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Diagnostics

Liquid mycobacterial culture outcomes after different sputum collection techniques before and during treatment

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ABSTRACT

Setting: Mycobacterial sputum culture is a key diagnostic and research tool.
Objective: To compare mycobacterial culture outcomes of three sputum collection methods.
Design: We compared culture results within sets of three sputum samples collected from 18 HIV-infected adult tuberculosis patients at regular intervals up to 84 days after treatment initiation. The first sputum was collected at home and brought to the clinic, where a second and third sputum were consecutively collected under supervision following mouthwash with bottled water and chlorhexidine solution respectively. All sputa were processed for liquid culture in duplicate.
Results: Out of 556 cultures 430 (77.3%), 91 (16.4%) and 35 (6.3%) were positive, negative or contaminated, respectively. The odds of contamination were higher with home collection and with water rinse than with chlorhexidine rinse (OR: 12.5, $p < 0.001$ and OR: 6.7, $p = 0.015$). Chlorhexidine rinse increased the odds of a negative culture compared to water rinse (OR: 3.5, $p = 0.002$). The odds of a positive culture were greater with water rinse than with home collection (OR: 2.5, $p = 0.005$). Water rinse significantly reduced time to culture positivity.
Conclusion: Compared to sputum collected at home, chlorhexidine rinse reduces culture contamination and water rinse increases the rate and viable mycobacterial load of positive cultures.

1. Introduction

Mycobacterial sputum culture remains a key tool for evaluating individuals with presumptive pulmonary tuberculosis (TB), particularly in settings with a high burden of HIV-coinfection and pauci-bacillary TB [1]. Mycobacterial sputum cultures performed at regular intervals quantify the reduction in sputum mycobacterial load and determine the time of culture conversion from positive to negative, which are critical to measure response to treatment in the evaluation of novel anti-TB agents and treatment regimens in clinical trials.

Sensitive culture methods such as liquid culture optimise recovery of mycobacteria but are prone to contamination by overgrowth with oral bacteria and fungi [2,3]. Current guidelines suggest that 2–6% contaminated liquid cultures represent an optimal balance between killing mycobacteria during sputum decontamination and obtaining clean cultures, which is challenging under successful TB treatment when sputum volumes decrease and only few viable bacteria remain.

Practical interventions such as instruction before sputum collection and basic hygiene measures have been shown to improve contamination rates [4]. Mouth rinsing with oral antiseptics before sputum collection with some, but not all, tested mouth wash procedures have shown reduced contamination [3,5,6], particularly in liquid culture [2] but possibly at the expense of *M tuberculosis* detection [3]. Most studies investigating sputum collection procedures have included few participants, did not include HIV-infected individuals or did not include samples collected during treatment.

Abbreviations: AP, Antero-posterior; BMI, Body Mass Index; CXR, Chest X-ray; HREC, Health Research Ethics Committee; PA, Postero-anterior; PBS, phosphate buffered saline; Xpert, Xpert MTB/RIF assay

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This study aimed to test the potential of two simple, low-cost interventions to improve the rate of positive mycobacterial cultures grown from clinical sputum specimens collected before and during treatment in a clinical trial in HIV-infected individuals.

2. Materials and methods

2.1. Participants and baseline assessments

Untreated individuals with a recently positive sputum Xpert MTB/RIF (Xpert; Cepheid, Paulshof, South Africa) at their community clinic were included if they were ≥18 years of age and had documented HIV-infection. Participants that were rifampicin-resistant (Xpert), pregnant, clinically unable to produce sputum spontaneously or were unlikely to comply with the protocol were not included. Participants were recruited at Task, Delft Community Health Centre, Cape Town, South Africa during the period of December 2013 to February 2015. Age, BMI, gender and CD4 count were recorded at the screening visit. A single spot sputum was collected to categorise participants as sputum smear-negative or positive (Auramine O-staining). A chest radiograph (CXR) was assessed for presence of cavitation and extent of disease by a single reader. The study was approved by the Health Research Ethics Committee of Stellenbosch University (N13/07/098). All participants gave written informed consent.

2.2. Sputum collection

Participants included in the study collected sputa once prior to initiating anti-TB treatment and then again on days 3, 7, 14, 28, 35, 56 and 84 after starting treatment. This is a typical sampling schedule in a clinical trial with a culture conversion endpoint. To allow direct comparison between collection methods we obtained three separate sputum specimens at each visit, collected spontaneously in the early morning, following a supervised mouthwash with bottled water at the site, and following a supervised mouthwash with a commercially available chlorhexidine solution in that sequence. At least 30 min passed between collections.

