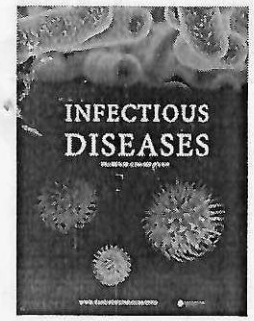

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RESEARCH ARTICLE

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Factors associated with hospitalization and mortality in adult and pediatric extrapulmonary tuberculosis at a tertiary care hospital in Central India

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ABSTRACT

Background: Comorbidities complicate the management of tuberculosis (TB) and have become an essential part of the end TB strategy to eradicate TB. However, pulmonary TB has received the most attention, and little is known about the impact of comorbidities and other factors on outcomes in patients with extrapulmonary tuberculosis (EPTB).

Objectives: Our aim was to analyze the factors associated with hospitalization and mortality in EPTB at a hospital in Central India, using non-TB patients with similar clinical presentations as a comparison.

Methods: Patients with presumptive EPTB were prospectively enrolled and followed up until the end of treatment or for at least 6 months. Detailed demographic and clinical information was collected for all participants, and patients were categorized as TB or non-TB using a composite reference standard. Multivariate logistic regression was used to analyze the impact of various clinical findings and risk factors on hospitalization and mortality.

Results: A total of 276 patients were categorized as TB cases and 175 as non-TB cases. Factors associated with hospitalization in children were younger age and non-adenitis site of disease. In adults, factors associated with mortality were older age, non-adenitis site of disease and HIV infection regardless of TB diagnosis, while diabetes mellitus increased the odds of mortality in EPTB patients.

Conclusion: Our results show that comorbidities increase the odds of death in both TB and non-TB patients in low-resource settings. This argues for a shift away from the traditional vertical management of diseases in these areas and supports a continued focus on building robust healthcare systems.

KEYWORDS
Extrapulmonary tuberculosis
childhood tuberculosis
non-communicable diseases
comorbidities
composite reference standard
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Introduction

The double burden of non-communicable diseases (NCDs) and infectious diseases in low-and middle-income countries has become prominent in recent years [1], adding to the complexity of tuberculosis (TB) management. HIV co-infection, malnutrition, diabetes mellitus (DM), smoking and harmful alcohol use have been identified as five major risk factors that increase the risk of developing TB disease, accounting for approximately 45% of new and relapsed TB cases globally each year [2]. Furthermore, comorbidities complicate the management of TB patients and increase the risk of hospitalization and death [3,4]. Therefore, managing TB comorbidities has become an essential part of the end TB strategy. In 2022, the WHO issued a framework for collaborative action on TB and comorbidities [2]. Recommended interventions include bilateral screening for several key risk factors, including DM and HIV in certain countries [5]. However, most of these studies and policies have focused on pulmonary tuberculosis (PTB), while extrapulmonary tuberculosis (EPTB) has been largely ignored. Although TB is primarily considered a pulmonary disease, an estimated 17% of the annual 10.6 million TB cases are extrapulmonary [6], with lymph nodes, pleura, bone and abdomen being the most common sites of infection. HIV and young age are associated with an increased risk of developing EPTB relative to PTB [7], but there is a scarcity in literature concerning the impact of risk factors on hospitalization and mortality in EPTB patients. This is particularly pronounced in children, where there is a significant knowledge gap [8]. Moreover, most studies analyzing the impact of various comorbidities and mortality in EPTB are retrospective studies [9–12] without information on non-TB patients presenting with similar clinical findings.

We aimed to study the clinical findings, comorbidities and risk factors associated with hospitalization and mortality in adult and pediatric EPTB at a tertiary care hospital in the low-resource and high TB burden setting of Central India, using non-TB patients with similar clinical presentation as a comparison.

Methods

This prospective cohort study was part of a larger study evaluating the diagnostic accuracy of the MPT64 antigen detection test for EPTB [13]. Patients with presumptive EPTB were prospectively enrolled between April 2018 and February 2020 at Chandrikaben Rashimikant Gardi Hospital (CRGH), associated with R.D. Gardi Medical College. Figure 1 shows the study design, inclusion, categorization and outcomes of participants in the study. We excluded patients for whom informed consent was not available. Patients who were lost to follow-up before 2 months were excluded from further analysis. Patients with known PTB at the time of inclusion were not enrolled in our study. However, in some EPTB cases, subsequent testing showed concomitant PTB.

The researchers (MRP and MK) worked closely with the clinicians involved in patient care regarding inclusion, baseline data collection and follow-up of study participants. If any queries arose during data curation or analysis, the responsible clinicians were contacted directly.

CRGH is a tertiary care hospital serving the semi-urban and rural population of Ujjain City, located in the state of Madhya Pradesh in Central India. In 2022, India had an estimated TB incidence rate of 199 per 100,000, with approximately 25% of cases being extrapulmonary [6]. The HIV and diabetes prevalence in the country in 2021 were 0.2% and 9.6% respectively [14,15].

Data collection

A pre-designed questionnaire, validated in previous studies [16], was used to collect detailed demographic information and clinical history of all recruited patients. Trained hospital staff interviewed patients in Hindi or in their local language. A senior researcher ensured that data collection met established standards by randomly joining the interviews. The presence of constitutional and local symptoms, known comorbidities and previous TB infections were explicitly asked. In the questionnaire, tobacco use and alcohol consumption were asked in the same question, so that we could not distinguish these two risk factors in our analysis.

The information from patient files was scanned, anonymized and transferred to an electronic version in Microsoft Excel. For internal validation, two researchers cross-checked 10% of the data entries.

All patients underwent a clinical examination, blood samples were collected and radiological investigations including chest X-rays and/or ultrasound were performed.

Samples from the various sites of infection were subjected to cytologic and/or histopathologic evaluation, Ziehl–Neelsen microscopy for the detection of acid-fast bacilli (AFB), *Mycobacterium tuberculosis* Lowenstein Jensen (LJ) culture and molecular testing with the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA,

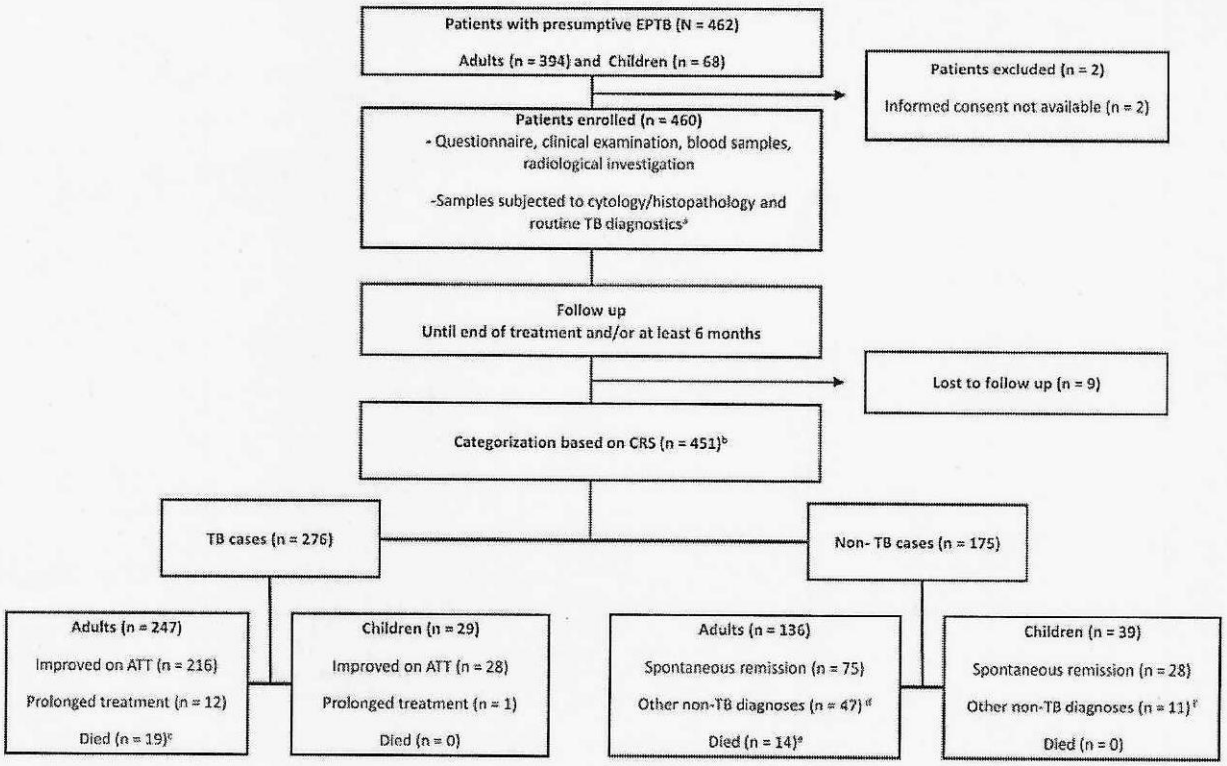

Figure 1. Flowchart of inclusion of patients, categorization and outcome. [a]Routine TB diagnostics included: Ziehl–Neelsen microscopy for the detection of acid-fast bacilli, *M. tuberculosis* culture and Xpert. [b]Categorization according to criteria shown in Table S1. [c]Sites: meningitis ($n = 10$), pleuritis ($n = 7$), ascites ($n = 1$), lymphadenitis ($n = 1$). [d]Non-TB diagnoses (adults): abscess ($n = 13$), malignancies ($n = 5$), benign tumor ($n = 9$), other bacteriological infection (including nontuberculous mycobacteria) ($n = 5$), cerebrovascular accident (CVA) or cardiac arrest ($n = 2$), chronic inflammation ($n = 3$), other neurological conditions ($n = 2$), liver cirrhosis with encephalopathy ($n = 1$), other (mastitis, prostate hyperplasia, intoxication, hyperglycemia, COPD, lumbar degenerative disc disease, leprosy, $n = 7$). [e]Cause of death/diagnoses associated with death in non-TB patients: malignancies ($n = 4$), CVA/cardiac arrest ($n = 5$), liver cirrhosis/encephalopathy ($n = 1$), other respiratory infection ($n = 1$), unknown ($n = 3$). [f]Non-TB diagnoses (children): abscess ($n = 4$), other neurological conditions ($n = 3$), benign tumor ($n = 1$), other bacteriological infection ($n = 1$), chronic inflammation ($n = 1$), liver cirrhosis with encephalopathy ($n = 1$).

United States—Xpert). Sputum was also collected for Xpert, LJ culture and AFB microscopy in most patients.

Definitions

DM was diagnosed by self-report or a random blood glucose (RBG) or 2-h plasma concentration after an oral glucose tolerance test greater than 11.1 mmol/l. An oral glucose tolerance test was performed if the RBG result was between 7.8 and 11.1 mmol/l, according to Indian guidelines [17]. HIV testing was performed in all patients with written informed consent.

Undernutrition was defined as a body mass index (BMI) <18.5 kg/m^2 (adults) or a score of 2 SD below the weight-for-age WHO child growth standards median (children) [18].

Anemia was defined as a hemoglobin of less than 13 g/dl in males, 12 g/dl in females and 11 g/dl in children. Information on the type of anemia was not available. An elevated total leukocyte count (TLC) was defined as more than 11,000/μl, while an elevated erythrocyte sedimentation rate (ESR) was defined as more than 10 mm/h for men and children, and more than 20 mm/h in women.

Follow-up and response to treatment

All patients were followed up regularly until the completion of treatment. During follow-up, clinical parameters were recorded to assess response to treatment, including signs and symptoms, weight and regression of local symptoms. Response to treatment/clinical improvement was considered if a minimum of three of the following four criteria were met: (1) improvement in systemic and local symptoms, (2) weight gain, (3) reduction in lymph node size or improvement in local findings and (4) improvement in VAS and EQ-5D. Thirteen TB patients received anti-tuberculous treatment (ATT) for a prolonged period. Patients who did not receive ATT were followed up for 6 months.

