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Evaluation of molecular diagnostic test for detection of adult pulmonary tuberculosis: A generic protocol

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***Background & objectives*: Tuberculosis (TB) continues to be the second most-leading cause of death due to a single infectious agent as of 2022 after COVID-19. Many affordable new molecular diagnostic tools are being developed for early and more accurate diagnosis, especially for low-resource settings in low- and middle-income countries. In this context, there is a need to develop a standardized protocol for validation of new diagnostic tools. Here, we describe a generic protocol for multi-centric clinical evaluation of molecular diagnostic tests for adult pulmonary TB.**

***Methods*: This protocol describes a cross-sectional study in TB reference laboratories in India. Adults (>18 yr) visitng the chest clinics or outpatient departments with symptoms of TB need to be enrolled consecutively till the required sample size of 150 culture positives and 470 culture negatives are met. *Mycobacterium tuberculosis* (Mtb) culture (mycobacteria growth indicator tube liquid culture) to be used under this protocol as the gold standard and Xpert MTB/RIF molecular test will be used as the comparator. The sputum samples will be tested by smear microscopy, Mtb culture, Xpert MTB/RIF and index molecular test as per the proposed algorithm. The specificity sensitivity, and positive/ negative predictive values are to be calculated for the index test with reference to the gold standard.**

***Discussion*: TB diagnosis poses many challenges as it differs with type of disease, age group, clinical settings and type of diagnostic tests/kits used. Globally, different protocols are used by several investigators. This protocol provides standard methods for the validation of molecular tests for diagnosis of adult pulmonary TB, which can be adopted by investigators.**

Key words Adult pulmonary TB - diagnosis - evaluation protocol - molecular test - multi-centric study - open RT-PCR

Tuberculosis (TB) is still a major health problem globally, with high rates of mortality as well as morbidity. Globally, 7.5 million people were estimated to be newly diagnosed with TB with an estimated death due to TB being 1.3 million in 2022[1]. The southeast Asia region reports the highest TB burden; India alone accounting for over a quarter (28%) of incident TB cases and a third (36%) of TB deaths[1]. Many people with TB are missed and remain undiagnosed by ongoing national programme; an estimated 4.1 million

globally, including 0.6 million in India[1], mostly among those with paucibacillary and non-pulmonary disease. Accurate and prompt diagnosis and treatment of TB cases are crucial to improve individual patient outcomes and prevention of onward community transmission. Reducing the vast TB burden and progressing towards elimination are global public health priorities. Internationally, the agreed targets for improved TB control globally are not achievable with the existing tools[2].

Currently the available tests for active TB diagnosis include smear microscopy, culture, molecular tests and chest radiography[3]. Smear microscopy although rapid has poor sensitivity ranging from 20 to 80 per cent[3]. *Mycobacterium tuberculosis* (MTB) culture, which is currently gold standard, suffers from a long incubation time (4 to 14 days for liquid culture and three to six weeks for solid culture)[4]. Progress in the molecular detection of TB is based on quicker and simpler nucleic acid amplification tests (NAAT) which have resulted in decreasing the time and increasing the sensitivity of diagnosis. Of these, Xpert MTB/rifampicin (RIF) (Cepheid Inc., CA, USA) is the most commonly used test with a short turnaround time (2-3 h). The pooled sensitivity and specificity of Xpert MTB/RIF, which included 70 studies are reportedly, 85 and 98 per cent, respectively[5]. The next generation of Xpert MTB/RIF and Xpert Ultra reportedly showed higher sensitivity of 88 per cent in a multi-country study[6].

The Truenat MTB assay (Molbio Diagnostics, New Delhi) is an indigenous molecular test that runs on a portable, battery-operated platform, and has a higher sensitivity of 88.3 per cent compared against the culture[7]. In 2020, the World Health Organization (WHO) recommended the Truenat MTB or MTB Plus assay as the preliminary diagnostic test for TB instead of smear microscopy/culture[8]. Despite many advantages, these closed NAAT systems have witnessed low uptake in many settings due to high cost of the machines and cartridges, lack of required infrastructure, requirement for specialized laboratories, trained personnel and other technical challenges[9]. The availability of open real-time polymerase chain reaction (RT-PCR)-based molecular kits for MTB detection reduces these limitations and decreases the cost substantially. WHO's End TB strategy emphasizes upon early diagnosis and intensified research and innovation. The global target set in 2023 during the second United Nations High-Level Meeting on TB includes 100 per cent coverage of rapid diagnostic testing for TB[1]. The aim of this protocol is to provide a standardized method to evaluate the diagnostic accuracy of any new NAAT test with reference to the gold standard culture and in comparison to the existing approved standard NAAT test.

