RISK 6 Transcriptomic Signature for Diagnosis and Treatment Monitoring of Extrapulmonary Tuberculosis

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Declaration

It is hereby declared that

1. The thesis submitted is my/our original work while completing my degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

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APPROVAL

We hereby declare that the thesis entitled **"RISK 6: Transcriptomic Signature for Diagnosis and Treatment Monitoring of Extrapulmonary Tuberculosis"** is from the student's own work and effort, and all other sources of information used have been acknowledged. This Thesis has been submitted with our approval.

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Ethics Statement

The samples for this thesis were obtained as part of an ongoing project "blooD-basEd DIagnostiCs of pAucibacillary Tuberculosis in bangladEsh (DEDICATE)", between icddr,b Dhaka, Bangladesh and Fondation Mérieux, Lyon, France. The DEDICATE study is evaluating multiple transcriptomic signatures among children with pulmonary TB and adults with extrapulmonary TB. Informed written consent and/or assent was obtained before enrollment of all participants. Consent forms and the study protocol were approved by the Research Review and Ethical Review committees of the Internal Review Board of icddr,b (# PR21102).

Abstract

Diagnosing extrapulmonary tuberculosis (EPTB) is challenging due to the diverse clinical manifestations of the disease and the requirement for invasive sampling and specialized processing. Moreover, current methods lack the ability to monitor or predict treatment efficacy even though standard EPTB treatment regimens often require extension. In this context, we aimed to evaluate the performance of the RISK6 transcriptomic signature, previously investigated solely in pulmonary TB, for EPTB diagnosis and treatment monitoring. 29 individuals (> 11 years old) presumed to have EPTB and 10 healthy controls, were enrolled from the clinical facility from March 2022 to 2023. Clinical samples from presumed cases underwent GeneXpert, culture, and, microscopy testing for the diagnosis of EPTB. Blood samples were obtained from all participants at enrolment (and post-treatment for confirmed cases) to assess the RISK6 signature score via RT-qPCR. RISK6 signature performance was evaluated using the receiver-operating characteristic curve (ROC AUC) and Student's t-test. Among 29 presumptive individuals tested, 15 were microbiologically confirmed for EPTB and 14 were unconfirmed (tested negative). Ongoing RISK6 score analysis is available for 15 confirmed cases at baseline (13 with post-treatment scores), 14 unconfirmed cases, and 10 controls. RISK6 effectively discriminated confirmed cases from controls (AUC of 92%, sensitivity 93%, specificity 80%) and unconfirmed cases (AUC of 84%, sensitivity 93%, specificity 73%). Furthermore, RISK6 scores for confirmed cases decreased significantly ($p < 0.001$) post-treatment. The findings in this study suggest that the RISK6 signature performance for EPTB meets the biomarker test criteria defined by the target product profile released by WHO. Minimally invasive, this signature can potentially transform EPTB diagnosis by providing an accessible testing option, even for challenging cases lacking sufficient evidence for biopsy. Additionally, the signature's post-treatment performance indicates a future role in helping clinicians elucidate treatment outcomes.

Keywords: Extrapulmonary tuberculosis; RISK6; Transcriptomic biomarker; Diagnosis, Treatment monitoring

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List of Acronyms

MTB	*Mycobacterium tuberculosis*
TB	Tuberculosis Disease
EPTB	Extrapulmonary Tuberculosis
mAGP	Mycolyl-arabinogalactan-peptidoglycan
TNF-α	Tumor necrosis factor-3
NALC	N-Acetyl-L-Cysteine
CSF	Cerebrospinal fluid
PBS	Phosphate buffer saline
AFB	Acid-Fast Bacillus
NAAT	Nucleic Acid Amplification Test
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assays
LTBI	Latent tuberculosis infection
RIF	Rifampicin
L-J	Löwenstein–Jensen
RD9	Region of detection 9
QFT-Plus	QuantiFERON TB GOLD PLUS
ELISA	enzyme-linked immunosorbent assay

PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RT-qPCR	Reverse transcriptase qPCR
ROC AUC	Area under receiver-operating characteristic curve

Chapter 1

Introduction

1.1 Background

Tuberculosis (TB), one of the leading causes of death from a single infectious agent, has plagued the world for millennia (World Health Organization, 2023). While TB is commonly associated with persistent coughing and lung involvement, it is important to recognize that TB can affect any region of the body. Mycobacterium tuberculosis (MTB), the causative agent of TB, can spread from the lungs, through the lymphatic system and bloodstream, to infect various tissues and organs (Figure 1). This phenomenon constitutes 15- 25% of all TB cases and is known as extrapulmonary tuberculosis (EPTB) (Rodriguez-Takeuchi et al., 2019). EPTB can occur concurrently with PTB, or by itself as MTB can exist latently in the lungs in the form of granulomas which can disseminate to other organs without pulmonary activation (Baykan et al., 2022).

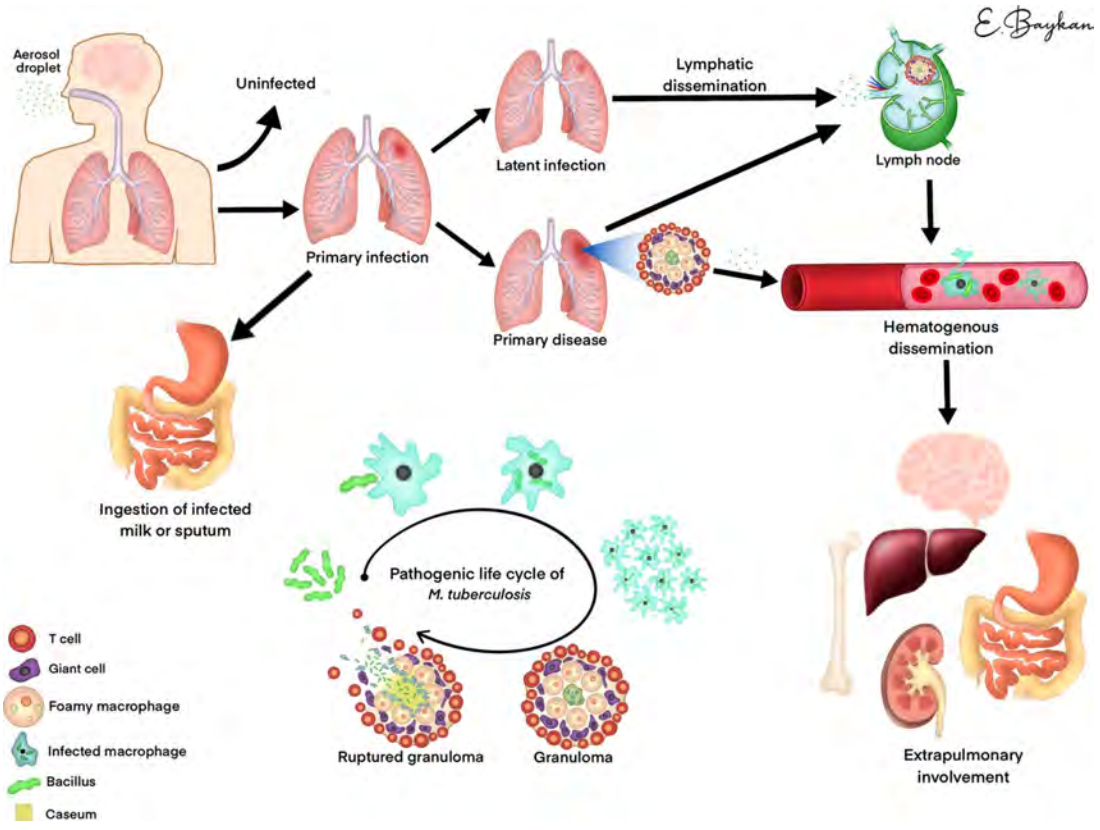


Figure 1: Extrapulmonary dissemination of MTB (Baykan et al., 2022).

1.2 Physiology

The ability of MTB to survive latently inside the host for indefinite amounts of time is partly owed to the remarkable and complex structure of its cell wall (Figure 3) (Marrakchi et al., 2014). A mycolyl-arabinogalactan-peptidoglycan (mAGP) complex forms a strng base to support the upper mycomembrane (Alderwick et al., 2015).The lipid fractions found in its cell wall, such as mycolic acids, cord factor, and wax-D, play crucial roles in their survival and virulence. Mycolic acids form a protective shell that prevents the bacteria from being attacked by lysozyme, cationic proteins, and oxygen radicals, while also protecting against complement deposition. Cord factor is responsible for the characteristic cord formation of Mycobacterium tuberculosis and is essential for its survival within host cells. Wax-D helps the bacteria to escape from phagocytic cells and macrophages. The high concentration of lipids in the cell wall provides resistance to antibiotics, lethal oxidation, osmotic lysis, and the action of acidic and alkaline compounds, while also affecting the permeability of the cell to stains and dyes. (Bandaru et al., 2020) The call wall also acts as an Achillis' heel as it extorts a high metabolic cost, and its development is the target of numerous anti-tubercular drugs.

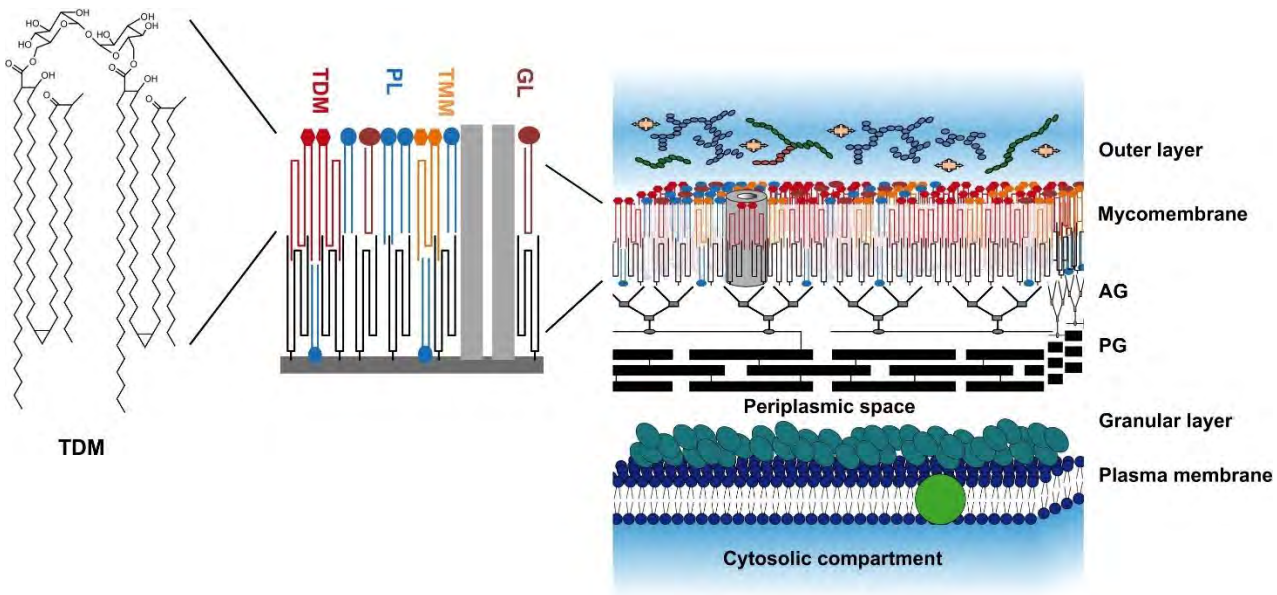


Figure 2 : Structure of MTB wall (Marrakchi et al., 2014).

1.3 Prevalence

According to the Global Tuberculosis Report 2023, 1.3 million people died from, and 7.5 million people were newly diagnosed with TB in 2022 alone. The global burden of TB is carried disproportionately, with 30 countries accounting for 87%, and alone Bangladesh alone accounting for 3.5% of the global TB burden (Figure 1) (World Health Organization, 2023). While 85% of TB patients can be cured with proper treatment, the mortality rate reaches 50% if treatment is not initiated (Tiemersma et al., 2011). Accurate diagnosis is essential for treatment initiation. However, EPTB presents unique challenges compared to PTB. Knowledge of clinical symptoms, microbiological diagnosis methods and treatment regimens are better established for PTB compared to EPTB. Therefore, despite advances in TB diagnosis and treatment, EPTB remains challenging to predict, diagnose, and monitor.

