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To cite this version:

HAL Id: hal-03131547
https://hal.archives-ouvertes.fr/hal-03131547
Submitted on 17 Oct 2022

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Laboratory development of a simple stool sample processing method diagnosis of pediatric tuberculosis using Xpert Ultra

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Abstract

Stool samples are alternatives to respiratory samples for bacteriological confirmation of childhood tuberculosis but require intensive laboratory processing before molecular testing to remove PCR inhibitors and debris. We aimed to develop a centrifuge-free processing method for use in resource-limited settings based on a sucrose-flotation method that showed good sensitivity for childhood tuberculosis diagnosis.

In an in vitro study using Xpert MTB/RIF Ultra on stool samples spiked with defined bacterial concentrations of *Mycobacterium tuberculosis* (*MTB*), we compared different simplification parameters to the reference sucrose-flotation method. Best methods were selected based on the rate of invalid/error results and on sensitivity, compared to the reference method on stools spiked at $10^3$ colony forming units (CFU)/g *MTB*. For final selection, we tested the best parameter combinations at $10^2$ CFU/g. Out of 13 different parameter combinations, three were tested at $10^2$ CFU/g. The best combination used 0.5g stool, manual shaking, no filtration, 30-minutes sedimentation, and a 1:3.6 dilution ratio. This method gave 10% invalid/error results and a sensitivity of 70% vs 63% at $10^3$ CFU/g and 53% vs 58% at $10^2$ CFU/g compared to the reference method.

This pre-clinical study was able to develop a centrifuge-free processing method to facilitate stool Xpert Ultra testing.

Keywords

Diagnosis, Xpert MTB/Rif Ultra, childhood tuberculosis, stool
1. Introduction

Tuberculosis (TB) is the main infectious cause of death worldwide [1]. It is estimated that 20 million children are exposed to TB each year with more than one million becoming sick, and 205,000 deaths were attributed to TB among children in 2018, making TB a global pediatric health emergency[2]. Modelling shows that death occurred almost exclusively in young children who did not receive treatment because they were not diagnosed[3]. This underdiagnosis is likely due to the paucibacillary nature of childhood TB and the difficulty of children to produce sputum, resulting in low diagnostic yield of existing tests [4].

Since 2013, WHO has recommended Xpert MTB/RIF, an automated nucleic acid amplification test that simultaneously detects *Mycobacterium tuberculosis* (MTB) and resistance to rifampicin, as the front-line test for TB diagnosis in children [5]. Despite the WHO recommendation, its uptake has been limited, notably due to the difficulty to collect sputum samples from children [6,7]. Pediatric sample collection methods such as early morning gastric aspirate or induced sputum are indeed operationally challenging, may be poorly accepted and are not available at primary health care centers in high burden and resource limited countries. To overcome the operational challenges of these methods, WHO recently endorsed alternative specimens such as stool samples for diagnosis of pediatric intrathoracic TB [8].

Stool is a non-invasive sample that enables to retrieve MTB present in the child’s respiratory tract system that has been swallowed [9]. Stools do not require sophisticated collection equipment [10]. However, they include PCR inhibitors that can result in invalid Xpert results, and debris that can lead to clotting and errors in the Xpert test run. Therefore, stool specimens need to be processed before Xpert testing [9,11,12].

There is a lack of standardized stool preparation and testing protocols, as highlighted in two recent meta-analysis, in which stool Xpert testing performance varied greatly between studies, depending on the processing method used, with sensitivity varying between 25 and 85% across studies [13,14]. Some methods are based on specimen concentration approaches after dilution in phosphate buffered saline (PBS), keeping the sediment for Xpert testing. Other methods use a flotation approach, keeping the supernatant for Xpert testing [7,15–22]. Sucrose flotation, a method mainly used for parasites’ eggs detection in feces, has been used for stool processing and testing with Xpert MTB/RIF in HIV-infected children [23]. This method consists in the addition of Sheather’s solution (56% sucrose solution) to the stool sample to enable isolation of the bacilli from stool particles by density gradient. It showed good sensitivity (62.1%) and specificity (99.6%) for confirmed TB [23]. The method requires several manipulations such as filtration and centrifugation, and therefore
requires a laboratory environment (at least biosafety level 2) and skilled personnel, which represent a major limitation in decentralizing stool Xpert testing at Primary Health Center (PHC) level [14]. In this in-vitro study, we aimed to simplify the original sucrose flotation stool processing method for Xpert MTB/RIF Ultra testing to make it suitable for use in low resource countries.

