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Supplementary appendix

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Performance of a stool-based quantitative PCR assay for the diagnosis of tuberculosis in adolescents and adults: a multinational, prospective diagnostic accuracy study

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Table of Contents

Supplemental Table 1. **Stool qPCR result agreement with sputum Xpert-Ultra, sputum culture and stool Xpert-Ultra in the complete cohort inclusive of cases and controls and by study site.**

Reference and Comparator tests	Stool qPCR Positive		Stool qPCR Negative		Agreement (p_0[1])
All Sites	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**% (95%CI)**
Sputum Xpert-Ultra	+	-	+	-	
n = 313	118 (37·6)	29 (9·3)	16 (5·1)	150 (47·9)	85·6 (81·2, 89·3)
Sputum Culture	+	-	+	-	
n = 250	104 (41·6)	29 (11·6)	7 (2·8)	110 (44·0)	85·6 (80·6, 89·7)
Stool Xpert-Ultra	+	-	+	-	
n = 377	114 (30·2)	35 (9·3)	11 (2·9)	217 (57·6)	87·8 (84·1, 90·9)
Eswatini					
Sputum Xpert-Ultra	+	-	+	-	
n = 113	59 (52·2)	7 (6·2)	9 (8·0)	38 (33·6)	85·8 (78·0, 91·7)
Sputum Culture	+	-	+	-	
n = 86	53 (61·6)	9 (10·5)	3 (3·5)	21 (24·4)	86·0 (76·9, 92·6)
Stool Xpert-Ultra	+	-	+	-	
n = 168	59 (35·1)	11 (6·5)	4 (2·4)	94 (56·0)	91·1 (85·7, 94·9)
Mozambique					
Sputum Xpert-Ultra	+	-	+	-	
n = 166	58 (34·9)	20 (12·0)	4 (2·4)	84 (50·6)	85·5 (79·3, 90·5)
Sputum Culture	+	-	+	-	
n = 132	50 (37·9)	18 (13·6)	2 (1·5)	62 (47·0)	84·8 (77·6, 90·5)
Stool Xpert-Ultra	+	-	+	-	
n = 162	53 (30·9)	24 (14·8)	4 (2·5)	81 (50·0)	82·7 (76·0, 88·2))
Tanzania					
Sputum Xpert-Ultra	+	-	+	-	
n = 34	1 (2·9)	2 (5·9)	3 (8·8)	28 (82·4)	85·3 (68·9, 95·0)
Sputum Culture	+	-	+	-	
n = 27	1 (3·1)	2 (6·3)	2 (6·3)	25 (84·4)	87·5 (71·0, 96·5)
Stool Xpert-Ultra	+	-	+	-	
n = 38	2	0	1	35	93·6 (82·5, 98·7)

Abbr: + denotes a positive result for the reference or comparator test in the same row and – denotes a negative result for the reference or comparator test in the same row. [1]The proportion of agreement observed was calculated as the sum of tests in agreement as negative or positive divided by the sum of all test results.

Supplemental Table 2. **Paired comparisons of stool qPCR and stool Xpert sensitivity compared to a complete microbiologic reference standard.**

Paired comparisons of stool qPCR and stool Xpert sensitivity compared to the Complete MRS[1]			
Positive by Complete MRS	**Stool qPCR Sensitivity**	**Stool Xpert-Ultra Sensitivity**	**p-value**
	% (95% CI)	% (95% CI)	
PLHIV (N = 79)	81·0 (70·6, 89·0)	68.4 (56·9, 78·4)	**0·016**
HIV Negative (N = 57)	89·5 (78·5, 96·0)	91·2 (80·7, 97·1)	1·00
Adult (N = 118)	86·3 (78·7, 92·0)	77·8 (69·2, 84·9)	**0·034**
Adolescent (N = 20)	75·0 (50·9, 91·3)	75·0 (50·9, 91·3)	1·00
Complete (N = 137)	84·7 (77·5, 90·3)	77·4 (69·4, 84·1)	**0·044**

[1]Complete Microbiologic Reference Standard (Complete MRS) Positive if sputum culture, sputum Xpert-Ultra and/or LAM in PLHIV when indicated are positive, valid results for all reference tests required for inclusion

Supplemental Table 3: Characteristics of participants with tuberculosis with a detectable *M tuberculosis*-specific DNA by a novel qPCR test and undetectable *M tuberculosis*-specific DNA by Xpert-Ultra performed on stool, Xpert-Ultra performed on sputum, *M tuberculosis*-specific culture performed on sputum, and a negative urine LAM in people living with HIV (n=11).

