High-sensitivity detection of *Mycobacterium tuberculosis* DNA in tongue swab samples

Alaina M. Olson[1], Rachel C. Wood[1], Kris M. Weigel[1], Alexander J. Yan[2], Katherine A. Lochner[1], Rane B. Dragovich[1], Angelique K. Luabeya[3], Paul Yager[2], Mark Hatherill[3], *Gerard A. Cangelosi[1]

[1]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, Washington, United States of America

[2]Department of Bioengineering, University of Washington, Seattle, Washington, United States of America

[3]South African Tuberculosis Vaccine Initiative, Institute of Infectious Disease & Molecular Medicine and Department of Pathology, Faculty of Health Sciences, University of Cape Town, South Africa

* Corresponding author

E-mail: gcang@uw.edu (GC)

Abstract

Tongue swab (TS) sampling combined with qPCR to detect *Mycobacterium tuberculosis* (MTB) DNA is a promising alternative to sputum testing for tuberculosis (TB) diagnosis. In prior studies, the sensitivity of tongue swabbing has usually been lower than sputum. In this study, we evaluated two strategies to improve sensitivity. In one, centrifugation was used to concentrate tongue dorsum bacteria from 2-mL suspensions eluted from high-capacity foam swab samples. The pellets were resuspended as 500-µL suspensions, and then mechanically lysed prior to dual-target qPCR to detect MTB insertion elements IS*6110* and IS*1081*. Fractionation experiments demonstrated that most of the MTB DNA signal in clinical swab samples (99.22% ± 1.46%) was present in the sedimentable fraction. When applied to archived foam swabs collected from 124 South Africans with presumptive TB, this strategy exhibited 83% sensitivity (71/86) and 100% specificity (38/38) relative to sputum MRS (microbiological reference standard; sputum culture and/or Xpert® Ultra). The second strategy used sequence-specific magnetic capture (SSMaC) to concentrate DNA released from MTB cells. This protocol was evaluated on archived Copan FLOQSwabs® flocked swab samples collected from 128 South African participants with presumptive TB. Material eluted into 500 µL buffer was mechanically lysed. The suspensions were digested by proteinase K, hybridized to biotinylated dual-target oligonucleotide probes, and then concentrated ~20-fold using magnetic separation. Upon dual-target qPCR testing of concentrates, this strategy exhibited 90% sensitivity (83/92) and 97% specificity (35/36) relative to sputum MRS. These results point the way toward automatable, high-sensitivity methods for detecting MTB DNA in TS.

Abstract word count: 248/250

Importance. Improved testing for tuberculosis (TB) is needed. Using a more accessible sample type than sputum may enable the detection of more cases, but it is critical that alternative samples be tested appropriately. Here, we describe two new, highly accurate methods for testing tongue swabs for TB DNA.

Importance word count: 47/150

Introduction

Screening and diagnosis of pulmonary tuberculosis (TB) remain urgent global health priorities. The standard patient sample for microbiological diagnosis of TB is sputum, a viscous expectorated material. The availability of alternatives to sputum would greatly facilitate testing for active TB in both clinical and community settings (1-4). One such alternative is tongue swab (TS) testing (5-12). In TS testing, the dorsum of the tongue is swabbed and the collected material is tested by qPCR for MTB DNA. Tongue swabbing is fast, painless, and does not require facilities for privacy or aerosol control. In a recent study of user preference, most health care workers expressed their preference for swabbing over sputum collection (13). Individuals who may struggle to produce sputum, such as children and people living with HIV (PLHIV), can easily provide swabs in any setting.

TS testing has exhibited varying sensitivities relative to sputum microbiological reference standards (sputum MRS), ranging from ~75% when using commercial sputum testing platforms (5, 6), to >90% when swabs are tested by manual, laboratory-based methods designed specifically for swab testing (5, 14). Specificity relative to sputum MRS consistently exceeds 95% (5, 6, 14). Recently, Steadman et al. (14) reported a laboratory-based method with high sensitivity. Their protocol incorporated heat, high-energy bead-beating with a BioSpec instrument, and high-volume qPCR targeting MTB insertion elements IS*6110* and IS*1081*. Although costly and potentially difficult to automate, this approach yielded high accuracy (93% sensitivity and 99% specificity relative to sputum MRS) when applied to symptomatic participants in a clinical setting in Uganda (14). Such observations illustrate the biological potential for TS as a non-sputum sample when appropriate analytical methods are used.

The current study evaluated two strategies to maximize TS sensitivity, using methods designed to be feasible to automate. The first strategy aimed to collect a greater proportion of the available MTB bacilli. Previous work suggested that only a fraction of available tongue

dorsum biomass was collected with nylon flocked swabs, and that a substantial portion is left behind (8). In the current study, tongue dorsum samples were collected with high-capacity open-cell foam swabs instead. Preliminary experiments showed that Medline Pint-Size™ DenTips® Oral Swabs collect more tongue dorsum biomass (measured as absorbance at 260 nm) than Copan FLOQSwabs® flocked swabs (p < 0.0001; Supplemental material S1). We conjectured that this would enable the collection of more MTB bacilli from tongue dorsa. However, increasing the size of the swab head presents a new challenge. Compared to the 0.5 mL buffer volume used with FLOQSwabs, a larger buffer volume of 2 mL is needed to release collected biomass into the foam swab sample eluate. To mitigate sample dilution, we evaluated cell concentration by centrifugation prior to cell disruption. Concentrated suspensions were mechanically lysed, and the crude lysate was amplified by a high-volume qPCR.

In this study, we applied this method to archived foam swab samples that were previously collected from a cohort of South African participants with presumptive TB (N = 124). Replicate FLOQSwabs from the same participants were previously tested on two commercial sputum testing platforms: Cepheid Xpert® MTB/RIF Ultra (Xpert Ultra) and Molbio Truenat® MTB Ultima (MTB Ultima). Xpert Ultra was 75.5% sensitive and 100% specific, and MTB Ultima was 71.6% sensitive and 96.9% specific, relative to sputum MRS (5). We investigated whether sensitivity was improved by the alternate sample collection and processing method using foam swabs. In the process, we also investigated whether the MTB DNA signal present in clinical swab samples resides in the sedimentable (particulate) fraction, rather than in the soluble (supernatant) fraction.