2. Material and methods

2.1. Study design

In a laboratory in vitro study, we modified sequentially different steps of the original sucrose flotation method (defined as the reference method for this study) and compared their performance on spiked human stools with pre-determined concentration of mycobacteria. The original method uses the following steps (parameters): i) adding 0.5g of stool in 10 ml Sheather’s solution, ii) vortex shaking, iii) filtration through funnel gauze, iv) centrifugation (100xg for 1 minute), v) mixing 0.5 mL of the top of the supernatant with 1.8 mL Xpert sample reagent (Fig 1). We tested the following parameters modification: i) increasing the stool volume to 1g; ii) using glass wool filtration for retrieving filtration; iii) replacing centrifugation by sedimentation, testing three different durations; and iv) modifying the dilution ratio with the sample reagent (Table 1). In all the evaluations the vortex was replaced by the manual shaking but the effect of the manual shaking was not individually assessed. We evaluated each index method focusing on the proportion of invalid or error results and sensitivity of Ultra to detect MTB on spiked stool samples in three different stages (Fig 2). In Stage 1, each index method with only one modified parameter was compared head-to-head with the reference method using Ultra on stool samples spiked at $10^3$ MTB colony forming units CFU/g. In Stage 2, the modified parameters showing the best results were tested in combination at $10^3$ MTB CFU/g. In Stage 3, the best parameter combinations were tested on stool samples spiked at $10^2$ CFU/g. Concentrations of $10^3$ and $10^2$ CFU/g correspond to smear-negative samples, consistent with the paucibacillary nature of samples from children. We chose those concentrations to ensure the detection using Ultra. Indeed, the level of detection (LOD) in stools using the Xpert MTB/Rif assay is around 6800 CFU/ml as compared to 131 CFU/ml with Xpert MTB/RIF in sputum samples. Since the LOD of Ultra is 10-fold lower (15.6 CFU/mL) than Xpert MTB/RIF in sputum, MTB detection in the range $10^2$-$10^3$ CFU/ml using Ultra should be feasible [11].
2.2. TB strains quantification

MTB strain H37Rv were ordered from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured on Löwenstein-Jensen and Middlebrook media. We used a real time qPCR targeting IS6110 elements based on primers and probe previously described to obtain pre-quantified MTB cell stocks for *in vitro* spiking [24,25]. This technique allows determining the amount of DNA present in a sample with very high precision. We used the quantified genomic DNA of *M. tuberculosis* H37Rv international standard (LCG ATCC) to generate a PCR standard curve using concentrations ranging from 1 to $10^4$ copies/µL. The H37Rv strain was diluted ten-fold and the DNA was extracted using the Genolyse Kit (Genotype – Hain). We performed all PCR reactions in 20 µl reaction volume containing 5 µl of DNA, 4 µL Light Cycler 380 Probe Mastermix (Roche Diagnostic), 1 µl of DMSO, 0.6 µl of each primer at 300 nM and 0.6 µl of dual labelled probe and QSP H2O with the following thermocycling conditions: 95°C for 15 minutes and amplification 95°C for 15 seconds following 60°C for 1 minute during 50 cycles. The qPCR was performed on a Light Cycler 380 (Roche Diagnostic). Determined concentrations were verified by counting colonies on solid media using a serial dilution process. The pre-quantified MTB cell stocks were stored at -20°C in Middlebrook 7H9 media (BD).

2.3. Specimen handling and spiking

We obtained de-identified stool samples from 136 patients aged from 2 to 90 years old without TB diagnosis hospitalized at Montpellier University Teaching Hospital. Stools were stored at 4°C for seven days maximum. Stool samples were divided in portions of 0.5 or 1 g and were spiked by adding 50 µL or 100 µL of a pre-quantified MTB cell stocks to obtain $10^3$ or $10^2$ MTB CFU/g and mixed using a wooden stick (Fig. S1). The pre-quantified MTB cell stocks were systematically vortexed before dilution and before spiking to avoid the clumps. The absence of clumps was also verified during the qPCR when the DNA concentration was controlled. A total of 827 tests were performed on the 136 stools.

