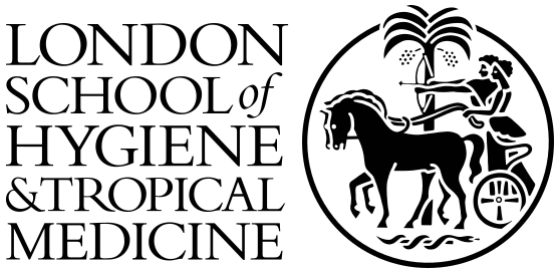

Same day tools, including Xpert Ultra and unstimulated IFN-γ, for the rapid diagnosis of pleural tuberculosis – a prospective observational study.

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Abstract

Introduction:

The diagnosis of pleural tuberculosis (TB) is problematic. The comparative performance of newer same day tools for pleural TB including Xpert MTB/RIF Ultra (ULTRA) has, hitherto, not been comprehensively been studied.

Methods:

Adenosine deaminase (ADA), Inter-Gam Ultrasensitive Rapid Immuno-suspension Assay (IRISA-TB), Xpert MTB/RIF, and ULTRA performance outcomes were evaluated in pleural fluid samples from 149 patients with suspected pleural TB. The reference standard was culture positivity (fluid, biopsy or sputum) and/or pleural biopsy histopathology (definite-TB). Those with non-TB were microbiologically test negative and were not initiated on anti-TB treatment. To determine the effect of sample concentration, 65 samples underwent pelleting by centrifugation followed by conventional Xpert MTB/RIF and ULTRA.

Results:

Of the 149 patients, 49 had definite-TB, 16 probable-TB (not definite but treated for TB) and 84 non-TB. ULTRA sensitivity (95% CI) and specificity was similar to Xpert MTB/RIF [37.5% (25.3-51.2) versus 28.6% (15.9-41.2)] and [98.8% (96.5-100) versus 98.8% (96.5-100)], respectively. Centrifugation did not significantly improve ULTRA sensitivity (29.5% vs. 31.3%, respectively). Adenosine deaminase and IRISA-TB sensitivity was 84.4% (73.9 – 95.0) and 89.8% (81.3– 98.3), respectively. However, IRISA-TB demonstrated significantly better specificity [96.4% vs. 87.5% (p= 0.034)], positive-predictive value [93.6% vs. 80.9 (p= 0.028)] and positive-likelihood ratio [25.1 vs. 6.8 (p= 0.032)] than ADA.

Conclusion:

Xpert ULTRA has poor sensitivity for the diagnosis of pleural TB. Alternative assays (ADA and IRISA-TB) are significantly more sensitive, with IRISA-TB demonstrating a higher specificity and rule-in value compared to ADA in this high TB and HIV-endemic setting.

Word Count: 240/250

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Introduction

Tuberculosis (TB) remains a global health problem with 10 million new cases attributable to the disease in 2017 (1). Although pulmonary TB is the predominant form of the disease, extra-pulmonary TB (EPTB) accounts for approximately 25% of active cases (2), with pleural TB being the a common manifestation of EPTB (3), if not the most common in several settings (4-6). The diagnosis of pleural TB is difficult due to the paucibacillary nature of the disease and the need for invasive sampling including blind or image-guided, or surgical open pleural biopsy (7). Diagnosis using pleural fluid is, in reality, the norm despite several drawbacks including limited sensitivity and specificity.

Xpert MTB/RIF a fully automated quantitative real-time PCR assay, until recently, the frontline test for TB in many endemic countries (8) had a poor yield in pleural TB (using pleural fluid) with a pooled sensitivity of ~25% when using culture and pleural biopsy as a reference standard (9-11).

