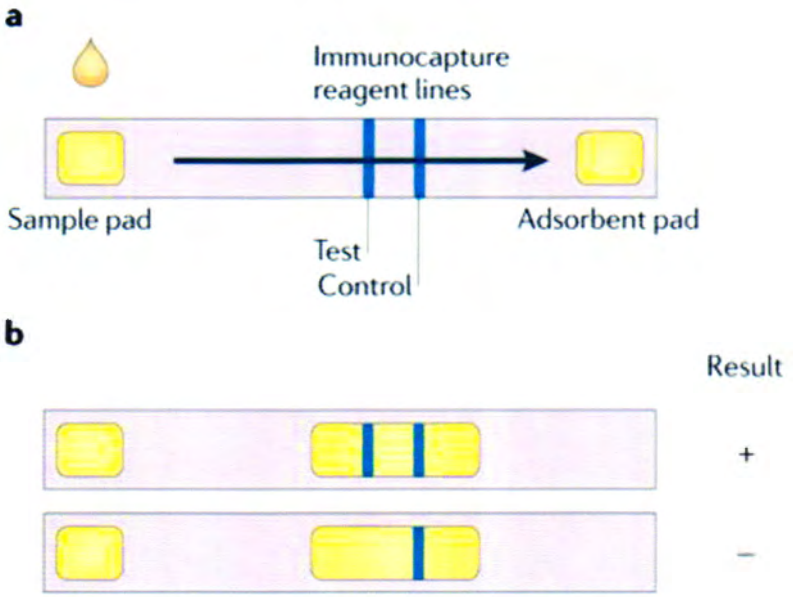


Fig 1.5 (a) The test sample is placed in the sample well(b)Positive and negative result indicated by immunochromatographic device (McNerney & Daley, 2011)

1.11 Drug Resistance in Tuberculosis

Drug resistance in mycobacterium tuberculosis is referred to decrease in sensitivity of TB bacilli to antituberculosis drugs (Willcox, 2012).Antituberculosis medications can be called as two-edge sword. On one end they eliminate infectious bacteria while on other side they select resistant microorganism for which those medication are no more effective (Johnson *et al.*, 2007). Multi drug resistance development in mycobacterium is an issue of great concern now a day. MDR-TB is a disease condition referring to the resistance to two chief first line antituberculosis agents such as rifampicin and isoniazid with or without resistance to other medication.This is due to the fact that no new drug has been particularly created after rifampicin (1967) for the treatment of this fatal ailment. Patients some time develop other forms such as extensively drug resistance TB and totally drug resistance TB

which are progressively more tough to treat so therapy diverts to less potent regimens with narrow spectrum (Koch & Wilkinson, 2014).

Global tuberculosis control programs has demonstrated that drug resistance Tuberculosis is at alarming stage and is currently a risk to various surveillance programs in numerous countries (Willcox, 2012).Globally according to an estimate new cases accounts for 3.3% of MDR-TB while 20% of already treated cases have develop MDR-TB. Approximately 480 000 new cases of MDR-TB have been reported around the world. Roughly around 190 000 people died of MDR-TB in 2014. About ¾ patients with MDR-TB lived in india South Africa China or Europe. Estimated 9.7% of the patients with MDR-TB have XDR-TB (WHO, 2015).

Inappropriate treatment, inadequate supply of drugs, poverty, improper regimens are some of the factors that contribute in the emergence of drug resistance tuberculosis (Urassa *et al.*, 2008).

Epidemiologically drug resistance in TB can be classified as

- Primary or transmitted resistance arises when the infected strain at the time of its first exposure with the subject is resistance to more than one antituberculosis medication which indicates that transmission of resistance take place within the community.
- Acquired resistance develops in response to use of inappropriate drugs resulting in emergence of resistance among patient's bacterial population (Zignol *et al.*, 2011).
- Initial: refers to the patients who refuse prior treatment,and have unclear medical history. In real these patients are case of true primary resistance and unpredictable amount of acquired resistance (Long, 2000).

Emergence of drug resistance has brought into consideration the requirement for rapid diagnostic procedures for drug resistance TB. Delay in diagnosis is related to adverse consequences and increasing risk of transmission within the community (Nicol, 2010).

1.12 Susceptibility Testing

Proper management of drug resistance TB demands precise and detailed information about the type of the resistance (Campbell *et al.*, 2011).Drug susceptibility in MTB can be estimated either by rate of growth or growth inhibition on a medium supplemented with drugs or by monitoring mutation in genes related to drug resistance using molecular methods (Kim, 2005).For detection of drug resistance various culture system and genotypic approaches are utilized. According to WHO recommendation liquid based culture and line probe assays serve as gold standard for testing susceptibility of MTB against first line drugs (Molicotti *et al.*, 2014).

1.12.1 Conventional Culture Methods

1.12.1.1 Indirect Proportion Method

For susceptibility testing WHO recommend proportion method using LJ media but other methods might also be approached such as resistance ratio method and absolute concentration. Other media such Middlebrook 7H10, 7H11, 7H12 may likewise be useful (Johnson *et al.*, 2007). For 1% idirect proportion method four quadrant petri dishes containing Middlebrook 7H10 agar media are used with three quadrants containing antibiotics and one quadrant which is kept drug free serve as a control. Inoculums dilutions of 10-2 and 10-4 are poured on each quadrant following incubation at 37 ± 1°C for 21 days (Babady & Wengenack, 2012).In order to figure out proportion of resistance number of colony on antibiotic containing media are compared with colony count on antibiotic free media. If calculated proportion is higher than the critical proportion, is an indication of

resistant while proportion value is found to be lower for susceptible isolates (Johnson *et al.*, 2007).

1.12.1.2 Liquid Culture Based Technique

Conventional drug susceptibility testing system involving bacterial isolation followed by DST offer inconvenience of demanding prolonged turnaround time of 10-12 weeks. Liquid based cultures techniques e.g radiometric BACTEC 460 TB system, BacT/ ALERT 3D, the Mycobacteria Growth Indicator Tube (MGIT) 960 and ESP Culture System II provide good alternative with turnaround time of 25-45 days (Gupta & Anupurba, 2010;Bwanga *et al.*, 2009).

1.12.1.2.1 BACTEC MGIT 960

Automated BACTEC MGIT 960 system is based on fluorometric technology. Detection is done on the basis of the oxygen utilization by bacterial population in the tube. Prepared kits are used for Susceptibility testing of RMP, INH, SM, EMB and pyrazinamide (PZA). Procedure involve the inoculation of 1:100 dilution (in case of PZA 1:10) of the TB strain on both drug free and drug carrying MGIT tubes. Result can be interpreted by comparing both drug free and drug containing MGIT tubes. Fluorescence in antibiotic containing tube is an indication of resistant strain. Normally test takes 7-10 days for completion (Richter *et al.*, 2009).

1.12.2 Molecular Detection of Resistance Markers

Molecular methods for drug susceptibility testing are more convenient compared to phenotypic one regarding their validity and turnaround time (Raizada *et al.*, 2014).Molecular studies suggest that most of the reported mutations (95%) regarding RIF resistance are associated with hot spot region (81 base pair) of rpoB gene (Morgan *et al.*, 2005). According

to WHO LPAs are recommended molecular techniques for MDR-TB monitoring in low income countries (Albert *et al.*, 2010).

1.12.2.1 Line-Probe Assays

Family of striped based assay that utilize PCR and reverse hybridization technology for rapid screening of mutations responsible for antibiotic resistance. Commercial kit comprise of GenoType1 MTBDR assay and INNO-LiPA Rif TB kit (Pai *et al.*, 2006).LPA give results within 24-48 hours and offer use of both specimen and culture isolates for testing (Albert *et al.*, 2010).The LiPA kit contain paper strip made up of nitrocellulose containing 10 immobilized probes. Technique involve events such as DNA isolation from culture or sample, amplifying rpoB gene hot spot region related to Rif resistance, hybridization of the replicated product and finally obtaining result by calorimetric development. If all wild type S probe are found to be positive along with all negative R probe isolate is referred to as RIF susceptible. Absence of one or more wild type S probe is an indication of RIF resistance (Fig 1.6) (Morgan *et al.*, 2005).

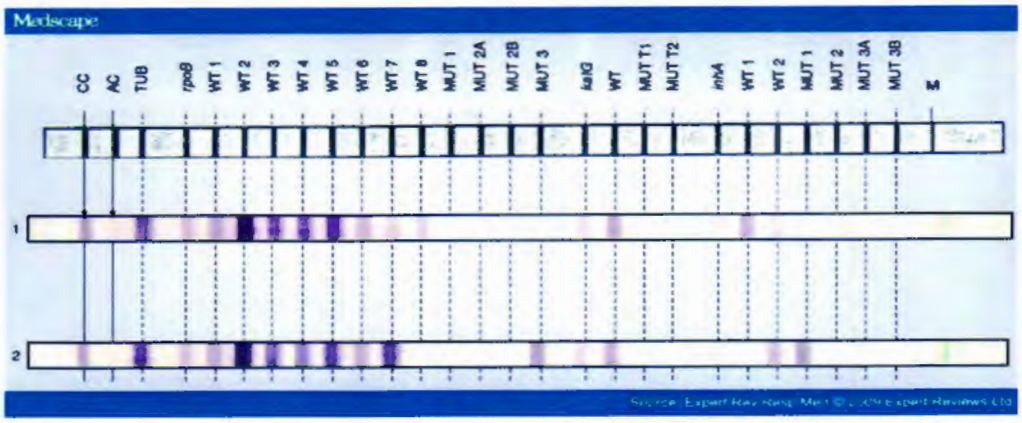


Fig 1.6 Representative patterns of a susceptible strain (lane 1) and multidrug-resistant strain (lane 2) (Richter et al., 2009)

The GenoType MTBDR assay, a commercial version of line probe assay introduce in 2004 has an edge over LiPA because of its efficiency to detect both mutation in rpoB gene

i.e. rifampicin and also mutation realted to katG gene i.e. isoniazid resistance (Raizada *et al.*, 2014).