2.4. Method selection and performance analysis

In each of the three stages we selected the best index methods (with one single modified parameter or combined modified parameter) using a drop-the-loser rule in two phases (Fig. 2 and S2). In phase 1, we performed 16 tests and selected index methods focusing on the proportion of invalid or error results. Index methods with Ultra results showing less than 20% invalid or errors, were selected for phase 2, while methods with more than 20% of invalid or errors (maximum threshold) were
dropped. In phase 2, we continued testing up to 30 tests and selected index methods that had <20% invalid or errors and an overall sensitivity difference with the reference method ≤10%. The thresholds of 20% of invalid or errors and of a 10% difference of sensitivity were arbitrarily chosen to avoid being too stringent or dropping a potentially good method that might perform better in vivo. This selection approach allowed us to drop ineffective methods early and to minimize the number of tests (Fig. 2 and Fig. S2).

For each method selected, we assessed the proportion of Ultra trace results, the mean cycle thresholds (CTs) and Ultra probes variance between samples: sample processing control (SPC), MTB repeat unit (IS6110-1080), \textit{rpoB} gene probes (\textit{rpoB}1, \textit{rpoB}2, \textit{rpoB}3 and \textit{rpoB}4) compared to the reference method, and the proportion of negative and invalid results for the SPC and each of the \textit{rpoB} probes. Indeed, an increase of mean SPC CT or a negative SPC result could be caused by PCR inhibitors; an increase of mean IS6110-1080 or \textit{rpoB} could be caused by PCR inhibitors or loss of bacteria during the sample processing; and an increase in variance between stool samples may illustrate a higher sensitivity of the method to PCR inhibitors that are present at different levels in different stools. Finally, we also evaluated the effect of the stool sample (inter-sample variability) on the SPC CTs values of the reference method and the optimized selected method.

2.5. PCR inhibition according to stool consistency

To assess the effect of stool consistency on the performance of the methods tested, we categorized the 136 stools in 4 groups: solid, semi solid, sticky and liquid based on a predefined visual consistency scale. We then compared the proportion of MTB detected, non-detected, errors and invalid results between the 4 groups, over the 827 tests performed.

2.6. Statistical analyses

We used an intention to diagnose approach to calculate the overall sensitivity that was defined as the detection of MTB, including traces, among all the spiked samples tested. Therefore, failed test reports (invalid or error) were counted as negative results. Xpert was not repeated on samples with invalid or error result.

Data were graphed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com and statistically analyzed using R (R Core Team, 2016) [26] through the interface RStudio (www.rstudio.com). MacNemar tests were used to compare MTB detection and trace results between the index and reference methods on the same stools. Paired t tests were used to compare mean CTs between the index and reference methods. Tests were two-
tailed and confidence intervals of proportions were 95%. A 0.05 significance level was used for all statistical analyses.

Because the study did not involve human subjects it was not submitted for ethics review.

3. Results

We assessed thirteen index methods on stool samples spiked at $10^3$ CFU/g. Of them, three were further tested with stools spiked at $10^2$ CFU/g.

3.1. Stage 1: Single parameter assessment - $10^3$ CFU/g

Of the nine tested index methods with single parameter modification (A to I in table 1), two were dropped after the first phase (Table 2): method G (ratio 1:1) and method H (ratio 1:2), that had 71.4% and 42.8% errors, respectively. The most common Ultra error was error 2008 due to clogging of the filter by debris in the sample. The other seven parameters were tested up to 30 samples in phase 2. All methods had less than 20% invalid/error results and an overall sensitivity not lower than 10% of the sensitivity of the reference method (Table 2).

3.1.1. Selection of the sedimentation time

The mean CTs of SPC were comparable with the reference method over the three sedimentation times ($p>0.05$) while mean CTs of IS1080-6110 were slightly lower at 1 hour and slightly higher at 30 minutes compared with the reference method (30 min vs reference, $p=0.03$, 1 h vs reference $p=0.08$ and 1.5 h vs reference $p=0.71$). This suggests an increase of PCR inhibitors or loss of bacteria during the sample processing with 30 minutes of sedimentation (Fig 3 and table S1). Consistently, compared to the reference method, the proportion of trace results tend to be higher for the 30-minute sedimentation (81% vs 53%; $p=0.06$), which was not the case for methods with 1 hour (48% vs 62%; $p=0.50$) and 1.5 hour sedimentation times (69% vs 62%; $p=0.34$) (Table 2). However, although the difference was not significant, of the three tested sedimentation times, 30 minutes was the only index method with a gain of sensitivity compared to the reference method. This is explained by the fact that the method using 30 minutes sedimentation time detected 3 positive stool samples that were not detected by the reference method.

