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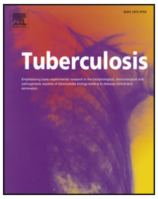

Diagnostics

Further evidence of *Mycobacterium tuberculosis* in the sputum of culture-negative pulmonary tuberculosis suspects using an ultrasensitive molecular assay

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ABSTRACT

Background: Rapid diagnosis of pulmonary tuberculosis (TB) is critical to TB control. However, many patients with paucibacillary TB disease remain undiagnosed. Current TB elimination goals require new tools to diagnose early disease. We evaluated performance of the Totally Optimized PCR (TOP) TB assay, a novel ultrasensitive molecular test.

Methods: We assessed analytical specificity against nontuberculous mycobacteria (NTM), and estimated the diagnostic accuracy of TOP in a pilot study in Brazil ($n = 46$) and a cross-sectional study in Boston ($n = 60$). We compared TOP results to culture and a composite reference standard (CRS).

Results: TOP exhibited no cross-reactivity against NTM. We tested 132 respiratory specimens from 106 patients with suspected pulmonary TB. The pilot demonstrated feasibility and 100% (95% CI 85–100) sensitivity in predominantly smear-positive specimens; TOP's specificity against solid media culture was low (58%, 37–77) but improved against a CRS (93%, 68–100). Similarly, when using the CRS in the Boston study, TOP (88%, 1–99) had greater sensitivity than solid or liquid media culture (25%, 3–65) and similar specificity (both 100%, 93–100).

Conclusions: The TOP assay enables detection of *M. tuberculosis* in culture-negative paucibacillary disease. While the use of TOP for the diagnosis of paucibacillary disease will require further clinical validation, its high sensitivity indicate a more immediate utility as a rule out TB test.

1. Introduction

Tuberculosis (TB) has surpassed HIV/AIDS as the leading cause of death by an infectious disease worldwide and remains a major threat to global health, productivity, and socioeconomic development [1]. Despite recent advances in diagnostic test development [2], it is estimated that at least one-third of individuals with TB disease are never diagnosed. This leads to poor outcomes and uninterrupted *Mycobacterium tuberculosis* transmission [3]. A 20% annual decline in the global TB incidence will be required to achieve the World Health Organization (WHO) stated goal of eliminating TB by 2050 [4,5], but the incidence is now decreasing by less than 2% per year. Improved diagnostics are

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needed to bridge this gap.

As characteristically seen in HIV-infected patients and children, the burden of undiagnosed disease is unevenly concentrated in so-called "special populations" [6]. This term is reserved for patients with a low bacterial count in sputum (e.g. paucibacillary TB disease). A key obstacle to augmenting case detection is that current sputum-based technologies establish a diagnosis at a relatively late stage (i.e. when disease severity is sufficiently advanced for mycobacterial growth in culture media). Despite their elevated cost, unacceptably long turnaround time, and need for laboratory biosafety containment, mycobacterial cultures remain the reference method for TB diagnosis because they: 1) remain the most sensitive diagnostic test available, 2) can demonstrate bacterial viability, and 3) allow for phenotypic drug susceptibility testing. However, culture yields suboptimal detection, particularly for patients with paucibacillary disease [7]. Historically, evidence of widespread culture-negative TB disease was reported in autopsy studies of HIV-infected persons in sub-Saharan Africa, pediatric TB investigations, and household contact studies [2,6]. More recently, evidence of active TB disease caused by non-culturable organisms has been significantly expanded using advanced imaging techniques [8] and enriched mycobacterial culture media [9,10], considerably complicating the diagnostic landscape [11].

Newly developed molecular assays significantly shorten the "sample-to-treatment" cascade of care and approach the sensitivity of culture. However, these tests are still best suited for detection of TB at later stages and have had limited impact in improving treatment outcomes [12–14]. These data suggest that a marked improvement in patient outcomes will likely require shifting diagnostic emphasis to earlier detection –i.e. before culture positivity [15]. However, efforts towards this goal are stymied by the continued use of cultures as the gold standard for TB diagnosis [6,16,17] and the associated methodological challenges that are required to overcome this limitation [18–21].

