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Novel and optimized diagnostics for pediatric TB in endemic countries: NOD-pedFEND study protocol

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Abstract

Background Pediatric tuberculosis is a major global public health challenge, with reliable diagnosis being a main obstacle to identifying and treating affected children. New and improved diagnostics, ideally on non-sputum samples, are urgently required, especially in the most vulnerable group of children under five years of age. Studies to date have been limited by small sample sizes and few bacteriologically-confirmed cases. Here, we describe the study protocol of the NIH-funded NOD-pedFEND study, which will be one of the largest diagnostic studies to date of children at greatest risk of tuberculosis.

Methods In this prospective observational cohort study, we aim to evaluate existing and novel diagnostic assays, including pathogen- and host-based tests and combinations of tests. A consecutive cohort of children under five years of age with signs and symptoms of tuberculosis is enrolled in Uganda and Peru. All children undergo an extensive baseline workup with signs- and symptoms recording, microbiological reference tests, chest X-ray and tuberculin skin test for rigorous classification according to internationally recognized microbiological, composite reference and strict standards. An array of samples is collected for investigational tests. Follow-up visits are conducted at 2 weeks, 2 months and 6 months. A small cohort of healthy controls is enrolled to evaluate the specificity of selected diagnostics. The study has been approved by the relevant institutional review boards.

Discussion With this large cohort study of children under five years of age, we aim to make an important contribution to the evaluation of new diagnostics for pediatric tuberculosis. By establishing a comprehensive biorepository, the study will also enable the assessment of novel tests as they become available during and after the study.

Keywords Tuberculosis, Children, Diagnosis, Cohort, Tests, Assays

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Background

For many years, pediatric tuberculosis (TB) incidence has been under-reported [1]. The World Health Organization currently estimates the annual incidence of TB in children at 1.3 million cases [2]. Significant advances in TB diagnostics made in the last decade have barely impacted pediatric TB case detection. Growth of *Mycobacterium tuberculosis* (*Mtb*) from induced sputa and gastric aspirates on liquid culture media remains the gold standard for TB diagnosis for children under five years of age [3]. In contrast to TB in adults, pediatric TB is mainly paucibacillary, i.e., characterized by low mycobacterial numbers, so that standard diagnostic methods used for diagnosing TB in adults, e.g., acid-fast bacilli smear microscopy on expectorated sputum and culture for *Mtb* have low sensitivity [4]. Moreover, microbiological test availability in high-burden settings is limited, and specimens for microbiological confirmation are often not collected. Therefore, the true disease burden is difficult to ascertain as most children with TB are not diagnosed by standard microbiological methods [5]. As a result, underdiagnosis is common in high-burden settings, leading to high mortality among children under five years of age [6]. TB is often recognized only post-mortem [7]. Depending on disease severity, age, the number of specimens collected, and specimen type, the proportion of children identified as having TB based on clinical grounds who are positive on TB culture is low, ranging anywhere from 5 to 40% [8]. Among the remaining children with microbiological culture results, a constellation of clinical, radiographic, exposure and immunological criteria is used to estimate the likelihood of having TB disease. Due to a lack of access to diagnostics and the imperfect reference standard, children with microbiologically unconfirmed TB represent the largest but also the most challenging group of children to manage in clinical and research settings [9].

Improved approaches to diagnose TB in young children are urgently needed. Among the high-priority needs are tests that 1) can be used at or near the point-of-care (POC); 2) are non-sputum-based; 3) can accurately identify children with TB currently classified as microbiologically negative.

Promising new diagnostics have been developed but not sufficiently evaluated in children under five years of age, a group at greatest risk of TB-associated morbidity and mortality and with disease that differs most from adults [10]. This protocol describes the design of a comprehensive study of children from Uganda and Peru with presumed TB that aims to address the most critical challenges and unmet needs in pediatric TB diagnosis by several different but inherently complementary approaches. The study will concurrently evaluate, optimize, and develop non-sputum-based diagnostics for children under five years of age.

The Feasibility of Novel Diagnostics for TB in Endemic Countries (FEND-TB) Consortium, funded by the U.S. National Institutes of Health (NIH), comprises a group of international clinical and laboratory investigators. The objective of the FEND-TB Consortium is to support the evaluation of early-stage TB diagnostic assays and strategies in the context of existing clinical algorithms in TB-endemic countries. FEND-TB conducts early-stage diagnostic accuracy and feasibility studies and then feeds back to assay developers to facilitate an efficient and iterative assay evaluation-assay revision process. An essential feature of the FEND-TB design includes a continuously enrolling pediatric protocol that will a) facilitate rapid evaluation of new investigational TB diagnostic assays as they become available, thereby allowing the FEND-TB consortium to respond nimbly to new scientific opportunities and b) allow for the collection of specimens for a biorepository from participants whose TB status is well characterized, for future use in the evaluation of new technologies and for use in evaluating the comparative performance of assays that enter evaluation at different times. The Novel and Optimized Diagnostics for Pediatric TB in Endemic Countries (NOD for TB) consortium, also funded by the NIH, aims to explore novel approaches for TB diagnosis in children, focusing on non-sputum point-of-care tests, increasing the proportion of bacteriologically-confirmed cases with more sensitive diagnostics and stratifying children with unconfirmed TB by their likelihood of having TB. Due to the overlap in study population and reference standard requirements, pediatric FEND and NOD were combined in the NOD-pedFEND study, for which the protocol is described here. Publication of this protocol can facilitate the harmonization with other pediatric diagnostic study protocols.