Mortality was defined as all-cause mortality. The cause of death determined by the clinicians or the diagnosis

that contributed the most to the patient's illness according to the patient files was recorded as the cause of death. No autopsies were performed.

Patient categorization

Patients were categorized as TB or non-TB based on a composite reference standard (CRS) (Table S1). Briefly, patients with a positive culture and/or Xpert result were categorized as bacteriologically confirmed TB cases. Clinically diagnosed patients had clinical findings suggestive of EPTB according to the local physicians and responded to ATT at 2–3 months and/or at the end of treatment. The majority of clinically diagnosed TB cases also had one or more additional criteria suggestive of TB (Table S1).

Patients were diagnosed as non-TB if they improved without ATT, or if clinicians made an alternative diagnosis.

The type of suspected EPTB was noted according to site of infection. In some subanalyses, we created a binary variable defined as adenitis and non-adenitis site of disease.

Ethical considerations

Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Medical Research Ethics in Norway, REK Helse Vest (2014/46/REK vest) and by the ethical committee in India (IEC 10/2018). None of the invasive procedures were performed for research purposes only.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, IBM, Armonk, NY) for Windows, version 29. Chi-square test was performed to assess differences in categorical variables. Logistic regression was used to analyze factors associated with hospitalization and mortality risk, and to adjust for covariates. Variables with significant crude odds ratios were included in the multivariate logistic regression models. A *P*-value of $<.05$ or a confidence interval not including 1 was considered statistically significant.

Results

A total of 460 patients with presumptive EPTB were included in the study (Figure 1). Based on a CRS, 247 adults and 29 children were categorized as TB patients and 136 adults and 39 children as non-TB patients. Nine patients (2%) were excluded from the analysis due to loss to follow-up. The overall mortality rate in adults was 8% in TB patients and 10% in the non-TB group (Table 1). No children died in our cohort.

Among adults, TB patients were younger and with a higher proportion of females, compared to the non-TB group (Table 1). In terms of comorbidities, the non-TB group had a significantly higher proportion of DM, hypertension and chronic obstructive pulmonary disease (COPD), while TB patients had a higher proportion of underweight and anemia. The prevalence of HIV co-infection was low in both TB and non-TB patients at 5% and 3%, respectively.

Systemic symptoms were more prevalent in TB patients; 82% had two or more systemic symptoms compared with 68% in the non-TB group ($P = .003$) (Table 1). The frequency of fever, weight loss, night sweats, loss of appetite and fatigue was higher in TB as compared to non-TB patients. In adults, lymphadenitis was the most common site of infection, accounting for 60% and 64% of cases in the TB and non-TB groups, respectively (Table 1). Pleuritis was more common in TB patients compared to non-TB patients, while the prevalence of meningitis was higher in the non-TB group. In children, the proportion of lymphadenitis patients was even higher at 90% in TB cases and 77% in non-TB cases.

Among children, TB patients were older, while no difference was found between TB and non-TB patients in terms of gender or undernutrition (Table 1). The proportion of patients with systemic symptoms was equally high in TB and non-TB patients. A higher proportion of TB patients had symptoms for more than 2 months compared to the non-TB group ($P = .03$) (Table 1).

When all TB patients were included, the proportion of patients with systemic symptoms was highest among TB pleuritis and TB meningitis with 92% and 95% of patients presenting with two or more constitutional symptoms, respectively (Table S2). In patients with TB lymphadenitis, the corresponding percentage was 74%.

In the non-TB group, about half of the patients ($n = 103$) improved without a specific diagnosis recorded by the clinicians (Figure 1). This group was significantly younger and consisted of more lymphadenitis, fewer hospitalized patients and a lower prevalence of comorbidities when compared to the remaining non-TB patients (Table S3).

The most common non-TB diagnoses were abscesses ($n = 17$), benign tumors ($n = 10$) and malignancies ($n = 9$). In this group, most patients died from

Table 1. Demographic and clinical findings in 451 study participants.

	Adults			Children		
	TB ($N=247$) *n* (%)	Non-TB ($N=136$) *n* (%)	*P*-Value	TB ($N=29$) *n* (%)	Non-TB ($N=39$) *n* (%)	*P*-Value
Age (years)						
<5				3 (10)	6 (15)	**.013**
5–9				6 (21)	20 (51)	
10–14				20 (69)	13 (33)	
15–29	136 (55)	49 (36)	**<.001**			
30–44	62 (25)	33 (24)				
>45	49 (20)	54 (40)				
Gender						
Female	138 (56)	63 (46)	.073	17 (59)	16 (41)	.151
Male	109 (44)	73 (54)		12 (41)	23 (59)	
Department						
Inpatient	123 (50)	65 (48)	.707	7 (24)	12 (31)	.547
Outpatient	124 (50)	71 (52)		22 (76)	27 (69)	
HIV positive	13 (5)	4 (3)	.291	0 (0)	0 (0)	–
DM	15 (6)	18 (13)	**.017**	0 (0)	0 (0)	–
Underweight[a]	172/239 (72)	70/132 (53)	**<.001**	12 (41)	23 (59)	.193
Hypertension	15 (6)	18 (13)	**.017**	0 (0)	0 (0)	–
COPD	12 (5)	16 (12)	**.013**	0 (0)	0 (0)	–
Previous TB	45 (18)	19 (14)	.286	7 (24)	0 (0)	**.001**
Tobacco/alcohol	80 (32)	51 (38)		0 (0)	0 (0)	
Anemia[b]	181 (73)	82 (60)	**.009**	21 (72)	33 (85)	.218
Site			**.004**			.336
Lymphadenitis	148 (60)	87 (64)		26 (90)	30 (77)	
Pleuritis	52 (21)	18 (13)		1 (3)	1 (3)	
Meningitis	20 (8)	25 (18)		1 (3)	7 (18)	
Gastrointestinal	8 (3)	1 (1)		0 (0)	0 (0)	
Other[c]	19 (8)	5 (4)		1 (3)	1 (3)	
All non-adenitis	99 (40)	49 (36)	.436	3 (10)	9 (23)	.173
Systemic symptoms						
Any	224 (91)	110 (81)	**.006**	24 (83)	35 (90)	.401
≥2 symptoms	202 (82)	93 (68)	**.003**	17 (59)	24 (62)	.808
Fever	168 (68)	77 (57)	**.026**	16 (55)	27 (69)	.234
Weight loss	165 (67)	66 (49)	**<.001**	17 (59)	20 (51)	.548
Loss of appetite	154 (62)	57 (42)	**<.001**	15 (52)	20 (51)	.971
Night sweats	85/245 (35)	32 (24)	**.024**	3 (10)	8 (21)	.241
Fatigue	195 (79)	92 (68)	**.015**	14 (48)	19 (49)	.971
Duration of symptoms ≥2 months	69 (28)	34 (25)	.535	10 (34)	5 (13)	**.033**
Elevated ESR[d]	205/234 (88)	112/131 (85)	.567	24 (83)	36 (92)	**.019**
Elevated TLC[e]	42/246 (17)	29 (21)	.307	3 (10)	11 (28)	.072
Outcome						
Death	19 (8)	14 (10)	.385	0	0	–

Statistically significant *P*-values shown in bold.
[a]Underweight was considered if BMI was <18.5 kg/m^2 (adults) or a score of 2SD below the weight-for-age WHO child growth standards median (children).
[b]Anemic (g/dl) (<12 females, <13 males or children less than 5 years <11.
[c]Other sites; TB: abscess ($n=5$), breast ($n=5$), urogenital ($n=4$), osteomyelitis ($n=3$), synovial fluid ($n=1$), larynx ($n=1$), skin ($n=1$). Non-TB: breast ($n=2$), urogenital ($n=2$), osteomyelitis ($n=2$).
[d]Elevated ESR: >10 mm/h for men and children and >20 for females.
[e]Elevated TLC: >11,000/μl.

cerebrovascular accidents (CVA)/cardiac arrest ($n=5$) and malignancies ($n=4$) (Figure 1).

Factors associated with hospitalization

About half of the adults in both groups were hospitalized, while the proportion of hospitalized children was lower (24%) among TB as compared to the non-TB group (31%). Among adult TB cases, male sex was associated with hospitalization, while no gender difference was observed in the non-TB group (Table 2). In both the TB and non-TB groups older age, being underweight, alcohol and/or tobacco use, and non-adenitis site of disease were all factors associated with hospitalization (Table 2). However, in multivariate analysis, only non-adenitis site of disease [adjusted OR 36 (95% CI 14 – 91)] was associated with significantly increased odds of hospitalization in TB patients. In the non-TB group, age [adjusted OR 1.05 (1.01 – 1.09)], underweight [adjusted OR 16 (95% CI 3–79)] and non-adenitis site of infection [adjusted OR 202 (95% CI 27–1524)] were independently associated with hospitalization after adjusting for age and comorbidities (Table 3). Systemic symptoms and elevated TLC were associated with hospitalization in both groups among adults.

In children, the odds of hospitalization decreased with increasing age in both groups (Table S4). In non-TB

Table 2. Factors associated with hospitalization in adults diagnosed with EPTB and non-TB conditions with similar clinical presentation.

	TB ($n=247$)				Non-TB ($n=136$)			
	OPD ($n=124$)	IPD ($n=123$)	cOR (95% CI)	*P*-Value	OPD ($n=71$)	IPD ($n=65$)	cOR (95% CI)	*P*-Value
Age (Median, range)	25 (15–68)	30 (15–90)	**1.03 (1.01–1.05)[a]**	**.002**	30 (15–80)	45 (17–80)	**1.04 (1.02–1.07)[a]**	**<.001**
Gender								
Female	82 (66)	56 (46)	1		37 (52)	26 (40)	1	
Male	42 (34)	67 (54)	**2.3 (1.4–3.9)**	**.001**	34 (48)	39 (60)	1.6 (.8–3.2)	.158
HIV positive	7 (6)	6 (5)	.9 (.3–2.6)	.787	1 (1)	3 (5)	3.4 (.3–33.0)	.296
DM	6 (5)	9 (7)	1.6 (.5–4.5)	.418	6 (8)	12 (19)	2.5 (.9–7.0)	.092
Underweight[b]	76/117 (65)	96 (78)	**2.0 (1.1–3.5)**	**.019**	29/67 (43)	41 (63)	2.2 (1.1–4.5)	**.024**
Hypertension	8 (6)	7 (6)	.9 (.3–2.5)	.803	7 (10)	11 (17)	1.9 (.7–5.1)	.230
COPD	1 (1)	11 (9)	**12.1 (1.5–95.1)**	**.018**	3 (4)	13 (20)	**5.7 (1.5–20.9)**	**.009**
Previous TB	29 (23)	16 (13)	.5 (.3–.957)	**.037**	9 (13)	10 (15)	1.3 (.5–3.3)	.649
Concomitant PTB	8 (7)	10 (8)	**1.3 (.5–3.4)**	.613	–	–	–	–
Tobacco/alcohol use	29 (23)	51 (41)	**2.3 (1.3–4.0)**	**.003**	18 (25)	33 (51)	**3.0 (1.5–6.3)**	**.003**
Anemia	87 (70)	94 (76)	1.4 (.8–2.3)	.267	37 (52)	45 (69)	**2.1 (1.02–4.2)**	**.043**
Survived	124 (100)	104 (85)	1		71 (100)	51 (78)	1	
Death	0 (0)	19 (15)	NA	–	0 (0)	14 (22)	NA	–
Site								
Lymphadenitis	115 (93)	33 (27)	1		69 (97)	18 (28)	1	
Non-adenitis	9 (7)	90 (73)	**34.8 (15.9–76.6)**	**<.001**	2 (3)	47 (72)	**90.1 (20.0–406.6)**	**<.001**
Clinical and laboratory characteristics								
Any systemic symptoms	109 (88)	115 (93)	2.0 (.8–4.9)	.136	53 (75)	57 (88)	2.4 (.971–6.0)	.058
<2 symptoms	30 (24)	15 (12)	1		30 (42)	13 (20)	1	
≥2 symptoms	94 (76)	108 (88)	**2.3 (1.2–4.5)**	**.016**	41 (58)	52 (80)	**2.9 (1.4–6.3)**	**.006**
Fever	68 (55)	100 (81)	**3.6 (2.0–6.4)**	**<.001**	29 (41)	48 (74)	**4.1 (2.0–8.5)**	**<.001**
Weight loss	73 (59)	92 (75)	**2.1 (1.2–3.6)**	**.008**	28 (39)	38 (58)	**2.2 (1.1–4.3)**	**.028**
Loss of appetite	61 (49)	93 (76)	**3.2 (1.9–5.5)**	**<.001**	21 (30)	36 (55)	**3.0 (1.5–6.0)**	**.003**
Night sweats	30 (24)	55 (45)	**2.5 (1.5–4.4)**	**<.001**	12 (17)	20 (31)	2.2 (.968–4.9)	.060
Fatigue	92 (74)	103 (84)	1.8 (.958–3.3)	.068	53 (75)	48 (74)	1.7 (.8–3.6)	.141
Duration of symptoms ≥2 months	36 (29)	33 (27)	.9 (.5–1.6)	.700	17 (24)	17 (26)	1.1 (.5–2.4)	.766
Elevated ESR[c]	98/116 (84)	107/118 (91)	1.8 (.8–4.0)	.145	57/69 (83)	55/62 (89)	1.7 (.6–4.5)	.325
Elevated TLC[d]	9 (7)	33 (27)	**4.6 (2.1–10.2)**	**<.001**	6 (8)	23 (35)	**5.9 (2.2–15.8)**	**<.001**
Elevated ADA[e]	3/3 (100)	56/64 (88)	NA	–	0/2 (0)	16/38 (42)	NA	–
Culture positive	39 (31)	19 (15)	.4 (.2–.7)	**.004**	–	–	–	
AFB positive	26 (21)	12 (10)	.4 (.2–.9)	**.017**	–	–	–	
Xpert positive	49 (40)	24 (20)	.4 (.2–.6)	**<.001**	–	–	–	