Participants were individually instructed how to produce and collect sputum by study personnel at each visit. This included education on the difference between sputum, nasal secretions and saliva. Participants were instructed not to touch the inside of the container or lid. The sample collected at home on the morning of the visit was kept at room temperature until arrival at the study site. Participants were requested to stand during the procedure, inhale deeply and hold their breath, followed by a deep cough upon exhalation. For mouth rinsing with water, we used commercially available still water (Aquellé, Franschhoek, South Africa) poured into 20 ml disposable plastic cups, just before the patient was requested to rinse their mouth for 60 s prior to giving a sputum specimen. The participant was then requested to gently release the sample by holding the container to his/her lower lip. Half an hour later, a similar process was undertaken with 10 ml chlorhexidine (Corsodyl, GlaxoSmithKline, Bryanston, South Africa), again rinsing for 60 s. Samples were accepted if there were no visible signs of oral material or food particles. Samples of < 5 ml or those that on visual examination appeared to contain salivary aspect were rejected and the participant requested to try again with a new pre-labelled specimen container until staff were satisfied that the best possible sample had been obtained. Sputum was collected in 50 ml sterile, wide-mouth, transparent, disposable, screw top plastic containers pre-labelled with the participant's ID and sputum type. After ensuring the container was tightly capped, labelled and the container ID matched the requisition form, containers were refrigerated at 2–8 °C until transport.

2.3. Sample processing

Samples were transported to the laboratory (Department of Biomedical Sciences, Stellenbosch University, Cape Town, South Africa) in cooled and temperature monitored boxes on the day of collection. Samples were transferred to sterile graded 50 ml centrifuge tubes and the volume of each was recorded. The quality was recorded as saliva, mucoid, mucopurulent, purulent or bloody by trained laboratory personnel. Decontamination was done with a final concentration of 1% NaOH-NALC (NAC-PAC® *RED* AFB Digestion and Decontamination System, Alpha-Tec Systems Inc., Vancouver, Canada) at room temperature for 20 min with vortexing every 5 min. The samples were then neutralised by addition of NPC-67 neutralising buffer (Alpha-Tec) and centrifuged for 15 min at 3000 g and 4 °C. The supernatant was discarded and resuspension buffer (Alpha-Tec) was added to the remaining pellet up to a final volume of 2 ml, of which 0.5 ml was used to inoculate two Mycobacteria Growth Indicator Tubes (MGIT; Becton-Dickinson, Sparks, Maryland, USA), each of which were incubated in automated BACTEC MGIT 960 instruments (Becton-Dickinson). Positive cultures were tested for contamination by blood agar and the presence of acid-fast bacilli confirmed by Ziehl-Neelsen staining and microscopy. A tube was considered negative if no growth was detected after 42 days. Contamination was reported when the tube was macroscopically contaminated, was blood-agar positive, fungal hyphae were observed on ZN smear or ZN-negative irrespective of the blood agar result and the presence of acid-fast bacilli. Time to culture positivity (TTP) was also recorded.

2.4. Scoring and statistical aspects

Each culture was reported as positive, negative or contaminated. Sputa were scored positive if at least one tube was positive, negative if at least one tube was negative but none were positive, or as contaminated if both tubes were contaminated. A score for the occasion was obtained from the three sputa submitted in analogous fashion. This was an observational study with no formal sample size estimation. We aimed for 100 triplets collected, taking into account occasional staff shortages and participants not returning to the site for sputum collections. Descriptive statistics were compiled for the outcomes of single cultures and for sputa, collected with different methods, and for occasions. For testing of associations, a mixed-effects generalised linear model from a binomial family with a logit link was used. The models were adjusted for day of collection as fixed effect and participant as random effect, to ensure that correlations between observations on a single individual were incorporated correctly. The estimates from these models were converted to odds ratios to aid interpretation of results.

3. Results

3.1. Participants, visits and sputum samples

We screened 26 HIV-positive and Xpert-positive individuals, of whom 18 were included. These participants had low BMI and CD4 counts (Table 1). Half of the participants were smear-positive. Most radiographs were abnormal but disease was rarely extensive. Ten to 16 participants completed each time point for a total of 94 visits in 92 of which all three sputa were collected. The majority of the 279 sputum samples were mucopurulent and had a volume of 1–4 ml (Table 2). The largest sputum volumes were collected at home without rinse. Blood admixture was rare.

