AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

COMMENTARY

Progress toward Developing Sensitive Non-Sputum-Based Tuberculosis Diagnostic Tests: the Promise of Urine Cell-Free DNA

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ABSTRACT A highly accurate, non-sputum-based test for tuberculosis (TB) detection is a key priority for the field of TB diagnostics. A recent study in the *Journal of Clinical Microbiology* by Oreskovic and colleagues (J Clin Microbiol 59:e00074-21, 2021, https://doi.org/10.1128/JCM.00074-21) reports the performance of an optimized urine cell-free DNA (cfDNA) test using sequence-specific purification combined with short-target PCR to improve the accuracy of TB detection. Their retrospective clinical study utilized frozen urine samples ($n = 73$) from study participants diagnosed with active pulmonary TB in South Africa and compared results to non-TB patients in South Africa and the United States in an early-phase validation study. Overall, this cfDNA technique detected TB with a sensitivity of 83.7% (95% CI: 71.0 to 91.5) and specificity of 100% (95% CI: 86.2 to 100), which meet the World Health Organization's published performance criteria. Sensitivity was 73.3% in people without HIV (95% CI: 48.1 to 89.1) and 76% in people with smear-negative TB (95% CI: 56.5 to 88.5). In this commentary, we discuss the results of this optimized urine TB cfDNA assay within the larger context of TB diagnostics and pose additional questions for further research.

Of the approximately 10 million people who develop tuberculosis (TB) each year, an estimated three million are not identified by national TB programs and many likely remain undiagnosed (1). Within active TB disease, various forms of TB present additional diagnostic difficulties. Extrapulmonary TB diagnosis typically requires non-respiratory specimens that may require taking invasive biopsy specimens, and existing diagnostic tests are not optimized for these samples (2). Children with TB typically have paucibacillary disease, so detecting TB bacilli in their respiratory samples is particularly difficult (3). Similarly, many people living with HIV (PLHIV) cannot produce respiratory specimens needed for standard TB testing (4), including nucleic acid amplification tests or mycobacterial culture.

There is an urgent need for accurate, rapid, point-of-care, non-sputum-based tests to diagnose TB (5). Over the last 10 years, a variety of primarily sputum-based molecular TB diagnostic tests have received World Health Organization (WHO) endorsement and have become commercially available, such as Xpert MTB/RIF (Cepheid, USA), Xpert MTB/RIF Ultra (Ultra) (Cepheid, USA), and Truenat MTB/RIF (Molbio Diagnostics, India) (6). Unique product delivery models have been explored in an attempt to increase access to these new TB diagnostic technologies (7, 8), although implementation and scale-up have posed major challenges (9). At present, however, most emerging TB technologies, including TB biomarkers generally (5) and circulating cell-free DNA (cfDNA), specifically (10, 11), do not meet the published diagnostic accuracy criteria specified in the WHO's target product profiles (TPPs) (12).

Citation MacLean E, Nathavitharana RR. 2021. Progress toward developing sensitive non-sputum-based tuberculosis diagnostic tests: the promise of urine cell-free DNA. J Clin Microbiol 59:e00706-21. https://doi.org/10.1128/JCM.00706-21.

Editor Christine Y. Turenne, University of Manitoba

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For the article discussed, see https://doi.org/10.1128/JCM.00074-21.

The views expressed in this article do not necessarily reflect the views of the journal or of ASM.

Accepted manuscript posted online 12 May 2021

Published 19 July 2021

There is considerable interest in the development of urine-based tests to decrease the TB diagnostic gap and reach the "missing millions" of undiagnosed people with TB. Urine is easy to collect, is low risk from an infection-control perspective, and is obtainable from almost all patients, including children and PLHIV. One commercially available lateral flow lipoarabinomannan (LAM) TB test, Determine TB LAM (Abbott [formerly Alere], USA), uses urine samples to detect TB in PLHIV. Determine TB LAM has received WHO endorsement for this use case, which is stronger for PLHIV who are inpatients and symptomatic or with a low-CD4 count than for PLHIV who are outpatients and/or have a higher CD4 count, due to differences in test sensitivity in these groups (13). A next-generation, higher-sensitivity urine LAM test, SILVAMP TB (Fujifilm, Japan), is currently under evaluation in several countries (14). Its use is also being explored in HIV-negative patients, although reported sensitivity is lower in this group (53.2%; 95% CI: 43.9 to 62.1) (15) compared to approximately 70.4% (95% CI: 53.0 to 83.1) in PLHIV (14)).