Statistically significant *P*-values and odds ratios shown with 95% CI in bold. Reference categories set to 1 if not specified. OPD; outpatient department, IPD; inpatient department, cOR; crude odds ratio, aOR; adjusted odds ratio.
[a]Difference in odds ratio per increase in years.
[b]Underweight was considered if BMI was <18.5 kg/m^2.
[c]Elevated ESR: >10 mm/h for men and >20 for females.
[d]Elevated TLC: >11,000/μl.
[e]Adenosine deaminase, elevated if >40 I/U (pleural fluids) or >10 (CSF). Results available for 107 participants.

cases, underweight and female gender were associated with hospitalization. None of the variables were independently associated with hospitalization when adjusting for age, gender and being underweight (Table 3). All children with disease outside the lymph nodes were hospitalized, and all of these patients had systemic symptoms.

Factors associated with mortality

Both TB and non-TB cases had a significantly higher risk of death at older ages. Interestingly, all cases that died were hospitalized. No children died in our study, so the mortality risk was only calculated in adults.

Concomitant HIV infection, COPD, tobacco and/or alcohol use and non-adenitis site of infection were associated with an unadjusted higher mortality risk in adults in both groups (Table 4). Furthermore, TB patients with DM comorbidity [OR 8 (95% CI 2–26)] and concomitant PTB [OR 4 (95% CI 1–14)] had a higher crude odds of dying compared to TB patients without these risk factors (Table 4). All TB cases who died had two or more systemic symptoms at inclusion.

After adjusting for age, site of infection and relevant comorbidities, the factors associated with an increased

Table 3. Multivariate analysis of factors associated with hospitalization in adult and pediatric patients diagnosed with EPTB or non-TB condition with similar clinical presentation.

	Adults							
	TB				Non-TB			
	cOR (95% CI)	*P*-Value	aOR[a] (95% CI)	*P*-Value	cOR (95% CI)	*P*-Value	aOR[b] (95% CI)	*P*-Value
Age	**1.03 (1.01–1.05)**	**.002**	1.00 (.976–1.03)	.780	**1.04 (1.02–1.07)**	**<.001**	1.05 **(1.01–1.09)**	**.024**
Male	2.3 (1.4–3.9)	**.001**	.9 (.3–2.5)	.935	1.6 (.8–3.2)	.158	–	
Underweight	1.9 (1.1–3.4)	**.019**	1.2 (.5–2.5)	.715	**2.6 (1.3–5.2)**	**.024**	15.8 **(3.1–79.1)**	**<.001**
COPD	**12.1 (1.5–95.1)**	**.018**	6.3 (.5–78.7)	.151	**5.7 (1.5–20.9)**	**.009**	.3 (.03–2.4)	.233
Previous TB	**.5 (.3–.957)**	**.037**	.6 (.2–1.5)	.267	1.3 (.5–3.3)	.649	–	
Tobacco/alcohol use	**2.3 (1.3–4.1)**	**.003**	.6 (.2–2.0)	.441	**3.0 (1.5–6.3)**	**.003**	1.8 (.5–6.3)	.357
Anemia	**1.3 (.8–2.3)**	**.267**	–		**2.1 (1.02–4.2)**	**.043**	1.8 (.6–5.8)	.325
Non-adenitis site	34.8 **(15.9–76.6)**	**<.001**	**36.0 (14.2–91.0)**	**<.001**	90.1 **(20.0–406.6)**	**<.001**	**201.8 (26.7–1523.9)**	**<.001**
	Clinical and laboratory characteristics							
	cOR (95% CI)	*P*-Value	aOR[c] (95% CI)	*P*-Value	cOR (95% CI)	*P*-Value	aOR[d] (95% CI)	*P*-Value
Fever	**3.6 (2.0–6.4)**	**<.001**	**2.2 (1.1–4.6)**	**.034**	**4.1 (2.0–8.5)**	**<.001**	**3.2 (1.4–7.7)**	**.008**
Weight loss	**2.0 (1.2–3.6)**	**.008**	**.9 (.4–1.8)**	.727	2.2 (1.1–4.3)	**.028**	1.2 (.5–3.1)	.651
Loss of appetite	**3.2 (1.9–5.5)**	**<.001**	**2.2 (1.1–4.6)**	**.027**	**3.0 (1.5–6.0)**	**.003**	1.6 (.6–4.1)	.297
Night sweats	**2.5 (1.5–4.4)**	**<.001**	**1.6 (.8–3.4)**	**.205**	2.2 (.968–4.9)	.060	–	
Elevated TLC	**4.6 (2.1–10.2)**	**<.001**	**3.5 (1.4–8.3)**	**.005**	**5.9 (2.2–15.8)**	**<.001**	**5.3 (1.8–15.6)**	**.002**
Culture positive	**.4 (.2–.7)**	**.004**	**.5 (.2–1.5)**	**.205**	–		–	
Xpert positive	**.4 (.2–.6)**	**<.001**	**.5 (.2–1.4)**	**.198**	–		–	
	Children							
	cOR (95% CI)	*P*-Value	aOR[e] (95% CI)	*P*-Value	cOR (95% CI)	*P*-Value	aOR[f] (95% CI)	*P*-Value
Age	**.763 (.585–.996)**	**.047**	**.7 (.5–.991)**	**.044**	.833 (.674–1.030)	.092	.8 (.6–1.0)	.061
Male	1.1 (.2–6.1)	.927	–		**.2 (.04–.76)**	**.036**	**.1 (.02–.951)**	**.044**
Underweight	2.2 (.4–12.3)	.383	5.4 (.5–58.4)	.165	**13.8 (1.5–122.0)**	**.019**	**20.9 (1.7–264.5)**	**.019**

Statistically significant values shown bold. Variables in multivariate regression are as follows:
[a]Age, site, underweight, COPD, alcohol/tobacco, gender and history of TB.
[b]Age, site, underweight, COPD, anemia and alcohol/tobacco.
[c]Age, fever, weight loss, loss of appetite, night sweats, elevated TLC, culture positive and Xpert positive.
[d]Age, fever, weight loss, loss of appetite and elevated TLC.
[e]Age and underweight.
[f]Age, gender and underweight.

mortality among TB patients were older age [adjusted OR 1.04 (95% CI 1.01–1.08)], non-adenitis site of infection [adjusted OR 52 (95% CI 4–634)], HIV [adjusted OR 15 (95% CI 2–121)] and DM [adjusted OR 6 (95% CI 1–31)] (Table 4).

In the non-TB group, age [adjusted OR 1.07 (1.01 – 1.13)], non-adenitis site of infection [adjusted OR 17 (95% CI 2–168)] and HIV [adjusted OR 53 (95% CI 2–1852)] were independently associated with increased odds of mortality (Table 4).

Since all patients who died were hospitalized, we analyzed the data for this subgroup by excluding non-hospitalized patients, finding a significantly increased risk of death in TB patients with older age [adjusted OR 1.05 (95% CI 1.01–1.09)], HIV co-infection [(15 (95% CI 2–140)] and positive culture [adjusted OR 9 (95% CI 2–49)] (Table S5).

Discussion

In this prospective cohort study, we found a mortality rate of 8% in EPTB patients, which is not significantly different from the mortality rate of 10% in adults with similar symptoms but with a diagnosis other than TB. The factors associated with increased mortality were similar in TB and non-TB patients. After adjusting for age and relevant covariates, we found an increased odds of mortality in patients with sites of infection other

Table 4. Factors associated with mortality in adults diagnosed with EPTB and non-TB conditions with similar clinical presentation.

