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Diagnostic Accuracy of Stool and Respiratory Sample-based Genexpert MTB/RIF assay for Diagnosis of Presumptive Tuberculosis among Children in Hospitals, Northwest, Ethiopia, 2024

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ABBREVIATIONS and ACRONYMS

APHI- Amhara public health institute

ATT- Anti-tuberculosis treatment

BAL- Bronchoalevelar lavage

CI- Confidence interval

CRS- composite reference standard

DMU- Debre Markos University

GA- Gastric aspirate

LJ- Lowenstein Jensen

MTB- *Mycobacterium tuberculosis*

MTB/RIF- *Mycobacterium tuberculosis*/rifampicin resistance

NALC-NaOH- N-acetyl-L-cysteine–sodium hydroxide

OADC- Oleic acid, albumin, dextrose and catalase

PANTA- polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin

PTB- Pulmonary tuberculosis

SOPs- standard operating procedures

SOS- Simple one step

SR-Sample reagent

TB- Tuberculosis

WHO- World health organization

Xpert assay- Gene Xpert MTB/RIF

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ABSTRACT

Background: Diagnosing pulmonary tuberculosis (pTB) in children is challenging due to the difficulties in acquiring respiratory specimens, which unspecific and paucibacillary disease presentation, and the lack of sensitive diagnostic assays with non-invasive sample collection methods. As a result, millions of children around the world get tuberculosis (TB) each year, which is a leading cause of morbidity and mortality.

Objective: The aim of this study was to assess the diagnostic accuracy of Stool and Respiratory Sample-based Genexpert MTB/RIF assay from presumptive TB among children in Northwest, Ethiopia.

Methods and Materials: Hospital based cross-sectional with diagnostic accuracy study was conducted on consecutively recruited presumptive TB children. Data were collected by sem-structured questionnaires. Single respiratory (5ml) and 3g stool specimen were collected Lowenstein Jensen (LJ) and Xpert assay. Laboratory SOPs were strictly followed to assure the quality of whole procedures. The diagnostic accuracy of stool Xpert was evaluated against respiratory specimen Xpert, culture and composite reference standards (CRS). Sensitivity, specificity, and predictive values for the stool Xpert assay were calculated with a 95% confidence interval (95% CI) with MedCal statistical software. Data were entered in EPIData V4.2 and exported to SPSS 25 for further analysis.

Results: A total of 557 children were recruited; 510 of whom had complete microbiological results. Overall, pTB was diagnosed in 52/510 (10.2%) of the children with presumptive TB. Of these, only four had microbiologically unconfirmed pTB, were clinically diagnosed with positive response to anti-TB and the remaining 48 were microbiologically confirmed (Positive Xeprt and LJ culture). Stool specimen Xpert had sensitivity of 93.8 %(95%CI: 82.8-98.6) and specificity of 99.8% (95%CI: 98.7–100) compared to culture; however, the sensitivity of stool was 88.5% (72-95.6) and specificity 100% (99.2-100) when compared to CRS. The Xpert on respiratory specimen had sensitivity and specificity of 95.8 % (85.8– 99.5) and 99.8% (98.7–100) to culture and 92.3 %(81.4-97.9) and 100% (99.2-100) compared to CRS.

Conclusion: The sensitivity and specificity of Xpert assay for stool specimen is almost similar to that of respiratory specimen. Stool specimen is a highly promising alternative specimen in the diagnosis of pTB in children when respiratory specimen is impossible.

Key words: Diagnostic accuracy, pulmonary tuberculosis, Xpert MTB/RIF, Stool, Children

1. INDUCTION

1.1 Background

Mycobacterium tuberculosis (MTB), a respiratory pathogen estimated to infect 25% of the world's population, is the cause of tuberculosis (TB), which is a transmissible disease that has killed more people throughout human history than any other microorganism [1]. TB is also the leading causes of illness and death worldwide [1]. TB remains a significant challenges in diagnosing and treating infections with childhood TB [2]. The main obstacles in diagnosing TB in children are the unspecific and paucibacillary nature of the illness presentation and the difficulty of obtaining respiratory samples [3]. Early and appropriate TB diagnosis in pediatrics is a key pillar of the World Health Organization's (WHO) End TB Strategy, where specific emphasis is placed on the discovery, development, and rapid uptake of new diagnostic tools and effective strategies for their implementation and scaling up of non-invasive sample collection method for MTB diagnosis [4].

Childhood TB is frequently diagnosed presumptively using nonspecific clinical, epidemiological, and radiological, tuberculin skin test, and other laboratory data. Child TB is often caused by a lower number of bacteria, and young children cannot freely provide respiratory samples (expectorated sputum, induced sputum, gastric aspirate, bronchoalveolar lavage (BAL) [5]. Obtaining respiratory samples for the diagnosis of TB is difficult, especially in younger children who cannot expectorate sputum. Gastric aspiration, an alternative method of acquiring a sample, is invasive and has a low yield, given the paucibacillary nature of TB in children [6]. In many resource-limited settings, including Ethiopia, a pediatric TB score chart is used to aid in the diagnosis of TB despite its widely varying sensitivity and specificity, especially in children [6], due to these reason some studies have documented stool as a possible specimen for detecting TB using GeneXpert MTB/RIF assay (Xpert assay) (Cepheid, Sunnyvale, CA, USA) among childhood pulmonary (pTB) [7] and indeed, stool has been explored as a potential sample option for diagnosing pediatric TB [3].

Young children are typically unable to generate a respiratory sample like sputum, gastric aspirate; BAL, for the diagnosis of pTB is frequently based on clinical examination. Sputum induction or gastric aspiration can be used to collect a sample for microbiological diagnosis, but these treatments are uncomfortable, stressful, and painful, and cannot be conducted at the lowest levels of the healthcare system, restricting children's access to pTB diagnosis. The difficult of respiratory sample collection and low bacillary load make microbiological confirmation of TB in children challenging [8] .

Stool-based tests for TB, in Xpert assay, offer several advantages in children, including ease of collection, non-invasiveness, and the ability to obtain multiple samples over time [9]. However, studies have reported a wide range of test sensitivities, indicating variability in their performance and WHO recommends for a study in large sample size and wider study area. Therefore, this study will assess diagnostic accuracy (sensitivity, specificity, positive and negative predictive value) of stool based Xpert assay for diagnosis of presumptive TB among Children in Amhara region health facilities, Northwest, Ethiopia.

1.2 **Statement of the problem**

In 2022, a projected 10.6 million individuals worldwide become infected with TB, with 1.3 million being children. Of these 1.3 million TB deaths, 208,000 (16%) were estimated to occur among children under 15 years old [10] and over 80% of these deaths were in young children (<5 years) [11] . Currently, 1.5 million youngsters acquire TB each year. They account for 1.1% of the total afflicted population in 2020, with just 36.5% notified to the appropriate authorities. According to the WHO Global TB Report, TB is one of the top ten causes of death among children [12]. Difficulties in clinical diagnosis of TB and microbiologic confirmation of TB in children are significant contributors to this disparity [13]. Once diagnosed, TB treatment outcomes are excellent with a mortality of <1%; hence, undiagnosed TB cases account the vast amount of pediatric TB deaths [11].

Children have an increased risk of TB progression and death, particularly in the absence of rapid diagnosis and missed or delayed diagnosis [14] and immunological immaturity [4, 15]. Roughly 96% of children dying from TB do not receive sufficient treatment, and among those under the age of five, 80% are not even diagnosed [4]. Nearly one- third of all child TB cases live in Africa where many have risk factors for a poor prognosis, such as HIV co-infection and severe malnutrition [16].

The 2018 United Nations General Assembly High Level Meeting on the fight against TB committed to diagnosing and treating 3.5 million children with TB by 2022; however, half of the incident TB cases in children are still not diagnosed nor reported annually [17].

Addressing the diagnostic challenge that pediatric TB poses is central and critical to progress in achieving targets for prevention, detection and treatment [14]. Due to diagnostic uncertainty of the sputum sample culture in children, the diagnosis of childhood TB mostly depends on clinical

assessment and radiological findings, and often the treatment is started based on clinical notion [18]. The collection of good quality sputum samples in children is tedious and low bacterial load in the sample makes isolation of the organism difficult [19].

It is known that good-quality sputum is 3.8 times more likely to isolate pathogenic bacteria than poor-quality sputum [18]. But it is difficult for children to expectorate sputum. Therefore early morning gastric aspirate along with induced sputum and BAL are considered better samples for childhood TB diagnosis [19]. Because of the invasiveness of these procedures, they cannot be performed in a primary health center that is an important and primary level of health care facility in Ethiopia [20]. In addition to the diagnosis of pulmonary TB in children, there is an essential need for the identification of multidrug resistant TB in children and high-risk groups to prevent the spread of drug resistant TB throughout the world [21].There is a need for rapid, reliable, accessible tests and the most easily obtainable type of sample for isolation and identification of MTB [19].

The stool sample is easy to obtain in children, can be collected at the primary health care level without the involvement of invasive procedures, and can be subjected to the culture and molecular diagnostic methods aiding in the diagnosis of pTB in children and their referral for appropriate treatment, thus reducing the morbidity and mortality due to TB [22]. In this study, the stool samples will be used for the identification of MTB, as mycobacterial DNA present in the sputum survives the passage through the gastrointestinal tract. The significance of Xpert assay in confirmation of TB is assessed by comparing the stool and induced sputum/gastric aspirate sample using Xpert assay, BACTEC *mycobacterial* growth indicator tube(MGIT 960) culture, and solid Lowstein Jensen(LJ) culture [15]. Nevertheless, few studies have reported the diagnostic accuracy of stool by using Xpert assay for childhood TB diagnosis with variable sensitivity and specificity. The diagnostic accuracy of stool Xpert requires further validation and adjustment utilizing a larger sample size. WHO recommends a diagnostic accuracy study of stool Xpert assay among children as compared with the respiratory samples to end TB strategy in 2035 [10].

The recently published by WHO indicates that the use of Xpert stool samples will be suggested for TB diagnosis in children in the new consolidated WHO guidelines [17]. Nonetheless, few studies have reported the validity of using Xpert on stool samples for childhood TB diagnosis. There is limited scientific literature comparing the accuracy of Xpert on stool samples in children. Therefore, our face-

to-face study was designed to analyze the diagnostic accuracy of Xpert assay on stool samples in children as compared to respiratory specimens in Hospitals northwest, Ethiopia, 2024.