In the second strategy, we focused on extracting and concentrating DNA after release from bacterial cells, rather than concentrating bacterial cells. After collection, tongue dorsum biomass on a FLOQSwab head is typically eluted into ~500 µL of TE buffer (5-9, 14). Because a FLOQSwab collects just ~80 µL of oral material, it is critical that all, or nearly all, of this material be included in the qPCR reaction. In past studies we have used ethanol precipitation of DNA for

this purpose (8, 9). However, this method is cumbersome and difficult to automate. Recently, Oreskovic et al. (15) reported the use of hybridization probes immobilized on magnetic beads to selectively capture and concentrate MTB DNA from dilute urine samples. This strategy, which we term sequence-specific magnetic capture (SSMaC), has several potential advantages. First, it is facile and amenable to automation in POC devices (16). For example, the fully automated, POC DASH™ (Digital Analyzer for Selective Hybridization) Rapid PCR system (Nuclein, Inc., Austin, TX, USA) incorporates a 10-min SSMaC step that concentrates SARS-CoV-2 RNA in swab samples, enabling qPCR detection with high sensitivity (17). Recently, a lab-based MTB-targeted version of the DASH assay was described for sputum testing (18). Second, in addition to concentrating target DNA, SSMaC enables partial purification of target DNA by removing qPCR inhibitors.

To evaluate the effectiveness of SSMaC for tongue swab testing, we optimized a previously reported (19) SSMaC protocol and applied it to archived clinical FLOQSwab samples that were collected from a cohort of South African participants with presumptive TB (N = 128). Replicate FLOQSwab samples from these participants were previously evaluated by Xpert Ultra and Molbio Ultima (5). The current study investigated whether samples from the same participants exhibited better sensitivity when analyzed by the optimized SSMaC-based method.

Materials and methods

Ethics. This study was approved by the University of Cape Town Human Research Ethics Committee (reference number 160/202) and the University of Washington Human Subjects Division (STUDY00001840).

Study setting and participants. Enrollment and sample collection were described previously (5). Enrollment began July 2021 and continued until March 2023 in Worcester, South Africa.

Foam swab collection. Participants with presumptive TB were enrolled in two cohorts in Worcester, South Africa, as described (5). When Cohort 2 (5) was enrolled, a subset (N = 156) provided foam swab samples (Pint-Size™ DenTips® Oral Swabs, MDS096202P; Medline Industries LP, Northfield, IL, USA) in addition to flocked swabs (regular FLOQSwabs® Flocked Swabs, 520CS01; Copan Italia, Brescia, IT). Foam swabs from some of these participants (N = 31) were used in unrelated studies, and one (N = 1) was lost during processing due to a broken collection tube. All remaining foam swabs (N = 124) were used in this study (Figure 1).

All swab samples were collected prior to sputum collection. Sample collection occurred over two days, with high-capacity foam swabs collected after two FLOQSwabs on Day 2. Foam swabs were brushed against the dorsum of the tongue for 15 sec and stored dry in 5 mL tubes (6101S, Globe Scientific Inc, Mahwah, NJ, USA). Swab shafts were cut with scissors to fit into the tubes. Samples were stored at -80 °C and shipped on dry ice to Seattle, Washington for testing at the end of the study.

Figure 1. Foam swab STARD Diagram
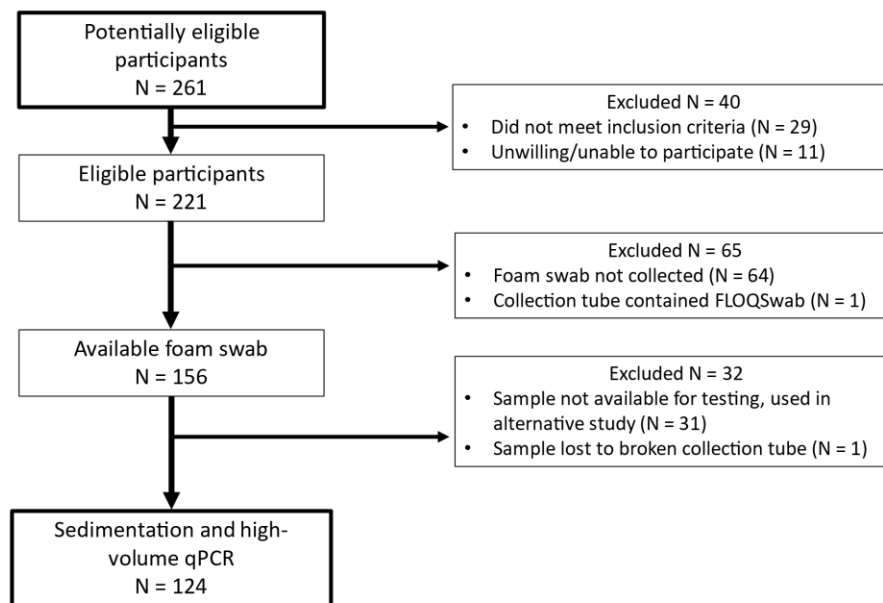
Foam swab processing. Foam swabs were kept at -80 °C until ready for processing. Swabs were removed from the freezer and immediately boiled at 100 °C in a water-filled heat block (88-860-023; Thermo Fisher Scientific Inc., Waltham, MA, USA) for 10 min to inactivate pathogens. Batches were limited to 4 tubes per block (88-860-107; Thermo Fisher Scientific Inc., Waltham, MA, USA), for a total of 8 at a time, to prevent excess temperature drops. 2 mL TE buffer (pH 8.0) was added to the sample followed by a 30 sec vortex at max speed (Vortex-Genie 2, SI-0236; Scientific Industries, Inc, Bohemia, NY, USA). During sample aspiration using a p1000 micropipette tip, the tip was used to compress the foam head against the wall of the tube to maximize liquid recovery. All of the recoverable liquid sample was transferred to a 2-mL screw-cap tube (02707355; Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were centrifuged at 17,320 RCF at room temperature for 2 min. After sedimentation, 1.5 mL supernatant was removed from the uppermost portion of the sample, avoiding the pellet, and then discarded. Samples were re-spun and aspirated again if the pellet was noticeably disturbed