In the absence of evidence that shorter sedimentation time would result in lower MTB detection and considering the convenience of using shorter sedimentation time for future implementation, we selected 30 minute sedimentation time for the combination of parameters.
3.2. Stage 2: Parameters combination assessment – $10^3$ CFU/g

Of the five combinations tested, three were succeed: method BD with 0.5g stool, no filtration, 30
minutes sedimentation time; method D combining 0.5g of stool, gauze filtration, and 30 minutes
sedimentation time; and method ABD combining 1g of stool, no filtration and 30 minutes
sedimentation time (Table 3). The proportion of trace results was not significantly different between
the ABD method and the reference method (55.6% vs 66.6%, p=0.55) and between the BD method
and the reference method (76.2% vs 58.2%, p=0.37). It tends to be higher with the D method (80.6%
vs 52.6%, p=0.06) (Table 3). This is likely due to the higher proportion of negative and invalid results
for each of the rpoB probes compared to the reference method (Table S2). Three positive stools
detected as trace with the method BD were not detected with the reference method.

The mean CTs SPC of methods BD (29.5 vs 29.6; p = 0.95), D (28.2 vs 29.1, p = 0.47) and ABD (29.1 vs
29.1, p = 0.99) did not differ with the mean CTs of the reference method. Compared to the reference
method, there was no difference of the mean CTs IS1080-6110 with the BD (25.2 vs 24.3; p value=
0.29) and ABD (23.9 vs 24.9, p = 0.15) index methods (Fig 4 and Table S2).

3.3. Stage 3: Parameters combination – $10^2$ CFU/g

Of the three combinations tested using stools spiked at $10^2$ CFU/g, only the index method BD had a
sensitivity not lower than 10% compared with the sensitivity of the reference method (53.3% vs
57.6%) (Table 3). Also, the proportion of trace results (81.3% vs 88.8%; p=0.94) and the mean CTs
SPC (28.6 vs 29.1; p=0.95) were comparable with results of the reference method. However, the
mean CTs IS1080-6110 were slightly lower (25.9 vs 27.5; p=0.045) (Fig 4 and Table S2). The similar
broad range of SPC CTs (25.4 to 39) between the index method BD on stools spiked at $10^3$ and $10^2$
CFU/g of MTB and the reference method (26.1 to 37.1) suggests that the index method BD is not
more susceptible to stool variation than the reference method (Fig S3).

We therefore selected the method BD, which removes the filtration and the centrifugation steps,
as the final optimized method (Fig 1).

3.4. Effect of the stool consistency on the PCR inhibition

Of 827 tests done in stage 1, 2 and 3, the majority were performed on liquid (32.6%) or sticky stools
(49.1%). There was no significant effect of sample consistency on the sensitivity of the Ultra assay.
However, we observed a higher proportion of invalid results with semi solid stools (18%) compared
with liquid (4%), sticky (6%) or solid (4%) stools (Table S3 and Fig S4). The proportion of error results
was similar between the group of solid and semi-solid stool samples (3.4%, 5/154) and the group of liquid and sticky stool samples (3.4%, 23/676). The most common error (code 2008 due to clogging of the cartridge filter) was the same between the two groups.

4. Discussion

Our study identified an optimized centrifuge-free stool processing method with a performance comparable to the original sucrose flotation method (Fig 5). Removing the filtration step led to more MTB detected in the sample while decreasing sample dilution with the sample reagent (SR) (1:3.5 vs 1:2 and 1:1) resulted in a significant increase of errors and invalid results. Using 1g of stool increased the sensitivity compared to 0.5 g, but when associated with sedimentation and the removal of filtration it led to a loss in sensitivity at both \(10^3\) and \(10^2\) CFU/g. This is likely to be due to the increase of PCR inhibitors with higher quantity of stool. Finally, replacing the centrifugation at 100 g for 1 minute by 30 minutes of sedimentation did not decrease the sensitivity, even after removing the filtration and when tested at \(10^2\) CFU/g.

