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Family Medicine and Population Health

Improving the management of hospitalized presumptive Xpert MTB/RIF-negative tuberculosis cases in Ethiopia

Nederlandse titel: Verbetering van de aanpak van Xpert MTB/RIF-negatieve gehospitaliseerde patienten met vermoeden van tuberculose in Ethiopië

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List of Abbreviations

AFB	Acid-Fast Bacilli
ART	Antiretroviral therapy
ASV	Amplicon Sequence Variants
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Corona Virus Infectious Disease-19
CRP	C-reactive protein
CT	Cycle threshold
CXR	Chest X-Ray
DADA2	Divisive Amplicon Denoising Algorithm 2
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
Fuji LAM	Fujifilm SILVAMP TB LAM
GVIF	Generalized variance-inflation factor
HIV	Human Immunodeficiency Virus
INH	Isoniazid
JUMC	Jimma University Medical Center
LF-LAM	Lateral Flow urine Lipoarabinomannan
LJ	Solid Lowenstein-Jensen
LOD	Low limit of detection
LRTI	Lower Respiratory trac Infection
LTBI	Latent Tuberculosis Infection
MDR-TB	Multi-Drug Resistance Tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
MRC	Mycobacteriology Research center
MTB	*Mycobacterium tuberculosis*
NAAT	Acid Amplification Tests
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
RSV	Respiratory Syncytial Virus

RRDR	Rifampicin resistance determining region
SDA	Sabouraud Dextrose Agar
STEP	Steps wise approach to non-communicable disease surveillance
TB	Tuberculosis
TB/HIV	Tuberculosis/Human Immunodeficiency Virus co-infection
TB-LAMP	Tuberculosis Loop-mediated Isothermal Amplification
WHO	World Health Organization
W4SS	WHO four-symptom screen
Xpert Ultra	Xpert MTB/RIF Ultra
16S rRNA	16S ribosomal RNA

Summary

Ethiopia is one of the 30 countries in the world with a high burden of tuberculosis (TB). While the country has made notable progress in TB management over the past decade, only 61% of pulmonary cases are bacteriologically confirmed. Consequently, empiric TB treatment was initiated in 39% of cases. While empiric treatment is beneficial for patients who truly have TB, it has been shown that it does not reduce overall mortality among people presenting with signs and symptoms of TB and may even increase the risk of death when other diagnoses are overlooked. To improve the management of presumptive TB cases, defined as persons presenting with signs or symptoms of TB in whom the presence of *Mycobacterium tuberculosis* could not be detected, it is important to understand the ability of the diagnostic algorithm to detect TB disease and ascertain the influence of empiric therapy on survival in people with a negative Xpert MTB/RIF result. It is also essential to accurately discriminate pulmonary TB from other pulmonary infections in ill, hospitalized cases in settings such as Ethiopia, where the health infrastructure is limited.

Therefore, the overall goal of this PhD study was to improve the management of hospitalized presumptive Xpert MTB/RIF-negative TB cases in Ethiopia by evaluating the role of empiric TB treatment in settings where Xpert MTB/RIF is used as a diagnostic test for people with presumptive pulmonary TB, the value of switching from the Xpert MTB/RIF assay to the more sensitive Xpert MTB/RIF ultra (Xpert Ultra) assay to diagnose pulmonary TB, and the role of chest X-ray and Lateral flow urine lipoarabinomannan (LF-LAM) in settings where Xpert Ultra is available. Moreover, in patients with presumptive TB in whom the presence of TB was excluded, we aimed to better understand the potential bacterial pathogen most likely to be responsible for the patients' lower respiratory tract infections (LRTIs) to develop optimal diagnostic and treatment strategies.

To achieve this goal, we enrolled a cohort of 250 people presenting with symptoms or signs of TB who had a negative Xpert MTB/RIF result, the initial diagnostic used in Ethiopia. Of these 250 participants, 125 were consecutive hospitalized patients initiated on empiric TB treatment and 125 participants consecutive hospitalized patients who were not started on TB treatment.

We first assessed the ability of diagnostic algorithms to detect TB disease in people who screened negative on the Xpert MTB/RIF assay and found that the clinical algorithm consisting of clinical

response to an antibiotic trial and chest X-ray findings led to identification of 15.6% of microbiologically confirmed TB cases. Even though confirmed TB was more prevalent in those patients in whom the physician decided to start empiric TB treatment (24.8% vs 6.4%), empiric TB treatment led to substantial overtreatment of TB and had no effect on 6-month survival (aOR 0.74, 95% CI: 0.1-2.7).

The use of the more sensitive Xpert Ultra assay diagnosed 89% of all bacteriologically confirmed cases of TB in our study population. The odds of confirmed TB were two to five times as higher in patients with chest X-ray findings suggestive of TB, but chest X-ray had poor sensitivity (69% when read by clinicians and 79.5% when read by a radiologist) and the addition of chest X-ray to a simplified TB risk prediction tool based on clinical symptoms did not improve its performance (AUC: 84.5% to 85.6% vs 84% to 86%). Among the HIV positive (n = 52) and severely ill (n = 16) patients, the LF-LAM assay failed to detect any case that was not detected by Xpert Ultra.

Among patients with presumptive TB in whom the presence of TB was excluded by the Xpert Ultra assay, 16S rRNA gene amplicon sequencing identified the pathogen responsible for the patients' LRTI in 6.0% (13/215) of cases. *Mycoplasma pneumoniae* (n = 7), *Bordetella pertussis* (n = 2), *Acinetobacter baumanii* (n = 2), and *Pseudomonas aeruginosa* (n = 2) appeared to be possible causes of the TB-like symptoms. In other patients, putative pathogens were present but the findings were similar in sputum samples from patients diagnosed with active TB (Xpert Ultra positive) and samples from patients without TB (Xpert Ultra negative). The presence of *Streptococcus (pseudo) pneumoniae* was associated with higher odds of radiological abnormalities (aOR 2.5, 95% CI 1.12–6.16), and the presence of *Streptococcus (pseudo) pneumoniae* (aOR 5.31, 95% CI 1.29–26.6) and *Moraxella catarrhalis/nonliquefaciens* (aOR 12.1, 95% CI 2.67–72.8) with higher odds of six-month mortality, suggesting that these pathogens may have clinical relevance.

In conclusion, even though the currently used clinical algorithm identified most of the bacteriologically confirmed cases, it has suboptimal performance when used in the context where Xpert MTB/RIF is the first diagnostic. The positive predictive value of the algorithm was low (24.8%), indicating a substantial number of false-positive results; it failed to detect 6.4% of people with active TB, leading to delayed or missed treatment initiation; and 75% of patients who started empiric TB treatment were culture-negative, suggesting overtreatment. Adding chest X-ray readings to the decision algorithm did not significantly improve clinicians' ability to predict bacteriologically confirmed TB beyond what is possible through clinical judgment. The use of LF-LAM did not yield any

benefit when used in combination with Xpert Ultra in HIV-positive presumptive pulmonary TB cases. Nevertheless, even in settings with access to Xpert Ultra, patients who are unable to produce sputum may still benefit from the use of the LF-LAM assay. Countries with high TB and HIV burdens, like Ethiopia, should urgently replace Xpert MTB/RIF with the highly sensitive Xpert Ultra assay for the diagnosis of TB. The use of LF-LAM in hospitalized HIV-positive patients with suspected TB should be avoided in settings where Xpert Ultra is available, but it may still be beneficial for those unable to produce sputum. To improve clinical judgment skills, National TB Program (NTP) should provide professional development training and keep healthcare providers updated on other bacterial pathogens causing LRTIs. The routine practice of administering an empiric TB treatment to patients negative for the Xpert Ultra test should be re-evaluated. Future research using tools with higher discriminatory power than 16S rRNA sequencing is needed to identify with greater accuracy the bacterial, viral, and fungal pathogens that may cause LRTI in Xpert Ultra-negative patients.

Samenvatting

Ethiopië is een van de 30 landen in de wereld met een hoge last van tuberculose (tbc). Hoewel het land de afgelopen tien jaar opmerkelijke vooruitgang heeft geboekt op het gebied van tbc-beheer, wordt slechts 61% van de gevallen met een vermoeden van tbc bacteriologisch bevestigd. Bijgevolg wordt in 39% van de gevallen een empirische tbc-behandeling gestart. Hoewel een empirische behandeling gunstig kan zijn voor patiënten die tbc hebben, is aangetoond dat het de algehele mortaliteit niet verlaagt onder gevallen met tekenen en symptomen van tbc en kan het zelfs het risico op overlijden verhogen wanneer andere diagnoses over het hoofd worden gezien. Om het beheer van vermoedelijke tbc-gevallen, gedefinieerd als personen met tekenen of symptomen van tbc bij wie de aanwezigheid van *Mycobacterium tuberculosis* niet kon worden gedetecteerd, te verbeteren, is het belangrijk om het vermogen van het diagnostische algoritme te begrijpen om tbc-ziekte te detecteren en de invloed ervan vast te stellen. Tevens is het essentieel om longtuberculose nauwkeurig te onderscheiden van andere longinfecties bij zieke, gehospitaliseerde gevallen in omgevingen zoals Ethiopië, waar de gezondheidsinfrastructuur beperkt is.

Daarom was het algemene doel van dit doctoraatsonderzoek om het beheer van in het ziekenhuis opgenomen vermoedelijke Xpert MTB/RIF-negatieve tbc-gevallen in Ethiopië te verbeteren aan de hand van drie methodes:

- Evalueren van de rol van empirische tbc-behandelingen in omgevingen waar Xpert MTB/RIF wordt gebruikt als een diagnostische test voor mensen met vermoedelijke longtuberculose,
- De waarde bepalen van overschakelen van de Xpert MTB/RIF-assay naar de meer gevoelige Xpert MTB/RIF ultra (Xpert Ultra)-assay om longtuberculose te diagnosticeren,
- Evalueren van de rol van röntgenfoto's van de borstkas en lipoarabinomannan (LF-LAM) in instellingen waar Xpert Ultra beschikbaar is.

Bovendien werd getracht bij patiënten met vermoedelijke tuberculose bij wie de aanwezigheid van tuberculose was uitgesloten, een beter begrip te krijgen van de potentiële bacteriële ziekteverwekker die hoogstwaarschijnlijk verantwoordelijk is voor de lage luchtweginfecties (LLWI's) om optimale diagnostische en behandelingsstrategieën te ontwikkelen.

Om dit doel te bereiken, werd een cohort van 250 mensen ingeschreven met symptomen of tekenen van tuberculose die een negatief Xpert MTB/RIF-resultaat hadden, de eerste diagnose die in Ethiopië wordt gebruikt. Van deze 250 deelnemers waren er 125 achtereenvolgende gehospitaliseerde patiënten die waren gestart met empirische tbc-behandeling en 125 achtereenvolgende gehospitaliseerde patiënten die niet waren begonnen met een tbc-behandeling.

Eerst werd het vermogen beoordeeld van diagnostische algoritmen om tbc-ziekte te detecteren bij mensen die negatief screenden op de Xpert MTB/RIF-assay. Zo werd ontdekt dat het klinische algoritme bestaande uit de klinische respons op een antibioticumonderzoek en bevindingen op röntgenfoto's van de borst leidde tot identificatie van 15,6% van microbiologisch bevestigde tbc-gevallen. Hoewel bevestigde tbc vaker voorkwam bij die patiënten bij wie de arts besloot om empirische tbc-behandeling te starten (24,8% versus 6,4%), leidde empirische tbc-behandeling tot substantiële overbehandeling en werd geen effect waargenomen op de overleving na 6 maanden (aOR 0,74; 95% BI: 0,1-2,7).

Het gebruik van de meer gevoelige Xpert Ultra-test diagnosticeerde 89% van alle bacteriologisch bevestigde gevallen van tuberculose in deze onderzoekspopulatie. De kans op bevestigde tuberculose was twee tot vijf keer zo hoog bij patiënten met thoraxfoto's die wijzen op tuberculose. Echter, thoraxfoto's zelf hadden een slechte gevoeligheid (69% wanneer gelezen door clinici en 79,5% wanneer gelezen door een radioloog) en de toevoeging van thoraxfoto's aan een vereenvoudigd tbc-risicovoorspellingsinstrument op basis van klinische symptomen verbeterde de prestaties niet (AUC: 84,5% tot 85,6% versus 84% tot 86%). Onder de hiv-positieve (n = 52) en ernstig zieke (n = 16) patiënten kon de LF-LAM-assay geen enkel geval detecteren dat niet door Xpert Ultra werd gedetecteerd.

Onder patiënten met vermoedelijke tuberculose bij wie de aanwezigheid van tuberculose was uitgesloten door de Xpert Ultra-assay, werd aan de hand van 16S rRNA-genampliconsequencing de ziekteverwekker gedetecteerd verantwoordelijk voor de LRTI van de patiënt in 6,0% (13/215) van de gevallen. *Mycoplasma pneumoniae* (n = 7), *Bordetella pertussis* (n = 2), *Acinetobacter baumanii* (n = 2) en *Pseudomonas aeruginosa* (n = 2) bleken mogelijke oorzaken van de tbc-achtige symptomen. Bij andere patiënten waren vermeende pathogenen aanwezig, maar de bevindingen waren vergelijkbaar in sputummonsters van patiënten met de diagnose actieve tuberculose (Xpert Ultra-positief) en monsters van patiënten zonder tuberculose (Xpert Ultra-negatief). De

aanwezigheid van Streptococcus (pseudo) pneumoniae was geassocieerd met een grotere kans op radiologische afwijkingen (aOR 2,5, 95% CI 1,12-6,16), en de aanwezigheid van Streptococcus (pseudo) pneumoniae (aOR 5,31, 95% CI 1,29-26,6) en Moraxella catarrhalis/nonliquefaciens (aOR 12,1, 95% BI 2,67–72,8) met een hogere kans op mortaliteit na zes maanden, wat suggereert dat deze pathogenen klinisch relevant zijn.

Conclusie: hoewel het klinische algoritme de meeste bacteriologisch bevestigde gevallen identificeerde, presteert het suboptimaal wanneer het wordt toegepast in een context waarin Xpert MTB/RIF de eerste diagnose is. De positief voorspellende waarde was laag (24,8%), wat wijst op een aanzienlijk aantal vals-positieve resultaten. Het klinisch algoritme slaagde er niet in om 6,4% van de mensen met actieve tuberculose te detecteren, wat leidde tot een vertraagde of gemiste start van de behandeling. Ongeveer 75% van de patiënten die met een empirische tuberculosebehandeling begonnen, waren cultuurnegatief, wat duidt op overbehandeling. Het toevoegen van röntgenfoto's van de thorax aan het beslissingsalgoritme verbeterde het vermogen van artsen om bacteriologisch bevestigde tuberculose te voorspellen niet significant meer dan wat mogelijk is via klinisch oordeel. Het gebruik van LF-LAM leverde geen enkel voordeel op bij gebruik in combinatie met Xpert Ultra bij HIV-positieve Xpert MTB/RIF-negatieve vermoedelijke gevallen van longtbc. Niettemin kunnen patiënten die geen sputum kunnen produceren, zelfs in omgevingen met toegang tot Xpert Ultra, nog steeds baat hebben bij het gebruik van de LF-LAM-test. Landen met een hoge tuberculose- en hiv-last zouden Xpert MTB/RIF moeten vervangen door de zeer gevoelige Xpert Ultra-test om patiënten die een tuberculosebehandeling nodig hebben beter te kunnen identificeren. Het routinematige gebruik van LF-LAM bij HIV-positieve patiënten met vermoedelijke tuberculose, waarbij Xpert Ultra beschikbaar is, blijft onzeker. Routinematig gebruik van een antibioticaonderzoek en empirische tuberculosebehandeling bij Xpert Ultra-negatieve patiënten moet opnieuw worden geëvalueerd om overmatig gebruik van geneesmiddelen en de ontwikkeling van antibioticaresistentie te voorkomen. Het nationale tbc-programma moet professionele ontwikkelingstrainingen bieden om de klinische beoordelingsvaardigheden te verbeteren, op bewijs gebaseerde besluitvorming te bevorderen en zorgverleners op de hoogte te houden van potentiële bacteriële pathogenen die verantwoordelijk zijn voor LLTI's vóór empirische tbc-behandeling.

Chapter 1: General Introduction

1.1 Etiology and Epidemiology of Tuberculosis

1.1.1 Etiology

On the evening of 24 March 1882 Robert Koch described *Mycobacterium tuberculosis* (MTB) as the etiology of tuberculosis (TB) for the first time at the Physiological Society in Berlin [1]. In recognition of the importance of this day, the International Union against TB and Lung Disease announced 24 March as the 'World TB Day' to raise awareness about the burden of TB worldwide [2].

Koch developed staining methods and solid culture media [3] and showed that MTB is an aerobic, non-motile, acid-fast, slow-growing (with a generation time of 15-20 hours), facultative intracellular pathogen that appears microscopically as straight or slightly curved rods [4, 5]. The thick, lipid rich cell wall provides many unique characteristics of mycobacteria is such as acid-fastness, extreme hydrophobicity, resistance to drying and acidity or alkalinity, as well as distinctive immunostimulatory properties [6, 7].

TB is caused mainly by MTB, and occasionally by other organisms of the MTB complex such as *M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti* and *M. mungi*) [6, 8-10]. *M. bovis* causes less than 2% of all TB cases [11]. *M. africanum* and *M. microti* mainly cause TB in some West African countries, where *M. africanum* (MTB complex lineage 5 and 6) can cause up to 40% of pulmonary TB [12]. Mycobacterial species other than MTB complex or Non-tuberculous Mycobacteria include *M. avium, M. simiae, M. kansasii,* and *M. haemophilu.* These mainly cause disease in immunocompromised persons [13, 14].

1.1.2 Transmission and pathogenesis

MTB is transmitted from person to person via inhalation of droplet nuclei of about 1-5 micron in diameter carrying viable TB bacilli. A droplet nucleus is propelled into the air by a person with pulmonary TB during coughing, sneezing, speaking, laughing or singing [15]. A cough episode may produce as many as 3000 infectious droplet nuclei. The droplet can remain suspended in the air for several hours [15, 16].

The probability of TB transmission depends on the susceptibility of the host, degree of infectiousness of the person with TB disease, and environmental factors such as temperature and humidity and ventilation, and the proximity, duration and frequency of exposure [17, 18]. Upon inhalation of the infectious droplets, most of the larger droplets become lodged in the upper respiratory tract, including nose and throat. The smaller droplet nuclei can reach the alveoli in the lungs, and establish MTB infection [19]. The alveolar macrophages endocytose the MTB bacilli in the lungs, bind the lipoarabinomannan antigen on the cell wall via mannose receptors, and bind opsonized bacilli via complement receptors [20]. Lysis of macrophages that engulf the MTB results in the formation of the Ghon focus, a localized granuloma within the lungs. From the Ghon focus, bacilli drain to the regional lymph nodes. The Ghon focus with associated tuberculous lymphangitis and involvement of the regional lymph nodes is called the primary complex [21]. In people with weakened immune systems, TB bacteria may spread via the blood. At this time the humoral immunity will not able to control the MTB infection because extracellular MTB is resistant to complement killing due to the high lipid content in its cell wall.

The course of the disease depends on the balance between the host cell mediated immunity and the pathogen [22]. An estimated one-quarter of world population is infected with MTB but in the majority (90-95%), cell mediated immunity either kills the bacilli or suppresses replication resulting in latent TB [4]. Most people with latent MTB infection have a lifetime risk of developing TB disease of 5 to 10% [23, 24]. People with HIV, diabetes, or immunosuppressive disorders, those who smoking or abuse alcohol, or with poor nutritional status have a higher risk of developing TB disease [25].

TB mainly affects the lungs and causes pulmonary TB. It can also affect other sites in the body called extra-pulmonary TB [26, 27]. People with extra-pulmonary TB are usually not infectious. People with pulmonary TB are usually infectious and can infect up to 10-15 other people through close contact over the course of a year [28].

1.1.3 Global burden of tuberculosis

According to the 2021 global TB report of the World Health Organization (WHO), 10 million people developed TB and 1.5 million died in 2020, of which 214,000 deaths occurred among people living with HIV [29]. Adults accounted for 89% and children (<15 years old) for 11% of people with TB [29].

The largest proportion of TB cases occurred in the South-East Asian Region (43%), followed by the African (25%), and the Western Pacific region (18%) [29]. Eight countries account for two-thirds of the total number of cases worldwide: India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%) [29].

The Stop TB strategy has resulted in remarkable progress in the past 2 decades (**Figure 1**) [30], but one third of cases remained undiagnosed in 2020. In addition, the strong association with HIV and the increasing burden of drug resistance puts TB among the serious threats that the world faces [31-33]. The impact of the coronavirus infectious disease-19 (COVID-19) pandemic on basic TB services [29] resulted in a drop in TB case notifications of 18% from 2019 to 2020 [34], undoing much of the progress made toward achieving the goal of ending the global TB epidemic. In Ethiopia, TB detection dropped by 11%, bacteriologically-confirmed TB by 12%, TB treatment success rate by 17%, and community health worker engagement in TB detection by 77% during the COVID-19 period [35].

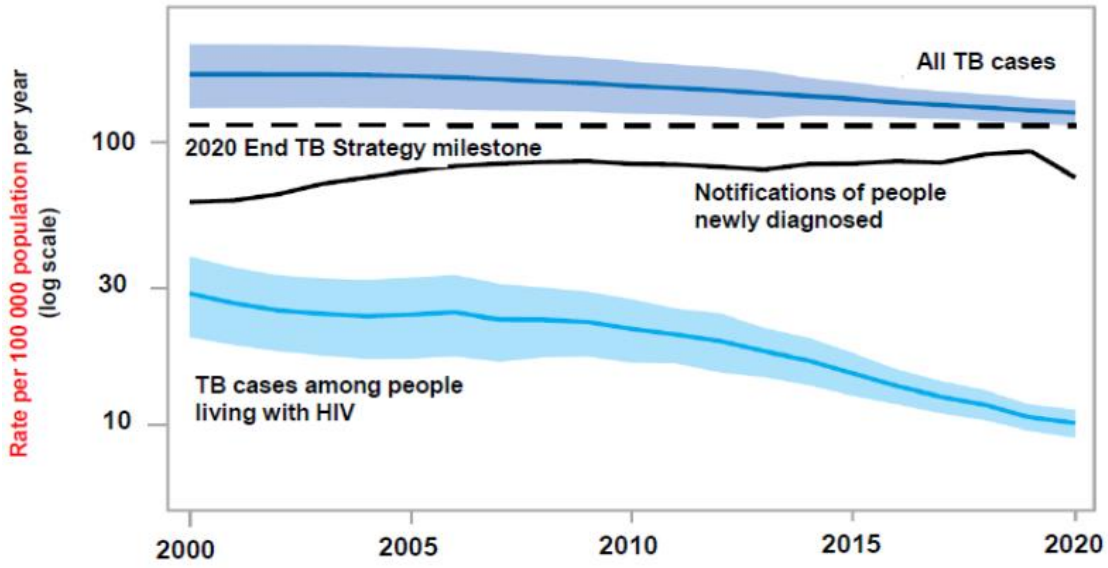

Figure 1: Changes in global TB incidence in the past two decades [29].

1.1.4 Tuberculosis in Ethiopia

1.1.4.1 Epidemiology of TB in Ethiopia

Ethiopia ranks 10th globally regarding the TB burden and is among the 30 high TB/HIV burden countries in the world. Between 2000 and 2020, TB incidence declined by 8-9% annually, from 421

per 100,000 to 132 per 100,000 population. TB mortality in the declined at a slower rate compared to incidence decline [36]. In 2020, 21,200 people died of TB. About 0.71% of new cases and 18% of previously treated cases have multidrug resistant TB (MDR-TB) [29] **(Table 1)**. Only 61% of all cases were bacteriologically confirmed, meaning that 39% of TB cases were started on empiric TB treatment.

Table 1. Statistics of TB in Ethiopia, 2021.

Estimated TB incidence rate[1]	132/100,000 (125-143)
Prevalence rate of smear positive pulmonary TB[2]	108/100,000 (73-143)
Prevalence of bacteriologically confirmed TB[2]	277/100,000 (208-347)
Estimated number of TB cases[1]	
Overall	151, 000 (106-205)
Male	83,000 (45-121)
Female	68,000 (37-100)
Children (0-14 years)	17,000 (11-23)
Adults (>15 years)	134,000 (86-184)
Total number of TB cases notified[1]	112, 600
% Of cases with known HIV status	81%
% Of pulmonary TB cases	70%
% Bacteriologically confirmed	61%
% Rifampicin resistant among new TB cases	0.71% (0.62-0.8)
% Rifampicin resistant among previously treated cases	12% (11-13)
Estimated number of deaths among HIV negative cases[1]	19, 000 (12-28)
Estimated number of deaths among HIV positive cases[1]	2,200 (1.3-3.2)

[1]WHO Global TB report 2021 [29], [2]National TB prevalence survey (2011) [37]. HIV, Human Immunodeficiency Virus.

In addition to HIV co-infection, which contributes to around 10% of notified TB cases in Ethiopia, other health and socio-economic conditions influence the epidemiology and treatment outcomes of TB. Undernutrition fueled by food insecurity is among the main attributing factors in Ethiopia. A study showed that 36% of patients with drug susceptible TB and 44% of those with drug resistant TB had acute severe malnutrition at time of diagnosis. Another study showed that 57% were underweight and 89% have anemia at the time of TB diagnosis [38].

People with uncontrolled Diabetes Mellitus (DM) are also an increased risk of developing TB, with 8.3% to 10% prevalence of DM among TB patients in two regions in Ethiopia [39, 40]. Finally, smoking increases TB risk and alcohol abuse increased both risk of TB and non-adherence to treatment [41, 42]. The 2015 national STEP survey in Ethiopia found that around 12% of adults engage in heavy episodic drinking [43].

1.1.4.2 Tuberculosis control efforts in Ethiopia

The national TB program has prioritized key strategic interventions in the 2021-2026 national TB strategic plan to reach the End TB targets set for 2030: 90% of all people with TB diagnosed and treated, and 90% of people diagnosed successfully complete treatment with services to ensure efficient management [44].

The National Strategy for TB control emphasizes strengthening the governance of TB programs and improving the quality of TB services to achieve early detection and treatment initiation. The strategy recommends the use of digital X-ray technologies, with artificial intelligence readings to fill radiology staffing gaps, collaboration and community participation, and strengthening of TB program management [44].

1.2 Diagnosis of tuberculosis

Diagnosis of active TB starts with screening of people with symptoms of TB [45]. The clinical presentation of TB is variable and depends on a number of factors including host immunity, presence of concomitant diseases, severity, site of infection (pulmonary TB or extra pulmonary TB), and microbial virulence. For pulmonary TB, chronic productive cough is the most common symptom, but may not be a prominent symptom in people living with HIV or severely ill patients [46]. Other symptoms include fever, loss of appetite, weight loss, weakness, night sweats, and hemoptysis [22, 47, 48]. In all people presenting with symptoms of pulmonary TB, diagnostic assays should be performed to confirm the presence of MTB through microbiological, molecular or immunological methods.

1.2.1 Microbiological methods

1.2.1.1 Smear microscopy

Smear microscopy was developed more than 100 years ago and is still the most used method to diagnose TB and monitor TB treatment response [49]. In both the Ziehl-Nelseen (ZN) and fluorescent auramine staining technique, mycobacteria retain primary color after exposure to decolorizing acid-alcohol. The ZN method stains the bacilli red, in the fluorochrome methods bacilli fluoresce yellow to orange [3, 50].

Smear microscopy is cheap ($3.1) [51] and highly specific (99.1%) [52], but its sensitivity is poor due to the low limit of detection (LOD) of 5000-10000 bacilli per milliliter of sputum. Compared to culture, the sensitivity of ZN smear microscopy is only 49% overall, 19.6% in children and 39.3% in people living with HIV [53-55]. Many cases are thus missed, especially among children and people living with HIV. While fluorescence microscopy requires less time and increases sensitivity by 10% [56, 57], its use is challenging in many limited-resource settings due to the inconsistent supply of reagents and maintenance cost. The concentration method also increases the sensitivity of ZN smear microscopy (by up to 9% in settings with high HIV prevalence), but this method is not standardized under program condition [53].

1.2.1.2 Mycobacterial culture

Culture is the reference standard for microbiological diagnosis of TB. MTB growth in solid or liquid medium only requires 10-100 viable bacteria per milliliter of sputum [58]. In 2007, the WHO recommended sputum culture for all smear negative pulmonary TB cases [59] and for monitoring response to treatment in drug resistant TB. Once a culture shows growth, rapid identification of MTB complex can be done using an immune-chromatographic assay [60] and drug susceptibility tests can be performed to diagnose the presence of drug resistant MTB [61].

Solid Lowenstein-Jensen (LJ) media is often used because of ease of preparation, low cost, and low contamination rate. Because solid culture takes on average four weeks for a positive result and six to eight weeks for a negative result, its usefulness in the decision-making process is limited [62, 63].

Liquid culture is the current gold standard method for bacteriological confirmation but is prone to contamination and it is relatively expensive [58, 64]. The Mycobacterial Growth Indicator Tube (MGIT) system uses highly enriched 7H9 media in a carbon-dioxide-sensing bottle for automated

detection of bacterial growth in the BACTEC MGIT 960 instrument (BD Diagnostics, Franklin Lakes, NJ, USA). The MGIT method has a 10% higher sensitivity compared LJ media and a shorter time to positivity, ranging from 14 to 21 days.

1.2.2 Molecular methods

1.2.2.1 Xpert MTB/RIF Assay

The Xpert MTB/RIF assay (Cepheid, Sunnyvale, USA) is a cartridge-based test that uses real-time PCR for detection of MTB DNA and rifampicin resistance directly from unprocessed sputum specimens [65]. Because the assay is fully automated for sample processing, DNA extraction, and amplification, it can be used at laboratories with medium level professional training and safety precautions, similar to smear microscopy [65, 66].

In 2010, the Xpert MTB/RIF assay was endorsed by the WHO for diagnosis of TB in people living with HIV and people at high risk of MDR-TB [67]. In 2013, the assay was recommended as the initial diagnostic test for all people with symptoms or signs of TB, independent of HIV status or MTB risk [68].

The development of Xpert MTB/RIF assay was a major step forward in improving the diagnosis of TB [69] given its high overall specificity (98%, 95% CI: 94-97%) and high sensitivity (85%, 95% CI: 82-88%) [70]. The use of Xpert MTB/RIF thus increases the proportion of bacteriologically confirmed TB cases [71], which reduces the proportion of patients receiving empirical TB treatment. For example, the proportion of clinically diagnosed culture confirmed cases dropped from 35% to 10% after the introduction of Xpert MTB/RIF in Uganda [72]. Similarly, the proportion of bacteriologically confirmed cases increased from 36% in 2016 to 61% in 2020 in Ethiopia following the implementation of the Xpert MTB/RIF assay [44].

Unfortunately, the sensitivity is lower in smear-negative culture-positive cases (67%, 95% CI: 62-72%) [73] and in children (58%, 95% CI: 39-65%) [74]. Consequently, a substantial number of TB cases, especially in people living with HIV and children, are still missed in settings where the Xpert MTB/RIF assay is implemented.

1.2.2.2 Xpert MTB/RIF Ultra Assay

To overcome the suboptimal efficacy sensitivity of xpert MTB/RIF, Xpert Ultra, a next generation Xpert MTB/RIF assay, was developed [75]. In contrast to the Xpert MTB/RIF, which uses only one PCR amplification target, the Xpert Ultra uses two multi-copy amplification targets (IS6110 and IS1081) and a larger PCR reaction chamber.

Xpert Ultra has a lower LOD than Xpert MTB/RIF (16 colony forming units [cfu]/mL versus 131 cfu/mL), almost equivalent to that of liquid MTB culture [75, 76]. In a multi-center study, the sensitivity of Xpert Ultra was higher than the sensitivity of Xpert MTB/RIF overall (88% vs 83%), among smear-negative culture positive cases (63% vs 46%), and in HIV-positive patients (90% vs 77%) [77]. These estimates were confirmed in a large systematic review: pooled sensitivity of 78% for Xpert Ultra vs 61% for Xpert MTB/RIF overall, and 88% vs 75% among people living with HIV [70]. The 13% increase in sensitivity in HIV-positive patients resulted in 10 extra TB cases detected per 1,000 patients living with HIV [76]. In an Indian TB study, replacing the Xpert MTB/RIF cartridge with the Xpert Ultra cartridge averted 0.5 TB deaths per 1,000 individuals [71].

The higher sensitivity of Xpert Ultra comes at a modest cost in specificity. In a systematic review, the pooled specificity was 95.8% for Xpert Ultra vs 98.8% for Xpert MTB/RIF in smear-negative, culture positive patients. The lower specificity was mainly observed among patients with a history of TB treatment [70], where the false-positive rate was 5% [78]. The reduced specificity results in one false positive case and thus one unnecessary treatment per 40 to 70 individuals [75]. Because of the lower specificity, an Xpert Ultra trace results is only considered positive for HIV positive patients [78].

In 2017, the WHO recommended replacing Xpert MTB/RIF by Xpert Ultra as the initial diagnostic test because of its superior performance in smear negative patients and people living with HIV [75]. Unfortunately, except for South Africa, the Xpert Ultra is not yet fully implemented as an initial diagnostic test in most high TB burden countries.

1.2.2.3 Truenat MTB Assays

In 2020 the WHO also approved Truenat MTB for early diagnosis of TB in adults and children with signs and symptoms of pulmonary TB [79]. The assay uses a chip-based real-time micro-PCR for the

semi-quantitative detection of MTB complex directly from sputum specimens and reports results in less than an hour. The assays use automated, battery-operated devices to extract, amplify and detect specific genomic DNA loci and can be used in laboratories with minimal infrastructure and minimally trained technicians [80].