Methods

Aims

Aim 1

Evaluate and develop novel assays that diagnose TB by detecting *Mtb* bacterial products in non-sputum body fluids.

Aim 2

Evaluate and develop novel assays that diagnose TB by detecting host biomarkers in non-sputum body fluids.

Aim 3

Identify combinations of assays that, applied together, could be used to risk-stratify children with unconfirmed TB.

Secondary aim

To gather parameter estimates for economic analyses.

Design

This study prospectively enrolls a consecutive cohort of children under five years of age in Uganda and Peru with presumed TB to evaluate a comprehensive array of non-sputum-based investigational assays. Participants from a previously established TB diagnostic study with a biorepository in Kenya ('retrospective cohort' [11]) round out the population available for NOD-pedFEND evaluations.

A case–control design is used to evaluate selected diagnostics at the early stage of development, with asymptomatic children included as healthy controls. This design allows us to efficiently evaluate potential diagnostic tests by limiting the number of participant specimens tested, reducing the time associated with real-time testing, and allowing the evaluation of tests that were not available at the time participants were enrolled.

Endpoints and reference standards

Children will be classified as having "confirmed TB" when *Mtb* is detected by Xpert MTB/RIF Ultra (Xpert Ultra), Mycobacteria Growth Indicator Tube (MGIT) or solid Löwenstein–Jensen (LJ) medium culture on one or more of the reference specimens (i.e., gastric aspirate, nasopharyngeal aspirate or stool). Participants without positive microbiological tests will be defined as having "unconfirmed TB" in the presence of clinical, radiological, immunologic features suggestive of TB, and positive response to treatment, or as children with "unlikely TB" if criteria for "unconfirmed TB" are not met according to the revised international case definitions for pediatric TB diagnostic research [12]. Using these published classifications enhances the comparability of the results to other studies.

All investigational tests will be assessed using a microbiological reference standard (MRS) and a composite reference standard (CRS). We will also use an extended Microbiological Reference Standard (eMRS), which includes positive Determine Abbott LAM for children living with HIV (CLHIV), and a strict reference standard (SRS), comparing confirmed TB cases to children with unlikely TB (see Fig. 1).

Furthermore, children with unconfirmed TB and children with unlikely TB will be characterized as having TB infection or not, based on tuberculin skin test (TST) results (Table 1). Immunological evidence of TB infection will be defined by a positive TST result with induration of ≥ 10 mm for HIV-negative children and ≥ 5 mm for children living with HIV and children with moderate or severe malnutrition. The nutritional status will be classified according to the WHO definitions [13]. Exposure to TB will be defined as reported exposure (household/close contact) to a patient with documented laboratory-confirmed TB within the preceding twelve months.

An independent expert panel of three clinicians with experience in pediatric TB diagnosis will be presented

