Detection and Quantification of *Mycobacterium tuberculosis* in the Sputum of Culture-Negative HIV-infected Pulmonary Tuberculosis Suspects: A Proof-of-Concept Study

Guillermo Madico¹,⁹, Moses Mpeirwe²,³, Laura White⁴, Solange Vinhas⁵, Beverley Orr⁶, Patrick Orikiriza², Nancy S. Miller⁶,⁷, Mary Gaedde⁵, Juliet Mwanga-Amumpaire⁵, Moises Palaci⁵, Barry Kreiswirth⁸, Joe Straight⁹, Reynaldo Dietze⁵, Yap Boum, II², Edward C. Jones-López¹,⁹*

¹ Section of Infectious Diseases, Department of Medicine, Boston Medical Center and Boston University School of Medicine, Boston, Massachusetts, United States of America, ² Epicentre, Médecins sans Frontières, Mbarara, Uganda, ³ Mbarara University of Science and Technology, Mbarara, Uganda, ⁴ Department of Biostatistics, Boston University School of Public Health, Boston, Massachusetts, United States of America, ⁵ Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitória, Brazil, ⁶ Clinical Microbiology Laboratory, Boston Medical Center, Boston, Massachusetts, United States of America, ⁷ Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts, United States of America, ⁸ Public Health Research Institute (PHRI) – Rutgers University, Newark, New Jersey, United States of America, ⁹ Thisis Diagnostics Inc., Boston, Massachusetts, United States of America

*edward.jones@bmc.org

Abstract

Rationale

Rapid diagnosis of pulmonary tuberculosis (TB) is critical for timely initiation of treatment and interruption of transmission. Yet, despite recent advances, many patients remain undiagnosed. Culture, usually considered the most sensitive diagnostic method, is sub-optimal for paucibacillary disease.

Methods

We evaluated the Totally Optimized PCR (TOP) TB assay, a new molecular test that we hypothesize is more sensitive than culture. After pre-clinical studies, we estimated TOP’s per-patient sensitivity and specificity in a convenience sample of 261 HIV-infected pulmonary TB suspects enrolled into a TB diagnostic study in Mbarara, Uganda against MGIT culture, Xpert MTB/RIF and a composite reference standard. We validated results with a confirmatory PCR used for sequencing *M. tuberculosis*.

Measurements and Results

Using culture as reference, TOP had 100% sensitivity but 35% specificity. Against a composite reference standard, the sensitivity of culture (27%) and Xpert MTB/RIF (27%) was...
lower than TOP (99%), with similar specificity (100%, 98% and 87%, respectively). In unadjusted analyses, culture-negative/TOP-positive patients were more likely to be older (P<0.001), female (P<0.001), have salivary sputum (P = 0.05), sputum smear-negative (P<0.001) and less advanced disease on chest radiograph (P = 0.05). *M. tuberculosis* genotypes identified in sputum by DNA sequencing exhibit differential growth in culture.

Conclusions
These findings suggest that the TOP TB assay is accurately detecting *M. tuberculosis* DNA in the sputum of culture-negative tuberculosis suspects. Our results require prospective validation with clinical outcomes. If the operating characteristics of the TOP assay are confirmed in future studies, it will be justified as a “TB rule out” test.

Introduction
Despite recent advances, tuberculosis (TB) remains a major global health problem with 9 million new cases and 1.4 million deaths in 2013.[1] Critically, the global incidence is decreasing by less than 2% per year, far from the 20% decline required to reach the World Health Organization (WHO) stated goal of eliminating TB by 2050.[2, 3] Patients with pulmonary TB represent ~75% of the global disease burden and contribute exclusively to transmission. Rapid, accurate and early detection of *Mycobacterium tuberculosis* (MTB) in the sputum of TB suspects, and active case finding are key components of the WHO strategy.[4, 5]

For decades, the rapid diagnosis of pulmonary TB has relied on sputum acid-fast bacilli (AFB) smear microscopy but its yield is low when compared to mycobacterial culture, which is considered the most sensitive method for diagnosis.[6] Recently developed molecular tests such as Xpert<sup>®</sup> MTB/RIF and GenoType<sup>®</sup> MTBDRplus provide a rapid alternative to culture in patients with high bacterial loads (i.e. sputum AFB smear-positive). However, their overall sensitivity (~90% against culture) in programmatic conditions has been lower than initially anticipated,[7] and particularly poor (~50%) in smear-negative/culture-positive individuals.[8–10] Other TB diagnostics under development suffer the common limitation of being less sensitive than cultures.[5, 11, 12]

For definitive diagnosis, reliance on cultures as the reference method is problematic because the process of decontaminating samples prior to culture is inherently detrimental to mycobacterial viability. As a result, the overall sensitivity of cultures is only 80–85% compared to a composite reference standard,[6] but significantly lower in clinical conditions where the bacterial load in sputum is low (i.e. paucibacillary TB disease) such as certain patients with HIV-infection,[13] children,[14] and extra-pulmonary TB.[15] Other individuals with active disease harboring non-culturable organisms in sputum include subjects with unstable latent TB infection and early sub-clinical disease that have “percolating” organisms,[16] and those with old untreated TB.[17, 18] In addition, “persistent” organisms after antituberculous therapy may represent the paucibacillary TB pool for poor treatment outcomes.[17] Without culture confirmation, paucibacillary TB is rarely identified leading to empirical treatment, over- or underdiagnosis, and increased morbidity and mortality.[19]

We have developed the “Totally Optimized PCR (TOP) TB assay”, a new nucleic acid amplification test (NAAT) that utilizes a combination of efficient sample processing, novel gene target selection, modern primer design techniques, and an extended PCR for selective target isolation and amplification. The assay is highly specific for Mycobacteria in the MTB
complex and therefore is not affected by background genomic noise, which enables detection with heightened sensitivity. We report here results of in silico and in vitro data. To compare accuracy of the TOP TB assay with culture and the leading molecular test (e.g. Xpert MTB/RIF), we then performed a cross-sectional evaluation using specimens from HIV-infected subjects enrolled into an existing prospective diagnostic study in Uganda.

