Centrifuge-free stool processing methods for Xpert MTB/RIF Ultra tuberculosis diagnosis in children in Uganda and Zambia: an observational, prospective, diagnostic accuracy study

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Summary

Background WHO recommends Xpert MTB/RIF Ultra (Ultra) for stool testing for tuberculosis diagnosis in children. Stool processing requires removal of debris and PCR inhibitors, frequently by using centrifugation, which can be an implementation barrier for low-income and middle-income countries (LMICs). We evaluated the diagnostic accuracy of Ultra on stool using three centrifuge-free processing methods, the simple one-step (SOS), stool processing kit (SPK), and the optimised sucrose flotation (OSF) methods against a microbiological reference standard (MRS).

Methods In this observational, prospective, multicountry, diagnostic accuracy study, we collected two respiratory samples and two stool samples in children younger than 15 years with presumptive tuberculosis in one hospital in Uganda and two hospitals in Zambia for Ultra testing and culture (on respiratory samples only). We defined positive MRS as positive culture or Ultra on respiratory sample and negative MRS as two negative respiratory samples by either culture or Ultra. We assessed the perception of the laboratory operators of test ease-of-use using a self-administered questionnaire at all sites. This study is registered with ClinicalTrials.gov (NCT04203628) and the Pan African Clinical Trial Registry (PACTR202006814433059).

Findings Of the 216 children enrolled between Jan 13, 2020, and Dec 31, 2021, 215 were included in the study and of these 104 (48·4%) were female and 211 (51·6%) were male, the median age was 1·8 years (IQR 1·1–4·8), 68 (31·6%) were HIV positive, and 38 (17·7%) were MRS positive. For one or both stool samples, depending on availability, the sensitivity of stool Ultra against MRS was 69·7% (95% CI 51·3–84·4) for SOS, 69·7% (51·3–84·4) for SPK, and 73·5% (55·6–87·1) for OSF (McNemar test p>0·6 for all), with a specificity above 96% for all methods. The SOS stool method was considered the easiest by six of seven operators because it required least manipulation and no additional reagents.

Interpretation Centrifuge-free stool processing methods could improve access to microbiological diagnosis of tuberculosis in LMICs. These results contributed to the WHO endorsement of the SOS and OSF methods.

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Introduction

WHO estimates that only 49% of the 1·25 million children with tuberculosis globally were notified to national programmes in 2022.[1] Mathematical models estimate that 96% of tuberculosis-related deaths are due to untreated and undiagnosed disease, especially in children younger than 5 years.[2] Clinicians encounter multiple challenges in confirming tuberculosis in children, including difficulties in respiratory sample collection (especially in children <5 years who cannot self-expectorate), and low bacterial load in respiratory samples due to the paucibacillary nature of pulmonary tuberculosis in children (resulting in low sensitivity of microbiological tests).[3] Typical respiratory samples for children such as gastric aspirate and induced sputum are poorly implemented in high-tuberculosis burden and low-income and middle-income countries (LMICs) due to operational and safety challenges. Consequently, most children treated for tuberculosis do not have a microbiological diagnosis.[4] Thus, there is an urgent need for a simple, child-friendly, non-invasive, and non-sputum-based specimen collection method for diagnosis of tuberculosis in children.

Using stool specimens to retrieve *Mycobacterium tuberculosis* from swallowed respiratory secretions is a non-invasive and simple method that can be used for rapid molecular testing, such as Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), which provides semi-quantitative *M tuberculosis* and rifampicin resistance results within 2 h.

Meta-analyses have shown pooled sensitivities of stool testing with Xpert MTB/RIF ranging between 50% and 68% and specificity above 98%,[5–8] with sensitivity ranging between 70% and 89% when using the current generation Xpert MTB/RIF Ultra (Ultra) cartridge.[9–11] In 2020, WHO recommended testing stool samples using Xpert MTB/RIF

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See Online for appendix 1

Research in context

Evidence before this study

Although WHO recommends Xpert MTB/RIF Ultra (Ultra) testing on stool for diagnosing tuberculosis in children unable to produce sputum, there is no published comprehensive comparative analysis of centrifuge-free stool processing methods, considering both diagnostic accuracy and feasibility for further deployment in primary health-care settings. We searched PubMed without any language restrictions for articles published between Jan 1, 2013, and Nov 30, 2023, using the terms "stool" and "tuberc*" and "child*" and "Xpert" and we found no studies comparing the diagnostic accuracy of available centrifuge-free stool processing methods. Existing evidence on stool Xpert testing performance varied greatly between studies depending on the processing method used but also on the study design and population, and only three studies described the detection yield of centrifuge-free methods. The absence of a standardised stool preparation and testing protocol prevents generalisation of diagnostic accuracy studies of stool Xpert for tuberculosis diagnosis. In addition, very little research has been conducted on the specific operational challenges faced by laboratory personnel when processing stool samples.

Added value of this study

This study builds upon this existing knowledge gap and is, to our knowledge, the first comparative evaluation of centrifuge-free stool processing methods and their potential impact to improve access to accurate tuberculosis diagnosis for children globally. This study compares three centrifuge-free stool processing methods, the simple one-step (SOS) method, the stool processing kit method, and the optimised sucrose flotation (OSF) method for diagnosing tuberculosis in children, addressing the limitations of traditional methods in low-income and middle-income countries. The results showed high sensitivity (70–73%) and specificity (>96%) of Ultra testing with these methods compared with the microbiological reference standard, offering a promising solution for tuberculosis diagnosis in children unable to produce sputum. The study also assessed the feasibility of these methods, with the SOS method being perceived as the simplest. The study employs a head-to-head design, allowing a direct comparison of the three centrifuge-free methods, ensuring a fair evaluation of their diagnostic accuracy. In addition to diagnostic accuracy, the study evaluates the feasibility of implementing these methods in routine laboratory settings, providing practical insights into their applicability and acceptance by laboratory personnel.

