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Non-invasive diagnosis of pediatric tuberculosis

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Abstract

Non-invasive diagnosis of pediatric tuberculosis

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Pulmonary tuberculosis (TB) is typically diagnosed by analysis of sputum samples. Sputum is a reliable specimen for TB diagnosis, but it has limitations. Requiring ill patients to cough up sputum can put health care workers at risk. The viscosity of sputum makes it difficult to work with. There are also patients, such as young children, who may have difficulty producing sputum on demand.

Oral swabs could provide a quick, simple, non-invasive alternative to sputum sampling. In a preliminary study, swabs were collected from 20 adult TB cases confirmed by Cepheid's GeneXpert (PCR) analysis of sputum. Eighteen of these subjects yielded swabs that were

positive for *Mycobacterium tuberculosis* (MTB) DNA in a manual quantitative PCR (qPCR) analysis. Control samples from 20 healthy adults were 100% negative.

The current study pursued two Aims. The first was to improve the sensitivity of oral swab analysis (OSA) by evaluating alternatives to the qPCR method used in the pilot study. Swabs were “spiked” with cultured MTB cells. Three systems were compared for their abilities to detect MTB: Qiagen’s QIAamp DNA Mini Kit followed by manual qPCR, Claremont BioSolution’s semi-automated Purelyse Kit followed by manual qPCR, and Cepheid’s fully automated GeneXpert. After adjustments to improve sensitivity, the Qiagen kit was chosen as the best method for use in the second Aim based on its sensitivity.

The second Aim was to complete a pilot evaluation of OSA applied to children with suspected TB in a high TB-burden setting. Oral swab samples were collected from children aged 0-12 years with TB-like symptoms who visited a clinic in South Africa. The children were clinically diagnosed with definite TB (sputum culture or GeneXpert confirmed, N=21), possible TB (negative by sputum testing but improved after TB treatment, N=42), or not TB (negative by sputum testing and improved without TB treatment, N=22). Swabs were analyzed using the optimized Qiagen protocol. MTB was detected in approximately 24% of the definite TB subjects, while in children above the age of 59 months, MTB was detected in 50% of the definite TB subjects. In these same populations, PCR analysis by GeneXpert of induced sputum detected 52% and 63% respectively.

The results show that *M. tuberculosis* can be detected on oral swabs using a variety of popular molecular analytical platforms. OSA may be most effective in children and adults aged five years and older.

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Chapter 1. INTRODUCTION

Tuberculosis (TB) is one of the deadliest communicable diseases with 9.6 million new cases and 1.5 million deaths worldwide in 2014 alone. This included an estimated one million new cases and 140,000 deaths in children (WHO, 2015). While recent efforts have decreased the incidence of TB, the disease still has a significant global burden.

Pulmonary tuberculosis is typically diagnosed using microbiologic, microscopic, or molecular techniques to analyze sputum samples (Lawn, 2013). This presents a number of problems. Requiring ill patients to cough up sputum can put health care workers who are exposed to the aerosolized bacterium at risk. The viscosity of sputum makes it difficult to analyze. There are also cases for which a sputum sample does not always provide an adequate diagnosis. In HIV-positive people, the sensitivity of traditional sputum-smear microscopy is only about 50 percent, and other methods such as chest X-rays add to the cost of diagnosis and lack specificity (Padmapriyadarsini et al, 2011; Cattamanchi et al, 2009). Many children under the age of five do not expectorate sputum, and even when sputum can be collected, it is often smear-negative for *Mycobacterium tuberculosis* (MTB) (Nelson et al, 2004). For these reasons a diagnostic test that does not require sputum is needed (Swaminathan et al, 2010).

OSA is a non-invasive diagnostic technique that involves swabbing a patient's inner cheek, extracting DNA from the swab, and using polymerase chain reaction (PCR) to detect the presence of MTB DNA (Wood et al., 2015). OSA shows great promise in TB diagnostics as a simpler, less invasive, and more uniform alternative to sputum. The current study expanded previous findings in adult HIV-negative TB suspects, by testing whether OSA can detect TB in very young children, some of whom may have an HIV-coinfection. The study also examined multiple extraction protocols to optimize the sensitivity of the test.

Chapter 2. REVIEW OF THE LITERATURE

Many studies have explored alternatives to sputum, usually with limited success. In one study, exhaled breath condensate (EBC), saliva, urine, and blood samples were collected from smear- and culture-positive TB patients to test for *M. tuberculosis* using the GeneXpert assay. Among these samples, the highest sensitivity was found in saliva samples (10 positives of 26 samples) (Shenai et al., 2013). In another study, stool samples were analyzed from children with culture-confirmed TB cases, detecting 8 of 17 cases (Nicol et al., 2013). Nasopharyngeal aspirates were collected and cultured with a similar diagnostic yield as induced sputum although the performance of this test was more variable than sputum (Franchi et al., 1998). Detecting lipoarabinomannan (LAM) in urine may hold promise for improving TB diagnostics specifically among individuals with HIV and low CD4 counts, but not among HIV-negative adults nor in children with or without HIV (Peter et al., 2015; Lawn et al., 2015; Nicol et al., 2014). A study showed that volatile organic compounds detected in an individual's breath may indicate pulmonary tuberculosis with a sensitivity of 84% and specificity of 64.7% when sputum culture, microscopy, and chest radiography were all positive or all negative, respectively (Philips et al., 2010).