3.2. Culture, visit and sputum scores

Results of single cultures (two per sputum), sputa (three collected per visit) and visits over time are displayed in Table 3. No visits negative on all samples were observed before day 56. Contaminated cultures were found at an expected rate for liquid cultures (6.3%) and appeared to occur randomly. The results according to sampling methods in the 92 visits with complete samples are displayed in Tables

Table 1
Clinical, microbiological and radiological features at baseline.

Demographics	*n (%) unless stated otherwise*
HIV-positive	18 (100)
CD4 count (cells/mm^3), median (IQR)	183 (88–253)
Male	9 (50)
Age (years), median (IQR)	36.5 (31.3–40.3)
Body mass index (kg/m^2), median (IQR)	19.3 (17.4–20.3)
Sputum	
Negative or scanty positive	9 (50)
Positive	9 (50)
1+	2 (11)
2+	5 (28)
3+	2 (11)
Chest radiograph	
Abnormal	16 (89)
No cavitation	2 (11)
Cavitation < 4 cm	7 (39)
Cavitation ≥4 cm	7 (39)
Diseased lung area < 25%	6 (33)
Diseased lung area < 26–50%	8 (44)
Diseased lung area ≥50%	2 (11)

Cavitation: sum of all cavities' maximum diameter on PA view.

Table 2
Sputum samples and outcomes.

	No rinse n (%)	Water rinse n (%)	Chlorhexidine rinse n (%)	Total n (%)
Sputum quantity*				
1 – 4 ml	3 (3.2)	66 (71.0)	59 (63.4)	128 (45.9)
5 – 9 ml	42 (45.2)	25 (26.9)	32 (34.4)	99 (35.5)
> 10 ml	48 (51.6)	2 (2.2)	2 (2.2)	52 (18.6)
Sputum quality*				
Mucopurulent	86 (92.5)	82 (88.2)	80 (86.0)	248 (88.9)
Bloody	7 (7.5)	3 (3.2)	0 (0)	10 (3.6)
Salivary	0 (0)	8 (8.6)	13 (14.0)	21 (7.5)
Sputum culture outcome*				
Contaminated	7 (7.6)	2 (2.2)	0 (0.0)	9 (3.3)
Negative	18 (19.6)	8 (8.7)	15 (16.3)	41 (14.9)
Positive	67 (72.8)	82 (89.1)	77 (83.7)	226 (81.9)

*In total 94 visits took place. One day 28 visit was missing the spontaneous sample and one day 84 visits was missing both the water and chlorhexidine rinse samples. Sputum quantity and quality include all sputa collected (total 279, 93 per method). The culture results include only complete sets of three sputa (total 276, 92 per method).

Table 4
Odds ratios (OR), their 95% confidence intervals and p-values of binary contaminated, positive or negative outcomes, comparing pairs of collection methods.

Contrast	OR	95% CI	p-value
Odds for contamination from 35 contaminated and 512 not contaminated cultures			
No wash vs CHX wash	12.5	3.5–79.6	0.0008
Water wash vs CHX wash	6.7	1.8–43.8	0.0146
No wash vs water wash	1.9	0.9–4.1	0.1122
*Odds for a positive culture from 430 positive and 126 not positive cultures**			
Water wash vs no wash	2.5	1.3–4.7	0.0051
CHX wash vs no wash	1.7	0.9–3.1	0.0892
Water wash vs CHX wash	1.5	0.8–2.8	0.2500
*Odds for a negative culture from 91 negative and 465 not negative cultures**			
CHX wash vs water wash	3.5	1.6–7.9	0.0019
No wash vs water wash	2.1	1.0–4.8	0.0670
CHX wash vs no wash	1.7	0.8–3.4	0.1630

Estimates are from generalised linear mixed-effects model with logit link of binary contaminated, positive or negative outcomes comparing pairs of collection methods. Models were adjusted for day of visit (fixed effect) and participant (random effect).
*Including contaminated.

2 and 3. Table 3 also summarizes TTP results.