Prior attempts at using urine as a sample for molecular TB tests have not yielded particularly sensitive results. A recent study examining urine Xpert MTB/RIF Ultra (Ultra) (Cepheid, USA) performance in Uganda displayed sensitivity of 17.2% (95% CI: 12.3 to 23.2) in HIV-negative participants, and sensitivity of 50.0% (95% CI: 28.2 to 71.8) in PLHIV with CD4 counts of $<$100 cells/μl (16). Urine TB-specific transrenal cfDNA, i.e., short fragments of DNA released by cellular breakdown that have filtered through the kidney barrier into the urine, has been used in a novel PCR-based assay in South Africa. Its overall sensitivity was 42.9% (95% CI: 35.4 to 50.5), and was marginally higher among PLHIV at 45.2% (95% CI: 34.4 to 56.5) than in HIV-negative participants at 40.0 (95% CI: 29.8 to 50.9 (17). However, none of these prior studies utilized sophisticated sample processing techniques to improve diagnostic accuracy, despite growing interest in preanalytical approaches to improve the yield of TB diagnostic tests.

Advances in specimen processing may solve part of the issue of low sensitivity in urine, including heightened understanding of the role of preanalytical variables (18), such as improved methods for extraction of cell-free genetic targets (compared in reference 19), and ultrasensitive hybridization DNA capture (20). This last technique was utilized to improve the performance of a urine based TB cfDNA test in a new study by Oreskovic and colleagues in this journal (21). Urine cfDNA is a difficult target to detect because of the low concentration of TB-specific fragments and short length ($<$100 bp); thus, steps that improve cfDNA capture should increase test sensitivity while keeping specificity high. The study team developed a DNA sequence-specific purification method that utilized oligonucleotide capture probes, targeting opposite strands of the double-stranded IS*6110* region, which were immobilized on magnetic beads. Beads were then added to urine samples. Combining sequence-specific purification with short-target PCR improved the capture of short (50 bp) TB cfDNA and lowered the limit of detection to $\leq$5 copies cfDNA/ml (21). This method was deployed using frozen urine samples from a small clinical study at Edendale Hospital, Pietermaritzburg, South Africa. Study participants were 49 adult patients with symptomatic active TB disease, defined by a positive Xpert MTB/RIF test result, as well as 10 control participants who were hospitalized with other non-TB diseases. Since the high TB burden at the enrollment site raised the risk of potentially enrolling people with undiagnosed TB as controls, the study team also enrolled 14 healthy adult control participants from the University of Washington, Seattle, USA, thus ensuring that one comparison group was truly TB-free.

The optimized cfDNA assay demonstrated a sensitivity of 83.7% ($n = 41/49$; 95% CI: 71.0 to 91.5) and specificity of 100% ($n = 24/24$; 95% CI: 86.2 to 100), meeting the WHO TPP criteria for a non-sputum biomarker-based detection test for active TB (12). For participants with positive Xpert MTB/RIF and culture, sensitivity increased to 88.2% ($n = 30/34$; 95% CI: 73.4 to 95.3). Some subgroup analyses were conducted, although these results should be interpreted with caution, as all were underpowered due to the small sample size. Among people with smear-positive TB, sensitivity was 100%, while among those with smear-negative TB, it declined to 76.0% ($n = 19/25$; 95% CI: 56.5 to

88.5). Sensitivity appeared higher for PLHIV than HIV-negative subjects (88.2%, 95% CI: 73.4 to 95.3 versus 73.3%, 95% CI: 48.1 to 89.1). CD4 count did not seem to modify performance, as sensitivity was similar in cases with CD4 counts of $\leq$200 cells/mm^3 and $>$200 cells/mm^3 (90.9% [$n = 20/22$; 95% CI: 72.2 to 99.4] versus 83.3% [$n = 10/12$; 95% CI: 55.2 to 97.0]; $P = 0.60$), but this will need to be further validated in larger studies.