during aspiration. The pellet was resuspended in the remaining ~500 μL supernatant. Bacilli were then mechanically lysed as follows. Working in batches of 16 (including one positive and negative extraction control swab each), 150 mg pre-weighed 0.1 mm glass disruption beads (9830; Research Products International (RPI), Mt. Prospect, IL, USA) were added to the sample tube containing the suspension. Samples were bead-beaten for three rounds of 1 min on with 1 min rests, using a high-energy cell disruptor (Mini-Beadbeater-16, 607; BioSpec Products, Inc., USA). Samples were spun down briefly. While avoiding the glass beads, the entire recoverable liquid volume was transferred to a 1.5-mL snap-cap tube (05408129; Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were stored at 4 °C briefly before plating for same-day qPCR.

Extraction controls were prepared using swabs collected from healthy volunteers in Seattle; the positive controls were spiked with 40,000 cells of log-phase cultured *M. tuberculosis* H37Ra (ATCC), in 40 μL PBSGT (1x Phosphate-Buffered Saline, 15% Glycerol, 0.05% Tween-80). All were stored at –80 °C prior to processing.

Foam swab testing. Fifty microliters of crude lysate were plated in technical duplicates, each mixed with 10 μL of master mix comprising 6 μL 10x KAPA3G HotStart Master (09084711103; Roche, Basel, CH), 500 nM final concentration of each primer (Integrated DNA Technologies, Coralville, IA, USA), 250 nM final concentration of each probe (Integrated DNA Technologies, Coralville, IA, USA), and 2.5 μL molecular grade water, for a final reaction volume of 60 μL. Thermal cycling was performed using the Bio-Rad CFX96 Deep Well Real-Time PCR Detection System (1854095, Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following cycling parameters from Steadman et al. (14): Initial denaturation at 98 °C for 3 min, followed by 45 cycles of, 1. Denaturation at 98 °C for 5 sec, 2. Annealing at 64 °C for 20 sec, and 3. Elongation at 72 °C for 30 sec, concluding with a final elongation at 72 °C for 1 min. All ramp rates were 2.5 °C per sec. Results were exported from the Bio-Rad CFX96 Deep Well machine

using a threshold of 1000 RFU to Microsoft Excel for further analysis and calculations. Cq and

quantification values were averaged between the two replicates of each sample.

FLOQSwab collection. Participants were enrolled and sampled as previously described

(5) and shown here in a STARD diagram (Figure 2).

Figure 2. FLOQSwab STARD Diagram
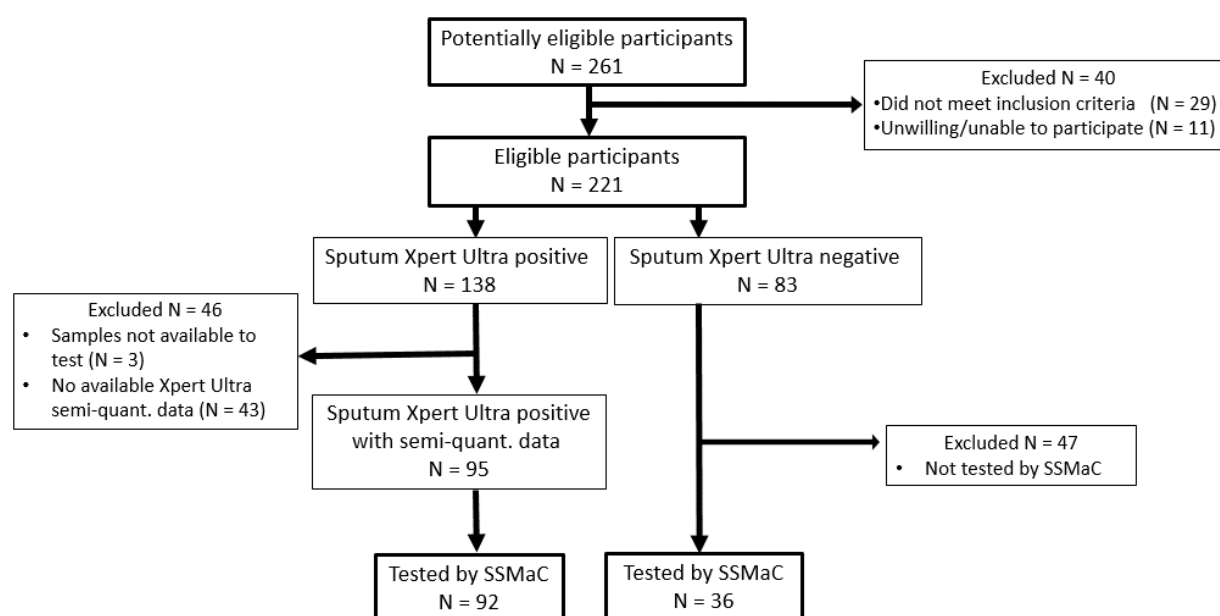
In Cohort 2 of the previous study (5), a subset of participants (N = 92) had available

sputum Xpert Ultra (SPUXU) semiquantitative data (trace, very low, low, moderate, high).

Samples from the 92 participants with SPUXU data were used for the current study. SPUXU

semi-quantitative (SQ) and quantitative Cq results were exported and cleaned by members of

the team at the clinical site. Of the 92 participants, 42 were SPUXU high, 16 medium, 23 low, 8

very low and 3 trace. Six of these 92 participants were sputum culture negative.

FLOQSwab sample processing by SSMaC. Samples for the current study were

processed in batches of 10 clinical samples alongside one positive and negative processing

control each for a total of 12 samples. The positive controls were swabs collected from healthy

donors in Seattle, WA and then spiked with 10 µL of buffer containing 10,000 cells of log-phase

cultured *Mycobacterium tuberculosis* H37Ra suspended in PBSGT. The negative controls were

swabs collected from healthy donors in the same way and stored without spiking.