High expenditure and sophisticated infrastructural issues question efficacy of line probe assay in high burden countries with low income (Pai *et al.*, 2006).

1.13 Aims and Objectives of the Study

The objective of this study was to investigate the potential role of Xpert system in the diagnosis of extrapulmonary tuberculosis in Pakistan. It will allow us to better insight the usefulness and efficiency of the Gene Xpert assay regarding recognition of MTB in extrapulmonary specimens. This study focused on comparison of Xpert with traditional diagnostic approaches used in Pakistan for tuberculosis detection.

Another aspect of this research is to determine the proportions and patterns of drug resistance using phenotypic DST method among EPTB patients in Pakistan. Another approach is to find out reliability of Xpert for screening of Rif resistance and comparing its efficacy with conventional phenotypic DST regarding detection of rifampicin resistance.

Chapter 2
Methodology

2. Materials and Methods

2.1 Sample Collection

A total of 597 clinical cases of extrapulmonary tuberculosis were selected from January to September at National Reference Laboratory Islamabad from different health facilities of Rawalpindi and Islamabad. The collected samples include 123 specimens of tissue (Tissue lymph node [n=44], Tissue pleura [n=7], Tissue from non specific site (NOS) [n=37], Fine needle aspirate from lymph node (FNA lymph node) [n=17], Fine needle aspirate from non specific site (FNA NOS) [n=18]) and 474 specimens of body fluid (CSF [n=116], Pleural fluid [n=192], Ascitic fluid [n=48], Synovial fluid [n=5], Fluid from non specific site (NOS) [n=18], Urine [n=17] and Pus NOS [n=87]).

2.2 Identity Number Allotment

All samples were assigned specific NRL number. Culture number, SM number, Xpert number and DST number were given according to the test performed.

2.3 Sample Handling

50 ml centrifuge tubes were used for sample collection. Those samples which were received in any other standard container were initially transferred to centrifuge tube.

2.3.1 Cerebrospinal fluid:

Body fluids such as cerebrospinal fluid (collected aseptically) were inoculated directly on culture media i.e. solid and liquid depending on the quantity of the sample and undergo further processing without decontamination step (Anargyros *et al.*, 1990).

2.3.2 Tissue/ Biopsies

Lymph node, Biopsies and other surgically eviscerated specimens (large sized) were homogenized using tissue grinder following NALC-NaOH procedure (De Wit *et al.*, 1992). Small sized tissues were cut into pieces in a sterile petri dish using sterile surgical blade.

2.3.3 Other Body Fluids

Urine and any other body fluids with volume more than 10 ml were centrifuged at 3000 Rcf for 10-20 min and sediment was resuspended in 1-2ml of distil water prior to decontamination step.

2.4 Sample Processing

2.4.1 Decontamiuation

Standard NaOH-NALC method was used for sample decontamination. The stock solutions that were used for digestion include Sodium Hydroxide (NaOH) and tri sodium citrate($Na_3C_6H_5O_7$).

2.4.1.1 NaOH-NALC Method

Equal volume (25ml) of Sodium Hydrooxide (5% NaOH) and Tri sodium citrate (2.9% $Na_3C_6H_5O_7$) was taken in a falcon tube containing NALC (N-acetyl L-cysteine) powder (0.25g). NALC-NaOH mixture was added in a volume equivalent to the quantity of the sample. Sample was then vortexed for about 15-30 sec and kept at room temperature for 15 minutes according to the SOPs.

2.4.2 Neutralization

After 15 minutes distilled water was added to the samples up to the top ring on the centrifuge tube and sample tubes were votexed for few seconds.

2.4.3 Centrifugation

Samples were then centrifuged at 3000 Rcf at 10°C for 20 minutes.

2.4.4 Resuspension

After centrifugation supernatant were discarded and sediment was resuspended by using 1-2 ml of distilled water (Yajko *et al.*, 1993).

2.5 Culture

Both solid (Lowenstein Jensen media) and liquid media were used for inoculation. Inoculation was done on prepared LJ media bottles and on 7ml MGIT tubes.

2.5.1 Inoculation On MGIT Tubes

MGIT tubes were labeled with specimen number. By using adjustable pipette 0.8 ml of MGIT growth supplement PANTA (PolymyxinB , Amphotericin B , Nalidixic acid , Trimethoprim , Azlocillin) was added to each MGIT tube. 0.5 ml of processed / concentrated specimen was transferred to the MGIT tube using sterile pipette. Separate pipette tip was used for each sample (Hillemann *et al.*, 2011; Pfyffer *et al.*, 1997).

2.5.2 Inoculation on Lowenstein Jensen Media

Media bottles were labelled with culture number. Using Pasteur pipette 2-3 drops of decontaminated samples was inoculated on solid media (Welch *et al.*, 1993).

2.5.3 Incubation

2.5.3.1 Incubation of 7ml MGIT tube

All inoculated MGIT tubes (7ml) were incubated in the BACTEC MGIT 960 instrument after scanning (Hillemann *et al.*, 2011).

2.5.3.2 Incubation of Lowenstein-Jensen Media

Media bottles were incubated at 37°C (Samra *et al.*, 2000) in slanting position for one week with lose screw caps. Following a week caps were tightened and bottles were placed upright.

2.5.4 LJ slopes Reading

Media bottles were checked for growth weekly on regular basis for 8 weeks (Samra *et al.*, 2000). During first week rapidly growing mycobacteria and contaminated slopes were removed In case of no colonies observed cultures were referred to as negative. Actual number of colonies was counted if number of colonies on a media was between1-9. Culture bottles with colonies 10-100, 100-200 and >200 were graded as 1+, 2+ and 3+ respectively. Following 8 weeks culture was alluded to as either positive or negative (Friedrich *et al.*, 2013).

2.5.5 MGIT Tube Reading

MGIT Tubes were monitored automatically by the instrument until positive. Once in a week positive cultures were removed from instrument. Final results for MGIT cultures were read following eight weeks (Pfyffer *et al.*, 1997; Chien *et al.*, 2000). Bright orange fluorescence at the bottom of the tube and an orange reflection at the meniscus were interpreted as positive culture. Tubes with very little or no fluorescence were perused as negative (Lemus *et al.*, 2004).

2.6 Auramine staining

2.6.1 Staining solution

Following prepared reagents were used for staining

- Auramine staining solution, 0.1%
- Acid-alcohol decolourizing solution, 0.5%
- Counterstaining solution (1.0% methylene blue)

2.6.2 Smear Preparation

Slides were labelled with appropriate culture number. Drop of decontaminated specimen was transferred using sterile pipette to make thin smear. Slides were left air dry at room temperature.

2.6.3 Staining Method

Dried slide were placed on a staining rack over a sink and were heat fixed by passing three times slowly through a flame of a spirit lamp. Small funnel containing a new filter paper was filled with Auramine solution. Filtered solution from the funnel was passed on to the slide covering the slide completely and was left for 20 min. After 20 min slides were washed with tape water. Acid alcohol was then poured onto the smear covering the entire slide and was allowed to act for 1-2 min. Slides were then rinsed with tape water and were then floaded with methylene blue (counter stain) for 1-2 min followed by washing with tape water. Slides were then shifted on a drying rack and were allowed air dried (Chaidir *et al.*, 2013).

2.7 Microscopy

Fluorescent LED microscope with objectives adapted for fluorescence of 20 x, and 40x/50x, and eyepieces of 10x was used for scanning of slides (Chaidir *et al.*, 2013).

2.7.1 Recording Results

According to the number of AFB observed under microscope results were recorded according to the grading scale for fluorescence microscopy set by WHO.

Union / WHO Scale x1000 field=HPF Result	Fluorescence (x200-250 magnification; 1 length=30 fields =300 HPF)	Fluorescence (x400 magnification; 1 length=40 fields =200 HPF)
Negative	Zero AFB/1 Length	Zero AFB/1 Length
Scanty	1-29 AFB/1 Length	1-19 AFB/1 Length
1+	1-9 AFB/1 field	20-199 AFB/1Length
2+	10-100 AFB/1 field	5-50 AFB/1 field
3+	>100 AFB/1 field	>50 AFB/1 field

Table 2.1 Grading scale for fluorescence microscopy (Khatun *et al.*, 2011)

2.8 Gene Xpert MTB/RIF Assay

2.8.1 Cartridge Preparation

Sample reagent was added in a 2:1 ratio to the decontaminated specimen present in the falcon tube. The closed tube was vortexed twice during a 15-min incubation period at room temperature. Using specific pipette 2 ml reagent-sample mixture was transferred to the Xpert test cartridge (Hillemann *et al.*, 2011; Lawn & Nicol, 2011).

