Thin layer agar-based direct phenotypic drug-susceptibility testing on sputum in Eswatini rapidly detects *Mycobacterium tuberculosis* growth and rifampicin resistance, otherwise missed by WHO endorsed diagnostic tests.

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Running title: TLA detects rifampicin resistance in Eswatini

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Abstract

BACKGROUND: Xpert®MTB/RIF rapidly detects resistance to rifampicin (RR), however this test misses the I491F-RR conferring *rpoB* mutation, common in Southern Africa. In addition, Xpert®MTB/RIF does not distinguish between viable and dead *Mycobacterium tuberculosis* (MTB).

OBJECTIVE: To investigate the ability of thin layer agar (TLA) direct drug-susceptibility testing (DST) to detect MTB and its drug-resistance profiles in field conditions in Eswatini.

DESIGN: Consecutive samples were tested in parallel with Xpert®MTB/RIF and TLA for rifampicin (1.0 µg/ml) and ofloxacin (2.0 µg/ml). TLA results were compared at the Reference Laboratory in Antwerp with indirect DST on Löwenstein-Jensen or 7H11 solid media and additional phenotypic and genotypic testing to resolve discordance.

RESULTS: TLA showed a positivity rate for MTB detection of 7.1% versus 10.0% for Xpert®MTB/RIF. Of a total of 4547 samples included in the study, 200 isolates were available for comparison to the composite reference. Within a median of 18.4 days, TLA detected RR with 93.0% sensitivity (CI-77.4-98.0) and 99.4% specificity (CI 96.7-99.9), versus 62.5% (CI 42.7-78.8) and 99.3% (CI 96.2-99.9) for Xpert®MTB/RIF. Eight isolates, 28.6% of all RR confirmed isolates, carried the I491F mutation, all detected by TLA. TLA also correctly identified 183 of the 184 ofloxacin-S isolates (99.5% specificity, CI 97.0-99.9).

CONCLUSIONS: In field conditions, TLA rapidly detects RR, and in this specific setting contributed to detection of additional RR patients over Xpert®MTB/RIF, mainly but not exclusively due to I491F. TLA also accurately excluded fluoroquinolones resistance.

Background

In 2019, globally about 10 million people developed tuberculosis (TB), and half a million people developed TB resistant to rifampicin (RR-TB) [1]. Even if rifampicin (RMP) drug-susceptibility testing (DST) coverage at TB diagnosis increased to 61%, a considerable number of patients with undetected RR-TB are still treated with an ineffective rifampicin-based treatment regimen. These patients are at high risk of treatment failure, and continue spreading RR-TB[1].

To enhance RR-detection, the End TB strategy recommends to improve case detection and DST coverage, also with the use of molecular techniques. The tests used most commonly to detect drug resistance are Xpert®MTB/RIF (Cepheid, USA), which simultaneously detects *Mycobacterium tuberculosis* complex (MTB) and RR, and line probe assays (LPA) such as Genotype MTBDR*plus,* (Hain, Lifescience, Germany), for isoniazid (INH) and RMP, and MTBDR*sl* for fluoroquinolone (FQ) and second-line injectables [2]. The roll-out of Xpert®MTB/RIF, also in peripheral laboratories, substantially decreased diagnostic delay [3][4]. However, these rapid molecular techniques miss specific *rpoB* mutants at positions outside the RR determining region of the *rpoB* gene (RRDR), which are associated with equally poor treatment outcomes as 'common' *rpoB* mutants [5]. One example is the I491F RR-conferring mutation, among the so called disputed mutations [6], which in Eswatini accounts for 56% of all RR-TB cases [7], reason of grave concern [8][9]. Furthermore, I491F is in most of the cases tested as false rifampicin sensitive (RS) in MGIT phenotypic DST (pDST) and is only detected by sequencing of the entire *rpoB* gene or slow pDST on solid medium, such as Löwenstein-Jensen (LJ) [9][10][11]. Isolates with these mutations are also partially missed by other non-commercial methods as microscopic observation direct susceptibility testing (MODS), with a reported sensitivity of 75% [12].