1.3 **Objectives of the study**

1.3.1 General objective

- ✓ To assess the diagnostic accuracy of Stool and respiratory sample based Xpert assay for detection of presumptive TB among Children in Hospitals, Northwest, Ethiopia, 2024.

1.3.2 Specific Objective

- ✓ To determine the sensitivity of stool based Xpert assay for the diagnosis of TB among children as compared respiratory samples in Hospitals, Northwest, Ethiopia, 2024.
- ✓ To determine the specificity of stool based Xpert assay for the diagnosis of TB among children as compared respiratory samples in Hospitals, Northwest, Ethiopia, 2024.
- ✓ To determine the Positive and negative predictive value of stool based Xpert assay for the diagnosis of TB among children as compared respiratory samples in Hospitals, Northwest, Ethiopia, 2024.

1.4 **Significance of the study**

The finding of this study was indicate the stool samples have been shown to contain MTB from swallowed sputum and can be obtained easily, with non-invasive technique and relatively safe to diagnose MTB among children in every health care facility without invasive procedures and training with Xpert assay. Due to this reason the finding of this innovative diagnostic strategies was addressed a promptly, the universal access to TB diagnosis, prevention and care in children and helps to reach the global TB end strategy.

The paucibacillary nature of disease in children and this challenge in obtaining respiratory samples due to difficulty spontaneously expectorating sputum also create obstacle towards microbiological confirmation. Hence new diagnostic strategy, based on easy to collect non-invasive samples, was improved clinical decision making and TB outcome in TB high burden settings in children. It prevents misdiagnosis of TB in low bacillary load patients like children.

Due to this explanation the outcome of the research was given a benefit for health care professionals in the lower health care system, policy makers, clinicians, physicians, research institutions, planners, communities, and children’s as a whole.

1.5 **Operational definition**

Respiratory sample: In this study was including only expectorated sputum, BAL and gastric aspirate.

Presumptive TB: In this study refers to children who have suggestive for pTB.

Confirmed TB:–in this study verified TB occurs when a child has symptoms consistent with pTB and the disease was confirmed microbiologically (positive respiratory sample for Xpert and/or LJ culture).

Unconfirmed TB: - in this study occurs when a child exhibits at least two of the following symptoms indicative of TB, presumptive TB clinically, a chest radiograph compatible with TB, or documented exposure to MTB and a positive response to anti-tuberculosis treatment (ATT) but TB illness was not confirmed microbiologically.

Unlikely TB: - no criteria for "Unconfirmed TB" was met and TB is not confirmed microbiologically (negative respiratory sample Xpert and/or LJ culture).

Composite reference standard: - is defined as either confirmed TB or unconfirmed TB in the definition of TB and cases that met "unlikely TB" criteria were classified as not had TB.

1. LITERATURE REVIEW

A study conducted in Dhaka, Bangladesh from 447 children, 29 (6.5%) were bacteriologically confirmed on induced sputum and 72 (16.1%) were bacteriologically confirmed in induced sputum and stool and Xpert on stool had sensitivity and specificity of 37.9% and 100.0%, respectively and Indonesia the sensitivity were increased the diagnosis in stool samples by 19-25% in the same study participants [6, 19]. A systematic Review and meta-analysis conducted in Boston, United States of America (USA) a pooled sensitivity and specificity was 57% and 98%against culture and 3% among children with clinically-diagnosed, unconfirmed TB [23].

A diagnostic usefulness of stool Xpert assay was conducted in India to test the applicability of stool Xpert in a clinical set-up; Xpert testing was performed in a pilot run of 36 samples. Stool Xpert yielded positive results in 11/15 (73%) PTB (7 smear-positive and 4 smear-negative) and 2/12 (17%) EPTB cases, while specificity was 100%, this study indicated that the utility of stool Xpert as a viable non-invasive, alternative sample for the diagnosis of paucibacillary TB [24] . Another study in the same country reported, the evaluation of the Xpert assay on stool samples for the diagnosis of pTB among pediatric populations revealed that about 13.33% of the pulmonary samples and, of them, 50% of the stool samples were positive by Xpert assay. The sensitivity and specificity of Xpert assay with stool and pulmonary samples were 50 (95% CI: 18.71–81.29%) and 100% (95% CI: 94.48–100%), respectively [18].

A study conducted in China, among a total of 141 children with active TB and 34 with non-TB respiratory tract infections were enrolled. Xpert-stool (60.3%, 85/141) and Xpert-Gastric aspirate (GA) (52.5%, 74/141) tests were similarly sensitive ($p = 0.187$). Among the subset of 48 children with confirmed TB, Ultra testing was equally sensitive on stool and GA samples (85.4%, 41/48). The agreement between Xpert-stool and Xpert-GA was moderate in children with active TB (kappa value = 0.527). After integrating Xpert-GA and Xpert-stool outcomes, 70.9% of the children were considered to have confirmed TB. The specificities of Xpert-stool and Xpert-GA were 97.1% (33/34) and 100% (34/34), respectively ($p = 0.314$) [25]. Similar study, conducted in Dushanbe, Tajikistan in the North and West part of China a large cohort of children from the GeneXpert stool test was positive in 69% of proven cases of TB, and there were very few false-positive tests. We also showed that this diagnostic

strategy was feasible to implement in a low-middle-income country with an inefficient health care delivery system [26].

Systematic review and Meta-analysis conducted in Italy reported that Pooled sensitivity for Xpert MTB/RIF assay for detecting pulmonary TB was 68.2% (95%CI: 61.1–74.7%) even if characterized by a high heterogeneity (I^2 =53.7%). Specificity was almost 100% (99%, 95%CI: 97– 100%; I2 = 45.7%). When divided for reference standard, in the six studies using sputum and nasogastric aspirate the accuracy was optimal (area under the curve (AUC) = 0.99, SE = 0.02), whilst in the studies using only sputum for TB detection the AUC was 0.85 (with a SE = 0.16) [27].

A diagnostic accuracy study were conducted in West Africa (tertiary hospitals in Benin, Mali and Ghana), the pooled sensitivity and specificity of stool Xpert verified by culture were 55.0% and 95.0% respectively. Against a composite microbiological reference standard (cMRS), the diagnostic yield of Xpert and culture were 67.7% and 70.9% respectively [22]. Other study conducted in Tanzania, the stool Xpert assay showed a sensitivity of 62.5% and specificity of 100% against the reference standard [7]. Prospective diagnostic accuracy study conducted in the Regional Referral Hospital in Mbarara, Uganda reported that against the microbiological reference standard, the sensitivity and specificity of stool Xpert assay was 50.0% (6/12, 95% CI 21.1–78.9%) and 99.1% (198/200, 95% 96.4–99.9%) respectively [16].

A similar study conducted in Uganda with a prospective observational study of a cohort of children in the aged 1 month to 14 years with presumptive TB the sensitivity and specificity of stool sample was 55.6% (21.2–86.3) and 98.2% (98.2–100) against Xpert MTB/RIF and culture in sputum. Only a minority of children had microbiologically confirmed TB with a higher proportion in children above 10 years and Abbassaia Chest Hospital, Cairo, Egypt Xpert was positive in 83.3% of confirmed TB as well as 1.6 and 0.8% of probable TB cases by patients and samples respectively and Xpert had a sensitivity of 83.33 and 80.56 % and specificity of 98.73 and 99.36 % by patients and samples respectively [28, 29]. Hospitalized, HIV-infected children aged 12 years or less enrolled in Kenya among 165 HIV infected children the sensitivities and specificity of stool Xpert were 63% and 98%which indicated stool Xpert increased sensitivity among children with severe immunosuppression 80% [30].

An institution-based cross-sectional study was carried out among consecutively recruited children with presumptive Pulmonary TB at Jimma University Medical Center, Ethiopia and the reports indicated that stool Xpert had sensitivity of 100% and specificity of 99.3% compared to culture; however, the

sensitivity was decreased to 50% when compared to CRS. The Xpert on gastric aspirate had sensitivity of 77.8% compared to culture and 40% compared to composite reference standard [20].Although this study concluded that the diagnostic yield of stool Xpert still requires further validation and optimization using larger sample size. Another study reported that the pooled sensitivity of Xpert MTB/RIF on stool specimens compared with bacteriologically confirmed tuberculosis with respiratory specimens was 0.50 with an I^2 of 86%, which was statistically significant ($P < .001$). The pooled specificity was 0.99 (I^2 = 0.0%; P = .44).but further studies could evaluate optimization as a diagnostic tool [31].

Still, there is a variability of literatures and limited data in Ethiopia on diagnostic accuracy of Xpert assay on stool specimens to detect pTB in children. Thus, we aimed to assess the diagnostic accuracy (sensitivity, specificity, positive and negative predictive values of Xpert assay on stool specimens in detecting bacteriologically confirmed pTB among children in the study area.

2. METHODS and MATERIALS

2.1 Study settings and area

Ethiopia is a low income country in Africa with **128,513,977** inhabitants https://www.worldometers.info/world-population/ethiopia accessed in February 16, 2024). Even if there is suboptimal decreasement of TB in Ethiopia, but, still present in a high-TB-burden country with an estimated 212, 220 new TB patients and 29,874 TB-related deaths in 2019, and among all forms of TB, 4% were children compared to the estimated 11% globally [32]. This study was conducted in Amhara National regional state from Felege Hiwot comprehensive specialized Hospital, Bahir Dar; Debre Markos comprehensive specialized hospital (DMCSH), and Finote selam General Hospital, (FSGH), and Injibara General Hospital. These Hospitals was selected according to the GeneXpert availability and the flow of high TB load patients in the hospitals and these area are categorized TB hot-spot area in the region [33].

3.2 **Study Period**

The study was conducted from March, 2024 to March, 30/2025.

3.4 **Study Design**

An institutional based cross- sectional was conducted.

3.5 **Source population**

All children (age ≤15 years) visiting inpatient and outpatient services in the study hospitals were considered the source population.

3.6 **Study Population**

All children (age ≤15 years) presenting with presumptive TB.

3.7 **Eligibility Criteria**

3.7.1 **Inclusion**

All children with presumptive TB persistent cough of ≥ 2 weeks, persistent fever, weight loss, chest pain, history of TB contact, night sweating, loss of appetite, weight loss, decrease activity, haemoptysis, previous TB history and history of TB contact in the family were included in the study. TB screening was done per standard of care and clinical presentations.

3.7.2 **Exclusion criteria**

Children who were on ATT, for > 72 hours before enrollment, critically ill children who didn't give pulmonary and stool samples and whose guardians weren't willing to participate in the study were excluded.

