Title
Nucleic acid amplification tests for diagnosis of smear-negative TB in a high HIV-prevalence setting: a prospective cohort study.

Permalink
https://escholarship.org/uc/item/2jg1p9rx

Journal
PloS one, 6(1)

ISSN
1932-6203

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Publication Date
2011-01-27

DOI
10.1371/journal.pone.0016321

Peer reviewed
Abstract

Background: Nucleic acid amplification tests are sensitive for identifying Mycobacterium tuberculosis in populations with positive sputum smears for acid-fast bacilli, but less sensitive in sputum-smear-negative populations. Few studies have evaluated the clinical impact of these tests in low-income countries with high burdens of TB and HIV.

Methods: We prospectively enrolled 211 consecutive adults with cough ≥2 weeks and negative sputum smears at Mulago Hospital in Kampala, Uganda. We tested a single early-morning sputum specimen for Mycobacterium tuberculosis DNA using two nucleic acid amplification tests: a novel in-house polymerase chain reaction targeting the mycobacterial secA1 gene, and the commercial Amplified® Mycobacterium tuberculosis Direct (MTD) test (Gen-Probe Inc, San Diego, CA). We calculated the diagnostic accuracy of these index tests in reference to a primary microbiologic gold standard (positive mycobacterial culture of sputum or bronchoalveolar lavage fluid), and measured their likely clinical impact on additional tuberculosis cases detected among those not prescribed initial TB treatment.

Results: Of 211 patients enrolled, 170 (81%) were HIV-seropositive, with median CD4+ T-cell count 78 cells/μL (interquartile range 29–203). Among HIV-seropositive patients, 94 (55%) reported taking co-trimoxazole prophylaxis and 29 (17%) reported taking antiretroviral therapy. Seventy-five patients (36%) had culture-confirmed TB. Sensitivity of MTD was 39% (95% CI 28–51) and that of secA1 was 24% (95% CI 15–35). Both tests had specificities of 95% (95% CI 90–98). The MTD test correctly identified 18 (24%) TB patients not treated at discharge and led to a 72% relative increase in the smear-negative case detection rate.

Conclusions: The secA1 and MTD nucleic acid amplification tests had moderate sensitivity and high specificity for TB in a predominantly HIV-seropositive population with negative sputum smears. Although newer, more sensitive nucleic acid assays may enhance detection of Mycobacterium tuberculosis in sputum, even currently available tests can provide substantial clinical impact in smear-negative populations.

Introduction

In 2009, 43% of the 4.6 million new pulmonary tuberculosis cases reported to the World Health Organization were diagnosed without microbiologic confirmation [1]. The failure to confirm a diagnosis with microbiologic testing can result in inappropriate management due to misdiagnosis and failure to initiate appropriate therapy early in the disease process. These disadvantages are especially relevant in low-income countries where supplementary imaging and laboratory data are usually not available to support an empiric diagnosis, and where late presentation for care is more common. A timely and accurate diagnosis of TB must be made if treatment is to be successful and person-to-person transmission reduced. Given the logistical challenges of making reliable sputum smear microscopy and culture facilities widely available, a simple, highly accurate, and rapid diagnostic test could have a dramatic
impact on worldwide TB transmission, morbidity, and mortality [2].

Nucleic-acid amplification tests (NAATs) targeting Mycobacterium tuberculosis (MTB) have enormous potential to improve TB case detection, with commercial NAATs, such as the GenProbe Amplified® Mycobacterium Tuberculosis Direct (MTD) Test (Gen-Probe Inc, San Diego, CA), reported to have nearly perfect sensitivity in sputum smear-positive patients and a sensitivity of 61–76% in smear-negative patients [3,4,5,6]. We have previously shown in a small study that a novel NAAT targeting a conserved region of the mycobacterial-genus secA1 gene and capable of identifying all clinically significant species of mycobacteria including M. tuberculosis, has a sensitivity of 100% and a specificity of 90% in sputum smear-negative TB suspects in Uganda, most of whom were HIV-seropositive [7]. In the current study, we evaluated the diagnostic accuracy, incremental value, and potential clinical impact of the secA1 and MTD NAATs in a population at high risk for both HIV and TB.