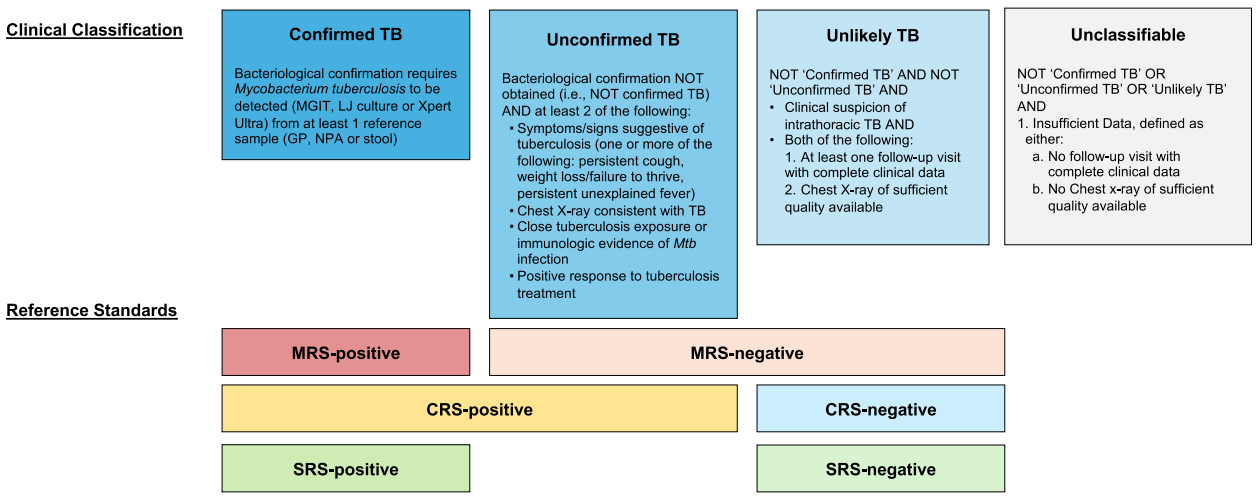

Fig. 1 Clinical classification definitions and reference standards. SRS = Strict Reference Standard, MRS = Microbiological Reference Standard, CRS = Composite Reference Standard, GA = gastric aspirate, NPA = nasopharyngeal aspirate. Participants MRS-positive meet the case definition of Confirmed TB. Participants MRS-negative meet the case definition of either Unconfirmed TB or Unlikely TB AND must have 6 negative tests for *Mtb*. Participants CRS-positive meet either case definitions of Confirmed TB or Unconfirmed TB. Participants CRS-negative meet the case definition of Unlikely TB. Participants SRS-positive meet the case definition of Confirmed TB (identical to MRS-positive). Participants SRS-negative meet the Clinical Case Definition of Unlikely TB AND must have 6 negative tests for *Mtb*. Immunological evidence of *Mtb* infection was defined by a positive TST

Table 1 Clinical case definitions, based on [12]

Clinical Case	Definition
Confirmed TB	Microbiological confirmation obtained; requires *Mtb* to be confirmed (culture or Xpert MTB/RIF assay) from at least 1 of the following: 1) Day 1 NPA MGIT culture 2) Day 2 NPA MGIT culture 3) Day 2 GA MGIT culture 4) Day 1 NPA LJ culture 5) Day 2 NPA LJ culture 6) Day 2 GA LJ culture 7) Day 1 NPA Xpert MTB/RIF Ultra 8) Day 2 NPA Xpert MTB/RIF Ultra 9) Day 2 GA Xpert MTB/RIF Ultra 10) Stool Xpert MTB/RIF Ultra
Unconfirmed TB	Microbiological confirmation NOT obtained (i.e., NOT 'Confirmed TB') AND at least 2 of the following: 1. Symptoms/signs suggestive of tuberculosis (one or more of the following: persistent cough, weight loss/failure to thrive, persistent unexplained fever) 2. Chest radiograph consistent with TB 3. Close tuberculosis exposure or immunologic evidence of *Mtb* infection 4. Positive response to tuberculosis treatment (requires documented positive clinical response on tuberculosis treatment) *Subcategories*[a]*:* - With *Mtb* infection (Immunological evidence of *Mtb* infection) - Without *Mtb* infection (no immunological evidence of *Mtb* infection)
Unlikely TB	NOT 'Confirmed TB AND NOT 'Unconfirmed TB' AND • Clinical suspicion of intrathoracic TB (i.e., compatible symptoms irrespective of duration) AND • Both of the following: 1. At least one follow-up visit with complete clinical data 2. Chest X-ray of sufficient quality available *Subcategories:* - With *Mtb* infection (Immunological evidence of *Mtb* infection (TST or IGRA positive) - Without *Mtb* infection (No immunological evidence of *Mtb* infection)
Asymptomatic	NOT ('Confirmed TB' OR 'Unconfirmed TB' OR 'Unlikely TB') AND • No symptoms/signs suggestive of tuberculosis *Subcategories:* - With *Mtb* infection (Immunological evidence of *Mtb* infection (TST or IGRA positive) - Without *Mtb* infection (No immunological evidence of *Mtb* infection)
Unclassifiable	NOT ('Confirmed TB' OR 'Unconfirmed TB' OR 'Unlikely TB' OR 'Asymptomatic') AND 1. Insufficient data, defined as either: a. No relevant follow-up visit with complete clinical data b. No Chest X-ray of sufficient quality available OR 2. Determined to be unclassifiable by the Expert Panel

Mtb Mycobacterium tuberculosis; *TB* tuberculosis; *NPA* nasopharyngeal aspirate; *GA* gastric aspirate; *MGIT* Mycobacteria Growth Indicator Tube, *LJ* Löwenstein–Jensen; *TST* tuberculin skin test; *IGRA* interferon gamma release assay

[a] For the purposes of determining immunological evidence of Mtb infection, only TST (no IGRA) is done on all participants.

[12]

with a summary of clinical findings in REDCap to assign the case definitions and determine alternative diagnoses (e.g., based on testing for malaria smear, blood culture results). As part of these clinical vignettes, reviewers will be presented with the majority radiological interpretation of the participants' chest X-rays by an additional three-member chest X-ray review panel. Reviewers will also be given a calculated Madalakas score which quantifies the degree of TB exposure [14].

Case summaries will be presented to two panel members, with a third member and, potentially, a team discussion employed to resolve disagreement until a majority opinion is reached by the expert panel. Additionally, automated classification of all participants will be done using R scripts, which algorithmically classify participants based on the NIH classifications (code available on request) [12].