We recently reported the diagnostic performance of a new nucleic acid amplification test the "Totally Optimized PCR (TOP) TB assay" in a cohort of HIV-infected patients with suspected pulmonary TB in Mbarara, Uganda [22]. Our results demonstrated that TOP enabled detection of *M. tuberculosis* DNA in the sputum with an estimated 2–3 fold heightened sensitivity relative to liquid mycobacterial cultures and Xpert MTB/RIF (Cepheid, Sunnyvale, CA), when compared to a composite reference standard that included *M. tuberculosis* sequencing. However, the generalizability of our initial study was limited because it included a single site, and we tested a convenience sample of sputum specimens processed for Xpert MTB/RIF. Furthermore, we did not provide sufficient data regarding the assay's analytical specificity against nontuberculous mycobacteria (NTM). In the present report, we provide additional evidence for the latter and show the diagnostic performance of TOP in two additional clinical studies.

2. Methods

2.1. Assessment of analytical specificity

To determine the analytical specificity of TOP against closely related mycobacteria, we sourced 20 species of NTM (provided by Richard Wallace, MD, University of Texas at Tyler, TX) that were selected because of their genetic proximity to TOP TB target primers.

2.2. Clinical studies

We evaluated the diagnostic performance of the TOP TB assay in discarded respiratory samples sourced from TB suspects undergoing a routine clinical evaluation at two clinical sites. We first performed a pilot study in Vitória, Brazil, to demonstrate feasibility and to evaluate assay performance in mostly sputum acid-fast bacilli (AFB) smear-positive specimens. We also conducted a study in hospitalized and ambulatory patients attending a large tertiary care hospital in Boston, Massachusetts. Except for Brazilian specimens where the AFB smear result was used to determine study eligibility, study personnel were blind to routine TB results; coded results were later linked via a study identification number. When available, we used clinical and microbiological information collected as part of routine care (e.g. out of study) to resolve discrepancies between culture and TOP results, and to prepare clinical vignettes (Supplementary Table 4).

Vitória, Brazil. The pilot study was conducted at the Núcleo de Doenças Infecciosas (NDI) in Vitória, the capital city of the State of Espírito Santo. The NDI has organized a network of five laboratories in the metropolitan region of Vitória that serves 16 TB clinics. With approximately 1400 cases/year, the TB incidence in Espírito Santo is 38/100,000 inhabitants. The prevalence of HIV infection in Espírito Santo is $< 1\%$ in the general population, and 7% in TB cases [23]. The NDI network performs universal solid culture (Ogawa-Kudoh media) testing on an average of 12,000 samples per year, of which 5–8% are culture-positive (700–850 new TB cases per year). Of these, approximately 80% are sputum smear AFB-positive and 20% are AFB-negative.

Boston, Massachusetts. This study was conducted at the Boston Medical Center (BMC) Clinical Microbiology Laboratory. BMC is a private, not-for-profit, 496-bed, academic medical center (www.bmc.org) that serves as the principal safety net hospital for the greater Boston area (57% of patients are from under-served populations and 32% do not speak English as their primary language). Each month, approximately 150–200 (90–150 respiratory) samples are tested for *M. tuberculosis* at the BMC laboratory, of which $\sim 2\%$ are culture-positive and reported to the Boston Public Health Commission (BPHC). BMC identifies an average of 2.6 new cases of TB per month (range 0–5), corresponding to 50–65% of notified TB cases in Boston. Over the three-year period preceding the study (2011–2013) [24], BPHC reported 40–45 new cases per year (incidence rate of 6.5–7.1 per 100,000 population) of which 60–70% were sputum smear AFB-negative/culture positive or AFB-negative/culture-negative; 65% had pulmonary TB, 80% were non-US born, 8–9% were HIV infected, and the majority (65–77%) were diagnosed while hospitalized [24].

2.3. Laboratory assessments

Both participating laboratories are state-of-the-art facilities with permanent trained personnel, established quality assurance and quality control protocols, and extensive experience in both TB clinical care and research. There were minor differences between sites in specimen handling prior to TOP testing and culture methods (Supplementary Table 1). Both participating laboratories followed standard mycobacterial laboratory methods as part of routine testing (see Supplementary Data: *Standard Laboratory Methods*).

