

1 **Stool Xpert MTB/RIF test for the diagnosis of childhood pulmonary**
2 **tuberculosis at primary clinics in Zimbabwe**

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26 **Abstract**

27 **Objective-** To evaluate the diagnostic performance of Xpert MTB/RIF on stool samples from
28 children clinically suspected of pulmonary tuberculosis at primary care clinics.

29 **Design-** A cross sectional diagnostic study enrolling 5- 16 year olds from whom one induced
30 sputum sample was tested for microbiological tuberculosis confirmation. Results of a single
31 stool sample tested on Xpert MTB/RIF were compared against microbiological confirmed
32 tuberculosis defined as a positive result on smear microscopy and/or culture and/or induced
33 sputum Xpert MTB/RIF. .

34 **Results-** Of the 222 children enrolled, 218 had complete microbiological results. Median age
35 was 10.6 (IQR: 8-13) years. Tuberculosis was microbiologically confirmed in 19 (8.7%) of
36 the 218 children. Of these, 5 (26%), 9 (47%) and 15 (79%) were smear, culture and induced
37 sputum Xpert MTB/RIF positive, respectively. Stool Xpert MTB/RIF testing detected 13
38 (68%) of the 19 microbiologically confirmed cases and was negative in 195 (98%) of 199
39 microbiologically negative cases. Stool Xpert MTB/RIF detected more of HIV-infected than
40 in HIV-uninfected children, 76.9% (10/13) versus 50% (3/6).

41 **Conclusion-** Stool Xpert MTB/RIF could be a potential alternative screening test for
42 children suspected to have tuberculosis if sputum is unavailable, particularly in HIV-infected
43 children. Strategies to optimise diagnostic yield of the stool Xpert MTB/RIF assay need
44 further study.

45

46 **Key words** - Genexpert, faecal specimen, children

47

48 **Introduction**

49 Childhood tuberculosis (TB) contributes about 10% of the total 9 million incident cases
50 worldwide, with an estimated 136 000 deaths in children in 2014 ⁽¹⁾. The burden of TB in
51 children is largely due to undiagnosed and late diagnosis of adult TB which creates a reservoir
52 for transmission to children ⁽²⁾.

53 The major challenge to timely diagnosis of TB in children is the difficulty in obtaining
54 spontaneously expectorated sputum, necessitating the use of sputum induction, which requires
55 skill and infrastructure. Further, sputum from children is often paucibacillary as children are
56 less likely to form cavitory lesions in lungs to contain the TB bacilli ^(2;3). Consequently, the
57 sensitivity of standard diagnostic tests, sputum microscopy and culture, is lower. A more easily
58 accessible sample that can be obtained with minimal skill at primary health care level and an
59 alternative diagnostic tool is therefore required for the diagnosis of TB in children.

60 *Mycobacterium tuberculosis* deoxyribonucleic acid (DNA) can be detected by an automated
61 molecular assay, Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA). The assay is automated
62 and includes sample processing, nucleic acid extraction, polymerase chain reaction and
63 detection of specific codon sequences of *Mycobacteria tuberculosis* within a closed system
64 ^(4;5). The assay has the advantage of producing a result in 3 hours with little hands on time in
65 sample processing.

66 The Xpert MTB/RIF assay has shown high sensitivity in previous studies when sputum ⁽⁶⁻⁸⁾,
67 gastric lavage ^(9;10), broncho-alveolar lavage ⁽¹¹⁾ and nasopharyngeal aspirates ⁽¹²⁾ were used.
68 As a result the assay has been endorsed by the World Health Organisation as an initial test for
69 diagnosing TB in children. However, invasive procedures are required to obtain such samples.

70 Stool is a potential alternative sample that can be collected non-invasively. Children tend to
71 swallow sputum when they cough and *Mycobacterium tuberculosis* DNA has been shown to
72 survive the harsh acidic and digestive environment of the gastro-intestinal tract.⁽¹³⁾ We
73 therefore evaluated the diagnostic performance accuracy of Xpert MTB/RIF using stool in
74 children with clinically suspected pulmonary TB presenting to primary care clinics in Harare,
75 Zimbabwe. The study aimed to determine the sensitivity and specificity of Xpert MTB/RIF
76 testing on stool against confirmed microbiological pulmonary TB determined by testing
77 induced sputum (IS) with microscopy, solid culture and Xpert MTB/RIF.