A multicenter study of 1807 adults presenting with symptoms suggestive of pulmonary TB disease to primary health care centers in Peru, India, Ethiopia and Papua New Guinea observed an overall pooled sensitivity of 36% in smear-negative cases and pooled specificity of 96% [81]. Sensitivities are thus lower when compared with Xpert MTB/RIF assay and especially compared to Xpert Ultra.

1.2.2.4 Loop-mediated isothermal amplification Assay

In 2016, the WHO recommended the Loop-mediated isothermal amplification (TB-LAMP) assay as a replacement test for sputum-smear microscopy in adults with signs and symptoms of TB [82]. TB-LAMP is a manual form of the Isothermal Nucleic Acid Amplification test (INAAT) that requires less than one hour to perform, does not require sophisticated instrumentation and can be used at the peripheral health center level with biosafety requirements similar to sputum smear microscopy. The TB-LAMP assay can detect MTB complex DNA directly from liquefied sputum samples without any prior DNA extraction, denaturation or enzymatic inactivation step [82, 83].

A multicenter study conducted on 1,777 adult participants with symptoms suggestive of TB in India, Uganda, and Peru showed 97.2% sensitivity among smear positive samples and 62% among smear-negative culture positive samples. Specificity ranged from 94.5% to 98.0% [84]. In a systematic review, the overall pooled sensitivity of TB-LAMP was 13.2% higher than sputum smear microcopy but similar among HIV-infected adults (63.8% vs 61.0%). Pooled specificity was 98.1% [85].

1.2.3 Immunological methods: lateral flow urine lipoarabinomannan Assay

The Lateral Flow urine Lipoarabinomannan (LF-LAM) (Alere, MA, USA) assay is an immune capture assay that detects the Lipoarabinomannan (LAM) antigen in urine. LAM is a component of the outer cell wall of the MTB complex and other pathogenic mycobacterium species [86]. When mycobacteria spread via the bloodstream to the kidneys, the kidney clears LAM into the urine [87]. The detection of mycobacterial LAM antigen in urine thus demonstrates the presence of MTB but cannot differentiate between the different mycobacterium species.

The overall pooled sensitivity of LF-LAM is 42% (40–64%), and the pooled specificity is 91% (78–93%) [88]. The LF-LAM assay is the only assay that has greater sensitivity for the diagnosis of TB among people living with HIV than among people who are HIV negative, most likely because people living with HIV are more likely than HIV-negative people to develop extra-pulmonary TB (40–80% vs 10–20%) [89], including spread via the blood flow to the kidney. The pooled sensitivity in people living with HIV is 45%, compared to 20% in HIV-negative individuals [90]. This disparity may be attributed to a compromised immune system and impaired clearance of pathogens from the body, which makes them more susceptible to disseminated TB.

When used in combination with other tests, LF-LAM can improve the diagnostic yield among people living with HIV by 14% as compared to Xpert MTB/RIF alone or by 36.6% as compared to clinical sign and symptoms alone [91]. Furthermore the use of LF-LAM can reduces mortality by 4% in HIV-positive hospitalized patients [92]. The assay can thus be used as a point-of-care rule-in test for TB in people living with HIV, especially in cases where a rapid TB diagnosis is critical for the patient's survival [93].

In 2015, the WHO recommended the use of LF-LAM to assist in the diagnosis of TB in HIV-positive adults with advanced HIV disease (CD4 cell count less than 200 cells/mm3) irrespective of signs and symptoms of TB or in people living with HIV who are seriously ill [80].

To improve the suboptimal sensitivity of the Alere LF-LAM, the Fujifilm SILVAMP TB LAM (Fuji LAM) assay has been developed. A study conducted in 968 South African HIV positive participants found a sensitivity of 70.4% for Fuji LAM compared to 42.3% for LF-LAM [94]. Fuji LAM has not yet been endorsed by the WHO.

1.2.4 C-reactive protein test

C-reactive protein (CRP) is a biomarker that is commonly used to detect inflammation and tissue damage. The liver produces this non-specific biomarker in response to various inflammatory events, such as infections, autoimmune diseases, and cancer. Advancements in CRP measurement techniques have led to the development of point-of-care assays that offer a quick and easy way to measure CRP levels from a small amount of blood obtained from a finger prick. These tests are inexpensive, simple to use, and provide results within minutes, making them useful in situations where prompt decision-making is critical for patient care [95, 96].

The WHO recommends that people with HIV who are not hospitalized undergo TB screening using the WHO four-symptom screen (W4SS) or CRP (with a threshold of 5 mg/L), followed by confirmatory testing if the screening is positive [97]. According to a recent study, using CRP to screen for TB would reduce the proportion of people with HIV requiring confirmatory testing as compared to symptom-based screening (28% vs. 86%). Furthermore, CRP-based TB screening detected 89% of all culture-positive TB cases and 94% of all Xpert MTB/RIF-positive TB cases [98]. When CRP and W4SS were used together, they produced a sensitivity and negative predictive value (NPV) of 100% among people with HIV and 93.3% and 90.0% among those who are HIV negative, respectively [99].

In countries where TB is prevalent, using CRP-based TB screening has the potential to revolutionize TB control strategies by identifying individuals who are at a higher risk of having active TB, reducing the need for confirmatory testing, and increasing access to TB preventive therapy for people with HIV [100].

1.2.5 Chest X-ray

A chest X-ray (CXR) identifies lung abnormalities that can be suggestive of TB including hilar adenopathy, miliary mottling in lung tissue, cavitation, and effusion in pleural and pericardial spaces [101] (**Table 2)**.

Table 2: Chest X-ray indicators of adult pulmonary TB [102].

Findings typical of TB	**Findings compatible with TB**
Air-space nodules/clustered nodules in upper/mid zones of the lungs	Consolidation/air-space nodules/clustered nodules in lower zones
Consolidation in upper/mid zones with ipsilateral lymph node enlargement	Equivocal nodules (miliary/air space)
Miliary nodules	Cavity with air-fluid level
Thick-walled cavity	Equivocal hilar prominence/widening of Para tracheal stripe
Cavity with surrounding consolidation	
Unilateral hilar/Para tracheal lymph node enlargement	
Effusion/empyema	

CXR has high sensitivity ranging from 70 to 92% for the diagnosis of pulmonary TB. Bilateral adenopathy occurs in 31%, cavitation in 56% and pleural effusion in up to 38% of cases of pulmonary TB [102, 103]. However, it has poor specificity (63%) [104]. CXR findings are normal in 15% of patients with microbiological confirmed TB and lymph node enlargement is seen up to 43% in patients not previously exposed to MTB.

Among people living with HIV, CXR has a poor sensitivity (43%) and very poor specificity (10%) [105] as many CXR abnormalities consistent with pulmonary TB are present also in other HIV-related lung pathologies [37, 106]. Moreover, there is significant intra-and-inter-observer variation in the reading of CXRs [107, 108].

Prior to the endorsement of Xpert MTB/RIF, WHO considered CXR to be an important tool in the diagnosis of pulmonary TB [59]. However, in the Xpert MTB/RIF era, CXR is promoted by the WHO to increase the pre-test probability of the Xpert MTB/RIF assay, lowering the number of people requiring a test and the additional costs [109].

1.3 Diagnosis of TB in Ethiopia

The 2018 national TB diagnostic algorithm recommended a molecular WHO-approved rapid diagnostics (mWRDs) test as the initial diagnostic test for all presumptive TB cases. In reality, smear microscopy is still used as an initial diagnostic test in most laboratories to avoid diagnostic delay when Xpert MTB/RIF service or other recommended mWRDs are not readily available for same-day results [110]. In the meantime, a sputum specimen should be sent for a rapid diagnostic test (e.g., Xpert MTB/RIF) for people at high risk of drug resistant-TB and people living with HIV. Further investigations include clinical response to broad spectrum antimicrobial treatment trial and CXR [110]

According to the national guideline, patients who are suspected to have TB but tested negative using the Xpert MTB/RIF test are treated with antibiotics that are effective against bacterial infections. However, these antibiotics have minimal activity against MTB. The duration of the antibiotic treatment is seven to ten days. If patients show an improvement in symptoms after receiving antibiotics, they are considered unlikely to have TB. However, patients who remain symptomatic after completing the antibiotic therapy are further investigated to determine the presence of TB or other diseases. In cases where patients are seriously ill and display danger signs

such as suggestive chest X-ray findings consistent with TB disease, physicians start TB treatment empirically [110].

1.4 Treatment of tuberculosis

Without treatment, the mortality rate from TB is high [36]. Natural history studies of TB conducted before drug treatment became available found that 20% of people with culture positive smear-negative pulmonary TB and 70% of individuals with sputum smear-positive pulmonary TB died within 10 years of diagnosis [111].

1.4.1 Treatment of bacteriological confirmed TB

The majority of TB cases can now be cured when treatment is provided and taken properly [112]. Effective treatment for drug-susceptible TB consists of an intensive phase of two month isoniazid, rifampicin, ethambutol, and pyrazinamide followed by a continuation phase of isoniazid and rifampicin for four months [36]. The continuation phase should be extended to seven months when smear microscopy or sputum cultures are positive after two months of treatment [113]. Treatment of drug-resistant TB is challenging for healthcare professionals. However, there is a new treatment approach called the BPaL regimen that has been recently recommended. This approach uses three drugs: bedaquiline, pretomanid, and linezolid, and is specifically designed for cases of drug-resistant TB that are resistant to at least one fluoroquinolone or injectable medication. Recent study have shown that this treatment has a very high success rate, with 90% of patients being cured 6 months after the end of treatment [114].

1.4.2 Empirical tuberculosis treatment

Empiric TB treatment is defined as the administration of TB treatment to a person in the absence of microbiological evidence of TB, i.e., in a person in whom the diagnosis of TB was made solely based on the presence of clinical symptoms and/or findings on CXR. In 2007, prior to the availability of the Xpert MTB/RIF assay, the WHO recommended empiric TB treatment in resource-constrained countries for people living with HIV who had two negative sputum smear microscopy tests, had CXR findings suggestive with TB, and did not respond to antibiotic treatment. This recommendation aimed to reduce the mortality of undiagnosed (and thus untreated) TB in this highly vulnerable population [59].

In settings where Xpert MTB/RIF is available, empiric treatment is still often used to reduce a risk of TB related mortality in Xpert MTB/RIF-negative patients whose clinical presentations strongly suggest pulmonary TB [72, 115]. For example, a study in South Africa observed that 49% of patients without bacteriological confirmation were started empiric TB treatment, mainly (77%) based on CXR findings [116]. In Uganda, a study found that 56% of HIV-positive smear-negative patients started empiric TB treatment without Xpert MTB/RIF testing even in places where Xpert MTB/RIF assay was available [117].

The use of empiric treatment in the Xpert MTB/RIF era is however controversial. Several clinical trials have been performed to study the effectiveness of empiric TB treatment in settings where Xpert MTB/RIF is available. A randomized controlled trial study conducted in South Africa involving 3,022 participants from 24 primary care clinics found that starting empiric TB treatment did not provide any survival benefits and even increased the risk of death in individuals who reported serious adverse events. Specifically, 61.7% of the participants in the intervention group and 11.4% in the control group began empiric TB treatment [118]. Another studies found that empiric treatment did not reduce mortality in the HIV positive people with suspected TB admitted to a referral hospital in a country with a high TB burden [119, 120].

Empiric TB treatment for people with a negative Xpert MTB/RIF may not only lack effectiveness but could also result in poor outcomes when other diagnoses are overlooked. A study conducted in Uganda among 631 hospitalized HIV-positive patients with coughs ≥ 2 weeks found that empiric treatment was associated with a higher rate of complications and increased mortality in the first two months of TB treatment. These findings emphasize the importance of an accurate diagnosis of TB and other potential causes of respiratory symptoms, particularly in high-risk populations such as HIV-positive individuals [121]. The risk and benefits of empiric TB treatment should always be carefully considered, and empiric TB treatment should only be initiated in situations where the benefits are likely to outweigh the risks, such as when there is a high risk of TB and diagnostic testing is not immediately available or when the patient has a high risk of disease progression or poor outcomes.

1.5 Etiological causes of LRTI in people with presumptive TB

In people with presumptive TB who test negative on the Xpert MTB/RIF assay, other disease-causing pathogens may be responsible for their symptoms. These can include a wide range of bacterial, viral, and fungal pathogens [122, 123]. MTB is indeed one of the bacterial pathogens that can cause respiratory symptoms [124]. However, there are many other bacterial pathogens most likely to be responsible to cause symptoms similar to TB, including *Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter spp., Streptococcus viridans, Pseudomonas aeruginosa, Escherichia coli*, and *Proteus spp.* [125-127]. The study conducted in Myanmar showed that a significant proportion (46%) of patients with LRTI symptoms were infected with potential bacterial or viral pathogens. This study used a real-time PCR assay to detect the presence of specific viral pathogens, including influenza A virus, influenza B virus, and respiratory syncytial virus (RSV). The results showed that among the 299 participants, 5.2% were positive for influenza A virus, 6.6% were positive for influenza B virus, and 4.4% were positive for RSV [128].

The most common atypical bacteria associated with LRTIs include *Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella pneumophila*, as well as some species of the genus Bordetella, such as *Bordetella pertussis*, which causes whooping cough [129]. A meta-analysis study of 15 studies showed that the overall prevalence of *Mycoplasma pneumoniae* was found to be 10.1% among the atypical bacteria studied, and *Bordetella pertussis* (12.4%). The prevalence rates for *Chlamydia pneumoniae* and *Legionella pneumophila* were lower but still significant [130].

The study conducted in Uganda revealed that fungal pathogens were relatively common cause of respiratory symptoms in individuals who have tested negative on the Xpert MTB/RIF assay and are suspected of having TB. The study used culture on Sabouraud Dextrose Agar (SDA) to identify fungal pathogens in 113 Xpert MTB/RIF-negative individuals. The results showed that disease-causing fungi were identified in 70.7% of the individuals. The most commonly identified fungal pathogens were *Candida albicans* (22.6%) and *Aspergillus species* (17.2%) [131]. Similarly, the study conducted in Ethiopia, used microscopy and culture to identify fungal pathogens, reported that 55.8% overall prevalence of potential fungal pathogens in Xpert MTB/RIF-negative presumptive TB cases [132]. A better understanding of the presnece of potential pathogesn responsable for LRTI in

patients in whom the presence of TB was excluded by the highly sensetive Xpert Ultra assay is important to develop evidence-based algorithm for the optimal management of these patients.

1.6 Knowledge gaps, rationale, overall goal and specific aims of the PhD

1.6.1 Knowledge gaps

While there have been many studies investigating Xpert MTB/RIF for the diagnosis of TB, little is known about the accuracy of the standard clinical algorithm of response to an antibiotic trial and chest X-ray findings in Xpert MTB/RIF-negative presumptive TB cases. The value of empiric TB treatment in settings where Xpert MTB/RIF or Xpert Ultra is available has not yet been investigated. Furthermore, very little is known on how to guide treatment other than empiric TB treatment in Xpert Ultra negative patients with respiratory symptoms residing in high TB burden countries in the African region.

The LF-LAM assay is an attractive tool for the diagnosis of TB in people living with HIV and critically ill hospitalized patients with suspected TB, as it improves case detection and reduces mortality [91, 133]. The diagnostic yield of the LF-LAM assay when used in combination with Xpert Ultra, the more sensitive second generation of Xpert, has not yet been investigated. Understanding the bacterial etiology of LRTIs in this population is important for optimal patient management. MiSeq 16S rRNA gene amplicon sequencing has not yet been applied to study the etiology of LRTI in hospitalized presumptive TB patients in whom MTB was not detected by the Xpert Ultra assay [134].

1.6.2 Overall goal of the PhD research

The overall goal of the study was to improve the management of hospitalized patients with Xpert MTB/RIF-negative presumptive TB in high-TB-burden countries in the African region.

1.6.3 Specific objectives

Aim 1: To assess the accuracy of a clinical algorithm consisting of an antibiotic trial and chest X-ray to identify people with TB disease and the role of empiric TB treatment in the management of hospitalized presumptive Xpert MTB/RIF-negative TB cases.

Aim 2: To assess the added value of chest X-ray (read by a clinician or radiologist) to identify cases of active TB among Xpert MTB/RIF-negative patients admitted to a hospital for symptoms of TB.

Aim 3: To assess the additional yield of the Lateral Flow Urine Lipoarabinomannan (LF-LAM) assay when used in combination with Xpert Ultra and the role of empiric TB treatment when Xpert Ultra is used as the initial diagnostic in hospitalized presumptive TB cases.

Aim 4: To identify possible bacterial LRTI pathogens in hospitalized patients who were initially suspected to have TB but later tested negative using the Xpert Ultra test. In addition, we aimed to compare the prevalence and distribution of respiratory bacterial pathogens in sputum samples that were Xpert Ultra positive and negative. Finally, the association between the presence of specific bacterial pathogenic taxa in Xpert Ultra negative sputum sample and clinical improvement on an antibiotic trial, chest X-ray findings, and 6-month survival was explored.

Chapter 2: Materials and Methods

2.1. Country background

Ethiopia, located in Horn of Africa, is the second largest and second populous country in the region, with an estimated 119,395,441 people of which 67% are below the age of 30 years [135]. Ethiopia is one of the poorest countries in the world, around 44% of its population lives in poverty due to a combination of natural disasters and man-made actions [136].

Healthcare delivery in Ethiopia is organized at three levels: primary healthcare units, secondary level, and tertiary level care. A primary health care unit is comprised of health posts, health centers, and a primary hospital. The unit provides basic preventive, promotive, curative, and rehabilitative care to about 60,000 to 100,000 people in its catchment area. The secondary level health care unit consists of a general hospital that renders services to about 1.5 million people. Tertiary hospitals provide advanced services to about five million people. TB diagnosis and treatment services are provided for free at all three levels of healthcare [137].

2.2. Study setting

The PhD study was performed at Jimma University Medical Center (JUMC), one of the oldest hospitals in Ethiopia and the only teaching and referral hospital in southwest Ethiopia. The 800-bed hospital has a catchment population of over 15 million people from both urban and rural communities. In 2021, about 255,000 patients were treated at the hospital; 220,000 as outpatient, 15,000 at the emergency unit, and 20,000 as inpatients [138]. Annually, about 2,800 TB patients are diagnosed at JUMC. At JUMC, the initial diagnostic for patients with clinical suspicion of TB is a Xpert MTB/RIF test.

The Mycobacteriology Research Center (MRC) at Jimma University was established in 2010 as an inter-university collaborative research project between Jimma University and a consortium of Flemish universities. The MRC is focused on providing services, research, and education in the field of mycobacteriology. Infrastructure is available at the MRC for solid and liquid (BACTEC MGIT 960 TB) culture, characterization of various mycobacterial strains, drug resistance testing (phenotypic

and genotypic), Xpert MTB/RIF assay (4 module instrument), fluorescence microscope, -80-degree Celsius freezer, refrigerated ultra-speed centrifuge, and incubators.

2.3. Study design and Sample size

This study enrolled hospitalized Xpert MTB/RIF-negative adults (≥18 years of age). The sample size was estimated to test the hypothesis that the proportion of bacteriologically confirmed cases is higher among Xpert MTB/RIF-negative TB patients started on empiric TB treatment compared to those not started on empiric treatment. To have 80% power to detect a 10% difference in the proportion of bacteriologically confirmed TB (4% versus 14%) a sample size of 250 participants (125 patients started on empiric TB treatment and 125 patients not started on empiric TB treatment) was required [139]. The study was not powered for the other aims, such as the effect on treatment outcomes due to the outcome (mortality) is not known at enrolment.

2.4. Study procedures: screening, enrollment and data collection

To identify eligible study participants, all Xpert MTB/RIF-negative patients were identified via the hospital laboratory's Xpert MTB/RIF register. A study nurse reviewed the file of Xpert MTB/RIF-negative patients admitted to the hospital to determine whether the physician in charge of patient care decided to treat or not to treat empirically with TB drugs. Within the first 48 hours following the physician's decision, patients were informed about the study, and informed consent was obtained until 125 patients initiated on empiric TB treatment and 125 patients not initiated on empiric TB treatment were enrolled.

For all study participants, three sputum samples were collected for MTB culture and Xpert Ultra. HIV-positive and severely ill patients (temperature >39^{0}C, respiratory rate >30 resp./min, cardiac rate >120 bmp, or unable to walk without help) [59] were also asked to provide a 10 ml urine sample for the LF-LAM assay (**Figure 2**).

Relevant socio-demographic and clinical data were collected via a pre-tested, structured questionnaire and retrieved from the participant`s medical records. If a chest X-ray was not done for routine care, participants were referred for a chest X-ray. We collected phone numbers from all study participants and their family members during enrollment. After six months of enrollment, we checked in with all participants - whether they were alive or deceased. For those who had started

TB treatment at the hospital after receiving a negative Xpert MTB/RIF result, medical records were reviewed to document their survival status. Additionally, for those who did not receive empiric TB treatment, phone calls were conducted to obtain self-reported survival information directly from participants or their family members. Notably, no participants were lost to phone calls during the follow-up process.

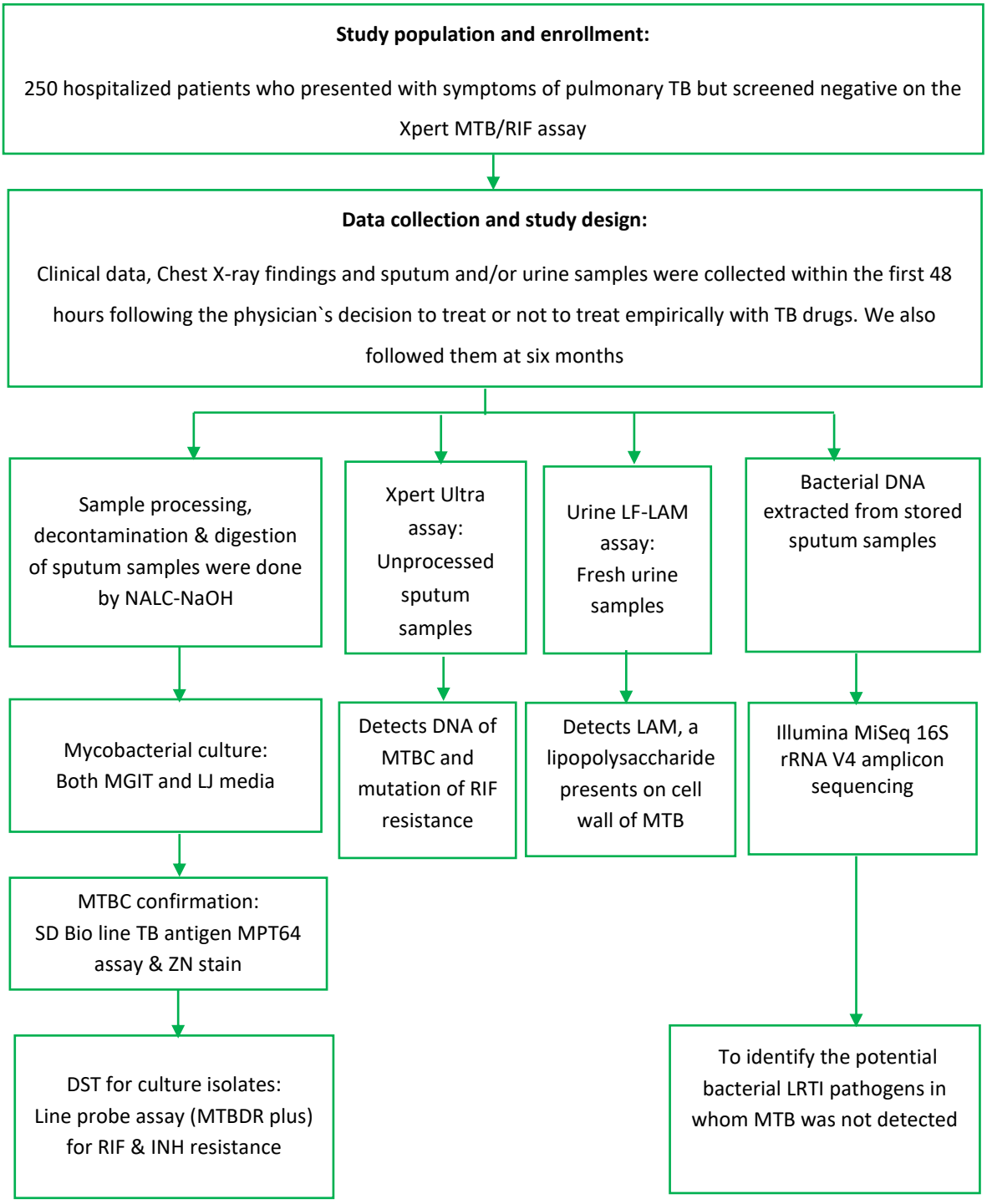

Figure 2: Flow chart showing the study population enrollment, data collection procedures, and laboratory tests performed. MGIT, Mycobacterial Growth Indictor Tube; and LJ, Lowenstein Jensen; ZN, Ziehl-Nelseen; RIF, rifampicin; INH, isoniazid; DST, Drug susceptibility testing; MTBC, *M. tuberculosis* complex; PTB, pulmonary tuberculosis; DNA, deoxyribonucleic acid; LRTI, Lower respiratory tract infections

2.5. Laboratory procedures

2.5.1. MTB culture and identification

An equal volume of sputum sample was mixed with N-Acetyl-L-Cysteine Sodium hydroxide (NALC-NaOH) with a final concentration of 1% NaOH in a 50ml falcon tube, vortexed, and incubated at room temperature (15-20^{0}C) for 15 minutes. Phosphate buffer (PBS) pH 6.8 was then added up to 50ml mark before centrifugation at 3,000xg for 15 minutes at 4^{0}C. The supernatant was decanted into a splash-proof container containing 5% hypochlorite disinfectant and sterile PBS was re-suspended the pellet in the volume of 2.5ml. For liquid culture, the Mycobacterial Growth Indictor Tube (MGIT) method was used. Each tube was labeled with the study identification number. The antibiotic supplement (PANTA) was prepared and reconstituted with 15ml MGIT growth supplement. To each MGIT tube, 0.8ml of the PANTA with the growth supplement mixture was added using a micropipette and 0.5ml of well-mixed sputum sediment was transferred to MGIT tubes using a sterile graduated pipette. The closed tubes were then placed in the BACTEC^M MGIT^{TM} 960 instrument and gently invested several times until signaling “positive” or until day 56 if no growth is detected [140].

For solid media culture, the locally prepared Lowenstein Jensen (LJ) tubes were labeled with patient identification number, 0.5ml of the sputum palette was spread evenly over the entire surface of the medium using a sterile graduated disposable pipette. The tube was left with the cup loosened for 2-3 days at 37^{0}C in a horizontal position, after which the cap was tightened securely and the tube was incubated in an upright position for six weeks and inspected for MTB growth at weekly intervals. Culture positive refers to a positive LJ/MGIT culture with a positive SD Bioline TB antigen MPT64 confirmatory test result and negative Brain Heart Infusion agar test. Culture negative denotes a negative LJ/MGIT culture or positive LJ/MGIT culture with a negative SD Bioline TB antigen MPT64 confirmation test result [141].

2.5.2. Xpert Ultra Assay

The Xpert Ultra assay was performed according to the manufacturer`s instructions. One ml of sputum sample was mixed with 2 ml of reagent, vortexed for at least 10 seconds, and incubated at room temperature for 10 minutes, after which it was again vortexed and incubated for another five minutes. The liquefied samples were aspirated using a sterile pipette up to the marked 2 ml line

and slowly transferred into the sample chamber of the Xpert Ultra cartridge labeled with the patient`s identification number. The Xpert Ultra test result was interpreted as MTB detected, MTB not detected, or trace call. For an invalid result, the Xpert Ultra test was repeated using the same sample. In the event of a "trace" being detected, a new sputum sample was collected for the second Xpert Ultra test. If the second test result was trace, the result was considered positive for HIV-positive patients and HIV-negative patients who have no history of TB [142].

2.5.3. LF-LAM Assay

The LF-LAM test [Abbott Laboratories, Lake Bluff, USA (formerly Alere Inc., Waltham, USA)] was performed manually by applying 60 µl of fresh urine to the sample pad at the bottom of the test strip using a micropipette. After 25 minutes of incubation at room temperature, the test strip was inspected visually. A positive result (a visible purple line) indicates that the LAM antigen of Mycobacteria is present in the sample, whereas a negative result (no visible purple line) indicates that it is not present or below the detection limit. To ensure assay validity, a procedural control bar is incorporated into the assay device. The test was read independently by two certified laboratory technologists working in the MRC in a blinded manner (blinded to each other and the sputum Xpert Ultra test result). The discrepancies between the two readers were resolved by the third laboratory staff. The test results were classified as positive if grade ≥1 or negative if there was no test band [91].

2.5.4. 16S rRNA gene amplicon sequencing

DNA was extracted from stored unprocessed sputum samples (stored-80^{o}C for 24 months) at the Mycobacteriology Research Center of Jimma University in Ethiopia using the Power Fecal DNA Isolation Kit (Qiagen) [143]. Illumina MiSeq 16S rRNA gene amplicon sequencing was performed at the Center for Medical Genetics of the University of Antwerp in Belgium using an in-house protocol for low biomass samples [144]. Briefly, 5 µl of bacterial DNA of the clinical samples was used to amplify the V4 region with an amplicon size of 254 bp of the 16S rRNA gene. Negative controls from the PCR (PCR grade water) and DNA extraction kit were included. Standard barcoded forward 515f (515f 5'-GTGCCAGCAGCCGCGGTAA-3') and reverse 806r (806r 5’-GGACTACGGGTATCTAAT-3) primers were used as described in [143]. PCR products were purified by the Agencourt AMPure XP Magnetic Bead Capture Kit (Beckman Coulter, Suarlee, Belgium). DNA concentrations were measured using the Qubit 3.0 fluorometer (Life Technologies, Ledeberg, Belgium). The library was prepared by

pooling all PCR samples in equimolar concentration and loaded onto a 0.8% agarose gel to remove residual primer dimers. The PCR product was purified by gel extraction using the NucleoSpin® gel and PCR clean-up (Macherey Nagel). The final DNA concentration of the library was measured with the Qubit 3.0 Fluorometer. The library was then denatured with 0.2 N NaOH, diluted to 7pM, treated with 10% PhiX control DNA [145], before being loaded onto a flow cell (MiSeq Reagent Kit 2×250 bp kit,v2 Chemistry) for paired-ended sequencing on the MiSeq Platform (Illumina San Diego, CA, United States).

2.6. Data analysis

Depending on the research questions, a different design was used to guide the analysis:

Aim 1: To test the hypothesis that the proportion of bacteriologically confirmed TB cases is higher among Xpert MTB/RIF-negative patients who have received empiric TB treatment than those who haven't, a cross-sectional study design was used. The characteristics of the study population were summarized using descriptive statistics. The proportion of patients with bacteriological confirmation of TB was compared between patients who received empiric TB treatment and those who did not receive empiric TB treatment. The ability of the clinical algorithm to predict the presence of bacteriologically confirmed TB was evaluated by calculating the positive predictive value. A logistic regression analysis was performed to determine the association between patient characteristics and bacteriologically confirmed active TB and the start of empiric TB treatment. Six-month treatment outcome was assessed and stratified based on empirical TB treatment status and bacteriological confirmation of TB.

Aim 2: Baseline characteristics of subjects and chest X-ray abnormalities were presented as frequencies and proportions. Kappa score was calculated to assess inter-reader agreement of the chest X-ray classification between clinicians and senior radiologists. Using bacteriologically confirmed TB as a reference standard, sensitivity, specificity, negative and positive predictive values for chest X-ray classification were estimated by clinicians and radiologists. Bivariate logistic regression models were used to determine the association between clinical variables and bacteriologically confirmed TB. Three multivariate models were constructed to assess the association between bacteriologically confirmed TB and clinical features (Model 1), clinical features and chest X-ray interpretation by the physician (Model 2), and clinical features and chest X-ray interpretation by the radiologist (Model 3). The prediction accuracy of the final models was

assessed through calibration and discrimination parameters. To assess the value of chest X-ray results in addition to clinical features, the three predictive models were converted to simplified risk scores. The diagnostic performance of predictive models in the diagnosis of active pulmonary TB was evaluated by calculating sensitivity, specificity, positive and negative predictive values.

Aim 3: Descriptive statistics were performed using frequencies and percentages to describe the characteristics of the study population. The yield of the Xpert Ultra and LF-LAM tests was determined by comparison to culture results. Six-month mortality was assessed among Xpert Ultra-negative participants and stratified by empirical treatment status. To identify factors associated with mortality in Xpert Ultra-negative patients, we calculated the odds ratio using a logistic regression model.

Aim 4: To identify possible bacterial lower respiratory tract infection (LRTI) pathogens in hospitalized patients who were initially suspected to have TB but later tested negative using the Xpert Ultra test, a cross-sectional study design was used. Processing and quality control of the sequencing reads were performed using the R package Divisive Amplicon Denoising Algorithm 2 (DADA2), version 1.6.0 to increase the sensitivity and specificity compared to OUT picking methods [144]. Processing of the amplicon sequence variants (ASVs), ASV read count aggregation on genus level, ASV annotation (e.g., classification), sample annotation with metadata, data visualization, and statistical analyses was done in R.

Bacteria were categorized as present in the sputum sample when they were present at ≥1% of the population. Bacteria were classified as potentially pathogenic, opportunistic (i.e., cause of disease in immunocompromised individuals including people living with HIV or elderly people) or not LRTI-causing based on literature review using PubMed, ScienceDirect, and Google Scholar and search terms pathogenic bacteria, opportunistic bacteria, bacterial genera, LRTI, and bacterial classification. When comparison of the 16S rRNA amplicon data could not classify the bacteria present to species level, the bacteria present were classified as potential LRTI pathogens.