Materials and Methods

Ethical approvals: The studies were approved by the Institutional Review Boards at Boston University Medical Center, Mbarara University of Science and Technology, and the Uganda National Council for Science and Technology. Samples were shipped to Boston under a Material Transfer Agreement for DNA sequencing.

TOP TB assay

The assay targets a gene (ponA1) involved in the assembly of peptidoglycans in the MTB bacterial wall.[29] The assay’s diagnostic primer set (3-ponA-F/R) targets sequences unique to all species in the MTB complex (Section IA, Fig A and Table A in S1 File). Amplicons generated by 3-ponA were detected using a capture-probe colorimetric assay, and the resultant Optical Densities (OD) provided a semi-quantitative measurement of MTB bacillary load.[21] A more detailed description of the TOP TB assay and its associated laboratory methods, including sample processing and DNA extraction, PCR amplification and amplicon detection are provided in S1 File (Sections IA and IB).

PCR genotyping

To establish the presence of MTB DNA, we tested all specimens with primer set 2-ponA-F/Ra (used for genotyping), which targets a section of ponA1 that is sufficiently distant (~1,100 bp) from the 3-ponA target (used for diagnosis) to remain unaffected by amplicons generated with primer 3-ponA (Section IC, Fig A and Table A in S1 File). 2-ponA PCR products were sequenced to distinguish among five possible genetic variants of MTB (genotypes 0T, 1T, 2, 3 and 4) (Section IC in S1 File). The sequencing nomenclature (Fig B in S1 File) and the genetic correspondence of 2-ponA genotypes to other familiar MTB whole genome genotyping methods are shown in the Appendix (Fig C and Table B in S1 File).

Clinical study

After completing pre-clinical studies, we tested a convenience sample of discarded sputum specimens obtained from participants enrolled into a cross-sectional TB diagnostic study in Uganda. Table C in S1 File summarizes: the study design, a description of the subjects, and methodology (including reference methods).

Setting. The study was conducted at the Epicentre/ Médecins sans Frontières Laboratory located at Mbarara University of Science and Technology in Mbarara, Uganda. With an estimated TB incidence of 166 cases per 100,000 inhabitants, Uganda is on the WHO list of high burden TB countries; the prevalence of HIV infection among TB patients is 48%. [1] Mbarara District is situated in the South Western (SW) zone of the Uganda National Tuberculosis and Leprosy Programme (NTLP). According to NTLP laboratory activity reports, 8,423 TB patients were registered in the SW zone (incidence rate 290 per 100,000). Of the 3701 TB suspects from Mbarara District, 668 (18%) were AFB smear-positive, and 68% were HIV infected.[22]

Study population. Participants for this study were enrolled into a prospective cross-sectional study designed to independently evaluate the diagnostic accuracy of a new AFB
smear microscopy method [23] and Xpert MTB/RIF, with liquid media culture (manual MGIT 960) as the reference method.[24] From September 4th 2012 to April 11th 2014, the parent study enrolled 1,047 (737 HIV-infected and 310 HIV-uninfected) consecutive TB suspects admitted to the wards or attending any of the outpatient clinics of the Mbarara Regional Referral Hospital or the Municipality Health Centre in Mbarara city. Eligible participants were adult (≥18 years), TB suspects (≥2 weeks of cough + at least one other symptoms of TB) [25] willing to follow the study protocol. Patients were excluded if they had received antituberculous drugs within three days, were too ill to consent, or presented with disseminated or extra-pulmonary TB without cough.

**Study design and measurements.** Participants had a standardized TB evaluation, HIV testing and provided three spontaneously expectorated (≥2 mL) sputum samples that included one early morning and two spot samples in a 24-hr period. One of the spot samples (selected by randomization) [24] was tested for direct AFB smear, Xpert MTB/RIF and culture.

**Sample handling prior to TOP processing and testing.** Specimens from the last 261 HIV-infected participants enrolled into the parent study were available for this study. TOP testing was done on the discarded portion of a pellet processed for Xpert MTB/RIF. A ~1mL aliquot was frozen at -80°C for two to six months prior to TOP testing; after thawing, the pellet was washed to remove N-acetyl-L-cysteine / sodium hydroxide solution, [26] processed for TOP and tested in a single batch at the Epicentre laboratory in Mbarara. Study personnel were blind to routine TB results; coded results were later linked via a study identification number.

**Standard Laboratory Methods.** The Epicentre/ Médecins sans Frontières Laboratory has quality assurance (QA) and quality control (QC) protocols, and well-trained personnel with extensive experience in laboratory based TB research. The appearance of sputa specimens was classified as purulent, mucopurulent, mucosalivary or, salivary by the microbiology technicians according to international laboratory guidelines.[27] We used the light-emitting diode-auremine fluorescence technique (Fluorescens<sup>®</sup> LED system, Bergman Labora, Danderyd, Sweden) for direct AFB microscopy on each specimen and reported the results according to the WHO grading scale.[28] The specimen was then decontaminated using the N-acetyl-L-cysteine (0.5%) / sodium hydroxide (1.5%) method.[26] For the reference culture method, we inoculated 500 μl into one manual-testing MGIT 960 (Becton, Dickinson, Franklin Lakes, NJ). We reported a negative culture result after 56 days of incubation at 37°C. Contamination in MGIT media was ruled out using Ziehl-Neelsen (ZN) microscopy and culture on blood agar. For all positive MGIT cultures, we differentiated between *M. tuberculosis* and non-tuberculous mycobacteria (NTM) using the SD TB Ag MPT64 Rapid system (SD Bioline, Kyongi-do, South Korea), following the manufacturer’s instructions. The GenoType Mycobacterium CM/AS identification kit (Hain Lifescience, Nehren, Germany) was used for identification of NTM. The Xpert<sup>®</sup> MTB/RIF assay (Cepheid, Sunnyvale, CA, U.S.A.) was performed according to the manufacturer’s instructions.