2.8.2 Cartridge Loading

Laptop and the gene XpertDx system was turned on. All the relevant information regarding the sample was entered in Gene XpertDx System software. Barcode on the Xpert MTB/RIF cartridge was scanned using scanner machine and cartridge was inserted in the Gene Xpert instrument module with blinking green light and the automatically generated results were read after 90 minutes. Positive result was indicated by the machine when five MTB rpoB gene specific probes were detected. Negative results by the machine were indication of MTB absence in the specimen. Rifampicin status of the sample was generated automatically by the system. After the test was finished cartridges were disposed off in appropriate waste container (Atehortua *et al.*, 2015).

2.9 Immunochromatographic Assay

Immunochromatography assay was performed in case of any doubtful positive result on both liquid and solid media. For sample preparation from solid culture a drop of distilled water was added in culture bottle while positive MGIT culture were directly used for

inoculation in sample well. Using Pasteur pipette water from positive culture was transferred to labelled ICT kit. Results were obtained after 15 minutes.

2.9.1 Result interpretation

ICT was referred to as positive if purple red band appear in both test and control zone and negative if purple red band appear in control zone but not on test zone (Abe *et al.*, 1999).

2.10 DST

2.10.1 Sample

Positive cultures of MTBC were further tested for drug susceptibility against rifampicin, isoniazid and ofloxacin. DST was performed within 15 days after the appearance of ist colony on the culture media. DST was not reported for the primary culture with < 5col. Primary cultures with more than 5col and less than 10col were sub cultured. Freshly subcultured M. tuberculosis strain H37Rv was used as control strain.

2.10.2 Proportion Method

2.10.2.1 McFarland Preparation

Suspension was made by adding 0.1 ml of (1%) Barium chloride solution to 9.9 ml of (1%) Sulfuric acid. The density of the resulting barium sulfate precipitate was used as a proxy to approximate the colony count of bacterial suspensions (Andrews, 2001).

2.10.2.2 Preparation of the Calibrated Bacterial Suspension

Colonies were picked from all over the culture using sterile loop and was shaken over the glass beads contained in the screw-capped glass tube. Loop was discarded and glass tube

was vortexed for 1 min. Using sterile Pasteur pipette 5ml of distilled water was added to each tube containing bacterial colonies from positive cultures and were allowed to stand for 10 min. Aseptically 2-3ml of supernatant was transferred to another tube with similar dimensions to the McFarland turbidity standard No. 1. Bacterial suspension was then allowed to settle and some of supernatant was discarded to concentrate the cell. Bacterial suspension was again vortexed for 30 sec. Few drops of distilled water were added to adjust the turbidity to aim the McFarland suspension.

2.10.2.3 Dilution of the Calibrated Bacterial Suspension

Using Calibrated bacterial suspension equivalent to McFarland No.1 tenfold serial dilutions were prepared down to a dilution of 10^{-2} and 10^{-4}. 0.5 ml of bacterial suspension was discharged in a glass tube containing 4.5 ml of distil water producing 10^{-1} dilution. 0.5 ml of bacterial suspension from 10^{-1} dilution was taken using sterile loop and was dropped off in a glass tube containing 4.5 ml of distilled water producing dilution 10^{-2}. 10^{-4} dilution was produced by taking 0.5ml of suspension from 10^{-2} and dissolving it in 4.5 ml distilled water making 10^{-3} and again taking 0.5 ml of suspension and adding it in 4.5 ml of distilled water in a glass tube.

2.10.2.4 Inoculation

Prepared drug containing and drug free media bottles were used. Media bottles were labelled with DST number. Media was decant and two slopes of medium without drug and two slopes of medium with drug were inoculated with 0.1 ml of the 2 chosen dilutions i.e. 10^{-2} and 10^{-4} (Canetti *et al.*, 1969).

2.10.2.5 Incubation

Inoculated slopes were incubated at 36 ± 1 °C in a horizontal position with loosen caps. After one week caps were tightly closed.

2.10.2.6 DST Reading

DST slopes were examined once in a week. During first week Contaminations and NTM were reported. Final result of DST was interpreted after 6 weeks of incubation (Gupta & Anupurba, 2010). Growth control were observed for growth that must have colony count between 100 to 300 and 5 to 100 colonies inoculated with 10^{-2} and 10^{-4} dilutions respectively. The usual criterion for resistance is 1% growth for all the drugs. The growth obtained on media containing drug was compared to growth obtained on the control inoculated with the 10^{-4} dilution. Strain was referred to as resistance to that specific drug, if the growth on the media containing one drug was ≥ to the growth on the 10^{-4} growth control. Similarly if the drug containing media had growth < to the growth on the 10^{-4} growth control, the strain was referred to as susceptible to that particular drug. Growth on culture media was graded as follow:

No growth	**0**
Fewer than 50 colonies	**Actual count**
50–100 colonies	**+**
100–200 colonies, light bacterial lawn	**++**
200–500 colonies, almost confluent	**+++**
>500 colonies, confluent growth	**++++**

2.10.3 MGIT DST

2.10.3.1 Reconstitution of Lyophilized Drugs

Lyophilized drugs were reconstituted by adding 4 ml of sterile distilled water to the drug powders.Bottles were closed and were allowed to stand for a few minutes and were then mixed thoroughly.

2.10.3.2 Addition of a Drug to the Medium

0.1ml (100 µL) of reconstituted drug solution was added into each of the labeled BACTEC MGIT 960 tubes.

2.10.3.3 Preparation of DST Inoculums

The day a MGIT tube was positive by the instrument is considered Day 0. DST was set up for the MGIT positive pure culture within 5 days. A tube that has been positive for more than 5 days was subcultured in a fresh MGIT 7ml tube. Tube having growth that were on day 1 and day 2 were vortexed and were allowed to stand for 5-10 min. Undiluted Supernatant was used for inoculation of drug set while tube having growth that were on day 3,4 or 5 were diluted with 4 ml of sterile saline added into 1.0 ml of positive broth producing 1:5 dilution which was then used for inoculation.

2.10.3.4 Inoculation and Incubation

MGIT tubes were labeled one for GC one for INH, one for RIF, and one for OFX. Each tube was supplemented with 0.8 mL of SIRE. Using calibrated micropipette 0.1ml (100 µl) from respective drugs stock solutions was added to the appropriate labeled tubes. GC was

kept drug free. Aseptically 0.5 ml of the well-mixed culture suspension was added into each of the drug containing tubes using a pipette. For the control, the test culture suspension was first diluted to 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline. 0.5 ml of this diluted suspension was added to the growth control tube. Inoculated tubes were allowed to stand for 30 min at room temperature before loading into the MGIT 960 instrument.

2.10.3.5 Reading

Once the test was complete (within 4 to 21 days), the instrument indicate that the results were ready. The instrument printout indicates susceptibility results for each drug. Results were qualitative: Susceptible (S), Resistant (R) or indeterminate (X) (Siddiqi, 2005).

2.11 Data Analysis

The collected data were entered into Microsoft Excel 2000 (version 9) software. Frequencies and percentages were calculated using SPSS v16 (Chicago, SPSS Inc) software. The sensitivity and specificity of each test were calculated according to following formula. (Iram *et al.*, 2015)

Sensitivity:

True positive (TP) x 100 / True positive (TP) + False negative (FN)

Specificity:

True negative (TN) x 100 / True negative (TN) + False positive (FP)

Chapter 3

Results

3. Results

3.1 Patient Enrollment and Sample Received

The study includes 597 specimens received from different health facilities of Rawalpindi and Islamabad (Table 3.1) between January 2015 and September 2015. The maximum number of samples received were from patients in the age group of 0-14 year(n=125) accounting 20.9%of the total followed by15-24 year (n=106), 25-34 year (n=90), 35-44 year (n=82), 45-54 year (n=74), 55-64 year (n=63) accounting 17.7 %, 15.0%, 13.7%, 12.3% and 10.5% respectively.Least number of specimens were from Patients with > 65 year (n=57) of age accounting 9.5 % of the total. The pleural fluids represented 32.2% (n=192) of the samples, CSF 19.4% (116), Pus 14.6% (n=87), Ascitic fluid 8% (n=48), Lymph node tissue 7.4% (n=44), Tissue from non specific site 6.2% (n=37), FNA from non specific site 3% (n=18), FNA from lymph node 2.8% (n=17), Urine 2.8% (n=17), Fluid from non specific site 1.5% (n=9), Pleural tissue 1.2% (n=7), and Synovial fluid 0.8% (n=5) (Table 3.2)

3.2 Sample Tested by Patient Type Based on Previous History of ATT

72% (n=430) of specimens tested were from the patients with no previous history of TB. Previously treated cases accounts 20.3% (n=121) of the total. 7.7% (n=46) cases were unclassified. Among new cases highest number of specimens were of Ascitic fluid accounting 85.4% (n=41) out of total 48 received Ascitic fluid samples similarly among new cases the

predominat specimen received were of lymph node with 15 (34.1%) out of 44 received number (Table 3.4).