77 TLA has previously been described as an affordable method to detect MTB [13][14]. This
78 technique has limited costs [15] and can provide results considerably faster than indirect
79 pDST methods. Direct Thin Layer Agar DST (referred as TLA below) is not among the non-
80 commercial methods recommended by WHO [16]. However, when TLA is used for
81 simultaneous MTB and drug resistance detection, clinical samples are inoculated
82 immediately after decontamination on the medium with and without antibiotics [17]
83 avoiding the intermediate step of MTB isolation, which eliminates the need for high-level
84 biohazard containment such as needed for indirect pDST [18].
85 These characteristics make this test suitable for low resource settings [19], although studies
86 on its applicability in routine practice are still limited. In settings where the prevalence of
87 I491F mutation is low, TLA has shown similar overall performance compared to indirect
88 MGIT-DST in terms of sensitivity, specificity and turnaround time [17][15]. However, the
89 ability of TLA to detect RR due to mutations outside the RRDR is unknown.
90 The primary objective of this study was to investigate the use of TLA as direct pDST test for
91 RR-TB detection in a peripheral laboratory, applied to smear-positive (Sm+) and smear-
92 negative (Sm-) sputum samples in a setting with high prevalence of the *rpoB* I149F mutation.
93 In addition, we describe the test performance for detection of MTB and resistance to
94 ofloxacin (OFX), as indicator for FQ resistance, key class of drug in the treatment of RR-TB.
95 DST results were evaluated in comparison to a composite reference standard that included
96 genotypic plus phenotypic testing.

97 ## Materials and Methods

98 **Study patients and test flow**

99 The study was conducted in the Nhlangano TB laboratory (NTBL), Eswatini, by Médecins Sans
100 Frontières-Switzerland and the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The

catchment area included 22 health clinics and 3 health centres (including Nhlangano) at up to 90 km of distance (map in Figure 1), from where samples were sent to NTBL in cold chain (2-8°C) without preservatives.

We included all samples collected from consecutive patients older than 15 years, who presented signs of presumptive TB, did not start TB treatment in the previous month, and consented to participate in this study. As per routine practice, patients were asked to produce two good quality sputum specimens (labelled as sample A and sample B) in 50 ml sterile screw-cap containers and, when collected at the clinics, sent at the health centres, where sample A was tested by Xpert®MTB/RIF following Cepheid procedures. Any patients Xpert®MTB/RIF positive for TB on sample A, regardless of the RMP result, had sample B sent to the National TB Reference Laboratory (NTBRL) in Mbabane for routine testing with liquid culture and pDST. In this case, sample C was collected for the purpose of this study. All leftovers from sample A, sample B, and sample C, if collected, were decontaminated and tested for the study with TLA, fluorescence microscopy (FM), Xpert®MTB/RIF and LJ culture at the NTBL. For any positive TLA plate, inoculated with either sample A, B or C, the corresponding LJ isolate was sent to ITM for extended phenotypic and genotypic testing. In case the LJ culture was contaminated or remained negative, an LJ subculture from the growth of the corresponding TLA plate was shipped.

Laboratory methods at NTBL

We performed TLA using 4-quadrant polystyrene plates prepared at NTBL. The medium contained 7H11 agar supplemented with a broad-spectrum antibiotic mixture to suppress contamination, as previously described [15][17]. TLA plates included: drug-free growth control (GC), p-Nitrobenzoic acid (PNB) (500 µg/ml), RMP (1.0 µg/ml) and OFX (2.0 µg/ml). The processed sputum sediment was resuspended with 2 ml phosphate buffer (PBS) and two

drops were inoculated per each well.

