Mycobacterium tuberculosis DNA detection on tongue swabs: Effect of anti-tuberculosis drug treatment

Lauren T. Sarkissian

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Public Health

University of Washington 2022

Committee:

Gerard A. Cangelosi

Christine Khosropour

David Horne

Program Authorized to Offer Degree:

School of Public Health

Department of Epidemiology

University of Washington

Abstract

Mycobacterium tuberculosis DNA detection on tongue swabs: Effect of anti-tuberculosis drug treatment

Lauren T. Sarkissian

Chair of the Supervisory Committee

Gerard A. Cangelosi

Department of Environmental Occupational Health Sciences

Oral swab analysis (OSA) can be used to detect *Mycobacterium tuberculosis* (MTB) DNA in patients with pulmonary tuberculosis (TB). No previous analyses have investigated the effect of anti-tuberculosis drug treatment on MTB detection via oral swabs. This study, using a cross-sectional design, evaluated the sensitivity and specificity of OSA post-treatment initiation relative to sputum culture. Oral swabs were collected from adults with pulmonary TB (age 18-50; N = 26-33) at months 0, 2, 4, and 6 post initiation of treatment at a clinical site in South Africa. Sputa were also collected and tested by Cepheid GeneXpert MTB/RIF Ultra (Xpert Ultra) and mycobacteriological culture. Treatment was effective in all patients as determined by sputum culture and clinical outcomes. MTB DNA was extracted from swabs and concentrated by ethanol precipitation. MTB DNA in the samples was detected by using a qPCR assay that is

specific to the *M. tuberculosis* complex IS6110 insertion element. OSA had a sensitivity of 74% and a specificity of 100% at month 0, with lower sensitivity and specificity at months 2 (sensitivity: 33%, specificity: 77%), 4 (sensitivity: NA, specificity: 89%), and 6 (sensitivity: NA, specificity: 76%). The reduced specificity at later months shows that OSA is susceptible to false-positive MTB DNA detection post-treatment, like sputum-based nucleic-acid amplification tests. These findings show that while OSA is promising for diagnosing TB, caution should be used when applying the method to patients post-treatment.

Introduction

Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* complex (MTBC) remains a leading cause of morbidity and mortality worldwide. In 2019, there were 10 million reported cases and 1.4 million deaths, 95% of which occurred in high-burden countries.[1] Co-infections, immunodeficiencies, and malnutrition are contributors to TB mortality in these regions.[1,2] Notably, individuals with HIV are 18 times more likely to develop active TB, and made up 8.2% of TB cases in 2019.[1,2] TB is primarily an infection of the lungs, which is referred to as pulmonary TB disease.[3] TB that infects areas other than the lungs is referred to as extrapulmonary TB disease.[4] Though TB is treatable, it requires adherence to a course of antibiotics that can last 6 months or more. Failure to adhere to treatment regimens can result in relapse or selection for multidrug-resistant tuberculosis (MDR-TB).[1,2]

Diagnosis of pulmonary TB is typically conducted by microbiologically assessing patient sputum.[5] Sputum samples undergo bacterial culture or nucleic acid-based

testing (most commonly GeneXpert or Xpert MTB/RIF or Ultra) to determine if a patient is TB-positive.[5] However, there limitations with sputum-based diagnostics. Sputum culture takes 2-6 weeks to yield results, and while sputum GeneXpert can provide diagnostics within 2 hours, it is less specific than culture.[6] Sputum collection can be hazardous to healthcare workers because of their exposure to aerosolized TB, resulting in significantly higher risk of TB compared to the general public.[7] Sputum is also difficult for patients to produce, particularly amongst children, people with HIV, and individuals whose TB infection is improving with antibiotics. In these cases, sputum production needs to be induced.[8] Another limitation of sputum-based diagnostic testing is its inability to test large groups of individuals easily and rapidly, which hinders case finding efforts.[9] These challenges with sputum-based diagnostics demonstrate the need for more efficient testing.