Materials and Methods

Ethics Statement

The Makerere University Faculty of Medicine Research Ethics Committee, the Mulago Hospital Institutional Review Board, the Uganda National Council for Science and Technology, and the University of California San Francisco Committee on Human Research approved the protocol. Some of these patients have been previously included in published studies [8,9,10,11,12,13,14,15].

Participants

Between September 2007 and May 2008, we prospectively enrolled consecutive inpatients ≥18 years of age who were admitted to Mulago Hospital in Kampala, Uganda, with cough ≥2 weeks duration into a protocol designed to provide a detailed evaluation (including HIV testing, chest radiography, sputum examination, and bronchoscopy) to identify the etiology of each patient’s pulmonary symptoms. Patients with a prior history of TB within two years and those receiving treatment for active TB at the time of hospitalization were excluded. For the current analysis, we included only patients without a positive sputum smear examination by the Ziehl-Neelsen technique, which was the standard method for smear examination at the time of the study.

Patient Data and Specimen Collection

After obtaining written informed consent from participants, study medical officers recorded demographic and clinical information following a standardized interview, physical exam, and frontal chest radiography. A laboratory technician gave standardized instructions on proper sputum submission [16], after which each patient provided two sputum samples (one spot sample on day one and one early-morning sample on day two) for microscopic examination and culture. The technician recorded the volume and quality of sputum according to technical guidelines [17]. Study medical officers referred HIV-seropositive patients with negative acid-fast bacilli (AFB) smears for bronchoscopy with bronchoalveolar lavage (BAL). Chest physicians performed bronchoscopy according to a standardized protocol that included airway inspection for Kaposi’s sarcoma lesions and collection of BAL fluid for microbiologic examination including mycobacterial smear and culture. Full details of bronchoscopy and examination of the clinical specimens have been previously described [11].

At discharge, we asked patients to return in two months for a follow-up clinical evaluation and repeat sputum examination if still symptomatic. After participants had completed all study proce-
consensus reading reached after joint review of discrepant results. The secA1 and MTD data were unblinded only after a final assignment had been made for all samples.

Statistical Analysis
We used STATA 11.0 (Stata Corporation, College Station, TX) for statistical analyses and defined a finding as significant if the probability of a two-tailed, type-I error (p-value) was <0.05. We calculated sensitivity, specificity, positive predictive value, and negative predictive value of each of the sputum NAATs in reference to two separate gold standards. For the primary analysis, we defined TB as present if a patient had ≥1 positive sputum or BAL mycobacterial culture. As a secondary analysis, we defined TB as present if cultures were positive or if a patient was clinically diagnosed with culture-negative TB at the 2-month follow-up visit.

Results
Participants
Four hundred seventy consecutive adult patients with cough ≥2 weeks were enrolled in the overall study. One hundred seventy-six were sputum AFB smear-positive by Ziehl-Neelsen staining, which had a sensitivity of 66%; 26 had only one sputum collected; eight patients had missing sputum samples; and seven patients were missing AFB results. Of the remaining 253 patients, 42 were excluded because culture results or final diagnoses were unknown, leaving 211 patients for this analysis (Figure 1).

Patients had a median age of 35 years (inter-quartile range (IQR) 28–42; Table 1). Slightly more patients were female (114, 54%) than male, and the majority had received antibiotics prior to hospital admission (139, 66%). Most patients were HIV-seropositive (170, 81%), with a median CD4+ T-cell count of 78 cells/µL (IQR 29–203 cells/µL); 94 (55%) of the HIV-seropositive patients were taking PCP prophylaxis, and 29 (17%) were taking antiretroviral therapy. Patients with HIV tended to be younger (median age 34 years, IQR 28–39) than patients without HIV (median age 48 years, IQR 34–62; p = 0.001), and more often female (57% vs. 41%; p = 0.072). Fifty-two of the 211 patients with negative sputum exams by conventional light microscopy had positive sputum smear examinations using concentrated fluorescence microscopy, but only 34 of these 52 patients (65%) had a confirmed TB diagnosis by mycobacterial culture or clinical follow-up. One hundred nine patients (52%) underwent bronchoscopy. No adverse events arose as a result of sputum collection or bronchoscopy. The clinical impacts of fluorescence microscopy

Figure 1. Patients enrolled, eligible, and analyzed.
doi:10.1371/journal.pone.0016321.g001
and of bronchoscopy in this population have been previously described [9,13].

