Extrapulmonary tuberculosis in Pakistan: Challenges in diagnosis and detection of drug resistance

Sabira Tahseen

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Name: Sabira Tahseen

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List of abbreviations:

AFB	Acid Fast bacilli	MTBC	Mycobacterium tuberculosis complex
ADA	Adenosine Deaminase	NAAT	nucleic-acid amplification tests
BCG	bacilli Calmette-Guérin	NGS	Next Generation Sequencing
CMU	Central Management Unit	NPV	Negative Predictive Value
CNSTB	Central Nervous System Tuberculosis	NTM	Non-Tuberculous mycobacteria
DNA	Deoxyribose nucleic acid	NTP	National tuberculosis program
DOT	Directly observed therapy	NTRL	National tuberculosis Reference laboratory
DST	drug-susceptibility testing	OATB	Osteoarticular tuberculosis
DRS	Drug resistance survey	PCR	Polymerase chain reaction
E	Ethambutol	pDST	phenotypic drug-susceptibility testing
EPTB	Extrapulmonary Tuberculosis	PMDT	Programmatic management of drug resistant TB
FATA	Federally Administered Tribal Areas	PPD	purified protein derivative
FQ	Fluoroquinolone	PPM	public–public and public–private mix
GB	Gilgit-Baltistan	PPV	Positive Predictive Value
GITB	Gastrointestinal TB	Pre-XDR	RR-TB plus resistance to FQ
GUTB	Genito urinary Tuberculosis	PTP	Provincial tuberculosis program
gDST	Genotypic Drug susceptibility testing	R	Rifampicin
H	Isoniazid	RR-TB	rifampicin-resistant Tuberculosis
HBC	High Burden countries	SLI	Second-line injectable
HIV	human immunodeficiency virus	SRL	Supranational Reference Laboratory
ICT	Islamabad Capital Territory	TAT	Turnaround time
IFN	Interferon	TB	Tuberculosis
IGRA	Interferon Gamma release assay	TBM	tuberculous meningitis
IPD	Individual Patient data	TBMU	Basic management unit (for TB)
K	= 1000	TCH	Teaching/tertiary health care hospital
KP	Khyber Pakhtunkhwa	TPE	Tuberculous Pleural effusion
LAM	Lipoarabinomannan	TST	Tuberculin skin test
LoD	Limit of detection	TNF	Tumor necrotic Factor
LPA	Line probe assay	WGS	Whole Genome Sequencing
LTBI	Latent TB Infection	WHO	World Health Organization
MDR-TB	Multi-drug resistant tuberculosis	WRD	WHO endorsed rapid diagnostics
MDR/RR-TB	Multidrug-resistant TB or rifampicin resistant Tuberculosis	XDR-TB	Extensively drug resistant tuberculosis
M. tuberculosis	Mycobacterium tuberculosis	Z	pyrazinamide
MGIT	Mycobacteria Growth Indicator Tube		

Definitions

Standard definitions used in the document are given below [1-4]

- **New TB case**: a newly registered episode of TB in a patient who has never been treated for TB or who has taken anti-TB medicines for less than 1 month [1, 2].

- **Previously treated TB case:** patients who have received 1 month or more of anti-TB medicines in the past. Previously treated cases may have been treated with a first-line regimen for drug-susceptible TB or a second-line regimen for drug-resistant forms (e.g., shorter MDR-TB regimen) [1, 2].

- A **bacteriologically confirmed TB** case is one from whom a biological specimen is positive by smear microscopy, culture, or a WHO-recommended rapid diagnostic (such as Xpert MTB/RIF) [1, 2].

- A **clinically diagnosed TB** case is a person who does not fulfill the criteria for bacteriological confirmation but has been diagnosed with active TB by a clinician or other medical practitioner who has decided to give the patient a full course of TB treatment. This definition includes cases diagnosed on the basis of X-ray abnormalities or suggestive histology and extrapulmonary cases without laboratory confirmation [1, 2].

- **Pulmonary tuberculosis** (PTB) refers to any bacteriologically confirmed or clinically diagnosed case of TB involving the lung parenchyma or the tracheobronchial tree [1, 2].

- **Extrapulmonary tuberculosis** (EPTB) refers to any bacteriologically confirmed or clinically diagnosed case of TB involving organs other than the lungs (e.g., pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges).

- **Extensive (or advanced) pulmonary tuberculosis (TB) disease:** Presence of bilateral cavitary disease or extensive parenchymal damage on chest radiography. In children aged under 15 years, advanced disease is usually defined by the presence of cavities or bilateral disease on chest radiography [1, 2].

- **Severe extrapulmonary TB:** presence of miliary TB or TB meningitis. In children aged under 15 years, extrapulmonary forms of disease other than lymphadenopathy (peripheral nodes or isolated mediastinal mass without compression) is considered as severe [1, 2].

- **Drug susceptibility testing (DST):** in vitro testing using either molecular, genotypic techniques to detect resistance-conferring mutations, or phenotypic methods to determine susceptibility to a medicine [3, 4].

- **Drug-susceptible TB (DS-TB)**: A bacteriologically confirmed or clinically diagnosed case of TB without evidence of infection with strains resistant to rifampicin and isoniazid [3, 4].

- **Rifampicin-susceptible, isoniazid-resistant TB (Hr-TB):** TB caused by *M. tuberculosis* strains resistant to isoniazid and susceptible to rifampicin [3, 4].

- **Rifampicin-resistant TB** (RR-TB): TB caused by *M. tuberculosis* strains resistant to rifampicin. These strains may be susceptible or resistant to isoniazid (i.e., MDR-TB), or resistant to other first-line or second-line TB medicines [3, 4].

- **Multidrug-resistant TB (MDR-TB):** is defined as disease due to *M. tuberculosis* that is resistant to isoniazid (H) and rifampicin (R) with or without resistance to other drugs [3, 4].

- **Pre-XDR-TB:** TB caused by *M. tuberculosis is* strains that fulfil the definition of multidrug resistant and rifampicin-resistant TB (MDR/RR-TB) and which are also resistant to any fluoroquinolone [3, 4].

- **XDR-TB**: TB caused by *M. tuberculosis* strains that fulfil the definition of MDR/RR-TB and which are also resistant to any fluoroquinolone and at least one additional Group A drug (Group A drugs are the most potent group of drugs in the ranking of second-line medicines for the treatment of drug-resistant forms of TB using longer treatment regimens and comprise levofloxacin, moxifloxacin, bedaquiline and linezolid) [3, 4].

Scientific environment

This thesis is a result of collaboration between the Centre for International Health (CIH) at the Department of Global Public Health and Primary Care, Faculty of Medicine, University of Bergen (UiB), Gulab Devi Chest Hospital, Lahore, Pakistan and the National TB Control Program (NTP), Islamabad, Pakistan.

The patient enrollment, clinical examinations, microbiology and histopathology examinations, and follow-ups of TB patients were done at the Gulab Devi Hospital. Drug susceptibility testing was done at the National TB reference laboratory, National TB Control Program. Studies from the prospective cohort are included in papers 2 and 3

The other two studies were retrospective. For paper-1, routine TB surveillance data was collected in collaboration with provincial (Punjab, Sindh, Khyber-Pakhtunkhwa, Balochistan) and regional (Azad Jammu Kashmir, Gilgit Baltistan, and Islamabad capital territory) tuberculosis Programs. For paper-4, routine drug susceptibility testing data from the National TB reference laboratory was analyzed.

The main supervisor and co-supervisors provided guidance and coauthored following

papers

1	Professor Tehmina Mustafa (Centre for International Health, Department of Global Public Health and Primary Care, UiB)	Main Supervisor	1,2,3,4
2	Professor Lisbet Sviland (Department of Clinical Medicine, UiB)	Co-supervisor	2,3
3	Dr Nauman Safdar	Co-supervisor	1,2,3

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Summary

Background: Pakistan is among the top five high-burden countries for tuberculosis (TB) and drug-resistant TB. Extrapulmonary TB (EPTB) accounts for 20% of all notified TB patients but there is little information on disease manifestations, bacteriological diagnoses, and prevalence of anti-TB drug resistance in EPTB.

Objective: The overall objective was to study EPTB in Pakistan, and evaluate the usefulness of conventional and new diagnostic tools in diagnosing and detecting drug resistance in EPTB patients. The first study primarily aimed to describe the disease manifestations among EPTB patients notified in Pakistan. The second study aimed to assess the performance of a rapid molecular assay, Xpert MTB/RIF (Xpert), and histological examination in the diagnoses of tuberculous lymphadenitis. The third study aimed to determine the prevalence of rifampicin and other anti-TB drug resistance in new EPTB patients and the fourth study aimed to assess the prevalence and genetic profile of isoniazid resistance and associated resistance to fluoroquinolone and pyrazinamide.

Material and Methods: In study-1, descriptive analysis was performed on a retrospective cohort of TB patients notified nationwide in 2016. Studies 2 and 3 were performed on a prospective cohort of patients presumed to have EPTB in a tertiary care hospital. In study-2, TB treatment naïve people with enlarged lymph nodes were included and excision biopsy specimens were tested by histology, Xpert, and culture. In study-3, patients with pleural effusion in addition to people in study-2 with enlarged lymph nodes were included. Pleural fluid sediments were tested for smear, Xpert MTB/RIF, and culture. Phenotypic drug susceptibility testing (DST) was performed using automated liquid DST (MGIT 960) and genotypic DST by line-probe assays (LPA). For study-4, a five-year retrospective DST data set was analyzed of TB patients tested in the National TB reference laboratory by phenotypic and/or genotypic DST methods.

Results: In study-1, individual patient data was collected of 54092 TB cases notified in 2016. Among 15790 EPTB patients included, the three most common forms of EPTB were

pleural (29.6%), lymphatic (22.7%), and abdominal TB (21.0%). Pleural TB was the most common among adults (34.2%) and abdominal TB in children (38.4%). The likelihood of having EPTB, was 1.1 times high for females, 2.0 times for children, and 3.3 times for residents of provinces in the Northwest. The treatment success rate for all types of EPTB included in the study was high except for TB meningitis.

In study-2, the performance of diagnostic tools in a cohort of 390 patients with enlarged lymph nodes was analyzed and among these 11 (2.8%) were positive by AFB microscopy, 124 (31.8%) by Xpert, 137 (35.1%) by culture, and histopathology was consistent with TB in 208 (53.3%). Using composite results, lymph node TB was diagnosed in 228 of which 78% were bacteriologically confirmed. Histopathology compared to Xpert had higher sensitivity (93 vs. 62%) but lower specificity (68 vs.83%) and in sub-group analysis, the sensitivity of Xpert was higher in patients with short clinical history.

In study-3, bacteriological diagnosis and prevalence of drug resistance was analyzed in 671 study participants. Bacteriologically confirmed TB was diagnosed in 255 and DST results were available for 72.5% (n=185) of EPTB patients. MDR-TB was reported in 2.2% (95% CI, 0.6–5.4), resistance to rifampicin in 2.7% (95% CI, 0.9–6.2), isoniazid in 7.6% (95% CI, 4.1–12.4), ethambutol in 1.1% (95% CI, 0.1–3.9), pyrazinamide in 2.2% (95%CI, 0.9–5.5) and fluoroquinolones in 6.0% (95% CI, 3.0–10.4). The sensitivity and specificity of LPA-DST was 100% and 98.8% respectively for rifampicin, isoniazid, and fluoroquinolone. Rifampicin results were available by all three methods for 82 patients and of these five were reported rifampicin resistant by Xpert and only one was confirmed by LPA and none by phenotypic DST.

In study-4, a retrospective DST data set of 11045 TB patients was analyzed. Both phenotypic and genotypic DST results were available for 80% of cases. A significant difference was reported between resistance detected by phenotypic and genotypic DST methods with 16% discordance in rifampicin and isoniazid. Among rifampicin-sensitive new EPTB, isoniazid resistance was 6.8% compared to 9.8% in PTB. The genetic profile

of isoniazid resistance was similar in PTB and EPTB but the difference was statistically significant between rifampicin-resistant and sensitive populations for isoniazid-resistant conferring mutations detected in 87% vs 71.6%, the prevalence of *katG* mutations in 76.1% vs 41.2% and *inhA in 7.6*% vs 30.2% respectively. A significantly higher levofloxacin resistance was seen associated with isoniazid resistance.

Conclusions: Pleural and lymphatic TB collectively comprised 50% of all notified EPTB cases. TB was diagnosed by Xpert alone in 54%, while in combination with histopathology in more than 95% of all TB lymph node cases. Rifampicin and isoniazid resistance was lower in the new EPTB compared to PTB; the difference was not statistically significant. The genetic profile of isoniazid resistance and associated fluoroquinolone and pyrazinamide resistance in EPTB was comparable with PTB.

Sammendrag

Bakgrunn: Pakistan er blant de fem landene med høyest forekomst av tuberkulose (TB) og legemiddelresistent TB. Ekstrapulmonær TB (EPTB) utgjør 20% av alle registrerte TB-pasienter, men det er lite informasjon om sykdomsmanifestasjon, bakteriologiske diagnoser og forekomst av legemiddelresistens i EPTB.

Mål: Det overordnede målet var å studere EPTB i Pakistan og evaluere nytten av konvensjonelle og nye diagnostiske verktøy for å diagnostisere og oppdage legemiddelresistens hos pasienter med EPTB. Hovedmålet med den første studien var å beskrive sykdomstegnene blant pasienter med EPTB. Målet med den andre studien var å vurdere den diagnostiske ytelsen av en rask molekylær analyse, Xpert MTB/RIF (Xpert), og histologisk undersøkelse for tuberkuløs lymfadenitt. Målet med den tredje studien var å studere forekomsten av rifampicin og annen legemiddelresistens mot TB hos nye pasienter med EPTB, og den fjerde studien hadde som mål å vurdere forekomsten og den genetiske profilen til isoniazidresistens samt tilhørende resistens mot fluorokinolon og pyrazinamid.

Materiale og metoder: I studie 1 ble det utført deskriptiv analyse av en retrospektiv kohort av TB-pasienter registrert nasjonalt i 2016. Studie 2 og 3 ble utført på en prospektiv kohort av pasienter med antatt EPTB ved et tertiært sykehus. I studie 2 ble personer som ikke hadde fått TB-behandling og hadde forstørrede lymfeknuter inkludert, og vevsprøver fra eksisjonsbiopsier ble undersøkt med histologi, Xpert og dyrkning. I studie 3 ble pasienter med pleural effusjon i tillegg til personene i studie 2 med forstørrede lymfeknuter, inkludert. Pleuravæske ble undersøkt for syrefastestaver (AFB)-mikroskopi, Xpert MTB/RIF og dyrkning. Fenotypisk legemiddelsusceptibilitetstesting (DST) ble utført ved bruk av automatisert flytende DST (MGIT 960), og genotypisk DST ble utført ved hjelp av line-probe-undersøkelser (LPA). I studie 4 ble et fem års retrospektiv DST-datasett analysert for pasienter som ble testet ved det nasjonale TB-referanselaboratoriet ved hjelp av både fenotypiske og genotypiske DST-metoder.

Resultater: I studie 1 ble individuelle pasientdata samlet inn fra 54 092 registrerte TB-

tilfeller i 2016. Blant disse hadde 15 790 pasienter EPTB. De tre vanligste formene for EPTB var pleural (29,6 %), lymfatisk (22,7%) og abdominal TB (21,0%). Pleural TB var den vanligste blant voksne (34,2%), mens abdominal TB var mest utbredt blant barn (38,4%). Sannsynligheten for å ha EPTB var 1,1 ganger høyere for kvinner, 2,0 ganger høyere for barn og 3,3 ganger høyere for innbyggere i provinsene nordvest i landet. Behandlingssuksessraten for alle typer EPTB som var inkludert i studien, var høy, bortsett fra for TB-meningitt.

I studie 2 ble ytelsen til diagnostiske verktøy analysert i en kohort på 390 pasienter med forstørrede lymfeknuter. Blant disse var 11 (2,8%) positive ved AFB-mikroskopi, 124 (31,8%) ved Xpert, 137 (35,1%) ved dyrkning, og histopatologi viste TB hos 208 (53,3%). Ved bruk av kombinerte resultater ble lymfeknutetuberkulose diagnostisert hos 228 pasienter, hvorav 78% ble bekreftet ved bakteriologiske tester. Sammenlignet med Xpert hadde histopatologi høyere sensitivitet (93% mot 62%), men lavere spesifisitet (68% mot 83%), og i undergruppeanalyser var sensitiviteten til Xpert høyere hos pasienter med kort klinisk historie.

I studie 3 ble bakteriologisk diagnose og forekomst av legemiddelresistens analysert hos 671 studiepersoner. TB ble bekreftet bakteriologisk hos 255, og DST-resultater var tilgjengelige for 72,5% (n = 185) av pasientene med EPTB. Multidrug resistant -TB ble rapportert hos 2,2% (95% CI, 0,6–5,4), resistens mot rifampicin hos 2,7% (95% CI, 0,9–6,2), isoniazid hos 7,6% (95% CI, 4,1–12,4), ethambutol hos 1,1% (95% CI, 0,1–3,9), pyrazinamid hos 2,2% (95% CI, 0,9–5,5) og fluorokinoloner hos 6,0% (95% CI, 3,0–10,4). Sensitiviteten og spesifisiteten til LPA-DST var henholdsvis 100% og 98,8% for rifampicin, isoniazid og fluorokinolon. DST-resultater for rifampicin var tilgjengelige for 82 pasienter ved alle tre metoder og av disse ble fem rapportert som rifampicinresistente ved Xpert, men bare én ble bekreftet ved LPA, og ingen ble bekreftet ved fenotypisk DST.

I studie 4 ble et retrospektivt DST-datasett med 11 045 TB-pasienter analysert. Både fenotypiske og genotypiske DST-resultater var tilgjengelige for 80 % av tilfellene. Det ble

rapportert en betydelig forskjell mellom resistens som ble påvist ved fenotypisk og genotypisk DST-metoder, med en uoverensstemmelse på 16% for rifampicin og isoniazid. Blant nye EPTB-pasienter med rifampicinsensitivitet ble isoniazidresistens påvist hos 6,8%, sammenlignet med 9,8% hos pasienter med PTB. Den genetiske profilen for isoniazidresistens var lik for PTB og EPTB, men forskjellen var statistisk signifikant mellom rifampicinresistente og rifampicinsensitive populasjoner når det gjaldt påviste mutasjoner knyttet til isoniazidresistens, med 87% mot 71,6% og forekomsten av katG-mutasjoner på 76,1% mot 41,2% og inhA-mutasjoner på 7,6% mot 30,2%, henholdsvis. Det ble observert en signifikant høyere forekomst av levofloxacinresistens i forbindelse med isoniazidresistens.

Konklusjoner: Pleural- og lymfatisk TB utgjorde til sammen 50% av alle registrerte tilfeller av EPTB. TB ble diagnostisert ved kun hjelp av Xpert i 54% av tilfellene, mens kombinasjonen av Xpert og histopatologi bekreftet diagnosen i over 95% av alle tilfeller med TB i lymfeknuter. Forekomsten av rifampicin- og isoniazidresistens var lavere blant nye EPTB-pasienter sammenlignet med PTB, men forskjellen var ikke statistisk signifikant. Den genetiske profilen for isoniazidresistens og tilhørende resistens mot fluorokinolon og pyrazinamid var sammenlignbar mellom EPTB og PTB.

List of publications

1. Tahseen S, Khanzada FM, Baloch AQ, Abbas Q, Bhutto MM, Alizai AW, Zaman S, Qasim Z, Durrani, Farough MK, Ambreen A, Safdar N, Mustafa T. Extrapulmonary tuberculosis in Pakistan- A nation-wide multicenter retrospective study. PLoS One. 2020 Apr 28;15(4): e0232134. doi: 10.1371/journal.pone.0232134. PMID: 32343714; PMCID: PMC7188211.

2. Tahseen S, Ambreen A, Ishtiaq S, Khanzada FM, Safdar N, Sviland L, Mustafa T. The value of histological examination in the diagnosis of tuberculous lymphadenitis in the era of rapid molecular diagnosis, *Scientific Reports* **volume 12**, Article number: 8949 (2022)

3. Tahseen S, Ambreen A, Masood F, Qadir M, Hussain A, Jamil M, Safdar N, Sviland L, Mustafa T. Primary drug resistance in extra-pulmonary tuberculosis: a hospital-based prospective study from Pakistan. Int J Tuberc Lung Dis. 2019 Aug 1;23(8):900-906. doi: 10.5588/ijtld.18.0531. PMID: 31533879.

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1. INTRODUCTION

Tuberculosis (TB) is one of the oldest diseases known to affect humans and studies of human skeletons have shown that it has affected humans for thousands of years [5-10]. TB is caused by the bacillus *Mycobacterium tuberculosis*, (*M. tuberculosis*). The causative organism remained unknown until 1882 when Dr Robert Koch announced his discovery [11, 12]. The disease is spread when people who are sick with TB, expel bacteria into the air by coughing. While pulmonary tuberculosis (PTB) is the most common presentation, TB can involve every organ system.

1.1.   Status of TB Epidemics

The first vaccination was introduced in 1921 and the first effective TB treatment drug was developed in the 1940s and an effective TB treatment regimen has been available since 1970 [13, 14].TB is now a preventable, treatable, and curable disease and despite 100 years of vaccination and 70 years of chemotherapy, TB still remains the world's leading cause of death from an infectious agent and claims more than a million lives each year with enormous impacts on families and communities [6-9, 15-17].

1.1.1. TB disease burden

About a quarter (2 billion) of the global population is estimated to be infected with *M. tuberculosis* [18, 19]. On average, only 5-10% of those who are infected develop active TB disease during their lifetime, with 3 to 5% developing TB in the first year following infection and an additional 3 to 5% any time after the first year [20-22]. TB is a prototypical disease of poverty [23, 24]. Despite causing up to half of all human deaths in Europe and North America over the past few centuries, TB today primarily affects the developing world [5, 6, 24]. An inverse linear association between TB incidence and per capita gross domestic product was shown in paired data analysis from 171 World Health Organization (WHO) member states [24, 25]. Every year an estimated 10 million (range, 8.9–11.0 million) people fall ill with TB. Incidence rates at the national level vary from less than 5 to more than 500 per 100K population per year. TB can affect anyone anywhere, but most

people who develop the disease (about 90%) are adults; (adult men, accounted for 56%, women for 32%, and children for 12% of all TB patients notified in 2019). Among all TB cases, 8.2% were among people living with HIV [26].

Thirty high TB burden countries (HBC) account for almost 86% of the global burden and two third of the total burden is born by eight countries including India (26%), China (8.5%), Indonesia (8.4%), Philippines (6.0%), Pakistan (5.8%), Nigeria (4.6), Bangladesh (3.6%) and South Africa (3.3%). This distribution predominantly tracks socio-economic status, with sub-Saharan Africa being one of the most intensely affected areas [27].

There were about 1.2 million TB deaths among HIV-negative people and an additional 0.2 million deaths among HIV-positive people, which is more than any other infectious disease. The only exception was during 2020-21 when a higher number of people died due to COVID-19 than to TB [5, 28].

Extrapulmonary tuberculosis (EPTB*)* refers to TB involving organs other than the lungs. The proportion of EPTB among TB cases varies depending on the country of origin and associated human immunodeficiency virus (HIV) coinfection [8, 29, 30].

Figure-1: Percentage of people with a new or relapse episode of TB who were diagnosed with extrapulmonary TB at country level, 2021 [28]
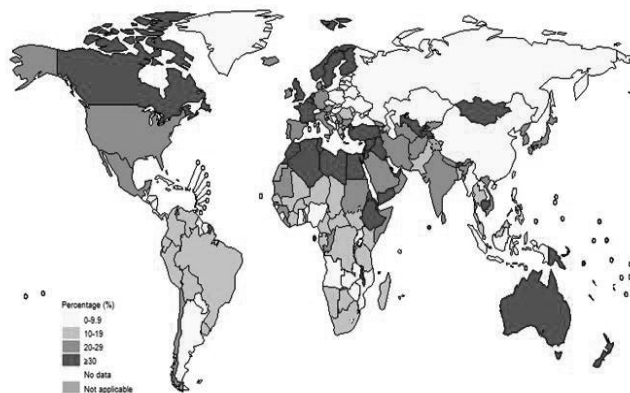
Drug resistance in TB: The emergence of drug resistance in *M. tuberculosis* was seen in the 1940s soon after the introduction of anti-TB chemotherapy [31]. The first outbreak of drug-resistant TB was reported in the United States in 1970. The global emergence of multi-drug resistance, initiated the post-antibiotic era, making TB essentially incurable in many parts of the world [31]. The advent of every new drug led to the selection of mutations conferring resistance to it. Rapid onset of isoniazid (H) resistance was seen among patients receiving monotherapy in clinical trials but not when H was given in combination with streptomycin or para-aminosalicylic acid [32]. Resistance to rifampicin (R) was observed soon after it was first administered [33]. Similarly, the emergence of resistance to bedaquiline and delamanid was reported soon after introduction [34].

Drug resistance in TB occurs through two main mechanisms: (i) primary or transmitted drug resistance, which occurs when resistant strains are transmitted to a new host, and (ii) secondary or acquired drug resistance, which occurs through the acquisition of drug-resistant conferring mutations to one or more drugs. For more than 10 years, estimates of the proportion of people diagnosed for the first time with multidrug-resistant TB (MDR-TB) or rifampicin-resistant TB (RR-TB) has remained at about 3–4% and for those previously treated for TB has stayed at about 18–21% [35]. This indicates that a substantive burden of drug-resistant TB is driven by ongoing transmission [36]. Nearly half a million individuals who developed tuberculosis in 2019 had rifampicin resistance (RR), a key first-line drug for the treatment of tuberculosis. The highest proportions (>50% in previously treated cases) of drug resistance are in countries of the former Soviet Union. Globally, 20.1% (95% CI: 15.5-25.0%) of MDR/RR-TB cases are resistant to fluoroquinolone (FQ), a core drug used in the treatment of drug-resistant tuberculosis.

Globally among R-sensitive TB, the prevalence of H resistance is estimated at 7.4% (95% CI 6.5%–8.4%) among new and 11.4% (95% CI 9.4%–13.4%) among previously treated TB patients [37].

1.1.2. Global plans and strategies to end TB

The WHO in 1993, declared TB 'a global emergency' with deaths from TB higher than any previous year in history [38]. Starting with the Global Plan to Stop TB 2001-2005 there have been at least three other global plans. Although progress was achieved, overall, these plans failed to reach and treat enough people with TB to make a success of the plan [38, 39]. WHO's post-2015 **End TB Strategy**, adopted by the World Health Assembly in 2014, aims to end the global TB epidemic as part of the newly adopted Sustainable Development Goals (SDG). It serves as a blueprint for countries to reduce TB incidence by 80%, and TB deaths by 90%, and to eliminate catastrophic costs for TB-affected households by 2030 [40]. The End TB strategy encompasses a package of interventions that can be fully adapted at the country level. The strategy provides a unified response to ending TB deaths, disease, and suffering. It builds on three strategic pillars underpinned by four key principles. The three pillars bring together critical interventions to ensure that all people with TB have equitable access to high-quality diagnosis, treatment, care, and prevention, without facing catastrophic expenditure or social repercussions. Pillar one of the End TB strategies puts patients at the heart of service delivery through integrated, patient-centered care and prevention. The four key components of pillar one are (i) Early diagnosis of TB including universal drug susceptibility testing (DST), and systematic screening of contacts and high-risk groups. (ii) Treatment of all people with TB including drug-resistant TB, and patient support. (iii) Collaborative TB/HIV activities and management of comorbidities. (iv) Preventive treatment of persons at high risk and vaccination against TB.

With regard to diagnosis and detection of drug resistance, the End TB strategy calls for WHO-endorsed rapid diagnostic tools (WRD) for early diagnosis and prompt TB treatment [40, 41], rapid DST for at least R for all bacteriologically confirmed TB patients, and DST for FQ for all RR-TB patients [42, 43].

Conventionally, the global strategy had prioritized the detection and treatment of persons with PTB based on the fact that TB is transmitted by airborne droplet nuclei and each

person with undiagnosed and untreated smear-positive TB causes 10-14 infections per year [20, 44]. In the absence of more sensitive diagnostic tools, HBC

continued to rely on AFB microscopy for the diagnosis of the most infectious TB patients [45]. EPTB in general was not and still is not given high priority on the public health agenda as it does not contribute significantly to the transmission of the disease [46].

1.1.2.1. Estimation of TB disease burden

Estimates of the burden of TB disease are measured in terms of incidence, prevalence, and mortality. To estimate incidence, four main methods are used by WHO; (i) TB prevalence surveys results, (ii) notifications in high-income countries, adjusted by a standard factor to account for under-reporting and under-diagnosis, (iii) national inventory studies and (iv) case notification data combined with an expert opinion about case detection gaps [47]. The focus of prevalence surveys is on bacteriologically confirmed PTB in adults, hence adjustments are needed to include children and EPTB. Furthermore, the estimation of incidence from prevalence is not straightforward as it requires assumptions about the duration of disease for different case categories. Mortality is obtained directly from national vital registration systems of mortality surveys or is derived indirectly from the incidence and case-fatality ratio [47, 48].

For **surveillance of drug resistance,** data is derived from two main sources: first is routine DST data (molecular or phenotypic) which constitute a continuous surveillance system for at least R, if results are available for the majority (80%) of bacteriologically confirmed PTB patients; second is periodic drug resistance surveys (DRS) in settings where routine testing capacity is insufficient [47]. Due to inherent challenges in collecting extrapulmonary samples, these patients are not included—in periodic surveys. The prevalence of resistance among EPTB is assumed to be similar to that among PTB patients [28].

1.1.3. Progress and challenges in TB control/end TB

Globally, the annual number of people diagnosed with TB and to have accessed TB

treatment has steadily grown from about 6 million in 2015 to 7.1 million in 2019, but still, as many as 30% of the estimated cases were missed out on diagnosis, and care [26]. TB incidence and deaths are falling, but not fast enough to reach the first milestone

of the End TB strategy. The Covid pandemic halted the progress in TB control with a decline in notifications down to 5.8 and 6.4 million in 2020 and 2021 respectively [5, 28]. Laboratory methods for the diagnosis of TB are continually evolving to achieve more rapid, accurate, and cost-effective results [49-52]. In parallel with the scale-up of more sensitive diagnostic tools, an increase in the proportion of bacteriologically confirmed PTB was reported from 57% in 2015 to 63% in 2021 [28].

A gradual increase is seen in the proportion of EPTB from 15% in 2015 to 17% of the incident TB cases notified globally in 2021. The proportion of EPTB ranges from 8% in the WHO Western Pacific Region to 24% in the Eastern Mediterranean Region [5, 26]. However, unlike PTB notifications, the proportion of bacteriological confirmation among notified EPTB cases is unknown with negligible attempts to monitor it at the country or regional level.

Table 1: Notifications of all forms of TB, new and relapse TB, bacteriologically confirmed PTB, and EPTB for WHO regions, 2021

WHO region	Total notified	TB N+R (n)	PTB N+R (n)	PTB B+ve N+R (%)	EPTB N+R (%)
African Region	1 508 787	1 479 535	1 281 737	66%	13%
Region of the Americas	227 592	213 212	183 202	79%	14%
South-East Asia Region	3 139 006	2 966 970	2 345 691	65%	21%
European Region	188 905	157 874	132 091	69%	16%
Eastern Mediterranean Region	503 760	497 895	383 193	57%	23%
Western Pacific Region	1 128 312	1 108 034	1 018 904	56%	8%
Global	**6 696 362**	**6 423 520**	**5 344 818**	**63%**	**17%**

PTB; Pulmonary TB, EPTB: Extra pulmonary TB, N; New. R: relapse, B+ve: Bacteriologically confirmed

A series of major historical landmarks for **TB treatment** followed the discovery of the first effective medications (streptomycin and para-aminosalicylic acid) in 1944. The revelation of "triple therapy" (streptomycin, para-aminosalicylic acid, and H) which assured cure in

1952, recognition that H and R could reduce the duration of treatment from 18 to 9 months in the 1970s and the observation in the 1980s that adding pyrazinamide (Z) to these drugs allowed cure in just 6 months [13, 14]. Most people (about 85%) who develop TB disease can be successfully treated with a 6-month drug regimen. The overall high treatment success has been sustained over several years. In 2019, the treatment success rate (TSR) was 86% which ranged from 74% in the Americas to 91% in the Eastern Mediterranean region [26].

Globally, testing of bacteriologically confirmed TB cases for R resistance, has increased from 7% in 2012 to 61% in 2019 (59% for new and 81% for previously treated TB patients) [26]. In parallel, MDR/RR-TB cases diagnosed and initiated on treatment increased from 122K in 2015 to 181K in 2019. Testing coverage for resistance to FQ forms a critical component of recommended treatment regimens for both R-resistant and R-sensitive TB [35]. Diagnostic algorithms for drug resistance detection are often driven by testing for resistance to R, with further DST only for RR-TB patients. As a result, H resistance among R-sensitive populations remains mostly undetected, and often not treated with the WHO-recommended modified regimen, thus risking poorer treatment outcomes and the development of further resistance [53].

For TB prevention, Bacillus Calmette–Guérin (BCG), an attenuated form of *M. bovis*, is the only proven **effective vaccine.** In 1921, the BCG vaccine was first used in humans, and in 1974, it was included in the infant immunization programs by the WHO and is one of the most commonly used vaccine globally [54]. BCG protects young children against TB meningitis and total coverage with BCG can prevent 40 -70% of deaths from TB in children and reduce total TB mortality by approximately 6% [55]. However, BCG does not reliably protect adults against classical PTB.

TB preventive treatment is recommended for people living with HIV and household contacts with people with bacteriologically confirmed PTB and others at risk of latent TB infection (LTBI) [18, 19]. In recent years, access to TB preventive treatment has increased, from 1 million in 2015 to 4.1 million in 2019 [26].

1.2.   Pathogen and pathogenesis of TB

1.2.1. Causative organism of TB

TB is caused by a group of nine closely related bacteria, collectively known as the *Mycobacterium tuberculosis complex* (MTBC) *that* causes TB in humans and animals [56-58]. The MTBC species share 99.9% sequence identity and are likely to have evolved from a single clonal ancestor [59, 60]. Population genomic studies suggest that

M. tuberculosis may have emerged ~70,000 years ago in Africa and subsequently disseminated along with anatomically modern humans, expanding globally during the Neolithic Age as human density started to increase [61].

Worldwide TB in humans is mainly caused by *M. tuberculosis* that results in high mortality and morbidity [7, 16, 17]. *M. africanum* causes human TB but is restricted to West Africa, where it accounts for up to 50% of cases [62, 63]. *M. canettii* is an extremely rare cause of human TB in the Horn of Eastern Africa [64]. Whereas *M. bovis* an animal-adopted strain, causes disease in cattle and spreads to humans through animal contact and consumption of unpasteurized milk [27, 65]. Other disease-causing species of MTBC are animal-adapted strains that range across different mammalian species [66, 67]. *M. tuberculosis* and

M. africanum are obligate human pathogens with limited survival outside the human body and no known animal reservoir [68, 69]. While it can infect most warm-blooded animals and is highly virulent for some, no animals can reproducibly transmit the infection to others. Consequently, the continued existence of *M. tuberculosis* depends on transmission among humans [69]. A direct link between virulence and transmission plays a role in causing active disease to transmit to secondary hosts [69, 70].

M. tuberculosis are slightly curved, rod-shaped bacilli, 0.2 - 0.5 microns in diameter; 2- 4 microns in length, have a thick lipid (mycolic acid) cell wall, is aerobic, non-motile and multiply slowly (~ every 18-24 hour) [71, 72]. Metabolism can slow to the point of dormancy and can remain in this state for decades [73].

1.2.2. Immune response against M. tuberculosis.

TB-transmission usually takes place through the airborne spread of droplet nuclei produced by patients with infectious PTB [6-9, 16]. When a person inhales droplet nuclei containing *M. tuberculosis*, it passes through the upper respiratory tract and bronchi to reach the alveoli of the lungs [9, 16, 74, 75]. The immune response in TB is complex, in the first encounter between the immune system and *M. tuberculosis*, the effectiveness of the innate immune response dictates the clinical outcome. If the innate immune response is strong enough and the bacillary load is low, the bacteria are likely to be eradicated [76-82]. Otherwise, *M. tuberculosis* are confined to the host cells, leading to latent infection. At this point, the adaptive immune response determines the progress of the disease, from an active to a progressive one [83].

The key players in the innate defense against *M. tuberculosis* are the alveolar macrophages and dendritic cells [83, 84]. These immune cells, recognize pathogen-associated molecular patterns in mycobacterial structures, activate signaling pathways, and lead to the production of pro-inflammatory cytokines (such as TNF, IL-1B, IL-12) and nitric oxide, and the pathogen is phagocytosed [85-88]. The infested macrophage either undergoes cell necrosis or apoptosis or can survive. If the cell undergoes necrosis, mycobacteria are released and may infect new macrophages or disseminate. In the case of apoptosis, the cell membrane is not compromised, and the bacteria are destroyed within the macrophage. If infected macrophages survive, the mycobacteria persist and may even proliferate before activation of an adaptive immune response [88-90].