However, more recently Cepheid developed the next-generation Xpert MTB/RIF Ultra (ULTRA) a multiplex nested PCR assay, which is WHO-endorsed as the new sputum-based frontline TB diagnostic test (12). Its key advantage is a higher sensitivity with the level of detection decreasing from ~130 to ~ 20 organisms/ ml of sample. This ~log difference in sensitivity provided hope that pauci-baciliary TB, including forms of EPTB like pleural TB, could now be more easily diagnosed (13). However, there are no comprehensive studies about the utility of ULTRA in pleural TB and none from endemic countries. A preliminary laboratory-based study detected 10 Ultra positives (sensitivity of ~47%) in selected culture positive pleural fluid samples; given that culture sensitivity is only ~40% the key drawback was one of selection and sampling bias (14). Thus, it is unknown how the performance of

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ULTRA compares with the conventional Xpert MTB/RIF assay for the diagnosis of pleural TB in unselected patients.

Given the drawbacks of microbiological tests and biomarkers to aid in pleural TB diagnosis, such as adenosine deaminase (ADA), have been extensively studied (7) though specificity may be limited at the 30IU/L cut-point (often used in clinical practice) (15). An alternative biomarker, interferon gamma (IFN-γ), an inflammatory cytokine secreted by macrophages and CD4 (+) T cells becomes highly compartmentalised in TB with pooled sensitivity and specificity estimates of 93% and 96%, respectively (3) and even higher sensitivities in high TB burden settings (16, 17). The Inter-Gam Ultrasensitive Rapid Immuno-Suspension Assay (IRISA-TB) is a recently validated and standardised same-day (1.5-hour turn-around time), low-cost immunoassay assay developed to measure unstimulated IFN-γ in EPTB. Its performance relative to ULTRA has, hitherto, not been evaluated.

To address these gaps in our knowledge we performed unbiased evaluation of the 4th generation Xpert cartridge (Xpert MTB/RIF), Xpert ULTRA, ADA and IRISA-TB in consecutively recruited patients in a prospective observational study using a comprehensive composite reference standard comprising culture and pleural biopsy histology.

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Methods

Patient recruitment, categorization and routine laboratory tests.

Patients with suspected pleural TB (any TB symptoms including any cough, fever, night sweats, loss of weight, haemoptysis and/or chest pain, and features consistent with a pleural effusion on chest x-ray) were prospectively recruited from Groote Schuur Hospital in Cape Town, South Africa. The University of Cape Town Human Research Ethics Committee approved the study (HREC: 421/2006 and 919/2014). All patients provided informed consent for study participation.

Pleural fluid was collected by ultrasound-guided pleurocentesis. A closed pleural biopsy, although not routine, was performed using an Abrams needle to aid in patient categorization. Biopsies were collected following aspiration of pleural fluid. Pleural fluid samples were subjected to routine biochemical and cytological analysis by the National Health Laboratory Services (NHLS). This included protein, albumin, ADA, glucose, differential cell counts, cytology, concentrated fluorescence smear microscopy, and liquid culture for M. tuberculosis using the MGIT 960 (Becton Dickinson, Sparks, Maryland). Pleural fluid ADA levels >30U/L, were reported as suggestive of pleural TB in accordance with national guidelines (18). The remaining fluid was bio-banked and frozen at -80°C and subsequently used for ULTRA, Xpert MTB/RIF and IRISA-TB analysis. Pleural biopsy samples were sent for histology and/or liquid culture. When possible, sputum was also collected for routine smear microscopy and liquid culture by the NHLS. HIV testing was performed in consenting patients.

Due to the limitations of a single pleural fluid TB culture for confirming a diagnosis, a composite reference standard was used for patient categorization (and this reference standard was used in all analyses presented). Patients were categorised as follows: (i) Definite-TB:

patients with at least one positive *M. tuberculosis* culture (pleural fluid, biopsy and/or sputum) and/or caseating granulomatous inflammation suggestive of TB on histological examination of pleural biopsy tissue, and with improvement on anti-TB treatment (all patients in this category received anti-TB treatment); (ii) Probable-TB: patients not meeting the criteria for definite-TB but with clinical and radiological indicators suggestive of TB and who were initiated on and responded to anti-TB treatment (all patients in this category received anti-TB treatment); (iii) Non-TB: patients with no microbiological or histological evidence of *M. tuberculosis* and/or an alternative diagnosis was available. These patients did not receive anti-TB treatment either at presentation or on follow-up.

