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► To cite this version:

Manon Lounnas, Abibatou Diack, Mark P. Nicol, Sara Eyangoh, Eric Wobudeya, et al.. Laboratory development of a simple stool sample processing method diagnosis of pediatric tuberculosis using Xpert Ultra. *Tuberculosis*, inPress, 125, pp.102002. 10.1016/j.tube.2020.102002. hal-03131547

HAL Id: hal-03131547

<https://hal.science/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

Manon Lounnas^{1,2} Abibatou Diack^{1,2}, Mark P Nicol^{3,4}, Sara Eyangoh⁵, Eric Wobudeya⁶, Olivier Marcy⁷, Sylvain Godreuil^{1,2} and Maryline Bonnet^{8#}

¹ UMR MIVEGEC IRD-CNRS-Université de Montpellier, IRD, Montpellier, France

² Département de Bactériologie, Centre Hospitalier Universitaire de Montpellier, Université de Montpellier, Montpellier, France

³ Division of Medical Microbiology and Institute for Infectious Diseases and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

⁴ School of Biomedical Sciences, University of Western Australia, Perth, Australia

⁵ Service de Mycobactériologie, Centre Pasteur du Cameroun, Réseau International des Instituts Pasteur, Yaounde, Cameroon

⁶ Mulago National Referral Hospital, Directorate of Paediatrics & Child health, Kampala, Uganda

⁷ University of Bordeaux, Inserm, French National Research Institute for Sustainable Development (IRD), UMR 1219, Bordeaux, France

⁸ IRD UMI 233 TransVIHMI- UM-INSERM U1175, Montpellier, France

#Corresponding author:

Maryline Bonnet: TransVIHMI, UMR UM-INSERM-IRD, 911 Avenue Agropolis, BP64501, 34394 Montpellier Cedex05, France. Tel: +3345067416491. Email: maryline.bonnet@ird.fr

1 **Abstract**

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

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

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

19 **Keywords**

20 Diagnosis, Xpert MTB/Rif Ultra, childhood tuberculosis, stool

21 **1. Introduction**

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

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

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

43 There is a lack of standardized stool preparation and testing protocols, as highlighted in two recent
44 meta-analysis, in which stool Xpert testing performance varied greatly between studies, depending
45 on the processing method used, with sensitivity varying between 25 and 85% across studies [13,14].
46 Some methods are based on specimen concentration approaches after dilution in phosphate
47 buffered saline (PBS), keeping the sediment for Xpert testing. Other methods use a flotation
48 approach, keeping the supernatant for Xpert testing [7,15–22]. Sucrose flotation, a method mainly
49 used for parasites' eggs detection in feces, has been used for stool processing and testing with Xpert
50 MTB/RIF in HIV-infected children [23]. This method consists in the addition of Sheather's solution
51 (56% sucrose solution) to the stool sample to enable isolation of the bacilli from stool particles by
52 density gradient. It showed good sensitivity (62.1%) and specificity (99.6%) for confirmed TB [23].
53 The method requires several manipulations such as filtration and centrifugation, and therefore

54 requires a laboratory environment (at least biosafety level 2) and skilled personnel, which represent
55 a major limitation in decentralizing stool Xpert testing at Primary Health Center (PHC) level [14].
56 In this *in-vitro* study, we aimed to simplify the original sucrose flotation stool processing method for
57 Xpert MTB/RIF Ultra testing to make it suitable for use in low resource countries.