Both traditional and new diagnostics struggle to provide a clear diagnosis of TB in children. Therefore, a panel of experts formed the descriptions in Table 1 to define the child's status of tuberculosis disease (Graham et al., 2012).

Table 1: Proposed clinical definitions for pediatric pulmonary tuberculosis.

| Clinical diagnostic Groups | Definition of Case Categories |
|---|---|
| Confirmed tuberculosis | Patients with suspected TB should be classified as “confirmed TB” when they present with: |
| | 1. At least 1 of the signs and symptoms suggestive of TB |
| | and 2. Microbiological confirmation is obtained |
| Probable tuberculosis | Patients suspected of TB should be classified as “probable TB” cases when they present with: |
| | 1. At least 1 of the signs and symptoms suggestive of TB |
| | and |
| | 2. Chest radiography is consistent with intrathoracic disease due to MTB |
| | and |
| | 3. There is at least 1 of the following: |
| | (a) a positive clinical response to anti-TB treatment |
| (b) Documented exposure to MTB | |
| (c) Immunological evidence of MTB infection | |
| Possible tuberculosis | Patients suspected of tuberculosis should be classified as “possible TB” when they present with at least 1 of the signs and symptoms suggestive of TB |
| | and either |
| | (1) One of the following: |
| | (a) A positive clinical response to anti-tuberculosis treatment |
| | (b) Documented exposure to MTB |
| | (c) Immunological evidence of MTB infection |
| | or |
| | (2) Chest radiography is consistent with intrathoracic disease |
| | NB: if at least 1 of (1) and (2) are both present, then this case should be classified as “probably tuberculosis” |
| Tuberculosis unlikely | Symptomatic but not fitting the above definitions and no alternative diagnosis established |
| Not tuberculosis | Fitting the diagnosis for tuberculosis unlikely but with an established alternative diagnosis |

Better diagnostics (including improved analytical methods as well as alternative sampling methods) could remove some of the uncertainty in the probable and possible definitions above, so that only true TB cases are being treated. This is especially important in reducing the spread of drug-resistant strains. It would also be beneficial for these new diagnostics to be easy to use and to quickly give results so that patients can be diagnosed and begin treatment in the same medical visit. In resource-limited settings particularly, this is needed to increase case finding and thereby decrease transmission and incidence of TB.

The Cepheid GeneXpert MTB/RIF assay has been endorsed by WHO for use in diagnosing TB using sputum samples and provides simple and rapid sample analysis (Lawn, 2013; Theron, 2014; WHO, 2011). However, the GeneXpert cannot be used at the point-of-care because of its reliance on a stable electrical supply and requirement of biosafety precautions similar to direct sputum microscopy (WHO, 2011).

Claremont BioSolutions has developed a rapid DNA extraction kit (PureLyse) that may be able to extract nucleic acids from oral swabs. Minimal training is required to use this kit, and the nucleic acid extraction takes approximately six minutes. Furthermore, no centrifugation is required and the device is battery-powered, making it ideal for low-resource settings (Niemz, 2011). This extraction kit represents a step toward point-of-care TB diagnostics. If OSA is to reach its full potential in TB diagnostics, a method for rapid, point-of-care swab analysis should be prioritized.

In a preliminary study, oral swabs were collected from confirmed TB patients and healthy controls (Wood et al., 2015). Twenty subjects in South Africa with confirmed pulmonary TB based on GeneXpert results, and twenty healthy control subjects in Seattle with no previous exposure to *M. tuberculosis*, were swabbed three times each. The control subjects were carefully

screened to ensure as little potential exposure to MTB as possible resulting in “pristine” negative controls. These samples were tested for *M. tuberculosis* DNA using PCR. A subject was considered positive by OSA if two or more swabs were positive.

As seen in Table 2, 90 percent of the 20 subjects with GeneXpert-confirmed TB were also positive by OSA in ≥ 2 out of 3 swabs. Ten of the 20 subjects (50%) with GeneXpert-confirmed TB were also confirmed using sputum smear microscopy. OSA yielded positive results for all of these smear positive subjects as well as eight more GeneXpert-confirmed cases. The healthy controls were all negative by OSA (Wood et al., 2015).

Table 2: OSA sensitivity and specificity relative to GeneXpert MTB/RIF among all subjects and among smear-positive subjects.

| | | Sensitivity OSA positive/total Xpert positive, % | Specificity OSA negative/total Xpert negative, % |
|------------------------|------------|---|---|
| All subjects | By subject | 18/20, 90.0% | 20/20, 100.0% |
| | By swab | 44/60, 73.3% | 60/60, 100.0% |
| Smear+ subjects | By subject | 10/10, 100.0% | 10/10, 100.0% |
| | By swab | 26/30, 86.7% | 30/30, 100.0% |

The results from this preliminary study are promising. However, the study had a small sample size and only looked at adult populations that were not HIV-positive. While the controls were roughly matched by age and gender, they were unmatched by location (South Africa cases and Seattle controls). The researcher was also not blinded to whether the samples were from cases or controls. Additional studies are needed to include larger samples sizes, an expansion of subjects beyond HIV-negative adults, and more closely matched control groups.