Samples previously processed for and tested by Molbio Truenat® MTB Ultima (5)

remained in excess (~494 µL) and were stored at -80 °C. These archived lysates were thawed

and ~474 µL aliquots were subjected to a 30 min proteinase K digestion at 55 °C. The enzyme

and any present DNA were then denatured for 10 min at 100 °C. The denatured DNA was

hybridized to biotinylated oligonucleotide capture probes for 20 min at 55 °C as previously

described (19) with modifications to probe sequences (Table S2). A second set of probes

targeting an additional insertion sequence, IS*1018*, was added in equimolarity to an IS*6110* set

based on sequences known to work in combination (for qPCR) as shown by Steadman et al.

(14) (Supplemental material 1). These sequences align with the sequences used for

downstream amplification. Twenty microliters of washed magnetic beads (Dynabeads™

MyOne™ Streptavidin C1, 65001; Thermo Fisher Scientific Inc., Waltham, MA, USA) were used

to capture the biotinylated oligonucleotides hybridized with MTB DNA. Magnetic concentration

was used to purify and concentrate MTB DNA 20-25-fold, resulting in 20 µL of eluate as

previously described (19). This process involved washing away parts of the sample not bound to

magnetic beads, including non-target DNA, cell debris, swab flocking, any carried-over

disruption beads from lysis, salts, surfactants, and other potential PCR inhibitors. Finally, a 5

min heat-block-incubation at 95 °C released the purified target DNA into 20 µL TE buffer.

Flocked swab sample qPCR reaction and analysis. Following SSMaC processing,

five microliters of concentrated bead eluate (MTB DNA) were amplified in a 20 µL qPCR

reaction adapted from Steadman et al. (14) (Supplemental material S2). Changes included

modifying the IS*1081* probes with the fluorescein (6-FAM) fluorophore in place of Yakima

Yellow®, and excluding RNase P primers and probe. Master mix was plated as 15 µL with the

following composition: Final concentrations of 500 nM for each primer and 250 nM for each

probe (see table for sequences), 2 µL KAPA3G HotStart Master, 10x concentrated, and 12.5 µL

molecular grade water. Each qPCR run included a standard curve of purified MTB DNA and

negative controls.

Reactions were run on an Applied Biosystems™ StepOnePlus™ thermal cycler

(4376600; Thermo Fisher Scientific Inc., Waltham, MA, USA), with the cycling parameters

described above. Normalization included exporting all runs at a 2239 ΔRn threshold. Results

were exported from the StepOnePlus™ software to excel, where calculations were executed.

Any Cq value below 40 was considered a positive result. For analyses of average Cqs, samples

that were undetected were assigned Cq values of 45.

Results

Evaluation strategies. The two new methods evaluated were 1) foam swabs combined

with sedimentation, and 2) FLOQSwabs processed by SSMaC. Different evaluation strategies

were used based on the availability of archived samples. To assess foam swabs with

sedimentation, percent sensitivity and specificity relative to sputum MRS were calculated and

assessed in relation to values previously reported using other methods (5). For FLOQSwabs

processed by SSMaC, we applied the new method to lysates previously processed for Molbio

Ultima testing (5). We asked whether sensitivity would be improved by using SSMaC to test the

remaining, larger fraction of lysate and compared the results to the original Molbio Ultima

results.

Foam swabs and sedimentation. Foam swabs collected from participants with

presumptive TB (N = 124) exhibited 82.6% sensitivity (71/86) and 100% specificity (38/38)

relative to sputum MRS (Table 1). These values were nominally higher than previously reported

for replicate FLOQSwabs collected from the same 124 participants and tested by Molbio Ultima and Xpert Ultra (5). However, the 95% confidence intervals of all three methods overlap and the differences were not statistically significant by paired z-test (p = 0.34 and 0.44, respectively).

Table 1. Sensitivities and specificities of methods paired to foam data

|	Foam Sedimentation (N = 124)		TS Molbio Ultima (N = 121)		TS Xpert Ultra (N = 121)	
% Sensitivity [95% CI]	71/86	82.6 % [72.87, 89.90]	66/86	76.7% [66.39, 85.18]	67/86	77.9% [67.67, 86.14]
% Specificity [95% CI]	38/38	100% [90.75, 100.00]	34/35	97.1% [85.08, 99.93]	35/35	100% [90.00, 100.00]

SPUXU data were available for a subset (N = 67) of the participants tested by foam swab and sedimentation (Table 2). The method performed best in participants with stronger SPUXU signals. It was insensitive when applied to samples from SPUXU "very low" and "trace" participants, as were the other two methods.

These results suggest that a significant portion of the MTB DNA signal present in clinical foam swab samples was in the sedimentable fraction, rather than in the supernatants. To assess this quantitatively, we conducted fractionation experiments in which we compared MTB DNA quantities measured by qPCR in concentrated samples relative to the supernatants. In this paired analysis (N = 6 samples), MTB DNA quantities in the pellets were consistently greater than those in the supernatants (p < 0.05, one-sided Wilcoxon Signed Rank test, W value = 0). On average, 99.22 ± 1.46% of the available MTB DNA signal was in the sedimentable fraction (Figure 3).