58

59 **2. Material and methods**

60 2.1. Study design

61 In a laboratory *in vitro* study, we modified sequentially different steps of the original sucrose
62 flotation method (defined as the reference method for this study) and compared their performance
63 on spiked human stools with pre-determined concentration of mycobacteria. The original method
64 uses the following steps (parameters): i) adding 0.5g of stool in 10 ml Sheather's solution, ii) vortex
65 shaking, iii) filtration through funnel gauze, iv) centrifugation (100xg for 1 minute), v) mixing 0.5 mL
66 of the top of the supernatant with 1.8 mL Xpert sample reagent (Fig 1). We tested the following
67 parameters modification: i) increasing the stool volume to 1g; ii) using glass wool filtration for
68 retrieving filtration; iii) replacing centrifugation by sedimentation, testing three different durations;
69 and iv) modifying the dilution ratio with the sample reagent (Table 1). In all the evaluations the
70 vortex was replaced by the manual shaking but the effect of the manual shaking was not individually
71 assessed. We evaluated each index method focusing on the proportion of invalid or error results
72 and sensitivity of Ultra to detect MTB on spiked stool samples in three different stages (Fig 2). In
73 Stage 1, each index method with only one modified parameter was compared head-to-head with
74 the reference method using Ultra on stool samples spiked at 10^3 MTB colony forming units CFU/g.
75 In Stage 2, the modified parameters showing the best results were tested in combination at 10^3
76 MTB CFU/g. In Stage 3, the best parameter combinations were tested on stool samples spiked at
77 10^2 CFU/g. Concentrations of 10^3 and 10^2 CFU/g correspond to smear-negative samples, consistent
78 with the paucibacillary nature of samples from children. We chose those concentrations to ensure
79 the detection using Ultra. Indeed, the level of detection (LOD) in stools using the Xpert MTB/Rif
80 assay is around 6800 CFU/ml as compared to 131 CFU/ml with Xpert MTB/RIF in sputum samples.
81 Since the LOD of Ultra is 10-fold lower (15.6 CFU/mL) than Xpert MTB/RIF in sputum, MTB detection
82 in the range 10^2 - 10^3 CFU/ml using Ultra should be feasible [11].

83

84 2.2. TB strains quantification

85 MTB strain H37Rv were ordered from the American Type Culture Collection (ATCC) (Manassas, VA)
86 and cultured on Löwenstein-Jensen and Middlebrook media. We used a real time qPCR targeting
87 IS6110 elements based on primers and probe previously described to obtain pre-quantified MTB
88 cell stocks for *in vitro* spiking [24,25]. This technique allows determining the amount of DNA present
89 in a sample with very high precision. We used the quantified genomic DNA of *M. tuberculosis* H37Rv
90 international standard (LCG ATCC) to generate a PCR standard curve using concentrations ranging
91 from 1 to 10⁴ copies/μL. The H37Rv strain was diluted ten-fold and the DNA was extracted using the
92 Genolyse Kit (Genotype – Hain). We performed all PCR reactions in 20 μL reaction volume containing
93 5 μL of DNA, 4 μL Light Cycler 380 Probe Mastermix (Roche Diagnostic), 1 μL of DMSO, 0.6 μL of each
94 primer at 300 nM and 0.6 μL of dual labelled probe and QSP H2O with the following thermocycling
95 conditions: 95°C for 15 minutes and amplification 95°C for 15 seconds following 60°C for 1 minute
96 during 50 cycles. The qPCR was performed on a Light Cycler 380 (Roche Diagnostic). Determined
97 concentrations were verified by counting colonies on solid media using a serial dilution process.
98 The pre-quantified MTB cell stocks were stored at -20°C in Middlebrook 7H9 media (BD).

99

100 2.3. Specimen handling and spiking

101 We obtained de-identified stool samples from 136 patients aged from 2 to 90 years old without TB
102 diagnosis hospitalized at Montpellier University Teaching Hospital. Stools were stored at 4°C for
103 seven days maximum. Stool samples were divided in portions of 0.5 or 1 g and were spiked by adding
104 50 μL or 100 μL of a pre-quantified MTB cell stocks to obtain 10³ or 10² MTB CFU/g and mixed using
105 a wooden stick (Fig. S1). The pre-quantified MTB cell stocks were systematically vortexed before
106 dilution and before spiking to avoid the clumps. The absence of clumps was also verified during the
107 qPCR when the DNA concentration was controlled. A total of 827 tests were performed on the 136
108 stools.

109

110 2.4. Method selection and performance analysis

111 In each of the three stages we selected the best index methods (with one single modified parameter
112 or combined modified parameter) using a drop-the-loser rule in two phases (Fig. 2 and S2). In phase
113 1, we performed 16 tests and selected index methods focusing on the proportion of invalid or error
114 results. Index methods with Ultra results showing less than 20% invalid or errors, were selected for
115 phase 2, while methods with more than 20% of invalid or errors (maximum threshold) were

116 dropped. In phase 2, we continued testing up to 30 tests and selected index methods that had <20%
117 invalid or errors and an overall sensitivity difference with the reference method $\leq 10\%$. The
118 thresholds of 20% of invalid or errors and of a 10% difference of sensitivity were arbitrarily chosen
119 to avoid being too stringent or dropping a potentially good method that might perform better *in*
120 *vivo*. This selection approach allowed us to drop ineffective methods early and to minimize the
121 number of tests (Fig. 2 and Fig. S2).