Table 3.1 Summary of referring health facilities

City	Health Facility name	# of EPTB Specimen	
		Number	%
Rwp&Isb	Military and allied Hospitals	279	46.7
Islamabad	PIMS	88	14.7
Islamabad	Poly clinic	20	3.3
Islamabad	KRL	9	1.5
Islamabad	FGH	8	1.3
Islamabad	ShifaInternationl	6	1.0
Rawalpindi	RLH	49	8.2
Rawalpindi	BBH	48	8.0
Rawalpindi	Holy Family Hospital	12	2.0
Muree	SAMLI	6	1.0
	Others	72	12.0
	Total	597	100

Table 3.2 Specimen type and age group of presumptive EPTB patients

Type of specimen	# tested (%)	0-14yrs	15-24yrs	25-34yrs	35-44yrs	45-54yrs	55-64yrs	>65yrs
Fluid (CSF)	116 (19.4)	58	12	16	10	4	7	9
Fluid (Pleural)	192 (32.2)	29	31	17	21	34	29	31
Fluid (Ascitic)	48 (8.0)	8	3	7	9	7	10	4
Fluid (Synovial)	5 (0.8)	1	0	0	1	1	1	1
Fluid (NOS)	9 (1.5)	2	3	2	0	0	1	1
Pus (NOS)	87 (14.6)	11	25	19	17	8	3	4
FNA (Lymph node)	17 (2.8)	2	5	5	2	3	0	0
FNA (NOS)	18 (3.0)	2	3	3	4	4	0	2
Tissue (Lymph Node)	44 (7.4)	5	11	8	6	8	5	1
Tissue (Pleura)	7 (1.2)	0	0	0	2	2	3	0
Tissue (NOS)	37 (6.2)	3	10	8	9	2	2	3
Urine	17 (2.8)	4	3	5	1	1	2	1
Total	597	125	106	90	82	74	63	57
Percentage %		20.9	17.7	15.0	13.7	12.3	10.5	9.5

Table 3.3 Sample tested by patient type based on previous history of ATT

Type of Specimen	# tested	Never treated No (%)	H/o of previous treatment No (%)	Not known No (%)
Fluid (CSF)	116	95 (81.8)	14 (12.0)	7 (6.0)
Fluid (Pleural)	192	146 (76.0)	40 (20.8)	6 (3.1)
Fluid (Ascitic)	48	41 (85.4)	5 (10.4)	2 (4.2)
Fluid (Synovial)	5	4 (80.0)	1 (20.0)	0 (0.0)
Fluid (NOS)	9	5 (55.6)	3 (33.3)	1 (11.1)
Pus (NOS)	87	52 (59.8)	27 (31.0)	8 (9.2)
FNA (Lymph node)	17	13 (76.5)	4 (23.5)	0 (0.0)
FNA (NOS)	18	15 (83.3)	2 (11.1)	1(5.6)
Tissue (Lymph Node)	44	20 (45.5)	15 (34.1)	9 (20.5)
Tissue (Pleura)	7	5 (71.4)	2 (28.6)	0 (0.0)
Tissue (NOS)	37	22 (59.5)	5 (13.5)	10 (27.0)
Urine	17	12 (70.6)	3 (17.6)	2 (11.8)
Total	597	430 (72.0)	121 (20.3)	46 (7.7)

3.3 Diagnostic Test Performed and Results Validity

Validity of results was examined for each diagnostic test by sample type. Out of 597, 591 (98.9%) samples were tested by Smear microscopy with 100% result validity among the tested number and 98.9% out of the total number of samples. Gene Xpert MTB/ Rif assay was performed on 564 samples (94.4%) out of which valid results were obtained from 561 samples representing 93.9% of the total received samples and 99.4% of tested number. 586 (98.1%) specimens were cultured and valid result was observed in 546 sample accounting 91.4% of tbe total samples received and 93.1% of the total specimen cultured. The proportion of invalid result was 3/564 and 40/586 for Gene Xpert Assay and culture respectively (Table 3.4)

Table 3.4 Summary of diagnostic test and valid results using AFB Smear, Xpert/MTB Rif Assay & culture

Type of specimen	# tested	%	AFB smear		Xpert MTB/Rif assay		Culture	
			# tested	# valid result	# tested	# valid result	# tested	# valid result
Fluid (CSF)	116	19.4	114	114	115	115	110	98
Fluid (Pleural)	192	32.2	191	191	188	188	191	181
Fluid (Ascitic)	48	8.0	48	48	48	48	47	45
Fluid (Synovial)	5	0.8	5	5	5	5	5	5
Fluid (NOS)	9	1.5	9	9	9	9	9	9
Pus (NOS)	87	14.6	85	85	78	77	86	84
FNA (Lymph node)	17	2.8	17	17	17	17	17	15
FNA (NOS)	18	3.0	18	18	18	18	18	17
Tissue (Lymph Node)	44	7.4	44	44	37	36	44	40
Tissue (Pleura)	7	1.2	7	7	7	7	7	6
Tissue (NOS)	37	6.2	36	36	26	26	35	30
Urine	17	2.8	17	17	16	15	17	16
Total	597	100.0	591	591	564	561	586	546
% among # tested			100	100	100	99.4	100	93.1%
% among Total	100		98.9	98.9	94.4	93.9	98.1	91.4

3.4 Diagnostic Categories for Extrapulmonary Tuberculosis

Patients were categorized as definite, probable and non TB cases on the basis of interpreted results from different diagnostic test used i.e. Smear, Culture and Gene Xpert MTB/Rif assay. (Table 3.5)

3.4.1 Definite TB case

In our studies TB cases that were positive on all three diagnostic tests or either found to be positive on Gene Xpert or cultures were all referred as definite TB cases. 44 cases were detected as positive on all three tests. 48 cases were positive on both Smear and Xpert with culture being either negative, not done or contaminated. 24 cases were only Xpert positive with smear result negative and culture either negative contaminated or not done. 22 cases were positive only on culture with Xpert results either negative or not done and smear result interpreted as negative. 9 cases were positive on both Xpert and culture but showed negative result on smear. 3 cases were both smear and culture positive with Xpert result read as either negative or not done.

3.4.2 Probable TB cases

7 cases that were positive on Smear but interpreted as negative or not done on culture and Gene Xpert were classified as probable TB cases.

3.4.3 Non TB Case

369 negative results on all three diagnostic tests were non TB cases. Similarly cases with negative or not done smear result along with negative, NTM, not done or contaminated result on culture and Gene Xpert being negative or not done were also non TB cases. There were 64 such cases.

3.5 EPTB Diagnosis by Specimen Type

Out of 597 received samples 591 were tested on smear microscopy. Gene Xpert was performed on 564 samples. There were 586 samples cultured out of which 329 were tested both on solid and liquid culture while 257 were only cultured on LJ due to shortage of MGIT tubes. Taking into consideration the individual performance of each test Xpert detected 126 (21.1%) positive cases, Smear was found to be positive for 107 cases (17.4%) while combined performance of liquid and solid culture was 13.0% detecting 78 positive cases. For samples such as CSF culture was found more efficient by detecting 12 (10.3%) cases compared with Gene Xpert and smear microscopy which recover 8 (6.8%) and 4 (3.4%) cases respectively whereas for samples such as pus smear microscopy and Xpert were found more efficient detecting 51 (58.6%) and 49 (56.3%) cases respectively compared with culture detecting only 22 (25.2%) positive cases (Table 3.6).

Table 3.5 Case definition for TB cases

Smear	Xpert	Culture	# of Cases	Case definition
+	+	+	44	Definite TB case
+	+	NEG	45	Definite TB case
+	+	C/CND	3	Definite TB case
NEG	+	NEG	22	Definite TB case
NEG	+	C/CND	2	Definite TB case
NEG	NEG	+	18	Definite TB case
NEG	+	+	9	Definite TB case
+	NEG	+	1	Definite TB case
+	Not Done	+	2	Definite TB case
NEG	Not Done	+	4	Definite TB case
+	Not Done	NEG	6	Probable TB case
+	NEG	NEG	1	Probable TB case
+	NEG	NTM	1	Non TB case
NEG	NEG	NTM	1	Non TB case
NEG	NEG	NEG	369	Non TB case
NEG	NEG	C/CND	39	Non TB case
NEG	Not Done	NEG	17	Non TB case
Not Done	NEG	NEG	2	Non TB case
Not Done	NEG	Not Done	3	Non TB case

3.6 Gene Xpert MTB/ Rif Assay Performance

Out of 597 enrolled cases 564 (94.4%) were tested using Gene Xpert. Due to error results were not obtained in 3 cases (0.5%). Out of 564 tested specimen Mycobacterium tuberculosis was detected in 126 cases (22.3%) and 438 (77.6%) sample were found to be Xpert negative. Out of 126 positive cases 16 (12.6%) were found to be Rifampicin resistance and in 4 (3.1%) cases Mtb was detected but Rifampicin result was indeterminate. Mycobacterium tuberculosis was detected with valid Rifampicin result in 122 cases (96.8%). As for the incidence by specimen the highest number of positive cases were of pus accounting 49 cases (62.8%) followed by lymph node in 18 cases (48.6%), FNA from non specific site in 8 cases (44.4%), pleural tissue in 3 cases (42.9%), tissue from non specific site in 9 cases (34.6%), FNA from lymph node in 5 cases (29.4%), fluid from non specific site in 2 cases (22.2%), synovial fluid in 1 case (20%), pleural fluid in 19 cases (10.1%), CSF in 8 cases (7%), ascitic fluid in 3 cases (6.3) and urine in 1 case (6.3). Out of 16 Rifampicin positive cases pleural tissue accounts highest percentage of 67% with 2 cases out of total 7 tested samples followed by tissue NOS with 3 cases (33%), pleural fluid with 4 cases (21%), FNA lymph node with 1 case (20%) and lymph node tissue with 3 cases (17%). Table 3.7

3.6.1 Gene Xpert Quantified Results

Out of 126 positive specimens most of the results are categorized as low (n=47; 37.3%) or very low (n=40; 31.7%). 34 (26.9%) were detected medium while very small number 5 (3.9%) out of 126 reported high bacterial load.