During incubation at 5% CO_2, plates were read at day 5, 7 10, 13, 15, 20, 25, 30, 35, 40, as previously described [15] with few modifications. In addition, the drug-containing wells were read on the day of GC positivity and on the next scheduled reading.

Plates were reported as positive for non-tuberculous mycobacteria (NTM) in case of mycobacterial growth in the quadrant containing PNB, while no or poor growth (≤ 3 colonies) on PNB with a positive GC compartment was considered positive for MTB [20]. Any growth on PNB was tested with the MPT64Ag test (SD Bioline).

Laboratory methods at the reference laboratory

Upon receipt of isolates at ITM, fresh subcultures were made on LJ medium, and indirect-DST was performed using the proportion method on LJ for RMP (40 µg/ml) and on 7H11 for OFX (2.0 µg/ml). Indirect solid DSTs were read blindly with respect to TLA.

All isolates with a discordant RMP result between any two of three tests (Xpert®MTB/RIF, TLA or indirect-DST) were further tested by MTBDR*plus,* had pDST done in MGIT (RMP 1.0 µg/ml), and the minimal inhibitory concentration (MIC) for RMP was determined on LJ (10-160 µl/ml). All isolates showing RR on any test and 100 isolates showing RS on all tests were investigated by Sanger sequencing of the *rpoB* target at ITM and/or by whole genome sequencing (WGS) performed at the Borstel Research Center (Germany), as previously described [21][22]. To constitute a composite reference standard for RMP resistance, MIC prevailed on pDST, and resistance found on any of the genotypic tests overrode any phenotypic result.

In case of discordance between TLA and indirect-DST for OFX, isolates were investigated by LPA MTBDR*sl* and sequencing (target genes and/or WGS). As composite reference standard, any resistance related to high confidence mutation found on any of the genotypic DST

overrode results showing susceptibility on another test.

Statistical analysis

For all tests conducted at the NTBL, we calculated the MTB positivity rate as the number of samples showing MTB over the total number of samples tested. The relative gain of TLA for detection of MTB over Xpert®MTB/RIF was calculated as the number of samples that were TLA positive but Xpert®MTB/RIF negative or inconclusive (including error, invalid or no result) divided by the total number of samples positive on Xpert®MTB/RIF. The relative gain of Xpert®MTB/RIF versus TLA was calculated as well. We also calculated the median turnaround time (TAT) between sample collection and inoculation and the median time from inoculation to each test result, and TAT for DST availability stratified by RMP resistance.

For all isolates received at ITM, we calculated the sensitivity and specificity (with 95% confidence intervals; CI) of TLA to detect resistance for RMP and specificity for OFX, against the composite reference standards. Implausible discordant results (e.g. non-RRDR mutation on WGS but detected by Xpert [23]), were considered administrative errors and excluded from the analysis.

All statistical analyses were performed with Stata 12 software (Stata Corporation, College Station).

Ethics approval

The study protocol was approved by the Institutional Review Board of ITM, the Ethics Committee of the University Hospital of Antwerp, Belgium, and the Ethics Committee of Eswatini.

Results

MTB detection

Between January 2014 and December 2016, 3097 patients provided a total of 4547 samples. The overall MTB positivity rate was 7.1% (322/4547) for TLA, and 10.0% (456/4547) for Xpert®MTB/RIF (Table 1). Among Sm+ samples, TLA positivity was 68.6% (138/201) versus 90.7% (176/194) for Xpert®MTB/RIF, compared to 3.7% (153/4107) versus 5.7% (234/4130) respectively for Sm- samples ($p<0.0001$ in both groups).

Among Xpert®MTB/RIF-negative or inconclusive samples (n=3972), the relative gain of TLA for detection of MTB, when performed as follow on test after Xpert®MTB/RIF was 7.5% (34/456), while the reverse, the relative gain of Xpert®MTB/RIF over TLA for MTB detection, was 55.9% (180/322).

The median turnaround time (TAT) between sample collection and TLA inoculation was 4 days (IQR 2-7), while the median time from inoculation to TLA positivity was 11 days (IQR 7-19).

