Oral Swab PCR as an Alternative to Sputum-based Methods for Diagnosis of Pulmonary Tuberculosis

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Abstract

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Background: Oral swab PCR (OSP) has been proposed as an alternative to sputum-based TB diagnosis of tuberculosis because it uses a non-invasive, convenient sample. Sputum can be difficult to obtain and handle, and an alternative sample could enable faster and less expensive PCR detection of *Mycobacterium tuberculosis* (MTB) bacilli in patient samples. OSP may also be an effective test for exposure to either MTB or non-tuberculous mycobacteria (NTM). The latter are environmentally transmitted and some species are opportunistic pathogens of humans. MTB and NTM bacilli may adhere to the oral epithelium, where they can be collected on a buccal swab.

Methods: Three swabs each were collected from 20 HIV-negative subjects with active, (GeneXpert-confirmed) pulmonary tuberculosis, and from 55 healthy, unexposed controls. To detect MTB, the oral swab samples were tested using an in-house polymerase chain reaction (PCR) protocol targeting the IS6110 insertion element, which is unique to the MTB complex. To detect NTM, select samples were tested using PCR protocols specific to certain species

(*Mycobacterium avium* complex, *Mycobacterium abscessus*, and *Mycobacterium kansasii*) and for the *Mycobacterium* genus.

Results: 44/60 (73.3%) swabs collected from case subjects were positive for MTB DNA by OSP. 18/20 (90%) case subjects yielded positive results (at least 2 positive swabs). 100% of control samples were negative. 15/15 (100%) and 6/6 (100%) swab samples tested negative for specific species of NTM, and for the *Mycobacterium* genus, respectively.

Conclusions: OSP appears promising as a simpler and more uniform alternative to sputum testing for pulmonary tuberculosis. Because OSP uses an easy, non-invasive sampling method that does not require active TB symptoms, it could potentially be used as an active case finding tool. The NTM results are a preliminary step toward improved assessment of NTM exposure using OSP, however further optimization is needed.

Specific Aims

Aim 1: Validate polymerase chain reaction (PCR) analysis of oral swab samples as a non-invasive means to detect *Mycobacterium tuberculosis* (MTB) pulmonary infection.

Hypothesis: Positive swab samples will occur at a higher frequency in the case group than in the control group.

Aim 2: Evaluate the utility of OSP for detection of exposure to non-tuberculous mycobacteria (NTM).

Chapter 1: Aim 1, Detection of MTB

Background

Tuberculosis (TB) remains a significant global health problem. In 2012, there were 8.6 million new cases and about 1.3 million deaths [1]. Exposure and subsequent infection is associated with person-to-person contact in settings such as public transportation, workplaces, schools, and households. In particular, occupations like health care [2] and mining put workers at an added risk due to contact with TB patients and close quarters [3]. The best available way to control transmission of MTB is by identifying and treating active TB cases [4]. Diagnosis of pulmonary TB is usually done by microbiological, microscopic, or molecular analysis of patient sputum. The most common microbiologic and microscopic techniques are smear microscopy and liquid or solid culture, though these methods lack sensitivity and specificity or are too slow, as in the case of culture, which can take 16-20 days. The GeneXpert automated nucleic acid amplification system is a molecular assay that tests for MTB presence and rifampicin resistance by targeting the *rpoB* gene sequence of the MTB genome. A recent meta-analysis estimated the sensitivity of the Xpert to be 90.4%, with a specificity of 98.4% [5]. However, the need for sputum as a diagnostic sample is a limiting factor. Sputum can be difficult to obtain from some patients, such as children and those with HIV infections, and its complexity and viscosity restrict test sensitivity, increase sample-to-sample heterogeneity, and increase costs and labor associated with laboratory testing [6]. Sputum collection can also be hazardous as producing it requires that the patient cough it up from deep in the lungs, a process that could spread the TB bacilli through the air and put health care workers and anyone else in the vicinity at risk of exposure and infection [2]. A sample that is easier than sputum to collect and handle would enable simpler and cheaper TB diagnosis by polymerase chain reaction (PCR) and other methods.

Moreover, because sputum is not easily collected in the absence of active TB symptoms, a less invasive alternative sample could facilitate more active TB case finding strategies [7].

The search for an alternative to sputum has been ongoing for years with limited success. A recent study applied the industry standard commercial PCR test, the GeneXpert MTB/RIF, to a variety of nontraditional samples obtained from smear and culture-confirmed, HIV-negative TB patients, including exhaled breath concentrate (EBC), saliva, blood, and urine. Sensitivities relative to culture ranged from 0% (EBC, 0/26) to 38.5% (saliva, 10/26), far below the 100% seen when the test was applied to sputum from the same patients [7]. Somewhat more promising results were published in another study that used Xpert to analyze stool samples from children with culture-confirmed pulmonary TB. Among HIV-positive children, the sensitivity relative to culture was 80% (4/5); however, in the absence of HIV coinfection sensitivity relative to culture was 33% (4/12). Xpert testing of sputum samples from these subjects was 65% sensitive (11/17), relative to culture [6].