	TB ($n = 247$)						Non-TB ($n = 136$)					
	Alive ($N = 228$) (%)	Dead ($N = 19$) (%)	cOR (95% CI)	*P*-Value	aOR (95% CI)[a]	*P*-Value	Alive ($N = 122$) (%)	Died ($N = 14$) (%)	cOR (95% CI)	*P*-Value	aOR (95% CI)[b]	*P*-Value
Age, years Median (range)	26 (15–75)	50 (19–90)	**1.07 (1.04–1.10)**	**<.001**	**1.04 (1.01–1.08)**	**.013**	33 (15–80)	60 (21–68)	**1.07 (1.03–1.11)**	**<.001**	**1.07 (1.01–1.13)**	**.017**
Male	98 (43)	11 (58)	1.8 (.7–4.7)	.214			63 (52)	10 (71)	2.3 (.7–7.9)	.169		
HIV positive	10 (4)	3 (16)	**4.1 (1.0–16.4)**	**.047**	**15.3 (1.9–121.6)**	**.010**	2 (2)	2 (14)	**10.0 (1.3–77.5)**	**.028**	**53.0 (1.5–1851.7)**	**.028**
DM	10 (4)	5 (26)	**7.8 (2.3–25.9)**	**<.001**	**5.9 (1.1–30.9)**	**.036**	14 (11)	4 (29)	3.1 (.9–11.2)	.086		
Underweight	155/220 (70)	17 (89)	3.6 (.8–15.9)	.095			62/118 (52)	8 (57)	1.2 (.4–3.7)	.745		
Previous TB	43 (19)	2 (11)	.5 (.1–2.3)	.374			17 (14)	2 (14)	1.0 (.2–5.0)	.971		
Concomitant PTB	14 (6)	4 (21)	**4.1 (1.2–13.9)**	**.025**	3.8 (.7–21.9)	.134			–			
Hypertension	12 (5)	3 (16)	3.4 (.9–13.2)	.080			14 (11)	4 (29)	3.1 (.9–11.2)	.086		
COPD	8 (3)	4 (21)	**7.3 (2.0–27.2)**		**3.6 (.7–18.6)**	**.129**	12 (10)	4 (29)	3.7 (.996–13.5)	.051	1.0 (.2–5.3)	.971
Tobacco and/or alcohol use	69 (30)	11 (58)	**2.3 (1.3–4.1)**	**.003**	**.6 (.2–2.0)**	**.571**	42 (34)	9 (64)	**3.4 (1.1–10.9)**	**.037**	1.4 (.3–6.7)	.647
Anemia	168 (74)	13 (68)	.8 (.3–2.1)	.619			70 (57)	12 (86)	**4.5 (1.0–20.7)**	**.057**	2.3 (.4–13.1)	.341
Sites												
Lymphadenitis	147 (64)	1 (5)	1		1		86 (70)	1 (7)	1		1	
Pleuritis	45 (20)	7 (37)	**22.9 (2.7–190.8)**	**.004**			14 (11)	4 (29)	**24.6 (2.6–236.2)**	**.006**		
Meningitis	10 (4)	10 (53)	**147.0 (17.1–1266.0)**	**<.001**			17 (14)	8 (57)	**40.5 (4.7–345.0)**	**<.001**		
Gastrointestinal	7 (3)	1 (5)	**21.0 (1.2–371.7)**	**.038**			1 (1)	0	–	–		
Other	19 (8)	0 (0)	–	–			4 (3)	1 (7)	**21.5 (1.1–409.8)**	**.041**		
All non-adenitis	81 (36)	18 (95)	**32.7 (4.3–249.2)**	**<.001**	**51.6 (4.2–634.2)**	**.002**	36 (30)	13 (93)	**31.1 (3.9–246.3)**	**<.001**	**17.4 (1.8–168.1)**	**.013**
Clinical findings and laboratory characteristics												
Systemic symptoms Present	205 (90)	19 (100)	NA				98 (80)	12 (86)	1.5 (.3–7.0)	.629		
≥2 symptoms	183 (80)	19 (100)	NA				81 (66)	12 (86)	3.0 (.6–14.2)	.158		
Fever	149 (65)	19 (100)	NA				68 (56)	9 (64)	1.4 (.5–4.5)	.543		
Weight loss	146 (64)	19 (100)	NA				56 (46)	10 (71)	2.9 (.9–9.9)	.081		
Loss of appetite	137 (60)	17 (90)	**5.6 (1.3–25.0)**	**.023**	3.4 (.6–18.9)[c]	.192	47 (39)	10 (71)	**4.0 (1.2–13.5)**	**.026**	2.9 (.8–11.1)[d]	.125
Night sweats	73/226 (32)	12/19 (63)	**3.6 (1.4–9.5)**	**.010**	3.0 (.9–10.0)[c]	.078	28 (23)	4 (29)	1.3 (.4–4.6)	.640		
Fatigue	178 (78)	17 (90)	2.4 (.5–10.7)	.255			80 (66)	12 (86)	3.2 (.7–14.7)	.145		
Duration of symptoms <2	164 (72)	14 (74)	1.1 (.4–3.1)	.915			91 (75)	11 (79)	1.3 (.3–4.9)	.721		
MTB culture positive	51/228 (22)	7/19 (37)	2.0 (.8–5.4)	.160					NA			
Xpert positive	68 /219 (31)	5/19 (26)	.8 (.3–2.3)	.668								
Elevated ESR[e]	187/216 (87)	18/18 (100)	NA				99 (81)	13 (93)	2.4 (.3–19.2)	.421		
Elevated TLC[f]	29/227 (13)	13/19 (68)	**14.8 (5.2–42.0)**	**<.001**	**7.0 (2.2–22.2)[c]**	**<.001**	21 (17)	8 (57)	**6.4 (2.0–20.4)**	**.002**	**4.9 (1.3–18.1)[d]**	**.016**

Statistically significant values shown in bold. Logistic regression: reference variable set to 1.
[a]Variables included in multivariate regression: age, HIV, DM, concomitant PTB, COPD, any tobacco/alcohol and site.
[b]Variables included in multivariate regression: age, HIV, COPD, site, any tobacco/alcohol, anemia.
[c]Variables included in multivariate regression: age, loss of appetite, night sweats and elevated TLC.
[d]Variables included in multivariate analysis: age, loss of appetite and elevated TLC.
[e]Elevated ESR: >10 mm/h for men and >20 for females.
[f]Elevated TLC: >11,000/μl.

than lymph nodes, older age and HIV co-infection in both groups, while DM comorbidity was associated with a significantly increased odds of mortality only in TB patients.

We found a low prevalence of DM in EPTB patients in our cohort; however, DM was significantly associated with mortality. DM is known to impair the cellular immune response and hyperglycemia affects almost all organs, increasing the risk of developing active TB disease [19]. Previous studies have shown that comorbidity with DM increases the risk of poor outcome in PTB patients, supporting the need for bidirectional screening [5]. However, few studies have investigated the association between DM and increased risk of death in EPTB patients [9]. A New Zealand study that examined the impact of DM in both PTB and EPTB patients, found older age and kidney function to be key factors for poor TB outcomes [3]. Keeping in mind that nephropathy is a common complication of DM, this could be one of the explanations for the increased mortality in TB-DM comorbid patients. In our study, only four patients were diagnosed with renal disease (one TB patient and three non-TB patients). Interestingly, two of these four patients died, both of whom were categorized as non-TB patients. Even though the number of DM patients in our study was relatively small, we found an independent association of DM with mortality risk when adjusting for age, site and other relevant comorbidities.

Although all patients in our cohort were included based on clinical suspicion of EPTB, the proportion of systemic symptoms and other baseline characteristics differed significantly between patients diagnosed with TB and those without a TB diagnosis. The TB group was younger and had more systemic symptoms than the non-TB group. These results are interesting because patients with some forms of EPTB, such as tuberculous lymphadenitis (TBLA) are considered to have few systemic symptoms [20]. Even when analyzing patients with adenitis separately, we found a high proportion of systemic symptoms in TBLA patients compared to non-TB adenitis patients. Given the diagnostic challenges in EPTB [21] and the large number of patients starting empirical ATT, particularly in resource-poor settings [22], clinical scoring systems to distinguish TB from non-TB would aid clinicians to initiate appropriate treatment. Although our results suggest that systemic symptoms are more common in adult EPTB patients compared to non-TB patients, the similar clinical presentations of the two groups highlight the need for improved diagnostic tests to confirm the diagnosis of EPTB.

We found a low mortality rate in EPTB patients compared to other studies reporting a mortality rate of 28–41% [11,23,24]. In our cohort, hospitalization, non-adenitis sites of infection and older age significantly increased the odds of a fatal outcome, regardless of TB diagnosis. When all-cause mortality was calculated separately for hospitalized patients, the mortality rate was 15% for TB patients and 22% for non-TB patients. The mortality rate for hospitalized non-TB patients is similar to other studies from high TB burden settings [25], while the mortality rate for EPTB patients is lower than a previous report of 34% from Mozambique [23]. The lower mortality rate in EPTB patients in our study may be due to the low HIV prevalence in India compared to many countries in sub-Saharan Africa.

Identification of patients at higher risk of unfavorable outcome is crucial for appropriate TB management. The Bandim TB Score already exists for PTB [26], but a similar scoring tool for EPTB is not available. We found that systemic symptoms increased the odds of dying in EPTB patients, but not in non-TB patients. This interesting finding could be explained by the most common causes of death being CVA or cardiovascular events in the non-TB group, which typically presents with scarce systemic symptoms. On the other hand, elevated TLC and ESR levels were highly associated with an increased mortality odds both in TB and non-TB patients. These blood tests are non-specific and reflect the systemic burden in these patients. Since the levels were elevated in both groups, these tests are not useful for the diagnosis of TB but may be useful in determining increased risk of poor outcome in TB patients. While our study does not analyze the predictive value of symptoms and clinical findings in relation to mortality, it does suggest that assessing the degree of systemic stress in EPTB patients may provide valuable information about mortality risk.

No children died in our cohort, which is surprising given the high mortality rates of pediatric tuberculous meningitis found in other studies [27,28]. The low mortality rate in our study could be explained by the relatively small pediatric sample size and the high proportion of TB lymphadenitis. In contrast to our results in adults, the proportion of pediatric patients with systemic symptoms was equally high in TB (83%) and non-TB (90%) patients and thus not helpful in distinguishing TB from non-TB, highlighting the need for improved laboratory tests to confirm pediatric EPTB. Factors associated with hospitalization in children were non-adenitis site of infection and young age. From a clinical perspective, this is to be expected as younger children are more

vulnerable to infections, and suspected non-adenitis sites of infection, i.e. tuberculous meningitis, tend to be more fatal. This is related to the immature immune system of young children, which increases the risk of disseminated forms of TB [29]. Interestingly, all pediatric EPTB patients in our cohort reported weight loss at the time of inclusion. In a recent study from Pakistan, weight loss was found to be associated with EPTB disease in children [30], but the association with hospitalization was not analyzed. Our results from the pediatric cohort need to be interpreted with caution as the sample size is small. However, the knowledge gap is pronounced in childhood TB [8,31], and reporting of pediatric findings is therefore important to improve diagnosis and management for this patient group.

In hospitalized EPTB patients, culture positivity was independently associated with a higher odds of death, which could be used as a proxy for a greater bacterial load. However, a similar association was not found for AFB microscopy, which is also linked with a high bacterial burden. Furthermore, no association was found between a positive Xpert result and hospitalization or mortality. This could be explained by the poor performance of these tests in the most lethal forms of EPTB, namely TB meningitis and pleural TB [32]. The low diagnostic yield of routine diagnostic tests in EPTB is a major challenge in the TB cascade of care [33], resulting in a high number of TB cases not being recognized or treated empirically [10]. Given that an Xpert result could be available within one hour, a timely diagnosis and thus a positive impact on TB outcomes would be expected. However, several studies have analyzed the impact of Xpert on mortality in high burden settings, without finding a significant reduction in mortality in TB patients [34]. The poor negative predictive value of diagnostic tests in EPTB forces clinicians in low-resource settings to treat presumptive TB patients empirically, which in turn makes it difficult to analyze the effect of new diagnostic tests on mortality rates [22]. In our study, a positive laboratory diagnosis of EPTB did not impact the odds of hospitalization or mortality when including all patients, however, among hospitalized adults culture positivity was associated with mortality.

HIV was associated with mortality in both TB and non-TB patients. This is consistent with literature, which reports that HIV increases the risk of death many-fold in TB patients [2]. Again, PTB has received most of the attention in TB research and the role of HIV in EPTB mortality is rather uncertain. While most studies show an increased risk of death in EPTB–HIV co-infected individuals, a systematic review found that these studies vary greatly and the risk of bias is too high to draw a valid conclusion [35], highlighting the need for more well-designed studies on this topic. In our cohort, HIV co-infection significantly increased the odds of death regardless of TB status, supporting the continued use of bidirectional screening for HIV and EPTB [36].

Undernutrition is considered a major risk factor for developing TB disease and has been shown to increase the risk of death in PTB [37], but may also be a consequence of TB disease [38]. Undernourished children are considered particularly vulnerable to TB disease as undernutrition weakens the immune system [39]. In our study, a significantly higher proportion of adults in the TB group had a low BMI, but this risk factor was not associated with hospitalization or mortality in either adults or children. This contrast previous literature on PTB, which found that undernutrition increases the risk of mortality [40]. This may be explained by the high prevalence of undernutrition and low mortality rate in our study. Although no association between poor outcome and undernutrition was found in our study, we report a high prevalence of undernutrition in patients with symptoms suggestive of EPTB, which supports further research on the role of nutrition in improving overall health in resource-poor settings.

The high proportion of anemia in the TB group compared to the non-TB group in our study is consistent with the literature reporting a high prevalence of anemia in EPTB patients [41], however, anemia could also be the result of other risk factors for TB such as excessive alcohol consumption and undernutrition. In children, the prevalence of anemia was similar in both TB and non-TB patients and was not associated with hospitalization in either group. Similarly in adults, anemia did not increase the odds of hospitalization or mortality in our study. Again, this could be explained by a high prevalence of anemia, probably related to the high proportion of undernutrition, and the low mortality in our study. This is unlike PTB, where clinical findings suggestive of anemia were used as part of a scoring system to stratify patients into different risk groups for poor outcome [42]. Although the prevalence of anemia was high among EPTB patients in our cohort, it was not independently associated with hospitalization or mortality.