The study participants, investigators, and all site staff that will be in contact with the participants will be blinded to the results of the new investigational diagnostics throughout the study. The study will be conducted and reported following Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines for reporting diagnostic accuracy studies [15, 16].

Setting

The study population for both retrospective and prospective components are children under five years of age with signs or symptoms suggestive of pulmonary TB. Children will be prospectively recruited from the following: Baylor Uganda Children's Clinical Center of Excellence (Kampala, Uganda), Jinja Regional Referral/Nalufenya Children's Hospital (Jinja, Uganda), Hospital Nacional Cayetano Heredia (Lima, Peru) and Hospital Nacional Docente Madre Niño San Bartolomé (Lima, Peru). Both inpatients and outpatients will be included. In addition, household contacts of adult index TB patients that will be identified within Rubaga, Kawempe, Nakawa, Makindye, and Central divisions of Kampala district (Uganda) will be screened for study eligibility.

For selected investigational assays, samples from a biorepository collected as part of the clinical trial *"Improving TB diagnosis in children with and without HIV in Kenya"* will also be used [11].

For the evaluation of selected tests in the early stages of development, a limited number of asymptomatic healthy controls will be recruited at the Ugandan sites.

Participants

To be eligible for enrolment, all the following inclusion criteria must be met:

- Age under five years;
- Written informed consent by parent/legal guardian;
- No treatment for TB disease or TB infection/exposure in the preceding 6 months;
- Weight > 3 kg.

Symptomatic children, prospective cohort

Children meeting above inclusion criteria will be offered enrolment into the cohort of symptomatic children if one or more of the following signs or symptoms suggestive of pulmonary TB are present:

- Persistent cough > 2 weeks, not responding to antibiotic therapy;
- Unexplained weight loss: > 5% reduction in weight compared with the highest weight recorded in last 3 months **OR** failure to thrive, defined as any of the following: clear deviation from a previous growth trajectory, documented downward crossing of percentile lines in the preceding 3 months, weight-for-age z score of ≤ -2 in the absence of information on previous/recent growth trajectory, or weight-for-height z-score of ≤ -2 in the absence of information on previous/recent growth trajectory,'
- Persistent unexplained fever (not responding to antibiotic therapy) or night sweats lasting > 1 week. Unexplained fever > 38 °C can be reported by a guardian or objectively recorded at least once

Children with additional evidence of extra-pulmonary TB will not be excluded.

Healthy controls, prospective cohort

Healthy controls will be recruited in Uganda for the evaluation of diagnostic assays at the earliest stages of development. To be eligible for enrolment as a healthy control, a child must not have had any of above signs or symptoms within the preceding 6 months and must not have a history of known TB exposure.

Exclusion criteria for both prospective groups

- A critical condition (if study procedures seem like an undue risk to child's life), such as hypovolemic shock or clinically relevant anemia (tachypnoea, tachycardia);
- Expected unavailability for follow-up study visits (e.g. travel);
- Parents/legal guardian unwilling or unable to provide written informed consent.

Retrospective cohort

The inclusion and exclusion criteria of the retrospective cohort have been described previously [11].

Informed consent

Written informed consent will be obtained from the parents or guardians of eligible children. In Uganda, some adult TB index cases will also be approached for consent to contact and evaluate members of their household for potentially eligible participants. Written informed consent for banking of specimens and their future use will be obtained from the parent or guardian.

Ethical considerations

The study will be conducted in compliance with the laws and regulatory requirements of Uganda and Peru. Local ethical approval has been obtained in Uganda from School of Biomedical Sciences Research Ethics Committee (SBS-REC-845) and Uganda National Counsel for Science and Technology (UNCST, HS1341ES) and in Peru from Universidad Peruana Cayetano Heredia Research Ethics Committee (Comité Institucional de Ética en Investigación – Humanos; 208,925) and the

Research Ethics Committees from Hospital Nacional Cayetano Heredia (078–191-22) and Hospital Nacional Docente Madre Niño San Bartolomé (019855–22), and local approval from the National TB program and local registration in PRISA repository (EI00003204) following local regulations before study commencement. Ethical approval has also been obtained from Rutgers University IRB (Pro2020000750) and the Oxford Tropical Research Ethics Committee (OxTREC 9–21). Participants will receive reimbursement for travel and meals, according to local practices and guidelines.

Study procedures

For an overview of the schedule of events, see supplementary Table 1. Detailed information on sample collection, processing, and lab procedures can be requested from the corresponding author.

Baseline visit

The following study procedures will be conducted at the baseline visit for all enrolled children (both symptomatic children and healthy controls):

- Data collection: standardized data will be collected, including demographic data, data on TB exposure (e.g., relationship and sleeping proximity to index TB case, laboratory results of index case), history of BCG vaccination, data on HIV status (including most recent CD4 count, data on anti-retroviral therapy if living with HIV, maternal HIV status), symptom history, recent medical treatments, data on TB history (including prior diagnoses, prior treatments).
- Physical examination will be performed by clinical members of the research team and will include assessment and data collection on findings of the clinical examination, including presence of a BCG scar, vital signs, and anthropometric measurements.
- HIV status will be recorded for all children. Children with unknown HIV status will be tested according to national guidelines.