SITE	GROUP	SEX	HIV	Stool qPCR Result	qPCR CT	CXR RESULTS
MZ	TB Case	Male	Negative	MTB Gene Amplified	32·60	Abnormal; consistent with TB
MZ	TB Case	Male	Negative	MTB Gene Amplified	34·14	Abnormal; consistent with TB
MZ	TB Case	Male	Negative	MTB Gene Amplified	33·23	Abnormal; consistent with TB
MZ	TB Case	Male	Negative	MTB Gene Amplified	34·17	Normal
MZ	TB Case	Male	Negative	MTB Gene Amplified	34·03	Abnormal; consistent with TB
MZ	TB Case	Female	Positive	MTB Gene Amplified	34·04	Not available
MZ	TB Case	Female	Positive	MTB Gene Amplified	31·64	Abnormal; consistent with TB
MZ	TB Case	Male	Negative	MTB Gene Amplified	29·22	Abnormal; consistent with TB
MZ	TB Case	Female	Negative	MTB Gene Amplified	32·70	Not available
MZ	TB Case	Male	Positive	MTB Gene Amplified	34·15	Abnormal; consistent with TB
MZ	TB Case	Male	Negative	MTB Gene Amplified	33·91	Abnormal; consistent with TB

Abbr: MZ; Mozambique, qPCR; quantitative polymerase chain reaction, CT; cycle threshold, CXR; chest x-ray; TB; tuberculosis

Supplemental Table 4. Characteristics of controls with detectable *M tuberculosis*-specific DNA by a novel stool qPCR test (n = 4) (A) and controls with detectable *M tuberculosis*-specific DNA by Xpert-Ultra (n = 2) performed on stool (B).

A.

SITE	Age	Sex	HIV status	Sputum Xpert-Ultra	Culture	Stool Xpert-Ultra	Asymptomatic at Baseline	Asymptomatic at 6 months	TB treatment initiation based on clinical diagnosis	qPCR CT Value
EW	<= 19	M	NR	NA	NA	MTB NOT detected	NA	YES	NO	36.55
EW	<= 19	M	NR	NA	NA	MTB NOT detected	YES	YES	NO	23.96
MZ	20 and above	M	NR	MTB NOT detected	NTM	MTB NOT detected	YES	YES	NO	34.08
MZ	20 and above	M	Pos	MTB NOT detected	CTD	MTB NOT detected	YES	NA	NO	34.69

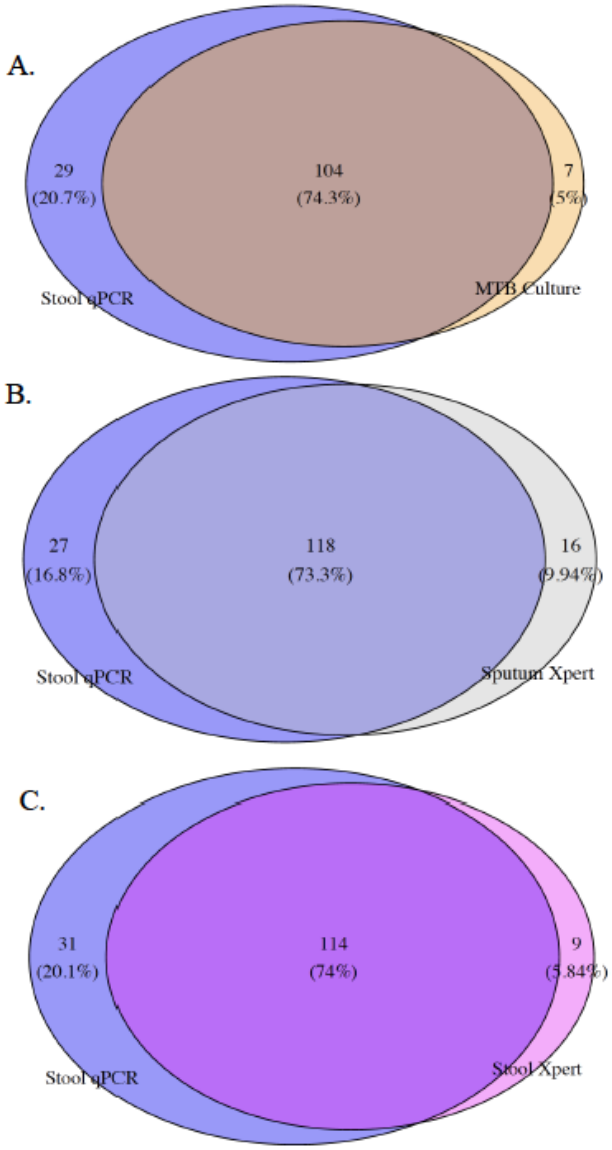
Abbr: MZ; Mozambique, EW; Eswatini, qPCR; quantitative polymerase chain reaction, NR; non-reactive, Pos; positive, CT; cycle threshold, MTB; Mycobacterium tuberculosis, NA; Not available, CTD; contaminated, NTM; non-tuberculous mycobacteria