STUDY DESIGN

Figure 1 illustrates the study design. Aim 1 tested OSA with three different platforms, QIAGEN's DNA Mini kit, Claremont Biosolution's Purelyse, and Cepheid's GeneXpert. Adjustments were made to increase the sensitivity of the QIAGEN and Claremont Biosolutions kits. Aim 2 used the optimized QIAGEN method to test samples from children with a potential pulmonary MTB infection.

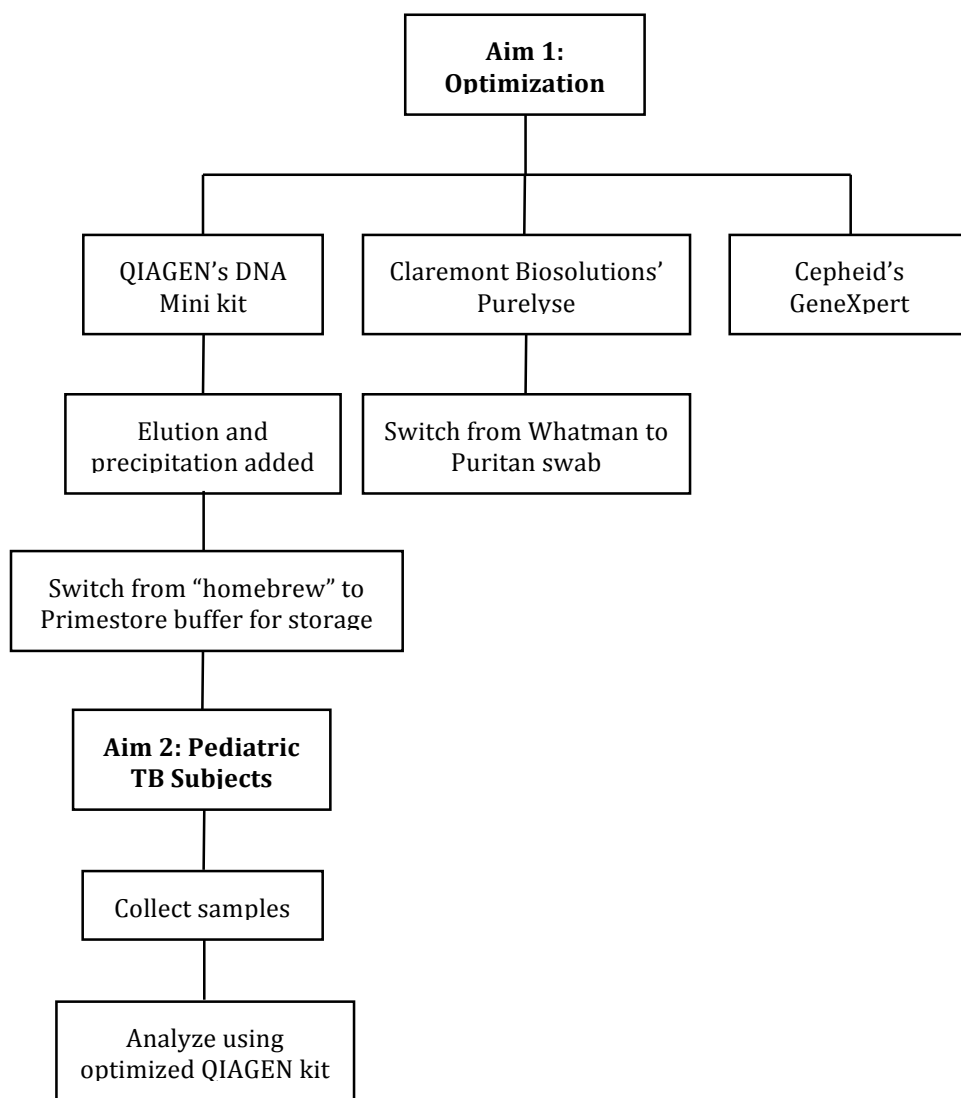


Figure 1: Study design

Chapter 3. AIM 1

Aim 1 was to determine if alternative extraction methods can improve the sensitivity of the verified OSA protocol used by Wood, et al. (2015). Because it is difficult to detect MTB in other types of samples from pediatric TB patients, it was important to apply the most sensitive possible analytical method to pediatric samples.

We hypothesized that modified PCR methods can improve upon the sensitivity, and/or ease of use, of the PCR method previously applied to oral swabs from adult pulmonary TB patients (Wood et al., 2015). To test this hypothesis, we used oral swabs spiked with *M. tuberculosis* cells to evaluate Claremont Biosolution's PureLyse protocol, Cepheid's GeneXpert, and an optimized QIAGEN protocol.