Dendritic cells are an important mediator between the innate and **adaptive immune response**. In addition to causing phagocytosis, dendritic cell migrates to the regional lymph nodes and present mycobacteria antigen to naïve T cells and activates CD4+ T cells, which then migrate to the lungs to impede mycobacterial progressive growth [68, 83, 91]. Two key cytokines are produced by activated T-cells, Interferon-gamma (**IFN-γ**) and Tumor necrosis factor alpha (TNF-α) [68, 92, 93]. **IFN-γ** plays a crucial role in macrophage activation and intracellular mycobacterial killing [93-95]. TNF-α has immune regulatory properties and plays a central role in granuloma formation and macrophage induction [93-

95]. Once *M. tuberculosis* colonizes the host, an inflammatory cellular infiltrate to these sites triggers the formation of **granulomas,** most prominently in the lungs. Cellular aggregates are constituted of macrophages, multinucleated giant cells, epithelioid and foamy cells, granulocytes, and lymphocytes [96-99].

1.2.3. Clinical outcomes of M. tuberculosis transmission

M. tuberculosis produces two distinct disease entities known as primary and post-primary TB [69, 98-100]. Both are necessary for the continued survival of *M. tuberculosis*. Primary TB mediates protective immunity to disseminated infection while post-primary TB causes tissue damage, that results in the formation of cavities [69, 98-101].

Primary TB occurs in immune-competent people when infected with *M. tuberculosis* for the first time. Primary TB typically develops and spreads to regional lymph nodes and then systemically for only a few weeks before regressing as immunity develops [100-102]. While the lesions may heal, they are seldom sterilized and organisms persist [69, 97]. A primary (Ghons) complex is formed, consisting of a granuloma, typically in the middle or lower zones of the lung (primary or Ghons focus) in combination with transient hilar and/or paratracheal lymphadenopathy and some overlying pleural reaction [68, 100-102]. The primary complex usually resolves within weeks or months, leaving signs of fibrosis and calcification detectable on chest X-ray [103-105]. The symptoms are mild, non-specific, and usually self-resolving [106, 107]. In general, the risk of disease progression is low other than in very young or immunosuppressed individuals and those using TNF-suppressing agents [95]. Infants (<2 years of age) are at the highest risk of disease development and potential dissemination [100-102, 108, 109]. Hematogenous dissemination of bacilli may occur shortly after primary infection or from any active disease site and may result in miliary TB. Miliary granulomas are 1–3 mm in diameter (the size of a millet seed), are widespread, and may be found in any visceral organ **[108-110].**

Post-primary TB, also known as adult-type or secondary TB, occurs in people who have developed immunity to primary TB. Post-primary TB is typically restricted to the upper

lobes of the lungs and does not involve lymph nodes or other organs [111]. About 90% of cases recover spontaneously without therapy. However, those who become ill account for 80% of all clinical cases and nearly 100% of transmission of infection. Little is known about bacterial or host features that allow transmission [97]. Lung cavitation and transmission is likely dependent on a robust immune response as elderly people and those infected with HIV tend to develop more disseminated disease with less cavitation [69, 97-99, 103, 104, 112].

Studies from the pre-antibiotic era suggested an incubation period of under six weeks for primary infection and 3-9 months for active TB. It was shown in longitudinal studies, that the majority of TB disease manifests soon after infection and rarely occurs more than two years after infection. The vast burden of global TB is, therefore, from recently transmitted infection [21]. The lung is the predominant site of infection with *M. tuberculosis* [6-9]. This may result in symptomatic, primary PTB disease usually in children or it may develop as post-primary PTB in adults [8, 107, 111]. *M. tuberculosis* may spread at the time of initial infection, reactivation, or reinfection (post-primary TB) directly from the lungs, through the lymphatic system or bloodstream, to other body sites thereby causing extrapulmonary manifestations [7, 9, 111, 113]. In 1948, a timetable for TB was explained by Walgreen A., he suggested that PTB manifests within a few months of primary infection, miliary and meningeal TB in 2-6 months, TB adenitis in 3-9 months, bones and joints after several years, whereas renal and genital TB may take over a decade [22].

Risk Factors: The risk of progression from exposure to *M. tuberculosis* bacilli to the development of active disease is governed by both exogenous and endogenous risk factors. Exogenous factors like bacillary load in the sputum and the proximity of an individual to an infectious TB patient play a key role. Endogenous risk factors and comorbidities such as HIV infection, diabetes, malnutrition, and tobacco and substance use disorders increase the risk of contracting TB and are associated with poorer TB treatment outcomes [5, 114].

1.2.4. Mechanism of Drug-Resistance in TB

Members of the genus Mycobacterium have long been noted for their intrinsic resistance to a wide array of antibiotics [115, 116]. The majority of drug resistance in clinical *M. tuberculosis strains* is attributed to chromosomal mutations in existing genes that are passed along from mother to daughter cells through vertical descent. Unlike many other bacterial pathogens, *M. tuberculosis rarely* recombines via lateral exchange of DNA and also *lacks* plasmids [117]. The primary vehicle driving drug resistance in

MTB is the acquisition of mutations in genes that code for drug targets or drug-activating enzymes [36, 118]. Drug resistance in *Mycobacteria* species can be driven by mutations in genomic regions or target genes that confer protection against anti-TB drugs. These are mainly in the form of single nucleotide polymorphism (SNPs), insertions or deletions (indels), and to a lesser extent, large deletions. The main mechanisms of drug resistance include drug target alteration, overexpression of drug target, disruption of pro-drug activation, and efflux pump activation [116, 117].

By 1998, resistance-conferring mutations were discovered for classical first and second-line TB drugs including H (alterations in genes *katG* and *inhA*); R (in *rpoB*); streptomycin (in *rrs* and *rpsL*); Z (in *pncA*); ethambutol (in *embB*); FQ (in *gyrA*); and kanamycin (in rrs) [117]. However, the targeted amplification and sequencing of known or suspected resistance genes revealed that these mechanisms were insufficient to explain all phenotypic resistance [119, 120]. Resistance mechanisms for several newer drugs including bedaquiline, delamanid, and pretomanid were discovered during a period when whole genome sequencing (WGS) was becoming routine. Several studies utilizing WGS analysis have been conducted to study drug resistance in *M. tuberculosis* [121]. Findings from these studies showed that genomic mutations in various regions of *M. tuberculosis are* responsible for phenotypically diverse drug responses. These studies also provided valuable insights into the evolution of drug-resistant *M. tuberculosis* and the mechanisms involved in maintaining its fitness to transmit within or across different geographical regions [120, 121].

Resistance could be present either at the onset of the disease as a result of the transmission of drug-resistant strains (primary drug resistance] or might emerge during the course of the disease due to inadequate treatment (acquired drug resistance) [122].

1.3. Clinical manifestations of TB

Following inhalation of *M. tuberculosis* an individual may have one of the following outcomes: i) fail to register an infection, ii) become infected but then clear the infection, iii) successfully contain the infection but continue to harbor bacilli in the absence of symptomatic disease (LTBI), or iv) develop progressive TB disease [7]. Most infections manifest as LTBI, a clinically asymptomatic, contained state; a smaller subset of infected individuals present with symptomatic active TB [6-9, 123]. Active TB at times may be subclinical with a risk of being missed in diagnosis [123, 124].

1.3.1. Pulmonary TB

PTB is typically classified as primary TB in children and post-primary TB in adults. However, owing to the changing epidemiology there is a considerable overlap in the radiologic presentations of these entities [125]. PTB in most cases, presents as a disease of the lung parenchyma and much less frequently as a disease of the tracheobronchial tree only [16, 110, 111, 126]. The classic clinical features of **parenchymal PTB disease** are chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, and hemoptysis [126]. A persistent non-remitting cough is the most frequently reported symptom [110]. TB symptoms are usually gradual in onset, however, in young children or immunocompromised individuals, it may have an acute onset [105, 106]. On rare occasions, patients with sub-pleural involvement may present with symptoms of chest pain and dyspnea [123, 127]. Chest X-ray findings are often typical with focal, diffuse, or reticulonodular opacities in the upper lobe, consolidation, cavities, nodules, miliary pattern, intrathoracic lymphadenopathy, and pleural effusion. However, some patients may also present with normal chest X-rays [16]. People presenting with any of these symptoms and/or a history of contact with infectious TB [108] and/or abnormal chest radiograph

raises suspicion of disease [103, 104, 112]. Symptoms of **Endobronchial TB** affecting the trachea and major bronchi are similar to those of parenchymal PTB, but wheezing and dyspnea are often more prominent on examination [128, 129]. It is often misdiagnosed as bronchial asthma or bronchial malignancy [128-130]. **Intra-thoracic lymph nodes** which form part of the primary (Ghon) complex are typically seen in young children [101, 102]. Peri-hilar and/or paratracheal lymph node enlargement with or without airway compression is the cardinal sign of disease. Symptoms are similar to other forms of PTB, but productive cough and hemoptysis are rare [125]. Diagnosis is complicated as the disease is often paucibacillary and young children are unable to expectorate [16, 102, 125]. Enlarged lymph nodes may obstruct large airways resulting in the collapse or hyperinflation of distal lung segments. Cold abscesses may form that may erode into surrounding structures like in the pericardium, esophagus, or airways leading to TB pericarditis, esophageal TB, lobar consolidation, and caseating pneumonia respectively [131].

1.3.2. Extrapulmonary TB

TB can involve organs other than the lungs e.g., pleura, lymph nodes, meninges, abdomen, joints, bones, genitourinary tract and skin [132-134]. Extrapulmonary involvement may present many years after exposure [8, 29, 30].

Extra-thoracic lymph node TB (LNTB): Extra-thoracic TB lymphadenitis is one of the most common forms of EPTB. The pathogenesis of TB lymphadenitis is less clearly understood but is generally thought to be a local manifestation of systemic disease [135, 136]. More than 40% of LNTB patients have radiological evidence of PTB suggesting the lymphatic spread of *M. tuberculosis* from the parenchyma of the lung. [137, 138]. *M. bovis*, was considered the more likely causative agent before the advent of pasteurization of milk [139]. The most commonly affected lymph nodes are in the cervical region, followed by the mediastinal, axillary, mesenteric, hepatic portal, peripancreatic, and inguinal lymph nodes [44, 140]. The disease is indolent and usually presents as a unilateral painless neck mass greater than 2×2 cm. It may be occur with or without fistula or sinus formation, or

cold abscess [136]. Constitutional symptoms are rare, except in individuals infected with HIV. Tenderness or pain is often associated with secondary bacterial infection [135, 138].

Pleural TB: TB is the leading cause of pleural effusions in TB-endemic countries [141, 142]. TB pleural effusion (TPE) is usually unilateral and variable in size. Concurrent parenchymal involvement is seen in 20 % of patients on chest X-rays and up to 80 % of patients on CT scans [141-144]. The typical presentation is acute with fever, cough, and localized pleuritic chest pain. TPE when part of a primary infection, is often self-limiting but in pregnancy may be associated with occult dissemination with potential risk to the fetus. As with all forms of EPTB, the incidence is higher in HIV-infected patients, with atypical symptoms, often with less pain, longer duration of illness, and more generalized signs [106, 144]. Previously, TPE was considered a delayed hypersensitivity reaction. With the advent of improved culture media, *M. tuberculosis* can be successfully grown in culture from as many as 70% of pleural fluids and tissues suggesting a direct infection of the pleura [141-143]. TPE thus needs to be included in the differential diagnosis for investigating any undiagnosed pleural effusion.

Gastrointestinal tuberculosis (GITB). Abdominal TB can occur due to ingestion of bacilli from swallowed sputum or infected milk, hematogenous spread, local extension from contiguous mediastinal lymph nodes to the esophagus, or spread from the gut or nodes to the peritoneum [145, 146]. GITB is commonly seen in populations with lower socioeconomic status, illiteracy, malnutrition, and HIV [147, 148]. It is uncommon in indigenous populations of developed countries [149]. The gastrointestinal tract can be involved anywhere along its length. The ileocecal area is the most common site [146, 147] and changes are described as ulcerative, hypertrophic, ulcero-hypertrophic, or fibrotic on imaging [150]. Proximal GIT is rarely involved [131, 151]. Stomach and duodenum when affected, may result in ulcers, which are clinically indistinguishable from peptic ulcers [148, 149]. Diagnosis of abdominal TB is often delayed as the clinical presentation is often non-specific and two-thirds of these patients have normal chest X-rays [147].

Central nervous system tuberculosis (CNSTB) is thought to be a two-step process. Hematogenous spread leads to a tuberculous focus (Rich focus) in the brain, which then invades and releases bacilli in the subarachnoid space [152-154]. In HIV-infected patients and young children, it is often associated with miliary disease [155]. The most common clinical manifestation of CNSTB is tuberculous meningitis (TBM) followed by CNS tuberculoma, and rarely as tuberculous encephalopathy and tuberculous radiculo-myelitis [156]. Early symptoms are non-specific, including a triad of fever, night sweats, weight loss, and headache of increasing intensity. As the disease progresses, patients become more confused with reduced consciousness and may present with hemiplegia, paraplegia, urinary retention (seen with spinal involvement), and pathognomonic sixth nerve palsy [155]. Neck rigidity is typically less pronounced than in acute bacterial meningitis. TBM is the most lethal form of TB. Almost a third of HIV-uninfected patients, and more than half of patients co-infected with HIV die from TBM, and despite treatment, half of the survivors suffer from permanent neurological impairment [72, 73, 125].

Osteoarticular tuberculosis (OATB): OATB results from hematogenous spread with a predilection for the spine and growing ends of long bones [36]. The infection usually remains dormant for 3-4 years before clinical disease. The spine is the most common site and bacilli target the red bone marrow resulting in gradual destruction of the bony tissue [157-159]. Once the vertebral integrity is lost, the structure collapses, and angulation (kyphosis) of the spine develops, which is sometimes followed by the fusion of vertebrae (ankylosis). Cold abscess formation or severe spinal angulation may cause compression of the spinal cord with neurological sequelae [159]. A paraspinal abscess can appear as a mass or psoas abscess that discharges in the groin [158]. The most common initial symptom of spinal TB is back pain, which may be present for weeks or months before diagnosis. An insidious, progressive back pain raises suspicion of tuberculous spinal infections.

Genito-urinary tuberculosis (GUTB); GUTB usually results from the reactivation of old, dormant tuberculous diseases [160]. The patients are often from a high-prevalence region with a history of PTB. The clinical presentation may vary from asymptomatic to non-

specific constitutional symptoms [161, 162]. Depending on the disease site, the patient may present with abdominal pain, abdominal mass, menstrual irregularities, infertility, obstructive uropathy, and abnormal renal function tests. Kidneys are most frequently affected and patients with renal TB have complaints of recurrent urinary tract infections, and sterile pyuria which does not respond to standard antibiotic therapy [161-163]. GUTB is often diagnosed late owing to insidious onset and delay may result in disease progression and irreversible damage.

Rare clinical manifestations of EPTB: Patients with TB affecting rare sites may present with unusual manifestations like swollen lip [164], severe pancytopenia as a result of the involvement of bone marrow [165], pain in the elbow [166, 167], pain in testicles [168], adrenalitis and prostatitis [169], cholecystitis [170], keratitis [171], uveitis [172], lupus vulgaris resulting in loss of vision and nose [173] and otitis media [174]. Diagnosis of TB is even more intricate with these unusual presentations.

1.4.   Diagnosis of TB and drug resistant TB

The microbiological detection of TB is critical to allow early diagnosis and initiate an effective treatment regimen as early as possible [42].

1.4.1. Laboratory methods for diagnosis of TB

Laboratory methods used for the diagnosis of TB are broadly classified into i) direct detection of mycobacteria and its products and ii) indirect measurement of the host humoral and cellular response against mycobacterium [51, 175]. A summary of diagnostic tools endorsed by the WHO for the diagnosis of TB and drug resistance is given in Table-2 [42, 176].

1.4.1.1.   Direct methods for detection of *M. tuberculosis*

1.4.1.1.1.   Direct AFB microscopy

Mycobacteria have thick lipid cell wall, and once stained resists decolorization with acid/alcohol [177, 178]. Direct microscopy is a fast and inexpensive method to identify acid-fast bacillus (AFB) [50, 179]. AFB microscopy is a relatively insensitive test with a

limit of detection (LoD) of 5000-10000 bacilli per milliliter of sputum. Traditionally, TB patients were broadly classified as "smear positive" or "smear negative", based on microscopy results. Due to low sensitivity, paucibacillary TB disease in people living with HIV, children, and extrapulmonary TB is often missed by microscopy [49, 52, 175].

1.4.1.1.2.   TB culture

Culture is more sensitive than smear microscopy with LoD of approximately 10-100 viable organisms/mL of specimen. It is also the only reliable mean to monitor the effectiveness of therapy in TB patients. A variety of solid (e.g., Löwenstein–Jensen, Ogawa, Middlebrook agar) and liquid media (7H9 broth) are available for the culture of mycobacteria from clinical specimens. Solid media can take 4–6 weeks (18–24-hour generation time) for culture to grow, and liquid media reduces this time by half. The Mycobacteria Growth Indicator Tube (MGIT) is a Middlebrooks 7H9 broth-based culture method, with an oxygen-sensitive fluorescent sensor to indicate microbial growth. A fully automated system BACTEC MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA), continuously monitors the fluorescence produced due to growing mycobacteria. TB culture using commercially available liquid media is the current gold standard method for the bacteriological confirmation of TB [42, 180]. However, delays in the diagnosis due to longer turnaround time, the cost of the test, and the need for sophisticated laboratory facilities, biosafety equipment, and highly trained staff limit its routine use in resource-limited settings [181, 182].

1.4.1.1.3.   Molecular methods for detection of MTB

Xpert® MTB/RIF assay (Cepheid, Sunnyvale, USA), is an automated cartridge-based real-time polymerase chain reaction (PCR) assay designed to simultaneously detect MTBC and RR within two hours. This was the first molecular-based assay endorsed by WHO in 2010, that has revolutionized TB control globally by contributing to the rapid diagnosis of TB and RR-TB disease [183, 184]. In 2017, Xpert® MTB/RIF Ultra ("Xpert Ultra") a next-generation assay with improved sensitivity was endorsed [185, 186]. Later more assays were recommended by WHO for the diagnosis of TB and drug resistance [187]. Xpert and

Xpert Ultra are also recommended for the diagnosis of TB and RR in children, using sputum, stool, nasopharyngeal, gastric aspirates, and various EPTB specimens [42].

1.4.1.1.4. Antigen detection

- **Lipoarabinomannan (**LAM) is the most promising mycobacterial antigen and diagnostic tests were among the first to move from the research to the commercial stage [21] [188]. LAM is the primary component of the *M. tuberculosis* cell wall and is released from metabolically active and degrading mycobacteria and excreted through urine filtration [175, 189, 190]. Immunochromatographic test (ICT) based serologic technique is used for detecting LAM antigen. LAM assay performs best in immunocompromised patients with disseminated TB, its use is therefore limited to the diagnosis of HIV-associated PTB and TB in children but is not applicable for pauci-bacillary EPTB [188-190]. The test has several potential advantages for rapid diagnosis of active TB as urine samples are simple to collect, process, and store, particularly in young children, who are often unable to produce sputum [175, 189].

- **Antigen detection using Immunohistochemistry (IHC) and immunocytochemistry (ICC) techniques.** These techniques are used to detect Mycobacterial antigens in formalin-fixed paraffin-embedded tissue sections, tissue aspirate, and body fluids. Different antigens used include BCG, MPT64, *M. tuberculosis* antigen 85, Antigen 5 (38kDa), LAM, ESAT6, HspX, Tb8.4, and the phospholipase C encoding A (PlcA) protein. The immunostaining technique has been claimed to be robust, rapid and to perform equally well in HIV-coinfected TB cases with atypical histological features. Immunostaining can be applied to a wide range of extrapulmonary clinical specimens with significantly high sensitivity (70-100%) and specificity (65-100%) compared to conventional AFB staining [175]. The improved sensitivity is partly attributed to the detection of intact as well as degraded bacilli [191-193]. The main disadvantage is invasive tissue sample collection and preparation [191-193].

1.4.1.2. Indirect methods for diagnosis of TB

1.4.1.2.1. Demonstration of granulomatous immune response

Granulomatous inflammation is a histologic pattern of tissue reaction that appears following cell injury. It was first recognized as a distinct entity in the early nineteenth century [194]. Granulomatous inflammation can be caused by a variety of conditions including infection, autoimmune, toxic, allergic, drug, and neoplastic conditions. The resultant granulomatous patterns vary in morphological features and are described as necrotizing granulomas, non-necrotizing granulomas, suppurative granulomas, diffuse granulomatous inflammation, and foreign body giant cell reaction. The difference narrows the pathologic and clinical diagnosis e.g., **necrotizing granulomas** are typically seen with mycobacterial infections and non-necrotizing granulomas with sarcoidosis [194-198]. Necrotizing granulomas caused by *M.tuberculosis* are classically characterized by central amorphous caseating granular debris with loss of cellular detail referred to as a tubercle, which is encircled by epithelioid cells, lymphocytes, histiocytes, fibroblasts, and occasionally Langhans' giant cells [196]. These histologic features are considered sufficiently characteristic for a reasonably accurate diagnosis of TB [196]. Since the very beginning, concerns had been raised about the use of histological features for the diagnosis of TB [199]. While caseating granulomas are typically seen in TB, noncaseating granulomas may also occur or classic histologic features may be missing altogether in immunosuppressed individuals [195]. Fine needle aspirate is increasingly being used for obtaining specimens for examination [200]. Routine histological and mycobacteriology analysis plays an important role in the diagnosis of TB [42, 201-203].

1.4.1.2.2. Cellular Immunity mediated response

TB infection can be demonstrated by skin tests and interferon-gamma release assay (IGRA). Both these tests evaluate the response mediated by T lymphocytes (cell-mediated immunity) to protein products of *M. tuberculosis* [204].

Skin tests are the prototype of cellular immunity-mediated tests. The **tuberculin skin test** (TST) is an intradermal injection of purified protein derivative (PPD) extracted from *M.*

tuberculosis cultures. The PPD is a crude antigenic mixture, which is shared among, *M. tuberculosis, M. bovis*, and other non-tuberculous mycobacteria (NTM) [205]. Inflammatory reaction, at the site of injection, results in the characteristic induration occurring after 24 hours. The TST results are interpreted by measuring this hypersensitivity reaction (delayed-type hypersensitivity) [180]. TST conversion usually occurs 3–6 weeks after exposure/infection. A positive TST may suggest an active TB, past infection, BCG vaccination, or sensitization by environmental mycobacteria [206].

Since TST was first introduced in 1890, it has undergone continual refinement in its formulation, standardization, and dosage [205, 207]. The PPD from the Statens Serum Institute (Copenhagen, Denmark) (PPD-RT23), is accredited and is used for the standardization of other PPDs [207, 208]. Until recently, the TST was the only tool for detecting LTBI [180]. Recently, various recombinant antigens such as ESAT-6, CFP-10, and MPB 64 have been developed to improve the sensitivity and specificity of TB skin tests. C-Tb (Statens Serum Institute, Copenhagen, Denmark) is a highly specific skin test for the diagnosis of LTBI which is designed to address some of the drawbacks of TST and Interferon-gamma release assay (IGRAs). C-Tb is applied and read in the same way as TST, but specificity is high and a universal 5 mm cut-point of induration is used for interpretation irrespective of the status of BCG, HIV, or both [209]. In TB endemic zone, the tuberculin test alone is not sufficient evidence to diagnose EPTB in adult patients.

Interferon-γ-release Assays (IGRA): In these assays, mycobacterial antigens such as ESAT-6 and CFP-10 are used to stimulate mononuclear cells from peripheral blood in-*vitro* and interferon-γ produced is measured [180]. This assay is commercially available as QuantiFERON-TB Gold (Cellestis, Australia) and T-SPOT TM. TB assay (Oxford Immunnotec, UK) [180, 210]. QuantiFERON-TB has a pooled sensitivity of 75% for patients with active TB including tuberculous pleuritis and offers a high specificity of 94% in patients with non-tuberculous mycobacteria infection [211]. The results are not confounded by BCG vaccination and the test also performs well in HIV co-infection, malnutrition, and young children with either latent or active TB. These tests are used for

the diagnosis of latent TB in many developed countries. In 2019, WHO extended its recommendation on the use of IGRAs for the diagnosis of LTBI in low and middle-income countries [212]. However, these assays cannot discriminate between latent infection and active disease, thereby limiting their use in high endemic countries [180, 210]. IGRAs have limited value to screen and rule out EPTB in high endemic countries, but the diagnostic accuracy of IGRAs for EPTB is not affected by the immune status of patients [213-216].

1.4.1.3. Adenosine Deaminase Activity (ADA)

Adenosine deaminase (ADA), is an enzyme produced by lymphocytes. Raised pleural fluid ADA is considered a useful biochemical marker for diagnosis of TPE, especially in high TB burden settings when associated with high protein and lymphocytosis. ADA has two iso-enzymes, ADA-1 produced by most cells, and ADA-2 which is produced by the monocytes and macrophages and is reported to be elevated in TB compared to other infections or malignancies [217]. Low pleural fluid ADA level is used to exclude the diagnosis of TPE, especially in patients with low pre-test probability [141]. Meta-analyses suggest good diagnostic performance for pleural fluid ADA [218-220]. Although raised levels do not establish a definite etiology of TPE, it is often used for clinical decisions to start anti-tuberculous therapy in the absence of bacteriological evidence. However, despite its widespread use, an optimum threshold value is not defined to discriminate TPE from other diseases [141, 221].

1.4.1.4. Antibody-based serological test

WHO in 2011 banned the use of commercially available antibody-based serologic tests for TB diagnosis due to inconsistency and imprecise accuracy and specificity [222].

1.4.2. *Laboratory methods for diagnosis of Drug-resistant TB*

The phenotypic and genotypic methods are used for the detection of drug resistance.

1.4.2.1. Phenotypic methods for detection of drug resistance

Culture-based phenotypic DST (pDST) methods are currently the gold standard for drug resistance detection. For pDST, mycobacteria are initially grown in a variety of solid or liquid culture media. Positive AFB culture growths are first identified for MTBC before

performing DST. Phenotypic methods are time-consuming, and require sophisticated laboratory infrastructure, qualified staff, and strict quality control [42, 43]. Rapid molecular-based DST is recommended by WHO as an initial test to detect drug resistance. However rapid tests for new and repurpose drugs recommended for MDR/RRTB treatment are not yet available. The diagnosis of extreme drug resistance TB (XDR-TB) is currently dependent on pDST for bedaquiline and linezolid [223].

1.4.2.2. Molecular methods for detection of drug resistance (gDST)

- **Line Probe Assay (LPA)**

LPAs are a family of DNA strip-based tests that detect mutations associated with drug resistance. In 2008, WHO endorsed the use of the first-line LPA (GenoType MTBDRplus) for the rapid detection of MDR-TB [224]. In 2016, second-line LPA, (GenoType MTBDR*sl*) was recommended by WHO as the initial test for MDR/RR-TB patients, to detect resistance to FQs and amikacin instead of pDST [225].

- *Xpert MTB/XDR Assay* **(Cepheid, Sunnyvale, USA).**

In 2021, WHO endorsed a cartridge-based Xpert MTB/XDR assay for detecting resistance to H, FQs, ethionamide, and second-line injectable drugs in less than 90min [42]. It is low complexity automated nucleic acid amplification test (NAAT) designed for the 10-colour GeneXpert instrument. This assay is intended for use as a reflex test on clinical specimens determined to be MTBC-positive. The technology has the advantage that it can be implemented in intermediate and peripheral laboratories [226, 227].

- **DNA sequencing using next-generation sequencing (NGS)**

NGS is a promising method for the detection of mutations associated with drug resistance for many anti-TB drugs with great potential to reduce the need for pDST for patient-care decisions and drug resistance surveys. NGS may be particularly useful for resistance detection to drugs for which pDST is unreliable or for settings with capacity issues [228]. The use of these technologies requires a standardized single reference source for the interpretation of mutations. To address this need, WHO has released a catalog of mutations in MTBC and their association with resistance [229]. Furthermore, the computational

expertise and resources required may limit the initial implementation of the current NGS system to central and well-performing regional laboratories [228, 230].

1.4.3. Challenges in the bacteriological diagnosis of TB

Challenges are encountered in the diagnosis of PTB and more often in EPTB.

1.4.3.1. Pulmonary TB

The diagnosis of PTB is often challenging in children and HIV-co-infected individuals due to paucibacillary disease, difficulties in expectoration, and atypical radiological abnormalities [231, 232]. Different procedures are used to obtain specimens from young children and also adults [233]. **Sputum induction** is performed using the ultrasonic nebulization of hypertonic saline. It is a relatively simple and safe procedure and is also suitable for use in resource-limited decentralized settings. It increases TB detection similar to more invasive techniques such as bronchoscopy [234-236]. **Gastric aspiration** is often used to obtain swallowed sputum from children and less frequently in adults. [237-239] **Bronchoscopy and biopsy** are used in patients having problems with diagnosis including endobronchial TB. [240, 241]

1.4.3.2. Extrapulmonary TB

Diagnosis of EPTB is more challenging, first a high index of clinical suspicion is required and patients with HIV coinfection, may have a nonspecific presentation [242], second, depending on the disease site invasive biopsies may be technically complicated to perform, third, EPTB disease is paucibacillary in the majority of the cases and often below the LoD of the diagnostic assays, making bacteriological diagnosis even more challenging [175, 243].

Table-2: Summary of diagnostic tools endorsed by WHO for diagnosis of TB and drug resistance [42, 176]

Category	Test	Year endorsed by WHO	TAT	Technology/ diagnosis	Recommended specimens	Advantages	Limitations
1. Conventional Diagnostic Test	AFB Microscopy	*	Same-Day	Staining/ AFB	Clinical Specimens Culture isolates	Inexpensive, rapid Minimal infrastructure, Recommended for monitoring of treatment.	Relatively insensitive, don't differentiate between dead and live bacilli, drug-susceptible and resistant, MTB and NTM
	LED-based FM	2009	Same-Day	Staining/ AFB	Clinical Specimens		
	Solid Culture	*	2-9wks		Clinical Specimen		
	Liquid culture	2007	1-3w	MTBC	Clinical Specimens	The gold standard, sensitive Diagnosis of CHTB and EPTB from paucibacillary samples, Differential Diagnosis of NTM infection.	BSL-3 laboratory required
2. Initial tests for diagnosis of tuberculosis with drug resistance detection	Xpert MTB/RIF assay LOD: 131cfu/ml	2010	Same-Day	NAAT/ MTBC Mutations associated with RIF resistance	Clinical Specimens (PTB & EPTB) Culture isolates	Low complexity – automated NAAT Sensitivity close to culture	Higher Infrastructure requirements compare to microscopy. Expensive, don't differentiate between dead and live MTBC bacilli
	Xpert MTB/RIF Ultra assay LOD: 16cfu/ml	2017	Same-Day			Simultaneous detection of RIF	
	Truenat MTB, MTB Plus, and MTB-RIF Dx assays	2020	Same-Day	MTBC	Clinical Specimens (PTB) Culture isolate	Low complexity Simultaneous detection of RIF	
	Moderate complexity automated NAATs						

Category	Test	Year	Time	MTBC	Clinical Specimen (sputum)	Clinical specimen	
3. Initial tests for diagnosis of TB without drug-resistance detection	TB-LAMP assay	2016	Same-Day				
	Urine LF-LAM	2016/2019	Same-Day	LAM	Urine	Performed on urine	Lack sensitivity
4. Follow-on diagnostic tests for detection of additional drug resistance	Xpert MTB/XDR Assay (Cepheid, Sunnyvale, USA).	2021	Same-Day	Mutations associated with H, Ethio, FQ, SLI		Minimal Infrastructure,	Requires a 10-color GeneXpert instrument.
	LPA						
	1. First-line LPAs	2008	2-Days	R and H	Clinical Specimen (AFB +ve) Culture isolates	Rapid detection of R and H	Medium complexity, Biosafety level 2 laboratory, AFB smear-positive specimen.
	2.Second-line LPAs GenoType MTBDRsl	2016	2-Days	FQ, SLI	Clinical Specimen (MTB+ve) Culture isolates	Rapid detection of FQ and SLI	
	3.High complexity reverse hybridization NAAT GenoScholar PZA-TB (Nipro, Osaka, Japan)	2021		Pyrazinamide	Culture isolates	The hybridization can be performed on the Twin Cubator instruments (Hain Lifescience, Germany) that are used for LPAs	High complexity
5. Phenotypic DST	Automated Liquid DST	2007		Phenotypic Resistance to First and second-line drugs	Culture isolates	Culture isolates	BSL-3 laboratory required

1.5.   Treatment of TB

The treatment of TB is centered on curing the individual patient, decreasing the transmission of TB bacteria to other people, and preventing the development of drug resistance during therapy [244].

1.5.1. Drug sensitive TB

Since 2010, WHO recommended a 6-month treatment regimen composed of R, H, Z, and ethambutol (E) for 2 months (2HRZE) followed by 4 months of R with H (4HR) is used globally for drug-sensitive TB [245]. The daily dosage of fixed-dose combination tablets is recommended with treatment support. In 2020, two new shorter treatment options were recommended by WHO including a 4-month regimen comprised of H, rifapentine, moxifloxacin, and Z for people aged 12 years or older with drug-susceptible PTB [2]. Rapid molecular-based diagnostic tools are recommended to test for resistance to R, H, and FQ [2]. The other 4-month treatment regimen (2HRZ(E)/2HR) is recommended for children and adolescents between 3 months and 16 years of age with non-severe TB [2, 246, 247]. The description of non-severe TB includes three EPTB disease forms (peripheral LNTB, intrathoracic LNTB without airway obstruction, and uncomplicated TPE) and PTB disease which is confined to one lobe of the lungs, is non-cavitary, without a miliary pattern and is paucibacillary [246]. A standard six-month treatment regimen is recommended for all other forms of EPTB except OATB and CNS TB for which longer therapy with adjunctive corticosteroids is suggested [2].

1.5.2. Drug resistant TB

1.5.2.1.   MDR/RR-TB Treatment

Until 2016, MDR-TB treatment was based on an injectable drug plus an FQ for a duration of 18–20 months or longer [248, 249]. In 2016, WHO grouped MDR-TB and RR-TB as MDR/RR-TB and for the first-time recommended use of a short treatment regimen in selected patients [250, 251]. The overall success of MDR TB treatment still remained far from the 2035 milestones [15]. In 2019, the first integrated recommendation for the

management and care of MDR/RR-TB was released [252]. Second-line treatment was substantially changed, based on evidence of improved therapeutic success with new regimens. Three new drugs (bedaquiline, delamanid, and pretomanid) and two repurposed drugs (linezolid and clofazimine) are now recommended for the treatment of drug-resistant TB. WHO new guidelines recommend shorter all-oral treatment for MDR/RR-TB patients [252]. These changes have also led to changes in the drug classification for MDR-TB, and in the definition of extensively drug-resistant tuberculosis (XDR TB) and pre-XDR TB [3, 248, 252, 253].

1.5.2.2. Rifampicin-sensitive and isoniazid-resistant TB treatment

In 2018, a 6-month treatment regimen including R, E, Z, and levofloxacin (LFX) was recommended for patients with confirmed R-sensitive, H-resistant TB [254].

1.5.3. TB preventive treatment

Among individuals infected with *M. tuberculosis*, there is a 5-10% risk of progressing to active TB. The risk is particularly elevated in children under the age of 5 years and people with compromised immunity. The recommended TB preventive treatment options include 6 or 9 months of daily H, 3 months of weekly rifapentine(P) plus H (3HP), and 3 months of daily HR. Alternative regimens include 1 month of daily rifapentine plus H and 4 months of daily R [255].

1.6. Situation of TB epidemics in Pakistan

Pakistan is a country in Southeast Asia with a population of 231 million (2021) and is part of the WHO Eastern Mediterranean region [28]. Pakistan's terrain is divided into three major topographic areas: the Northern highlands, the Indus River plain in the east, and the Baluchistan plateau in the west. The country is administratively divided into four provinces namely, Punjab, Sindh. Khyber Pakhtunkhwa (KP) and Balochistan, two regions including Azad Jammu Kashmir (AJK) and Gilgit Baltistan (GB), and Islamabad Capital Territory (ICT). The average population density is 287/sq. km. which varies greatly between provinces and districts. Sixty-six percent of the population lives in rural areas and

40% of the urban population lives in slums. Pakistan is a lower middle-income country and an estimated 4% of the population is living below the international poverty line. The prevalence of undernourishment is 12% and 0.1% of the population is infected with HIV, 12% of females and 13% of males have diabetes whereas smoking is prevalent in 3% of females and 38% of males [256]. The HIV epidemic is concentrated in key populations including people who inject drugs [257, 258].

1.6.1. TB burden in Pakistan

Pakistan ranks 5th among HBC and has an estimated 611K (445-803K) incident TB cases, and 48K people died of TB in 2021 [28]. A TB disease prevalence survey was conducted in 2010-11 to estimate the burden of TB in adult population over 15yrs [47, 48, 259]. Estimated TB cases are attributed to five risk factors including undernourishment (133k), smoking (34k), diabetes (30K), HIV(10k), and alcohol (4.8K) [28]. A fifteen-fold higher prevalence of TB is reported in HIV-infected injectable drug users compared to the general population in Pakistan [260].

Pakistan is also among the top five high drug-resistant TB burdens countries. The first population-based national drug resistance survey (DRS) on smear-positive PTB patients was conducted in 2012–13. Any resistance to R and MDR was reported in 4.2% and 3.7% of the new PTB cases and 19.8% and 18.1% among previously treated cases [261]. Among R-sensitive patients' resistance to LFX and Z was reported at 10.3% and 0·5% respectively. Among RRTB, resistance to LFX and Z was reported in 21.8% (13·1–30·5) and 39.5% (95%CI 30.1–48.9) respectively [262].