When inoculation was performed within 4 days, or since 5 days or later from sample collection, the TLA positivity rate for Sm+ samples decreased from 72.6% to 59.0% (p=0.053), while the negative rate increased from 20.1 % to 28.3.8% (p=0.16), though not reaching statistical significance. Contamination varied from 7.4% to 12.1% (p=0.27).

Results available for evaluation of TLA for detection of drug resistance

Out of 322 MTB positive TLA plates, 214 (66.5%) had the corresponding LJ slants sent to ITM (Figure 3). At ITM, 200 (93.5%) of the isolates grew after subculturing. Four (2.0%) isolates were classified as administrative errors (table S1), leaving 196 (98%) isolates available for analysis.

RMP resistance detection

Of 196 isolates included and with valid indirect-DST results at ITM, 168 (85.7%) had a valid initial Xpert®MTB/RIF result. For another 28 (14.3%) samples Xpert®MTB/RIF results were either indeterminate or not available, while TLA was concordant with indirect- DST (Table 2). After resolution versus the composite reference standard, a total of 168 (85.7%) isolates were finally classified as RS and 28 (14.3%) RR. Of the 168 samples with valid Xpert®MTB/RIF result, 157 (93.5%), were concordant between Xpert®MTB/RIF and TLA (142 RS and 15 RR), while eleven samples (6.5%) had discordant results between the two tests (Table 2). Of the 142 RS TLA and Xpert®MTB/RIF concordant samples, one was RR by indirect-DST, making a total of 12 (6.1%) discordances for the 196 isolates tested between any two of the three tests.

By Xpert®MTB/RIF , 9 (5.4%) isolates were falsely reported as RS and one falsely showed RR, giving a sensitivity of 62.5% (15/24; CI 42.7-78.8) and specificity of 99.3% (143/144; CI 96.2-99.9). On TLA, RR was missed in two isolates and in another isolate TLA falsely showed RR. Hence, the sensitivity of TLA to detect RR was 93.0% (26/28; 95% CI 77.4-98.0) and specificity 99.4% (167/168; 95% CI 96.7-99.9).

Most discrepancies between TLA and Xpert®MTB/RIF RMP results were due to the presence of non-RRDR mutations outside the Xpert®MTB/RIF target (five I491F and one V170F mutation), or wild type (WT) *rpoB* genes with variable phenotypic results. All non-RRDR mutations were detected by TLA, and had a RMP-MIC ≥ 160 µg/ml. While V170F in one isolate was detected by MGIT, only 2 of 5 I491F mutations were detected by the liquid medium (Table 2).

In addition to the five I491F mutants found among TLA-Xpert®MTB/RIF discrepant results, another three I491F mutants were detected: one I491F in combination with M434I was RR

by all tests, two (one showing I491F mutation alone, and one in combination with M434I and S450L) had no Xpert®MTB/RIF RMP DST results (MTB not detected or RMP indeterminate). So, in total, 8/28 (28.6%) of the confirmed RR isolates had the I491F mutation, all of them showing also a S315T mutation in *katG* by WGS.

Two paired isolates from the same patient were RR by all phenotypic tests and Xpert®MTB/RIF yet had a WT *rpoB* sequence (Table 2). Two additional isolates were consistently phenotypic RR (indirect-DST LJ, MIC-LJ, MGIT and TLA in one isolate), while all molecular assays (Xpert®MTB/RIF, LPA, Sanger sequencing and WGS) suggested a WT *rpoB* gene. In both isolates, WGS detected a P631S mutation in the *ponA1* region (Table 2).

One isolate, carrying the H445L elusive mutation was RS by Xpert®MTB/RIF and RR by indirect-DST LJ.

Overall, the median time to detect RR was 18.4 days, similar to 17.0 days for resistance conferred by I491F alone (p=0.8) and 18.3 days for isolates carrying other RR conferring mutations, but significantly longer than the 12.2 days for RS isolates (p=0.03) (Figure 2).