Given the unmet clinical and public health needs, it is urgent to rigorously validate promising new molecular tests in a well-designed prospective study in routine clinical practice. Here, we describe a generic protocol for the diagnostic evaluation of new molecular test kit(s) compared to the microbiological reference standard for detection of adult pulmonary TB. Since the addition of drug resistance testing to every sample increases the test cost and logistics, this protocol focuses on the detection of MTB only. A second step of drug resistance may be introduced for samples detected to be MTB positive.

Material & Methods

Study design: We propose a cross-sectional diagnostic test study to determine the sensitivity and specificity of a new index molecular test for detection of pulmonary TB in individuals with presumptive TB diagnosis using mycobacteria growth indicator tube (MGIT) culture as the microbiological reference standard.

Study population: The study should include presumptive adult pulmonary TB cases attending hospital outpatient department (OPDs)/clinics/district microscopy centres (DMCs) and directly observed therapy short-course centres. All such consecutive cases willing to provide consent should be enrolled in the study.

Definition of presumptive tuberculosis (TB): Individuals with any of the following symptoms and signs can be considered as individuals with 'presumptive TB'[10]: cough for two weeks or more, fever for two weeks or more, significant weight loss, haemoptysis and any abnormality in chest radiograph (one or more of the following findings by standardized interpretative criteria: cavitary lesion(s), apical infiltrates, hilar lymphadenopathy, new infiltrates and other suggestive radiological findings).

Study sites: A multi-centric study for the assessment of index test(s) needs to be undertaken independently; a minimum of four centres from different geographical regions representative of Pan-India to be included for the assessment of each index test. Preferably, the centres approved by the national TB elimination programme

(NTEP) to be selected for the study. These centres must be involved in routine diagnosis of TB with facility for culture and molecular testing. Different operators need to be assigned to Xpert MTB/RIF (used in current TB programme in India) and the index test (the one to be assessed) to avoid bias at the level of laboratories. Similarly, the results of MGIT culture will be recorded independently by different laboratory personnel.

Inclusion criteria: The following criteria are recommended for choosing the study participants: *(i)* aged >18 yr; *(ii)* with presumptive pulmonary TB; *(iii)* individuals consenting for study participation; *(iv)* individuals willing to provide sputum specimen.

Exclusion criteria: The follwong exclusion criteria are recommended: *(i)* individuals unable to produce 2 sputum samples of >3 ml; *(ii)* individuals receiving one or more anti-TB medication for >96 h before screening as they would be likely to produce dead bacilli in their sputum, which could be detected by the new molecular assay but not growing in culture.

Diagnostic tests:

(i) Reference test: MGIT culture (gold standard)/ reference standard
(ii) Index test: new NAAT test under evaluation
(iii) Comparator: standard NTEP-approved NAAT test (Xpert MTB/RIF used currently in the TB control programme in India)

As the index test would be a NAAT-based molecular diagnostic test, it would be necessary to compare the performance of a NAAT test with another standard NAAT-based test. Since Xpert MTB/RIF is the test in use under 'National TB Elimination Programme' (NTEP) and WHO-approved, Xpert MTB/RIF would be used as a comparator in such a study.

Index tests: The new molecular diagnostic tests using open RT-PCR-based kits needs to be compatible with various commercially available RT-PCR systems (*e.g.* BIORAD CFX96, ABI7600, Roche LightCycler® 480, *etc*). The index-test kit needs to include gene-specific probes for detection of amplified target DNA sequences of MTB complex. The target genes can be identified by a signal emitted by the reporter dyes or fluorophores [e.g. Texas Red/Cy5, FAM (5-carboxyfluorescein)/HEX (phosphoramidite)] attached to the probes. After extraction of DNA from the sputum samples, 5 to 10 µl of the extracted DNA template needs to be added to the reaction mix and run on an RT-PCR machine for up to 40 cycles or as specified by the manufacturer.