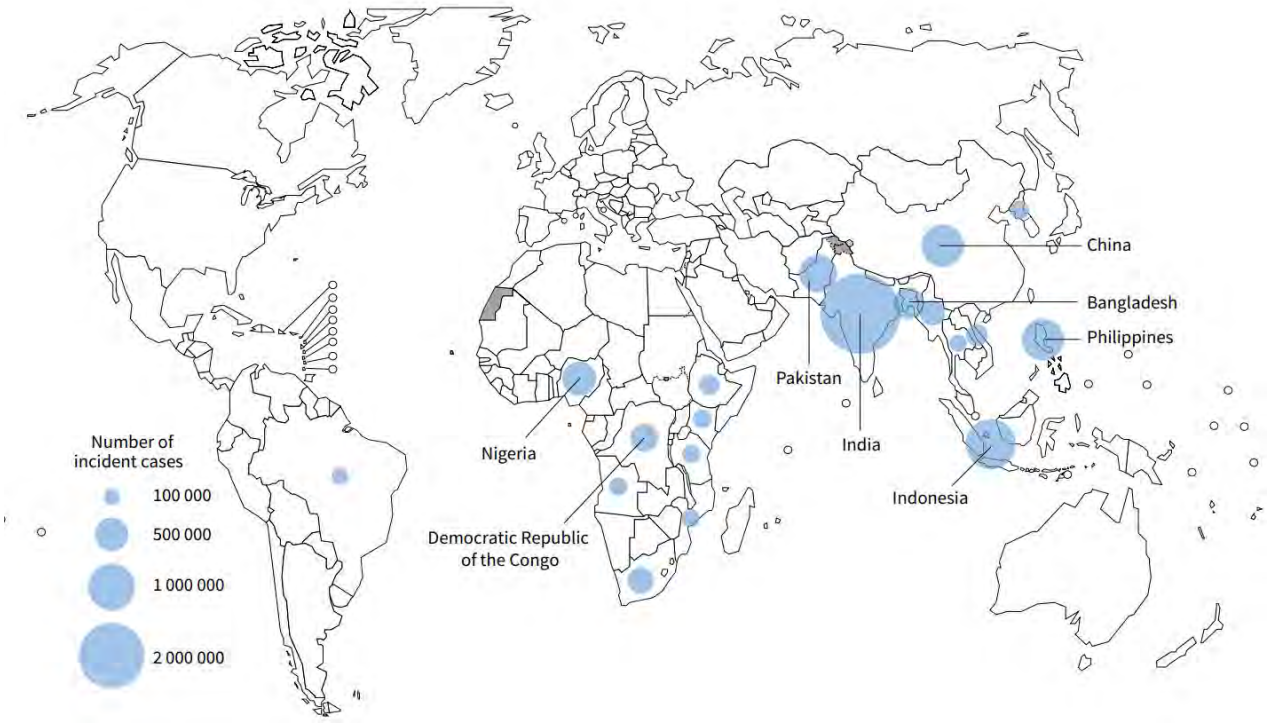


Figure 3: Estimated number of incident TB cases in 2022, for countries with at least 100 000 incident cases.

1.4 Challenges in EPTB Diagnosis and Treatment monitoring

The diagnosis of EPTB presents significant challenges. Firstly, EPTB can manifest as different forms such as lymphadenitis, pleural, skeletal, central nervous system, abdominal, gastrointestinal, peritonitis, genitourinary, miliary, pericarditis and as associated with tumor necrosis factor-3 (TNF-α) inhibitors (Bilchut et al., 2022). The different forms of EPTB produce diverse and nonspecific symptoms which often mimic other diseases, leading to delayed diagnosis. Patients may experience respiratory symptoms such as pleural effusion, gastrointestinal issues like ascites or differential diagnoses with Crohn's disease and amebiasis, neurological manifestations like CSF lymphocytic pleocytosis, musculoskeletal problems such as joint inflammation or vertebral osteomyelitis, and lymphatic system involvement with chronic lymphadenopathy. Additionally, HIV infection and a tuberculosis-

endemic country of origin can be risk factors (Peirse & Houston, 2017). The second challenge lies in sample collection. Infection sites are often inaccessible and obscure requiring invasive procedures such as biopsy or aspiration in order to obtain the specimen. Additionally, due to the paucibacillary nature (low bacterial load) and because the distribution of bacterium is not uniform in the affected sites, collected samples often fail to have the minimum levels of detectable bacilli. The third challenge lies in the limitations of currently established diagnostic methods, most of which are designed for sputum samples for PTB diagnosis. The sensitivity and specificity for the tests vary depending on the type of TB (Lee, 2015).

EPTB treatment often requires prolonged therapy beyond standard regimens. However, existing diagnostic tools are insufficient for accurately monitoring treatment progress and predicting treatment outcomes. This can lead to suboptimal treatment decisions, increased risk of treatment failure, and potential for drug resistance development. As a result, there is an urgent need for more effective diagnostic tools to guide EPTB treatment and improve patient outcomes.

1.5 Laboratory Diagnosis of EPTB

1.5.1 Sample Processing

Patient samples are decontaminated and/or processed to increase specificity and decrease contamination before proceeding with a test. Sputum processing often involves decontamination using Sodium Hydroxide and Sodium Citrate along with liquification using N-Acetyl-L-Cysteine (NALC), followed by centrifugation to collect the bacilli. While variations exist, the general principles are similar and one method is followed for all sputum samples once selected.

For EPTB, each unique sample type requires a different approach. Some of the unique steps for the common sample types are highlighted in Table 1.

Table 1: Unique processing steps for different EPTB samples

Sample Type	Unique steps
Tissues and Lymph Node	Cutting the tissue sample into small pieces using forceps and surgical blade. Using a tissue grinder/ homogenizer.
Cerebrospinal fluid (CSF)	Volumes larger than 5 ml requires concentrating using a centrifuge. Volumes between 1 and 5 ml needs to be used directly without processing. Volumes smaller than 1ml requires addition of phosphate buffer saline (PBS).
Urine	40-200 ml of Urine needs to be concentrated using centrifuge. first morning samples need to be collected for 3-6 days.
Bone, and other rarer sample types	Multiple approaches varying between labs

For tissues, the collected samples are often too large to homogenize entirely and a portion needs to be cut out. Like during sample collection, there are risk of selecting a portion without bacilli. Urine, CSF samples have very low detection rates even after concentrating.

Finally, biosafety level 2 labs, cabinets, and highly skilled technicians are needed to process EPTB samples.

1.5.2 Acid-Fast Bacillus (AFB) Microscopy

A hallmark characteristic of MTB is its thick waxy cell wall composed of mycolic acid. The thickness makes them slow-growing, yet resistant

Acid-fastness is the ability to resist discoloration from acids during the Ziehl-Neelsen staining process. Although acid-fastness is not unique to MTB alone, correlation with patient history and identification of the rod-shaped structure under a microscope gives this test a high level of specificity (Reynolds et al., 2009). Once a cornerstone of tuberculosis diagnosis and research, it is being phased out in favor of more sensitive nucleic acid amplification tests. It is still regularly used in resource limited settings for PTB diagnosis due to the low cost, fast results and relatively low amount of capital required for equipment. The general drawbacks include low sensitivity (5000 cfu/ml), handling of contagious samples, narrow collection window for sputum, and inter-operator variance in reading slides. Due to the lower number of bacilli in the EPTB samples. sensitivity is around 0-40% and a negative result cannot rule out the presence of MTB (Chakravorty et al., 2005).

1.5.3 Culture

Culture tests are considered the gold standard for PTB diagnosis due to their excellent sensitivity, with lowest levels of detection reaching 10 CFU/ml. Once positive, the culture growth can be used to perform drug susceptibility testing which is an added benefit. Two major drawbacks, are time and the requirement of live bacilli. Solid culture in Lowenstein Jensen slants can take up to 60 days due to the slow growing nature of MTB. While automated liquid culture systems can shorten this time to around 42 days, they are expensive

to setup, and perform. EPTB samples are mostly transported from tertiary health care settings to laboratories, in varying conditions and different transport mediums if any. In a few cases, EPTB samples are used to perform other tests, and considered for TB testing only after other tests turn negative. The few bacilli that are present in the sample might be killed or become non-culturable during these processes. Culture sensitivity for EPTB varies widely depending on the sample type, with lower rates reported for tuberculous pericarditis (10-11%), meningitis (24-29%), and lymphadenitis (5-14%) compared to abdominal TB (28-50%) (Peoples, 2014).

1.5.4 Nucleic Acid Amplification Tests (NAATs)

Polymerase chain reaction (PCR) tests offer higher sensitivity and specificity with rapid turnaround time compared to AFB and Culture. However, traditional PCR tests require trained staff and dedicated, contamination free areas and biosafety cabinets to prepare master mixes, and manual extraction of DNA. Point of cares and small healthcare facilities such as TB screening centers do not have these facilities.

Cartridge based NAATs such as the GeneXpert system is widely used in place of traditional PCR tests due to numerous advantages. Sputum samples do not require separate processing and extraction, but instead only require 15 mins with the included sample processing reagent. Results can be generated in one and a half hours, with simultaneous semi-quantification of bacterial load and resistance to the rifampicin drug. The latest iteration of the GeneXpert test (Xpert MTB/RIF Ultra) has detection levels as low as 11.8 CFU/ml, but the non-ultra version (Xpert MTB/RIF) has a limit of detection of 131 CFU/ml. However, as PCR does not require live bacilli, sensitivity of both GeneXpert tests often exceed culture sensitivity for EPTB.

Studies have reported favorable performance compared to current diagnostic methods for EPTB diagnosis (Lawn & Zumla, 2012; Uddin et al., 2023).

For EPTB diagnosis, NAATs, have the same disadvantages which are common to all tests reliant on presence of bacilli: requirement to handle contagious samples, the paucibacillary nature of the EPTB samples, and different EPTB samples requiring a different pre-processing technique before it can be used with the GeneXpert procedure. Additionally, components such blood and bile from the samples can inhibit the PCR reactions giving false negative results. This gives GeneXpert tests for EPTB lower sensitivity than for Sputum. Sensitivities reported by different studies vary greatly between EPTB sample types, with pooled sensitivity varying from 31% in pleural fluid to 97% in bone or joint fluid (Denkinger et al., 2014; Kohli et al., 2018).

1.5.5 Host biomarkers

Most host biomarker studies have been performed with PTB patients, and limited information is available for performance among individuals with EPTB (Nogueira et al., 2022). Developing accurate for active TB as a whole has been challenging. Antibodies, a biomarker used widely in other diseases due to the ease of testing, has been inconsistent for TB, with limited utility. On the other hand, cytokine responses have found a niche in latent TB diagnosis. Interferon-gamma (IFN-γ) release assays (IGRAs) are able to diagnose current or previous MTB infection. However, they are not accurate in diagnosing active TB, not can they differentiate between prior, latent and active TB (Goletti et al., 2018; Petruccioli et al., 2020). Multiple cytokines are being combined into specific biosignatures and shows promise. Omics approaches which include genomics, transcriptomics, proteomics and metabolomics take a comprehensive approach towards biomarker discovery. Omics approaches are costly

and not possible to implement in most high-prevalence settings due to the unavailability of technological and analytical infrastructure.

Unlike omics approach as a whole, the signatures discovered through transcriptomics can be implemented in resource limited settings as only quantitative PCR is needed once a transcriptomic signature is discovered. Moreover, transcriptomic blood gene expression signatures for TB, such as ACS 16 (Zak et al., 2016), ACS 11 gene (Darboe et al., 2018), RISK11(Scriba et al., 2021), RISK6, the 3 gene signature by Sweeney et al (Sweeney et al., 2016), are better studied and have shown better diagnostic performance compared to non-transcriptomic host biomarkers.

1.6 RISK6 signature

The RISK6 transcriptomic signature was discovered in search of a signature that is robust, parsimonious and deployable at the point-of-care (Penn-Nicholson et al., 2020). It has been validated among PTB patients for predicting incident TB, as a triage test and for monitoring treatment response in multiple countries including Bangladesh (Bayaa et al., 2021; Penn-Nicholson et al., 2020). RISK6 consists of 6 genes that make 9 transcript pairs. Each transcript pair contains one transcript that is upregulated in progressors and one transcript that is downregulated in progressors, compared to healthy controls (Figure 4). As this designed was developed with point-of-care use in mind from the beginning, all performance training and assessment were performed using a standardized protocol and locked-down algorithm.

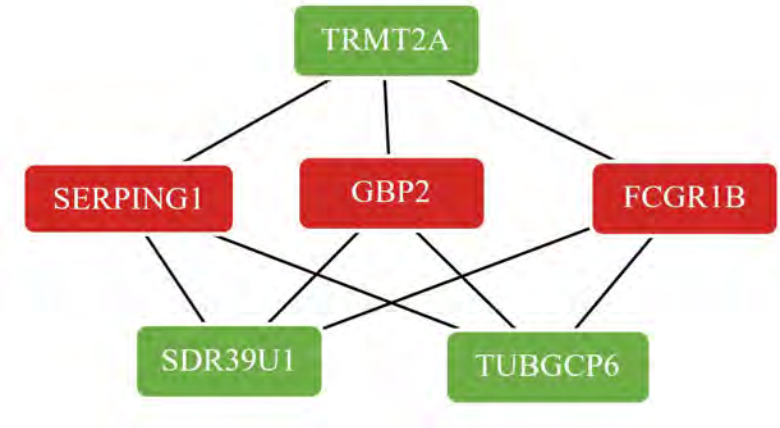


Figure 4: 9 RISK6 transcript pairs consisting of 6 genes. Transcripts upregulated in progressors, compared to healthy controls, are in red nodes and downregulated transcripts are in green nodes.