The difference in the abundance of bacterial LRTI pathogens detected was compared between Xpert Ultra positive and negative samples using Chi-Squared test. Logistic regression analysis was performed to determine the association (odds ratio (OR) and its 95% confidence interval) between (potential) bacterial LRTI pathogens and response to an antibiotic trial, findings on chest X-ray and

6-month survival status. For each (potential) bacterial LRTI pathogen identified and each outcome of interest, a separate model was built. For each model, the adjusted OR was estimated by including patient characteristics that were associated with the outcome of interest at p-value < 0.2 in bivariate analysis. Generalized variance-inflation factor (GVIF) was estimated to check multicollinearity. Backward stepwise model reduction was performed using the likelihood ratio test with a p-value cut-point of 0.1.

2.7. Ethical considerations

The Jimma University Institute of Health Ethical Review Board approved the studies with Ref. No: IHRPGD/397/2018. A discussion was held with experts from the TB program at JUMC's TB treatment center, and then a similar discussion was held with clinicians on the ward. Participant recruitment began after obtaining informed consent from each study participant. Participants were also informed of the right to withdraw from the study without compromising their future care. Physicians treating patients with bacteriologically confirmed TB were informed of the result.

Table 3. Summary of study population, sample size, and diagnostic methods used for the 4 aims of the study.

Aims	Study population	Assessments used in analysis	Publication status
1. To assess the ability of clinical algorithms (antibiotic trials and chest radiography) to identify TB disease and the role of empiric TB treatment in the management of hospitalized presumptive Xpert MTB/RIF-negative TB cases.	250 hospitalized Xpert MTB/RIF-negative patients: 125 initiated on empiric TB treatment 125 in whom TB treatment was not started	Both LJ and MGIT sputum cultures, and Xpert Ultra	Published on IJID in 2020 [146]
2. To assess the added value of chest X-ray (read by a clinician or radiologist) to identify cases of active TB among people admitted to a tertiary hospital with symptoms of TB who had a negative Xpert MTB/RIF result.	247 hospitalized Xpert MTB/RIF-negative patients	Sputum culture, Xpert Ultra and, chest X-ray	Published on ERJ Open research in 2021 [147]

3.	To assess the additional yield of the Lateral Flow Urine Lipoarabinomannan (LF-LAM) assay in combination with Xpert Ultra and the role of empiric TB treatment when Xpert Ultra is used as the initial diagnostic in hospitalized presumptive TB cases.	250 hospitalized Xpert MTB/RIF-negative presumptive TB cases	Sputum culture, Xpert Ultra, and urine LF-LAM (from 52 HIV-positive and 16 severely ill HIV-negative participants)	Published on Scientific reports in 2021 [148]
4.	To identify possible bacterial LRTI pathogens in hospitalized patients who were initially suspected to have TB but later tested negative using the Xpert Ultra test, we aimed to compare the prevalence and distribution of respiratory bacterial pathogens in sputum samples that were Xpert Ultra positive and negative. Finally, the association between the presence of specific bacterial pathogenic taxa in Xpert Ultra negative sputum sample and clinical improvement on an antibiotic trial, chest X-ray findings and 6-month survival was explored.	215 Xpert Ultra negative and 35 Xpert Ultra positive patients	MiSeq 16S rRNA gene amplicon sequencing	Published on Microbiol Spectr. 2024 [149]

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Chapter 3: Paper 1

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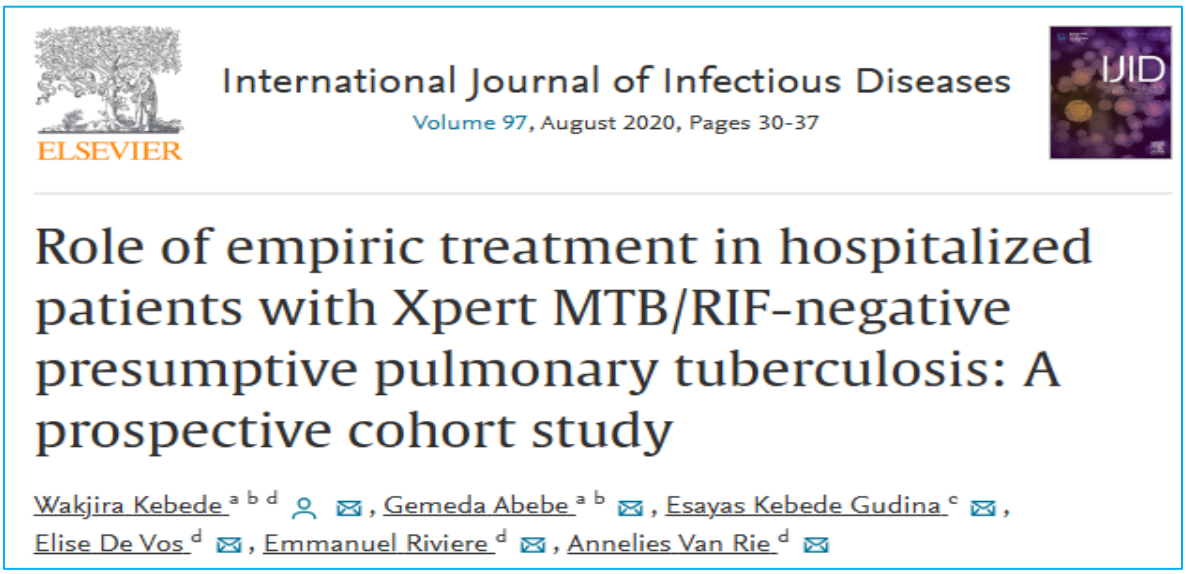

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My role in this work: I, as the first author, collaborated with my promotors, Prof. Annelies Van Rie and Prof. Gemeda Abebe, conceived the study. Together with Prof. Esayas Kebede Gudina, I supervised the data collectors during participant enrollment. I performed laboratory experiments and obtained all relevant data from patient medical records. The statistical analysis was done by me and the results were interpreted with my promoters. I wrote the first draft of the manuscript, revised it based on input from co-authors, and completed the manuscript submission process.

3.1. Abstract

Introduction: Empiric treatment allows the rapid initiation of treatment in absence of microbiological confirmation of tuberculosis (TB). The ability of clinical algorithms to accurately identify TB disease and the role of empiric treatment on survival in people with a negative Xpert MTB/RIF (Xpert) result remains poorly documented.

Methods: 125 hospitalized Xpert-negative patients initiated on empiric TB treatment based on a clinical algorithm (response to antibiotic trial and chest X-ray) and 125 hospitalized Xpert-negative patients in whom TB treatment was not started were enrolled. Sputum samples were evaluated for *Mycobacterium tuberculosis* by liquid and solid culture. All study participants were followed up for 6 months to document survival. The positive predictive value of the clinical algorithm for diagnosis of bacteriological confirmed TB in hospitalized Xpert-negative patients was evaluated. Logistic regression was used to identify factors associated with bacteriological confirmation and empiric TB treatment initiation.

Results: Xpert-negative inpatients in whom empiric TB treatment was initiated were more likely to have microbiological confirmed TB compared to those in whom empiric TB treatment was not started (24.8% vs 6.4%, p = 0.0001). Six-month risk of death was 5.2%, but the risk was twice as high in people with bacteriological confirmation of TB (10.3% vs 4.3%, p = 0.12). Even though cardinal symptoms of TB were associated with both bacteriological confirmation and a decision to start empiric treatment, the predictive value of the clinical algorithm was poor (positive predictive value = 24.8%) and empiric treatment did not affect 6-month risk of death (5.6% vs 4.8%, p = 0.78).

Conclusion: Even though a clinical algorithm identifies the majority of confirmed TB cases among Xpert-negative hospitalized patients, empiric treatment did not impact survival and resulted in substantial overtreatment. The more sensitive Xpert Ultra assay should be rolled out to eliminate the need for empiric TB treatment.

Keywords: TB algorithm; Clinical diagnosis; Empiric TB treatment; Xpert MTB/RIF-negative; Xpert MTB/RIF ultra; Ethiopia.

3.2. Introduction

Tuberculosis (TB) remains a worldwide public health concern. In 2019, an estimated 10 million people developed TB and 1.3 million died from the disease, including about 300, 000 people with Human Immunodeficiency Virus (HIV) infection [1, 2]. The World Health Organization (WHO) reported that 30 high TB burden countries collectively account for 87% of TB cases globally. Ethiopia has the 10th highest burden of TB, with 114, 233 cases and an estimated incidence rate of 151 TB cases per 100,000 people in 2018 [1].

In Ethiopia, an estimated one-third of new TB cases remain undetected, in part due to the continued use of smear microscopy in most clinics, even though smear microscopy has a poor sensitivity compared to culture [3-6]. To improve the detection of *Mycobacterium tuberculosis* (*Mtb*), the WHO recommended the Xpert MTB/RIF (Xpert) assay in 2011 as the initial diagnostic test for all patients presenting with signs or symptoms of TB [7]. The Xpert assay has a high accuracy to detect *Mtb,* with 85% pooled sensitivity of 85% overall and 67% in smear-negative culture-positive samples, and a pooled specificity of 98% [8]. Unfortunately, due to budgetary reasons, the use of the Xpert assay as an initial diagnostic test in Ethiopia continues to be restricted to referral and teaching hospitals [9].

Because the sensitivity of the 1st generation Xpert assay is still suboptimal in smear-negative sputum samples [10, 11] the 2018 Ethiopian TB treatment guideline recommends that an antibiotic trial is performed when a TB diagnosis is in doubt. A course of broad-spectrum antibiotics with negligible *Mtb* activity is given to symptomatic Xpert-negative patients. Patients who respond well to the antibiotic trial are considered not to have TB, while those who remain symptomatic after completion of the antibiotics trial undergo evaluation by chest X-ray after which a physician may decide to empirically start to TB treatment if the clinical picture and/or X-ray is suggestive of TB disease [12].

While empiric treatment (i.e., initiation of TB treatment without bacteriological confirmation) is beneficial for patients who truly have TB [13-15], a randomized controlled trial showed that empiric TB treatment does not reduce overall mortality in people living with advanced HIV disease (16) and some studies found that empiric treatment may even increase the risk of death when other diagnoses are overlooked in people living with HIV [17-18].

Little is known about the accuracy of a clinical algorithm (consisting of an antibiotic trial and chest X-ray) and the role of empiric TB treatment in the Xpert era in the general population of people with presumptive TB. This study aimed to assess the value of a clinical algorithm and empiric TB treatment in the management of Xpert-negative presumptive pulmonary TB cases hospitalized at a referral hospital in southwest Ethiopia.

3.3. Materials and methods

3.3.1. Study setting

The study was conducted at Jimma University Medical Center in southwest Ethiopia, an 800-bed facility that serves a catchment population over 20 million individuals from both urban and rural communities. In 2018, about 255 000 patients were treated at the hospital; 220 000 as outpatient, 15 000 at emergency unit, and 20 000 as inpatient [19]. Since 2018, Xpert is the initial diagnostic test for TB used at the hospital and about 2800 TB cases are notified annually.

3.3.2. Sample size

To test the hypothesis that the proportion of bacteriologically confirmed cases is higher among Xpert-negative TB patients started on empiric TB treatment compared to those not started on empiric treatment, we estimated that a sample size of 250 participants (125 patients started on empiric TB treatment and 125 patients not started on empiric TB treatment) was required to have 80% power to detect a 10% difference in the proportion of bacteriologically confirmed TB (4% versus 14%) [20], the study was not powered to detect a difference in survival between groups.

3.3.3. Study design and procedures

All Xpert-negative adults (≥ 18 years of age) who presented to Jimma University Medical Center with presumptive TB were identified via the hospital laboratory Xpert register. Those who were admitted for hospitalization were followed by study nurses until the health care worker in charge of patient care decide to treat the patients with broad spectrum antibiotics. Within the first 48 hours following the health worker`s decision, informed consent was obtained, two sputum samples were collected for liquid and solid *Mtb* culture, and a structured questionnaire was administered by trained nurses to collect socio-demographic and clinical data. The medical file was reviewed for chest X-ray findings and to document the type of antibiotics prescribed. All study participants were followed up at 6 months to determine survival status.

3.3.4. Laboratory procedures

Mtb cultures were performed in the Mycobacteriology Research Center, established in 2010 as an inter-university collaborative research project between Jimma University and a consortium of Flemish Universities. An equal volume of sputum sample was mixed with N-Acetyl-L-Cysteine Sodium hydroxide with a final concentration of 1% NaOH in a 50mL falcon tube, vortexed, and incubated at room temperature for 15 minutes. Phosphate buffer saline pH 6.8 was added up to the 50mL mark before centrifugation at 3,000 x g for 15 minutes at 4°C. The supernatant was decanted into a splash-proof container containing 5% hypochlorite disinfectant and the pellet was re-suspended in 2.5mL sterile PBS. For liquid culture, the Mycobacterial Growth Indicator Tube (MGIT) method and BACTEC™ MGIT™ 960 instrument was used as per manufacturer`s instructions. Each tube was gently inverted several times until signaling "positive" or until day 42 if no growth was detected.

For solid media culture, 0.5mL of the sputum pellet was spread evenly over the entire surface of the Lowenstein Jensen medium using a sterile graduated disposable pipette. The tube was left with the cup loosened for 2-3 days at 37°C in horizontal position, after which the cap was securely tightened, and the tube was incubated in upright position and inspected for *Mtb* growth at weekly intervals for 6 weeks. *Mtb* growth was confirmed by smear microscopy using the Ziehl-Neelsen staining and Capilla test [21].

When both liquid and solid cultures were contaminated, a single Xpert MTB/RIF ultra (Xpert Ultra) was performed on a stored sputum sample according to manufacturer's instructions [22]. One milliliter of sputum sample was mixed with 2mL of reagent, vortexed for at least 10 seconds and incubated at room temperature (15-20^{0}C) for 10 minutes, after which it was again vortexed and incubated for another five minutes. The liquefied samples were aspirated using a sterile pipette up to the marked 2mL line and slowly transferred into the sample chamber of the Xpert Ultra cartridge labeled with the patient's identification number. The lid was then firmly closed and the cartridge inserted into a GeneXpert instrument [22].

3.3.5. Data analysis

Study population characteristics were summarized using descriptive statistics of frequency, mean, and standard deviation or median and inter-quartile ranges (IQR) as appropriate. Characteristics were tabulated overall and by empiric TB treatment status. The proportion of Xpert-negative

patients with bacteriological confirmation of TB was compared between patients who did and did not receive empiric TB treatment. The ability of the clinical algorithm (antibiotic trial and chest X-ray in case of non-response to antibiotics) to predict the presence of diagnose bacteriologically confirmed TB in hospitalized Xpert-negative patients was assessed by calculating the positive predictive value.

Logistic regression analysis was performed to determine the association (odds ratio and its 95% confidence interval) between patient characteristics and bacteriologically confirmed active TB and between patient characteristics and empiric TB treatment initiation. Characteristics that showed an association in the pair wise analysis ($p < 0.20$) were included in the multivariable logistic regression model. Six-month survival was assessed and stratified by empiric TB treatment status and bacteriological confirmation of TB. Data analysis was performed using R Statistical software version 3.6.1 [23].

3.3.6. Ethical clearance

The study was approved by the ethical review board of the Institute of Health, Jimma University (protocol no: IHRPGD/397/2018). Written informed consent was obtained from all patients. Clinicians treating patients in whom TB was bacteriologically confirmed were notified of the result.

3.4. Results

3.4.1. Characteristics of the study population

Between December 25, 2018 and July 26, 2019, consecutive Xpert-negative adults were screened for eligibility until the target of 125 empirically treated and 125 untreated patients were enrolled (**Figure 1**). Median age of participants was 39 years (IQR 28-50), about half were male (55.2%), had rural residency (57.6%), and were illiterate (47%) (**Table 1**). Only 18% of patients had a history of prior TB treatment. One in three patients (33.6%) was underweight (BMI<18.5kg/m^2). Among the 243 (97.2%) patients with documented HIV status, 21.4% were HIV positive, of whom 48.1% had CD4 count ≤ 200 cells/µl, and most (86.5%) were already on antiretroviral therapy (ART) for a mean of 7 years (±1.85). All patients presented with cough, fever, night sweats and shortness of breath. Most (66%) patients had prolonged cough for a mean duration of 4 weeks (± 2.6) and about half (54.8%) also had prolonged fever for a mean duration of 15 days (± 8.3). Almost all patients

(232/250, 92.8%) had already visited another health facility before admission at the Jimma University hospital.

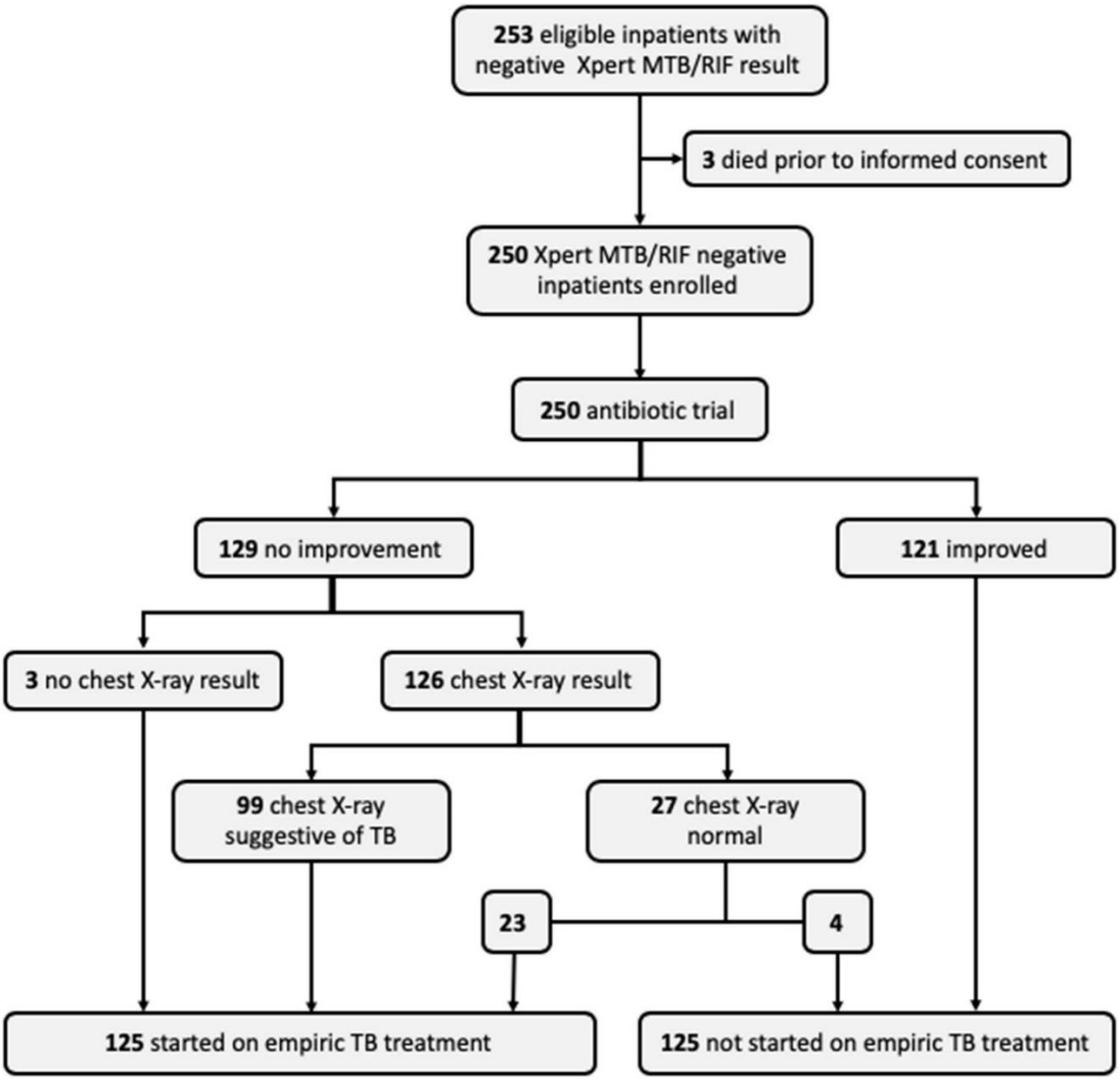

Figure 1: Flow chart of enrollment, result of antibiotic trial, chest X-ray findings and empiric TB treatment status among 253 Xpert MTB/RIF-negative adults hospitalized at Jimma University Medical Center, Ethiopia.

Table 1. Demographic and clinical characteristics of Xpert MTB/RIF-negative patients hospitalized at Jimma University Medical Center, Ethiopia.

Characteristics, n (%)		**All (N = 250)**	**Empiric TB treatment (N = 125)**	**No empiric TB treatment (N= 125)**
Age (years, median, IQR)		39 (28-50)	35 (25-50)	40 (30-53)
Sex	Male	138 (55.2)	70 (56.0)	68 (54.4)
	Female	112 (44.8)	55 (44.0)	57 (45.6)
Educational level	Illiterate	117 (46.8)	56 (44.8)	61 (48.8)
	Primary	51 (20.4)	25 (20.0)	26 (20.8)
	Secondary & above	82 (32.8)	44 (35.2)	38 (30.4)
Marital status	Single	59 (23.6)	37 (29.6)	22 917.6)
	Married	154 (61.6)	70 (56.0)	84 (67.2)
	Widowed/divorced	37 (14.8)	18 (14.4)	19 (15.2)
Residence	Urban	106 (42.4)	53 (42.4)	53 (42.4)
	Rural	144 (57.6)	72 (57.6)	72 (57.6)
Health facilities visited prior to hospital admission	Local government hospitals	126 (50.4)	63 (50.4)	63 (50.4)
	Primary care center	58 (23.2)	30 (24.0)	28 (22.4)
	Private Clinic	48 (19.2)	26 (20.8)	22 (17.6)
	None	18 (7.2)	6 (4.8)	12 (9.6)
History of TB treatment	No	205 (82.0)	97 (77.6)	108 (86.4)
	Yes	45 (18.0)	28 (22.4)	17 (13.6)
BMI	≤18.5Kg/m^2	84 (33.6)	46 (36.8)	38 (37.4)
	>18.5Kg/m^2	166 (66.4)	79 (63.2)	87 (69.6)
Severely ill	No	232 (92.8)	109 (87.2)	123 (98.4)
	Yes	18 (7.2)	16 (12.8)	2 (1.6)
Chest pain	No	82 (32.8)	15 (12.0)	67 (53.6)
	Yes	168 (67.2)	110 (88.0)	58 (46.4)
Weight loss	No	111 (44.4)	36 (28.8)	75 (60.0)
	Yes	139 (55.6)	89 (71.2)	50 (40.0)

Table 1: Continued.

Characteristics, n (%)		All (N = 250)	Empiric TB treatment (N = 125)	No empiric TB treatment (N= 125)
Duration of cough	< 14 days	85 (34.0)	23 (18.4)	62 (49.6)
	≥ 14 days	165 (66.0)	102 (81.6)	63 (50.4)
Duration of fever	< 14 days	137 (54.8)	54 (43.2)	83 (66.4)
	≥ 14 days	113 (45.2)	71 (56.8)	42 (33.6)
Duration of night sweats	< 14 days	130 (52.0)	55 (44.0)	75 (60.0)
	≥ 14 days	120 (48.0)	70(56.0)	58 (40.0)
Duration of shortness of breath	< 14 days	123 (49.2)	41 (32.8)	82 (65.6)
	≥ 14 days	127 (50.8)	84 (67.2)	43 (34.4)
HIV status	Negative	191 (76.4)	92 (73.6)	99 (79.2)
	Positive	52 (20.8)	26 (20.8)	26 (20.8)
	Unknown	7 (2.8)	0 (0.0)	7 (5.6)
CD4 count (n= 52)	≤ 200 cells/µl	25 (48.1)	14 (53.8)	11 (42.3)
	> 200 cells/µl	27 (51.9)	12 (46.2)	15 (57.7)
On ART		45 (86.5)	21 (80.8)	24 (92.3)
Duration on ART (years, mean, ±SD)		7 (±1.85)	8.9 (± 5)	6.8 (± 4.7)

HIV = Human Immunodeficiency virus, ART = antiretroviral therapy, TB = tuberculosis. All participants had symptoms of cough, fever, night sweat and shortness of breath.

3.4.2. Response to antibiotic trial, chest X-ray result and empiric TB treatment initiation

In line with the national guideline [12], all study participants received an antibiotic trial. Most common antibiotics used were ceftriaxone and azithromycin (112/250, 44.8%), amoxicillin (76/250, 30.4%) or vancomycin and doxycycline (62/250, 24.8%). The decision to initiate empiric treatment was predominantly guided by poor response to antibiotic treatment and not by chest X-ray findings, as only 4 of the 129 patients with poor response to antibiotic treatment were not initiated on empiric TB treatment while 23 of the 27 patients with a normal chest X-ray were initiated on empiric TB treatment (**Figure 1**).

3.4.3. Bacteriological confirmation of TB and its prediction by the clinical algorithm

Bacteriological confirmation was made in 35 patients by culture and in an additional 4 patients by Xpert Ultra when both the liquid and solid culture was contaminated (**Table 2**). In none of these 4 cases was the result on Xpert Ultra a trace result. The proportion of bacteriologically confirmed TB cases was almost 4 times higher among Xpert-negative patients who received empirical TB treatment (24.8%, 95% CI 17.5%, 33.3%) compared to those patients who were not started on empiric TB treatment (6.4% 95% CI 2.8%, 12.2%) (P ≤ 0.001).

Table 2. Laboratory results of hospitalized Xpert-negative patients stratified by empiric TB treatment status

	All (n = 250)	**Empiric TB treatment (n= 125)**	**No empiric TB treatment (n =125)**
Mtb culture			
LJ and/or MGIT positive	35 (14.0)	27 (21.6)	8 (6.4)
LJ and MGIT contaminated sputum Xpert Ultra positive	4 (1.6)	4 (3.2)	0 (0.0)
Sputum Xpert Ultra negative	7 (2.8)	3 (2.4)	4 (3.2)
LJ and MGIT negative	204 (81.6)	91 (72.8)	113 (90.4)

Mtb =*Mycobacterium tuberculosis*, LJ = Lowenstein–Jensen medium, MGIT = Mycobacteria Growth Indicator Tube.

Of the 125 people started on empiric treatment, 94 (75.2%) were not found to have active TB (**Table 3**). The positive predictive value of the clinical algorithm was low (24.8%, 95 CI: 24.5% -25.0%), resulting in substantial overtreatment. The clinical algorithm failed to detect 6.4% of people who responded to the antibiotic trial but were found to have bacteriologically confirmed TB, representing 22% (8/35) of all bacteriologically confirmed patients in the study. According to the guidelines, these patients were left untreated for TB.

Table 3. Bacterial confirmation by empiric TB treatment status.

Clinical algorithm		Sputum *Mtb* culture		Total
		Positive	**Negative**	
	Empiric TB treatment started	31 (79.5%)	94 (44.5%)	125 (50.0%)
	Empiric TB treatment not started	8 (20.5%)	117 (55.5%)	125 (50.0%)
	Total	**39**	**211**	**250**

3.4.4. Factors associated with bacteriological confirmation and initiation of empiric TB treatment.

The presence of prolonged cough (aOR 3.2, 95% CI: 1.1-10.9), prolonged shortness of breath (aOR 2.5, 95% CI: 1.1-6.2), body mass index ≤ 18.5kg/m^2 (aOR 4.8, 95% CI: 2.1-11.3) and age 41 to 64 years (aOR 0.3, 95% CI: 0.07-0.8) were independently associated with bacteriologically confirmed active TB (**Table 4**).

Table 4. Association between socio-demographic and clinical factors and bacteriologically confirmed TB among 250 Xpert-negative patients hospitalized at Jimma University Medical Center in Ethiopia.

Characteristics		***Mtb* culture confirmed (n =39)**	***Mtb* culture not confirmed (n =211)**	**OR (95% CI)**	**aOR (95% CI)**
Age	18-40 years	32 (82.0)	112 (53.1)	Ref.	Ref.
	41-64 years	4 (10.3)	71 (33.6)	**0.2** (0.05-0.5)	**0.3** (0.07-0.8)
	≥ 65 years	3 (7.7)	28 (13.3)	0.4 (0.08-1.2)	0.5 (0.09-1.9)
Sex	Male	24 (61.5)	97 (46.0)	Ref.	
	Female	15 (38.5)	114 (54.0)	0.7 (0.4-1.5)	
Educational level	Illiterate	11 (28.2)	105 (49.8)	Ref.	Ref.
	Primary	8 (20.5)	43 (20.4)	1.8 (0.6- 4.7)	1.3 (0.4-3.9)
	Secondary or above	20 (51.3)	63 (29.9)	**3.0** (1.4-6.9)	2.4 (0.8-6.8)
Marital status	Single	17 (43.6)	42 (19.9)	Ref.	Ref.
	Married	17 (43.6)	137 (64.9)	**0.3** (0.1-0.6)	0.5 (0.2-1.3)
	Widowed or di-vorced	5 (12.8)	32 (15.2)	0.4 (0.1-1.1)	0.5 (0.1-1.8)

Table 4: Continued.

Characteristics		***Mtb* culture confirmed (n =39)**	***Mtb* culture not confirmed (n =211)**	**OR (95% CI)**	**aOR (95% CI)**
Residence	Urban	15 (38.5)	91 (43.1)	Ref.	
	Rural	24 (61.5)	120 (56.9)	1.2 (0.6-2.5)	
HF visited prior to hospital admission	No prior visit	3 (7.8)	15 (7.1)	Ref.	
	Local government hospital	10 (25.6)	116 (55.0)	0.4 (0.1-2.1)	
	Primary health center	14 (35.9)	44 (20.9)	1.7 (0.4-8.1)	
	Private Clinic	12 (30.7)	36 (17.1)	1.6 (0.4-7.6)	
Severely ill	No	34 (87.2)	198 (93.8)	Ref.	
	Yes	5 (12.8)	13 (6.2)	2.2 (0.6-6.3)	
HIV status	Negative	28 (71.8)	170 (80.5)	Ref.	
	Positive	11 (28.2)	41 (19.4)	1.6 (0.7-3.3)	
BMI	> 18.5Kg/m^2	14 (35.9)	152 (72.0)	Ref.	Ref.
	≤ 18.5Kg/m^2	25 (64.1)	59 (28.0)	**4.6** (2.3-9.6)	**4.8 (2.1-11.3)**
Weight loss	No	9 (23.1)	102 (48.3)	Ref.	Ref.
	Yes	30 (76.9)	109 (51.7)	**3.1** (1.5-7.2)	1.5 (0.6-3.9)
Chest pain	No	6 (15.4)	76 (36.0)	Ref.	Ref.
	yes	33(84.6)	135 (64.0)	**3.0** (1.3-8.5)	1.6 (0.5-5.0)
History of TB treatment	No	31 (79.5)	174 (82.5)	Ref.	
	Yes	8 (20.5)	37 (17.5)	1.2 (0.5-2.7)	
Duration of fever	< 14 days	18 (46.2)	119 (56.4)	Ref.	
	≥ 14 days	21 (53.8)	92 (43.6)	1.5 (0.7-3.0)	
Duration of cough	< 14 days	5 (12.8)	80 (37.9)	Ref.	Ref.
	≥ 14 days	34 (87.2)	131 (62.1)	**4.1** (1.7-12.4)	**3.2 (1.1-10.9)**
Duration of night sweats	< 14 days	19 (48.7)	111 (52.6)	Ref.	
	≥ 14 days	20 (51.3)	100 (47.4)	1.1 (0.5-2.3)	
Duration of shortness of breath	< 14 days	11 (28.2)	112 (53.1)	Ref.	Ref.
	≥ 14 days	28 (71.8)	99 (46.9)	**2.9** (1.4-6.3)	**2.5 (1.1-6.2)**

OR= Odds Ratio, aOR= adjusted Odds Ratio, CI = Confidence Interval, Ref = Reference category, BMI = Body mass index.

Factors independently associated with empiric TB treatment initiation were the presence of weight loss (aOR 2.4, 95% CI: 1.3-4.6), chest pain (aOR 5.6, 95% CI: 2.8-12.5), being severely ill (aOR 11.0, 95% CI: 2.2 - 86.8), having prolonged cough (aOR 2.3, 95% CI: 1.2-4.5) or prolonged dyspnea (aOR 3.1, 95% CI: 1.4-5.2) and age 41 to 64 years (aOR 0.4, 95% CI: 0.2-0.9) (**Table 5**).

Table 5: Factors associated with initiation of empiric TB treatment among Xpert-negative patients hospitalized at Jimma University Medical Center in Ethiopia.

Characteristics		**Empirically treated for TB (n = 125)**	**Not empirically treated for TB (n = 125)**	**OR (95% CI)**	**aOR (95% CI)**
Age	18-40 years	78 (62.4)	66 (52.8)	Ref.	Ref.
	41-64 years	30 (24.0)	45 (36.0)	**0.6** (0.4-0.9)	**0.4 (0.2-0.9)**
	≥ 65 years	17 (13.6)	14 (11.2)	1.0 (0.4-2.3)	0.7 (0.2-1.9)
Sex	Male	68 (54.4)	70 (56.0)	Ref.	
	Female	57 (45.6)	55 (44.0)	1.1 (0.6-1.8)	
Educational level	Illiterate	55 (44.0)	61 (48.8)	Ref.	
	Primary	25 (20.0)	26 (20.8)	1.1 (0.5-2.0)	
	Secondary & above	45 (36.0)	38 (30.4)	1.3 (0.7-2.3)	
Marital status	Single	37 (29.6)	22 (17.6)	Ref.	Ref.
	Married	70 (56.0)	84 (67.2)	**0.5** (0.3-0.9)	0.5 (0.2-1.2)
	Widowed or divorced	18 (14.4)	19 (15.2)	0.6 (0.2-1.3)	0.4 (0.1-1.3)
Residence	Urban	53 (42.4)	53 (42.4)	Ref.	
	Rural	72 (57.6)	72 (57.6)	1.0 (0.6-1.7)	
HF visited prior to hospital admission	No prior visit	6 (4.8)	12 (9.6)	Ref.	
	Local government hospital	63 (50.4)	63 (50.4)	2.0 (0.7-6.0)	
	Primary care center	30 (24.0)	28 (22.4)	2.1 (0.7-6.8)	
	Private Clinic	26 (20.8)	22 (17.6)	2.4 (0.8-7.8)	
Severely ill	No	109 (87.2)	123(98.4)	Ref.	Ref.
	yes	16 (12.8)	2 (1.6)	**9** (2.5-57)	**11 (2.2-86.8)**

Table 5: Continued.