Oral swabs have been proposed as an alternative to sputum-based testing for TB infection.[5] Oral swabs are a more convenient sampling method; they are safer to collect by healthcare workers and better tolerated by a variety of patients.[9] Samples can also be collected in non-clinical community settings as well, allowing for higher throughput diagnostics.[9] Tongue swabs have been shown to recover MTBC DNA that has accumulated on the tongue dorsum, possibly as a result of being expectorated from the lungs.[9,10] Tongue swabs are collected by scraping the front ~2/3 of the patient's tongue dorsum while rotating and applying light pressure, for 15-20 seconds.[10] DNA is then extracted from the swabs, and qPCR detects and quantifies the amount of MTBC DNA.[9]

No previous analyses have investigated the impact of anti-TB drug treatment on the efficacy of oral swabs for TB diagnosis. The goal of this study was to define the

sensitivity and specificity of oral swabs along the TB treatment course, using sputum culture (the diagnostic gold standard), as a reference. As such, the study will determine sensitivity and specificity of OSA at baseline, and months 2, 4, and 6 post-initiation of treatment. Additionally, the sensitivities and specificities of OSA will be compared to sputum GeneXpert, using sputum culture as a reference. Evaluating the impact of anti-TB drugs on MTBC DNA recovery from oral swabs will be helpful for understanding how oral swabs can be implemented within clinical settings and for treatment monitoring. Expanding swift and well-tolerated diagnostic options for TB will help reduce TB incidence and help mitigate MDR-TB infections worldwide.

Methods

Study design and population

This cross-sectional study enrolled adult (18-50 years), HIV uninfected, MTBC sputum culture-confirmed, drug-sensitive pulmonary TB subjects in Worcester, South Africa at a well-known clinical trial site maintained by the South African Tuberculosis Vaccine Initiative (SATVI). Worcester is a semi-rural town in Western Cape with 350,000 people and a TB incidence that exceeds 800 cases per 100,000 people.[11] Study participants received care provided free of charge by the South African TB Control Programme. Participants received the standard-of-care, a 6-month course that included rifampin, isoniazid, pyrazinamide, and ethambutol, referred to as [2(HRZE/4(HR)]. Care was provided in a directly observed treatment, short course (DOTS) TB treatment regimen, in which optimal adherence is supported by patient education, pill counts, and telephonic contacts.[1,2] Participants who developed MDR-TB or experienced TB

treatment failure were excluded from further study participation and referred to the appropriate health service.

Subjects were compensated for their time and participation, and written informed consent was collected from each subject. Study protocols were approved by the Human Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town and the Human Subjects Division at the University of Washington.

Sample acquisition

Each participant had their tongue swabbed by clinical staff member with two separate Copan FLOQSwabs at four time points over their treatment course, as follows: month 0 (baseline, treatment initiation), month 2, month 4, and month 6 (treatment conclusion). Copan FLOQSwabs were swabbed back and forth over the participant's tongue dorsum while applying a light pressure for 15-20 seconds and then put into a 2 mL screw-cap tube with 500 µL phosphate-buffered saline (PBS) for storage as described in Wood et. al, 2021 and Weigel et. al 2013.[10,12] PBS was chosen for storage for compatibility with a concurrent molecular viability testing (MVT) study.[12] Samples collected by SATVI were stored frozen at -80℃ within 8 hours of collection and then shipped to the University of Washington in Seattle for analysis. Once the samples were received by the University of Washington in Seattle, they were recoded for blinded testing and analysis.

Laboratory analysis

Data were generated using an optimized qPCR assay that is specific to the *M. tuberculosis* IS6110 insertion element, as previously described.[13] Clinical samples were prepared for qPCR by using a modified QIAGEN QIAamp DNA mini kit protocol and

ethanol precipitation to isolate and concentrate the DNA extracted from the Copan FLOQSwabs, as previously described.[13] The samples were then split into two aliquots of ~250 µL each: one was stored at -80 °C, and the other was used for qPCR analysis. Each qPCR run included a positive extraction control spiked with 10 µL of 10^3 MTB strain H37Ra, and a non-template negative qPCR control. Each 25 µL reaction consisted of 5 µL of extracted DNA, 12.5 µL (1x) of Luna Universal qPCR Master Mix, 1.125 µL (0.45 µM) of forward primer, 3.375 µL (1.35 µM) reverse primer, 0.625 (0.25 µM) FAM/MGBNFQ probe, and 2.375 µL of H_2O.[14]