1.7 Objectives

A biomarker capable of predicting, screening, and monitoring extrapulmonary tuberculosis (EPTB) would be a crucial step in eradicating TB. Given RISK6's performance in pulmonary TB patients, its potential for EPTB diagnosis and management is significant. If RISK6 demonstrates similar effectiveness in EPTB patients, it could be a valuable tool in combating this challenging form of tuberculosis. In this regard, for the first time, we explore the RISK6 signature among EPTB with the following objectives:

Primary Objective: Performance of RISK6 signature for screening and diagnosing EPTB.

Secondary Objective: Potential of EPTB Treatment monitoring with RISK6 signature.

Chapter 2

Methods

2.1 Study Design and Enrolment

In this observational study, 29 individuals who were presumed to have EPTB and 10 asymptomatic healthy controls were enrolled between January 2021 and December 2023 from the TB Screening and Treatment Centre of icddr,b in Dhaka, Bangladesh.

Participants were enrolled only if they provided written informed consent, were over 11 years old, and deemed able to provide adequate amount of blood.

EPTB samples and 6 ml blood were collected from presumptive EPTB patients with signs and symptoms suggestive of EPTB during enrollment (T0).

EPTB samples were tested to confirm the disease and participants were sorted into the confirmed group if any microbiological test gave a positive result and into the unconfirmed group if all microbiological tests were negative. Confirmed participants were contacted for progress of treatment over phone 3 months after treatment initiation (T1) and 6 ml of blood was collected at the end of treatment (T2).

Healthy controls provided only blood samples as they were asymptomatic with no suggestion of TB.

2.2 Collection of samples

EPTB Samples were sent directly to the mycobacteriology lab of icddr,b from tertiary care facilities across the country. The requested test was performed, and any leftover sample was used to perform the additional biological tests. In addition, 6 ml of blood was collected in Tempus^{TM} blood collection tubes during enrolment. 3 ml of the blood was used for RISK6 signature evaluation and 3 ml for a prototype test utilizing a different signature. Individuals enrolled as controls provided 7 ml blood during enrolment: 3 ml for transcriptomic signatures, and 4 ml for latent tuberculosis detection.

2.3 Laboratory procedures

2.3.1 GeneXpert MTB/RIF

EPTB samples were processed according to the sample type, as mentioned in table 1. The sample was then mixed with sample processing reagent supplied with the GeneXpert cartridges in a 1:2 ratio and vortexed. After letting sediments settle for 15 minutes, 2 ml of the inactivated mixture was pipetted into the test cartridge, which was then inserted in the testing platform (Boehme et al., 2010).

The GeneXpert system reported results as MTB Detected, MTB Not Detected, and invalid.

If MTB was Detected it also reported the burden levels as High, Medium, Low, and Very Low, depending on cycle count where threshold levels were reached (ct value). It also reported the resistance to rifampicin (RIF) as RIF Resistance detected, RIF resistance not detected or RIF resistance indeterminate. Invalid results could arise due to sample processing errors, inhibitors in the sample, or defects in the cartridge. Indeterminate RIF results arise due to insufficient bacterial loads. Invalid and indeterminate results were repeated. The process is represented in Figure 1.

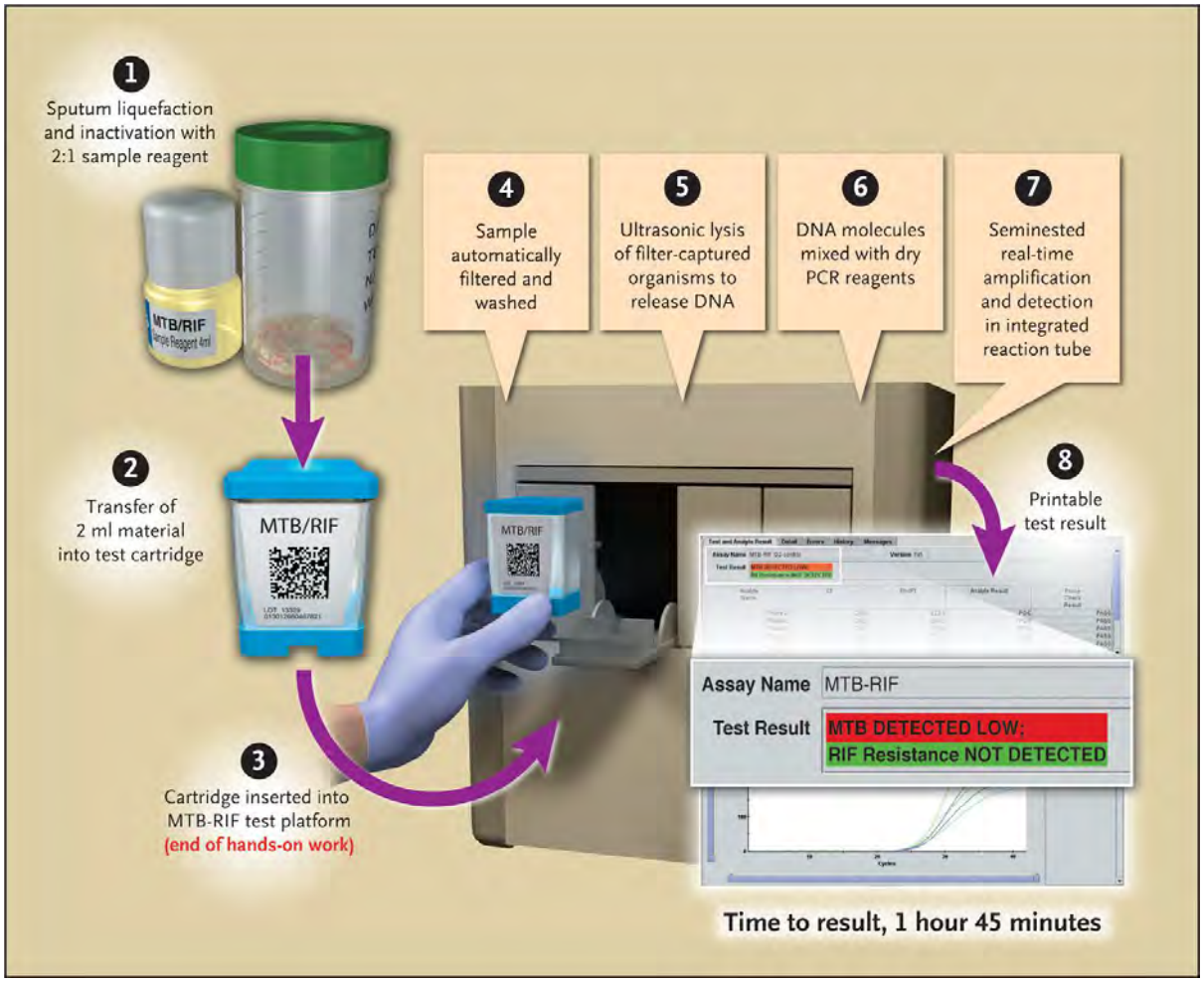


Figure 5: Procedure for GeneXpert MTB/RIF. The assay automatically filters, washes and lyses the sample, performs a semi-nested , multiplex, real-time PCR and reports the results.(Boehme et al., 2010).

2.3.2 Löwenstein–Jensen Culture

Löwenstein–Jensen (LJ) slants were prepared with Malachite gree, egg albhumin, egg white, L asparagine, potato flour, potassium dihydrogen phosphate, magnesium sulphate and glycerol. Samples were processed using the NaOH-NaLC method and inoculated on two LJ slants. Slants were Incubated for up to 8 weeks at 37 °C. Readings were taken every week to look for visible growth of mycobacterial colony or contaminations. If contamination was

found, processed and stored leftover sample was decontaminated using NaOH and reinoculated on fresh LJ slants. If visible mycobacterial growth was found, the culture was reported positive, or PCR was performed from the colony if growth was not clearly mycobacterial. If no growth or contamination was found for 8 weeks in either slant, the culture was reported as MTB not found.

Figure 6: a) LJ culture showing nascent MTB colonies. b) LJ culture showing contamination growth.

Growths were confirmed with RD9 (Region of detection 9) PCR after extraction of DNA using Heat shock and sonication. In brief, colonies were homogenized and placed in 95°C for 5 mins, followed by ultrasonication at 2o kHz for 15 minutes. Supernatant was collected for PCR. This crude method works as the amount of DNA obtained from the culture isolates is very high. A typical RD9 PCR gel run is shown in figure.

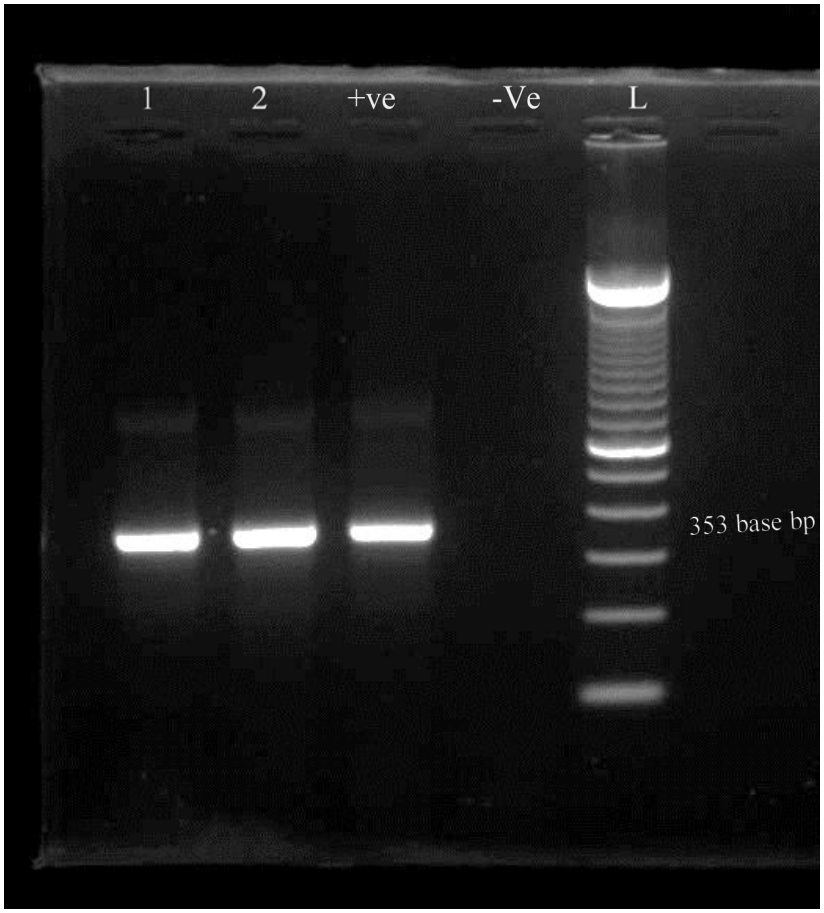


Figure 7: Agarose gel run result of RD9 PCR. Well 1 and 2 are samples showing presence of RD9 region, well 3 has the positive PCR control (MTB strain H37RV) well -ve is the negative PCR control and well L has a 100 bp DNA ladder. The RD9 region is 353 base pair.

2.3.3 Acid-fast Bacillus Microscopy/ Ziehl-Neelsen Staining

Processed samples were smeared onto a labelled slide using a loop. The slide was placed on a warmer for 10 mins to fix the stain. The smear was covered with 1% Carbol Fuchsin stain and heated to 60 °C for 3 to 5 minutes using a flame on cotton swab with alcohol. Distilled water was used to wash away excess Carbol Fuchsin from the slides. The slides were then flooded with 25% sulfuric acid for up to 3 minutes. Distilled water was used again to was the

excess acid off of the slides. The slides were dried and observed under a light microscope. Results were reported as follows:

Table 2: AFB grading chart

Number of AFB's seen	**Result and grading**	**Number of fields to be examined**
None in 100 fields	Negative	100
1-9 in 100 fields	Scanty, Report exact number of AFB	200
10-99 in 100 fields	1+	100
1-10 per field	2+	20
>10 per field	3+	20

2.3.4 QuantiFERON TB GOLD PLUS (QFT-Plus)

Healthy controls underwent latent tuberculosis infection (LTBI) testing using the QFT-Plus IGRA. Blood samples were drawn into Nil, TB1, TB2, and Mitogen tubes designed to assess the immune response to MTB antigens. The TB1 and TB2 tubes specifically stimulate CD4+ and CD8+ T cells, respectively. After 16-24 hours of incubation at 37 °C, the tubes were centrifuged at 2500g for 15 minutes to obtain plasma.

The IFN-γ levels in the plasma were measured using an enzyme-linked immunosorbent assay (ELISA) as part of the QFT-Plus test. The Nil control measured baseline IFN-γ levels, while

the Mitogen control served as a positive control to confirm immune functionality. The test results were interpreted based on the manufacturer's guidelines, with an IFN-γ concentration of ≥0.35 pg/mL in the TB antigen tubes considered a positive result, signalling an immune response consistent with LTBI.