**Analytical strategy**

We report the results according to the Standards for Reporting of Diagnostic Accuracy (STARD) guidelines.[29, 30] We calculated the diagnostic cut-off for TOP OD using a cut-off value of three standard deviations above the mean of the OD values of negative controls (e.g. laboratory cut-off). [31] As a sensitivity analysis, we used receiver operating curve (ROC) tools to determine the cut-off that simultaneously maximized sensitivity and specificity (e.g. ROC cut-off). We estimated per-patient sensitivity and specificity using culture as the reference standard, using all available results to adjudicate TB status. We also estimated per-patient sensitivity and specificity using a Composite Reference Standard (CRS) that included culture, MTB
sequencing (e.g. 2-ponA genotyping), a NAAT other than TOP (e.g. Xpert MTB/RIF) and AFB smear, as described. [15, 32–34] We analyzed patient characteristics according to TOP and culture results using Kruskal-Wallis (for continuous data) and Fisher’s exact test (for categorical data), and compared groups using Wilcoxon and Fisher’s exact tests. Variables with p < 0.1 and those considered to be clinically significant were included in multivariate logistic and ordinal logistic regression models. In the former we consider correlates with culture+/TOP+ compared to culture-/TOP+ individuals. In the latter, we compare all three outcomes ordinaly. For these models we group X-ray results into two categories: Normal/Minimal versus Moderate/Far Advanced. The models controlled for age, sex, previous TB treatment, sputum appearance, sputum volume (only for first model), and X-ray (2 category).

**Results**

**Preclinical studies**

In the preclinical phase, TOP TB’s primer set 3-ponA (used for diagnosis) demonstrated: i) excellent analytical sensitivity when clinical sputum samples were “spiked” with *Mycobacterium bovis* Bacille Calmette-Guérin (Fig D, top in S1 File); ii) semi-quantitative detection capability over a range of MTB loads (Fig D, bottom in S1 File); iii) high analytical specificity, testing negative against a panel of 18 common respiratory bacteria and other microorganisms (Fig E in S1 File), and; iv) high specificity against non-tuberculous mycobacteria (Fig F in S1 File). The 2-ponA primer set (used for sequencing) demonstrated a ~8–10% lower analytical sensitivity but similar analytical specificity in the preclinical phase of testing (data not shown).

**Clinical study**

We then evaluated the TOP TB assay in 261 HIV-infected pulmonary TB suspects enrolled into the parent study between October 2, 2013 and April 11, 2014 (Fig 1). Table 1 shows characteristics of the study cohort according to culture and TOP TB assay results.

As shown in Fig 2, 48/261 (18%) patients were culture-positive, all of which were also TOP-positive. Seventy-four (28%) were culture-negative (N = 64) or contaminated (N = 10) and were TOP-negative; 139 (53%) were culture-negative (N = 137) or contaminated (N = 2) but TOP-positive (Fig 2a). The distribution of TOP ODs by culture and AFB smear are shown in Fig 2b and 2c, respectively. Of the 139 culture-negative/TOP-positive samples, 2-ponA sequencing confirmed the presence of MTB DNA in 128 (92%). The sensitivity and specificity of TOP and Xpert MTB/RIF compared to culture or a CRS are shown in Table 2; the breakdown of results included in the CRS is shown in Table D in S1 File. We were unable to sequence MTB from 11/139 (8%) culture-negative/ TOP-positive specimens with low TOP OD values (median 0.13, IQR 0.11–0.34).

In univariate analyses that compared culture-positive/TOP-positive vs. culture-negative/ TOP-positive patients (Table 1), the latter were more likely to be older (P < 0.001); women (P < 0.001); have a salivary sputum (P = 0.05); have a previous history of TB disease (P = 0.05), and have early TB disease as measured by sputum AFB smear grade (P < 0.001) and chest radiograph (P = 0.05). In a multivariate analysis comparing culture+/TOP+ to culture-/TOP+ patients, age (p = 0.003) and gender (p = 0.002) remained statistically significant. In a comparison of all three TOP/culture categories from Table 1, multivariate results revealed that age (p = 0.02) and gender (p < 0.001) were statistically significant and previous TB treatment was marginally significant (p = 0.10).

The cut-off for TOP OD values was determined to be 0.0854 using the laboratory criterion (e.g. +/- three standard deviations criterion). When we used ROC analysis with 100 random observations, we found the cut-off to be 0.088 leading to reclassification of only 4 individuals.
The area under the ROC curve was 0.86 for culture and 0.95 for the CRS using TOP OD as the diagnostic test (Fig G in S1 File).

**M. tuberculosis sequencing results**

The relative frequency and distribution of 2-ponA genotypes differed significantly according to TOP OD values (Fig 3 and Table 2; P = 0.005); in particular, genotype 4 strains were mostly restricted to culture-negative samples with low ODs (Fig 3). As shown in Fig 4, 2-ponA genotypes had variable growth in culture (P = 0.002).

