Detecting Mycobacterium tuberculosis DNA by PCR of swabs from Tuberculosis Patients

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Method Article

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Abstract

Diagnosis of pulmonary tuberculosis \(TB) usually includes laboratory analysis of sputum, a viscous material derived from deep in the airways of patients with active disease. As a diagnostic sample matrix, sputum can be difficult to collect and analyze. An alternative, less invasive sample matrix could greatly simplify TB diagnosis. We hypothesized that Mycobacterium tuberculosis cells or DNA accumulate on the oral epithelia of pulmonary TB patients, and can be collected and detected by using oral \(buccal) swabs combined with PCR detection of M. tuberculosis DNA. A case-control study \(Wood RC et al, SREP-14-09269B, 2015) supported the efficacy of this approach. Oral swab samples are non-invasive, non-viscous, and easy to collect with or without active TB symptoms.

Procedure

A. Prepare for buccal swab collection 1. Prepare lysis and transport buffer under sterile conditions as follows \(final concentrations listed). EDTA, 50 mM Tris, pH 8.0, 50 mM Sucrose, 50 mM NaCl, 100 mM SDS 1% w/v 2. Filter-sterilize the buffer and aliquot it into sterile 2 mL screw-cap tubes with O-rings 500 µL per tube. **B. Sample collection** 1. Use Whatman OmniSwabs. 2. Instruct subjects to not brush teeth, use mouthwash, eat, or drink for 30 min prior to sample collection. 3. Do not touch or handle the collection end of the swab during removal from packaging. 4. Firmly brush the swab along the inside of the subject’s cheek 7-8 times \(about 10 seconds). 5. After collection, eject the head of the swab into a tube containing the sterile lysis buffer. 6. Collect negative field samples as appropriate. Expose these control swabs to the air then eject into the tube with lysis buffer; otherwise, handle in the same manner. 7. Label each swab sample with the Subject ID and the Swab ID. 8. Store the samples \(500 µL lysis buffer with ejected swab head) at -80 °C as soon as possible, and within 8 hours of collection. 9. Use dry ice if shipping is necessary. **C. DNA extraction using QIAGEN QIAamp DNA mini kit \(alternative protocols may also be used)** 1. Before opening the tube, heat each sample \(lysis buffer with swab head) in a water bath at 95 °C for 10 min. 2. Vortex and then centrifuge briefly to remove drops from inside the lid. 3. Add 20 µL QIAGEN protease stock solution and 600 µL Buffer AL to the sample. Mix immediately by vortexing for 15s. 4. Incubate at 56 °C for 10 min. Briefly centrifuge to remove drops from inside the lid. 5. Add 600 µL ethanol to the sample, and mix again by vortexing. Briefly centrifuge at low speed to remove drops from inside the lid. 6. Use a pipette to remove 700 µL of the fluid mixture from step 5, leaving the swab head behind in the tube. Apply this fluid to the QIAamp Mini spin column \(in a 2 mL collection tube provided by the kit) without wetting the rim. Close the cap, and centrifuge at 6000 x g \(8000 rpm) for 1 min. Aspirate to fully remove the filtrate from the collection tube, and place the spin column back in the same collection tube. 7. Repeat step 6 by applying up to 700 µL of the remaining mixture from step 5 to the QIAamp Mini spin column. 8. Repeat step 6 for a third time if any of the mixture from step 5 remains. 9. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g \(8000 rpm) for 1 min. Aspirate to fully remove the filtrate from the collection tube, and place the spin column back in the same collection tube. 10. Carefully open the QIAamp Mini spin column and add 500 µL Buffer AW2 without wetting the rim. Close the cap and

centrifuge at full speed \(20,000 x g; 14,000 rpm) for 3 min. 11. Aspirate the filtrate from the collection tube and replace the QIAamp Mini Spin column. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover. 12. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube, aspirate any remaining filtrate from the collection tube, and discard. Carefully open the QIAamp Mini spin column and add 50 µL Buffer AE to the center of the silica column. Incubate at room temp \(15-25 °C) for 2 min and then 42 °C for 3 min, and centrifuge at full speed \(20,000 x g; 14,000 rpm) for 1 min. Discard column, retaining eluted DNA. 13. Store eluted DNA at 4 °C for immediate PCR analysis, after which, store at -20 °C. Note: Refer to QIAGEN QIAamp® DNA Mini and Blood Mini Handbook for manufacturer's instructions and information on handling and storage of reagents. **D. qPCR Analysis \(alternative methods may also be used).** 1. Make the PCR Master Mix under sterile conditions in accordance with manufacturer's guidelines. Primer sequences are listed in Wood RC et al, Scientific Reports, 2015. 2. Load 20 µL aliquots of the PCR Master Mix into individual wells of a 96-well plate. 3. For maximum sensitivity, run the ethanol precipitation protocol \(step E below) prior to testing samples. 4. Add 5.0 µL of DNA or 5.0 µL H2O \(for negative controls) to the individual wells. 5. Proceed with analysis using the following reaction protocol: a. Incubate at 95 °C for 10 min. b. 45 cycles of: i. 95 °C for 15 seconds \(denaturation). ii. 60 °C for 1 minute \(annealing/extension). 6. The qPCR analysis was performed using the Applied Biosystems StepOnePlus Real-Time PCR system. **E. Ethanol precipitation \(for maximum sensitivity)** 1. Add concentrated NaCl to the DNA solution to a final concentration of 0.2 M and vortex. 2. Add polyacryl to 15 ng/µL and vortex. This can improve yield and also helps by providing a visible pellet during the procedure. 3. Add 2 volumes of ice-cold 200 proof molecular grade ethanol and mix well. Store on ice for 1 hour to precipitate DNA. 4. Pellet DNA by centrifuging at >20000g for 20 min at 0-4 °C. 5. Remove supernatant. 6. Wash with 1 mL 70% ethanol and re-pellet DNA for 2 minutes at max g at 4 °C. -Invert 3-4x to wash. 7. With a pipette, carefully remove all of the supernatant, without disturbing the now loose pellet. 8. Speed-vac pellet until no fluid remains, about 30-40 min. 9. Fully resuspend the pellet in 5 µL AE buffer, rolling the liquid along the back wall and bottom of the tube. 10. Vortex lightly 2s and briefly centrifuge to ensure DNA and AE buffer are together at tube bottom. 11. Incubate at room temp for 1-1.5 hours. 12. Vortex at half speed \(5) for 10 min. 13. Use the sample immediately for PCR, and add 20 µL of pre-prepared PCR Master Mix to each individual sample. 14. Vortex at half speed for 5 min, covered with foil. 15. Spin briefly, and transfer all to tube/plate for PCR.