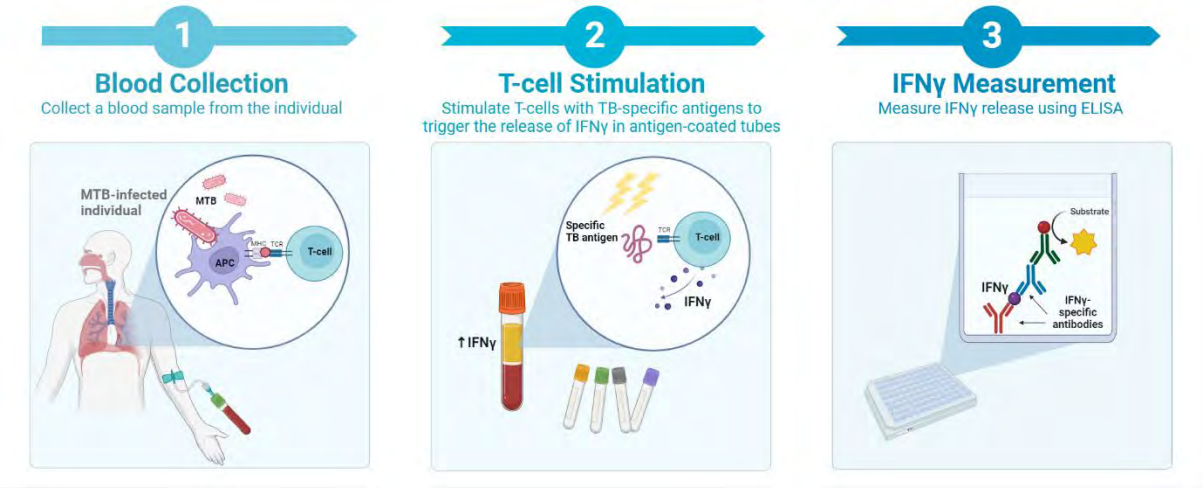


Figure 8: QFT-Plus principle.

2.3.5 RNA Extraction

RNA was extracted from blood collected in Tempus tubes using TRIzol reagent. 6 ml collected blood is drawn into Tempus tubes containing 3ml of Tempus reagent making a total of 9 ml. 45 ml was used to extract RNA for RISK6 signature analysis.

In brief, the steps were:

Prewashing the Tempus blood by removing excess Tempus reagent:

In a 50 ml conical tube, 1.5ml of Phosphate buffer saline was added to 4 ml of Tempus tube blood. The mixture was then vortexed and centrifuged for 20 mins at 4000g at 4°C, after which the supernatant was discarded.

Addition of TRIzol for phase separation:

1.33 ml cold TRIzol was added to the pellet and transferred into a microcentrifuge tube. After 5 minutes of incubation at room temperature, 0.3 ml of chloroform was added. After 2 minutes of incubation at room temperature the samples were centrifuged at 12000 rpm for 10 minutes. The upper aques layer containing RNA was transferred to a new tube.

Precipitating the RNA:

100% isopropanol equal in volume to the collected aquas phase was added to the new tube and incubated for 60 minutes at -20°C. After incubation, the samples were centrifuged at 15000 rpm for 10 mins at 4°C. Total RNA formed a white gel like pellet at the bottom and the supernatant was discarded.

Washing salts from RNA pellet:

1 ml of 70% ethanol was added to the pellet and centrifuged 5 minutes at 12000 rpm after a 10-minute incubation. The supernatant was carefully discarded by pouring followed by aspiration using a micropipette.

Solubilizing the RNA:

The pellet was resuspended in 27 μL DEPC treated water by pipetting and placing in a heat block for 8 minutes at 56 °C.

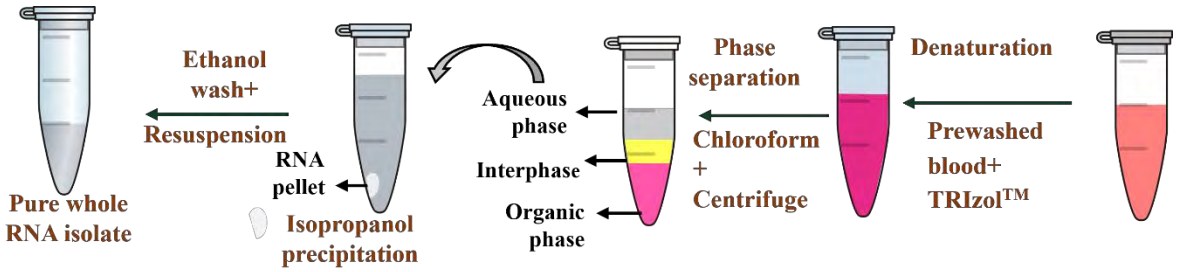


Figure 9: RNA extract obtained from prewashed Tempus tube blood using TRIzol.

2.3.6 RISK6 PCRs

Generating cycle threshold values (Ct) for the 6 genes in order to calculate RISK6 score was performed in 3 steps.

1) CDNA synthesis using RT-PCR

Extracted RNA was converted to first-strand cDNA using RT-PCR. 6 µL of the extracted RNA was mixed with RT Buffer mix and RT enzyme mix and run for 60 minutes at 37 °C. A 5-minute denaturation at 95 °C was used at the end to deactivate the reverse transcriptase enzyme.

Random primers allowed the RT enzyme to amplify all the RNA present in the sample.

The RT-PCR product was diluted to a ratio of 1:5 before proceeding further.

2) Preamplification using pooled primer mix

In order to increase the signal intensity and reduce background noise in the final qPCR, a preamplification PCR using pooled primers were performed. The pooled primers targeted the all 6 genes in the RISK6 signature: GBP2, TUBGCP6, TRMT2A, SDR39U1, FCGR1B, and SERPING1.

2.5 µL of the CDNA was added to 2.5 µL pooled primer mix and 5 µL TaqMan® Universal PCR Master Mix. The PCR was run for 16 cycles.

Only the 6 genes required by the signature were thus targeted and amplified.

The PCR parameters were:

Table 3: Preamplification PCR parameters

Parameter	**Polymerase activation***	**PCR Cycles (16 cycles)**		**Storage**
	Hold	**Denature**	**Anneal/extend**	**Hold**
Temperature	95°C	95°C	60°C	4°C
Time (mm:ss)	10:00	00:15	04:00	∞

The pre amplified products were diluted to a ratio of 1:5 followed by 1:5 again for a final dilution of 1:25 to proceed further.

3) Gene Expression Assay

The diluted pre-amplified sample was then used for final quantitative PCR. FAM-dye TaqMan assays for the 6 genes were run separately in 12 reactions (Figure 9). qPCR for each gene was run twice and the average of the Ct value was taken as the final Ct value for the gene. If Ct value differed by more than 2, it was considered invalid and the PCRs were repeated.

The RISK6 signature is self-normalizing hence, no separate target amplification was needed to normalize the Ct values. Negative PCR controls were included for only one

plate each day, regardless of the number of PCR plate runs performed. No positive controls were needed as all PCRs are expected to generate fluorescent activity regardless of the risk of TB.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk FCGR1B EPA 001	Unk GBP2 EPA 001	Unk SERPING1 EPA 001	Unk SDR39U1 EPA 001	Unk TRMT2A EPA 001	Unk TUBGCP6 EPA 001	Unk FCGR1B EPA 001	Unk GBP2 EPA 001	Unk SERPING1 EPA 001	Unk SDR39U1 EPA 001	Unk TRMT2A EPA 001	Unk TUBGCP6 EPA 001
B	Unk FCGR1B EPA 002	Unk GBP2 EPA 002	Unk SERPING1 EPA 002	Unk SDR39U1 EPA 002	Unk TRMT2A EPA 002	Unk TUBGCP6 EPA 002	Unk FCGR1B EPA 002	Unk GBP2 EPA 002	Unk SERPING1 EPA 002	Unk SDR39U1 EPA 002	Unk TRMT2A EPA 002	Unk TUBGCP6 EPA 002
C	Unk FCGR1B EPA 003	Unk GBP2 EPA 003	Unk SERPING1 EPA 003	Unk SDR39U1 EPA 003	Unk TRMT2A EPA 003	Unk TUBGCP6 EPA 003	Unk FCGR1B EPA 003	Unk GBP2 EPA 003	Unk SERPING1 EPA 003	Unk SDR39U1 EPA 003	Unk TRMT2A EPA 003	Unk TUBGCP6 EPA 003
D	Unk FCGR1B EPA 004	Unk GBP2 EPA 004	Unk SERPING1 EPA 004	Unk SDR39U1 EPA 004	Unk TRMT2A EPA 004	Unk TUBGCP6 EPA 004	Unk FCGR1B EPA 004	Unk GBP2 EPA 004	Unk SERPING1 EPA 004	Unk SDR39U1 EPA 004	Unk TRMT2A EPA 004	Unk TUBGCP6 EPA 004
E	Unk FCGR1B EPA 005	Unk GBP2 EPA 005	Unk SERPING1 EPA 005	Unk SDR39U1 EPA 005	Unk TRMT2A EPA 005	Unk TUBGCP6 EPA 005	Unk FCGR1B EPA 005	Unk GBP2 EPA 005	Unk SERPING1 EPA 005	Unk SDR39U1 EPA 005	Unk TRMT2A EPA 005	Unk TUBGCP6 EPA 005
F	Unk FCGR1B EPA 006	Unk GBP2 EPA 006	Unk SERPING1 EPA 006	Unk SDR39U1 EPA 006	Unk TRMT2A EPA 006	Unk TUBGCP6 EPA 006	Unk FCGR1B EPA 006	Unk GBP2 EPA 006	Unk SERPING1 EPA 006	Unk SDR39U1 EPA 006	Unk TRMT2A EPA 006	Unk TUBGCP6 EPA 006
G	Unk FCGR1B EPA 008	Unk GBP2 EPA 008	Unk SERPING1 EPA 008	Unk SDR39U1 EPA 008	Unk TRMT2A EPA 008	Unk TUBGCP6 EPA 008	Unk FCGR1B EPA 008	Unk GBP2 EPA 008	Unk SERPING1 EPA 008	Unk SDR39U1 EPA 008	Unk TRMT2A EPA 008	Unk TUBGCP6 EPA 008
H	Unk FCGR1B EPA 009	Unk GBP2 EPA 009	Unk SERPING1 EPA 009	Unk SDR39U1 EPA 009	Unk TRMT2A EPA 009	Unk TUBGCP6 EPA 009	Unk FCGR1B EPA 009	Unk GBP2 EPA 009	Unk SERPING1 EPA 009	Unk SDR39U1 EPA 009	Unk TRMT2A EPA 009	Unk TUBGCP6 EPA 009

Figure 10: PCR plate Setup for analysing 8 samples. 6 genes along with a replicate for each gene are tested from each sample.

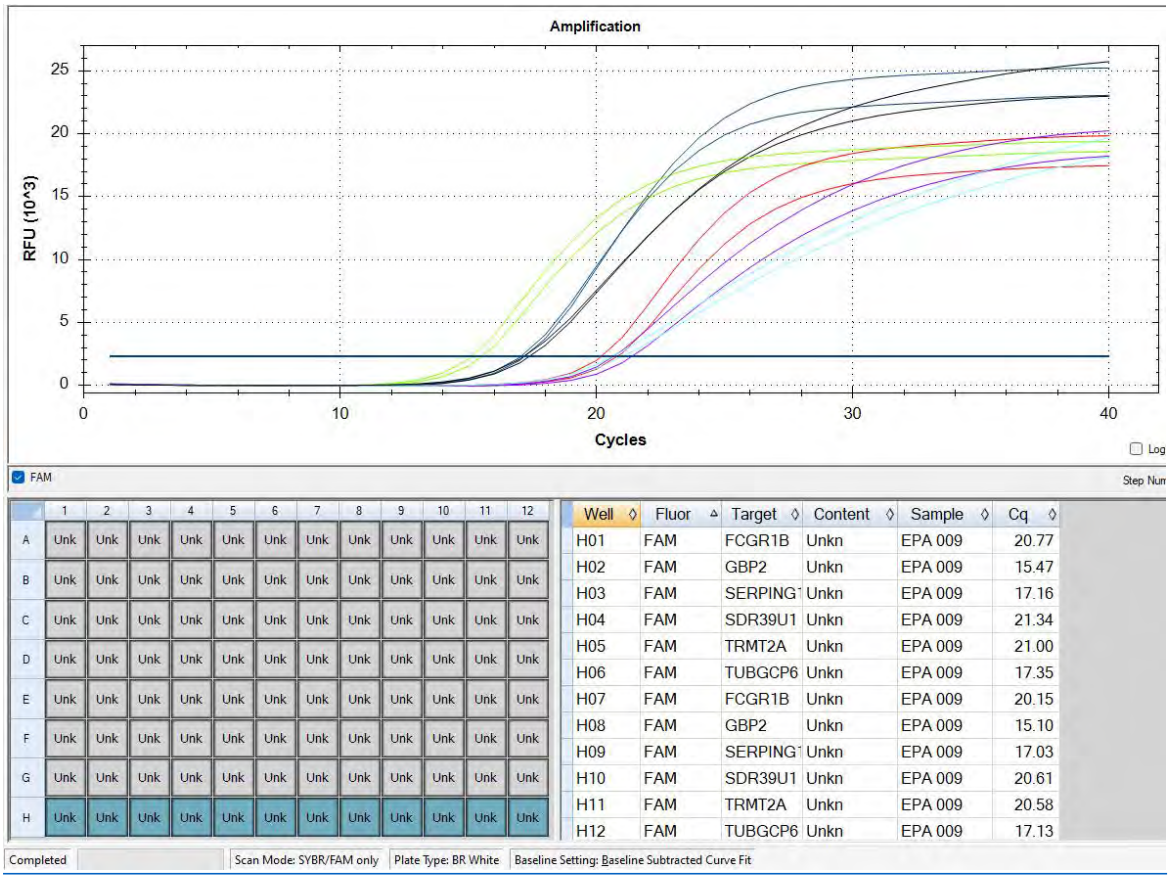
The qPCR parameters were:

Table 4: Gene expression assay qPCR parameters

Parameter	**Polymerase activation**	**PCR Cycles and reading (40 cycles)**		
	Hold	**Denature**	**Anneal/extend**	**FAM reading**
Temperature	95°C	95°C	60°C	-
Time (mm:ss)	10:00	00:15	01:00	-

2.4 RISK6 Score Calculation

Risk6 scores were calculated as described by Penn-Nicholson et al (Penn-Nicholson et al., 2020). The mean Ct value for each gene (example shown in figure) was used to compute the raw differences in Ct for the 9 transcript pairs, producing the log-transformed ratio of expression. The ratios were compared to ratios in a look-up table for each pair of the 9 transcript pairs, obtaining a corresponding score. The average of the 9 scores were was the finals score. If any gene failed to amplify and produce a Ct value, that gene and corresponding transcript pairs were excluded from the final score calculation as the signature is robust. These steps were automated using the R script available at: https://bitbucket.org/satvi/risk6.



Well	Fluor	Target	Content	Sample	Cq
H01	FAM	FCGR1B	Unkn	EPA 009	20.77
H02	FAM	GBP2	Unkn	EPA 009	15.47
H03	FAM	SERPING1	Unkn	EPA 009	17.16
H04	FAM	SDR39U1	Unkn	EPA 009	21.34
H05	FAM	TRMT2A	Unkn	EPA 009	21.00
H06	FAM	TUBGCP6	Unkn	EPA 009	17.35
H07	FAM	FCGR1B	Unkn	EPA 009	20.15
H08	FAM	GBP2	Unkn	EPA 009	15.10
H09	FAM	SERPING1	Unkn	EPA 009	17.03
H10	FAM	SDR39U1	Unkn	EPA 009	20.61
H11	FAM	TRMT2A	Unkn	EPA 009	20.58
H12	FAM	TUBGCP6	Unkn	EPA 009	17.13

Figure 11: Ct value of each gene and replicate for each sample. The mean Ct value of the replicates is taken. Replicate pairs for one sample have been coloured and singled out for ease of visualization.

2.5 Data Analysis

Data was entered and stored in REDcap. Performance of RISK6 was evaluated using area under receiver-operating characteristic curve (ROC AUC) analysis and Student's t-test. p value < 0.05 was considered statistically significant. Youden index was calculated to find the cut off value with optimal sensitivity and specificity. Data was analyzed using R version 4.3.2 (2023-10-31 ucrt). readxl, dplyr, tidyr, and ggplot2 packages were used for data reading, manipulation and visualization. pROC package was used to generate ROC curves. ggplot2 package was used to generate violin plots.

Chapter 3

Results

3.1 General characteristics of enrolled participants

Characteristic	**Confirmed**, N = 15[1]	**Unconfirmed**, N = 14[1]	**Control**, N = 10[1]	**Overall**, N = 39[1]
Sex				
Male	8 (53.3%)	7 (50.0%)	6 (60.0%)	21 (53.8%)
Female	7 (46.7%)	7 (50.0%)	4 (40.0%)	18 (46.2%)
Age Category				
0-25	4 (26.7%)	5 (35.7%)	1 (10.0%)	10 (25.6%)
25-35	6 (40.0%)	3 (21.4%)	2 (20.0%)	11 (28.2%)
35-45	2 (13.3%)	2 (14.3%)	1 (10.0%)	5 (12.8%)

21 (53.4%) out of 39 of enrolled participants in the study were Male. Age ranged from 16 to 70 years. Confirmed EPTB cases were predominantly under 35 years old. Out of 29 enrolled presumptive cases, 15 were Diagnosed microbiologically with EPTB. 14 remained as unconfirmed as MTB was not detected in either GeneXpert, Culture or AFB tests. In addition, 10 asymptomatic, healthy persons were enrolled as asymptomatic controls.

The most common EPTB specimen were urine (30.4`%) followed by abscesses (17.4%), pus (17.4%). And lymph Nodes (13.0%). However, 6/7 Urines samples tested negative for EPTB, while all abscesses, all lymph nodes and 3/4 pus samples tested positive.

Out of 15 confirmed individuals, 2 had High burden in GeneXpert, 3 had Medium burden, 3 had Low burden, 6 had Very Low burden, and 1 sample had Trace levels of MTB. 7 out of the 15 confirmed individuals were Positive in Culture tests while only 1 person was found to be Positive in AFB test and had a grade of 1+. These results are shown in Table 5 along with the Mean RISK6 transcriptomic scores. Baseline (T0) Risk6 scores are available for all 15 individuals while End of Treatment (T2) scores are available for 13 among the confirmed individuals. Individuals enrolled as asymptomatic controls did not undergo any microbiological testing. 7 individuals, out of 10 enrolled, tested positive for latent tuberculosis infection.

Table 5: General characteristics and Test Results by enrolment group.

45-60	3 (20.0%)	3 (21.4%)	6 (60.0%)	12 (30.8%)
>60	0 (0.0%)	1 (7.1%)	0 (0.0%)	1 (2.6%)
EPTB specimen			N/A	
Abscess	4 (30.8%)	0 (0.0%)		4 (17.4%)
Colonic Ulcer	0 (0.0%)	2 (20.0%)		2 (8.7%)
Lymph Node	3 (23.1%)	0 (0.0%)		3 (13.0%)
Pleural Fluid	2 (15.4%)	0 (0.0%)		2 (8.7%)
Pus	3 (23.1%)	1 (10.0%)		4 (17.4%)
Urine	1 (7.7%)	6 (60.0%)		7 (30.4%)
Wound Swab	0 (0.0%)	1 (10.0%)		1 (4.3%)
Culture Result			N/A	
Positive	7 (46.7%)	0 (0.0%)		7 (24.1%)
Negative	8 (53.3%)	14 (100.0%)		22 (75.9%)
GeneXpert Burden		N/A	N/A	
High	2 (13.3%)			2 (13.3%)
Medium	3 (20.0%)			3 (20.0%)
Low	3 (20.0%)			3 (20.0%)
Very Low	6 (40.0%)			6 (40.0%)
Trace	1 (6.7%)			1 (6.7%)
Microscopy Result			N/A	
Positive	1 (6.7%)	0 (0.0%)		1 (3.4%)
Negative	14 (93.3%)	14 (100.0%)		28 (96.6%)
QFT Result	N/A	N/A		
Positive			7 (70.0%)	
Negative			3 (30.0%)	
Risk6 Score at Baseline	0.7 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	
Risk6 Score After treatment	0.5 ± 0.2	NA ± NA	NA ± NA	

[1]n (%); Mean ± SD; N/A = Not Applicable

3.2 RISK6 Score Analysis

The asymptomatic control group (EPA), consisting of 10 individuals, had a median RISK6 score of 0.453. The presumed EPTB but unconfirmed group (EPN), with 14 individuals, had a median score of 0.464. The 15 confirmed EPTB cases (EPC) had a median score of 0.75.

Among the 15 cases, scores after treatment completion are available for 13 individuals, with a median score of 0.552 (Figure 11).

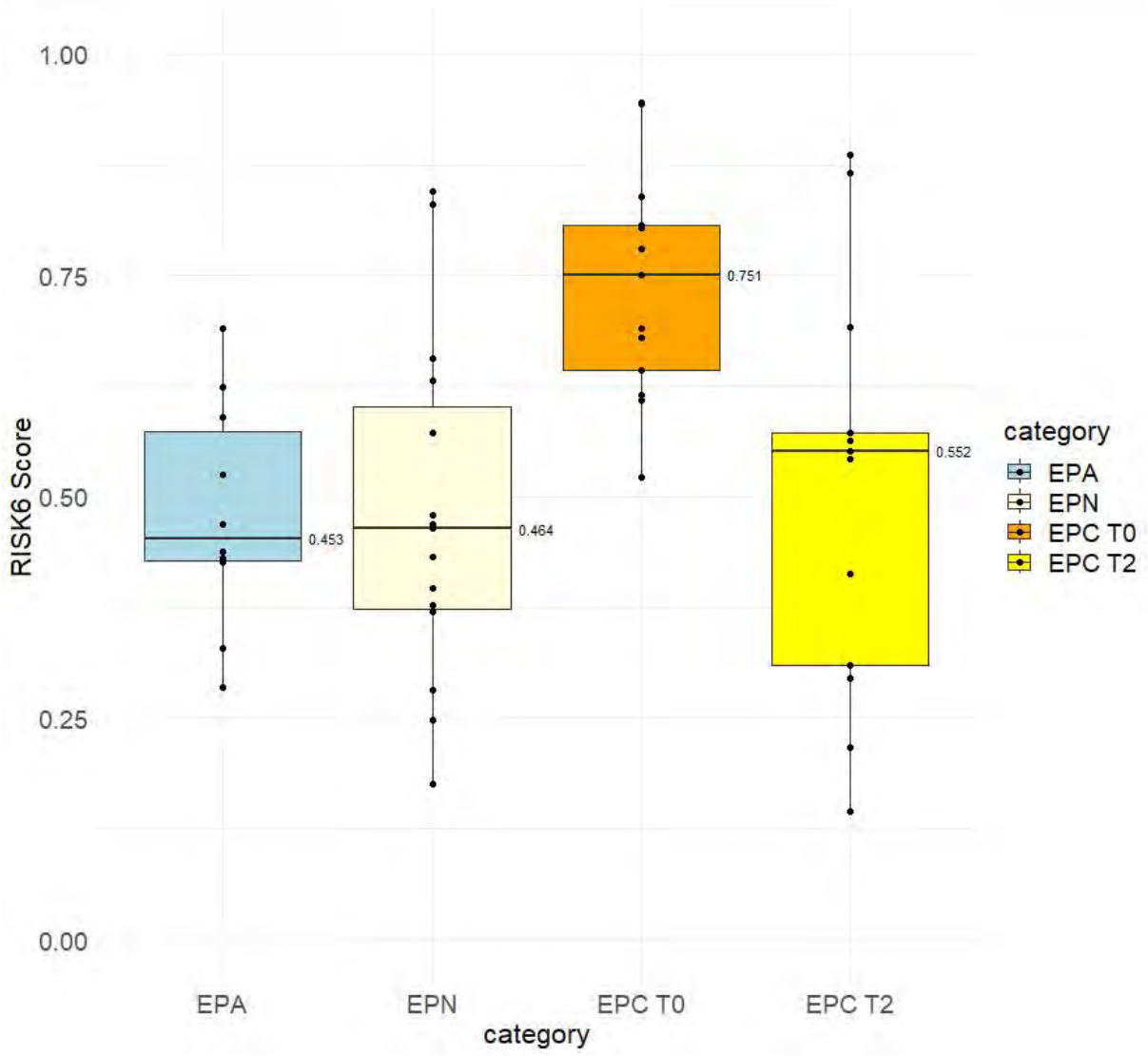


Figure 11: Box Plots for RISK6 scores of EPA, EPN, EPC T0, and EPC T2 samples.

There was no median score difference between EPC group who were positive in culture test (0.751) vs EPC group who were negative in culture test (0.748) (Figure 12). However, the variance in scores were much larger for culture negative patients.