78 **Methods**

79

80 Participants

81 Participants were recruited from eight primary care clinics in Harare between September 2013
82 and October 2014, with guardian consent and participant assent. These were children aged 5 to
83 16 years presenting with a chronic cough of more than 2 weeks and any one of the classical
84 signs and symptoms of TB, including weight loss, loss of appetite, persistent fever without an
85 apparent cause, night sweats or history of close contact with a TB index patient, defined as
86 living in close proximity (sharing a room within a household) with an adult diagnosed with
87 tuberculosis within the preceding twelve months. Those aged 0 to 5 years were not included as
88 there was insufficient evidence on safety of sputum induction in this age-group, at the time of
89 seeking study approvals, to satisfy the Harare City Health approval committee. Evidence had
90 been especially requested for this vulnerable age-group as sputum induction is not commonly
91 practised in Zimbabwe. Participants were excluded if they had an already confirmed TB
92 diagnosis, were on anti-TB treatment for more than 72 hours prior to enrolment, required
93 emergency medical attention or were resident outside Harare.

94

95 Study procedures

96 A structured questionnaire was used to collect data on demographics and physical examination.
97 HIV status was obtained from documented proof of test result. In case of unknown HIV status,
98 participants were offered the test through the clinic's HIV testing protocols. All participants
99 were asked to provide a stool sample and to undergo sputum induction to obtain one sputum
100 sample.

101 Sputum induction was performed by a trained nurse following the method described by Zar *et*
102 *al.*,⁽¹⁴⁾ except where it was contraindicated⁽¹⁵⁾. The procedure was performed in an open area
103 after a 2-3 hour fast. Two doses of 100µg salbutamol were administered via an aero-chamber
104 to prevent bronchoconstriction, followed by nebulisation with 5% hypertonic saline using a
105 portable nebulisation unit, for five minutes or until the participant started to cough. Oxygen
106 saturation was monitored during and 30 minutes after the procedure. Induced sputum (IS) was
107 collected into a sterile container, using nasopharyngeal suctioning with a sterile mucus
108 extractor if required. If the participant failed to produce sputum the procedure was repeated
109 once, five minutes after the first attempt.

110 The stool sample (about 5g) was collected into a wide mouthed specimen collection jar on spot
111 or submitted the following day. Samples were transported at 4-8⁰C to the laboratory within 8
112 hours of collection.

113 Anti-TB treatment was commenced by the residing medical officer at the clinic following
114 national TB guidelines, where diagnosis is based on clinical history and examination and/or
115 chest radiography and/or microbiological results. Study test results on microbiological
116 confirmation were made available to the medical officer to assist them in participant
117 management.

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119 Laboratory methods

120 The sputum sample was processed for microscopy, culture and Xpert MTB/RIF (Cepheid,
121 Sunnyvale, CA, USA) testing within 24 hours of collection. It was decontaminated by adding
122 an equal volume of 4% sodium hydroxide for 15 minutes and neutralised with phosphate
123 buffered saline (PBS). The mixture was centrifuged at 3200g for 20 minutes to obtain a sputum
124 sediment which was re-suspended in 1.5ml PBS. A drop was used to make smears stained
125 initially using Auramine O method for fluorescent microscopy and confirmed by ZN staining.
126 All smears were re-read by a second reader blinded to the first reading. Discordant results were
127 resolved through a third reading by a different reader. A 0.1ml of IS sediment was cultured on
128 Lowenstein Jensen slopes at 37⁰C for eight weeks with weekly monitoring for growth. At least
129 one colony on Lowenstein Jensen slope was considered to be positive *Mycobacterium*
130 *tuberculosis* growth, confirmed by ZN stain and speciation using the SD Bioline MPB64
131 antigen rapid test. Discordant ZN positive/MPB64 antigen negative samples were speciated
132 using colony morphology, growth at different temperatures and growth on paranitrobenzoic
133 acid containing Lowenstein Jensen slopes. Confirmed cultures underwent phenotypic direct
134 susceptibility testing on Lowenstein Jensen slopes for 4weeks at 37⁰C. For Xpert MTB/RIF
135 (Cepheid, Sunnyvale, CA, USA) testing, 0.5ml of IS sediment was mixed 1.5ml Xpert
136 MTB/RIF reagent and mixture tested according to manufacturer's instructions.