Enrolment: Spectrum bias can be avoided by enrolling a consecutive series of individuals adequately representing the population in which the test will be used. All eligible individuals presenting to the hospital OPD or DMC or clinic of the selected site are to be screened for TB to identify presumptive TB cases. After obtaining written informed consent from such individuals, demographic details of the individuals including their name, age, gender, address, contact details, prior history of TB treatment, clinical history, diagnosis of HIV and radiological findings should be documented in a case record form as per the NTEP norms. All screening evaluations to be completed and reviewed to confirm that potential participants met all the eligibility criteria.

Sample size: The anticipated sensitivity of an index test needs to be kept at 90 per cent and specificity 99 per cent. The aim of the study would be to estimate the sensitivity with an absolute precision of 5 per cent (L=0.05) on either side with a confidence level of 95 per cent and the specificity with an absolute precision of one per cent (L=0.01). A higher precision for specificity would be required to minimize false positivity. The formulas used for calculating sample size for estimating sensitivity and specificity are as follows:

For sensitivity: $n \geq \frac{z^2_{1-\alpha/2} \times S_N(1-S_N)}{L^2 \times \text{Prevalence}}$

where,

$z_{1-\alpha/2}$ = 1.96 for 95 per cent level of confidence

S_N = anticipated sensitivity

L = Absolute precision as desired on either side (half-width of CI) of sensitivity (margin of error)

Prevalence = Anticipated prevalence of the disease

For specificity: $n \geq \frac{z^2_{1-\alpha/2} \times S_P(1-S_P)}{L^2 \times (1-\text{Prevalence})}$

where,

$z_{1-\alpha/2}$ = 1.96 for 95 per cent level of confidence

S_P = Anticipated specificity,

L = Absolute precision as desired on either side (half-width of CI) of specificity (margin of error)

Prevalence = Anticipated prevalence of the disease

The prevalence of culture positives among presumptive cases in hospital setting has been reported to be 24 per cent[11] (Prevalence = 0.24). With this assumption, the minimum sample size requirement has been calculated as 577 for estimating sensitivity and 501 for estimating specificity with the required precision. The loss due to indeterminate samples is expected to be five per cent. Thus, the sample size required is as follows:

(i) For sensitivity: 608
(ii) For specificity: 528
(iii) The higher of these two is 608.

Thus, a total of 610 consecutive cases meeting the inclusion and exclusion criteria would be enrolled and their sputum samples would be tested as per the algorithm as shown in the Figure. At the expected rate of 24 per cent culture positivity among presumptive TB cases in hospital OPD-setting, this number of enrolled cases would be expected to provide nearly 146 positives and 464 negatives for MTB by the gold standard culture. These can be approximated to 150 and 470, respectively. Enrolment would be continued till the required number of participants are covered.

Sample collection, processing and storage: Two sputum samples, each of minimum 3 ml, to be collected from each presumptive TB case: one spot and one morning sample. Chances to have lesser bacterial load in spot sample would prevail. Since morning sputum sample carries higher bacterial load, two samples are usually collected to have sufficient sputum volume and bacterial load. Especially in a validation study involving multiple tests, it would be preferable to collect two samples for processing culture and molecular tests.

Sputum samples collected from each individual with presumptive TB need to be processed as per the algorithm depicted (Figure) under standard biosafety conditions. Briefly, the sputum samples need to be partially homogenized using sterile beads. Approximately, 1 ml from each spot and morning sample to be pooled under sterile conditions. From the pooled sample, one ml may be used for molecular testing following the manufacturer's instructions for both standard NAAT (Xpert MTB/RIF) and index test(s).

The remaining portion of each sputum specimen should be used for direct smears and decontamination by *N*-acetyl-l-cysteine–sodium hydroxide (NaLC-NaOH) method[12]. The resulting deposit is to be used for concentrated smear preparation and inoculation into two MGIT960 tubes. Lowenstein–Jensen (LJ) slopes may also be used for culture, only for storage or retesting purpose (in case of contamination of all MGIT tubes). All positive cultures can be identified using the rapid immunochromatographic test (ICT) within five days of instrument positivity; interpretation of the results to be done as per the manufacturer's instructions. For each paticipant, one positive culture and two decontaminated samples will be stored at −80°C for potential future use if necessary. The extracted DNA may also be stored at −20°C until the end of the study to resolve any discrepant results.