Although the optimized method showed comparable performance than the reference method, it had a higher rate of trace call (76.2% vs 58.2%) when assessed on stools spiked at \(10^3\) CFU/g of MTB. In our study setting using spiked samples with MTB, 3 trace results out of a total of 16 trace results were only detected by the optimized method suggesting that the higher proportion of trace might be due to a better sensitivity of the method. This will need to be verified under clinical conditions.

The variations of CTs SPC across stools was comparable between the reference and the optimized methods suggesting that the sensitivity of the optimized method was equally affected by the difference in stool composition than the reference method. This result is consistent with the results from other studies showing that stool samples have higher heterogeneity in sample composition compared to other samples, which can result in variation of sensitivity between stools [11,27].

In stools spiked at \(10^2\) CFU/g both methods showed a very high proportion of trace calls (81.3% vs 88.8%). Trace call is a new result category linked to the addition of two different multi-copy amplification targets (IS6110 and IS1081) in the Xpert Ultra assay to increase its sensitivity but that doesn’t amplify the \(rpoB\) probes. Trace calls can be true positive results from the detection of very low bacterial concentrations but they can be also false positive results from the detection of persisting DNA from dead bacteria in samples from previously treated patients [28]. Therefore, they should be interpreted with caution taking into consideration patients’ past medical history[29,30].

Among MTB positive results from respiratory samples, recent studies report a proportion of trace
calls ranging between 15 and 44% [31–33]. However our *in vitro* study used spiked samples and the presence of NDA in the sample was controlled. Therefore, trace calls were true positive results. To explain the higher proportion of trace calls observed in stool Ultra positive samples in our study, we could hypothesize that the stool processing reduces the concentration of DNA but we could also question if the *rpoB* probes are not more vulnerable to PCR inhibitors that are common in stool samples. Since we evaluated this proportion only on stools spiked with low quantity of MTB, further evaluation with broader MTB load range is needed to confirm this trend. Although there is a risk of false positive results with trace calls, in particular among recently previously treated patients, this is unlikely to affect the diagnosis of TB in children who are in majority new cases. As recommended by WHO, a trace-positive result is sufficient to initiate anti-tuberculosis therapy in children[34]. Finally, the study results may suggest that there is no significant effect of the stool consistency on the sensitivity and proportion of invalid results. This is in line with the study recently published by Walters et al showing no association between MTB detection and stool consistency. However the authors noted that none of the liquid stools generated a positive Xpert result. This can be explained by the large proportion of stools collected in diapers, a higher proportion of inhibitors in diarrheal stools and a higher dilution of low concentration of MTB DNA[16]. In our study, we did not observe higher PCR inhibition in liquid stools but further evaluation using stool from children with presumptive TB would be needed. Indeed, the sample of stools tested in our *in vitro* study were not representative of stools samples from children living in limited resource countries.

This *in vitro* study has several limitations. First, the fact that we added artificially mycobacteria to the stools could make the detection easier as compared to stool samples from TB patients where the tubercle bacilli binds to debris or cells in stool samples during the digestive process. This might result in an artificial increase of the sensitivity and affect the reproducibility of the *in vitro* study results in clinical conditions as previously reported by Beutler et al [27]. To overcome this bias the criteria for the selection of the methods in our study were first based on the proportions of invalid results or errors that are not impacted by the spiking approach itself but more by the physical components of stool specimens. Also, the second selection criterion was the difference in sensitivity as compared to the reference method, which is also less expected to be affected by this bias, rather than the sensitivity itself. Second, this study used small sample sizes leading to wide confidence intervals of the sensitivity. Third, although the study assessed the potential effect of stool consistency on the performance assessment, stools were primarily obtained from adults living in France and results might be different when using stools from children living in resource limited
countries where the prevalence of diarrheal disease is higher and for which the diet might be different.