**Discussion**

Our study provides strong evidence that the TOP TB assay accurately detects trace amounts of MTB DNA in the sputum of HIV-infected TB suspects who otherwise may yield a negative culture. Currently a culture diagnosis is the optimal reference standard for diagnosis. In the absence of culture to determine specificity, we established the validity of positive diagnosis using a composite reference standard and, most importantly, we sequenced MTB from culture-negative specimens. We used a reproducible genotyping method that is supported by the
Table 1. Characteristics of 261 HIV-infected pulmonary tuberculosis suspects in Mbarara, Uganda by *M. tuberculosis* culture and TOP TB assay results.

| Characteristic                  | Overall | Culture positive | Culture negative | P value |
|--------------------------------|---------|-----------------|-----------------|---------|
|                                | N       | TOP positive    | TOP positive    | TOP negative | Overall¹ | Two-way² |
| N                              | 261     | 48              | 139             | 74       | <0.001   |
| Age (years)                    | 39.0 [30.5–47.0] | 33.5 [30.0–49.40] | 42.0 [33.0–49.0] | 38.5 [31.0–48.8] | 0.002   | <0.001   |
| Female sex                     | 138 (53) | 12 (25)         | 78 (56)         | 48 (65)  | <0.001   | <0.001   |
| Previous TB treatment*         | 33 (13) | 3 (9)           | 23 (17)         | 7 (9)    | 0.04+    | 0.05+    |
| Years since previous TB treatment | 9.3 [5.9–10.9] | 5.4             | 9.3 [5.9–10.9]  | 11.9 [10.3–12.0] | 0.28#   | 0.16#    |
| CD4 (cells/mL)*                | 322 [104–495] | 182 [54–338]    | 343.5 [93–457]  | 355 [158–590] | 0.06    | 0.13     |
| Sputum volume (mL)             | 3 [2–5] | 4 [3–5]         | 3 [2–4.5]       | 3 [2–5]  | 0.33#    | 0.16#    |
| Purulent sputum appearance^     | <0.001+ | 0.05+           |                  |          |          |
| Chest radiograph*              | Normal  | 29/103 (28)     | 2/17 (12)       | 14/47 (30) | 13/39 (33) | 0.04+    | 0.05+    |
| Scanty                         | 9 (3)   | 8 (17)          | 1 (1)           | 0 (0)    |          |
| 1+                             | 10 (4)  | 10 (21)         | 0 (0)           | 0 (0)    |          |
| 2+                             | 7 (3)   | 6 (13)          | 1 (1)           | 0 (0)    |          |
| 3+                             | 13 (5)  | 13 (27)         | 0 (0)           | 0 (0)    |          |
| Sputum MGIT culture            | Positive| 48 (18)         | 48 (100)        | 0/139 (0) | 0/74 (0)  | -        | -        |
| Contaminated                   | 12 (5)  | -               | 2/139 (1)       | 10/74 (14) |          |
| MGIT DTP (days)                | 19 [13–33] | 19 [13–33]      | NA              | NA       | -        |
| Xpert Mtb/RIF *                | Positive| 50/259 (19)     | 45/47 (96)      | 3/139 (2) | 2/73 (3)  | <0.001+  | <0.001+  |
| Indeterminate                  | 4/259 (2) | 1/47 (2)        | 1/139 (1)       | 2/73 (3)  |          |
| M. tuberculosis 2-ponA genotype| d¹      | 5 (2)           | 4 (8)           | 1 (1)    | 0        | -        | 0.005+   |
| 1T                             | 6 (2)   | 3 (6)           | 3 (2)           | 0        |          |
| 1                             | 2 (1)   | 0               | 2 (1)           | 0        |          |
| 2                             | 40 (15) | 16 (33)         | 24 (17)         | 0        |          |
| 3                             | 93 (36) | 18 (38)         | 74 (53)         | 1 (1)    |          |
| 4                             | 27 (10) | 3 (6)           | 24 (17)         | 0        |          |
| Neg                           | 88 (34) | 4 (8)           | 11 (8)          | 73 (99)  |          |

Values are median [interquartile range] or number (percentage), unless otherwise specified

MGIT = Mycobacterial Growth Indicator Index (BACTEC 960, Becton Dickinson, U.S.A.); DTP = Days-to-positive; AFB = Acid-fast bacilli

¹ P overall = Comparison between three groups

² P two-way = Comparison between culture-positive/ TOP-positive vs. culture-negative/ TOP-positive

* Missing information: Previous TB treatment (1); CD4 cell count (85); Time since previous TB treatment (20); Chest X-ray extent of disease (154), cavitation (153); Sputum AFB smear (1); Xpert MTB/RIF (2)

^ Purulent sputum category includes purulent and muco-purulent; Mucoid category includes mucoid and muco-salivary

* Fisher’s exact test.

# Kruskal-Wallis test or Wilcoxon test.

DOI:10.1371/journal.pone.0158371.t001
genetic signature of a global collection of MTB clinical isolates representing all major phylogenetic lineages.