Implications of all the available evidence

These findings suggest that stool specimens, processed through centrifuge-free methods, could substantially improve access to tuberculosis diagnosis in children, particularly in regions with limited health-care resources. The non-invasive nature of stool sample collection makes it a child-friendly and feasible alternative to sputum or gastric aspirate samples. The introduction of centrifuge-free methods has operational implications, potentially making tuberculosis diagnosis more accessible at lower-level health-care facilities with limited laboratory capacity. Based on preliminary results from our study the SOS and OSF methods have been included into the WHO and StopTB Partnership Global Laboratory Initiative technical manual. This study substantially contributes to the field of paediatric tuberculosis diagnosis by introducing innovative, practical, and effective stool processing methods, potentially shaping the future landscape of tuberculosis diagnostics for children worldwide.

as a primary diagnostic test for childhood pulmonary tuberculosis diagnosis; this recommendation was updated to include Ultra in 2022.[12] At that time, all published stool processing methods included complex pre-processing and centrifugation before analysis to remove PCR inhibitors and macroscopic particles. This approach limited use at low resource health-care facilities with reduced laboratory capacity.[11] To address this challenge, different groups have developed simple centrifuge-free methods for LMICs: the optimised sucrose flotation (OSF) method (developed by the TB-Speed Stool Study Group),[13] a stool processing kit (SPK; developed by FIND),[14] and the simple one-step (SOS) stool method (developed by KNCV Tuberculosis Foundation).[15] The OSF is based on Sheather's solution to create a sucrose density gradient to separate *M tuberculosis* from debris;[13] the SPK method uses a stool processing buffer with the Ultra sample reagent to inactivate PCR inhibitors and sample filtration using glass wool;[14] and the SOS method uses sedimentation by gravity, allowing *M tuberculosis* to release from the stool matrix and float in the supernatant.[15]

In this study, we evaluated the three centrifuge-free stool processing methods for Ultra testing in children with presumptive tuberculosis. To complete the clinical validation of the OSF method we also included the centrifuge-based classic sucrose flotation (CSF) method as a comparator. The primary objective was to evaluate the diagnostic accuracy against a microbiological reference standard (MRS). Secondary objectives included the (1) head-to-head comparison of the sensitivity and specificity of the three centrifuge-free methods; (2) comparison of the sensitivity and specificity of the OSF versus the CSF; (3) diagnostic accuracy of the stool processing methods using a composite reference standard (CRS); (4) diagnostic accuracy of the stool processing methods using only the first collected stool specimen and two stool specimens; (5) agreement for *M tuberculosis* detection between the different processing methods; and (6) feasibility of the three centrifuge-free stool processing methods.

Methods

Study design and population

We did an observational prospective, multicountry, diagnostic accuracy study with a two-stage sequential design between Jan 13, 2020, and March 31, 2022. The first stage

(Jan 13, 2021, to Jan 6, 2022) included a cohort of children younger than 15 years with presumptive intrathoracic tuberculosis (appendix 1 p 3) to primarily estimate the specificity of the four stool methods with Ultra among children who did not have tuberculosis. To include sufficient children with confirmed tuberculosis, in the second stage (Jan 7, 2021, to March 31, 2022), the same children with microbiologically confirmed tuberculosis based on one positive Ultra result from any respiratory sample were included, primarily to evaluate sensitivity. Children with a tuberculosis treatment history in the past 3 months or extrapulmonary tuberculosis only were excluded. This design is appropriate for evaluating diagnostic tests when the prevalence of the disease is low in the cohorts studied.[16] The study was conducted in outpatient and inpatient units of three referral hospitals: Mbarara Regional Referral Hospital (Mbarara, Uganda), Lusaka University Teaching Hospital (Lusaka, Zambia), and the Arthur Davison Children's Hospital (Ndola, Zambia; added from Aug 6, 2021).

A medical doctor performed a clinical evaluation, followed by sample collection for microbiological investigations over 2 consecutive days, chest x-ray, and HIV testing for children with unknown HIV status. All children had a follow-up visit after 2 months for clinical evaluation and additional tests if required clinically.

Study enrolment was interrupted between April 1 and June 22, 2020, due to complete lockdown due to the COVID-19 pandemic in Uganda and Zambia but follow-up visits were maintained at the study site whenever possible. Alternatively, follow-up visits were done by study nurses by telephone and families were instructed to bring the child to the nearest health facility, where appropriate vital signs measurements and clinical assessments could be made (appendix 2).

The study received approval from the Ugandan National Council for Science and Technology (reference UNCST, HS 2676) and Mbarara University Research Ethics Committee (MUSTREC 1/7, 23/04-19), the University of Zambia Biomedical Research Ethics Committee (UNZABREC, 034-2019), the INSERM Ethics Committee (TB-Speed Stool processing), and the WHO Research Ethics Review Committee (TB-Speed Stool processing). We obtained written informed consent from parents or caregivers of children, from laboratory operators for the feasibility study, and assent from children older than 7 years.

This study is registered with ClinicalTrials.gov (NCT04203628) and the Pan African Clinical Trial Registry (PACTR202006814433059).