Seventy-five of the 211 patients (36%) had culture positive TB. An additional 17 patients who had negative mycobacterial cultures met clinical criteria for TB, while for 44 patients, an in-person clinical assessment could not be made because no follow-up evaluation occurred at two months. Thus, a total of 92 (55%) out of 167 patients had culture-confirmed or clinically confirmed TB. Among 193 patients with known vital status at two months, 46 (24%) had died, including 41 (26%) HIV-positive and 5 (14%) HIV-negative patients (mortality difference 12%, 95% CI -1 to +25, p = 0.13).

Table 1. Demographic and clinical characteristics of the 211 study participants.

| Characteristics                       | All patients (n = 211) | HIV-seropositive (n = 170) | HIV-seronegative (n = 41) |
|---------------------------------------|------------------------|-----------------------------|---------------------------|
| Median age, years (IQR)               | 35 (28–42)             | 34 (28–39)                  | 48 (34–62)                |
| Women                                 | 114 (54)               | 97 (57)                     | 17 (41)                   |
| Taking antibiotics prior to admission| 139 (66)               | 115 (68)                    | 24 (59)                   |
| Median CD4+ T-cell count, cells/μL (IQR) | - -                    | 139 (29–203)               | - -                       |
| Taking co-trimoxazole prophylaxis on admission | - -                   | 94 (55)                    | - -                       |
| Taking antiretroviral therapy on admission | - -                   | 29 (17)                    | - -                       |
| Culture-confirmed TB                  | 75 (36)                | 67 (39)                     | 8 (20)                    |
| Clinically confirmed or culture-confirmed TB | 92 (55)*              | 81 (61)**                   | 11 (32)**                 |
| Two-month mortality                   | 46 (24)*               | 41 (26)**                   | 5 (14)**                  |

Abbreviations: IQR, inter-quartile range; N, number; TB, tuberculosis.

Legend: *Characteristics presented as number and column percentage unless otherwise indicated. Missing or indeterminate results reduced the number at risk in the superscripted subsets as follows: **N = 167; ***N = 133; ****N = 34; †N = 193; ††N = 157; †††N = 36.

doi:10.1371/journal.pone.0016321.t001

Results of Sputum NAATs

Using mycobacterial culture results as the gold standard, the sensitivity of MTD for diagnosing TB was 39% (95% Confidence Interval (CI) 28–51), which was 15% higher (95% CI 2–27, p = 0.019) than that of secA1, which had a sensitivity of 24% (95% CI 15–35; Table 2). The specificities of MTD (95%, 95% CI 90–98) and secA1 (95%, 95% CI 90–98) were the same (specificity difference 0%, 95% CI 0–0).

Table 2. Diagnostic performance of nucleic acid amplification tests for TB, compared to both the primary and secondary reference standards.

| Index Test Results |
|-------------------|
| Reference Positive (N) | Reference Negative (N) | Percent Sensitivity (95% CI) | Percent Specificity (95% CI) |
| Mycobacterial culture reference standard | 75 | 136 | |
| MTD | |
| Positive | 29 | 7 | 39% (28–51) | 95% (90–98) |
| Negative | 46 | 129 | |
| Indeterminate | - | - | |
| secA1 | |
| Positive | 18 | 7 | 24% (15–35) | 95% (90–98) |
| Negative | 57 | 128 | |
| Indeterminate | - | 1 | |
| Clinical TB reference standard | 92 | 75 | |
| MTD | |
| Positive | 29 | 2 | 32% (22–42) | 97% (91–100) |
| Negative | 63 | 73 | |
| Indeterminate | - | - | |
| secA1 | |
| Positive | 18 | 1 | 20% (12–29) | 99% (93–100) |
| Negative | 74 | 74 | |
| Indeterminate | - | - | |

Abbreviations: CI, confidence interval; IQR, inter-quartile range; MTD, Gen-Probe Amplified Mycobacterium Tuberculosis Direct test; secA1, secretory gene A1 target test; TB, tuberculosis.

doi:10.1371/journal.pone.0016321.t002
CI −4.3 to +4.3, p = 1.0). The positive predictive value for MTD was 81% (95% CI 64–92), and the negative predictive value was 74% (95% CI 67–80). The secA1 had a positive predictive value of 72% (95% CI 51–88), and a negative predictive value of 69% (95% CI 62–76). One patient had an indeterminate secA1 result secondary to PCR inhibition that could not be overcome by dilution.