3.8 Sample size calculation

The sample size was calculated using the Buderer's formula for sensitivity (**$z^2_{1-\alpha/2}$ sensitivity (1-sensitivity) /d2*prevalence**) and specificity **($z^2_{1-\alpha/2}$ specificity (1-specificity)/d^2(1-prevalence**) [34]. The study conducted by Dubale et al.in similar settings reported the sensitivity of 50% and specificity of 99.3% in the diagnostic accuracy of stool Xpert MTB/RIF and a prevalence of 13.2% was used based on a hospital at southern Ethiopia for novel diagnostics for children [20]. With an absolute precision of 12%, at a 95% level of confidence and the calculated sample size was 506. After adding 10% non-respondent rate the final sample size was **557**.

3.9 **Sampling technique**

All participants meeting the criteria were enrolled consecutively until the sample size was attained.

3.10 Study Variables

3.10.1 Depenedent variable

Diagnostic accuracy of stool Xpert (Yes or No)

3.10.2 Independent variables

Sensitivity

Specificity

Positive and negative predictive value and other variables were assessed, Human immunodeficiency virus (HIV), nutritional status (severe acute malnutrition ((SAM)), normal and moderate), and BCG vaccination status, sociodemographic factors (age, sex, residence, and clinical factors).

3.11 **Data collection**

On the day of recruitment, trained physicians and pediatric nurses were collected data from children at selected hospitals in northwest Ethiopia, using semi-structured questionnaires on sociodemographic and clinical variables. Data were collected through face-toface interviews with guardians/caregivers/parents that provide informed consent and assent for the study, as well as a requested to provide respiratory and stool samples for the evaluation of pTB. Children were considered regardless of their ability to generate sputum; induced sputum otherwise, gastric aspirate was performed on children's who were unable to produced sputum sample.

3.12 **Specimen collection, processing and transport**

In this study, the Laboratory professionals and trained nurses were collected the gastric aspirate and induced sputum/sputum and considered as the pulmonary sample, as children had difficulty to expectorate and tend to swallow the sputum. Single-induced sputum or gastric aspirate (a minimum of 5ml) for culture and Xpert assay were collected. One Stool sample (Approximately 3g) was collected from children who were satisfying the inclusion criteria by the nurses and pediatrician. The samples were collected early morning gastric aspirates using nasogastric tubes from children who were unable to produce sputum. All children were asked to provide one stool specimen for Xpert testing. Specimens were collected in wide-mouthed and screw-capped containers and stored at the health facility. The radiological investigation (chest X-ray) was done for all the recruited children. Also, the immunization status was recorded for all. Samples were transported into the laboratory and processed immediately under the biosafety guidelines [35]. In case of delay, samples were processed within 7 days of collection; in case the samples were not processed on the same day of collection, they were stored at 2–8°C. Laboratory personnel who preform laboratory analysis were blinded to the patients' clinical status.

3.13 **Laboratory Xpert assay**

The respiratory sample was subjected to Xpert MTB/RIF according to the manufacturer's instructions (Cepheid, Sunnyvale, California, USA) described in the package insert by trained laboratory professionals with standard operating procedures (SOPs). Xpert assay was performed at hospitals laboratory for both respiratory and stool samples as part of routine practice. While TB culture for respiratory samples were transported and processed in reference laboratory. Briefly, clinical samples

were added to sample reagents using a ratio of 2:1 with sample reagent to specimen. Approximately, 5ml of a respiratory sample (gastric aspirate or induced sputum/ sputum) were taken to which twice the volumes, that is, 10ml of sample reagent were added. The mixture were mixed, vortexed, incubated, and processed according to the manufacturer's instruction. The respiratory sample was decontaminated and concentrated using N-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH) method before further processing.

Stool samples were subjected to stool processing before decontaminating the sample with the NALC–NaOH method [36]. Briefly, one teaspoon (˜3 g) stool sample were mixed with 3 to 4ml of phosphate buffer saline and mixed thoroughly with vortexed until homogenized and filtered through a single layer of gauze. The filtrate was decontaminated with the NALC–NaOH method [32]. This mixture was then incubated for 15 min at room temperature to allow the particulate matter to settle. The supernatants from each processed stool sample was then collected in another container and mixed with sample reagent according to the manufacturer's instructions (2:1 ratio of Xpert reagent to sample). The mixture from each specimen was then vortexed and incubates for a further 15 min at room temperature; 2 ml were transferred to a GeneXpert cartridge and analysed using Xpert. Stool Xpert results were interpreted the same way as sputum Xpert results. In case of invalid results, the stool was reanalyzed using Xpert, and the final reports drawn up based on the obtained result.

3.14 **TB culture**

Culture is used as gold standard method for the diagnosis of TB [37] and used this method as reference standard for the study. Culture was performed using Lowenstein-Jensen (LJ) media slants in accordance with SOP in a contained Biosafety Level 3 laboratory at reference laboratory. Before culture, the samples were subjected to a harsh decontamination procedure (using 4% sodium hydroxide) that liquefied the organic debris and eliminated the unwanted normal flora. The respiratory samples were inoculated by using LJ solid culture and liquid culture, incubated. LJ, two drops of processed and suspended tablet was inoculated onto two LJ slopes and incubated at 35-37^{oC} for a maximum of 56 days. For MGIT inoculation, PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) were added to limit bacterial overgrowth with OADC (oleic acid, albumin, dextrose and catalase) supplement to enhance the growth of MTB. MGIT media was inoculated with 500 µl of suspended tablet and incubated as the same as LJ for a maximum of 42 days. Children were

classified into three categories based on their clinical, radiological, and laboratory results that were confirmed, unconfirmed and unlikely TB.

Microbiologically confirmed TB was defined as a positive result on a respiratory and stool specimen using Xpert and culture. Unconfirmed or a negative microbiological result was defined as a respiratory specimen that was negative for both using Xpert and culture. A culture was considered contaminated following observation of overgrowth of microorganisms that were lacking characteristics of mycobacteria.

3.15 **Data quality control**

Before data collection period, data collectors were trained about the objective of the research. The investigators were given the training about the aim of the study and data collection system and laboratory analysis by using semi-structured questionnaires in a half-day period. The questionnaires were prepared in English, translated into Amharic and back translated into English to check consistency. Pre-test was carried out in 5% of the sample size in Denebcha primary hospital to familiarize the interviewer with the instrument and to check the coherence of the questionnaires. To keep the quality of data, principal investigator was checked the laboratory standard operating procedures (SOPs) for sample collection, processing, storage, identification and the questionnaires for its completeness in each day in person and communicating phone.

3.16 **Ethical approval and consent to participate**

Ethical clearance to conduct this study was obtained from Debre Markos University, College of medicine and Health sciences ethical committee and conducted in accordance with the principles of the Declaration of Helsinki. Prior to the study, the medical director of the selected hospitals were provided written informed consent to conduct the study in the hospital. Written informed consent was provided by parents or guardians and participant assent from older children. Parents or guardians were clearly informed the study objectives and procedures prior to sample collection. Participation in this study was voluntary based. All information obtained in this study were kept confidential and used only in this study purpose. Positive results were immediately communicated to the attending physician for the study participants to receive appropriate treatment.

3.17 **Data analysis and Presentation**

Data were coded and entered in the Statistical Package of Epidata v4.2. The data were exported to SPSS V25 for further analysis. Baseline demographic characteristics were analysed using percentages for categorical variables, and medians and interquartile ranges (IQRs) for continuous variables. Sensitivity, specificity and predictive values for the stool Xpert were calculated using respiratory samples Xpert assay and culture as gold standard. 95% confidence intervals (95%CI) was determined using one of the following reference standards: i) LJ culture alone; ii) LJ culture and/or Xpert (proven tuberculosis); and iii) CRS (both confirmed and unconfirmed TB). To estimate the tests' sensitivity, specificity, and predictive values, MedCal statistical software was used (https://www.medcalc.org/calc/diagnostic_test.php). The 95% CI was used to identify the differences and similarities between the two procedures (stool Xpert and respiratory sample Xpert). Non-overlapping 95% CIs showed a difference between the two procedures (stool Xpert vs. respiratory sample Xpert vs LJ culture), and vice versa.

3.18 **Dissemination of results**

The result of this study will be disseminated to the clinicians, laboratory professionals, health workers, policy makers, research institutions, Amhara regional health bureau, Debere Markos University, college of Medicine and Health sciences Ethiopian public Health Institute (EPHI). Finally the results were published in international indexed journals to enrich the international communities in the world.

3. RESULTS

4.1 Socio demographic and Clinical Characteristics of the children

A total of 557 children with presumptive pTB were recruited to the study. Out of these, 47 were excluded in the final analysis: due to 29 were not given to stool specimens, 8 were diagnosed with extra pTB and 10 were unable to give expectorated sputum and refused to give gastric aspirate. We included the remaining 510 children's for whom we analyzed 86 expectorated sputum, 423 gastric aspirate, 1 BAL and 510 stool specimens were used to perform all the required tests (**Fig 1**).

Two hundred eighty four (55.7%) of 510 children were males and the majority (41.7%) of the age of the children were between 5 and 9 years old. Three hundred thirty (64.7%) of the children were rural dwellers (**Table 1**).

Table 1 . Demographic characteristics of children with their MTB diagnostic results.

Characteristics	Respi Xpert +ve n (%)	Respi Xpert–ve n (%)	Stool Xpert +ve n (%)	Stool Xpert -ve n (%)	Respi LJ +ve n (%)	Respi LJ +ve n (%)	p- value
Gender							
Male	21 (4.1)	263 (51.6)	22 (4.6)	253 (52.8)	22 (4.3)	262 (51.4)	0.111
Female	26 (5.1)	200 (39.2)	24 (5)	180 (37.6)	26 (5.1)	200 (39.1)	
Residence							
Urban	15 (2.9)	165 (32.4)	15 (3.1)	155 (32.4)	15 (2.9)	165 (32.4)	0.032
Rural	32 (6.3)	298 (58.4)	31 (6.5)	278 (54.5)	33 (6.5)	297(58.2)	
Age							
<5 years	21(4.1)	163 (32)	21(4.4)	155 (32.4)	20 (3.9)	164 (32.2)	0.183
5-9 years	14 (2.7)	199 (39)	13 (2.7)	194 (40.5)	15 (2.9)	198 (38.8)	
10-15 years	12 (2.3)	101(19.8)	12 (2.4)	84 (17.5)	13(2.5)	100 (19.6)	
Income							
< 1500 ETB	10 (2)	123 (24.1)	9 (1.9)	120 (25.1)	10 (2)	123 (24.1)	0.004
>=1500 ETB	38 (7.5)	339 (66.5)	37 (7.7)	313 (61.4)	38 (7.5)	339 (66.5)	

Respi=Respiratory sample, LJ= Lowenstein Jensen, MTB= *Mycobacterium tuberculosis*

Concerning the clinical manifestation of the children and their diagnostic accuracy of stool and respiratory Xpert the characteristics of the participant, 31 (6.1%) had HIV positive, had cough for >2 weeks, 379 (74%) had fever, 319 (62.5%) had night sweat 322 (63%) had loss of appetite, 302 (59.2%) had hemoptysis, 90 (17.6%) weight loss, 250 (49%) had decreased activity, 243 (47.6%) had previous TB history, 33 (6.5%) and 41 (8%) had TB contact history in the family. The majority of the children, 341 (66.9%), were vaccinated with BCG. Sixteen of the children (3%) were severely malnourished (**Table 2**).