Characteristics		Empirically treated for TB (n = 125)	Not empirically treated for TB (n=125)	OR (95% CI)	aOR (95% CI)
HIV status	Negative	99 (79.2)	92 (78.0) *	Ref.	
	positive	26 (20.8)	26 (22.0)	0.9 (0.5-1.7)	
Body mass index	> 18.5Kg/m^2	79 (63.2)	87 (69.6)	Ref.	
	≤ 18.5Kg/m^2	46 (36.8)	38 (30.4)	1.3 (0.7-2.3)	
Weight loss	No	36 (28.8)	75(60.0)	Ref.	Ref.
	Yes	89 (71.2)	50 (40.0)	**3.7** (2.2-6.3)	**2.4** (1.3-4.6)
Chest pain	No	15 (12.0)	67 (53.6)	Ref.	Ref.
	Yes	110 (88.0)	58 (46.4)	**8.4** (4.6-16.6)	**5.6 (2.8-12.5)**
History of TB	No	97 (77.6)	108 (86.4)	Ref.	
treatment	Yes	28 (22.4)	17 (13.6)	1.8 (0.9-3.6)	
Duration of fever	< 14 days	54 (43.2)	83 (66.4)	Ref.	Ref.
	≥ 14 days	71 (56.8)	42 (33.6)	**2.6** (1.6-4.3)	1.6 (0.7-3.0)
Duration of cough	< 14 days	23 (18.4)	62 (49.6)	Ref.	Ref.
	≥ 14 days	102 (81.6)	63 (50.4)	**4.3** (2.5-7.8)	**2.3 (1.2-4.5)**
Duration of night	< 14 days	55 (44.0)	75 (60.0)	Ref.	Ref.
sweats	≥ 14 days	70 (56.0)	50 (40.0)	**1.9** (1.2- 3.2)	0.6 (0.3-1.4)
Duration of short-	< 14 days	41 (32.8)	82 (65.6)	Ref.	Ref.
ness of breath	≥ 14 days	84 (67.2)	43 (34.4)	**3.9** (2.3- 6.6)	**3.1 (1.4- 5.2)**

7 Unknown HIV status, OR= Odds Ratio, aOR= adjusted Odds Ratio, CI = Confidence Interval, Ref = Reference category.

3.4.5. Six-month mortality status by bacteriological confirmation and empiric TB treatment status

Overall, the six-month risk of death was 5.2% (**Table 6**). Risk of death was more than twice as high in people with bacteriological confirmation of TB (10.3% vs 4.3%). While clinically significant, this difference did not reach statistical significance (p = 0.12), possibly due to the low number of deaths observed in the study (13/250). Six-month risk of death was not affected by empiric treatment, as 5.6% of those empirically treated and 4.8% of those not started on TB treatment died (p = 0.78).

Table 6. Six-month mortality stratified by bacteriological *Mtb* confirmation status and by empiric TB treatment status

	Started on empiric TB treatment		**Not started on empiric TB treatment**	
Outcomes	**Survived**	**Died**	**Survived**	**Died**
All	**118/125 (94.4%)**	**7/125 (5.6%)**	**119/125 (95.2%)**	**6/125(4.8%)**
Bacteriologically confirmed TB*	28/31 (90.3%)	3/31 (9.7%)	7/8 (87.5%)	1/8 (12.5%)
No active TB	90/94 (95.7%)	4/94 (4.3%)	112/117 (95.7)	5/117 (4.3%)

*Bacteriological confirmation by liquid culture, solid culture or positive Xpert Ultra on stored sputum sample.

3.5. Discussion

In this study of hospitalized adults with presumptive pulmonary TB and a negative Xpert result, the presence of microbiologically confirmed TB was 4 times higher (24.8% vs 6.4%) in patients in whom the clinician decided to start empiric TB treatment based on a clinical algorithm consisting of an antibiotic trial and chest X-ray compared to people not started on empiric TB treatment. While this may support the use of such clinical algorithm, the positive predictive value of the clinical algorithm in this population of severely ill patients of whom 21% were HIV positive was low (24.8%). This resulted in high rates of overtreatment, with 75.2% of Xpert-negative patients receiving empirical TB treatment in the absence of bacteriological confirmation of active TB. Furthermore, the clinical algorithm failed to detect some patients with bacteriologically confirmed TB. Overall, 6.4% of people who responded to the antibiotic trial and were therefore not started on empiric TB treatment had bacteriologically confirmed TB. As these patients remained untreated, they may put their family members and the community at risk of *Mtb* infection. The optimism about empiric treatment was further diminished by the lack of impact of empiric TB treatment on six-month risk of death, as 5.6% of those empirically treated and 4.8% of those not started on TB treatment died. The six-month risk of death was however more than twice as high (10.3% vs 4.3%) in people with bacteriological confirmation of TB compared to those without bacteriological confirmation. While clinically significant, this difference was statistically not significant, possibly due to small number of deaths observed in the study.

For decades, clinicians have used empiric TB treatment to overcome the limited sensitivity of smear microscopy and to avoid the negative consequences of delays in treatment initiation, especially in high HIV/TB prevalent areas [13, 24]. In 2007, the WHO recommended that, in the absence of rapid and simple tools to diagnose TB, algorithms can assist clinical decision-making in HIV-prevalent and resource-constrained settings, to expedite the diagnostic process and minimize incorrect diagnosis and mortality. The WHO recommended that seriously ill patients with symptoms suggestive of TB should be treated empirically with broad-spectrum antibiotics because the benefits outweigh the risks and that all those who do not respond to the antibiotic trial are started on empiric TB treatment [25]. Cross-sectional studies in sub-Saharan Africa reported a positive predictive value of an antibiotic trial and chest X-ray algorithm in smear-negative patients ranging from 36.7% to 59% [24, 26, 27]. In our study, the positive predictive value of the clinical algorithm to detect the presence of bacteriological TB was lower (24.8%), even though cardinal symptoms of TB such as weight loss, prolonged cough and prolonged shortness of breath were associated with a decision to initiate empiric TB treatment. A lower positive predictive value of the clinical algorithm in the Xpert era is likely due to the higher sensitivity of the Xpert assay compared to smear microscopy.

The proportion of bacteriologically confirmed TB cases among empirically treated patients with smear-negative presumptive TB has varied between studies. A study from Uganda found that 23% of empirically treated smear-negative patients were culture positive [13], with higher rates observed in a Uganda (35.3%) and Korea (31%) (24, 28). In the Xpert era, studies have reported that 4% to 10% of Xpert-negative patients who received empiric treatment were found to be culture positive [15, 20]. In our study, 24.8% of empirically treated Xpert-negative patients were bacteriologically confirmed. The relatively high proportion may be due to the fact that we restricted study participation to individuals admitted to a referral hospital whereas the other studies were performed at primary care facilities.

Overall, most (27/35) bacteriologically confirmed cases in our study population were identified by the clinical algorithm, suggesting a high value of the use of the clinical algorithm. However, one in five (22%) of bacteriologically confirmed patients were missed by the algorithm and among those started on TB treatment, three in four (75%) were culture negative. Ideally, the rate of overtreatment, i.e., the proportion of people treated for TB who do not have active TB, is low in order to not overburden health care systems, avoid unnecessary toxicity and pill burden of TB treatment, and to avoid negative health consequences of missing another diagnosis. Among empirically treated

smear negative patients, the proportion who were not bacteriologically confirmed has varied from 49% in South Africa to 75.5% at a referral hospital in Addis Ababa, Ethiopia [27, 29]. A certain level of overtreatment may be acceptable if this result in a reduction in mortality. Unfortunately, empiric treatment of Xpert-negative patients in our study was not associated with a reduction in mortality as 5.6% of empirically treated patients and 4.8% of those untreated died. Our result extends the observations of no survival benefit of empiric TB treatment among people living with HIV [16 -18] to the population of all people (independent of HIV status) with presumptive TB admitted to a referral hospital in a high TB burden country.

Our study had some limitations. First, because we limited enrollment to hospitalized Xpert-negative patients, our results cannot be generalized to an outpatient setting. Second, we were not able to investigate the presence of pathogens other than *Mtb* to explore what the cause of illness was in the study participants without bacterial confirmation of TB. Third, despite the presence of a negative culture, it is still possible that some patients truly had pulmonary or extrapulmonary TB, which may have resulted in an overestimation of the proportion of overtreatment. Finally, while we observed a clinically significant difference in six-month survival between people with and without bacteriological confirmation, this difference was not statistically significant as the study was not powered to investigate factors associated with risk of death.

In conclusion, while the use of a clinical algorithm of antibiotic trial and chest X-ray by experienced clinicians can identify the majority of active TB cases among hospitalized Xpert-negative individuals, the use of empiric treatment may result in substantial overtreatment without an impact on survival. Given the higher mortality among bacteriologically confirmed patients, all patients with presumptive TB should gain access to the more sensitive Xpert MTB/RIF ultra-assay to better identify those patients in need of TB treatment.

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Chapter 4: Paper 2

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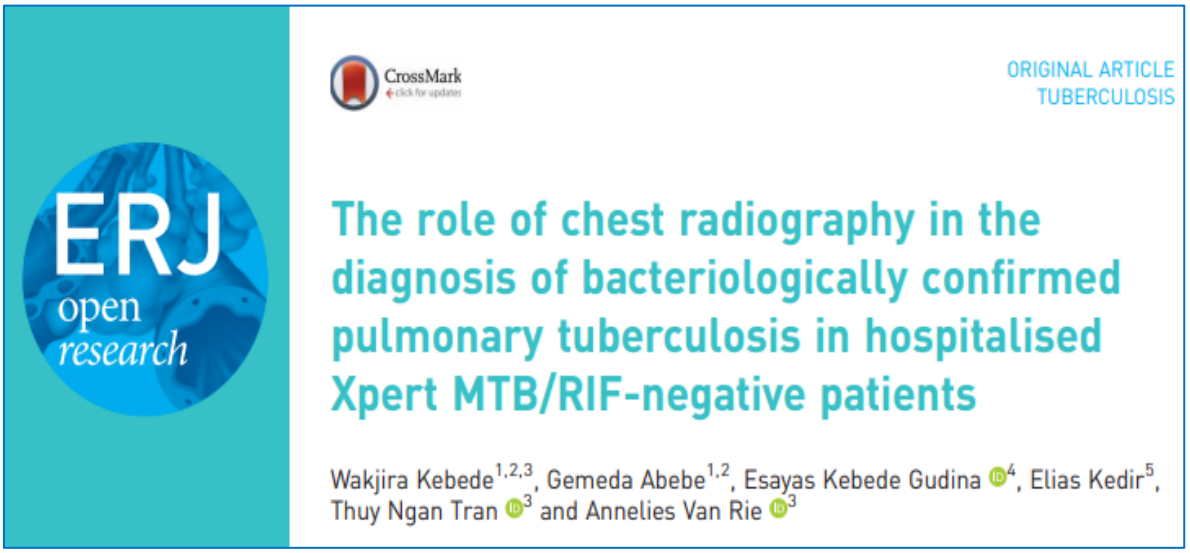

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My role in this work: As the first author, I am working closely with Prof. Annelies and Prof. Gemeda to design the study. I supervised the data collectors along with Prof. Esayas K. Gudina, performed sputum culture and Xpert Ultra assays for MTB diagnosis, conducted statistical analysis with Thuy Ngan Tran, and interpreted the findings with Prof. Annelies Van Rie. I wrote the initial draft of the article, incorporated feedback from all co-authors, and completed the manuscript submission process.

4.1. Abstract

Introduction: The role of a chest radiography to diagnose active tuberculosis (TB) in symptomatic patients who have a negative Xpert MTB/RIF (Xpert) test result is unclear. This study aimed to assess the performance of chest radiography and the value of chest radiography findings for a prediction tool to identify cases of active pulmonary TB among symptomatic, Xpert-negative hospitalized patients.

Methods: Xpert-negative patients hospitalized between January and July 2019 at Jimma University Medical Center in Ethiopia were assessed by mycobacterial culture and CXR. CXR was interpreted by a clinician for clinical decision making and by a radiologist for research purposes. Using bacteriological confirmation as the reference standard, the performance of chest radiography to diagnose active TB was assessed by the area under the receiver operating characteristic (AUC) curve, predictors of active TB were identified using bivariate and multivariate logistic regression analyses.

Results: Of 247 Xpert-negative patients, 38% and 40% were classified as suggestive of TB by clinician and radiologist, respectively. Of the 39 (15.8%) bacteriologically confirmed cases, 69% and 79% were classified as having chest radiography findings suggestive of TB by clinician or radiologist, respectively. While there was a strong association between bacteriologically confirmed TB and chest radiography classified by clinician as suggestive of TB (aOR 2.7, 95% CI: 1.2-6.6), chest radiography with signs typical of TB (aOR 5.3, 95% CI: 2.1-14.4) or compatible with TB (aOR 5.1, 95% CI: 1.3-20.0), the positive predictive value of the chest radiography was low (27% and 34% for classification by clinician and radiologist, respectively). The addition of chest radiography findings by clinician or radiologist to clinical characteristics did not improve the performance of the prediction tool, with similar risk classification distribution, AUCs and negative and positive prediction values.

Conclusions: Despite the strong association between chest radiography findings and active TB among hospitalized Xpert negative individuals**,** chest radiography findings did not improve the performance of a risk prediction tool based solely on clinical symptoms. Countries with a high TB/HIV burden should urgently replace Xpert by the more sensitive Xpert ultra-assay to improve the diagnosis of active TB.

4.2. Introduction

Tuberculosis (TB) remains a major public health problem, with an estimated 10 million new active TB cases and 1.3 million deaths in 2019 [1]. Of the 10 million people diagnosed globally with active TB in 2019, 87% occurred in 30 high TB burden countries. Ethiopia has the 10th highest burden of TB with 114, 233 cases corresponding to an estimated incidence rate of 151 TB cases per 100. 000 people [1, 2].

The World Health Organization (WHO) strongly recommends the use of Xpert MTB/RIF (Xpert) assay (and since 2017 the Xpert Ultra assay) as the initial diagnostic test for all adults and children with signs and symptoms of TB [3]. The WHO also conditionally recommends that Xpert (or Xpert Ultra) be used for further testing of smear-negative specimens in resource-constrained countries where multi-drug resistant TB (MDR-TB) or Human Immunodeficiency Virus (HIV) are of lesser concern [4]. Despite the progress made in bacteriological diagnosis of active TB following the implementation of the Xpert assay, an estimated one in three TB cases globally remain undiagnosed.

In the pre-Xpert era, the WHO viewed chest radiography as an important tool to diagnose pulmonary tuberculosis (PTB) when the diagnosis cannot be confirmed bacteriologically [5]. Several studies indeed demonstrated that a chest radiography is highly sensitive and can enhance the diagnosis of TB, especially in severely ill and HIV-positive patients, albeit with low specificity [6, 7] due to the presence of similar chest radiography findings in people with respiratory infections other than TB [8]. Furthermore, important variation between clinicians and radiologists in chest radiography interpretation can affect its specificity [9, 10].

In 2007, the WHO recommended that a chest radiography should be used in people presenting with symptoms of TB after an initial negative bacteriological test, a course of broad-spectrum antibiotics and a second negative round of bacteriological testing [5]. In 2008, the WHO also recommended the use of chest radiography directly after an initial negative smear microscopy test [11].

In the Xpert era, there has been a lot of interest in the use of chest radiography as a triaging tool to limit the use of the relatively expensive Xpert assays. Little attention has been paid to the role of a chest radiography in people with presumptive TB who have a negative initial Xpert assay result. This study aimed to assess added value of chest radiography (read by clinician or radiologist) to identify

cases of active TB among people admitted to a tertiary hospital with symptoms of TB who had a negative Xpert result.

4.3. Methods

4.3.1. Study setting

The study was conducted from January to July 2019 at Jimma University Medical Center (JUMC) referral hospital that provides inpatient and outpatient services to the over 15 million people living in southwest Ethiopia [12]. At the JUMC, the Xpert test is used as the initial diagnostic in people presenting with symptoms or signs of PTB. When the Xpert assay is negative for *Mycobacterium tuberculosis* (*M. tuberculosis*), the clinician can request a chest radiography, paid by the individual seeking care, for clinical decision-making [13].

4.3.2. Study population and data collection

This analysis is a secondary analysis of a study that aimed to assess the role of empiric treatment in hospitalized adult (age ≥ 18 years) patients with symptoms of PTB and negative Xpert result [14]. In the parent study, 125 patients treated for active TB and 125 patients in whom the physician decided not to treat for TB were enrolled. All patients were assessed by chest radiography and sputum mycobacterial culture prior to TB treatment initiation. CXRs were not performed at the end of treatment.

Mycobacterial culture was used as the gold standard for diagnosis of TB. About 0.5mL of the pellet of a sputum sample subjected to N-acetyl-l-cysteine-sodium hydroxide was inoculated on the BACTEC MGIT 960 system and incubated for up to 42 days; another 0.5mL of the pellet was inoculated on solid Lowenstein-Jensen (LJ) media at 37˚C for 56 days. *M. tuberculosis* growth was confirmed by the Ziehl-Neelsen stain and Capilla test. Cultures with non-acid fast bacilli growth on blood agar were re-decontaminated and re-incubated [15]. In case of contamination of both solid and liquid culture, a single Xpert Ultra test was performed on a stored sputum sample according to manufacturer's guideline or explained in literature [16, 17]. Participants were classified as cases of bacteriologically confirmed TB when *M. tuberculosis* complex grew on liquid and/or solid culture media or when *M. tuberculosis* was detected on Xpert Ultra.

Relevant socio-demographic and clinical data were collected via a pre-tested structured questionnaire and retrieved from the participants' medical records. Data collected were age, sex, educational level, marital status, body mass index (BMI), weight loss, chest pain, loss of appetite, history of TB treatment, presence and duration of cough, fever, night sweats, and shortness of breath, HIV status, and CD4 count.

The classification of the chest radiography by a treating clinician as 'suggestive for TB' or 'not suggestive for TB' as part of routine clinical care was retrieved from the medical records. For research purpose, a senior radiologist who was blinded to the chest radiography reading by the clinician and the patient's clinical and laboratory data, classified the chest radiography as normal, compatible with TB (enlarged hilar nodes, pneumonic lesion, atelectasis, mass lesion, or miliary abnormalities) or typical for TB (nodular, alveolar, or interstitial infiltrates affecting the zones above the clavicles or upper zones of the lungs) using a standardized classification system [18].

4.3.3. Data analysis

Baseline characteristics of study participants and chest radiography abnormalities are reported as frequencies and proportions. Kappa (κ) score was calculated to assess the inter-reader agreement of chest radiography classification between clinicians and senior radiologist. For this analysis, the radiologist's readings of 'typical of TB' and 'compatible with TB' were combined into a single 'suggestive of TB' category to create a binary classification of 'suggestive of TB' or 'not suggestive of TB'. Using bacteriologically confirmed TB as the gold standard, sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were estimated for chest radiography classification by clinician and radiologist.

Bivariate logistic regression models were used to determine the association between clinical variables (sex, symptoms of TB, BMI, and HIV infection), radiological findings (by clinician or radiologist), and bacteriologically confirmed pulmonary TB. Three multivariate models were built to assess the association between bacteriologically confirmed TB and clinical characteristics (model 1), clinical characteristics and chest radiography interpretation by clinician (model 2) and clinical characteristics and chest radiography interpretation by radiologist (model 3). Variables included in multivariate logistic regression model were those with p-value < 0.2 in bivariate analysis. Generalized variance-inflation factor (GVIF) was estimated to check multicollinearity among variables in multivariate

models [19]. Backward stepwise model reduction was performed using the likelihood ratio test with a cut-point of p-value 0.1.

The predictive accuracy of the final models was assessed by calibration and discrimination parameters. Model discrimination was estimated by the area under a receiver operating characteristic curve (AUC) with a range from 0.5 indicating no discrimination to 1.0 indicating perfect discrimination. Model calibration was assessed visually with a calibration plot and by comparing the predicted and observed probabilities using the Hosmer-Lemeshow test ($p > 0.05$ indicates good fit). The models were internally validated using a non-parametric bootstrap approach with 1,000 random bootstrap samples. The extent of performance over-optimism when the models are applied to new patients in a similar population was measured by the average AUC difference between the bootstrap samples and the original full sample.

To assess the value of chest radiography results in addition to clinical characteristics, the three prediction models were transformed to simplified risk scores. For each model, the coefficient of each variable was divided by the smallest coefficient and rounded to the nearest integer to give the weight for each variable in the simplified risk score. For each of the three models, a risk score was calculated for each individual participant and each patient's risk of bacteriologically confirmed TB was classified into one of four risk categories (very low, low, moderate, and high risk).

Finally, using different cut-offs, the diagnostic performance of chest radiography in diagnosing active PTB was assessed by calculating sensitivity, specificity, positive and negative predictive values. All data analyses were performed using the R Statistical software version 3. 6.1.

4.3.4. Ethical considerations

The study was approved by the institutional review board (IRB) of Jimma University Institute of Health (Ref. no. IHRPGD 397/2018). Written informed consent was obtained from all study participants. Patients with bacteriologically confirmed TB on study samples were started on TB treatment.

4.4. Results

4.4.1. Baseline characteristics

Of 250 patients enrolled in the parent study, 247 patients with data on both CXR and bacteriological evaluation were included in this analysis **(Figure 1).** Baseline characteristics of the study population

are shown in **(Table 1).** Median age was 39 years, about half (55.5%) were male, most were married (61.5%), had a primary school education (66.8%) and were rural residents (57.9%). One in three (34.0%) were underweight (BMI ≤ 18.5kg/m^2) and among the 240 (97%) patients tested, 52 (21.7%) were HIV positive. All participants self-reported one or more TB symptoms, including weight loss (55.0%), chest pain (66.8%), prolonged cough (≥ 2 weeks) (65.6%), fever (45.3%), night sweats (48.2%) and shortness of breath (50.6%). Few (18.2%) self-reported a history of TB treatment.

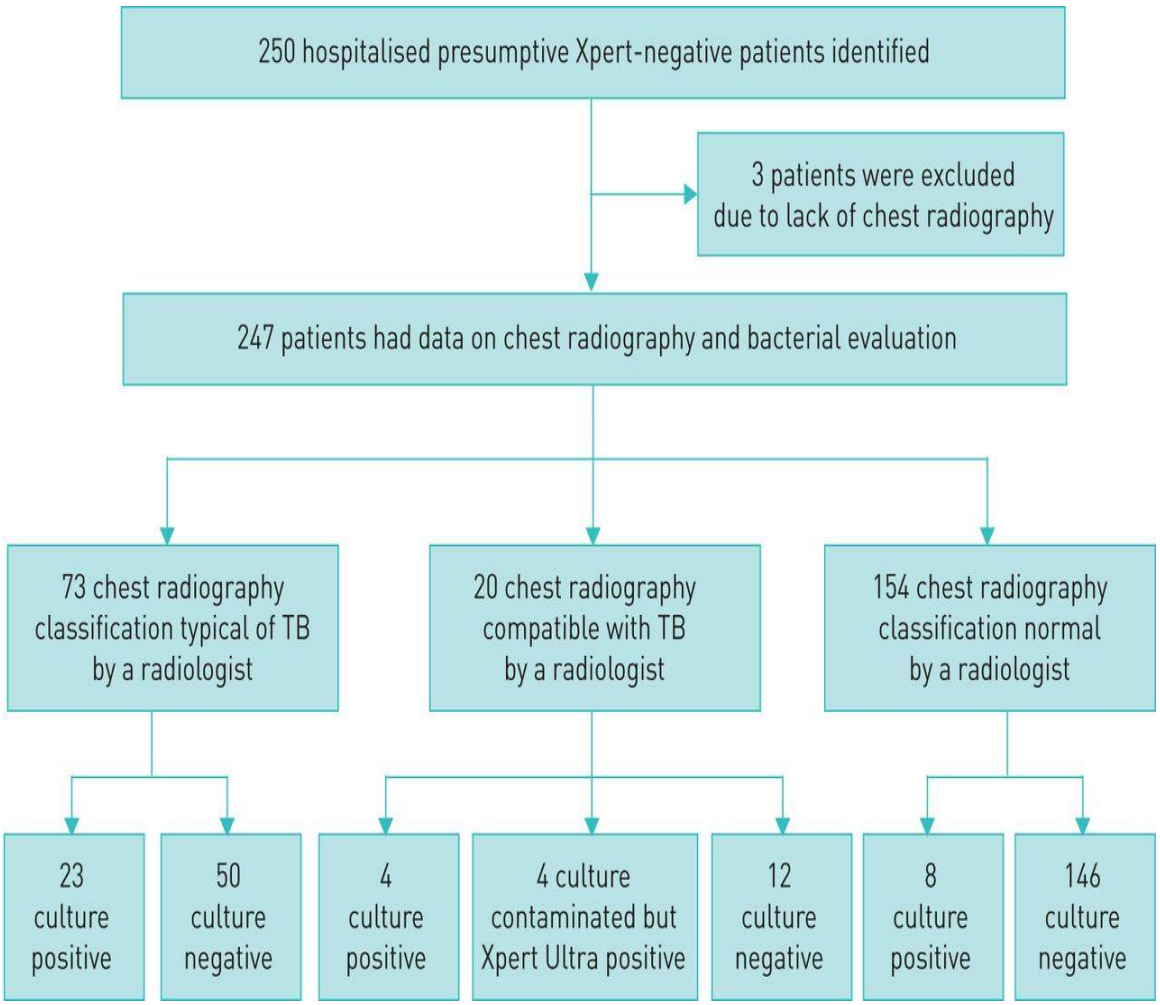


Figure 1. Flow chart of enrolment, chest radiography classification by radiologist and results of laboratory tests among 247 Xpert-negative adults hospitalized at Jimma University Medical Center, Ethiopia. TB: Tuberculosis.

Table 1. Characteristics of 247 Xpert-negative presumptive tuberculosis (TB) cases hospitalized at Jimma University Medical Center, southwest Ethiopia.

Characteristics	Category	Number
Age	18 -40 years	141 (57.1%)
	≥41years	106 (42.9%)
Sex	Female	110 (44.5%)
	Male	137 (55.5%)
Marital status	Single	58 (23.5%)
	Married	152 (61.5%)
	Widowed or divorced	37 (15.0%)
Educational level	Illiterate	114 (46.2%)
	Primary	51 (20.6%)
	Secondary & above	82 (33.2%)
Residence	Urban	104 (42.1%)
	Rural	143 (57.9%)
Severely ill	Yes	17 (6.9%)
History of TB treatment	Yes	45 (18.2%)
Body mass index (BMI)	>18.5kg/m^2	163 (66.0%)
	≤ 18.5Kg/m^2	84 (34.0%)
HIV status*	Negative	188 (78.3%)
	Positive	52 (21.7%)
CD4 count (n = 52)	≤ 200 cells/µl	25 (48.1%)
	>200 cells/µl	27 (51.9%)
Loss of appetite	Yes	199 (80.6%)
Weight loss	Yes	136 (55.0%)
Chest pain	Yes	165 (66.8%)
Cough of ≥ 2 weeks	Yes	162 (65.6%)
Fever of ≥ 2weeks	Yes	112 (45.3%)
Night sweat of ≥ 2weeks	Yes	119 (48.2%)
Shortness of breath of ≥ 2weeks	Yes	125 (50.6%)

*7 missing HIV test result, HIV = Human Immunodeficiency Virus.

4.4.2. Chest radiography findings

According to the clinician who read the chest radiography as part of routine clinical care, 99 (40.1%) participants had a chest radiography suggestive of TB and 148 (59.9%) a CXR not suggestive of TB. According to the radiologist, 73 (29.6%) patients had typical chest radiography findings for TB (31 cavitary lesion, 26 pleural effusion and 16 consolidation), 20 (8.1%) had a chest radiography compatible with TB (14 miliary disease, 4 fibrosis and 2 hilar adenopathy) and 154 (62.3%) had a chest radiography not suggestive of active TB (**Table 2**). The agreement between the classification of chest radiography reading by clinician suggestive of TB vs not suggestive of TB) and radiologist (typical or compatible with TB vs not suggestive of TB) was good [87.1% agreement; κ score = 0.728, $p < 0.001$] (**Table 3**).

Table 2: Chest radiography classification by clinicians and radiologist, stratified by bacteriological TB confirmation status in 247 Xpert-negative patients hospitalized at Jimma University Medical Center, Ethiopia.

Chest radiography findings	All	Confirmed for TB	Not confirmed for TB
Patients n	**247**	**39**	**208**
CXR classified by clinicians			
Not suggestive of TB	148 (59.9%)	12 (30.8%)	136 (65.4%)
Suggestive of TB	99 (40.1%)	27 (69.2%)	72 (34.6%)
CXR classified by a radiologist			
Not suggestive of TB	154 (62.3%)	8 (20.5%)	146 (70.2%)
Typical of TB	73 (29.6%)	23 (59.0%)	50 (24.0%)
Cavitary lesion	31 (12.6%)	11 (28.2%)	20 (9.6%)
Pleural effusion	26 (10.5%)	6 (15.4%)	20 (9.6%)
Consolidation	16 (6.5%)	6 (15.4%)	10 (4.8%)
Compatible with TB	20 (8.1%)	8 (20.5%)	12 (5.8%)
Miliary disease	14 (5.7%)	6 (15.4%)	8 (3.8%)
Fibrosis	4 (1.6%)	1(2.6%)	3 (1.4%)
Hilary adenopathy	2 (0.8%)	1 (2.6%)	1 (0.5%)

Data are presented as n (%), unless otherwise stated.

Table 3. The inter-reader agreement between the clinicians and a radiologist on the chest radiography classification.

	Radiograph read by a radiologist[#]			Kappa	P value
	Typical or compatible with TB	Not suggestive of TB	Total		
Radiograph read by clinicians[¶]					
Suggestive of TB	80 (86.0%)	19 (12.3%)	99 (40.1%)	0.728	< 0.001
Not suggestive of TB	13 (14.0%)	135 (87.7%)	148 (59.9%)		
Total	93 (37.7%)	154 (62.3%)	247 (100%)		

Data are presented as n (%), unless otherwise stated. TB: tuberculosis. #: radiologist classified chest radiography for research purpose, using the standardized methodology to classify each chest radiograph [18]. Clinicians classified the chest radiography as part of routine care, for patient management.

Of the 39 participants with bacteriologically confirmed PTB, the radiologist classified 23 (59.0%) patients' chest radiography as having typical chest radiography findings of TB, 8 (20.5%) as having signs compatible with TB and 8 (20.5%) as having no abnormalities suggestive of TB (**Table 2**). All eight patients with normal chest radiography but bacteriologically confirmed PTB were HIV-positive. The clinician classified the CXR of 27 (69.2%) of the 39 patients with bacteriologically confirmed as suggestive of TB, and 12 (30.8%) as not suggestive of TB. The sensitivity of the clinician's chest radiography reading to identify a case of bacteriologically confirmed TB was 69% (95% CI: 63-90), specificity 65% (95% CI: 58-71), PPV 27% (95% CI: 22-33), and NPV 92% (95% CI: 88-95). The sensitivity of the radiologists reading to identify cases of bacteriologically confirmed TB was 79.5% (95% CI: 64-90), specificity 70% (95% CI: 64-76), PPV 34% (95% CI: 28-39), and NPV 95% (95% CI: 90-97).

4.4.3. Ability of chest radiography to diagnose bacteriologically confirmed TB among patients with a negative Xpert result

Eight clinical variables and chest radiography reading (by clinician or radiologist) were associated with bacteriologically confirmed PTB in bivariate logistic regression **(Table 4).** All variables in the initial multivariate models showed GVIFs <2 (1.06-1.46), indicating absence of multicollinearity. After backward stepwise model reduction, model 1 (clinical parameters only) comprised five variables: age, loss of appetite, BMI, prolonged cough, and prolonged shortness of breath. In model 2 (clinical parameters and clinician chest radiography reading), four clinical variables (age, loss of

appetite, BMI, and prolonged cough) and chest radiography reading were included. Compared to patients with a chest radiography not suggestive of TB, the odds of bacteriologically confirmed TB were 2.7 (95% CI: 1.2-6.6) times higher for patients with a chest radiography suggestive of TB. In model 3 (clinical parameters and chest radiography reading by radiologist), four clinical variables (age, BMI, loss of appetite, and prolonged cough) and chest radiography reading were included. Compared to patients with a chest radiography not suggestive of TB, the odds of bacteriologically confirmed TB were 5.3 (95% CI: 2.1-14.4) times higher for patients with typical chest radiography signs of TB and 5.1 (95% CI: 1.3-20.0) times higher for patients with chest radiography signs compatible with TB.

Table 4 Association between potential predictor variables and bacteriologically confirmed tuberculosis (TB) among 247 symptomatic hospitalized patients with negative Xpert MTB/RIF result.