When clinically-defined TB was used as the reference standard, both tests had slightly lower sensitivities but higher specificities. MTD had a sensitivity of 32% (95% CI 22–42) and secA1 a sensitivity of 20% (95% CI 12–29), with a sensitivity difference of 12% (95% CI 2–22, p = 0.019). Again, the specificity of MTD (97%, 95% CI 91–100) and secA1 (99%, 95% CI 93–100) were similar (specificity difference 1.3%, 95% CI −2.6 to +5.3, p = 1.0). The positive predictive value of MTD was 94% (95% CI 79–99), and the negative predictive value was 54% (95% CI 45–62). The secA1 had a positive predictive value of 95% (95% CI 74–100) and a negative predictive value of 50% (95% CI 42–58).

Both the secA1 and the MTD tests had a higher sensitivity in HIV-seropositive patients than HIV-seronegative patients, using culture as the reference standard, although the precision of the estimates among HIV-seronegatives was limited by the small number of patients (n = 41, Table 3). MTD had a sensitivity of 42% (95% CI 30–55) among HIV-seropositive patients and a sensitivity of 13% (95% CI 0–53) among HIV-seronegative patients (difference in sensitivity, 29%, 95% CI −7 to +65, p = 0.14). The secA1 had a sensitivity of 25% (95% CI 16–38) among HIV-seropositive patients, and 13% (95% CI 0–53) among HIV-seronegative patients (difference in sensitivity, 13%, 95% CI −19 to +44, p = 0.67). There were no major differences in sputum quality, volume, smear-grade, or culture-grade between patients with and without HIV (data not shown).

### Potential Clinical Impact of Nucleic Acid Amplification Testing

At hospital discharge, only 25 (33%) of 75 AFB smear-negative patients ultimately diagnosed with culture-positive TB had been prescribed TB treatment. Had results of the more sensitive of the two NAATs, the MTD, been made available to clinicians within 24 hours of sputum collection, 18 additional TB patients could have been correctly started on TB treatment, while only two patients with negative mycobacterial cultures would have been inappropriately started on treatment. Thus, availability of MTD results would have lead to an absolute increase in sensitivity for TB of 24%, and a relative increase of 72% in early TB case detection among the Ziehl-Neelsen AFB-smear-negative population (43 TB patients if MTD results had been available compared to 25 TB patients when MTD results were not available). Instead of waiting for culture results to initiate TB treatment, these patients could have been started a median of 27 days (range 15–42 days) earlier.

Unfortunately, 10 of these 18 smear-negative, MTD-positive, culture-positive TB patients died a median of 8.5 days (range 4–13 days) after enrollment, and it is unknown whether earlier initiation of treatment for these patients would have improved their outcomes.

### NAAT Performance and Impact among Patients Smear-negative by Fluorescence Microscopy

Among 159 patients with negative fluorescence microscopy results, the MTD was 29% (95% CI 17–44) sensitive in reference to mycobacterial culture, while secA1 was only 8% sensitive (95% CI 2–20). Both NAATs were equally specific in fluorescence microscopy smear-negative patients (98%, 95% CI 94–100).