Chapter 3.1. METHODS

MTB was cultured in the laboratory by inoculating Middlebrook 7H9 broth with MTB cells and incubating the culture for two to three weeks. A spectrophotometer was used to measure an OD of the culture. Using the OD and the conversion factor for MTB, an estimation was calculated for the number of MTB cells per mL of 7H9. Using this estimation, cells were diluted with the 7H9 to the following concentrations: 10^4 , 10^3 , 10^2 , 10 cells per 5 μL .

Oral swabs were collected from healthy volunteers in Seattle. The swabs were brushed up and down along the inner cheek ten times. For each concentration of cells, three swabs were spiked by pipetting 5 μL of the ten-fold dilution of cells directly onto the swab tip. The tip was then ejected into a tube containing buffer. Negative controls were also collected by swabbing a volunteer's mouth and placing the swab into lysis buffer without spiked MTB cells. Samples were stored at $-80\text{ }^\circ\text{C}$ until ready for analysis.

Three extraction methods were chosen for this comparison: QIAGEN's QIAamp DNA Mini kit, Claremont BioSolution's Purelyse kit, and Cepheid's GeneXpert.

In initial optimization experiments, various buffers and swabs were evaluated. The yield from the Whatman OmniSwab and the Puritan PurFlock was compared when using the Claremont kit. Because the Whatman swab had already been validated in the pilot study by Wood, et al. (2015) and in a TB study in monkey populations (Wilbur, et al., 2012), the use of this swab was continued in the QIAGEN protocol. The Puritan Purflock outperformed the Whatman Omniswab with the Claremont kit as shown in Figure 4, and was therefore chosen for use in the Claremont protocol.

The pilot study used a "homebrew" lysis buffer for storing and shipping swab samples. Collaborators in Cape Town, South Africa collected pediatric samples for Aim 2 of this project and used Longhorn Diagnostic's PrimeStore buffer for sample storage and shipment. Therefore, the "homebrew" lysis buffer and Longhorn Diagnostic's PrimeStore buffer were compared using the QIAGEN kit. The PrimeStore buffer was expensive in comparison to the homemade lysis buffer (Primestore: \$12.50/sample, homemade: <\$0.01/sample). Therefore, it was only evaluated with the QIAGEN kit.

The QIAGEN QIAamp DNA Mini kit was chosen because its efficacy was verified in the pilot study (Wood, et al., 2015). This extraction method uses a column with a silica membrane to which the DNA adheres until it is eluted with an alkaline buffer. To optimize the sensitivity of this extraction, the elution volume and incubation period were increased and an ethanol precipitation was added as described in the following protocol.

In this protocol, Whatman Omniswabs were ejected into 500 μ L of sterile lysis buffer. As in the pilot study, the samples were heated to 95 °C for ten minutes to inactivate and disrupt

MTB cells and then cooled on ice for three minutes before opening. The initial step in the QIAGEN instructions was omitted because it called for suspending the samples in buffer, and the samples were already in buffer (QIAGEN, 2016). The QIAGEN instructions were then followed until its final step. This step was altered from both the QIAGEN instructions and the pilot study protocol. Samples bound to DNA columns were incubated at room temperature for three minutes and then at 42 °C for three minutes with 150 µL of AE buffer and subsequently eluted. This step was repeated for a total elution volume of 300 µL. The AE buffer raises the pH, thereby releasing the bound DNA from the silica membrane. Adjusting the final step allowed more contact time of the buffer with the membrane, ensuring equilibrium between the bound and unbound states was reached. The increased elution volume and repeated elution were changes intended to maximize yield. An ethanol precipitation was then used to concentrate and further purify the DNA.

The precipitated DNA pellet was resuspended in 5 µL of AE buffer so that the entire sample could be analyzed using a probe-based qPCR protocol targeting the IS6110 insertion sequence. The qPCR analysis used a 25 µL reaction containing 1x LightCycler Master Mix (Roche), 2 mM MgCl₂, 45 µM forward primer, 1.35 µM reverse primer, 0.25 µM FAM/MGBNFQ probe, 7.75 µL H₂O, and 5 µL resuspended DNA. The Applied Biosystems StepOnePlus Real-Time PCR system was used with an initial incubation at 95°C for 10 minutes and 45 cycles of 95°C for 15 seconds (denaturation) and 60°C for 60 seconds (annealing/extension). Two negative PCR controls were included in each run to ensure no contamination occurred during the PCR setup.

Claremont's Purelyse kit was chosen because of its potential to be incorporated into a future point-of-care oral swab test. This kit uses a small battery-powered device that blends the

sample with silica beads, resulting in efficient lysis of tough-walled MTB cells. The DNA adheres to the silica beads and is then eluted with an alkaline buffer. In this protocol, Puritan Purflock swabs were ejected into a 700 μL 3% PVI solution, vortexed for one minute and incubated at room temperature for six and a half minutes. The fluid solution was then removed to a new tube and the swab was discarded. Seven hundred μL of 2x binding buffer was added, and the manufacturer's instructions were then followed through the elution. After the elution, an ethanol precipitation was used so that the entire sample could be resuspended and analyzed by qPCR.

Cepheid's GeneXpert was chosen as an automated analysis method since it is already in place in many diagnostic laboratories around the world. Whatman Omniswabs were ejected into 800 μL of sterile lysis buffer. 1600 μL of GeneXpert's Sample Reagent were added, and the manufacturer's instructions were followed. This automated system completed the DNA extraction, ran a PCR, and provided an output of the results.