Table 2: Clinical characteristics of the study participants with their diagnostic accuracy of MTB.

Variables	Respi Xpert +ve n (%)	Respi Xpert–ve n (%)	Stool Xpert +ve n (%)	Stool Xpert -ve n (%)	Respi LJ +ve n (%)	Respi LJ +ve n (%)	p- value
Cough							
< 2weeks	13 (2.5)	118 (23.1)	13 (2.7)	104 (21.7)	13 (2.5)	118 (23.1)	0.52
>= 2weeks	34 (6.7)	345 (67.6)	33 (6.9)	329 (68.7)	35 (6.9)	344 (67.5)	
Persistent fever							
< 2weeks	15 (2.9)	174 (34.1)	16 (3.3)	159 (33.2)	16 (3.1)	173 (33.9)	0.86
>= 2weeks	32 (6.3)	287 (56.3)	30 (6.3)	272 (56.7)	32 (6.3)	287 (56.3)	
Night sweat							
Yes	35 (6.9)	287 (56.3)	32 (6.7)	269 (56.2)	34 (6.7)	288 (56.5)	0.32
No	12 (2.4)	176 (34.5)	14 (2.9)	164 (34.2)	14 (2.7)	174 (34.1)	
Appetite loss							
Yes	28 (5.5)	274 (53.7)	29 (6.1)	255 (53.2)	28 (5.5)	274 (53.7)	0.58
No	19 (3.7)	189 (37.1)	17 (3.5)	178 (37.2)	20 (3.9)	188 (36.9)	
Hemoptysis							
Yes	13 (2.5)	77 (15.1)	15 (3.1)	66 (13.8)	15 (2.9)	75 (14.7)	0.00
No	34 (6.7)	386 (75.7)	31(6.5)	367 (76.6)	33 (6.5)	387 (75.9)	
Weight loss							
Yes	31 (6.1)	219 (42.9)	32 (6.7)	204 (42.6)	32 (6.3)	218 (42.7)	0.004
No	16 (3.1)	244 (47.9)	14 (2.9)	229 (47.8)	16 (3.1)	244 (47.8)	
Decrease activity	35 (6.9)	208 (40.8)	35((7.3)	198 (41.3)	37 (7.2)	206 (40.4)	0.000
Yes	12 (2.4)	255 (50)	11 (2.3)	235 (49.1)	11 (2.4)	256 ((50)	
No							
HIV							
Yes	0 (0)	31 (6.1)	0 (0)	27 (5.6)	0 (0)	31 (6.1)	0.68
No	39 (7.6)	364 (71,4)	38 (7.9)	342 (71.4)	40 (7.8)	383(75.1)	
Unknown	8 (1.6)	68 (13.3)	8 (1.7)	64 (13.4)	8 (1.6)	68 (13.3)	

BCG							
Yes	29 (5.7)	312 (61.2)	27 (5.6)	292 (62)	30 (5.9)	311 (61)	
No	14 (2.7)	126 (24.7)	15 (3.1)	120 (25.1)	14 (2.7)	126 (24.7)	0.024
Unknown	4 (0.8)	25 (4.9)	4 (0.8)	21 (4.4)	4 (0.8)	25 (4.9)	
Comorbidity							
Yes	7(1.4)	57 (11.2)	5 (1)	54 (11.3)	7 (1.4)	57 (11.2)	0.75
No	40 (7.8)	406 (79.6)	41 (8.6)	379 (79.1)	41 (8)	405 (79.4)	
Previous TB							
Yes	3 (0.6)	30 (5.9)	3 (0.6)	29 (6.1)	3 (0.6)	30 (5.9)	0.68
No	45 (8.8)	425 (83.3)	43 (9)	397 (82.9)	45 (8.8)	425 (83.3)	
TB contact							
Yes	8 (1.6)	33 (6.5)	8 (1.8)	33(6.9)	8 (1.6)	33 (6.5)	0.024
No	39 (7.6)	430 (84.3)	38 (7.9)	400 (83.5)	40 (7.8)	429 (84.1)	
X-ray investigation							
Suggestive	12 (2.4)	14 (2.7)	11(2.3)	15(3.1)	12 (2.4)	14 (2.7)	0.000
Non suggestive	15 (2.9)	397 (77.8)	16 (3.3)	369 (77)	17 (3.3)	395 (77.4)	
Not done	20 (3.9)	52 (10.2)	19 (4)	49 (10.2)	19 (3.7)	53 (10.4)	

4.2 Diagnostic characteristics of TB in the children

Finally, specimens from the remaining 510 children were used to perform all the required tests in this study. *Mycobacterium Tuberculosis* was detected in 48/510 (9.4%) by solid LJ culture on respiratory specimens and 47/510 (9.2%) on Xpert assay were confirmed. In one of the children's gastric aspirate LJ culture was positive while the Xpert MTB/RIF assay from the same specimen was negative. However, in one of the children, Xpert assay from the gastric aspirates was negative while LJ culture results were positive. All the 86 expectorated sputum specimens were negative by both Xpert assay and LJ culture. Stool Xpert testing revealed that 46/479 (9.6%) MTB positive cases were detected. Out of the stool specimen examined by diagnostic methods, 2 were LJ culture positive on respiratory samples and one Xpert assay positive whereas stool Xpert negative. The results of respiratory sample Xpert assay and stool Xpert assay as compared to respiratory sample LJ culture amongst study children were explained (**Table 3**). We actively interviewed children and reviewed the medical records of the remaining 462/510 (90.6%) of children who were microbiologically negative. Of these, 4/462 (0.9%) were clinically diagnosed as TB by the clinicians. Among the 4 clinically diagnosed TB cases, 4/4 (100%) showed clinical improvement after ATT. Out of the 4 cases who have improved clinically, all

of them had radiological findings suggestive for pTB and hereafter were classified as “unconfirmed pTB’’. Nonetheless, nobody of the children with clinically diagnosed TB (unconfirmed TB) had positive stool Xpert result in the study. TB Confirmed positive results means that one of the diagnostic techniques either Xpert assay or LJ culture positive, whereas the remaining children 458/462 (99.1%) of the cases, TB was ruled out negative and an alternative diagnosis was made, and treatment were given according to the case identified and later, they were classified as “unlikely TB” (Fig 1).

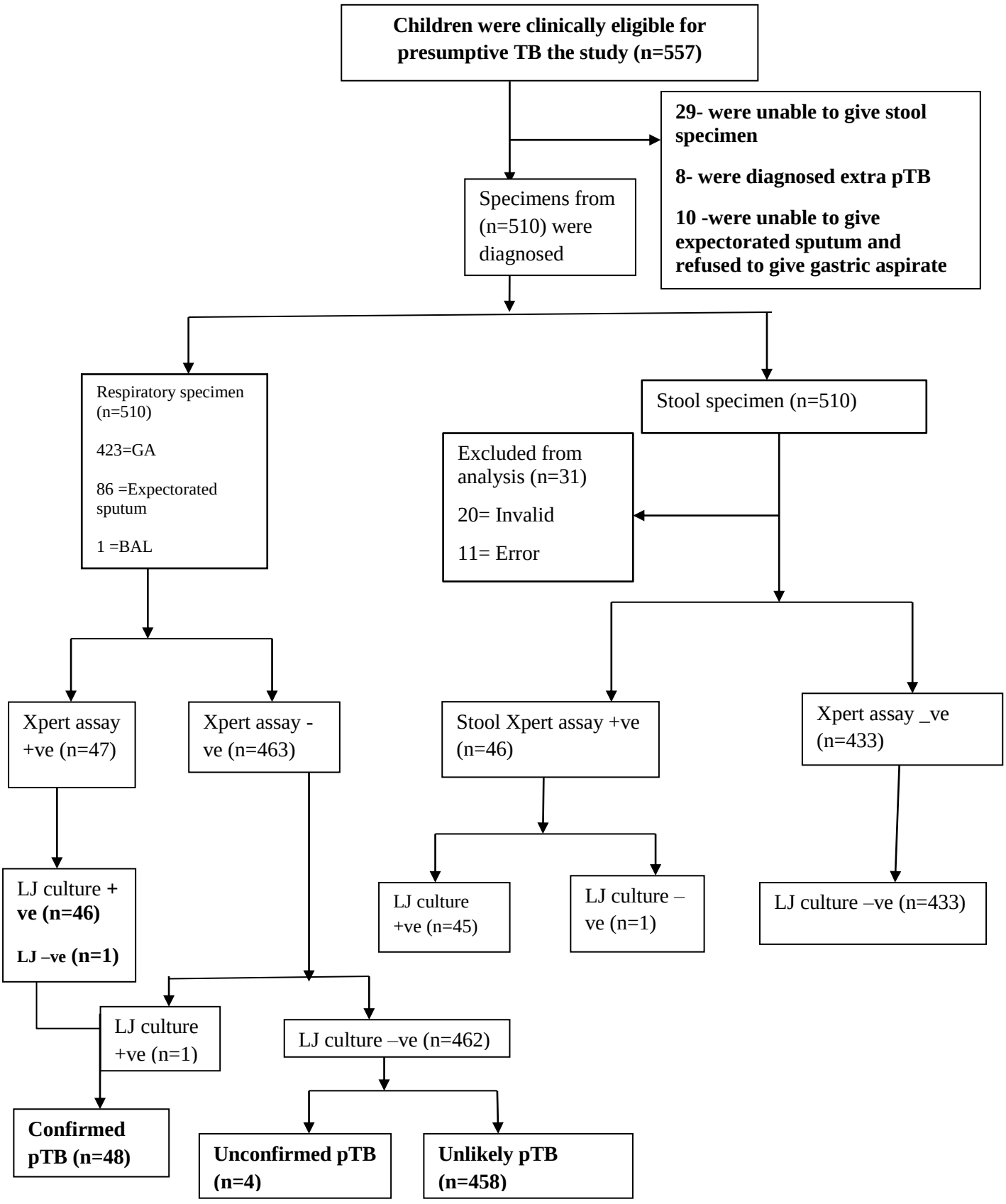
Children were clinically eligible for presumptive TB the study (n=557)
29- were unable to give stool specimen
8- were diagnosed extra pTB
10 -were unable to give expectorated sputum and refused to give gastric aspirate
Specimens from (n=510) were diagnosed
Respiratory specimen (n=510)
423=GA
86 =Expectorated sputum
1 =BAL
Stool specimen (n=510)
Excluded from analysis (n=31)
20= Invalid
11= Error
Xpert assay +ve (n=47)
Xpert assay -ve (n=463)
Stool Xpert assay +ve (n=46)
Xpert assay _ve (n=433)
LJ culture + ve (n=46)
LJ –ve (n=1)
LJ culture +ve (n=45)
LJ culture –ve (n=1)
LJ culture –ve (n=433)
LJ culture +ve (n=1)
LJ culture –ve (n=462)
Confirmed pTB (n=48)
Unconfirmed pTB (n=4)
Unlikely pTB (n=458)