Our results show that comorbidities increase the odds of death in both TB and non-TB patients. This underscores the increasing importance of the double burden of NCDs and infectious diseases in developing countries

[1]. Our findings suggest that prevention and appropriate management of comorbidities would reduce mortality independent of a TB diagnosis. This argues for a move away from the historically vertical management of diseases in resource-poor settings and strive towards the UN Sustainable Development Goal of achieving universal health coverage by focusing on building robust healthcare systems.

The strengths of this study are the prospective cohort design, the relatively high number of TB and non-TB patients, and the low number of patients lost to follow-up. In addition, a wide range of diagnostic tools for TB were available, including culture, Xpert and response to treatment, which constitutes a CRS. One limitation is that the diagnosis of DM was largely based on an elevated RBG and postprandial blood glucose test to categorize patients into DM and non-DM. This could both overestimate and underestimate the prevalence of DM in our cohort, as a single measurement of elevated RBG could represent transient hyperglycemia due to infection in the body [43]. Another limitation is the small sample size in some of the subanalyses, especially in the pediatric cohort, yielding low power to these calculations. Finally, the generalizability of the results is limited as this is a single center study in India among a predominantly rural population and may not be transferrable to other settings and populations.

In conclusion, mortality in our cohort was low irrespective of EPTB diagnosis. Age, site of infection and comorbidities were associated with increased odds of hospitalization and mortality in both TB and non-TB patients. Systemic symptoms, HIV and DM were independently associated with mortality in EPTB patients. Our research highlights the significant impact of the double burden of TB and NCDs in low-and middle-income countries and supports the continued focus on strengthening healthcare systems to achieve universal health coverage.

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Author contributions

OMBH performed data curation, formal analysis, wrote the first draft and finalized the manuscript. MK performed data collection, investigations, sampling and reviewed the manuscript. EG and SH contributed to data analysis and critically reviewed the manuscript. MRP organized the study, contributed towards data collection, sampling and edited the manuscript. TM conceptualized the study, acquired funds for the study, developed the methodology, developed the analysis plan, supervised and edited the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Disclosure statement

The authors have no conflict of interest to declare.

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Data availability statement

Data generated and analyzed during the study is available in the article and supplementary material. Further details are available from the corresponding author upon a reasonable request.

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ORIGINAL ARTICLE

Diagnosing adult and pediatric extrapulmonary tuberculosis by MPT64 antigen detection with immunohistochemistry and immunocytochemistry using reproduced polyclonal antibodies

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Abstract

Diagnosing extrapulmonary tuberculosis (EPTB) is challenging. Immunohistochemistry or immunocytochemistry has been used to diagnose tuberculosis (TB) by detection of MPT64 antigen from various extrapulmonary specimens and has shown good diagnostic performance in our previous studies. The test can distinguish between disease caused by *Mycobacterium tuberculosis* (Mtb) complex and nontuberculous mycobacteria and can be applied on formalin-fixed paraffin-embedded tissue. As the antibodies previously used were in limited supply, a new batch of polyclonal antibodies was developed for scale-up and evaluated for the first time in this study. Our aim was to assess the diagnostic accuracy of the MPT64 test with reproduced antibodies in the high burden settings of Pakistan and India. Patients were enrolled prospectively. Samples from suspected sites of infection were collected and subjected to histopathologic and/or cytologic evaluation, routine TB diagnostics, GeneXpert MTB/RIF (Xpert), and the MPT64 antigen detection test. Patients were followed until the end of treatment. Based on a composite reference standard (CRS), 556 patients were categorized as TB cases and 175 as non-TB cases. The MPT64 test performed well on biopsies with a sensitivity and specificity of 94% and 75%, respectively, against a CRS. For cytology samples, the sensitivity was low (36%), whereas the specificity was 81%. Overall, the MPT64 test showed higher sensitivity (73%) than Xpert (38%) and Mtb culture (33%). The test performed equally well in adults and children. We found an additive diagnostic value of the MPT64 test in conjunction with histology and molecular tests, increasing the yield for EPTB. In conclusion, immunochemical staining with MPT64 antibodies improves the diagnosis of EPTB in high burden settings and could be a valuable addition to routine diagnostics.

Keywords: extrapulmonary tuberculosis; childhood tuberculosis; antigen detection test; immunohistochemistry; immunocytochemistry; composite reference standard

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No conflicts of interest were declared.

Introduction

Diagnosing tuberculosis (TB) has been identified as the weakest link in the TB cascade of care [1], representing a major barrier for achieving the UN sustainable development goals of eradicating TB [2]. Diagnostic challenges are particularly high for extrapulmonary TB (EPTB) due to the varied clinical presentations, the paucibacillary nature of the disease, and the difficulty in obtaining appropriate specimens for analysis [3]. The performance of routine TB diagnostics such as culture and molecular tests is poor in

cell smears and biopsies, which are important samples for laboratory diagnosis of various forms of EPTB. Even the WHO recommended GeneXpert MTB/RIF (Xpert) assay (Cepheid, Sunnyvale, CA, USA) has a sensitivity ranging between 50% and 82% for EPTB depending on the site of disease and type of specimen, using culture as reference standard [4]. In addition, not all sample types can be used in the Xpert instruments, particularly formalin-fixed paraffin-embedded tissue, for which a simple processing method for molecular testing is not widely available [5]. Therefore, EPTB is often diagnosed based on cytology or histopathology alone, with an emphasis on features of granulomatous inflammation. However, these features may also be present in conditions and diseases other than TB [6], and many TB patients may have atypical histopathologic or cytologic changes, making diagnosis difficult [7–9]. Furthermore, the distinction between disease caused by *Mycobacterium tuberculosis* (Mtb) and nontuberculous mycobacteria (NTM) is not possible based on histopathology or cytology. Suboptimal EPTB diagnosis leads to widespread empirical treatment, resulting in either over- or under-treatment. This causes unnecessary patient costs and morbidity, and increases the risk of developing drug resistance to key anti-TB chemotherapy.

To address the need for improved diagnosis of EPTB in cell smears and biopsies, an antigen detection test has been developed for detection of the Mtb specific antigen MPT64 by immunohistochemistry (IHC) or immunocytochemistry (ICC). This antigen is a protein secreted by the Mtb complex and cannot be detected in NTM infections [10,11]. Immunohistochemical and immunocytochemical staining with MPT64 antibodies has shown promising diagnostic performance in a variety of extrapulmonary specimens in case–control laboratory studies and has been validated in cohort-based diagnostic accuracy studies [12–17]. The test method is rapid and robust, and its relative simplicity makes it suitable for implementation in low-resource settings with modest laboratory facilities. Recent results from validation of the MPT64 test in routine diagnostics in the resource-limited and high TB burden areas of Zanzibar and Tanzania show sensitivity and specificity of 69–92% and 78–95%, respectively, against a composite reference standard (CRS) [18–20]. However, the antibodies evaluated in previous studies were in limited supply. As sufficient supply is a prerequisite for a test to be used in routine diagnostics, a strategy has been developed for producing larger volumes of the MPT64 polyclonal antibodies, which can facilitate the use of this test on a large scale [21]. The aim of this study was to assess the performance of the MPT64 test with the reproduced polyclonal antibodies for the diagnosis of EPTB in the high TB burden settings of Pakistan and India, and to compare the results with routine diagnostics including Xpert.

Materials and methods

Ethical considerations

The study was approved by the Regional Committee for Medical Research Ethics in Norway, REK Helse-Vest (2014/46/REK vest), the Ethical Committee in India (IEC 10/2018), and the Institutional Review Board, Al-Aleem Medical College, Lahore, Pakistan (GDEC/234/18). Written informed consent was obtained from all participants. None of the invasive procedures was performed for research purposes only.

Sample inclusion

Samples were collected from patients at two tertiary care hospitals, Chandrikaben Rashmikant Gardi Hospital, associated with R.D. Gardi Medical College (RDGMC) in Ujjain, India, and Gulab Devi Hospital (GDH) in Lahore, Pakistan. New presumptive EPTB patients were prospectively enrolled from the inpatient or outpatient departments of the respective hospitals between April 2018 and February 2020 (Figure 1). Exclusion criteria were insufficient material for examination or receiving anti-tuberculous treatment (ATT) at enrollment or within the past year. Seven biopsies were retrospectively included.

Demographic information was collected using a questionnaire. Patients were interviewed in Hindi, Urdu, or their local language. A clinical examination was performed, and blood samples were taken from all participants.

Patients were followed up regularly until completion of the treatment, or for at least 6 months. During follow-up, clinical parameters were recorded to assess the response to treatment.

Sample collection and processing

Samples were collected from the suspected sites of EPTB infection. Material for fine needle aspirates (FNA) from lymph nodes and superficial masses was collected using a 22G needle on a 10 ml syringe. Four smears were prepared, one for cytology, one for acid-fast bacilli (AFB) detection, and two on flex slides for ICC. The slides for ICC were fixed in absolute ethanol for 1 h and stored at −20 °C until further processing. In addition, the material from the syringe was used for Xpert and

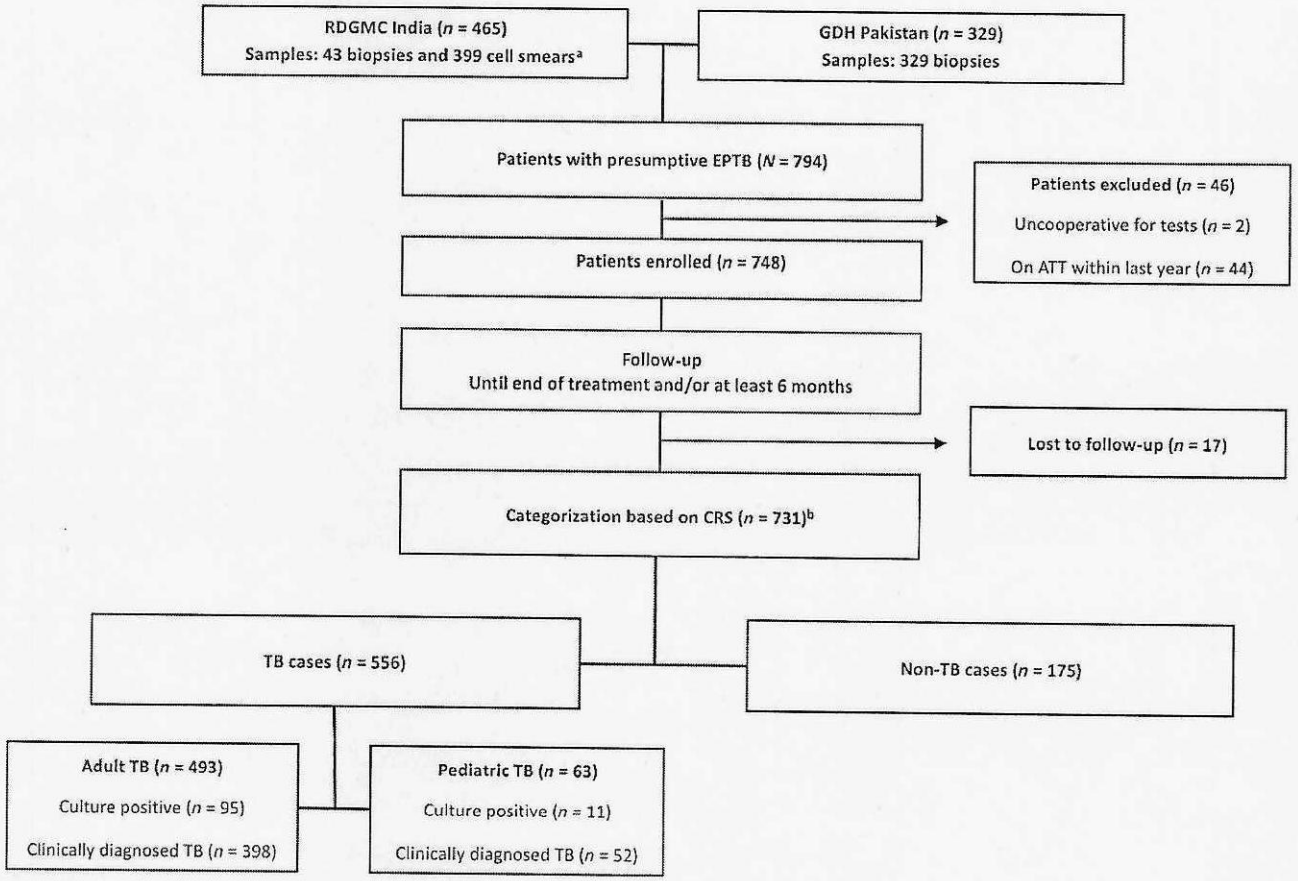

Figure 1. Flowchart of inclusion of patients and categorization. [a]Thirteen cases with both biopsy and cytology samples. [b]Categorization criteria: Culture positive; positive LJ culture or MGIT result. Clinically diagnosed; started on ATT and with a good response at 2/3 months or at end of treatment. Non-TB; improvement without ATT or an alternative diagnosis made by the clinician. Non-TB diagnoses are shown in supplementary material, Table S1. ATT, anti-tuberculous treatment; CRS, composite reference standard; EPTB, extrapulmonary tuberculosis; GDH, Gulab Devi Hospital; LJ, Lowenstein–Jensen; MGIT; mycobacteria growth indicator tube; RDGMC, R.D. Gardi Medical College.