122 For each method selected, we assessed the proportion of Ultra trace results, the mean cycle
123 thresholds (CTs) and Ultra probes variance between samples: sample processing control (SPC), MTB
124 repeat unit (IS6110-1080), *rpoB* gene probes (*rpoB1*, *rpoB2*, *rpoB3* and *rpoB4*) compared to the
125 reference method, and the proportion of negative and invalid results for the SPC and each of the
126 *rpoB* probes. Indeed, an increase of mean SPC CT or a negative SPC result could be caused by PCR
127 inhibitors; an increase of mean IS6110-1080 or *rpoB* could be caused by PCR inhibitors or loss of
128 bacteria during the sample processing; and an increase in variance between stool samples may
129 illustrate a higher sensitivity of the method to PCR inhibitors that are present at different levels in
130 different stools. Finally, we also evaluated the effect of the stool sample (inter-sample variability)
131 on the SPC CTs values of the reference method and the optimized selected method.

132

133 2.5. PCR inhibition according to stool consistency

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

138

139 2.6. Statistical analyses

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

144 Data were graphed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San
145 Diego, California USA, www.graphpad.com and statistically analyzed using R (R Core Team, 2016)
146 [26] through the interface RStudio (www.rstudio.com). MacNemar tests were used to compare MTB
147 detection and trace results between the index and reference methods on the same stools. Paired t
148 tests were used to compare mean CTs between the index and reference methods. Tests were two-

149 tailed and confidence intervals of proportions were 95%. A 0.05 significance level was used for all
150 statistical analyses

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

152

153 **3. Results**

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

156 3.1. Stage 1: Single parameter assessment - 10^3 CFU/g

157 Of the nine tested index methods with single parameter modification (A to I in table 1), two were
158 dropped after the first phase (Table 2): method G (ratio 1:1) and method H (ratio 1:2), that had
159 71.4% and 42.8% errors, respectively. The most common Ultra error was error 2008 due to clogging
160 of the filter by debris in the sample. The other seven parameters were tested up to 30 samples in
161 phase 2. All methods had less than 20% invalid/error results and an overall sensitivity not lower than
162 10% of the sensitivity of the reference method (Table 2).

163

164 3.1.1. Selection of the sedimentation time

165 The mean CTs of SPC were comparable with the reference method over the three sedimentation
166 times ($p>0.05$) while mean CTs of IS1080-6110 were slightly lower at 1 hour and slightly higher at
167 30 minutes compared with the reference method (30 min vs reference, $p=0.03$, 1 h vs reference
168 $p=0.08$ and 1.5 h vs reference $p=0.71$). This suggests an increase of PCR inhibitors or loss of bacteria
169 during the sample processing with 30 minutes of sedimentation (Fig 3 and table S1). Consistently,
170 compared to the reference method, the proportion of trace results tend to be higher for the 30-
171 minute sedimentation (81% vs 53%; $p=0.06$), which was not the case for methods with 1 hour (48%
172 vs 62%; $p=0.50$) and 1.5 hour sedimentation times (69% vs 62%; $p=0.34$) (Table 2). However,
173 although the difference was not significant, of the three tested sedimentation times, 30 minutes
174 was the only index method with a gain of sensitivity compared to the reference method. This is
175 explained by the fact that the method using 30 minutes sedimentation time detected 3 positive
176 stool samples that were not detected by the reference method.

177 In the absence of evidence that shorter sedimentation time would result in lower MTB detection
178 and considering the convenience of using shorter sedimentation time for future implementation,
179 we selected 30 minute sedimentation time for the combination of parameters.

180

181 3.2. Stage 2: Parameters combination assessment – 10³ CFU/g

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

191 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
192 29.1, p= 0.99) did not differ with the mean CTs of the reference method. Compared to the reference
193 method, there was no difference of the mean CTs IS1080-6110 with the BD (25.2 vs 24.3; p value=
194 0.29) and ABD (23.9 vs 24.9, p= 0.15) index methods (Fig 4 and Table S2).