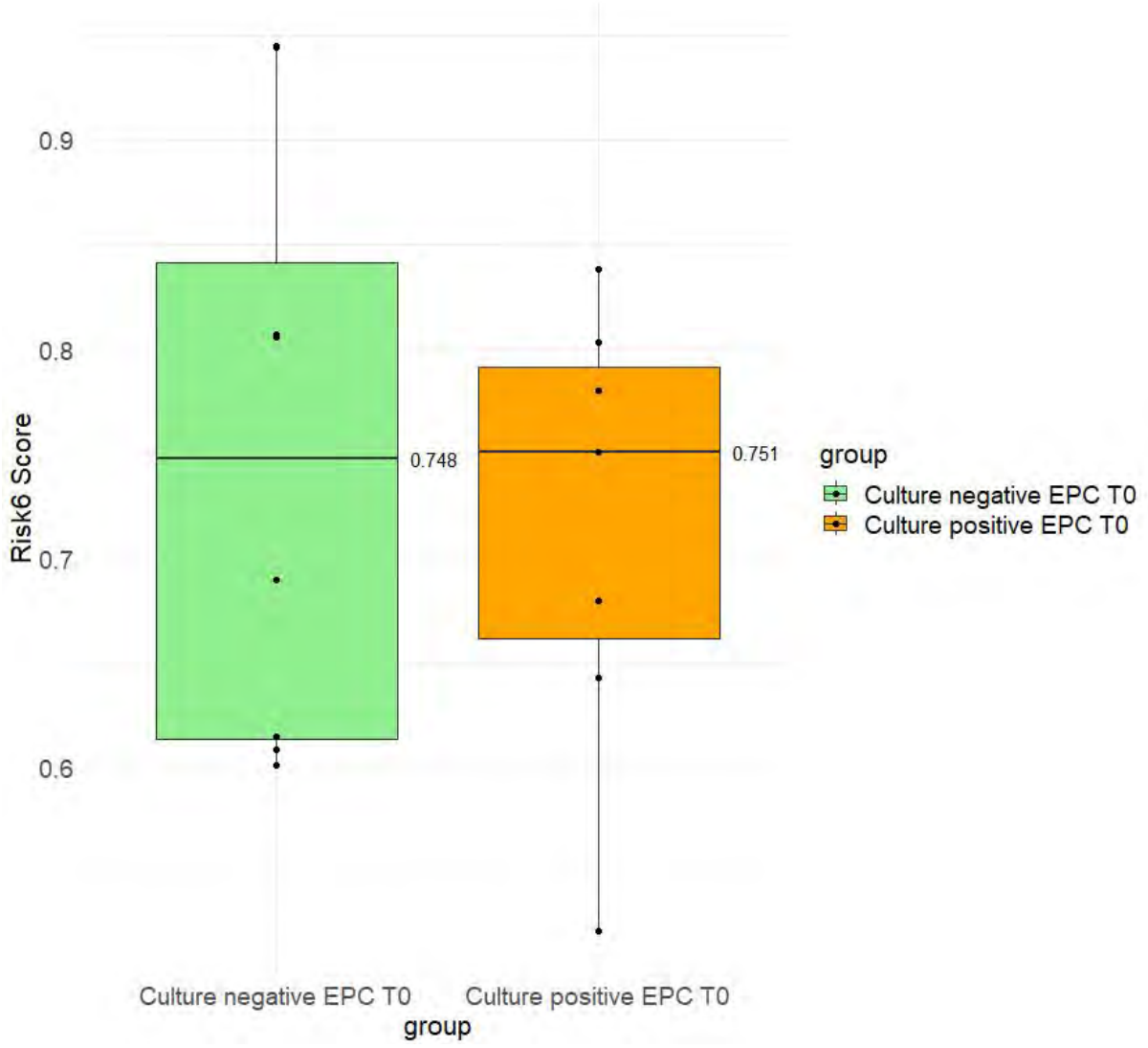


Figure 12: Box Plots for Culture positive EPC T0 and Culture negative EPC T0.

Among 13 available RISK6 scores among patients who completed treatment, median RISK6 scores decreased from 0.751 at baseline during diagnosis (EPC T0) to 0.552 after treatment completion (EPC T2). This overall decrease was statistically significant ($p < 0.001$). The

individual score changes are linked in figure 13. Scores remained similar for 3 patients, increased for 1, and decreased for 9 patients.

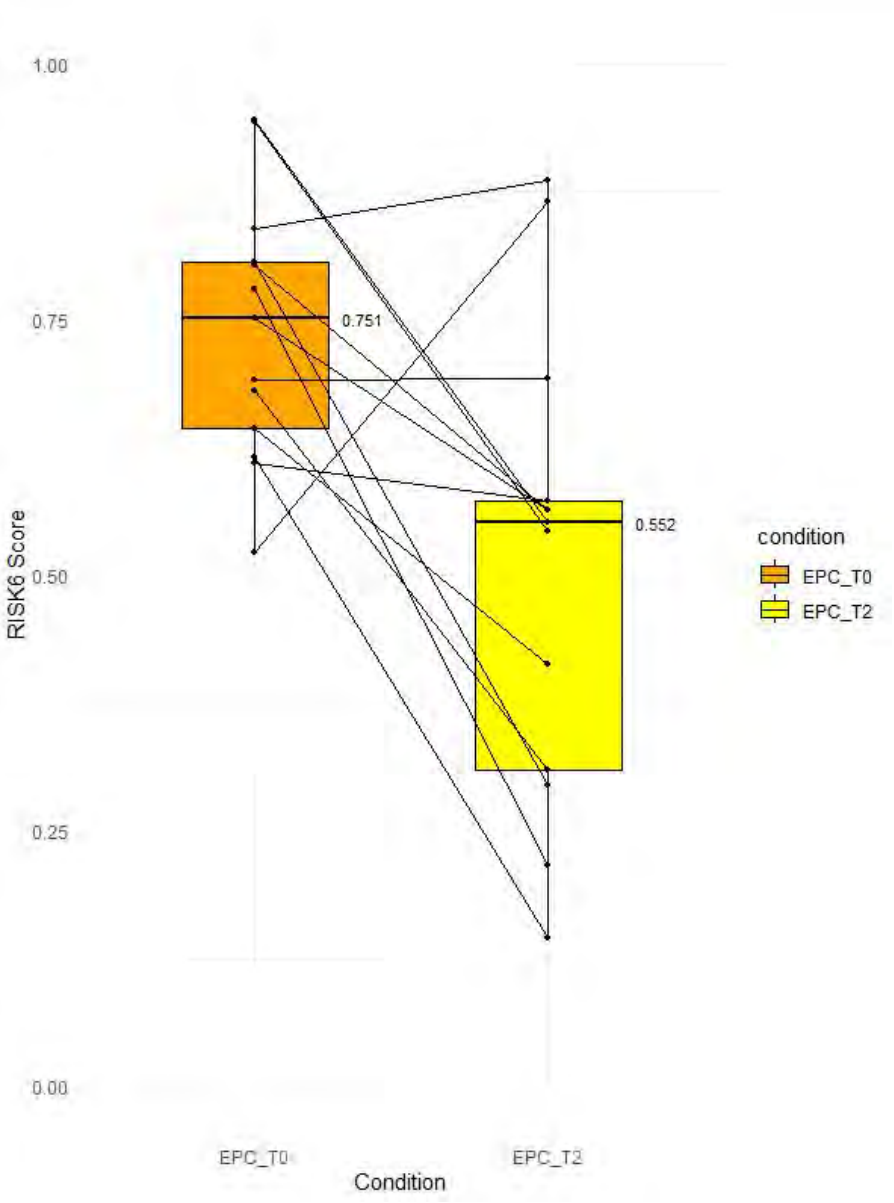


Figure 13: Paired Box Plots for RISK6 scores of EPC T0 and EPC T2 samples.

RISK6 effectively discriminated confirmed EPTB cases from healthy controls, achieving an AUC of 92% (Figure 14). The Youden index cutoff point was 0.596 with sensitivity and specificity of 93% and 80% respectively. For discrimination between confirmed EPTB cases

from unconfirmed ones the AUC was 84%. There were two Youden index cutoff points, a threshold value of 0.587 had sensitivity of 93% and specificity of 73% and a threshold value of 0.50 had a sensitivity of 100% with a specificity of 67%.

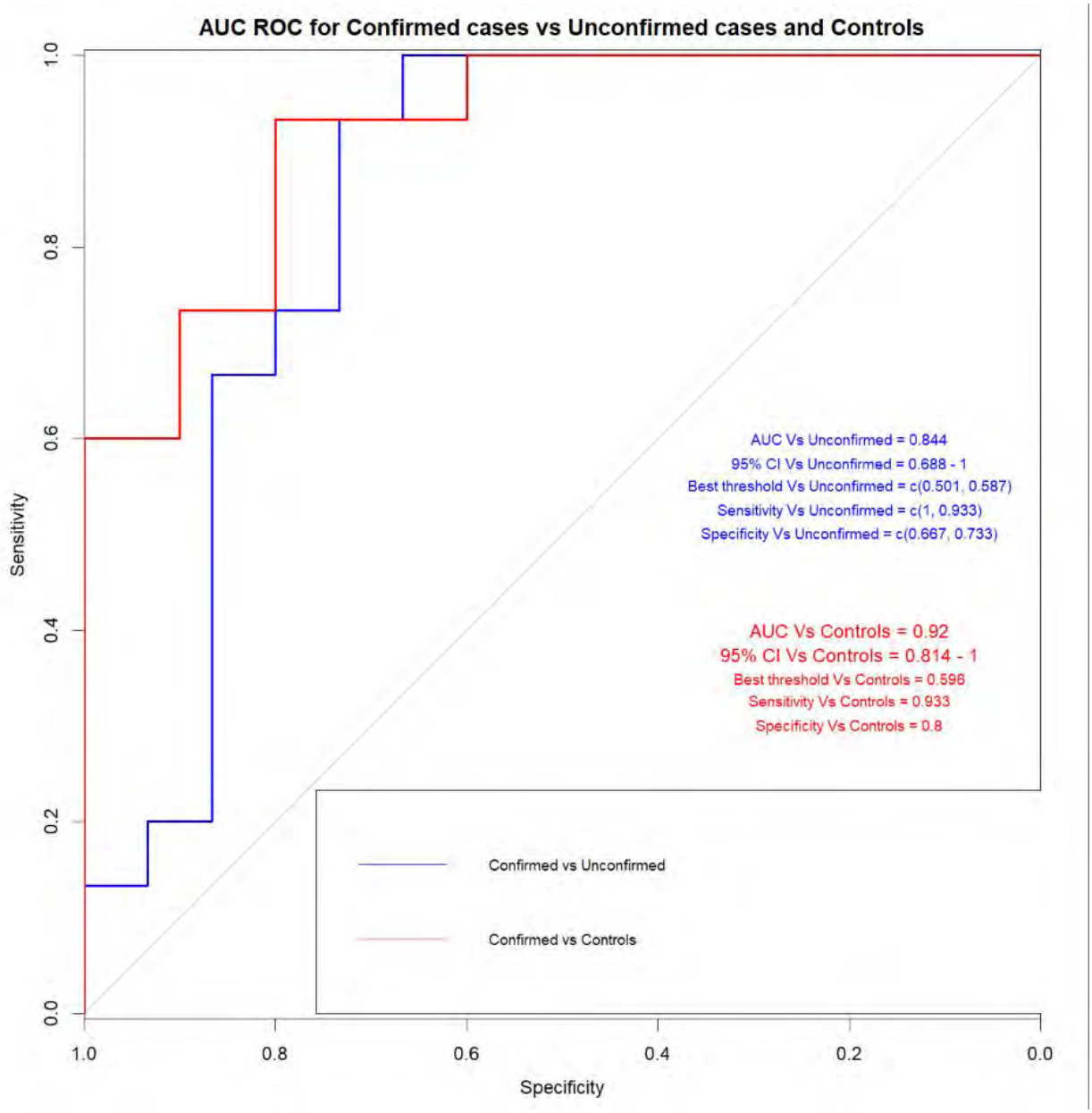


Figure 14: AUC ROC for Confirmed cases vs Unconfirmed cases and Controls.

After the end of treatment, RISK6 scores returned towards the baseline score of controls as represented in figure 15. The AUC vs controls and unconfirmed cases was 0.53 and 0.55

respectively.