IFN-γ measurement

Interferon-gamma concentrations were measured in pleural fluid supernatants using the IRISA-TB Assay (IRISA-TB; Antrum Biotech Pty Ltd., Cape Town, South Africa) according to the manufacturer's instructions. The assay was performed in duplicate and the average value reported. Pleural fluid supernatant was prepared by centrifuging 1ml of pleural fluid at 3000×g for 15 minutes.

ULTRA and Xpert MTB/RIF assays

Both the ULTRA and Xpert MTB/RIF assays were performed using 1ml of pleural fluid diluted with 2 ml of Xpert sample buffer followed by vigorous mixing. ULTRA and Xpert MTB/RIF cartridges were run on a GeneXpert 4-module machine (Cepheid, Dx System Version 4.7b). To evaluate the effect of sample concentration on ULTRA and Xpert MTB/RIF sensitivity, a median (IQR) of 10 (5-10) ml pleural fluid was centrifuged at 3000×g for 15 minutes and the corresponding pellet was resuspended in 1ml of PBS. The sample was

then processed as described for unconcentrated samples. PCR inhibition was evaluated by comparing the PCR cycle-threshold (Ct) values of the internal positive control (lyophilized *Bacillus atrophaeus* subsp. *globigii* spores) from neat and concentrated samples. The limit of detection (LOD) of ULTRA and Xpert MTB/RIF was determined in triplicate by serially diluting H37*Rv* CFUs (0 to 125 CFU/ml) into 1ml aliquots of non-TB pleural fluid sample. The limit of detection (LOD) of ULTRA and Xpert MTB/RIF was determined by spiking 1ml pleural fluid samples with known concentrations of H37Rv (0 to 125 CFU/ml). An H37Rv stock solution was aspirated several times using a fine gauge needle to prevent aggregation, followed by performing serial dilutions into a 0.25% Tween-80/PBS solution, as performed in previous studies (17, 19, 20). Equal volumes of each dilution were then added to 1ml of non-TB pleural fluid samples in triplicate. The CFU/ml of each dilution was confirmed by enumeration on OADC-enriched 7H10 agar.

Statistical analysis

Diagnostic accuracy, including 95% confidence intervals (95% CIs), was assessed using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the receiver operator curve (AUROC) in definite-TB and non-TB groups. Unpaired and paired categorical variables were compared using the $\chi 2$ and McNemar test, respectively. Continuous variables were compared using Student's t-test where appropriate. The Mann-Whitney and Wilcoxon Rank Sum test was used for unpaired and paired non-parametric continuous variables, respectively. Statistical analyses were performed using GraphPad Prism (version 6.0), Medcalc Version 18.6 and Microsoft Excel. PPV, NPV and likelihood ratios were compared by DEAD, Semiquant (https://semiquant.shinyapps.io/DEAD/).

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Results

Clinical and demographic data

A total of 165 patients were recruited into the study; 49 subjects had definite-TB, 84 were classified as non-TB and 16 subjects were classified as probable TB. Those with non-TB effusions had a spectrum of malignant and non-malignant diagnoses including lymphoma, adenocarcinoma, small cell carcinoma, and parapneumonic effusion. An additional 16 patients had insufficient clinical data to be categorized in the above groups and were subsequently excluded. A study overview is provided in Figure 1. Demographic and clinical data are summarized in Table 1.