3.6.2 EPTB Cases Diagnosed on Gene Xpert in Reference to Sex and Age Group

Out of 564 specimens tested on Gene Xpert 307 specimens were of males and 257 samples belongs to females with predominant age group of 0-14 year in both cases i.e. 65 and 59 specimen of female and male respectively. Positivity rate in case of females was observed more 64 cases (24.9%) out 257 tested samples as compared to male with 62 (20.1%) positive cases of Mtb out of 307 tested specimen. It was observed that females with age between 34 and 44 were more affected with 9 positive cases out of 19 accounting 47.3% while for male the more targeted age group was 15 to 24 year of age with 14 (30.4%) positive cases out of 46. Least effected female were with ages more than 65 years i.e. no positive case out of 22 tested specimens similarly in case of males least positive cases were observed in age group between 55 and 64 with 2 (6.8%) positive cases out of 29 tested samples.In our studies overall among both male and female patients with ages between 15 and 24 were more prone to the disease with positivity rate of 38.9% i.e. 37 positive cases out 95 tested specimen while least affected age group was 55 to 64 with 3 (4.8%) positive cases out of 62. (Table 3.8)

Table 3.7 EPTB specimen tested and Gene Xpert MTB/Rif Assay result

Type of specimen	# tested	Percentage	# MTB +	% positive	# RR	%RR
Fluid (CSF)	115	20.3	8	7.0	0	0
Fluid (Pleural)	188	33.3	19	10.1	4	21
Fluid (Ascitic)	48	8.5	3	6.3	0	0
Fluid (Synovial)	5	0.8	1	20.0	0	0
Fluid (NOS)	9	1.5	2	22.2	0	0
Pus (NOS)	78	13.8	49	62.8	3	6
FNA (Lymph node)	17	3.0	5	29.4	1	20
FNA (NOS)	18	3.1	8	44.4	0	0
Tissue (Lymph Node)	37	6.5	18	48.6	3	17
Tissue (Pleura)	7	1.2	3	42.9	2	67
Tissue (NOS)	26	4.6	9	34.6	3	33
Urine	16	2.8	1	6.3	0	0
Total	564	100.0	126	22.3	16	12.6

3.7 Diagnostic Yield of Smear, Gene Xpert and Culture

All three diagnostic tests performed with valid interpreted result i.e. positive or negative in case of smear, not detected or MTB in case of Gene Xpert and positive, negative or NTM in case of culture were observed in 512 specimens out of 597 received samples. Out of 512 specimens smear microscopy detected 93 (18.1%) specimens as positive. 121 (23.6) specimens were positive on Gene Xpert while 72 (14.0) specimens were culture positive. For pus samples Xpert show the highest recovery rate of 65.7% by detecting 48 positive cases out of 73 compared with culture and smear microscopy which detected 21(28.7%) and 45 (61.4%) cases respectively. Gene Xpert detected 19 (10.6%) pleural fluid samples as positive while only 12 (6.7) were found positive on smear and 15 (8.3) on culture showing the higher sensitivity of Gene Xpert Assay for these samples. Gene Xpert was found more sensitive for lymph node specimens detecting MTB in18 (56.2%) cases out of 32 while smear was positive for only 12 (37.5%) cases and only 11 (34.3%) cases were culture positive. Sensitivity of Xpert, Smear microscopy and Culture for specimen such as synovial fluid, fluid from non specific site, lymph node aspirate, and urine was similar with recovery rate of 20%, 22.2%, 26.6% and 7.1% respectively. Efficiency of Xpert was observed more for specimens such as Ascitic fluids (6.6%), Fine needle aspirate (47%), pleural tissue (33.3%), and Tissue NOS (35%) compared with smear microscopy and culture. Recovery rate for CSF samples by culture were found to be higher i.e. 12.3% compared with Gene Xpert detecting 8 (8.2%) out of 97 specimens while Smear microscopy detect only 4 cases (4.1%). (Table 3.9)

Table 3.9 Diagnostic yield of Smear, Gene Xpert and Culture

Type of specimen	# Specimen tested (all three)	Smear + (%)	Xpert + (%)	Culture + (%)
Fluid (CSF)	97	4 (4.1)	8 (8.2)	12 (12.3)
Fluid (Pleural)	179	12 (6.7)	19 (10.6)	15 (8.3)
Fluid (Ascitic)	45	2 (4.4)	3 (6.6)	1 (2.2)
Fluid (Synovial)	5	1 (20)	1 (20)	1 (20)
Fluid (NOS)	9	2 (22.2)	2 (22.2)	2 (22.2)
Pus (NOS)	73	45 (61.4)	48 (65.7)	21 (28.7)
FNA (Lymph node)	15	4 (26.6)	4 (26.6)	4 (26.6)
FNA (NOS)	17	5 (29.4)	8 (47)	2 (11.7)
Tissue (Lymph Node)	32	12 (37.5)	18 (56.2)	11 (34.3)
Tissue (Pleura)	6	1 (16.6)	2 (33.3)	1 (16.6)
Tissue (NOS)	20	4 (20)	7 (35)	2 (10)
Urine	14	1 (7.1)	1 (7.1)	1 (7.1)
Total	512	93 (18.1)	121 (23.6)	72 (14.0)

3.8 Gene Xpert Versus Smear Microscopy

Comparison of results of Gene Xpert with fluorescence smear microscopy indicates that out of 597 specimens 93 cases were found positive on both Gene Xpert and smear microscopy. Gene Xpert picks additional 33 positive cases that yield negative result on Smear microscopy (Table 3.10.2). Out of these 33 cases 22 were also positive on culture. There were also 3 cases (1 pleural fluid and two pus specimens) that were positive on smear but were interpreted as negative on Gene Xpert. (Table 3.10.1) Comparing these 3 cases with culture results indicate that 1 case was found negative on culture as well while other 2 were indicated as NTM on culture after confirmation by ICT. (Table 3.10)

3.8.1 Sensitivity and Specificity

Sensitivity of Gene Xpert was found to be 96.8% compared with smear microscopy with calculated sensitivity of 73.8% while specificity of Gene Xpert was 92.8% while for smear microscopy it was 99.3% (Table 3.10.3)

Table 3.10 Comparison of Gene Xpert result with Smear result (Summary)

	Xpert MTB Result				
Smear Result	**MTB**	**Not Detected**	**Error**	**Not Done**	**Total**
Positive	93	3	0	8	104 (17.4%)
Negative	33	427	3	24	487 (81.6%)
Not done	0	5	0	1	6 (1.0%)
Total	126 (21.1%)	435 (72.9%)	3 (0.5%)	33(5.5%)	597(100.0%)

Table 3.10.1 Comparison of Gene Xpert result with smear positive result

	Xpert MTB Result				
Type of specimen	**MTB**	**Not Detected**	**Error**	**Not Done**	**Total**
Fluid (C5F)	4	0	0	0	4
Fluid (Pleural)	11	1	0	0	12
Fluid (Ascitic)	2	0	0	0	2
Fluid (Synovial)	1	0	0	0	1
Fluid (NOS)	2	0	0	0	2
Pus (NOS)	44	2	0	5	51
FNA (Lymph node)	4	0	0	0	4
FNA (NOS)	5	0	0	0	5
Tissue (Lymph Node)	12	0	0	3	15
Tissue (Pleura)	1	0	0	0	1
Tissue (NOS)	6	0	0	0	6
Urine	1	0	0	0	1
Total	93	3	0	8	104

Table 3.10.2 Comparison of Gene Xpert with smear Negative results

	Xpert MTB Result				
Type of Specimen	**MTB**	**Not Detected**	**Error**	**Not Done**	**Total**
Fluid (CSF)	4	105	0	1	110
Fluid (Pleural)	8	168	0	3	179
Fluid (Ascitic)	1	45	0	0	46
Fluid (Synovial)	0	4	0	0	4
Fluid (NOS)	0	7	0	0	7
Pus (NOS)	5	24	1	4	34
FNA (Lymph node)	1	12	0	0	13
FNA (NOS)	3	10	0	0	13
Tissue (Lymph Node)	6	18	1	4	29
Tissue (Pleura)	2	4	0	0	6
Tissue (NOS)	3	16	0	11	30
Urine	0	14	1	1	16
Total	33	427	3	24	487

Table 3.10.1 Sensitivity and Specificity of Gene Xpert VS Smear

	Gene Xpert	Smear
Sensitivity	96.8 %	73.8 %
Specificity	92.8 %	99.3

3.10 Comparing Gene Xpert with Culture in Smear Positive Cases

Out of 104 smear positive cases MTB was detected in 44 cases on both Gene Xpert and culture. There was only 1 case missed by Gene Xpert that was positive on both culture and smear. This case was classified as new case of TB. There were such 45 cases with negative culture result but positive on both smear and Xpert, of which 22 were new cases, 21 were with previous TB history and 2 were unclassified. (Table 3.12.1)