Symptomatic children will undergo the following study procedures at the baseline visit:

- Chest X-ray will be performed with two views, antero-posterior and lateral. Radiographs will be read by the treating or study clinician according to routine practice and findings will be recorded on the patient clinical record. For establishment of clinical case definitions, all radiographs will be digitalized and stored. These will be reviewed by two independent and blinded readers who are experienced in reviewing pediatric chest radiographs. The overall quality of the chest radiographs will be indicated. In case of discordant reading, a 3rd expert reader will be used to achieve consensus.
- Nasopharyngeal aspirate (NPA): two NPAs will be collected (target volume 2 mL) for testing with Xpert Ultra, MGIT, and LJ culture. Following enrolment, the first NPA will be collected. The following day, the second NPA will be obtained. Specimens will be obtained after insertion of 2 drops of saline into each nostril by suctioning using a sterile catheter.
- Gastric Aspirate: One early morning gastric aspirate will be collected (target volume 3 mL) for testing with Xpert Ultra, MGIT, and solid medium LJ TB culture. The procedure will be done as per WHO guidance. Specimens will be collected after a minimum fasting period of 3 h using a nasogastric tube. Sterile water or normal saline will be used for lavage only if no gastric contents can be aspirated.
- Stool: specimens will be collected from diapers or in a stool collection hat and placed in a container for transfer to the laboratory. If the child does not pass stool during the visits, the caregiver will receive a container to collect stool at home (up to seven days window).
- Upper airway swab: up to twelve upper airway (e.g. oral, nasal) swabs will be collected and transferred to the laboratory.
- Saliva will be collected by holding a SalivaBio Children's Swab (for children 6 months – 5 years old) or a SalivaBio Infant's Swab (for children < 6 months old) in their oral cavity for approximately five minutes, allowing it to soak up saliva.
- Nasal cells will be collected from the outside wall of the nostril just past the inferior turbinate, using a Cytopak Cytosoft brush, and transferred to the laboratory in RNA stabilization buffer for storage.
- Urine: specimens will be collected using a specimen collection cup or an adhesive plastic urine collection bag and transferred to a specimen container prior to transfer to the laboratory. If urine collection is difficult, a window of maximum five days is allowed. Urine will be tested with a dipstick, after which the remaining urine will be used or stored for testing with the investigational assays.
- Blood: serum, plasma and whole blood collection will be performed by trained members of the clinical or research staff according to WHO recommended limits for blood volume per child of 3 mL/kg in 24 h [17].

Specimens will be refrigerated or kept at room temperature as appropriate until transported to the laboratory as soon as possible. Following transport of collected

specimens, all processing, aliquoting and storage of samples will take place in the TB laboratories of Makerere University in Uganda, and Universidad Peruana Cayetano Heredia in Peru.

Follow-up visits

Symptomatic children will have follow-up visits at 2 weeks (visit 2), 2 months (visit 3) and 6 months (or end of TB treatment; visit 4) after enrolment. Unscheduled visits can be performed when needed.

The following study procedures will be conducted at visit 2 (2 weeks):

- Physical examination, including weight
- Symptom review
- Documentation of any provided treatment
- If on TB therapy, review of compliance
- TST
- Sample collection: upper airway swabs, saliva, blood and urine

The following study procedures will be conducted at visit 3 (2 months) and visit 4 (6 months or end of treatment):

- Physical examination, including weight
- Symptom review
- Documentation of any provided treatment
- If on TB therapy, review of compliance

Children in the symptomatic arm who have not received TB therapy during the course of the study will be followed for up to 24 months with phone surveys at 12 months, 18 months and 24 months for symptom review. Any child with symptoms or suspicion of TB on phone follow-up will return to the clinic for repeat baseline procedures.

Study procedures healthy controls

Asymptomatic children will only undergo the following study procedures at baseline:

- TST
- Stool: specimens will be collected as described for the symptomatic group.
- Blood collection: whole blood will be collected for plasma separation for the cf-DNA investigational assays. Additionally, whole blood will be collected in PAXgene tubes for the Nanostring investigational assays. The total blood volume will be adjusted based on weight of the child, not to exceed the WHO recommended limits.

The cohort of healthy controls will also have follow-up visits at 2 weeks (visit 2), 2 months (visit 3) and 6 months (visit 4) after enrolment. During these follow-up visits, the following study procedures will be conducted:

- Physical examination, including weight
- Symptom review
- Documentation of any provided treatment

Diagnostic assays to be evaluated

A range of novel diagnostic assays, both pathogen-based and host-based, will be evaluated in this study. These include but are not limited to the following: FujiLAM on urine, Xpert Ultra on upper airway swabs, Xpert Ultra on stool after simple one-step stool processing [18], TB host-response cartridge (Cepheid), C-reactive protein, *Mtb*-specific amplification and sequencing, sequencing of host cell-RNA and microbiome, NanoString (on stimulated and unstimulated blood) and MesoScale multiplexed cytokine assay on plasma (MSD).