Mtb culture. Fluids were aspirated aseptically and transported to the laboratory for Xpert and Mtb culture, and cell smears were prepared for AFB smear and ICC.

Fresh biopsies were cut in half, one half was fixed in formalin and sent for histopathology and IHC, whereas the other half was sent for AFB microscopy, Mtb culture, and Xpert. Most biopsies were already formalin-fixed in blocks, from which 4-μm-thick sections were prepared for histopathology and IHC. At RDGMC, sputum was also collected from most of the patients for AFB microscopy, Xpert, and Mtb culture.

Diagnostic tests

The Ziehl–Neelsen (ZN) staining method was used for the detection of AFB. Slides for cytologic and histopathologic examination were stained with May–Grünwald–Giemsa and hematoxylin–eosin stains, respectively. Cytomorphology compatible with TB was granulomatous inflammation with or without necrosis, or necrosis without predominance of neutrophils or caseous necrosis. In biopsies, granulomatous inflammation with or without necrosis, or caseous necrosis was considered consistent with TB.

Mycobacterial cultures were performed in the RDGMC or GDH microbiology laboratories. Samples were inoculated on Löwenstein–Jensen (LJ) medium and incubated at 37 °C for 8 weeks. At GDH, both LJ medium and mycobacteria growth indicator tube (MGIT) were used. Xpert was also performed at both sites according to WHO protocols [22].

Immunostaining with MPT64

Pathologists (MRP, MK, and SI) were trained in immunostaining and slide evaluation. The production

of the anti-MPT64 antibodies used in our study is described in detail by Hoel *et al* [21]. In brief, rabbits were immunized to produce polyclonal anti-MPT64 antibodies, and the performance of various adjuvants–antigen combinations was assessed by IHC screening using serial dilutions to identify the optimal working dilution. To overcome the limitation of batch-to-batch variability, the best performing individual sera with antibodies were pooled to produce one large volume of reproduced polyclonal antibodies. The antibodies are commercially available (anti-MPT64, Cat# 157698, Ximbio, London, UK) [23].

In preparation for immunostaining, formalin-fixed biopsies were deparaffinized with xylene before tissue sections and cell smears were rehydrated through decreasing concentrations of alcohol and washed with distilled water (see supplementary material, Protocols for immunochemical staining). Antigen retrieval was performed on biopsy specimens by heat-induced antigen retrieval with citrate buffer, pH 6.2 using a microwave oven (300–400 W) for 15 min. To prevent endogenous peroxidase, tissue sections and cell smears were incubated with peroxidase block (hydrogen peroxide), 3% bovine serum albumin, and 10% normal goat serum. Slides were washed with buffer (Tris buffer, pH 7.6) between each step.

Immunostaining was performed as described previously [12,16], but with some modifications (see supplementary material, Protocols for immunochemistry for full details). Slides were incubated for 1 h with polyclonal anti-MPT64 primary antibodies at dilutions of 1/300 for IHC at RDGMC and 1/250 for IHC at GDH, and 1/200 for ICC together with the Dako Envision + System HRP kit (K4009, Dako, Glostrup, Denmark). In-house methods were used to decide the optimal working dilutions at the different study sites depending on sample type and local conditions. A horseradish peroxidase-conjugated secondary anti-rabbit antibody was then applied for 45 min. The substrates 3-amino-9-ethylcarbazol or diaminobenzidine were applied to the slides for 10 min for cell smears and for 15 min for biopsies. This was followed by counterstaining with hematoxylin and mounting with Immu-Mount (Thermo Fisher Scientific, Waltham, MA, USA) or DPX (Merck KGaA, Darmstadt, Germany). The slides were washed with a wash buffer between incubation steps.

Evaluation of immunostaining

Immunostained slides were evaluated by RDGMC and GDH pathologists (MRP, MK, and SI), who were blinded for the categorization of patients. Slides were screened at ×10 magnification, and a more detailed assessment was performed at ×40 magnification. Slides were classified as positive if brown-reddish granular staining was seen intracytoplasmically in inflammatory cells or extracellularly in necrotic areas (Figure 2).

Categorization of patients

A CRS was used to categorize study participants into the culture confirmed TB, clinically diagnosed TB and non-TB groups based on various diagnostic criteria (Figure 1). In brief, culture-positive patients showed a positive result on LJ medium or MGIT. Clinically diagnosed cases had signs and symptoms suggestive of EPTB and started ATT with a good response at 2/3 months and/or at the end of treatment. Non-TB cases either improved without ATT or the clinician made an alternative diagnosis (supplementary material, Table S1).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 28. The chi square test was performed to assess differences in categorical variables. Sensitivity, specificity, positive predictive values, negative predictive values, and diagnostic accuracy were calculated using cross-tabulations. A p value <0.05 was considered statistically significant. The STARD guidelines for the reporting of diagnostic accuracy studies were followed as closely as possible [24].

Results

Study population

A total of 748 patients were enrolled in the study, and based on the CRS, 556 cases were categorized as TB patients [493 adults and 63 pediatric cases (<15 years of age)], whereas 175 cases were categorized as non-TB patients (139 adults and 36 pediatric cases) (Figure 1). In the TB group, 106 cases were culture confirmed, whereas 450 patients were clinically diagnosed. A significantly higher proportion of TB patients were female. Among adults, TB patients were younger compared with non-TB patients (Table 1). The HIV prevalence was low in both groups.

Adenitis was the most common form of infection in both groups, accounting for 71% and 61% of TB and non-TB cases, respectively (Table 1). Most patients in

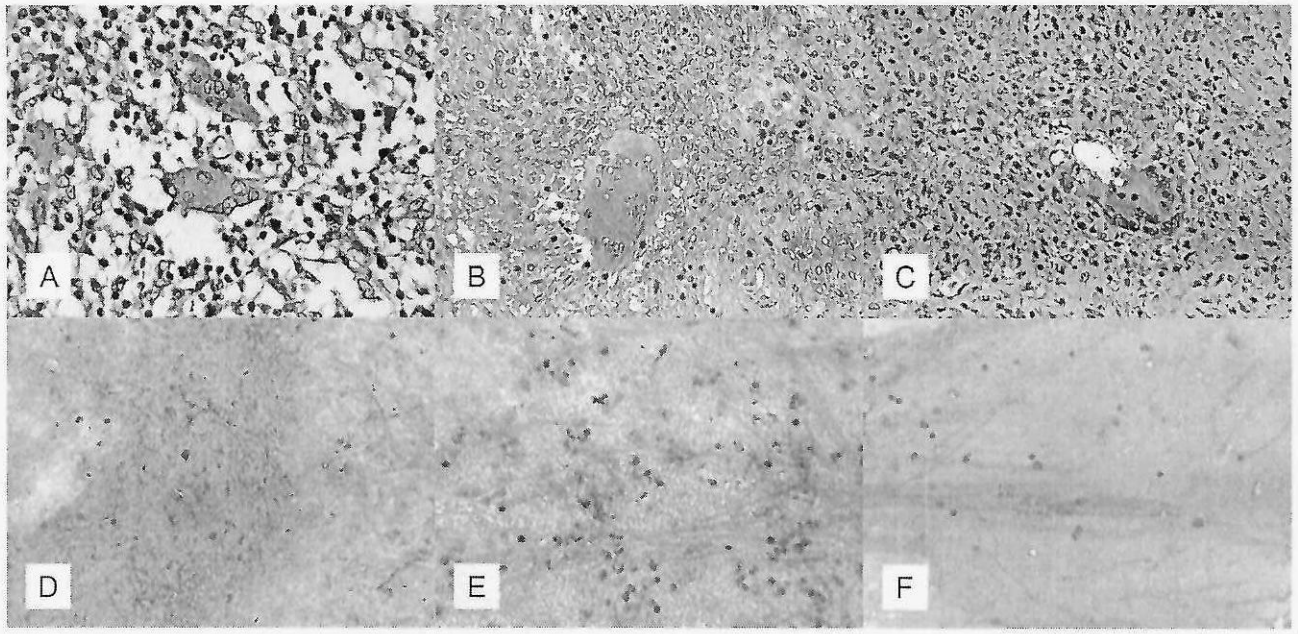

Figure 2. Positive MPT64 staining shown as brown-reddish granular staining. (A) Positive immunohistochemical staining in Langhans type giant cells and lymphocytes from a biopsy of breast tissue. (B) Granuloma with positive immunohistochemical staining of giant cells, epithelioid cells, lymphocytes, and necrotic area in a biopsy from the omentum. (C) Positive immunohistochemical staining with strong signal in Langhans type giant cells, lymphocytes, and necrotic area in biopsy from peripheral lymph node. (D) Positive immunocytochemical staining in lymphocytes and necrotic background in fine needle aspirate from peripheral lymph node. (E) Positive immunocytochemical staining in necrotic area in fine needle aspirate from peripheral lymph node. (F) Positive immunocytochemical staining in lymphocytes in pleural fluid.

the non-TB group improved without ATT and without a reported alternative diagnosis. The most common non-TB diagnoses were abscess ($n = 20$), benign tumors ($n = 10$), and malignancies ($n = 8$) (supplementary material, Table S1).

Performance of all diagnostic tests on biopsies

In biopsies, the MPT64 test showed good diagnostic performance, with an overall sensitivity of 94% (95% CI: 91–97) against a CRS (Tables 2 and 3). The sensitivity was 49% (95% CI: 39–59) for Mtb culture and 69% (95% CI: 56–80) for Xpert. Most biopsies were from lymph nodes and pleural tissue, where the MPT64 test demonstrated good true positive rates of 96% and 94%, respectively (Table 2). The overall specificity of the test in biopsies was 75% (95% CI: 43–95). Positive predictive values, negative predictive values, and diagnostic accuracy were calculated based on the cohort prevalence and are presented in supplementary material, Table S2. For biopsy specimens, the correlation between a positive MPT64 test result and TB histology was good at 95% (supplementary material, Table S3).

Comparison of adult and pediatric biopsy cases

In biopsies, the performance of the MPT64 test was similar between adults and children, with a sensitivity of 94% (95% CI: 91–97) for adults and 95% (95% CI: 83–99) for children (Table 3). Likewise, the performance of ZN, Xpert, and Mtb culture did not differ significantly between adults and children.