Performance outcomes of IRISA-TB

The median (IQR) of the IFN-γ levels (n=149) were significantly higher in definite-TB compared to non-TB pleural effusions: [198.7pg/ml (93.4-298.2) vs. 0.0pg/ml (0.0-0.0), $p<0.0001$; Figure 2A]. Using Definite and Non-TB groups, a ROC curve-derived rule-in cut-point of 20.5pg/ml (Figure 2B, the sensitivity (95% CI), specificity, PPV and NPV of IRISA-TB was 89.8% (81.3-98.3), 96.4% (92.4-100), 93.6% (86.6-100) and 94.2% (89.2-99.1), respectively. Table 2 compares the diagnostic accuracy of IRISA-TB with other same-day diagnostics in the definite-TB versus non-TB groups.

Performance outcomes of pleural fluid ADA

Median (IQR) of the ADA levels (n=140) were approximately 5 times higher in definite-TB compared to non-TB effusions [55.6 (41.7-65.9) vs. 12.0 (1.0-22.4) U/L, $p<0.0001$]. Using a clinical cut point of >30 U/L (18), the sensitivity (95%CI), specificity, PPV and NPV of

ADA were 84.4% (73.9-95.0), 87.5% (79.9-95.1), 80.9% (69.6- 92.1) and 90.0% (83.0-97.0), respectively (Table 2). The scatter plot and ROC of ADA are shown in Figures 2A and 2B.

Performance outcome of ULTRA and Xpert MTB/RIF

The sensitivity (95%CI) of ULTRA (n=149) was marginally better than Xpert MTB/RIF [37.5% (23.8-51.2) vs. 28.6% (15.9-41.2), p=0.393; Table 2]. Pleural fluid concentration did not significantly improve the sensitivity of either ULTRA or Xpert MTB/RIF, [29.5% vs. 31.3% and 29.5% vs. 33.4%, respectively; Table 3]. The median (IQR) cycle-threshold values of the Xpert MTB/RIF internal positive control was significantly different between neat and concentrated samples, [26.3 vs. 25.55, p=0.0483] but not when using ULTRA (Figure S1). ULTRA had a lower LOD compared to Xpert MTB/RIF (18.7 CFU/ml vs. ≥76.2 CFU/ml of pleural fluid, respectively; Figure S2). Furthermore, the lowest dilution to provide a trace positive result by ULTRA was 8.8 CFU/ml.

Discussion

Given that a reliable same-day diagnostic tool for pleural TB is still lacking, we prospectively evaluated the utility of ADA, IRISA-TB, Xpert MTB/RIF and the recently released ULTRA assay for the diagnosis of pleural TB. Our key findings were that (i) ULTRA sensitivity was no better than the conventional Xpert MTB/RIF despite a lower *in vitro* limit-of-detection, (ii) the ULTRA sensitivity was not improved by pelleting of larger volumes of pleural fluid, (iii) ADA and IRISA-TB had significantly higher sensitivity for pleural TB compared to molecular tests, and (iv) compared to ADA, IRISA-TB had significantly better specificity and positive predictive value making it the ideal rule in test for pleural TB (though it also had a very high NPV in a high burden setting thus prompting clinicians when to search for alternative diagnoses that may mandate pleural biopsy and thoracoscopy). There are a number of strengths and novel aspects of our study. It is the first study to comprehensively evaluate Xpert Ultra in patients with suspected pleural TB (and directly against the Xpert G4 cartridge), it is the largest study to date (149 participants) to evaluate a nucleic acid amplification test, ADA, and unstimulated IFN-γ in tandem, the first study to evaluate Ultra for pleural TB in the context of HIV co-infection, and evaluated an updated version of the IFN-γ assay. The use of a composite reference standard (culture and histopathology) better reflects the true performance of each assay (as culture alone is an imperfect gold standard in this context).