Chapter 3.2. FINDINGS

A number of changes were made to the QIAGEN and Claremont Biosolutions kits. The successful changes are summarized in Table 3 along with the result of each protocol adjustment. The Cepheid kit is not listed in this table for two reasons. First, no adjustments were necessary for the kit to successfully detect MTB. Second, because this is an automated system, there is little flexibility in the protocol for changes to be made.

Table 3: Comparison between Wood, et al. method and optimized protocol.

| Extraction method | Change to protocol | Result |
|--------------------------|--|--|
| QIAGEN | Elution changed from 50 μ L to 300 μ L and incubation time doubled | More sensitive, included in optimized protocol |
| | Ethanol precipitation added | More sensitive, included in optimized protocol |
| | Switch from “homebrew” lysis buffer to Longhorn Diagnostic’s Primestore buffer | More sensitive, included in optimized protocol |
| Claremont Biosolutions | Switch from “homebrew” lysis buffer to 3% PVI solution | No change in sensitivity, included in optimized protocol for disinfection purposes |
| | Switch from Whatman Omniswab to Puritan Purflock swab | Increased sensitivity, included in optimized protocol |

Figures 2, 3, and 4 show comparisons of the quantification cycle (Cq) values from the protocol adjustments that improved the sensitivity of the kits. Cq values are the number of cycles it takes to amplify the nucleic acid segment enough to be detected by the PCR machine. Therefore, a lower Cq value means that there is more of the organism present in the sample.

In Figure 2, the protocol that was used by Wood et al. is compared to the modified protocol, which increased the elution volume from 50 to 300 μ L, increased the incubation time for the elution, and added an ethanol precipitation (N=3). As described earlier, the changes to the elution steps allowed for a better removal of nucleic acids from the column membrane. The ethanol precipitation was added so that rather than running a 5 μ L portion of the 300 μ L elution volume, the entire sample could be run in a single reaction. These steps improved the sensitivity of the protocol.

Figure 3 shows the comparison between the Whatman Omniswab and the Puritan Purflock swab when using the Purelyse kit to extract the DNA (N = 3 each; with 250 cfu). The

Puritan PurFlock swab resulted in lower Cq values than the Whatman Omniswab. The different pH of the buffers for the QIAGEN kit and Purelyse kit may explain why the Whatman swab performed poorly with the Purelyse kit.

Figure 4 shows the comparison between the homebrew lysis buffer and Longhorn Diagnostic's Primestore buffer. The Primestore buffer was discovered by accident when we received pediatric samples from South Africa and discovered that the samples were stored in Primestore buffer rather than our "homebrew" lysis buffer. The Primestore and "homebrew" buffer were compared side-by-side to ensure that the pediatric samples could still be tested with the QIAGEN kit. The PrimeStore buffer gave better results than the "homebrew" HBEA buffer (N = 3 each; with 250 cfu).

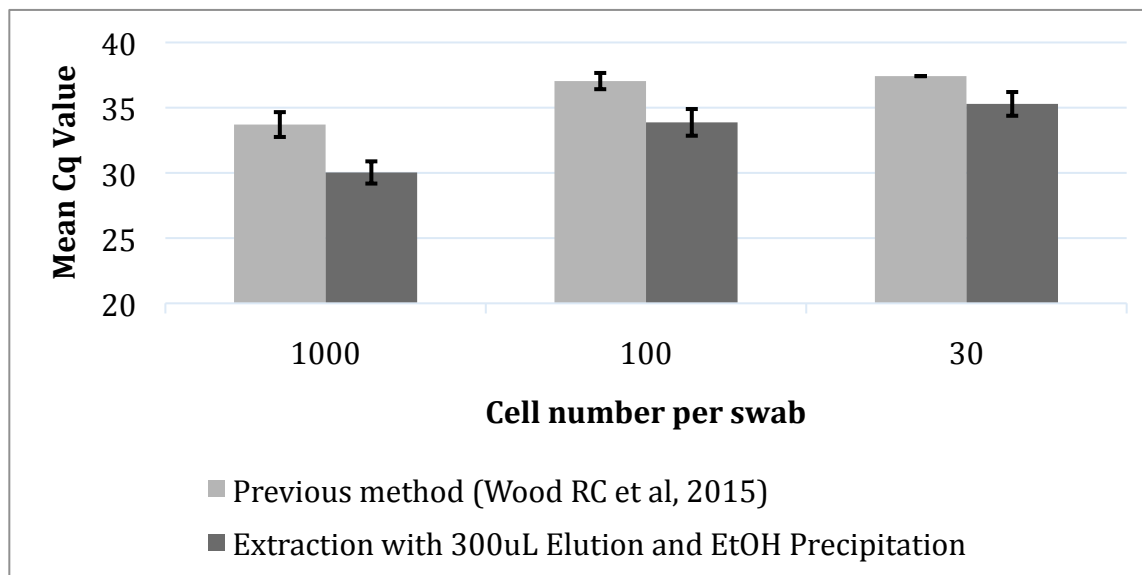


Figure 2: Comparison between the Wood, et al. protocol and the modified QIAGEN protocol.

