Xpert MTB/RIF Ultra is highly sensitive for the diagnosis of tuberculosis lymphadenitis in an HIV-endemic setting

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Abstract

Background: Tuberculosis lymphadenitis (TBL) is the most common extrapulmonary TB (EPTB) manifestation. Xpert MTB/RIF Ultra (Ultra) is a World Health Organization-endorsed diagnostic test, but performance data for TBL, including on non-invasive specimens, are limited.

Methods: Fine needle aspiration biopsies (FNABs) from outpatients (≥18 years) with presumptive TBL (n=135) underwent: 1) routine Xpert (later Ultra once programmatically available), 2) a MGIT 960 culture (if Xpert- or Ultra-negative, or rifampicin-resistant), and 3) study Ultra. Concentrated paired urine underwent Ultra. Primary analyses used a microbiological reference standard (MRS).

Results: In a head-to-head comparison (n=92) of FNAB study Ultra and Xpert, Ultra had increased sensitivity [91% (95% confidence interval 79, 98) vs. 72% (57, 84); p=0.016] and decreased specificity [76% (61, 87) vs. 93% (82, 99); p=0.020], and detected patients not on treatment. HIV nor alternative reference standards affected sensitivity and specificity. In patients with both routine and study Ultras, the latter detected more cases [+20% (0, 42); p=0.034] and, further indicative of potential laboratory-based room-for-improvement (e.g., specimen processing optimisation), false-negative study Ultras were more inhibited than true-positives. Study Ultra false-positives had less mycobacterial DNA than true-positives [trace-positive proportions 59% (13/22) vs. 12% (5/51); p<0.001]. “Trace” exclusion or recategorization removed potential benefits offered over Xpert. Urine Ultra had low sensitivity [18% (7, 35)].

Conclusions: Ultra on FNABs is highly sensitive and detects more TBL than Xpert. Patients with FNAB Ultra-positive “trace” results, most of whom will be culture-negative, may
require additional clinical investigation. Urine Ultra could reduce the number of patients needing invasive sampling.

250/250
**Background**

Tuberculosis (TB) is a leading cause of morbidity and mortality globally. In 2019, extrapulmonary TB (EPTB) represented 16% of new TB cases reported [1] and, in HIV-positive populations, can account up to 50% of all TB cases [2]. TB lymphadenitis (TBL) accounts for 35% of all EPTB [3, 4]. South Africa, with high TB and HIV burden [1], is particularly affected by EPTB and TBL.

TBL is typically diagnosed by examining fine needle aspiration biopsies (FNABs) from affected lymph nodes. This requires specialised sampling and facilities, and tests have suboptimal sensitivity [5]. One widely-used test is Xpert MTB/RIF (Xpert; Cepheid, USA); a semi-automated real-time PCR that rapidly detects *Mycobacterium tuberculosis* complex (MTBC) DNA and rifampicin resistance [6, 7]. A systematic review and meta-analysis showed heterogeneity in the sensitivity of FNAB Xpert vs. microbiological [83% (95% confidence interval: 71, 91)] and composite reference standards [81% (72, 88)] [8]. Specificities were 94% (88, 97) and 99% (95, 100), respectively [8]. Most EPTB diagnostic algorithms recommend culture after a negative Xpert [9], however, this creates delay. Better TBL tests are needed.

One potential test is Xpert MTB/RIF Ultra (Ultra), which offers improved sensitivity over Xpert for pulmonary TB, partly enabled by, in addition to *rpoB*, amplification of multi-copy insertion elements (IS6110, IS1081) [10]. Data on Ultra for TBL are emerging: one retrospective evaluation tested ten Xpert-negative, culture-positive FNABs and found half to be Ultra-positive [11]; another retrospective evaluation (n=25) reported sensitivity and specificity of 94% (71-77) and 100% (63-72), respectively [12]; and a prospective evaluation (n=73) reported a sensitivity and specificity of 78% (40-97) and 78% (66-87), respectively.
No studies included head-to-head Xpert and Ultra data. Additionally, since Ultra’s advent, algorithms for TBL diagnosis remain essentially unchanged from the Xpert era – culture is still recommended in Ultra-negative patients. Whether this is needed or, conversely, if culture is needed to confirm positive Ultra results due to specificity concerns associated with the new trace semi-quantitation category [10, 14], requires investigation.

Lastly, FNABs are rarely collected in primary care; patients are referred to district or tertiary facilities, resulting in care cascade gaps [15]. If an Ultra has high sensitivity and specificity on an easily accessible fluid like urine, the need for invasive sampling could be mitigated; potentially drastically reducing provider and patient economic and time costs, including those associated with referral. To our knowledge, urine Ultra for TBL specifically is unevaluated.

We evaluated the head-to-head diagnostic accuracy of Xpert and Ultra on FNABs, and Ultra on urine in patients with presumptive TBL in a tertiary hospital setting in an HIV-endemic in South Africa. We hypothesised Ultra would show improved sensitivity compared to Xpert.
Methods and materials

Ethics statement

The study was approved by the Stellenbosch University Human Research Ethics Committee and Tygerberg General Hospital (TGH) (both N16/04/050).