Performance of all diagnostic tests on cytology samples

Against a CRS, the sensitivity and specificity of the MPT64 test for cytology specimens were 36% (95% CI: 29–43) and 81% (95% CI: 73–87), respectively (Tables 2 and 3). The test performed best in FNA samples from lymph nodes (sensitivity of 43%), whereas the true positive rate was lower in samples from pleural fluid and cerebrospinal fluid (CSF) (Table 2). Specificity was good in CSF samples at 91% and in FNA at 80% (Table 2). Overall, the sensitivity of the MPT64 test in cytology samples was better than the routine diagnostic tests ZN microscopy, Mtb culture, and Xpert, which had true positive rates of 15% (95% CI: 10–20), 26% (95% CI: 20–32), and

Table 1. Demographic and baseline characteristics of 731 study participants

	Adults			Children		
	TB ($N = 493$)	Non-TB ($N = 139$)	*p* value	TB ($N = 63$)	Non-TB ($N = 36$)	*p* value
	n (%)	*n* (%)		*n* (%)	*n* (%)	
Age (years)						
<5				2 (3)	6 (17)	<0.001
5–9				16 (25.5)	18 (50)	
10–14				45 (71.5)	12 (33)	
15–29	293 (59)	46 (33)	<0.001			
30–44	128 (26)	35 (25)				
>45	72 (15)	58 (42)				
Gender						
Female	275 (56)	61 (44)	0.013	43 (68)	14 (39)	0.004
Male	218 (44)	78 (56)		20 (32)	22 (61)	
HIV positive*	11/457 (2)	4/137 (3)	0.757	0 (-)	0 (-)	-
Site of infection						
Lymphadenitis	340 (69)	80 (57.5)	0.012	56 (89)	27 (75)	0.071
Pleuritis	78 (16)	26 (18.5)	0.418	2 (3)	1 (3)	1.0
Meningitis	19 (4)	25 (18)	<0.001	1 (2)	7 (19)	0.003
Gastrointestinal	10 (2)	1 (1)	0.471	-	-	-
Other†	46 (9)	7 (5)	0.107	4 (6)	1 (3)	0.650
Sample type‡						
Biopsy	304 (62)	16 (12)	<0.001	41 (65)	1 (3)	<0.001
Cytology	202 (41)	123 (88)		22 (35)	35 (97)	
Diagnostic tests						
Xpert positive§	96/250 (38)	0/125 (0)	<0.001	11/32 (34)	0/35 (0)	<0.001
Culture positive‖	95/284 (33)	0/123 (0)	<0.001	11/34 (32)	0/33 (0)	<0.001
ZN positive¶	59/446 (13)	1/130 (1)	<0.001	7/58 (12)	0/35 (0)	0.033

TB and non-TB cases as per CRS. Significant *p* values are shown in bold.
HIV, human immunodeficiency virus; Xpert, GeneXpert MTB/RIF; ZN, Ziehl–Neelsen.
*Results available from 680 samples.
†TB adults: abscess ($n = 24$), urogenital ($n = 8$), osteomyelitis ($n = 6$), breast ($n = 3$), skin ($n = 3$), pericarditis ($n = 1$), synovial fluid ($n = 1$). TB children: abscess ($n = 2$), urogenital ($n = 1$), laryngitis ($n = 1$). Non-TB adults: abscess ($n = 3$), urogenital ($n = 2$), osteomyelitis ($n = 1$), breast ($n = 1$). Non-TB children: osteomyelitis ($n = 1$).
‡Among CRS positive adults, 13 samples have both cytology and biopsy results, thus total percentage exceeds 100% for this group.
§Valid results available from 442 samples.
‖Valid results available from 474 samples.
¶Valid results available from 669 samples.

29% (95% CI: 23–36), respectively (Table 3). When a positive Xpert result and/or a positive MPT64 result was added, the sensitivity increased to 50% (95% CI: 44–57), while the specificity was 82% (95% CI: 75–88) when using CRS as the reference standard (Table 3). The added diagnostic yield of the MPT64 test and Xpert combined in cytology samples was 95% (95% CI: 85–98) in culture positive samples.

Surprisingly, the correlation between cytomorphology suggestive of tuberculous lymphadenitis (TBLA) and a positive MPT64 result was low (45%) (supplementary material, Table S3).

Comparison of adult and pediatric cytology cases

In cytology specimens, the MPT64 test showed a non-significantly higher sensitivity in adults compared with pediatric cases [37% (95% CI: 30–44) versus 24% (95% CI: 8–47)] (Table 3) while the specificity was higher among pediatric samples [78% (95% CI: 69–86) versus 91% (95% CI: 75–98)]. The performance of ZN, Xpert, and Mtb culture was similar in the two groups.

Comparison of diagnostic tests

The Venn diagram (Figure 3) shows a direct comparison of the diagnostic tests for EPTB in samples with valid results from all three tests. In cytology samples, the tests identified different patients, with 39 MPT64 positive cases being negative on Xpert and Mtb culture, while 23 patients were positive on all three tests. In biopsies with valid results from all tests, all Xpert and/or culture positive samples were also positive with the MPT64 test, with an additional six cases diagnosed with the MPT64 test only.

Table 2. Results of diagnostic tests in pediatric and adult extrapulmonary samples

	ZN	Culture	Xpert	MPT64
TB samples ($N = 556$)	66/503 (13)	106/318 (33)	107/282 (38)	392/539 (73)
All biopsy samples ($N = 345$)	33/293 (11)	52/106 (49)	42/61 (69)	320/339 (94)
Adult biopsies	27/257 (11)	45/93 (48)	36/51 (71)	282/299 (94)
Pediatric biopsies	6/36 (17)	7/13 (54)	6/10 (60)	38/40 (95)
Sites, all ages				
Lymph node biopsies	29/232 (13)	50/91 (55)	25/39 (64)	244/255 (96)
Pleural biopsies	3/28 (11)	0/7 (0)	6/9 (67)	31/33 (94)
Other sites*, biopsies	1/33 (3)	2/8 (25)	11/13 (85)	45/51 (88)
All cytology samples ($N = 224$)	33/224 (15)	55/212 (26)	65/221 (29)	76/212 (36)
Adult cytology samples	32/202 (16)	51/191 (27)	60/199 (30)	71/191 (37)
Pediatric cytology samples	1/22 (5)	4/21 (19)	5/22 (23)	5/21 (24)
Sites, all ages				
FNA lymph nodes	28/148 (19)	42/137 (31)	50/149 (34)	61/142 (43)
Pleural effusions	4/46 (9)	7/45 (16)	8/44 (18)	13/45 (29)
CSF	0/20 (0)	2/20 (10)	3/20 (15)	3/15 (20)
Other sites†, cytology	1/10 (10)	4/10 (40)	4/9 (44)	3/10 (30)
Non-TB samples ($N = 175$)	1/165 (0.6)	0/156 (0)	0/160 (0)	31/156 (20)
All biopsy samples ($N = 17$)	0/7 (0)	0/2 (0)	0/3 (0)	3/12 (25)
Adult biopsies	0/7 (0)	0/2 (0)	0/3 (0)	2/11 (18)
Pediatric biopsies	-	-	-	1/1 (100)
Sites, all ages				
Lymph node biopsies	0/1 (0)	-	-	0/1 (0)
Pleural biopsies	0/4 (0)	0/1 (0)	0/2 (0)	1/4 (25)
Other sites†, biopsies	0/2 (0)	0/1 (0)	0/1 (0)	2/7 (29)
All cytology samples ($N = 158$)	1/158 (0.6)	0/154 (0)	0/157 (0)	28/144 (19)
Adult cytology samples	1/123 (0.8)	0/121 (0)	0/122 (0)	25/112 (22)
Pediatric cytology samples	0/35 (0)	0/33 (0)	0/35 (0)	3/32 (9)
Sites, all ages				
FNA lymph nodes	1/106 (0.9)	0/102 (0)	0 (0)	20/101 (20)
Pleural effusions	0/19 (0)	0/19 (0)	0/18 (0)	6/19 (32)
CSF	0/32 (0)	0/32 (0)	0/32 (0)	2/23 (9)
Other sites§, cytology	-	-	-	0/1 (0)

TB and non-TB cases as per CRS. Data are *n*/*N* (%). Thirteen TB cases had MPT64 results from both biopsy and cytology samples.
CSF, cerebrospinal fluid; Culture, *Mycobacterium tuberculosis* culture; FNA, fine needle aspirate; Xpert, GeneXpert MTB/RIF; ZN, Ziehl–Neelsen.
*Abscess ($n = 24$), urogenital ($n = 9$), bones ($n = 6$), gastrointestinal ($n = 6$), skin ($n = 3$), pericardial ($n = 1$), larynx ($n = 1$), breast ($n = 1$).
†Ascites ($n = 5$), pus ($n = 3$), abscess ($n = 1$), synovial fluid ($n = 1$).
†Abscess ($n = 3$), bones ($n = 2$), urogenital ($n = 2$), breast ($n = 1$).
§Ascitic fluid ($n = 1$).

Discussion

This is the first study to evaluate the diagnostic accuracy of the MPT64 antigen detection test on cytology and biopsy specimens by ICC and IHC using a reproduced batch of polyclonal antibodies. Against a CRS, the overall sensitivity of the test was better than Xpert and culture. The test performed best on biopsy specimens with a sensitivity of 94%, and results were equally good in adults and children. In cell smears from pus, FNA, and effusions, the sensitivity of the MPT64 test was low but higher than Xpert and culture against a CRS. The specificity of the MPT64 test was particularly good in pediatric cytology specimens (91%), and lower in adults (78%). Thus, the MPT64 test in combination with routine diagnostics can increase the diagnostic yield for EPTB in high burden settings.

Although primarily considered a pulmonary disease, about 17% of the estimated 10.6 million annual global TB cases are extrapulmonary [25]. However, this may be a low estimate because of the difficulty in confirming EPTB diagnosis and the reporting of coexisting extrapulmonary and pulmonary TB disease as pulmonary TB only in many high burden countries. Furthermore, the proportion of EPTB is increasing in many regions [26,27]. In Western Europe, for example, up to 53% of TB cases are extrapulmonary [27]. Even though EPTB is not contagious, it still causes significant morbidity and has serious economic consequences for patients and their families [28,29]. Improving EPTB diagnosis is therefore important in both low- and high-prevalence areas.

Table 3. Diagnostic validation of tests for EPTB, compared to a CRS and culture

	Against CRS			Against culture		
	n	Sensitivity (95% CI)	Specificity (95% CI)	*n*	Sensitivity (95% CI)	Specificity (95% CI)
Biopsy samples						
All patients						
Xpert	64	69 (56–80)	100 (29–100)	35	71 (29–96)	29 (13–49)
Culture	108	49 (39–59)	100 (16–100)	–	–	–
ZN microscopy	300	11 (8–15)	100 (53–100)	94	20 (10–35)	90 (78–97)
MPT64	351	94 (91–97)	75 (43–95)	107	100 (93–100)	2 (0.05–10)
Xpert and/or MPT64	353	94 (91–97)	75 (43–95)	107	100 (93–100)	2 (0.05–10)
Adults						
Xpert	54	71 (56–83)	100 (29–100)	29	80 (28–99)	29 (13–51)
Culture	95	48 (38–59)	100 (16–100)	–	–	–
ZN microscopy	264	11 (7–15)	100 (59–100)	83	13 (4–27)	88 (74–96)
MPT64	310	94 (91–97)	82 (48–98)	94	100 (92–100)	–[†]
Xpert and/or MPT64	312	94 (91–97)	82 (48–98)	94	100 (92–100)	–[†]
Pediatric cases						
Xpert	10	60 (26–88)	–[*]	6	50 (1–99)	25 (1–81)
Culture	13	54 (25–81)	–[*]	–	–	–
ZN microscopy	36	17 (6–33)	–[*]	11	80 (28–99)	100 (54–100)
MPT64	41	95 (83–99)	0 (0–97)	13	100 (59–100)	17 (0.4–64)
Xpert and/or MPT64	41	95 (83–99)	0 (0–98)	13	100 (59–100)	17 (0.4–64)
Cytology samples						
All patients						
Xpert	378	29 (23–36)	100 (98–100)	362	93 (82–98)	96 (93–98)
Culture	366	26 (20–32)	100 (98–100)	–	–	–
ZN microscopy	382	15 (10–20)	99 (97–100)	366	47 (32–59)	98 (96–99)
MPT64	356	36 (29–43)	81 (73–87)	340	46 (33–60)	75 (70–80)
Xpert and/or MPT64	382	50 (44–57)	82 (75–88)	366	95 (85–98)	74 (69–79)
Adults						
Xpert	321	30 (24–37)	100 (97–100)	308	92 (81–98)	95 (92–98)
Culture	312	27 (21–34)	100 (97–100)	–	–	–
ZN microscopy	325	16 (11–22)	99 (96–100)	312	49 (35–63)	98 (96–99)
MPT64	303	37 (30–44)	78 (69–85)	290	46 (32–61)	73 (67–78)
Xpert and/or MPT64	325	52 (45–59)	80 (71–86)	312	94 (84–99)	72 (66–77)
Pediatric cases						
Xpert	57	23 (8–45)	100 (90–100)	54	100 (40–100)	98 (89–100)
Culture	54	19 (5–42)	100 (89–100)	–	–	–
ZN microscopy	57	5 (0.1–23)	100 (90–100)	54	25 (0.6–81)	100 (93–100)
MPT64	53	24 (8–47)	91 (75–98)	50	50 (7–93)	87 (74–95)
Xpert and/or MPT64	57	36 (17–59)	91 (77–98)	54	100 (40–100)	86 (73–94)

CI, confidence interval; CRS, composite reference standard; EPTB, extrapulmonary tuberculosis; Xpert, GeneXpert MTB/RIF; ZN, Ziehl-Neelsen.
[*]No non-TB cases in pediatric biopsy cases for Xpert, culture, and ZN when using a CRS.
[†]No non-TB cases in this group.