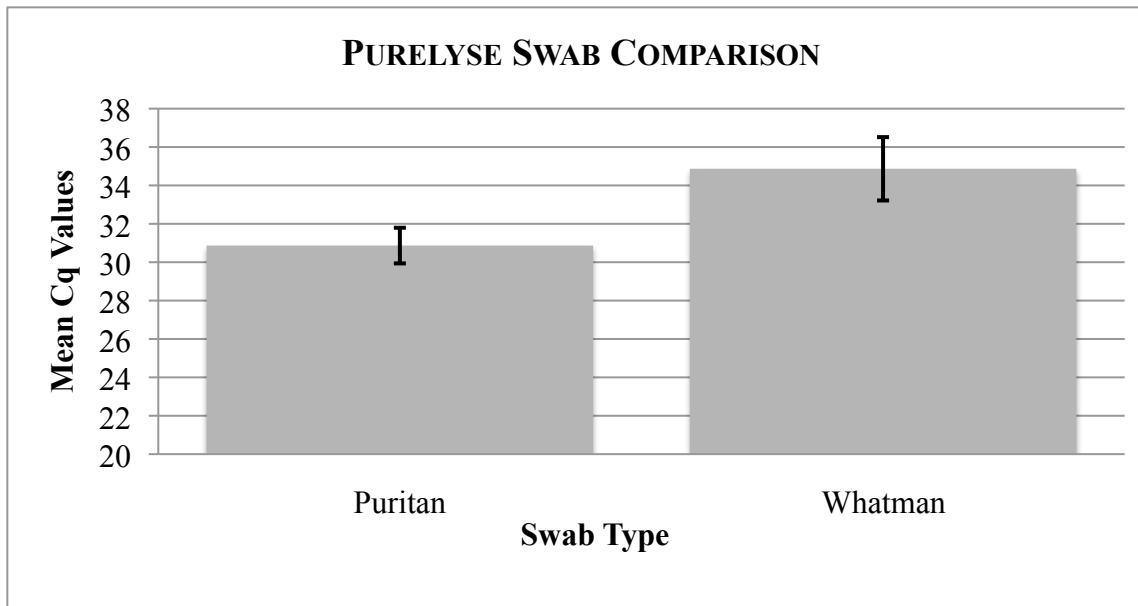


Figure 3: Comparison between the Puritan Purflock and Whatman Omniswab using the Purelyse extraction kit.

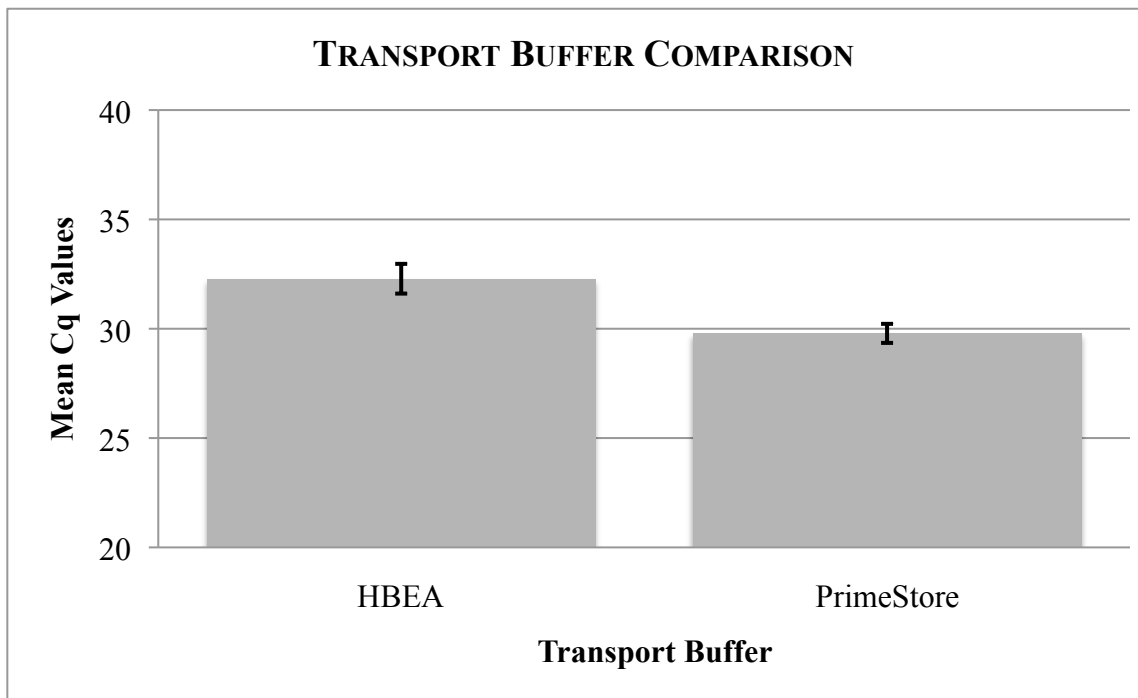


Figure 4: Comparison between the homebrew lysis buffer (HBEA) and the Longhorn Primestore buffer using the QIAGEN extraction kit.