Patient recruitment

135 outpatients (≥18 years) with presumptive TBL (swollen lymph node) undergoing routine referral and investigation at a tertiary referral clinic at TGH in Cape Town, South Africa, were consecutively recruited from 25 January 2017-12 March 2019 and gave FNABs and urine. Patients who received TB treatment ≤60 days prior were excluded.

Fine needle aspirate collection

FNABs were collected by multiple needle passes using a 23-gauge needle and 10 ml syringe. While the needle was inserted, negative suction with a cutting motion was applied for aspiration. The first two passes were used for routine cytology. From each pass, two slides were prepared: the first air-dried for Rapidiff staining and the second spray-fixed for Papanicolaou staining (~25 μl total volume used per pass) (Figure 1). The remaining syringe contents were flushed into 1.5 ml TB transport medium [16]. The third pass (5-50 μl) was collected into 700 μl 5% saline (Ysterplaat Medical Supplies, Cape Town, South Africa).

Xpert, Ultra, and culture

Routine testing: Xpert (version 1; Cepheid, USA) was done programmatically from 25 January 2017–9 April 2018 by the government programmatic laboratory [National Health Laboratory Service (NHLS)] who did Ultra (version 1) thereafter [17]. Sample reagent (2 ml; Cepheid, USA) was added to 500 μl of aspirate-containing 1.5 ml TB transport medium (4:1...
ratio) and 2 ml of the mixture used for Xpert or Ultra [18, 19]. Per the algorithm, if a specimen was Xpert- or Ultra-positive and rifampicin-susceptible, culture was not done. If Xpert- or Ultra-negative, or Xpert- or Ultra-positive and rifampicin-resistant, 500 μl aspirate-containing TB transport medium was inoculated into a MGIT960 liquid culture without NALC-NaOH decontamination (Figure 1). If a non-actionable (not positive or negative) [14] Xpert or Ultra occurred, the remaining 500 μl TB transport medium was used to repeat the test.

*Study testing:* The third pass in 700 μl saline was tested with Ultra (cartridge version 3; study Ultra) using a 2:1 sample reagent ratio [19]. Study Ultra was done irrespective of whether routine Xpert or Ultra was done.

*MTBC typing and drug susceptibility testing:* MTBDRplus was done on culture-positive isolates for speciation and drug susceptibility testing.

*Urine Ultra*

5-20 ml urine stored at -80 °C were centrifuged (1811xg, 10 min, room temperature) and the supernatant removed until 700 μl remained, which was tested with Ultra (2:1 sample reagent volume ratio) [19].

*Patient treatment and follow-up*

Treatment decisions were programmatic without study involvement (no study results reported for patient management). Attempts were made to telephonically follow-up patients at least 12 weeks after recruitment at which point TB treatment initiation status were recorded and, if treatment started, treatment response was queried. Patients were lost-to-follow-up if at least two calls were unsuccessful, and messages were unreturned for each timepoint.
Definitions

Patient groups: Patients were designated definite, probable, or non-TB using different reference standards. For the microbiological reference standard (MRS), definite TB was culture-positive and/or cytology-positive on FNABs, and non-TBs culture- and cytology-negative on FNABs. Unclassifiable patients had no positive MRS test, culture contaminated or not done, and cytology not done. Supplementary Table 1 has the extended microbiological standard (eMRS), composite reference standard (CRS), and probable TB definitions.

Other definitions: Xpert or Ultra actionable results for TB were MTBC-detected and rifampicin-susceptible, rifampicin-resistant or rifampicin-indeterminate, or MTBC not detected [14]. For culture, actionable results were positive or negative for MTBC. For cytology, the presence or absence of granulomatous inflammation was recorded.

Statistical analysis

We included patients in head-to-head analyses if they had actionable routine index test (Xpert or Ultra), study Ultra, and culture results (or, if culture was non-actionable, a cytology result was available). Proportion tests [20] were done using STATA version 16.0 (StataCorp, College Station Texas, USA) and GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, USA). Venn diagrams were made with InteractiVenn [21]. Differences in diagnostic accuracy metrics were calculated using proportion tests or McNemar’s test as appropriate. STARD guidelines were followed [22]. We excluded the probable TBs from the primary analysis due to few patients meeting this definition.
Results

Patient characteristics

Of 135 patients, 44% (59/135) were definite TB and 56% (75/135) non-TBs per the MRS. Characteristics are compared in Table 1.