Sample handling prior to TOP testing. In both laboratories, once the routine work was completed, if the remaining volume of the sample was ≥ 1 mL, it was coded and stored unprocessed in a refrigerated cabinet until for up to 1 week (as per routine clinical care). In Brazil, extracted DNA after TOP processing was frozen at $-80\,^{\circ}$C for two months until TOP testing in a single batch at the NDI without knowledge of culture results. In Boston, eligible samples were picked up weekly and subjected to the TOP sample processing method within 24–48 h after receipt in the research laboratory. TOP testing was done in weekly batches without knowledge of smear and cultures results.

TOP TB assay. A detailed description of TOP TB assay methods, including primer and probe design, sample processing and DNA extraction, PCR amplification and amplicon detection has been published [22]. Briefly, the assay targets a gene (*ponA1*) involved in the assembly of peptidoglycans in the *M. tuberculosis* bacterial wall [25]. The assay's diagnostic primer set (3-ponA-F/R) targets sequences unique to all species in the *M. tuberculosis* complex. Amplicons generated by 3-ponA are detected using a capture-probe colorimetric assay, and the resultant Optical Densities (OD) provide a semi-quantitative measurement of bacillary load [22,26]. To establish the presence of *M. tuberculosis* DNA, specimens that were detected positive using the diagnostic primer set (3-ponA) were confirmed with a second primer set (2-ponA-F/R) used for genotyping. 2-ponA PCR products were sequenced (Genewiz, NJ, USA) to distinguish among five possible genetic variants of *M. tuberculosis* (0^T, $1^T/1$, 2, 3 and 4) that we have correlated to other widely accepted molecular genotyping methods (RFLP, Spoligotyping, SNiPs), as previously described [22].

2.4. Analytical strategy

We report the results according to the Standards for Reporting of Diagnostic Accuracy (STARD) guidelines [27,28]. We calculated the diagnostic TOP OD cut-off for each study separately to account for the inherent variability of the ELISA-type colorimetric detection method used. 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) [29], after validating the latter method using receiver operating curve (ROC) tools in a prior study [22]. For the Brazilian pilot study that was biased toward mostly smear AFB+ samples, we first performed a per-specimen analysis and correlated TOP ODs to culture and AFB smear results (we did not perform a similar analysis in Boston because of the limited number of culture-positive specimens). For both studies, we estimated per-patient sensitivity and specificity compared to 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 AFB smear, response to antituberculous treatment, mycobacterial culture and *M. tuberculosis* sequencing (e.g. 2-ponA genotyping), as recommended [17,18,30]. In Boston, we analyzed patient characteristics according to TOP and culture results using Wilcoxon test (for continuous data) and Fisher's exact test (for categorical data).

Ethical approvals. The studies were approved by the Comité de Ética em Pesquisa do Hospital Universitário Cassiano Antônio de Moraes and the Institutional Review Board at Boston University Medical Center. Samples were shipped to Boston for genotyping under a Material Transfer Agreement.

3. Results

3.1. Analytical specificity studies

TOP TB's primer set 3-ponA (used for diagnosis) demonstrated no cross-reactivity against a panel of 20 species of NTM (Fig. 1). As shown, the TOP PCR product was amplified only with *M. tuberculosis* DNA and no targeted DNA sequences (185 bp) were amplified when using DNA from NTM species. The 2-ponA primer set (used for sequencing) demonstrated similar analytical specificity (data not shown).

3.2. Clinical studies

We evaluated the diagnostic accuracy of the TOP assay in two separate cohorts of subjects with suspected pulmonary TB in Vitória and Boston. In all, the study included a total of 132 specimens from 106 subjects (Fig. 2). Supplementary Table 1 summarizes key design features of the two studies.

3.3. Brazil pilot study

From January 28 to April 11, 2014, we obtained 73 samples from 46 patients; 26 (36%) patients provided a single specimen, and 47 (64%) provided > 1 sample. One sample was excluded (missing TOP result). The median age of patients was 35 years (range 10–76), 65% were male and the median sample volume was 1 mL (range 0·5-2). In a *per-specimen* analysis (Fig. 3a), 37 (51%) samples were culture-positive, of which 34 were also TOP-positive. Twenty-three (32%) were negative for *M. tuberculosis* by both methods, including three (all smear AFB+) that grew NTM species (*M. abscessus, M. avium and M. kansasii*). Twelve (17%) samples (five smear AFB-positive and seven smear AFB-negative) were TOP-positive but culture-negative (n = 11) or contaminated (n = 1). TOP results by culture and by AFB smear results are shown in Fig. 3b and c, respectively.