There have been previous reports of MTB DNA detection in buccal swabs and other oral samples [8, 9]. OSP was initially developed as a test for exposure to MTB in a study investigating genetic susceptibility factors to TB infection and disease among native South Americans. The study found that 37 of 202 (18%) of the samples collected were positive for MTB [10]. In another study, OSP was used to detect MTB among free-ranging non-human primates because traditional TB diagnostics were not practical in a field setting. It was found that those living in regions with higher human TB prevalence were more likely to be positive for MTB [11]. Another study used OSP to confirm MTB infections in laboratory macaques experimentally infected with simian-human immunodeficiency virus (SHIV). The use of OSP allowed for the detection of the pathogen itself rather than relying on the host's compromised immune response, which precluded the use of standard tuberculin skin test diagnostics. 7/7 (100%) of oral swabs were positive for MTB in

the animals investigated, in addition to 6/7 (86%) positive liver swabs and 5/7 (71%) lung swabs. This study indicates that MTB may be present in the mouth in cases of extrapulmonary tuberculosis as well as pulmonary [12]. These studies did not attempt to correlate MTB DNA detection in humans with reference (clinical or laboratory) TB diagnosis, or a control group. Reference standards are essential to rule out false positive results due to field or laboratory contamination of samples. However, the validity of this technique is supported by the propensity of most bacteria to adhere to surfaces, especially mammalian cells, and adhesion of mycobacterial species to epithelial cells has been previously shown. *Mycobacterium leprae*, the causative agent of leprosy, has been found to adhere to nasal and alveolar epithelial cells [13]. *Mycobacterium avium* is a species of environmental mycobacteria that is an opportunistic pathogen, ubiquitous in the environment, and readily forms biofilms on impervious surfaces such as in drinking water systems [14, 15]. It also has been shown to adhere to the bronchial epithelium where it can form biofilms and develop some resistance to antibiotic treatment [16].

As a further step toward validating OSP an alternative sample that is easy to collect and has a diagnostic yield approaching that of sputum, we conducted PCR analysis of oral (buccal) swabs collected from confirmed TB patients and healthy controls. We compared the ability of OSP to detect positive TB cases among cases already confirmed by a standard method; GeneXpert MTB/RIF conducted on patient sputum, as well as to a matched group of healthy controls. We also assessed patient and clinical parameters relative to OSP results.

Materials and Methods

Study design & population

In this matched case-control study, 20 recently diagnosed TB patients in Worcester, South Africa were selected to be the case group and matched to 20 of the 55 healthy control subjects in

Seattle, WA, USA. The subjects were matched on gender and 10-year age range. All case subjects were diagnosed with the GeneXpert system and were HIV-negative. Three swabs were collected about 24 hours apart from each subject and within a period of seven days. The swabs were collected from the case subjects before they had been on treatment for greater than seven days. All control subjects were confirmed healthy and unexposed via questionnaire. The questionnaire determined control subject eligibility based on domestic risk factors including previous diagnosis of TB, previous positive purified protein derivative (PPD) skin test or Interferon-Gamma Release Assay (IGRA) blood test, travel to high-burden countries, time spent with homeless populations, time spent with immigrant groups, and time spent on American Indian reservations.

Subjects in the case group were actively recruited through a pre-screening process using notification registers at TB clinics in Worcester, South Africa. The first swab was collected at the South African Tuberculosis Vaccine Initiative trial house, and the subsequent two swabs were collected at the subject's home or workplace. They were compensated with 150 rand (about $15 US) after the first swab, and 100 rand (about $10 US) after the second and third swabs. The control subjects were passively recruited through flyers and email notifications. The researchers followed up with the eligibility questionnaire for screening controls. The control swabs were collected at either the subject's workplace, or the researcher's office. The controls were compensated with a $5 coffee card after each swab. Written, informed consent was collected from each subject. The Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC Ref No. 266/2013) and the Human Subjects Division at the University of Washington approved this study (HSD Protocol No. 45151).

Sample acquisition

The swabs (OmniSwab, Whatman) were brushed along the inside of the subject's cheek for about 10 seconds (7-8 times) to collect cells and saliva. The head of the swab was ejected into a tube containing 500 µL of a sterile lysis buffer [11]. Negative field samples (swabs briefly exposed to the air at the sampling site and put into lysis buffer) were collected with every case sample and with every three control samples. Samples were stored at -80 °C and case samples were shipped from Worcester, South Africa to Seattle, WA, USA on dry ice. All qPCR analyses were conducted in Seattle.