FNAB index test results

76% (103/135) of patients had routine Xpert requested [6/103 (6%) not done] and 24% (32/135) routine Ultra requested [3% (1/32) not done]. Non-actionable results for routine Xpert, routine Ultra and study Ultra were 0% (0/97), 6% (2/31), and 3% (4/135), respectively. 41% (40/97) of routine Xpers were positive (remainder negative). For routine Ultra, 38% (11/29) were positive and, for study Ultra, 74/313 positive (56%; p=0.070 vs. routine Ultra) (Figure 2). In a head-to-head comparison of patients with actionable results from each test (study Ultra, routine Xpert, culture, cytology) 37% (22/59), 8% (5/59), 20% (12/59) and 24% (14/59) were positive by each test (Figure 3A; study Ultra had the highest yield). 12% (7/59) of these patients with at least one positive result were exclusively detected by study Ultra (cytology exclusively detected two). This proportion detected only by study Ultra (and hence were negative by routine Xpert and/or cytology) increased to 22% (13/59) when culture results, which are not available for rapid clinical decision making, were omitted.

Diagnostic accuracy and yield of study Ultra and routine Xpert on FNABs

Overall: When Ultra was compared head-to-head to Xpert using the MRS (n=92) (Table 2), Ultra had improved sensitivity [91% (95% confidence interval: 79, 98) vs. 72% (57, 85); p=0.016] and decreased specificity [76% (61, 87) vs. 93% (82, 99); p=0.020]. Ultra’s positive predictive value (PPV) [79% (66, 89) vs. 92% (78, 98); p=0.114] and negative predictive value (NPV) were like Xpert’s [90% (76, 97) vs. 77% (64, 87); p=0.105]. Conclusions were unchanged for non-head-to-head comparisons or those that used the eMRS or CRS (Table 2,
Supplementary results), which included patients with probable TB (Supplementary Table 2). Compared to MTBDR plus on isolates, no false-negative or false-positive Ultra rifampicin-resistance results occurred, however, numbers were small, precluding precise accuracy estimates (Supplementary Results).

HIV: Sensitivities and specificities did not differ in HIV-positives vs. -negatives for study Ultra or routine Xpert (Table 2). Within HIV-positives, Ultra had improved sensitivity [97% (82, 100) vs. 76% (56, 96); p=0.022] and similar specificity [79% (59, 92) vs. 93% (76, 99); p=0.127] to Xpert.

Trace semi-quantitation exclusion or reclassification: When study Ultra traces were excluded, sensitivity [-1% (-17, 11); p=0.836] and specificity [+7% (-9, 24); p=0.400] were unchanged. When trace results were reclassified as negative, sensitivity decreased [-13% (-25, 1); p=0.014] and specificity increased [+9% (-2, 19); p=0.046] (Table 2).

Ultra PCR inhibition: An analysis of sample processing control (SPC) C_T values (Supplementary Figure 1; higher values indicate more inhibition) showed more inhibition in study Ultra positives than -negatives [25.80 (IQR: 24.78-27.33) vs. 25.20 (24.55-26.05); p=0.024]. Furthermore, false-negatives were more inhibited than true-positives [26.10 (25.10-28.60) vs. 25.10 (24.00-25.50); p=0.001]; suggesting inhibition contributes to diminished sensitivity.

Relationship with bacterial load: Neither Study Ultra nor routine Xpert C_T correlated with bacillary load measured using culture time-to-positivity (Supplementary Figure 2) in FNABs.

Comparison of study Ultra true-positive and false-positives

False-positives had less bacterial load than true-positives [IS6110/IS1081 C_T 19.00 (IQR: 16.40-21.60) vs. 24.85 (19.88-28.15); p<0.001], a greater proportion were hence “trace” [59% (13/22) vs. 12% (6/51); p<0.001] (Table 4). Less inhibition was also observed for the
former group [SPC C_T 25.05 (24.45-25.95) vs. 26.10 (25.10-28.60); p=0.005]. More study
Ultra true-positives were on treatment at follow-up than Ultra false-positives [92% (44/48)
vs. 27% (6/22); p<0.001] as more true-positives were positive using a routine test than the
false-positives [98% (50/51) vs. 27% (6/22); p<0.001]. The proportions of patients with
previous TB in false- vs. true-positives were similar [27% (6/22) vs. 35% (18/51); p=0.503].
The characteristics of true- and false-positives are in Table 4 and false-positives per patient
information in Supplementary Table 3.

Study vs. routine Ultra FNAB results
Concordance: In patients who received both study and programmatic Ultras, 55% (17/31)
were study Ultra-positive and 35% (11/31) routine Ultra-positive. The former detected +20%
(95% confidence interval: 0, 42) more TBL (Table 3).
PCR inhibition: SPC C_T analysis showed no difference between study and routine Ultra
[25.10 (IQR: 24.35-25.85) vs. 25.50 (24.20-26.50); p=0.081] (Supplementary Figure 1A).