Table 2. Sensitivities for each method stratified by sputum Xpert Ultra semi-quantitative (SPUXU SQ) results

SPUXU SQ (N)	Foam Sedimentation		TS Molbio Ultima		TS Xpert Ultra	
	N	% Sensitivity [95% CI]	N	% Sensitivity [95% CI]	N	% Sensitivity [95% CI]
High (30)	30/30	100.00% [88.43, 100.00]	29/30	96.67% [82.78, 99.92]	30/30	100.00% [88.43, 100.00]
Medium (12)	12/12	100.00% [73.54, 100.00]	11/12	91.67% [61.52, 99.79]	12/12	100.00% [73.54, 100.00]
Low (17)	13/17	76.47% [50.10, 93.19]	11/17	64.71% [38.33, 85.79]	11/17	64.71% [38.33, 85.79]
Very low (5)	3/5	60.00% [14.66, 94.73]	3/5	60.00% [14.66, 94.73]	3/5	60.00% [14.66, 94.73]
Trace (3)	0/3	0.00% [0.00, 70.76]	0/3	0.00% [0.00, 70.76]	0/3	0.00% [0.00, 70.76]
All	**(58/67)**	**86.57% [76.03, 93.67]**	**(54/67)**	**80.60% [69.11, 89.24]**	**(56/67)**	**83.58% [72.52, 91.51]**

Figure 3. Paired qPCR results (MTB DNA Quantity pg/µL) for foam swab samples with (black bars) and without (gray bars) sedimentation concentration
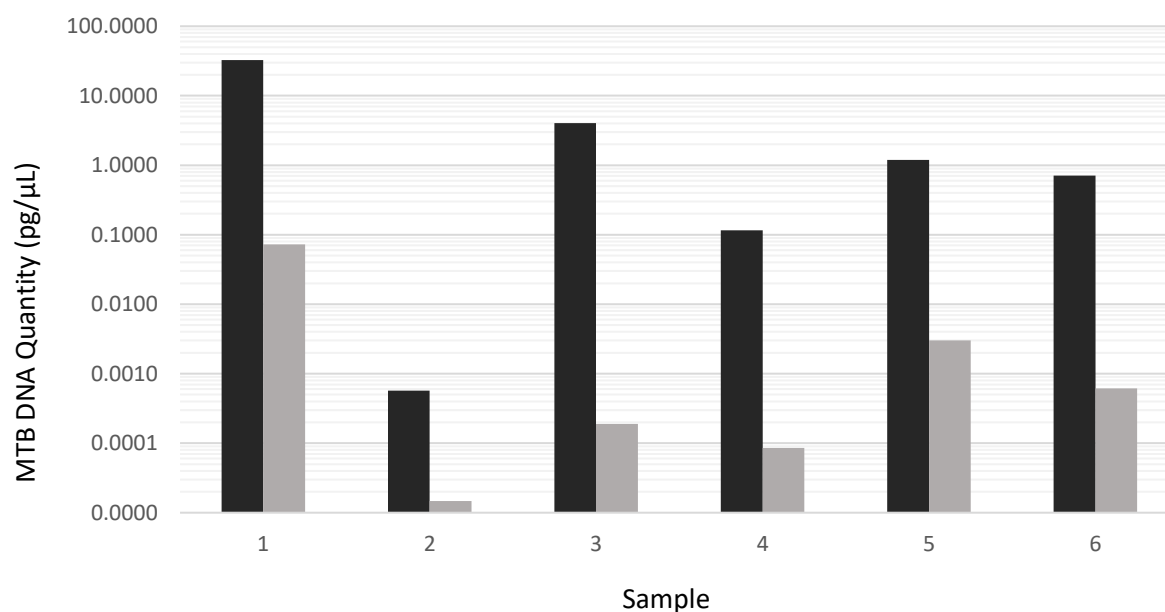
SSMaC. SSMaC was performed on the lysates from 92 sputum MRS-positive and 36 sputum MRS-negative participants. The sensitivity was 83/92 (90.2%) relative to sputum MRS, a significant improvement over the sensitivity of Molbio Ultima testing (z-test, $p = 0.04$). It was also better than the sensitivity exhibited by Xpert Ultra testing of replicate swabs from these participants, although that difference was not significant ($p = 0.19$) (Table 4, bottom line). The 95% confidence intervals for all three methods overlap with varying severity.

When the results were stratified according to SPUXU SQ, SSMaC consistently trended towards higher sensitivities across all categories of sputum positivity (Table 4). The specificity of SSMaC was also high at 97.22% as compared to TS Molbio Ultima (100%) and TS Xpert Ultra (100%) (Table 5).

Table 4. Percent sensitivity of TS methods relative to SPUXU SQ

Sputum Xpert Ultra Semi-Quantity (N)	TS Xpert Ultra		TS Molbio Ultima		TS SSMaC	
	N	% Sensitivity [95% CI]	N	% Sensitivity [95% CI]	N	% Sensitivity [95% CI]
High (42)	42/42	100.00% [91.59, 100.00]	40/42	95.24% [83.84, 99.42]	42/42	100.00% [91.59, 100.00]
Medium (16)	16/16	100.00% [79.41, 100.00]	14/16	87.50% [61.65, 98.45]	15/16	93.75% [69.77, 99.84]
Low (23)	16/23	69.57% [47.08, 86.79]	15/23	65.22% [42.73, 83.62]	19/23	82.61% [61.22, 95.05]
Very Low (8)	3/8	37.50% [8.52, 75.51]	4/8	50.00% [15.70, 84.30]	6/8	75.00% [34.91, 96.81]
Trace (3)	0/3	0.00% [0.00, 70.76]	0/3	0.00% [0.00, 70.76]	1/3	33.33% [0.84, 90.57]
All (92)	**77/92**	**83.70% [74.54, 90.58]**	**73/92**	**79.35% [69.64, 87.08]**	**83/92**	**90.22% [82.24, 95.43]**

Table 5. Percent specificity of methods paired by SSMaC

TS Xpert Ultra		TS Molbio Ultima		SSMaC Concentration	
N	% Specificity [95% CI]	N	% Specificity [95% CI]	N	% Specificity [95% CI]
36/36	100.00% [90.26-100.00]	36/36	100.00% [90.26-100.00]	35/36	97.22% [85.47-99.93]

Table 6. SSMaC % sensitivity by HIV Status

HIV Status	N	% Sensitivity [95% CI]	Two population z-test *p* value	Cq Avg ± Stdev	One-tailed, unpaired t-test *p* value
Positive	24/30	80.00% [61.43, 92.29]	0.0434	36.32 ± 8.08	<0.0001
Negative	60/64	93.75% [84.76, 98.27]		30.20 ± 5.64	

Table 7. Foam swab % sensitivity by HIV Status

HIV Status	N	% Sensitivity [95% CI]	Two population z-test *p* value	Cq Avg ± Stdev	One-tailed, unpaired t-test *p* value
Positive	16/27	59.26% [38.80, 77.61]	0.0005	32.05 ± 7.07	0.0061
Negative	55/59	93.22% [83.54, 98.12]		28.06 ± 6.72	

Associations with HIV coinfection status. Sensitivities of both the SSMaC (Table 6)
and foam swab (Table 7) methods were higher in participants without HIV coinfections
compared to PLHIV. This observation was statistically significant as suggested by two-
population z-tests (*p* = 0.04 and 0.0005, respectively). Both methods also had lower average
Cqs (stronger MTB DNA signals) among those without HIV. One-tailed, unpaired t-tests
suggested significant differences (*p* < 0.0001 and *p* = 0.0061, respectively).