OFX drug resistance detection

Overall, of the 196 isolates included in the analysis, 185 had valid indirect-DST results in ITM. One isolate, carrying mutation D94N, was OFX-R 2.0 µg/ml on indirect-DST and correctly detected by TLA. A total of 184 isolates were classified as OFX-S, all except one correctly identified by TLA (183/184; specificity 99.5%; CI 97.0-99.9 (Table 3), including three isolates OFX-R by indirect-DST but susceptible by molecular tests. One isolate was false OFX-R, but WT for all molecular tests.

Discussion

This study evaluated the TLA performance for MTB detection and direct-DST for RMP, alongside OFX testing. In Nhlangano, a peripheral and low resource setting, TLA showed a

241 relatively high positivity rate for MTB detection, albeit below the ≥83% between smear
242 positive samples reported by other studies [13][14]. In our study culture positivity was
243 slightly affected by delay in sample processing, while contamination rate did not significantly
244 increase. These results suggest that TLA may be suitable in laboratories at peripheral levels,
245 where samples collected from remote areas are transported for testing.
246 TLA performed excellent to detect RR after a median of 18.2 days.
247 In our study, more than 80% of the discordances between initial Xpert®MTB/RIF and TLA
248 were resolved in favor of TLA, which also correctly detected all I491F mutations, which
249 accounted for almost half of all discordances.
250 In Eswatini the prevalence of I491F is reason of concern. This mutation is regularly missed by
251 Xpert®MTB/RIF, limitation that persists in the new version Xpert®MTB/RIF Ultra [24], with
252 patients misdiagnosed as having RS-TB and receiving repeated rounds of ineffective first line
253 treatment. The national drug-resistance survey carried out in Eswatini in 2017-2018 [7] has
254 shown that the prevalence of I491F in MDR isolates has reached 56% compared to 30%
255 detected by the survey from 2009[9][11]. In our study, I491F caused 28.6% of all RR, although
256 our findings may not be representative for the entire country. Indeed, WGS analysis showed
257 that isolates with I491F mutations belong to a particular outbreak clone that evolved over
258 time and acquired further resistances to first- and second-line drugs [25][26]. Thus, isolates
259 with this particular mutation, not detected by standard diagnostic tests, is an enormous
260 public health problem. To improve rapid detection of these missed RR cases, the new
261 algorithm proposed by the National TB Program in 2019 includes starting empirical MDR-TB
262 treatment for all detected INH-R cases, while pursuing pDST on solid medium and sequencing
263 of the *rpoB* gene to determine RR [27]. This approach is supported by our results, where all
264 the isolates with I491F mutations carried also mutations in *katG* gene, correlated to INH

265 resistance. In this algorithm, TLA could play a role to rapidly detect these RR cases at
266 peripheral laboratory equipped for moderate hazardous containment (BSL2 level) [18],
267 while waiting for sequencing results.

268 Partial fitness loss for isolates carrying the I491 mutation has been proposed as a reason for
269 false RS results in MGIT due to the short incubation time (maximum 14 days) [10][5]. Despite
270 the relatively short turnaround time on TLA (median 17.0 days), none of the I491F strains
271 were false RS on TLA, compared to three of the five MGIT tested. The time to detection for
272 the I491F mutants did not differ from the ones carrying other RR-conferring mutations, albeit
273 collectively the *rpoB* mutants grew significantly slower than *rpoB* wildtype isolates on
274 primary isolation.

275 Besides I491F, another non-RRDR mutation (V170F) detected in one isolate, was missed by
276 Xpert®MTB/RIF and LPA, yet detected by TLA. This mutation is globally less frequent [28], is
277 reliably detected by pDST including MGIT, and has not yet led to known micro-epidemics.