Urine Ultra yield, sensitivity and specificity, and non-actionable results
Urine Ultra had low sensitivity [18% (7/35)] and high specificity [98% (88, 100)] (head-to-
head comparisons with FNAB study Ultra in Supplementary Table 4). Of concentrated
urines tested with (n=84), 8% (7/84) were non-actionable and 100% (7/7) of these resolved to
actionable when unconcentrated urine was tested (one unconcentrated urine was now Ultra-
positive). None of the 18 HIV-negative patients had any positive urine Ultra. 12% (7/57)
HIV-positives were urine Ultra-positive (six of seven detected by both positive MRS and
study Ultra FNAB result; Figure 3C). In other words, when urine Ultra was attempted
amongst HIV-positives, 11% (7/64, 3 of which were trace) were positive, meaning that
universal concentrated urine Ultra testing in HIV-positives with presumptive TBL could
reduce the number of FNABs required for TB diagnosis as few are non-actionable.
Patient treatment status at follow-up

96% (130/135) of patients were followed-up [median (IQR: 37 (16-65) weeks since recruitment] and 52% (68/130) initiated treatment. Of the 48% (62/130) not initiated on treatment, 11% (7/62) were definite TB and 89% (55/62) non-TB, of which 57% (4/7) and 29% (16/55) were study Ultra-positive, respectively. In these definite TBs not on treatment yet detected by study Ultra, 50% (2/4) were detected by routine Ultra or Xpert, meaning that study Ultra was the only rapid PCR test available in half of these cases (these two definite-TBs were pre-treatment loss-to-follow-ups). Regarding the clinical status of patients initiated on treatment, 94% (64/68) reported treatment completion and, of these, 94% (60/64) reported being clinically well. 3% of the patients followed-up (4/130) died [one of these four was study Ultra-positive (routine Ultra-negative, Xpert-negative)] and 100% (4/4) of decedents were non-TB) and not placed on treatment.
Discussion

Our key findings are: 1) study Ultra on FNABs had, compared to Xpert, improved sensitivity and decreased specificity, and outperformed routine Ultra (tests unaffected by HIV, alternative reference standards, and probable TBs); 2) approximately 3 in 10 study Ultra-positives had not been placed on treatment, indicating opportunities to improve TBL treatment with Ultra; 3) excluding study Ultra trace results improved specificity (more so than reclassifying to negative) without large sensitivity costs relative to treating Ultra trace results as positive; 4) Urine Ultra had low sensitivity but could reduce the proportion of presumptive TBL patients who require a FNAB in our setting, and 5) Ultra false-negative results are associated with PCR inhibition. These data show high sensitivity of Ultra on FNABs for TBL with the inclusion of trace-positive results (without which sensitivity benefits over Xpert are not seen).

Ultra on FNABs had increased sensitivity than Xpert, suggesting Ultra is a rapid initial test for TBL. Ultra did still not detect, however, approximately 1 in 10 TBL cases; indicating a sustained need for more sensitive tests (especially those that use non-invasive specimens) and a continued role for reflex tests for downstream testing of Ultra-negative FNABs.

Importantly, like was done previously for Xpert [23], we showed one likely cause of Ultra false-negativity is increased PCR inhibition (which could be caused by mucopurulent or viscous samples as seen in sputum [23]), suggesting that optimised specimen processing workflows to better remove interfering agents are still needed to boost sensitivity.

Notably, Ultra had suboptimal specificity (two in ten MRS-negative people were study Ultra-positive). One reason may be that culture and cytology have limitations as reference standards for EPTB [8]. Notably, this finding mirrors prior work on TBL that used tissue in addition to fluid biopsies for Ultra, where a specificity of 78% vs. culture was observed [13].
However, when compared to an eMRS including microbiological tests such as FNAB culture as well as culture and Ultra on non-site-of-disease fluids, FNAB Ultra specificity was 100% in that study. In contrast, we applied microbiological tests only to FNABs and did not exhaustively sample anatomical sites [24], which might underestimate specificity.

Ultra false-positivity was more frequent in patients with less mycobacterial DNA and, in contrast to pulmonary TB, FNAB Ultra false-positivity was not associated with prior TB [14]. The true nature of these Ultra “false-positives” in EPTB requires clarification and is an important topic for future research (in our setting, most “false-positive” patients with presumptive pulmonary TB remain well without treatment) [25, 26]. Such “false-positive” results could be caused by *M. tuberculosis* in FNABs that are not culturable using conventional methods like MGIT960. For example, in animal models, *M. tuberculosis* DNA in lymph nodes is detectable during re-activation of TB, despite no pathological evidence of disease and no culturability. *M. tuberculosis* is hypothesised to then disseminate throughout the body from the lymph node [27]. Moreover, we observed no correlation in bacterial load measured using between Ultra and culture, further supporting the presence of *M. tuberculosis* DNA in the absence of culturability.

Critically, if Ultra trace results were excluded or reclassified to elevate specificity, Ultra would lose sensitivity benefits versus Xpert, however, this sensitivity loss was less for the former than the latter strategy; suggesting exclusion is the preferred strategy for handling trace results.

When routine and study Ultra concordance were analysed, study Ultras had higher yield. This may be due to specimen processing (e.g., more sample reagent is used for routine Ultras compared to study Ultras) or cartridge version differences (which includes extending product
stability and improving product manufacturability without affecting assay performance) but is overall indicative of an area to improve the diagnosis of TBL within the programme.