Procedures

Sample collection

On the day of enrolment and the day after, one respiratory sample, either a gastric aspirate, sputum, or nasopharyngeal aspirate depending on enrolment sites, was collected for tuberculosis culture and Ultra (appendix 1 p 10). On the day of enrolment and the day after, one stool (20 mL) was directly collected into a stool container or transferred from nappies using a wooden stick or container spoon for Ultra testing. For outpatients unable to produce stool on site, parents were instructed on how to collect stool at home and to bring the sample on the next day. All specimens were processed either fresh or after up to approximately 72 h storage at 2–8°C.

Sample processing

All laboratory procedures were performed at each study site. To minimise sample bias from varying bacterial concentrations, each stool specimen was homogenised and divided into four samples. Each sample was processed using the different methods in a randomised order defined by a masked statistician (appendix 1 pp 6–7). The operator always sampled the specimen using a so-called north, south, west, and east approach (appendix 1 pp 4–6). In case of insufficient stool sample to prepare four aliquots, OSF and CSF methods were prioritised to have enough samples for the validation of the OSF method.

Stool processing was performed following previously described procedures (appendix 1 pp 3–4).[13–15] Laboratory technicians were trained on each processing method by members of the groups that developed the methods—ie, KNCV Tuberculosis Foundation, FIND, and TB-Speed—either onsite or remotely via videoconference. Operators underwent a standardised proficiency test individually including observation of practices and correction of mistakes.

Processed stools were tested using Ultra. In case of an indeterminate result (ie, invalid, error, or no result), the Ultra test was repeated once using the same sample.

Gastric aspirate, sputum, or nasopharyngeal aspirate were cultured using mycobacteria growth indicator tubes (MGIT; Bactec 960 Becton and Dickinson, NJ, USA) and Lowenstein–Jensen slopes and were also tested with Ultra (appendix 1 pp 6–7).

See Online for appendix 2

Index case and reference standard definitions

Patients were classified with a positive index test (stool Ultra) if either of the two stool samples had *M tuberculosis* detected (including trace results), and negative if there was at least one negative Ultra stool result without any positive result.

A positive MRS was defined by any positive *M tuberculosis* culture or Ultra result from respiratory samples. A negative MRS was defined by negative culture or Ultra results from at least two different samples without any positive result. Patients with one or two negative results from one sample only were considered as unclassified MRS.

The CRS used the published Clinical Case Definitions for Classification of Intrathoracic Tuberculosis Disease, classifying the patients as confirmed, unconfirmed, and unlikely tuberculosis with the use of an independent validation committee.[17] Patients who did not fit any of the specified criteria were kept as unclassified. Confirmed (corresponding to children with a positive MRS) and unconfirmed tuberculosis were grouped together as a positive CRS and unlikely tuberculosis classified as negative CRS.

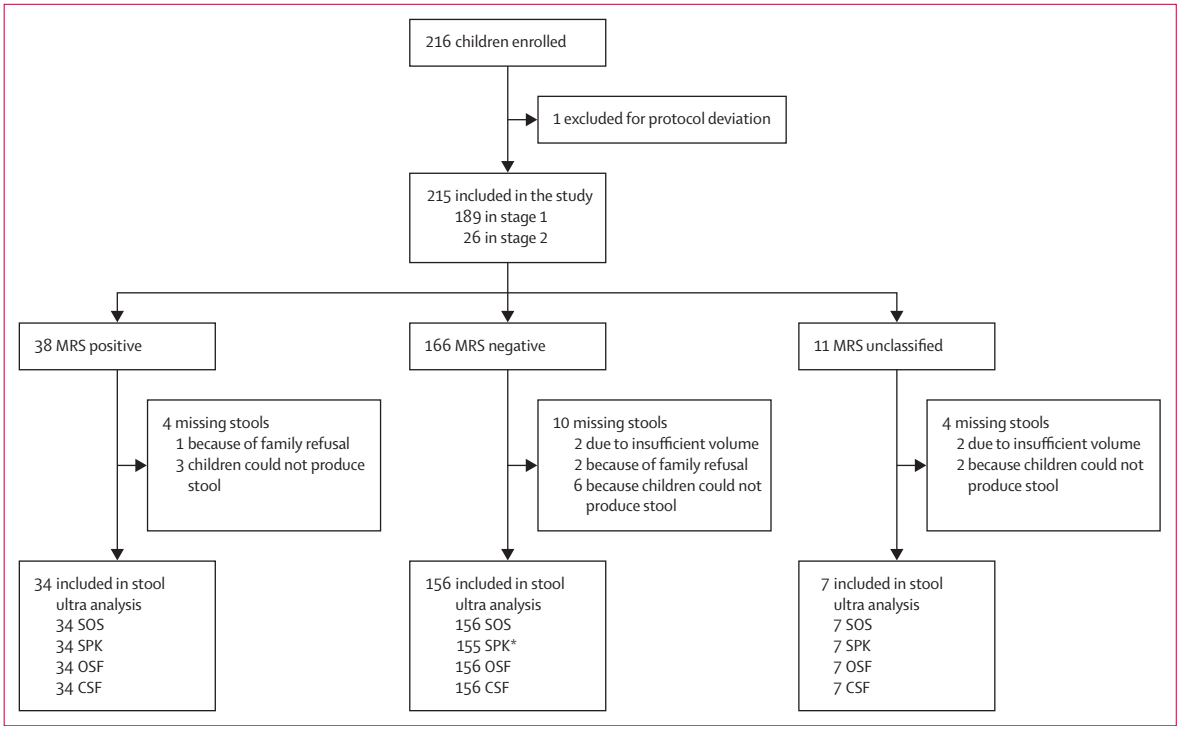

Figure 1: **Study profile**
CSF=classic sucrose flotation. MRS=microbiological reference standard. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit. *Stool sample from one child was not tested with SPK due to a kit shortage.