Discussion

Many exploratory evaluations of TS have used in-house manual nucleic acid
amplification tests (NAATs), primarily qPCR, to detect MTB DNA in tongue swab samples (7-9,
14, 20-22). Some of these methods exhibited >90% sensitivity and specificity relative to sputum
MRS (8, 9, 14). These observations illustrate the potentially high accuracy of TS testing relative
to sputum testing. Other evaluations used the commercial Cepheid Xpert MTB/RIF® Ultra
automated qPCR system (Xpert Ultra) (6, 23-26). These approaches generally exhibited lower
sensitivity, possibly because the Xpert Ultra is engineered to test sputum rather than swabs.
There remains a need for new methods that are 1) highly sensitive; and 2) amenable to
automation for future POC testing. This study evaluated two such methods.

One method used a high-capacity foam swab that collects more material from the
tongue dorsum than a Copan FLOQSwab, based on observations by spectrophotometry. Using

A_{260} as a measure of biomass picked up from the tongue, foam swabs outperformed FLOQSwabs in a preliminary experiment (Supplemental material S1). We hypothesized that the higher-capacity foam swabs may also collect more TB bacilli from the tongue. To process the foam swabs, we employed a sedimentation step to concentrate the TB cells in a pellet prior to lysis. The sensitivity of the foam swabs was higher than those of the FLOQSwabs when tested by either the Molbio Ultima or Xpert Ultra.

We employed an updated SSMaC protocol in the second method to explore whether further processing of residual crude lysates would detect a greater number of TB positive participants. We concentrated the remaining lysate from FLOQSwabs previously evaluated by Molbio Ultima (5) using optimized SSMaC and tested them by manual qPCR. The resulting sensitivity matched or outperformed those previously reported (5). As reported previously using other methods (9, 20), both new methods exhibited higher sensitivity in HIV-uninfected people.

Figure 4 shows the assay input fraction for the different TS tests evaluated in this study. Using the method described previously (5), Molbio Ultima tested only 1.2% of the total sample volume (Figure 4C). In contrast, SSMaC and foam swab sedimentation tested larger fractions (20% and 25%, respectively). This could help to explain the improvement in sensitivity observed in the latter two methods. Both new methods also appeared to be more sensitive than Xpert Ultra testing of oral swabs. This sensitivity enhancement cannot be attributed to sample volume, because Xpert Ultra tests 100% of the sample (Figure 4A), but may be explained by the limitations of using a commercial platform that is optimized to analyze sputum samples to test swab samples.

Figure 4. Sample volume to assay input volume flow chart
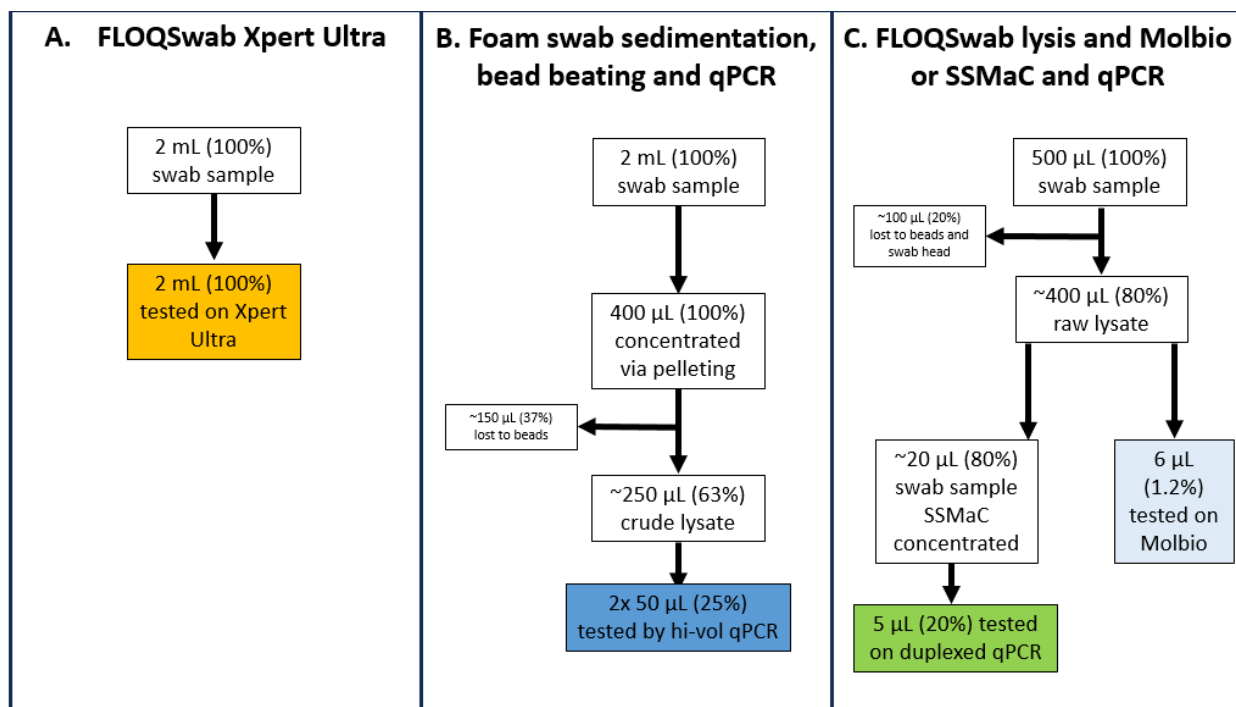
Prior to this analysis, it was not known whether the MTB DNA signal in oral swab samples was associated with bacterial cells, extracellular fractions, or both. When the concentrate and supernatant fractions were tested side by side by qPCR, the concentrated fraction had a significantly larger proportion of the detectable MTB DNA. Therefore, the majority of the TB signal observed in TS assays is associated with particulate material. These results do not allow us to definitively discern whether the MTB DNA resides within intact bacilli or is associated extracellularly with bacterial cells or other particulate material. Nevertheless, they indicate that simple sample concentration approaches such as centrifugation or filtration can be effective in tongue swab sample processing for TB detection.