Few studies examined Ultra on urine [28-30] and none in patients investigated for TBL. Urine Ultra may obviate the need for invasive sampling (and hence referral to a specialised facility, and associated costs and delays). Despite concentration [31], low yield and sensitivity were observed for urine Ultra, suggesting it could marginally reduce FNAB collection (approximately 1/10). Such a strategy is undermined by elevated non-actionable result rates and cost effectiveness, including the number-needed-to-test, would require prospective investigation and modelling, however, we expect the utility of such an approach to be further enhanced with better urine tests (we did not have access to FujiFILM SILVAMP) [32].

These results have strengths and limitations. Our study was pragmatic and routine culture not always done and, although our MRS included cytology, multiple cultures (including on specimens from other anatomical sites) may improve specificity estimates. Furthermore, multiple FNAB passes were done to obtain adequate volumes that could have introduced sampling variation, however, FNABs were collected using a standardised protocol by a single health worker. Additionally, a third of the FNAB collected for routine testing was used for Ultra and had more sample reagent (SR) buffer added for testing (4:1 ratio of SR buffer to sample; the programmatic standard-of-care) compared to study Ultras, in which the full sample collected was used and a 2:1 ratio of SR buffer added. A different cartridge version (version 1) was used in routine testing whereas study Ultra used a later version (version 3), however, in an internal head-to-head version evaluation done by the programmatic laboratory (as part of separate study), no sensitivity differences between different versions were observed (most changes between versions were done to improve stability and optimise
manufacturing) meaning that it is unlikely that these version differences in routine vs. study

Ultra accounted for any meaningful performance differences. Thus, the differences observed

primarily appear to be due to sampling and specimen processing differences, which improved

sensitivity and yield, however, our study was not designed to quantify the contribution of

each component that deferred between routine and study Ultra testing.

In conclusion, in a routine clinical setting in patients with presumptive TBL, Ultra detects

more TBL than Xpert and would result in more people placed on treatment. This is driven by

the added benefit of trace results. Furthermore, programmatic Ultra testing can be optimised

on the diagnostic laboratory front as study Ultra had better performance than that done

routinely. Urine Ultra could reduce invasive sampling and associated delays but there

remains a need for better urine-based tests for TBL. We recommend that a positive FNAB

Ultra result be used to initiate treatment, however, patients with a negative Ultra still require

confirmatory testing and many patients with a trace-positive result will be culture-negative.

Our study supports Ultra’s use for TBL diagnosis.
References


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Figure legends

Figure 1: Specimen collection and diagnostic testing in participants with presumptive TB lymphadenitis. Abbreviations: FNAB, fine needle aspirate; TB, tuberculosis; Ultra, Xpert MTB/RIF Ultra; Xpert, Xpert MTB/RIF.

Figure 2: Overview of different FNAB-based test results. Tests done as part of the routine diagnostic algorithm (Xpert later replaced by Ultra, cytology, and culture) and the study (Ultra) are shown. Study Ultra detected TB in most culture-positive FNABs and some culture-negative FNABs. Italicised text indicates programmatic testing (programmatic algorithm adherence imperfect). Data are n/N (%). Abbreviations: RIF, rifampicin; TB, tuberculosis; Ultra, Xpert MTB/RIF Ultra; Xpert, Xpert MTB/RIF.

Figure 3: Venn diagrams showing positive results from different FNAB tests (after the 104th participant, Ultra was routinely done instead of Xpert) and urine Ultra. (A) Study Ultra, routine Xpert, culture and cytology results in 59 patients. Study Ultra was positive in seven FNABs undetected by routine Xpert. (B) Routine Ultra results relative to Study Ultra, routine Ultra, culture, and cytology in 19 patients. Study Ultra was exclusively positive in 36% (7/19) FNABs not detected by routine Ultra, culture and cytology, and had the highest yield. (C) Urine Ultra results relative to FNAB study Ultra and the MRS in 57 HIV-positive patients (Urine Ultra negative in all HIV-negatives). Urine Ultra detects less TBL than FNAB study Ultra but could obviate the need for TB diagnostic FNABs in some patients. Data are n/N (%). Abbreviations: FNAB, fine needle aspirate; MRS, microbiological reference standard; TB, tuberculosis; Ultra, Xpert MTB/RIF Ultra; Xpert, Xpert MTB/RIF.
Patient with presumptive TB lymphadenitis undergoing routine investigation

Multiple syringe passes of the lymph node

1st and 2nd Pass (~50 μl each)

Cytology (~25 μl each)

2 smears (~12.5 μl each)

Xpert or Ultra (500 μl)

Negative Xpert or Ultra

MGIT960 Culture

Positive Xpert or Ultra, rifampicin-resistant

MGIT960 Culture

Positive Xpert or Ultra, rifampicin-susceptible

No MGIT960 Culture

Routine testing

3rd Pass (~50 μl)

Urine (5-20 ml)

Saline (700 μl)

Concentration by centrifugation

Ultra (700 μl)

Initial patient follow up (with a second one if patient started treatment)

Study testing
Patients with presumptive TB lymphadenitis (n=135)