Validation of laboratory methods

Before testing the swab samples from the subjects, all laboratory methods were confirmed for greatest possible sensitivity and yield. Previous reports of OSP used a standard phenol-chloroform DNA extraction procedure [11] in order to obtain purified DNA for PCR testing. This protocol can take 2-3 days, and the DNA yield can be variable in that it is highly reliant on user competency. However, it is relatively inexpensive, utilizes harsh chemicals to ensure complete lysis of the *Mycobacterium* cells, and had been proven effective through previous reports. Alternatives were investigated to determine if DNA yield comparable to the phenol-chloroform could be achieved through a faster, and less user-dependent method. Several alternative methods were found in literature reviews, and the QIAGEN QIAamp DNA mini kit spin column protocol was determined to be a potential alternative [17]. To test this, whole MTB, strain H37Ra, cells were spiked at known amounts of 10^3, 10^2, and $10^{1.5}$ into swab samples. Each dilution was prepared and extracted in triplicate using the two extraction protocols and yields were compared by quantification cycle (Cq) value from qPCR.

Sensitivity of the PCR assay [18] was confirmed through testing a standard curve of known amounts of purified MTB DNA from the H37Ra strain. Starting amounts of 1000 pg/µL were diluted ten-fold down to 0.001 pg/µL, for a series of 7 dilutions. 1000 pg/µL corresponds

with 10^6 genomes per PCR, while 0.001 pg/μL corresponds with 1 genome per PCR. This PCR protocol targets the IS6110 sequence, of which there are 17 copies on the H37Ra strain, though the number of copies on a given MTB strain can range from 1 or 2 to about 30. The standard curve was included with each PCR (5-12 samples) to ensure the consistent functioning of the assay and the reagents.

Laboratory analysis

Prior to opening the sample tube and initiating the DNA extraction, each sample of 500 μL buffer and swab was heated to 95 °C for 10 minutes to inactivate and disrupt MTB cells. The first step of the QIAamp protocol was omitted because it called for the addition of a buffer, which was unnecessary as the swabs were already in the lysis buffer. To begin the extraction, materials in the samples were suspended in the lysis buffer through vortexing and agitation. Subsequent steps followed the manufacturer's instructions. The final step was altered so that the samples were incubated at 42 °C for 3 minutes and at room temperature for 2 minutes before elution with 50 μL of AE buffer, which raises the pH so that the DNA is released from the silica membrane. This increased the contact time of the buffer on the silica in order to maximize the yield. At least one negative extraction sample (lysis buffer, no swab) was extracted alongside every batch of samples, which ranged in size from 5 to 12 samples.

In a first-pass (partial volume) analysis, aliquots (5 μL) of the 50 μL DNA eluates were analyzed using a probe-based qPCR protocol targeting a 74 bp amplicon on the IS6110 insertion sequence [18]. qPCR analysis was completed on a 25 μL reaction mixture that consisted of 1x LightCycler Master Mix (Roche), 2 mM $MgCl_2$, 0.45 μM forward primer, 1.35 μM reverse primer, 0.25 μM FAM/MGBNFQ probe, 7.75 ul H_2O, and 5 μL DNA. qPCR was performed on the Applied Biosystems StepOnePlus Real-Time PCR system using the following reaction protocol: initial incubation at 95 °C for 10 min and 45 cycles of 95 °C for 15 seconds (denaturation) and 60 °C for

1 min (annealing/extension) [18]. At least 2 negative PCR controls (5 µL of H_2O instead of DNA) were included in each run to ensure no contamination occurred during PCR setup. For our purposes, negative MTB results are defined by a lack of amplification after 45 cycles.

A second-pass (full volume) analysis was also conducted on select swab samples from both the case and control groups to determine if sensitivity and positivity could be increased. Eluates of case samples that tested negative in partial volume analysis were concentrated approximately 9-fold by ethanol precipitation of the 45 µL eluate volume that remained after partial volume analysis [19]. Precipitates were resuspended in 5 µL of AE buffer. For all case samples that tested positive after full volume analysis, matched control subject samples were retested using the same ethanol precipitation protocol. Negative precipitation control samples (45 µL of AE buffer, no swab) were precipitated alongside the samples. The same PCR protocol as described above was used to reanalyze the samples.

Confirmation of extraction efficacy in controls

In order to confirm that the extraction procedure was effective, and that DNA was being obtained, select control samples were tested as a positive control for the *Streptococcus* genus. A SYBR-based qPCR protocol targeting the *tuf* gene [20] was used, with a 20 µL reaction mixture consisting of 0.8ul each forward and reverse primers, 10 µL of 2x QIAGEN QuantiFast SYBR Green master mix, 2 µL of DNA template, and 6.4 µL H_2O.

Statistical analyses.

STATA 12.1 and VassarStats were used for all the statistical analyses, which include the Fisher's exact test, and sensitivity and specificity calculations, respectively.