In a *per-patient* analysis (n = 46) that included all available results per participant (i.e. including both study and routine clinical care results), 22 (48%) patients were culture-positive, all of which were also TOP-positive, and 14 (30%) were negative on both tests. Of the remaining 10 (22%) patients with culture-negative/TOP-positive results, two had a culture-positive sister sample, and one had a clinical diagnosis of TB that improved after treatment (Supplementary Table 3). Of the other seven patients, 2pon-A genotyping confirmed the presence of *M. tuberculosis* DNA in six (one sample was unavailable). The clinical history of these seven individuals with discordant results is shown in Supplementary Table 4. The per-patient sensitivity and specificity of TOP in Brazil compared to culture vs. the CRS are shown in Table 1.

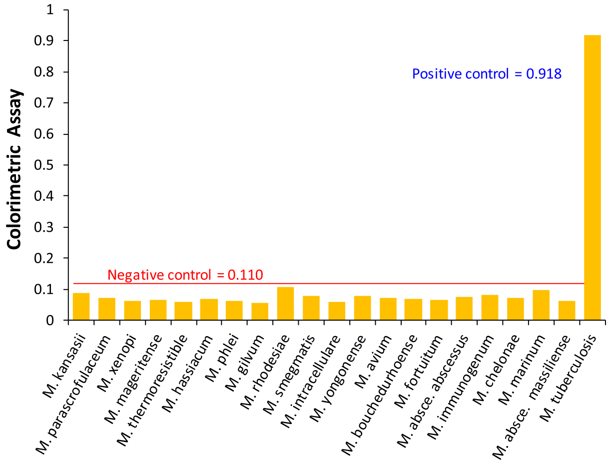
3.4. Boston study

Between February 21 and August 1, 2014, we obtained 60 respiratory samples (64% sputum, 35% induced sputum and 1% endotracheal aspirates) sourced from 60 patients. Supplementary Table 2 shows demographic characteristics of the study population. The median age of patients was 54 years (range 17–92), 63% were male, 50% were non-US born, 12% were HIV-infected, and 38% were current or former smokers. The median specimen volume was 1 mL (range 0·5-3). Of the 60 patients, 2 (3%) were culture positive; of these, one was TOP-positive (smear AFB-negative) and one was a bloody sample that was TOP-negative (smear AFB-positive). Fifty-two (87%) subjects tested negative on both tests, including three who were culture-positive for NTM (two *M. avium* and one *M. kansasii*). The remaining six patients were culture-negative/TOP-positive, of whom two had a culture-positive sister sample (Supplementary Table 3); genotyping results confirmed *M. tuberculosis* DNA in all six (see Supplementary Table 4 for clinical histories). The sensitivity and specificity of TOP compared to culture in the Boston study vs. the CRS are shown in Table 1.

4. Discussion

This study adds to published evidence that the TOP TB assay accurately detects *M. tuberculosis* DNA in respiratory samples from individuals with suspected TB with otherwise negative TB results based on culture alone. In the absence of culture to determine specificity, we validated our results using a CRS that included sequencing *M. tuberculosis* directly from specimens. Further, the analytical and clinical specificity of the assay demonstrate no cross-reactivity against closely related NTM species, further strengthening our results. These findings are consistent with both old and new data demonstrating the limitations of mycobacterial cultures to diagnose individuals with paucibacillary TB disease.

For decades, rapid detection of pulmonary TB has relied on sputum AFB smear microscopy, but its yield is low when compared to mycobacterial cultures [7]. Recently developed molecular tests such as Xpert MTB/RIF and GenoType MTBDRplus provide a rapid alternative to culture for patients with high bacterial loads (i.e. sputum smear AFB-positive). However, their overall sensitivity (~90% against culture) in programmatic conditions has been lower than initially anticipated [31], and particularly poor (~50%) in patients with paucibacillary TB disease [17,32–34]. A newer and improved version of the Xpert MTB/RIF assay (e.g. Xpert MTB/RIF Ultra) has demonstrated improved sensitivity, but it still remains below that of culture [35]. Therefore, a critical diagnostic gap in many programmatic settings remains. Without microbiological confirmation, the diagnosis of paucibacillary TB disease is rarely definite, forcing clinicians to employ empirical treatment