Characteristics		**Bacteriologically confirmed for TB**		**OR (95 % CI)**	**P value**	**Model 1** aOR (95% CI)	**Model 2** aOR (95% CI)	**Model 3** aOR (95% CI)
		Yes	No					
Patient n		**39**	**208**					
Age	≥41 years	7 (17.9)	99 (47.6)	Ref.		Ref.	Ref.	Ref.
	18-40 years	32 (82.1)	109 (52.4)	4.2 (1.8-10.6)	0.001	**6.5 (2.6-18.0)**	**5.5 (2.2-15.2)**	**4.9 (2.0-13.7)**
Sex	Female	15 (38.5)	95 (45.7)	Ref.				
	Male	24 (61.5)	113 (54.3)	1.3 (0.7-2.8)	0.407			
Cough of ≥ 2	No	5 (12.8)	80 (38.5)	Ref.		Ref.	Ref.	Rf.
weeks	Yes	34 (87.2)	128 (61.5)	4.3 (1.7-12)	0.003	**3.7 (1.4-12.1)**	**3.3 (1.2-11.1)**	**2.8 (0.95-9.5)**
Fever of ≥ 2	No	18 (46.2)	117 (56.2)	Ref.				
weeks	Yes	21 (53.8)	91 (43.8)	1.5 (0.8- 3.0)	0.281			
Night sweat of ≥ 2	No	19 (48.7)	109 (52.4)	Ref.				
weeks	Yes	20 (51.3)	99 (47.6)	1.2 (0.6- 2.3)	0.673			
Shortness of	No	11 (28.2)	111 (53.4)	Ref.		Ref.		
breath of ≥ 2	Yes	28 (71.8)	97 (46.6)	2.9 (1.4-6.4)	0.005	**2.5 (1.1-6.2)**		
weeks								

Table 4: Continued.

Characteristics		Bacteriologically confirmed for TB		OR (95 % CI)	P value	Model 1	Model 2	Model 3
						aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
Loss of appetite	No	2 (5.1)	46 (22.1)	Ref.		Ref.	Ref.	Ref.
	Yes	37 (94.9)	162 (77.9)	5.3 (1.5-3.1)	0.025	**9.2 (2.3-61.9**)	**8.5 (2.2-56.8)**	**5.8 (1.5-39.3)**
Weight loss	No	9 (23.1)	102(49.0)	Ref.				
	Yes	30(76.9)	106(51.0)	3.2 (1.5-7.5)	0.003			
Chest pain	No	6 (15.4)	76(36.5)	Ref.				
	Yes	33(84.6)	132) 63.5)	3.2 (1.4- 8.7)	0.013			
History of TB treat-	No	31(79.5)	171 (82.2)	Ref.				
ment	Yes	8 (20.5)	37 (17.8)	1.2 (0.5- 2.7)	0.686			
Severely ill*	No	34 (87.2)	196 (94.2)	Ref.				
	Yes	5 (12.8)	12 (5.8)	2.4 (0.7- 6.9)	0.12			
Body mass index	>18.5kg/m^2	14 (35.9)	149 (71.6)	Ref.		Ref.	Ref.	Ref.
	≤ 18.5Kg/m^2	25 (64.1)	59 (28.4)	4.5 (2.2 - 9.5)	4.17e-^{05}	**5.0 (2.3-11.7)**	**4.4 (2.0-10.2)**	**3.9 (1.7-9.2)**
HIV status*	Negative	28 (71.8)	160 (79.6)	Ref.				
	Positive	11 (28.2)	41 (20.4)	1.5 (0.7 - 3.3)	0.247			
chest radiography classified by clinicians	Not suggestive of TB	12 (30.8)	136 (65.4)	Ref.		Not included	Ref.	
	Suggestive of TB	27 (69.2)	72 (34.6)	4.3 (2.1-9.21)	0.0001		**2.7 (1.2-6.6)**	
chest radiography classified by radiolo- gist	Not suggestive of TB	8 (20.5)	146 (70.2)	Ref.		Not included	Not included	Ref.
	Compatible with TB	8 (20.5)	12 (5.8)	12.2 (3.9-39.2)	1.83e-^{06}			**5.1 (1.3-20.0)**
	Typical of TB	23 (59.0)	50 (24.0)	8.4 (3.7-21.1)	1.48e-^{05}			**5.3 (2.1-14.4)**

Data are presented as n (%), unless otherwise stated. aOR: adjusted odds ratio. #: defined as patients with a fever of >39°C, tachycardia >120 beats·min^{-1}, or tachypnoea >30 breaths·min−1 [5]; *7 missing HIV test result.

To contribution (weighted points) of the variables to the risk scores for the different models was estimated and a risk score calculated for each individual patient using clinical parameters only (model 1), clinical parameters plus chest radiography reading by clinicians (model 2), and clinical parameters plus chest radiography reading by radiologist (model 3) (**Table 5**). The distribution of risk scores and classification in 4 risk categories for bacteriologically confirmed TB (very low, low, moderate and high risk) for each of the three models is presented in **Table 6**. For all three models, 0% of patients with a very low risk, and about 70% of patients with high risk (74% for model 1, 67% for model 2 and 70% for model 3) had bacteriologically confirmed TB.

Table 5: Weighted points assigned to each of the independent variables significantly associated with bacteriologically confirmed tuberculosis (TB) in multivariate analysis

Characteristics	**Model 1**		**Model 2**		**Model 3**	
	Regression coefficient	Contribution to risk score	Regression coefficient	Contribution to risk score	Regression coefficient	Contribution to risk score
Intercept	-7.1		-6.7		-6.57	
Age (18-40 years)	1.8684	2	1.7057	2	1.5845	2
Cough of ≥ 2 weeks	1.3070	1	1.2016	1	1.0239	1
Shortness of breath of ≥ 2weeks	0.9208	1	-	-		
Body mass index ≤ 18.5 Kg/m^2	1.6167	2	1.4884	1	1.3576	1
Loss of appetite	2.2156	2	2.1376	2	1.7605	2
chest radiography classified as suggestive of TB by clinician	Not included	Not included	1.0026	1	Not included	Not included
chest radiography classified as typical of TB by radiologist	Not included	Not included	Not included	Not included	1.6275	2
chest radiography classified as compatible with TB by radiologist	Not included	Not included	Not included	Not included	1.6679	2

Model 1: risk score 2 if age (18–40 years) +1 if cough of ⩾2 weeks +1 if shortness of breath of ⩾2 weeks +2 if body mass index ⩽18.5 kg·m−2 +2 if loss of appetite. Model 2: risk score 2 if age (18–40 years) +1 if cough of ⩾2 weeks +1 if body mass index ⩽18.5 kg·m−2 +2

if loss of appetite +1 if chest radiography classification suggestive of TB by clinicians. Model 3: risk score 2 if age (18–40 years) +1 if cough of ⩾2 weeks +1 if body mass index ⩽18.5 kg·m−2 +2 if loss of appetite +2 if chest radiography classification typical/compatible of TB by radiologist. Shortness of breath of ⩾2 weeks was excluded due to lack of association in models 2 and 3.

Table 6: Distribution of risk scores and risk category among all 247 symptomatic hospitalized patients with negative Xpert result and among those with bacteriologically confirmed tuberculosis.

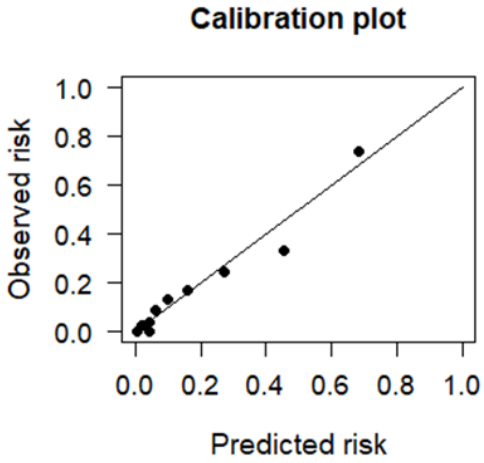
	Risk score	**All patients**		**Bacteriologically confirmed cases**		**Risk category**	**All patients**		**Bacteriologically confirmed cases**	
		N	%	N	**%**		N	%	N	%
Model 1	0	1	0	0	0	Very low	29	12	0	0
	1	3	1	0	0					
	2	25	10	0	0					
	3	38	15	1	3	Low	140	56	10	7
	4	57	23	3	5					
	5	45	18	6	13					
	6	50	20	12	24	Moderate	59	24	15	25
	7	9	4	3	33					
	8	19	9	14	74	High	19	8	14	74
Model 2	0	0	0	0	0	Very low	34	13	0	0
	1	7	3	0	0					
	2	27	11	0	0					
	3	50	20	3	6	Low	120	49	6	5
	4	70	28	3	4					
	5	45	18	9	20	Moderate	72	29	19	26
	6	27	11	10	37					
	7	21	9	14	67	High	21	9	14	67
Model 3*	0	0	0	0	0	Very low	6	2	0	0
	1	6	3	0	0					
	2	26	11	1	4	Low	177	72	7	4
	3	51	21	2	4					
	4	51	21	2	4					
	5	49	20	2	4					
	6	19	8	7	37	Moderate	41	17	16	39
	7	22	7	9	41					
	8	23	9	16	70	High	23	9	16	70

* Typical and compatible chest radiography are mutually exclusive categories.

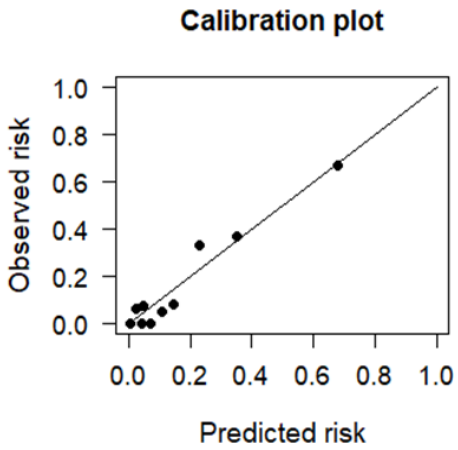
The calibration plots of models 1, 2 and 3 showed good calibration with predicted probabilities reasonably similar to the observed probabilities across the distribution of risk (**S1. Figure: A, B and C**). The AUCs of the final multivariate models 1, 2 and 3 were 0.842 (95% CI: 0.77-0.90), 0.845 (95% CI: 0.77- 0.91), and 0.857 (95% CI: %, 0.78-0.92) respectively **(S2 Figure)**. After bootstrapping, the estimated optimism in the three model's AUC was shown to be very low (mean range 0.0227-0.0239) and the calibration plot showed that predicted probabilities and observed probabilities were almost similar (**S3 Figure: A, B and C).**

The simplified risk prediction tools built based on the three models yielded very similar AUC to that of the final multivariable models, with little difference between the three models: AUC = 0.835 (95% CI: 0.77-0.90) for risk tool 1, AUC = 0.845 (95% CI: 0.78-0.91) for risk tool 2, and 0.887, (95% CI: 0.78-0.92) for risk tool 3 (**S4 Figure: A, B and C**).

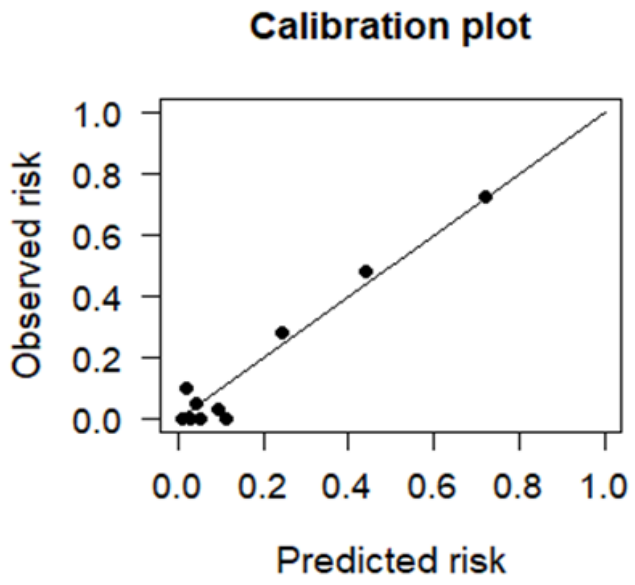
The sensitivity, specificity and predictive values for specific risk score cutoffs are presented in **Table 7**. If, for model 1 (clinical parameters only), a cut-off 8 (high risk vs other categories) is chosen as the threshold for empiric treatment decision making, the NPV and specificity for confirmed pulmonary TB were high (89% and 98%, respectively) but the sensitivity and PPV were relatively low (36% and 74%, respectively). Adding chest radiography reading by clinician or radiologist did not improve the performance of the risk prediction tool, with similarly low sensitivity and PPV.



A. Model 1: Based on clinical parameters only

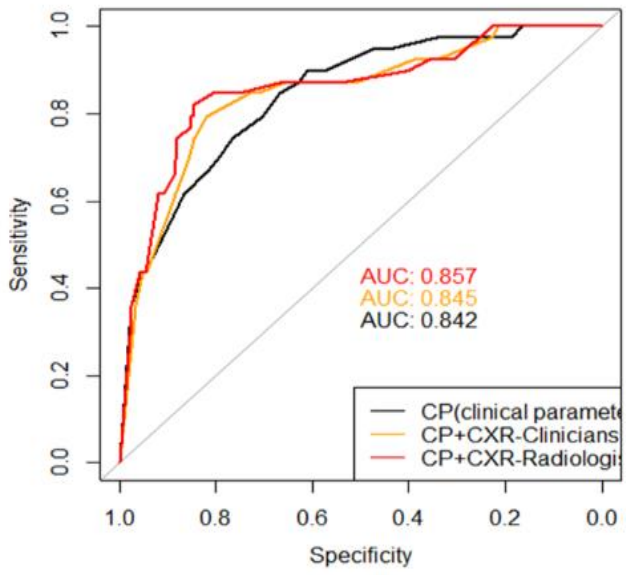


B. Model 2: Based on clinical parameters + chest X-ray read by clinicians

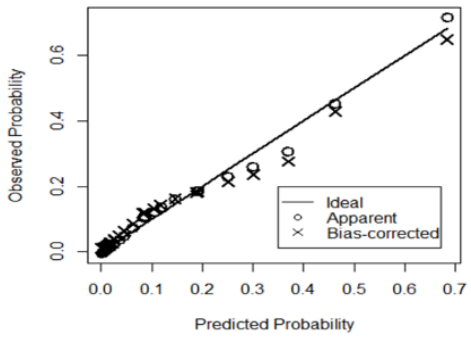


C. Model 3: Based on clinical parameters plus chest X-ray read by a radiologist

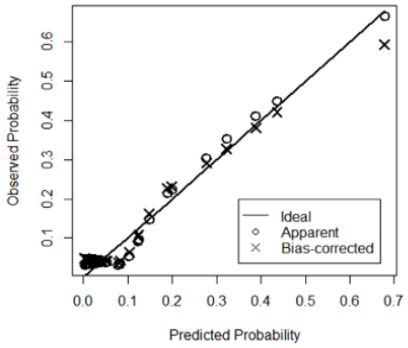
Supplementary Figure1 Calibration plot showed the agreement between observed and predicted probabilities for active pulmonary TB (before bootstrapping).



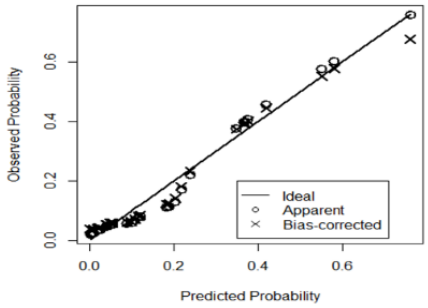
Supplementary Figure 2: Receiver operating characteristic (ROC) curve of the ability of clinical symptoms (age (18-40 years), cough of ≥ 2weeks, shortness of breath of ≥ 2weeks, loss of appetite, and low body mass index (≤ 18.5kg/m2) and CXR read by clinician or radiologist to predict the presence of active pulmonary TB.



A. Model 1: Clinical parameters only.

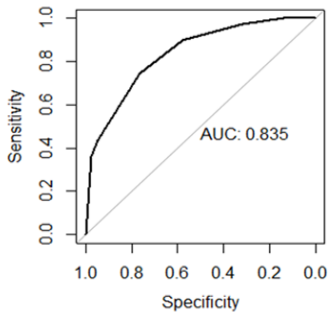


B. Model 2: Clinical parameters + chest X-ray read by clinician.

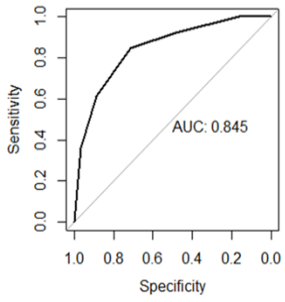


C. Model 3: Clinical parameters plus chest radiography read by radiologist.

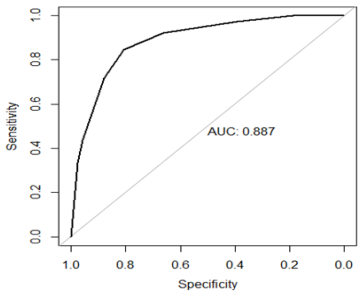
Supplementary Figure 3. Calibration plot showed the agreement between ideal, apparent and corrected biased to predict active pulmonary TB (after bootstrapping).



A. Model 1: AUC = 0.835, (95% CI: 0.77-0.90)



B. Model 2: AUC = 0.8454, **(**95% CI: 0.78-0.91)



C. Model 3: AUC= 0.88 (95% CI: 0.83-0.94)

Supplementary Figure 4. The simplified risk score developed based on clinical parameter (model 1), clinical parameter plus chest radiography read by clinicians (model 2), and clinical parameter plus chest radiography read by radiologist (model 3).

Table 7. The performance of chest radiography to diagnose active pulmonary tuberculosis among 247 Xpert-negative patients using bacteriological confirmation as a reference standard.

	N (%)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Clinical parameters only[#]					
Cut-off score ≥ 3	218 (88.3)	100 (91-100)	14 (10-19)	18(13-24)	100(88-100)
Cut-off score ≥ 6	78 (31.6)	74 (58-87)	76 (70-82)	37 (26-49)	94 (89-97)
Cut-off score 8	19 (7.8)	36 (21-53)	98 (94-99)	74 (49-91)	89 (84-93)
Clinical parameters[¶] + chest radiography classified by clinicians					
Cut-off score ≥ 3	213 (86.2)	100 (91-100)	16 (12-22)	18 (13-24)	100(90-100)
Cut-off score ≥ 5	93 (37.7)	85 (69-94)	71 (64-77)	35 (26-46)	96 (92-99)
Cut-off score 7	21 (8.5)	36 (21-53)	97 (93-99)	67 (43-85)	89 (84-93)
Clinical parameters[¶] + chest radiography classified by radiologist					
Cut-off score ≥ 2	241 (97.6)	100 (91-100)	3 (1-6)	16 (12-21)	100(54-100)
Cut-off score ≥ 6	64 (26.0)	82 (66-92)	85 (79-89)	50 (37-63)	96 (92-98)
Cut-off score 8	23 (9.3)	41 (26-58)	97 (93-99)	70 (47-87)	90 (85-93)

PPV: positive predictive value; NPV: negative predictive value. #: age (18–40 years), cough of ⩾2 weeks, loss of appetite, body mass index ⩽18.5 kg·m^{-2} and shortness of breath of ⩾2 weeks; ¶: age (18–40 years), cough of ⩾2 weeks, loss of appetite and body mass index ⩽18.5 kg·m^{-2}.

4.5. Discussion

Among hospitalized patients with a negative Xpert result, we found that 15.8% had bacteriologically confirmed TB, about 40% had radiological signs of TB, and about 79.5% of patients with bacteriologically confirmed TB had chest radiography findings suggestive of pulmonary TB. Despite the strong association between signs of TB on a chest radiography and bacteriological confirmed TB, the addition of chest radiography findings to selected clinical symptoms did not improve the performance of a risk prediction tool. Availability of chest radiography findings for Xpert negative patients assessed for TB at a tertiary care hospital in a high TB burden country is thus unlikely to substantially improve the performance of empiric TB treatment decision-making based solely on clinical patient characteristics, which is common practice in many high TB burden low resource countries.

In the pre-Xpert era, the WHO recommended the use of chest radiography in severely ill or smear-negative patients, especially in resource-constrained settings [20, 21]. This recommendation was based on the high sensitivity of chest radiography findings for diagnosis of bacteriologically confirmed TB among smear negative patients, with observed sensitivity ranging between 53.3% (95% CI: 28-79) and 77% (95% CI: 63-87) in prior studies [22-24]. In the Xpert era, chest radiography has been promoted in triaging algorithms to reduce the need for Xpert assays [25] and to improve case detection in TB prevalence surveys [7, 26], but not yet as a follow up test in Xpert negative patients. We observed sensitivity of the chest radiography to diagnose bacteriologically confirmed TB felt similar to that in smear negative individuals, with sensitivity estimates of 69% when the chest radiography was read by clinicians and 79.5% when read by a radiologist, no other studies have reported on the sensitivity of chest radiography among Xpert negative patients.

Because empiric treatment did not impact six months survival in thus population of hospitalized patients with Xpert negative PTB [14], we assessed if the addition of chest radiography findings to clinical characteristics in risk prediction models could improve the identification of patients with bacteriologically confirmed TB. Prediction models using clinical variables only (loss of appetite, BMI, prolonged cough, and shortness of breath and age) or a combination of clinical characteristics and chest radiography findings (as defined by clinician or radiologist) were developed, internally validated, and used to categorize patients into four distinctive risk groups ranging from low to very high risk of bacteriologically confirmed TB. We showed that the performance of the prediction tools containing the chest radiography information was not superior in AUC or risk category distribution compared to the prediction tools containing only information on clinical patient characteristics. These results suggest that the inclusion of chest radiography image read by clinicians or radiologist is unlikely to improve the performance of empiric TB treatment in these population. Whether the use of computer-aided reading to classify chest radiography findings could increase the role of chest radiography in Xpert negative individuals should be explored in future studies.

To improve diagnostic accuracy and early treatment of all TB cases, greater emphasis should be placed on use Xpert Ultra, the most sensitive bacteriological assay. For example, even though the Xpert Ultra was already recommended by WHO in 2017, the Global Fund 2020 procurement for Ethiopia consisted of 80% first generation Xpert assays and only 20% Xpert ultra-assays [27, 28]. Where procurement of Xpert Ultra is not possible, the clinical risk prediction tool suggests that

targeting empiric treatment to those at moderate or high risk of bacteriological TB may be an effective strategy, but this would need to be confirmed in prospective studies [3].

Our study had some limitations. First, the subjects included in this study were recruited from a single tertiary hospital. Validation of our findings in other hospital settings and outpatient settings are needed as the findings may differ at non-tertiary hospital settings and are likely to differ in low TB burden settings due to differences in population characteristics, empiric treatment decisions, and available resources. Second, only a small number of HIV positive patient were included in the study, precluding our ability to perform a stratified analysis by HIV status. This may be important given that all patients with bacteriologically confirmed TB and a negative chest radiography in our study were HIV positive. Larger studies powered to assess the potential role of chest radiography in HIV positive and HIV negative individuals separately are thus needed. Finally, we assessed the role of chest radiography in patients with a negative first generation Xpert assay. Given the negligible impact, one would predict a similar negative finding among patients with a negative Xpert Ultra result, but this should be confirmed in a prospective study.

In conclusion, despite a strong association between chest radiography findings and presence of active pulmonary TB, the addition of chest radiography findings did not improve the performance of clinical parameters traditionally used for empiric TB treatment decision-making in Xpert negative patients. These findings call for the urgent global implementation of the rapid and highly sensitive Xpert Ultra test in routine clinical practice to improve diagnostic accuracy and early treatment of all TB cases.

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Chapter 5: Paper 3

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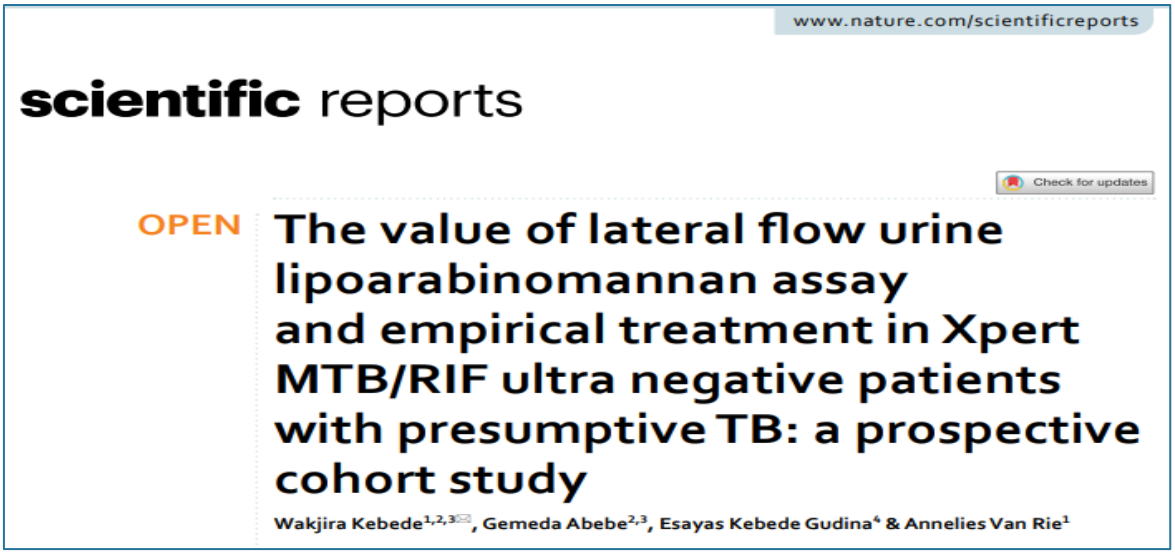

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My role in this work: As the first author, I collaborated with Prof. Annelies Van Rie and Prof. Gemeda Abebe to design the study. I supervised the teams responsible for collecting data and conducted laboratory tests to diagnose MTB. I performed a comprehensive statistical analysis and interpreted the results with my promoters. Additionally, I drafted the manuscript and revised it based on feedback from all co-authors. Finally, I completed the manuscript submission process.

5.1. Abstract

Introduction: The value of Lateral Flow urine Lipoarabinomannan (LF-LAM) assay and the role of empiric tuberculosis (TB) treatment in the era of the highly sensitive Xpert MTB/RIF Ultra (Xpert Ultra) assay is unclear. This study aimed to assess the additional yield of LF-LAM assay when used in combination with Xpert Ultra, and the role of empiric TB treatment when Xpert Ultra used as the initial diagnostic in presumptive TB cases admitted to a tertiary hospital in Ethiopia.

Methods: We performed a secondary analysis of a cohort of hospitalized Xpert MTB/RIF-negative patients. Sputum samples were examined for *Mycobacterium tuberculosis* by culture and Xpert Ultra. In HIV positive and severely ill patients, a urine sample was collected for the LF-LAM assay. Treatment outcome was assessed six months after enrollment. Logistic regression was used to identify factors predictive of deaths among Xpert Ultra negative patients.

Results: The Xpert Ultra assay diagnosed 31 of the 35-culture positives among the 250 hospitalized Xpert MTB/RIF-negative participants. The LF-LAM assay did not identify any case not detected by Xpert Ultra among the 52 (21.4%) participants living with HIV and the 16 patients with severe disease. Among Xpert Ultra negative patients, those who received empirical TB treatment had a similar odd of death (aOR 0.74, 95% CI: 0.1–2.7) as those not started on TB treatment. Low body mass index (≤ 18.5 kg/m2) was the only significant predictor of death in Xpert Ultra negative patients (aOR 4. 0, 95% CI: 1.08–14.6).

Conclusion: In this prospective cohort, LF-LAM did not improve the diagnostic yield when used in combination with Xpert Ultra. Empiric TB treatment for Xpert Ultra negative presumptive TB cases was not associated with death at six months. Future studies in diverse settings should be to determine the optimal management of Xpert Ultra negative patients.

Key words: Presumptive TB; *Mycobacterium tuberculosis*; TB case findings; Xpert MTB/RIF-negative; urine-LAM; Xpert MTB/RIF Ultra; Ultra; Empiric treatment.

5.2. Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB) complex [1]. With an estimated 10 million active TB cases, of which 10% are co-infected with HIV, and 1.3 million deaths in 2019, TB remains a public health problem worldwide [2]. In 2019, Ethiopia had the 10th highest incidence of TB globally, with 114, 233 new cases and an incidence rate of 151 cases per 100,000 people [2, 3].

Even though the World Health Organization (WHO) endorsed the molecular Xpert MTB/RIF assay almost a decade ago [4], many primary care clinics in high burden countries still rely on smear microscopy as the initial diagnostic test. Because of the low sensitivity of smear microscopy, smear negative patients with TB symptoms are often empirically started on TB treatment [5]. Even when the Xpert MTB/RIF assay is available, empirical treatment remains highly prevalent in high TB burden countries [6]. The use of empirical treatment can be justified in these settings given the suboptimal performance of the Xpert assay, especially among smear negative individuals (sensitivity of 67%) and in people living with HIV (sensitivity of 80%) [7, 8].

In 2017, the WHO recommended replacing Xpert MTB/RIF by Xpert MTB/RIF Ultra (Xpert Ultra) as the initial diagnostic test because of its superior performance in smear negative patients and patients living with HIV [9]. A meta-analysis of 19 studies found an overall pooled sensitivity of 84% the Xpert Ultra compared to 69% for Xpert MTB/RIF [10]. In smear-negative TB cases, Xpert Ultra was 17% more sensitive compared to Xpert MTB/RIF; in people living with HIV Xpert Ultra was 13% more sensitive [11-13]. Whether empiric TB treatment is still justified in high TB burden settings when Xpert Ultra is used as the initial diagnostic has not yet been investigated.

The lateral flow urine lipoarabinomannan assay (LF-LAM) is another TB diagnostic tool that was recently endorsed but is not yet commonly used in high TB burden countries [14]. In inpatient settings, LF-LAM can assist in the diagnosis of TB in people living with HIV. Among people living with HIV who present with signs and symptoms of TB to an inpatient setting, the pooled sensitivity of LF-LAM was 52% (40–64%) and pooled specificity 87% (78–93%). When used in combination with other tests, LF-LAM can improve the diagnostic yield by 14% (compared to Xpert MTB/RIF alone) or 36.6% (compared to clinical sign and symptoms alone) [15, 16]. It has also been shown that the use of LF-LAM can improve the survival of patients hospitalized with advanced HIV disease [pooled risk

ratio for mortality 0.85 (0.76–0.94)] [14, 17, 18]. The yield of LF-LAM when used in combination with Xpert Ultra has not yet been assessed.

In this study, we aimed to assess the additional yield of LF-LAM assay when used in combination with Xpert Ultra, and the role of empiric TB treatment in Xpert Ultra negative patients with symptoms of TB admitted to a tertiary hospital in Ethiopia.

5.3. Materials and methods

5.3.1. Study setting

The study was conducted at Jimma University Medical Center, a tertiary hospital located in Jimma, Oromia Region, Ethiopia. Patients are referred to the Center from health centers, district hospitals and private health facilities in Jimma Zone and district hospitals in neighboring regions, resulting in a catchment population of over 20 million. The hospital has about 800 inpatient beds and about 20,000 inpatients, 220,000 outpatient, and 15,000 emergency cases visit the hospital annually [19]. During the study period (December 2018–July 2019), the Xpert MTB/RIF assay was used as the initial diagnostic test on sputum from presumptive pulmonary TB cases [20]. The decision to start TB treatment was made by physicians based on clinical findings (TB symptoms and their severity), response to antibiotics, chest X-ray findings (routinely performed), and HIV status and CD4 count. The Mycobacteriology Research Center of Jimma University, which is located in close proximity of the medical center, serves as the TB reference laboratory for southwest Ethiopia.

5.3.2. Study population and data collection

This analysis is a secondary analysis of a cohort study that aimed to determine the role of empiric treatment among hospitalized adults (age ≥ 18 years) with symptoms of pulmonary TB (current cough, night sweats, fever, and weight loss) who could produce a sputum sample and tested negative on the Xpert MTB/RIF assay [21]. For the cohort study, all consecutive hospitalized Xpert MTB/RIF-negative patients were followed to determine if they were started on TB treatment. The first 125 patients who received empiric TB treatment and the first 125 patients not started on empiric TB treatment were offered study participation. Of those approached, two declined and three were excluded because they died prior to informed consent. A structured questionnaire was administered by trained nurses to collect demographic and clinical characteristic. Medical records of the patients were reviewed to document HIV status, CD4 count, and improvement of TB symptoms

on broad spectrum antibiotics treatment. Two sputum samples were collected in the first 48 h following enrolment for a single Xpert Ultra assay, a liquid culture using the Mycobacteria Growth Indicator Tube (MGIT) system and a solid culture on Lowenstein Jensen (LJ). Drug susceptibility testing for first line anti-TB drugs (rifampicin and isoniazid) was done by Line Probe assay (LPA). HIV-positive and severely ill patients (temperature > 39 °C, respiratory rate > 30 resp. /min, cardiac rate > 120 bpm, or unable to walk without help) [22] were asked to provide a 10 ml urine sample for a LF-LAM assay. All laboratory tests were performed at the Mycobacteriology Research Center of Jimma University.

5.3.3. Laboratory procedures

5.3.3.1. Mycobacterial culture

Sputum sample decontamination and digestion, inoculation of concentrated sputum samples on LJ and MGIT media were performed using standard procedures [26]. The mycobacterial culture was classified as positive when growth was detected by LJ and/or MGIT with a positive SD Bioline TB antigen MPT64 confirmatory test and a negative blood agar test. Culture negative denotes the absence of growth on both LJ and MGIT culture or positive LJ/MGIT culture with a negative SD Bioline TB antigen MPT64 result.

5.3.3.2. Xpert MTB/RIF Ultra test

Xpert Ultra test was performed according to manufacturer's instruction [24]. Briefly, 1 ml of sputum was added to 2 ml of reagent in a 15-ml Falcon tube, vortexed for at least 10 s and incubated at room temperature for 10 min. The mixture was then added to the Xpert Ultra assay cartridge and placed in the instrument. The Xpert Ultra test was repeated using the same sample in cases of an invalid result. In cases of "trace" result, a new sputum sample was collected for a second Xpert Ultra test. If the second test was positive (trace or higher), the result was considered positive for patients living with HIV and for HIV-negative patients without a history of TB treatment.