	Unlikely tuberculosis (n=129)	Confirmed plus unconfirmed tuberculosis* (n=76)	Unclassified* (n=10)	Overall (n=215)
Age, years	1·8 (1·1–4·1)	1·8 (1·1–5·1)	1·8 (1·4–2·5)	1·8 (1·1–4·8)
<5	97 (75·2%)	56 (73·7%)	9 (90·0%)	162 (75·3%)
5–10	18 (14·9%)	9 (11·8%)	1 (10·0%)	28 (13·0%)
>10–15	14 (10·8%)	11 (14·5%)	0	25 (11·6%)
Female	62 (48·1%)	36 (47·4%)	6 (60·0%)	104 (48·4%)
Male	67 (51·9%)	40 (52·6%)	4 (40·0%)	211 (51·6%)
HIV positive	49 (38·0%)	18 (23·7%)	1 (10·0%)	68 (31·6%)
HIV negative	80 (42·0%)	58 (76·3%)	9 (90·0%)	147 (68·4%)
Weight for height Z score less than minus 2 standard deviations (age <5 years)	58/97 (59·8%)	37/56 (66·1%)	2/9 (22·2%)	97/162 (60·6%)
Weight for age Z score less than minus 2 standard deviations (age ≥5 years)	7/18 (38·9%)	5/8 (62·5%)	0/1	12/27 (44·4%)
Positive culture result	0	22 (28·9%)	0	22 (10·2%)
MRS positive	0	38 (50·0%)	0	38 (17·7%)
History of tuberculosis contact	9/125 (7·2%)	21/75 (28·0%)	0/7	30/207 (14·5%)

Data are median (IQR), n (%), or n/N (%). *As per the Clinical Case Definitions for Classification of Intrathoracic Tuberculosis Disease.

Table 1: **Demographics and clinical characteristics of enrolled children according to the classification by the composite reference standard**

Feasibility assessment of centrifuge-free stool processing methods

We assessed the perception of seven laboratory operators using a self-administered questionnaire containing open and multiple-choice questions after sites included 30 patients in Mbarara and Lusaka and within 3 months after the site opened in Ndola. We collected their opinion on ease-of-use, quality of the instructional material, and perceived feasibility at each step for the methods with a Likert scale (totally agree, partially agree, partially disagree, and totally disagree). We asked operators to rank each method from 1 to 3 (best to worst) with respect to (1) the method they would recommend and (2) the method that was the easiest to perform. If they considered two or more methods equally difficult to perform, they used the same number.

	Unlikely tuberculosis (n=129)	Confirmed plus unconfirmed tuberculosis (n=76)	Unclassified (n=10)	Overall (n=215)
Number of respiratory samples				
One	9 (7·0%)	8 (10·5%)	8 (80·0%)	25 (11·6%)
Two	120 (93·0%)	68 (89·5%)	2 (20·0%)	190 (88·4%)
Two respiratory samples				
Two sputa	6/120 (5·0%)	0/68	0/2	6/190 (3·1%)
Sputum plus gastric aspirate	2/120 (1·7%)	0/68	0/2	2/190 (1·0%)
Sputum plus nasopharyngeal aspirate	7/120 (6·8%)	2/68 (2·9%)	0/2	9/190 (4·7%)
Two gastric aspirates	63/120 (52·2%)	45/68 (66·2%)	2/2 (100%)	110/190 (57·9%)
Gastric aspirate plus nasopharyngeal aspirate	42/120 (35·0%)	21/68 (30·9%)	0/2	63/190 (33·2%)
One respiratory sample only				
Sputum or nasopharyngeal aspirate	0/9	1/8 (12·5%)	7/8 (88·5%)	8/25 (32·0%)
Gastric aspirate	9/9 (100%)	7/8 (88·5%)	1/8 (12·5%)	17/25 (68·0%)
Stool sample collection				
One	17/123 (13·8%)	7/72 (9·7%)	4/6 (66·7%)	28/201 (13·9%)
Two	106/123 (86·2%)	65/72 (90·3%)	2/6 (33·3%)	173/201 (86·1%)

Data are n (%) or n/N.

Table 2: **Sample characteristics per patient and classification by the composite reference standard**

	M tuberculosis detected	*M tuberculosis* not detected	Indeterminate
Confirmed tuberculosis (n=38)			
SOS (n=34)	23	10	1
SPK (n=34)	23	10	1
OSF (n=34)	25	9	0
CSF (n=34)	23	11	0
Unconfirmed tuberculosis (n=38)			
SOS (n=37)	1	36	0
SPK (n=37)	1	36	0
OSF (n=37)	0	36	1
CSF (n=37)	0	37	0
Unlikely tuberculosis (n=129)			
SOS (n=120)	3	116	1
SPK (n=119)	5	109	5
OSF (n=120)	2	116	2
CSF (n=120)	1	117	2
Unclassified (n=10)*			
SOS (n=6)	0	6	0
SPK (n=6)	0	6	0
OSF (n=6)	0	6	0
CSF (n=6)	0	6	0

Data are n. CSF=classic sucrose flotation. *M tuberculosis*=*Mycobacterium tuberculosis*. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit. *Four patients voluntarily withdrew, three died, two were lost to follow-up, and one completed follow-up.