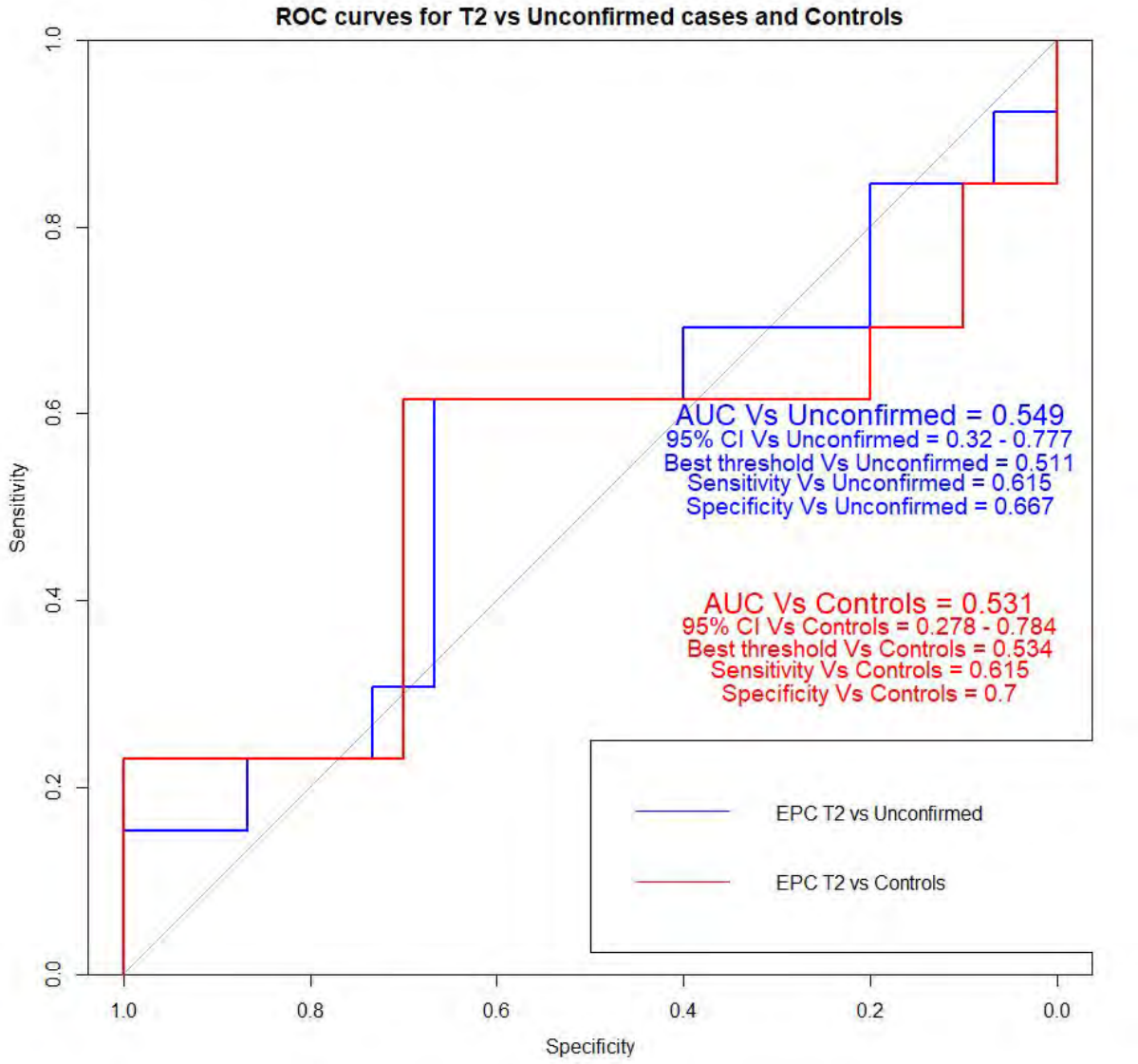


Figure 15: ROC AUC for Confirmed cases (T2) vs Unconfirmed cases and Controls.

Healthy controls with LTBI had an AUC of 0.619 vs healthy controls without LTBI (Figure 16). RISK6 distinguished LTBI with a sensitivity of 100% and specificity of 33%. Due to the low sample size, the 95% CI ranges from 0.156-1 and makes this finding statistically insignificant.

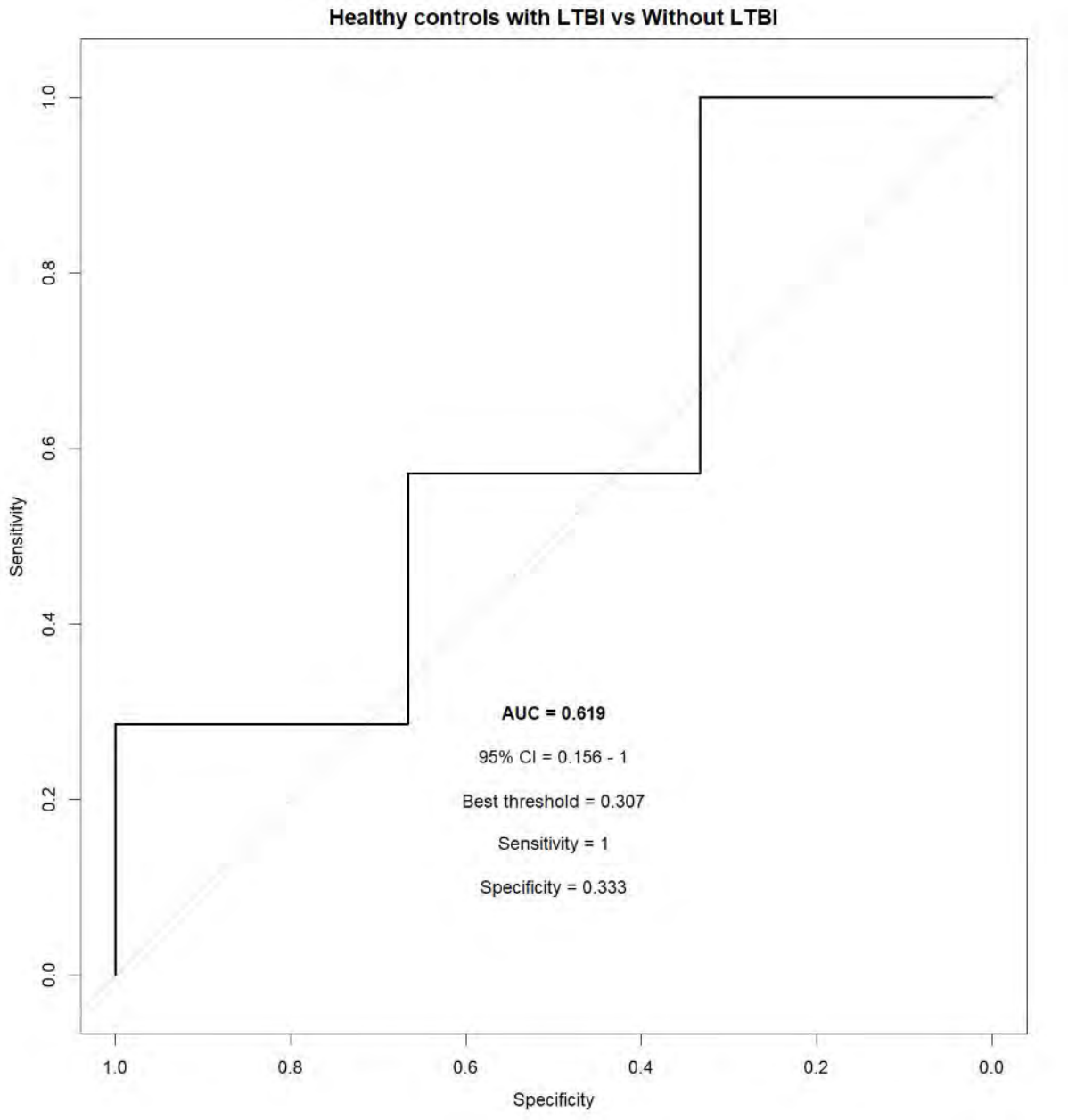


Figure 16: ROC AUC of Healthy controls with and without LTBI.

Chapter 4

Discussion

A host biomarker-based test is needed for EPTB due to the limitations of existing methods that rely on the presence of, or biomarkers based on MTB. These limitations are reflected in the results. Out of 15 confirmed EPTB patients, only 1 tested positive in AFB microscopy, and even then, with a low score of 1+. The high number of bacilli needed for confirmation through microscopy is simply not present in the majority of EPTB specimens. Culture, a gold standard for TB diagnosis, confirmed only 7 out of the 15 cases. 10 out of the 15 GeneXpert positive cases had burden levels of Low, Very Low, and Trace, reinstating that most EPTB samples have a low bacterial load. Although culture tests are technically more sensitive than GeneXpert assays for sputum samples, EPTB samples often require more extensive processing, which can increase the risk of damage to the limited number of bacilli present. Furthermore, while sputum samples are typically collected in and transported from designated treatment and screening centers in a standardized manner, EPTB samples originate from tertiary care points across the country, sometimes even from various diagnostic laboratories where sample portions have been used for other tests and called back by the physicians for forwarding towards EPTB testing. This complex sample collection situation increases the likelihood of contamination, exposure to conditions detrimental to the culture viability of bacilli, and the potential reduction of tissue where bacterial growth was most apparent. While detrimental, these factors are often unavoidable as recollection of samples can be highly inconvenient to the patient if not impossible. These challenges further emphasize the need for a test that can screen for TB without requiring to detection of the bacilli.

The RISK6 signature demonstrated its potential as a screening tool in this study. The baseline (T0) median RISK6 score for confirmed EPTB cases (EPC) was 0.75, significantly higher than the median of the healthy control group (EPA) at 0.453. The RISK6 signature exhibited a remarkable AUC of 0.92 (95% CI 81.4- 1) in discriminating confirmed EPTB cases from

healthy controls. With a cutoff value of 0.531, it had a sensitivity of 93% and a specificity of 80%. Similarly, when RISK6 was evaluated in Bangladesh for PTB, confirmed PTB vs healthy donors reached an AUC of 90.1% (95% CI 80.7–99.4) (Bayaa et al., 2021). The RISK6 signature exhibited a remarkable AUC of 92% in discriminating confirmed EPTB cases from healthy controls. With a cutoff value of 0.531, it had a sensitivity of 93% and a specificity of 80%.

The unconfirmed group, who had similar symptoms and level of clinical suspicion for EPTB, is important to consider when interpreting the accuracy of the signature, as people without any symptoms are unlikely to be referred for EPTB testing. Even against the unconfirmed group, RISK6 discriminated against confirmed cases with a high AUC of 0.84. There are two Youden index cutoff points to consider when discriminating with the unconfirmed group, a threshold value of 0.587 gave a sensitivity of 93% and specificity of 73% and a threshold value of 0.50 gave a sensitivity of 100% with a specificity of 67%. For a screening test, a higher sensitivity is appreciated, however, minimum WHO target profile criteria require a specificity of 70% for a tuberculosis screening test. (Organization, 2014). While the threshold level of 0.587 meets the criteria of both sensitivity and specificity, sacrificing 3% of sensitivity allows a 7% increase in sensitivity according to the results from the samples available for this analysis. Although the controls and the unconfirmed group had practically identical median RISK6 scores, some scores for unconfirmed cases were very high. Two particular cases, N04 and N05 had very high RISK6 scores of 0.845 and 0.830 respectively. N04 had pleural effusion, high fever, night sweats, and a contact history with PTB patient. A sputum sample was obtained from this patient as it was not possible to collect pleural fluid, which could be a reason behind negative microbiological tests. N05 had symptoms of gastrointestinal EPTB suspicion, but stool samples have one of the lowest sensitivities out of all EPTB samples with studies reporting as low as 20% (Talib et al., 2019). It is thus possible

that some cases might be true EPTB cases, but were categorized as unconfirmed due to the limitation of current methods.

Interestingly, there was no significant difference in the median RISK6 scores between culture-positive (0.751) and culture-negative (0.748) confirmed EPTB cases. This finding suggests that RISK6 is not influenced by culture positivity, making it a valuable diagnostic tool even when culture results are negative. The finding also reaffirms that cultural results may frequently be negative for genuine EPTB cases. Since EPTB often presents with low bacterial loads, this feature of RISK6 enhances its relevance for cases that are difficult to confirm microbiologically. (Chakravorty et al., 2005; Orvankundil et al., 2019)..

Among the 10 healthy controls, 7 tested positive for LTBI using the QFT-Plus test. RISK6 distinguished LTBI-positive controls from LTBI-negative ones with an AUC of 0.619. The wide confidence intervals (95% CI: 0.156-1) suggest that the finding is not statistically significant, which is expected as the sample size is small. It is still interesting to speculate, that inability to discriminate between LTBI infection could be a benefit. IGRA tests like QFT-Plus cannot distinguish between LTBI and ATB (Metcalfe et al., 2011; Sester et al., 2011). If RISK6 treats LTBI the same as people with no infection, the combination of both could prove useful in discriminating LTBI from active TB. IGRA positivity with a low RISK6 score would confirm LTBI, and IGRA positivity with a high RISK6 score would indicate active TB.

Post-treatment RISK6 scores in confirmed cases (EPC T2) decreased significantly and returned to baseline-level scores seen in controls (EPA). The AUC for discriminating between post-treatment confirmed cases and healthy controls or unconfirmed cases was 0.53 and 0.55, respectively, indicating that RISK6 loses discriminatory power after successful

treatment. This aligns with the expectation that the inflammatory or immune response, which RISK6 measures, diminishes as the infection resolves. While the overall results are aligned with the expected decrease in RISK6 score, 2 individuals did not experience a significant change (Less than 0.1 change) and the scores increased for 1 individual, C15, from 0.522 to 0.870. Individual C15 had symptoms of a cold abscess, joint pain, polyuria, and hematuria. C15 also had a previous history of pulmonary TB which the patient reported to be treated, suggesting dissemination of granulomas in various parts of the body which activated into the current episode of EPTB. C15 had a relatively high bacterial load indicated by Medium GeneXpert burden and culture positivity from a urine sample. The patient also required a treatment extension to 1 year from the standard 6 months before the physician considered him cured. RISK6 scores are expected to decrease with treatment progression, if scores increase instead, they are expected to indicate treatment failure(Penn-Nicholson et al., 2020). For C15, if the RISK6 score was measured a few months after treatment began, it might have been predicted that the patient needed an extension in advance. This result could also indicate a potential disruption in the responses measured by the RISK6 signature when the diseases progress past a certain point, limitations for individuals with a previous TB episode, or for TB episodes resulting from reactivation rather than new infection.