TOP OD	Mycobacterial Species	Culture ID
0.089	M. kansasii	MK-1037
0.070	M. parascrofulaceum	MO-5495
0.061	M. xenopi	MO-5452
0.065	M. mageritense	MO-5569
0.060	M. thermoresistible	MO-3080
0.067	M. hassiacum	MO-5426
0.063	M. phlei	MO-16:53109
0.057	M. gilvum	MO-3761
0.108	M. rhodesiae	MO-5403
0.078	M. smegmatis	MO-5511
0.060	M. intracellulare	MA-16:53588
0.078	M. yongonense	MA-7437
0.071	M. avium	MA-8495
0.068	M. bouchedurhonense	MA-6521
0.066	M. fortuitum	MF-4621
0.075	M. abscessus sub. abscessus	MC-10021
0.080	M. immunogenum	MO-5515
0.071	M. chelonae	MC-16:53526
0.098	M. marinum	MM-780
0.061	M. abscessus sub. Massiliense	MC-16:53493
0.918	M. tuberculosis	BCG
0.110	Negative control	N/A

Fig. 1. Analytical Specificity of the TOB TB Assay against Selected Non-Tuberculosis Mycobacteria (NTM). This panel includes 20 different species of NTM provided by Richard Wallace, MD (University of Texas at Tyler, TX). The list of species was selected by their genetic proximity to TOP TB's diagnostic target (3ponA). For each NTM species, a loopful of culture was harvested into DNA extraction solution (Epicentre, Madison WI) and processed for DNA extraction as described [22]. DNA preparations (~ 10^5 to 10^7 colony-forming units; high copy numbers) were mixed with human DNA preparation (0.5 µg from a healthy volunteer) before testing to simulate clinical sample conditions. Positive (~ 25 CFU of *M. tuberculosis*; low copy number) and negative controls containing 0.5 µg of background human DNA were included. TOP TB assay conditions and primers were used to generate amplicons that were detected using the same capture-probe colorimetric assay used for the TOP TB assay, with resultant Optical Densities (OD) [22].

algorithms that lead to over- or under diagnosis, unnecessary drug toxicity, and increased morbidity and mortality [1,36].

Results from this study add credence to our previous finding of 72% TOP-positivity (compared to 18% culture-positivity) in the sputum of HIV-infected patients with suspected pulmonary TB in Mbarara, Uganda [22] –a population at a particularly high pre-test probability of TB disease. The pilot study in Brazil demonstrated feasibility in predominantly sputum smear AFB-positive specimens but provided limited information on overall diagnostic performance. In contrast, diagnostic accuracy of TOP in Boston was almost identical to that reported in Uganda, despite extreme differences in disease prevalence. The clinical histories of patients with discordant culture-negative/TOP-positive results in this study were highly suspicious for TB disease, as indicated by repeated evaluations for TB over time, individual risk factors for TB disease, and imaging findings consistent with TB. Furthermore, of the 16 individuals with discordant results, four had a previous or concurrent culture-positive sample or a clinical diagnosis of TB. Of the eight patients with culture-positive NTM disease, six were TOP-negative and two were TOP-positive. Based on the clinical history of the latter two patients, we suspect a mixed mycobacterial infection, where *M. tuberculosis* likely represented a minority population, as suggested in a recent study with Xpert MTB/RIF that coincidentally included the same