5.3.3.3. LF-LAM test

The urine Determine TB-LAM test (Abbott Laboratories, Lake Bluff, USA; formerly Alere Inc, Waltham, USA) was performed by applying 60µL of fresh urine to the sample pad at the bottom of the test strip using a micro-pipette. After 25 min of incubation at room temperature, the test strip was inspected visually. If any band is observed, the intensity was scored as grade 1, 2, 3 or 4 as compared

with the reference card. The test was read independently by two certified laboratory professionals in blinded manner (blinded to each other and the sputum Xpert Ultra and culture results). LAM test results were classified as positive if grade ≥ 1 and as negative if no test band was observed [16].

5.3.4. Data analysis

Descriptive statistics were performed using frequencies and percentages to describe the characteristics of the study population. The yield of Xpert Ultra and LAM tests was determined by comparison to mycobacterial culture results. Mortality in the 6 months post assessment was assessed among Xpert Ultra negative participants, stratified by empiric treatment status. To identify factors associated with mortality among Xpert Ultra negative patients, we calculated the odds ratio using a logistic regression model. Data analysis was performed using R Statistical software version 3.6.1.

5.3.5. Ethical considerations

Ethical clearance was obtained from the Ethical Review Board of Institute of Health of Jimma University (Ref. No: IHRPGD/397/2018). Written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations. Clinicians treating study participants in whom TB was bacteriologically confirmed were notified of the result.

5.4. Results

5.4.1. Characteristics of the study population

All 250 patients admitted to the hospital with signs or symptoms of TB who were enrolled in the parent study were included in the secondary analysis. By design, all study participants had a negative Xpert MTB/RIF result (**Figure 1**). The majority were 40 years of age or younger (144/250, 57.6%), female (138/250, 55.2%), and rural residents (154/250, 57.6%). Almost all patients (232/250, 92.8%) had already visited another health facilities for similar complaints before their current hospitalization. HIV status was documented for 243 (97.2%) participants of which 21.4% were positive (**Table 1**). All participants had symptoms of current cough, fever, night sweat and shortness of breath; 165 (66%) had a cough for ≥ 14 days, 45 (18%) had a history of previous TB treatment and (93/250, 37.6%) had a chest X-ray finding typical or compatible with TB.

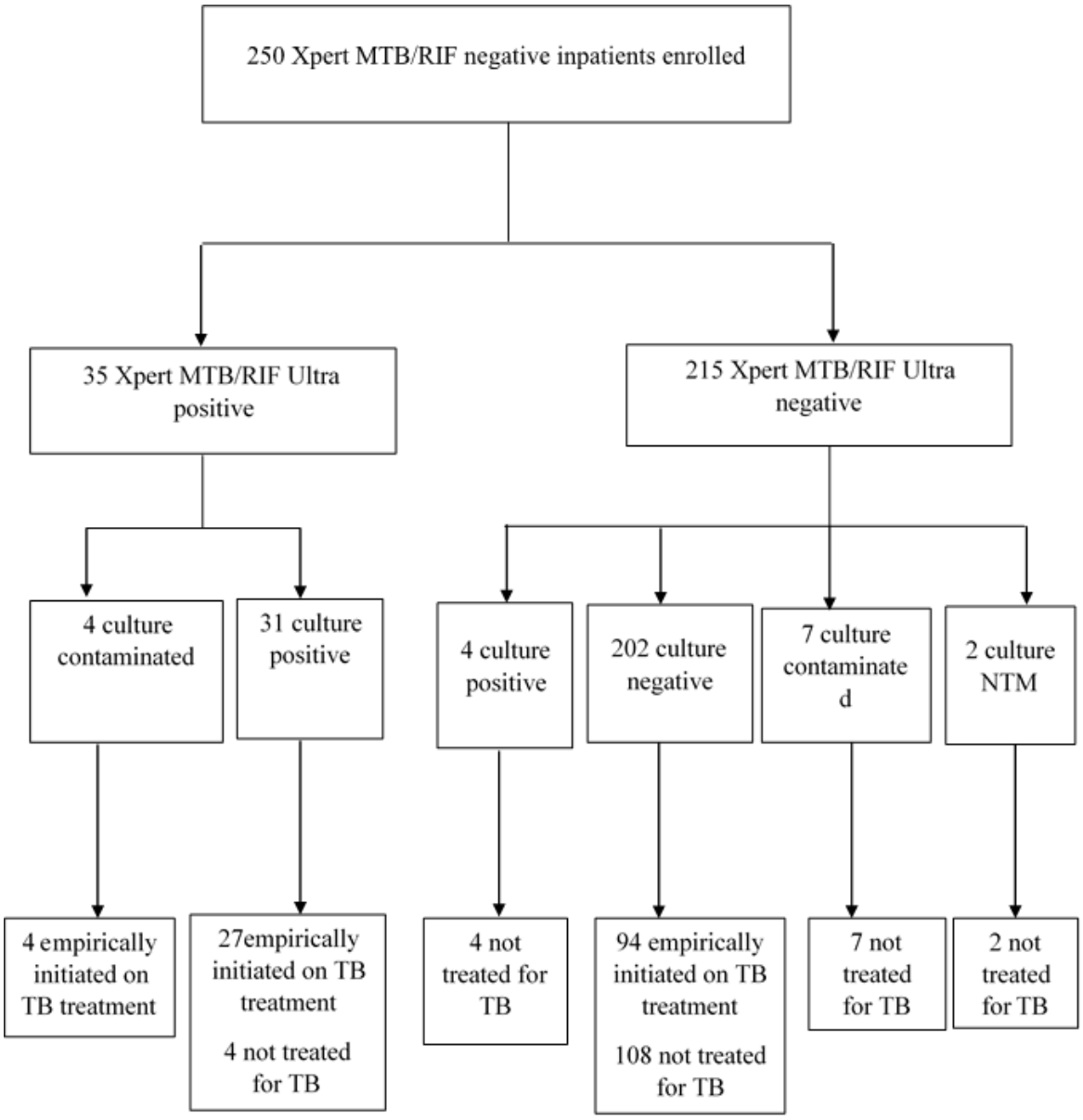

Figure 1. Patient enrollment flowchart showing the number of patients enrolled, Xpert Ultra result, and culture result and treatment status distribution. *TB, tuberculosis; MTB, Mycobacterium tuberculosis; NTM, non-tuberculosis mycobacteria.*

Table 1. Demographic and clinical characteristics of 250 Xpert MTB/RIF-negative patients hospitalized at Jimma University Medical Center, Oromia, Ethiopia.

Characteristics	Category	All	Xpert ultra test results	
			Positive	Negative
Patients n		250	35	215
Age	≤ 40 years	144 (57.6)	29 (82.9)	115 (53.5)
	> 40 years	106 (42.4)	6 (17.1)	100 (46.5)
Sex	Female	138 (55.2)	22 (62.9)	116 (54.0))
	Male	112 (44.8)	13 (37.1)	99 (46.0)
Educational status	Illiterate	116 (46.4)	8 (22.9)	108 (50.2)
	Primary level	51 (20.4)	8 (22.9)	43 (20.0)
	Secondary & above	83 (33.2)	19 (54.2)	64 (29.8)
Residence	Urban	106 (42.4)	15 (42.9)	91 (42.3)
	Rural	144 (57.6)	20 (57.1)	124 (57.7)
Health facilities visited prior to hospital admission	Local government hospitals	126 (50.4)	9 (25.7)	117 (54.4)
	Primary care center	48 (19.2)	10 (28.6)	38 (17.7)
	Private Clinic	58 (23.2)	13 (37.1)	45 (20.9)
	None	18 (7.2)	3 (8.6)	15 (7.0)
Body mass index	≤ 18.5 kg/m^2	84 (33.6)	22 (62.9)	62 (28.8)
	> 18.5 kg/m^2	166 (66.4)	13 (37.1)	153 (71.2)
HIV status	Negative	191 (76.4)	24 (68.8)	167 (77.7)
	Positive	52 (20.8)	11 (31.4)	41 (19.1)
	Unknown	7 (2.8)	0 (0.0)	7 (3.2)
CD4 count (n = 52)	≤ 200 cells/ml	24 (46.2)	10 (90.9)	14 (26.9)
	> 200 cells/ml	28 (53.8)	1 (9.1)	27 (73.1)
History of TB treatment	No	205 (82.0)	27 77.1)	178 (82.8)
	Yes	45 (18.0)	8 (22.9)	37 (17.2)
Clinical severity	Non-severe	232 (92.8)	30 (85.7)	202 (94.0)
	Severe	18 (7.2)	5 (13.9)	13 (6.0)
Weight loss	No	111 (44.4)	9 (25.7)	102 (47.4)
	Yes	139 (55.6)	26 (74.3)	113 (52.6)
Pleuritic chest pain	No	82 (32.8)	5 (14.3)	77 (35.8)
	Yes	168 (67.2)	30 (85.7)	138 (64.2)

Table 1: Continued.

Characteristics	Category	All	Xpert ultra test results	
			Positive	Negative
Loss of appetite	No	49 (19.6)	2 (5.7)	47 (21.9)
	Yes	201 (80.4)	33 (94.3)	168 (78.1)
Duration of cough	< 14 days	85 (34.0)	5 (14.3)	80 (37.2)
	≥ 14 days	165 (66.0)	30 (85.7)	135 (62.8)
Duration of fever	< 14 days	137 (54.8)	17 (48.6)	120 (55.8)
	≥ 14 days	113 (45.2)	18 (51.4)	95 (44.2)
Duration of night sweats	< 14 days	130 (52.0)	18 (51.4)	112 (52.1)
	≥ 14 days	120 (48.0)	17 (48.6)	103 (47.9)
Duration of shortness of breath	< 14 days	123 (49.2)	10 (28.6)	113 (52.6)
	≥ 14 days	127 (50.8)	25 (71.4)	102 (47.4)

Data are presented as n (%), unless otherwise stated.

5.4.2. Results of *Mtb* culture and Xpert Ultra assay

Of the 250 cultures, 35 (14.0%) were positive for *Mtb* complex and 2 (0.8%) for non-tuberculosis mycobacteria (NTM), 202/250 (80.8%) were *Mtb* culture negative and 11/250 (4.4%) were contaminated (**Table 2**).

Table 2: Xpert Ultra and LAM test results compared with mycobacterial culture for the diagnosis of pulmonary TB among Xpert MTB/RIF-negative patients hospitalized at Jimma University Medical Center, Ethiopia

	Mycobacterial culture results				
	LJ/MGIT positive	LJ/MGIT negative	LJ/MGIT contaminated	NTM	Total
Xpert Ultra					
Mtb detected*	27 (77.2)	0 (0.0)	4 (36.4)	0 (0.0)	31 (12.4)
Mtb Trace	4 (11.4)	0 (0.0)	0 (0.0)	0 (0.0)	4 (1.6)
Mtb not detected	4 (11.4)	202 (100)	7 (63.6)	2 (100)	215 (86.0)
Total	**35**	**202**	**11**	**2**	**250**
LF-LAM in HIV positive participants					
Positive	6 (60)	0 (0.0)	0 (0.0)	0 (0.0)	6 (11.5)
Negative	4 (40)	39 (100)	2 (100)	1 (100)	46 (88.5)
Total	**10**	**39**	**2**	**1**	**52**

Data are presented as n (%), unless otherwise stated. *All were sensitive to rifampicin. *Mtb, Mycobacterium tuberculosis*; NTM, non-tuberculosis mycobacteria, LJ, Lowenstein Jensen medium, MGIT, Mycobacteria Growth Indicator Tube, TB, Tuberculosis; LF-LAM, Lateral Flow urine Lipoarabinomannan.

The Xpert Ultra assay was positive for *Mtb* complex in 35/250 (14%), of which 4 (11.4%) had a trace call. The Xpert Ultra result was negative in 215 (86.0%) participants, including four samples that were repeated because of an error result on the initial test (**Table 2**). The Xpert Ultra assay was positive in 4 patients with a contaminated culture. One rifampicin resistant strain identified by LPA was not identified as rifampicin resistant by Xpert Ultra.

Participants whose sputum cultures were contaminated (n = 11) or positive for NTM (n = 2) were excluded from this analysis. Of the remaining 237 participants, 35 (14.8%) had culture-confirmed TB. The Xpert Ultra assay detected 31 (88.6%) of the 35 culture-confirmed cases. The 4 culture-confirmed cases that were missed by Xpert Ultra were HIV negative. The assay was positive in 4 (36.4%) of the 11-culture contaminated and was negative in all 202 culture-negative participants. The 4 Xpert Ultra positive culture-contaminated cases had very low mycobacterial burden as demonstrated by a high cycle threshold (Ct) value (Ct value of the lowest probe ≥ 29.2, **S1.Table)**, were all HIV negative, and none of these cases had a history of prior TB treatment.

Of the eight Xpert MTB/RIF negative patients not started on empiric treatment, four were started on TB treatment based on a positive culture and four based on a positive Xpert Ultra.

S1 Table. The cycle threshold (Ct) values and patient outcome at six months of the 4 Xpert Ultra positive culture negative cases.

Code	**Ct value**				**Outcome at six months**
	rpoB1	**rpoB2**	**rpoB3**	**rpoB4**	
Patient 1	30.8	29.6	31.8	33.8	Survived
Patient 2	30.4	29.8	31.4	32.3	Survived
Patient 3	29.2	28.8	30.7	32.4	Survived
Patient 4	29.7	29.0	31.3	33.5	Survived

5.4.3. The yield of LF-LAM test

Among 52 HIV positive participants assessed by LF-LAM, 6 (11.5%) LF-LAM tests were positive (**Fig. 2**). Six of the 10 (60.0%) culture positive Xpert Ultra positive cases were detected by LF-LAM. In addition, 3 of 16 (19%) of severely ill HIV negative patients assessed by LF-LAM had a positive LF-LAM result, including two Xpert Ultra positive but culture negative participants. None of the LF-LAM positive cases were Xpert Ultra negative. As such, the LF-LAM assay did not increase the diagnostic yield when used in combination with Xpert Ultra.

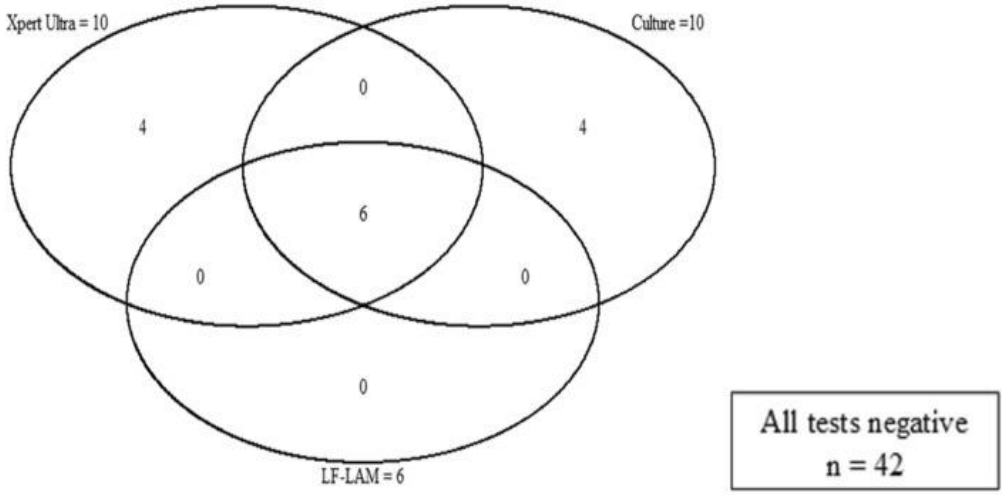

Figure 2: Sputum culture, Xpert Ultra and urine LF-LAM results in 52 HIV-positive Xpert MTB/RIF-negative hospitalized patients. Xpert Ultra, Xpert MTB/RIF Ultra; LF-LAM, Lateral Flow Urine Lipoarabinomannan.

5.4.4. Role of empiric TB treatment in Xpert Ultra negative patients

Of the 215 (86%) Xpert Ultra negative participants, 94 (43.7%) were diagnosed clinically and empirically started on TB treatment, and 121 (56.3%) were clinically considered not to have TB and were not initiated on TB treatment. Of the 94 Xpert Ultra negative participants started on empiric TB treatment, 90 (95.7%) were alive and 4 (4.3%) had died in the 6 months following assessment. Of the 121 Xpert Ultra negative patients not started on TB treatment, 115 (95.0%) were alive and 6 (5.0%) had died in the 6 months following assessment (**Table 3**). Among Xpert Ultra negative patients, empirical TB treatment had similar odds of death but the small sample size resulted in wide 95% confidence intervals (aOR 0.74, 95% CI: 0.1–2.7).

Table 3: Role of empiric TB treatment in the Xpert Ultra era among Xpert MTB/RIF-presumptive TB cases hospitalized at Jimma University Medical Center, Oromia, Ethiopia

TB treatment status & outcomes	All patients	Xpert Ultra test results	
		Positive	Negative
Empirically initiated on TB treatment	125/250 (50.0)	31/35 (86.6)	94/215 (43.7)
Outcome			
Alive	118/125 (94.4)	28/31 (90.3)	90/94 (95.7)
Died	7/125 (5.6)	3/31 (9.7)	4/94 (4.3)
Not initiated on TB treatment	125/250 (50.0)	4/35 (11.4)	121/215 (56.3)
Outcome			
Alive	119/125 (95.2)	4/4[#] (100.0)	115/121 (95.0)
Died	6/125 (4.8)	0/4 (0.0)	6/121 (5.0)
Total	250	35	215

Data are presented as n (%), unless otherwise stated. #Both liquid (MGIT) and solid (LJ) cultures were contaminated, but 2 of the 4 cases were LF-LAM positive.

5.4.5. Predictor of 6-month mortality among Xpert Ultra negative patients

In regression analysis, it was found that underweight or body mass index (BMI) ≤ 18.5 kg/m2 was significant predictor of mortality among Xpert Ultra negative patients (aOR 4.0, 95% CI: 1.08–14.6) (**Table 4**).

Table 4: Patient characteristics associated with mortality among Xpert Ultra negative patients hospitalized at Jimma University Medical Center, Oromia, Ethiopia

Characteristics	Category	Outcome		Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
		Died	Alive		
Patients n		**10**	**205**		
Age	≤ 40 years	5(50.0)	108(52.7)	Ref	
	> 40 years	5 (50.0)	97 (47.3)	1.11 (0.3-4.1)	
Sex	Female	5 (50.0)	112 (54.6)	Ref	
	Male	5 (50.0)	93 (45.4)	1.2 (0.3-4.4)	
Educational status	Secondary & above	1 (10.0)	43 (21.0)	Ref	
	Primary level	3 (30.0)	60 (29.3)	2.1 (0.26-44.2)	
	Illiterate	6 (60.0)	102 (49.8)	2.5 (0.41-48.5)	
Residence	Urban	5 (50.0)	87 (42.4)	Ref	
	Rural	5 (50.0)	118 (57.7)	0.73 (0.2-2.7)	
Clinical severity	Non-severe	9 (90.0)	190 (94.1)	Ref	Ref
	Severe	1 (10.0)	12 (5.9)	1.7 (0.19-10.7)	0.2 (0.03-5.8)
Body mass index	> 18.5 kg/m^2	4 (40.0)	150 (73.2)	Ref	Ref
	≤ 18.5 kg/m^2	6 (60.0)	55 (26.8)	4.0 (1.12-16.5)	**4.0 (1.1-14.6)** *
HIV status [a]	Negative	6 (60.0)	160 (78.0)	Ref	Ref
	Positive	4 (40.0)	38 (18.5)	2.8 (0.7-10.3)	2.1 (0.4-8.7)
Weight loss	No	4 (40.0)	99 (48.3)	Ref	Ref
	Yes	6 (60.0)	106 (51.7)	1.4 (0.38-5.61)	0.6 (0.1-2.7)
Loss of appetite	No	1 (10.0)	46 (22.4)	Ref	Ref
	Yes	9 (90.0)	159 (77.6)	2.6 (0.47-48.6)	0.3 (0.02-2.3)
Pleuritic chest pain	No	4 (40.0)	74 (36.1)	Ref	Ref
	yes	6 (60.0)	131 (63.9)	0.84 (0.23-3.4)	1.2 (0.2-6.7)
History of TB treat-	No	9 (90.0)	169 (82.4)	Ref	
ment	Yes	1(10.0)	36 (17.6)	0.5 (0.02-2.9)	
Duration of fever	< 14 days	6 (60.0)	115 (56.1)	Ref	Ref
	≥ 14 days	4 (40.0)	90 (43.9)	0.85 (0.21-3.07)	1.1 (0.2-6.0)

Table 4: Continued.

Characteristics	Category	Outcome		Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
		Died	Alive		
Duration of cough	< 14 days	4 (40.0)	77 (37.6)	Ref	Ref
	≥ 14 days	6 (60.0)	128 (62.4)	0.9 (0.24–3.62)	0.8 (0.1–3.9)
Duration of night sweats	< 14 days	6 (60.0)	107 (52.2)	Ref	Ref
	≥ 14 days	4 (40.0)	98 (47.8)	0.72 (0.18–2.62)	2.0 (0.4–11.7)
Duration of shortness of breath	< 14 days	5 (50.0)	109 (53.2)	Ref	Ref
	≥ 14 days	5 (50.0)	96 (46.8)	1.13 (0.30–4.19)	0.5 (0.09–2.7)
Culture results	Negative	9 (90.0)	202 (98.5)	Ref	
	Positive	1 (10.0)	3 (1.5)	7.48 (0.35–65.5)	
Empiric treatment	No	6 (60.0)	115 (56.1)	Ref	Ref
	Yes	4 (40.0)	90 (43.9)	0.85 (0.21–3.07)	0.74 (0.1–2.7)

Data are presented as n (%). Significant values are in bold. CI confidence interval, *p value = 0.041. a, 7 missing HIV test results.

5.5. Discussion

In this prospective cohort study, we confirmed the high sensitivity of Xpert Ultra among cases of presumptive TB, as 89% (31 of the 35) of the culture positive cases missed by Xpert MTB/RIF were positive on Xpert Ultra. We demonstrated that the use of empirical treatment may no longer be justified in Xpert Ultra negative presumptive pulmonary TB patients as it does not impact the odds of death, and that LF-LAM does not increase the diagnostic yield among HIV positive and severely ill patients when Xpert Ultra is used as the initial assay.

The observed high sensitivity of Xpert Ultra is in line with a meta-analysis, where 87.2% of TB culture positive patients were positive on Xpert Ultra test [12] and an overall estimated pooled sensitivity of 90.9% (95% credible interval 86.2–94.7) [25]. Similar to other studies, we also observed potentially false positive Xpert Ultra results, as 4 cases were Xpert Ultra positive by culture negative. False positive Xpert Ultra results have been associated with a recent history of TB treatment [11]. In our study, the Xpert Ultra positive culture negative cases had no history of previous TB treatment. Interestingly, two of the four cases had a positive LF-LAM result, suggesting that these cases may actually be true positive instead of false positive cases of TB.

The LF-LAM assay detected 60.0% of the culture positive cases among people living with HIV, similar to findings of a study performed in four countries in sub-Saharan Africa where a 60.0% sensitivity of LF-LAM was observed among culture positive cases [26]. In contrast to studies performed during the Xpert MTB/RIF era, where addition of LF-LAM to Xpert MTB/RIF assay increased the yield [15, 16], the LF-LAM assay did not improve diagnostic yield in our cohort as all LF-LAM positive patients were also positive on Xpert Ultra. This suggests a limited role for LF-LAM in patients where Xpert Ultra can be used as the initial diagnostic as all LF-LAM negative cases would have to be assessed by Xpert Ultra due to poor LF-LAM sensitivity, and all LF-LAM positive cases would need to be assessed by Xpert Ultra for rifampicin resistance. A multicenter study in settings where Xpert Ultra is used as the initial diagnostic is needed to determine the role of LF-LAM in the diagnostic algorithm, especially for patients who cannot produce a sputum sample.

In high TB burden settings, treatment decisions often continue to rely on clinician's judgement and chest X-ray findings, even when it has been shown that empiric treatment does not result in survival benefit [6, 21]. In this study, we found that the Xpert Ultra assay detected almost all culture positive cases, questioning the value of reliance on empiric TB treatment for patients with negative Xpert Ultra results. Furthermore, adjusted analysis did not reveal six month decreased risk of death due to empirical TB treatment among Xpert Ultra negative patients, as risk of death was similar between those who did and did not receive empiric TB treatment (aOR 0.74, 95% CI: 0.1–2.7). These results suggest that the recommendation by Kendall et al. and by Decroo et al. that clinicians should continue to prescribe TB treatment for Xpert MTB/RIF-negative patients whose clinical presentations strongly suggest pulmonary TB in order to minimize a risk of TB related mortality [6, 7] may no longer hold when Xpert Ultra is used as the initial diagnostic. All participants in whom the clinician started empiric TB treatment had been assessed by chest X-ray and had received a 'trial' of antibiotics (ceftriaxone and azithromycin, amoxicillin or vancomycin and doxycycline) to which they had not responded favorably. Given that empiric TB treatment in these Xpert Ultra negative patients did not decrease the risk of death, our results suggest that prescribing empiric treatment should be re-assessed as this may pose an unnecessary burden the health care system and may expose patients to unnecessary treatment. Future research should investigate which alternative diagnoses should be considered in these patients in order to develop evidence-based guidelines for the management of Xpert Ultra negative patients.

Our study had some limitations. First, we limited enrollment to hospitalized Xpert MTB/RIF-negative patients, which limits generalizability of the outpatient settings. Second, the prevalence of HIV in our cohort was relatively low (21.4%), which may limit generalizability to high HIV burden settings. Third, we excluded patients who could not provide a sputum sample. As empiric treatment and LF-LAM assay may still be of value for patients unable to produce sputum, future studies should assess the value of LF-LAM for the diagnosis of HIV positive TB in people with presumptive TB who cannot produce a sputum sample, including people with presumptive extra pulmonary TB (EPTB). Similarly, the role of empirical treatment in patients with presumptive EPTB should be investigated. Fourth, our study was not powered to assess factors associated with mortality among people with presumptive TB who had a negative Xpert Ultra assay. Consequently, the effect estimates of the association between empiric treatment and mortality in Xpert Ultra negative was imprecise. Furthermore, some participants may have had culture negative pulmonary TB although the prevalence of our cohort is likely very low as two cultures were performed in addition to Xpert MTB/RIF and Xpert Ultra. Lastly, we did not assess the presence of other respiratory pathogens to explore the cause of symptoms or death in the Xpert Ultra negative patients.

In conclusion, Xpert Ultra assay provides a sensitive, specific and rapid diagnosis of TB among presumptive pulmonary TB cases. Among patients with a negative Xpert Ultra result, the use of LF-LAM test did not yield additional cases and empiric TB treatment was not associated with mortality at six months. Future studies should be performed to establish guidelines for the management of Xpert Ultra negative patients.

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Data availability: All data generated or analyzed during this study are included in the article and its supplementary information files. The raw data generated in this study can be obtained by reasonable request to the corresponding author.

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Chapter 6: Paper 4

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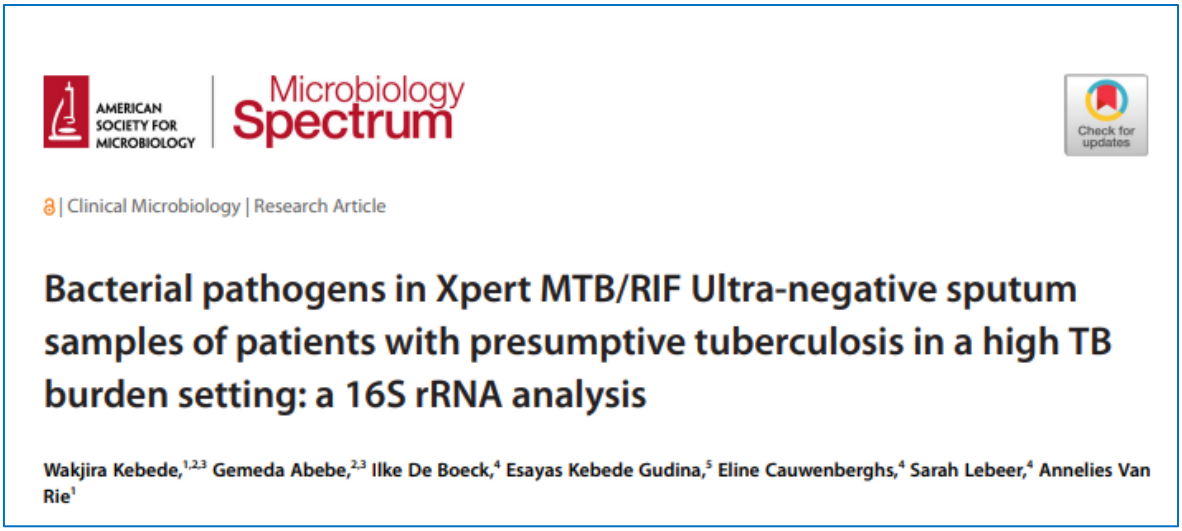

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Author Contributions: The study was designed by W. Kebede, G. Abebe, and A. Van Rie. W. Kebede, G. Abebe, and E.K. Gudina coordinated the data collection at the site. DNA extraction and 16S rRNA gene amplicon sequencing was performed by W. Kebede, Ilke De Boeck, and Eline Cauwenberghs. Data analysis was done by W. Kebede, Ilke De Boeck, Eline Cauwenberghs, and A. Van Rie. The first draft of the paper was written by W. Kebede, and all authors reviewed the paper, provided comments, and approved the final version of the manuscript for submission.

6.1. Abstract

Introduction: In patients with presumptive tuberculosis (TB) in whom the diagnosis of TB was excluded, understanding the bacterial etiology of lower respiratory tract infections (LRTIs) is important for optimal patient management.

Methods: A secondary analysis was performed on a cohort of 250 hospitalized patients with symptoms of TB. Bacterial DNA was extracted from sputum samples for Illumina 16S rRNA sequencing to identify bacterial species based on amplicon sequence variant level.

Results: The bacterial pathogen most likely to be responsible for the patients' LRTI could only be identified in a minority (6.0%, 13/215) of cases based on 16S rRNA amplicon sequencing: *Mycoplasma pneumoniae* (n = 7), *Bordetella pertussis* (n = 2), *Acinetobacter baumanii* (n = 2), and *Pseudomonas aeruginosa* (n = 2). Other putative pathogens were present in similar proportions of Xpert Ultra-positive and Xpert Ultra-negative sputum samples. The presence of *Streptococcus (pseudo) pneumoniae* appeared to increase the odds of radiological abnormalities (aOR 2.5, 95% CI 1.12–6.16) and the presence of *S. (pseudo) pneumoniae* (aOR 5.31, 95% CI 1.29–26.6) and *Moraxella catarrhalis/nonliquefaciens* (aOR 12.1, 95% CI 2.67–72.8) increased the odds of 6-month mortality, suggesting that these pathogens might have clinical relevance.

Conclusions: *M. pneumoniae, B. pertussis,* and *A. baumanii* appeared to be the possible causes of TB-like symptoms. *S. (pseudo) pneumoniae* and *M. catarrhalis/nonliquefaciens* also appeared of clinical relevance based on 16S rRNA amplicon sequencing. Further research using tools with higher discriminatory power than 16S rRNA sequencing is required to develop optimal diagnostic and treatment strategies for this population.

IMORANCE: The objective of this study was to identify possible bacterial lower respiratory tract infection (LRTI) pathogens in hospitalized patients who were initially suspected to have TB but later tested negative using the Xpert Ultra test. Although 16S rRNA was able to identify some less common or difficult-to-culture pathogens such as *Mycoplasma pneumoniae* and *Bordetella pertussis*, one of the main findings of the study is that, in contrast to what we had hypothesized, 16S rRNA is not a method that can be used to assist in the management of patients with presumptive TB having a negative Xpert Ultra test. Even though this could be considered a negative

finding, we believe it is an important finding to report as it highlights the need for further research using different approaches.

Key words: Diagnostics, Presumptive TB cases, *M. tuberculosis*, Bacterial etiology, LRTIs, Sequencing, Ethiopia.

6.2. Introduction

Lower respiratory infections (LRIs), which include bronchitis, bronchiolitis, and pneumonia, are one of the most common diseases, with 489 million LRI episodes occurring annually worldwide (1, 2). Globally, LRIs are the fourth leading cause of death claiming 2.4 million lives in 2019 (2). In Ethiopia, LRIs are the main reason for hospital admissions and the third leading cause of death, accounting for 8.2% of all deaths in 2019 (3).

LRIs are caused by a range of pathogens, including bacteria, viruses, and fungi (3–5). The main bacterial etiologies of LRIs are *Mycobacterium tuberculosis* (Mtb), *Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter species (spp.), Streptococcus viridans, Pseudomonas aeruginosa, Escherichia coli, and Proteus spp.* (4, 6–8). Atypical pathogens that can cause LRIs are *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Legionella pneumophila* (9).

In most low- and middle-income countries, people presenting with prolonged cough are first investigated for tuberculosis (TB) using smear microscopy or a rapid molecular test, such as the Xpert MB/RIF assay. When Mtb is not detected, clinicians often prescribe a trial of broad-spectrum antibiotics (10, 11). This can be problematic as empiric use of antibiotics does not always result in clinical improvement and may drive the emergence of antibiotic resistance (12). When broad-spectrum antibiotics fail to improve clinical symptoms, empiric TB treatment is often initiated even though studies have shown that empiric TB treatment does not affect survival (13–15) and may even increase mortality (16).

In 2017, the World Health Organization (WHO) endorsed the Xpert Ultra assay (17) given its excellent performance for the diagnosis of TB, with a pooled sensitivity of 88% (95% CI: 85% to 91%) and specificity of 96% (95% CI: 94% to 97%) compared to liquid culture (18, 19). With a high negative predictive value (98.1%), patients presenting with symptoms of TB whose sputum sample is Xpert

Ultra negative are thus highly unlikely to suffer from pulmonary TB (20). This raises the question whether empiric TB treatment for patients with negative Xpert Ultra test results is the correct management. A better understanding of the etiological cause of respiratory symptoms in patients presenting with symptoms of TB whose sputum sample is negative on Xpert Ultra is important to develop evidence-based algorithms for the optimal management of this patient population.