The natural history of pulmonary TB in HIV-uninfected adults is traditionally viewed as a sub-acute or chronic illness whose progression is accompanied by increasing bacterial loads in...
sputum, paralleling worsening disease severity on chest radiography.\[4, 6\] The paradigm states that patients with early TB are often smear- and culture-negative, and with an increase in severity, most patients become smear-negative/culture-positive first, and eventually, smear- and culture-positive. However, a variety of epidemiologic studies including household contact investigations, molecular epidemiology and TB screening studies have demonstrated that the rate of disease progression in humans can be highly variable, perhaps as a consequence of low or stagnant MTB bacterial loads in sputum.\[4, 35, 36\] Furthermore, effective diagnostic and treatment programs that seek out cases to identify patients with most advanced disease (i.e. AFB smear- and culture-positive) may produce an epidemiological shift that results in the remaining populations with suspected TB of having a higher prevalence of early TB disease (i.e. smear-negative/culture-negative), that are the most difficult to confirm bacteriologically.\[4\] For example, in the U.S. during the 1980s, 90% of TB cases were confirmed by culture but this proportion decreased to 77% by 2013; \[37\] in some settings (e.g. Boston, MA and Alberta, Canada), ~50% of notified TB cases are culture-negative.\[38, 39\] Therefore, culture-negative TB disease is a global problem resulting from both biological and epidemiological factors, for which there are currently limited solutions beyond the initiation of empirical antituberculous treatment based on clinical algorithms.\[40\]

Table 2. Per-patient sensitivity and specificity of the TOP TB assay, Xpert MTB/RIF and culture in 261 HIV-infected tuberculosis suspects according to a reference standard established by M. tuberculosis culture or a Composite Reference Standard (CRS) in Mbarara, Uganda.

| Diagnostic Method | MTB detected (N) | MTB not detected (N) | Sensitivity | Specificity | PPV | NPV |
|-------------------|-----------------|----------------------|-------------|-------------|-----|-----|
|                   | n/N % (95% CI)  | n/N % (95% CI)      | n/N % (95% CI) | n/N % (95% CI) |
| Culture reference standard | 48 213¹ | 50 209 | 45/47 96% (84, 94) | 207/212 98% (94, 99) | 45/50 90% (77, 96) | 207/209 99% (96, 100) |
| TOP TB assay      | 187 74         | 177 84               | 48/48 100% (93, 100) | 74/213 35% (28, 41) | 48/137 26% (20, 33) | 74/74 100% (95, 100) |
| Composite reference standard ³ | 48 213 | 50 209 | 48/177 27% (21, 34) | 84/84 100% (96, 100) | 48/48 100% (93, 100) | 84/211 40% (33, 47) |
| Culture           | 48 213         | 48/177 27% (21, 34) | 81/83 98% (91, 100) | 48/50 96% (85, 99) | 81/209 39% (32, 46) |
| Xpert MTB/RIF     | 50 209         | 48/176 27% (21, 35) | 81/83 98% (91, 100) | 48/50 96% (85, 99) | 81/209 39% (32, 46) |
| TOP TB assay      | 187 74         | 176/177 99% (97, 100) | 73/84 87% (77, 93) | 176/187 94% (89, 97) | 73/74 99% (93, 100) |

Definition of abbreviations: CI = Confidence interval; CRS = Composite reference standard; MTB = Mycobacterium tuberculosis; NPV = Negative predictive value; PPV = Positive predictive value

¹ Includes 12 patients with contaminated culture results
² Two Xpert MTB/RIF results were missing and 4 had indeterminate result (N = 259)
³ Composite Reference Standard (CRS) included M. tuberculosis culture, M. tuberculosis sequencing (e.g. 2-ropA genotyping), a NAAT other than TOP (e.g. Xpert MTB/RIF), and AFB smear.\[15\] The breakdown of CRS results is shown in Table S4 (Appendix)

doi:10.1371/journal.pone.0158371.t002

TOP TB enables enhanced detection of MTB in the sputum of TB suspects with HIV/AIDS, perhaps one of the largest and most vulnerable (together with children) populations with paucibacillary TB disease. In the early phase of assay development, our in vitro results suggested an analytical sensitivity of 1–4 colony-forming units (CFU) of MTB per mL, a level of detection greater than culture (e.g. 10–100 CFU/ml). With nullification of culture as the reference method, we anticipated challenges in validating the accuracy of our results. Several analytical methods have been recommended when dealing with imperfect reference methods such as mycobacterial cultures.\[15, 32, 33, 41\] The use of one of these—“discrepant analysis”, or the use of a third test, is limiting because usually it is not applied consistently across all the specimens that are being examined, only the ones where the new test result conflicts with the “gold standard”.\[32, 33, 41\] Our methods minimize the limitations of using discrepant analysis to evaluate NAATs because we tested all specimens, and because of the complete lack of overlap
(lack of ‘dependence’) between the 3-ponA (diagnostic) and 2-ponA (genotyping) primers. Furthermore, the use of both clinical and epidemiologic data to act further as a referee, the latter demonstrating variability of genotypes according to TOP OD values. The first group (far left) includes 74 subjects that were culture-negative and TOP-negative (3-ponA primer). N denotes the number of subjects in each TOP OD group; n denotes the number of subjects with a positive 2-ponA genotype in each group. A 2-ponA genotype could not be identified in 8% (4/48) culture-positive/TOP-positive samples and 8% (11/139) culture-negative/TOP-positive samples. A 2-ponA genotype was identified in 1% (1/74) of culture-negative/TOP-negative samples.

doi:10.1371/journal.pone.0158371.g003

Our results suggest that the TOP assay is sufficiently sensitive to overcome well-recognized difficulties with sputum procurement, such as inadequate specimen volumes and/or poor quality of specimens (e.g. excess saliva) – a problem that is thought to diminish diagnosis of TB in women disproportionately. [44–47] This also raises the possibility of using sputum to diagnose paucibacillary TB when non-pulmonary clinical specimens (blood, gastric aspirates, urine, stool) have otherwise been thought necessary to establish the diagnosis. [5, 12, 48] Interestingly,
our sequencing data establish a potential link between diagnosis, epidemiology and pathogenic behavior of MTB in humans. In particular, a high frequency of genotype 4 MTB isolates was noted in HIV-infected patients with low TOP ODs in Uganda. We had rarely observed this variant in the global collection of clinical isolates before these studies were started, raising the possibility that genotype 4 strains are uniquely adapted to HIV-infected hosts and cause predominantly culture-negative TB disease.