Data Analyses

After cycling, results were analyzed using Microsoft Excel. Test accuracy of OSA was determined by calculating sensitivity and specificity at baseline (month 0), and months 2, 4, and 6 post initiation of treatment, using sputum culture as a reference at the same time points (ex. OSA month 0 compared with sputum culture month 0, OSA month 2 compared with sputum culture month 2). The test accuracy of GeneXpert was calculated at months 0, 2, 4, and 6 post initiation of treatment relative to sputum culture. Sputum culture is considered the gold standard for diagnosing pulmonary TB. Therefore, OSA and GeneXpert concordance and discordance was determined relative to sputum culture. Individuals who are negative by sputum culture but positive by OSA or GeneXpert were classified as a false positive result. To gain more insight into OSA performance, samples that yielded a false positive result at any time point were investigated to determine clinical symptoms, sputum smear microscopy results, and appeared to be false positive at other time points along the treatment course. This

descriptive analysis explored the relationship between persistent patient symptoms and false positivity. A secondary analysis was completed to investigate whether the high specificity of OSA was due to method success or because of low sensitivity. For this analysis, the sensitivity of sputum GeneXpert was artificially lowered by applying a Cq cutoff that yielded a similar sensitivity to OSA.

Results

Patient Characteristics

There were 25 patients enrolled in this study. Due to the COVID-19 pandemic, there was a loss in follow up amongst study participants over time. This caused incomplete participant datasets across the treatment course (months 0, 2, 4, and 6), meaning many participants were not tested by OSA, sputum GeneXpert, or sputum culture at various time points. This resulted in a range of N values for the resulting analyses, as outlined in Fig. 1 in the appendix. The mean participant age was 40 years, with a range of 21-57 years. At enrollment, there were 19 male participants and 7 female participants.

Diagnostic Accuracy of Oral Swabs

A quantification cycle (Cq) cutoff of 38 was applied to reduce false positivity due to method error.[13] Across all time points (month 0, 2, 4, and 6) sputum GeneXpert had a higher sensitivity than OSA, but OSA had a higher specificity than sputum GeneXpert (see Table 1). A Cq cutoff of 32 was also applied to the OSA data to determine if specificity could be improved. Overall, when applying a Cq cutoff of 32 to OSA qPCR, sensitivity decreased and as a result specificity increased compared to the 38 Cq cutoff.

Though the specificities for OSA appear higher than that of sputum GeneXpert, crude calculations of sensitivity and specificity alone do not indicate if the high specificity of OSA is due to method success or because of low sensitivity.

Table 1. Sensitivity and Specificity of OSA (Cqcutoffs 32, 38) and GeneXpert Relative to Culture*

	Sputum GeneXpert		OSA (32 Cq cutoff)		OSA (38 Cq cutoff)	
Month	sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
0	26/26 100% 95% CI: [1.0, 1.0]	2/5 40% [-0.03, 0.83]	15/27 55% [0.37, 0.74]	5/5 100% [1.0, 1.0]	20/27 74.07% [0.57, 0.91]	5/5 100% [1.0, 1.0]
2	3/3 100% 95% CI: [1.0, 1.0]	3/13 23% [0.002, 0.46]	NA	12/13 92% [0.78, 1.07]	1/3 33% [-0.20, 0.87]	10/13 77% [0.54, 1.0]
4	NA	11/19 57.90% 95% CI: [0.36, 0.80]	NA	17/18 94% [0.84, 1.05]	NA	16/18 89% [0.74, 1.03]
6	NA	16/19 55% 95% CI: [0.58, 0.94]	NA	20/21 95.20% [0.86, 1.04]	NA	16/21 76.19% [0.58, 0.94]

*Numerators for sensitivity calculations are the amount of true positives, and the denominators are the number of true positives plus true negatives. Numerators for specificity calculations are the number of true negatives, and the denominators are the number of true negatives plus false positives. The NA values at months 2, 4, and 6 are instances where the sensitivity calculations were not calculable due to positivity rates, which is detailed in Fig 1 in the appendix.