16S rRNA gene amplicon sequencing is a culture-free method to identify and compare bacterial diversity and microbial composition of a sputum sample. 16S rRNA detects both culturable and non-culturable microorganisms (21) and is a less costly method for studying microbial diversity compared to whole genome sequencing and metagenomic approaches (22). When informative, implementation of a 16SRNA assay could help clinicians make decisions and implement effective therapeutic strategies, as has been done for patients with non-cystic fibrosis and chronic obstructive pulmonary disease (COPD) (23, 24). 16S rRNA gene amplicon sequencing has not yet been applied to study the prevalence of bacterial pathogens in sputum samples of patients presenting with symptoms of TB in whom the Xpert Ultra test result was negative.

This study aimed to use Illumina MiSeq 16S rRNA V4 amplicon sequencing to determine the putative etiology of LRTI in hospitalized presumptive TB patients in whom Mtb was not detected by the Xpert Ultra assay, as this information could result in the development of an assay to guide the management for this population. In addition, we aimed to compare the prevalence and distribution of respiratory bacterial pathogens in sputum samples that were Xpert Ultra positive and negative to assess whether differences in microbial composition observed by 16S rRNA could be a marker of the etiology of the respiratory symptoms. Finally, the association between the presence of specific bacterial pathogenic taxa in Xpert Ultra negative sputum sample and clinical improvement on an antibiotic trial, chest X-ray findings, and 6-month survival was explored to assess the clinical relevance of the bacterial composition of the sputum sample.

6.3. Materials and Methods

6.3.1. Study site, design and data collection

We performed a secondary analysis of a cohort study that aimed to determine the impact of empiric TB treatment on mortality among hospitalized patients who tested negative on the Xpert MTB/RIF assay (13). In this cohort study, sputum samples were collected before antibiotic trials were started

from 250 adults (age ≥18 years) with symptoms of pulmonary TB (current cough, night sweats, fever, and weight loss) who were hospitalized between December 2018 to July 2019 in the Jimma Medical Center in Ethiopia. At the Jimma University Mycobacteriology Research Center, the TB reference laboratory for Southwest Ethiopia, sputum samples were decontaminated and evaluated for the presence of Mtb by liquid culture using the Mycobacteria Growth Indicator Tube (MGIT) BACTEC MGIT 960 System (Becton Dickinson, Sparks, MD, USA), solid Lowenstein-Jensen (LJ) media culture, and the Xpert Ultra assay (Cepheid, Sunnyvale, CA, USA) (25, 26). The Xpert Ultra test was repeated on the same sample in case of an invalid result and repeated on another sample in case of a "trace" result. Ethical clearance was obtained from the Ethical Review Board of Institute of Health, Jimma University, with Ref. No: IHRPGD/397/2018. Written informed consent was obtained from all study participants.

A structured questionnaire was used to collect demographic and clinical data; medical records were reviewed for HIV status, chest X-ray findings, and response to antibiotic treatment. All study participants were followed up for 6 months to determine survival status.

6.3.2. 16S rRNA gene amplicon sequencing

DNA was extracted from stored unprocessed sputum samples (stored at −80°C for 24 months) at the Mycobacteriology Research Center of Jimma University in Ethiopia using the commercially available PowerFecal DNA Isolation Kit (Qiagen) (27). MiSeq preparations were done in the Lab of Applied Microbiology and Biotechnology (Belgium) using an in-house optimized protocol for low-biomass samples (28), and dual-index paired-end Illumina MiSeq 16S rRNA V4 region with an amplicon size of 254-bp sequencing was performed at the Center for Medical Genetics of the University of Antwerp (Belgium), as described (28).

6.3.3. Statistical analysis

Processing and quality control of the sequencing reads were performed using the R package Divisive Amplicon Denoising Algorithm 2 (DADA2), version 1.6.0., to increase the sensitivity and specificity compared to OUT picking methods (28). At the genus level, we processed amplicon sequence variants (ASVs) and aggregated ASV read counts. We annotated ASVs and added metadata to samples using R. Statistical analyses and data visualization was performed using R.

Bacteria were categorized as present in the sputum sample when they were present at ≥1% of the population. Bacteria were then classified as potentially pathogenic, opportunistic (i.e., cause of disease in immunocompromised individuals, including people living with HIV or elderly people), or not LRTI-causing based on literature review using PubMed, Science Direct, and Google Scholar and using search terms pathogenic bacteria, opportunistic bacteria, bacterial genera, LRTI, and bacterial classification (**Table S4**). When comparison of the 16S rRNA amplicon data could not classify the bacteria present to species level, the bacteria present were classified as potential LRTI pathogens.

The difference of bacterial LRTI pathogens detected was compared between Xpert Ultra positive and negative samples using Chi-Squared test. Logistic regression analysis was performed to determine the association (odds ratio [OR] and its 95% CI) between (potential) bacterial LRTI pathogens and response to an antibiotic trial, findings on chest X-ray, and 6-month survival status. For each (potential) bacterial LRTI pathogen identifiedand each outcome of interest, a separate model was built. For each model, the adjusted OR was estimated by including patient characteristics that were associated with the outcome of interest at P-value < 0.2 in bivariate analysis. Generalized variance-inflationfactor was estimated to check multicollinearity. Backward stepwise model reduction was performed using the likelihood ratio test with a P-value cut-point of 0.1.

6.4. Results

6.4.1. Cohort characteristics

Of the 250 Xpert MTB/RIF-negative participants presenting with symptoms suggestive of pulmonary TB, 35 (14%) were diagnosed with pulmonary TB (Xpert Ultra and culture positive) and 215 (86%) were not diagnosed with pulmonary TB (211 Xpert Ultra negative and culture negative; 4 Xpert Ultra negative and contaminated cultures). Among the 215 Xpert Ultra negative patients, 17.2% (*n* = 37) had a history of TB treatment, 20.2% (*n* = 42) were living with HIV, 13.5% (*n* = 29) were elderly (age ≥65 years), 6.3% (*n* = 13) were severely ill, 1.9% (*n* = 4) had diabetes mellitus (DM), and 5.1% (*n* = 11) had a diagnosis of COPD. Most patients had a normal chest X-ray (*n* = 150, 70.8%) and about half (*n* = 117, 54.4%) improved clinically after a trial of broad-spectrum antibiotics. Compared to participants with a positive Xpert Ultra test result, those with a negative Xpert Ultra test were less likely to have prolonged symptoms or comorbidity (diagnosis of DM or COPD) and were more likely to be older, underweight or overweight, have a normal chest X-ray and improve clinically after a trial of antibiotics (**Table 1**).

Table 1 Characteristics of 250 hospitalized adults with symptoms of Pulmonary TB who tested negative on Xpert MTB/RIF, stratified by Xpert Ultra results.

Characteristics	**Category**	**Xpert Ultra Negative** n (%)	**Xpert Ultra Positive** n (%)
All patients		**215 (86)**	**35 (14)**
Age	18- 40 years	113 (52.6)	31 (88.6)
	41-64 years	73 (34.0)	2 (5.7)
	≥65 years	29 (13.5)	2 (5.7)
Sex	Female	117 (54.4)	21 (60.0)
	Male	98 (45.6)	14 (40.0)
Residence	Urban	92 (42.8)	14 (40.0)
	Rural	123 (57.2)	21 (60.0)
Body mass index	Underweight (<18.5 kg·m−2)	99 (46.0)	10 (28.6)
	Normal (18.5-24.9 kg·m^{-2})	61 (28.4)	23 (65.7)
	Overweight (>25-29.9 kg·m^{-2})	55 (25.6)	2 (5.7)
Co-morbidities	Diabetes mellitus	4 (1.9)	3 (8.6)
	Chronic obstructive pulmonary disease	11 (5.1)	7 (20.0)
HIV status*	HIV infected	42 (20.2)	10 (28.6)
	HIV negative - severely ill[#]	13 (6.3)	5 (14.3)
	HIV negative - not severely ill	153 (73.6)	20 (57.1)
History of TB treatment	No	178 (82.8)	27 (77.1)
	Yes	37 (17.2)	8 (22.9)
Clinical improvement on antibiotic trial	No	98 (44.6)	31 (88.6)
	Yes	117 (54.4)	4 (11.4)
Symptoms at presentation	Cough ≥ 2 weeks	134 (62.3)	31 (88.6)
	Shortness of breath ≥ 2 weeks	101 (47.0)	26 (74.3)
	Night sweat ≥ 2 weeks	102 (47.4)	18 (31.4)
	Fever ≥2 weeks	94 (43.7)	19 (54.3)
	Weight loss	112 (52.1)	27 (77.1)

Table 1: Continued.

Characteristics	Category	Xpert Ultra Negative n (%)	Xpert Ultra Positive n (%)
Symptoms at presentation	Loss of appetite	168 (78.1)	33 (94.3)
	Chest pain	137 (63.7)	31 (88.6)
Radiological findings	Normal	150 (70.8)	4 (11.4)
	Cavitary lesion	20 (9.4)	11 (31.4)
	Pleural effusion	20 (9.4)	6 (17.1)
	Consolidation	10 (4.7)	6 (17.1)
	Miliary disease	8 (3.8)	6 (17.1)
	Fibrosis	3 (1.4)	1 (2.9)
	Hilary adenopathy	1 (0.5)	1 (2.9)

*HIV status missing for 7 Xpert Ultra negative patients, #Severely ill defined as temperature > 39°C, respiratory rate > 30 resp./min, cardiac rate > 120 bpm, or unable to walk without help.

6.4.2. Bacterial composition of sputum samples via 16S rRNA sequencing

Haemophilus, Streptococcus, and Moraxella were among the most prevalent genera in the sputum samples of all study participants (**Fig. 1**). One or more potential bacterial LRTI pathogens were present at ≥1% in 79.1% (170/215) Xpert Ultra negative samples and 82.8% (29/35) Xpert Ultra positive samples (*P* = 0.615). In Xpert Ultra negative samples, *Haemophilus* spp. (*n* = 105, 48.7%), *Staphylococcus* spp. (*n* = 77, 35.8%), *S. pneumoniae/pseudopneumoniae* (*n* = 56, 26.0%), *Moraxella catarrhalis/nonliquefaciens* (*n* = 47, 21.9%), *M. pneumoniae* (*n* = 7, 3.3%), and *Bordetella pertussis* (*n* = 2, 0.9%) were detected on amplicon sequence variant (ASV) level. In addition, one or more opportunistic pathogens were identifiedin 40.8% of the 71 Xpert Ultra negative sputum samples collected from elderly patients or patients living with HIV: *Rothia aeria* (*n* = 24, 33.8%), *Acinetobacter baumannii* (*n* = 2, 2.8%), *Streptococcus pyogenes* (*n* = 1, 1.4%), and *P. aeruginosa* (*n* = 2, 2.8%). Except for *Mtb,* similar proportions of (potential) bacterial pathogens and opportunistic pathogens were detected in the Xpert Ultra positive sputum samples (**Table 2**). Multiple potential bacterial LRTI pathogens were more often identifiedin Xpert Ultra MTB-negative sputum samples (58.1%, 125 of 215) than in Xpert Ultra MTB-positive sputum samples (34.3%, 12 of 35) (*P* = 0.01).

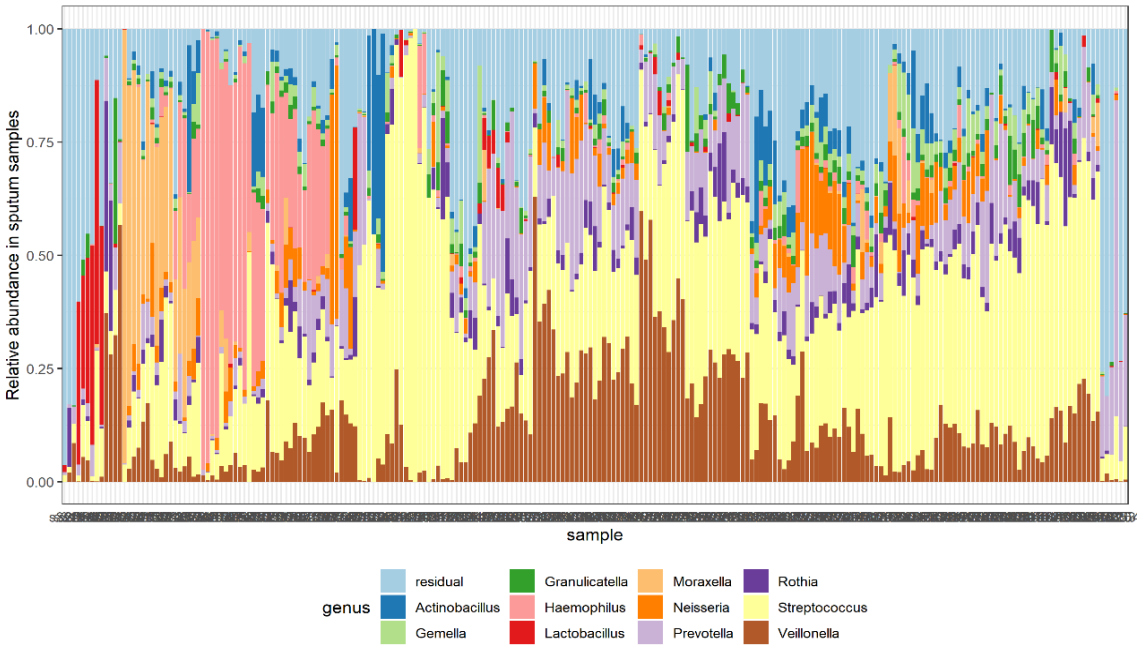

Figure 1: Distribution of the most abundant bacterial genera in sputum samples identified by 16S rRNA gene amplicon sequencing in 250 Xpert MTB/RIF-negative presumptive TB cases.

Table 2: Bacterial pathogens identified by 16S rRNA gene amplicon sequencing in sputum samples stratified by Xpert MTB/RIF Ultra results.

	Xpert Ultra negative	**Xpert Ultra positive**
All patients	**215 (86)**	**35 (14)**
Bacterial pathogens		
Haemophilus spp.	105 (48.7)	18 (51.4)
Staphylococcus spp.	77 (35.8)	12 (34.3)
Streptococcus pneumoniae/pseudopneumoniae	56 (26.0)	8 (22.9)
Moraxella catarrhalis/nonliquefaciens	47 (21.9)	9 (25.7)
Mycoplasma pneumoniae	7 (3.3)	0 (0.0)
Mycobacterium tuberculosis	0 (0.0)	5 (14.3)
Bordetella pertussis	2 (0.9)	0 (0.0)
HIV positive or elderly patients	**71**	**12**
Opportunistic pathogens		
Rothia aeria	24 (33.8)	3 (25.0)
Pseudomonas aeruginosa	2 (2.8)	0 (0.0)
Acinetobacter baumannii	2 (2.8)	0 (0.0)
Streptococcus pyogenes	1(1.4)	1(8.3)

6.4.3. Association between bacterial pathogens in sputum sample and response to antibiotic trial

The sputum samples were collected from 215 patients with an Xpert Ultra negative result before they received a seven-day antibiotic trial of [ceftriaxone and azithromycin (43.7%, *n* = 94), amoxicillin (30.2%, *n* = 65), or vancomycin plus doxycycline (26.1%, *n* = 56)] **-Fig. S1**). Only 54.4% (*n* = 117) improved clinically after the antibiotic trial. Of 98 patients failing to respond to antibiotics, 21 started empiric TB treatment, whereas 77 did not. Of these 77 patients, the likely causative pathogen could be identifiedin the sputum sample of 7 (9%): *M. pneumoniae* (*n* = 2) *B. pertussis* (*n* = 2), *Acinetobacter baumanii* (*n* = 2), and *P. aeruginosa* (*n* = 1). In addition, 4 (5%) patients were diagnosed with bacteriologically confirmed TB during the 6-month follow-up period. For most 66 (86%) patients, the cause was their prolonged respiratory symptoms and their failure to respond to antibiotics remained unclear.

When adjusted for patient characteristics associated with poor response to an antibiotic trial (age ≥65 years, HIV status, history of TB treatment, presence of prolonged cough, fever, chest pain, or weight loss- **Table S1**), the presence of *S. pneumoniae/pseudopneumoniae* (aOR 3.31, 95% CI 1.68–6.72), *Haemophilus* spp. (aOR 2.08, 95% CI 1.16–3.78), *M. catarrhalis/nonliquefaciens* (aOR 4.24, 95% CI 2.04–9.27), or *M. pneumoniae* (aOR 8.78, 95% CI 1.34–173.4) was associated with poor clinical response to an antibiotic trial (**Table 3**).

Table 3: Association between abundant (potential) bacterial pathogens and response to antibiotic trial among 215 symptomatic hospitalized patients with a negative sputum Xpert MTB/RIF Ultra result

Bacterial pathogens		**Good response to antibiotic trial**	**Poor response to antibiotic trial**	**Crude OR (95% CI)**	**Adjusted OR* (95% CI)**
All patients		**117 (54.4)**	**98 (45.6)**		
Haemophilus spp.	Absent	70 (59.8)	40 (40.8)	ref	ref
	Present	47 (40.2)	58 (59.2)	**2.16(1.25-3.75)**	**2.08(1.16-3.78)**
Staphylococcus spp.	Absent	81 (69.2)	57 (58.2)	ref	
	Present	36 (30.8)	41 (41.8)	1.62(0.92-2.84)	
Moraxella catarrhalis/ nonliquefaciens	Absent	102 (87.2)	66 (67.3)	ref	ref
	Present	15 (12.8)	32 (32.7)	**3.30(1.68-6.70)**	**4.24 (2.04-9.27)**

Table 3: Continued.

Streptococcus pneumoniae/ pseudopneumoniae	Absent	102 (87.2)	57 (58.2)	ref	ref
	Present	15 (12.8)	41 (41.8)	**4.89(2.53-9.85)**	**3.31(1.68- 6.72)**
Mycoplasma pneumoniae	Absent	116 (99.1)	92 (93.9)	ref	ref
	Present	1 (0.9)	6 (6.1)	**7.50(1.26- 144)**	**8.78(1.34-173.4)**
Bordetella pertussis	Absent	117 (100)	96(98.0)	ref	
	Present	0 (0.0)	2 (2.0)	7.01(4.8^{e-64}-NA)	
Opportunistic bacterial pathogens					
HIV positive or elderly patients		31	40		
Rothia aeria	Absent	20 (64.5)	27 (57.4)	ref	
	Present	11 (35.5)	13 (32.5)	0.87 (0.32-2.37)	
Pseudomonas aeruginosa	Absent	30 (96.8)	39 (97.5)	ref	
	Present	1 (3.2)	1 (2.5)	0.77 (0.03-19.9)	
Acinetobacter baumannii	Absent	29 (93.5)	40 (100)	ref	
	Present	2 (6.5)	0 (0.0)	$4.6e^{-08}$ (NA-1.9^{e+108})	

*****Adjusted for age ≥ 65 years, HIV-status, history of TB-treatment, presence of prolonged cough, fever or chest pain, NA: Infinitive number.

6.4.4. Association between bacterial pathogens in sputum sample and baseline chest X-ray findings

Overall, 29.2% of patients with an Xpert Ultra negative sputum sample had an abnormal chest X-ray. When adjusted for patient characteristics associated with the presence of an abnormal chest X-ray (rural residence, presence of prolonged cough, fever, chest pain, or shortness of breath-**Table S2**), the odds of an abnormal chest X-ray were higher in the presence of *S. pneumoniae/pseudopneumoniae* (aOR 2.5, 95% CI 1.12–6.16) and lower in the presence of *M. catarrhalis/nonliquefaciens* (aOR 0.37, 95% CI 0.19–0.74) in the sputum (**Table 4**).

Table 4: Association between the presence of (potential) bacterial pathogens and chest X-ray findings among 215 symptomatic hospitalized patients with negative Xpert Ultra result

Bacterial pathogens		**Chest X-ray findings***		**Crude OR (95% CI)**	**Adjusted OR (95% CI) #**
		Normal	**Abnormal**		
All patients		150 (70.8)	62 (29.2)		
Haemophilus spp.	Absent	81 (54.0)	26 (41.9)	ref	
	Present	69 (46.0)	36 (58.1)	1.62 (0.89-2.97)	
Staphylococcus spp.	Absent	95 (63.3)	41 (66.1)	ref	
	Present	55 (36.7)	21 (33.9)	0.88 (0.46-1.63)	
Streptococcus pneu-	Absent	117 (78.0)	40 (64.5)	ref	ref
moniae/ pseudopneu-	Present	33 (22.0)	22 (35.5)	1.95 (1.01-3.72)	**2.5(1.12-6.16)**
moniae					
Moraxella catarrhalis/	Absent	112 (74.7)	54 (87.1)	ref	ref
nonliquefaciens	Present	38 (25.3)	8 (12.9)	0.43 (0.17-0.95)	**0.37(0.19-0.74)**
Mycoplasma pneu-	Absent	145 (96.7)	60 (96.8)	ref	
moniae	Present	5 (3.3)	2 (3.2)	0.96 (0.13-4.62)	
Bordetella pertussis	Absent	149 (99.3)	61 (98.4)	ref	
	Present	1 (0.7)	1 (1.6)	2.44 (0.09-62.4)	
Opportunistic bacterial pathogens					
HIV positive or elderly patients		51	20		
Rothia aeria	Absent	33 (64.7)	14 (70.0)	ref	
	Present	18 (35.3)	6 (30.0)	0.78 (0.24-2.33)	
Pseudomonas aeru-	Absent	51 (100)	18 (90.0)	ref	
ginosa	Present	0 (0.00)	2 (10.0)	4.43 (1.1^{e-108}- NA)	
Acinetobacter bau-	Absent	50 (98.0)	19 (95.0)	ref	
mannii	Present	1 (2.0)	1 (5.0)	2.63 (0.1- 68.8)	
Streptococcus py-	Absent	51 (100)	19 (95.0)	ref	
ogenes	Present	0 (0.00)	1 (5.0)	1.54 (2.5^{e-122} -NA)	

Adjusted for rural residence, presence of prolonged cough, fever, chest pain or shortness of breath; *3 patients missing CXR diagnosis; NA: Infinitive number.

6.4.5. Association between bacterial pathogens in sputum sample and survival status at six months

Among the 215 patients with a Xpert Ultra negative sputum, nine (4.2%) died: three while hospitalized and six after discharge. When adjusted for patient characteristics associated ($P < 0.2$) with survival status at 6 months (rural residence, body mass index, and HIV status-**Table S3**), the presence of *Streptococcus pneumoniae/pseudopneumoniae* (aOR 5.31, 95% CI 1.29–26.6), *M. catarrhalis/nonliquefaciens* (aOR 12.1, 95% CI 2.67–72.8), and *M. pneumoniae* (aOR 34.5, 95% CI 4.79–292.3) were associated with mortality (**Table 5**). The presence of multiple pathogens was not associated with mortality among Xpert Ultra MTB-negative (OR 2.61, 95% CI 0.52–12.8) or Xpert Ultra MTB-positive patients (OR 2.10, 95% CI 0.25-17.1).

Table 5: Association between the presence of potential bacterial LRTI pathogens in sputum and mortality among 215 symptomatic hospitalized patients with negative Xpert Ultra result

Bacterial pathogens		**Survival status**		**Crude OR (95% CI)**	**Adjusted OR* (95% CI)**
		Alive	**Died**		
Haemophilus spp.	Absent	107 (51.9)	3 (33.3)	ref	
	Present	99 (48.1)	6 (66.7)	2.1 (0.55-10.4)	
Staphylococcus spp.	Absent	132 (64.1)	6 (66.7)	ref	
	Present	74 (35.9)	3 (33.3)	0.8 (0.42-1.48)	
Streptococcus pneu-	Absent	156 (75.7)	3 (33.3)	ref	ref
moniae/ pseudo-	Presen	50 (24.3)	6 (66.7)	**6.2 (1.58-30.4)**	**5.31 (1.29-26.6)**
pneumoniae					
Moraxella catarrh-	Absent	165 (80.1)	3 (33.3)	ref	ref
alis/ nonliquefaciens	Present	41 (19.9)	6 (66.7)	**8.0 (2.03-39.4)**	**12.1(2.67 -72.8)**
Mycoplasma pneu-	Absent	202 (98.1)	6 (66.7)	ref	ref
moniae	Present	4 (1.9)	3 (33.3)	**25.2 (4.24-143)**	**34.5(4.79-292)**
Bordetella pertussis	Absent	205(99.5)	8 (88.9)	ref	
	Present	1 (0.5)	1 (11.1)	25.6 (0.95-689)	

Table 5: Continued.

Bacterial pathogens		Survival status		Crude OR (95% CI)	Adjusted OR* (95% CI)
		Alive	Died		
Opportunistic bacteria pathogen					
HIV positive or elderly patients		66	5		
Rothia aeria	Absent	44 (66.7)	3 (60.0)	ref	
	Present	22 (33.3)	2 (40.0)	1.33 (0.16-8.61)	
Pseudomonas aeruginosa	Absent	65 (98.5)	4 (80.0)	ref	
	Present	1 (1.5)	1 (20.0)	16.2 (0.57-468)	
Acinetobacter baumannii	Absent	64 (96.9)	5 (100)	ref	
	Present	2 (3.03)	0 (0.0)	3.38 (NA-3.3 $^{e+183}$)	
Streptococcus pyogenes	Absent	65 (98.5)	5 (100)	ref	
	Present	1 (1.5)	0 (0.0)	8.3 (NA- 1.2 $^{e+206}$)	

*****Adjusted for rural residence, body mass index or HIV-status. NA: Infinitive number, bold face shows association

6.5. Discussion

In this study, we aimed to investigate the bacterial etiology of LRTI in patients presenting with symptoms of TB who had a very low probability of having TB given their sputum's negative Xpert Ultra result based on 16S rRNA sequencing. The presence of potential bacterial pathogens in the sputum samples was identified and compared with their prevalence in Xpert Ultra positive sputum samples. We could determine the presence of most likely causal pathogen in only 13 of the 215 patients, as described in Table 2, with 7 cases of *M. pneumoniae*, 2 cases of *B. pertussis*, 2 cases of *A. baumannii*, and 2 cases of *P. aeruginosa*.

Overall, one or more (potential) bacterial LRTI pathogens were present in 80% of sputum Xpert Ultra negative samples. The most common pathogenic bacterial ASVs detected were *Haemophilus* spp*., Staphylococcus* spp*., Streptococcus pneumoniae (pseudo) pneumoniae,* and *M. catarrhalis/nonliquefaciens*, present in >20% of patients. Thechallenge in attributing LRTI to the presence of these pathogens is further highlighted by the observation that one or more of these (potential) bacterial LRTI pathogens were also present in about 82.8% of Xpert Ultra positive sputum samples and that, except for a higher prevalence of *Mtb* in Xpert Ultra positive sputum

samples, the bacterial populations were almost similar for Xpert Ultra negative and positive samples.

This 80% prevalence of one or more potential bacterial LRTI pathogen is higher than what has been reported in Cameron and Cambodia based on culture methods, where bacterial LRTI pathogens was reported in 44% and 46.8% of presumptive TB cases, respectively (7, 25). The high prevalence of (potential) bacterial LRTI pathogens in patients with confirmedTB is in line with prior reports that co-detection with other bacterial pathogens is common in patients diagnosed with pulmonary TB (26, 27). In Cambodia, co-detection with another potential bacterial LRTI pathogens was observed in 33% of patients diagnosed with pulmonary TB by sputum culture (7). In Nigeria, 50% of sputum samples collected from patients with TB grew both *Mtb* and other bacteria implicated in LRTI as the same as in this paper (28). The higher prevalence may be explained by the use of 16S rRNA gene amplicon sequencing, which can identify both culturable and unculturable bacteria, providing a complete picture of the bacterial community of sputum samples (29, 30).

Among the patients with an Xpert Ultra negative sputum result, 29.2% had an abnormal chest X-ray, which is similar to the findings from a study in South Africa where 27.2% of Xpert Ultra negative patients had abnormal findings on chest X-ray (31). We also found that the presence of *Streptococcus pneumoniae/pseudopneumoniae* in the Xpert Ultra negative sputum samples increased the odds of an abnormal chest X-ray (aOR 2.5, 95% CI 1.12–6.16), whereas the presence of *M. catarrhalis/nonliquefaciens* decreased the odds of abnormal chest X-ray findings (aOR 0.37, 95% CI 0.19–0.74).

In our study population, just over half (54.4%) of patients with an Xpert Ultra negative sputum result improved on an antibiotic trial. Patients for whom *M. catarrhalis/nonliquefaciens, Streptococcus pneumoniae/pseudopneumoniae, M. pneumoniae,* and *Haemophilus* spp. was detected in the sputum sample had higher odds of poor response to an antibiotic trial, even after adjusting for patient characteristics. This may be due to the presence of drug-resistant bacteria (32). Three of the four pathogens associated with failure to improve on an antibiotic trial were also associated with an increased odds of mortality in the 6 months following the initial assessment: *S. pneumoniae/pseudopneumoniae* (aOR 5.31, 95% CI 1.29–26.6), *M. catarrhalis/nonliquefaciens* (aOR 12.1, 95% CI2.67–72.8), and *M. pneumoniae* (aOR 34.5 95% CI 4.79–292.3).

The main strength of the study was the use of 16S rRNA sequencing for the firsttime to detect bacterial LRTI pathogens in sputum samples of patients presenting with symptoms of TB who had a very low probability of having TB as *Mtb* was not detected by the highly sensitive Xpert Ultra assay. Another strength is the prospective collection of comprehensive clinical data. This allowed an assessment of the associations between the bacterial community and patient outcomes. Our study also had some limitations. First, this was a hospital-based study, limiting generalizability to outpatient settings. Second, bacterial sputum culture was not available in our resource-poor study setting, and assessment of sputum quality using Gram staining to determine the extent of oral floracontamination was not performed. A positive result from 16S rRNA gene sequencing may indicate either infection or colonization of the normal respiratory flora (29). Third, despite using the DADA2 algorithm with ASVs to increase the sensitivity and specificitycompared to OUT picking methods, the 16S rRNA amplicon sequencing of the V4 region could not always discriminate accurately up to species level. For instance, of the *Haemophilus* spp*., Haemophilus influenza*type b and non-typable *Haemophilus* are causal pathogens for LRTI (33). However, *Haemophilus parainfluenza*is a common isolate from the healthy nasopharynx as well as *H. influenzae*type b. Non-typable *H. influenzae*can be found in sputum cultures of nearly half of adults with chronic bronchitis (34). Finally, because it is unclear which level of abundance a pathogen is clinically relevant, we reported any presence above 1%. This may have resulted in the inclusion of minority populations of pathogenic bacteria that are not of clinically important.

Among the overall 30 types of the *Staphylococcus* spp*., S. aureus* is a common cause of pneumonia, but it is also frequently isolated in respiratory samples from healthy individuals as a colonizing bacterium (35). Of the *Streptococcus* spp*., S. pneumoniae* is a well-established cause of LRTI, but the role of *S. (pseudo) pneumoniae* is less certain, although it has been reported in COPD (36). *M. nonliquefaciens* frequently colonizes the upper respiratory tract and is usually non-pathogenic, rarely causing invasive disease (37). *M. catarrhalis* also commonly colonizes the healthy airways (28), but it can cause pneumonia in children and adults with underlying chronic lung disease (38). Third, although viral and fungal communities can cause LRTI, they cannot be detected in sputum samples when using 16 s rRNA. Finally, as drug susceptibility tests were notperformed, the presence of antibiotic resistance as a cause for poor response to an antibiotic trial or mortality could not be assessed.

In conclusion, the study found that 16S rRNA could identify the bacterial pathogen responsible for LRTI in 6.0% of Xpert Ultra negative patients but was not specificenough to differentiatebetween carriage and disease-causing pathogens in 80% of cases, making this approach not appear to be clinically useful. The presence of *M. pneumonia* was associated with 34 times greater odds of mortality and the presence of *S. pneumoniae (pseudo) pneumoniae* or *M. catarrhalis/nonliquefaciens* increased the odds of mortality rate by 5 to 12 times, respectively, suggesting clinical relevance of these pathogens. Further research using tools with higher discriminatory power that can also detect viruses and fungi is required to guide the management of Xpert Ultra negative patients.

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Kebede, G. Abebe and A. Van Rie designed the study. W. Kebede, G. Abebe, E.K. Gudina, and A. Van Rie coordinated the study and the data collection at the site. Ilke De Boeck, Eline Cauwenberghs, Sarah Lebeer, and W. Kebede performed DNA extraction for 16S rRNA gene amplicon sequencing. W. Kebede, Ilke De Boeck, Eline Cauwenberghs, and A. Van Rie analyzed the data. W. Kebede and A. Van Rie wrote the firstdraft. All authors have reviewed the paper and provided comments, and have approved the finalversion of the manuscript for submission.

Author contributions

Wakjira Kebede, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft | Gemeda Abebe, Conceptualization, Data curation, Funding acquisition, Methodology, Supervision, Writing-review and editing | Ilke De Boeck, Data

curation, Formal analysis, Investigation, Software, Writing-review and editing | Esayas Kebede Gudina, Methodology, Project administration, Supervision, Writing-review and editing | Eline Cauwenberghs, Formal analysis, Investigation, Visualization, Writing-review and editing | Sarah Lebeer, Investigation, Methodology, Resources, Visualization, Writing-review and editing | Annelies Van Rie, Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing-review and editing.

Data availability statement: The sequence data used and/or analyzed during the current study are included as supplemental material.

Additional files: The following material is available online.

Supplemental Material

Data Set S1 (Spectrum02931-23-s0001.csv). Sequence data.
Supplemental material (Spectrum02931-23-s0002.docx). Additional experimental details, figures,and tables.