Our study has limitations. Our results were obtained by testing a convenience sample of low-volume, discarded, stored sputum specimens from an existing diagnostic clinical study; therefore, performance of the TOP assay may have been underestimated. The selection of the study population was solely based on when the TOP TB assay was ready for clinical testing (October 2013) rather than selection bias, as shown by the results of the parent study that included the entire population of HIV-infected patients. [24] The lack of clinical follow-up of subjects limits the clinical interpretation of certain results. For example, TOP was positive in numerous patients with a current or past history of treatment for TB, which complicated the clinical interpretation of negative cultures; interestingly, a similar phenomenon has recently been described with Xpert MTB/RIF, although a discrepant analysis was not performed.[49, 50] A positive TOP result likely represented either residual (dead) MTB DNA, viable but non-culturable organisms or bacterial persistence after treatment, the latter a potential harbinger of TB recurrence and/or risk of drug resistance.[17, 51, 52] Admittedly, detection of trace amounts of MTB DNA may be due to bacterial "spillage" from a dormant lung foci or low level...
bacterial replication that may not require treatment. The colorimetric readout uses a study-spe-
cific cut-off value to establish the "Limit of Blank", a key assay parameter.[31] Finally, in its
current embodiment, the TOP TB assay does not include provisions for detecting drug-resis-
tant TB. However, the primary global need is for a rapid and reliable triage test.[5, 12]

Conclusions
Culture-negative TB is widespread, resulting from several biologic and epidemiologic factors.
By shifting diagnostic emphasis to early detection, the TOP TB assay broadens sensitive and
accurate detection of MTB across the entire clinical spectrum of TB disease. Our findings will
require validation with clinical outcomes obtained prospectively. If the operating characteristics of the TOP assay are confirmed in future studies, it would be justified as a triage or "TB
rule out" test.

Supporting Information
S1 File. Supplementary Appendix.
1. Experimental Laboratory methods
   a. Technical aspects of TOP TB assay
   b. TOP TB assay laboratory methods
2. M. tuberculosis sequencing and genotype nomenclature
3. Supporting Figures
   • Fig A: DNA sequence alignment of M. tuberculosis ponA1
   • Fig B: Sequence representation of five possible 2-PonA genotypes of M. tuberculosis clini-
cal isolates
   • Fig C: Genetic correspondence of M. tuberculosis 2-ponA genotypes with other common
   whole-genome genotyping methods
   • Fig D: Analytical sensitivity of 3-ponA primer
   • Fig E: Analytical specificity of 3-ponA primer
   • Fig F: Specificity of TOP TB assay against non-tuberculous mycobacteria
   • Fig G: Receiver operating curve (ROC) analysis of TOP TB assay results according to cul-
ture or composite reference standard
4. Supporting Tables
   • Table A: Description of 3-ponA and 2-ponA primers
   • Table B: Genetic correspondence of M. tuberculosis 2-ponA genotypes with other com-
   mon whole-genome genotyping methods
   • Table C: Summary of Uganda clinical study
   • Table D: Breakdown of results for the Composite Reference Standard
   • Table E: Sensitivity analysis using TOP TB assay cut-off determined by ROC analysis
5. References
   (DOC)
Acknowledgments

The authors would like to acknowledge Dr. Joel Bazira, MBChB, MMED, PhD, Head Department of Microbiology Faculty of Medicine Mbarara University of Science and Technology for his support. We thank personnel at Boston Medical Center’s offices of Development (Kirsten Hinsdale and Hugh Keeping) and Boston University’ Office of Technology Development (Michael Pratt) for their continued support. We also wish to thank Kathy Eisenach, PhD and Kevin Fennelly, MD, MPH for their critical input and reviewing the manuscript, and David Hamer, MD, Tamar Barlam, MD, Peter Rice, MD, Jerrold Ellner, MD and Robert Wilkinson, MD for reviewing the manuscript.

The results shown here were presented in part at the 1st Massachusetts-South Africa Conference for Technology Transfer & Global Innovation, Stellenbosch, South Africa 8–11 June 2015.

Author Contributions

Conceived and designed the experiments: GM LW YB EJ-L. Performed the experiments: GM MM PO JM-A YB EJ-L. Analyzed the data: GM MM LW MG BK YB EJ-L. Wrote the paper: SV BO NSM MP JS GM MM LW PO MG JM-A BK YB EJ-L RD. Participated in early development of the TOP TB assay: SV BO NSM MP JS RD. Provided M tuberculosis clinical isolates from the Public Health Research Institute strain bank: BK.

References

1. WHO. Global Tuberculosis Report 2014. Geneva, Switzerland: World Health Organization, 2014 Contract No.: WHO/HTM/TB/2014.08.

2. WHO. The End TB Strategy. Global strategy and targets for tuberculosis prevention, care and control after 2015. Geneva: World Health Organization, 2014.

3. Lonnroth K, Castro KG, Chakaya JM, Chauhan LS, Floyd K, Glaziou P, et al. Tuberculosis control and elimination 2010–50: cure, care, and social development. Lancet. 2010; 375(9728):1814–29. Epub 2010/05/22. doi: 10.1016/S0140-6736(10)60483-7 PMID: 20488524.

4. WHO. Systematic screening for active tuberculosis: principles and recommendations. Geneva, Switzerland: World Health Organization, 2013 Contract No.: WHO/HTM/TB/2013.04.