Laboratory procedures: All conventional test procedures for smear, culture (solid and liquid) and Xpert MTB/RIF will be performed as per the NTEP national laboratory guidelines[12] and laboratory manual of the Indian Council of Medical Research (ICMR)-NIRT[13]. The standard operating procedure (SOP) for index test(s) provided by the manufacturer(s) including the use of positive and negative controls need to be followed. All procedures for preparation of media, reagents, washing, decontamination, disposal and storage to be performed according to the SOP of ICMR-NIRT.

Tests: *(i)* Smear microscopy: one direct smear and one concentrated sputum smear
(ii) MGIT culture: two tubes per specimen – a total of four MGIT tubes for two specimens per participant
(iii) Identification of culture: rapid immunochromatographic test (ICT) of MGIT culture
(iv) Xpert MTB/RIF: one test per participant.

Quality control (QC) measures: All sites should ensure high quality of laboratory procedures, data recording and documentation without any deviation from the protocol. All the study sites should participate in internal quality control (IQC) as well as external quality assurance (EQA) for all methods. The standard manuals of the global laboratory initiative[14] and the WHO practical manual[15] to be adapted to the local laboratory requirements.

Culture: Positive (reference strain H37Rv or H37Ra) and negative controls for MGIT and LJ cultures need to be tested as per the NTEP guidelines. It is required that MGIT time to the detection QC for MTB reference strain is performed every month/new lot of reagents/ machine service. Sterility and performance testing of