137 Stool samples were processed within two days of collection as described by Nicol *et al.* ⁽¹⁶⁾.
138 Briefly, an aliquot of 0.15g was measured into a screw capped centrifuge tube using a sterile
139 disposable plastic loop. The loop was left inside the tube, 2.4ml of PBS added and the mixture
140 vortexed to remove stool particles from the loop and emulsify the sample. The loop was
141 removed and sample mixture left undisturbed at room temperature for 20 minutes after which

142 two aliquots of 1ml supernatant were removed. One aliquot was used for immediate testing and
143 another stored for repeat testing if required. Prior to testing, the processed stool sample was
144 centrifuged at 3200g for 15 minutes, sediment re-suspended in 1ml PBS, mixed with 2ml Xpert
145 MTB/RIF reagent and tested according to the manufacturer's (Cepheid, Sunnyvale, CA, USA)
146 instructions. Interpretation of results was auto-generated by the Xpert MTB/RIF system, and
147 personnel carrying out the index test were blinded to sputum smear and culture test results.

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149 Data analysis

150 The sample size was based on an anticipated sensitivity of the stool MTB/RIF test of 80%,
151 level of precision set at 10% and an anticipated loss to follow up of 4%. At the time of
152 computation there was no data on prevalence of TB in children in our setting, therefore
153 prevalence was set at 29% adopted from a community study of paediatric TB contacts in South
154 Africa⁽¹⁷⁾.

155 Data was entered into EpiData version 3.1 (Odense, Denmark) and analysed using STATA
156 version 13.0 (StataCorp, Texas, USA). Descriptive statistics were used to characterise the study
157 population. The Chi square test was used to test for associations. The performance of stool
158 Xpert MTB/RIF was assessed as the proportions of stool Xpert MTB/RIF positive cases
159 amongst microbiologically defined TB status and clinically diagnosed TB cases.
160 Microbiological TB was defined as a positive result on either ZN smear microscopy and/or
161 culture and/or Xpert MTB/RIF test on IS. Indeterminate TB status results and samples without
162 complete data on index and reference tests were excluded from analysis. The study proposed
163 to evaluate the performance of stool Xpert MTB/RIF in TB clinical case definitions as defined
164 by Graham et al., 2012⁽¹⁸⁾. However, the number of children reporting having a chest X-ray
165 done was minimal (n = 24) to provide meaningful classification.

166 Ethical approval for the study was obtained from the Harare City Health Department, Joint
167 Parirenyatwa Hospital and College of Health Sciences Research Ethics Committee and by the
168 Medical Research Council of Zimbabwe.

169

170 **Results**

171 A total of 222 participants were recruited. Four participants were excluded from the analysis:
172 one participant was withdrawn by the guardian, two participants were unable to provide

173 induced sputum (IS) samples and one participant had an IS Xpert MTB/RIF test that produced
174 an error result despite repeated testing.

175 The median age of the 218 remaining participants was 10.6 (IQR: 8 -13) years and 44% were
176 male. The median z-scores for weight-for-age and height-for-age were -1.09 (IQR: -1.99 to -
177 0.46) and -1.88 (IQR: -3.70 to -0.73) respectively. HIV status was available for 201 (91%)
178 participants, of whom 111 (51%) were HIV infected, with 55 (49.5%) taking antiretroviral
179 therapy (ART). The distribution of demographic characteristics between microbiological TB
180 and no TB groups is shown in Table 1.