Table 3: **Study profile compared with the composite reference standard**

Statistical analysis

The study sample size was calculated separately for specificity and sensitivity against the MRS. With an expected specificity of Ultra in stool of 90%, the minimum sample size to estimate specificity with 5% precision was 140 children with negative MRS. Inflating this number by 15% for attrition and considering that 10% of children with presumptive tuberculosis will be MRS positive, we needed to enrol a total of 177 children with presumptive tuberculosis in stage 1. With an expected sensitivity of Ultra in stool of 60%,[11] the minimum sample size to estimate sensitivity with 10% precision was 93 children with a positive MRS. We expected to enrol 17 patients with an MRS positive from stage 1. Therefore, 86 children with presumptive tuberculosis and a positive Ultra result needed to be enrolled in stage 2 (enrichment cohort).

Data were analysed using the R software (version 4.4.0).

Patients and sample characteristics were summarised using percentages for categorical variables and median with IQR for continuous variables.

For diagnostic accuracy, sensitivity, specificity, and positive and negative predictive values and their 95% CIs were estimated for Ultra performed on stools processed with the four different methods on any stool sample collected using the MRS and CRS as reference standard, excluding children with unclassified MRS and CRS for each analysis, respectively. This was done in a per-protocol analysis for which indeterminate Ultra results were excluded from analysis. We computed the predictive values using the prevalence of tuberculosis in the prospective cohort of children with presumptive tuberculosis based on the MRS and CRS.[18]

As a secondary analysis we repeated the per-protocol analysis using only the first stool sample collected. We performed an intention-to-diagnose analysis in which indeterminate stool Ultra results were considered as false negative if the MRS was positive, or false positive if the MRS was negative (worst case scenario), as previously described.[19] Sensitivities and specificities of the SOS, SPK, and OSF methods were compared head-to-head using the McNemar test for matched data. We also constructed Venn diagrams

	Centrifuge-free methods			CSF
	SOS	SPK	OSF	
Microbiological reference standard				
True positive	23	23	25	23
False positive	3	6	2	1
False negative	10	10	9	11
True negative	151	144	151	153
Sensitivity	69·7% (51·3–84·4)	69·7% (51·3–84·4)	73·5% (55·6–87·1)	67·6% (49·5–82·6)
Specificity	98·1% (94·4–99·6)	96·0% (91·5–98·5)	98·7% (95·4–99·8)	99·4% (96·4–100·0)
Positive predictive value*	83·0% (47·7–98·4)	70·4% (38·2–92·2)	88·5% (53·6–99·6)	93·4% (56·4–100·0)
Negative predictive value*	96·0% (89·6–99·0)	95·9% (89·4–99·0)	96·5% (90·3–99·2)	95·7% (89·3–98·9)
Composite reference standard				
True positive	23	24	25	23
False positive	3	5	2	1
False negative	46	46	45	48
True negative	116	109	116	117
Sensitivity	33·3% (22·4–45·7)	34·3% (23·3–46·6)	35·7% (24·6–48·1)	32·4% (21·8–44·5)
Specificity	97·5% (92·8–99·5)	95·6% (90·1–98·6)	98·3% (94·0–99·8)	99·2% (95·4–100·0)
Positive predictive value*	85·6% (54·3–98·6)	77·8% (48·0–95·1)	90·4% (60·4–99·6)	94·4% (62·6–100·0)
Negative predictive value*	76·5% (66·3–84·9)	76·4% (66·0–84·9)	77·3% (67·1–85·6)	76·6% (66·4–84·9)

Data are n or % (95% CI). Comparison of sensitivity using McNemar test in per-protocol analysis against the microbiological standard, no correction was used for multiple statistic comparison: SOS versus OSF (p=1·00), OSF versus SPK (p=1·00), SOS versus SPK (p=1·00), and CSF versus OSF (p=0·62). Comparison of specificity using McNemar test in per-protocol analysis against the microbiological standard: SOS versus OSF (p=1·00), OSF versus SPK (p=0·13); SOS versus SPK (p=0·50), and CSF versus OSF (p=1·00). CSF=classic sucrose flotation. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit. *Positive and negative predictive values have been adjusted on prevalence from the prospective cohort.

Table 4: **Diagnostic accuracy of the stool processing methods combined with Xpert MTB/RIF Ultra on any stool collected against the microbiological and composite reference standards**

	Centrifuge-free methods			CSF
	SOS	SPK	OSF	
Microbiological reference standard				
True positive	19	22	24	21
False positive	1	2	1	0
False negative	13	10	8	12
True negative	146	129	146	152
Sensitivity	59·4% (40·6–76·3)	68·8% (50·0–83·9)	75·0% (56·6–88·5)	63·6% (45·1–79·6)
Specificity	99·3% (96·3–100·0)	98·5% (94·6–99·8)	99·3% (96·3–100·0)	100·0% (97·6–100·0)
Positive predictive value*	92·2% (51·9–100·0)	86·0% (49·9–99·2)	93·8% (59·3–100·0)	100·0% (61·7–100·0)
Negative predictive value*	94·7% (88·0–98·3)	95·9% (89·4–98·9)	96·7% (90·6–99·3)	95·3% (88·7–98·6)
Composite reference standard				
True positive	19	23	24	21
False positive	1	1	1	0
False negative	46	38	42	49
True negative	114	102	113	116
Sensitivity	29·2% (18·6–41·8)	37·7% (25·6–51·0)	36·4% (24·9–49·1)	30·0 (19·6–42·1)
Specificity	99·1% (95·3–100·0)	99·0% (94·7–100·0)	99·1% (95·2–100·0)	100·0% (96·9–100·0)
Positive predictive value*	93·8% (59·4–100·0)	94·6% (66·0–100·0)	94·9% (65·6–100·0)	100·0% (67·3–100·0)
Negative predictive value*	75·7% (65·6–84·1)	78·0% (67·8–86·1)	77·6% (67·5–85·8)	76·1% (66·0–84·4)