Manipulating Sputum GeneXpert Sensitivities

Cq cutoffs were applied to sputum GeneXpert to assess if the high specificity of OSA was due to method success or because of low sensitivity. In the original analysis, sputum GeneXpert had a sensitivity of 100%, and OSA (Cq 38) had a sensitivity 74%. Therefore, a Cq cutoff of 16.3 was applied to sputum GeneXpert to yield an artificial sensitivity of around 64%. This cutoff was applied at months 0, 2, 4, and 6 to observe how the sensitivities and specificities of sputum GeneXpert changed. It was hypothesized that if sputum GeneXpert's sensitivities were lowered to approximate that of OSA, then the specificities of the two methods would resemble each other. After running the analysis, it was observed that the sensitivities of sputum GeneXpert

decreased overall, and the specificities increased, following the same trend as OSA (table 2a and 2b). This indicates that OSA may not actually be more specific than sputum GeneXpert, but rather appears to be more specific because of low sensitivity. This analysis was repeated using OSA at the 32 Cq cutoff, which yielded the same results as using the OSA 38 Cq cutoff (table 2a and 2b).

Table 2a. Adjusted Sens/Spec Graph Comparing OSA (cutoff 38) to Sputum GeneXpert (cutoff 16.3)

	Sputum GeneXpert (cutoff 16.3)		OSA (cutoff 38)	
Month	sensitivity	specificity	sensitivity	specificity
0	16/25 64% 95% CI: [0.45, 0.83]	6/6 100% [1.0, 1.0]	20/27 74.07% [0.57, 0.91]	5/5 100% [1.0, 1.0]
2	1/2 50% 95% CI: [-0.19, 1.19]	10/13 77% [0.54, 1.0]	1/3 33% [-0.20, 0.87]	10/13 77% [0.54, 1.0]
4	NA	16/16 100.00% 95% CI: [1.0, 1.0]	NA	16/18 89% [0.74, 1.03]
6	NA	17/18 94% [0.84, 1.05]	NA	16/21 76.19% [0.58, 0.94]

Table 2b. Adjusted Sens/Spec Graph Comparing OSA (cutoff 32) to Sputum GeneXpert (cutoff 16.2)

	Sputum GeneXpert (cutoff 16.2)		OSA (cutoff 32)	
Month	sensitivity	specificity	sensitivity	specificity
0	11/26 42% 95% CI: [0.23, 0.61]	5/5 100% [1.0, 1.0]	15/27 55% [0.37, 0.74]	5/5 100% [1.0, 1.0]
2	NA	11/13 85% 95% CI: [0.65, 1.04]	NA	12/13 92% [0.78, 1.07]
4	NA	16/16. 100.00% 95% CI: [1.0, 1.0]	NA	17/18 94% [0.84, 1.05]
6	NA	16/17 94% 95% CI: [0.83, 1.05]	NA	20/21 95.20% [0.86, 1.04]

Descriptive analyses of False Positives

Next, we investigated whether there were correlations between OSA false positivity and patient clinical and laboratory parameters including clinical symptoms, sputum smear microscopy results, and whether participants had multiple false positives. In total, 21 people were false-positive by sputum GeneXpert at one or more time points. Of these individuals, three were positive by both OSA and sputum GeneXpert (one individual at M4, one at M2, and one at M6). Two participants were false-positive by sputum GeneXpert and OSA at multiple time points. One of these participants was false-positive by sputum GeneXpert at M0, 2, 4 and 6. The other participant was false-positive by sputum GeneXpert at M2 and M6. Details of these participants can be found in Fig. 2. Further investigation was done on these participants to see if their symptoms or sputum smear results indicated persistent TB infection and treatment failure. None of the participants who were false-positive by OSA or sputum GeneXpert reported abnormal respiratory, GI, neurological, or musculoskeletal symptoms. Two participants who were false-positive by sputum GeneXpert and OSA had scanty smear results (5-49 AFB in one length, 200x)[15], the rest had no indication of any acid-fast bacilli. Thus, a relationship between false positivity and sputum smear results or persistent TB symptoms was not detected.