From the 104th patient, Ultra was done routinely instead of Xpert

**Xpert results**

- Actionable Xpert results: 97/103 (94%)
  - Xpert-positive: 40/97 (41%)
  - Xpert-negative: 57/97 (59%)

**Culture results**

- Culture-negative: 45/54 (83%)
  - Study Ultra:
    - Positive: 9/45 (20%)
      - RIF-resistant: 1/9 (11%)
      - RIF-susceptible: 3/9 (33%)
      - RIF-indeterminate: 5/9 (56%)
    - Negative: 36/45 (80%)

- Cytology:
  - Positive: 4/45 (9%)
    - Ultra-positive: 2/4 (50%)
    - Ultra-negative: 2/4 (50%)
  - Negative: 41/45 (91%)

**Ultra results**

- Actionable Ultra results: 29/32 (91%)
  - Ultra-positive: 11/29 (38%)
  - Ultra-negative: 18/29 (62%)

- Culture-negative: 17/18 (94%)
  - Study Ultra:
    - Positive: 7/17 (41%)
      - RIF-resistant: 0/7 (0%)
      - RIF-susceptible: 0/7 (0%)
      - RIF-indeterminate: 7/7 (100%)
    - Negative: 10/17 (59%)

- Cytology:
  - Positive: 0/1 (0%)
    - Ultra-positive: 0/1 (0%)
    - Ultra-negative: 0/1 (0%)
  - Negative: 1/1 (100%)

**Routine Xpert requested**

- 103/135 (76%)

**Routine Ultra requested**

- 32/135 (24%)

**Culture results**

- Culture-positive: 3/3 (100%)
  - Study Ultra:
    - Positive: 2/3 (67%)
      - RIF-resistant: 1/2 (50%)
      - RIF-susceptible: 0/2 (0%)
      - RIF-indeterminate: 1/2 (50%)
    - Negative: 1/3 (33%)

- Cytology:
  - Positive: 0/1 (0%)
    - Ultra-positive: 0/1 (0%)
    - Ultra-negative: 0/1 (0%)
  - Negative: 1/1 (100%)

**Not done**

- 6/103 (6%)

- 1/32 (3%)

**RIF-susceptible**

- 10/11 (91%)

**Culture-positive**

- 1/1 (100%)

**Not done**

- 6/103 (6%)

- 1/32 (3%)

**RIF-susceptible**

- 10/11 (91%)

**Culture-positive**

- 1/1 (100%)
''One routine Xpert-positive, rifampicin (RIF)-susceptible patient had a contaminated culture but was study
Ultra-positive, RIF-resistant and 32 routine Xpert-positive, rifampicin (RIF)-susceptible patients had no culture
per the **Figure 1** algorithm.

''One routine Ultra was trace-positive RIF-indeterminate.

Culture should not normally be requested per the routine algorithm for these patients but was nevertheless
done.

Ultra results under cytology subheadings (in the last row of boxes) are routine and not study Ultras.

Missing data: In patients with a routine Xpert-negative result, one had a contaminated culture and two were
culture not done. Two routine Ultras were non-actionable. Three FNABs did not have cytology done.
Figure 3

A

B

C

Culture
(3/19)
16%
Cytology
(5/19)
26%
Routine Ultra
(2/19)
11%
Study Ultra
(8/19)
42%
Culture
(14/59)
24%
Cytology
(12/59)
20%
Routine Xpert
(5/59)
8%
Study Ultra
(22/59)
37%
MRS
(28/57)
49%
FNAB study Ultra
(35/57)
61%
Urine Ultra
(7/57)
12%
Table 1: Demographic and clinical characteristics by microbiological reference standard status. Definite TBs were more likely to be younger, have an involved neck or breast lymph node (vs. another anatomical site) and, if HIV-positive, a lower CD4 count than non-TBs. Data are n (%) or median (IQR).