Data are n or % (95% CI). Comparison of sensitivity using McNemar test in per-protocol analysis against the microbiological standard, no correction was used for multiple statistic comparison: SOS versus OSF (p=0·22), OSF versus SPK (p=1·00), SOS versus SPK (p=0·62), and CSF versus OSF (p=0·25). Comparison of specificity using McNemar test in per-protocol analysis against the microbiological standard: SOS versus OSF (p=1·00), OSF versus SPK (p=1·00), SOS versus SPK (p=1·00), and CSF versus OSF (p=1·00). CSF=classic sucrose flotation. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit. *Positive and negative predictive values have been adjusted on prevalence from the prospective cohort.

Table 5: **Diagnostic accuracy of the stool processing methods combined with Xpert MTB/RIF Ultra on the first stool collected against the microbiological and composite reference standards**

including stools tested by the SOS, SPK, and OSF methods to illustrate the number of stools with a positive Ultra result by any of the three centrifuge-free methods compared with the MRS result. The same approach was used to compare the OSF and CSF methods. No correction was used for multiple statistic comparison. We used the Clopper–Pearson method to compute 95% CI.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 216 children enrolled between Jan 13, 2020, and Dec 31, 2021, 215 were included in the study. Median age was 1·8 years (IQR 1·1–4·8), 104 (48·4%) were female, 211 (51·6%) were male, 68 (31·6%) were HIV positive, and 109 (57·6%) of 189 had moderate to severe malnutrition (figure 1; table 1; appendix 1 pp 9–10). All children had at least one respiratory sample collected and 190 (88·4%) had two respiratory samples (table 2; appendix 1 p 10).

Overall, 173 (86·1%) of 201 children had two stool samples collected and 28 (13·9%) had one sample only, resulting in a total of 374 stool samples For the children with two stool samples, 159 (73·9%) were processed with SOS, 156 (72·6%) were processed with SPK, 165 (76·7%) were processed with OSF, and 168 (78·1%) were processed with CSF. For the children with only one stool sample 38 (18·5%) were processed with SOS, 40 (18·6%) were processed with SPK, 32 (14·9%) were processed with OSF, and 29 (13·5%) were processed with CSF (appendix 1 p 13). Due to low volume of some stool samples, it was not always possible to prepare 4 samples from each stool, resulting in a difference of samples processed with different methods: 356 (95·2%) samples processed with SOS, 352 (94·1%) samples processed with SPK, 362 (96·8%) samples processed with OSF, and 365 (97·6%) samples processed with CSF. All samples were tested with Ultra.

For all methods, less than 1·5% error and less than 3% invalid Ultra results were recorded, except for the SPK method, which showed 30 (8·5%) invalid Ultra results of 352 tests. *M tuberculosis* was detected in 40 (11·2%) of 356 stool samples from 26 (13·2%) of 197 children for SOS, 43 (12·2%) of 352 stool samples from 29 (18·8%) of 196 children for SPK, and 44 (12·1%) of 362 stool samples from 27 (13·7%) of 197 children for the OSF method. The proportion of trace results was 15 (37·5%) of 40 positive tests for SOS, 16 (36·4%) of 44 for SPK and 11 (25·0%) of 44 for OSF (appendix 1 pp 13–14).

Overall, 204 children had a valid MRS, including 38 (18·6%) MRS positive and 166 (81·4%) MRS negative (figure 1) and 11 (5·1%) of 215 children could not be classified using the MRS, because they had only one negative culture or Ultra respiratory samples result. 205 children had a valid CRS including 38 (18·5%) confirmed tuberculosis, 38 (18·5%) unconfirmed tuberculosis, and 129 (62·9%) unlikely tuberculosis, and ten children (4·7%) of 215 could not be classified (table 3; appendix 1 p 15).

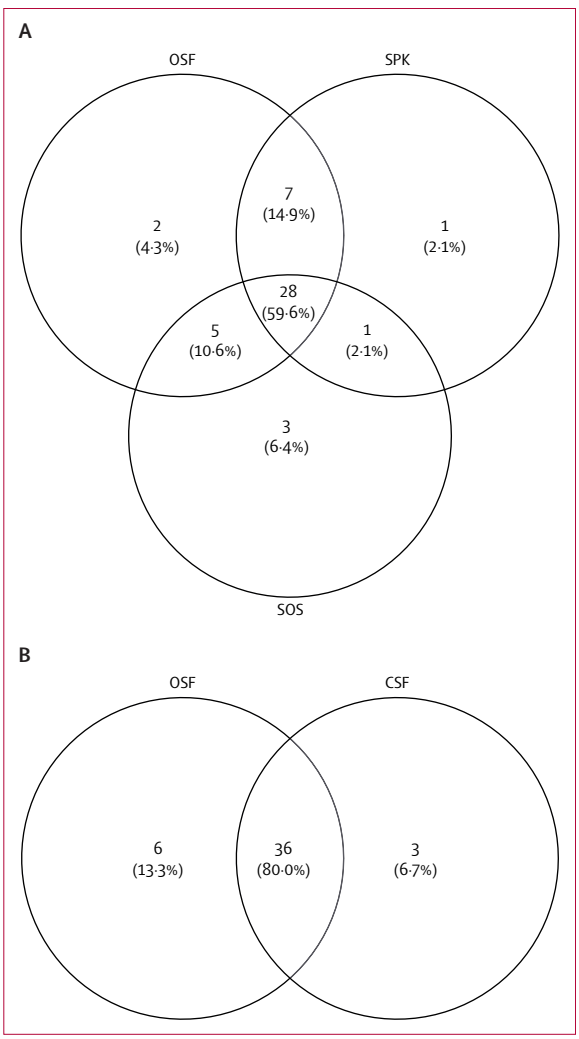

Figure 2: **Venn diagram showing intersection of Xpert MTB/RIF Ultra positive results on stool tests using the three centrifuge-free stool processing methods**