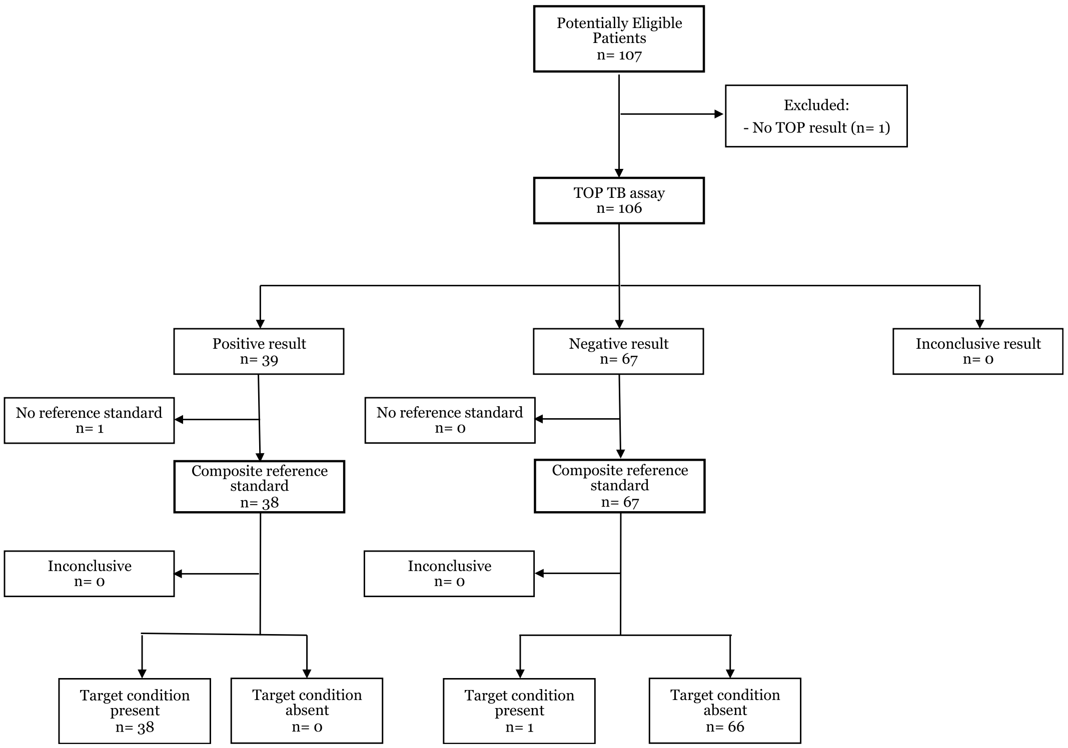


Fig. 2. Study profile.