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<thead>
<tr>
<th>Demographics</th>
<th>Overall (n=135)</th>
<th>Definite-TB (n=59)</th>
<th>Non-TB (n=75)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36 (29-46.5)</td>
<td>34 (27-41)</td>
<td>39 (31.5-47.5)</td>
<td>0.019</td>
</tr>
<tr>
<td>Female</td>
<td>72/135 (53)</td>
<td>30/59 (51)</td>
<td>42/75 (56)</td>
<td>0.553</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>77/133 (58)</td>
<td>35/58 (60)</td>
<td>41/74 (55)</td>
<td>0.569</td>
</tr>
<tr>
<td>CD4 count (cells/µl)</td>
<td>183 (66-304)</td>
<td>147 (43-281)</td>
<td>219 (156-358)</td>
<td>0.012</td>
</tr>
<tr>
<td>Previous TB</td>
<td>42/135 (31)</td>
<td>19/59 (32)</td>
<td>22/75 (29)</td>
<td>0.720</td>
</tr>
<tr>
<td>Pulmonary TB</td>
<td>38/42 (90)</td>
<td>17/59 (29)</td>
<td>20/75 (27)</td>
<td>0.807</td>
</tr>
<tr>
<td>Extrapulmonary TB</td>
<td>4/42 (10)</td>
<td>2/59 (3)</td>
<td>2/75 (3)</td>
<td>0.807</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Involved site</th>
<th>Overall (n=135)</th>
<th>Definite-TB (n=59)</th>
<th>Non-TB (n=75)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neck</td>
<td>92/134 (67)</td>
<td>53/59 (90)</td>
<td>39/75 (52)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thorax</td>
<td>16/134 (12)</td>
<td>4/59 (7)</td>
<td>12/75 (16)</td>
<td>0.102</td>
</tr>
<tr>
<td>Breast</td>
<td>9/134 (7)</td>
<td>0/59 (0)</td>
<td>9/75 (12)</td>
<td>0.006</td>
</tr>
<tr>
<td>Other</td>
<td>17/134 (13)</td>
<td>2/59 (3)</td>
<td>15/75 (20)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Missings data: HIV, two; CD4, four; lymph node site, one. One patient was unclassifiable based on case definitions. "Other" sites include arm (n=3), leg (n=3), groin (n=7), and head (n=4).
Table 2: Diagnostic accuracy analyses (non-head-to-head above, head-to-head below) of routine Xpert and study Ultra on FNABs using a MRS compared to routine Xpert but lower specificity. The relative performances of Xpert and Ultra had similar patterns by HIV status and versus the eMRS or CRS (Supplementary Table 2). Data are %, 95% CI, and n/N.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>HIV-negative</th>
<th>HIV-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=96</td>
<td>n=50/96 (62)</td>
<td>n=49/96 (57)</td>
</tr>
<tr>
<td></td>
<td>n=30</td>
<td>n=57/96 (63)</td>
<td>n=57/96 (63)</td>
</tr>
<tr>
<td>Non-head-to-head</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xpert</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>72 (57, 84)</td>
<td>91 (82, 99)</td>
<td>92 (73, 99)</td>
</tr>
<tr>
<td>Specificity</td>
<td>33/46</td>
<td>33/56</td>
<td>32/46</td>
</tr>
<tr>
<td>PPV</td>
<td>86 (58, 95)</td>
<td>92 (80, 98)</td>
<td>91 (72, 99)</td>
</tr>
<tr>
<td>NPV</td>
<td>35/53</td>
<td>35/53</td>
<td>35/53</td>
</tr>
<tr>
<td>Xpert</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>72 (57, 84)</td>
<td>91 (82, 99)</td>
<td>92 (73, 99)</td>
</tr>
<tr>
<td>Specificity</td>
<td>33/46</td>
<td>33/56</td>
<td>32/46</td>
</tr>
<tr>
<td>PPV</td>
<td>86 (58, 95)</td>
<td>92 (80, 98)</td>
<td>91 (72, 99)</td>
</tr>
<tr>
<td>NPV</td>
<td>35/53</td>
<td>35/53</td>
<td>35/53</td>
</tr>
<tr>
<td>Ultra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76 (61, 81)</td>
<td>79 (66, 89)</td>
<td>67 (51, 80)</td>
</tr>
<tr>
<td>Specificity</td>
<td>42/46</td>
<td>42/56</td>
<td>35/42</td>
</tr>
<tr>
<td>PPV</td>
<td>87 (55, 92)</td>
<td>86 (55, 91)</td>
<td>79 (59, 90)</td>
</tr>
<tr>
<td>NPV</td>
<td>39/72</td>
<td>39/72</td>
<td>39/72</td>
</tr>
<tr>
<td>A if traces excluded</td>
<td>-1 (1, 1)</td>
<td>-3 (1, 1)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>p=0.0142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A if traces reclassified</td>
<td>+18 (8, 29)</td>
<td>+13 (8, 28)</td>
<td>+14 (7, 9)</td>
</tr>
<tr>
<td>p=0.0011</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head-to-head</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xpert</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>72 (57, 84)</td>
<td>91 (82, 99)</td>
<td>92 (73, 99)</td>
</tr>
<tr>
<td>Specificity</td>
<td>33/46</td>
<td>33/56</td>
<td>32/46</td>
</tr>
<tr>
<td>PPV</td>
<td>86 (58, 95)</td>
<td>92 (80, 98)</td>
<td>91 (72, 99)</td>
</tr>
<tr>
<td>NPV</td>
<td>35/53</td>
<td>35/53</td>
<td>35/53</td>
</tr>
<tr>
<td>Ultra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>76 (61, 81)</td>
<td>79 (66, 89)</td>
<td>67 (51, 80)</td>
</tr>
<tr>
<td>Specificity</td>
<td>42/46</td>
<td>42/56</td>
<td>35/42</td>
</tr>
<tr>
<td>PPV</td>
<td>87 (55, 92)</td>
<td>86 (55, 91)</td>
<td>79 (59, 90)</td>
</tr>
<tr>
<td>NPV</td>
<td>39/72</td>
<td>39/72</td>
<td>39/72</td>
</tr>
<tr>
<td>A if traces excluded</td>
<td>-1 (1, 1)</td>
<td>-3 (1, 1)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td>p=0.0142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A if traces reclassified</td>
<td>+18 (8, 29)</td>
<td>+13 (8, 28)</td>
<td>+14 (7, 9)</td>
</tr>
<tr>
<td>p=0.0011</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ if traces reclassified</td>
<td>-13 (±23, 11) p=0.014 6</td>
<td>+9 (±2.19) p=0.046 6</td>
<td>+5 (±11.20) p=0.576 6</td>
</tr>
</tbody>
</table>