Discussion

We evaluated the sensitivity and specificity of oral swabs over the TB treatment course and compared it with sputum GeneXpert and sputum culture. We found that like other nucleic acid-based tests, OSA can be prone to false positivity especially as the treatment course progresses (Fig. 1 in Appendix). Though OSA appeared to be more

specific than GeneXpert along the treatment course, our secondary analyses showed that this may be a product of poor OSA method sensitivity. Issues with manual DNA extraction methods may contribute to false positivity rates, as laboratory handling error can lead to sample contamination. The descriptive analyses show that the increased specificity of OSA compared to sputum GeneXpert during treatment may be a byproduct of low method sensitivity, as there also appears to be little relationship with persistent symptoms and false positivity rates. These analyses had limitations because patient clinical follow-up was hindered by the COVID-19 pandemic. Many participants did not complete a month 6 clinical evaluation, meaning their symptoms could not be assessed against the false positive results yielded by sputum GeneXpert and OSA. Because OSA does not assess microbial viability, residual TB DNA in the airways may be contributing to false positivity. Future studies may use oral swabs in tandem with molecular viability testing to address these issues.[12,16] Because of OSA's false positivity rate, it may be unreliable for assessing infection and TB treatment response.

This study had several limitations. Ongoing issues with COVID-19 impacted patient enrollment and sample acquisition (both sputum and oral swabs) at all time points, resulting in incomplete sample sets for participants, reducing the overall sample size of the study. This study also lacks generalizability, because it was limited to HIV-negative adults in Worcester, South Africa. Future studies are needed to assess the performance of oral swabs to diagnose TB and monitor TB treatment within other populations and geographical regions. These studies ought to explore how individuals with coinfections like HIV, other comorbidities, as well as other demographic and

lifestyle characteristics impact oral swab efficacy as a diagnostic and treatment monitoring tool.

Overall, this this was the first study to investigate how oral swabs perform along the TB treatment course. This study is an important step in characterizing how oral swabs respond to anti-tuberculosis drugs, and if they can be improved to monitor the TB treatment course.

References

1. *Global Tuberculosis Report 2020*. World Health Organization; 2020.

2. Tuberculosis (TB). Accessed September 18, 2021. https://www.who.int/news-room/fact-sheets/detail/tuberculosis

3. Pulmonary tuberculosis - Symptoms, diagnosis and treatment | BMJ Best Practice US. Accessed May 24, 2022. https://bestpractice.bmj.com/topics/en-us/165

4. Core Curriculum on Tuberculosis: What the Clinician Should Know. :211.

5. Luabeya AK, Wood RC, Shenje J, et al. Noninvasive Detection of Tuberculosis by Oral Swab Analysis. Miller MB, ed. *J Clin Microbiol*. 2019;57(3). doi:10.1128/JCM.01847-18

6. Friedrich SO, Rachow A, Saathoff E, et al. Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. *Lancet Respir Med*. 2013;1(6):462-470. doi:10.1016/S2213-2600(13)70119-X

7. Uden L, Barber E, Ford N, Cooke GS. Risk of Tuberculosis Infection and Disease for Health Care Workers: An Updated Meta-Analysis. *Open Forum Infect Dis*. 2017;4(3):ofx137. doi:10.1093/ofid/ofx137

8. Narasimhan P, Wood J, MacIntyre CR, Mathai D. Risk Factors for Tuberculosis. *Pulm Med*. 2013;2013:1-11. doi:10.1155/2013/828939

9. Valinetz ED, Cangelosi GA. A Look Inside: Oral Sampling for Detection of Non-oral Infectious Diseases. McAdam AJ, ed. *J Clin Microbiol*. 2021;59(10). doi:10.1128/JCM.02360-20

10. Wood RC, Andama A, Hermansky G, et al. Characterization of oral swab samples for diagnosis of pulmonary tuberculosis. Subbian S, ed. *PLOS ONE*. 2021;16(5):e0251422. doi:10.1371/journal.pone.0251422

11. Fighting TB – South Africa's 'insidious epidemic' | University of Oxford. Accessed March 7, 2022. https://www.ox.ac.uk/news/2015-05-28-fighting-tb-%E2%80%93-south-africa%E2%80%99s-%E2%80%98insidious-epidemic%E2%80%99

12. Weigel KM, Jones KL, Do JS, et al. Molecular Viability Testing of Bacterial Pathogens from a Complex Human Sample Matrix. Manganelli R, ed. *PLoS ONE*. 2013;8(1):e54886. doi:10.1371/journal.pone.0054886