### Table 3. Diagnostic performance of nucleic acid amplification tests for TB stratified by HIV status, compared to the primary reference standard only.

| Index Test Results | Reference Positive (N) | Reference Negative (N) | Percent Sensitivity (95% CI) | Percent Specificity (95% CI) |
|--------------------|------------------------|------------------------|-----------------------------|-----------------------------|
| Mycobacterial culture reference standard | 75 | 136 | | |
| HIV-seropositive patients | 67 | 103 | | |
| MTD | | | 42% (30–55) | 93% (87–97) |
| Positive | 28 | 7 | | |
| Negative | 39 | 96 | | |
| Indeterminate | - | - | | |
| secA1 | | | 25% (16–38) | 93% (86–97) |
| Positive | 17 | 7 | | |
| Negative | 50 | 95 | | |
| Indeterminate | - | 1 | | |
| HIV-seronegative patients | 8 | 33 | | |
| MTD | | | | |
| Positive | 1 | 0 | 13% (0–53) | 100% (89–100) |
| Negative | 7 | 33 | | |
| Indeterminate | - | - | | |
| secA1 | | | | |
| Positive | 1 | 0 | 13% (0–53) | 100% (89–100) |
| Negative | 7 | 33 | | |
| Indeterminate | - | - | | |

Abbreviations: CI, confidence interval; IQR, inter-quartile range; MTD, Gen-Probe Amplified® Mycobacterium Tuberculosis Direct test; secA1, secretory gene A1 target test; TB, tuberculosis.

doi:10.1371/journal.pone.0016321.t003

PLoS ONE | www.plosone.org 5 January 2011 | Volume 6 | Issue 1 | e16321
Among 48 fluorescence smear-negative, mycobacterial culture-positive patients, 12 (25%) patients were empirically initiated on TB treatment. Had MTD results been available to clinicians, an additional 10 (21%) patients could have been prescribed treatment (22 TB patients if MTD results had been available compared to 10 TB patients when MTD results were not available), an 83% relative increase in the proportion of fluorescence microscopy smear-negative cases initiated on treatment.

Discussion

Adoption of new technologies for TB diagnosis in highly endemic areas is a challenge because of the start-up and training costs. Yet, increased use of molecular tests for TB would permit more expeditious initiation of treatment and other measures to reduce TB transmission, which could eventually pay large dividends for global TB control [22,23]. Although studies suggest that NAATs are cost-effective for TB diagnosis even in low-income countries [24,25], this technology has been used to only a limited extent to evaluate smear-negative TB suspects in sub-Saharan Africa (Table 4). These studies have reported variable sensitivity depending on HIV prevalence, but have consistently shown that these assays can identify a substantial proportion of smear-negative TB patients. Delays in adopting NAATs for TB reflect ongoing operational and feasibility concerns in low-income settings [26], but a lack of information about the clinical impact and incremental value of these tests beyond the standard diagnostic algorithm of sputum smear microscopy and clinician judgment may also have contributed to the slow uptake of this technology [21,27].

In a prospective diagnostic cohort study of patients suspected of TB in a low-income country with a high prevalence of TB and HIV, we have shown that NAATs identify many smear-negative TB patients whom clinicians would otherwise fail to diagnose. Although the sensitivities of the NAATs evaluated in our study were modest compared to mycobacterial culture, we found that, if routinely applied, same day NAAT would have decreased time-to-treatment initiation in smear-negative TB patients by almost four weeks. Because smear-negative TB may account for up-to-half of all TB cases in sub-Saharan Africa [1], our data suggest that finding a way to put nucleic acid tests into everyday use could increase TB case detection in the region by 20% or more.

We undertook this study because prior data from a small cohort of smear-negative patients suggested that the secA1 assay might have a high sensitivity in smear-negative patients. In the current study, however, the in-house secA1 PCR assay was less sensitive than a commercial amplification assay at identifying MTB in AFB-smear-negative sputum, although both assays were equally specific. These results are likely explained by the fact that secA1 is a single-copy gene, whereas the MTD test targets rRNA, which is present in multiple copies per organism. Because the comparative advantage of the secA1 assay actually lies in its ability to distinguish multiple mycobacterial species [19], it may be better suited for use in lower TB incidence settings, where non-tuberculous mycobacterial pathogens are common and speciation is clinically important.