The ULTRA cartridge, which incorporates a larger input sample volume and two different multi-copy amplification targets (*IS6110* and *IS1081*) results in approximately 10-fold improvement in the lower limit of detection *in vitro* using spiked *M.tb* (13), yet the sensitivity in clinical samples remained suboptimal and not much different from the conventional

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MTB/RIF cartridge. This is most likely due to the immune-mediated and paucibacilliary nature of pleural TB, which remains below the detection limit (even) of ULTRA. The only published data on ULTRA in pleural TB comes from a low burden setting using samples from different extra-pulmonary sites, and that only included a small number (n=24) of pleural fluid samples (14). Furthermore, culture positivity was used as the reference standard resulting in sample and selection bias (only 40% of pleural TB is culture positive), which may have overestimated test specificity. Given that culture positivity self-selects for higher burden of microbiological disease, restricting analysis to this sub-group is also likely to overestimate sensitivity. We have also confirmed the limit of detection of the ULTRA was 10-fold lower than Xpert MTB/RIF (8.8 vs. 76.2 CFU/ml, respectively). Pleural fluid is known to have inhibitory molecules which can affect molecular assays (21). However, no PCR inhibition of the positive internal control was seen when using the ULTRA assay, whereas inhibition was seen with the Xpert MTB/RIF assay (but not in a previous study that we performed, presumably due to a sample size effect) (17). Pellet-based concentration of the pleural fluid by centrifugation and resuspension did not improve sensitivity of either ULTRA or Xpert MTB/RIF. The median time to positivity was 22 days, indicating a low bacterial load within the fluid. Furthermore, ULTRA-positive culture-positive samples tended to have a shorter time to positivity than the ULTRA-negative culture-positive ones (data not shown), confirming the perception that pleural fluid is highly paucibacillary (and concentrating of 10ml of pleural fluid is unlikely to improve performance despite ULTRA being more sensitive). Moreover, concentrating volumes larger than 10 ml is unlikely to improve sensitivity as centrifuging as much 100ml of fluid does not improve the diagnostic yield of culture (22), which has a similar limit of detection as ULTRA (13, 23). This is in contradistinction to TB meningitis and genitourinary/ disseminated TB in advanced HIV, where concentrating the (CSF or urine) fluid improves the sensitivity Xpert MTB/RIF (24,

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25). This is likely because TB serositis is more of an immune-reactive disease characterised by a hypersensitivity reaction to TB antigens in addition to mycobacterial invasion of the pleural space. Thus, whilst we have previously shown that CSF (24) and urine centrifugation (25) may improve sensitivity, concentration in the case of a hypersensitivity reaction will have little effect given the very low burden of mycobacteria or TB antigen. The same phenomenon is likely to explain the lack of a concentration effect in TB pericarditis as we have previously shown (26).

In high TB settings such as South Africa, a high ADA level (30 IU/L cut-point) is frequently used to guide initiation of anti-TB treatment. In this study, using the accepted laboratory cut-point of 30 IU/L, about one fifth of the TB patients would have been missed, and close to 1 in every 10 non-TB patients would have erroneously been initiated on unnecessary anti-TB treatment. In high prevalence settings, ADA has satisfactory diagnostic performance but in lower settings the PPV is not clinically useful (27). The most recent meta-analysis reported a sensitivity and specificity of 86% and 88%, respectively (28), confirming the misclassification bias and that 1 out of every 9 or 10 non-TB patients would be erroneously placed on anti-TB treatment (at a 10% disease prevalence this would amount to about 10 additional false TB starts in every 100 patients suspected with pleural TB). The specificity of ADA can be improved if the proportion of lymphocytes are taken into account (27). However, this was not routinely requested by the attending clinician and was not expressly part of our study protocol. Furthermore, a significant proportion (~25%) of pleural effusions are neutrophil predominant (29) and lymphocyte counts in pleural fluid are not widely accessible (for the same reason it is not frequently requested in our setting). As such, this analysis was not performed.