13. Shapiro AE, Olson AM, Kidoguchi L, et al. Complementary non-sputum diagnostic testing using oral swabs and urine LAM testing for TB in people with HIV. Published online March 7, 2022:2022.02.16.22271079. doi:10.1101/2022.02.16.22271079

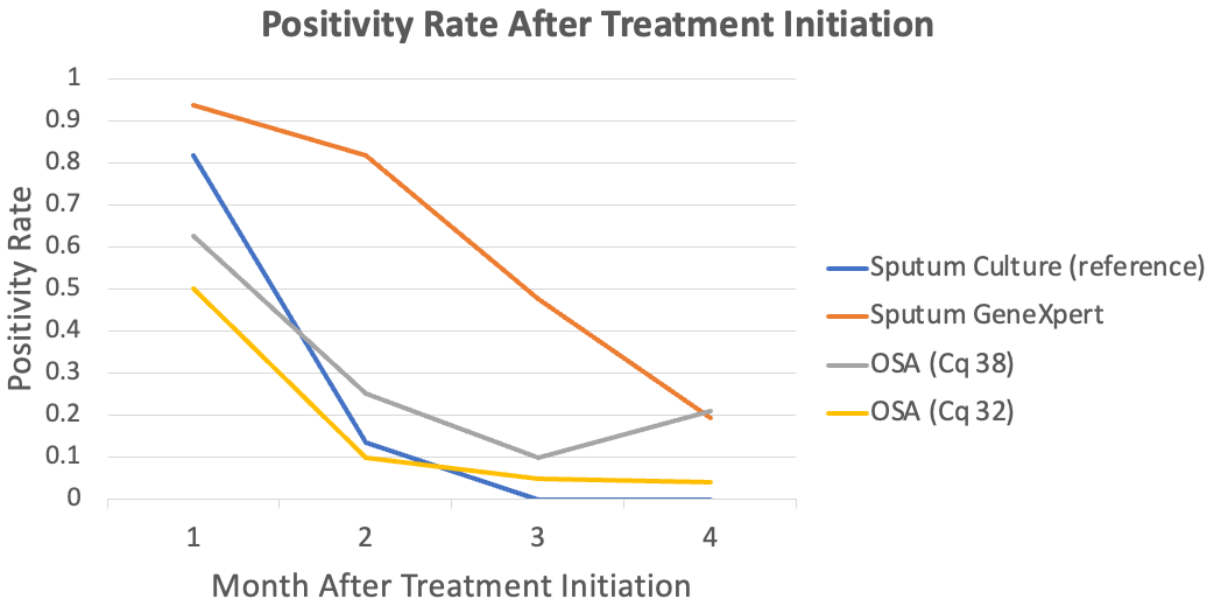
14. Halsey TA, Edwards J, Cunningham PL, et al. Combined Real-Time PCR and rpoB Gene Pyrosequencing for Rapid Identification of Mycobacterium tuberculosis and Determination of Rifampin Resistance Directly in Clinical Specimens. *J Clin Microbiol*. 2010;48(4):1182-1188. doi:10.1128/JCM.02149-09

15. Lumb R, Van Deun A, Bastian I, Fitz-Gerald M, Pathology SA, Stop TB Partnership (World Health Organization). *Laboratory Diagnosis of Tuberculosis by Sputum Microscopy: The Handbook*.; 2013.

16. Cangelosi GA, Meschke JS. Dead or Alive: Molecular Assessment of Microbial Viability. Drake HL, ed. *Appl Environ Microbiol*. 2014;80(19):5884-5891. doi:10.1128/AEM.01763-14

Appendix

Figure 1: Positivity rate of Sputum GeneXpert, Sputum Culture, and Oral Swab PCR post treatment initiation. Followed by a chart of participant counts at each time point per method.



Month	Total N		
	GeneXpert	Culture	OSA
0	31	32	32
2	16	16	16
4	19	19	18
6	21	21	21

Figure 2: False positive counts of sputum GeneXpert and OSA and where OSA + sputum GeneXpert positivity overlaps.

False Positivity Counts by Diagnostic Type			
Month	OSA (Cq 38) N=9	Sputum GeneXpert N=21	OSA + Sputum GeneXpert N=2
0	0	3	0
2	3	7	1
4	1	7	1
6	5	5	0