Results

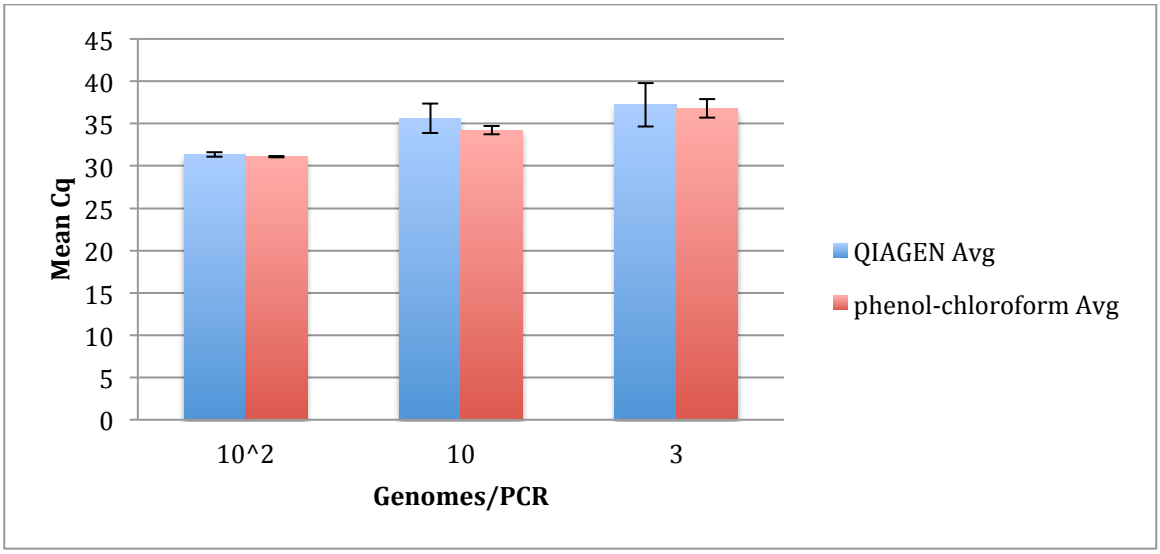
Study population

This study included 20 case subjects and 20 control subjects. The case subjects were newly diagnosed TB patients, all of whom were positive for TB by GeneXpert. The control subjects were determined to be healthy and unexposed to TB via questionnaire, and were presumed to be negative for TB by GeneXpert. Each group included 8 women and 12 men; all over the age 21. The median age of the women in the case group was 38, IQR (31-51) and 40, IQR (33-51) in the control group. The median age of the men in the case group was 45, IQR (35-56), and in the control group, it was 42, IQR (38-54).

Validation of laboratory methods

Results from the tests to compare the phenol-chloroform extraction protocol to the QIAGEN QIAamp DNA mini kit extraction are summarized in Figure 1.

Figure 1. Average Cq values of the DNA extracted from known dilutions of TB cells using the phenol-chloroform protocol and the QIAGEN QIAamp DNA mini kit protocol.



The Cq values can be used to approximate the starting quantities of DNA, and lower Cq values indicate higher amounts of DNA in a sample, whereas higher values indicate less DNA. These results show that there is little difference between the two extraction procedures, and the QIAGEN kit was chosen for extracting swab samples.

Tests to confirm the sensitivity of the PCR reaction showed that DNA diluted to 0.001 pg/μL, or 1 genome per reaction, was consistently detected.

Confirmation of extraction efficiency

Testing by OSP revealed the control subjects to be negative in all three swab samples per subject in both the partial volume and full volume analyses. 15/15 (100%) of control swab samples that were tested for the *Streptococcus* genus were positive, while all negative controls tested negative, confirming the presence of bacteria commonly found in the oral microbiome.

Comparative results

Of the 60 swab samples collected from the case group, 44 (73.3%) were positive *for M. tuberculosis* DNA after full volume analysis. Of the 20 case subjects, 18 (90%) yielded at least 2 positive swabs. Two case subjects yielded no positive swab samples. Acid-fast smear results were also available for 17 of the case subjects, of which 10 (58.8%) were positive. Results of sputum smear microscopy (cases only) and OSP are shown in Table 1. Sensitivity of OSP performance relative to Xpert is summarized for all subjects and for sputum smear-positive subjects in Table 2. Specificity of OSP was calculated relative to the presumed disease-negative status of matched healthy controls.

Table 1. Sputum smear and OSP results. Smear microscopy was conducted on sputa from 17 of the 20 cases. Case and control subjects each provided 3 oral swab samples designated A, B, and C. All subjects were confirmed TB-positive by GeneXpert.