Supplementary files

Table S1: Association between patient characteristics and response to an antibiotic trial.

Characteristics	Category	Clinical improvement on antibiotic trial	No clinical improvement on antibiotic trial	Crude OR (95% CI)	P value
Age (in years)	18- 40	66 (56.4)	47 (47.9)	ref	
	41-64	38 (32.4)	35 (35.7)	1.2 (0.71-2.34)	0.394
	≥65	13 (11.2)	16 (16.4)	1.7 (0.76-3.98)	0.192
Sex	Female	65 (55.5)	52 (53.1)	ref	
	Male	52 (44.5)	46 (46.9)	1.1 (0.64-1.89)	0.715
Residence	Urban	50 (42.7)	42 (42.8)	ref	
	Rural	67 (57.3)	56 (57.2)	0.9 (0.57-1.71)	0.986
Body mass index	<18.5	58 (49.5)	41 (41.8)	ref	
	18.5-24.9	31(26.5)	30 (30.6)	1.3 (0.72-2.60)	0.337
	>25-29.9	28 (24.0)	27 (27.6)	1.3 (0.70-2.65)	0.358
HIV status*	Negative	96 (83.5)	70 (75.2)	ref	
	Positive	19 (16.5)	23 (24.7)	1.6 (0.84-3.30)	0.144
History of TB treatment	No	91 (77.8)	87 (88.8)	ref	
	Yes	26 (22.2)	11 (11.2)	0.4 (0.19-0.92)	0.036
Weight loss	No	47 (40.2)	56 (57.1)	ref	
	Yes	70 (59.8)	42 (42.9)	0.5 (0.29-0.86)	0.0136
Cough	<2 weeks	39 (33.3)	42 (42.8)	ref	
	≥2weeks	78 (66.7)	56 (57.2)	0.6 (0.38-1.16)	0.152
Shortness of breath	<2 weeks	58 (49.6)	56 (57.2)	ref	
	≥ 2 weeks	59 (50.4)	42 (42.8)	0.7 (0.42-1.26)	0.268
Night sweat	<2 weeks	56 (47.9)	57 (58.1)	ref	
	≥ 2 weeks	61 (52.1)	41 (41.8)	0.6 (0.38-1.13)	0.133
Fever	<2 weeks	62 (53.0)	59 (60.2)	ref	
	≥ 2 weeks	55 (47.0)	39 (39.8)	0.7(0.43-1.28)	0.289
Loss of appetite	<2 weeks	27 (23.1)	20 (20.4)	ref	
	≥ 2 weeks	90 (76.9)	78 (79.6)	1.1 (0.61-2.26)	0.637
Chest pain	<2 weeks	35 (29.9)	43 (43.9)	ref	
	≥ 2 weeks	82 (70.1)	55 (56.1)	0.5 (0.30-0.95)	0.034

** HIV status missing for 7 Xpert Ultra negative patients*

Table S2: Association between patient characteristics and chest X-ray findings among 215 symptomatic hospitalized patients with negative Xpert Ultra result.

Characteristics	Category	Chest X-ray findings		COR (95% CI)	P value
		Normal	Abnormal		
Age (in years)	18- 40	81 (54.0)	29 (46.8)	ref	
	41-64	49 (32.7)	24 (38.7)	1.36 (0.71-2.61	0.342
	≥65	20 (13.3)	9 (14.5)	1.25 (0.40-3.01)	0.616
Sex	Female	81 (54.0)	35 (56.5)	ref	
	Male	69 (46.0)	27 (43.5)	0.9 (0.5-1.64)	0.744
Residence	Urban	68 (45.3)	22 (35.5)	ref	
	Rural	82 (54.7)	40 (64.5)	1.5 (0.82-2.81)	0.188
Body mass index	<18.5	72 (48.0)	25 (40.3)	ref	
	18.5-24.9	43 (28.7)	18 (29.0)	1.20 (0.58-2.45)	0.608
	>25-29.9	35 (23.3)	19 (30.7)	1.56 (0.75-3.21)	0.224
HIV status*	Negative	114 (79.2)	49 (80.3)	ref	
	Positive	30 (20.8)	12 (19.7)	0.93 (0.42-1.93)	0.851
History of TB treatment	No	124 (82.7)	51 (82.3)	ref	
	Yes	26 (17.3)	11 (17.7)	1.02 (0.45-2.19)	0.943
Weight loss	No	84 (56.0)	19 (30.6)	ref	
	Yes	66 (44.0)	43 (69.4)	2.88 (1.55-5.49)	0.0001
Cough	<2 weeks	64 (42.7)	17 (27.4)	ref	
	≥2weeks	86 (57.3)	45 (72.6)	1.96 (1.04-3.83)	0.0394
Shortness of breath	<2 weeks	86 (57.3)	27 (43.5)	ref	
	≥ 2 weeks	64 (42.7)	35 (56.5)	1.74 (0.96-3.18)	0.0686
Night sweat	<2 weeks	80 (53.3)	31 (50.0)	ref	
	≥ 2 weeks	70 (46.7)	31 (50.0)	1.14 (0.63-2.07)	0.659
Fever	<2 weeks	90 (60.0)	29 (46.8)	ref	
	≥ 2 weeks	60 (40.0)	33 (53.2)	1.7 (0.94-3.11)	0.0788
Loss of appetite	<2 weeks	33 (22.0)	13 (21.0)	ref	
	≥ 2 weeks	117 (78.0)	49 (79.0)	1.06 (0.52-2.25)	0.8687
Chest pain	<2 weeks	69 (46.0)	9 (14.5)	ref	
	≥ 2 weeks	81 (54.0)	53 (85.5)	5.01 (2.4-11.5)	4.65e-05

** HIV status is missing for six patients with normal chest X-ray findings and one with abnormal chest X-ray findings.*

Table S3: Association between patient characteristics and six-month mortality among 215 symptomatic hospitalized patients with negative Xpert Ultra result.

Characteristics	Category	Survival status		Crude OR (95% CI)	P value
		Alive	Died		
Age (in years)	18- 40	109 (52.9)	4 (44.4)	ref	
	41-64	69 (33.5)	4 (44.4)	1.5 (0.36-6.87)	0.527
	≥65	28 (13.6)	1 (11.2)	0.9 (0.04-6.90)	0.981
Sex	Female	113 (54.9)	4 (44.4)	ref	
	Male	93 (45.1)	5 (55.6)	1.5 (0.39-6.28)	0.542
Residence	Urban	86 (41.7)	6 (66.7)	ref	
	Rural	120 (58.3)	3 (33.3)	0.3 (0.07-1.39)	0.155
Body mass index ($kg \cdot m^{-2}$)	<18.5	97 (47.1)	2 (22.3)	ref	
	18.5-24.9	58 (28.1)	3 (33.3)	2.5 (0.40-19.4)	0.322
	>25-29.9	51 (24.8)	4 (44.4)	3.8 (0.03-1.39)	0.130
HIV status*	Negative	161 (80.9)	5 (55.6)	ref	
	Positive	38 (19.1)	4 (44.4)	3.3 (0.8-13.4)	0.078
History of TB treatment	No	171 (83.0)	7 (77.7)	ref	
	Yes	35 (17.0)	2 (22.3)	1.4 (0.20-6.06)	0.685
Weight loss	No	100 (48.5)	3 (33.3)	ref	
	Yes	106 (51.5)	6 (66.7)	1.88 (0.48-9.12)	0.378
Cough	<2 weeks	78 (37.8)	3 (33.3)	ref	
	≥2weeks	128 (62.2)	6 (66.7)	1.2 (0.31-5.90)	0.784
Shortness of breath	<2 weeks	109 (52.9)	5 (55.6)	ref	
	≥ 2 weeks	97 (47.1)	4 (44.4)	0.8 (0.21-3.48)	0.876
Night sweat	<2 weeks	107 (51.9)	6 (66.7)	ref	
	≥ 2 weeks	99 (48.1)	3 (33.3)	0.5 (0.11-2.10)	0.393
Fever	<2 weeks	116 (56.3)	5 (55.6)	ref	
	≥ 2 weeks	90 (43.7)	4 (44.4)	1.0 (0.24-4.0)	0.964
Loss of appetite	<2 weeks	46 (22.3)	1 (11.2)	ref	
	≥ 2 weeks	160 (77.7)	8 (88.8)	2.3 (0.40-43.2)	0.437
Chest pain	<2 weeks	75 (36.4)	3 (33.3)	ref	
	≥ 2 weeks	131 (63.6)	6 (66.7)	1.1 (0.29-5.54)	0.851
Empiric TB-treatment	No	117 (56.8)	4 (44.4)	ref	
	Yes	89 (43.2)	5 (55.6)	1.6 (0.42- 6.80)	0.469

Table S4: Classification of bacteria as pathogenic or opportunistic (i.e., causative of LRTI in people living with HIV or the elderly) when detected in clinical sputum specimens of patients with clinical suspicion of LRTIs.

Authors	Year of publication	Type of samples used	Methods	Identified bacterial pathogens	Opportunistic bacteria	Ref.
Zacharioudakis IM, et al.	2021	Sputum	BioFire pneumonia pannel	*Streptococcus pneumonia, Staphylococcus Spp., Klebsiella pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Klebsella aerogenes, Mycoplasma spp.*	*Rothia aeria Streptococcus pyrogens, Streptococcus constellatus*, *Acinetobacter baumannii, Pseudomonas aeruginosa*	[39]
Buchan BW, et.	2022	Sputum, Broncho alveolar lavage tracheal aspirate)	BioFire Film Array pneumonia panel and Unyvero LRT, BAL panel	*Streptococcus pneumonia, Staphylococcus Spp., Legionella pneumophila, Klebseilla pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Klebsella aerogenes, Mycoplasma spp.*	*Streptococcus pyrogens*, *Pseudomonas aeruginosa, Acinetobacter baumannii*	[40]
Alby K, et al	2018	Sputum and endotracheal aspirates	BioFire Film Array pneumonia panel	*Pseudomonas aeruginosa, Klebseilla pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Proteus spp.*	*Streptococcus pyrogens, Streptococcus constellatus*, *Acinetobacter baumannii*	[41]
Kamel NA, et al	2022	Broncho alveolar lavage	FilmArray Pneumonia Panel plus	*Streptococcus pneumonia, Staphylococcus Spp., Klebseilla pneumoniae, Haemophilus influenzae, Escherichia coli, Moraxella catarrhalis, Serratia marcecens*	*Acinetobacter baumannii, Pseudomonas aeruginosa*	[42]
Yoo IY, et al	2020	Sputum	FilmArray Pneumonia Panel plus	*Streptococcus pneumonia, Staphylococcus Spp., Klebseilla pneumoniae, Haemophilus influenzae, Escherichia coli, Klebsella aerogenes, Klebsiella oxytoca.*	*Acinetobacter baumannii, Pseudomonas aeruginosa*	[43]
Xie G, et al	2021	Sputum, bronchoalveolar lavage fluid, lung tissue by transbronchial lung biopsy, pleural effusion, and blood	Metagenomic next-generation sequencing	*Streptococcus pneumonia, Streptococcus pseudopneumonia, Staphylococcus Spp., Entrococcus faecium, Pseudomonas aeruginosa, Klebseilla pneumoniae, Haemophilus influenzae, Acinetobacter baumannii, Moraxella catarrhalis, Klebsella aerogenes.*	*Rothia aeria, Streptococcus pyrogens, Acinetobacter baumannii*	[44,45]
Huang J, et al.	2020	Sputum and bronchoalveolar lavage fluid	Metagenomic next-generation sequencing	*Streptococcus pneumonia, Haemophilus influenzae, Streptococcus pseudopneumonia,*	*Rothia aeria, Acinetobacter baumannii*, *Pseudomonas aeruginosa*	[46]

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Chapter 7: General discussion

Screening all patients who report cough of any duration is important for the diagnosis of TB and to reduce TB transmission and mortality [1]. Smear microscopy is still the main method used for diagnosis of TB in primary health centers and district hospitals in many resource-constrained countries, including Ethiopia. The sensitivity of smear microscopy is low, especially in children and people living with HIV, and it only detects about one-third of patients with culture-positive TB [2, 3]. When using smear microscopy as the initial diagnostic, the WHO algorithm recommends that all individuals with a smear microscopy negative result are evaluated for the clinical response to broad-spectrum antibiotic trials and CXR. In cases where the clinical suspicion of TB remains high, empiric TB treatment is recommended [4] because will cure TB and prevent further transmission in those cases where the diagnosis was missed [5]. A randomized controlled trial however showed that empiric TB treatment does not reduce mortality [6], and some studies found that empiric treatment may even increase the risk of death when other pulmonary infections are overlooked [7].

In 2010, the WHO endorsed the Xpert MTB/RIF assay for the diagnosis of pulmonary TB and in 2017 the even more sensitive was endorsed the Xpert Ultra assay improves the detection of active TB cases in smear-negative, culture-positive patients by 17% [8, 9]. Little is known about the value of an antibiotic trial, CXR and empiric treatment when Xpert MTB/RIF or Xpert Ultra is used as the initial diagnostic for TB. We therefore performed a study to answer questions relevant to TB control in resource limited settings such as Ethiopia.

7.1. What is the value of an antibiotic trial and chest X-ray in settings where Xpert MTB/RIF is used as the first diagnostic?

Our study found that, even though the clinical algorithm identified most (79.5%) of the bacteriologically confirmed cases, the clinical algorithm has suboptimal performance when used in the context where Xpert MTB/RIF is the first diagnostic: the positive predictive value was relatively low (24.8%), 6.4% of bacteriologically confirmed TB cases were missed, and 75% of those who started on empiric TB treatment were culture-negative. The positive predictive value (PPV) observed was lower than the 37.5% PPV of the clinical algorithm in smear-negative HIV-positive patients [10], likely due to the higher sensitivity of the Xpert MTB/RIF assay compared to smear microscopy. The high

proportion of patients without bacteriological confirmed TB that were started on empiric TB treatment was comparable to other studies conducted in African countries, where 49% to 75.5% of smear-negative patients started on empirical TB treatment were not bacteriologically confirmed [11-13].

CXRs can identify changes in the lungs such as nodules, cavities, or infiltrates that are highly suggestive of TB [14]. The added value of CXR to identify cases of active TB cases in the era of Xpert MTB/RIF assays remained unclear. We found that, among the 15.8% of patients in whom Xpert MTB/RIF was false negative, those with a CXR suggestive of TB had two to five times higher odds of having microbiologically confirmed TB compared to patients with a CXR not suggestive of TB. Unfortunately, the addition of CXR findings did not improve the performance of a risk prediction tool based on clinical symptoms. This finding was in line with a study conducted in Uganda, which found that the addition of CXR reading did not complement Xpert MTB/RIF in the diagnosis of smear-negative pulmonary TB among TB/HIV co-infected adults [15]. In our study population, the sensitivity of CXR to diagnose bacteriologically confirmed TB was relatively high: 69% when the CXR was read by a clinician and 79.5% when read by a radiologist, similar to the 80% sensitivity found in a study conducted in Ethiopia among smear-negative individuals [16, 17]. Artificial Intelligence (AI)-assisted X-ray reading could change the role of CXR in the future, particularly in resource-limited settings where access to trained radiologists and diagnostic tests may be limited [18]. Several studies have evaluated the performance of AI-assisted X-ray reading for TB diagnosis, with promising results. For example, a study published in The Lancet Digital Health found that an AI algorithm trained on over 23, 954 chest X-rays achieved a sensitivity of 90% and specificity of 70% for detecting TB, compared to a sensitivity of 75% and specificity of 56% for human readers [19]. This may not only improve the performance of CXR in Xpert MTB/RIF negative patients but could also improve the use of CXR as a triaging tool to limit the use of the relatively expensive Xpert MTB/RIF assay in resource poor settings such as Ethiopia.

7.2. What is the additional yield of Xpert Ultra compared to Xpert MTB/RIF?

Our study confirmed the high sensitivity of Xpert Ultra among cases of presumptive Xpert MTB/RIF, as 89% of the culture-positive cases missed by Xpert MTB/RIF were detected by Xpert Ultra. The high sensitivity of Xpert Ultra we observed is in line with a meta-analysis where 87.2% of TB culture-positive patients were positive on the Xpert Ultra test [20] and an overall estimated pooled

sensitivity of 90.9% [21]. Similar to other studies, we also observed the occurrence of false-positive Xpert Ultra results [22], as the Xpert Ultra assay was positive in four patients with negative cultures. In contrast to other studies, where false-positive Xpert Ultra results were more likely in patients who have recently completed TB treatment [23], none of the Xpert Ultra positive culture-negative cases in our study had a history of prior TB treatment.

7.3. What is the additional yield of LF-LAM when used in combination with Xpert Ultra Assay?

The LF-LAM test detects the presence of LAM, a component of the cell wall that can be released into the urine. The test is simple and rapid, and can be performed at the point of care, making it a useful tool for diagnosing TB in resource-limited settings. However, the sensitivity of the LF-LAM test varies depending on the stage of the disease and the patient population being tested [24]. In patients with advanced HIV disease, the sensitivity of the LF-LAM test is around 60% [25]. Studies found that the addition of LF-LAM to the Xpert MTB/RIF assay increased the overall detection yield of TB by 14% compared to using the Xpert MTB/RIF assay alone, whereas when LF-LAM was used in combination with clinical signs and symptoms alone, the detection yield of TB increased by 36.6% compared to relying solely on clinical evaluation [26, 27]. In contrast, our study found that adding LF-LAM to Xpert Ultra did not yield any benefit. Nevertheless, even in settings with access to Xpert Ultra, patients who are unable to produce sputum may still benefit from the use of the LF-LAM assay.

7.4. What is the role of empiric TB treatment on the survival of bacteriologically not confirmed TB cases?

For decades, clinicians have used empiric TB treatment to overcome the limited sensitivity of smear microscopy and to avoid the negative consequences of delays in treatment initiation [28, 29]. The optimism about empiric TB treatment was diminished by its lack of impact on survival as shown in clinical trials conducted in individuals with advanced HIV disease in the settings with no or limited access to Xpert MTB/RIF [6, 7, 30]. Data on the impact of empiric treatment in settings where Xpert MTB/RIF is used as the initial diagnostic was lacking. A secondary analysis of data on smear-negative HIV-positive individuals in Uganda suggested that in high TB burden settings, empirical treatment

may still be justified in patients with a negative Xpert MTB/RIF result because a negative Xpert MTB/RIF test does not lower the post-test probability below the treatment threshold [31].

In our study population, where Xpert MTB/RIF was used as the initial diagnostic, a clinical poor response to an antibiotic trial was the key reason to initiate empiric TB treatment, with 96.9% of Xpert MTB/RIF-negative patients who did not improve clinically after a course of antibiotics being started on empiric TB treatment. This is similar to a study in Uganda, where 92% of Xpert MTB/RIF negative patients who did not improve on an antibiotic trial-initiated empiric TB treatment [32]. CXR findings were not a determining factor in the decision to start empiric TB treatment, with 85.2% of patients initiated on empiric TB treatment having a normal CXR. This is different from what was observed in South Africa, where 50% of smear-negative patients initiated empirical TB treatment based on CXR abnormalities [12].

In our study, empiric TB treatment was not associated with a reduction in mortality, as 5.6% of empirically treated cases and 4.8% of those untreated died. This result is important as it expands on findings of lack of impact on mortality in smear-negative patients with advanced HIV disease [6, 7, 30]. Our findings were confirmed by a large retrospective analysis of register data in Kenya, where mortality was higher among patients started on empiric treatment (clinically diagnosed) compared to bacteriologically (Xpert MTB/RIF) diagnosed patients (9.9% vs 4.5%; aHR 5.16, 95% CI 2.17 - 12.3) [5].

As expected, we also found no impact on mortality of empiric TB treatment in patients with negative Xpert Ultra results, with similar risk of death between those who did and did not receive empiric TB treatment. Our results advocate for a re-assessment of the practice of prescribing empiric treatment in Xpert MTB/RIF or Ultra-negative patients as this poses a burden on the health care system, exposes patients to unnecessary TB treatment and may even increase mortality. Furthermore, the use of antibiotic trials to guide empiric TB treatment may contribute to the global antimicrobial resistance development with adverse individual and public health consequences. Our data suggests that to improve TB treatment outcomes of patients presenting with symptoms of TB, highly sensitive assays such as Xpert Ultra, screening for comorbidities, and management of other respiratory infections should be prioritized.

7.5. What is the bacterial composition in sputum samples from Xpert Ultra negative patients?

Based on the very high sensitivity and negative predictive value for the diagnosis of TB, a negative Xpert Ultra result strongly suggests that the patient is unlikely to have active pulmonary TB. These patients are thus likely to have other etiological agents causing their respiratory symptoms. Determining the cause of LRTI is thus important to guide appropriate treatment in this population. We therefore performed a first exploratory study to determine the bacteriological composition in sputum samples of Xpert Ultra-negative patients.

Using 16S RNA amplicon sequencing, the pathogen most likely to be responsible for the patients' LRTI could only be identified in 6.0% of Xpert Ultra negative patients and included *Mycoplasma pneumoniae, Bordetella pertussis*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. At least one bacterial pathogen associated with LRTI was detected by 16S RNA sequencing in 79.1% of Xpert Ultra-negative patients and 82.9% of Xpert Ultra-positive patients, with no significant difference in the prevalence of bacterial LRTI pathogens between patients who tested negative versus positive on Xpert Ultra ($p = 0.065$). This prevalence is higher than what was reported in Cameroon (44%) and Cambodia (46.7%) [33, 34], likely because the 16S rRNA gene amplicon sequencing used in our study provides a more complete picture of the microbial community in sputum samples compared to traditional culturing methods used in the other studies.

Even though the prevalence and composition were similar between Xpert Ultra negative and positive samples, the presence of certain pathogens was associated with clinical outcomes. First, the presence of *Streptococcus pneumoniae/pseudopneumoniae* in sputum samples from Xpert Ultra-negative patients was associated with an increased likelihood of abnormal CXR findings, indicating a potential contribution to respiratory symptoms. Second, three bacterial pathogens associated with failure to improve with an antibiotic trial (*Moraxella catarrhalis/nonliquefaciens, Streptococcus pneumoniae/pseudopneumoniae*, and *Mycoplasma pneumoniae*) were also associated with an increased risk of mortality within 6-months following initial assessment.

7.6. Conclusions and recommendations

In settings where Xpert MTB/RIF is used as the initial diagnostic test, the use of a clinical algorithm consisting of an antibiotic trial and CXR identifies most active TB cases but results in substantial empiric overtreatment without an impact on survival in hospitalized patients. The positive predictive value of the currently used clinical algorithm for identifying patients with active TB was low and failed to detect people with active TB. Adding CXR reading to the decision algorithm did not significantly improve clinicians' ability to predict bacteriologically confirmed TB beyond what is possible through clinical characteristics. In people presenting with symptoms of pulmonary TB, the Xpert Ultra assay provides a more sensitive diagnosis of TB than the Xpert MTB/RIF assay and empiric TB treatment of Xpert-Ultra negative patients did not improve survival. The use of the first-generation urine LF-LAM did not improve the diagnostic yield when used in combination with Xpert Ultra in hospitalized HIV-positive patients. Taken together, these findings suggest that countries with high TB and HIV burdens should urgently replace smear microscopy and Xpert MTB/RIF with the highly sensitive Xpert Ultra assay to better identify those patients in need of TB treatment. Routine use of an antibiotic trial and empiric TB treatment in Xpert Ultra negative patients should be advised against as this not only results in overuse of TB drugs, placing an unnecessary strain on health systems and patients, but may also contribute to the growing global burden of antibiotic resistance. Future studies should investigate whether there is still a role for empiric TB treatment in very select populations of Xpert Ultra negative patients and whether the addition of LF-LAM increases the yield of TB diagnosis in patients with symptoms or signs of TB who cannot produce a sputum sample, including people with presumptive extrapulmonary TB.

While our results strongly advocate against the routine use of empiric TB treatment in people with a negative Xpert Ultra result, our analysis of the bacterial composition of the sputum in this population was unable to guide the development of an alternative management strategy. The likely causative agent could not be identified in 6% of patients. 16S rRNA did identified one or more potential bacterial LRT pathogens in one-third of Xpert Ultra-negative patients and detection of certain specific bacterial species was linked to significantly higher mortality, suggesting a clinical relevance. Future research using tools with higher discriminatory power than 16S rRNA sequencing are needed to identify with greater accuracy of the bacterial, viral and fungal pathogens that may cause LRTI in Xpert Ultra negative patients. These data could then be used to determine which alternative diagnoses should be considered in Xpert Ultra-negative patients and to develop evidence-based

guidelines for the management of Xpert Ultra-negative patients in countries with limited diagnostic resources.

7.7. References

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Chapter 8: Policy Brief: September 2023

Submitted to National TB program (NTP), Minister of Health, Ethiopia

Improving the Management of Hospitalized Presumptive Xpert MTB/RIF-negative Tuberculosis Cases in Ethiopia

This policy brief discusses the benefits of using a more sensitive diagnostic test called Xpert Ultra for diagnosing pulmonary tuberculosis (TB) in Xpert MTB/RIF-negative presumptive TB cases. It also explores the impact of empiric TB treatment on the survival of people with presumptive TB cases and the role of chest X-rays and lateral flow urine lipoarabinomannan (LF-LAM) assays in settings where Xpert Ultra is available. Furthermore, it emphasizes the importance of understanding the potential bacterial pathogens responsible for lower respiratory tract infections (LRTIs) in individuals who are suspected to have TB but have been ruled out for the disease. This understanding is crucial for developing effective diagnostic and therapeutic approaches.

Based on data from the PhD project (February 2019–July 2022) at Jimma Medical Center, Ethiopia

8.1. Introduction/Problem/Context

Although Ethiopia has made significant progress in controlling tuberculosis (TB) over the past decade, only 69% of pulmonary TB cases are confirmed by bacterial testing. As a result, empiric TB treatment was initiated in 31% of cases [1,2]. While empiric treatment is beneficial for patients who truly have TB [3,4]. it can also lead to delayed or missed diagnoses, suboptimal management of other diseases, and compromised patient outcomes when sensitive diagnostics are overlooked [5]. To improve the management of presumptive TB cases, defined as persons presenting with signs or symptoms of TB but not confirmed by bacterial testing, it is important to understand the ability of the diagnostic algorithm to detect TB disease and ascertain the influence of empiric therapy on survival

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in people with a negative Xpert MTB/RIF result. Furthermore, it is essential to accurately differentiate pulmonary TB from other pulmonary infections in ill, hospitalized patients.

8.2. About the study

The study took place at Jimma Medical Center, an 800-bed facility in southwest Ethiopia that provides to over 20 million people from both urban and rural areas. The research involved 250 patients who were admitted to the hospital and initially tested negative for TB using the Xpert MTB/RIF assay. Out of these patients, 125 were put on empirical TB treatment based on a clinical algorithm, while the other 125 were not given any TB treatment. All 250 patients, who were adults aged 18 or above, had symptoms of pulmonary TB such as coughing, fever, night sweats, and weight loss. Samples of their sputum were collected before broad spectrum antibiotics were administered. The TB reference laboratory for Southwest Ethiopia, the Jimma University Mycobacteriology Research Center, evaluated the sputum samples for the presence of *M. tuberculosis* using the MGIT, LJ, and Xpert Ultra assay. In addition, the laboratory examined other bacterial components in the sputum of Xpert Ultra-negative patients by 16S rRNA gene amplicon sequencing.

8.3. Results

8.3.1. Antibiotic trial and chest X-ray may offer limited additional diagnostic value in the management of Xpert MTB/RIF or Xpert Ultra negative TB cases

Although the clinical algorithm was able to correctly identify most (79.5%) of the cases of TB confirmed by laboratory tests, its performance is not optimal when used as the initial diagnostic tool alongside Xpert MTB/RIF[6]. The algorithm produces a relatively low positive predictive value of 24.8%, which indicates a significant number of false-positive results. Additionally, the algorithm failed to detect 6.4% of individuals with active TB, leading to delayed or missed treatment initiation. About 75% of patients who received empirical TB treatment showed negative culture results, implying that they may have been over-treated. The inclusion of chest X-ray readings in the decision

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algorithm did not significantly enhance clinicians' ability to predict confirmed TB cases beyond what can be achieved through clinical judgment alone [7].

8.3.2. The Xpert Ultra assay demonstrated an increased yield compared to the Xpert MTB/RIF assay for detection of active TB

The Xpert Ultra assay is a more sensitive test (89%) compared to the Xpert MTB/RIF, which allows for more precise detection of Mycobacterium tuberculosis DNA in sputum samples. By using Xpert Ultra, healthcare professionals can enhance the accuracy diagnosing pulmonary TB, which means that individuals with TB can be identified more quickly and accurately, resulting in timely initiation of treatment. Early detection and treatment of TB are crucial in preventing the spread of the disease and reducing its impact on individuals and communities [8].

8.3.3. The LF-LAM assay, when combined with the Xpert Ultra assay, did not provide any additional benefits in hospitalized HIV-positive patients who were suspected of having TB

The LF-LAM test is especially useful in settings where access to advanced diagnostic tools may be limited. However, the test's sensitivity varies depending on the stage of the disease and the patient population being tested. In patients with advanced HIV disease, the sensitivity of the LF-LAM test is around 60%. Studies have shown that adding LF-LAM to the Xpert MTB/RIF assay can increase the overall detection yield of TB by 14% compared to using the Xpert MTB/RIF assay alone. However, our study found that adding LF-LAM to Xpert Ultra did not provide any additional benefits. Nevertheless, even in settings where Xpert Ultra is available, patients who are unable to produce sputum may still benefit from the use of the LF-LAM assay.

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8.3.4. The use of empiric TB treatment does not significantly affect the survival of bacteriologically unconfirmed TB cases

In our study, we investigated whether starting TB treatment without confirming the diagnosis would impact the survival of patients. We used a diagnostic test called Xpert MTB/RIF as the first step to diagnose TB. If patients tested negative for TB using this test but did not show improvement after taking antibiotics, they were then started on empiric treatment. Surprisingly, 85.2% of patients who were started on TB treatment based on clinical judgment had normal chest X-ray findings. Among patients who tested negative for TB using Xpert MTB/RIF and did not show clinical improvement after taking antibiotics, 96.9% were started on empiric TB treatment. However, our findings showed that starting treatment for TB without confirming the diagnosis did not lead to a decrease in mortality rates. Both the group of patients who received empiric TB treatment and the group who did not receive it had similar mortality rates of 5.6% and 4.8%, respectively. Additionally, we observed that starting empiric TB treatment did not affect mortality rates in patients who tested negative for TB using the Xpert Ultra test. In conclusion, our study suggests that the use of empiric TB treatment does not significantly affect the survival of bacteriologically unconfirmed TB cases.

8.3.5. The bacterial composition in sputum samples from Xpert Ultra negative patients

Identifying the bacterial pathogen responsible for lower respiratory tract infections (LRTIs) in individuals with presumed TB is essential. Accurate identification of the causative agent helps in providing targeted treatment and preventing unnecessary use of antibiotics. Additionally, it assists in developing effective diagnostic algorithms and therapeutic approaches tailored to specific pathogens, ultimately improving patient outcomes. In one-third of Xpert Ultra-negative patients, 16S rRNA sequencing identified one or more potential bacterial LRT pathogens, and detecting specific bacterial species was linked to significantly higher mortality, indicating clinical relevance.

8.3.6. Recommendation for policy implications

These findings indicate that countries with a high prevalence of TB and the human immunodeficiency virus (HIV) like Ethiopia should prioritize the adoption of the Xpert Ultra assay over the Xpert MTB/RIF test. Adopting the highly sensitive Xpert Ultra assay as the primary diagnostic tool for TB

can allow more accurate identification of individuals who require TB treatment, reducing the risk of delayed or missed diagnoses.

On the other hand, the routine use of LF-LAM in hospitalized HIV-positive patients with suspected TB should be avoided when the Xpert Ultra assay is available. The reason for this recommendation is that incorporating LF-LAM into standard diagnostic protocols where Xpert Ultra is already being used remains uncertain. Therefore, it is advisable to rely on the highly sensitive Xpert Ultra assay for accurate TB diagnosis in these cases. However, caution should be exercised even in settings with access to Xpert Ultra, patients who are unable to produce sputum may still benefit from the use of the LF-LAM assay.

Additionally, the routine practice of administering an antibiotic trial and empiric TB treatment in patients who test negative with the Xpert Ultra assay should be re-evaluated. This means that if a patient's Xpert Ultra test result is negative for TB, healthcare providers should reconsider automatically prescribing antibiotics and initiating TB treatment without further evaluation. This re-evaluation is necessary to ensure that patients are not unnecessarily exposed to antibiotics and potential side effects when they may not have active TB.

Understanding the cause of respiratory symptoms in TB patients with a negative Xpert Ultra sputum sample is crucial for developing evidence-based algorithms for optimal management. 16S rRNA sequencing helps identify specific bacteria causing LRTIs. The presence of certain bacterial species is associated with higher mortality rates in LRTI patients. Therefore, to improve clinical judgment skills, NTP should provide professional development training and keep healthcare providers updated on other bacterial pathogens causing LRTIs.

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Curriculum vitae

Wakjira Kebede Deyyas was born in West Oromia Region, Ethiopia in 1987. He received a Bachelor degree in Pharmacy from Jimma University in 2010, and a Master degree in Immunology from the University of Gondar in 2014, Ethiopia. Since then, he has been working as a lecturer at Jimma University. In November 2019, Wakjira started his PhD study at the Faculty of Medicine and Health Sciences of the University of Antwerp, Belgium, funded by the VLIR-UOS Network scholarship Program. His research focuses on operational research on TB/HIV, aimed at improving the management of hospitalized presumptive TB cases in Ethiopia. This research is specifically targeted towards those who present with symptoms of TB but test negative for Xpert MTB/RIF, which is a commonly used diagnostic test for TB. Throughout his PhD journey, Wakjira has actively participated in national and international conferences, where he presented his research work in person or through virtual platforms.