Figure 1. **Diagnostic flow diagram reporting of the children characteristics in the study.**

Error/invalid results were documented in 31/510 (6.1%)) of Xpert assay performed on stool. Due to different factors the tests didn't repeat in these invalid and error cases in stool specimen (a shortage of Xpert cartridges and reagents). Of the 479 stool specimens with valid Xpert results, 46/479 (9.6%, 95 %CI; 7.5-13.4) were MTB positive. Stool Xpert was detected 45 respiratory LJ culture confirmed TB cases and three positive cases missed by stool Xpert assay. Furthermore, respiratory Xpert assay also detected one MTB positive cases which was found to be negative on stool Xpert assay (**Table 3**).

Table 3. Prevalence of positive Respi Xpert and stool Xpert associated to Respi culture for MTB

Respi sample Gene Xpert	**Respi LJ culture**			**Kappa value**
	MTB Positive n (%)	**MTB Negative n (%)**	Total n (%)	
MTB Positive n (%)	46 (9)	1 (0.2)	47 (9.2)	0.96
MTB Negative n (%)	2 (0.4)	461 (90.4)	463 (90.8)	
Total	48 (9.4)	462(90.6)	510(100)	
Stool Sample GeneXpert	**Respi LJ culture**			Kappa value
	MTB Positive n (%)	**MTB Negative n (%)**	Total n (%)	
MTB Positive n (%)	45(9.4)	1(0.2)	46(9.6)	0.95
MTB Negative n (%)	3(0.6)	430(89.8)	433(90.4)	
Total n (%)	48(10)	431(89.8)	479(100)	

Respi=Respiratory Sample, MTB= *Mycobacterium tuberculosis*, LJ= Lowenstein-Jensen

4.3 Diagnostic accuracy of PTB using respiratory and stool specimens

Of the 48 children with bacteriologically confirmed pTB, Xpert on respiratory specimen had a diagnostic positive of 47/510 (9.2%, 95% CI; 7.02-12.28), whereas Xpert on stool identified pTB in 46 (9.6%) children. Compared to Xpert culture on respiratory specimen demonstrated a higher diagnostic outcome by detecting 48 children among 510 presumptive TB cases. Of the 479 children in the total presumptive TB suspected Xpert assay on stool specimens identified pTB in 46 which was comparative with the other diagnostic procedures.

Using culture of the respiratory specimen (gastric aspirate and expectorated samples) as the reference standard, stool Xpert had sensitivity of 93.8% (95% CI: 82.8–98.6) and specificity of 99.8% (95% CI:

98.7–100), with positive predicative value (PPV) and negative predictive value (NPV) 97.8 % (95%CI;86.4-99.7) and 99.3% (95% CI; 98-99.8) respectively, whereas respiratory sample Xpert assay had sensitivity of 95.8% (95% CI: 85.8– 99.5) and specificity of 99.8% (95% CI: 98.7–100) with PPV and NPV of 97.9 %(95% CI;86.6-99.7 and 99.6% (95% CI;98.3-99.9) respectively (**Table 4**).

Table 4 . Diagnostic accuracy of Respiratory sample and stool Xpert assay using LJ culture as a reference standard in northwest, Ethiopia 2025.

Diagnostic test	Diagnostic accuracy (Culture as reference standard)			
	Sensitivity % (95%CI)	Specificity% (95% CI)	PPV % (95% CI)	NPV % (95%CI)
Respi Xpert assay	95.8%(95% CI:85.8– 99.5)	99.8% (95% CI: 98.7–100)	97.9% (95% CI;86.6-99.7	99.6% (95% CI;98.3-99.9)
Stool Xpert assay	93.8%(95% CI: 82.8–98.6	99.7% (95% CI: 98.7–100)	97.8% (95%CI;86.4-99.7)	99.3% (95% CI; 98-99.8)

Respi= Respiratory Sample, CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value

The Xpert MTB/RIF assay is primarily used for detecting MTB in respiratory samples, but research is ongoing regarding its use in stool samples for the diagnosis of pTB, particularly for certain populations like children or immunocompromised individuals and its association with respiratory Xpert assay and stool tests is still under investigation for the two procedures .In this study using Xpert assay of respiratory sample (bacteriological confirmation) as one of the reference standards explained in the supplementary (Table 5) below as compare to stool.

Table 5. Prevalence of positive stool Xpert associated to Respi Xpert assay for MTB

Stool Sample GeneXpert	Respi Xpert assay (reference standard)			P value
	MTB Positive n (%)	MTB Negative n (%)	Total n (%)	
MTB positive n (%)	44(9.2)	2(0.4)	46(9.6)	0.000
MTB negative n (%)	3 (0.6)	430 (89.8)	433(90.4)	
Total n (%)	47 (9.8)	432(90.2)	479 (100)	

Respi=Respiratory Sample, MTB= *Mycobacterium tuberculosis*, LJ= Lowenstein-Jensen, n=number

Stool Xpert had 93.6% sensitivity, 99.5% specificity, PPV 95.7% and NPV 99.3% respectively (**Table 6**). This indicated that, stool Xpert assay demonstrates excellent diagnostic performance, characterized by high sensitivity and specificity, along with strong predictive values. These attributes underscore its utility as a reliable diagnostic tool for detecting MTB, complementing with bacteriological confirmation provided by respiratory samples.

Table 6. The diagnostic accuracy of stool and respiratory sample Xpert assay using respiratory sample Xpert assay (bacteriological confirmations) as one of the reference standards in northwest, Ethiopia, 2025

Diagnostic Test	Diagnostic accuracy (respiratory Xpert assay as reference standard)			
	Sensitivity %(95%CI)	Specificity %(95% CI)	PPV% (95% CI)	NPV %(95%CI)
Stool Xpert assay	93.6%(95% CI; 82.5-98.7)	99.5%(95% CI; 98.3-100)	95.7%(95% CI;84.4-98.9)	99.3%(95% CI; 98-99.8)

CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value

Besides, the sensitivity, specificity, PPV, and NPV of stool and respiratory sample Xpert assay were also calculated using a reference standard to CRS which were patient classification considered as (confirmed, unconfirmed and unlikely). In this study, using stool samples for PTB diagnosis in children offers a promising alternative sample to conventional diagnostic methods, particularly in children which have challenging for expectorating respiratory samples. While stool tests were complement existing diagnostic specimen, they ideally used in conjunction with CRS for optimal accuracy. Our study indicated that, stool specimen is essential to enhance the diagnostic capabilities childhood TB and reliability of stool testing for PTB in pediatric populations. Stool and respiratory sample Xpert assay for MTB detection rate as compared to CRS is depicted in supporting information (**Table 7**).

Table 7. Stool Xpert, respi Xpert and respi culture MTB detection rate compared to composite reference standard (confirmed and unconfirmed TB).

Respi culture	**Patient classification as CRS**				P value
	Confirmed pTB n (%)	Unconfirmed PTB n (%)	Unlikely pTB	Total n (%)	
MTB growth observed n(%)	48(9.4)	0	0	48 (9.4)	0.000
MTB growth not observed n (%)	0	4 (0.8)	456 (89.4)	460 (90.2)	

Total n (%)	48 (9.4)	4 (0.8)	456 (89.4)	510 (100)	
Respi Xpert assay					
MTB Positive n (%)	47 (9.2)	0	0	47 (9.2)	
MTB Negative n (%)	1 (0.2)	4 (0.8)	458 (89.8)	463 (90.8)	0.000
Total n (%)	48 (9.4)	4 (0.8)	458 (89.8)	510 (100)	
Stool Xpert assay					
MTB positive n (%)	46 (9.6)	0	0	46 (9.6)	0.000
MTB Negative n (%)	2 (0.4)	4 (0.8)	427 (89.1)	433 (90.4)	
Total n (%)	48 (10)	4 (0.8)	427 (89.1)	479 (100)	

Respi=Respiratory Sample, MTB= *Mycobacterium tuberculosis*, *n=number*

Accordingly, stool Xpert had a sensitivity of 88.5 %(72-95.6) specificity of 100 %(99.1-100), PPV of 100% (95% CI; 92-100) and NPV of 98.6% (95% CI; 97.5–99.5) against CRS. The corresponding sensitivity, specificity, PPV and NPV for respiratory sample Xpert assay was 90% (95% CI; 78.9–96.8), 100% (95% CI; 99.2–100), 100 % (92.3-100) and 98.6% (95% CI; 97.2–99.5) respectively compared to CRS (**Table 8**).