		Sample[1]			Matched healthy controls		
Subject	Smear	A (Cq)	B (Cq)	C (Cq)	A (Cq)	B (Cq)	C (Cq)
1	+	Negative	38.56*	38.8	Negative	Negative	Negative
2	ND[2]	Negative	37.76	33.1	Negative	Negative	Negative
3	negative	Negative	Negative	Negative	Negative	Negative	Negative
4	negative	Negative	Negative	Negative	Negative	Negative	Negative
5	negative	36.95	37.96	32.15	Negative	Negative	Negative
6	negative	35.31*	Negative	36.05*	Negative	Negative	Negative
7	+++	35.96	27.97	30.98	Negative	Negative	Negative
8	+++	35.82	34.52	33.46	Negative	Negative	Negative
9	+++	35.09	38.68	31.24	Negative	Negative	Negative
10	+	36.93	40.92	Negative	Negative	Negative	Negative
11	+++	39.79	38.36	36.31	Negative	Negative	Negative
12	++	39.41*	40.61	Negative	Negative	Negative	Negative
13	negative	Negative	37.49	40.49	Negative	Negative	Negative
14	ND	36.04	33.7	34.35	Negative	Negative	Negative
15	negative	Negative	41.48	39.36	Negative	Negative	Negative
16	+++	39.87*	39.41*	36.6*	Negative	Negative	Negative
17	negative	Negative	40*	37.85	Negative	Negative	Negative
18	ND	34.07	Negative	38.54	Negative	Negative	Negative
19	+++	37.46	38.38	36.59	Negative	Negative	Negative
20	++	Negative	36.49	34.7	Negative	Negative	Negative

[1]Results of partial volume analysis are shown except values marked by asterisk (*), which are full volume results.

[2]ND, not determined.

Table 2. OSP sensitivity and specificity relative to Xpert among all subjects and among smear positive subjects.

		Sensitivity (OSP positive/total Xpert positive, %, 95% CI)	Specificity (OSP negative/total Xpert negative, %, 95% CI)
All subjects	By subject	18/20, 90.0% (66.9-98.2)	20/20, 100.0% (80.0-100)
	By swab	44/60, 73.3% (60.1-83.5)	60/60, 100.0% (92.5-100)
Smear+ subjects	By subject	10/10, 100.0% (65.5-100)	10/10, 100.0% (65.5-100)
	By swab	26/30, 86.7% (68.4-95.6)	30/30, 100.0% (85.9-100)

Analysis of factors potentially related to swab negativity

Further analysis was undertaken to determine whether the 2 subjects who remained negative after full volume analysis did so by chance or if there was a biological or medical explanation. Additionally, positivity by date of swab collection was analyzed to determine if there was an association between early collection and negativity. Viewing Table 1, it appears that a majority of the negative case swab samples are clustered in the first 6 subjects, who were assigned a subject ID by date of enrollment in the study. In Figure 2, the swab sample results are displayed as the rate of positive swab samples by individual date.

Figure 2. Percent of positive swab results by the date they were collected.

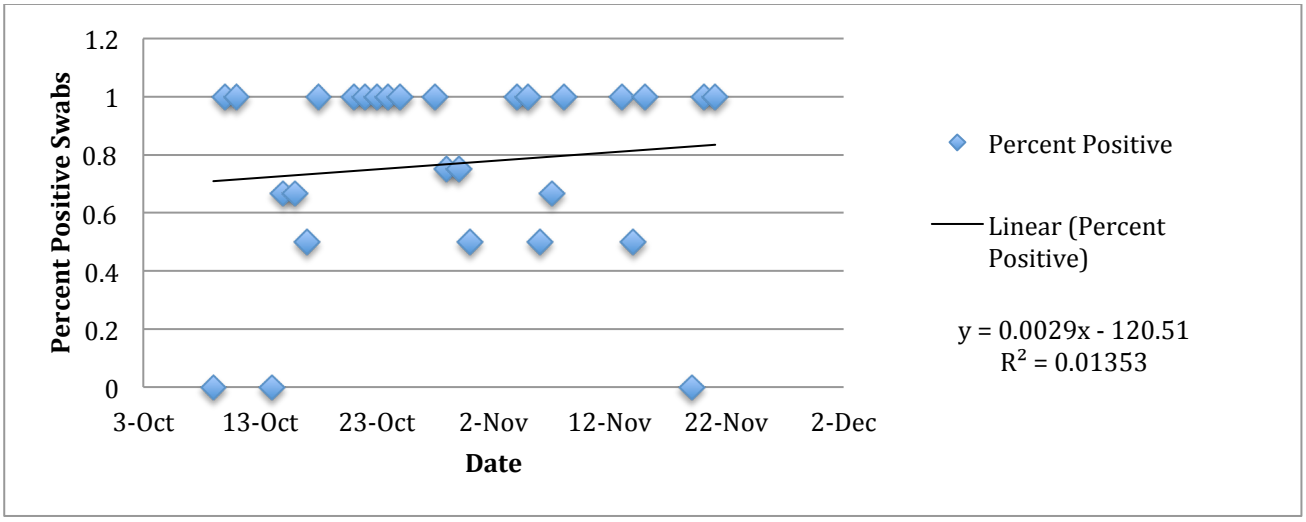


Figure 2 shows that on the earlier dates, the percent positivity was slightly lower than in the later dates; however, there were more swabs collected per day in some of the earlier dates than in the later dates, which could potentially skew the data. Therefore, while user proficiency may have had some impact on the swab result, it is unlikely that any improvements in technique that may have occurred throughout the duration of the study had a substantial impact on OSP results.