3.11 Comparing Gene Xpert with Culture in Smear Negative Cases

369 cases out of 597 received specimens were found negative on all three diagnostic tests. Comparing Gene Xpert result with culture in smear negative cases revealed that Gene Xpert pick additional 22 cases which were found negative on both culture and smear depicting its usefulness in diagnosis of smear negative TB cases. Out of these 22 cases 17 patients were new TB cases 4 with previous TB history and one unclassified patient. There were such 18 cases recovered by culture which were negative on both smear and Gene Xpert. History of ATT of these 18 cases revealed 1 never treated and 17 previously treated patients. (Table 3.12.2)

Table 3.12.1 Comparison of Gene Xpert and Culture results with Smear Positive results

Smear Positive

	Culture					
Xpert	**MTB**	**NEG**	**CONT**	**NTM**	**CND**	**Total**
MTB	44	45	2	1	1	93
Not Detected	1	1	0	1	0	3
Not Done	2	6	0	0	0	8
Total	47	52	2	2	1	104

Table 3.12.2 Comparison of Gene Xpert and culture results with Smear Negative results

Smear Negative

	Culture					
Xpert	**MTB**	**NEG**	**CONT**	**NTM**	**CND**	**Total**
MTB	9	22	2	0	0	33
Not Detected	18	369	34	1	5	427
Error	0	3	0	0	0	3
Not Done	4	17	2	0	1	24
Total	31	411	38	1	6	487

3.12 Comparison of Rifampicin Results on Xpert Vs Phenotypic DST

Among all enrolled patients Xpert detected rifampicin in 16 cases (5 previously treated, 10 never treated and one unclassified) while phenotypic DST was positive for 10 cases (3 previously treated, 4 never treated, and 3 unclassified). Rifampicin results for both tests were available for 47 cases. 6 cases were found resistant on both phenotypic and genotypic DST including 1 pleural fluid, 3 pus samples, 1 lymph node tissue and 1 pleural tissue.Out of 16 Rifampicin positive cases on gene Xpert for10 cases DST was not performed because of negative culture results. Out of 10 rifampicin resistant cases resulted positive on phenotypic DST for 4 cases Xpert result was not available. There was one case that was RIF negative on Gene Xpert but was found NTM on phenotypic DST. We do not find any case resistant on Gene Xpert and susceptible on phenotypic DST and vice versa (Table 3.13).

Table 3.13 Comparison of Rifampicin results on Xpert Vs Phenotypic DST

	DST (MGIT + LJ)				
Gene Xpert	**Rif resistant**	**Rif susceptible**	**Not done**	**NTM**	**Total**
Rif resistant	6*	0	10*	0	16
Rif susceptible	0	40	65	1	106
Not Done	4	15	452	0	471
Indeterminate	0	0	4	0	4
Total	10	55	531	1	597

***EPTB specimen Rifampicin resistant on both Phenotypic and genotypic DST**

Type of Specimen	Rif resistance (genotypic & phenotypic DST)
Fluid (Pleural)	1
PUS (NOS)	3
Tissue (Lymph Node)	1
Tissue (Pleura)	1
Total	6

3.13 Prevalence of MDR in EPTB Specimen

Phenotypic DST for rifampicin and isoniazid resistance was conducted on 66 samples.Resistance to rifampicin was observed in 10 cases (15.1%)including 1 pleural fluid sample, 3 pus, 4 lymph node tissues, 1 pleural tissue and 1 tissue NOS. Highest rifampicin resistance of 40% was observed in lymph node specimens followed by pus (30%) and 10% for pleural fluid, pleural tissue and tissue from non specific site. Resistance to Isoniazid was observed in 14 cases (21.2%) with tissue lymph node accounting highest number of resistance i.e. 35.71% (n=5) followed by pus sample 21.4% (n=3), 14.2% (n=2) for pleural fluid and tissue NOS and 7.1% (n=1) for CSF and pleural tissue. While MDR TB was reported in 13.6% cases (n=9) with lymph node TB being the predominant type with 4 cases (44.4%) out of 9 positive cases. We also found out of 10 cases which show resistance to Rifampicin 9 were also resistance to isoniazid excluding one pus sample which show mono resistance to rifampicin but susceptible to isoniazid. Similarly mono resistance to isoniazid was observed in 5 cases which were found susceptible to rifampicin. (Table 3.14)

3.14 Prevalence of Ofloxacin Resistance in EPTB Specimen

Taking into account Phenotypic DST results conducted on 65 samples Resistance to ofloxacin in rifampicin susceptible cases was found to be 9.2% while ofloxacin resistance in rifampicin resistance cases was found to be 40%.These 4 cases show additional INH resistance as well (Table 3.15)

Table 3.14 Prevalence of MDR in EPTB Specimen

Type of specimen	DST available (R+INH)	Rifampicin resitance (%)	Isoniazid resistance (%)	MDR (%)
Fluid (CSF)	10	0 (0.0)	1 (7.1)	0 (0.0)
Fluid (Pleural)	12	1 (10)	2 (14.2)	1 (11.1)
Fluid (Ascitic)	0	0 (0.0)	0 (0.0)	0 (0.0)
Fluid (Synovial)	0	0 (0.0)	0 (0.0)	0 (0.0)
Fluid (NOS)	1	0 (0.0)	0 (0.0)	0 (0.0)
Pus (NOS)	19	3 (30)	3 (21.4)	2 (22.2)
FNA (Lymph node)	3	0 (0.0)	0 (0.0)	0 (0.0)
FNA (NOS)	2	0 (0.0)	0 (0.0)	0 (0.0)
Tissue (Lymph Node)	12	4 (40)	5 (35.7)	4 (44.4)
Tissue (Pleura)	1	1 (10)	1 (7.1)	1(11.1)
Tissue (NOS)	5	1 (10)	2 (14.2)	1 (11.1)
Urine	1	0 (0.0)	0 (0.0)	0 (0.0)
Total	66	10 (15.1%)	14 (21.2%)	9 (13.6%)

Table 3.15 Prevalence of Ofloxacin resistance in EPTB specimen

Type of Specimen	# DST (R +O)	Rifampicin susceptible (n=54)		Rifampicin resistance (n=10)	
		OfloxacinSusceptible	Ofloxacin Resistant	Ofloxacin Susceptible	Ofloxacin Resistant
Fluid (CSF)	10	10	0	0	0
Fluid (Pleural)	11	7	2	1	1
Fluid (Ascitic)	0	0	0	0	0
Fluid (Synovial)	0	0	0	0	0
Fluid (NOS)	1	1	0	0	0
Pus (NOS)	19	14	2	3	1
FNA (Lymph node)	3	3	0	0	0
FNA (NOS)	2	2	0	0	0
Tissue (Lymph Node)	12	7	1	2	2
Tissue (Pleura)	1	0	0	0	0
Tissue (NOS)	5	4	0	0	0
Urine	1	1	0	0	0
Total	65	49 (90.7%)	5 (9.2%)	6 (60.0%)	4 (40.0%)