Table 8. **The diagnostic accuracy of respiratory Xpert, stool Xpert assay and respiratory LJ culture compared to patient classification as CRS in northwest, Ethiopia 2025**

Diagnostic Test	Diagnostic accuracy (Patient classification as standard)			
	Sensitivity %(95%CI)	Specificity %(95% CI)	PPV %(95% CI)	NPV% (95% CI)
Respi GeneXpert	90% (78.9-96.8)	100% (99.2-100)	100%(92-100)	98.6%(97.5-99.5)
Stool Xpert	88.5%(72-95.6)	100%(99.1-100)	100%(92.3-100)	98.6%(99-100)
Respi culture	92.3%(81.4-97.9)	100%(99.2-100)	100%(92.6-100)	99.1%(97.8-99.6)

Respi= Respiratory Sample, CI = confidence interval, PPV = positive predictive value, NPV = negative predictive value, CRS = composite reference standard

4. Discussion

Diagnosing of PTB in children who cannot expectorate sputum is problematic with TB high demographic risk of exposure, Ethiopia is among the country with a high TB and TB/HIV problem among 30 high TB burden countries and many undiagnosed cases are available in this countries which are difficult to end TB control strategy milestones in 2035 even if rapid microbiological detection methods exist [6, 36]. National guidelines recommend Xpert assay for childhood TB diagnosis, but obtaining respiratory samples is challenging in children [38]. Therefore, a non-invasive, non-sputum-based alternative specimen is essential for improving care and diagnosis in this population and recently, stool specimens have been suggested as a viable initial sample for diagnosing PTB using Xpert assay [39]. The effectiveness of stool examination for diagnosing childhood PTB has been assessed in a few studies but, not recognized in conventional testing methods and molecular diagnostics techniques [39]. In the current study, we have shown that stool Xpert assay performs a consistent result to respiratory Xpert assay and culture in children with presumptive PTB. Other studies have also reported stool specimen had similar promising results [20, 24, 36].

We found that stool Xpert assay had sensitivity and specificity of 93.8% and 99.7% respectively for MTB detection in among children with microbiologically confirmed from respiratory specimens in LJ culture and 88.5% for those with microbiologically and/or clinically diagnosed TB as compared to CRS. Studies in Ethiopia [20], Egypt [28], Kenya [40], and Pakistan [41] reported similar high sensitivities (83.3% to 100%) for stool Xpert compared to respiratory culture. The sensitivity of unconfirmed TB cases of clinically diagnosed TB was negative; this may be due to the presence of paucibacillary nature of disease in this population.

The specificity of stool Xpert in our study aligned with previous research in Ethiopia, South Africa, and Egypt, which reported specificities between 99.3% and 99.7% [20, 28, 36, 42]. Additionally, the specificity when compared to CRS was consistent with a study from Pakistan [41]. Notably, one culture-positive respiratory specimen and two for stool specimens tested negative for both respiratory and stool Xpert assay, this is possibly due to the presence of viable bacilli and the high capacity of detecting MTB in culture since LJ culture is the gold standard for the diagnosis of MTB.

In this study, only one stool Xpert result was negative when compared to all respiratory Xpert positive cases, indicating that stool could serve as a viable potential alternative specimen as sputum for routine PTB diagnosis in every health facilities. Whereas gastric aspiration, induced sputum, and BAL can be

employed to gather pulmonary specimens from patients who cannot expectorate sputum, these methods are invasive, painful and necessitate trained healthcare professionals to obtain specimens. In contrast, stool is a noninvasive sample that is safe, easy to collect, and shows potential for detecting MTB and easily performed in every health facilities. Additionally, the stool Xpert test uses a streamlined protocol adapted from gravity sedimentation technique and KNCV Footing bases, give valid Xpert results of stool with a simple protocol [43, 44], which eliminates labor-intensive steps like homogenization, sanitization, and centrifugation of the stool specimen that are typically required in respiratory specimens [45, 46]. This approach significantly reduces sample processing time, lessens the workload for laboratory personnel, and lowers costs for the patients.

In the current study, regrettably, both stool and respiratory Xpert did not detect any of the four clinically diagnosed PTB cases. Additional consistent studies have also reported similar poor diagnostic performance of stool Xpert in children with clinically diagnosed (microbiologically negative) TB [6, 20]. This may highlight a limitation of both stool and respiratory Xpert, as patients with clinically diagnosed but unconfirmed TB is more prone to having paucibacillary disease nature, specimen collection, laboratory diagnostic procedures, long preservation and transportation time and a very low mycobacterial load. Children with a strong clinical suspicion of TB, even when stool and respiratory Xpert results are negative, should begin ATT with clinical decisions until more effective and sensitive diagnostic tools become available.

In this study, older children are able to expectorate sputum and produce more adult-type sputum. Consequently, from this sputum 4.7% of the expectorated sputum samples were positive for *M. tuberculosis* during the study which contradict from other studies conducted in Ethiopia [20]. This is different from other studies conducted in the same population group, because during the study period was trying to give advance counseling to children and their parents to expectorate quality sputum samples and we did also asses the quality of the sputum sample in the current study.

In comparison to other specimen types, stool specimens have not been thoroughly evaluated for their effectiveness and optimization in conventional testing and molecular diagnostics techniques. As a result, the value of stool specimens in diagnosing pTB is not widely acknowledged in the lower health care systems. In this study, we assessed the performance of stool-based Xpert for diagnosing TB in children within high TB burden settings in Ethiopia. However, the sensitivity (93.6%) and specificity (99.5%) of stool Xpert assay somewhat similar result as compared to that of respiratory sample Xpert

sensitivity (95.8%), and specificity (99.8%) as result this finding was given assurance and stool-based testing continues to provide considerable benefits and promising specimen, especially in cases where obtaining sputum or respiratory tract samples is challenging or impractical to diagnose TB in children. This finding was similar to other studies reported [20, 31, 40]. Hence, the similar diagnostic usefulness implies that both stool and respiratory specimen Xpert assays could be trusted for clinical decision-making, allowing healthcare providers to accurately diagnose and treat MTB based on the results from stool specimens. The current study has some limitations. We frequently faced challenges related to the storage of specimens due to a shortage of Xpert cartridges for long time. Additionally, a limitation of this study was that we collected only a single respiratory and stool specimen for testing, rather than successive specimens. Collecting multiple samples could have potentially increased the yield MTB and accuracy of the tests conducted.

5. **Conclusions**

This study highlights that, the comparable sensitivity and specificity of stool and respiratory specimen by Xpert assays were observed, combined with the practical advantages of stool collection, points out stool specimen is a promising potential alternative specimen for diagnosing pTB, especially in children who face challenges in providing conventional respiratory specimens. This approach could enhance early detection of MTB. The collection of stool samples presents several advantages. Firstly, it is a simpler and more straightforward process compared to obtaining respiratory specimens, which can be invasive and uncomfortable for children. This ease of collection not only enhances patient compliance but also reduces the risk of complications associated with more invasive procedures. In addition this, the study also give lessons, stool collection is relatively safe and can be performed in a variety of settings, including primary health care facilities. This accessibility makes it an attractive option for implementation at the lowest levels of the healthcare system, where resources may be limited. By utilizing stool samples for PTB diagnosis, healthcare providers can improve the overall diagnostic capacity and avoids misdiagnosis of PTB, particularly in underserved areas where access to more complex testing methods may be restricted. In this study, we recommend that the Ministry of Health of Ethiopia consider stool as a promising specimen for the detection of TB in children, distribute guideline give trainings for the lower health care systems stool is a potential specimen when obtaining respiratory specimens are impractical.

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7. APPENDIXES

7.1 Stool sample collection, Transport and storage

Stool collection is frequently done by caretakers or patients themselves, depending on the child's age. Ideally, the collecting occurs at the health care facility. Nonetheless, obtaining a specimen on demand is typically problematic; thus, stool is collected at home, and the patient or caregiver must return to the hospital for specimen submission.

7.2 Instructions for the patient or caregiver on how to collect the stool sample

1. Ideally, collect the stool sample during the first daily bowel movement. If possible, first empty the bladder, to avoid mixing urine with the stool sample.

2. Put some clean plastic sheeting on the spot where the stool will be dropped, to ensure the collection of a clean sample. Avoid contamination of the plastic with soil, detergent or disinfectant from the toilet.

3. If the stool sample needs to be collected from a child that uses a diaper (i.e. a nappy), then either collect the stool directly from the diaper as soon as possible after defecation, or put a plastic sheet in the diaper to avoid (prolonged) contact between the stool and the surface of the diaper (diapers may contain substances that inhibit the test).

4. Fill the stool container with the stool sample up to half full, using the spoon provided with some types of containers, a clean plastic bag, a clean piece of cardboard or a clean spoon. Do not fill the container to the brim. Only a small amount of stool is required for testing (3 g is sufficient for both testing and retesting if the first test is unsuccessful).

5. Close the container tightly, place the container in the plastic bag provided (preferably a self-sealable bag) and close the bag. Leave the absorbent material in the plastic bag so that this material can absorb any substances that may leak out of the container.

6. As soon as the stool sample has been collected, store the plastic bag containing the stool container in a clean, cool place (e.g. in a fridge if possible), avoiding exposure to direct sunlight. Do not freeze the sample.

7. Take the plastic bag containing the stool container to the health care facility, preferably on the same day that you collected the stool sample

For transport and storage of stool specimens, the same conditions apply as for transport and storage of sputum specimens for Xpert testing. Thus, between collection and testing, stool specimens can be kept at a maximum of 35 °C for up to 3 days, followed by a maximum of 7 days at 2–8 °C. Ideally, stool sample containers should be kept at 2–8 °C while being sent to the laboratory and should then be stored in the refrigerator (at 2–8 °C) until testing can be performed and for a long delay will be stored in -20°C.

7.3 **Stool processing for Xpert assay by simple –one step method**

The simple one step (SOS) stool processing method uses one step to release *M. tuberculosis* from stool. Particulate matter is sediment by gravity, and it is assumed that this allows TB bacilli to float to the top of the watery solution because of their lipid-containing cell wall and will be applied in this research.

7.4 **Procedure**

Before stool processing, the Bristol stool scale is used to determine the consistency of the stool specimen. For stool with the appearance of Bristol type 1 to 5 (formed stool), 0.8 g or a thumbnail-sized amount of stool (Fig. 1) is directly transferred from the stool container into the SR bottle using a wooden stick or applicator. For feces with the appearance of Bristol types 6 and 7 (liquid stool), remove 2 mL of SR from the SR bottle and transfer 2 mL of stool to the SR bottle with a balloon pipette. For all types of stools, the SR is vigorously shaken for 30 seconds before incubating for 10 minutes at room temperature. This step is repeated once.

After confirming that all solid particles and debris have settled, transfer 2 mL of supernatant from the SR bottle to the Xpert MTB/RIF or Xpert Ultra cartridge. The cartridge is then placed in the GeneXpert device. The GeneXpert instrument is used as directed by the manufacturer, and the Xpert results are interpreted accordingly. A full SOP for doing the SOS stool processing procedure may be found in the KNCV stool toolbox on their website. The GeneXpert instrument is used as directed by the manufacturer, and the Xpert results are interpreted accordingly. The KNCV stool toolbox on the website has a thorough SOP for doing the SOS stool processing approach.