Available clinical information on the case subjects included disease status, certain relevant conditions, and acid-fast smear test results. Four of the 20 case subjects were being treated for diabetes mellitus at the time they were referred to the TB clinic. Of these 4 subjects, 3 were OSP-negative in the partial volume analysis (subjects 3, 4, and 6 in Table 1), and 2 (subjects 3 and 4) remained negative in the full volume analysis. These factors were tested for association with OSP results using the Fisher's exact test. The Fisher's exact test is useful for determining significance of an association, and is commonly used for small sample sizes, making it an appropriate test. For the association between OSP and diabetes, the null hypothesis is that diabetes status does not affect the outcome of the OSP results, or that the two proportions are independent from each other. This statement can be applied to the other factors tested for an association with OSP results, smear results and gender. Results are considered significant is the p-value is less than 0.05 ($\alpha = 0.05$). The results are summarized in Table 3.

Table 3. Associations between OSP results and diabetes, smear data, and gender, for both partial volume and full volume analyses.

Fisher's exact test	p-value
OSP and diabetes	
Partial Vol.	0.013
Full Vol.	0.032
OSP and smear	
Partial Vol.	0.25
Full Vol.	0.154
OSP and gender	
Partial Vol.	0.014
Full Vol.	0.147

As seen in Table 3, the p-value is less than 0.05 in both the partial volume and full volume scenarios when looking at the association between OSP and diabetes. Therefore, the null hypothesis is rejected and it appears that there is an association between diabetes and OSP results. In the case of OSP and smear, the p-value is greater than 0.05 using data from both the partial volume and full volume analyses, so we fail to reject the null hypothesis. Finally, the association between OSP and gender was investigated, and is significant using partial volume analysis data, though it is no longer significant using the full volume analysis data. In this case, there is no known biological or medical reason why one gender would be more likely to have a specific OSP result, and this association is likely to have arisen by chance due to the small sample size.

Discussion

Healthy control subjects were included in the study to test for false-positive PCR results arising from laboratory contamination. Given the novelty of the sampling method under investigation, we could not in advance exclude the possibility that healthy but recently exposed

people, or latently infected people, might occasionally yield positive OSP results. Therefore, Seattle was chosen for control subject recruitment because of its very low incidence of TB (5.5 cases per 100,000) [21].

The results indicate that oral swabs have promise as alternative samples for laboratory detection of MTB DNA. For any applications of OSP, the inclusion of the full volume analysis is recommended as it was shown to increase the sensitivity of the test. In contrast to earlier studies, which measured the sensitivity of saliva, blood, urine, EBC, or stool analyses relative to culture, [6, 7] the present analysis measured the sensitivity of OSP among TB patients with a GeneXpert confirmed diagnosis. Therefore, sensitivities are difficult to compare between studies. Nonetheless, OSP performed well relative to GeneXpert, especially when multiple swabs were analyzed at full volume. If larger studies validate these findings, then OSP may be an advantageous sample technique for tuberculosis diagnosis and case finding. The buccal swab is very easy to collect, requiring just seconds and no specialized equipment or invasive techniques. Relative to sputum, buccal swab samples are more uniform in volume and composition, and less viscous and heterogeneous. These characteristics may enable simpler and less expensive diagnostic approaches. In addition, symptoms (sputum production) are not required for swab collection.

After the partial volume analysis, four subjects were found to be negative for MTB. Two of these subjects subsequently tested positive in at least two swab samples during the full volume analysis. In both analyses an association was observed between OSP negativity and ongoing treatment for diabetes mellitus. Because these patients were already under care for a condition that is known to increase risk of active TB, their disease may have been detected at a relatively early stage with low bacillary load [22]. This is supported by the fact that 3 of the 4 diabetes patients had a negative smear test. Data was not available for the 4th diabetes patient. Smear

positivity is often associated with a higher bacillary load [23], consistent with the notion that these patients were diagnosed in the early stages of TB disease, when they would have had lower bacillary load in their sputum.

Additionally, diabetes is associated with changes in oral mucosa. Diabetics sometimes exhibit reduced salivary flows and are more susceptible to oral infections, which could correlate with elevated amounts of oral microbial flora, including native flora [24, 25]. This could decrease opportunities for non-native MTB to adhere to buccal cells. These factors could decrease the amount of MTB cells in the mouth and inhibit detection by OSP. Although this association was significant, the sample size was too small to draw definitive conclusions.

Bacillary load in sputum may not reflect bacillary load in the mouth, as the bacteria could be in suspension or within monocytes and macrophages in the sputum, whereas in the mouth they could adhere to the oral epithelium. Therefore, OSP may be able to detect MTB in the mouth that is not reflective of the concentration of bacilli in sputum. Additional studies are needed to fully evaluate this possibility, and to better understand the interactions of MTB with other microorganisms and epithelial cells in the mouth.