195

196 3.3. Stage 3: Parameters combination – 10² CFU/g

197 Of the three combinations tested using stools spiked at 10² CFU/g, only the index method BD had a
198 sensitivity not lower than 10% compared with the sensitivity of the reference method (53.3% vs
199 57.6%) (Table 3). Also, the proportion of trace results (81.3% vs 88.8%; p=0.94) and the mean CTs
200 SPC (28.6 vs 29.1; p=0.95) were comparable with results of the reference method. However, the
201 mean CTs IS1080-6110 were slightly lower (25.9 vs 27.5; p=0.045) (Fig 4 and Table S2). The similar
202 broad range of SPC CTs (25.4 to 39) between the index method BD on stools spiked at 10³ and 10²
203 CFU/g of MTB and the reference method (26.1 to 37.1) suggests that the index method BD is not
204 more susceptible to stool variation than the reference method (Fig S3).

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

207

208 3.4. Effect of the stool consistency on the PCR inhibition

209 Of 827 tests done in stage 1, 2 and 3, the majority were performed on liquid (32.6%) or sticky stools
210 (49.1%). There was no significant effect of sample consistency on the sensitivity of the Ultra assay.
211 However, we observed a higher proportion of invalid results with semi solid stools (18%) compared
212 with liquid (4%), sticky (6%) or solid (4%) stools (Table S3 and Fig S4). The proportion of error results

213 was similar between the group of solid and semi-solid stool samples (3.4%, 5/154) and the group of
214 liquid and sticky stool samples (3.4%, 23/676). The most common error (code 2008 due to clogging
215 of the cartridge filter) was the same between the two groups.

216

217 **4. Discussion**

218 Our study identified an optimized centrifuge-free stool processing method with a performance
219 comparable to the original sucrose flotation method (Fig 5). Removing the filtration step led to more
220 MTB detected in the sample while decreasing sample dilution with the sample reagent (SR) (1:3.5
221 vs 1:2 and 1:1) resulted in a significant increase of errors and invalid results. Using 1g of stool
222 increased the sensitivity compared to 0.5 g, but when associated with sedimentation and the
223 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
224 to the increase of PCR inhibitors with higher quantity of stool. Finally, replacing the centrifugation
225 at 100 g for 1 minute by 30 minutes of sedimentation did not decrease the sensitivity, even after
226 removing the filtration and when tested at 10^2 CFU/g.

227 Although the optimized method showed comparable performance than the reference method, it
228 had a higher rate of trace call (76.2% vs 58.2%) when assessed on stools spiked at 10^3 CFU/g of MTB.
229 In our study setting using spiked samples with MTB, 3 trace results out of a total of 16 trace results
230 were only detected by the optimized method suggesting that the higher proportion of trace might
231 be due to a better sensitivity of the method. This will need to be verified under clinical conditions.
232 The variations of CTs SPC across stools was comparable between the reference and the optimized
233 methods suggesting that the sensitivity of the optimized method was equally affected by the
234 difference in stool composition than the reference method. This result is consistent with the results
235 from other studies showing that stool samples have higher heterogeneity in sample composition
236 compared to other samples, which can result in variation of sensitivity between stools [11,27].

237 In stools spiked at 10^2 CFU/g both methods showed a very high proportion of trace calls (81.3% vs
238 88.8%). Trace call is a new result category linked to the addition of two different multi-copy
239 amplification targets (IS6110 and IS1081) in the Xpert Ultra assay to increase its sensitivity but that
240 doesn't amplify the *rpoB* probes. Trace calls can be true positive results from the detection of very
241 low bacterial concentrations but they can be also false positive results from the detection of
242 persisting DNA from dead bacteria in samples from previously treated patients [28]. Therefore, they
243 should be interpreted with caution taking into consideration patients' past medical history[29,30].
244 Among MTB positive results from respiratory samples, recent studies report a proportion of trace

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

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

277 countries where the prevalence of diarrheal disease is higher and for which the diet might be
278 different.

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

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

303 An important step toward a possible implementation of stool testing with Ultra at low level of
304 health care facility requires standardization of testing procedures. Further head to head
305 comparison of the performances of these different methods in one study to overcome the bias
306 due to the use of different study populations and methodology across studies evaluating stool
307 processing methods is needed [13,14]. This evaluation needs also to incorporate the assessment
308 of the feasibility and acceptability pf the methods by health care workers from low level of health
309 care facilities.

310

311 **Funding**

312 This work was part of the TB-Speed project funded by Unitaid. The funders had no role in study
313 design, data collection and interpretation, or the decision to submit the work for publication.

314

315 **Availability of data and materials**

316 The datasets used and analyzed during the current study are available from the corresponding
317 author on reasonable request.