1.6.2. Progress and challenges in TB control effort.

In Pakistan, the *implementation* of the DOTS strategy started in 2001 [263]. In the initial five years, TB care services were established, integrated within the public health sector. In the next five years, the TB program focused on improving the quality of diagnostic services, and expansion of DOTS coverage in the private sector [264]. Starting in 2009, services were expanded to include programmatic management of drug-resistant TB, scale-

up of rapid molecular diagnosis, culture, and DST services, private sector engagement, childhood TB, and active case findings [265, 266].

1.6.2.1.   TB care and diagnostic services

The TB diagnosis relied totally on AFB microscopy for the initial 15 years of DOTS implementation. Xpert was introduced in 2011, very soon after its endorsement by WHO. However, its expansion and uptake was gradual and the diagnostic algorithm was modified over time in parallel with improved coverage [265]. Xpert was initially recommended for people at risk of DR-TB and those with immunocompromised conditions, in 2015, recommendations were expanded to cover the diagnosis of TB in children and EPTB, and in 2017 for R testing of all bacteriologically confirmed TB patients and for diagnosis of people with abnormal chest X-rays. Finally, in 2021, Xpert testing was recommended for all patients with signs and symptoms of TB [265]. R-sensitive TB patients are initiated on standard six-month TB treatment and are not tested for resistance to other drugs. RR-TB patients diagnosed are referred to specialized programmatic management of drug resistant TB (PMDT) treatment sites. The clinical specimen is collected for comprehensive DST to investigate for additional drug resistance and RR-TB patients are initiated on second-line TB treatment.

1.6.2.2.   TB surveillance system

In 2019 there were more than 1400 TB management units (TBMU) and 1,746 laboratories, including 411 in the private sector, offering TB diagnosis. Xpert testing facilities were available in 327 of the laboratories [266]. Each TBMU maintains a standard TB register, in which individual patient data (IPD) is recorded of all notified TB patients. At the end of each quarter, all reporting TBMUs prepare TB notification reports in a standard format. The facility reports are consolidated into a district report and further into the provincial report. Provincial TB programs, validate and submit these reports to the surveillance unit of the National Tuberculosis Program (NTP). In these reports, TB cases notified are stratified by PTB (bacteriologically confirmed and clinically diagnosed) and EPTB.

However, EPTB cases are not stratified by laboratory diagnosis, and information on EPTB disease sites is not included. Treatment outcomes are reported after a year in the same quarter and are presented collectively for PTB and EPTB incident TB cases.

Similarly, separate quarterly reports are generated by the PMDT treatment site for notified RR-TB patients started on second-line TB treatment. Information on the disease site (PTB and EPTB) is not included in this report. For RR-TB, treatment outcome reports are generated after two years.

1.6.2.3. TB Notification, treatment coverage, and Outcomes

A gradual increase in TB notification was seen between 2001-2015. TB notifications remained stagnant at 164-167/100K during 2016-18 followed by a decline to 147/100Kpopulation in 2019. [266] This declined further down to 120/100k during the COVID-19 pandemic, with partial recovery in 2021 to 147/100k [28]. In parallel with enhanced engagement with the private health sector for TB control, a gradual increase in private sector contribution to TB notification was noted from 27% in 2016 to 40% in 2021 [28, 267]. A gradual increase in the proportion of EPTB was noted during the first decade of DOTs implementation and is constant at around 20% since 2013. In Table-3, a comparison of TB notification by province/region is shown for 2021 compared to 2016 [28, 267].

Table-3: TB notification of pulmonary and extrapulmonary TB in 2016 and 2021

	Tuberculosis		Pulmonary TB		Extrapulmonary TB		EPTB%	
	2016	2021	2016	2021	2016	2021	2016	2021
Pakistan	**356390**	**338844**	**285068**	**278567**	**71322**	**60277**	**20%**	**18%**
Punjab	218284	208353	183141	180473	35143	27880	16%	13%
Sindh	67987	67218	55878	54414	12109	12804	18%	19%
Balochistan	10137	11616	7616	8834	2521	2782	25%	24%
KP	45159	42569	28360	27925	16799	14644	37%	34%
FATA*	4126		2745		1381		33%	
AJK	5663	4848	4113	3597	1550	1251	27%	26%
GB	2767	3064	2080	2532	687	532	25%	17%
ICT	2267	1176	1135	792	1132	384	50%	33%

*FATA districts were merged in KP province in 2018

Among PTB cases, the proportion of bacteriologically confirmed is almost constant at around 50%. The proportion of bacteriologically confirmed PTB cases being tested for R resistance is gradually improving in parallel with improved coverage of Xpert testing. In 2019, 59% of the new and 89% of previously treated PTB cases were tested for R-resistance [26]. A gradual decline in the proportion of RR-TB was noted from 19.8(2013) to 7.3% (2019) among previously treated TB patients [26, 265]. Simultaneously a gradual increase was seen in MDR/RR-TB patients initiated on second-line treatment from 200 in 2009 to approximately 3000 annually. Among MDR/RR-TB patients, almost 40% are pre-XDR TB [28, 268].

Since 2013, TSR for drug-sensitive TB is maintained at above 90% [28]. With the introduction of bedaquiline and other new drugs containing shorter treatment regimens for MDR/RR-TB patients an improvement in TSR is noted from 64% in 2015-2017 to 73% in 2018-19 [28], alongside reports of acquired resistance to bedaquiline [269, 270].

1.6.3. Problem statement

In Pakistan one in every five TB patients notified has EPTB but there is limited information on

- Prevalence of different disease manifestations of EPTB.
- Performance of WRD in the diagnosis of EPTB.
- Prevalence of drug resistance in TB treatment naïve EPTB patients.
- Prevalence and genetic profile of H resistance in EPTB compared to PTB patients.

2. OBJECTIVES

The overall aim of the studies was to describe disease manifestations, evaluate the performance of diagnostic methods and the prevalence of drug resistance in EPTB patients in Pakistan

Study-1

The primary aim of the study was to describe the disease manifestations of EPTB in Pakistan. The secondary objectives included, the study of demographic characteristics and treatment outcomes of EPTB patients in routine settings.

Study-2

This study aimed primarily to assess the diagnostic value of rapid molecular methods (Xpert MTB/RIF) and histological examination in the diagnosis of LNTB. The secondary objectives aimed to evaluate the performance characteristics of molecular and histology examination against culture and treatment outcomes of patients diagnosed using different methods.

Study- 3

This study aimed to determine the prevalence of drug resistance in new EPTB patients. The secondary objectives included the evaluation of the performance of Xpert in the diagnosis of LNTB and TPE and the performance of LPA compared to pDST in the detection of resistance to R, H, and FQ.

Study-4

The primary aim was to study the prevalence of H-resistance in EPTB and the secondary aim was to study the genetic profile of phenotypic H-resistant strains and associated LFX and Z resistance in R-resistant and R-sensitive populations among PTB and EPTB patients.

3. MATERIAL AND METHODS

3.1.   Study setting, design, and population

All four studies were conducted in Pakistan. A brief overview of the status of TB epidemics, TB care services, and TB control program in Pakistan is given in the introduction. A summary of the study settings, design, population, sample size, data collection method, and ethical considerations is given in Table-4. Additional details are given below

Study-1: TB patients notified in the routine program setting were studied. During the study period (2016), there were 1345 TBMUs with AFB microscopy in all, and 73 in addition, had on-site Xpert testing facilities [267]. In 2016, 68% (n=366061) of the estimated incident TB cases were notified with 27% contribution by the private sector. Among TB cases notified 20% had EPTB. Stratified convenient sampling was used for site selection and paper TB registers were collected from 50 TBMU in 32 districts including 29 primary (16 public, 13private), 11 secondary (all public), and 10 tertiary hospitals (8 public, 2 private) covering all provinces and regions.

Studies 2 and 3: Both studies were nested in a large research project aimed at improving the diagnosis of EPTB under routine programmatic conditions in Pakistan. The study was conducted at Gulab Devi Hospital (GDH) a tertiary care hospital specialized in TB care. Presumptive TB and TB patients are often referred to GDH for consultation and treatment from other cities and districts. After the diagnosis, TB patients are offered options for treatment in TBMU close to their residence. For this study, eligible patients with presumptive LNTB and TPE were recruited from OPD. Among TB patients diagnosed those who opted for treatment at GDH were followed till completion of treatment.

Study 4: TB patients referred in routine program setting to NTRL for DST. Patients with valid R and H DST results were included. Duplicates were excluded and results of both phenotypic and gDST were included.

Table-4: Summary of study settings, design, population, and sample size of four studies contained in the thesis

	Study-1	Study-2	Study-3	Study-4
Objective	Disease manifestation. demography and treatment outcomes of EPTB patients notified in 2016 in Pakistan	The added value of histological examination compared to the molecular method of diagnosis	Primary drug resistance in EPTB	H resistance in EPTB. Genetic profile of H resistance and associated FQ and Z resistance in EPTB compared to PTB
Study setting	Multi-centric TB Management unit including 37 public and 13 in the private sector	Single center Tertiary care hospital specialized in TB care	Single center Tertiary care hospital specialized in TB care	Single reference center NTRL Islamabad, Pakistan with a referral for DST from 50% of the country
Study Design	Retrospective Cohort Descriptive	Prospective Cohort Diagnostic accuracy	Prospective Cross-sectional Prevalence	Retrospective Cross-sectional Observational
Data collection	Paper TB registers with individual TB patient data registered at health facilities selected using stratified convenient sampling	Electronic and paper clinical record forms of patients presumed to have TB lymphadenitis.	Electronic and paper clinical record forms of patients presumed to have TB lymphadenitis and TB pleural effusion	Electronic laboratory registers of NTRL
Study population	All types of notified PTB and EPTB patients in 2016 at selected health facilities	Presumptive TB patient with lymphadenopathy	Presumptive TB patients either with lymphadenopathy or pleural effusion	PTB and EPTB patients tested in NTRL with DST results for R and H.
Study period	One year (2016)	April 2016 to August 2017	April 2016 to August 2017	Five years (2015-2019)
Sample size	54092 notified TB patients, including 38,302 PTB and 15,790 EPTB	412 presumptive TB patients with lymphadenopathy	671 presumptive TB patients including 412 having lymphadenopathy and 259 with pleural effusion	11045 TB patients, including 9647 PTB and 1398 EPTB
Ethical consideration:	IRB-CMU(ATM)* Islamabad, Pakistan	NBC-Pakistan and REK VEST – Norway	NBC-Pakistan and REK VEST - Norway	IRB-CMU (ATM)* Islamabad, Pakistan

*Institutional review board, common management unit Aids TB and Malaria, ** National bioethics committee Pakistan*** Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest). University of Bergen (Postboks 7804, 5020 Bergen, Norway).

3.2.   Laboratory methods and reference standard

Laboratory methods used in four studies are summarized in Table-5.

Study-1: During the study period, AFB microscopy was a used as frontline diagnostic test in routine, and Xpert testing was offered to only a select group of patients at risk of drug resistance.

Study-2: Excision biopsy specimens of lymph nodes were processed for histopathology and mycobacteriology including AFB smear, Xpert, and culture.

Study-3: Excision biopsy specimen of lymph node (same patients as in study-2) and pleural fluid specimens were examined at GDH laboratory for mycobacteriology and histopathology (Table-5). For pDST, *M. tuberculosis* isolates were sent to NTRL. DST was performed on all MTBC culture isolates using phenotypic automated liquid (MGIT) and genotypic (LPA) DST methods.

Study-4: All clinical specimens were processed for culture and specimens from known RR-TB patients were processed in parallel for rapid gDST (LPA). Automated liquid pDST (MGIT) was performed on all MTB culture isolates. In addition, gDST was performed on culture isolates if the clinical specimen was not tested or results were not available.

Reference standard: For diagnostic accuracy, a reference standard was used to evaluate the performance of the index test [42]. For study-2, the performance of Xpert and histopathology was evaluated against culture. For study-3 performance of LPA was evaluated against liquid DST as the reference standard.

Quality control: For study-1, quality assessment of AFB microscopy by blinded rechecking was implemented in routine settings.

All DSTs (studies 3 and 4) were performed at NTRL Recommended quality control measures were followed and control strains were tested with each batch of DST [271]. For external quality assessments, NTRL regularly participated and qualified in annual proficiency testing conducted by the TB supranational TB reference laboratory at institute of tropical Medicine, Antwerp, Belgium.

Table 5: Summary of the laboratory methods in four studies

	Lab Methods Description	Study-1	Study-2	Study-3	Study-4
TB Diagnosis					
1	AFB microscopy				
1a	Auramine O-stain. Stained smears are examined using a Light emitting diode (LED) fluorescence microscope, or	Yes	Yes for AFB microscopy of clinical samples		Yes for AFB microscopy of clinical samples
1b	Ziehl-Neelsen Stain. Stained smears were examined using a bright field microscope	Yes	Yes, for identification of AFB in culture growth		Yes, for identification of AFB in culture growth
2	Rapid molecular Assay: Xpert MTB/RIF v4.0 (Cepheid, Sunnyvale, CA, USA)	*	Yes		**
3	Culture				
3a	Solid media –Lowenstein Jensen,	No	Yes-One slope		Yes-One slope
3b	Automated liquid MGIT 960; Becton Dickinson, Sparks, MD, USA	No	Yes-One tube		Yes-One tube
4	Identification of cultural growth	No	Yes, for MTBC identification in all AFB+ culture isolates		Yes, for MTBC identification in all AFB+ culture isolates
5	**Histopathology** Hematoxylin and Eosin-stained section	***	Yes	NA	NA
Drug susceptibility testing					
1	Phenotypic DST (MGIT 960) Becton Dickinson, Sparks, MD, USA) R (1.0ug/ml), H (0.1ug/ml), Z (100ug/ml), and ofloxacin (2ug/ml) during 2015–17 and LFX (1.0ug/ml) during 2018–19	No	Yes- on all MTBC culture isolate		Yes- on all MTBC culture isolate
2	Genotypic DST (Line probe assay)				
2a	MTBDRplus V2.0 (Hain Lifescience, Nehren, Germany)	No	Yes-MTB culture isolate		Yes, clinical specimen or MTBC culture isolate
2b	MTBDRsl V2.0 (Hain Lifescience, Nehren, Germany)	No	Yes-MTB culture isolate		

*In selected at-risk population ** done for some at NTRL, information not used for the study

*** Information not recorded in the TB register

3.3. Case definitions and statistical analysis

Case definitions: A TB case and drug resistance were defined based on composite results. [272]. For **study-1,** the TB case was defined based on AFB microscopy or clinical diagnosis. A bacteriologically confirmed TB case was defined based on positive AFB microscopy. For **study-2**, the TB case was defined based on composite results of Xpert, culture, and histopathology. Bacteriologically confirmed TB case was defined based on a positive culture and/or Xpert result. Histopathology consistent with TB was defined as morphological features showing well or poorly-formed granuloma and/or caseous necrosis with or without Langhans-type giant cells. **For study-3 and 4, d**rug resistance was defined based on composite results of pDST and gDST.

Data management and Statistical analysis: Data collected in paper form was entered electronically. For study-1, data on the EPTB disease site, if was found missing for more than 20% of the EPTB patients notified by any TBMU, the facility was revisited to collect missing information from hospital records.

For study 1,2,3, data was entered in EpiData Manager v4.2 (Epi-Data Association, Odense, Denmark). Data analyses was done on Stata v13 (Stata Corporation, College Station, TX, USA).

Logistic regression was performed to calculate odds ratios (OR) and 95% confidence intervals for comparisons between groups, while mean, median, and quartiles were analyzed for quantitative variables. Two sample proportion test was used to analyze differences in proportions between groups. A p-value of <0.05 was considered statistically significant. Cross-tabulation was used to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

Ethics statement: Ethical approval was obtained for all four studies (Table-4). For the prospective cohort, (Study 2,3) participants were enrolled after informed consent. All data sets were de-identified before analysis.

4. RESULTS

4.1.   Extrapulmonary TB in Pakistan

4.1.1. *Disease manifestations of EPTB*

The TB registers were collected from 50 healthcare facilities with records of 54,092 TB patients notified in 2016. EPTB was reported in 29.2% of all TB patients, The most common EPTB disease manifestations were pleural (29.6%), lymphatic (22.7%), and abdominal TB (21.0%). Pleural TB was significantly more common in males (34.5 vs 25.1%, $P < .001$) and lymphatic TB in females (26.6 vs 18.4%, $P < .001$). Except for pleural TB, a higher number of females were notified within each EPTB disease form. A steady increase in pleural and OATB was noted with advancing age simultaneously with the decline in lymphatic and abdominal TB.

4.1.2. *Demographic characteristics of EPTB patients*

Among the study population, 30 % of females and 27.9% of males had EPTB. The median age was 24 years for EPTB and 30 years for PTB patients. Peak notification (27%) for PTB and EPTB was seen in the age group of 15-24yrs with females being in the clear majority among both disease types (F: M 1.4:1). Children under 15 years had the highest prevalence of EPTB (43.3%). The odds of having EPTB (OR), was 1.1 for females, 2.0 for children, and 3.3 for residents of the Northwest part of the country.

4.1.3. *Treatment outcomes of EPTB patients*

TSR, for all forms of EPTB collectively was higher compared to PTB. TSR for pleural, lymphatic, and abdominal TB was 90% or above but was significantly lower for TBM (74%) with a high mortality rate and loss to follow-up among notified cases.

4.2.   Diagnosis of lymph node TB

A total of 412 people with enlarged lymph nodes presumed to have TB were recruited in the study. Excision biopsy specimens were examined for bacteriology and histology. Using composite reference standard, 228 patients were diagnosed having LNTB.

4.2.1. Performance of laboratory methods in diagnosis of LNTB

Among 228 LNTB cases, 178 were **bacteriologically confirmed** including 11 (4.8%) positive by AFB microscopy, 124 (54.4%) by Xpert, and 137 (60.1%) by culture including 83(46.6%) both by Xpert and culture. LNTB disease in the majority (92%) of the cases was paucibacillary and MTB detected by Xpert was low to very low. Culture recovery was higher in specimens with higher bacillary load and in patients with short clinical history (45/55=81.8%) compared to those who were sick for more than three months (22/36=61%). Among bacteriologically confirmed, 89% (n=158) had histology consistent with TB.

Among 208 LNTB having **histology consistent with TB**, 92% had necrotizing granulomatous inflammation, 6% had caseous necrosis without granuloma and 2% had granulomatous inflammation without necrosis. Among this group, 76% of LNTB were bacteriologically confirmed. Histopathology versus Xpert had higher sensitivity (93 vs. 62%) but lower specificity (68 vs. 83%) against culture.

4.2.2. Demography and clinical symptoms

The median age of LNTB patients was 19 yrs. and 61% were females. Among LNTB patients, 38% were sick for 3 months or less, 35% for more than 3 months and history was not available for others. Along with lymphadenopathy, 5.2% of LNTB patients presented with abscess, and 3% had sinus formation. Of 147 LNTB patients interviewed, 89% complained of fever, 52% had a current cough, 37% weight loss, 30% appetite loss, and 14%-night sweats. Of 157 chest X-rays examined, intrathoracic lymph nodes were reported in 30%, abnormal chest X-rays suggestive of TB in 10%, and pleural effusion in two.

4.2.3. Treatment outcomes of LNTB

Of 183 LNTB patients followed till completion of treatment, slow clinical response was reported in 27% and treatment was extended beyond six months. In a subgroup of patients with histology suggestive of TB, no difference in treatment response was seen between bacteriology-positive and negative cases. Whereas among bacteriologically-confirmed TB, a significantly slower clinical improvement was reported in patients lacking granulomatous

response compared to those having histology consistent with TB.

4.3.   Primary drug resistance in extrapulmonary TB

A total of 671 TB treatment naïve people, including 412 with lymphadenopathy and 259 with pleural effusion were recruited in the study. The proportion of females and children among LNTB was 60% and 27% and among TPE groups was 35% and <1% respectively.

4.3.1. Bacteriological diagnosis

Among **lymph nodes** examined**,** 43% (178) were bacteriologically confirmed including 136 positives on culture, 124 by Xpert including 82 by both methods. The performance of Xpert against culture in the diagnosis of LNTB had a sensitivity of 60.3%, specificity of 88.8%, PPV of 66.1%, and NPV of 86.1%.

Among **pleural fluids** examined, 29.7% (77) were bacteriologically confirmed, including 69 positives on culture and 18 by Xpert including 10 by both. The performance of Xpert against culture in the diagnosis of TPE had a sensitivity of 14.5%, specificity of 95.8%, PPV of 55.5%, and NPV of 75.5%.

4.3.2. Primary drug resistance

Among 205 culture-positive EPTB cases, DST results were available for 90.2% including pDST for 182 and gDST for 184 patients. Any resistance to R was reported in 2.7% (95% CI, 0.9–6.2), H in 7.6% (95% CI, 4.1– 12.4), E in 1.1% (95% CI, 0.1–3.9), Z in 2.2% (95% CI, 0.9–5.5) and FQ in 6.0% (95% CI, 3.0–10.4). Resistance to second-line injectable drugs was not reported in any case. MDR-TB was reported in four (2.2%, 95% CI, 0.6–5.4) and pre-XDR TB in two patients. Among R-sensitive patients, H resistance was reported in 5.5% (95% CI, 2.9 -10.0) and FQ resistance in 5.0% (95% CI, 2.5 - 9.4).

4.3.3. Genotypic DST performance and drug resistance profile

Among 185 cases, RR was detected in five including four strains resistant by both MGIT and LPA with resistance conferring mutations in *rpoB* S531L in all four. One RR was detected by LPA, with a mutation in E510H/L511P.

Of 14 H-resistance cases by MGIT, 13 were reported resistant by LPA with mutations in *katG* S315T in seven (four MDR-TB and three non-RR-TB strains) and *inhA* promoter region in six (six non-MDR-TB strains). FQ resistance was reported by MGIT in nine and 11 by LPA, with a mutation in *gyrA* D94G in five (one MDR-TB and four non-MDR-TB), A90V in four (one MDR-TB and three non-RR-TB), and S91P and D94H in one each (both non-RR-TB). The sensitivity and specificity of LPA against liquid DST for detection of R, H, and FQ resistance were 100% and 99%.

R results were available for 82 cases by all three DST methods. Among these, five were reported RR by Xpert, of these four were sensitive both by MGIT 960 and LPA and only one was resistant by LPA (MTBDRplus)

4.4. Isoniazid resistance, genetic profile, and associated resistance

We analyzed a large five-year DST data set from 11045 TB patients (PTB=9647, EPTB=1398) tested in NTRL Pakistan. The proportion of females, children (<15yrs), and new TB cases was 48.6%. 6.1% and 44% among PTB and 49.7%. 17% and 91.7% respectively in EPTB patients.

EPTB disease site: Among 1398 EPTB patients included, 41% had LNTB, 20.5% TPE, 6.2% TBM, 1.0% had pericardial TB, 0.5% had GUTB, 2.9% other disease sites and in 27.9% EPTB disease site was not specified.

4.4.1. Rifampicin and isoniazid resistance

Of all cases included, 79.5% (n=8787, PTB=7551, EPTB=1236) of the isolates were tested by both pDST and gDST methods. A significant difference was seen in the proportion of R and H-resistance detected by pDST and gDST methods (*P* < .001) among PTB isolates. Among the study population, 55.4% of PTB (n=7551) and 9.5% of EPTB were RR. Among RR, 94.9% (95% CI, 94.2–95.5) of PTB and 90.7% (95% CI, 83.9–95.2) of EPTB were MDR and among R-sensitive, 11.6% (955 CI, 10.5–12.7) of PTB and 6.7% (95% CI, 5.4–8.3) of EPTB were resistant to H.

4.4.2. Genotypic profile of isoniazid resistance

Rifampicin resistant TB. Among 3971 MDR-PTB isolates, 13.1% (519) of the strains were genotypic wild type (WT) on LPA. Genetic mutations were detected in *katG* in 76.1%, *inhA* in 7.6%, and combined *katG* and *inhA* mutations in 3.1%. Among 107 MDR-EPTB isolates, 8.4% were genotypic WT, mutations were detected in *katG* in 79.4% and *inhA* in 10.3%, and double mutation in 2 isolates.

Rifampicin sensitive TB. Among 389 H-resistant PTB isolates, 29.6% (115) were genotypic WT, and resistance-conferring mutations were detected in the *katG* gene in 40.6%. *inhA in* 29.6%, and double mutation in one isolate. Among 75 H-resistant EPTB isolates, 22.7% were WT on LPA. H-resistant conferring mutations were detected in *katG* in 44%, and *inhA* in 33.3%, with no double mutation.

Within both RR and R-sensitive populations, significant differences were not reported in H genetic profile between PTB and EPTB, new and retreatment, or in annual trends

4.4.3. Associated levofloxacin and pyrazinamide resistance

Rifampicin resistant TB. Among MDR-PTB isolates (n = 3969), resistance to LFX, Z and combined LFX and Z were reported in 49%, 47%, and 27.9%, compared to 23.8%, 6.4%, and 3.0%. in RR-TB isolates not associated with H-resistance (n = 214). Similarly, among MDR-EPTB (n = 107) isolates, resistance was reported in 48.6%, 61.7%, and 33.0% compared to 9.1%, 0%, and 0% in RR-TB not associated with H resistance (n = 11). The difference in resistance was not significant between PTB and EPTB, new and previously treated TB.

Rifampicin sensitive TB. Among 389 R-sensitive H-resistant PTB isolates, resistance to LFX, Z, and combined LFX and Z, isolates were reported in 25.2%, 12.2%, and 6.4% respectively compared to 13.0%, 3.7%, and 1.0 in 2976 H sensitive isolates. Among 75 H-resistant EPTB isolates, resistance was reported respectively in 6.7%, 8.8%, and 0% compared to 7.2%, 2.8%, and 0.2% in 1042 H-sensitive EPTB isolates. Significant differences were not seen in the annual trend of resistance to LFX and Z.

Figure-2: Key finding of four studies included in thesis

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                                        ┌──────────┐
                                        │ Study-1  │
                                        └──────────┘
  ┌──────────────────────────────────────────┐
  │ The three most common prevalent EPTB      │
  │ disease manifestations in Pakistan are     │
  │ pleural (29.6%), lymphatic (22.7%) and     │
  │ abdominal TB (21.0%)                        │
  └──────────────────────────────────────────┘
                                        ┌──────────┐
                                        │ Study-2  │
                                        └──────────┘
  ┌──────────────────────────────────────────┐
  │ Xpert MTB/RIF assay diagnosed TB in 54%    │
  │ and combined with histological examination,│
  │ in more than 95% of the of all TB lymph    │
  │ nodes cases                                 │
  └──────────────────────────────────────────┘
                                        ┌──────────┐
                                        │ Study-3  │
                                        └──────────┘
  ┌──────────────────────────────────────────┐
  │ In TB treatment-naïve EPTB patients, any   │
  │ resistance to rifampicin was 2.7% (95% CI  │
  │ 0.9–6.2), isoniazid 7.6% (95% CI, 4.1–     │
  │ 12.4), pyrazinamide 2.2% (95% CI, 0.9–5.5) │
  │ and fluoroquinolones 6.0% (95% CI, 3.0–    │
  │ 10.4).                                      │
  └──────────────────────────────────────────┘
                                        ┌──────────┐
                                        │ Study-4  │
                                        └──────────┘
  ┌──────────────────────────────────────────┐
  │ H-resistance among R-sensitive new EPTB    │
  │ patients was 6.8% (95% CI, 5.4-8.5). The   │
  │ genetic profile of H-resistance was similar│
  │ in PTB and EPTB. There was a significant   │
  │ difference in H resistance profile between │
  │ RR and R-sensitive TB and a higher LFX and │
  │ Z resistance associated with H resistance  │
  └──────────────────────────────────────────┘
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5. DISCUSSION

5.1.   Methodological considerations

5.1.1. Study design, study population, and follow-up period

Study-1: A retrospective cohort study design was considered suitable to study a large number of EPTB patients for disease manifestations. Data collection was started in 2018, and a cohort of 2016 was considered most appropriate for the study as this was the most recent cohort to have completed treatment by that time point. Standard TB registers were collected with IPD including age, sex, previous history of TB treatment, laboratory results, and treatment outcomes.

The main advantage of the retrospective study design was that it allowed a large sample of TB patients with representation from all provinces/regions and all tiers of health care to be entered into a study. The study design had the additional advantage of time and cost efficiency. Second, as standard TB registers were used as a data source, information on the same variables was available for the majority of the patients. Lastly, observer bias was possibly minimal as TB data was collected in a routine setting independently of any specific hypothesis. The key weaknesses of this design included, first, incomplete or missing records in some e.g., disease site was not recorded in 10% of EPTB patients. Second, as it was an observational study, it was not possible to differentiate between causal links or simple associations for any reported significant association e.g., high notification of abdominal TB in children in the Northwest part of the country.

Study-2 and 3: Both studies were nested in a larger research project designed as a prospective cohort study. The main advantage of the study was that all patients were pre-screened and only TB treatment naïve patients were included. Excision biopsy was performed on all and specimens were systematically tested using Xpert, culture, and histological examination (in the case of lymph nodes). The main limitations of the study design were that, it was a single-center study, second as it was conducted in a tertiary care setting, where a considerable number of patients were referred for consultation or second

opinion with diagnostic tests already performed in some. All these lead to slow recruitment in the study.

Study-2: Other than mentioned above, the additional strength of this study was that the STARD (STAndard for reporting diagnostic accuracy studies) checklist for the reporting of diagnostic accuracy was followed as closely as possible [273, 274]. Composite results were used for final diagnosis and treatment outcomes were studied for subgroups of patients reported positive by different diagnostic methods. The key weaknesses were incomplete clinical and radiological data as only $2/3^{rd}$ of the patient consented to in-depth interviews and treatment at the study site and all chest X-rays were not available in the hospital record at the time of the second reading.

Study-3: The main limitation of the study was that over 17 months, only 671 eligible people (412 with enlarged lymph nodes and 259 with pleural effusion) were recruited. Furthermore, among 255 bacteriologically confirmed TB cases, DST results were available for 72.5% of patients.

Study-4: For this laboratory-based surveillance study, cross-sectional study design and retrospective sampling were considered suitable. PTB and EPTB patients, for whom DST was performed during 2015-19, using pDST and/or gDST method were included. Only one unique isolate per TB patient was included in the final data set. The main strength of the study design was that retrospective sampling allowed analysis of large DST data set for H resistance, genetic profile, and trend over time. Furthermore, for almost 80% of the patients, both pDST and gDST results were available. The main weakness of the retrospective study design was that TB patients were classified as new and previously treated based on information in the laboratory request form with possibilities of errors and misclassification. Lastly, as it was a laboratory-based study, patients were not followed for treatment outcomes.

5.1.2. Laboratory methods and reference standard

Study-1: AFB microscopy was the only method used for TB diagnosis. More than 50% of PTB and almost all EPTB patients were diagnosed clinically and initiated on TB treatment with the possibility of over and under-diagnosis.

Study-2: Slightly more than half of all LNTB patients were diagnosed by Xpert. Xpert Ultra with better sensitivity was not yet launched at the time of study and GeneXpert MTB/RIF version G4 was used with the possibility that Ultra would have higher bacteriological confirmation compared to our results.

Study-3: Xpert MTB/Rif was performed on clinical samples whereas LPA and pDST were performed on the same culture isolates. GeneXpert MTB/RIF version G4 is known to report false RR in specimens with a low bacillary load. We reported a very low sensitivity and specificity of Xpert for RR detection, our conclusions most likely will not apply to Xpert Ultra, which is claimed to have overcome the issues of false RR.

Study-4: Composite results of pDST and gDST (LPA) were used to define resistance, but further investigation was not done to analyze the reason for discordance.

Reference standard:

The gold standards are seldom perfect and absolute predictors of disease. In medicine and statistics, a gold standard test refers to a diagnostic test or benchmark that is the best available under reasonable conditions [275]. For evaluating new tools for the diagnosis of TB and drug resistance, culture and the phenotypic liquid DST is considered the gold standard. However, culture is known to be an imperfect reference standard [42, 276] as mycobacteria may fail to grow on culture due to various reasons [277, 278]. Similarly, pDST is also challenged as the gold standard for resistance detection and it is reported to miss clinically and epidemiologically highly relevant resistance [279, 280]. The use of a reference standard which is not perfect is likely to have added bias to our analysis for performance characteristics of the index test with the risk of underestimating the specificity by regarding the true positive result as false positive (study-2).

In study-4, we reported significant differences between pDST and gDST in resistance detection to R and H.

For analysis of performance characteristics, index and reference tests need to undertake simultaneously to avoid biases caused by changes in true disease status. [42]. In our study, although the same specimen was used for all tests, however in study-3, Xpert was performed on clinical samples whereas both pDST and gDST (LPA) were performed on culture isolates. In study-4, LPA was performed on clinical samples for the majority of the cases and pDST was performed on culture isolates. This probably was the reason for good agreement between pDST and LPA in Study-3 but not in study-4.

5.1.3. Reliability and validity

The quality of the findings is largely dependent on the quality of the data. Reliability is about the consistency of a measure, and validity is about the accuracy of a measure [281].

5.1.3.1. Internal validity

Internal validity defines the extent to which the observed results represent the truth in the study population and, thus, are not due to methodological errors.

Study-1: For site selection, we purposefully went for oversampling of EPTB to study a wider spectrum of EPTB disease manifestations. There was thus a higher proportion of EPTB in our study sample (29%) compared to national notification (20%) in the same year. Selection bias as a result of non-random selection of health facilities and information missing on disease sites for 8.5% of EPTB patients, is likely to have skewed our findings to some extent. Furthermore, almost all EPTB patients were clinically diagnosed, the possibility of overdiagnosis and detection bias resulting in higher notification of abdominal TB in the Northwest province cannot be excluded.

Study-2-3: The study was conducted in a single specialized tertiary care hospital for TB with the possibility of recruiting patients having a higher risk of TB and drug-resistant TB. A structured questionnaire was used for interviewing TB patients to collect information on symptoms and duration of illness, diagnostic and treatment delays. However, only 64% of

patients participated in the in-depth interview with the possibility that 36% of the missing data and/or recall bias among interviewed might have skewed the results.

Study-2: Internal validity of the study is likely to be high, as only lymph node excision biopsy specimens were studied. We reported a significantly higher sensitivity of Xpert in patients with short compared to those with a long history of illness. There is a possibility that information on the duration of illness missing for 34% of patients may have biased the results and interpretation. Furthermore, the dropout of 20% of the EPTB patient from the treatment cohort may have biased treatment outcomes findings. Moreover, subgroups of patients analyzed for differences in treatment outcomes were very small in number for precise estimations e.g., there were only four bacteriologically confirmed LNTB patients with non-necrotizing granuloma.

To study the prevalence of concomitant PTB, chest X-rays were reread and 10% of patients were reported having abnormalities suggestive of TB. AFB microscopy was performed on sputum for diagnosis of PTB. Among all LNTB patients, only one was reported to have concomitant PTB based on AFB microscopy, with the possibility of underdiagnosis of concurrent PTB due to the use of a less sensitive diagnostic method.

Study-3: Patients with a history of previous TB treatment were excluded. The possibility of misclassifying a patient as a new TB case due to recall bias or purposeful intent to hide previous TB treatment (social desirability bias) cannot be excluded. Moreover, due to low enrolment, the sample size reached for bacteriologically confirmed TB cases with DST results was not powered sufficiently for a very precise estimation of R, H, and FQ resistance in LNTB and pleural TB patients.

Study-4; A constant annual trend of H resistance among new PTB and EPTB patients reflects good internal validity. Clinical data was collected from information provided in the laboratory request form in routine practice. Clinical information was missing in some and the possibility of errors in recorded information and misclassifying some TB patients cannot be excluded.

5.1.3.2. External Validity

External validity refers to the extent to which the results of a study are generalizable to patients in daily practice, especially for the population that the sample is thought to represent.

Study-1: Health facilities with a higher proportion of EPTB notifications were selected. Moreover, the majority of the EPTB patients were diagnosed clinically with the possibility of the reported prevalence of various EPTB disease manifestations being different from the true prevalence.

Study 2: The research study was conducted in a tertiary hospital specialized in TB care and only excision biopsy specimens of lymph nodes were examined for diagnosis of TB, which limits generalizing findings of this study to other specimen types and other EPTB disease sites and other settings.

Study-3: Similar to the limitations mentioned above (Study-2), there is also a possibility that a higher proportion of patients at risk of drug resistance were included in the study population. We studied drug resistance in patients with lymph node TB and pleural TB, which limits generalizing the prevalence of drug resistance to the population level and other types of EPTB disease.

Study-4: For this study, DST data was collected from NTRL. As only a select group of known RRTB patients or those at risk of drug resistance or difficult to diagnose are mostly referred to this laboratory there is a strong possibility that reported H and FQ resistance among the R-sensitive study population was higher compared to TB patients at population level.

Reported prevalence and genetic profile of H-resistance in R-sensitive population were consistent between study-3 and study-4 suggesting good external validity

5.2. Discussion on main findings

5.2.1. *Extrapulmonary TB in Pakistan.*

In 2016, a total of 366,061 all-type TB patients were notified in Pakistan, and of these, we studied IPD of 54,092 TB cases including 15,790 having EPTB. The population studied comprised 84.6% adults and 15.4% children (<15yrs).