B.

SITE	Age	Sex	HIV status	Sputum Xpert-Ultra	Culture	Stool qPCR	Asymptomatic at Baseline	Asymptomatic at 6 months	TB treatment initiation based clinical diagnosis	Xpert-Ultra SQ
TZ	<= 19	F	NR	NA	NA	MTB Gene NOT Amplified	YES	NA	NO	Trace
EW	20 and above	F	Pos	NA	NA	MTB Gene NOT Amplified	YES	YES	NO	Trace

Abbr: TZ; Tanzania, EW; Eswatini, qPCR; quantitative polymerase chain reaction, MTB; Mycobacterium tuberculosis, NA; Not available, NR; non-reactive, Pos; positive, SQ; semi-quantitative

Supplemental Figure 1 A-C: Proportional Venn diagrams of the stool qPCR as compared to individual diagnostic tests.

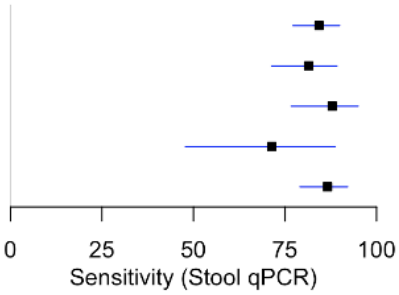


Supplemental Figure 1 Legend: Among participants with a diagnosis of tuberculosis and when both tests were performed, the stool qPCR results were compared to other tests of interest. Additive value was determined by the number of participants detected by stool qPCR, but not detected by the comparator test. A. Participants with tuberculosis and available *M tuberculosis* culture result from sputum and qPCR result from stool where the test result was positive by either or both tests (N = 140). B. Participants with tuberculosis and available Xpert-Ultra result from sputum and qPCR result from stool where the test result was positive by either or both tests (N = 163). C. Participants with tuberculosis who completed stool Xpert-Ultra and stool qPCR and where either test or both tests had positive results (N = 154).

Supplemental Figure 2 A-B. Diagnostic Accuracy of the stool the novel qPCR and stool Xpert-Ultra performed on stool specimen for the diagnosis of tuberculosis when compared to the complete microbiologic reference standard.

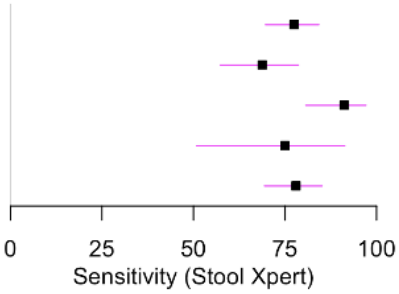
A.