The study had several limitations that should be addressed in follow-up studies. First, only HIV-negative, adult case subjects were included. HIV status and age can affect bacillary load, which could influence OSP detection. Additionally, the sample size was too small to enable complete assessment of factors associated with OSP negativity. Moreover, the case and control groups were not well matched in terms of ethnicity, location, and infectious disease exposure. Although these factors are unlikely to account for the high incidence of *M. tuberculosis* DNA detection in cases relative to controls, a control group more similar to the case group and located in the same or a similar community would strengthen future studies. Finally, the study did not explore alternative sample collection and analysis methods. Some altered laboratory methods

could improve sensitivity. Before extraction, pelleting out the mycobacterial cells could help separate them from host cells and DNA present in the sample, which could inhibit PCR. Additionally, other PCR methods could be explored, such as nested PCR or reverse touch-down PCR that could increase sensitivity. Additional follow-up studies could use OSP to test household contacts of TB patients to explore the use of OSP as a case finding tool or test of exposure.

Aim 1 Conclusions

The results suggest that OSP is a promising alternative to traditional sputum-based testing for TB diagnosis. Due to the risks and complications associated with sputum, oral swabs as diagnostic samples could be beneficial among all patients, though they could be particularly beneficial to patients in whom sputum production is difficult. With further development of sample collection and analysis methods, OSP could simplify the molecular diagnosis of TB and potentially be used for active TB case finding, made possible by the simple, non-invasive nature of the sampling method. Faster identification and treatment of active TB could help control the spread of the disease.

Chapter 2: Aim 2, Detection of NTM

Background

In addition to MTB, selected swabs collected for this study were also analyzed by PCR for non-tuberculous mycobacteria (NTM). NTM are relatives of TB and some are opportunistic pathogens of humans. They are ubiquitous in the environment and can be transmitted to humans through exposure to contaminated soil, water, biofilms, and food [26, 27], although precise routes of exposure are not known in most cases. They are hardy and often resistant to chlorination and biocides increasing the potential for them to be present in municipal drinking water systems [28]. These mycobacterial species can cause disease in susceptible individuals, such as the immune-compromised, elderly, and people with other pulmonary infections or conditions like cystic fibrosis [29]. Some NTM cause extrapulmonary disease, though pulmonary disease is the most common with symptoms that can resemble tuberculosis. In some cases, NTM disease can complicate tuberculosis diagnosis, particularly in resource-limited settings where identification of the bacteria to the species level may not be possible [28].

NTM have been previously isolated from TB patients. In a study investigating the frequency of isolating NTM from children in rural South Africa with possible pulmonary tuberculosis, 109/1732 (6%) of children produced 114 NTM isolates. Of those, 95% were symptomatic and 5 were co-infected with tuberculosis. 63% of the NTM isolates were identifiable, with *M. intracellulare* (41%), *M. gastri* (6%), *M. avium* (4%), and *M. gordonae* (4%) being the most frequency occurring [28].

Additionally, it has been shown that the prevalence of pulmonary NTM (PNTM) disease in the United States is increasing. Estimates using data from Medicare beneficiaries show an increase in prevalence from 20/100,000 in 1997 to 47/100,000 in 2007. However, these figures are likely to be a conservative estimate of the actual number of cases due to previous reports that

the coding used in the study (International Classification of Diseases, 9th revision) to identify cases meeting American Thoracic Society (ATS) criteria may miss 25-75% of all cases. Additionally, prevalence of PNTM disease varies by region, ethnicity, and gender. Women tend to have more PNTM disease than men, and the prevalence is higher in western and southeastern states. Asians/Pacific Islanders are two times more likely than whites to suffer from PNTM, while blacks are half as likely as whites [27]. In a study investigating NTM disease prevalence and risk factors in Oregon from 2005-2006, the species that caused the greatest number of cases of pulmonary and extrapulmonary disease were *M. avium* complex, *M. abscessus/chelonae*, *M. fortuitum*, *M. marinum*, *M. kansasii*, *M. mucogenicum*, and *M. xenopi* [30].

In this study, none of the subjects in either the case or control group were diagnosed with NTM disease; therefore, positive tests would indicate that the subjects had been exposed to NTM. There is a need for increased assessment of NTM exposure and methods for detecting NTM to better understand routes of exposure and disease risks. Specifically, PCR assays were developed to test for *M. avium* complex (MAC), *M. abscessus*, *M. kansasii*, and the *Mycobacterium* genus. These species were selected based on the NTM species most commonly isolated and implicated in disease among the populations of Worcester, South Africa, and Seattle, USA. Among the four most frequently detected NTM isolates in South Africa, *M. intracellulare* and *M. avium* are both in the *M. avium* complex, and thus would be detectable with the same primer set. *M. gastri* is not pathogenic, and thus is of less interest. *M. kansasii* was also selected because it was the only other species outside of MAC that is both commonly implicated in disease in the United States and was identified in South African children [28], though in that study, it was one of the least frequently detected isolates. This will serve as a preliminary step toward identifying NTM species using OSP and evaluating the utility of OSP as a test for exposure.