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182 Microbiologically confirmed tuberculosis

183 The overall prevalence of TB by microbiological confirmation, defined as a positive result on
184 either ZN smear microscopy and/or culture and/or Xpert MTB/RIF test on IS, was 8.7%
185 (19/218). Five (26%) out of the 19 microbiological confirmed TB cases were less than 10 years
186 old. Of the 19 microbiologically confirmed TB cases, 5 (26%) were smear positive, 9 (47%)
187 were culture positive and 15 (79%) were Xpert MTB/RIF positive on IS. Only two (10.5%) out
188 of 19 microbiologically confirmed TB cases were positive by all 3 microbiological tests (Figure
189 1). Amongst the culture positive children, only 2 (22%) were smear positive. Rifampicin
190 resistance was not detected in any of the TB cases either by direct susceptibility testing or Xpert
191 MTB/RIF testing. A positive HIV status was documented in 13 (68%) of the 19 microbiological
192 TB cases. Of these 13 children, 7 (54%) children were on ART and 11 (85%) children tested
193 positive with Xpert MTB/RIF sputum (Figure 1).

194 A total of 32 (14.7%) out of 218 children were commenced on anti-tuberculosis treatment, 19
195 (59.4%) of whom were microbiologically confirmed and 13 (40.6%) clinically diagnosed by
196 the attending clinician.

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198 Diagnostic performance of the stool Xpert MTB RIF

199 Xpert MTB/RIF on stool was positive in 17 (8.3%) of 218 children. Of these, 13 (76.5%) had
200 microbiologically confirmed TB. Xpert MTB/RIF stool detected 60%, 66.7% and 86.7% of
201 smear positive, culture positive and IS Xpert MTB/RIF positive cases of TB respectively. Of
202 the 13 microbiologically confirmed TB cases with HIV, Xpert MTB/RIF on stool identified 10
203 (76.9%) cases. Stool Xpert MTB/RIF detected 13 (68%) of the 19 microbiological confirmed

204 TB cases and was negative in 195 (98%) of the 199 children without microbiological
205 confirmation (Table 2). Notably, stool Xpert MTB/RIF performed better in children aged 10
206 years and older compared to those less than 10 years, detecting 71.4% (10/14) and 60% (3/5),
207 respectively. Thirteen children with a clinical diagnosis and commenced on anti-tuberculosis
208 treatment included two (50%) of the four children with a positive stool Xpert MTB/RIF test
209 and without microbiological confirmation. These two children were both HIV-infected and
210 older than 10years (Table 2).

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227 **Discussion**

228 The main finding of this study is that Xpert MTB/RIF testing on stool samples identified 68%
229 of microbiologically confirmed TB and was negative in 98% of children with negative

230 microbiologically results. In addition, similar to findings by Nicol *et al.*,⁽¹⁶⁾ this study showed
231 that Xpert MTB/RIF on stool was more sensitive in HIV-infected than in HIV-uninfected
232 children. Although the test failed to identify 32% of TB cases, it may be of more value in HIV-
233 infected children where there is a high risk of acquiring TB, as well as high risk of disease
234 progression. The routinely available test at primary care centres is smear microscopy which
235 has a sensitivity of less than 10-15% in children with proven TB⁽¹⁹⁻²¹⁾ and performs even more
236 poorly in those with HIV infection⁽²²⁾.

237 The Xpert MTB/RIF is currently being rolled out in many parts of southern Africa for use as
238 an initial test for the diagnosis of TB. The test detects 2 to 3 times more cases than smear
239 microscopy in children^(7-9;20) and additionally provides rapid diagnosis in less than 3 hours.
240 However, its limitation, as well as that of microscopy, is that it requires a sputum sample which
241 is difficult to obtain freely expectorated from children. At primary care level induction of
242 sputum from children is rarely attempted and when performed the quality of specimen varies
243 with personnel which results in variable test sensitivity. Findings by Weday *et al.*, 2014
244 showed that testing of stool samples from children using Xpert MTB/RIF test detected all of
245 the sputum smear positive cases⁽²³⁾. In this study, we have shown that stool Xpert MTB/RIF
246 test identified most of the IS Xpert MTB/RIF positive cases suggesting that when a sputum
247 sample is not readily available a stool sample which is easier to collect may be used with Xpert
248 MTB/RIF test. However, the diagnostic yield of stool Xpert MTB/RIF test still requires
249 optimisation.