5. Pai M, Schito M. Tuberculosis Diagnostics in 2015: Landscape, Priorities, Needs, and Prospects. J Infect Dis. 2015; 211(suppl 2):S21–S8. Epub 2015/03/15. doi: 10.1093/infdis/jiu803 PMID: 25765103; PubMed Central PMCID: PMC4366576.

6. ATS/CDC/IDSA. Diagnostic standards and classification of tuberculosis in adults and children. Am J Respir Crit Care Med. 2000; 161:1376–95. PMID: 10764337

7. Boehme CC, Nabet P, Hilleman D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampicin resistance. N Engl J Med. 2010; 363(11):1005–15. Epub 2010/09/10. doi:10.1056/NEJMoa0907847 PMID: 20825313; PubMed Central PMCID: PMC2947799.

8. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2014; 1:CD009593. Epub 2014/01/23. doi: 10.1002/14651858.CD009593.pub3 PMID: 24448973.

9. Chang K, Lu W, Wang J, Zhang K, Jia S, Li F, et al. Rapid and effective diagnosis of tuberculosis and rifampicin resistance with Xpert MTB/RIF assay: a meta-analysis. J Infect. 2012; 64(6):580–8. Epub 2012/03/03. doi:10.1016/j.jinf.2012.02.012 PMID: 22381459.

10. Bamard M, Gey van Pittius NC, van Helden PD, Bosman M, Coetzee G, Warren RM. The Diagnostic Performance of the GenoType MTBDRplus Version 2 Line Probe Assay Is Equivalent to That of the Xpert MTB/RIF Assay. J Clin Microbiol. 2012; 50(11):3712–6. Epub 2012/09/14. doi:10.1128/JCM.01958-12 PMID: 22972826; PubMed Central PMCID: PMC3486209.

11. UNITAID. Tuberculosis. Diagnostics Technology and Market Landscape. 4th Edition. 2015. Switzerland: World Health Organization, 2015.

12. Dorman S. Advances in the diagnosis of tuberculosis: current status and future prospects. Int J Tuberc Lung Dis. 2015; 19(5):504–16. Epub 2015/04/14. doi:10.5588/ijtld.15.0048 PMID: 25868017.

13. Getahun H, Harrington M, O’Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes.
14. Perez-Velez CM, Marais BJ. Tuberculosis in children. N Engl J Med. 2012; 367(4):348–61. Epub 2012/07/27. doi: 10.1056/NEJMra1008049 PMID: 22830465.

15. Denkinger CM, Schumacher SG, Boehme CC, Dendukuri N, Pai M, Steingart KR. Xpert MTB/RIF assay for the diagnosis of extrapulmonary tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2014; 44(2):435–46. Epub 2014/04/04. doi: 10.1183/09031936.0007814 PMID: 24696113.

16. Esmail H, Barry CE 3rd, Young DB, Wilkinson RJ. The ongoing challenge of latent tuberculosis. Philos Trans R Soc Lond B Biol Sci. 2014; 369(1645):20130437. Epub 2014/05/14. doi: 10.1098/rstb.2013.0437 PMID: 24821923; PubMed Central PMCID: PMC4024230.

17. Salgame P, Geada S, Collins L, Jones-Lopez E, Ellner JJ. Latent tuberculosis infection—Revisiting and revising concepts. Tuberculosis (Edinb). 2015; 95(4):373–84. doi: 10.1016/j.tube.2015.04.003 PMID: 26038289.

18. Stead WW. Pathogenesis of the sporadic case of tuberculosis. N Engl J Med. 1967; 277(19):1008–12. Epub 1967/11/09. doi: 10.1056/NEJM196711092771906 PMID: 6059578.

19. Sterling T JC, Jayathilake K, Gotuzzo E, Veloso V, Cortes C, Padgett D, Crabtree-Ramirez B, Shepherd B, McGowan C., editor Culture-negative TB is associated with increased mortality in HIV-infected persons. Conference on Retroviruses and Opportunistic Infections; 2015 February 23–26, 2015; Seattle, Washington.

20. Kieser KJ, Boutte CC, Kester JC, Baer CE, Barczak AK, Meniche X, et al. Phosphorylation of the Peptidoglycan Synthase PnpA1 Governs the Rate of Polar Elongation in Mycobacteria. PLoS Pathog. 2015; 11(6):e1005010. doi: 10.1371/journal.ppat.1005010 PMID: 26114871; PubMed Central PMCID: PMCPMC4483258.

21. Denis M, Soumet C, Legeay O, Arnauld C, Bounaix S, Thiery R, et al. Development of a semiquantitative PCR assay using internal standard and colorimetric detection on microwell plate for pseudorabies virus. Mol Cell Probes. 1997; 11(6):439–48. Epub 1998/03/17. doi: 10.1006/mcpr.1997.0139 PMID: 9500814.

22. Ministry of Health RoU. National tuberculosis and leprosy programme, South Western Uganda annual report. 2008.

23. Fennelly KP, Morais CG, Hadad DJ, Vinhas S, Dietze R, Palaci M. The small membrane filter method of microscopy to diagnose pulmonary tuberculosis. J Clin Microbiol. 2012; 50(6):2096–9. Epub 2012/03/17. doi: 10.1128/JCM.00572-12 PMID: 22422854; PubMed Central PMCID: PMC3372110.

24. Boum Y 2nd, Kim S, Orikiriza P, Acuna-Villaorduna C, Vinhas S, Bonnet M, et al. Diagnostic Accuracy of the Small Membrane Filtration Method for Diagnosis of Pulmonary Tuberculosis in a High HIV Prevalence Setting. J Clin Microbiol. 2016. doi: 10.1128/JCM.00017-16 PMID: 27030493.