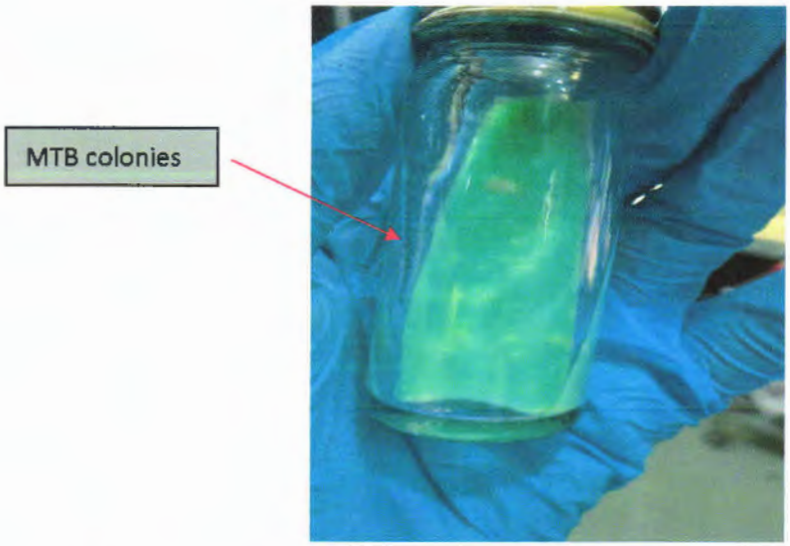


Fig 3.1 Mycobacterium Tuberculosis colonies on Lowenstein–Jensen Media

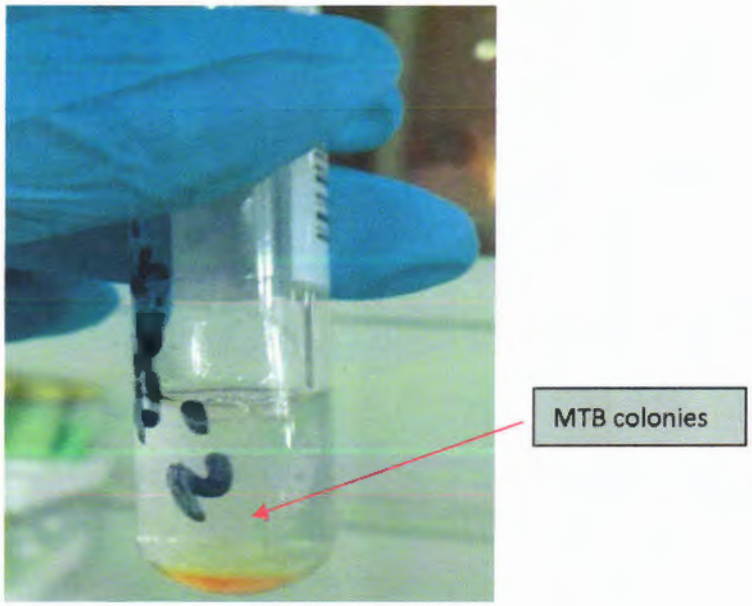


Fig 3.2 MGIT 7ml containing suspended MTB colonies

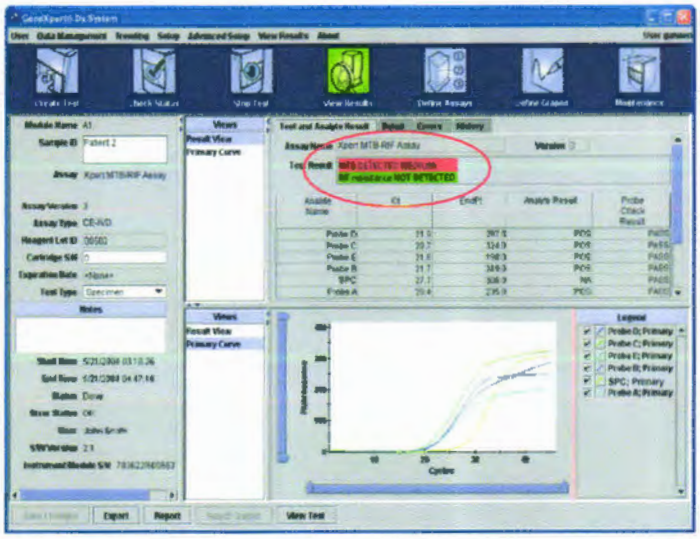
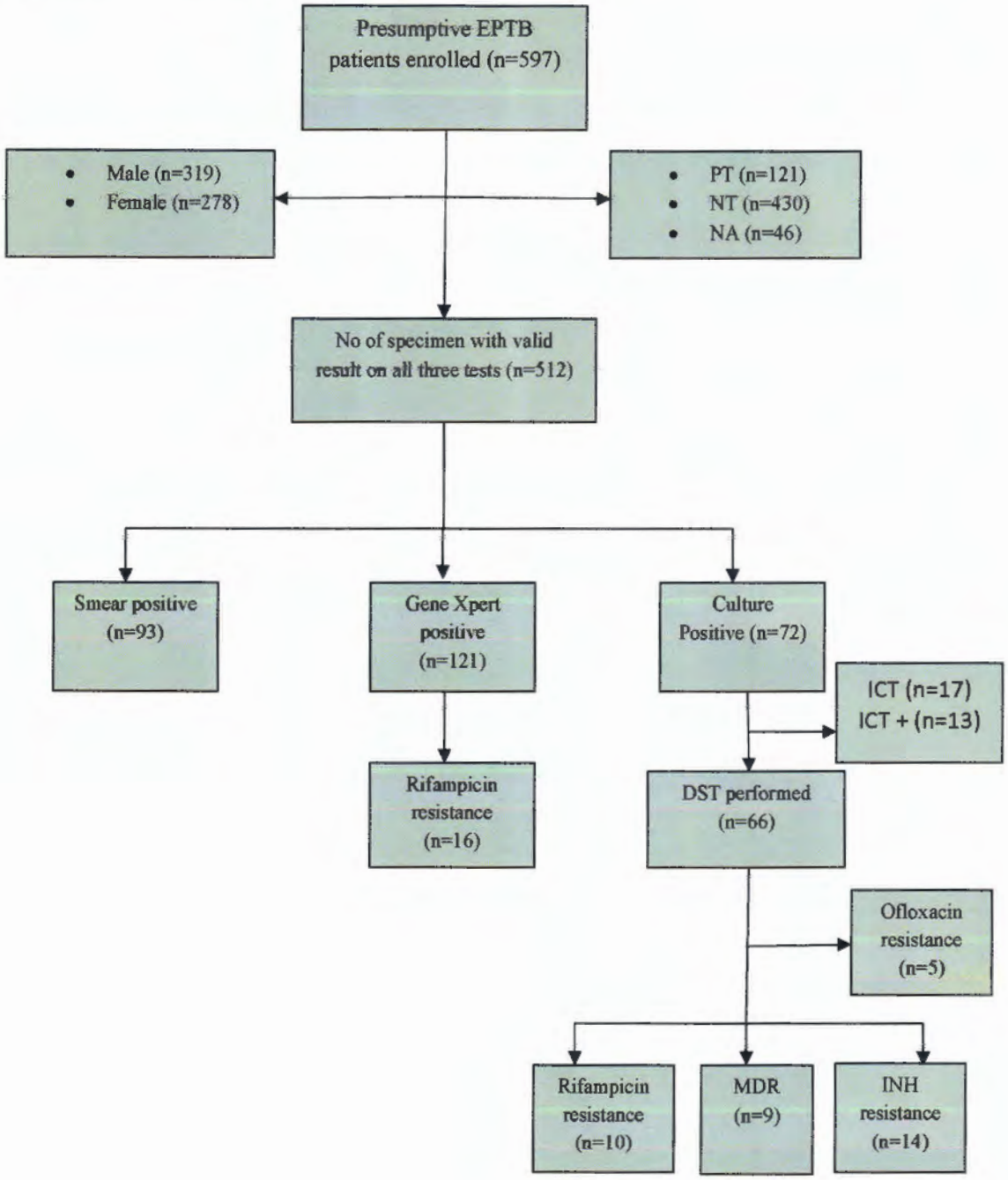


Fig 3.3 Gene Xpert software indicating MTB and Rifampicin status

Fig 3.4 Immunochromatographic device indicating positive and negative results

Fig 3.5 Summary of enrolled cases, Positive results of Smear, Gene Xpert and Culture. DST results for antituberculosis drugs.



Chapter 4
Discussion

4.0 DISCUSSION

Confirmation of extrapulmonary form of tuberculosis is always difficult due to variety of non specific manifestations presented by this type, Specimen's low bacillary load, Trouble in getting additional sample for verification, low performance of nucleic acid based technique due to presence of inhibitors in the specimen that pose hindrance and inadequacy of universally applicable sample processing procedures for all EPTB specimens (Solovic *et al.*,2013; Chakravorty *et al.*, 2005).

Traditional methods for tuberculosis diagnosis like microscopy and culture require large bacillary burden to end up positive. Additionally usual cultures demand more sophisticated laboratory setup and more turnaround time in comparison with molecular methods with proven high sensitivity and rapid diagnostic yield. Recently various PCR based methods using diverse MTB targets have been illustrated. Gene Xpert is one of them which demand minimal technical skills, has convenience of using unprocessed specimen and involves 2 hour TB diagnosis along with evaluation of RIF resistance status (Hillemann *et al.*, 2011;Iram *et al.*, 2015). In the present study, we have assessed the analytical accuracy of Xpert MTB/RIF test both for EPTB cases and in contrast with usual diagnostic methods.

4.1 Diagnostic Efficacy of Gene Xpert for Extrapulmonary specimens

Taking into account the individual performance of Gene Xpert in our study out of 564 tested specimen Mycobacterium tuberculosis was confirmed in 126 cases (22.3%) and 438 (77.6%) sample were negative for MTB on Xpert. Out of 126 positive cases 16 (12.6%) were found to be Rifampicin resistance and in 4 (3.1%) cases MTB was detected but

Rifampicin result was indeterminate. Iram *et al*, in 2015 through their study show recovery rate of 22.5% by Gene Xpert for EPTB samples which is almost equal to ours.

Estimated bacterial load in Gene Xpert positive specimen was indicated as low in 37.3% (47/126) specimens. Very low in 31.7% (40/126) sample, medium in 26.9% (34/126) cases and high in 3.9% (5/126) specimen indicating the paucibacillary nature of the extrapulmonary specimens. Nhu *et al* in 2014 also indicate almost similar qualitative estimation of bacterial load with most of positive samples with low bacterial burden i.e. very low (49.5%), low (42.2%), medium (8.3%).

Various European studies suggest more susceptibility of young females and older males for tuberculosis infection (Crampin *et al.*, 2014). Our findings using Gene Xpert support this fact. Positivity rate in younger female was observed more than male. Female in age group between ages 0 and 14 and 15-24 were more prone to the disease with 18.4% and 46.9% positive cases respectively compared with male of same ages with positivity rate of 10.1% and 30.4%. Considering Gene Xpert results for older age groups we concluded males with age more than 65 were more vulnerable to disease with 11.7% positive cases in comparison with females where we did not find any positive case. . A study conducted by Sreeramareddy *et al*, in 2008 also refers to the more susceptibility of younger females for EPTB similarly Webster & Shandera in 2014 through their studies show greater EPTB infection in females under age of 45.

4.2. Gene Xpert Performance in Comparison with Culture and Smear

Out of 512 tested specimens MTB were detected by Gene Xpert for 23.6% cases compared to culture and fluorescence microscopy with positivity rate of 14% and 18.1%

respectively indicating comparable efficacy of Gene Xpert for EPTB samples in our study. Here Gene Xpert could detect 9.6% & 5.5% more positive cases compared to culture and FM microscopy respectively.