This work has promising implications. The sequence-specific purification of urine TB cfDNA seems to lead to improved PCR sensitivity and specificity that is higher than that observed in previous urine molecular diagnostic studies, and may meet TPP performance criteria. Urine is a simple sample to collect and is easily accessible; this is an important factor for many underserved TB populations, particularly those with paucibacillary disease, including children and those with extrapulmonary disease. If larger studies confirm high test sensitivity in people without HIV, a urine TB cfDNA test could be more widely applicable than current urine LAM assays. It is also plausible that this urine cfDNA preparation method may be more broadly applicable to other diseases. Of note, centralized testing capacity has increased in many settings due to the COVID-19 pandemic (22–24) and, in association with promotion of the updated WHO Essential Diagnostics List (25), this may bode well for increased molecular testing of diseases such as TB going forward.

However, although the authors note that this is foundational work that could be further simplified to develop a future rapid test, this optimized testing approach would currently only be deployable in settings with centralized testing, which poses limitations for its applicability as a TB diagnostic test. Obtaining urine cfDNA is a high-complexity process; specialized reagents, lab equipment, and highly trained technicians are all necessary to complete the sample preparation in its current iteration, but recent increases in testing capacity may help improve access to this kind of centralized assay. Other high-throughput TB assays are in the late stages of development and are demonstrating high sensitivity and specificity, although these are focused on respiratory samples (26), so the niche for another centralized assay will have to be determined. Nonetheless, there is an urgent need for accurate non-sputum-based tests, and if processed-urine tests perform sufficiently well that they detect smear-negative and Ultra-negative TB, there could be many use cases for such an assay. Another issue that must be explored is the potential for lower cfDNA specificity in high HIV- and high TB-endemic areas, particularly in patients with a history of treated TB. As observed with Ultra in South Africa, decreased specificity due to residual DNA may be a problem for complex decision making related to treatment initiation in people with a history of TB treatment who are being evaluated for recurrent TB (27).

As this is an early-phase study, many questions remain. Large, prospective studies in a variety of settings will be important to understand urine cfDNA's performance, and to determine the optimal use case (e.g., triage versus diagnosis) and placement of such a test (e.g., inpatient or outpatient settings). Elucidating the accuracy of cfDNA as a biomarker for extrapulmonary TB will be critical. One study of next-generation LAM testing showed that LAM may be highly sensitive for certain types of extrapulmonary TB (28). If LAM, a bacterial cell wall component, is found in urine of people with extrapulmonary TB, cfDNA may be present in such cases as well. Determining whether urine cfDNA reliably decreases with anti-TB therapy will also be important. If so, it may have potential as a treatment monitoring tool or test of cure, which is a substantial gap in our current TB diagnostic armamentarium (29).

TB diagnostic innovation is needed more than ever. As a result of the COVID-19 pandemic, large declines in TB diagnosis and treatment initiation (an average of 23% compared to 2019) have been reported in high-TB-incidence countries (30). Simultaneously, the COVID-19 pandemic has also shown us that major advances in the development and implementation of rapid, accurate diagnostics are possible in a short time frame, including a disposable, high sensitivity, molecular self-test (31); use of a variety of specimens, including saliva and different types of swabs (32); and massive expansion of diagnostic

manufacturing and infrastructure capacities (33). TB and other infectious diseases stand to benefit from these advances if they can be translated with similar urgency and investment.

An accurate TB test that utilizes urine and is not limited to PLHIV has long been awaited, particularly in groups that have so far been left behind, such as children and others affected by extrapulmonary TB. Furthermore, if urine cfDNA testing could be deployable in peripheral settings and scaled up at the point-of-care, it could be a game-changer in efforts to find the "missing millions." It is, however, essential to remember that tests alone are not silver bullets (34). Rather, tests must be delivered as part of high quality care within the TB care cascade (35) if they are to prevent morbidity and mortality (36, 37).

ACKNOWLEDGMENT

E.M. is supported by a doctoral fellowship from the Fonds de Recherche du Québec–Santé.

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