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IRISA-TB sensitivity, like that of ADA, was significantly higher than ULTRA highlighting that tests requiring to express detection of *M.tb* will always struggle to reach the sensitivity of immunodiagnostic tests (which can greatly reduce the need for invasive biopsy procedures). Indeed, we have confirmed our previous findings, and those of others, that IFN-γ is both a good rule-in and rule-out diagnostic test for pleural-TB (16, 17, 30-32). We used the IRISA-TB kit, which is both rapid and inexpensive and can be used in most resource poor settings where other routine ELISAs are performed; moreover, it gets around the hurdle of long assay times and high cost of research-based kits, which remain un-validated in a clinical setting. The latter is important as EPTB compartments have high concentrations of interfering heterophile molecules and thus kit-based variation in sensitivity can be considerable (33-35). Interferon gamma levels were also found to be elevated in three non-TB patients. Two of these three patients showed similar histopathology and ADA levels but there was no alternative clinical diagnosis to explain the IFN-γ results with the available clinical information. One drawback of using immunodiagnostic tests is the lack of antimicrobial susceptibility data, which requires either a culture isolate or positive nucleic acid amplification test. However, the diagnostic yield in pleural fluid for both is low making this concern redundant. The diagnostic yield can be improved with pleural biopsy specimens (30). Indeed, in the current study pleural fluid culture sensitivity (45%) was lower compared to biopsy culture sensitivity (82%; data not shown). Recently, Christopher *et al*, showed a 30% increase in Xpert MTB/RIF sensitivity when using pleural tissue in addition to pleural fluid i.e. macerated pleural tissue was used in the Xpert assay (36). This approach was not undertaken in our study but would have still meant that ULTRA sensitivity would have been in the region of ~50%. Further studies are required to interrogate this issue though its

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292 importance is mitigated by the fact that pleural biopsy is not routinely performed in most TB
293 endemic settings.

294 There are several limitations of our study. There was a low proportion of HIV-infected
295 patients and many patients with unknown HIV status. However, the HIV prevalence rates
296 among TB patients in the Western Cape Province are known to be lower than the rest of
297 South Africa (37) and patients often refuse testing. Nevertheless, our findings were still
298 derived in a TB-endemic sitting with a relatively high HIV co-infection rate where Beijing
299 strains predominate, and thus should ideally be confirmed in other settings. A further
300 limitation is that we did not evaluate the potential impact on morbidity and length of hospital
301 stay of ADA, IRISA-TB, Xpert MTB/RIF and ULTRA compared to empiric treatment.
302 However, our study design did not lend itself to deriving these measures (it would have
303 required a RCT) and an interventional study design would have been difficult to interpret
304 because of high rates of empiric treatment. Lastly, as TB-IRISA was performed on frozen
305 pleural fluid samples, it is possible that cell lysis due to freeze/thaw may have resulted in
306 slightly inflated IFN-γ levels, when compared to freshly run samples. However, we believe
307 this effect to be negligible based on correspondence with the manufacturer, and as IFN-γ
308 protein is rapidly released from cells (other methods of IFN-γ detection, such as flow
309 cytometry, require protocols that inhibit the secretion of intracellular cytokines to reliably
310 detect them). The effect of freeze thaw on ADA is also uncertain.

311 In conclusion, despite a better limit of detection than the conventional Xpert MTB/RIF
312 cartridge, ULTRA has poor sensitivity for the diagnosis of pleural TB. Biomarkers, such as
313 ADA and IRISA-TB are significantly more sensitive, with IRISA-TB demonstrating a higher
314 specificity and rule-in value, compared to ADA, in a TB and HIV-endemic setting.

315

316 **Acknowledgements:**

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317 K Dheda and some of the work presented here was supported by SA MRC (RFA-EMU-02-
318 2017), EDCTP (TMA-2015SF-1043 & TMA- 1051-TESAII) and the SANBIO.

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431 **Tables:**

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Table 1: Baseline characteristics of the definite, probable, and non-TB groups.