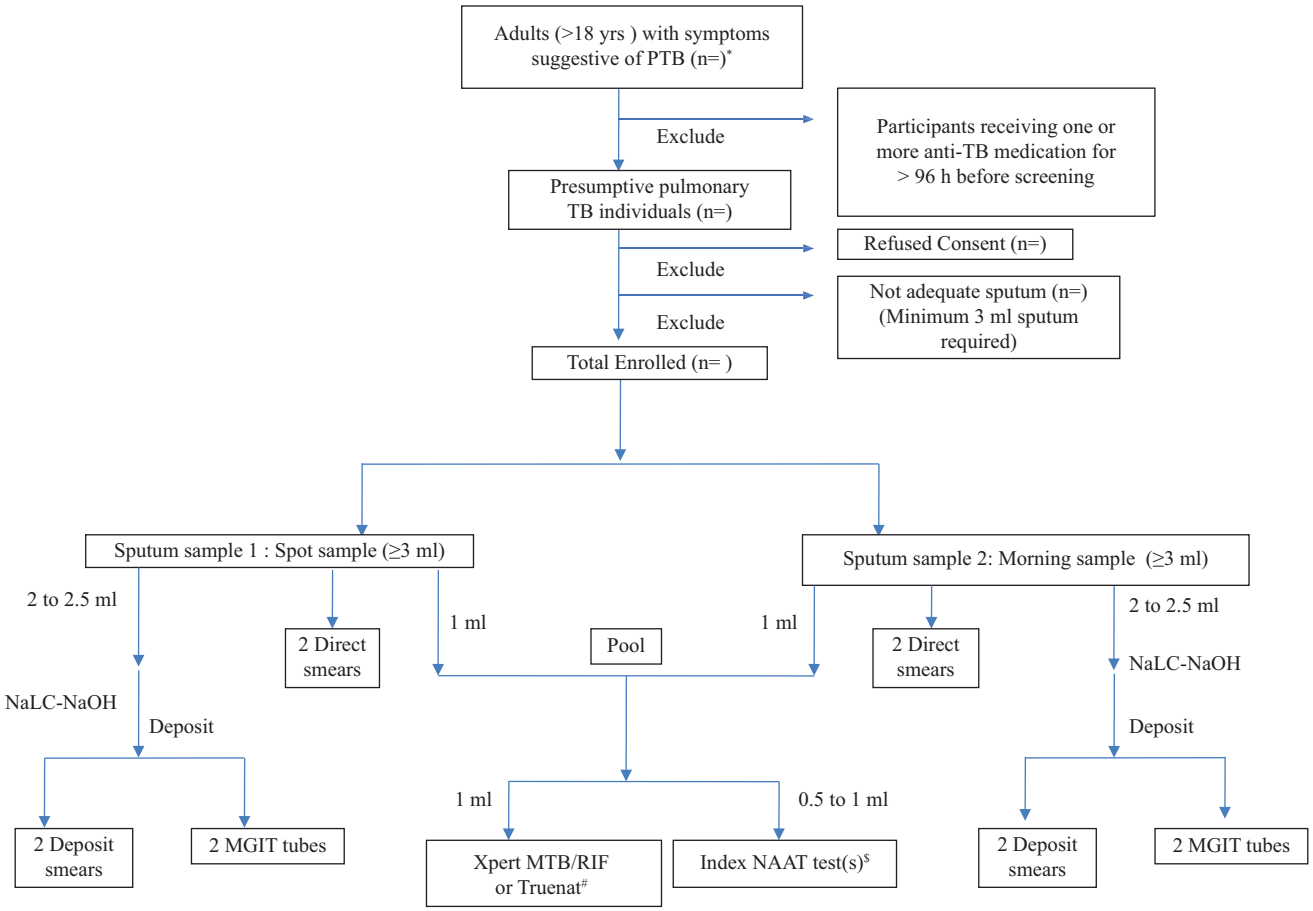

Figure. Schematic flow through of the proposed protocol. *Screening: Medical history & clinical examination as per NTEP guidelines. #Comparator: GeneXpert as recommended by NTEP. $Index Test: New NAAT Test under evaluation. Storage: Sites to process samples for additional testing and storage as per their routine practice (1-MGIT/1-LJ as per practice). One positive culture and 2 decontaminated samples per patient will be stored at -80ºC for later use, if necessary 2 DNA samples will be stored at -20ºC for resolution of discrepant results. PTB, pulmonary tuberculosis; ATT, anti-tuberculosis treatment; MGIT, mycobacteria growth indicator tube; MTB, *Mycobacterium tuberculosis*; RIF, rifampicin; NAAT, nucleic acid amplification test.

culture media should be performed with every new batch or lot.

Drug sensitivity testing (DST): The standard American Type Culture Collection strains will be used for each drug as reference control. QC will be performed everytime with a new batch of drugs prepared, after servicing of the instrument and after long gap of setting up DST.

Smear: A positive (MTB H37Rv) control smear and a negative control (*e.g. Escherichia coli*) smear should be included in each batch of tests daily and with new lots of reagents.

ICT Identification of *Mycobacterium tuberculosis* (MTB) complex: Culture of MTB reference strain in MGIT broth could be used as a positive control. Culture of Mycobacteria other than TB (*e.g.* a well-characterized strain of *Mycobacterium avium* complex/ *Mycobacterium kansasii*) in MGIT broth could serve as a negative control.

Molecular diagnostics: For molecular diagnostics, IQC would include a control supplied by the manufacturer. EQA for molecular diagnostics is available in India and offered by the Central TB Division through its national reference laboratories (*https://www.naateqa.in/Presentation*). EQA programme for Xpert MTB/RIF would contain at least five samples to test the reproducibility, repeatability and accuracy of the assay and need to be carried out at least once a year by the participating laboratories. Each panel for Xpert MTB/RIF consists of *(i)* MTB not detected, *(ii)* MTB detected – low, *(iii)* MTB detected – medium and *(iv)* MTB detected – high. All the laboratories participating in the study would be part of the EQA programme.

Table I. Primary data analysis

Cases	Interpretation of NAAT under evaluation
Index positive/ culture positive*	True positive irrespective of smear or standard NAAT test result
Index negative/ culture negative	True negative irrespective of smear or standard NAAT test result
Index negative/ culture positive*	It should be considered as false negative for the index test
Index positive/ culture negative	It should be considered as false positive for the index test

*A study participant will be considered as culture positive only if two or more MGIT cultures (out of four culture tubes) show growth of MTB complex; all other results will be counted as culture negative for primary analysis. MGIT, mycobacteria growth indicator tube; MTB, *Mycobacterium tuberculosis*; NAAT, nucleic acid amplification test

Data analysis plan: If the index test produces an error or indeterminate results, one repeat may be allowed. Samples yielding both contaminated and NTM cultures would be excluded from analysis. The diagnostic accuracy of the index test with reference to culture will be estimated based on the primary analysis (Table I). Interpretation of smear and culture results will be based on the definitions given in Supplementary Tables I and II.