Specimen storage for investigational tuberculosis diagnostic assays

Samples will be stored for future testing using other investigational in vitro TB diagnostic assays as identified by the FEND-TB Consortium. The following types of specimens may be stored, establishing a biorepository: serum, plasma, whole blood, QuantiFERON-TB Gold Plus cell pellets, urine, stool, upper airway swabs, saliva, nasal cells, nasopharyngeal aspirates, gastric aspirates, and mycobacterial isolates. Specimens will be stored for up to fifteen years after the end of the study. Should further storage be required, an amendment will be submitted to the IRBs. Developers with promising diagnostics are encouraged to contact the study team through https://www.fend-tb.org.

Digital chest x-ray images

In this study, chest X-rays, stored digitally for the establishment of clinical case definitions, will be evaluated with CAD software programs as selected by the FEND consortium.

Analysis of biorepository samples from Kenyan cohort

In the study *"Improving TB diagnosis in children with and without HIV in Kenya"*, samples from 300 Kenyan children were stored in a biorepository. Included participants were classified as confirmed TB ($n=32$), unconfirmed TB, unlikely TB, or unclassifiable [11]. Samples from this biorepository will also be used for evaluation of investigational assays.

Collection of cost data for economic analysis

Cost data for performing conventional and investigational tuberculosis diagnostic tests will be collected through inspection of laboratory budgets and through direct observation and time analysis of staffing needs, workflow, and resources consumed. These data will be used to model the effect of novel diagnostic tests on health-economic and patient-relevant outcomes.

Clinical management

Any child with a diagnosis of TB disease made by local clinicians will be treated according to the national TB program guidelines. Children in whom active TB is ruled out will receive preventive treatment according to national guidelines. Children with a positive malaria smear or newly diagnosed HIV infection will receive treatment as per national guidelines. The research team will not interfere with participant treatment.

Status and timeline

Recruitment of participants commenced in August 2021 in Uganda, and in January 2023 in Peru. Enrollment is expected to be completed in 2025.

Sample size

The overall enrolment target for the symptomatic cohort in Uganda is 800 children, which we expect to result in approximately 80 children with confirmed TB, 360 children with unconfirmed TB and 360 children with unlikely TB. The recruitment target for healthy controls is 80. For Peru, the enrolment target is 200 to 400 children. With the minimum enrollment of 200 we expect to result in approximately 20 children with confirmed TB, 90 children with unconfirmed TB and 90 children with unlikely TB. With 400 Peruvian children enrolled we expect to double the numbers in all groups. The Kenyan repository has specimens from 300 children, including 32 with confirmed TB, 135 with unconfirmed TB, and 134 with unlikely TB.

The focus of this study is to estimate the diagnostic accuracy of various assays. With this sample we are not powered to compare the diagnostic accuracy of assays to each other. Discordance between assays will have to be large and an assay will have to be substantially better than its comparator to detect differences with high power. However, we will be able to provide estimates of differences in candidate and comparator assays that will provide important data and be helpful for designing future confirmatory trials.

With a total of 100 children with confirmed TB tested on an assay from Uganda ($N=80$) and Peru ($N=20$) and assuming an estimated sensitivity (i.e., observed) of 66% (the WHO minimum target product profile for a diagnostic test [19]), the 95% confidence interval (CI; using Wilson's score method) will have a total width of 18.3% (−9.7% to +8.5% of the point estimate). When the sensitivity estimate increases, the 95% CI will narrow. The maximum possible width of the 95% CI for sensitivity estimated with 100 participants with confirmed TB will be 19.8% (when the estimate is 50%). For a triage test the WHO target product profile for sensitivity is > 90%. With a 90% sensitivity estimate the 95% CI would have a narrower width of 11.9% (−7.4% to +4.5% of the point estimate).

Specificity estimates of pathogen-based tests are expected to be substantially higher (i.e., ≥ 90%) and will have much narrower confidence intervals. For assays tested in all prospectively enrolled participants, with a total of 200 enrolled in Peru we anticipate that 450 (combining all sites) will be classified as unlikely TB, and with a specificity estimate of 90%, the 95% CI will have width of 5.6% (−3.1% to +2.4% of the point estimate).

For assays tested in both prospectively enrolled participants and the biorepository samples we will able to more precisely estimate sensitivity and specificity.

Data analysis plan

The analysis and investigative team will follow the STARD 2015 guidelines for the analysis and reporting of diagnostic accuracy studies [15]. This will ensure that our protocol, data collection and analysis provide complete and valid reporting on test performance.