This study had several limitations. The sample size was limited, and samples were processed in a non-blinded manner. The collection of foam swab samples was done after the collection of two FLOQSwabs. Although it is assumed that flocked swabs do not collect all available material from the tongue dorsum, we cannot know if the foam swabs would have performed differently if collected first. Samples were frozen for several months and shipped to a

lab for processing; different results may have been observed if samples were tested fresh on site.

Despite these limitations, the results support strategies that may be useful for developing automated, POC technologies for TS. All the elements included in SSMaC are automatable and promising for developing a dedicated device. For example, the DASH system incorporates SSMaC and is designed specifically for testing swabs. Although centrifugation is challenging to automate, a POC filtration system like the one used in Xpert Ultra can achieve the same effect.

In conclusion, these results confirm and extend evidence from others (14) that TS testing can be highly sensitive and specific when purpose-designed methods are used to process and test the samples. With further development and automation, TS can become a valuable addition to the TB control toolkit, enabling high throughput, broadly tolerated sampling of virtually any individual in any setting. TS has the potential to simplify TB diagnosis, improve the care of many TB patients, and enable active case-finding strategies that will reduce TB transmission.

Acknowledgements

Literature cited

1. Pai M, Dewan PK, Swaminathan S. 2023. Transforming tuberculosis diagnosis. Nat Microbiol 8:756-759.
2. Branigan D. 2022. 2022 Tuberculosis Diagnostics Pipeline Report. Group TA,
3. Reid M, Agbassi YJP, Arinaminpathy N, Bercasio A, Bhargava A, Bhargava M, Bloom A, Cattamanchi A, Chaisson R, Chin D, Churchyard G, Cox H, Denkinger CM, Ditiu L, Dowdy D, Dybul M, Fauci A, Fedaku E, Gidado M, Harrington M, Hauser J, Heitkamp P, Herbert N, Herna Sari A, Hopewell P, Kendall E, Khan A, Kim A, Koek I, Kondratyuk S, Krishnan N, Ku C-C, Lessem E, McConnell EV, Nahid P, Oliver M, Pai M, Raviglione M, Ryckman T, Schäferhoff M, Silva S, Small P, Stallworthy G, Temesgen Z, van Weezenbeek K, Vassall A, Velásquez GE, Venkatesan N, Yamey G, Zimmerman A, et al. Scientific advances and the end of tuberculosis: a report from the Lancet Commission on Tuberculosis. The Lancet doi:10.1016/S0140-6736(23)01379-X.
4. Nooy A, Ockhuisen T, Korobitsyn A, Khan SA, Ruhwald M, Ismail N, Kohli M, Nichols BE. 2024. Trade-offs between clinical performance and test accessibility in tuberculosis diagnosis: a multi-country modelling approach for target product profile development. Lancet Glob Health 12:e1139-e1148.
5. Wood RC, Luabeya AK, Dragovich RB, Olson AM, Lochner KA, Weigel KM, Codsi R, Mulenga H, Vos Md, Kohli M, Penn-Nicholson A, Hatherill M, Cangelosi GA. 2024. Diagnostic accuracy of tongue swab testing on two automated tuberculosis diagnostic platforms, Cepheid Xpert MTB/RIF Ultra and Molbio Truenat MTB Ultima. Journal of Clinical Microbiology 62:e00019-24.
6. Andama A, Whitman GR, Crowder R, Reza TF, Jaganath D, Mulondo J, Nalugwa TK, Semitala FC, Worodria W, Cook C, Wood RC, Weigel KM, Olson AM, Lohmiller Shaw J, Kato-Maeda M, Denkinger CM, Nahid P, Cangelosi GA, Cattamanchi A. 2022. Accuracy of Tongue Swab Testing Using Xpert MTB-RIF Ultra for Tuberculosis Diagnosis. J Clin Microbiol doi:10.1128/jcm.00421-22:e0042122.
7. Shapiro AE, Olson AM, Kidoguchi L, Niu X, Ngcobo Z, Magcaba ZP, Ngwane MW, Whitman GR, Weigel KM, Wood RC, Wilson DPK, Drain PK, Cangelosi GA. 2022. Complementary Nonsputum Diagnostic Testing for Tuberculosis in People with HIV Using Oral Swab PCR and Urine Lipoarabinomannan Detection. J Clin Microbiol 60:e0043122.
8. Wood RC, Andama A, Hermansky G, Burkot S, Asege L, Job M, Katumba D, Nakaye M, Mwebe SZ, Mulondo J, Bachman CM, Nichols KP, Le Ny A-LM, Ortega C, Olson RN, Weigel KM, Olson AM, Madan D, Bell D, Cattamanchi A, Worodria W, Semitala FC, Somoskovi A, Cangelosi GA, Minch KJ. 2021. Characterization of oral swab samples for diagnosis of pulmonary tuberculosis. PLOS ONE 16:e0251422.
9. Luabeya AK, Wood RC, Shenje J, Filander E, Ontong C, Mabwe S, Africa H, Nguyen FK, Olson A, Weigel KM, Jones-Engel L, Hatherill M, Cangelosi GA. 2019. Noninvasive Detection of Tuberculosis by Oral Swab Analysis. Journal of Clinical Microbiology 57:e01847-18.
10. Nicol MP, Wood RC, Workman L, Prins M, Whitman C, Ghebrekristos Y, Mbhele S, Olson A, Jones-Engel LE, Zar HJ, Cangelosi GA. 2019. Microbiological diagnosis of pulmonary tuberculosis in children by oral swab polymerase chain reaction. Scientific Reports 9:10789.
11. Wood RC, Luabeya AK, Weigel KM, Wilbur AK, Jones-Engel L, Hatherill M, Cangelosi GA. 2015. Detection of Mycobacterium tuberculosis DNA on the oral mucosa of tuberculosis patients. Sci Rep 5.