This study is limited by its relatively small sample size (n= 39), especially for the sub-sections discriminating by LTBI or culture positivity. Additionally, the limitations of microbial tests make it challenging to fully validate the performance of RISK6 in diagnosing EPTB without following up with patients who were categorized as unconfirmed. True evaluation as a Triage test also requires a prospective study design which was not implemented for this project. Treatment monitoring capability is somewhat hindered due to not including an intermediary blood collection timepoint (T1) between diagnosis and treatment completion. Future studies should have a prospective study design to find out

which individuals with high RISK6 scores develop EPTB and have a representative distribution of EPTB specimen types.

The RISK6 signature was designed to and needs to be, adopted into near point-of-care formats before it will become useful in high-burden, resource-constrained settings like Bangladesh. It will not be feasible to perform manual extraction and RT-qPCRs to screen for patients, as the infrastructure and human resource requirements would make RISK6 score calculation as restrictive as currently established diagnostic tests. Cepheid, the manufacturer of GeneXpert has incorporated the 3 gene signature by Sweeney et al, into the new GeneXpert MTB Host Response Assay. In a recent multi-country study for PTB, this assay achieved an AUC of 0.89 (95% CI 0·86–0·91) with a sensitivity of 90.3% and specificity of 62.6% which does not need the minimum specificity targets for a non-sputum-based triage test for pulmonary tuberculosis (Gupta-Wright et al., 2024). If RISK6 is adopted to the GeneXpert platform it will be easy to deploy as GeneXpert has established a significant presence in TB diagnostics. QuantuMDx has already incorporated RISK6 into their newly developed TB Host Immune Response Assay (TB-HIRA) and working towards incorporating it in a portable cassette-based format "Q-POC" for use at the point of care. These upcoming solutions should be evaluated among EPTB as well, and not PTB alone.

The RISK6 transcriptomic score demonstrated strong discriminatory power in differentiating confirmed EPTB cases from both unconfirmed cases and healthy controls. Meeting the WHO target product profile performance for a triage test, the findings in this study show that RISK6 shows promise as a clinical tool for combating EPTB. This biomarker has the potential to transform EPTB diagnosis by enabling physicians to screen for the disease even when there is insufficient clinical suspicion to warrant a biopsy. Its ability to monitor treatment response, and track disease resolution further emphasizes its clinical potential. Overall, this study

highlights the need for improved diagnostic tools in the fight against EPTB and supports the continued investigation and development of RISK6 into a point-of-care test.

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Appendix A.

Table A1: Assays used for RISK6 genes

Gene Symbol	Gene name	Assay ID
GBP2	guanylate binding protein 2	Hs00894846_g1
SERPING1	serpin family G member 1	Hs00934329_m1
FCGR1B	Fc fragment of IgG receptor Ib	Hs02341825_m1
SDR39U1	Short chain dehydrogenase/reductase family 39U member 1	Hs01016970_g1
TRMT2A	tRNA methyltransferase 2 homolog A	Hs01000041_g1
TUBGCP6	tubulin gamma complex associated protein 6	Hs00363509_g1

Table A2: Threshold values, sensitivity, and specificity for figure 12

AUC VS Unconfirmed				AUC VS Controls			
	Threshold	Sensitivity	Specificity		Threshold	Sensitivity	Specificity
1	-Inf	1.00000000	0.00000000	1	-Inf	1.00000000	0.0
2	0.2116145	1.00000000	0.06666667	2	0.3068824	1.00000000	0.1
3	0.2650160	1.00000000	0.13333333	3	0.3777314	1.00000000	0.2
4	0.3257148	1.00000000	0.20000000	4	0.4280712	1.00000000	0.3
5	0.3732153	1.00000000	0.26666667	5	0.4338350	1.00000000	0.4
6	0.3865725	1.00000000	0.33333333	6	0.4530194	1.00000000	0.5
7	0.4141820	1.00000000	0.40000000	7	0.4950093	1.00000000	0.6
8	0.4480485	1.00000000	0.46666667	8	0.5236079	0.93333333	0.6
9	0.4665415	1.00000000	0.53333333	9	0.5578804	0.93333333	0.7
10	0.4745091	1.00000000	0.60000000	10	0.5956500	0.93333333	0.8
11	0.5008315	1.00000000	0.66666667	11	0.6049462	0.86666667	0.8
12	0.5469669	0.93333333	0.66666667	12	0.6120959	0.80000000	0.8
13	0.5865494	0.93333333	0.73333333	13	0.6192030	0.73333333	0.8
14	0.6049462	0.86666667	0.73333333	14	0.6329361	0.73333333	0.9
15	0.6120959	0.80000000	0.73333333	15	0.6611713	0.66666667	0.9
16	0.6231867	0.73333333	0.73333333	16	0.6848522	0.60000000	0.9
17	0.6369199	0.73333333	0.80000000	17	0.6901715	0.60000000	1.0
18	0.6490444	0.66666667	0.80000000	18	0.7205059	0.53333333	1.0
19	0.6674900	0.66666667	0.86666667	19	0.7651909	0.46666667	1.0
20	0.6849362	0.60000000	0.86666667	20	0.7912119	0.40000000	1.0
21	0.7205059	0.53333333	0.86666667	21	0.8044918	0.33333333	1.0
22	0.7651909	0.46666667	0.86666667	22	0.8065109	0.26666667	1.0
23	0.7912119	0.40000000	0.86666667	23	0.8225896	0.20000000	1.0
24	0.8044918	0.33333333	0.86666667	24	0.8911222	0.13333333	1.0
25	0.8065109	0.26666667	0.86666667	25	0.9444588	0.06666667	1.0
26	0.8183992	0.20000000	0.86666667	26	Inf	0.00000000	1.0
27	0.8341524	0.20000000	0.93333333				
28	0.8414884	0.13333333	0.93333333				
29	0.8942678	0.13333333	1.00000000				
30	0.9444588	0.06666667	1.00000000				
31	Inf	0.00000000	1.00000000				

Questionnaire for Study participants:

Questionnaires

Clinical evaluation of biomarker-based diagnosis of child pulmonary TB and extrapulmonary TB

Study conducted by the International Centre for Diarrhoeal Diseases Research, Bangladesh

Hospital ID ☐☐ **Patient ID:** ☐☐☐☐☐☐

Interviewer ID: ☐☐ **Interview Date: ...../...../..... (DD/MM/YY)**

Hospital Registration No...... **Date of sample collection ...../...../..... (DD/MM/YY)**

A. Particulars of the patient:

A1. Name : _____ A2. Contact no: 1. _____

2. _____

A3. Date of birth ...../...../..... (DD/MM/YY) A4. Age (year): ☐☐

A5. Sex : 1= male; 2 = female ☐ A6. Dwelling: 1=rural; 2=urban ☐

A7. Height (cm) _____ A8. Weight (kg) _____

A9. Educational Status (year) [If illiterate code "00"] ☐☐

A10. Religion ☐
1 = Islam
2 = Hinduism
3 = Buddhism
4 = Christianity
5 = Others (specify) _____

A11. Marital Status ☐
1 = Single (Never married)
2 = Married
3 = Divorced
4 = Separated
5 = Widow
6 = Others (specify) _____

A12. Occupation ☐
01 = Farmer, Agriculture
02 = Day laborer
03 = Petty business
04 = Small business
05 = Business
06 = Government Service
07 = Private Service
08 = Rickshaw puller, Van driver
09 = Student
10 = Garments / factory worker
11 = Housewife
12 = Unemployed / Retired
13= Teacher
14= Doctor
77 = Others (specify) _____

A13. **Present Address:**

Village/ House No: ----------

Road No ----------

P.O----------

Thana: ----------

District:----------

A14. **Permanent Address:**

Village/ House No:----------

Road No ----------

P.O----------

Thana: ----------

District: ----------

B. Clinical Presentation:

B1. Cough ☐ B 1.1 Cough > 3 weeks ☐

1 = yes; 2 = no; 1 = yes; 2 = no

(If "yes" go to next question. If "no" go to question B 2)

B 1.2 Cough in days ---------- B 1.3 Sputum 1 = yes; 2 = no ☐

B 1.4 Haemoptysis ☐ B2. BMI < 17.0 ☐

1 = yes; 2 = no; 3= not applicable 1 = yes; 2 = no

B3. Fever ☐ B3.1 Fever in days ----------

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to question B4)

B3.2 Character of Fever ☐ B4. Evening rise in temperature ☐

1 = High grade and intermittent 1 = yes; 2 = no

2 = High grade and continuous

3 = Low grade and intermittent

4 = Low grade and continuous

B 5. Night sweating 1 = yes; 2 = no ☐ B 6. Chest pain 1 = yes; 2 = no ☐

B 7. Loss of appetite ☐ B8. Shortness of breath ☐

1 = yes; 2 = no 1 = yes; 2 = no

B9. Significant weight loss ☐ B 9.1 Duration of weight loss (days) _____

1=yes; 2=no

C. Treatment History:

C1. Did you have the same symptoms prior to this episode? ☐ C 1.1 When (year) ----------

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to question C 2)

C2. Did you have other symptoms of lung disease prior to this episode (chest pain, shortness of breath)? ☐ C 2.1 When (year) ----------

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to question C 3)

C3. Previously diagnosed as TB ☐ C 3.1 Where ----------

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to section D)

C 3.2 How many times ☐

C 3.3 Type of TB? ☐

1=Pulmonary TB

2=Extra-pulmonary TB (pls. Specify site.....)

C 4. Received treatment for TB ☐
1 = yes; 2 = no
(If "yes" go to next question. If "no" go to section D)

C 4.1 If yes, Treatment regimen ☐

1 = Category I (Isoniazid, Rifampicin, Ethambutol, Pyrazinamide)

2 = Category II (Isoniazid, Rifampicin, Ethambutol, Pyrazinamide, and Injection Streptomycin)

3 = Others (pls. specify)

_____

_____

C 5. Treatment Completed ☐
1 = yes; 2 = no
(If "yes" go to next question. If "no" go to C 6)

C 5.1 If yes, What was the outcome ☐

1=Cured

2=Treatment complete

3 =Lost to follow-up/Default

4=Treatment failure

5= Others (pls. specify) _____

C 6 Duration of treatment> 1 month ☐
1 = yes; 2 = no

C 6.1 Ever injection taken > 1 month ☐

1 = yes; 2 = no

D. Family History:

D1. Family members ☐☐

D2. TB patients in family ☐

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to section E)

D 2.1 If yes, No of TB patients in the family ☐

D 2.2 Status of the TB patient in family ☐

1 = On treatment

2 = Cured/treatment complete

3 = Treatment failure

4 = Treatment default

5 = Others (pls. specify) _____

D 2.3 Age of the TB patient (years) ☐☐

D 2.4 Relationship with subject: _____

E. Personal History:

E1. Smoking ☐

E 1.1 If current or previous history of smoking,

1 = yes; 2 = never; 3= previous smoker

(If 1 or 3 go to next question. If 2 go to question E 2)

no. of sticks/day ----------

E 1.2 How long (years) ----------

E2. Exposure to TB patient ☐

1 = yes; 2 = no

(If "yes" go to next question. If "no" go to question E 3)

E 2.1 If yes, Relationship with subject.....

E 2.2 Duration of exposure (days) ☐☐

E3. Drug abuse? ☐

1 = yes; 2 = never; 3= previous abuser

(If 1 or 3 go to next question. If 2 go to question E 4)

E 3.1 If current or previous h/ of drug abuse, injectable drug? ☐

1 = yes; 2 = no

E 3.2 How many times/week _____

E 3.3 How long? _____ (years)

E4. Alcohol ☐

1 = yes; 2 = never; 3= previous alcoholic

(If 1 or 3 go to next question. If 2 go to question E 5)

E 4.1 If current or previous h/ of alcohol intake,, how many times/week _____

E 4.2 How long (years) ----------

E5. Diabetes ☐

1 = yes; 2 = no; 3= not known

(If 1 go to next question. If 2 or 3 go to question E 6)

E5.1 How long (years) ----------

E 5.2 If yes, drugs taken ☐

1 = yes; 2 = no

E 5.2 Name of drugs _____

E6. History of incarceration? ☐

1 = yes; 2 = no

E 6.1 How many times? _____

E 6.2 How long? _____ (years)

Responsible Officer: .....