Among the adult population, pleural (29.6%), lymphatic (22.6%), and abdominal TB (21.0%) collectively contributed to more than 70% of all notified EPTB cases. Except for abdominal TB, the distribution pattern of EPTB was similar across provinces and also consistent with reports from other countries [46, 282-291]. In children, compared to adults, abdominal TB (38.4% vs 15.8%) and CNS TB (6.0% vs 4.2%) cases were higher, TPE (14.0% vs 34.2%) and OATB (3.9% vs 11.0%) cases were less frequent, whereas LNTB was equally frequent (22.4% vs 22.7%). The majority of children with abdominal TB (80%) were notified by health facilities in the Northwest part of Pakistan. On the contrary, abdominal TB is rarely reported in high-income countries [46, 285, 292]. In hospital-based small studies from different countries, abdominal TB was reported in 6.8% (Afghanistan), 12.8% (India), 14.8% (Nepal), 15.4% (Australia), and 17.5% (Saudi Arabia) of EPTB patients [282-284, 288, 289]. With over 10 million stunted children, the drivers of TB in children do exist in Pakistan [293]. However, malnutrition alone does not explain the high notification of abdominal TB in the Northwest as malnutrition is more prevalent in the Southeast part of the country. Compared to LNTB and TPE, diagnosis of abdominal TB is more challenging. Diagnostic workup may require resources for radiology, endoscopy, and/or surgical procedure and examination of fluid or tissue biopsy specimens for bacteriology and histopathology. However, a definite diagnosis is not guaranteed in the end. In low-income settings like Pakistan, there is a possibility that due to a lack of resources, facilities, and expertise, children with unexplained chronic diarrhea are started empirically on TB treatment. To understand better, there is a need to study the characteristics of children diagnosed with abdominal TB and evaluate clinical practices

and criteria used for the diagnosis and possible overdiagnosis.

In our study cohort, intra-thoracic LNTB was reported in 1% of children, whereas it is reported more frequently in young ages by others [46, 285]. Low notification in our study settings was likely due to delay in diagnosis, lack of training, lack of access to radiology, or under-recording. WHO has recently issued guidelines for short 4-month TB treatment for minimal TB disease in children having intrathoracic and peripheral lymph nodes. If diagnosed early in the disease, children can benefit from the recommended short TB treatment [3].

TBM was notified, in 6.0% of children and 4.2% of adults which was higher compared to high-income countries [46, 285, 286], but lower in comparison to low-income countries with high HIV prevalence [292, 294]. We collected data from 50 health facilities which included ten tertiary care hospitals and TBM cases notified by seven of these hospitals were lower than the average of the study population. Low notification of TBM is most likely a reflection of weak linkages within tertiary care hospitals and gaps in notifications by pediatrics and neurology departments. Furthermore, low notifications of TBM can also be either due to patients dying of TBM before reaching any healthcare facility or before the diagnosis, or when seeking care from the private sector which is not formally engaged by NTP.

We reported OATB in 9.3% (1483) of EPTB patients with spinal TB being the most frequent. Our findings are consistent with reports from high-income countries [46, 285-287] but the prevalence of OATB is lower compared to Benin (25.4%) and Ghana (17.5%) [292, 294]. Furthermore, patients with OATB had higher median age compared to other forms of EPTB, consistent with the timetable suggested by Walgreen A in 1948 [22].

In TB-burdened countries, EPTB is reported as an important cause of pyrexia of unknown origin (PUO) [295]. In our study cohort, disease site was not recorded for 8.5% of patients otherwise classified as EPTB. There is a possibility of incomplete data recording in some cases but the possibility of patients with PUO being given empiric TB treatment based on

clinical evaluation alone or with a positive TST or IGRA in the absence of any clue of the disease site cannot be excluded.

EPTB can affect any part of the body, and due to the heterogeneity in disease location, and difficulties in obtaining specimens, the definite diagnosis can be very challenging. In high-income countries, 50–60% of the notified EPTB cases are bacteriology confirmed [285, 287, 288]. In our study cohort compared to 86% of PTB, only 8.8% of EPTB patients were investigated by bacteriology and only 0.6% of EPTB were bacteriologically confirmed. Our findings were consistent with reports from other low-income countries [292]. In the absence of bacteriological confirmation possibility of overdiagnosis of EPTB cannot be excluded. During the study period, Xpert testing facilities in Pakistan were only a few. With improved access to rapid molecular diagnostics, bacteriological diagnosis among EPTB is likely to improve but skills and resources required for obtaining specimens are likely to remain a bottleneck for improving laboratory confirmation of EPTB in resource-poor countries.

In our study cohort, fewer patients with EPTB (4.0%) compared to PTB (10.2%) had a history of previous TB treatment. A high TSR among EPTB patients was consistent with other studies [46, 292]. TSR for pleural, lymphatic, and abdominal TB was high but significantly low for TBM (74.3%), it is therefore not advisable to lump different EPTB disease forms as a single entity [285]. However, in routine surveillance systems, the outcome of all forms of EPTB is reported as a single entity, as a result, unfavorable outcomes of life-threatening forms like TBM are masked by the overall high TSR among prevalent forms of EPTB and are thus ignored.

5.2.2. Diagnosis of lymph node TB

LNTB is the second most prevalent manifestation of EPTB in Pakistan (study-1). We studied the performance of the histologic examination and Xpert in 412 TB treatment naïve people presumed to have LNTB. LNTB was diagnosed in 228, including 69% (158) by both bacteriology and histopathology, 9% (20) by bacteriology, and 22% (50) by

histopathology only.

In our cohort, LNTB was diagnosed in 58.4% of all presumptive TB recruited in the study. Using similar diagnostic approaches, a higher proportion of LNTB patients were diagnosed among two cohorts investigated in Ethiopia (80%) and Pakistan (74%) [296, 297]. All three studies were conducted in a tertiary care setting with the possibility of recruitment of highly selected patients. However, there were important differences between populations studied at three sites. Compared to our cohort, which comprised all TB treatment naïve and only one HIV reactive patient, the prevalence of HIV was high (9%) in one and a history of previous TB treatment (9%) in the other cohort. The associated risk factors likely contributed to a higher TB diagnosis with a high proportion of AFB positive in the other two studies (37% and 15%) compared to 4% in our cohort. [296, 297].

We evaluated the performance of Xpert against culture and reported sensitivity and specificity of 62.4% and 83.3% compared to pooled sensitivity and specificity of 82.4%, and 80.3% of 11 similar studies with 786 lymph nodes [201, 203, 298-300]. The plausible explanation of lower sensitivity in our cohort was the high proportions of specimens with low bacillary load as among all 124 Xpert positive lymph nodes only 11 were AFB smear positive. In our study, the smear results correlated well with the Xpert cycle threshold (Ct) value. Compared to the suggested cut-off of Ct-value of <21 for AFB smear-positive, MTB detected was either low (Ct 22-28) or very low(Ct >28) in 113 smear-negative LNTB cases [301]. Despite fewer AFB-positive specimens, compared to a similar study from Pakistan, we reported comparable sensitivity (62 vs. 65) and specificity (83 vs. 80%) with a higher PPV (67 vs. 54%) most likely attributed to better culture performance. New generation Xpert Ultra is expected to improve sensitivity [302, 303] but that is likely to be at the cost of some loss of specificity [299]. Specimens that are positive on Xpert but negative on culture, are interpreted as false positives and often attributed to previous TB treatment [304]. However, we only recruited TB-treatment naïve people and to better understand false-positive results, we analyzed the performance of Xpert in the study population

stratified by duration of illness. Xpert performed better (sensitivity 76% and PPV-82%) in patients with a history of short illness (< 3 months), compared to those who were sick for longer than 3 months (Sensitivity of 52% and PPV of 61%). A plausible explanation for this difference is that in patients with a longer history of illness, effective host immune responses played a role in the containment of the disease and viability of MTB. As a result, while MTB-DNA was still detected by molecular assay but MTBC was not grown on culture. Concerns have been raised by others on the accuracy of the culture results and its use as the reference standard and a higher heterogeneity is reported in the performance of Xpert in lymph nodes compared to other EPTB specimens [299, 305].

In our cohort, among bacteriologically confirmed LNTB patients, (positive by Xpert and/or culture) histopathology suggestive of TB was reported in 89% (n=158). The findings are consistent with reports from other low HIV settings [297, 306], but were higher compared to HIV prevalent settings [307]. A diminished capacity to mount a CD4+ T-cell response correlates with a reduced granuloma-forming capacity [308], and TB without tubercles is often reported in HIV-positive patients [309]. In our cohort, among bacteriologically confirmed LNTB, the granulomatous response was not reported in 20 cases. In Pakistan, HIV prevalence is low, but undernourishment is estimated to contribute to 20% of the TB disease burden. The underlying malnutrition causing diminished CD4+T-cell response and lack of granulomatous response is a plausible explanation [26]. Although lack of TB-specific histology can also be a consequence of misidentification or methodological error [198, 310], however, a delay in treatment response was observed in these patients (OR:4.07) suggesting weak immune response as a possible cause [37]. On the contrary, in LNTB patients with TB-specific histologic response, no difference was reported in treatment response between bacteriology-negative and positive patients.

We reported histology consistent with TB in 208 LNTB patients with necrosis in 98% and bacteriology confirmation in 75%. Similar studies from Morocco and Peru have reported necrosis in 84-85% with bacteriological confirmation in 75% of LNTB with TB-specific

histology [306, 307]. Consistent with these findings, a significant proportion of necrotizing granulomas are reported to have no obvious infectious etiology [311]. Xpert Ultra with better LoD is likely to improve bacteriological confirmation of TB, however, challenges would remain in situations where the biopsy specimen is either very small or histological examination is preferred over bacteriology or the specimen is preserved immediately in formalin for histology. Recent advances like the diagnosis of TB in formalin-fixed paraffin-embedded tissue by detecting TB-specific antigens [193, 312], and MTB by Xpert are likely to improve bacteriological diagnosis of TB without the need for splitting specimens for bacteriology and histology examination [313].

International standards of TB care (ISTC) recommend testing appropriate specimens for microbiology and histopathology for evaluating possible EPTB patients [314-316]. Bacteriology has an advantage over histopathology in the definite diagnosis of TB. Xpert has the advantage of rapid diagnosis of TB and resistance to R. Though culture is of value, long reporting time and the need for sophisticated laboratory facilities make it a non-viable option for the majority of the patients in HBC. In our study cohort, rapid molecular assay diagnosed 54% of the LNTB cases, consistent with reports from other studies [297, 306, 307]. However, with reliance on Xpert MTB/RIF, almost half of the LNTB cases are likely to be missed. In high TB and low HIV prevalent settings like Pakistan, with a high pre-test probability of TB, diagnosis can be made with certainty based on specific granulomatous inflammation in the absence of positive bacteriology.

5.2.3. *Primary drug resistance in extrapulmonary TB*

In this study, we evaluated primary drug resistance in EPTB patients. Only TB treatment naïve patients either with lymphadenopathy or pleural effusion were recruited. Among 671 presumptive TB cases, 255 were bacteriologically confirmed and comprehensive DST results were available for 185(72.5%) cases.

Any resistance to R was reported in 2.7% and MDR in 2.2%. Compared to the prevalence of RR and MDR in smear-positive PTB patients in National DRS, point estimates were lower in EPTB but the difference was not statistically significant [261, 262].

Similarly, the proportion of H, LFX, and PZA resistance in rifampicin-sensitive EPTB was comparable to PTB (Table-6). Our findings were consistent with estimates of drug resistance reported in new TB patients in similar studies [317, 318].

Table-6: Estimated prevalence of drug resistance in rifampicin-resistant and sensitive populations in study-3 and study-4 compared to National drug resistance survey 2012-13.

Study		DRS	Study-3	Study-4	Study-4
Year		2012-13	2016-17	2015-19	2015-19
Drug resistance in Rifampicin sensitive					
Population	TB site	PTB	EPTB	EPTB	PTB
	% new	89%	100%	95%	74%
Isoniazid	Isolates tested (no)	1486	180	1118	3366
	% Resistant	8.7%	5.6%	6.7%	11.6%
	(95% CI)	(7.4-10.3)	(3.0-9.9)	(5.4-8.3)	(10.5-12.7)
Levofloxacin	Isolates tested (no)	1401	180	1117	3365
	% Resistant	10.3%	5.0%	7.2%	14.4%
	(95% CI)	(7·1–13·5)	(2.7-9.2)	(5.8-8.8)	(13.3-15.7)
Pyrazinamide	Isolates tested (no)	1397	180	1019	3048
	% Resistant	0.5%	0.6%	2.8%	3.7%
	(95% CI)	(0·1–0·8)	(0.1-3.0)	(1.9-4.1)	(3.1-4.5)
Drug resistance in Rifampicin resistant					
Population	TB site	PTB	EPTB	EPTB	PTB
	% new	53.7%	100%	68%	26%
Isoniazid	Isolates tested (no)	106	5	118	4185
	% Resistant	90.6%	80.0%	90.7%	94.9%
	(95% CI)	(83.5-94.8)	(29.9-98.9)	(83.6-95.0)	(94.1-95.4)
Levofloxacin	Isolates tested (no)	99	5	118	4183
	% Resistant	21.8%	40.0%	44.9%	47.7%
	(95% CI)	(13·1–30·5)	(11.8-76.9)	(36.2-53.9)	(46.2-49.3)
Pyrazinamide	Isolates tested (no)	103	5	104	3696
	% Resistant	39.5%	39.5%	55.8%	44.8%
	(95% CI)	(30·1–48·9)	(23.1-88.2)	(46.2-64.9)	(43.2-46.4)

We tested all culture isolates by gDST (LPA) and pDST (MGIT 960) and reported a high sensitivity (100%) and specificity (99%) of LPA for R, H, and FQ, consistent with other reports [49, 319, 320]. A perfect sensitivity of gDST (LPA) in our study is most likely attributed to the fact that both pDST and gDST were performed on the same culture isolates. Among phenotypically susceptible strains, resistance-conferring mutations were

detected in four including one each for R and H and two for FQ. A borderline resistance-conferring mutation for R at *rpoB*511, a low-level mutation for H at *inhA* c-15t, one low-level conferring mutation at *gyrA* A90V, and a high-level resistance (D94G) for FQ. R resistance-conferring mutation at *rpoB* 511 has been reported previously for discordance in results between Xpert and LJ DST [261].

Contrary to high agreement rates between pDST and LPA results, discordance was reported between Xpert MTB/Rif V4.0 and pDST. Of eleven RR detected by Xpert, culture was positive in only five, and four were susceptible by pDST and LPA. False RR by Xpert V4.0 is reported in paucibacillary clinical samples from children [321], and TB cases diagnosed during active case finding [322]. Although further investigation was not done to investigate discordant results, the difference in the proportion of RR in clinical specimens with a very low level of MTB (9/75,12%) compared to those with low or medium MTB (2/77, 2.6%) suggests false RR results. Among eleven RR by Xpert, only one RR case was confirmed by LPA, false RR was reported in four, and six RR results could not be verified due to negative cultures. The possibility of other pre- and post-analytic errors resulting in false results cannot be excluded with certainty. WHO recommends repeat testing on fresh samples when R resistance is detected in patients considered to be at low risk of MDR-TB [323]. These recommendations are difficult to follow in EPTB patients, especially in situations in which repeat testing entails a repeat of surgical or other invasive procedures. Next-generation Xpert Ultra is claimed to reduce false RR results along with improved sensitivity [324, 325].

Among bacteriologically confirmed, 70% (124/178) of LNTB and 23.4% (18/77) of TPE were positive by Xpert. With Xpert Ultra, bacteriological diagnosis is likely to improve but resistance detection will continue to lag behind TB diagnosis and resistance detection would still have to depend on good culture yield.

5.2.4. Isoniazid resistance, genetic profile, and associated resistance

We studied a large DST data set from 11045 TB patients including 12.7% EPTB patients registered in NTRL during 2015–2019. Among the population studied, 91.8% of EPTB compared to 44% of PTB had no history of prior TB treatment. We reported, among the R-sensitive population, H resistance of 6.8% (95%CI, 5.4–8.5) in new EPTB patients which was significantly lower ($P < .001$) compared to 9.8% (95%CI; 8.7–11.1) in new PTB patients. H resistance in new EPTB patients is consistent with results reported in study-3 and new PTB (Table-6) in Pakistan [261, 326, 327]. The significantly higher H resistance reported among PTB patients was likely due to the referral to NTRL of the selective PTB patients at risk of drug resistance for DST as opposed to EPTB patients who were mostly referred for the diagnosis of TB (Table-6).

Of all TB patients, 79.5% (8787) of the *M. tuberculosis* isolates (EPTB =1236) were tested by both pDST and gDST methods. Using composite results RR was reported in 4303 patients with 15.8% discordance in results between the two methods, 13.2% of RR were reported susceptible by MGIT (pDST) and 2.5% by LPA (gDST). In the study population, compared to population-based DRS (2012-13), RR missed by pDST was higher (13.2% vs 4%) [261]. This difference was most likely due to the use of automated liquid DST (MGIT) in routine, which is known to miss more RR compared to solid DST on the Lowenstein Jenson media used in DRS [279, 328]. On the contrary, a lower proportion of RR cases were missed by gDST (2.5% vs 7.7%), which most likely is a reflection of the current diagnostic algorithm in which Xpert is used for diagnosis of RR and patients reported R-sensitive are not investigated further. The limitations of the currently available pDST and gDST in detecting R resistance are well recognized [279, 328, 329]. Selection of either one of the two DST methods for routine practice is likely to result in important diagnostic and clinical implications. In our study population, without gDST, 10.1% (411/4078) of the MDR would have been reported as H-resistant TB. In a study from South Africa, 15% of H-resistant isolates were initially tested negative for RR by WHO-endorsed all three

commercial tests. However, based on deep sequencing results and identification of resistance-conferring mutation at *rpoB* Ile491phe these were reclassified as MDR [330]. Similarly in another study, misclassification of MDR-TB as H-resistant TB was demonstrated, and the impact of treating these patients with WHO recommended FQ containing regimen for H-resistant TB was strongly argued [329]. Furthermore, undiagnosed RR and not H-resistant TB was suggested as a possible reason for the reported higher failure rate with standard first-line drug regimens [53].

In our data set, compared to RR, a higher proportion of H resistance (14.5%; n = 660) was missed by gDST. Our findings are consistent with the results of a recent study from the eastern DRC, in which H resistance by gDST (LPA) was reported in 55% of the RR cases detected by Xpert MTB/RIF [331].

We studied 4542 phenotypic resistant isolates for molecular markers. Among these, H resistance-conferring mutations were identified in 85.5%, with the representation of high-level resistant conferring mutation (*katG) in 72.7%,* low-level resistant conferring mutation (*inhA)in 9.9% and* combined mutations (*katG and inhA)* in 2.8%. Our findings are consistent with the published data [332-334] but with a lower proportion of combined mutations [333, 335]. We reported a significant difference in the genetic profile of H resistance between RR (n = 4078) and R-sensitive (n = 464) populations, H resistance conferring mutations were detected in 87.1% vs 71.6%, moreover, high representation of *katG* and combined *inhA* and *katG* was seen associated with RR and higher representation of *inhA* were reported in R-sensitive. Within RR and R-sensitive populations, H resistant genetic profile was comparable between PTB and EPTB isolates. Our findings are consistent with the international collection of over 5,000 strains showing a substantially higher representation of *katG* among MDR strains (89%) compared to H resistance among R-sensitive (61%) isolates. [336].

Published data on the influence of genotype on treatment outcomes of H-resistant TB are conflicting. A study from Vietnam, suggest that *katG* mutations and not *inhA* are associated with unfavorable treatment outcomes [337]. In a study from South Africa, the association

of any specific H resistance-conferring mutation with poor treatment outcomes was not reported and a higher TSR was reported in patients with *katG* mutations when treated with high-dose H compared to standard dose [338]. The clinical benefits of high-dose H are also reported in patients with drug-resistant TB [339-341].

In Pakistan, one of the key challenges for treating MDR-TB and H-resistant TB is high FQ resistance [262]. In our study population, FQ resistance was higher among both RR and R-sensitive populations compared to the results of population-based DRS(Table-6) [262, 333]. However, our findings are consistent with a laboratory-based similar study from Pakistan [342]. Both among RR and R-sensitive TB populations, a significantly higher LFX and Z resistance was reported in association with H resistance. Results were compatible between PTB and EPTB in RR patients. Our findings are consistent with the results of a recent study [335]. A high FQ resistance in Pakistan limits the usage of the WHO-recommended regimen for H-resistant TB. However, an almost equal representation of *katG* and *inhA* mutations in H-resistant TB, suggests the need to study treatment outcomes with standard treatment in H-resistant TB associated with specific mutations. It has been more than a decade since Xpert was introduced and still in 2021, only 69% of the new PTB were tested for R. Challenges to universally test all bacteriologically confirmed populations for H and FQ resistance are likely to be more intense. Although a new GeneXpert cartridge for genotypic testing of H and FQ resistance is now available [343]. But universal testing for all TB patients in HBC will require besides resources for cartridges, a huge investment for placing next-generation modules at the same level of health care as R testing. Among bacteriologically confirmed PTB and many EPTB patients, those with paucibacillary disease are less likely to be reported interpretable valid results by this assay. In the country context of Pakistan, even with systematic testing, about 30 percent of the H resistance is likely to be missed by molecular methods. Furthermore, due to high FQ resistance, a significant proportion of H-resistant patients will still not stand eligible for FQ containing regimen. More patients will benefit if additional resources are invested in finding TB and RR-TB cases.

5.3.   Conclusion

In Pakistan, the three most common disease manifestations of EPTB are pleural, lymphatic, and abdominal TB which collectively comprise more than 70% of all notified EPTB patients. Children under fifteen and residents of the Northwest part of the country have higher odds of presenting with EPTB. Similar to PTB, treatment success is high among EPTB patients except for TB meningitis.

Diagnosis of EPTB is challenging because of the paucibacillary nature of the disease. The performance of currently available rapid molecular diagnostic tools is sub-optimal. However, in compliance with ISTC recommendations for evaluating possible EPTB patients, more than 95% of LNTB can be diagnosed with the combined use of Xpert and histological examination.

Among new EPTB patients having LNTB and TPE, the prevalence of MDR and RR-TB was 2.2% and 2.7%. Among R-sensitive, H resistance was 5.6% which was not statistically different from the estimated prevalence in new PTB. There was a good agreement between culture-based automated liquid DST and LPA but false RR were reported by Xpert in paucibacillary specimens warranting caution in clinical decisions based on Xpert RR results.

H resistance in new EPTB patients in the retrospective data set was 6.7% which was not statistically different from H resistance reported in new EPTB patients in study-3. The genetic profile of H resistance in EPTB was compatible with PTB within RR and R-sensitive populations with significantly higher representation of *katG* (high level resistant conferring mutation) among RR and *inhA* in R-sensitive population and significantly high FQ and Z resistance in association with H resistance.

5.4.   Future Perspective

End TB strategy calls for diagnosis of TB using WRD of more than 90% and universal DST for 100% of TB patients by 2025. Globally in 2021, only 38% of the TB patients were tested using WRD, and 69% of the new bacteriologically confirmed PTB were tested for RR among notified [40]. While the End TB targets are laudable but currently are lagging far behind the target. Globally 20% of the notified TB cases have EPTB but EPTB notification is not tracked at the same level as PTB and information is missing on the proportion of EPTB patients tested using WRD, bacteriological diagnosis, and RR among notified EPTB cases. End TB targets for diagnosis and universal DST cannot be achieved without improving bacteriological diagnosis and drug resistance detection for EPTB in parallel with PTB.

The focus of TB programs has remained on PTB due to its infectiousness and resultant transmission. There is a need, in parallel to address challenges concerning diagnosis, drug resistance detection, and treatment of patients with EPTB. In HBC, there are knowledge gaps on manifestations of EPTB disease, clinical and diagnostic approaches used for diagnosis, bottlenecks in implementing ISTC, delays in diagnosis, and out-of-pocket expenses incurred by patients.

At the country level, small studies and surveys and a more granular data analysis at the facility level can help understand issues specific to EPTB care. Concerted efforts, using a multidisciplinary approach, clear guidance on the diagnosis and management of EPTB affecting different sites, improved access to new diagnostic tools, capacity building of clinicians on EPTB management, wider use of simpler techniques like a fine-needle aspiration to obtain specimens, training of laboratory technicians on the processing of extrapulmonary specimens, effective linkages for referral of patients and transport of specimens and comprehensive surveillance system to enable tracking of EPTB by disease site and laboratory diagnosis, can collectively contribute in improving TB diagnosis and care in patients with extrapulmonary disease.

With better access and use of WRD, the feasibility of achieving testing targets, improvement in bacteriological diagnosis, and rifampicin testing are high for patients with PTB, except those with paucibacillary disease e.g., children and people living with HIV. For EPTB, diagnostic yields will improve with wider use of molecular tools like Xpert Ultra, but bacteriological diagnosis among EPTB is likely to lag behind PTB due to complex diagnostic challenges and resource requirements beyond just a sensitive diagnostic tool. Among EPTB, the improvements in bacteriological diagnosis would vary based on disease sites and the simplicity of procedures required for sample collection. Peripheral lymph node and pleural TB are the two most commonly reported sites of EPTB globally and sampling is comparatively simple. In Pakistan, these two sites collectively make up to 50% of EPTB cases, and in the best-case scenario, if specimens from the majority of these patients are systematically tested using WRD, up to 50% bacteriological confirmation of LNTB and less for TPE can be achieved but diagnostic challenges would likely continue for other more insidious EPTB disease forms. Furthermore, with the use of Xpert ultra, bacteriological diagnosis of paucibacillary EPTB is likely to improve by virtue of trace calls without R testing results. As a consequence, DST for R will lag much behind the bacteriological diagnosis of EPTB, and RR and other drug resistance will remain undiagnosed in many of these patients. Access to culture-based DST is limited and not a feasible alternative option for patients in HBC. The establishment of a sentinel surveillance system with the availability of onsite molecular and culture facilities at sentinel sites can contribute to some extent in generating reliable surveillance data on ongoing resistance to anti-TB drugs for geographically-defined PTB and EPTB populations.

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7. PUBLISHED PAPERS

7.1.   Paper-1

PLOS ONE

Extrapulmonary tuberculosis in Pakistan- A nation-wide multicenter retrospective study

Sabira Tahseen[1,2☯ *], Faisal Masood Khanzada[1☯], Aurangzaib Quadir Baloch[3‡], Qasim Abbas[4‡], Mansoor Manzoor Bhutto[5¤‡], Ahmad Wali Alizai[6‡], Shah Zaman[7‡], Zahida Qasim[8,9‡], Muhammad Najeeb Durrani[10‡], M. Khalid Farough[11‡], Atiqa Ambreen[12‡], Nauman Safdar[13‡], Tehmina Mustafa[2,14☯]

1 National TB Reference Laboratory, National TB Control Program, Islamabad, Pakistan, 2 Centre for International Health, Department of Global Public Health and Primary Care, University of Bergen, Bergen, Norway, 3 National TB Control Program, Ministry of National Health Services Regulation and Coordination Islamabad, Islamabad, Pakistan, 4 Department of Health, Provincial TB Control Program, Government of Khyber Pakhtunkhwa, Peshawar, Pakistan, 5 Provincial Reference Laboratory, Provincial TB Control Program, Sindh, Karachi Pakistan, 6 Department of Health, Provincial TB Control Program, Government of Balochistan, Quetta, Pakistan, 7 Department of Health, Provincial TB Control Program, Government of Gilgit Baltistan, Gilgit, Pakistan, 8 Department of Pathology, District Head Quarter Hospital Mirpur, Azad Jammu Kashmir, Pakistan, 9 Department of Health Azad Jammu Kashmir, District Head Quarter Hospital Mirpur, Azad Jammu Kashmir, Pakistan, 10 Department of Health, Ministry of National Health Services Regulation and Coordination, Islamabad, Pakistan, 11 TB Project, Green Star Marketing, Karachi, Pakistan, 12 Department of Microbiology, Gulab Devi Hospital, Lahore, Pakistan, 13 Global Health Directorate, Indus Health Network, Karachi, Pakistan, 14 Department of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway

☯ These authors contributed equally to this work.
¤ Current address: Sindh Health Care Commission, Karachi, Pakistan
‡ These authors also contributed equally to this work.
* sabira.tahseen@gmail.com

Abstract

Background

Pakistan is fifth among high burden countries for tuberculosis. A steady increase is seen in extrapulmonary tuberculosis (EPTB), which now accounts for 20% of all notified TB cases. There is very limited information on the epidemiology of EPTB. This study was performed with the aim to describe the demographic characteristics, clinical manifestations and treatment outcomes of EPTB patients in Pakistan.

Method

We performed descriptive analysis on routinely collected data for cohorts of TB patients registered nationwide in 2016 at health facilities selected using stratified convenient sampling.

Findings

Altogether 54092 TB including 15790 (29.2%) EPTB cases were registered in 2016 at 50 study sites. The median age was 24 years for EPTB and 30 years for PTB patients. The crude prevalence of EPTB in females was 30.5% (95%CI; 29.9–31.0) compared to 27.9% (95%CI; 27.3–28.4) in males. The likelihood of having EPTB (OR), was 1.1 times greater for females, 2.0 times for children, and 3.3 times for residents of provinces in the North-West.

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The most common forms of EPTB were pleural (29.6%), lymphatic (22.7%) and abdominal TB (21.0%). Pleural TB was the most common clinical manifestation in adults (34.2%) and abdominal TB in children (38.4%). An increase in the prevalence of pleural and osteoarticular and decline in lymphatic and abdominal TB was observed with advancing age. Diversity in demography and clinical manifestations were noted between provinces. The treatment success rate for all type EPTB was significantly high compared to bacteriology confirmed PTB with the exception of EPTB affecting CNS with a high mortality rate.

Conclusions

The study provides an insight into demography, clinical manifestations and treatment outcomes of EPTB. Further studies are needed to explain significant diversities observed between provinces, specific risk factors and challenges concerning EPTB management.

Introduction

Extrapulmonary tuberculosis (EPTB) represents 15% of the incident TB cases notified globally. The proportion of patients who present with extrapulmonary manifestations varies from 8% in the WHO Western Pacific region, 17% in South East Asia and 24% in the Eastern Mediterranean Region [1, 2]. There are many forms of extrapulmonary manifestations of TB, affecting every organ system in the body. Considerable differences are reported in susceptibility to different sites of EPTB by age, race/ethnicity, sex, and country of origin [3, 4, 5]. The proportion of EPTB among cohort of notified TB cases is reported and monitored every quarter at the country level and by WHO annually for all member countries, regions and global trends. Cohort of notified incident TB cases is analyzed for demographic characteristics collectively for PTB cases and for treatment outcomes, stratified by bacteriology confirmed and clinically diagnosed TB cases only. However, due to limited data variables used for routine quarterly TB surveillance reports, stratified cohort analysis for EPTB is not possible [6]. Routine surveillance data is used by a few, mostly high-income countries to study the epidemiology of EPTB including demography, clinical manifestations, bacteriological diagnosis and notification trends [3–5, 7, 8].

Pakistan is among the top five high burden countries (HBC). Implementation of the DOTS strategy for TB started in 2001 and within five years expanded to cover most of the public health sector. National TB control program (NTP) subsequently focused on the expansion of DOTs coverage in the private health sector, childhood TB and programmatic management of drug-resistant TB [9, 10]. Regardless, the management of EPTB remained mostly neglected, a steady increase is seen in absolute numbers of EPTB and proportion among notified TB cases from 17.4% (45,537) in 2011 to 20% (71,322) in 2016 [1,9]. Diversity is noted in the proportion of EPTB between provinces. Besides a few hospital-based studies, there is very little information on the epidemiology of EPTB disease in Pakistan [11, 12]. We performed a descriptive analysis on a nationwide sample to determine the demographic characteristics, clinical manifestations and treatment outcomes of EPTB patients in Pakistan.

Study design and methodology

Study design

This is a multicenter retrospective observational study, performed on routinely collected data for cohorts of TB patients registered nationwide in 2016 at health facilities selected using stratified convenient sampling.

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Study setting

Pakistan is a country in South Asia, with an estimated incidence of 510K TB cases and having a low prevalence of HIV. In 2016, 68% of the estimated TB cases were notified and 20% of these presented with EPTB [1]. The private health sector including general practitioners (GPs) engaged in TB care, contributed to 27% of all notified TB cases. By 2016, for TB diagnosis, microscopy services were established at about 1300 health care facilities (HCF), GeneXpert (Cepheid, Sunnyvale, CA, USA) at 73 HCF and TB culture in 16 laboratories [1, 10]. Xpert MTB/RIF (Xpert) testing is recommended for the diagnosis of EPTB since 2013 [1, 13] but TB culture facilities are offered mostly for drug-resistant TB patients. Histopathology services although limited to tertiary hospitals in public sector but are offered widely by commercial clinical laboratories.

Standard recording and reporting tools are used [6] and data of each notified TB patient is recorded in TB registers at each HCF. A separate TB register is maintained at district level for recording TB cases notified by GPs. Although there are no specific columns in TB registers for recording EPTB disease site or laboratory test results other than bacteriology but the staff is generally guided to record these details in a column for remarks.

Pakistan is administratively divided into four provinces; Punjab (PJB), Sindh (SND), Khyber Pakhtunkhwa (KP) and Balochistan (BTN), three regions namely Federally administered tribal area (FATA), Gilgit Baltistan (GB), and Azad Jammu Kashmir (AJK) and Islamabad capital territory (ICT). FATA districts were merged into KP province in 2019. SND and PJB are large provinces comprising >70% of the total country population and geographically located in South-East, whereas KP, FATA, BTN, and GB are in North-West of the country (S1 Fig).

Data source and collection

We used TB registers with records of TB patients notified in 2016 for the study. For data collection, HCFs were selected using stratified convenient sampling. Separate lists were first obtained of HCFs in each province and region along with the numbers of PTB and EPTB cases notified by each in 2016. HCFs were than stratified into Level-I (Primary HCF/clinics), Level-II (secondary HCF/specialized TB hospitals) and Level-III (tertiary hospitals). Based on EPTB case notification, a shortlist was prepared by selecting the top five HCF/ tier for each province and three/ tier for the region. This list was then handed over to the respective TB program for data collection with guidance to further select from this list two HCFs/tier/province and one HCF/tier/ region, based on the quality and completeness of the data recorded in the TB registers. Data collection was started in 2018 and all HCF staff was guided to check for data completeness and to record missing data before making copies of the TB registers.

For a sampling of TB cases notified by private GPs, the two implementing partners working respectively in 13 and 79 districts were asked to provide a copy of the district TB registers. Private GP clinics were classified as level-I HCF.

Data management

TB registers were received at NTP office Islamabad. A specially designed electronic file, (developed in EpiData Manager V4.4.2.1) was used for data entry. Case-based data was entered (through EpiData Entry Client v4.4.3.1, EpiData Association, Odense, Denmark) for variables including age, sex, history of TB treatment, disease site, laboratory results and treatment outcomes. All TB cases registered between 1st January and 31st December 2016 were included in the study. After entry, data was checked for completeness and if for any HCF, EPTB disease sites recorded were less than 80% of the registered cases, respective TB program was requested again to collect missing information where possible.

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Standard definitions were used for defining children (0-14yrs), adult ($\geq$15yrs), new, previously treated, bacteriology confirmed (B+) and clinically diagnosed TB cases based on data recorded in the TB registers [6]. Cases were categorized by major disease sites, reported as either PTB or EPTB. The PTB group comprised cases with PTB listed as the only disease site. The EPTB cases were grouped according to extrapulmonary disease site: pleural, lymphatic intra-thoracic, lymphatic extra-thoracic, osteoarticular spine and osteoarticular other than the spine, the central nervous system including meninges, abdominal including peritoneal and disseminated TB including miliary. Extrapulmonary manifestations if specified other than sites mentioned above were listed as "others" and if not specified were listed as EPTB not specified (NOS).

Data were analyzed using STATA® v13.1 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845, USA). Logistic regression was performed to calculate odds ratios (OR) and 95% confidence intervals for comparisons between groups, while mean, median and quartiles were analyzed for quantitative variables. Two sample proportion test was used to analyze differences in proportions between groups. A p-value of $p < 0.05$ was considered statistically significant.

Ethics statement: For confidentiality, patient identifiers were not entered into the database used for the study. The study protocol was approved by the "Institution review board" of HIV, TB and malaria programs, Islamabad, Pakistan and Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest).

Results

Altogether 50 TB registers were collected including 37 HCF (35 public and 2 private) and 13 district TB registers. Only one implementing partner provided records of TB patients notified by GPs in 13 districts. The study population covered 32 districts and included samples from all four provinces, three regions, and federal capital. Study sites included 29 level-I (16 public, 13 district GP clusters), 11 level-II (all public) and 10 level-III HCF (8 public, 2 private). Details of study sites are shown in S1 and S2 Tables.

Reported tuberculosis cases

Altogether 54,092 TB including 38,302 PTB and 15,790 EPTB (29.2%) patients were registered at selected 50 study sites in 2016. Of all TB cases notified country wide in 2016, the proportion included in the study sample from each province/region is shown in S2 Table. Twenty one TB cases were recorded having concurrent EPTB and PTB and these were counted with EPTB cohort. Age was missing for 22 TB including three EPTB patients. The disease site was not specified for 1399 EPTB cases (8.5%). Treatment outcome was not recorded for 3222 TB (7.0%) including 694 EPTB (4.9%) patients.

The characteristics of PTB and EPTB patients included in the study are shown in Table 1.

Demographic characteristics

The median age of patients with EPTB was 24yrs compared to 30yrs for PTB. Overall 52.4% of EPTB and 49.2% of PTB patients were females. Peak TB notification was seen in the age group of 15-24yrs (26.6%) with females being in the clear majority among both PTB (F: M 1.4:1) and EPTB patients (F:M 1.4:1). A decline in female TB notifications was observed with increasing age (Fig 1).

The crude prevalence of EPTB in females was 30.5% (95%CI; 29.9–31.0) compared to 27.9% (95%CI; 27.3–28.4) in males. Compared to the total study sample, the proportion of EPTB was higher among TB patients notified in North-West provinces/regions (BTN, KP, FATA, AJK) and ICT and are shown in Table 2 and S2 Table.