The sensitivity of the MTD test was also relatively low in our study and lower than that reported in previous studies in similar populations [26]. This may reflect the effects of freezing, storage, thawing and delayed processing on the integrity of nucleic acids (especially RNA) in the sputum specimens. More importantly and especially noteworthy for future demonstration studies of NAATs in sputum smear-negative populations, the lower sensitivity we observed could be explained by the higher sensitivity of sputum smear microscopy (33% in the previous study vs. 66% in our study) and mycobacterial culture (71% in the previous study vs. 94% in our study) used to define the eligible population [26]. Use of poor-quality microscopy to select eligible patients, or poor-quality cultures to define the reference standard are well-known biases in diagnostic studies [28,29], biases which are best avoided entirely by also reporting measures of clinical impact. Thus, it is important that MTD detected a substantial proportion of untreated cases of smear-negative TB, whether TB was defined clinically or microbiologically, and whether smear-negativity was defined using conventional light or concentrated fluorescence microscopy.

There were some limitations to the study. First, NAATs were performed in a research laboratory far from the clinical site, which prevented assessment of the accuracy or feasibility of the tests in the routine operational setting, and may have resulted in a lower sensitivity for the reasons noted above. Second, for specimens that required dilution because of PCR inhibition and proved to be negative after 3-to-100 fold dilution using the secA1 assay, improved sensitivity may have been seen if we had been able to eliminate the inhibition and run the assay at higher concentrations. Third, although the sensitivities of the NAATs in this study were much higher among HIV-seropositive patients than among HIV-seronegative patients, we had insufficient power to determine whether these differences were statistically significant. This will be

### Table 4. Diagnostic performance of sputum nucleic acid amplification testing for TB diagnosis among smear-negative TB suspects in sub-Saharan Africa.

| Location (Year) | Test | HIV Prevalence | Percent Sensitivity (95% CI) | Reference |
|-----------------|------|---------------|----------------------------|-----------|
| Zambia (2001)   | IS6110 PCR | 75%\(^\dagger\) | 40 (25–57) | [26] |
| Zambia (2001)   | Gen-Probe AMTD | 75%\(^\dagger\) | 60 (43–75) | [26] |
| South Africa\(^a\) (2010) | Cepheid GeneXpert MTB/RIF | 66%\(^\dagger\) | 73 (65–79) | [6] |
| Uganda (2009)   | secA1 PCR | 46% | 100 (63–100) | [7] |
| Kenya (2004)    | Roche Amplicor MTB | 35% | 82%\(^\dagger\) (76–87) | [30] |

Abbreviations: IS, insertion sequence; PCR, polymerase chain reaction.

\(^{a}\)Estimated from population-specific HIV prevalence rather than directly measured;

\(^{\dagger}\)Also includes specimens from Azerbaijan, India, and Peru;

\(^{1}\)Sensitivity based on 3 PCR specimens.

doi:10.1371/journal.pone.0016321.t004
an important issue for future studies to address. Finally, neither of the reference standards used in this study included liquid mycobacterial culture, which might have provided higher, more accurate estimates of NAAT specificity and positive predictive value, and lower estimates of sensitivity and negative predictive value. In summary, we carried out a prospective diagnostic cohort study to evaluate the accuracy and potential effectiveness of nucleic acid amplification testing for TB diagnosis in a hospitalized population with a high incidence of HIV, and found modest sensitivity, excellent specificity, and a large potential clinical impact. Future studies should identify feasible ways to implement nucleic acid testing and evaluate them with attention not only to their diagnostic accuracy but also to their clinical and public health impact.

Acknowledgments

The authors wish to acknowledge the patients who participated in this study; the staff and administration of Mulago Hospital, including Dr. Samuel Yoo, who performed bronchoscopy and helped assign final diagnoses for enrolled patients; and the clinical, research, and administrative staff of the MU-UCSF Research Collaboration, especially Alfred Andama, Florence Nankya, Rachel Kyeyune, John Kiidha, Patrick Byanyima, and Kate Nabakibbi.

Authors Contributions

Conceived and designed the experiments: JLD LH AC JAK. Performed the experiments: JLD WW AC CH PSC PM JAK. Analyzed the data: JLD WW HM CH PSC PM JK. Contributed reagents/materials/analysis tools: JLD LH AC HM JAK. Wrote the paper: JLD CM JAK. Edited and reviewed final draft: JLD LH WW HM AC CH CM PSC PM JAK.

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