(A) Between the three centrifuge-free stool processing methods on samples from children with a positive MRS. (B) Between the OSF and CSF on samples from children with a positive MRS, which includes only children for whom all samples were tested by the two methods. CSF=classic sucrose flotation. MRS=microbiological reference standard. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit.

The per-protocol sensitivity of Ultra compared with the MRS using at least one stool sample was 69·7% (95% CI 51·3–84·4) for SOS, 69·7% (51·3–84·4) for SPK, 73·5% (55·6–87·1) for OSF, and 67·6% (49·5–82·6) for CSF. Specificity was more than 96% for all methods (table 4; appendix 1 p 12). There was no significant difference in sensitivity and specificity between the three centrifuge-free processing methods, and between OSF and CSF. Compared with CRS, the sensitivity was 33·3% (22·4–45·7) for SOS, 34·3% (23·3–46·6) for SPK, 35·7% (24·6–48·1) for OSF, and

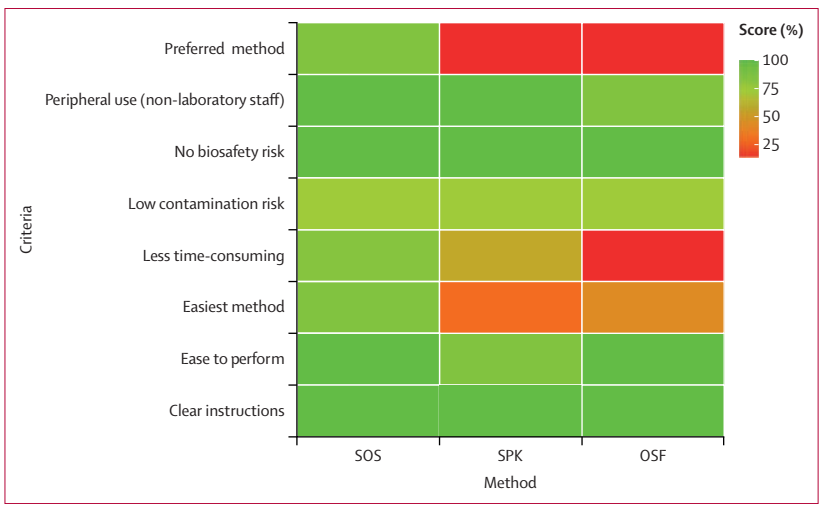

Figure 3: **Heat map of operator responses to feasibility assessment of SOS, SPK, and OSF stool processing methods**
The heat map visualises the distribution of operator responses across key criteria for each stool processing method. Colour intensity represents the proportion of positive responses for each criterion. OSF=optimised sucrose flotation. SOS=simple one-step. SPK=stool processing kit.

32·4% (21·8–44·5) for CSF with a specificity of more than 95% for all methods. The sensitivity of the four methods was similar when using an intention-to-diagnose approach against the MRS, but the specificity was reduced to 93·6% (88·5–96·9) for SOS, 81·3% (74·2–87·1) for SPK, 92·3% (86·9–96·0) for OSF, and 96·2% (91·8–98·6) for CSF (appendix 1 p 16). The specificity of the SPK method was significantly lower than that of the SOS (81·3% *vs* 93·6%, p=0·0031) and OSF (92·3%, p=0·0085) methods.

When considering only the first stool sample, against the MRS, sensitivity was 59·4% (95% CI 40·6–76·3) for SOS, 68·8% (50·0–83·9) for SPK, 75·0% (56·6–88·5) for OSF, and 63·6% (45·1–79·6) for CSF, and specificity was more than 98% for all methods (table 5; appendix 1 p 12). There was no significant difference in sensitivity and specificity between the three centrifuge-free processing methods. When considering children with two stool samples available for testing, sensitivity was 69·0% (49·2–84·7) for SOS, 69·0% (49·2–84·7) for SPK, 70·0% (50·6–85·3) for OSF, and 66·7% (47·2–82·7) for CSF, and specificity was above 95·4% for all methods (appendix 1 p 17).

In children with positive *M tuberculosis* testing, no rifampicin resistance was identified by Ultra and five had indeterminate resistance due to trace result.

Among patients with a positive MRS, 28 (59·6%) of 47 Ultra positive stool samples by any of the three centrifuge-free methods were positive by all three methods, 13 (27·7%) by two methods, and six (12·8%) by one method only (figure 2A). Among children with a negative MRS, none were positive with the three centrifuge-free methods, one was detected by OSF and SOS, one was detected by the OSF method, two by the SOS method, and six by the SPK method (appendix 1 p 18). Among patients with a positive MRS, of the 45 Ultra positive stool samples by either the OSF or CSF methods, 36 (80·0%) were positive by both methods, six (13·3%) by OSF only, and three (6·7%) by CSF only (figure 2B).