Table 1. Characteristics of patients having extrapulmonary compared to pulmonary tuberculosis notified by study sites in 2016.

|	Pulmonary TB n(%)	Extra pulmonary PTB n(%)	OR (95% CI)
TB cases notified	38302	15790	
Median Age (IQR)	30(19,50)	24(15,39)	
Sex			
Male	19455(50.8)	7519(47.6)	Ref
Female	18847(49.2)	8271(52.4)	1.1 (1.1–1.2)
Age Group*			
0–14	4731(12.4)	3607(22.8)	1.69(1.60–1.79)
15–24	9932(25.9)	4474(28.3)	Ref
25–34	6474(16.9)	2874(18.2)	0.99 (0.93–1.04)
35–44	4720(12.3)	1649(10.4)	0.78 (0.73–0.83)
45–54	4946((12.9)	1351(8.6)	0.61 (0.57–0.65)
55–64	4075(10.6)	958(6.1)	0.52 (0.48–0.56)
65+	3405(8.9)	874(5.5)	0.57 (0.52–0.62)
Place of origin			
PJB	17950(46.9)	5580(35.3)	Ref
SND	12294(32.1)	3514(22.3)	0.9 (0.9–1.0)
KP	3872(10.1)	3930(24.9)	3.3 (3.1–3.4)
BTN	1310(3.4)	801(5.1)	2.0 (1.8–2.1)
FATA	466(1.2)	472(3.0)	3.3 (2.9–3.7)
GB	1256(3.3)	437(2.8)	1.1 (1.0–1.2)
AJK	577(1.5)	347(2.2)	1.9 (1.7–2.2)
ICT	577(1.5)	709(4.5)	4.0 (3.5–4.4)
Previous TB treatment			
No	34235(89.4)	15072(95.4)	Ref
Yes	3894(10.2)	627(4.0)	0.4(0.3–0.4)
NA	173(0.5)	91(0.6)	1.2(1.0–1.5)
Health Care Provider			
Public	16684(43.6)	9206(58.3)	Ref
Private	21618(56.4)	6584(41.7)	0.5(0.5–0.6)
Health Care facility Level			
PHC(All)	16800(43.6)	6128(38.8)	Ref
SHC	9950(26.0)	4687(29.7)	1.3 (1.2–1.4)
TCH	11552(30.2)	4975(31.5)	1.2 (1.1–1.3)

PJB-Punjab, SND-Sindh, KP-Khyber Pakhtunkhwa, BTN-Balochistan, AJK-Azad Jammu Kashmir, GB-Gilgit Baltistan, FATA-federally administered tribal areas, ICT-Islamabad Capital territories. PHC-Primary health care, SHC-secondary health care facility, TCH-Tertiary care hospital
* 22 cases with missing records for age excluded from this analysis

The odds of patients presenting with EPTB, compared with PTB, were 1.1 times as high for females, 2.0 times for children, 3.0 times for residents of KP and FATA and 2.0 times for BTN and ICT compared to PJB. Female to male ratio (FMR) and age-specific female likelihood to present with EPTB in each province /region is shown in Table 2 and S3 Table. A higher odds (OR = 1.3) for EPTB were reported among females in the age group of 25-34yrs and among residents of SND province (Fig 1 and S3 Table). No differences were observed in the likelihood of female presenting with EPTB seeking health care from the public (OR = 1.14, 95% CI: 1.08–1.19) compared to private sector (OR = 1.18, 95% CI: 1.11–1.24). Overall, 15.4% (8338) of all

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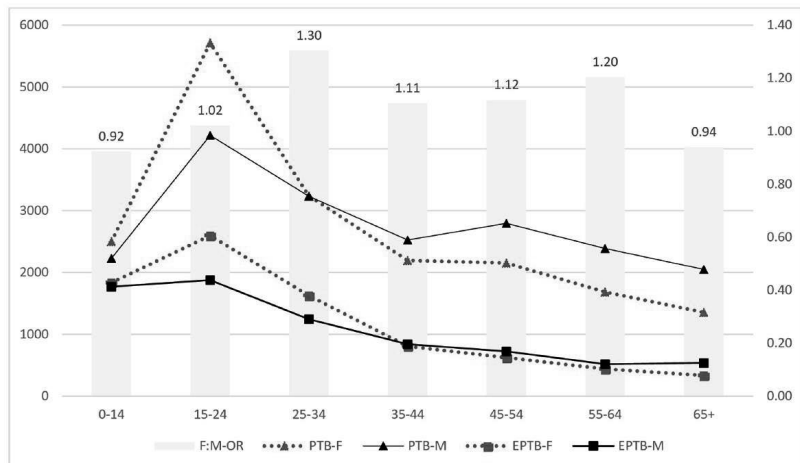
Fig 1. Age and sex-specific pulmonary and extrapulmonary tuberculosis case notifications and age-specific odds of female for having extrapulmonary tuberculosis. PTB-M; Male pulmonary tuberculosis patients, PTB-M- Female pulmonary tuberculosis patients, EPTB-F, Female extrapulmonary tuberculosis patients, EPTB-M, Male extrapulmonary tuberculosis patients, F: M-OR: Female to male odds ratio for EPTB. TB cases notified are shown on the primary axis and OR on the secondary axis.

TB, 22.8% (3607) of EPTB and 12.4% (4731) of PTB patient were children (Table 1). More than 40% of EPTB patients in BTN, KP, FATA, and GB were children compared to around 10% in PJB, SND, and AJK. The crude prevalence of EPTB in children was 43.3% and was highest compared to all other age groups. A decline in the proportion of EPTB was observed with increasing age (Table 3). A similar pattern was observed across all province/regions with the exception of SND having the highest proportion of EPTB among 15-34yrs (S4A–S4H Table).

Extrapulmonary manifestations

The median age was youngest (17yrs) for patients with abdominal TB and oldest (34yrs) for patient with osteoarticular TB other than spine. The site-specific median age for the cohort of EPTB patients studied is shown in Table 3 and S4A–S4H Table.

Among all EPTB patients (15790), frequency of different manifestations were, pleural (29.6%), lymphatic (22.6%), abdominal (21.0%), osteoarticular (9.4%), central nervous system (CNS) (4.6%), other (3.7%) and disseminated TB (0.6%). The EPTB disease site was not recorded in 8.5% of patients. Frequency of different EPTB disease forms in children (3607) compared to adults (12180) were, abdominal (38.4% vs 15.8%), lymphatic (22.4% vs 22.7%), pleural (14.0% vs 34.2%), osteoarticular (3.9% vs 11.0%), CNS (6.0% vs 4.2%) and not specified (12.1 vs 7.4%) (Table 3).

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Table 2. Pulmonary and extrapulmonary tuberculosis case notified, female to male ratio and odds of female for having extrapulmonary TB.

		Pakistan	PJB	SND	KP	BTN	FATA	GB	AJK	ICT
All form TB	All	54092	23530	15808	7802	2111	938	1693	924	1286
	Female	27118 (50.1%)	11759(50.0%)	7746(49.0%)	4026(51.6%)	1149(54.4%)	440(46.9%)	952(56.2%)	466(50.4%)	580(45.1%)
	Male	26974(49.9%)	11771(50.0%)	8062(51.0%)	3776(48.4%)	962(45.6%)	498(53.1%)	741(43.8%)	458(49.6%)	706(54.9%)
	FMR	1.0	1.0	1.0	1.1	1.2	0.9	1.3	1.0	0.8
PTB	All	38302	17950	12294	3872	1310	466	1256	577	577
	Female	18847(49.2%)	8819(49.1%)	5763(46.9%)	2044(52.8%)	734(56.0%)	234(50.2%)	707(56.3%)	302(52.3%)	244(42.3%)
	Male	19455(50.8%)	9131(50.9%)	6531(53.1%)	1828(47.2%)	576(44.0%)	232(49.8%)	549(43.7%)	275(47.7%)	333(57.7%)
	FMR	1.0	1.0	0.9	1.1	1.3	1.0	1.3	1.1	0.7
EPTB	ALL	15790	5580	3514	3930	801	472	437	347	709
	Female	8271(52.4%)	2940(52.7%)	1983(56.4%)	1982(50.4%)	415(51.8%)	206(43.6%)	245(56.1%)	164(47.3%)	336(47.4%)
	Male	7519(47.6%)	2640(47.3%)	1531(43.6%)	1948(49.6%)	386(48.2%)	266(56.4%)	192(43.9%)	183(52.7%)	373(52.6%)
	FMR	1.1	1.1	1.3	1.0	1.1	0.8	1.3	0.9	0.9
EPTB% (95%CI)	ALL	29.2% (28.8–29.6)	23.7% (23.2–24.3)	22.2% (21.6–22.9)	50.4% (49.3–51.5)	37.9% (35.9–40.1)	50.3% (47.1–53.6)	25.8% (23.7–28.0)	37.6% (34.4–40.8)	55.1% (52.4–57.9)
	Female	30.5% (30.0–31.1)	25.0% (24.2–25.8)	25.6% (24.6–26.6)	49.2% (47.7–50.8)	36.1% (33.3–39.0)	46.8% (42.1–51.6)	25.7% (23.0–28.6)	35.2% (30.9–39.7)	57.9% (53.8–62.0)
	Male	27.9% (27.3–28.4)	22.4% (21.7–23.2)	19.0% (18.1–19.9)	51.6% (50.0–53.2)	40.1% (37.0–43.3)	53.4% (48.9–57.9)	25.9% (22.8–29.2)	40.0% (35.4–44.6)	52.8% (49.1–56.6)
FM OR for EPTB		1.14 (1.09–1.18)	1.15 (1.08–1.22)	1.47 (1.36–1.58)	0.91 (0.83–0.99)	0.84 (0.71–1.01)	0.77 (0.59–0.99)	0.99 (0.79–1.23)	0.82 (0.62–1.07)	1.22 (0.98–1.53)

PJB-Punjab, SND-Sindh, KP-Khyber Pakhtunkhwa, BTN-Balochistan, AJK- Azad Jammu Kashmir, GB-Gilgit Baltistan, FATA-Federally administered tribal areas, ICT-Islamabad Capital territories, FMR-Female to male ratio, PTB-Pulmonary tuberculosis EPTB-extrapulmonary tuberculosis, FM OR-Female to male odds ratio

Among all ages, notification of pleural TB compared to abdominal TB was significantly higher ($P < .001$) in South Eastern provinces, PJB (36.2 vs12.0%), SND (33.9 vs 17.1%) and AJK (39.2 vs 16.4%) whereas, abdominal TB was more common ($P < .001$) in North West provinces including KP (34.4 vs 23.4%), FATA (49.8 vs 22.0) and GB (46 vs10.1%) (Table 3 and S4A–S4H Table).

Among adult population, pleural TB was the commonest form (range 16.9–55.6%) across all provinces/regions. However the second commonest was lymphatic TB in PJB, SND, GB and ICT (21.3–26.7%), compared to abdominal TB in KP, BTN and FATA (range 15.3–25.3%). Among children the most commonly reported extrapulmonary manifestation was lymphatic TB in PJB, SND and AJK (>40%), abdominal TB in KP (44%), FATA and GB (>70%) and meningitis (33%) in BTN (S4A–S4H Table).

A steady decline in lymphatic and abdominal TB and an increase in pleural and osteoarticular TB was observed with advancing age (Table 3 and Fig 2).

Lymphatic TB was significantly more common in females (26.6 vs 18.4%, $P < .001$). A higher number of females were observed within each EPTB disease form, with the exception of pleural TB which was more common in males (34.5 vs 25.1%, $P < .001$) (Table 3).

Bacteriological diagnosis

Results of AFB smear and/or Xpert were recorded for 86.1% PTB and 8.8% EPTB patients. Among notified cases, 49.3% of PTB (18872) and 0.55% (87) EPTB patients were bacteriology confirmed. Culture and/or histopathology results were not recorded in the TB register.

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Table 3. Extrapulmonary manifestation of tuberculosis by sex, age group and place of residence among the cohort of TB patients.

	Site-NOS	Pleural	LN-EXT	LN-INT	Abdomen	OAS	OAOS	CNS	Dis/mil	Other	Tot. EPTB	Tot. TB	EPTB%
All	1339 (8.5)	4668 (29.6)	3400(21.5)	181(1.1)	3313(21.0)	910(5.8)	573(3.6)	725(4.6)	94(0.6)	587(3.7)	15790	54092	29.2%
Median Age (IQR)	20 (10,35)	28 (19,45)	22 (15,32)	21 (16,32)	17 (6,30)	34 (23,50)	30 (19,48)	21 (12,40)	24 (18,40)	26 (17,38)	24 (15,39)	28 (18,46)	
Sex													
Female	693(8.4)	2,073 (25.1)	2,093 (25.3)	104(1.3)	1,786 (21.6)	491(5.9)	295(3.6)	365(4.4)	45(0.5)	326(3.9)	8271	27,118	30.5%
Male	646(8.6)	2,595 (34.5)	1,307 (17.4)	77(1.0)	1,527 (20.3)	419(5.6)	278(3.7)	360((4.8)	49(0.7)	261(3.5)	7519	26,974	27.9%
FMR	1.07	0.8	1.6	1.35	1.17	1.17	1.06	1.01	0.92	1.25	1.1	1.01	
Age group[*]													
0–14	437 (12.1)	504(14.0)	768(21.3)	41(1.1)	1385(38.4)	74(2.1)	67(1.9)	218(6.0)	12(0.3)	101(2.8)	3607	8338	43.3%
15–24	334(7.5)	1,423 (31.8)	1,151 (25.7)	70(1.6)	795(17.8)	182(4.1)	148(3.3)	182(4.1)	35(0.8)	154(3.4)	4,474	14,406	31.1%
25–34	214(7.4)	905(31.5)	681(23.7)	27(0.9)	488(17.0)	202(7.0)	108(3.8)	94(3.3)	11(0.4)	144(5.0)	2,874	9,348	30.7%
35–44	129(7.8)	546(33.1)	336(20.4)	18(1.1)	250(15.2)	145(8.8)	75(4.5)	60(3.6)	14(0.8)	76(4.6)	1,649	6,369	25.9%
45–54	110(8.1)	493(36.5)	227(16.8)	10(0.7)	180(13.3)	123(9.1)	88(6.5)	58(4.3)	9(0.7)	53(3.9)	1,351	6,297	21.5%
55–64	63(6.6)	397(41.4)	128(13.4)	9(0.9)	115(12.0)	98(10.2)	51(5.3)	55(5.7)	4(0.4)	38(4.0)	958	5,033	19.0%
65+	52(5.9)	400(45.8)	108(12.4)	6(0.7)	98(11.2)	86(9.8)	36(4.1)	58(6.6)	9(1.0)	21(2.4)	874	4,279	20.4%
All≥15yrs	902(7.4)	4164 (34.2)	2631(21.6)	140(1.2)	1926(15.8)	836(6.9)	506(4.2)	507(4.2)	82(0.7)	486(4.0	12180	45732	26.6%
Place of residence													
PJB	463(8.3)	2019 (36.2)	1305 ((23.4)	86(1.5)	672(12.0)	291(5.2)	288(5.2)	200(3.6)	10(0.2)	246(4.4)	5580	23530	23.7%
SND	101(2.9)	1190 (33.9)	1002(28.5)	32(0.9)	602(17.1)	272(7.7)	94(2.7)	80(2.3)	21(0.6)	120(3.4)	3514	15808	22.2%
KP	454 (11.6)	918(23.4)	635(16.2)	57(1.5)	1350(34.4)	163(4.1)	101(2.6)	90(2.3)	30(0.8)	132(3.4)	3930	7802	50.4%
BTN	204 (25.5)	83(10.4)	59(7.4)	5(0.6)	124(15.5)	56(7.0)	37(4.6)	205 (25.6)	12(1.5)	16(2.0)	801	2111	37.9%
FATA	7(1.5)	104(22.0)	47(10.0)	(0.0)	235((49.8)	24((5.1)	8(1.7)	19(4.0)	1((0.2)	27(5.7)	472	938	50.3%
GB	5(1.1)	44(10.1)	105(24.0)	(0.0)	201(46.0)	31(7.1)	25(5.7)	13(3.0)	3(0.7)	10(2.3)	437	1693	25.8%
AJK	9(2.6)	136(39.2)	65(18.7)	1(0.3)	57(16.4)	26(7.5)	12(3.5)	13(3.7)	5(1.4)	23(6.6)	347	924	37.6%
ICT	96(13.5)	174(24.5)	182(25.7)	(0.0)	72(10.2)	47((6.6)	8(1.1)	105 (14.8)	12(1.7)	13(1.8)	709	1286	55.1%

EPTB-Extrapulmonary TB, Site-NOS-EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intrathoracic, OAS-Osteoarticular spine, OAOS-Osteoarticular other than the spine, CNS-Central Nervous system, Dis/Mil–Disseminated /Miliary TB. PJB-Punjab, SND-Sindh, KP-Khyber Pakhtunkhwa, BTN-Balochistan, AJK-Azad Jammu Kashmir, GB-Gilgit Baltistan, FATA-Federally administered tribal areas, ICT-Islamabad Capital territories.

[*] Age was missing from 22 records including 3 EPTB patients

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Treatment outcomes

Overall treatment success for EPTB cases was higher compared to both bacteriology confirmed and clinically diagnosed PTB. The treatment success was close to 90% for people having pleural, lymphatic and abdominal TB. Treatment success was significantly lower ($P < .001$) for patients with TB meningitis compared to other forms (Table 4).
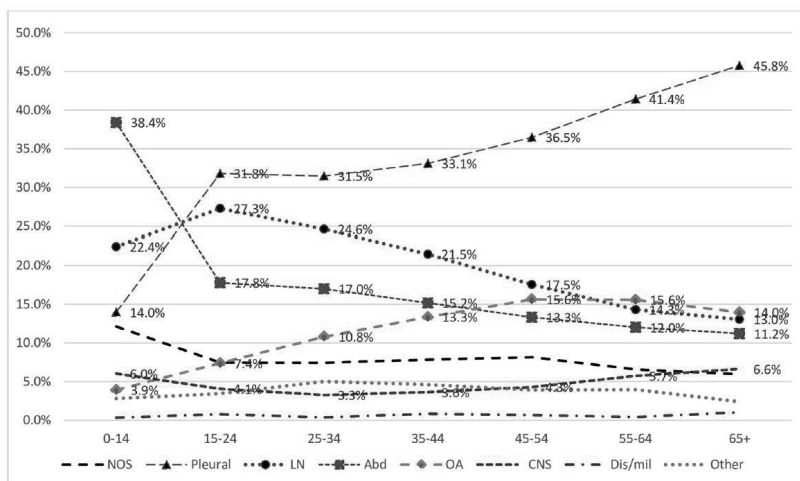
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Fig 2. Frequency of different extrapulmonary manifestations by age group. NOS: EPTB site not specified, LN-lymphatic, Abd-abdomen; OAS-Osteoarticular, CNS-Central Nervous system, Dis/mil-Disseminated /Miliary.

Discussion

To our knowledge, this is the first multicenter study where routine TB program data is used to describe demographic characteristics, clinical manifestations and treatment outcomes of EPTB patients in Pakistan. We performed a record review of 54,092 TB patients including 15,790 EPTB cases, notified in 2016 by HCF conveniently selected from all tiers of health care covering all provinces/regions in the country. The study population comprised 21.6% of EPTB and 13.1% of PTB cases notified countrywide in 2016. We purposefully went for oversampling of EPTB to include jurisdictions that appeared to be particularly sensitized to the issue and potentially would provide complete records to study a wider spectrum of EPTB manifestations. There was thus a higher proportion of EPTB in the study sample (29%) compared to national notification (20%) in the same year [1].

The variations in proportion of EPTB among TB cases notified in different provinces/ regions is already observed in routine TB notification data. Proportion of EPTB in countries neighboring Pakistan, varies from 5% in China (HBC), 18% in India (HBC), 25% in Iran and 29% in Afghanistan [2]. Interestingly compared to national average, proportion of EPTB is higher in provinces/regions in the North West (KP, FATA, BTN) neighboring Afghanistan and Iran and a relative lower in proportion in the South East provinces (PJB &SND) neighboring India. In TB population studied, proportion of EPTB although was higher but provincial ranking was consistent with national notification trend. There are some evidences suggesting that PTB incidence decreases as geographical altitude increases [14–18] without effect on EPTB incidence [18]. In Pakistan, provinces and regions in North West with higher proportion of EPTB among notified TB cases, show a trend of lower PTB case notification rate

Table 4. Treatment outcomes of patients having bacteriology confirmed pulmonary tuberculosis, clinically diagnosed pulmonary tuberculosis and extrapulmonary tuberculosis.

	PULMONARY TB		EXTRA PULMONARY TB								
	Bacteriology confirmed	Clinically diagnosed	All	Pleural	Lymphatic	Abdominal	Osteo-Articular	CNS	Diss./ Mil	Other	NOS
TB cases(n)	18872	19430	15790	4668	3581	3313	1483	725	94	587	1339
Successfully Treated*	14959:79.3% (78.7–79.8)	16803:86.5% (86.0–87.0)	14125:89.5% (89.0–89.9)	4233:90.7% (89.8–91.5)	3217:89.8 (88.8–90.8)	3018:91.1% (90.1–92.0)	1288:86.9% (85.0–88.5)	539:74.3% (71.0–77.5)	78:83.0% (73.8–89.9)	496:84.5% (81.3–87.3)	1256:93.8% (92.4–95.0)
Lost to Follow up*	1147: 6.1% (5.7–6.4)	844:4.3% (4.1–4.6)	653:4.1% (3.8–4.5)	207:4.4% (3.9–5.1)	135:3.8% (3.2–4.4)	66:2.0% (01.5–2.5)	70:4.7% (3.7–5.9)	110:15.2% (12.6–18.0)	9:9.6% (4.4–17.4)	28:4.8% (3.2–6.8)	28:2.1% (1.4–3.0)
Treatment-Failure*	430;2.3% (2.1–2.5)	71;0.4% (0.3–0.5)	33;0.2% (0.1–0.3)	6;0.1% (0.05–0.3)	8;0.2% (0.09–0.4)	14;0.4% (0.2–0.7)	3;0.2% (0.04–0.6)	1;0.1% (00.0–0.7)	0;0.0% (0.0–0.03)	1;0.2% (0.0–0.9)	0;0.0% (0.0–.0.3)
Died*	616;3.3% (3.0–3.5)	410;2.1% (1.9–2.3)	207;1.3% (1.1–1.5)	67;1.4% (1.1–1.8)	33;0.9% (00.6–1.3)	40;1.2% (0.9–1.6)	15;1.0% (0.6–1.7)	32;4.4% (3.0–6.2)	4;4.3% (1.2–10.5)	3;0.5% (0.1–1.5)	13;1.0% (0.5–1.7)
Transferred out *	382;2.0% (1.8–2.2)	112;0.6% (0.5–0.7)	78;0.5% (0.4–0.6)	16;0.3% (0.2–0.6)	29;0.8% (0.5–1.2)	5;0.2% (0.05–0.4)	12;0.8% (0.4–1.4)	8;1.1% (0.5–2.2)	0;0.0% (0.0–0.03)	1;0.2% (0.0–0.9)	7;0.5% (0.2–1.1)
Not evaluated/ recorded*	1338;7.1% (6.7–7.5)	1190;6.1% (5.8–6.5)	694;4.4% (4.1–4.7)	139;3.0% (2.5–3.5)	159;4.4% (3.8–5.2)	170;5.1% (4.4–5.9)	95;6.4% (5.2–7.8)	35;4.8% (3.4–6.7)	3;3.2% (0.7–9.0)	58;9.9% (7.6–12.6)	35;2.6% (1.8–3.6)

*Data shown are number of patients;% (95%CI)

CNS-Central Nervous system, Diss/Mil-Disseminated/Miliary TB, NOS-Site not specified

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compared to national average (S2 Table). However, differences in notifications of PTB and EPTB cannot be explained based on this correlation alone and further studies including sub national surveys are needed to determine prevalence of TB, coverage and accessibility to health care services, health seeking behaviors of patients with extrapulmonary manifestations compared to pulmonary symptoms and practices of doctors managing EPTB in different geographical settings.

In total study population, male and female were equal in number but differences were observed in gender distribution both among PTB and EPTB cases notified in different provinces. Similar to the global trends higher number of male among PTB cases and higher odds of female presenting with EPTB was seen in residents of South-Eastern provinces (SND, PJB) and ICT.[1–5,7,8] In contrast, a higher female to male ratio was seen among PTB patients from North-Western provinces (KP, BTN), consistent with trend seen in routine notification [19,20] and similar to the pattern in bordering Afghanistan [2]. However likelihood of both sexes presenting with EPTB manifestations was similar in these regions (BTN, KP, FATA, and AJK). Differences in the relative frequency of EPTB by sex, race, ethnicity, and provenance have been reported by others [3–5, 8, 21–23]. Gender differences in notification rates are known to reflect differences in TB epidemiology, and/or gender differences in access to care [24]. In Pakistan, based on TB prevalence survey findings, it is estimated that males have 1.8 times higher burden of PTB [1,2,25] but gender differences are minimal among notified TB cases and prevalence to notification ratio clearly explains gaps in case detection of PTB in males. However as opposed to PTB, the true burden and gaps in detection of EPTB cannot be determined objectively as EPTB is typically excluded from the measurement of prevalence [2]. Regardless, variation in case notification rates between provinces, similarity was seen in adult case notification pattern, with higher number of females among notified PTB and EPTB

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patients in age group of 15-34yr, which was similar to the PTB notifications pattern reported in Europe in middle of last century.[24]. Pakistan has one of the highest levels of child and maternal under nutrition worldwide and large social and geographical inequalities are noted in child and maternal nutrition [26,27]. According to WHO estimates approximately 150,000 incident TB cases in Pakistan are associated with malnutrition [2]. Further studies are needed to establish evidence based linkages between TB in young females to poverty, under nutrition, sex-specific social and biological characteristics.

Consistent with other studies, we reported a strong association between EPTB and children, [3, 4, 8] with EPTB affecting children twice as frequently as adults (OR = 2.0). The most intriguing finding was high proportion of children (n = 1385, 38.4%) having abdominal TB and more than 80% of these children belonged to provinces in North-West (KP, FATA, and GB). Beside children, higher rates of abdominal TB was also reported in adult TB patients from KP and FATA. While surveillance data analysis of EPTB patients from high income countries have reported low prevalence (<5%) of abdominal TB [3,4, 8], but prevalence varying from 6.8% (Afghanistan), 12.8% (India), 14.8% (Nepal), 15.4% (Australia) and 17.5% (Saudi Arabia) are reported mostly in hospital-based small studies [28–32]. The drivers of TB in children including the high levels of chronic and acute malnutrition do still exist in Pakistan, with over 10 million stunted children [27]. The malnutrition alone, however, does not explain the higher prevalence of abdominal TB among children in the North-West regions as a higher prevalence of stunting (> national average of 40.2%) is also reported in SND in the South. There is a need to study high rates of abdominal TB in children and in particular clinical criteria used for diagnosis and prevailing empiric TB treatment practices.

Consistent with trend reported in other countries, the two most common extrapulmonary manifestations reported in our study population were pleural and lymphatic, collectively making 50% of all EPTB cases [3–5, 7, 8, 21, 22, 28–32]. Similarity in frequency of different EPTB manifestations was noted in adult population, with pleural TB being the most common across all provinces/regions. Although lymphatic TB was the second commonest form, but only a very few patients (1%) were reported having intra-thoracic lymphatic TB. Contrary to our findings, high predilection for intra-thoracic lymphatic TB in young ages is reported by others [3, 4]. Likely reasons for under reporting in our settings include lack of training and/or access to radiology facilities or under-recording of findings in TB registers.

We reported osteoarticular TB in 9.3% (1483) of EPTB patients and spinal TB was noted to be more common compared to other forms of bones/joint TB. The median ages of patients with osteoarticular TB was higher compared to all other forms of EPTB. Our findings are consistent with prevalence reported in high income countries [3, 4, 5, 7] but prevalence is lower compared to Benin (25.4%) and Ghana (17.5%) [8, 33].

Among EPTB patients in our study, 6.0% of children and 4.2% adults were reported having CNS TB, frequency was higher compared to high-income countries [3,4,7] but lower than reported by studies in low income and high HIV prevalence countries [8,33]. Among ten tertiary care hospital included in our study, with the exception of three, proportion of CNS TB reported by each was lower than total average. Based on these findings, possibility of reporting a higher prevalence of CNS TB due to selection bias can be argued, nevertheless it does indicates serious gaps in notifications and weak linkages between reporting units and other specialties treating CNS TB in tertiary care settings. Beside weak linkages, other possible reasons for underreporting includes patients with serious forms of TB dying before reaching HCF or dying undiagnosed in hospitals.

We reported a low frequency of "other" group of EPTB manifestations including genitourinary tuberculosis (3.7%) in population studied. Although possibility of underreporting cannot be excluded, but our findings are consistent with reports from high income countries, where a

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relative higher predilection of genitourinary tuberculosis is reported in whites compared to non-whites. [3, 5, 23]

EPTB disease site was not recorded in 8.5% of patients included in the study. In TB-burdened countries EPTB is one of the important causes of fever of unknown origin [34]. In TB registers where disease site was recorded for majority of the notified EPTB cases, one plausible explanation for missing details is, that these patients were most likely initiated on empiric TB treatment for fever of unknown origin without further investigations either due to lack of diagnostic facilities or resources.

Of all EPTB cases included in the study, a high majority of the patients were successfully treated consistent with findings from other studies [4, 8]. Proportion of patients with history of previous treatment was low in EPTB (4.0%) compared to PTB (10.2%) cohort, which most likely was one of the reasons for a higher treatment success rate compared to PTB. However treatment outcomes are usually reported, using standard classifications; and "treatment completion" is considered a successful outcome [6, 8]. Possibilities cannot be excluded of reporting treatment completion for patients without clinical improvement particularly in case of undiagnosed drug resistance or disease other than EPTB, as well as for patient with clinical improvement who were initiated on empiric TB treatment for trivial nonspecific infection.

Furthermore, treatment outcomes of different non-pulmonary manifestations of TB cannot be lumped together as if they are a single entity [3]. Treatment success rate were higher for pleural, lymphatic and abdominal TB (90%), but was significantly lower for CNS TB (74.3%, 95%CI 71.0–77.5%). Reported mortality in patients with CNS TB was 4.4% but more than 15% were reported lost to follow up with possibility of underreporting of mortality rate. The poor outcome of CNS TB and other severe forms of TB are masked by the overall high treatment success and are thus ignored.

EPTB can affect any part of the body, and due to the heterogeneity in clinical manifestations, and difficulties in obtaining specimens, the definite diagnosis can be especially challenging. In high-income countries, 50–60% of the notified EPTB cases are bacteriology confirmed [3, 5, 8, 32]. On the contrary, in our study, less than 10% of patient were tested and only 0.5% of the notified EPTB cases were bacteriology confirmed, similar to reports from other low income countries [8]. Besides lack of resources and access to specialized facilities, likely reasons for reliance on clinical diagnosis, includes low sensitivity of widely available AFB microscopy, limited access and complexity associated with TB culture testing and lack of surveillance for EPTB. Possibilities of over and under-diagnosis of EPTB cannot be excluded in these scenarios. EPTB in general is not given high priority on the public health agenda, probably as it does not contribute significantly to the transmission of the disease [4]. With availability of simpler, more sensitive molecular diagnostics [35] there is need for systematic efforts to increase testing of all EPTB specimens for definite diagnosis of TB and drug resistance. This can be achieved by a more decentralized access to new diagnostic tools, multidisciplinary engagements with effective linkages, clear guidance on diagnosis and management of EPTB, capacity enhancement of clinical and laboratory staff and finally comprehensive surveillance system to monitor EPTB notifications in real time.

The main limitations of our study includes use of stratified convenient sampling and voluntary data collection, which makes it quite possible that our findings are not completely representative of the entire country. Secondly, routine clinical records were used with possible multiple problems in the information recorded. Third, almost all of the notified EPTB cases were clinically diagnosed with possibilities of different protocols being used for diagnosis across country.

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Conclusion

The study provides an insight into the demography, prevalent clinical manifestations and treatment outcomes of EPTB. Further studies are needed to explain significant differences observed between provinces and associated specific risk factors. From a public health perspective, there is a need to focus on EPTB accounting 20% of the TB disease burden, and address challenges concerning the quality of diagnosis and treatment of patients presenting with extra-pulmonary manifestations of TB.

Supporting information

S1 Fig. Pakistan map showing provincial and regional boundaries.
(PDF)

S1 Table. Characteristics of the selected health care facilities and TB cases notified. EPTB–Extra pulmonary tuberculosis, PTB-Pulmonary tuberculosis, HCF-Health care facility, HCP-Health care provider, Pub-public, Pvt–private, THC-Tertiary health care, SHC–Secondary health Care, PHC-Primary health care, GP–General Practitioner, GX- GeneXpert, HP- histopathology, PR- Paper register, ER- electronic file, PJB-Punjab, SND- Sind, KP-Khyber Pakhtunkhwa, BTN-Balochistan, AJK -Azad Jammu Kashmir, GB-Gilgit Baltistan, FATA-Federally administered tribal area, ICT-Islamabad capital Territory.
(PDF)

S2 Table. National and study sites TB case notifications in 2016 by province and region.
PTB- Pulmonary tuberculosis, EPTB–Extra pulmonary tuberculosis, N = New TB case, R = Relapse TB case, GB–Gilgit Baltistan, KPK–Khyber Pakhtunkhwa, AJK-Azad Jamu Kashmir, FATA- Federally administered tribal area, ICT–Islamabad capital Territory, GP- General practioner.
(PDF)

S3 Table. Age and sex specific pulmonary and extrapulmonary tuberculosis notifications and odds of female (OR) for having extrapulmonary tuberculosis by place of residence.
F-EPTB = Female Extrapulmonary TB, F-PTB = Female Pulmonary TB cases, M-EPTB = Male Extrapulmonary TB, M-PTB = Male Pulmonary TB cases.
(PDF)

S4 Table. A: Notified tuberculosis cases and extra-pulmonary manifestations of tuberculosis by sex, age groups and health facilities in Punjab, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. B: Notified tuberculosis cases and extrapulmonary manifestation of tuberculosis by sex, age groups and health facilities in Sindh, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. C: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and health facilities in Khyber Pakhtunkhwa, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. D: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and

health facilities in Balochistan, Pakistan, during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. E: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and health facilities in Federally Administered Tribal Areas, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. F: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and health facilities in Gilgit Baltistan, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. G: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and health facilities in Azad Jammu & Kashmir, Pakistan, during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB. H: Notified tuberculosis cases and extrapulmonary manifestations of tuberculosis by sex, age groups and health facilities in Islamabad capital Territory, Pakistan during 2016 EPTB–Extra pulmonary tuberculosis, Site-NOS: EPTB site not specified, LN-EXT-lymphatic extra-thoracic, LN-INT-Lymphatic intra thoracic, ABD-Abdomen, OAS–Osteoarticular spine, OAOS-Osteoarticular other than spine,CNS-Central nervous system, DIS/MIL–Disseminated /Miliary TB.
(PDF)

Acknowledgments

Author Contributions

Conceptualization: Sabira Tahseen, Faisal Masood Khanzada, Aurangzaib Quadir Baloch, Tehmina Mustafa.

Data curation: Sabira Tahseen, Faisal Masood Khanzada.

Formal analysis: Sabira Tahseen, Faisal Masood Khanzada.

Investigation: Qasim Abbas, Mansoor Manzoor Bhutto, Ahmad Wali Alizai, Shah Zaman, Zahida Qasim, Muhammad Najeeb Durrani, M. Khalid Farough, Atiqa Ambreen, Nauman Safdar.

Methodology: Sabira Tahseen, Tehmina Mustafa.

Project administration: Sabira Tahseen, Faisal Masood Khanzada.

Resources: Aurangzaib Quadir Baloch.

Software: Faisal Masood Khanzada.

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Supervision: Sabira Tahseen, Tehmina Mustafa.

Validation: Sabira Tahseen, Faisal Masood Khanzada.

Writing – original draft: Sabira Tahseen.

Writing – review & editing: Sabira Tahseen, Faisal Masood Khanzada, Aurangzaib Quadir Baloch, Qasim Abbas, Mansoor Manzoor Bhutto, Ahmad Wali Alizai, Shah Zaman, Zahida Qasim, Muhammad Najeeb Durrani, M. Khalid Farough, Atiqa Ambreen, Nauman Safdar, Tehmina Mustafa.

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7.2. Paper-2

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The value of histological examination in the diagnosis of tuberculous lymphadenitis in the era of rapid molecular diagnosis

Sabira Tahseen[1,2✉], Atiqa Ambreen[3], Sheeba Ishtiaq[4], Faisal M. Khanzada[2], Nauman Safdar[1,5], Lisbet Sviland[6,7] & Tehmina Mustafa[1,8]

Extrapulmonary tuberculosis often poses a diagnostic challenge. This study aimed to assess the value of histological examination in diagnosing tuberculous lymphadenitis (LNTB) when performed simultaneously with rapid molecular assay (Xpert MTB/RIF) testing. People presumed to have LNTB were prospectively enrolled in a tertiary care hospital. Excision biopsy was performed and tested by histology, Xpert, and culture. Of 390 lymph nodes, 11 (2.8%) were positive by AFB microscopy, 124 (31.8%) by Xpert, 137 (35.1%) by culture, and histopathology was consistent with TB in 208 (53.3%). Altogether, LNTB was diagnosed in 228 and bacteriologically confirmed TB in 178 cases. Against culture, histopathology versus Xpert had higher sensitivity (93 vs. 62%) but lower specificity (68 vs. 83%). In patients with short clinical history, a significantly higher number of Xpert-positive specimens were culture-positive. Among patients with histology suggestive of TB, no difference was seen in response to treatment between bacteriology positive and negative, but a significant slow response was noted in bacteriology confirmed TB with nonspecific histology. In a country like Pakistan, with high TB and low HIV prevalence, diagnosis is possible for more than 95% of LNTB when Xpert and histopathology examination is used in combination, compared to less than 60% by Xpert alone.