Demographic data	**Definite TB (n = 49)**	**Non-TB (n = 84)**	**Probable TB (n = 16)**	**P-Values**
Median Age (IQR)	39[ᵃ] (28 - 57)	61[ᵃᵇ] (54 - 69)	47[ᵇ] (38 - 53)	[ᵃ]: p < 0.0001 [ᵇ]: P < 0.0001
Sex				
Male	32 (21.5%)	54 (36.2%)	10 (6.7%)	
Female	17 (11.4%)	30 (20.1%)	6 (4.0%)	
HIV-infected				
Yes	9 (6.5%)	4 (2.9%)	4 (2.9%)	
No	29 (20.9%)	45 (32.4%)	8 (5.8%)	
Unknown	5 (3.6%)	15 (10.8%)	1 (0.7%)	
Not tested	5 (3.9%)	13 (9.4%)	1 (0.7%)	
Median CD4 count* (cells/ml) (IQR)	102** (73 - 232)	117 (39 - 493)	163 (57 - 462)	
Previous TB				
Yes	9 (6.0%)	9 (6.0%)	5 (3.4%)	
No	32 (21.5%)	61 (40.9%)	7 (4.7%)	
Unknown	8 (5.4%)	14 (9.4%)	4 (2.7%)	

Continuous data analysed by unpaired t-test; categorical data analysed by Chi-squared test. Superscript symbols: [a] and [b]: were used to indicate which groups were being compared for statistical analysis. * CD4 counts are available for all HIV infected individuals unless otherwise stated. **One definite HIV infected TB patient did not have an available CD4 count result. As such the median CD4 counted is reported for 8 patients. See table S1 for number of Definite TB participants that were culture and histological positive; culture-negative and histological positive; culture positive and histological positive; culture positive with no histology requested and histology positive with culture requested.

441 **Table 2: Accuracy of Xpert G4, ULTRA, IRISA-TB, and ADA for the diagnosis pleural tuberculosis**

	Sensitivity % (CI) n/N	Specificity % (CI) n/N	Positive Predictive Value % (CI) n/N	Negative Predictive Value % (CI) n/N	Positive Likelihood Ratio (CI)	Negative Likelihood Ratio (CI)	Diagnostic odds Ratio (CI)
Xpert ULTRA	37.5[bd] (23.8 – 51.2) 18/48	98.8[f] (96.5 - 100) 83/84	94.7[j] (84.7 – 100) 18/19	73.5[nk] (65.3 – 81.6) 83/113	31.5 (4.3 – 228.6)	0.6[qt] (0.5 - 0.8)	49.8 (6.4 – 389.4)
Xpert MTB/RIF	28.6[ac] (15.9 – 41.2) 14/49	98.8[e] (96.4 - 100) 83/84	93.3[i] (80.7 - 100) 14/15	70.3[ml] (62.1 – 78.6) 83/118	24.0 (3.2 – 177.0)	0.7[sr] (0.6 - 0.9)	33.2 (4.2 – 262.3)
IRISA-TB - Cut point 20.5 pg/ml	89.8[ab] (81.3 – 98.3) 44/49	96.4[g] (92.4 – 100) 81/84	93.6 [h] (86.6 – 100) 44/47	94.2[kl] (89.2 – 99.1) 81/86	25.1[p] (8.2 – 76.7)	0.1[qs] (0.0 - 0.2)	237.6 (54.2 – 1041.3)
ADA - Cut point 30 IU/ml	84.4[cd] (73.9 – 95.0) 38/45	87.5[feg] (79.9 – 95.1) 63/72	80.9 [hij] (69.6 - 92.1) 38/47	90.0[mn] (83.0 - 97.0) 63/70	6.8[p] (3.6 – 12.6)	0.2[rt] (0.1 - 0.4)	38.0 (13.1 – 110.4)
P-value	[a]; [b]; [c] and [d]: p < 0.0001	[e]: p=0.004 [f]: p=0.005 [g]: p = 0.034	[h]: p = 0.028 [i]: p = 0.071 [j]: p = 0.032	[k]; [l] and [m]: p < 0.0001 [n]: p = 0.00013	[p]: p = 0.032	[q]; [r] and [s]: p < 0.0001 [t]: p = 0.0006	

442 A positive *M.tb* pleural fluid, biopsy and/or sputum culture and/or histology in keeping with *M.tb* infection used as a reference for Definite TB. No

443 microbiological or histological evidence of *M.tb* and/or an alternative diagnosis was available was defined as Non-TB. CI: confidence interval, ADA:

444 adenosine deaminase. IRISA-TB IFN-γ cut-point of 20.5 pg/ml. ADA clinical cut point of 30 U/L is used for clinical decision-making. Superscript symbols: [a];

445 [b]; [c]; [d]; [e]; [f]; [g]; [h]; [i]; [j]; [k]; [l]; [m]; [n]; [p]; [q]; [r]; [s] and [t]: were used to indicate which groups were being compared for statistical analysis.