318

319 **Authors' contribution**

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

323

324 **Declaration of competing interest**

325 The authors declare no financial conflicts of interest.

326

327 **Acknowledgement:**

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

336

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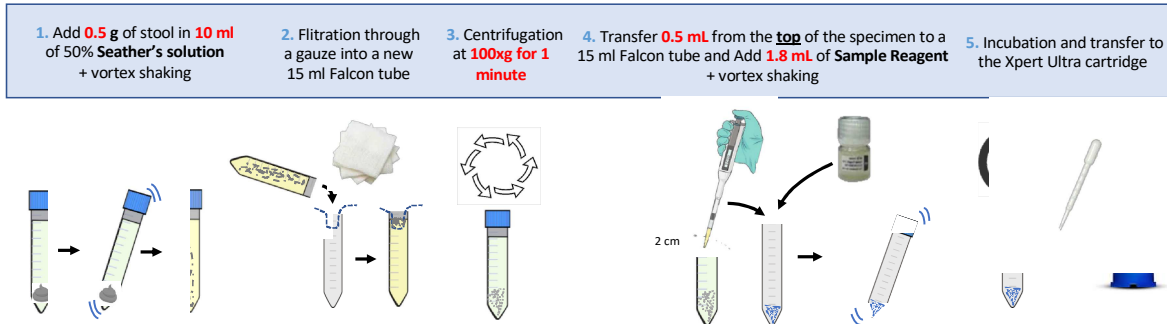
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450 **Tables and Figures**

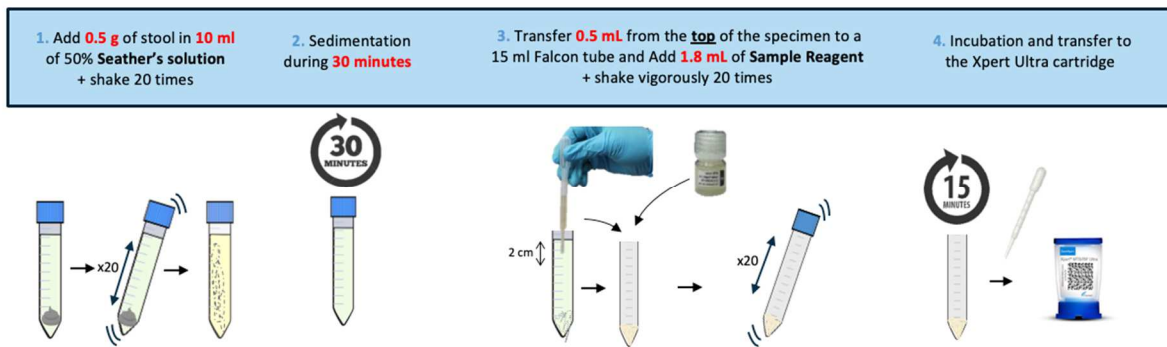
451 **Figure 1. Procedure step by step of the sucrose flotation method**

452 **A/ Original (reference) method**



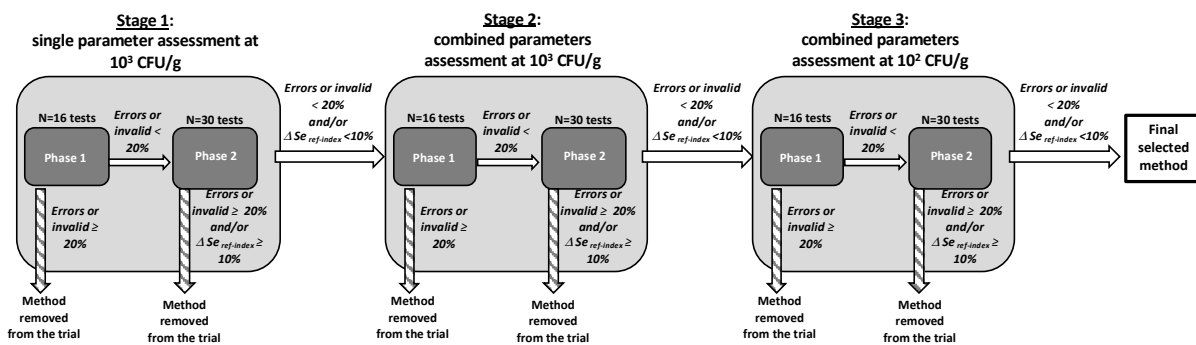
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454 **B/ Optimised method**



455

456 **Figure 2. Method selection study design**



457

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

459 16 tests are performed on 8 stools and 30 tests are performed on 15 stools.