Of the seven operators who answered the feasibility questionnaire, six reported all centrifuge-free methods as simple to perform. One operator partially disagreed regarding the SPK method. All operators indicated no additional biosafety risk as compared to Ultra from sputum (figure 3; appendix 1 p 19). The most cited barriers for implementation of SPK and OSF under routine conditions were the need for additional supplies or reagents. When asked whether the procedure could be performed by non-laboratory staff, five agreed for SOS, four for SPK, and two for OSF. Overall, six of seven operators considered the SOS method the easiest. The remaining operator identified the OSF as the easiest method.

Discussion

In this multicountry diagnostic accuracy study, three centrifuge-free stool processing methods resulted in high Ultra sensitivity (70–73%) and specificity (>96%) against a robust MRS. Sensitivity was more than 59% for all processing methods when testing only one stool sample, which is likely to be the specimen collection approach used under programmatic conditions. Sensitivity might be even higher if the methods were compared with a reference standard using specimen collection methods and testing used under programmatic conditions. All methods were considered safe to be used by a laboratory technician in a microscopy-level laboratory and the SOS method appeared to be the simplest method.

Based on preliminary results from our study and another study (NCT04899076), WHO recommended that the SOS and OSF methods could be used (the SPK device is not, and will not become, commercially available), depending on local preferences and laboratory infrastructure.[12] Subsequently, WHO and the StopTB Partnership Global Laboratory Initiative issued a technical manual for the use of the SOS and OSF methods.[20]

The proportion of Ultra error results was very low for the three centrifuge-free stool processing methods, but SPK had a higher proportion of invalid results as compared to the other methods. This finding was mainly due to difficulties with the use of the integrated filter in the syringe. Only 60% of positive stool samples were identified by all three methods. The OSF method was as accurate as the CSF method, supporting results from the in vitro development study.[13]

The sensitivity of the centrifuge-free stool processing methods with Ultra (70–73%) that we report here is at the top end of the 95% CIs of pooled sensitivity (95% CI 39·1–71·7) of Ultra on stool from a 2022 Cochrane meta-analysis of centrifuge-based methods using a similar MRS.[21]

The MRS used in our study was robust, with 88·4% of children able to provide two respiratory specimens and the combination of two highly sensitive tests (MGIT culture and Ultra).[22] Another strength of the study was the use of a

head-to-head study design that evaluated the different methods using the same study population, methodology, and study procedures, supporting the validity of the results as compared to findings from meta-analysis that pooled data from studies with a high degree of heterogeneity.[11]

Feasibility of the stool processing methods will play a key role in the capacity to scale up stool testing. The SOS stool method was perceived by six of seven laboratory technicians as the simplest method in terms of supplies and sample manipulation. These results are consistent with recent feasibility and acceptability data of the SOS method at secondary and tertiary health facilities in Viet Nam under programmatic conditions.[23] Additional information has shown the robustness of the SOS method.[24]

Our study has several limitations. First, the proposed sample size of participants with a positive MRS could not be reached due to low recruitment, resulting in wide 95% CIs around the sensitivity estimates. Second, the study took place in reference hospitals where most children were hospitalised. This setting can explain the high proportion of successful stool collections, which may not reflect what could be achieved in an ambulatory setting. In the TB-Speed decentralisation study, stool collection was successful in 77% of children at district hospitals and 62% at primary health-care levels.[25] Third, the feasibility assessment questionnaire was completed by experienced laboratory personnel, which might not reflect the true feasibility at lower health-care facility levels. Additional feasibility and acceptability data of the stool processing methods at these low levels of care are needed.

Stool samples are a promising alternative sample collection method for low levels of health care and centrifuge-free simplified specimen processing methods are likely to facilitate the use of Ultra on stool for diagnosis of tuberculosis in children unable to produce sputum. It is worth noting that stool could also be a good sample for diagnosis of gastrointestinal luminal tuberculosis, but this was not evaluated in this study. This approach is a major advance to increase access to microbiological diagnosis of tuberculosis in high burden settings and LMICs that face operational challenges in using respiratory specimen collection methods. However, not all children with a positive MRS were identified by Xpert from stool. Considering this limitation and knowing that most children with tuberculosis will still be diagnosed clinically, it is important to maintain a comprehensive approach to childhood tuberculosis diagnosis and strengthen capacity for clinical diagnosis in parallel with implementation of stool testing.[3]

Contributors

MB, OM, and EW conceived and designed the study. ML coordinated and contributed to the development of the trial protocol. MB, OM, EW, SB, KK, RK, PN, MR, PDH, EWT, BN, JM-A, MPN, and SE contributed to the development of the trial protocol. OM, MB, and EW led the study at international level. JM-A led the study in Uganda. BN and CC led the study in Zambia. ML coordinated study implementation and laboratory aspects at international level, KK coordinated laboratory aspects in Zambia, and RK coordinated laboratory aspects in Uganda. PN and MR provided material and training for the SPK method. PDH and EWT provided material and training for the SOS method. SE and MPN provided scientific guidance and expertise for the study, through the Scientific Advisory Board. JM-A, CC, and BN implemented the study and enrolled participants. SB and ML did the statistical analysis. ML and SB verified the data and prepared the report. MB, ML, SB, OM, and EW contributed to the interpretation of the results. ML, MB, and ENM wrote the first draft. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Study data will not be publicly available. Data could be made available by the sponsor (Inserm) to any interested researcher. De-identified participant data and a data dictionary can be made available and shared under a data transfer agreement. Requests for access to the TB-Speed Stool study data should be sent to the corresponding author. The study protocol, statistical analysis plan, and informed consent forms are available in appendix 2.

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