Only absolute positives and negatives by culture are to be considered for primary analysis. Ambiguous culture results (only one culture positive and/or late growth without sign of contamination) should not be considered. In order to resolve discrepancies due to experimental errors, secondary analysis may be performed as per Table II using a comprehensive reference standard[7,16] (demonstration of MTB by standard NAAT or smear).

Listing of constraints such as number of errors or indeterminates by the index test(s), procedural/lab specific/instrument specific/site specific limitations of the index test(s) need to be documented (*e.g.* requirement of excessive consumables or exclusive instruments /dedicated man power *et*c). These constraints would be given due consideration while making policy recommendations. Level at which the index test may be employed, *e.g*. DMC, TB unit and district TB centre or reference lab (IRL/NRL) would also be evaluated based on the constraints such as steps required for extraction and amplification, requirements of additional consumables, biosafety level, skilled staff and time taken.

Statistical analysis: The index molecular test needs to be evaluated for its specificity, sensitivity, accuracy positive as well as negative predictive value. A 95 per cent confidence interval need to be calculated for each of the above indicators as given below:

95 % CI for sensitivity = sensitivity ± 1.96 ($SE_{sensitivity}$)

95 % CI for specificity = specificity ± 1.96 ($SE_{specificity}$)

The sensitivity and specificity calculation framework to be used with reference to MGIT culture is given below as a Table III.

$$\%\,\text{Sensitivity}\frac{\text{True positives}}{\text{Total positives by MGIT culture}}\times 100=\left[a/(a+c)\right]\times 100$$

$$\%\,\text{Specificity}\frac{\text{True negatives}}{\text{Total negatives by MGIT culture}}\times 100=\left[d/(b+d)\right]\times 100$$

The positive and negative predictive values will be calculated with reference to MGIT culture as given below:

Table II. Secondary data analysis

Cases	Further action performed to resolve discrepancies
Culture positive but index negative	Secondary analysis -See if standard NAAT test (Xpert MTB/RIF) is positive: If standard NAAT test is also positive, it will be considered false negative for index test -If standard NAAT test is negative, culture results should be verified with smear from culture (to rule out contamination). Possibility of NTM culture should be ruled out by performing ICT from MGIT culture. -If smear is AFB positive, it will be considered as false negative for index test. If smear is AFB negative, this will be true negative for index test
Culture negative but index positive	Secondary analysis -If standard NAAT is positive, it will be considered as positive for index test -If standard NAAT (Xpert MTB/RIF) is also negative, it will be considered as false positive for index test

RIF, rifampicin; AFB, acid-fast bacilli; NTM: nontuberulous mycobacteria; ICT, immunochromatographic test

Table III: 2×2 contingency table

Index NAAT test	MGIT culture	
	Positive	Negative
Positive	a	b
Negative	c	d

% Positive predictive value (PPV) of index NAAT test in presumptive TB cases:

$$\%\,PPV \frac{\text{True positives}}{\text{Total positives by index Test}} \times 100$$
$$= \left[a / (a+b)\right] \times 100$$

% Negative predicted value (NPV) of index NAAT test in presumptive TB cases:

$$\%\,NPV = \frac{\text{True negatives}}{\text{Total negatives by index test}} \times 100$$
$$= \left[d / (c+d)\right] \times 100$$

Descriptive statistics on participant characteristics and estimates of diagnostic accuracy should be reported separately for relevant subgroups serving as a proxy for disease spectrum or severity. The acceptable kappa coefficient range would be 0.81-1, indicating almost perfect agreement between the index test and the standard test. Standards for Reporting of Diagnostic Accuracy Studies guidelines[17] would be followed for reporting the findings.