The sensitivity of the MPT64 test in biopsies was good (94%), confirming previous studies [12,13]. The MPT64 test performed better than AFB microscopy, Xpert, and Mtb culture with sensitivities of 11%, 49%, and 69%, respectively, against a CRS. We hypothesize that the superior sensitivity of MPT64 compared with Xpert and Mtb culture in our study could be explained by antigen accumulation in the host cells at the site of infection. This phenomenon has been described as a central process in TB pathogenesis and tissue destruction [30]. MPT64 antigen has been found to accumulate in granuloma structures, suggesting a role in the persistence of chronic infection [31]. Thus, the accumulated antigen in the lesions is the most plausible explanation for the superior performance of the MPT64 antigen detection test.

The advantage of the MPT64 test on biopsy specimens is not only in differentiating TB from other granulomatous diseases, but also in the detection of other diagnoses such as cancers by the histopathologic evaluation of the lesions. In a high TB burden setting, the main differential diagnoses for conditions with granulomatous inflammation are fungal infections, NTM infections, and sarcoidosis, with very different treatment options [6,32]. Especially, NTM infections have become relevant in recent years due to the high

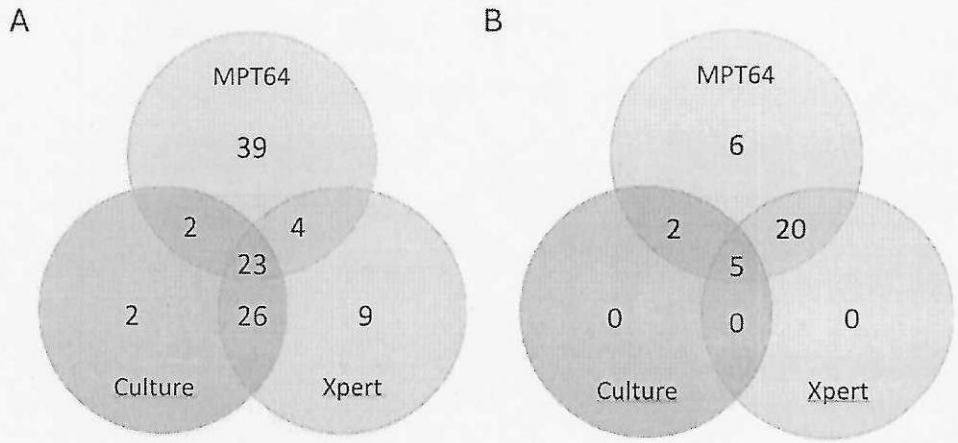

Figure 3. Head-to-head comparison of diagnostic tests for extrapulmonary tuberculosis. Venn diagrams including samples with valid results from all three tests. (A) Cytology samples ($n = 105$). Ziehl–Neelsen (ZN) microscopy was positive in 30 samples. (B) Biopsy samples ($n = 33$). ZN microscopy was positive in three samples.

prevalence in HIV-infected individuals. As the MPT64 antigen is specific to the Mtb complex and is not present in NTM infections, the test could be useful to distinguish these diseases. Furthermore, our study showed a high correlation between histopathologic patterns suggestive of TB and a positive MPT64 result, suggesting that the MPT64 test in conjunction with histopathologic descriptions could aid the pathologist in diagnosing EPTB.

The MPT64 test may be useful in diagnosing 'incidental' TB, which commonly occurs in high prevalence settings due to the nonspecific clinical presentation of the various forms of EPTB [33,34]. Postoperative biopsies are often fixed in formalin and therefore not suitable for Xpert investigation [5] and culture but can be evaluated by the MPT64 test. Furthermore, with logistic support, sampling could be done in peripheral areas and, after fixation, the slides could be transported to a central laboratory for evaluation. These unique features make the MPT64 test a useful supplement to routine diagnostics for EPTB.

The sensitivity of the MPT64 test in cytology specimens was low in our study (36%), contrasting the results of our previous studies, which showed sensitivities of 69–92% [18–20]. This could be explained by the difference in primary polyclonal antibodies. In all previous studies, we used an in-house polyclonal antibody which was available in limited amounts. With the intention for scale-up of the test, we reproduced the primary antibody and produced a single batch of this new polyclonal antibody in large amounts [21]. Since polyclonal antibodies recognize and bind to different epitopes of the antigen in question, the performance of the reproduced primary antibodies may differ from those previously used. In addition, the performance of the MPT64 test may be affected by differences in the *in vivo* epitope expression due to differences in fixation techniques and sample preparation. Formalin fixation is known to alter the three-dimensional conformation the epitopes in biopsies [35] and, although antigen retrieval steps reverse this, it can still affect the epitope expression. For cytology specimens, alcohol fixation and the long storage time in a freezer could affect the epitope affinity. Although the overall performance of the MPT64 test on cytology samples was low, it still performed better than the routine diagnostic tests AFB microscopy, culture, and Xpert.

Fine needle aspiration cytology (FNAC) has become an important tool in diagnosing TB, especially in TBLA. However, the traditional definition of cytomorphology suggestive of TB is challenged by studies finding a high proportion of TB patients without the typical finding of granulomatous inflammation on FNAC [36]. Particularly, FNAC samples showing necrosis without granulomas are a major challenge for the pathologist to rule out TB in an endemic setting. The MPT64 test correctly identified a large proportion (76%) of TB patients with necrosis without granulomatous inflammation on cytology (supplementary material, Table S3) and combining the MPT64 result with cytologic descriptions increased the diagnostic yield (supplementary material, Table S4). Surprisingly, the specificity of the test was low in FNA samples with cytology suggestive of TB, adding little value in confirming TB in this patient group

(supplementary material, Table S3). In a TB endemic setting, some false positive results could be due to concomitant TB antigens in the samples of non-TB cases. Furthermore, the natural course of TBLA may be relapsing and remitting [37], which could lead to misclassification of non-TB patients. However, despite its limitations, FNAC is an important tool in the diagnosis of EPTB in a high burden setting due to its simplicity and good sensitivity.

The performance of the MPT64 test was similar between adult and pediatric cases in our study. Childhood TB has been largely neglected in the literature and poses a particular diagnostic challenge [38,39]. The potential role of the MPT64 test as a confirmatory test for pediatric TBLA has been suggested based on promising results [19,20]. In our study, the sensitivity of the MPT64 test for diagnosing TBLA in children was only 25% (supplementary material, Table S4), but the specificity was excellent at 96%, suggesting an additive value of the MPT64 test in diagnosing pediatric TBLA.

The MPT64 test performed better than Xpert in our study, compared with a CRS. WHO-endorsed rapid molecular tests such as the Xpert/Xpert Ultra and TrueNat (Molbio, Goa, India) are recommended for the timely diagnosis of TB [40]. However, these tests do not perform equally well on all sample types, limiting the use for most forms of EPTB [4]. The MPT64 test, although not as rapid as Xpert, could provide a result within 1–2 days, which could significantly reduce the diagnostic delay in EPTB. We found that adding the results of Xpert and the MPT64 test increased the diagnostic yield for all types of EPTB. Our findings support the use of the MPT64 test along with molecular testing to increase sensitivity in presumptive EPTB while still providing a rapid diagnosis.

The MPT64 test, Xpert, and culture identified different patients and thus complemented each other in cytology samples. Early bacterial multiplication before sufficient antigen accumulation could explain some of the cases with a positive culture result but negative immunostaining. By combining molecular tests detecting DNA with antigen detection tests, the specimens are subjected to two fundamentally different diagnostic methods, which could increase the diagnostic yield for EPTB.

We found good correlation of test performance on biopsies between the two study sites, suggesting that the MPT64 test is robust and reliable between sites (supplementary material, Table S5). The test depends on invasive sampling and basic laboratory facilities being available, which are not always present in rural health care facilities in endemic countries. However, prioritizing the strengthening of laboratory structures would improve the health care services in general, and increase the diagnostic possibilities for a wide range of diseases. Furthermore, the immunochemical staining technique is a valuable asset in the diagnosis of other conditions, most importantly cancers.

The major strengths of this study are the high number of EPTB cases enrolled at two centers with good laboratory facilities in high TB burden settings, the long follow-up period, and inclusion of clinically diagnosed cases. However, there are limitations to our study. The use of a CRS to compensate for the imperfection of culture in diagnosing EPTB comes with the risk of misclassification. Especially the use of the rather broad antibiotic rifampicin in first-line ATT could lead to patients with other bacterial infection being misclassified as TB patients. In addition, many cases labeled as non-TB lacked an alternative diagnosis, which could particularly affect the specificity of the test. Furthermore, not all samples were subjected to the same number of reference tests, which could overestimate and underestimate the diagnostic performance of the test. In addition, the COVID-19 pandemic temporarily disrupted the supply of key reagents for MPT64 immunochemistry, resulting in prolonged storage of samples in a freezer, which could influence epitope presentation.

In conclusion, diagnosing EPTB is challenging and there is a need for improved diagnostics, especially for childhood EPTB. We have shown that the MPT64 antigen detection test has a higher diagnostic yield than Xpert and culture in diagnosing EPTB in high burden settings against a CRS. The test performed particularly well on biopsy specimens and may aid pathologists to diagnose EPTB in conjunction with routine TB diagnostics and histopathology. Although sensitivity was low in cytology samples, the high specificity in pediatric FNA specimens suggests a role for the MPT64 test to support the diagnosis of childhood TB. We found an additive value of combining the MPT64 test and Xpert in diagnosing EPTB. Further feasibility studies and cost–benefit analyses are needed to determine the potential role of the MPT64 test in the routine diagnosis of EPTB.

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Author contributions statement

OMBH performed data curation, formal analysis, wrote the first draft and finalized the manuscript. MK performed data collection, investigations, sampling, interpretation of the staining and reviewed the manuscript. SI performed data collection, description and interpretation of the staining and reviewed the manuscript. AA assisted with data collection, microbiological analysis and edited and reviewed the manuscript. MRP organized the study, contributed toward data collection, sampling, description and interpretation of the staining and edited the manuscript. TM conceptualized the study, acquired funds for the study, developed the methodology, developed the analysis plan, supervised and edited the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Data availability statement

Data generated and analyzed during the study are available in the article and supplementary material. Further details are available from the corresponding author upon reasonable request.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1. CRS and categorization of patients

Table S2. Diagnostic validation of tests for EPTB in adults and children

Table S3. Positive MPT64 results and their correlation to cytomorphologic and histopathologic descriptions

Table S4. Diagnostic accuracy of cytology, routine TB diagnostics, and the MPT64 test on FNA samples in adults and children

Table S5. Comparison of MPT64 diagnostic accuracy in biopsies from Pakistan and India

Protocols for immunochemical staining at RDGMC and GDH