Various studies report considerable capacity of Xpert MTB/RIF test for non sterile fluids like pus, lymph nodes and urine and slightly low sensitivity for sterile specimen. Relating this statement with our results we find good diagnostic potential with good sensitivity for non sterile specimen like pus and lymph node and comparatively low sensitivity of Xpert for sterile specimens like CSF. But against this statement our yield of Xpert was better when compared with culture and FM microscopy in pleural fluids which are assumed to be sterile.

Supporting our results of Gene Xpert for pus sample with high detection rate of 65.7% compared to culture (28.7%) and FM smear microscopy (61.4%) Iram *et al* in 2015, Moure et al in 2012 and Srwar *et al* in 2014 also reported highest yield of positivity for pus samples. Our Xpert results for lymph node specimens were also better with recovery rate of 56.2% than culture (34.3%) and smear (37.5%). A published work by Ghariani *et al* during the year 2015 also report similar results like ours.

Gene Xpert detected 19 (10.6%) pleural fluid samples as positive while only 12 (6.7%) were found positive on smear and 15 (8.3%) on culture showing the higher sensitivity of Gene Xpert Assay for these samples. Our results were better than comparative analysis conducted by Moure et al in 2012 and similar work by Rufai *et al* during 2015 which show low sensitivity of Gene Xpert for pleural fluids compared to conventional techniques. For CSF we obtain better recovery (12.3%) by culture method which was 4.1% and 8.2% better than Gene Xpert and Smear results respectively indicating that low yield specimens narrow down the diagnostic potential of the Xpert assay (Moure *et al.*, 2012). High detection

accuracy value by Xpert compared to culture than our for CSF with sensitivity >85% were reported by study carried out by Tortoli *et al.*

Comparing Gene Xpert results in smear positive cases with culture our results indicated that Xpert missed only one case which was positive on culture while culture was negative for 45 cases which were positive on both other techniques. Similarly comparing Gene Xpert results with culture in smear negative cases we found 18 cases were missed by Gene Xpert while 22 cases remain undiagnosed by culture. Lower efficiency in detection of these 19 cases by Gene Xpert in this scenario could be explained by low bacterial load contained in EPTB specimens (Moure *et al.*, 2012). Reduction in detection of Xpert can be correlated with culture grades which were found to be scanty for 15 out of 19 cases. Similarly inability of culture to detect 67 cases shown positive by Gene Xpert can be explained by the low bacillary load in extrapulmonary specimen, clumbs formation and uneven arrangement of bacilli. Additionally there is more posibility of killing of viable bacilli during NALC-NaOH decontamination step during culture processing (Iram *et al.*, 2015). Correlating these culture negative cases with Gene Xpert results we found most of cases 45 out of 67 were detected low and very low. Only 3 cases were detected high. Another reason for culture negative can be the previous medical history of the patients. Out of 67 culture negative cases 25 were previously treated patients which could be indicated as false positive by Gene Xpert Because of inability of assay to differentiate among mycobacterium tuberculosis which are dormant, viable and non viable bacilli shed in response to use of antituberculosis drugs used during patient treatment (Friedrich *et al.*, 2013).

4.3 Sensitivity and Specifieity of Gene Xpert in Comparison with FM smear and Culture

The lower limit of detection for Gene Xpert assay is 131 CFU/ml and analytical sensitivity of 5 genome copies (Weyer *et al.*, 2013). The test is highly specific for DNA from intact mycobacterium tuberculosis as washing step eliminates contamination from free DNA.

Our findings include high sensitivity of Gene Xpert in comparison with FM smear microscopy but low specificity. The sensitivity of Gene Xpert and FM smear microscopy were 96.8% and 73.8% respectively while specificity was 99.3% for FM smear microscopy and 92.8% for Gene Xpert. High sensitivity of Gene Xpert compared to FM microscopy has been reported by other studies as well. Tortoli *et al* reported 48% sensitivity of FM microscopy when compared with Gene Xpert. Findings of Alvarez-Uria *et al* also include better performance of Gene Xpert than Fluorescent microscopy. Results of study conducted in Kenya by Kiptoo *et al* in 2014 also indicate high sensitivity of Xpert 83.7% compared to Fluorescent microscopy (68.3%) and specificity of 87.9% lower than Fluorescent mieroscopy (93.3%).

In our study sensitivity of culture was even lower than FM microscopy when compared with Gene Xpert. We calculated lower sensitivity of culture 44% compared to Gene Xpert which was found to be 74%. Specificity of culture was found 95.1% which was higher than Gene Xpert which show 85% specificity in our research work. Against our finding various study conclude from their studies high sensitivity and specificity of Gene Xpert system than culture. Findings of Hillemann *et al* include high sensitivity 77.3% and high specificity as well against culture as a reference standard. Similarly in other published work Vadwai *et al* reported high sensitivity of 81% for Gene Xpert and high specificity of 99.6% compared to culture used as reference standard. High detection rate in above mentioned study was due to that the fact that study was conducted on diagnosed cases of TB

while we consider TB suspects in our research. Low specificity of Xpert assay like in our case has been also reported in many studies. Ghariani *et al* reported sensitivity of 94.9% by Gene Xpert for culture positive cases and specificity of 37.9%. Iram *et al* in 2015 also reported high sensitivity (100%) low specificity (86%) of Xpert assay for extrapulmonary specimen when culture was used as a reference standard.

Referring to our results regarding low specificity of Xpert assay we can conclude using reference standard with low sensitivity might result into lower specificity of the test (Ghariani *et al*., 2015). For diagnosis of EPTB, culture and smear microscopy are imperfect choices to be used as reference standard due to paucibacillary nature of specimens. Assuming to correct identification of TB by Xpert in a sample with a negative culture the interpreted results will be false positive that would result into underrated Xpert's actual specificity (Denkinger *et al*., 2014). So we can conclude absence of an inappropriate reference standard might have distorted the true specificity of Xpert test (Friedrich *et al*., 2013).

4.4 Comparison of Rifampicin Results on Xpert Vs Phenotypic DST

Outcomes of first national anti-tuberculosis drug resistance survey in Pakistan suggest high sensitivity but low specificity of phenotypic DST than that of Xpert (Tahseen *et al*.,2016). Xpert has been reported as having 100% sensitivity in detecting RMP resistance (Carriquiry *et al*., 2012). But in our studies there was no difference observed between phenotypic and genotypic confirmation as we do not find any case resistant on Gene Xpert and susceptible on phenotypic DST and vice versa. One NTM reported by phenotypic DST was negative on Gene Xpert showing high specificity of Gene Xpert result in our study. Because of small size of specimen tested for rifampicin resistance, performance of Xpert assay for RIF-resistance detection compared to phenotypic DST cannot be assessed.

4.5 Prevalence of MDR and Ofloxacin resistance in EPTB Specimen

Substantial proportion of strain showing resistance to rifampicin are mostly resistance to INH as well indicating that resistance to this first line drug (rifampicin) is referred to as surrogate for MDR-TB (Coovadia *et al.*, 2013). Our finding supports this fact as 9 out of 10 rifampicin resistance cases were also INH resistance. MDR was observed in 13.6% cases that was almost similar to one reported by Maurya *et al* 13.4% for extrapulmonary specimen. Out of 9 MDR patients 7 were male while 2 were female while Ullah *et al* reported high proportion of MDR in female gender. This difference is due to small sample size in our case. Various studies show patients with previous history of drugs are more prone to MDR TB (Maurya *et al.*, 2012) but in our studies Multi-drug resistant (MDR) EPTB was detected in both new and retreatment patientsas 3 cases were new patients while 3 were previously treated patient and 3 with unclear medical history.

Ofloxacin is used for treatment of MDR-TB which is a powerful fluoroquinolone (Verma *et al.*, 2011). A study conducted in China during 2009 correlate fluoroquinolone resistance association with resistance to at least one first-line drug (Xu *et al.*, 2009). In our study Ofloxacin mono resistance was 9.2 % in general and 40% among MDR isolates while Daniel et al reported Fluoroquinolone resistance of 7.5% in general and 11.8% among MDR-TB isolates.

4.6 Conclusion

We conclude that rapid detection of extrapulmonary tuberçulosis could significantly improved by proper execution of Gene Xpert MTB/RIF assay. This instrument has influential utility for identification of MTB in smear negative and culture negative cases as it has

outperformed FM microscopy by 5.6% and culture by 9:6% in our study. Our study demonstrated good sensitivity but low specificity of Xpert assay which indicate using reliable reference standard with high sensitivity is very important to access the actual diagnostic performance of test.

Various features like simplicity, safe use, automation, high sensitivity for the detection of bacilli DNA and drug resistance make this test appealing tool for EPTB diagnosis specially when techniques like culture and smear require more turnaround time and rely on high bacillary load to become positive. In resource-constrained settings this platform is exceptionally an optimistic approach for close to patient TB diagnosis.

4.7 Future Prospective

This assay has revolutionize the diagnosis of tuberculosis thus we hope in future rapid diagnosis and targeted treatment will contribute to terminate tuberculosis epidemic. Time has reached to look beyond evaluation of Xpert's accuracy alone rather analyze actual contribution of the test in decision making and advancement in patient's outcome. There is a need to assess contribution of Xpert in early diagnosis of extrapulmonary tuberculosis and initiation of suitable treatment and thus ultimately results in decreased mortality and morbidity. This advancement in field of TB diagnosis will help to identify ideal implementation approaches for new tools.

Chapter 5
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