Stool has recently been recommended by WHO as a sample to be used for molecular TB diagnosis in children but there is still no recommendation on how samples should be processed before Xpert testing [8]. It is therefore urgent to identify a stool processing method that would both optimize the performance of stool Xpert Ultra testing and be feasible in resource-limited settings at lower level of health care facility, in order to improve access to molecular testing. Despite its limitation, our in vitro study provided a head-to-head comparison of the performances of different alternative optimized methods with the original sucrose-flotation method. This study using standardized evaluation plan of analytic performances provided a pre-clinical validation of a simple, affordable and centrifuge-free stool processing method with good performance. However, this method need to be evaluated among children with presumptive TB.

Other power-free stool processing methods are under development. A proof-of-concept study assessing a method based on simple a sample dilution in PBS and then in the Xpert sample reagent (two Steps method) showed 89% concordance of Xpert MTB/RIF results between stools and respiratory samples in children with presumptive TB (three positive results in respiratory samples were also positive in stool and out of 26 negative results in respiratory samples, 5 were positive in stools) [20]. An even simpler method without the PBS dilution step (Simple One-step Stool method) is under evaluation. Another processing method based on the use of a stool processing buffer and a specific filter configuration showed good results with Xpert MTB/RIF in a proof-of-concept study among children with presumptive TB (17/20, 85% sensitivity and 20/20, 100% specificity compared to Xpert MTB/RF in respiratory samples) but had a lower sensitivity (4/16, 25%) when compared to culture on one respiratory sample in a larger study of children with presumptive TB [9,16]. This highlights challenges in identifying better stool processing methods and the need for more sensitive rapid molecular assays to improve the utility of stool for the diagnosis of intrathoracic TB in children from resource-limited settings[23].

An important step toward a possible implementation of stool testing with Ultra at low level of health care facility requires standardization of testing procedures. Further head to head comparison of the performances of these different methods in one study to overcome the bias due to the use of different study populations and methodology across studies evaluating stool processing methods is needed [13,14]. This evaluation needs also to incorporate the assessment of the feasibility and acceptability pf the methods by health care workers from low level of health care facilities.
Funding
This work was part of the TB-Speed project funded by Unitaid. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution
ML and AD conducted the experiments and collected data. ML designed the work, performed the data analysis and wrote the manuscript. MN, SE, EW, OM and SG reviewed the study design, the data and the manuscript. MB supervised the work.

Declaration of competing interest
The authors declare no financial conflicts of interest.

Acknowledgement:
The authors would like to acknowledge the members of the Scientific Advisory Board who gave oversight on the design of the study and reviewed study progress: Luis Cuevas (Liverpool School of Tropical Medicine, UK), Christophe Delacourt (Hôpital Necker-Enfants Malades, France), Stephen Graham (University of Melbourne, Melbourne, Australia), Malgorzata Grzemska (WHO, Switzerland), Anneke Hesseling (Stellenbosch University, Cape Town, South Africa), Elizabeth Maleche Obimbo (University of Nairobi, Kenya), Abdulai Abubakarr Sesay (CISMAT-SL, Sierra Leone), as well as Chishala Chabala (University of Zambia) who represented other TB-Speed investigators at Scientific Advisory Board meetings.
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https://doi.org/10.1128/JCM.01012-12.


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Tables and Figures

Figure 1. Procedure step by step of the sucrose flotation method

A/ Original (reference) method

1. Add 0.5 g of stool in 10 ml of 50% Seather’s solution + vortex shaking
2. Filtration through a gauze into a new 15 ml Falcon tube
3. Centrifugation at 100xg for 1 minute
4. Transfer 0.5 ml from the top of the specimen to a 15 ml Falcon tube and add 1.8 ml of Sample Reagent + vortex shaking
5. Incubation and transfer to the Xpert Ultra cartridge

B/ Optimised method

1. Add 0.5 g of stool in 10 ml of 50% Seather’s solution + shake 20 times
2. Sedimentation during 30 minutes
3. Transfer 0.5 ml from the top of the specimen to a 15 ml Falcon tube and add 1.8 ml of Sample Reagent + shake vigorously 20 times
4. Incubation and transfer to the Xpert Ultra cartridge