25. WHO. Treatment of tuberculosis guidelines. 2010.

26. Kent PT, Kubica GP. Public Health Mycobacteriology—A Guide for the Level III Laboratory. Atlanta, GA: U. S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, 1985.

27. Rieder HL C T, Myking H, Urbanczik R, Laszlo A, Kim SJ, Van Deun A T A. The public health service national tuberculosis reference laboratory and the national laboratory network: minimum requirements, role and operation in a low-income country. Paris, France: International Union Against Tuberculosis and Lung Disease, 1998.

28. WHO. Strategic and Technical Advisory Group for Tuberculosis: report on conclusions and recommendations. Geneva, Switzerland: World Health Organization, 2009.

29. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al. Towards complete and accurate reporting of studies of diagnostic accuracy: The STARD Initiative. Ann Intern Med. 2003; 138(1):40–4. Epub 2003/01/07. PMID: 12513043.

30. Fontela PS, Pant Pai N, Schiller I, Dendukuri N, Ramsay A, Pai M. Quality and reporting of diagnostic accuracy studies in TB, HIV and malaria: evaluation using QUADAS and STARD standards. PLoS One. 2009; 4(11):e7753. doi: 10.1371/journal.pone.0007753 PMID: 19915664; PubMed Central PMCID: PMCPMC2771907.

31. Armbuster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008; 29 Suppl 1:S49–52. Epub 2008/10/15. PMID: 18852857; PubMed Central PMCID: PMC2556583.

32. Dendukuri N. Evaluating Diagnostic Tests in the Absence of a Gold Standard. Advanced TB diagnostics course, Montreal, July 2011.

33. Reitsma JB, Rutjes AW, Khan KS, Coomarasamy A, Bossuyt PM. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. J Clin Epidemiol. 2009; 62(8):797–806. Epub 2009/05/19. doi: 10.1016/j.cej.2009.02.005 PMID: 19447581.
Diagnosis of Culture-Negative Pulmonary Tuberculosis

34. Naaktgeboren CA, Bertens LC, van Smeden M, de Groot JA, Moons KG, Reitsma JB. Value of composite reference standards in diagnostic research. BMJ. 2013; 347:f5605. doi: 10.1136/bmj.f5605. PMID: 24162938.

35. Guwatudde D, Nakakeeto M, Jones-Lopez EC, Maganda A, Chiunda A, Mugerwa RD, et al. Tuberculosis in household contacts of infectious cases in Kampala, Uganda. Am J Epidemiol. 2003; 158(9):887–98. Epub 2003/10/31. PMID: 14585767; PubMed Central PMCID: PMC2869090.

36. Sloot R, Schim van der Loeff MF, Kouw PM, Borgdorff MW. Risk of tuberculosis after recent exposure. A 10-year follow-up study of contacts in Amsterdam. Am J Respir Crit Care Med. 2014; 190(9):1044–52. Epub 2014/09/30. doi: 10.1164/rccm.201406-1159OC PMID: 25265362.

37. CDC. Reported Tuberculosis in the United States, 2013. Atlanta, GA, U.S. In: Department of Health and Human Services C, editor. Atlanta 2014.

38. BPHC. Tuberculosis Impact in Boston Residents: 2013. In: Commission. BPH, editor. Boston2013.

39. Health A. Tuberculosis in Alberta Surveillance Report (2010 to 2012). In: Health OotCMOo, editor. 2014.

40. Lawn SD, Ayles H, Egwaga S, Williams B, Mukadi YD, Santos Filho ED, et al. Potential utility of empirical tuberculosis treatment for HIV-infected patients with advanced immunodeficiency in high TB-HIV burden settings. Int J Tuberc Lung Dis. 2011; 15(3):287–95. PMID: 21333094.

41. Caliendo AM, Gilbert DN, Ginocchio CC, Hanson KE, May L, Quinn TC, et al. Better tests, better care: improved diagnostics for infectious diseases. Clin Infect Dis. 2013; 57 Suppl 3:S139–70. doi: 10.1093/cid/cit578 PMID: 24200831; PubMed Central PMCID: PMC3820169.

42. Salminia H, Fairfax MR, Lephart PR, Schreckenberger P, DesJarlais SM, Johnson JK, et al. Evaluation of the FilmArray Blood Culture Identification Panel: Results of a Multicenter Controlled Trial. J Clin Microbiol. 2016; 54(3):687–98. doi: 10.1128/JCM.01679-15 PMID: 26739158; PubMed Central PMCID: PMCPMC4767991.

43. Khan MS, Dar O, Sismanidis C, Shah K, Godfrey-Faussett P. Improvement of tuberculosis case detection and reduction of discrepancies between men and women by simple sputum-submission instructions: a pragmatic randomised controlled trial. Lancet. 2007; 369(9577):1955–60. Epub 2007/06/15. doi: 10.1016/S0140-6736(07)60916-7 PMID: 17560448.

44. Holmes CB, Hausler H, Nunn P. A review of sex differences in the epidemiology of tuberculosis. Int J Tuberc Lung Dis. 1998; 2(2):96–104. PMID: 9562118.

45. Kana BD, Gordhan BG, Downing KJ, Sung N, Vostroknutova G, Machowski EE, et al. The resuscitation-promoting factors of Mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro. Mol Microbiol. 2008; 67(3):672–84. doi: 10.1111/j.1365-2958.2007.06078.x PMID: 18186793; PubMed Central PMCID: PMCPMC2229633.

46. Nathan C, Barry CE 3rd. TB drug development: immunology at the table. Immunol Rev. 2015; 264 (1):308–18. doi: 10.1111/imr.12275 PMID: 25703568; PubMed Central PMCID: PMCPMC4339218.