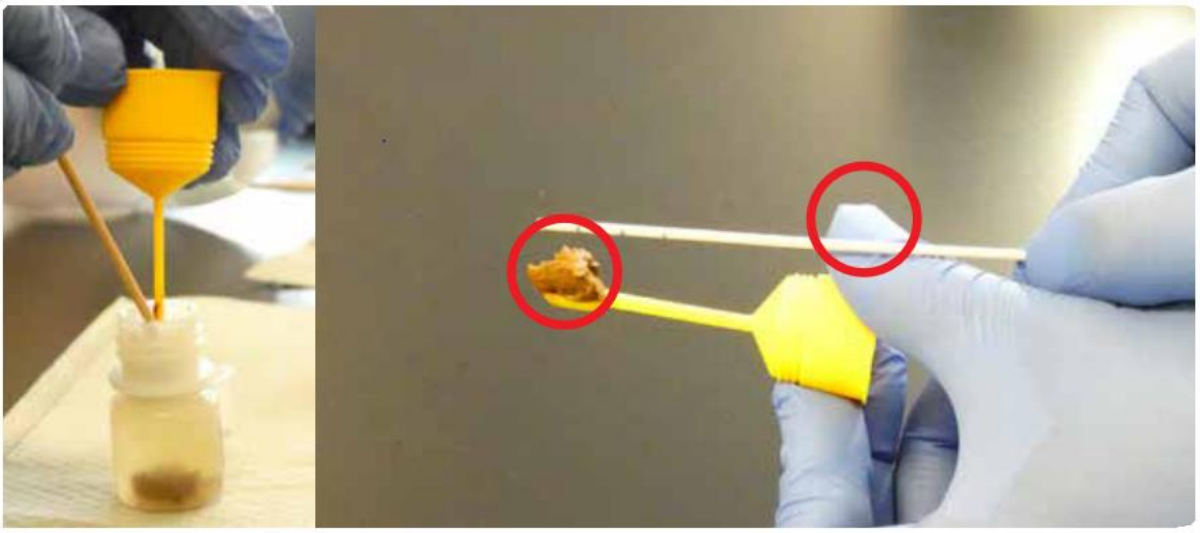

Fig 1: In the Simple- one step stool processing method, 0.8 g or an amount of stool equal to the size of a

thumbnail is used.

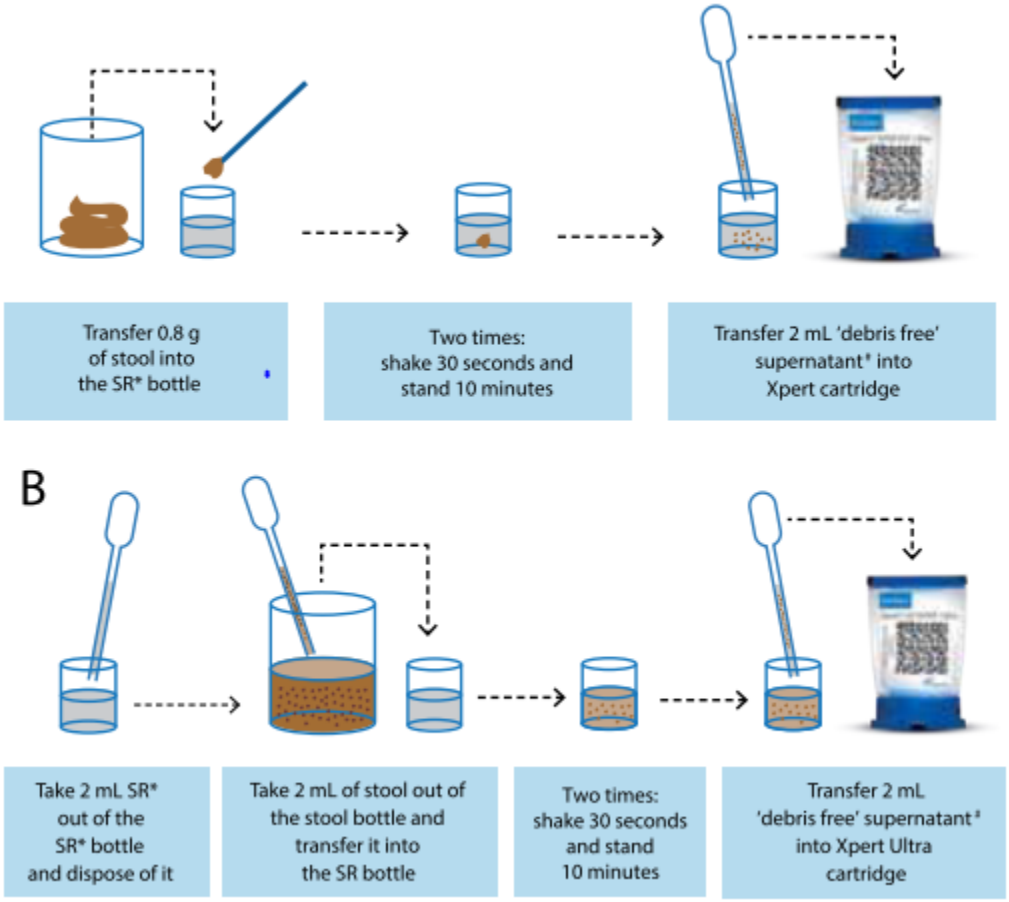

Figure 2. Schematic overview of the SOP of the SOS stool processing method and the Xpert MTB/RIF or Xpert Ultra assay for different types of stools

7.5: **Sputum/Gastric Aspirate Specimen Collection, Transport and Storage for Xeprt MTB/RIF assay**

Collect raw sputum or sputum sediment samples following institution's standard procedures. The minimum volume of the respiratory sample for Respiratory samples for culture and Xpert assay respectively.

Sputum sediment: Store suspended sediments at 2–8 °C for up to seven days. Raw sputum: Transport and store specimens at 2–8 °C before processing whenever possible. If necessary, sputum specimens can be stored at a maximum of 35 °C for up to three days and then at 2–8 °C for an additional seven days.

7.6 **Assay Procedures**

The MTB/RIF Assay requires at least 0.5 mL of resuspended sputum sediment after digestion, decontamination and concentration.

1. Wear protective disposable gloves.
2. Label each Xpert MTB/RIF Assay cartridge with the sample ID.
3. Transfer at least 0.5 mL of the total suspended sediment to a conical, screw-capped tube for the Xpert MTB/RIF Assay using a transfer pipette. Alternatively, the entire sediment can be processed in the original tube.
4. Using a transfer pipette, transfer 1.5 mL of Sample Reagent to 0.5 mL of resuspended sediment. For larger volumes of sediment, add Sample Reagent equal to three times the volume of the resuspended sediment.
5. Recap the tube and shake vigorously 10 to 20 times or vortex for at least 10 seconds
6. Incubate sample for a total of 15 minutes at 20-30°C.
7. Between 5 and 10 minutes into the incubation period, shake vigorously 10 to 20 times or vortex for at least 10 seconds.

7.7 **Culture by LJ and BACTEC MGIT 960**

Culture and antibiotic susceptibility testing of *M. tuberculosis* strains are performed according to conventional methods. The processed specimens are inoculated on 2 Lowenstein Jensen (L-J) slants. The L-J slants are incubated at 37°C for 8 weeks and examined once every week for any growth of visible mycobacterial colony as well as contamination. After getting sufficient culture growth, a standard suspension of *M. tuberculosis* isolates are inoculated onto L-J media containing antimicrobial agents and also onto control L-J media without any antimicrobial agent. Isolates are considered resistant to a particular concentration of drug when 1% or more colonies grow on the drug-containing medium when compared to the drug-free medium.

7. 7 English **version Informed consent form for study participant's parents/guardian**

Introduction: Good morning/afternoon/evening.

My name is _____ and I am the site coordinator for a study conducted by Debre Markos University (DMU) standardizes the diagnostic accuracy of stool to diagnose TB in children as compared to respiratory samples. The study is supported by DMU. The project is being conducted at selected health facilities in Amhara region and the facility

you are attending is among the ones selected. The main purpose of this project is to optimize and standardize the stool to diagnose TB. If this works diagnosing TB in children becomes much easier and there is no need for invasive methods to obtain sputum. Sputum is normally used to test for TB but for small children or very sick children it is difficult to provide a sputum sample. Children swallow the sputum and therefore currently using a tube the sputum sample is taken through the nose form the stomach, this is not a pleasant procedure for the children. This project will provide vital information to help in the generation of standard operating procedures (SOPs) for this method for future scale-up in Ethiopia and in other countries. You are asked to participate because your child was identified as having TB by the clinician in this facility.

Study Procedure: We would like to ask you if you are willing for your child to take part in the study. You will be given stool containers and sputum cup to collect the stool and the respiratory samples. We would also like to record some information from the child to help us conduct the analysis, like age, sex, medical condition of the child including the test results obtained from all tests for TB requested by the clinician and the stool results and information on the decision by the clinician on whether or not your child has TB and based on what that decision was taken. We will investigate using a machine called GeneXpert in the selected health facilities, whether we can find TB in the stool and the respiratory sample of the child. Your child is not the only one in this facility who has been asked to participate; all children who are suspected of presumptive TB patients will be asked.

Risk and Benefit: There will not be any risk for you and your child if you choose to be part of the project. There may not be direct benefit to you or your child as a participant. However, your participation can help to make diagnosing of TB for children in Amhara region and other countries simpler in the future. If you choose not to participate in the study, your child will still receive the standard care and treatment as per the national guidelines. There is no any type of benefit in the participation the project.

Confidentiality: All the information you give will be kept strictly confidential. We will keep the records in a safe place and only investigator/s will be allowed to look at them. Yours and your child personal identifying information will not be used and shared outside of the study team. We will not use your/your child's name and details for the analysis of the data. Any publications that come out of the study will not mention any details that will identify you/your child. The study results will facilitate inclusion of simplified child TB diagnostic approaches in the local treatment policy.

Voluntary Participation: You/your child is here for TB diagnosis for initial tests, but we want to take two samples (respiratory and stool) to do further studies to improve TB detection for other children. Participation in this study is voluntary, you are free to choose for your child to be part of the study and you can decline to participate or remove yourself/your child from the study at any time. You may refuse to answer any of the questions and you may halt your participation at any time. If you decide not to participate in this assessment, or drop out at a later time, your decision will not affect your child's treatment at the facility in any way. If you agree to participate, we would like to ask you to let us take a respiratory and stool sample from your child. This sample will be used to perform additional test in the laboratory to find the best sample to detect TB in children. The results from this study will not have direct effect on the treatment course for your child.