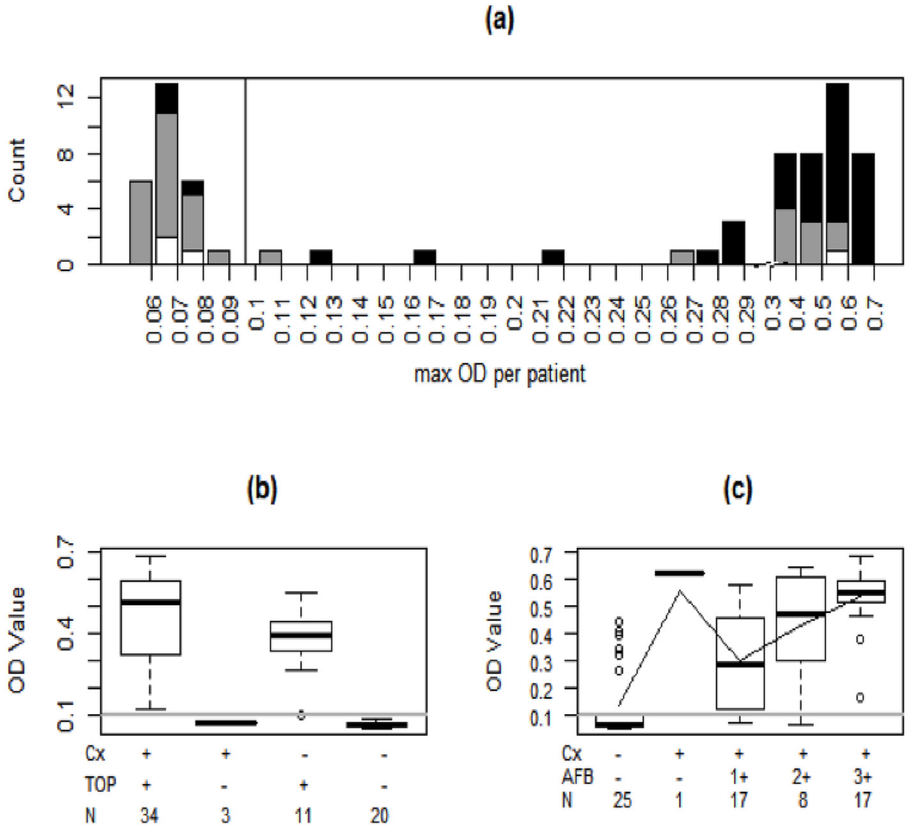


Fig. 3. TOP TB assay results in 72 specimens (46 subjects) from Vitória, Brazil. Four subjects with nontuberculous mycobacteria were excluded from graphs b and c for plotting purposes. **(a)** Vertical line denotes the cut-off TOP OD (0.0984) for a positive test. Histograms represent the number of subjects with culture-positive (black), culture-contaminated (white) and culture-negative (grey) results, by TOP OD values. The X-axis is zoomed in at the lower end of TOP OD values (0.100–0.300) for ease of comparison with the Uganda study [22]. **(b)** Group TOP OD values according to culture (Cx) and TOP results (group means are 0.47, 0.07, 0.38 and 0.06, from left to right). The mean TOP OD of culture-positive/TOP-positive (0.47) and culture-negative/TOP-positive samples was similar (0.38, P = 0.07). **(c)** Median TOP ODs paralleled sputum acid-fast bacilli (AFB) smear microscopy grades (Kruskall Wallis P < 0.001), supporting semi-quantitative performance of the TOP TB assay. A smoothing spline fit to the data is shown. When compared to culture, the per-specimen sensitivity and specificity of the TOP TB assay in Brazil was 92% (34/37, 95% CI 77%–98%] and 66% (23/35, 95% CI 48–80), respectively.

Table 1
Per-patient sensitivity and specificity of the TOP TB assay according to a reference standard established by *Mycobacterium tuberculosis* culture or a Composite Reference Standard in Brazil and Boston studies.

Study site	Culture Reference Standard				Composite Reference Standard[a]			
	MTB detected	MTB not detected	Sensitivity (95% CI)	Specificity (95% CI)	MTB detected	MTB not detected	Sensitivity (95% CI)	Specificity (95% CI)
Brazil (n = 46)								
TOP TB assay	22/22	14/24	100% (85–100)	58% (37–77)	31/31	14/15	100% (89–100)	93% (68–100)
Culture	–	–	–	–	22/31	15/15	65% (46–80)	100% (78–100)
Boston (n = 60)								
TOP TB assay	1/2	52/58	50% (1–99)	90% (78–96)	7/8	52/52	88% (47–100)	100% (93–100)
Culture	–	–	–	–	2/8	52/52	25% (3–65)	100% (93–100)

Definition of abbreviations: CI = Confidence interval; MTB = *Mycobacterium tuberculosis*.

[a] Composite Reference Standard (CRS) included *M. tuberculosis* culture, response to antituberculous treatment, AFB smear and *M. tuberculosis* sequencing (e.g. 2-ponA genotyping) [17]. The breakdown of CRS results is shown in Appendix (Supplementary Table 3).

two countries as this study [37]. Finally, we used a reproducible genotyping method that is based on the genetic signature of a global collection of *M. tuberculosis* isolates representing all major phylogenetic lineages [22]. The variable frequency of 2-ponA variants between Mbarara, Vitória and Boston add yet another layer of evidence. Taken together, these data suggest that TOP TB enables accurate detection of *M. tuberculosis* DNA in several categories of culture-negative paucibacillary TB patients including those with HIV/AIDS, extra-pulmonary TB, and old untreated TB (as indicated by apical fibrous scarring on chest radiograph) [38,39].