For each assay, we will provide a STARD diagram, describing the numbers according to each reference standard classification and investigational assay results. This will include the number and proportion for whom the assay result was not obtained or was non-determinate and the reasons why. The overall number and proportion with non-determinate results and 95% CIs will be estimated.

The sensitivity and specificity against each reference standard of each investigational assay will be considered separately, and performance will be assessed among the cohorts of children, and by comparing sensitivity and specificity in children living with HIV and children HIV exposed but uninfected to children without HIV. The likelihood ratio positive and likelihood ratio negative will also be estimated.

When estimable (which will depend on whether the unconfirmed TB group is included in the evaluation of the investigational assay), positive and negative predictive values will be calculated. For the analysis of the diagnostic accuracy of investigational assays early in development or being optimized and tested on the asymptomatic group, the number and proportion of asymptomatic participants testing positive and negative will also be estimated. For

the analysis of diagnostic accuracy of upper airway swabs and stool with Xpert Ultra, we will estimate the relative diagnostic yield of each specimen and incremental yield of multiple specimens.

To address the potential issue of lower specificity of ultrasensitive assays, children with unlikely TB and asymptomatic controls will be further characterized by univariable and multivariable analysis using the TB exposure score, data on HIV status, age, clinical data at baseline and follow-up, final outcome, TST and malaria testing, and chest X-ray and blood culture results if applicable, to determine factors associated with positivity on each assay. For the analysis of diagnostic accuracy of next-generation LAM tests, we will compare accuracy against that of the Determine TB LAM Ag.

For assays that are still in development, testing will be done on a subset of participants as follows: all participants who are MRS-positive, an equal number of participants who meet the definition of unlikely TB, and an equal number of asymptomatic healthy controls (a sampling ratio of 1:1:1 using simple random sampling within site). So that the distribution of characteristics is similar in children with unlikely TB and healthy children, children in whom testing will be done will be randomly selected from within strata defined by HIV status and age, and, if feasible, also by malnutrition status. These earlier stage assays will not be tested on participants with unconfirmed TB at this stage of development.

All investigational assays will be run on the same sample or sets of samples to minimize variation. If there are insufficient sample volumes and multiple specimens of the type required are available, we will minimize bias by using randomization to determine which samples to allocate to each test.

Precision of estimation of sensitivity and specificity will use the 95% stratified Wilson CI [20], which adjusts for multi-center enrollment by clinic. When evaluation of an assay is based on a subsample and sampling probabilities differ within a group being analyzed, weights may be used for estimation. Recognizing that there may be differences in diagnostic accuracy characteristics of the candidate tests due to processing and storage of the specimens taken from the repository but that we are underpowered to test for meaningful differences, where applicable we will estimate differences in sensitivity and specificity by whether the specimens were from the repository or prospectively tested as an exploratory analysis.

Subgroups

In addition to analyses using all samples tested on an investigational assay, analyses by the following subgroups are of interest and will provide critical preliminary information about investigational assays for key subpopulations:

1) Geographical region (Uganda, Peru, Kenya)
2) HIV status (HIV+, HIV−, HIV exposed uninfected)

Additional subgroup analyses may be included in the statistical analysis plan.

Latent class analysis

Unconfirmed TB is the largest TB diagnostic category in children, yet this group is largely ignored in diagnostic development because, by definition, this group cannot be identified with a diagnostic gold standard using currently available tests. In the context of an imperfect reference standard, latent class analysis (LCA) is increasingly recognized as an important analytical methodology to estimate the accuracy of novel, imperfect diagnostics [21]. It is based on the assumption that the observed diagnostics all measure one or more common latent variables. Latent class models include disease prevalence as a parameter. Therefore, the resulting estimates of diagnostic accuracy are portable to different settings unlike when a composite reference standard is used. These models are very flexible allowing for the addition of covariates, e.g., HIV status, age, TB exposure score or malnutrition that influence either diagnostic accuracy or disease prevalence. Using LCA, we will be able to analyze the entire cohort of children with confirmed TB, unconfirmed TB and unlikely TB to estimate the probability that each child has TB. This probability when confirmed would potentially allow targeting of anti-TB therapy to the currently unconfirmed cases most likely to benefit.

We will use LCA to fit a model for the joint probability of each of the different assays, expressed as a function of the sensitivity and specificity of each assay, and combinations of assays and the prevalence of the latent variables. These unknown parameters will be expressed as functions of the covariates listed above using a series of logistic regression models. Further, among children that are TB positive according to the latent class model, we will use Gaussian random effects to model conditional dependence between sub-groups of tests that are based on a common biological mechanism, e.g., relying on the bacterial load, and therefore at risk of given a false negative result. Among children that are TB negative according to the latent class model, conditional dependence will be modeled among those diagnostics that are known to have poor specificity. We will carry out a series of sensitivity analyses with different conditional dependence structures that are biologically plausible and study the variation in estimates of sensitivity and specificity across them. Second, we will use external information, i.e., the

TB exposure score, to see if it agrees well with the predictions from our model. This approach will allow us to stratify the children with unconfirmed TB into children with high probability for TB and children with low probability of having TB.