If you have any questions, related to the study please contact the principal investigator of this research Mr. Habtamu Belew or co-investigator of this research

Habtamu Belew, DMU, (Debre Markos, Ethiopia, P.O. Box 269) PI of the project **Phone**: +251918591570 or: Email: merahabtamu29@gmail.com

For any questions related to the study or you experienced any adverse event before/during/after participation in the study tell for and if you have any ethical complaint and issues about by our right, contact Ethic Review Office of Debre Markos University college of Health Sciences Ethical committee at the following address. Tel address:

Do you agree for your child to participate in the project?

☐ Yes, I agree for my child to participate in the study

☐ No, I do not wish for my child to participate in the study Participant's parents/guardians' statement:

The above survey has been explained to me and my child, and I agree that my child to take part in the survey. I understand that this is my choice and that if I change my mind, I can decide to end the interview at any time.

Participant's parents/guardians name and signature_____ Date_____

Interviewer name and signature_____ Date_____

7.8 **English Version Questionnaire**

Questionnaire Procedure: There are some questions assessing the research question of the diagnostic accuracy of stool and respiratory sample based Xpert assay to diagnose presumptive TB among children, in Amhara Region. I would like to ask you to give your genuine and honest answers on the questions forwarded. If you need clarification, please ask me at any time. As a participant of this study, you are expected to give Stool and respiratory sample (sputum or gastric aspirate). Being asked to give a sample does not necessarily mean that you have the disease. When you are found to be positive for the microorganism, you will be informed by the health worker and receive proper treatment.

S.no	Part I: Sociodemographic Variables	Answers	Remark
	Name of the Hospital	_____	
	Medical registration number(MRN)	_____	
100	Sex	1. Male 2. Female	
101	Age	_____	
102	Residence	1. Urban 2. Rural	
103	Religious	1. orthodox 2. Muslim 3. Others	
104	Monthly income of the family	1. <1500 EBR 2. ≥1500ETB	
105	Type of transport to reach health facility	1. Car 2. Bajaj 3. On foot 4. others	

105	Part II: Clinical Variables		
106	Vaccination status for BCG and scar	1. Yes 2. No	
107	HIV	1. Positive 2. Negative	
108	Cough	1. <2wk 2. ≥2wk	
109	Fever	1. <2wk 2. ≥2wk	
110	Night sweat	1. Yes 2. No	
111	Loss of appetite	1. Yes 2. No	
112	Hemoptysis	1. Yes 2. No	
113	Significant weight loss	1. Yes 2. No	
114	Decreased activity	1. Yes 2. No	
115	Previous history of TB	1. Yes 2. No	
116	History of TB contact in the family	1. Yes 2. No	
	Part III: Nutritional status		
117	Nutritional edema	1. Yes 2. No	
118	Malnutrion	1. Normal 2. Moderate 3. SAM	
119	Overweight/ obesity	1.Overweigh and obesity 2. Normal 3.Thinness 4.Severe thinness	
	Part IV: Laboratory investigation		
120	Chest x-ray	1. Suggestive for TB 2. Non suggestive TB	
121	Respiratory specimen type	1. Sputum 2. Gastric aspirate 3. Other_____	
122	Xpert result for respiratory sample	1. *M.tuberculosis* detected 2. *M.tuberculosis* not detected 3. Invalid	
123	Rifampicin resistance for resp in Xpert Resp= **respiratory sample (sputum/ Gastric aspirate)**	1. Detected 2. Not detected 3. Invalid	
124	INH resistance	1. Detected 2. Not detected 3. Invalid	

125	Other_____	1. Detected 2. Not detected 3. Invalid	
122	Stool sample for Xpert	1. *M.tuberculosis* detected 2. *M.tuberculosis* not detected 3. Invalid	
123	Rifampicin resistance for stool	1. Detected 2. Not detected 3. Invalid	
124	INH resistance	1. Detected 2. Not detected 3. Invalid	
125	Others specify_____	1. Detected 2. Not detected 3. Invalid	

TB culture Result

LJ culture result for *M.tuberculosis* in Resp	Result (one Tick)						Contaminated
	Neg	<10	+(10-99)	++(≥100)	+++(Innumerable/confluent growth	NTM	

LJ culture result for *M.tuberculosis* in stool	Result (one Tick)						Contaminated
	Neg	<10	+(10-99)	++(≥100)	+++(Innumerable/confluent growth	NTM	

Phenotypic drug susceptibility test (DST)

Result for	1st line drug					2nd line drug	Other
	RMP	INH	STM	ETB	PZA		
Resp sample							

Result for	1st line drug					2nd line drug	Other
	RMP	INH	STM	ETB	PZA		
Stool sample							

INH=Isoniazid, RMP=Rifampicin, STM= streptomycin, ETB=Ethambutol, PZA=Pyrazinamide

7.9 Amharic version questionnaire

የጥያቄዎች የአሰራር ቅደም ተከተል፡ ከዚህ በታች የጥናታችን ፅንሰ ሀሳብ የያዙ ጥቂት ማስታወቂያችን አዘጋጅተናል፡፡ የጠያቂያችን አላማም ሰገራን እና የሞነፈሻ ናሙና በማስቀም የሳንባ ነቀርሳን ከህጻናት ላይ በፍጥነት ለመመርመር የናሙና አሰራጭ ለሚፈላለግ ታስቦ የታለመጥናት ነው፡ ፡ ሁለቱ ናሙናዎችም የሚሰሩበት መመርመሪያ ጅን ኤክስፐርት እና ልጀ ካልቸር ነው፡ ፡ ስለዚህ ከዚህ በታች ያሉትን ጥያቄዎች ለማስያቅ እፈልጋለሁ እና እርስዎም ጥያቄዎችን ለመመለስ በቅንነትና በታማኝነት እና ባለመሰላቀል ስለተሳተፉ እናመሰግናለን፡፡ ለጥናቱ የሚያገለግሉ ሁለት ናሙናዎችን ይሰጡሉ፡ ፡ ናሙና ሰጡችሁ ማለት ደግሞበሽታዉአለባችሁ ማለት አይደለም ፡ አጋጣሚሁኖ በሽታዉከተገኝባችሁምአስፈላጊዉን ሜዳህኒት እስክድኑ ድረስ በጤና ባለሞያወች እንክብካቤና ክትትል እንድትስጡይሆናል፡ ፡

ተ.ቁ	ክፍል I: **ስለማላዮ የሚከይቁ ጥያቄዎች**	ምልሶች	ልየታ
100	የበሽተኛዉማላያ ቁጥር	_____	
101	ፆታ	1. ወንድ 3. ሴት	
102	እድሜ	_____	
103	ሞሪያ	1. ከተማ 2. ገጠር	
104	ሀይሞት	1. ተዋህዶ ኦርቶዶክስ 2 .ሞስሊም 3 .others	
	ክፍል II: ክሊኒካል ማስየቆች		
105	የቢሲጅ ክትባት ሁኔታ	1 አለ 2. የለም	
106	ኤችአይቪ	1. አለ 2. የለም	
107	ሳል	1. <2 ሳምንት 2. ≥2ሳምንት	
108	ሞቀት	1. <2 ሳምንት 2. ≥2ሳምንት	
109	ሜታ ሜታ ላበት	1. አለ 2. የለም	
110	የምግብ ፍላጎት ሞቀነስ	1. አለ 2. የለም	
111	ደምየ ተቀላቀለ አክታ	1. አለ 2. የለም	
112	የክብደት ሞቀነስ	1. አለ 2. የለም	
113	እንቅስቃሴ ሞቀነስ	1.አለ 2. የለም	
114	ክዚህ በፌት ቲቢ ተይዞ ነበር	1. አለ 2. የለም	
115	በቤተሰብ ዉስጥ ቲቢ ነበር	1. አለ 2. የለም	
	ክፍል III: የምግብ ሁኔታ		
116	በምግብ እጥረት የሞጣ እብጠት	1. አለ 2. የለም	
117	የምግብ እጥረት	1. ኖርሜል 2. ሞከለኛ 3. በጣምየ ታመመ	

118	ክ ማከን በ ላ ይ ዉፍረ ት	1.ውፍረ ት አ ለ 2. ኖ ር ማል 3.ቅ ጭ ፣ 4.በ ጣም ቅ ጭ ፣	
	Part IV: **የ ላ ቦ ራ ቶ ሪ ምር ሙራ ን በ ተ ማ ላ ክ ተ**		
119	ቸ ስ ት ኤ ክ ስ ሬ	1. እ ን ድ ኬ ቲ ቨ ፎ ር ቲ ቢ 2. የ ለ ም	
120	በ ምር ሙራ ዉ ማ ሳ ረ ት	1. ቲ ቢ አ ለ 2. ቲ ቢ የ ለ ም	
121	Specimen Type በ አ ክ ታ ዉ ላ ይ በ ጅ ን ኤ ክ ስ በ ር ት	1. ተ ገ ኝ ቶ ል 2. ሽ ል ተ ገ ኘ ም 3. ውጤ ቱ አ ል ተ ረ ጋ ገ ጠ ም	
122	የ ሰ ገ ራ ጅ ን ኤ ክ ስ በ ር ት	1. ተ ገ ኝ ቶ ል 2. ሽ ል ተ ገ ኘ ም 3. ውጤ ቱ አ ል ተ ረ ጋ ገ ጠ ም	
123	ሜ ዳ ህ ኒ ት የ ተ ላ ሚ ቲ ቢ(ሪ ፍ)	1. ተ ገ ኝ ቶ ል 2. ሽ ል ተ ገ ኘ ም 3. ውጤ ቱ አ ል ተ ረ ጋ ገ ጠ ም	
124	በ አ ክ ታ ዉ ላ ይ ል ጀ ካ ል ቸ ር	1.አ ለ 2. የ ለ ም	
125	በ አ ክ ታ ዉ ላ ይ ባ ክ ቴ ክ ሚ ሯ ት 960	1.አ ለ 2. የ ለ ም	
126	የ ሰ ገ ራ ል ጀ ካ ል ቸ ር	1. አ ለ 2. ለ ም	
127	ሜ ዳ ህ ኒ ት የ ተ ላ ሚ ቲ ቢ	1.ሰ ስ ብ ቲ ብ ል 2.የ ተ ላ ሚ ቲ	**Phenotypic**