While promising, our results will require clinical validation with patient outcomes as the biological and clinical significance of detecting *M. tuberculosis* DNA in culture-negative TB suspects is not well understood and rife with speculation [40]. Currently, the most widely held opinion is that such results represent non-viable bacilli that are clinically insignificant because DNA-positive/culture-negative discordant results have been most frequently reported in individuals during or after completing antituberculous therapy [40–42]. However, several lines of evidence suggest that this interpretation may be an oversimplification, particularly when semi-quantitative assays such as TOP indicate trace amounts of *M. tuberculosis* DNA are present (i.e. confirming paucibacillary disease), or no previous history of TB treatment is elicited. First, mycobacterial culturing techniques were specifically developed several decades ago to provide a nutrient rich, oxygen and temperature-controlled medium artificially optimized for mycobacterial growth; therefore, their yield is susceptible to disruption by various conditions such as chemicals, antituberculous treatment, and as-yet poorly understood phenotypic or genotypic changes altering mycobacterial growth dynamics. For example, the sample decontamination process that is required prior to standard cultures is inherently detrimental to mycobacterial viability, likely compromising their yield in paucibacillary samples [7]. Second, *M. tuberculosis* is known to enter into a persister-like phenotype shortly after initiation of antituberculous treatment. Although *M. tuberculosis* with the persister phenotype is nonculturable with standard culture media, its viability is restored with enriched media [9,43]. Lastly, there is extensive precedent, extending back over 20 years, of numerous molecular assays with analytical and clinical sensitivity that is superior to that of culture for multiple microorganisms and sample types [44]; early results for many of these tests faced similar claims of non-viability, only to later replace culture as the preferred diagnostic method [45,46].

Furthermore, these is a growing body of data challenging the notion that mycobacterial growth is required to define clinically relevant TB disease. In a study that used advanced imaging techniques, patients with initially culture-positive results who remained persistently Xpert-positive at end of antituberculous treatment (even when clinical and microbiological cure was achieved), were at increased risk for TB relapse [8]. In South Africa, Chengalroyen et al. recently confirmed the existence of differentially detectable *M. tuberculosis* populations in the sputum of patients with suspected TB that were not culturable with standard mycobacterial culture methods [9,10]. Other individuals with active disease harboring non-culturable organisms include those with unstable latent TB infection or persons with early sub-clinical disease who have "percolating" organisms [47], and those with old untreated TB [39]. Taken together, these data have significantly complicated the TB diagnostic landscape and show the need for caution when interpreting DNA-positive/culture-negative results in patients with a high-pretest probability of disease [11].

4.1. Limitations

Our study has limitations. The two studies reported here used minor differences in sample handling and laboratory methods, which may have introduced some measure of variability in assay performance when compared to culture. In Boston, there were a small number of culture positive samples; and overall, four of the specimens we tested were culture-positive/TOP-negative (three of which had a TOP-positive sister sample) as a result of including challenging specimens such as low-volume samples and one hemorrhagic sample in Boston (PCR inhibition could have led to a false-negative result). Also, the colorimetric readouts used different, study-specific cut-off values to establish the "Limit of Blank", a key assay parameter [29]. Future development work will need to incorporate the TOP assay into a standardized molecular platform. Importantly, we did not follow participants with TOP-positive/culture-negative results for clinical outcomes; therefore, we do not know the clinical significance of such results. Admittedly, detection of trace amounts of *M. tuberculosis* DNA may be due to bacterial "spillage" from a dormant lung focus or low-level bacterial replication that may not require treatment. Finally, the TOP TB assay does not include provisions for detecting drug-resistant TB, but the primary global need is for a rapid and reliable triage test with high sensitivity [2,6].

5. Conclusions

Because of limitations in existing technologies, including mycobacterial cultures, microbiological confirmation of paucibacillary TB disease remains a key diagnostic gap. By shifting diagnostic emphasis to early detection, the TOP TB assay broadens sensitive and accurate detection of *M. tuberculosis* across the clinical spectrum of pulmonary TB disease. While use of the TOP assay for definitive diagnosis of active disease will require prospective validation with clinical outcomes, its operating characteristics suggest that it may have more immediate utility as a triage or "TB rule out" test.

Author contributions

Conception and Design: GM, NM, MP, RD and EJ-L; Acquisition of Data: GM, SV, BO, MG and EJ-L; Analysis and Interpretation: GM, LW, RD and EJ-L. Also, MM, PO, JM-A and YB participated in early development of the TOP TB assay. All authors contributed to either drafting or revising this manuscript and gave final approval.

Declarations of interest

Drs. Madico and Jones-López are co-inventors of TOP TB assay's primers and probes. This invention is owned by Boston Medical Center, which has submitted an international patent application. Dr. Madico, Dr. Jones-López and Mr. Joe Straight were co-founders and shareholders of Thisis Diagnostics Inc., a start-up company created in 2013 to develop and commercialize the TOP TB assay. Thisis Diagnostics Inc. was dissolved in late 2018.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.03.007.

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