Figure 2. Method selection study design

Stage 1: single parameter assessment at $10^3$ CFU/g

Errors or invalid ≤ 20% and/or Δ Se ref-index ≤ 10%

Stage 2: combined parameters assessment at $10^3$ CFU/g

Errors or invalid ≤ 20% and/or Δ Se ref-index ≤ 10%

Stage 3: combined parameters assessment at $10^3$ CFU/g

Errors or invalid ≤ 20% and/or Δ Se ref-index ≤ 10%

Final selected method

Each stage includes two phases based on a drop-the-loser rule. N correspond to the number of tests,

16 tests are performed on 8 stools and 30 tests are performed on 15 stools.
### Table 1. Parameters tested for the optimization of the reference method

<table>
<thead>
<tr>
<th>Reference method step by step</th>
<th>Alternative parameters assessed</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5g of stool</td>
<td>A- 1g of stool</td>
<td>If we add more stool we add more MTB but more PCR inhibitors</td>
</tr>
<tr>
<td>Gauze filtration</td>
<td>B- No filtration</td>
<td>The sedimentation might be sufficient to let the large particles settle with no need of filtration</td>
</tr>
<tr>
<td></td>
<td>C- Glass wool filtration</td>
<td>According Banada et al. (2016) the glass wool can be used to filter stool debris more efficiently than the other types of filter materials tested (including cotton, Whatman filters, filter pads, gauze, and glass filter pads)</td>
</tr>
<tr>
<td>Centrifugation at 100g for 1 minute</td>
<td>D- Sedimentation for 30 minutes</td>
<td>We selected three different times of sedimentation, 30’, 1h and 1h30 based on feasibility and preliminary tests performed in our lab</td>
</tr>
<tr>
<td></td>
<td>E- Sedimentation for 1 hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F- Sedimentation for 1.5 hours</td>
<td></td>
</tr>
<tr>
<td>Mix 0.5 mL of stool mix with 1.8 mL of sample reagent (ratio 1:3.6; sample: SR)</td>
<td>G- Ratio 1:2; 1 mL of sample mixed with 2 mL of SR</td>
<td>With sputum, the recommended sample reagent/sample pellet ratio by the Xpert assay package is 1:2 or 1:3. We will test if decreasing the amount of added sample reagent could improve the sensitivity without adding invalid results</td>
</tr>
<tr>
<td></td>
<td>H- Ratio 1:1; 1 mL of sample mixed with 1 mL of SR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I- Ratio 1:2b: 0.8 mL of sample mixed with 1.6 mL</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Results of method assessment according selection criteria on the 9 alternative parameters tested at $10^3 \text{ CFU/g}$

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of tests</th>
<th>Proportion of Invalid</th>
<th>Proportion of Error</th>
<th>Overall Sensitivity (CI95)</th>
<th>Proportion of Trace among MTB detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A - 1g of stool</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>13.3%</td>
<td>0.0%</td>
<td>82.7% (63.5-93.4)</td>
<td>58%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>13.3%</td>
<td>0.0%</td>
<td>63.3% (43.9-79.4)</td>
<td>58%</td>
</tr>
<tr>
<td><strong>B - No Filtration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>15.6%</td>
<td>0.0%</td>
<td>78.1% (59.5-90.0)</td>
<td>56%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>12.5%</td>
<td>0.0%</td>
<td>87.5% (70.96)</td>
<td>61%</td>
</tr>
<tr>
<td><strong>C - Glass wool filtration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>0.0%</td>
<td>0.0%</td>
<td>91.6% (71.5-98.5)</td>
<td>56%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>83.8% (61.8 - 94.5)</td>
<td>64%</td>
</tr>
<tr>
<td><strong>D - Sed 30'</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>4.5%</td>
<td>0.0%</td>
<td>79.5% (57.3-89.3)</td>
<td>81%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>9%</td>
<td>3.3%</td>
<td>73% (43.9 - 79.4)</td>
<td>53%</td>
</tr>
<tr>
<td><strong>E - Sed 1h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>0.0%</td>
<td>0.0%</td>
<td>76.7% (48.8-86.5)</td>
<td>48%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>86.7% (61.8 - 94.5)</td>
<td>62%</td>
</tr>
<tr>
<td><strong>F - Sed 1h30</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>0.0%</td>
<td>0.0%</td>
<td>86.7% (61.8.4-94.5)</td>
<td>69%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>86.7% (61.8.4-94.5)</td>
<td>62%</td>
</tr>
<tr>
<td><strong>G - Ratio 1:2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>16</td>
<td>0.0%</td>
<td>42.8%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>H - Ratio 1:1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>16</td>
<td>0.0%</td>
<td>71.4%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>I - Ratio 1:2b</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index method</td>
<td>30</td>
<td>0.0%</td>
<td>0.0%</td>
<td>93.3% (72.4-98.6)</td>
<td>68%</td>
</tr>
<tr>
<td>Reference method</td>
<td></td>
<td>7.7%</td>
<td>3.9%</td>
<td>86.7% (68.4 - 95.6)</td>
<td>58%</td>
</tr>
</tbody>
</table>