460 Table 1. Parameters tested for the optimization of the reference method

| Reference method step by step | Alternative parameters assessed | Rationale |
|---|---|--|
| 0.5g of stool | A- 1g of stool | If we add more stool we add more MTB but more PCR inhibitors |
| Gauze filtration | B- No filtration C- Glass wool filtration | <ul style="list-style-type: none"> - The sedimentation might be sufficient to let the large particles settle with no need of filtration - 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) |
| Centrifugation at 100g for 1 minute | D- Sedimentation for 30 minutes E- Sedimentation for 1 hour F- Sedimentation for 1.5 hours | We selected three different times of sedimentation, 30', 1h and 1h30 based on feasibility and preliminary tests performed in our lab |
| Mix 0.5 mL of stool mix with 1.8 mL of sample reagent (ratio 1:3.6; sample: SR) | G- Ratio 1:2; 1 mL of sample mixed with 2 mL of SR H- Ratio 1:1; 1 mL of sample mixed with 1 mL of SR I- Ratio 1:2b: 0.8 mL of sample mixed with 1.6 mL | 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. |

461
462

463 Table 2. Results of method assessment according selection criteria on the 9 alternative parameters
 464 tested at 10³ CFU/g

| | | Number of tests | Proportion of Invalid | Proportion of error | Overall sensitivity (CI95) | Proportion of Trace among MTB detected |
|----------------------------------|------------------|-----------------|-----------------------|---------------------|----------------------------|--|
| A - 1g of stool | Index method | 30 | 13.3% | 0.0% | 82.7% (63.5-93.4) | 58% |
| | Reference method | | 13.3% | 0.0% | 63.3% (43.9-79.4) | 58% |
| B - No Filtration | Index method | 30 | 15.6% | 0.0% | 78.1% (59.5-90.0) | 56% |
| | Reference method | | 12.5% | 0.0% | 87.5% (70-96) | 61% |
| C - Glass wool filtration | Index method | 30 | 0.0% | 0.0% | 91.6% (71.5-98.5) | 56% |
| | Reference method | | 0.0% | 0.0% | 83.8% (61.8 - 94.5) | 64% |
| D - Sed 30' | Index method | 30 | 4.5% | 0.0% | 79.5% (57.3-89.3) | 81% |
| | Reference method | | 9% | 3.3% | 73% (43.9 - 79.4) | 53% |
| E - Sed 1h | Index method | 30 | 0.0% | 0.0% | 76.7% (48.8-86.5) | 48% |
| | Reference method | | 0.0% | 0.0% | 86.7% (61.8 - 94.5) | 62% |
| F - Sed 1h30 | Index method | 30 | 0.0% | 0.0% | 86.7% (61.8.4-94.5) | 69% |
| | Reference method | | 0.0% | 0.0% | 86.7% (61.8.4-94.5) | 62% |
| G - Ratio 1:2 | Index method | 16 | 0.0% | 42.8% | ND | ND |
| | Reference method | | 0.0% | 0.0% | ND | ND |
| H - Ratio 1:1 | Index method | 16 | 0.0% | 71.4% | ND | ND |
| | Reference method | | 0.0% | 0.0% | ND | ND |
| I - Ratio 1:2b | Index method | 30 | 0.0% | 0.0% | 93.3% (72.4-98.6) | 68% |
| | Reference method | | 7.7% | 3.9% | 86.7% (68.4 - 95.6) | 58% |

465 *ND = No data

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

467 Methods that had more than 20% of error or invalid were dropped after 16 tests and the overall
 468 sensitivity and proportion of trace was not assessed.