Cohort and Reference Standard	TP	FN	Sensitivity (95% CI)
Complete MRS	118	22	84·3% (77·2, 89·9)
PLHIV, Complete MRS	66	15	81·5% (71·3, 89·2)
HIV NR, Complete MRS	51	7	87·9% (76·7, 95·0)
Adolescent, Complete MRS	15	6	71·4% (47·8, 88·7)
Adult, Complete MRS	103	16	86·6% (79·1, 92·1)



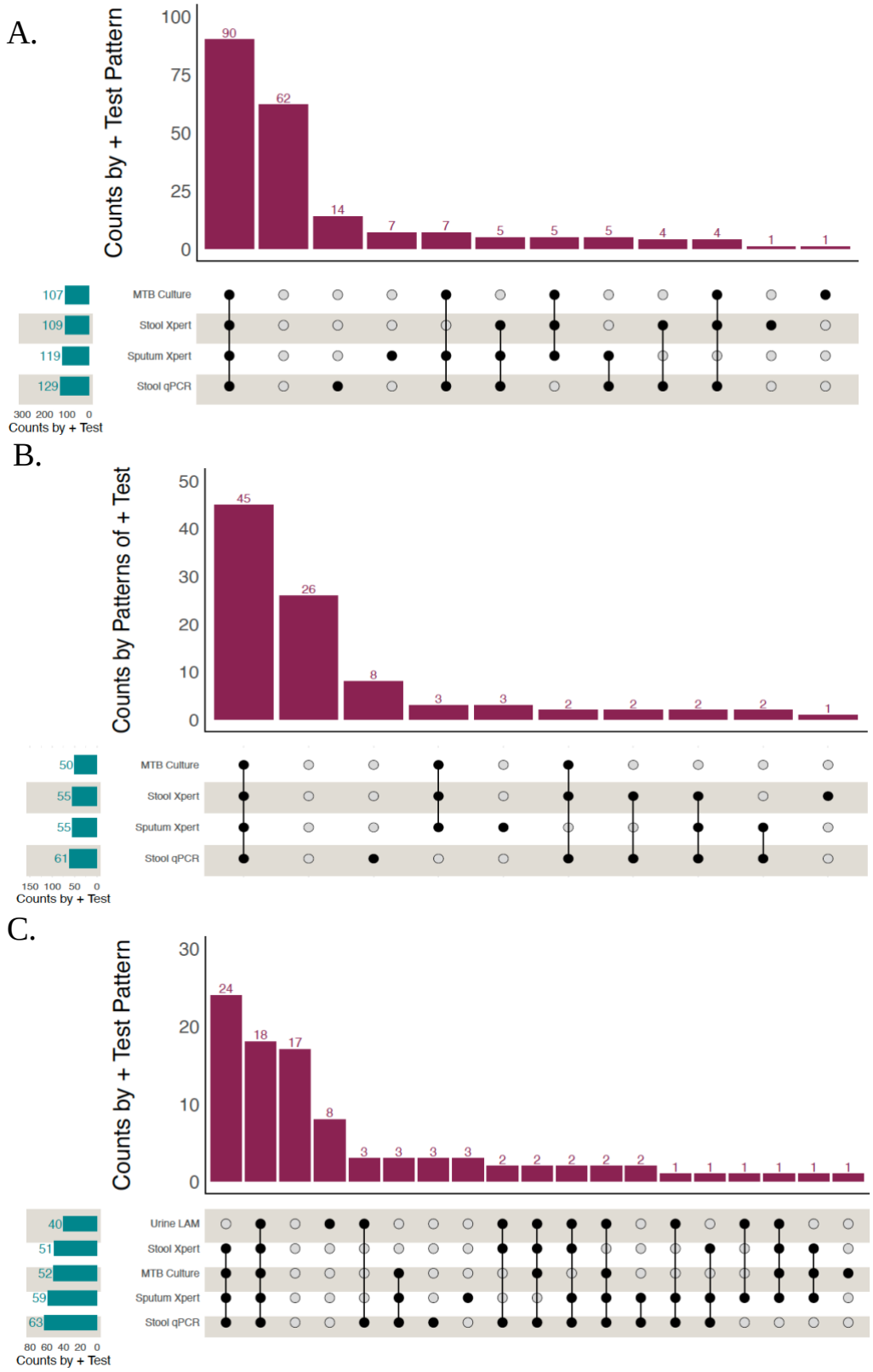
B.