504 Missing data in the non-head-to-head table: Unclassifiable Ultra, n=1; non-actionable Ultras, n=4; HIV, n=2.
505 Within column p-values: Xpert vs. Ultra within an analysis (non-head-to-head, head-to-head) in patients of the same HIV status (overall, negative, positive).
506 Within row p-values: HIV-negative vs. HIV-positive within an analysis (non-head-to-head, head-to-head).
507 Abbreviations: CI, confidence interval; CRS, composite reference standard; eMRS, extended microbiological reference standard; FNABs, fine needle aspirate biopsies; MRS, microbiological reference standard; NPV, negative predictive value; PPV, positive predictive value; Ultra, Xpert MTB/RIF Ultra; Xpert, Xpert MTB/RIF.
Table 3: Study and routine Ultra concordance in patients with both tests done on FNABs.

More patients were positive by study Ultra (55%) compared to routine Ultra (35%), corresponding to a 20% incremental yield. Study Ultra had no non-actionable results (column not shown).

<table>
<thead>
<tr>
<th>Routine Ultra</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>Non-actionable</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Δ Study Ultra vs. routine Ultra +20% (95% confidence interval; CI: 0.42) p=0.034

Non-actionable Ultra results included ‘Error’ (n=1) and ‘No result’ (n=1).

Abbreviations: Ultra, Xpert MTB/RIF Ultra; FNABs, fine needle aspirate biopsies.
Table 4: Comparison of patient and microbiology characteristics by whether study Ultra was TP or FP per the MRS. FPs were less likely to have been placed on treatment, had less bacterial load, and were less likely to have been detected by routine Xpert and routine Ultra than TPs. An individual breakdown of each Ultra-positive, MRS-negative patient is in Supplementary Table 3. Data are n (%) or median (IQR).

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Ultra TPs</th>
<th>Ultra FPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-positive</td>
<td>31/51 (61)</td>
<td>13/22 (59)</td>
</tr>
<tr>
<td>CD4 count (cells/µl)</td>
<td>147.0 (32.00-281.30) (n=30)</td>
<td>208.0 (101.3-286.0) (n=12)</td>
</tr>
<tr>
<td>Previous TB</td>
<td>18/51 (35)</td>
<td>6/22 (27)</td>
</tr>
<tr>
<td>Patients initiated on TB treatment after 12-week follow-up</td>
<td>44/48 (92)</td>
<td>6/22 (27)</td>
</tr>
<tr>
<td>If on treatment, did the patient report improved health?</td>
<td>43/44 (98)</td>
<td>6/6 (100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study Ultra result information</th>
<th>Ultra TPs</th>
<th>Ultra FPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB CT&lt;sub&gt;min&lt;/sub&gt;</td>
<td>25.70 (20.20-28.20) (n=45)</td>
<td>25.70 (20.40-29.10) (n=9)</td>
</tr>
<tr>
<td>IS6110/IS1081 CT</td>
<td>19.00 (16.40-21.60) (n=51)</td>
<td>24.85 (19.88-28.15) (n=22)</td>
</tr>
<tr>
<td>Trace semi-quantitation category</td>
<td>6/51 (12)</td>
<td>13/22 (59)</td>
</tr>
<tr>
<td>SPC CT</td>
<td>26.10 (25.10-28.60) (n=51)</td>
<td>25.05 (24.45-24.95) (n=22)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Routine Xpert or routine Ultra information</th>
<th>Ultra TPs</th>
<th>Ultra FPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Xpert</td>
<td>31/42 (74)</td>
<td>3/11 (27)</td>
</tr>
<tr>
<td>Positive Ultra</td>
<td>7/7 (100)</td>
<td>3/10 (30)</td>
</tr>
</tbody>
</table>
Missing data: CD4 count, n=2; patients who were lost to follow-up, n=3; unclassifiable routine Xpert results, n=3. True positive in routine Xpert era not done, n=1; True positive in routine Ultra era non-actionable, n=1; False positive in routine Ultra not done, n=1.

Abbreviations: FP, false-positive; IS6110/IS1081 Cₚ, cycle threshold value for the Xpert MTB/RIF Ultra IS6110/IS1081 probe; rpoB Cₚmin, minimum cycle threshold value from the Xpert MTB/RIF (Ultra) rpoB probes; TP, true-positive; Ultra, Xpert MTB/RIF Ultra. *Study Ultra results were not reported for potential patient management.