If one considered each of these sputa as an independent event, thus ignoring participant ID and time points as potential confounders, a valid attempt at analysing diagnostic accuracy would be to look at the sensitivity and specificity of each method to identify a positive visit as such. In such an analysis, contaminated sputa would be false positives (flagging positive on the instrument without evidence of *M tuberculosis*), and negative sputa would either be true or false negatives. Analysed in such a manner, sensitivity and specificity of no-wash, water wash and chlorhexidine wash would be 85.0% and 41.7%, 97.6% and 75.0%, and 89.5 and 100%, respectively, with water wash appearing as the most likely winner.

3.3. Statistical analysis

As a more appropriate method for assessing associations, we modelled binary culture outcomes using generalised linear mixed-effects models from a binomial family with a logit link. Visit time points were included as fixed effect and participant ID as random effect to ensure appropriate inference. Table 4 shows estimates converted to odds ratios and corresponding confidence intervals. This analysis suggests that chlorhexidine mouth wash reduced the chance of culture contamination by 12.5 times compared to home collection and 6.7 times compared to

Table 3
Culture, sputum and visit results.

	Day 0 n	Day 3 n	Day 7 n	Day 14 n	Day 28 n	Day 35 n	Day 56 n	Day 84 n	Total n (%)
Visit	***16***	***14***	***10***	***11***	***10***	***11***	***11***	***11***	***94 (100)***
Positive	16	14	10	11	10	11	9	7	88 (93.5)
Negative	0	0	0	0	0	0	2	4	6 (6.5)
Contaminated	0	0	0	0	0	0	0	0	0 (0)
Sputum	***48***	***42***	***30***	***33***	***29****	***33***	***33***	***31****	***279 (100)***
Positive	44	41	30	30	26	25	21	12	229 (82.1)
Negative	2	1	0	1	2	6	11	18	41 (14.7)
Contaminated	2	0	0	2	1	2	1	1	9 (3.2)
Cultures	***96***	***84***	***60***	***64***	***58***	***66***	***66***	***62***	***556 (100)***
Positive	86	81	55	54	51	49	36	18	430 (77.3)
Negative	3	2	1	3	4	16	22	40	91 (16.4)
Contaminated	7	1	4	7	3	1	8	4	35 (6.3)
Time to culture positivity in days, mean (standard deviation)									
No rinse	6.0 (3.2)	9.8 (4.0)	11.2 (7.9)	15.4 (6.9)	15.0 (4.4)	18.4 (6.5)	17.7 (7.7)	26.3 (10.8)	12.7 (7.7)
Water rinse	7.9 (3.5)	9.8 (3.5)	12.1 (4.2)	14.1 (4.4)	16.3 (5.3)	19.9 (8.0)	16.4 (8.2)	18.7 (8.0)	13.4 (6.7)
Chlorhexidine rinse	9.0 (6.1)	11.6 (5.9)	13.8 (4.1)	15.1 (3.0)	20.9 (8.1)	22.7 (9.4)	25.4 (5.7)	23.9 (7.1)	15.9 (8.4)

Visit results are a composite score from three sputa, sputa are a composite score of 2 cultures. *1 incomplete visit with missing spontaneous sample on day 28 and 1 incomplete visit with missing water rinse and chlorhexidine rinse sample on 84.

water rinse. However, chlorhexidine mouth wash had a 3.5 times higher chance of obtaining a negative culture than a water mouth wash. Water wash, in turn, had 2.5 times better odds than home collection of obtaining a positive culture. TTP was lowest in samples collected after water rinse. To analyse the increase of TTP over time we made a linear mixed-effects model of the interaction between collection method and day of collection, adjusting for correlated measures per individual. This showed that compared to water rinse the daily increase in TTP is 0.079 days more in home collected samples (95% CI: 0.020–0.137; p = 0.0096) and 0.091 days more in samples collected following chlorhexidine wash (95% CI: 0.035–0.147; p = 0.0016). This means that the samples collected after water rinse contain significantly more mycobacteria viable in liquid culture than those collected with the other methods.

Finally, we tested if sputum outcomes of contaminated, positive or negative, adjusted for collection method, collection visit and participant ID, were associated with factors listed in Tables 1 and 2. Of gender, age, BMI, smear status (positive or negative), cavitation (≥4 cm or less), diseased area (> 25% or less), sputum quality (mucopurulent or other) and sputum volume (≥5 ml or less than 5 ml), only negative and positive smears were significantly associated with obtaining negative (OR: 7.0; p = 0.0049) and positive sputa (OR: 8.5; p = 0.0002), respectively. Younger age (OR: 1.01 for each year younger, p = 0.0053) was associated with culture contamination.