Every year 10 million people fall ill with tuberculosis (TB). TB is caused by *Mycobacterium tuberculosis* (MTB) which primarily affects the lungs causing pulmonary TB (PTB) but can also affect other organs, causing extrapulmonary tuberculosis (EPTB). In 2020 EPTB accounted for 18% of 5.8 M TB cases notified globally[1]. Commonest disease manifestations of EPTB include lymph nodes, pleura, abdominal, and osteoarticular system[2]. Patients with EPTB often have constitutional symptoms, and specific symptoms based on the tissue or organ affected[2,3]. The diagnosis of EPTB often poses challenges either because of its occult nature, difficulty in obtaining samples, or the paucibacillary nature of the disease[3]. Specimen from the extrapulmonary disease site is usually obtained by aspiration or biopsy, and the laboratory diagnosis of tuberculosis is made by histological and bacteriological examination[2–4]. Histologic diagnosis is based on identifying granulomatous inflammation commonly characterized by aggregates of epithelioid histiocytes, with a peripheral cuff of lymphocytes and plasma cells. The epithelioid cell may also coalesce to form multinucleated giant cells, and central necrosis is distinctly present in necrotizing granulomas. *Mycobacteria* species are the most common etiologies of necrotizing granulomas[4,5]. The sensitivity of histological features has been reported to vary from 59 to 88% for lymph node TB (excisional biopsy) against a composite reference standard[6]. The histologic examination has limitations in the diagnosis of TB; the granulomatous response may fail to elicit in immunocompromised individuals with TB; furthermore,

[1]Department of Global Public Health and Primary Care, Centre for International Health, University of Bergen, Bergen, Norway. [2]National TB Reference Laboratory, National Tuberculosis Control Program Pakistan, Block E and F, EPI building, Near NIH, Chak Shahzad, Islamabad, Pakistan. [3]Department of Microbiology, Gulab Devi Hospital, Lahore, Pakistan. [4]Department of Pathology, Gulab Devi Hospital, Lahore, Pakistan. [5]Social and Health Inequalities Network (SHINe) Pakistan, Islamabad, Pakistan. [6]Department of Clinical Medicine, Faculty of Medicine, University of Bergen, Bergen, Norway. [7]Department of Pathology, Haukeland University Hospital, Bergen, Norway. [8]Department of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway. ✉email: sabira.tahseen@gmail.com

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granulomatous responses are also seen in infectious other than tuberculosis, autoimmune, toxic, allergic, and neoplastic conditions[4–6]. For bacteriological diagnosis of EPTB, acid-fast bacilli (AFB) microscopy has a limited value because of its low sensitivity. The culture is regarded as the gold standard for the diagnosis of TB; still, its use is limited by the requirements of sophisticated laboratory infrastructure, freshly collected specimens to ensure bacilli's viability, and a long turnaround time[2,6]. Molecular methods for the diagnosis of TB have evolved over the last two decades[7,8]. The first automated real-time nucleic acid amplification (NAA) technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance, Xpert MTB/RIF* assay (Cepheid, Sunnyvale, CA, USA), was endorsed by WHO in 2010[9]. The Xpert MTB/RIF assay(Xpert) is sensitive and more rapid than conventional methods, with higher feasibility for implementation close to the point of care because of minimal infrastructure requirement[7,8]. The policy recommendations have evolved with increasing use and evidence[10–13]. In 2013, WHO, for the very first time, recommended the use of Xpert for the diagnosis of TB in selected extrapulmonary specimens, including lymph nodes[14]. In 2017, WHO endorsed the next-generation Xpert MTB/RIF ultra * assay with improved detection limit and sensitivity similar to solid culture[15]. In light of additional evidence, in 2021, WHO updated its guidelines and recommended using Xpert and Xpert Ultra as initial diagnostic tests rather than AFB microscopy and or culture for pulmonary and extrapulmonary specimens[16–18].

International Standard for Tuberculosis care (ISTC) for a patient with presumed EPTB recommends obtaining appropriate specimens for microbiology testing and histologic examination[19,20]. The laboratory diagnosis of EPTB in high TB burden countries relied on clinical judgment and identifying granulomatous inflammation in biopsied samples rather than bacteriological examination because of the lack of appropriate diagnostic facilities[2]. With the advancement in molecular technology, access to rapid and more sensitive diagnostic tools for TB has dramatically improved. Tuberculosis disease cannot be ruled out with a negative smear and NAA test result. For this reason, histopathology examination and culture are used in conjunction with more rapid AFB smear and NAA tests in developed countries[6,7]. However, clear guidance is lacking on the use of histologic examination to diagnose TB. We conducted this study to evaluate the added value of histological examination in the diagnosis of TB in patients presumed to have TB lymphadenitis by comparing histologic and molecular diagnosis using culture as the gold standard. We also evaluated the difference in clinical outcomes of LNTB patients diagnosed by bacteriology and/or histologic features.

Study setting, population, and methodology

This study was part of a larger research project aimed at improving the diagnosis of EPTB under routine programmatic conditions in Pakistan[21,22].

Study setting. Pakistan is a high TB and low HIV prevalent country[1]. The study was conducted in a private, not-for-profit tertiary care hospital located in the capital city of the largest province. The hospital specializes in TB care services; presumptive and already diagnosed TB patients are referred to Gulab Devi Hospital (GDH) for consultation and second opinion. Annually, almost 6000 TB patients are registered for TB treatment at GDH, and many others, after the diagnosis, are referred back for treatment to TB clinics closer to their residence. Routinely, all patients presumed to have EPTB are investigated for concomitant PTB by sputum AFB smear microscopy examination. For presumptive TB lymphadenitis, an excision biopsy is performed for diagnosis. The hospital has laboratory facilities for histopathology and mycobacteriology, including AFB microscopy, Xpert MTB/Rif, solid and liquid culture, and *M. Tuberculosis* isolates are sent to National TB Reference Laboratory Islamabad for drug susceptibility testing (DST)[21]. Laboratory reports are issued routinely within 24–48 h for AFB smear and Xpert and within 4–6 days for a histopathology examination.

Study population and methodology. Patients of all ages and gender presenting in out-patient clinics with enlarged lymph nodes presumed to have LNTB were evaluated for inclusion. Patients with a history of previous TB treatment and/or an already established diagnosis of LNTB were excluded. Eligible patients were enrolled prospectively after informed consent.

All enrolled patients were asked to submit sputum samples. An excision biopsy of the lymph nodes was performed. Among patients diagnosed with LNTB, those who opted for treatment at GDH were interviewed using a structured questionnaire and followed till completion of the first-line anti TB treatment (FL-ATT) as described previously[22]. Chest X-rays available in files of the enrolled patient were reread for the evidence of intrathoracic lymph nodes, PTB, and any other abnormality.

Laboratory methods. The LN biopsy specimen, immediately after excision, was divided into two; one half was placed in 10 ml physiologic saline (0.9%), and the other half was placed in buffered formalin and then transferred to the laboratory.

The LN biopsy specimen in saline was processed for AFB smear, Xpert, and culture for bacteriology examination. The biopsy specimen in saline was first minced in a manual sterile tissue homogenizer (15 ml; 19×155 mm), then transferred to a sterile 50 ml tube, processed without decontamination, and then centrifuged (3000 g for 15 min). The sediment was used for smear, Xpert, and culture examination[21,23]. AFB smears were stained with Auramine-O and examined using a light-emitting diode(LED) fluorescence microscope[24]. Xpert was performed using manufacturer protocols[25]. For culture, two slopes of Lowenstein-Jensen (LJ) medium and one Mycobacteria Growth Indicator Tube (MGIT 960; Becton Dickinson, Sparks, MD, USA) were inoculated[23].

The LN biopsy specimen in buffered formalin was processed for histopathology examination, and sections were stained with hematoxylin and eosin (H&E); a histopathologist at GDH examined the sections. For this study, the H&E stained slides of all LNs reported having morphological features consistent with TB were reexamined. Histopathology reports were structured into four groups (i) well-formed granulomas with no necrosis.

(ii) well-formed granulomas (predominantly)with necrosis (iii) ill-formed granulomas with necrosis (iv) caseous necrosis with no granuloma[26].

The sputum samples were examined by AFB microscopy only.

Definitions. Histopathology consistent with TB was defined as morphological features showing well or poorly formed granuloma and/or caseous necrosis with or without Langhans type giant cell. Bacteriology confirmed LNTB (B + ve LNTB) was defined as MTB positive by Xpert and/or culture. The composite reference standard (CRS) for TB was defined as MTB positive by Xpert and/or culture and/or histopathology consistent with TB.

Duration of illness was calculated from the first symptom, and a cut-off of three months was used to stratify short from long periods of illness. Successful treatment outcome was defined as clinical response with standard of care when treatment was given for six months and extended treatment if given for more than six months. Patients whose clinical response was documented in the treatment card at the end of five months but who didn't come for the last follow-up were also counted as successfully treated with standard treatment.

Data management and statistical analysis. Data was entered in EpiData Manager v4.2 (Epi-Data Association, Odense, Denmark) and analyzed using Stata v13 (Stata Corporation, College Station, TX, USA). Data on demography, clinical symptoms, duration of illness, treatment duration, and the outcome was extracted from the questionnaire and patient treatment card. Only cases with interpretable histology and bacteriology examination results were analyzed, and cases with tissue other than lymph nodes or neoplastic disease on histological examination were excluded from the analysis.

Categorical variables were reported as frequencies and percentages. The performance of the Xpert and histology was calculated against culture as a microbiological reference standard. Cross-tabulation was used to calculate sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV). Two sample/group proportion tests were used to compare the response to treatment and analyzed using binary logistic regression to calculate the odds ratio (OR) and 95% confidence interval (95% CI). P value < 0.05 was considered statistically significant.

Ethical considerations. The study protocol was approved by the National Bioethics Committee of Pakistan (Islamabad, Pakistan) and the Regional Committee for Medical and Health Research Ethics, Western-Norway (REK Vest). University of Bergen (Postboks 7804, 5020 Bergen, Norway). Eligible study participants were enrolled after informed consent. The de-identified data was used for analysis. The design and reporting of the study followed the guidelines for reporting diagnostic accuracy studies (2015).

Results

Altogether 412 patients with enlarged lymph nodes were enrolled from April 2016 to August 2017. Twenty-two cases, including four, reported having no tissue, 13 with tissue other than lymph nodes, and five with the neoplastic disease were excluded (Figure 1). Among 390 patients included, AFB microscopy was positive in 11 (2.8%), Xpert detected MTB in 124 (31.8%), culture was positive in 137 (35.1%), and histopathology was consistent with TB in 208 (53.3%). LNTB was diagnosed in 228 (58.5%) based on CRS, and bacteriology confirmed LNTB in 178 (45.6%) cases.

Demography and clinical features. Demographic features are shown against the diagnostic profile of LNTB patients in Table 1. The median age was 19 yrs, and 61% were females. All patients presented with lymphadenopathy; 12 (5%) patients also had abscess formation, and 7 (3%) had discharging sinuses. Of patients diagnosed with LNTB (n = 228), HIV status was known for one HIV reactive and 71 non-reactive patients. Among LNTB patients, 183 opted for treatment at GDH, 147 consented to in-depth interviews, and chest X-rays were available in the files of 153 patients. Among LNTB patients, 38% (n = 87) were sick for 3 months or less and 35% (n = 80) for more than 3 months, and history was not recorded by the remaining 27% (n = 61). Of the 147 LNTB patients interviewed, symptom reported included fever (89%), current cough (52%), weight loss (37%), appetite loss (30%) and night sweat (14%). Among 153 chest X-rays reread, 30% reported intrathoracic lymph nodes, 10% had abnormal shadows suggestive of PTB, two showed pleural effusion, and three had other abnormalities, whereas, in 54% (n = 83), no abnormality was seen (Table 1).

Laboratory results. *AFB microscopy.* Of 390 LN tissues examined, 11 were positive for AFB microscopy, and 64% were scant positive. All 11 AFB positive LN were positive for MTB by Xpert, and eight were culture positive (Figure 2). Sputum was examined from 224/228 LNTB patients; only one was reported AFB smear-positive.

Xpert MTB/RIF results and performance. Of 390 LN examined, interpretable Xpert results were reported in 379 (97%). MTB was detected in 124 LN, and in 93%, MTB detected was low or very low in quantity based on the cut-off threshold value (Table 2). Of 124 MTB positive, 66.9% (n = 83) were culture positive, ranging from 100% in high, 75% in medium, 70% in low, and 63% with a very low quantity of MTB. The Se, Sp, PPV, and NPV of molecular diagnosis against culture were 62%, 83%, 67%, and 80%, respectively. A significantly higher Se ($P = 0.012$) and PPV ($P = 0.028$) for Xpert was reported in subgroups of patients having short clinical history (3 month or less) compared to those ill for longer than three months at the time of presentation.

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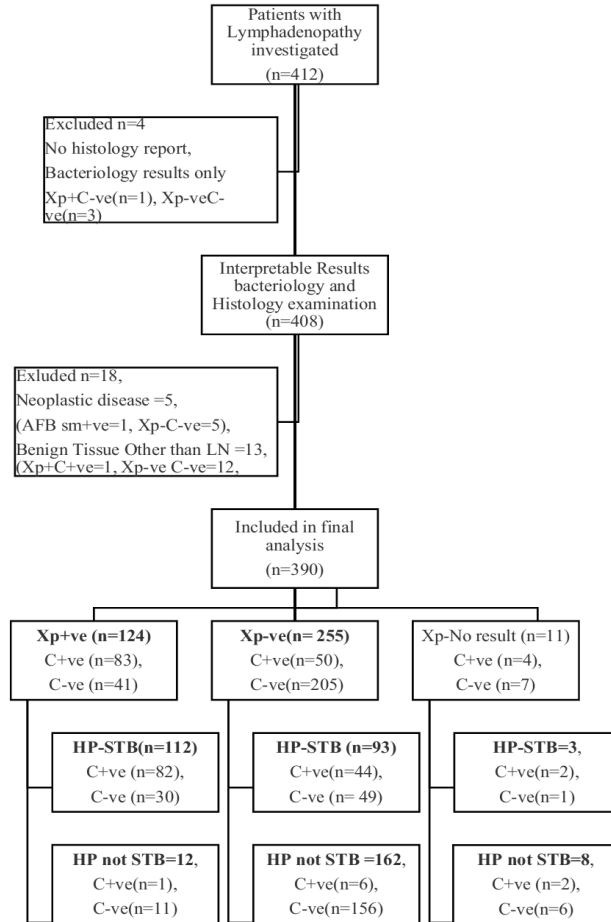
Xpert: Xpert MTB/RIF, HP-STB: Histopathology suggestive of tuberculosis, HP Not STB: Histopathology not suggestive of tuberculosis, C-ve: culture-negative, C+ve: culture positive

Figure 1. Flow diagram showing people investigated for enlarged lymph nodes presumed to have TB and the rapid molecular assay, histology, and culture examination results.

	All (n)	Morphological features on histology examination					Bacteriology results			
		HP-0 n (%)	HP-1 n (%)	HP-2 n (%)	HP-3 n (%)	Hp-4 n (%)	Xp+ve C+ve n (%)	C+ve (only) n (%)	Xp+ve (only) n (%)	XP−ve C−ve n (%)
All LNTB	228	20 (9%)	4 (2%)	109 (48%)	82 (36%)	13 (6%)	83 (36%)	54 (24%)	41 (18%)	50 (22%)
Median age (yrs.)	19	15	31.5	19	19.5	25	19	19	19.5	19
Female	140	12 (9%)	4 (3%)	71 (51%)	45 (32%)	8 (6%)	48 (34%)	36 (26%)	26 (19%)	30 (21%)
Age-Group (yrs.)										
<5 yrs	6	3 (50%)	0	1 (17%)	2 (33%)	0%	1 (17%)	1 (17%)	3 (50%)	1 (17%)
5–14 yrs	58	7 (12%)	0	32 (55%)	16 (28%)	3 (5%)	18 (31%)	7 (12%)	7 (12%)	26 (45%)
>15 yrs	164	10 (6%)	4 (2%)	76 (46%)	64 (39%)	10 (6%)	64 (39%)	33 (20%)	31 (19%)	36 (22%)
Duration of illness										
≤3 months	90	5 (6%)	2 (2%)	40 (44%)	36 (40%)	7 (8%)	45 (50%)	14 (16%)	10 (11%)	21 (23%)
>3 months	77	3 (4%)	1 (1%)	50 (65%)	19 (25%)	4 (5%)	22 (29%)	22 (29%)	14 (18%)	19 (25%)
NA	61	12 (20%)	1 (2%)	19 (31%)	27 (44%)	2 (3%)	16 (26%)	18 (30%	17 (28%)	10 (16%)
HIV status										
Positive	1	0	0	1 (100%)	0	0	0	1 (100%)	0	0
Non-Reactive	70	6 (9%)	1 (1%)	29 (41%)	31 (44%)	3 (4%)	19 (27%)	22 (31%)	10 (14%)	19 (27%)
Unknown	157	14 (9%)	3 (2%)	79 (50%)	51 (32%)	10 (6%)	64 (41%)	31 (20%)	31 (20%)	31 (20%)
Local Symptoms										
LAP only	209	20 (10%)	4 (2%)	102 (49%)	74 (35%)	9 (4%)	74 (35%)	52 (25%)	38 (18%)	45 (22%)
LAP and abscess	12	0	0	3 (25%)	6 (50%)	3 (25%)	8 (67%)	2 (17%)	1 (8%)	1 (8%)
LAP and sinus	7	0	0	4 (57%)	2 (29%)	1 (14%)	1 (14%)	0	2 (29%)	4 (57%)
Constitutional Symptoms										
Patient interviewed	147	6 (4%)	3 (2%)	77 (52%)	52 (35%)	9 (6%)	56 (38%)	33 (22%)	18 (12%)	40 (27%)
Fever	131	6 (5%)	2 (2%)	66 (50%)	49 (37%)	8 (6%)	50 (38%)	30 (23%)	17 (13%)	34 (26%)
Cough	76	3 (4%)	2 (3%)	37 (49%)	29 (38%)	5 (7%)	25 (38%)	23 (30%)	12 (16%)	16 (21%)
Night sweats	21	1 (5%)	0	9 (43%)	9 (43%)	2 (10%)	8 (38%)	6 (29%)	2 (10%)	5 (24%)
Weight loss	55	1 (2%)	0	24 (44%)	25 (45%)	5 (9%)	21 (38%)	14 (25%)	5 (9%)	15 (27%)
Appetite loss	44	1 (2%)	0	24 (55%)	14 (32%)	5 (11%)	18 (41%)	11 (25%)	4 (9%)	11 (25%)
Chest X-Ray findings										
X-Ray Reread	153	5 (3%)	3 (2%)	86 (56%)	50 (33%)	9 (6%)	59 (39%)	34 (22%)	21 (14%)	39 (25%)
PTB-suggestive	14	1 (7%)	0	6 (43%)	5 (36%)	2 (14%)	4 (29%)	3 (21%)	4 (29%)	3 (21%)
Intra-thoracic LN	51	1 (2%)	0	34 (67%)	14 (27%)	2 (4%)	24 (47%)	12 (24%)	4 (8%)	11 (22%)
Pleural effusion	2	0	0	2 (100%)	0	0	1 (50%)	0	0	1 (50%)
Other abnormalities	3	0	0	3 (100%)	0	0	2 (66%)	0	0	1 (33%)
No abnormality	83	3 (4%)	3 (4%)	41 (49%)	31 (37%)	5 (6%)	28 (34%)	19 (23%)	13 (16%)	23 (28%)

Table 1. Demographic characteristics and clinical features of lymph node tuberculosis patients and results of histopathology and bacteriology examination. HP-0: histological, not suggestive TB, HP-1: Well-formed granulomas no necrosis, HP-2: well-formed granuloma with necrosis, HP-3: ill-formed granulomas with necrosis, HP-4: Caseous necrosis only. LAP: Lymphadenopathy, XP: Xpert MTB/RIF, C: culture, LN: Lymph node. Xp+ve C+ve: MTB detected by Xpert MTB/RIF and grown on culture. Xp+ve only: MTB detected by Xpert MTB/RIF and no growth on culture. C+ve only: MTB not detected or results not interpretable, MTB grown on culture. XP−ve C−ve: MTB-Not detected or results not available on Xpert MTB/RIF and no growth on culture.

Histological examination results and performance. Histopathology suggestive of TB was reported in 208 cases, including necrotizing granulomatous inflammation in 191 (92%) and caseous necrosis without granuloma in 13 (6%), and granulomatous inflammation without necrosis in four cases (2%) (Table 3).

Among 208LN with histopathology suggestive of TB, 82(39%) were positive both by Xpert and culture, 30(14%) by Xpert only, 46(22%) by culture only, and 50 were negatives by both (Figure 2).

In Table 3, the performance of histological examination is shown against culture, Xpert, and B + LNTB. The Se, Sp, PPV and NPV for histological diagnosis against B + LNTB was 89%, 76%, 76%,and 89% respectively. Performance of histological examination was higher against culture compared to Xpert as the reference standard, but the difference was not statistically significant for Se ($P = 0.384$), Sp ($P = 0.341$), PPV ($P = 0.149$), and NPV ($P = 0.422$).

Histopathology was not consistent with TB in 20/178 B + LNTB cases. In these cases, reactive lymphoid hyperplasia was reported in 14, chronic nonspecific inflammation in four, acute suppurative inflammation, and lymphoproliferative disorder in one each.

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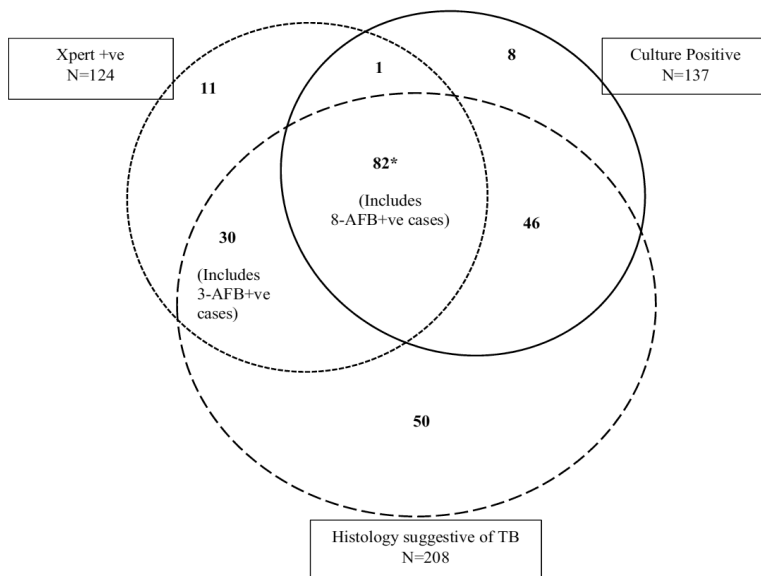
*Includes one LNTB patient with concomitant AFB smear positive pulmonary TB.

Figure 2. Venn diagram showing Xpert MTB/RIF, culture, and histology results of 228 tuberculous lymphadenitis patients diagnosed using composite reference standard.

Xpert MTB/RIF	Duration of illness /Culture results								
	Any duration of illness			≤3 Months		>3 Month		No information	
	All n	Positive n	Negative n	Positive n	Negative n	Positive n	Negative n	Positive n	Negative n
All tested	390	137	253	59	44	44	47	34	162
Interpretable results (n)	379	133	246	59	44	42	46	32	156
MTB-detected									
High	1	1	0	1	0	0	0	0	0
Medium	8	6	2	2	0	3	0	1	2
Low	53	37	16	24	5	7	5	6	6
Very low	62	39	23	18	5	12	9	9	9
MTB-detected-All	124	83	41	45	10	22	14	16	17
MTB not detected	255	50	205	14	34	20	32	16	139
Performance characteristics % (95%CI)									
Sensitivity	62.4%	(54.3–70.4)		76.3%	(64.0–85.3)	52.4%	(37.7–66.6)	50.0%	(35.2–67.5)
Specificity	83.3%	(79.4–88.1)		77.3%	(63.0–87.1)	69.6%	(55.2–80.9)	89.1%	(84.6–93.7)
Positive predictive value	66.9%	(58.1–74.3)		81.8%	(69.7–89.8)	61.1%	(44.9–75.2)	48.5%	(33–64.4)
Negative predictive value	80.4%	(76.8–85.9)		70.8%	(56.8–81.8)	61.5%	(47.9–73.5)	89.7%	(85.2–94.1)

Table 2. Performance of Xpert MTB/Rif assay against culture in people with short (≤3 months) versus long clinical history (>3 months). The difference in the performance of Xpert between subgroups of patients having short clinical history compared to those with a longer history of illness, sensitivity ($P = 0.012$), specificity ($P = 0.409$), PPV ($P = 0.028$), and NPV ($P = 0.327$).

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Histopathology	Bacteriology results							
		Culture		Xpert		Bacteriology	
	Total reported n	Positive n	Negative n	Positive n	Negative n	Positive* n	Negative n
Total reported	390	137	253	124	255	178	212
Suggestive of TB							
WFG no necrosis	4	2	2	2	2	4	0
WFG with necrosis	109	68	41	54	52	82	27
IFG with necrosis	82	50	32	45	37	61	21
C Necrosis (only)	13	8	5	11	2	11	2
Suggestive of TB (all)	208	128	80	112	93	158	50
Not suggestive of TB	182	9	173	12	162	20	162
Performance characteristics %(95%CI)							
Sensitivity	93%	(87.9–96.5)		90%	(83.9–94.4)	89%	(83.3–92.6)
Specificity	68%	(62.4–73.8)		64%	(57.5–69.2)	76%	(70.3–81.6)
Positive predictive value	62%	(54.8–67.9)		55%	(47.8–61.3)	76%	(69.7–81.3)
Negative predictive value	95%	(90.9–97.4)		93%	(88.3–96.0)	89%	(83.6–92.8)

Table 3. Performance of histological examination against culture, Xpert MTB/Rif assay, and bacteriology in the diagnosis of tuberculous lymphadenitis. HP: Histopathology, WFG: Well-formed granuloma, IFG: Ill-formed granuloma, C. Necrosis: Caseous necrosis, TB: tuberculosis. *Bacteriology positive defined as positive on Xpert and/or Culture.

Among Xpert positive, the culture positivity was 73.2%(95%CI; 64.3–80.6) in LN tissues showing TB specific morphological features, compared to 8.3% (95% CI;1.5–35.3) in tissues showing non-specific morphology ($P < 0.001$).

Drug resistance.   On phenotypic DST, rifampicin resistance was reported in two and mono resistance to isoniazid in six LNTB patients[21]. Of rifampicin-resistant, one case was negative and the other positive by AFB microscopy and Xpert. Rifampicin resistance was not detected by Xpert in both cases. Of six INH-resistant cases, all LNTB cases were AFB microscopy negative, and four were MTB positive by Xpert.
The necrotizing granulomatous lesion was reported histologically in all eight drug-resistant cases.

Treatment outcomes.   Of all LNTB patients diagnosed, 183 were registered for FL-ATT at GDH. Of these, 90% (n = 165) completed treatment, 9.3% (n = 17) were lost to follow up and one was declared treatment failure. Of 165 who completed treatment, 73% (n = 121) were treated successfully with a six-month standard treatment, and 27% (n = 45) after an extended treatment due to delayed clinical response. Predictors of slow treatment response have been reported previously[22]. Treatment outcomes and delayed treatment response in subgroups of TB patients stratified by diagnostic results are shown in Table 4. Two rifampicin-resistant and 5/6 INH-resistant LNTB patients were started on standard FL-ATT. One rifampicin-resistant TB patient was lost to follow up after two months, and the other completed treatment in 6 months. Of five INH-resistant LNTB patients, two completed treatment successfully in six months, whereas treatment was extended for 2, 3, and 6 months in the other three patients.

Discussion

LNTB is the second most prevalent manifestation of EPTB in Pakistan[27]. We studied the performance of the histological examination against culture as the gold standard compared to the rapid molecular assay (Xpert) to evaluate the added value of histopathology in the diagnosis of LNTB. People presumed to have LNTB who had no history of previous TB treatment were enrolled. Among presumptive LNTB patients, 390 had interpretable results for both bacteriological and histological examinations. LNTB was diagnosed in 228, including 69% (158) by both bacteriology and histopathology, 9% (20) by bacteriology, and 22% (50) by histopathology suggestive of TB.
In our study, three diagnostic tools were used: histology, Xpert, and culture. Measured by positive culture prevalence of TB was 35% compared to the median prevalence of 30% for 484 LN tissues tested in 10 studies[17]. In our cohort, using CRS, TB was diagnosed in 58.4% (228); among LNTB patients diagnosed, only one patient was HIV reactive, and 4.8% (11) AFB positive. Using the same diagnostic approaches, a higher proportion of TB cases were diagnosed among people investigated in other studies[28,29]. In a study from Ethiopia, LNTB was diagnosed in 80% of patients examined, and among LNTB cases, 9% were HIV positive, and 37% were AFB positive[28]. Similarly, in another study from Pakistan, LNTB was diagnosed in 74% of the people investigated, and 9%of the LNTB patients had a history of previous treatment, with 15% AFB positive[29]. As all three studies were conducted in tertiary care centers, there is a possibility that highly selected patients were being referred to these centers. In addition, a high HIV prevalence and history of previous TB treatment contributed to an even higher prevalence of TB in the study population.

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	LNTB patient diagnosed	Enrolled on Treatment	Treatment completed		Duration of treatment				OR	95%CI	P value
					6 months		>6 months				
	n	n	n	%	n	%	n	%			
All Patient	228	183	165	90%	121	73%	44	27%			
Gender											
Male	88	69	60	87%	48	80%	12	20%	Ref		
Female	140	114	105	92%	73	70%	32	30%	1.75	0.78–4.1	0.14
Age Group											
< 15yrs	64	49	46	94%	36	78%	10	22%	Ref		
>15yrs	222	179	161	90%	119	74%	42	26%	1.27	0.56–3.20	0.55
Local clinical Features											
LAP	209	167	152	91%	110	72%	42	28%	Ref		
LAP + Abscess/ sinus	19	16	13	81%	11	85%	2	15%	0.48	0.05–2.30	0.34
Duration of illness											
3 months or shorter	90	86	75	87%	52	69%	23	31%	Ref		
Longer than 3 months	77	76	72	95%	53	74%	19	26%	0.81	0.37–1.76	0.56
Culture Results											
Positive	137	109	100	92%	73	73%	27	27%	Ref		
Negative	89	74	65	88%	48	74%	17	26%	0.96	0.44–2.1	0.90
Xpert Results											
MTB-Detected	124	96	86	90%	64	74%	22	26%	Ref		
MTB-Not detected	99	84	77	92%	55	71%	22	29%	1.16	0.55–2.46	0.66
Bacteriology confirmed* LNTB											
HP Suggestive of TB	158	128	115	90%	88	77%	27	23%	Ref		
HP Not suggestive of TB	20	9	9	100%	4	44%	5	56%	4.07	0.80–21.80	0.034
Histopathology suggestive of LNTB											
B. Positive *	158	128	115	90%	88	77%	27	23%	Ref		
B.Negative	50	46	41	89%	29	71%	12	29%	1.35	0.55–3.2	0.46
Rifampicin sensitive LNTB											
Isoniazid Sensitive	109	92	84	91%	62	74%	22	26%	Ref		
Isoniazid Resistant	6	5	5	100%	2	40%	3	60%	4.23	0.44–52.7	0.10

Table 4. Diagnostic results and treatment outcomes and duration of first-line TB treatment of Tuberculous lymphadenitis patients enrolled at Gulab Devi Hospital. LAP: Lymphadenopathy, MTB: *Mycobacterium tuberculosis*, *Bacteriologically confirmed/B.Positive: Positive on Xpert and/or culture, B.Negative: MTB not detected by Xpert and culture.

The Xpert MTB/RIF performance in the diagnosis of EPTB in different studies has been evaluated in systematic reviews and meta-analyses[10–13,16,17]. We reported the Se, Sp, PPV, and NPV of Xpert against the culture of 62.4%, 83.3%, 66.9%, and 80.4%, respectively. Systematic review of eleven similar studies (n = 786 LN specimens) reported a pooled Se, Sp, PPV, NPV of 82.4%, 80.3%, 31.6% and 97.6%[17]. We reported a lower sensitivity and NPV and higher specificity and PPV than pooled performance. However, our study results were within the range for Se (50–100%) and Sp (0–100%) reported by individual studies. The lower sensitivity in our cohort was likely due to the exclusion of retreatment TB cases and low HIV prevalence, with the possibility that a higher proportion of study participants had a bacillary load in LN much below the detection limit of microscopy. The smear results in our cohort correlated well with the Xpert cycle threshold (Ct) value. With a Ct value of < 21 as the cutoff for AFB smear-positive, in our cohort, MTB detected by Xpert in 113 AFB smear-negative LN specimens, was low (Ct 22–28) or very low (Ct > 28) in quantity[30]. Despite fewer AFB positives specimens, compared to another study from Pakistan, we reported similar sensitivity (62 vs. 65) and specificity (83 vs. 80%) with a higher PPV (67 vs. 54%), most likely due to better performance of culture.

In our cohort, the Xpert improved bacteriological diagnoses of LNTB by eleven folds compared to microscopy (124 MTB + ve vs.11AFB + ve) but still could diagnose less than 60% of all TB cases. Xpert Ultra is expected to improve sensitivity in such paucibacillary specimens[31,32]. Ultra is reported to have higher sensitivity and lower specificity than Xpert, but the certainty of the evidence is low as the number of studies and patients tested are small[17].

Compared to other EPTB specimens, a higher heterogeneity in Xpert performance is reported in lymph node tissues[16,17]. Xpert positive results, which are culture-negative, are interpreted as false positive, and low specificity and PPV are often attributed to previous TB treatment[33]. For lymph node TB, concerns are raised about the accuracy of the culture results used as the reference standard[16,17]. To understand false-positive Xpert results in our cohort of new LNTB patients, we analyzed the performance of Xpert stratified by duration of illness. Overall performance was better with significantly higher Se (P = 0.012) and PPV (P = 0.028) in patients who presented

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early (Se-76%, PPV-82%) compared to those who presented after a delay of more than 3 month (Se = 52%, PPV = 61%). The host immune responses playing a role in the containment of the disease and the viability of MTB in patients ill for a longer period is the most likely explanation for the detection of MTB-DNA by Xpert, but failure to grow in culture. Future studies in pulmonary and extrapulmonary TB patients are needed to better understand this phenomenon. Besides delays in seeking treatment, other factors that may affect the culture results include variation in the quality and quantity of LN specimens processed for culture[16,17].

In our study cohort, histology consistent with TB was reported in 208, and necrosis was present in 98% of the LN tissue examined. Compared to our findings, necrosis was reported in 84–85% of histologically diagnosed TB cases in studies from Morocco and Peru[34,35]. In our study, *M. Tuberculosis* infection was confirmed in 75% by culture and/or Xpert, consistent with the results of the other two studies[34,35]. In contrast, four cases with non-necrotizing granuloma were all bacteriologically confirmed in our cohort. It has been reported that a significant proportion of necrotizing granulomas appear infectious with no obvious infectious etiology[36]. We studied treatment response and outcomes in LNTB patients with histopathology suggestive of TB; no difference was reported in treatment outcomes and duration between positive and negative bacteriology cases. In high TB and low HIV prevalent settings like Pakistan, with a high pre-test probability of TB, diagnosis can be made with certainty in tissues showing granulomatous inflammation even in the absence of positive bacteriology.

Among 178 bacteriological confirmed TB cases in our study cohort, histopathology was suggestive of TB in 89% (n = 158), consistent with the results of studies conducted in low HIV prevalent settings[29,34] but was higher compared to 79% in a study conducted in high HIV setting[35]. The human TB granuloma is the product of a robust cellular immune response to bacterial components. A diminished capacity to mount a CD4+ T-cell response correlates with a reduced granuloma-forming capacity[37]. Tuberculosis without tubercles has been reported in HIV-positive patients[38]. Among 20 LNTB cases in our study cohort, HIV status was known for six non-reactive patients. In Pakistan, HIV prevalence is low, but undernourishment is estimated to contribute to 20% of all TB cases[1]. It is plausible that underlying malnutrition was responsible for diminished CD4 + T-cell activity and the absence of granulomatous immune response in these cases. We studied the clinical response to TB treatment among B + ve LNTB patients; a significantly delayed response was observed in patients with non-TB specific morphology (OR:4.07), suggesting weak immune responses[37]. Other reasons that also need consideration for missing granulomatous inflammation in tissue sections include an error made in selecting tissue for histology sections or examining a single section, which is not a true representative of the diseased tissue[5,39].

In our cohort, the two rifampicin-resistant cases were not detected by Xpert, We also studied the treatment outcome of drug-resistant LNTB patients. Of two rifampicin-resistant patients, one was successfully treated with six-month standard FL-ATT, with a possibility that excision biopsy completed removed diseased lymph nodes, consistent with published evidence of the role of surgery in improving treatment outcomes of LNTB patients[40]. On the contrary, the delayed clinical response was reported in 3/5 INH-resistant TB patients (OR = 4.23), consistent with unfavourable treatment outcomes reported in INH-resistant PTB patients[41]. Xpert has an advantage over histopathology in detecting rifampicin resistance; However, in our cohort, drug resistance, including rifampicin resistance, was diagnosed by phenotypic DST. Culture will still be required in cases where Xpert fails to detect TB, drug resistance TB, or non-tuberculous mycobacteria need to be investigated.