Safety considerations

There will be a risk of minor physical discomfort and, on occasion, swelling or bruising, and a very small risk of infection at the needle-insertion site during blood draws. However, this risk is minimal and not greater than the risk posed during blood draws for routine care investigations. Gastric aspirates, nasopharyngeal aspirates and upper-airway swabs may cause some discomfort to the participants but in general are well-tolerated by children as they are routinely used in clinical settings. Collection of stool shall not pose a risk to the participants. For urine collection, some children might feel some irritation on the skin at the site where the urine bag will be placed but this is uncommon and should resolve in a few days. Collection of nasal cells is expected to only cause minor discomfort. Participants eligible for X-rays as part of their TB diagnosis will be subjected to levels of radiation that are internationally accepted as standard medical practice.

Data management plan and monitoring

Stored study specimens and digital chest X-rays will be labeled with the participant's study identification number. Study personnel outside of the clinical site will not have access to information that will allow the study identification number to link to individuals. The label will not contain identifying information. The collection of participant data will be done using both paper tools and electronic case report forms (eCRFs). eCRFs will be developed and integrated in the HIPAA-compliant and highly secure Research Electronic Data Capture (REDCap) database. Study staff will be provided individual login details to facilitate their access and entry of collected participant data as needed. Data will be routinely reviewed at the back end by the project data management team who will promptly send out data queries where clarifications or missed data entries are needed.

Study records will be secured in locked file cabinets at all collaborating sites and electronic files will be secured on password-protected computers and institutional intranet drives. Communications with the sites will only use the study identification number to identify participant records. All study staff will receive training in research subjects' protection and confidentiality. Should there be a decision to withdraw, the child's legal guardian has the right to decide whether data and samples from his/her child are to be irreversibly anonymized. Personal and medical information regarding study participants will not be released to anyone other than authorized study personnel without written permission of the participant.

Monitoring of the study processes will be the responsibility of the Principal Investigator. On-site study monitoring will be performed regularly by the internal monitor/QA coordinator in accordance with established policies at all study sites. External study monitors assigned by the sponsor may regularly visit the site to; a) verify compliance with human subjects and other research regulations and guidelines b) assess adherence to the study protocol, study-specific procedures, and local counseling practices, and c) confirm the quality and accuracy of the information collected at the study site and entered into the study database.

Discussion

This study aims to provide important contributions to the evaluation of new diagnostics for pediatric TB. A large cohort of children underfive years of age with signs or symptoms consistent with TB will be included, representing an extremely vulnerable and high-risk group. Children living with and without HIV, and exposed but uninfected children will be recruited from study sites in two different continents. All children will undergo extensive baseline investigations for rigorous classification according to internationally recognized microbiological and composite reference standards. By establishing a comprehensive well-characterized biorepository, the study will also enable the assessment of novel tests as they become available during and after the course of the study.

Abbreviations

CI	Confidence interval
CLHIV	Children living with HIV
CRS	Composite reference standard
eCRF	Electronic case report form
eMRS	Extended Microbiological Reference Standard
FEND-TB	Feasibility of Novel Diagnostics for TB in Endemic Countries
LCA	Latent class analysis
LJ	Löwenstein–Jensen
MGIT	Mycobacteria Growth Indicator Tube
MRS	Microbiological reference standard
Mtb	Mycobacterium tuberculosis
NIH	U.S. National Institutes of Health
NPA	Nasopharyngeal aspirate
NOD for TB	Novel and Optimized Diagnostics for Pediatric TB in Endemic Countries
POC	Point-of-care
REDCap	Research Electronic Data Capture
SRS	Strict reference standard
STARD	Standards for Reporting of Diagnostic Accuracy Studies
TB	Tuberculosis
TST	Tuberculin skin test
Xpert Ultra	Xpert MTB/RIF Ultra

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12887-025-05554-3.

Supplementary Material 1

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Authors' contributions
RS, ED, AP, MR, SK, ND, PS, DA, SD, JE, MJ and AK made substantial contributions to the conception and design of the work. EB, GK, EN, FB, NK, NM and CU made substantial contributions to the acquisition of the data. RS, EB, FB, NK and NM drafted the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Local ethical approval has been obtained in Uganda from School of Biomedical Sciences Research Ethics Committee (SBSREC) and Uganda National Counsel for Science and Technology (UNCST) and in Peru from Universidad Peruana Cayetano Heredia Research Ethics Committee (Comité Institucional de Ética en Investigación – Humanos) and the Research Ethics Committees from Hospital Nacional Cayetano Heredia and Hospital Nacional Docente Madre Niño San Bartolomé, and local approval from the National TB program and local registration in PRISA repository (Nº: EI00003204) following local regulations before study commencement. Ethical approval has also been obtained from Rutgers University IRB and the Oxford Tropical Research Ethics Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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