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Improved method for collection of sputum for tuberculosis testing to ensure adequate sample volumes for molecular diagnostic testing

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ABSTRACT

The quality and quantity of sputum collected has an important impact on the laboratory diagnosis of pulmonary TB. We conducted a pilot study to assess a new collection cups for the collection of sputum for the diagnosis of pulmonary tuberculosis. The pilot study utilized the standard collection cup in South Africa demonstrating a mean collection volume of 2.86 ± 2.36SD ml for 198 samples; 19% of the specimens contained <1 ml and 12% contained >5 ml. We designed and tested two novel sputum cups with a narrow bottom section and clear minimum and maximum markings to allow patients and clinicians to know whether sufficient sputum volume has been produced. The cups differed in their shape and manufacturing approach. The two options also support different mixing approaches being considered for a highly sensitive companion TB-screening assay being developed at Northwestern University (XtracTB assay). Sputum was collected from 102 patients at Nolungile Youth Centre, Khayelitsha, Cape Town, South Africa for a total of 204 samples. The mean volumes collected from the two cups were 2.70 ± 0.88SD ml and 2.88 ± 0.89SD ml. While the mean volumes of current and novel cups are similar, the volume ranges collected with the novel cups were narrower, and 98% of the specimen volumes were within the target range. Only 4 samples contained >5 ml, but none were >6 ml, and none of the specimens contained <1 ml. The number of coughs that produced the samples, patient HIV and TB status plus qualitative descriptions of the sputum specimens were also evaluated.

1. Background

Tuberculosis (TB) infected over 10.4 million people in 2015 resulting in 1.8 million deaths with the majority of infections occurring in low and middle income countries (Global Tuberculosis Report, 2015, 2016). Rapid and sensitive diagnostic tests for TB are critical for providing care to patients and reducing transmission. Nucleic acid tests (NATs) provide faster results than culture and with higher sensitivity compared to sputum smear microscopy. Therefore, the CDC and WHO recommend that NAT be performed on a respiratory specimen from patients with signs and symptoms of TB (Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis, 2009). The leading commercial molecular test, Cepheid's Xpert MTB/RIF, has a sensitivity of 98% when compared to the gold standard culture with smear positive specimens, but sensitivity of smear negative specimens is only 67% (Steingart et al., 2014). Suboptimal sensitivity may reduce test impact, as clinicians will use empiric treatment in test-negative patients (Theron et al., 2014) or alternatively TB in patients with paucibacillary TB. Some children and people living with HIV may be missed entirely.

For a molecular test to match culture's sensitivity, it must detect ≤10 genomic copies of *Mycobacterium tuberculosis* DNA (the analytical limit of detection of culture), process ≥1 ml of sputum to ensure that a sufficient number of tubercle bacilli are in the specimen, and efficiently remove sputum-associated inhibitors from this large sample. The recently developed XtracTB Screening assay was designed to meet these specifications by selectively purifying MTB DNA utilizing a novel sputum thinning and lysis step combined with DNA sequence specific capture yielding a test sensitivity that surpasses all commercially available MTB molecular tests and is equivalent to culture, the current reference standard (Reed et al., 2016). The assay correctly identified 97% of a blinded panel of smear negative and smear positive specimens and demonstrated an analytical sensitivity of below 10 CFU/ml.

To achieve this level of performance, a high quality sample is required, and the sample must be liquefied and homogenized. Additionally, for test settings that lack biosafety precautions such as a biosafety cabinet, it is necessary to completely sterilize the sample prior to handling by the healthcare worker. A high quality sample requires sufficient volume that is properly produced from deep in the lungs rather than

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saliva or nasal mucus. In addition, the process must be as simple as possible to avoid potential errors.

Current sputum collection cups (Fig. 1) were not designed for simple estimation of the amount of sputum produced. For unconcentrated smear microscopy, the specimen volume is not critical. For molecular detection technologies, however, the amount of sputum can have a direct impact on the sensitivity of the test. Typically, (e.g Cepheid GeneXpert TB test), a buffer is added to thin the sputum to the point where it can be pipetted. The thinning buffer is added at a 2:1 or 3:1 ratio thus significantly diluting the sample (Product Insert for Cepheid GeneXpert TB Test, 2015; Tang et al., 2015). The collection cups currently in use, however, do not provide any indication of the volume of sample produced making accurate buffer addition difficult. In the standard cylindrical cup, it can be difficult to discern between 1 and 5 ml (Fig. 1).

This report shows that with the appropriate cup design and markings, patients can reliably produce at least 1 ml of sputum (100% of samples tested) and not >5 ml (98% of samples tested). The current standard cup samples showed a similar mean volume collected, but with significant variation with 19% of the samples below 1 ml and 12% above 5 ml. The two novel cups produced sputum volumes which were statistically similar to each other. The study population included HIV+, HIV-, TB+, and TB presumptive patients. No statistical difference was noted in these populations. Further, we examined the color, quality, number of coughs required and whether the sample was the first or second sample collected. Only color showed any statistical difference in the volume collected. The single most important variable to assuring an appropriate sputum volume is a cup design that allows users to clearly see whether they produced the correct amount of sputum.