250 The inability of stool Xpert MTB/RIF to detect six of the children with microbiological
251 confirmation may possibly be due to the presence of polymerase chain reaction inhibitors
252 present in stool samples which may have interfered with detection of TB in cases. Of late, there
253 is emerging evidence that the use of larger sample volumes of 2cm³⁽²⁴⁾ and 0.6g⁽²⁵⁾ and pre-
254 treatment with a stool processing buffer to inactivate polymerase chain reaction inhibitors⁽²⁵⁾
255 achieves greater diagnostic yield of stool Xpert MTB/RIF test.

256 Stool Xpert MTB/RIF test performed poorly in the clinically diagnosed children. This finding
257 concurs with that of Detjen *et al.*, 2015 in a review of Xpert MTB/RIF testing in children using
258 sputum and gastric aspirates, where the assay showed poor performance in clinically defined
259 probable TB cases⁽²⁶⁾. This potentially indicates a limitation of the assays' detection limit
260 rather than of the sample used as clinical microbiologically negative TB cases are more likely
261 to have paucibacillary disease. The two clinically diagnosed children identified by stool Xpert

262 MTB/RIF were both HIV-infected, again indicating the potential use of the test in HIV
263 infection.

264 The limitation of our study was the lower than expected TB prevalence, which limits study
265 power. Secondly, we used one stool sample collected at random. The use of a second sample
266 has been shown to have incremental value in detecting additional cases ^(6;7;21). Care should be
267 taken when interpreting our findings given that the performance of stool Xpert MTB/RIF was
268 assessed in microbiologically confirmed TB and in children 5 to 16 years old. Different results
269 may be obtained for clinical cases and children less than 5 years in whom TB disease
270 presentation is markedly different.

271 In conclusion, the stool Xpert MTB/RIF holds promise as an alternative test to Xpert MTB/RIF
272 sputum testing, particularly in HIV-infected children. Stool collection is easier and relatively
273 safe compared to sputum collection and may potentially have diagnostic utility if sensitivity is
274 optimised. Further studies are required to determine optimal stool collection times, optimal
275 sample volumes, the need for a second sample, concentration techniques that allow maximum
276 yield and test combination algorithms.

277

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282 M Chipinduro developed the study concept and proposal, supervised the study and was
283 responsible for the main writing of the paper. K Mateveke supervised the data analysis. B
284 Makamure supervised the microbiological tests. RA Ferrand assisted with proposal writing and
285 revised the manuscript writing critically for intellectual content. E Gomo was the academic
286 supervisors for M Chipinduro and supervised the whole research process from proposal writing
287 to manuscript writing. All authors read and approved the final manuscript.

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290 Conflict of interest

291 The authors declare no conflict of interest.

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375 Tables

376 Table 1: Baseline characteristics for TB and non TB patients

Characteristic	TB patients (n = 19) n (%)	No TB patients (n = 199) n (%)	p value
Age, median (IQR)	13 (9 -15)	11 (8 -13)	
Sex			
Female	12 (63)	111 (56)	0.56
Age			
5- < 10	5(26)	95 (48)	0.07
≥10-16	14 (74)	104 (52)	0.07
History of contact			
Yes	4 (21)	47 (24)	0.77
History of TB			
Yes	2 (11)	15 (8)	0.65
HIV status			
Positive	13 (68)	^a 98 (55)	0.28
On ART			
Yes	7 (54)	48 (49)	0.74
Presenting symptoms			
fever	14 (74)	^b 91 (47)	0.03
weight loss	^c 13 (81)	^d 109 (60)	0.10
night sweats	^e 8 (44)	^f 76 (40)	0.74

This table includes 218 children (19 microbiologically confirmed TB patients and 199 non TB patients) and excludes 4 children without microbiological results.