Cohort and Reference Standard	TP	FN	Sensitivity (95% CI)
Complete MRS	107	31	77·5% (69·7, 84·2)
PLHIV, Complete MRS	55	25	68·8% (57·4, 78·7)
HIV NR, Complete MRS	52	5	91·2% (80·7, 97·1)
Adolescent, Complete MRS	15	5	75·0% (50·9, 91·3)
Adult, Complete MRS	92	26	78·2% (69·4, 85·1)



Supplemental Figure 2 Legend: (A) Sensitivity of the stool qPCR test compared with the complete microbiologic reference standard (Complete MRS). (B) Sensitivity of the stool Xpert test compared with the Complete MRS. Abbr: Complete MRS = complete microbiologic reference standard; PLHIV; people living with HIV, TP; true positive, FN; false negative.

Supplemental Figure 3 A-C. Comparative and combined test positivity rates in participants with tuberculosis (including the sub-group living with HIV)



Supplemental Figure 3 Legend A. Comparative and combined test positivity rates in all participants with tuberculosis. Among participants with tuberculosis, 205 completed all diagnostic tests; of these, 143 participants were positive by at least one test. This figure displays the counts of individual test positives (horizontal bars) and the counts of patterns of test positives (vertical bars and dot and lines). **B. Comparative and combined test positivity rates in participants without HIV.** Among participants with tuberculosis and without HIV, 94 completed all diagnostic tests; of these, 68 participants were positive by at least one test. This figure displays the counts of individual test positives (horizontal bars) and the counts of patterns of test positives (vertical bars and dot and lines). **C. Comparative and combined test positivity rates in participants with tuberculosis living with HIV.** Among participants with tuberculosis and living with HIV, 95 participants completed all diagnostic tests inclusive of urine LAM; of these, 78 participants were positive by at least one test. This figure displays the counts of individual test positives (horizontal bars) and the counts of patterns of test positives (vertical bars and dot and line)**.**

Supplemental Materials: Quantitative Polymerase Chain Reaction (qPCR) for stool samples assay protocol

1.0 Prepare 900 µMol/L primers, 100 µMol/L probe, and internal control and MTB standards reagents prior to decrease contamination.

2.0 Prepare the master mix for 400 wells for both the internal control and the MTB plates separately *(Supplemental Table 4).*

3.0 Using a multichannel pipette, aliquot 5µl of the organism specific mastermix to all wells and add 2 µl nuclease free water to the no template control (NTC) wells.

4.0 Cover the plate with aluminum foil. With a pipette, add 2 µl of unknown DNA sample to each well (in duplicates) by piercing through the foil for each well.

5.0 Add 2 µl of prepared standards and then cover the pierced wells with a strip of foil to avoid contamination due to open wells.

6.0 Centrifuge the plate for 1 minute at 1000RPM to settle the reagents.

7.0 Remove the aluminum foil and cover the plate with adhesive optical film, then centrifuge the plate at 1000 RPM for 2 minutes.

8.0 The plate is ready for amplification, put in the Quant Studio machine. The mode should be set to fast, whilst the hold and PCR stage should be 95 ℃ × 20 seconds and 95 ℃ × 1 seconds and 60 ℃ × 20 seconds respectively for 40 cycles. The fluorescence will be captured during the cycle.

9.0 After completion of the run, the test validity should have a correlation value of above 0.95. The standards should be about 3-4 ct values apart.

This table shows the Master Mix constituents with the required volumes and concentrations for both the MTB and internal control plates.

Samples per well: 7 µl Total	**For 100 wells**	**For 400 wells**	**For 800 wells**
Taqman 2x Fast mix: 3.5 µl	350µl	1400µl	2800 µl
Forward primer (900 µM): 0.007 µl	0.7 µl	2.8 µl	5.6 µl
Reverse primer (900 µM): 0.007 µl	0.07 µl	2.8 µl	5.6 µl
Probe (100 µM): 0.0175 µl	1.75 µl	7 µl	14 µl
Water: 1.47 µl	147 µl	588 µl	1176 µl

To prepare standards, dilute 1:1000 by adding 1 µl of IC/ H37Rv DNA in 999 µl of nuclease free water. Make 10-fold dilutions by taking 100 µl of standard 1 into 900 µl nuclease free water. Change tips between each dilution and vortex at maximum speed for 3 seconds in order to mix well. Repeat the above step for the total of 5 standards. Notably, the internal control id run in single and not duplicates.