4. Discussion

This study compared sputum collection at the clinic following water or chlorhexidine rinse, respectively, to spontaneous collection at home without mouth rinse in 18 HIV-positive participants on several occasions during the first 12 weeks of anti-TB treatment. We analysed 556 culture results and found that chlorhexidine significantly lowered the chance of culture contamination compared to both alternative methods but increased the chance of negative cultures compared to water rinse. Water rinse, in turn, increased the chance of obtaining positive cultures compared to no rinse and it significantly decreased TTP, but it did not decrease the chance of culture contamination. In summary, samples collected with water rinse had more viable bacteria compared to chlorhexidine rinse, which caused samples with very low loads to turn negative, as well as to no rinse, which caused samples with very high loads to have contaminated outcomes. To our knowledge this is the first published report simulating clinical trial conditions that includes a large proportion of samples collected under treatment.

The question is thus which method can be recommended for use in a clinical trial? The least promising method is spontaneous sputum collected at home. It is believed that samples collected straight after waking may originate in the lower airways and thus contain more mycobacteria because clearance of deep respiratory secretions is less effective when the patient was asleep. Sputum collected in this manner had the highest volumes in our study, but also the greatest rate of contamination and the least chance to recover positive cultures. Samples collected at home were likely to be kept at room temperature for a longer time compared to samples collected at the clinic that were refrigerated straight away. In order to choose between water and chlorhexidine rinse at the clinic, the observation to be made is that contamination and recovery rates, albeit not always showing statistical significance, seem to move in opposite directions. Water rinse appears to provide the best compromise between obtaining a good rate of positive cultures at a low contamination rate. This is supported by the significantly lower TTP in samples collected after water rinse, which indicates superior quality of these samples for liquid mycobacterial culture. Moreover, simplicity and low cost make water rinse our recommended method of sputum collection in a clinical trial. Although our study did not test this method, water rinse might also be recommended for collection at home.

HIV-positive TB patients were chosen for this study as such individuals are not generally considered good candidates for clinical trials of novel anti-TB medication in which the conversion of positive cultures over time is an important endpoint. HIV-positive TB patients are expected to have low volumes of pauci-bacillary sputum. This study shows that such patients should not be *a priori* excluded from such trials but rather given a chance to participate if they fulfil the other entry criteria.

The fact that a disinfectant used for mouth rinsing can significantly reduce the sputum culture contamination rate supports that contaminants in sputum samples are originating from the oral cavity that can be reached with a mouth wash, and not from the lower airways or from the collection procedure itself. Chlorhexidine has no mycobactericidal activity [7,8] and consequently a significant reduction of positive cultures could not be found. One could argue that the sputum collection after chlorhexidine rinsing may have been disadvantaged as it was always collected after the water rinse and an interval of 30 min between different rinses may not have been long enough to recover the reservoir of mycobacteria for a next sample. This is unlikely, however, as a large series (n = 3,077) of sputum triplicates collected 30 min apart in a clinical trial showed no difference between first, second and third sputa for rates of positive, negative and contaminated cultures [9]. Moreover, any benefit of the water rinse may have been "carried forward" to the time of the chlorhexidine rinse – especially if no food was eaten in the interim. Limitations of this study are the relatively low number of participants and that it did not include tests on solid media, but these are less prone to contamination and would have made it more difficult to show an impact. Also, we did not include a "coached no-rinse sample" collected under supervision at the site as a control. This would have taken the study beyond the capability of most participants to collaborate.

In summary, water wash was not as good as chlorhexidine in reducing contamination but it was the most likely method of obtaining a positive culture and would be our method of choice for sputum collection in a clinical trial if one sample can be collected. If more than one sample is collected, a combination of methods might be considered.

5. Definitions

- *Early morning sputum* – specimen collected without rinsing the mouth with water or any other liquids before sputum production (i.e. no-wash sample)
- *Water rinse at study site* – specimen collected after rinsing the mouth with water
- *Chlorhexidine rinse at study site* – specimen collected after rinsing the mouth with an antiseptic mouthwash

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

ML was responsible for data collection and interpretation, drafting and revision of the manuscript. AD and SF designed the study, collected and interpreted data and revised the manuscript. CK and LvdM assisted with data collection and interpretation, and manuscript revision. NM assisted with manuscript drafting and revision. AV was involved with collection and interpretation of data. LP assisted with the revision of the manuscript. LvdM performed the statistical analyses.

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Appendix A. Supplementary data

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