469

470

471 Table 3. Results of method assessment according selection criteria on combined parameters

472 tested at 10³ and 10² CFU/g

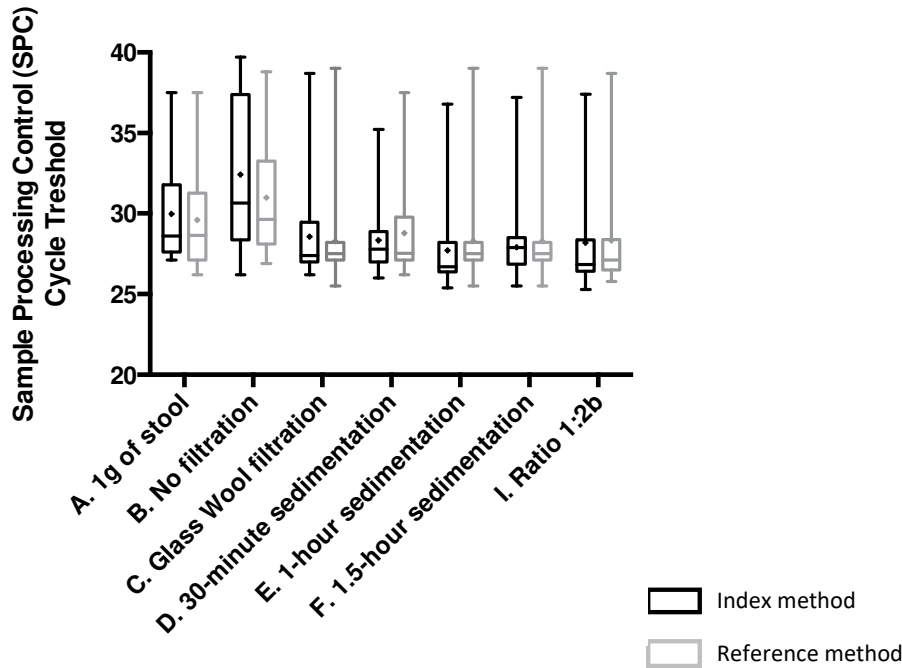
| | | Number of tests | Proportion of Invalid | Proportion of error | Overall Sensitivity (IC95) | Proportion of Trace among MTB detected |
|--|------------------|-----------------|-----------------------|---------------------|----------------------------|--|
| 10³ CFU/g | | | | | | |
| BD - No filtration + Sed 30' + ratio 1:3.6 | Index method | 30 | 10.0% | 0.0% | 70% (50.4-84.5) | 76.2% |
| | Reference method | | 13.3% | 3.3% | 63.3% (43.9-79.4) | 58.2% |
| D - Gauze filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 3.3% | 0.0% | 80% (59.5-90.0) | 80.6% |
| | Reference method | | 6.9% | 3.3% | 72.7.6% (57-85) | 52.6% |
| CD - Glass wool filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 0.0% | 0.0% | 80% (59.5-90.0) | ND |
| | Reference method | | 3.3% | 0.0% | 96.8% (87.8 - 100.0) | ND |
| ABD - 1g + No filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 5.0% | 0.0% | 90% (72-99) | 55.6% |
| | Reference method | | 0.0% | 0.0% | 100% (79 - 100) | 66.6% |
| ADI - 1g + Sed 30' + Ratio 1:2b | Index method | 16 | 0.0% | 37.5% | ND | ND |
| | Reference method | | 0.0% | 0.0% | ND | ND |
| 10² CFU/g | | | | | | |
| BD - No filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 10.7% | 0.0% | 53.3% (38.8-78.1) | 81.3% |
| | Reference method | | 7.1% | 0.0% | 57.6% (37.4-75.1) | 88.8% |
| D - Gauze filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 10.0% | 0.0% | 28.6% (13.9-48.8) | ND |
| | Reference method | | 7.1 % | 0.0% | 57.6% (37.4 - 75.1) | ND |
| ABD - 1g + No filtration + Sed 30'+ ratio 1:3.6 | Index method | 30 | 11.1% | 0.0% | 11.1% (1.9-36.1) | ND |
| | Reference method | | 0.0% | 5.6% | 22% (7.30 - 48.0) | ND |

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

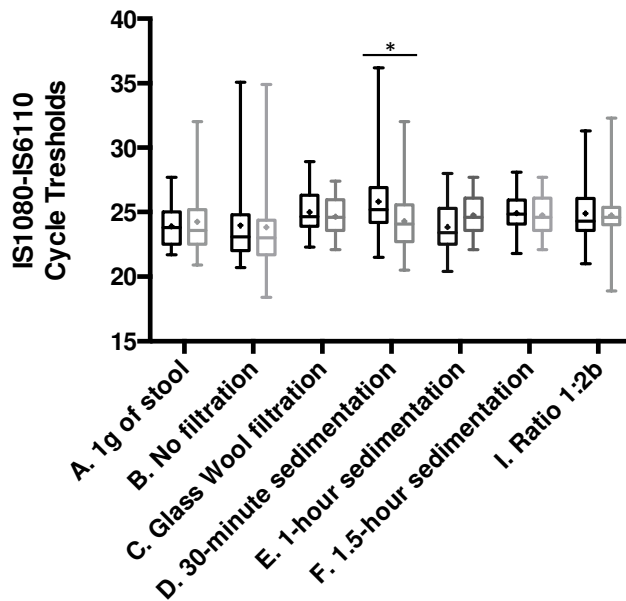
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475 Figure 3. SPC CTs (A) and IS1080-6110 CTs (B) over selected parameters tested at 10³ CFU/g