446 **Table 3: Sensitivity and specificity of ULTRA and Xpert MTB/RIF assay using unprocessed**

	ULTRA		Xpert MTB/RIF	
	Sensitivity % (CI) n/N	Specificity % (CI) n/N	Sensitivity % (CI) n/N	Specificity % (CI) n/N
Fluid	29.5 (13.3 - 53.2) 5/17	100 (89.6 - 100) 34/34	29.5 (13.3 - 53.2) 5/17	100 (89.9 - 100) 34/34
Concentrated	31.3 (14.2 - 55.6) 5/16[a]	100 (89.6 - 100) 33/33	33.4 (15.2 - 58.3) 5/15[b]	100 (89.3 - 100) 32/32

447 **and concentrated (pellet centrifugation) pleural fluid to diagnose pleural tuberculosis**

448 Two aliquots of a median volume of 10 ml of pleural fluid was centrifuged at 3000 × g for 15 min
449 with the pellet resuspended in sterile PBS, followed by Xpert MTB/RIF and ULTRA. A positive *M.tb*
450 pleural fluid, biopsy and/or sputum culture and/or histology in keeping with *M.tb* infection used as a
451 reference for Definite TB. No microbiological or histological evidence of *M.tb* and/or an alternative
452 diagnosis was available was defined as Non-TB. CI: confidence interval. [a]: Error (n=1) in the
453 concentrated ULTRA. [b]: Error (n=2) in the concentrated Xpert MTB/RIF.

454

Figure Legends:

Figure 1. Study overview of patient groups, investigations performed, and tests undertaken.

[a] No biopsy taken, n=10; [b] Fluid smear not requested, n=40; [c] Sputum smear not requested, n=1; [d] Histology not requested, n=11; [e] Fluid culture not requested, n=25; [f] Sputum culture not requested, n=3; [g] Biopsy culture not requested, n=66; [h] ADA levels not requested, n=25; [I] Contamination, n=1; [j] Biopsy sample sub-optimal for histology, n=13; [k] Errors, n=2. [m] Insufficient clinical data for final diagnosis. Participants classifications: Definite-TB: at least one positive *M. tb* culture (pleural fluid, biopsy and/or sputum) and/or caseating granulomatous inflammation suggestive of TB on histological examination of pleural biopsy tissue, and with improvement on anti-TB treatment; Probable-TB: patients not meeting the criteria for definite-TB but with clinical and radiological indicators suggestive of TB and who were initiated on and responded to anti-TB treatment, Non-TB: patients with no microbiological or histological evidence of *M. tb* and/or an alternative diagnosis was available.

Figure 2: A) Scatter plot of IFN-γ levels using IRISA-TB and adenosine deaminase ADA) using pleural fluid from patients with Definite TB and Non-TB pleural effusions. *Mann-Whitney. ROC derived cut point of 20.5 pg/ml IFN-γ (indicated by – – -) for IRISA-TB and ADA cut point of 30 IU/l (indicated by ·····). **B): Area under the receiver operator characteristic (ROC) curves for IRISA-TB and adenosine deaminase (ADA).** Area under the curve; IRISA-TB: 0.94 and ADA: 0.88, respectively. The ROC curves where generated using the Definite and Non-TB groups with the chosen cut point for IRISA-TB indicated with an arrow. No significant difference was observed between the two ROC curves when using the Hanley & McNeil method.