^a Excluded 20 no TB patients without documented proof of HIV test, ^bExcluded 5 no TB patients who did not know history of fever, excluded ^c3 and ^d18 TB and no TB patients respectively who did not know history of weight loss, excluded ^e1 and ^f11 TB and no TB patients respectively who did not know history of night sweats

TB is Tuberculosis (pulmonary), HIV is human immunodeficiency virus and ART is anti-retroviral therapy

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388 Table 2: Diagnostic performance of stool Xpert MTB/RIF in microbiologically determined TB status
 389 and in clinically diagnosed TB cases

		Microbiological TB Result N = 218									
		Positive					Negative				
		All	HIV+	HIV-	<10yr	≥10yr	All	HIV+	HIV-	<10yr	≥10yr
		N	N=13	N=6	s	s	N=19	*N=9	*N=8	s	s
		=19	n(%)	n(%)	N=5	N=14	9	8	1	N=95	N=10
		n(%)			n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	4
											n(%)
Stool	+	13	10	3	3	10	4	4	0	1	3
Xpert		(68)	(76.9	(50)	(60)	(71.4)	(2.0)	(4.1)	(0.0)	(1.1)	(2.9)
MTB/RIF))))))))))
F	-	6	3	3	2	4	195	94	81	94	101
Test		32.6	(23.1	(50)	(40)	(28.6)	(98)	(95.9)	(100)	(98.9)	(97.1)
))))))))))

		Clinically Diagnosed TB				
		All	HIV+	HIV-	<10yr	≥10yr
		N=13	^N=7	^N=	s	s
		n(%)	n(%)	3	N=6	N=7
				n(%)	n(%)	n(%)
Stool	+	2	2	0	0	2
Xpert		(15.4	(28.6	(0.0)	(0.0)	(28.6)
MTB/RIF)))))
F	-	11	5	3	6	5
Test		(84.6	(71.4	(100	(100)	(71.4)
)))))

TB is tuberculosis (pulmonary), HIV is human immunodeficiency virus, + is positive, - is negative and n is the number of correct results expressed as a percentage (%).

* 20 children without HIV test result excluded; ^ 3 children without HIV test result excluded. Two of the 4 children with a negative microbiological test result and stool Xpert MTB/RIF positive received a clinical diagnosis.

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397 Figure Legend

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399 Figure 1: Venn diagram for positive smear microscopy, culture and Xpert MTB/RIF on induced
400 sputum (IS). This diagram includes 19 children with microbiological tuberculosis. Numbers in
401 brackets indicate HIV infected cases. All the 19 microbiological confirmed children received anti-
402 tuberculosis treatment.

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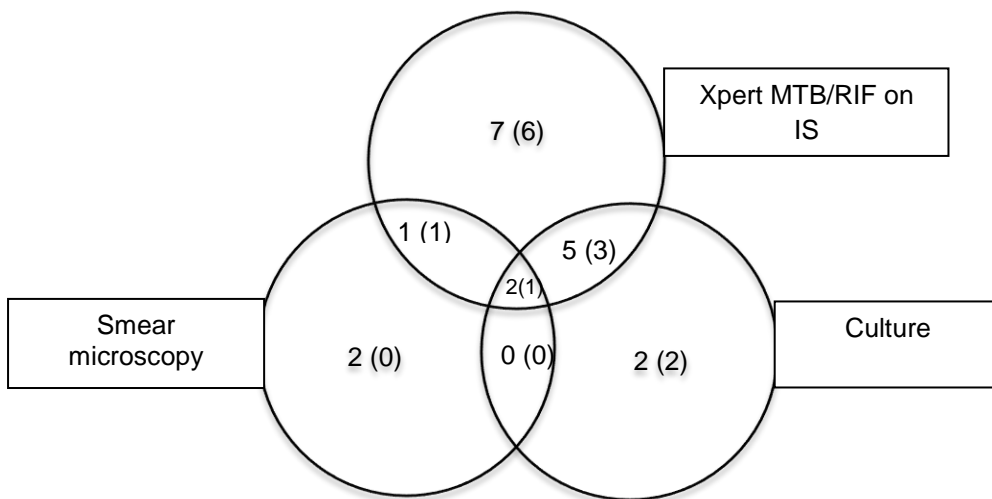
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424 **Figure 1**



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