The main shortcomings of our study are; first, it was a single-center study conducted in a specialized tertiary care setting; secondly, only excision biopsy specimens were tested. Therefore, our results and conclusion cannot be generalized to all settings and all types of LN specimens. Third, Xpert MTB/RIF was used with lower sensitivity than the next-generation Xpert Ultra. Fourth, the number of patients was small in some of the subgroups analyzed, and confidence intervals were wide, with a risk of imprecision in "Results". Fifth, clinical and radiological data were available for less than 80% of LNTB patients, which may have biased the analysis. Lastly, although 10% of the patients had a chest X-ray suggestive of TB, sputum was examined by AFB microscopy only, with the possibility of under-diagnosis of concomitant PTB.

Xpert has the advantage of rapid diagnosis of TB and resistance to rifampicin. Though culture is of value, long reporting time and limited cultural facilities make it a non-viable option for the majority of the patients in high burden countries. In our study cohort, molecular assay diagnosed 54% of the LNTB cases, consistent with findings from other studies[29,34,35]. With reliance only on Xpert, almost half of the TB cases are likely to be missed; Our finding reinforces the ISTC and the need for clinicians to adhere to standards for evaluating possible EPTB and testing of appropriate specimens for microbiology and histopathology examination[19,20]. In situations where the specimen is not tested, or the molecular assay fails to diagnose TB for any reason, the histopathology examination is of value in suggesting the diagnosis of LNTB. Recent advances to diagnose TB in formalin-fixed paraffin-embedded tissue by detecting TB-specific antigens[26,42] or MTB by Xpert are likely to improve bacteriological diagnosis of TB without the need for a separate specimen for molecular testing[43].

Adhering to ISTC would remain a challenge, especially for clinicians working outside tertiary care settings in resource-constrained high disease burden countries like Pakistan. There is a need to improve access to diagnostic services for EPTB specimens by capacity development of an intermediate tier by training clinicians on the use of simpler techniques like fine-needle aspiration and laboratory technicians on handling LN aspirate/biopsy specimen and testing EPTB specimens by Xpert, linking intermediated level with laboratories at a higher level and effective mechanism to transport biopsy specimen/cytology smears[2,27].

Conclusion

Diagnosis of EPTB is challenging because of the paucibacillary nature of the disease, the performance of all diagnostic tools currently available is not optimal and negative results don't rule out TB. For patients presumed to have LNTB, the biopsy specimen should be tested for microbiology and histopathology examinations. In

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countries like Pakistan with high TB and low HIV prevalence, diagnosis of more than 95% of LNTB cases is possible when Xpert testing is combined with histopathology examination, compared to less than 60% by Xpert alone.

Data availability
All relevant data is within the manuscript.

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Author contributions
S.T. and T.M.: Conception and design of the work. S.T., A.A., S.I., N.S.: Acquisition of data. S.T., F.M.K., T.M., L.S.; Analysis and interpretation of data. S.T. wrote the initial draft, T.M. and L.S. critically reviewed and revised the manuscript. All authors reviewed and approved the content in the submitted manuscript.

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Correspondence and requests for materials should be addressed to S.T.

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7.4.   Paper-4

PLOS ONE

Isoniazid resistance profile and associated levofloxacin and pyrazinamide resistance in rifampicin resistant and sensitive isolates/ from pulmonary and extrapulmonary tuberculosis patients in Pakistan: A laboratory based surveillance study 2015-19

Sabira Tahseen[1,2]*, Faisal Masood Khanzada[1], Alamdar Hussain Rizvi[1], Mahmood Qadir[1], Aisha Ghazal[1], Aurangzaib Quadir Baloch[3], Tehmina Mustafa[2,4]

1 National TB Reference Laboratory, National TB Control Program, Islamabad, Pakistan, 2 Centre for International Health, Department of Global Public Health and Primary Care, University of Bergen, Bergen, Norway, 3 National TB Control Program, Ministry of National Health Services Regulation and Coordination, Islamabad, Pakistan, 4 Department of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway

* sabira.tahseen@gmail.com

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Abstract

Background

Pakistan is among top five high burden countries for tuberculosis and drug resistant TB. Among rifampicin sensitive new pulmonary TB (PTB), prevalence of isoniazid resistance is 8.3% (95%CI: 7.0–10.7) and resistance to fluoroquinolone is higher (11·1%, 95%CI: 7·8–14·3) than isoniazid resistance.

Method

Five year retrospective data (2015–2019) of drug susceptibility testing (DST) for *Mycobacterium tuberculosis* isolates, performed using recommended phenotypic (pDST) and/or genotypic (gDST) methods was analyzed stratified by rifampicin results for isoniazid resistance profiles and associated levofloxacin and pyrazinamide resistance.

Findings

DST data was analyzed from 11045 TB patients. Isolates were tested using pDST (87%), gDST (92%) and both methods (79.5%). For both rifampicin and isoniazid, a significant difference ($P < .001$) was noted between resistance detected by pDST and gDST. Among isolates, tested by both methods (8787), 49% were resistant to rifampicin and 51.7% to isoniazid with discordance in resistant results of 15.8% for each, with 13.2% (570) of rifampicin resistance reported sensitive by pDST and 14.2% (660) of isoniazid resistance missed by gDST. Estimated isoniazid resistance among rifampicin sensitive new PTB, extrapulmonary TB and previously treated PTB was 9.8% (95%CI: 8.7–11.1), 6.8% (95%CI: 5.4–

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8.5) and 14.6% (95%CI: 11.8–17.9) respectively. Significant differences were reported between the genotypic profile of isoniazid resistance associated with rifampicin-resistant and sensitive isolates including detectable mutations (87% vs 71.6%), frequency of *inhA* *(7*.6% and 30.2%) *and katG* mutations (76.1% vs 41.2%) respectively. Among rifampicin resistant and sensitive isolates, a significantly higher level of resistance to levofloxacin and pyrazinamide was seen associated with isoniazid resistance.

Conclusion

There are risks and many challenges in implementing WHO recommended treatment for isoniazid resistant tuberculosis. The laboratory based surveillance can complement random surveys in country specific planning for TB diagnostics and appropriate treatment regimens.

Introduction

Isoniazid (INH) is one of the most important first-line medicines for the treatment of active tuberculosis (TB) with high bactericidal activity and a good safety profile [1–3]. Together with rifampicin (RMP), the two drugs represent the cornerstone of World health organization (WHO) recommended first-line treatment regimen used worldwide [4]. INH is also used in high dose in short course second line treatment regimens for drug resistant TB (DRTB) [5]. INH is critical not only for the treatment of active TB, but it is also highly effective in preventing disease and is the most commonly used medicine for latent TB infection [6]. INH resistance can thus undermine the effectiveness of treatment for both TB disease and infection.

Culture-based phenotypic testing is the current reference method for testing anti-TB medicines. It relies on testing at critical concentrations of drugs, that is, the lowest concentration of an anti-TB medicine that inhibits the in vitro growth of 99% of phenotypically wild-type strains of *Mycobacterium tuberculosis* (Mtb) [7]. In 2008, WHO endorsed the first genotypic drug susceptibility testing (DST) method for the rapid detection of multidrug-resistant (MDR) TB. INH resistance has been associated with multiple genes, most frequently *katG* and *inhA* [8–11]. The reported frequency of mutations varies in different geographical regions [11]. A number of studies have reported that the mutation at codon 315 in *katG* gene is often associated with a high level of INH resistance whereas mutations in *inhA* gene are associated with low-level resistance [8, 9].

Globally there are an estimated 10 million incident TB cases and among these, 7.0 million were notified in 2018. A high treatment success rates of at least 85% for new TB cases is regularly reported by all countries [7]. The estimated global prevalence of INH resistance among RMP sensitive new and previously treated TB (RsHr-TB) is 7.4% (95%CI: 6.5%–8.4%) and 11.4% (95%CI:9.4%–13.4%) respectively [12]. People infected with a TB strain that is resistant to INH, are reported to have a higher rate of unfavorable treatment outcomes with standard first line treatment [13].

In 2019, WHO issued guidelines recommending a modified 6-month treatment regimen containing RMP, ethambutol (EMB), pyrazinamide (PZA) and levofloxacin (LFX) for people with RsHr-TB. Exclusion of resistance to RMP is strongly recommended and empirical treatment is not advised. Fluoroquinolone (FQ) and PZA testing prior to treatment, is also advised where possible in order to prevent the acquisition of additional drug resistance. In addition, information on the specific INH mutations (*katG or inhA*) and overall host acetylator status at country or regional level are considered useful for regimen design [5].

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Pakistan is country in South Asia with 212 M population and is among top five high burden countries (HBC) for TB and drug resistant TB (DRTB) [7]. TB disease burden is estimated at 562K incident TB cases at 265 /100K population. DOTs is the official strategy for TB control in the country and six month treatment regimen containing RMP throughout was adopted starting from 2012. Treatment success rate is maintained above 90% for new TB patients. In 2018, 64% of the estimated TB cases were notified including 80% pulmonary TB (PTB) [7]. First population based drug resistance survey (DRS) for smear positive PTB patients was conducted in 2012–13 and prevalence of RMP resistance was estimated at 4.2% and MDR at 3.7% in new cases. Any resistance to INH not associated with RMP resistance (RsHr-TB) was reported in 8.3% (95%CI: 7.0–10.7) of new and 7.1% (95%CI: 4.0–11.4) in previously treated PTB patients [14]. Subsequently, DRS isolates were also tested for FQ and PZA resistance among RMP resistant and sensitive population as part of a multicounty surveillance project and FQ resistance was reported respectively in 21·8% (95%CI;13·1–30·5)and 11·1%(95%CI:7·8–14·3) and PZA resistance in 39.5% (30.1–48.9) and 0.5%(0.1–0.8) [14, 15]. However there is limited published data on molecular markers for INH resistance [12]. We analyzed routine laboratory data to study diagnostic and clinical implications of the prevalent phenotypic and genotypic profile of INH resistance and LFX and PZA resistance associated with INH resistance in RMP resistant (RrHr) and sensitive (RsHr) isolates from pulmonary and extrapulmonary TB (EPTB) patients.

Study setting, design and methodology

Study setting

In Pakistan, Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) testing services are decentralized, patient reported to have RMP resistance are referred to specialized DRTB treatment sites and specimens are than referred for DST. Specimen transport is well established between DRTB treatment sites and culture and DST laboratories. National TB Reference laboratory (NTRL) is located in Islamabad, the federal capital of the country and receive clinical specimens or culture isolates, referred routinely from treatment sites across Punjab province and three territories including Islamabad, Azad Jammu Kashmir and Gilgit Baltistan together covering more than 50% of the country population. NTRL offer diagnostic and DST services for patients already diagnosed as RMP resistant by Xpert MTB/RIF or who are at risk of drug resistance or presumed to have TB specially PTB in children and EPTB.

Study design

This is a retrospective five-year (January 2015-December 2019) laboratory based surveillance study. All confirmed Mtb isolates from patients having PTB or EPTB, tested either using phenotypic DST (pDST) and/or genotypic method (gDST) with DST results reported for both RMP and INH were included.

Laboratory methods

Throughout the study period, gDST for RMP, INH and FQ was performed by Line Probe assay(LPA), using GenoType MTBDRplus and MTBDRsl version 2.0 (Hain Lifescience, Nehren, Germany). MGIT 960 automated system (BD, Sparks, MD, USA) was used to perform pDST at recommended critical concentrations for RMP (1.0ug/ml), INH (0.1ug/ml), PZA (100ug/ml) and ofloxacin (2ug/ml) during 2015–17 and LFX (1.0ug/ml) during 2018–19 [16].

All clinical samples were processed for culture and pDST was performed for all confirmed Mtb isolates. LPA was introduced in late 2015 and gDST was increasingly performed directly

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on clinical samples from known RMP resistant patients. Culture isolates were used for gDST for patients having RMP resistance with invalid results on direct testing, known RMP sensitive or with unknown RMP status. Sequencing was not performed as facilities were not established.

All recommended quality control measures for DST were followed during study period including, testing of known sensitive and resistant control strains with each batch of DST [17] and regular participation in annual external quality assessments conducted by WHO collaborating center (Supra National TB reference laboratory, ITM, Antwerp, Belgium) with successful certification for first and second line DST.

Data management

Case based data was extracted from computerized laboratory information system of NTRL in CSV format. Data was then checked for duplications, for each patient only first pDST and /or gDST results reported either from same or paired samples were included. Duplicate or sequential DST results from same patient were excluded. After cleaning, all personal identifiers were removed before analysis.

Data analysis

Data were analyzed using STATA1 v13.1 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845, USA). Mean, median and quartiles were analyzed for quantitative variables and 95% confidence intervals for comparisons between groups. Two sample proportion test was used to analyze differences in proportions between groups. A p-value of <0.05 was considered statistically significant.

Study population was analyzed for demographic characteristics of patients, previous history of TB treatment, province of residence, referring health facilities and AFB smear results. All phenotypic and genotypic results were first analyzed independently for proportion of RMP and INH resistance. Further analysis was done on large subset of Mtb isolates tested by both DST methods. LPA results were interpreted based on recommended guidelines [18]. Results were compared for agreement and discordance between two DST methods for RMP and INH. For final interpretation, resistance conferring mutations to RMP and INH were considered to be true resistance, even if phenotypic testing showed susceptibility. Based on the final interpretations, prevalence of INH and RMP resistance, genetic profile of INH resistance and associated resistance to LFX and PZA was analyzed stratified by RMP result, disease site and previous history of TB treatment. Genetic profiles of INH resistant isolates was studied for the frequency of mutations known to confer resistance among samples displaying either phenotypic and/or genotypic resistance to INH. The INH resistant isolates were sorted into four groups: Group1: phenotypic resistant but genotypic wild type (WT), Group 2: isolates with *inhA* mutation/s only, Group 3: isolates with *katG* mutation only and Group 4: isolates with combined *katG* and *inhA* mutations. Annual trends were analyzed for RsHr-TB, genetic profile of INH resistance and associated FQ and PZA resistance.

Patient having no or unknown previous history of ATT were categorized as new and those with history of previous treatment or on treatment for more than a month as previously treated. For estimation of INH resistance among previously treated, data was analyzed only from those patient with history of WHO recommended TB treatment for new TB patients. Annual trend for INH resistance was analyzed for years with DST results available by both methods for more than 100 patients. For FQ resistance, all strains reported resistant to OFX (2015–17), LFX (2018–19) or showing FQ conferring mutations on LPA were considered LFX resistant. Results were compared with the prevalence estimates of resistance reported in DRS [14] and primary drug resistance in EPTB [19].

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Ethics statement

The study protocol was approved by IRB of Common unit for HIV/AIDS, TB and Malaria program, Islamabad, Pakistan. The antimicrobial resistance was analyzed in Mtb strains isolated routinely in the laboratory for diagnostic purposes. To maintain confidentiality of the patients, de-identified data was used for analysis.

Results

Study population

During the study period (January 2015 to December 2019), altogether 11,680 DSTs were reported, 635 were duplicate and/or sequential DST, which were excluded, and final analysis was performed on 11,045 DST results from same number of patients (Fig 1). Patients included were referred from 86 health facilities in 29 districts. Altogether 65.8% patients were referred by tertiary care, 30.2% by secondary and 3.9% by primary health care facilities and 83.8% of patient referral was from health facilities managing DRTB (S1 Table).

Median age of TB patients was 30 years (32 years for PTB and 23 years for EPTB), 7.5% were children (<15yrs), 48.8% females and 86% were resident of Punjab province. Of all patients, 87.3% presented with PTB, 56% of PTB and 8.3% of EPTB patients had a history of previous TB treatment. Among previously treated PTB, 44.8% had history of TB treatment regimen for new patients, 11.8% for retreatment and 22.4% for DRTB (S1 Table). Details of pulmonary and extrapulmonary specimens processed for testing is given in S2 Table.

Rifampicin and isoniazid resistance

Rifampicin and isoniazid resistance by DST methods. Of all 11045 TB patients, pDST results were available for 9620 including 14.1% (1352) EPTB, gDST for 10212 including 12.6% (1282) EPTB and both pDST and gDST results for 8787 patients including 14.1% (1236) EPTB. RMP and INH resistance detected by pDST and gDST is shown in Table 1 and annual trend in S3 Table. Among PTB isolates, a significant difference ($P < .001$) was seen between resistance detected by pDST and gDST for RMP (49.8 vs 56.4%) as well as INH (58.4 vs 49.2%) respectively but difference was not significant among EPTB isolates (Table 1).

Correlation between genotypic and phenotypic DST results. Of all isolates tested by both DST methods (n = 8787), an agreement in results was reported for both RMP and INH in 81% (7117), RMP in 92.3% (8109) and INH in 91.9% (8071) of isolates (Table 2; S3 and S4 Tables). A significant difference was seen between RMP resistance ($P < .001$) detected by pDST (42.5%, 95%CI; 41.5–43.5) and gDST (47.7%, 95%CI; 46.7–48.8) and INH resistance ($P < .001$) detected by pDST (51.1%, 95%CI; 50.0–52.1) and gDST (44.2%:95%CI 43.1–45.2). Compared to pDST proportion of INH resistance by gDST was significantly lower among RMP resistant (97.3 vs 83.1%) and RMP sensitive (16.9 vs 8.7%) isolates. Taking both DST results into account, altogether RMP resistance was detected in 4303 (49%, 95%CI; 47.9–50.0) isolates with 678 (15.8%) discordant results including 570(13.2%) reported sensitive by pDST and 108(2.5%) by gDST. INH resistance was detected in 4542(51.7%, 95%CI; 50.6–52.7) isolates with 716(15.8%) discordant result including 660(14.2%) reported sensitive by gDST and 56 (1.2%) by pDST. (Table 2; S4 Table).

Isoniazid resistance in rifampicin sensitive TB. Among RMP sensitive new PTB (n = 2489) and EPTB (n = 1058) patients, INH resistance (RsHr-TB) was reported in 9.8% (95%CI 8.7–11.1) and 6.8% (95%CI 5.4–8.5) respectively and 14.6% (95%11.8–17.9) among patients treated previously for new TB (n = 547) (Table 3). A stable annual trend of RsHr-TB was seen in new PTB and EPTB patients (Fig 2). A significant difference was reported in
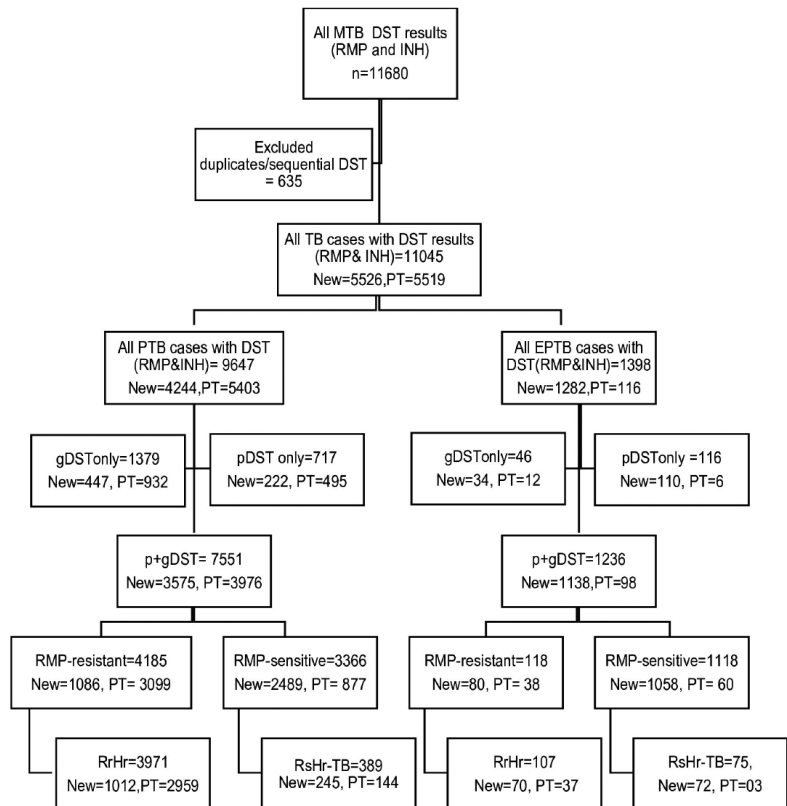
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Fig 1. Flow diagram showing drug susceptibility testing of *Mycobacterium tuberculosis* isolates by disease site, history of TB treatment and DST method, National TB reference laboratory, Pakistan 2015–2019. RMP-rifampicin, INH-isoniazid, R-resistant, S-sensitive, pDST-phenotypic drug susceptibility testing; gDST-Genotypic drug susceptibility testing, Rr-rifampicin resistant, Rs-rifampicin sensitive, Hr-isoniazid resistant, Hs-Isoniazid sensitive.

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proportion of RsHr-TB between new PTB and EPTB ($P = 0.004$) and new and previously treated PTB patients ($P<0.01$). Among new PTB, significant differences were not seen between children and adults, male and females and resident of Punjab and other regions (Table 3).

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Table 1. Isoniazid and rifampicin resistance detected by phenotypic and genotypic DST methods in Mtb isolates from pulmonary and extrapulmonary TB patients, National TB reference laboratory, Pakistan 2015–19.

Isolates tested	All TB (n = 11045)			Pulmonary TB (n = 9647)			Extrapulmonary TB (n = 1398)		
	pDST	gDST	p+gDST	pDST	gDST	p+gDST	pDST	gDST	p+gDST
	9620	10212	8787	8268	8930	7551	1352	1282	1236
Any rifampicin resistance									
RMP resistant-n	4225	5146	4303	4117	5027	4185	108	119	118
RMP-resistant-%	43.9%	50.4%	49.0%	49.8%	56.3%	55.4%	8.0%	9.3%	9.5%
(95%CI)	(42.9–44.9)	(49.4–51.4)	(47.9–50.0)	(48.7–50.9)	(55.2–57.3)	(54.3–56.5)	(6.6–9.6)	(7.7–11.0)	(8.0–11.3)
Any isoniazid resistance									
INH resistant-n	5025	4557	4542	4828	4393	4360	197	164	182
INH resistant-%	52.2%	44.6%	51.7%	58.4%	49.2%	57.7%	14.6%	12.8%	14.7%
(95%CI)	51.2–53.2	43.7–45.6	50.6–52.7	57.3–59.5	48.2–50.2	56.6–58.9	12.7–16.6	11.0–14.7	12.8–16.8
Isoniazid resistance associated with rifampicin resistance (MDR)									
RrHr/Rr-TB-n	4117/4225	4112/5146	4078/4303	4010/4117	4008/5027	3971/4185	107/108	104/119	107/118
RrHr/Rr-TB %	97.4%	79.9%	94.8%	97.4%	79.7%	94.9%	99.1%	87.4%	90.7%
(95%CI)	(96.9–97.9)	(78.8–81.0)	(94.1–95.4)	(96.0–97.9)	(78.6–80.8)	(94.2–95.5)	(94.9–100)	(80.0–92.8)	(83.9–95.2)
Isoniazid resistance associated with rifampicin susceptible (RsHr)									
RsHr/Rs-TB-n	908/5395	445/5066	464/4484	818/4151	385/3903	389/3366	90/1244	60/1193	75/1118
RrHr/Rs-TB-%	16.8%	8.8%	10.3%	19.7%	9.9%	11.6%	7.2%	5.2%	6.7%
(95%CI)	(15.8–17.9)	(8.0–9.6)	(9.5–11.3)	(18.5–20.9)	(8.9–10.8)	(10.5–12.7)	(5.9–8.8)	(1.7–6.8)	(5.4–7.2)

N = number isolates tested, n = number resistant, pDST = Phenotypic drug susceptibility testing, gDST = Genotypic drug susceptibility testing

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Molecular characterization of isoniazid resistant isolates

Among all RrHr-TB isolates, INH conferring mutations were detected in 87.1% (95%CI: 86.0–88.1) compared to 71.6% (95%CI: 67.2-75-6) in RsHr-TB ($P < .001$). A significant difference ($P < .001$) was also seen in frequency distribution for both *katG* (76.1% and 41.2%) and *inhA* (7.6% and 30,2%) mutations between RrHr-TB and RsHr-TB respectively (Table 4).

Table 2. Correlation between phenotypic and genotypic drug susceptibility testing results for rifampicin and isoniazid in Mtb isolates from all type TB patients, National TB reference Laboratory Pakistan 2015–19.

		All	Rifampicin phenotypic(MGIT960) and genotypic(LPA) DST results							
			pRgR	pRgS	pSgR	pSgS	pRgNA	pSgNA	pNAgR	pNAgS
Isoniazid phenotypic(MGIT960)and genotypic (LPA) DST results	All	11045	3625	108	570	4484	492	341	951	474
	pRgR	3826	3097	66	352	311				
	pRgS	660	431	38	59	132				
	pSgR	56	9		26	21				
	pSgS	4245	88	4	133	4020				
	pRgNA	539					485	54		
	pSgNA	294					7	287		
	pNAgR	675							628	47
	pNAgS	750							323	427
Isoniazid Resistant	RrHr%		97.6%	96.3%	76.7%		98.6%		66.0%	
	RsHr%					10.3%		15.8%		9.9%
	95%CI		97.0–98.0	90.8–99.0	73.0–80.0	9.5–11.3	97.1–99.4	12.1–20.2	62.9–69.0	7.4–13.0

p-phenotypic, g-genotypic, DST-drug susceptibility testing, S-sensitive, R-Resistant, NA-not available.

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Table 3. Isoniazid resistance among rifampicin sensitive new and previously treated pulmonary and extrapulmonary TB patients, national TB reference laboratory, Pakistan 2015–2019.

	Pulmonary TB			Extrapulmonary TB		
	New	PT*	p-value	New	PT*	p-value
All Patients	245/2489;9.8%	80/547;14.6%	<0.01	72/1058;6.8%	3/60;5.0%	0.588
	(8.7–11.1)	(11.8–17.9)		(5.4–8.5)	(1.0–13.9)	
Gender						
Male	119/1263;9.4%	45/290;15.5%	0.002	28/533;5.3%	1/31;3.2%	0.608
	(7.9–11.2)	(11.5–20.2)		(3.5–7.5)	(0.08–16.7)	
Female	126/1226;10.3%	35/257;13.6%	0.122	44/525;8.4%	2/29;6.9%	0.776
	(8.6–12.1)	(9.7–18.4)		(6.2–11.1)	(0.8–22.8)	
Age Group						
Children(<15yrs)	36/302;11.9%	7/31;22.6%	0.09	11/184;6.0%	0/6;0.0%	0.536
	(8.5–16.1)	(10.0–41.1)		(3.0–10.4)	(0.0–45.9)	
Adult	204/2147;9.5%	72/508;14.2%	0.002	61/869;7.0%	3/54;5.6%	0.694
	(8.3–10.8)	(11.3–17.5)		(5.4–8.9)	(1.1–15.4)	
Age NA	5/40;12.5%	1/8;12.5%		0/5;0.0%	0/0	
	(4.2–26.8)	(0.03–52.7)		0.00%	0.00%	
Place of Residence						
Punjab province	211/2041;10.3%	64/446;14.3%	0.015	61/871;7.0%	3/48;6.3%	0.853
	(9.1–11.7)	(11.2–18.0)		(5.4–8.9)	(1.3–17.2)	
Outside Punjab	34/448;7.6%	16/101;15.8%	0.01	11/187;5.9%	0/12;0.0%	0.387
	(5.3–10.4)	(9.3–24.4)		(3.0–10.3)		
AFB smear Results						
Positive	95/903;10.5%	65/385;16.9%	0.001	8/128;6.3%	0/17;0.0%	0.287
	(8.7–12.7)	(13.3–21.0)		(2.7–11.9)	(0.0–19.5)	
Negative	41/516;7.9%	7/86;8.1%	0.949	19/303;6.3%	2/34;5.9%	0.927
	(5.9–10.6)	(3.3–16.1)		(3.8–9.6)	(0.7–19.7)	
Not available	109/1070;10.2%	8/76;10.5%	0.934	45/627;7.2%	1/9;11.1%	
	(8.4–12.2)	(4.7–19.7)		(5.3–9.5)	(0.2–48.2)	

Number shown are number isoniazid resistant/ number rifampicin susceptible isolates tested, % isoniazid resistant (95%CI)

*PT; previously treated with treatment regimen recommended for new TB patients

Rifampicin resistant TB. Among 3971 RrHr-PTB isolates, 13.1% (519) were wild type (WT) on LPA. Genetic mutations associated with INH resistance were detected in *inhA* in 7.6% and *katG* in 76.1%, and combined *katG* and *inhA* mutations in 3.1%. Among 107 RrHr-EPTB isolates, 8.4% (9) were genotypic WT, mutations were detected in *inhA* in 10.3%, *katG* in 79.4%, and double mutation in 2 isolates (1.9%) only. A significant difference was not seen between PTB and EPTB (Table 4), new and previously treated (S5 Table) and annual trends (Fig 3; S6 Table).

Among all MDR isolates (PTB and EPTB), the most frequent mutation were reported in codon S315T1 (97.2%, 3028/3112) in *katG* gene and *C-15T* in *InhA* promoter region (92.3%, 287/311) (Table 4).

Rifampicin sensitive TB. Among 389 RsHr-PTB isolates, 29.6% (115) were genotypic WT, INH conferring mutations were detected in *inhA in* 29.6%, *katG* gene in 40.6% and double mutation in only one isolate. Among 75 RsHr-EPTB isolates, 22.7% (17) were WT on LPA, INH conferring mutations were detected in *inhA* in 33.3%, *katG* in 44%, with no double mutation. Significant differences were not noted between isolates from PTB and EPTB (Table 4),
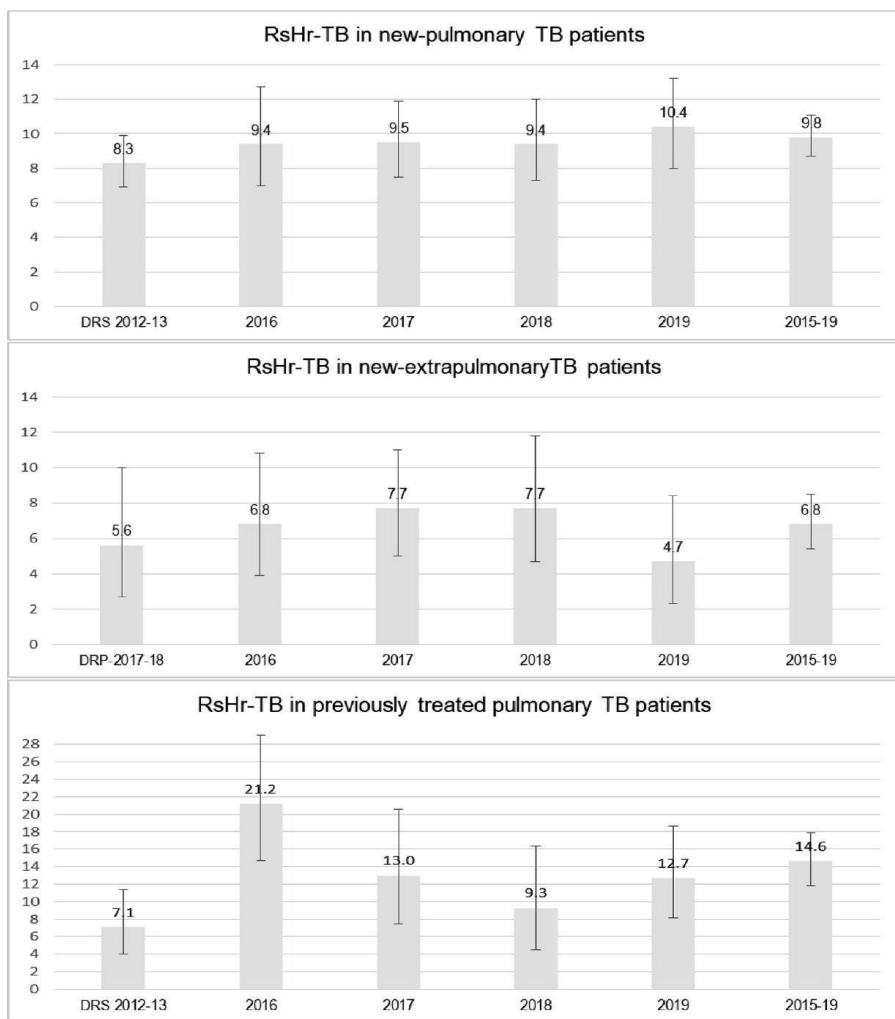
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Fig 2. Trend of isoniazid resistance in rifampicin sensitive new pulmonary TB, new extrapulmonary TB and previously treated pulmonary TB patients, National TB reference laboratory, Pakistan, 2015–19. RsHr; Rifampicin sensitive isoniazid resistant.

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Table 4. Molecular characterization of isoniazid resistance in rifampicin resistant and sensitive Mtb isolates from pulmonary and extrapulmonary TB patients, National TB reference laboratory Pakistan 2015–19.

	All-TB		RrHr-TB		RsHr-TB		RrHr-TB				RsHr-TB			
							PTB		EPTB		PTB		EPTB	
INH resistant isolates	N = 4542		N = 4078		N = 464		N = 3971		N = 107		N = 389		N = 75	
gWTpNWT	660	14.5%	528	12.9%	132	28.4%	519	13.1%	9	8.4%	115	29.6%	17	22.7%
Any Mutation	3882	85.5%	3550	87.1%	332	71.6%	3452	86.9%	98	91.6%	274	70.4%	58	77.3%
katG_S315T	3213	70.7%	3028	74.3%	185	39.9%	2943	74.1%	85	79.4%	153	39.3%	32	42.7%
katG_315	90	2.0%	84	2.1%	6	1.3%	84	2.1%		0.0%	5	1.3%	1	1.3%
inhA_c-15t	411	9.0%	287	7.0%	124	26.7%	277	7.0%	10	9.3%	101	26.0%	23	30.7%
inhA_-15	17	0.4%	8	0.2%	9	1.9%	8	0.2%		0.0%	9	2.3%		0.0%
inhA_t-8c	10	0.2%	8	0.2%	2	0.4%	8	0.2%		0.0%	1	0.3%	1	1.3%
inhA_-8	5	0.1%	3	0.1%	2	0.4%	2	0.1%	1	0.9%	2	0.5%		0.0%
inhA_t-8a	6	0.1%	3	0.1%	3	0.6%	3	0.1%		0.0%	2	0.5%	1	1.3%
inhA_-15,-8	1	0.0%	1	0.0%	0	0.0%	1	0.0%		0.0%		0.0%		0.0%
inhA_c-15t & t-8c	1	0.0%	1	0.0%	0	0.0%	1	0.0%		0.0%		0.0%		0.0%
katG_S315T inhA_c-15t	84	1.8%	84	2.1%	0	0.0%	84	2.1%		0.0%		0.0%		0.0%
katG_S315T inhA_t-8c	27	0.6%	27	0.7%	0	0.0%	25	0.6%	2	1.9%		0.0%		0.0%
katG_S315T inhA_t-8a	4	0.1%	4	0.1%	0	0.0%	4	0.1%		0.0%		0.0%		0.0%
katG_315 inhA_c-15t	6	0.1%	5	0.1%	1	0.2%	5	0.1%		0.0%	1	0.3%		0.0%
katG_S315T inhA_-15	3	0.1%	3	0.1%	0	0.0%	3	0.1%		0.0%		0.0%		0.0%
katG_S315T inhA_-8	3	0.1%	3	0.1%	0	0.0%	3	0.1%		0.0%		0.0%		0.0%
katG_S315T inhA_a-16g	1	0.0%	1	0.0%	0	0.0%	1	0.0%		0.0%		0.0%		0.0%
Summary														
gWTpNWT–n %	660	14.5%	528	12.9%	132	28.4%	519	13.1%	9	8.4%	115	29.6%	17	22.7%
(95%CI)		(13.5–15.6)		(11.9–14.0)		(24.4–32.8)		(12.0–14.2)		(3.9–15.4)		(25.1–34.4)		(13.8–33.8)
p-value			<0.01				0.153				0.225			
katG mutation-n %	3303	72.7%	3112	76.3%	191	41.2%	3027	76.2%	85	79.4%	158	40.6%	33	44.0%
(95%CI)		(71.4–74.0)		(75.0–77.6)		(36.6–45.8)		(74.9–77.5)		(70.5–86.6)		(35.7–45.7)		(35.5–55.9)
p-value			<0.01				0.443				0.584			
inhA mutation- n %	451	9.9%	311	7.6%	140	30.2%	300	7.6%	11	10.3%	115	29.6%	25	33.3%
95%CI		(9.1–10.8)		(6.8–8.5)		(26.0–34.4)		(6.8–8.4)		(5.2–17.7)		(25.1–34.3)		(22.9–45.2)
p-Value			<0.01				0.300				0.523			
Double Mutation- n %	128	2.8%	127	3.1%	1	0.2%	125	3.1%	2	1.9%	1	0.3%	0	0.0%
95%CI		(2.4–3.3)		(2.6–3.7)		(0.0–1.2)		(2.6–3.7)		(0.2–6.6)		(0.007–1.4)		(0.00–46.9)
p-value			0.187				0.478				0.129			

RrHr-Rifampicin resistant Isoniazid resistant, RsHr-Rifampicin sensitive Isoniazid resistant

new and previously treated (S5 Table) and annual trends. A more stable annual trend was seen for *katG* compared to *inhA* mutations. (Fig 3; S6 Table).