COMPARISON OF OPTIMIZED SENSITIVITIES

Table 4 compares the sensitivities of each kit. Because a different gene is targeted by Cepheid's GeneXpert, the Cq values cannot be compared. Instead, the table shows the concentration of cells that were spiked onto the swab in the left column and in the next columns, how many samples that tested positive for MTB with each kit. The QIAGEN and Claremont kits had similarly high sensitivity. Cepheid detects MTB at the higher concentrations but was not as sensitive as the other two kits.

Table 4: Comparison of optimized sensitivities among QIAGEN, Claremont Biosolutions, and Cepheid kits.

| System used: | QIAGEN DNA Mini | Claremont Purelyse | Cepheid GeneXpert |
|--------------------------|------------------------|---------------------------|--------------------------|
| Gene targeted: | IS6110 | IS6110 | rpoB |
| 10 ⁴ cfu/swab | 6/6 (100%) | 6/6 (100%) | 6/6 (100%) |
| 10 ³ cfu/swab | 5/6 (83%) | 6/6 (100%) | 6/6 (100%) |
| 10 ² cfu/swab | 6/6 (100%) | 5/6 (83%) | 2/6 (33%) |
| 10 ¹ cfu/swab | 5/6 (83%) | 4/6 (67%) | 0/6 (0%) |
| 0 cfu/swab | 0/6 (0%) | 0/6 (0%) | N/D |

Chapter 4. AIM 2

The purpose of Aim 2 was to determine if OSA can be used to detect TB in pediatric cases. We hypothesized that MTB is detectable by OSA in children. In order to test this hypothesis, we used the optimized QIAGEN protocol to extract nucleic acids and qPCR to detect MTB DNA from suspected pulmonary TB pediatric samples.

Chapter 4.1. METHODS

Collaborators at the University of Cape Town, South Africa collected samples from 85 suspected pulmonary tuberculosis patients (ages 2 months to 12 years, median 25 months) (University of Cape Town HREC REF: 045/2008, University of Washington IRB REF: 49234). These children came to the clinic with symptoms suggestive of pulmonary TB, and they and their parent(s) were given information regarding this study. If the child and their parent(s) volunteered and gave informed consent, the child was enrolled in the study. The children's mouths were swabbed using a Whatman Omniswab. The swab was gently brushed up and down the inner cheek for approximately ten seconds, then ejected into a tube containing 1.5 mL of Primestore buffer (Longhorn Diagnostics), and stored at -80 °C. Four swabs per child were collected, two from each cheek. A swab was collected from each cheek prior to induced sputum collection, and then at least four hours passed before the procedure was repeated. Induced sputum was cultured and tested with Cepheid's GeneXpert. Swab samples were shipped to the University of Washington in Seattle on dry ice and again stored at -80 °C until analyzed.

The optimized QIAGEN protocol described above was used to extract DNA from the samples. However, a few adjustments to this protocol had to be made. The samples were shipped in 1.5 mL of buffer rather than 500 µL. The volume of AL buffer and ethanol used in the initial steps had to be increased in proportion to this higher sample volume. The sample was also split to reserve half the volume in case contamination during the extraction process required reanalysis of samples. Quantitative PCR was run in the manner described above.

The oral swabs were considered positive if they amplified within 39 cycles of the qPCR. If amplification occurred after 39 cycles, the sample was considered negative. This threshold was chosen by using a single sample from 71 of the children as a training set. Because the negative

controls were collected in South Africa where TB is highly prevalent, we did not expect our negatives to be as “pristine” as those that were collected in Seattle and used in the preliminary Wood, et al. study. When analyzing the training set, we determined that our analysis may pick up background levels of MTB in the negative controls after 39 cycles of qPCR. Twelve samples returned Cq values greater than 39. Three of the 12 occurred in children without TB, seven with possible TB, and two with definite TB. The two children with definite TB had three additional swabs with Cq values below 39 while those children with possible TB or with no TB had no additional positive swabs. If a child produced at least one positive swab ($Cq < 39$), we considered them positive for TB.

We compared our results with the clinical diagnoses that fit the definitions found in Table 5.

Table 5: Definitions of clinical diagnosis.

| Classification | Criteria |
|-----------------------|---|
| Definite TB | Isolation of <i>M. tuberculosis</i> in at least 1 culture of sputum/NPA or site-specific clinical specimen |
| Probable TB | the chest radiograph or extrapulmonary specimen suggests a diagnosis of TB AND there is a clinical response to anti-tuberculous therapy AND 1 of the following: a positive TST (induration of ≥ 5 mm in HIV-infected children or ≥ 10 mm in HIV negative children), a history of close contact with an adult case of TB residing in the same household or in close proximity to the child, recent loss of weight or failure to gain weight within the last 3 months, positive AFB smear but culture negative; histological evidence of TB on biopsy |
| Possible TB | Symptoms suggestive of TB and are commenced on TB treatment but do not meet the criteria for definite or probable TB |
| Disease other than TB | Do not meet the criteria for definite or probable TB AND the child is not commenced on anti-tuberculous therapy AND there is evidence of clinical improvement at 2 month clinical follow up. |