2. Methods

2.1. Current cup testing

To understand the range of sputum volume collected using the collection cup, we weighed (Mettler Toledo, Model: AE200) three existing empty sputum cups to obtain an average empty cup weight (A49A; Turner Plastic, Cape Town, South Africa) and then weighed the cups as they were returned to the National Health Laboratory Service (NHLS) at C18, Groote Schuur Hospital, Cape Town, ZA for TB testing. Ninety-three percent (184/198) of samples were collected in the hospital and 7% were collected at outlying clinics (14 of 198). Standard sputum production instructions were given to patients (National Health Laboratory Service Handbook, 2015). No patient demographic data or sputum observations were recorded. All sputum collected for both the current cup and novel cups was eventually used for standard GeneXpert TB testing.

2.2. Novel sputum cup designs

Our approach starts with a specially designed sputum collection cup (Fig. 22) that allows patients to easily understand the amount of sputum to produce. Two different versions of the cup corresponding to two different methods of mixing, heating and manufacturing approaches (injection vs. blow molding). The minimum line represents the volume (1 ml) required to attain the desired limit of detection and the maximum line represents the most sputum that can be reliably processed with one XactTB reagent thinning tablet (5 ml). By using one reagent tablet per reaction the process is simplified for the lab worker and costs are reduced compared to requiring multiple tablets and variable amounts of a liquid buffer. “MIN” and “MAX” lines provide feedback to the patient so that they can provide an appropriate sputum amount.

The novel sputum cups were designed at Northwestern University. The injection molded version was manufactured by Protolabs (Maple Plain, Minnesota, USA), and the blow molded version was manufactured by Flexcraft (Neptune City, New Jersey, USA). Both cups were molded from polypropylene thermoplastic. The inside of the cups was treated with a hydrophobic coating (Aculon Multi-Surface Hydrophobic Treatment, San Diego, CA, USA) by pouring the hydrophobic agent into the cup, turning the cup to assure all surfaces were wetted, and pouring off the excess coating and allowing the cups to dry at room temperature overnight. The hydrophobic treatment causes the sputum sample to slide down the sides of the cups and pool at the cup's bottom rather than sticking to the sides. The printing on the cups was done by Dr. Graphx (Chicago, Illinois, USA). The markings on the cup represent approximately 1.4 ml to the bottom of the minimum line and 4.6 ml to the bottom of the maximum line. This was done to better assure a minimum of 1 ml and a maximum of 5 ml were actually collected. Both cups used foam lined caps from SKS Bottle and Packaging (Watervliet, New York, USA). Prior to the study, the cups were numbered, weighed and their masses were recorded (Mettler model: ML802/01). Samples were then collected at two clinics in the Western Cape, South Africa and transported to the Khayelitsha NHLS lab in Cape Town, ZA using their standard sample transport system and methods.

2.3. Statistical approach

For all analyses a one way Analysis of Variations (ANOVA) analysis to determine whether the volume data was significantly different across the variables considered. A p value of 0.05 was selected as the level of significance. Excel (Microsoft Corporation, Redmond, WA, USA) was used to conduct the analysis.

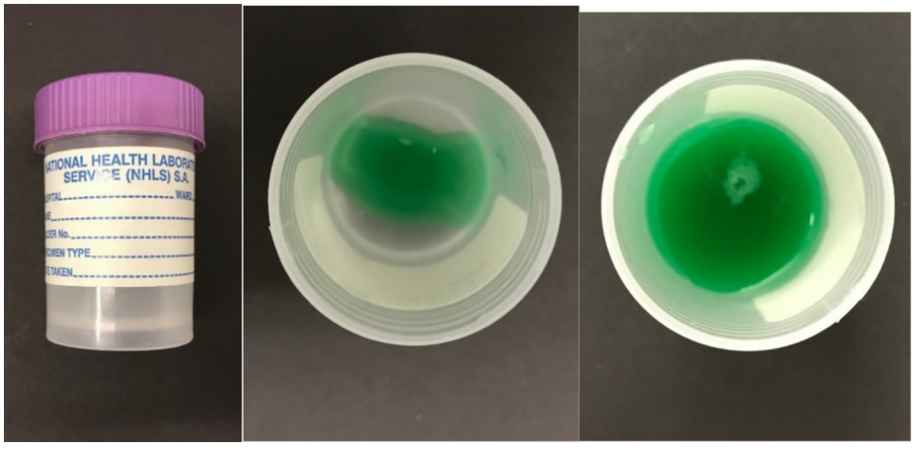

Fig. 1. Typical sputum cup empty, 1 ml and 5 ml of dyed artificial sputum highlighting the challenge of determining with an appropriate amount of sputum was produced and the appropriate amount of thinning agent for tests like the Cepheid GeneXpert TB test.