A. SPC CTs



B. IS1080-6110 CTs



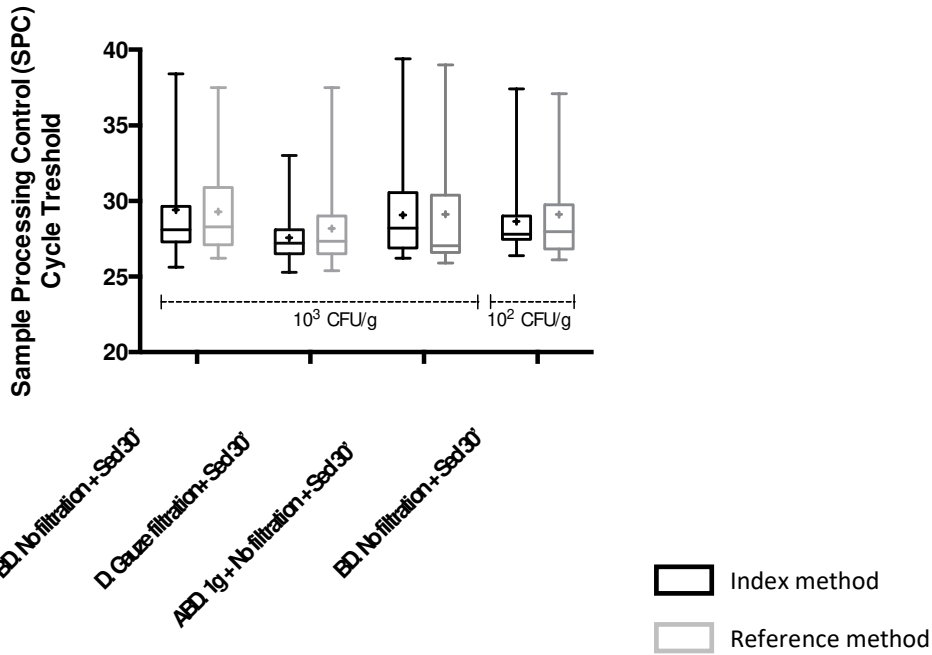
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477 The boxplots show the median Ct values and the interquartile ranges. The whiskers go down to the
478 smallest value and up to the largest. The mean is represented as a '+'. The asterisks indicate
479 statistically significant differences ($p <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

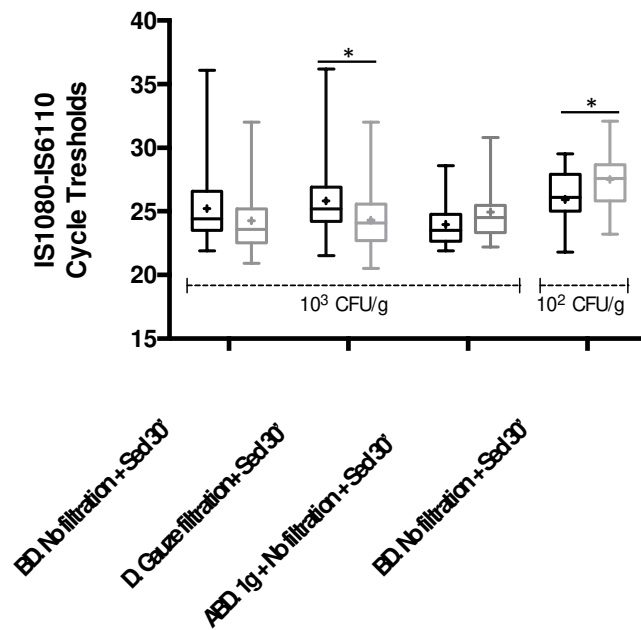
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481 Figure 4. SPC CTs (A) and IS1080-6110 CTs (B) over selected parameters tested at 10^3 and 10^2 CFU/g

A. SPC CTs



B. IS1080-6110 CTs



482

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

486

487