Ethics and dissemination: The study should be compliant with the ICMR-National Ethical Guidelines for Biomedical and Health Research involving Human Participants. The institutional ethics committee of each participating site should be intimated about the study for necessary approval prior to initiating the study.

(i) Written informed consent should be obtained from the participants prior to the study.

(ii) Two sputum specimens need to be collected from presumptive TB cases as per the algorithm (Figure). No additional sputum or any other specimens will be collected. Probability of harm or discomfort anticipated in the research is expected to be nil or very unlikely.

(iii) The confidentiality of research participants should be ensured by encrypting the personal identifiers of the participants.

(iv) Dignity of research participants will be prioritized.

(v) No compensation to be provided to the participants since there would be no additional cost or travel involved in sample collection for the study. Participants to be compensated for travel and time only if they are asked to pay additional visits exclusively for the sake of the study and not during regular treatment visits.

(vi) The investigators should not have any conflicts of interest (financial or non-financial). Affiliations in any forms and sources of funding must be declared in advance.

(vii) The findings of the study should be made accessible through reports.

(viii) The anti-tuberculosis treatment (ATT) decisions will not be made based on the result of the index test under evaluation, rather on the basis of routine clinical and laboratory tests only (smear, solid/liquid culture, standard NAAT results and clinical workup).

(ix) Follow-up visits will be required for a few discrepant cases to exclude TB as per NTEP-guideline.

(x) There should be no delay in reporting and participants should be given the best possible diagnostic investigations.

(xi) Sputum samples collected should not be stored for any future research. The stored pellets will only be needed to resolve discrepant cases. One positive culture and two DNA samples per participants may be stored at −80°C for later usage.

(xii) At all the sites all study participants should be followed up till the final diagnosis is made and an individual is initiated on appropriate treatment as per the NTEP norms. Those found to be MTB positive by standard NAAT test may be put on ATT by a medical officer of the study site as per the NTEP guidelines.

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Supplementary Table I. Definitions for 'per specimen' analysis

Definitions	Description
Smear-positive specimen	≥1+ smear (≥10/100 fields)
Scanty-positive specimen	1-9 AFB per 100 fields
Culture-positive specimen	MGIT culture growth <42 days*, confirmed by smear from culture and ICT for MTB complex
Culture-negative specimen	MGIT culture that do not exhibit growth within 42 days, and no organism seen on the Gram and ZN-stained smears
Contaminated culture	MGIT: Instrument flashes positive but without detection of AFB. However, other organisms must be demonstrated on Gram stain from the culture Specimens with >2 contaminated cultures will not be included in result analysis
Other molecular test results	As per the manufactures instruction/software if the controls are valid, then proceed to results and interpretations. If negative or positive controls are invalid, all of the specimens and controls from that run must be reprocessed, beginning with sample preparation

*In majority of clinical samples, growth of MTB is detected within 28 days of incubation. AFB, acid fast bacilli; MTB, *Mycobacterium tuberculosis*; MGIT, mycobacteria growth indicator tube; ICT, immuno-chromatographic test; ZN; Ziehl-Neelsen
Source: Ref 14

Supplementary Table II. Operational case definitions

Definition	Description
Smear-positive case	≥1 smears of 1+ or more (out of 4 smears) or ≥2 scanty-positive (out of 4 smears)
Smear-negative case	1 scanty positive smear or only negative smears
Culture positive case	≥2 positive out of 4 MGIT culture tubes All MGIT-positive tubes will be confirmed by smear from culture and ICT for MTB complex NTM: Patient samples which show growth of mycobacteria other than MTB complex only will not be included in analysis Patients >2 contaminated cultures will be excluded from analysis
Culture-negative case	≥3 negative out of four MGIT culture tubes (culture does not exhibit growth within 42 days, and no organism seen on the Gram and ZN-stained smears)
Indeterminate cases (excluded)	Patients with <2 specimens examined; smear-positive, culture-negative cases Cases with >2 contaminated cultures (out of 4 MGIT cultures) Cases with mycobacterium other than tuberculosis only and standard or index NAAT positive Indeterminate cases will be excluded from analysis

NAAT, nucleic acid amplification test; MTB, *Mycobacterium tuberculosis*; NTM, non-tuberculous mycobacteria.
Source: Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, *et al*. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 2010; *363* : 1005-1015