Associated levofloxacin and pyrazinamide resistance

Among RMP resistant PTB isolates, resistance to LFX and PZA was reported respectively in 47.7% (95%CI: 46.2–49.2) and 44.8% (95%CI: 43.2–46.4) compared to 14.4% (13.3–15.7) and 3.7%, (95%CI: 3.0–4.5) in RMP sensitive. Similar pattern was seen in EPTB (Table 5; S7 Table).

Rifampicin resistant TB. Among RrHr-PTB isolates(n = 3969), resistance to LFX, PZA and combined LFX and PZA was 49%, 47% and 27.9%, which was higher compared to
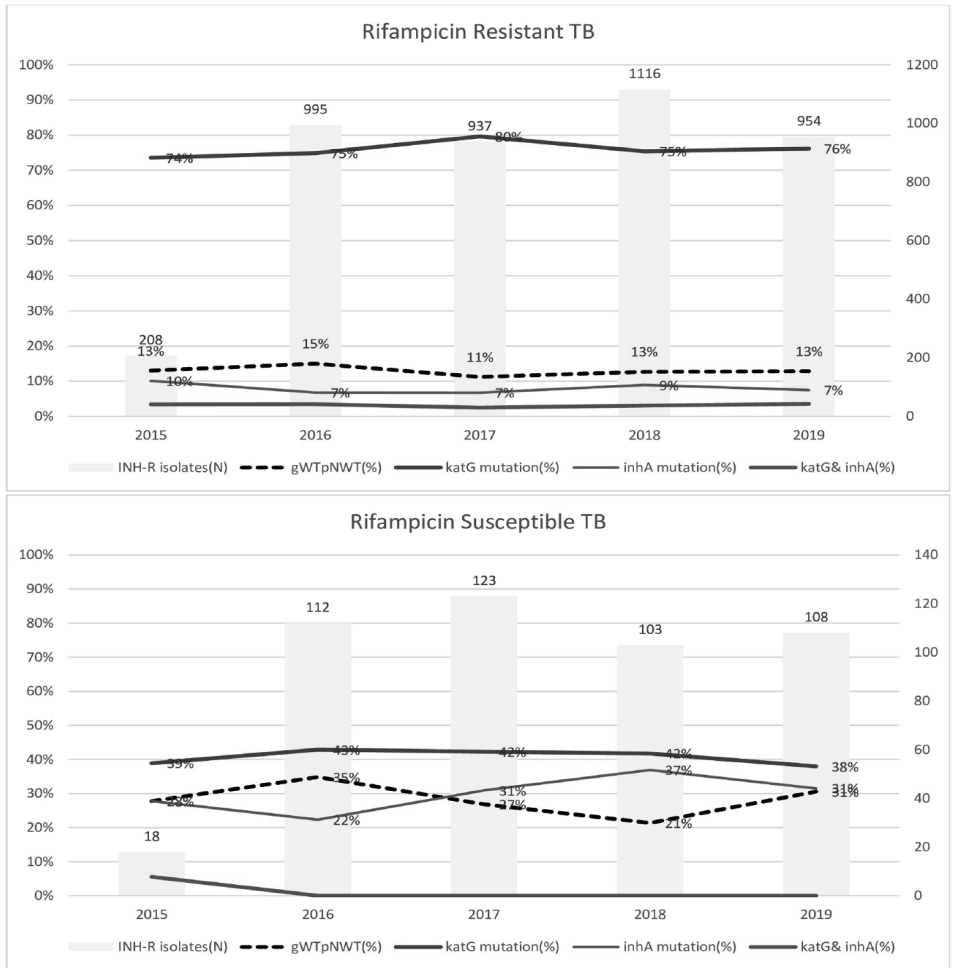
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Fig 3. Trend of isoniazid resistance profiles associated with rifampicin resistant and rifampicin sensitive isolates, National TB reference laboratory, Pakistan 2015–19.

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Table 5. Levofloxacin and pyrazinamide resistance associated with isoniazid resistance in rifampicin resistant and sensitive *Mycobacterium Tuberculosis* isolates from pulmonary and extrapulmonary Tuberculosis patients, National TB reference laboratory 2015–2019.

Isoniazid resistance profile	Rifampicin Resistant TB					Rifampicin Sensitive TB				
	Pulmonary TB		Extrapulmonary TB		p-value	Pulmonary TB		Extrapulmonary TB		p-value
	n/N	%(95%CI)	n/N	%(95% CI)		n/N	%(95% CI)	n/N	%(95% CI)	
Levofloxacin Resistance										
All Isolates	1997/ 4183	47.7 (46.2– 49.2)	53/ 118	44.9 (35.7– 54.3)	0.548	486/ 3365	14.4 (13.3– 15.7)	80/ 1117	7.2 (5.7– 8.8)	<**0.001**
H sensitive (gWTpWT)	51/214	23.8 (18.3– 30.1)	1/11	9.1(0.2– 41.3)	0.259	388/ 2976	13.0 (11.8– 14.3)	75/ 1042	7.2 (5.7– 8.9)	<**0.001**
H Resistant-All	1946/ 3969	49.0 (47.5– 50.6)	52/ 107	48.6 (38.8– 58.5)	0.935	98/ 389	25.2 (21.0– 29.8)	5/75	6.7(2.3– 14.9)	<**0.001**
▪ gWTpNWT	213/ 518	41.1 (36.8– 45.4)	5/9	55.6 (21.2– 86.3)	0.381	38/ 115	33.0 (24.6– 42.4)	1/17	5.9(0.01– 28.7)	0.002
▪ *inhA* mutation	138/ 300	46.0 (40.3– 51.8)	2/11	18.2(2.3– 51.8)	0.069	24/ 115	20.9 (13.9– 29.4)	1/25	4.0(0.1– 20.4)	0.046
▪ *katG* mutation	1504/ 3026	49.7 (47.9– 51.5)	44/ 85	51.8 (40.7– 62.7)	0.703	36/ 158	22.8 (16.5– 30.1)	3/33	9.1(1.9– 24.3)	0.076
▪ **Double Mutation**	**91/125**	**72.8 (64.1– 80.4)**	**1/2**	**50.0 (1.2– 98.7)**	0.474	0/1	0.0(0.0– 97.5)	0/0		NA
Pyrazinamide Resistance										
All Isolates	1655/ 3696	44.8 (43.2– 46.4)	58/ 104	55.8 (45.7– 65.5)	0.026	114/ 3048	3.7(3.0– 4.5)	29/ 1019	2.8 (1.9– 4.1)	0.174
H sensitive (gWTpWT)	13/202	6.4(3.5– 10.8)	0/10	0.0 (0.0– 30.8)	0.406	72/ 2704	2.7 (2.1– 3.3)	23/ 951	2.4 (1.5– 3.6)	0.618
H Resistant- All	1642/ 3494	47.0 (45.3– 48.7)	58/ 94	61.7 (51.1– 71.5)	0.005	42/ 344	12.2(8.9– 16.1)	6/68	8.8 (3.3– 18.2)	0.424
▪ gWTpNWT	133/ 445	29.9 (25.7– 34.4)	2/6	33.3(4.3– 77.7)	0.857	16/99	16.2(9.5– 24.9)	1/15	6.7 (0.2– 31.9)	0.336
▪ *inhA* mutation	84/266	31.6 (26.0– 37.5)	4/10	40.0 (12.2– 73.8)	0.576	5/105	4.8(1.6– 10.8)	5/24	20.8(7.1– 42.2)	0.008
▪ *katG* mutation	1354/ 2676	50.6 (48.7– 52.5)	51/ 76	67.1 (55.4– 77.5)	0.005	21/ 139	15.1(9.6– 22.1)	0/29	0.0 (0.0)	0.025
▪ **Double Mutation**	**71/107**	**66.4 (56.6– 75.2)**	1/2	50.0(1.3– 98.7)	0.672	0/1	0.0(0.00– 97.5)	0/0		NA
Combined Levofloxacin and Pyrazinamide Resistance										
All Isolates	981/ 3696	26.5 (25.1– 28.0)	31/ 104	29.8 (21.2– 39.6)	0.453	31/ 3047	1.0(0.6– 1.4)	2/ 1018	0.2(0.03– 0.80)	0.013
H sensitive (gWTpWT)	6/202	3.0(0.01– 6.4)	0/10	0.0(0.0– 30.8)	0.578	9/ 2703	0.3(0.2– 0.6)	2/950	0.2(0.02– 0.8)	<**0.001**

(*Continued*)

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Table 5. (Continued)

Isoniazid resistance profile	Rifampicin Resistant TB				Rifampicin Sensitive TB			
	Pulmonary TB		Extrapulmonary TB	p-value	Pulmonary TB		Extrapulmonary TB	p-value
	n/N	%(95%CI)	n/N	%(95% CI)		n/N	%(95% CI)	n/N	%(95% CI)	
H Resistant- All	975/ 3494	27.9 (26.4– 29.4)	31/ 94	33.0 (23.6– 43.4)	0.277	22/ 344	6.4(4.1– 1.0)	0/68	0.0(0.0)	0.032
▪ gWTpNWT	56/445	12.6(9.6– 16.3)	1/6	16.7 (0.4– 64.1)	0.764	10/99	10.1(5.0– 17.8)	0/15	0.0 (0.0)	0.198
▪ inhA mutation	55/266	20.7 (16.0– 26.0)	0/10	0.0(0.0– 30.8)	0.108	1/105	1.0(0.02– 5.1)	0/24	0.0(0.0)	0.623
▪ katG mutation	805/ 2676	30.1 (28.3– 31.9)	29/ 76	38.2 (27.2– 50.0)	0.130	11/ 139	7.9(4.3– 13.7)	0/29	0.0 (0.0)	0.117
▪ Double Mutation	59/107	55.1 (45.2– 64.8)	1/2	50.0 (12.6– 98.7)	0.886	0/1	0.0(0.0– 97.5)	0/0		NA

n-number of clinical isolates resistant to Levofloxacin and/or Pyrazinamide, N-Number of Isolates tested, WT-wild type, g-genotypic, p-phenotypic

reported resistance in RrHs-TB (n = 214) of 23.8%, 6.4% and 3.0%. Similarly among RrHr-EPTB (n = 107) reported resistance of 48.6%, 61.7% and 33.0% was higher compared to 9.1%, 0% and 0% in RrHs-TB (n = 11) (Table 5).Within RrHr-TB, significant difference in resistance was not seen between PTB and EPTB (*P* = .08) (Table 5), new and previously treated TB (*P* = .956) (S7 Table) and annual trends (S8 Table).

Rifampicin sensitive TB. Among RsHr-PTB(n = 389), resistance to LFX, PZA and combined LFX and PZA, isolates was reported in 25.2%, 12.2% and 6.4% respectively compared to 13.0%, 3.7% and 1.0 in RsHs-PTB(n = 2976). Among RsHr-EPTB (N = 75)isolates, resistant was reported respectively in 6.7%, 8.8% and 0% compared to 7.2%, 2.8% and 0.2% in RsHs-EPTB (N = 1042).No significant changes were see in the annual trend of resistance to LFX and PZA. (S8 Table).

Isoniazid conferring mutations and Levofloxacin and pyrazinamide resistance. Among RrHr-PTB isolates, a relative higher resistance to LFX was seen in isolates with double mutations and relative higher resistance to PZA and combined LFX and PZA resistance was seen associated with *katG* and double mutations. (Table 5; S7 Table; S1 Fig).

Discussion

We analyzed a large DST data set from 11045 patients registered during 2015–2019 in NTRL, Pakistan. During the study period, pDST was performed on 87%, gDST on 92% and 79.5% of isolates were tested by both methods. We analyzed results of 8787 Mtb isolates including 1236 from EPTB patients, tested by both phenotypic and genotypic methods. Key findings of our analysis include significant difference in resistance detected by WHO recommended pDST (MGIT 960) and gDST (LPA) methods for both RMP and INH, a significantly lower sensitivity of gDST to detect INH resistance in RMP sensitive TB, difference in genetic profiles of INH resistance associated with RMP resistant and sensitive TB, a higher level of LFX and PZA resistance associated with INH resistance in RMP resistant and sensitive population. Additionally

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we noted a stable annual trend of INH resistance in RMP sensitive new TB patients, INH resistance profiles, associated FQ and PZA resistance and INH resistance missed by gDST method.

We reported, an INH resistance of 9.8% (95%CI; 8.7–11.1) in new and 14.6% (95%CI: 11.8–17.9) in previously treated RMP sensitive PTB patients. Among new PTB, a higher point estimates compared to the estimates of DRS (8.3%, 95%CI: 7.0–10.7) conducted in 2013 [14] can be explained based on the possibility of a selected higher risk individuals among new RMP sensitive patients referred for testing in routine settings. However a stable RsHr resistance trend was seen during the study period with an insignificant increase only in 2019. Among previously treated, compared to DRS, the proportion of RsHr-TB was significantly higher but fluctuations were seen in annual trends. Fluctuation in resistance was most likely due to variation in proportion of relapse vs failures of previous treatment among patient tested. However, a higher RsHr reported among previously treated was consistent with findings of other similar studies from Pakistan [20–22]. Among children with PTB (n = 302), estimated RsHr-TB of 11.9%(8.5–16.1), was higher but not statistically different from adults(p = .189) and was consistent with the global estimates of 12.1% (95%CI:9.8% to 14.8%) among all childhood TB cases [23]. However prevalence estimates in children can also be argued as not being a true representative of childhood TB population as most likely specimens from sicker children having access to specialized TB care and diagnostic facilities were tested. Among RMP sensitive new EPTB patient (n = 1058), INH resistance of 6.8% (95%CI; 5.4–8.5) was significantly lower compared to PTB ($P < .001$) in same study population but was consistent with primary drug resistance (5.6%, 95CI; 2.7–10.0) reported in EPTB [19] and PTB in Pakistan [14, 20, 24]. Most of the EPTB patients in contrast to PTB, were referred for diagnosis of TB and only a few had history of previous TB treatment (<10%), with possibility of these estimates being a true reflection of RsHr in EPTB at population level.

Among Mtb isolates tested by both methods (n = 8787), discordance in RMP results was reported in 7.7% of all and 15.8% (678/4303) of RMP resistant isolates. Discordance in RMP results was also reported previously in random population-based DRS (2013) in which all samples were tested in parallel using Xpert MTB/Rif and Lowenstein Jensen (LJ) media and sequencing was performed to confirm and resolve discordance in RMP results [14]. However in DRS, discordance of 11.7% was reported among all RMP resistant isolates, including 4% (4/104) missed on pDST and 7.7% (8/104) by gDST. Contrary to DRS, in this study 13.2% (n = 570) were missed by MGIT (pDST) and 2.5% (n = 108) by LPA (gDST). The most plausible explanation for a higher number of observed missed RMP resistance by pDST (13.2% vs 4%) in study sample was due to use of automated liquid DST (MGIT), which is known to miss higher proportion of RMP resistant cases compared to LJ media [25, 26]. On the other hand the lower proportion of RMP resistant cases missed by gDST (7.7% vs 2.5%) was most likely an effect of the current diagnostic algorithm followed in programme settings in which patients reported RMP sensitive are initiated on standard first line treatment and are not investigated further unless they fail to respond or fail treatment.

Recently conducted systematic review and meta-analysis, reported significant higher failure rate among INH resistant compared to susceptible TB patients when treated with standard first-line drugs regimens [13]. Limitations of currently available pDST and gDST in detecting RMP resistance are well recognized [25–27]. Selection of either one of the two DST method for routine practice is likely to result in important diagnostic and clinical implications. In our study population, without genotypic DST, 10.1% (411/4078) of the MDR would have been reported as RsHr-TB and treatment in these instances with standard first line treatment regimen would likely have resulted in suboptimal treatment outcomes. In a recently published study, a non-negligible extent of misclassifying MDR-TB as INH-resistant TB is demonstrated and impact of treating patients with missed RMP resistance for RsHr-TB with WHO

recommended FQ containing regimen is strongly argued [27]. In another study from South Africa 15% of INH resistant isolates initially tested negative for RMP resistance by all three WHO-endorsed commercial tests were reclassified as MDR on identification of resistance conferring mutation (*rpoB* Ile491phe) using deep sequencing [28].

In our data set, contrary to RMP, a higher proportion of INH resistance (14.5%; n = 660) was missed by gDST. Our finding are consistent with the results of a recent study from eastern DRC, in which INH resistance was reported in only 55% of the RMP resistant cases detected by Xpert MTB/RIF on subsequent testing by LPA, raising an argument on use of RMP resistance as surrogate marker for MDR [29].

We studied 4542 INH resistant isolates for molecular markers and mutations causing INH resistance were identified in 85.5% with frequency of *katG*, *inhA* and combined *katG and inhA* mutations in 72.7%, 9.9% and 2.8% respectively. Our findings are consistent with the published data [11, 12, 30] but with a lower proportion of combined *katG* and *inhA* mutations in our population [12, 31]. INH resistance profiles when studied, stratified by RMP results, significant differences were reported between RrHr-TB (n = 4078) and RsHr-TB(n = 464) with regard to proportion of INH conferring mutation detected (87.1% vs 71.6%) and frequency of the mutations in *inhA* (7.6 vs 30.2%), *katG* (76.3 vs 41.2%) and combined *inhA* and *katG* (3.1% vs 0.2%). The distribution of INH resistance mutations among RsHr-TB is less well mapped globally, however our findings are consistent with the estimates from an international collection of over 5,000 strains with reported frequency of S315T *katG* in 88.7% of MDR and 61.3% of RsHr-TB showing substantially higher representation of *katG* among MDR strains [32].

In our study population of RsHr-TB, 41% had mutation in *katG*, 30% in *inhA* and only one isolates had combined mutation. Published data on the influence of genotype on treatment outcomes of RsHr-TB are conflicting, study from Vietnam, suggest that *katG* mutations and not *inhA* are associated with unfavorable treatment outcomes [33]. Whereas in a study from South Africa, no evidence was found suggesting that specific isoniazid resistance conferring mutations are associated with poor treatment outcomes, and results showed that patients with *katG* mutations had greater odds of successful outcome when treated with high-dose isoniazid compared to those who received standard dose [34].Few studies have also evaluated the effectiveness of high-dose isoniazid in patients with DRTB and limited data available suggest clinical benefit without a higher toxicity [35, 36]. A recent study has demonstrated that high dose INH in MDR patients with *inhA* mutations has similar magnitude of bactericidal activity as with standard doses in drug-susceptible TB patients [37].

In Pakistan, one of the key challenge for treating MDR-TB and RsHr-TB is high FQ resistance. We reported a higher FQ resistance in RMP resistant and sensitive isolates compared to population level resistance [12, 15] but was consistent with previous laboratory based study from Pakistan [38]. In RMP resistant and sensitive population, significantly higher resistance to LFX and PZA was noted associated with INH resistance and was statistically higher for all INH resistance profiles. However a relative higher resistance was seen associated with combined *katG* and *inhA* mutations, consistent with findings of a recent study [31]. A high LFX resistance and lower frequency of *katG* mutations in RsHr-TB in our study population imply consideration for high dose INH rather than LFX as a treatment option.

The End TB Strategy released in 2015 calls for the early diagnosis of TB including universal DST [39]. Xpert MTB/RIF assay was endorsed by WHO in 2010, however even in 2018, among bacteriologically confirmed TB patients, only 51% globally and 45% in Pakistan were tested for RMP resistance [7]. Diagnostic and operational challenges to implement universal INH testing and treatment for INH resistant TB, are more complex, because of the larger population of RMP sensitive patients and complex laboratory capacity required for testing in the

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absence of a rapid and convenient diagnostic platform for detection of INH resistance and challenges faced in decentralization of LPA in most of HBC settings. Additionally in Pakistan country context, even with systematic testing, 30 percent of the INH resistance are likely to be missed if tested by LPA only, second, there is lack of laboratory capacity to systematically exclude RMP resistance which are missed by Xpert MTB/Rif, third, high LFX resistance makes FQ testing mandatory for all and lastly, FQ resistance in a substantial number of RsHr-TB patients will render them ineligible for recommended treatment.

Currently HBC are struggling to develop capacity for comprehensive second line DST including new and repurpose drugs [5] and to establish sequencing capacity to diagnose RMP and other drug resistance not detected by routine tests. Plans to implement universal testing and treatment for INH resistant TB with existing capacity are likely to overwhelm laboratory systems at the cost of compromising services for RMP resistant TB patients. A new GeneXpert cartridge for testing INH and FQ resistance is expected to be available in near future [40, 41], however countries will need to invest in procurement of next generation modules and to make it available at same level of health care as RMP testing for detection of INH and FQ resistance in RMP sensitive TB patients in parallel with plans to implement treatment for INH resistance. In addition performance evaluation of new diagnostic tests in both RMP resistant and sensitive population in different geographical settings also needs consideration.

The value of any such laboratory study would be greatly increased if treatment outcomes could be linked to the resistance profile. Studies are also needed to fill knowledge gap in isoniazid acetylator status of the population in HBC and to evaluate effectiveness, optimal dosing and potential toxicity of high-dose isoniazid to offer simple treatment options applicable in program settings.

We studied retrospective data and possibility of errors in routinely collected patient information cannot be excluded. Furthermore findings of this study cannot be generalized to the population level. However laboratory based surveillance can complement random survey as analysis provides information on drug resistance pattern in a large dataset of DRTB patients at time of treatment initiation and can guide in country specific planning for TB diagnostics, diagnostic algorithm and appropriate treatment regimens for drug resistance in TB patients.

Supporting information

S1 Table. Demographic and clinical characteristics of pulmonary and extrapulmonary tuberculosis patients tested for drug susceptibility at national TB reference laboratory Pakistan, 2015–2019.
(PDF)

S2 Table. Specimen types from pulmonary and extrapulmonary tuberculosis patients processed for drug susceptibility testing, national TB reference laboratory Pakistan, 2015–19.
(PDF)

S3 Table. Annual trend and difference in isoniazid and rifampicin resistance detected by phenotypic and genotypic DST methods, in pulmonary and extrapulmonary tuberculosis patients, national TB reference laboratory Pakistan 2015–19. R-rifampicin, H-isoniazid, r-resistant, s-susceptible, DST-drug susceptibility testing, p-phenotypic; g-genotypic.
(PDF)

S4 Table. Rifampicin and isoniazid resistance trend and correlation between phenotypic and genotypic drug susceptibility testing, national TB reference Laboratory, Pakistan 2015–2019. RMP-rifampicin, INH-isoniazid, R-resistant, S-sensitive, pDST-phenotypic, drug

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susceptibility testing; gDST-Genotypic drug susceptibility testing.
(PDF)

S5 Table. Molecular characterization of isoniazid resistance in Mtb isolates from pulmonary and extrapulmonary TB patients, stratified by rifampicin resistance and history of TB treatment, national TB reference laboratory, Pakistan 2015–2019. INH-isoniazid.
(PDF)

S6 Table. Annual trend of genotypic profiles of isoniazid resistance associated with rifampicin resistant and sensitive Mtb isolates from new and previously treated TB patients, national TB reference laboratory, Pakistan, 2015–19. RMP-rifampicin, INH-isoniazid, PT-Previously treated.
(PDF)

S7 Table. Levofloxacin and pyrazinamide resistance associated with isoniazid resistance in rifampicin resistant and sensitive Mtb isolates from pulmonary and extra pulmonary TB patient, stratified by history of TB treatment, national TB reference laboratory Pakistan, 2015–19. n-Number of isolates resistant to Levofloxacin and/or Pyrazinamide, N- Number of isolates tested INH-isoniazid.
(PDF)

S8 Table. Annual trend of levofloxacin and pyrazinamide resistance associated with isoniazid resistance in rifampicin resistant and sensitive Mtb isolates from pulmonary and extrapulmonary TB patients, national TB reference laboratory Pakistan, 2015–19.
LFX-Levofloxacin, PZA-Pyrazinamide, n = Number of isolates resistant, N = Number of isolates tested.
(PDF)

S1 Fig. Levofloxacin, pyrazinamide and combined levofloxacin and pyrazinamide resistance associated with isoniazid sensitive, isoniazid resistant and specific isoniazid conferring mutations in rifampicin resistant and sensitive isolates from pulmonary and extrapulmonary TB patients. National TB reference Laboratory, Pakistan 2015–2019. Rr-Rifampicin resistant, Rs-Rifampicin sensitive, Hr-Isoniazid resistance, Hs-Isoniazid sensitive, isoniazid sensitive.
(TIF)

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Author Contributions

Conceptualization: Sabira Tahseen, Faisal Masood Khanzada, Aurangzaib Quadir Baloch, Tehmina Mustafa.

Data curation: Sabira Tahseen, Faisal Masood Khanzada.

Formal analysis: Sabira Tahseen, Faisal Masood Khanzada, Tehmina Mustafa.

Investigation: Faisal Masood Khanzada, Alamdar Hussain Rizvi, Mahmood Qadir, Aisha Ghazal.

Methodology: Sabira Tahseen, Faisal Masood Khanzada, Tehmina Mustafa.

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Project administration: Sabira Tahseen.

Resources: Aurangzaib Quadir Baloch.

Supervision: Sabira Tahseen, Faisal Masood Khanzada, Alamdar Hussain Rizvi, Tehmina Mustafa.

Validation: Sabira Tahseen, Faisal Masood Khanzada.

Visualization: Sabira Tahseen.

Writing – original draft: Sabira Tahseen.

Writing – review & editing: Sabira Tahseen, Faisal Masood Khanzada, Alamdar Hussain Rizvi, Mahmood Qadir, Aisha Ghazal, Aurangzaib Quadir Baloch, Tehmina Mustafa.

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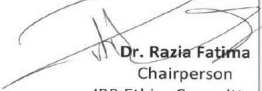
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8. APPENDICES

8.1.   Appendix-1: Ethical approval retrospective study

Ethics Committee CMU (TB, Malaria and HIV/AIDS)

Ministry of National Health Services, Regulations and Coordination, Islamabad, Pakistan

F. No. IRB - CMU - 2019 - 07

July 05 , 2019

Dr. Sabira Tahseen
NTA, NRL
Islamabad.

Subject: **CMU-ERC-07: DEMOGRAPHIC AND CLINICAL CHARECTERISTIC OF PATIENTS WITH EXTRA PULMONARY TUBERCULOSIS IN PAKISTAN:**

Dr. Sabira Tahseen,

I am pleased to inform you that the above mentioned project has been cleared by Institutional Review Board (IRB) Ethics Committee, Common Unit (HTM), Islamabad, Pakistan.

Kindly keep the IRB Ethics Committee updated with the progress of the project and submit the formal final report on completion

Yours Sincerely,

Dr. Razia Fatima
Chairperson
IRB Ethics Committee

8.2. Appendix-2: Ethical approval prospective research project (REK)

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK vest	Trine Anikken Larsen	55978497	15.01.2015	2014/46/REK vest
			Deres dato:	**Deres referanse:**
			27.12.2014	

Vår referanse må oppgis ved alle henvendelser

Tehmina Mustafa
Lungeavdelingen/ Institutt for global helse og samfunnsmedisin

2014/46 Forbedring av tuberkulosediagnostikk

Forskningsansvarlig: Helse Bergen HF
Prosjektleder: Tehmina Mustafa

Vi viser til søknad om prosjektendring datert 27.12.2014 for ovennevnte forskningsprosjekt. Søknaden er behandlet av leder for REK vest på fullmakt, med hjemmel i helseforskningsloven § 11.

Vurdering

Omsøkt endring

Det skal inkluderes nye medarbeidere i prosjektet.

Prosjektleder ønsker å inkludere flere deltakere i studien.

Vurdering

Det skal inkluderes flere prosjektmedarbeidere i studien.

Gjennomføringen av den diagnostiske testen for ekstrapulmonal tuberkulose skal utføres i ytterligere tre land. Dette gjelder Pakistan, India og Pakistan. Det samme samtykkeskrivet som er brukt i Zanzibar, skal også brukes ved innhenting av samtykke i disse landene. I motsetning til Zanzibar, skal det ikke samles inn biologisk materiale. Forskergruppen skal også innhente etisk godkjenning i de respektive landene.

REK vest har ingen innvendinger til omsøkt endring og godkjenner prosjektendringen.

REK vest vil imidlertid gjøre oppmerksom på at endringssøknad skal som hovedregel utformes på norsk. Dersom prosjektet i hovedsak skal utføres i utlandet, kan søknaden utformes på engelsk, jf. forskrift om organisering av medisinsk og helsefaglig forskning § 7. I dette prosjektet foregår en del av prosjektet i utlandet, mens en annen del foregår i Norge. I utgangspunktet skulle endringssøknaden vært utformet på norsk. REK vest godkjenner likevel i dette tilfellet at endringssøknaden er utformet på engelsk, men legger samtidig til grunn at fremtidige endringssøknader i dette prosjektet skal utformes på norsk.

Vedtak
REK vest godkjenner prosjektendringen i samsvar med forelagt søknad.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK vest. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK vest, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.
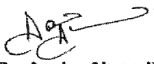
Med vennlig hilsen

Ansgar Berg
Prof. Dr.med
komitéleder

Trine Anikken Larsen
førstekonsulent

Kopi til: *postmottak@helse-bergen.no*

8.3. Appendix-3: Ethical approval prospective research project (NBC)

National Bioethics Committee (NBC) Pakistan

Ref: No.4-87/16/NBC-198/RDC/ 2 922   Date: 08 March, 2015

Patron
Minister of State, Ministry of
National Health Services Regulations
and Coordination

Chairperson
Secretary, Ministry of NHSR&C,
Government of Pakistan

Vice Chairperson,
Director General, Ministry of
NHSR&C, Government of Pakistan

Secretariat
Pakistan Medical Research Council

Members Ex-Officio

President, College of Physicians and
Surgeons of Pakistan

President, Pakistan Medical and
Dental Council, President

President, Pakistan Association of
Family Physicians

Executive Director, Pakistan
Medical Research Council ,
Member/Secretary

WHO Country Representative

President, Supreme Court Bar
Association

Surgeon General /DGMS (IS)
Pakistan Army

Director General Health, Punjab

Director General Health, Sindh

Director General Health, Khyber
Pakhtun Khwa

Director Health Services, FATA

Director General Health,
Balochistan

Director General Health, AJK

Director Health Services,   Gilgit
Baltistan

Registrar, Pakistan Nursing Council

Members

Prof. Dr. Farhat Moazam
(Chairperson HCEC)

Prof. Dr. Aasim Ahmad
(Chairman REC)

Prof. Dr Munir Akhtar Saleemi
Prof. Dr Abdul Razzaq Sabir
Dr Aamir Mustafa Jafarey
Dr Asmatullah
Dr Farah Qadir
Dr. Salman Ahmed Tipu
Dr. Saima Pervaiz Iqbal
Dr. Ambreen Munir
Dr. Jamshed Akhtar
Dr. Farkhunda Ghafoor
Prof. Dr. Zafar Hayat

Dr Tehmina Mustafa
Center for International Health,
The Department of Global Public Health and
Primary Care,
University of Bergen,
Overlege Danielsens Hus, 4th floor
Årstadveien 21
N-5009 Bergen
Norway

Subject: **Improving diagnosis of extrapulmonary tuberculosis by implementation of a sensitive and specific assay in routine tuberculosis diagnostics (NBC-198).**

Dear Dr Tehmina Mustafa,

I am pleased to inform you that the above mentioned project has been cleared by "Research Ethics Committee of the National Bioethics Committee".

Kindly keep the National Bioethics Committee Secretariat updated with the progress of the project and submit the formal final report on completion.

Yours sincerely

(Prof Dr. Aasim Ahmad)
Chairman
NBC-Research Ethics Committee

NBC Secretariat:
_____
Pakistan Medical Research Council, Shahrah-e-Jamhuriat, Off Constitution Avenue, Sector G-5/2, Islamabad
www.nbcpakistan.org.pk, www.pmrc.org.pk, e-mail: nbcpakistan.org@gmail.com, pmrc.rdc@gmail.com Tel: 92-51- 9207386, 9206092, Fax 9216774

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8.4.   Appendix-4: Ethical approval retrospective study

Ethics Committee CMU (TB, Malaria and HIV/AIDS)

Ministry of National Health Services, Regulations and Coordination, Islamabad, Pakistan

F. No. IRB - CMU - 2018 - 02

December 03, 2018

Dr. Sabira Tahseen,
National TB Control Program,
Islamabad.

Subject: **CMU-ERC-02: Molecular Characterization and Prevalence of Drug Resistance associated with Rifampicin Resistance and Susceptible Strains**

Dear Dr. Sabira Tahseen,

I am pleased to inform you that the above mentioned project has been cleared by Institutional Review Board (IRB) Ethics Committee, Common Unit (HTM), Islamabad, Pakistan.

Kindly keep the IRB Ethics Committee updated with the progress of the project and submit the formal final report on completion

Yours Sincerely

Dr. Razia Fatima
Chairperson
IRB Ethics Committee

Errata for
Extrapulmonary tuberculosis in Pakistan: Challenges
in diagnosis and detection of drug resistance

Sabira Tahseen

Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

_____ 1-10-2023

(date and sign. of candidate)

_____ 04.10.23

(date and sign. of faculty)

Errata

Title page: Capital letter incorrect use "Tuberculosis" corrected to "tuberculosis"

Page 10: Capital letter incorrect use "Provincial" corrected to "provincial"

Page 10: Capital letter incorrect use "Regional" corrected to "regional"

Page 10: Capital letter incorrect use "Tuberculosis" corrected to "tuberculosis"

Page 10: Capital letter incorrect use "the National TB Reference Laboratory" corrected to "the National TB reference laboratory"

Page 14: Full stop misplaced "(95% CI, 0.6–5.4).," corrected to "(95% CI, 0.6–5.4),"

Page 22: Capital letter incorrect use "Multidrug-resistant" corrected to "multidrug-resistant"

Page 22: Capital letter incorrect use "Union" corrected to "union"

Page 23: Full stop missing "management of comorbidities (iv)" corrected to "management of comorbidities. (iv) "

Page 24: Comma missing "prevalence surveys results (ii)" corrected to "prevalence survey results, (ii)"

Page 24: Extra word "diagnosis, and (iii) national" corrected to "diagnosis, (iii) national"

Page 26: Capital letter incorrect use "Form of *M. Bovis*," corrected to "form of *M. bovis*,"

Page 27: Missing word "*tuberculosis*" corrected to "*M. tuberculosis*"

Page 32: Comma missing "register an infection ii) become infected" corrected to "register an infection, ii) become infected"

Page 32: Acronym not used "Pulmonary Tuberculosis" corrected to "Pulmonary TB"

Page 33: Acronym not used "Extrapulmonary Tuberculosis" corrected to "Extrapulmonary TB"

Page 34: repetition "often diagnosed late owing to insidious onset and delay in diagnosis may result in" corrected to "often diagnosed late owing to insidious onset and delay may result in"

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Page 35: Capital letter incorrect use "Central Nervous System Tuberculosis" corrected to "Central nervous system tuberculosis"

Page 35: Capital letter incorrect use "Osteoarticular Tuberculosis" corrected to "Osteoarticular tuberculosis"

Page 35: Capital letter incorrect use "Genito-urinary Tuberculosis" corrected to "Genito-urinary tuberculosis"

Page 36: Capital letter incorrect use "keratitis [171], Uveitis" corrected to "keratitis [171], uveitis"

Page 37: Word missing "is even more intricate with unusual presentation." corrected to "is even more intricate with these unusual presentations."

Page 37: Spelling error "It is also the only reliable means to monitor" corrected to "It is also the only reliable mean to monitor"

Page 38: Word misuse "claimed to be robust and rapid and to perform equally well" corrected to "claimed to be robust, rapid, and perform equally well"

Page 43: Comma missing "below the LoD of the diagnostic assays making bacteriological diagnosis even more" corrected to "below the LoD of the diagnostic assays, making bacteriological diagnosis even more"

Page 46: Word missing "without a miliary pattern and paucibacillary" corrected to "without a miliary pattern and is paucibacillary"

Page 48: Comma missing and word misuse "were established and integrated within" corrected to "were established, integrated within"

Page 49: Letter missing "Each TBM maintains" corrected to "Each TBMU maintains"

Page 52: Comma missing "included the study" corrected to "included, the study"

Page 53: Extra word "phenotypic and gDST performed were included" corrected to "phenotypic and gDST were included"

Page 54: Word misplaced "All types of PTB and EPTB patients were notified in 2016 at selected health facilities" corrected to "All types of notified PTB and EPTB patients in 2016 at selected health facilities "

Page 55: Full stop missing "reference standard" corrected to "reference standard."

Page 55: Capital letter incorrect use and comma missing "Supra National TB reference laboratory institute of tropical Medicine, Antwerp, Belgium" corrected to "TB supranational reference laboratory at institute of tropical Medicine, Antwerp, Belgium"

Page 57: Word misplaced "Histopathology is consistent with TB and was defined as" corrected to "Histopathology consistent with TB was defined as"

Page 61: Word incorrect "was resistant to LPA (MTBDRplus)" corrected to "was resistant by LPA (MTBDRplus)."

Page 79: Extra Space and full-stop misplaced "with standard first-line drug regimens. [53]." Corrected to "with standard first-line drug regimens [53]."

Page 79: Word missing "We studied 4542 phenotypic isolates for" corrected to "We studied 4542 phenotypic-resistant isolates for"

Page 80: Full stop misplaced "in patients with drug-resistant TB.[339-341]" corrected to "in patients with drug-resistant TB [339-341].

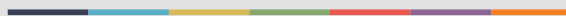Page 174: Semicolon incorrect use "Appendix-2;" corrected to "Appendix-2:"

Page 176: Semicolon incorrect use "Appendix-3;" corrected to "Appendix-3:"

Page 177: Semicolon incorrect use "Appendix-4;" corrected to "Appendix-4:"

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