Chapter 4.2. FINDINGS

PEDIATRIC SAMPLE RESULTS

Table 6: Summary of Results comparing OSA vs. sputum analysis among pediatric suspected TB subjects.

| | N | Sputum analysis (Culture and/or GeneXpert Positive) | OSA Positive |
|--|----|---|--------------|
| Treated for TB (definite and possible) | 63 | 21 | 7 |
| Not TB (not treated) | 22 | 0 | 1 |

Table 6 shows a summary of the results. Analysis of induced sputum by culture and/or GeneXpert identified 21 (33%) of the subjects that were treated for TB while OSA identified only 7 (11%). However, not all children that were treated for TB had the disease. Therefore, it is useful to look specifically at those that had definite TB. In Table 7, the sensitivity and specificity of OSA and sputum culture are compared to the combined clinical and laboratory diagnosis of definite TB and no TB.

Table 7: Sensitivity and Specificity of OSA and sputum culture analysis as compared to definite TB diagnosis.

| For children with definite TB (sputum confirmed) and no TB of all ages | | |
|---|-------------|--------------------------------|
| | OSA | Sputum culture analysis |
| Sensitivity | 5/21 (24%) | 12/21 (57%) |
| Specificity | 21/22 (95%) | 22/22 (100%) |
| For children aged 59 months and above | | |
| Sensitivity | 4/8 (50%) | 5/8 (62.5%) |
| Specificity | 4/5 (80%) | 5/5 (100%) |

Chapter 5. DISCUSSION

The diagnostic yield of OSA in the pediatric samples is low for both OSA and sputum culture compared to the combined definite and probable TB subjects that were treated for the disease, and with OSA, it is especially low in children under 59 months. In children above the age of 59 months, the performance of OSA was similar to sputum analysis. Cohen's Kappa Coefficient measures the agreement between two tests. High coefficients (approaching 1) represent significant agreement (Landis et al, 1977). When comparing sputum culture and OSA with all of the definite TB and no TB subjects, Cohen's Kappa is .2 (low agreement). However when looking at children above 59 months, the coefficient is .8 which represents substantial agreement. Below 59 months, the coefficient is -.1, showing no agreement.

OSA detected MTB in only a small number of samples in pediatric cases. However, we saw substantial agreement between OSA and sputum culture analysis when looking at children

aged 59 months or above. OSA may be most effective in children above approximately five years of age. Future OSA studies may want to focus their research on older children rather than using resources to sample infants and very young children unless case detection can be improved in younger populations through better sampling and processing techniques.

This study only contained nine HIV+ individuals. OSA detected TB in the two HIV+ subjects with definite TB (sputum confirmation) as well as in a child with possible TB (improved after treatment for TB). A study containing a larger sample size may discover that OSA is as effective as or more effective than sputum analysis in HIV co-infected children.

The ease with which oral swabs can be collected is also a great strength. Many clinical settings in which TB is prevalent have limited resources. An oral swab can be collected without special facilities such as isolation rooms.

Well-characterized mutations in the *rpoB* gene are the most common mechanism conferring MTB resistance to rifampin, an important antibiotic for TB treatment. However, in this study, the qPCR detecting the presence of MTB DNA (following Qiagen and Purelyse protocols) targets the insertion sequence called IS6110. This insertion sequence is repeated multiple times in most MTB strains' genomes allowing for enhanced sensitivity. However, by targeting the *rpoB* gene instead (or in addition to IS6110), OSA could also detect rifampin resistance in the infecting bacteria. Drug resistance is an important factor to consider in TB diagnostics and targeting this gene would provide live-saving information to health care providers.

This study evaluated the GeneXpert and found its sensitivity was less than that of the QIAGEN and Claremont Biosolutions kits when applied to oral swabs. However, the GeneXpert could potentially be used with oral swabs in the future, after additional development of methods

for applying oral swab samples to GeneXpert cartridges. Cepheid's GeneXpert is in use in many high TB burden areas. If OSA could be used with the GeneXpert, it would be easier to integrate the use of OSA into clinics. Moreover, Cepheid is developing a new cartridge called the GeneXpert Ultra. The Ultra is projected to have better sensitivity than the current GeneXpert. It may be possible to adapt OSA to the GeneXpert Ultra to achieve higher sensitivity than that seen in this study.

Collecting samples for OSA requires very little training. However, for the test to work properly, a swab must be rubbed along the inner cheek of a patient. To ensure that a subject has been swabbed correctly, we are currently investigating means of testing for the presence of nucleic acid sequences that are ubiquitous in people's mouths (but seldom found elsewhere). If successful, we will be able to check the quality of the swabbing protocol in addition to detecting MTB.

Chapter 6. CONCLUSION

OSA has the potential to be a simpler, non-invasive alternative to sputum-based tests. A variety of extraction methods can be used to analyze oral swab samples, and this analysis identifies methods which improve upon those used by Wood et al., 2015 Pediatric TB continues to present difficulties for diagnostics. However, OSA may provide an improved or supplementary method to increase diagnostic yield for children over 5 years old with pulmonary TB.

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