Materials & Methods

For Aim 2, samples from five of the same subjects that had been collected and extracted using the same procedures as described in Aim 1 were tested for the three NTM species and *Mycobacterium* genus. Individual SYBR-based qPCR protocols were used for the NTM species and *Mycobacterium* genus testing. Table 4 shows information for each specific assay including target gene and product size. There were three different primer sets investigated for detection of the *Mycobacterium* genus and they are their source are displayed as well.

Table 4. PCR assays used for NTM detection.

Species	Target Gene	Amplicon (bp)	Source
M. avium complex	ITS	164	[31]
M. kansasii	ITS	152	[31]
M. abscessus	*dnaJ*	203	[31]
Mycobacterium genus	*16S rRNA*	66	[31]
Mycobacterium genus	*hsp65*	304	[32]
Mycobacterium genus	*hsp65*	228	[33]

The same PCR mix was used for each of the three species-specific NTM assays, and the *Mycobacterium* genus assay from Esfahani et al, 2012 [31]. PCR was performed on a 25 µL reaction mixture that contained 1 µL of each primer, 12.5 µL of 2x QIAGEN QuantiFast SYBR Green master mix, 5.5 µL of H_2O, and 5 µL of DNA template. In the PCR reaction for the *Mycobacterium* genus from Kim et al, 2010 [32], a reaction mixture of 20 µL contained 0.8 µL forward primer, 1.4 µL reverse primer, 10ul of 2x QIAGEN QuantiFast SYBR Green master mix, 5.8 µL H_2O, and 2 µL DNA template. The PCR assay from Khan & Yadav, 2004 [33]was investigated, but determined to be unsuitable, as it did not amplify *M. kansasii*. The assay for the *Mycobacterium* genus described in Kim et al, 2010, was determined to be the best due to sensitivity and specificity considerations (the protocol described in Esfahani et al, 2012 was not

specific enough and amplified species outside the *Mycobacterium* genus) and was used to test samples.

The limits of detection for each NTM PCR protocol was determined through the use of a standard curve with 7, 10-fold dilutions, from 1000 pg/μL to 0.001 pg/μL of purified DNA from cultures grown in the lab.

Results

The samples tested for the presence of NTM were from the control group, and it was found that 15/15 (100%) tested negative for each of the 3 specific species of interest, and 6/6 (100%) were negative for the *Mycobacterium* genus.

The lowest observed limits of detection were in the protocols for *M. avium* complex and *M. abscessus* at 0.1-0.01 pg/μL (100-10 genomes per PCR reaction). The lowest amount of DNA detected in the protocol for *M. kansasii* was inconsistent, ranging from 1-0.01 pg/μL (1000-10 genomes per PCR reaction). The protocol for the *Mycobacterium* genus had a limit of detection of 0.1 pg/μL (100 genomes per PCR reaction).

Discussion

The results of testing for NTM are not necessarily indicative of NTM absence, as the limits of detection for each of the PCR assays were higher than the assay for MTB. While larger amounts of DNA would certainly be detectable using any of these assays, none were considered sensitive enough for continued use, since it was not known how much bacteria to expect in any of the samples. However, these results are useful for identification of gene sequences and protocols specific to the amplification of certain species of Mycobacteria, and further optimization of PCR protocols will be continued to increase sensitivity.

Aim 2 Conclusions

In this preliminary investigation, PCR assays for specific Mycobacterial species were identified and tested but no NTM species were detected in any of the samples. There will need to be further optimization of these assays before retesting any of the samples to increase the likelihood of detecting NTM. This could be a useful step in validating the use of OSP as a test for exposure to NTM, which would facilitate follow-up studies analyzing risk factors associated with NTM exposure.

Final Conclusions

The results of this study support the hypothesis that swabs positive for MTB would occur at a higher frequency among newly diagnosed TB patients than in unexposed, healthy controls. The controls were 100% negative for MTB, while in the case group, 44/60 (73.3%) swabs and 18/20 (90%) of subjects were positive. This shows that OSP has great potential for real-world applications as a diagnostic or case finding tool.

It could be used in conjunction with other existing diagnostic tests, or used as an alternative to sputum-based testing, particularly among patients who have difficulty producing sputum. Additionally, it could be applied as a case finding tool in settings such as households or workplaces to test for additional cases when an individual in that setting is diagnosed. Faster identification and diagnosis could lead to earlier treatment and prevent further spread of the disease in this instance. Easier detection can help reduce the disease burden.

The validation of OSP as a test for exposure to MTB and NTM is still in development, though preliminary steps have been made toward this aim.

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