12.   Church EC, Steingart KR, Cangelosi GA, Ruhwald M, Kohli M, Shapiro AE. 2024. Oral swabs with a rapid molecular diagnostic test for pulmonary tuberculosis in adults and children: a systematic review. The Lancet Global Health 12:e45-e54.

13.   Codsi R, Errett NA, Luabeya AK, Van As D, Hatherill M, Shapiro AE, Lochner KA, Vingino AR, Kohn MJ, Cangelosi GA. 2023. Preferences of healthcare workers using tongue swabs for tuberculosis diagnosis during COVID-19. PLOS Global Public Health 3:e0001430.

14.   Steadman A, Andama A, Ball A, Mukwatamundu J, Khimani K, Mochizuki T, Asege L, Bukirwa A, Kato JB, Katumba D, Kisakye E, Mangeni W, Mwebe S, Nakaye M, Nassuna I, Nyawere J, Nakaweesa A, Cook C, Phillips P, Nalugwa T, Bachman CM, Semitala FC, Weigl BH, Connelly J, Worodria W, Cattamanchi A. 2024. New Manual Quantitative Polymerase Chain Reaction Assay Validated on Tongue Swabs Collected and Processed in Uganda Shows Sensitivity That Rivals Sputum-based Molecular Tuberculosis Diagnostics. Clin Infect Dis 78:1313-1320.

15.   Oreskovic A, Panpradist N, Marangu D, Ngwane MW, Magcaba ZP, Ngcobo S, Ngcobo Z, Horne DJ, Wilson DPK, Shapiro AE, Drain PK, Lutz BR. 2021. Diagnosing Pulmonary Tuberculosis by Using Sequence-Specific Purification of Urine Cell-Free DNA. J Clin Microbiol 59:e0007421.

16.   Xianyu Y, Wang Q, Chen Y. 2018. Magnetic particles-enabled biosensors for point-of-care testing. TrAC Trends in Analytical Chemistry 106:213-224.

17.   Achenbach CJ, Caputo M, Hawkins C, Balmert LC, Qi C, Odorisio J, Dembele E, Jackson A, Abbas H, Frediani JK, Levy JM, Rebolledo PA, Kempker RR, Esper AM, Lam WA, Martin GS, Murphy RL. 2022. Clinical evaluation of the Diagnostic Analyzer for Selective Hybridization (DASH): A point-of-care PCR test for rapid detection of SARS-CoV-2 infection. PLoS One 17:e0270060.

18.   Reed JL, Basu D, Butzler MA, McFall SM. 2017. XtracTB Assay, a Mycobacterium tuberculosis molecular screening test with sensitivity approaching culture. Sci Rep 7:3653.

19.   Yan AJ, Olson AM, Weigel KM, Luabeya AK, Heiniger E, Hatherill M, Cangelosi GA, Yager P. Detection of Mycobacterium tuberculosis from tongue swabs using sonication and sequence-specific hybridization capture. PLoS ONE, in press.

20.   LaCourse SM, Seko E, Wood R, Bundi W, Ouma GS, Agaya J, Richardson BA, John-Stewart G, Wandiga S, Cangelosi GA. 2022. Diagnostic performance of oral swabs for non-sputum based TB diagnosis in a TB/HIV endemic setting. PLoS One 17:e0262123.

21.   Song Y, Ma Y, Liu R, Shang Y, Ma L, Huo F, Li Y, Shu W, Wang Y, Gao M, Pang Y. 2021. Diagnostic Yield of Oral Swab Testing by TB-LAMP for Diagnosis of Pulmonary Tuberculosis. Infection and drug resistance 14:89-95.

22.   Kang Y, Koo B, Kim O-H, Park J, Kim H, Lee H, Kim M, Jang Y, Koo Y, Shin Y, Lee SW, Kim S-H. 2021. Gene-Based Diagnosis of Tuberculosis from Oral Swabs with a New Generation Pathogen Enrichment Technique in Real-World Practice. SSRN Electronic Journal doi:10.2139/ssrn.3781639.

23.   Flores JA, Calderon R, Mesman AW, Soto M, Coit J, Aliaga J, Mendoza M, Leon SR, Konda K, Mestanza FM, Mendoza CJ, Lecca L, Murray MB, Holmberg RC, Pollock NR, Franke MF. 2020. Detection of Mycobacterium Tuberculosis DNA in Buccal Swab Samples from Children in Lima, Peru. The Pediatric Infectious Disease Journal 39.

24.   Mesman AW, Calderon R, Soto M, Coit J, Aliaga J, Mendoza M, Franke MF. 2019. Mycobacterium tuberculosis detection from oral swabs with Xpert MTB/RIF ULTRA: a pilot study. BMC Res Notes 12:349-349.

25.   Lima F, Santos AS, Oliveira RD, Silva CCR, Gon+ºalves CCM, Andrews JR, Croda J. 2020. Oral swab testing by Xpert-« MTB/RIF Ultra for mass tuberculosis screening in prisons. Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 19:100148.

26.   Cox H, Workman L, Bateman L, Franckling-Smith Z, Prins M, Luiz J, Van Heerden J, Edries LAT, Africa S, Allen V, Baard C, Zemanay W, Nicol MP, Zar HJ. 2022. Oral swabs tested with Xpert

MTB/RIF Ultra for diagnosis of pulmonary tuberculosis in children: a diagnostic accuracy study.
Clin Infect Dis doi:10.1093/cid/ciac332.