*ND = No data

Sed: sedimentation; MTB: Mycobacterium tuberculosis; IC: confidence intervals

Methods that had more than 20% of error or invalid were dropped after 16 tests and the overall sensitivity and proportion of trace was not assessed.
Table 3. Results of method assessment according selection criteria on combined parameters tested at $10^3$ and $10^2$ CFU/g

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of tests</th>
<th>Proportion of invalid</th>
<th>Proportion of error</th>
<th>Overall Sensitivity (IC95)</th>
<th>Proportion of Trace among MTB detected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$10^3$ CFU/g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD - No filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>10.0%</td>
<td>0.0%</td>
<td>70% (50.4-84.5)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>13.3%</td>
<td>3.3%</td>
<td>63.3% (43.9-79.4)</td>
</tr>
<tr>
<td>D - Gauze filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>3.3%</td>
<td>0.0%</td>
<td>80% (59.5-90.0)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>6.9%</td>
<td>3.3%</td>
<td>72.7.6% (57-85)</td>
</tr>
<tr>
<td>CD - Glass wool filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>0.0%</td>
<td>0.0%</td>
<td>80% (59.5-90.0)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>3.3%</td>
<td>0.0%</td>
<td>96.8% (87.8 - 100.0)</td>
</tr>
<tr>
<td>ABD - 1g + No filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>5.0%</td>
<td>0.0%</td>
<td>90% (72-99)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>100% (79 - 100)</td>
</tr>
<tr>
<td>ADI - 1g + Sed 30' + Ratio 1:2b</td>
<td>Index method</td>
<td>16</td>
<td>0.0%</td>
<td>37.5%</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>0.0%</td>
<td>ND</td>
</tr>
<tr>
<td><strong>$10^2$ CFU/g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD - No filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>10.7%</td>
<td>0.0%</td>
<td>53.3% (38.8-78.1)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>7.1%</td>
<td>0.0%</td>
<td>57.6% (37.4-75.1)</td>
</tr>
<tr>
<td>D - Gauze filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>10.0%</td>
<td>0.0%</td>
<td>28.6% (13.9-48.8)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>7.1 %</td>
<td>0.0%</td>
<td>57.6% (37.4 - 75.1)</td>
</tr>
<tr>
<td>ABD - 1g + No filtration + Sed 30' + ratio 1:3.6</td>
<td>Index method</td>
<td>30</td>
<td>11.1%</td>
<td>0.0%</td>
<td>11.1% (1.9-36.1)</td>
</tr>
<tr>
<td></td>
<td>Reference method</td>
<td></td>
<td>0.0%</td>
<td>5.6%</td>
<td>22% (7.30 - 48.0)</td>
</tr>
</tbody>
</table>

Sed: sedimentation; MTB: Mycobacterium tuberculosis; IC: confidence intervals
Figure 3. SPC CTs (A) and IS1080-6110 CTs (B) over selected parameters tested at $10^3$ CFU/g

A. SPC CTs

B. IS1080-6110 CTs

The boxplots show the median Ct values and the interquartile ranges. The whiskers go down to the smallest value and up to the largest. The mean is represented as a ‘+’. The asterisks indicate statistically significant differences ($p < 0.05$).
Figure 4. SPC CTs (A) and IS1080-6110 CTs (B) over selected parameters tested at $10^3$ and $10^2$ CFU/g.

A. SPC CTs

B. IS1080-6110 CTs

The boxplots show the median Ct values and the interquartile ranges. The whiskers go down to the smallest value and up to the largest. The mean is represented as a ‘+’. The asterisks indicate statistically significant differences ($p < 0.05$).