278 In our study, one (16.7%) of the isolates carrying a H445L elusive mutation, showed also WT
279 minority population. RR was detected by TLA, LJ-based pDST and Sanger sequencing, but
280 missed by rapid molecular tests and MGIT. The mutation was detected by WGS only at low
281 frequency. Indeed, heteroresistance may be the cause of false-susceptible results. Tests have
282 different limits of detection for minority populations: as low as 1% for phenotypic tests, 5%
283 for MTBDR*plus*, 20-40% for Xpert®MTB/RIF classic, 20% for Sanger sequencing [29] and 1%
284 for WGS [30], depending on coverage depth. In addition, detection of minority populations
285 and variants causing resistance can be challenged by preselection during primary culture
286 isolation and multiple subcultures [31].

287 Surprisingly two isolates, phenotypically RR by indirect-DST, MGIT and LJ-MIC and in one case
288 by TLA, were WT *rpoB* Sanger sequencing while WGS showed presence of mutation P631S in

289 *ponA1*. Polymorphisms in this region, that encodes for a protein involved in mycobacterial
290 growth and cell wall synthesis, seem to constitute and advantage for growth in presence of
291 rifampicin and modifies susceptibility to this drug [32][33]. Even if rare, the role of this
292 mutation and other non-*rpoB* resistance conferring-mechanisms, should be further
293 investigated.
294 Of the three TLA-OFX-S isolates found OFX-R on indirect-DST yet WT by sequencing, two
295 (paired isolates from the same patient), were borderline resistant by the indirect-DST, as
296 they showed the same number of colonies in the drug-free and -containing tubes, one of
297 them positive only with three colonies. Although these borderline results could be the cause
298 of discordances, indirect-DST could not be repeated. For the third, no obvious explanation
299 for the discrepancy could be found.
300 Laboratories in peripheral settings usually lack high-risk TB facilities with stable power
301 supply, negative pressure and complex equipment that is regularly maintained, which limits
302 the implementation of phenotypic testing, especially indirect-DST [34]. TLA direct-DST,
303 similarly to MODS, poses less biosafety risk, lowering requirements to moderate-risk facilities
304 to contain biohazard potentially created by samples centrifugation and vortex [18]. In a head-
305 to-head comparison, TLA showed to be superior to MODS in detecting resistance due to
306 I491F mutation [12].
307 Our study is the first to test TLA for RMP and OFX in a high endemic setting at district level,
308 although numbers did not suffice for full assessment of OFX performance.
309 A drawback pointed out for direct-TLA DST is the lack of standardization of the inoculum [35],
310 although we did not find an association of bacterial load (as determined by smear
311 microscopy) and false DST results. Secondly, the direct nature of TLA testing complicates
312 appropriate quality controls, for which fresh sputum is needed, as manipulation of strains

313 would increase biosafety requirements. However the use of samples spiked with avirulent
314 strains, compatible with BSL2 hazard containment, could be considered for this purpose [36].
315 For 4 isolates (2.0%), discordant results could not be explained, and for this reason
316 considered as administrative errors. These errors are not infrequent in diagnostic
317 laboratories [37] that handle high amount of tests.
318 The TLA technique requires multiple readings of the plates, which is time consuming. Our
319 cumulative data suggests that the workload could be reduced by decreasing the number of
320 readings, as readings at day 28 and 35 provided relatively low incremental yield. For future
321 testing, reading at day 7, 14, 21 and 42 may be logistically more feasible, as well as the use
322 of a redox indicator allowing for macroscopic reading, as suggested by recent studies
323 [38][39].
324 Our study presented some limitations. It was not possible to link on site multiple samples
325 collected from the same patient, so that a per patient analysis of the results was not possible.
326 In addition, confirmatory tests were performed only on isolates showing discordances. TLA
327 showed to correctly exclude resistance in all susceptible cases, however our samples did not
328 include a sufficient number of isolates resistant to OFX to fully evaluate TLA performance. In
329 addition, molecular tests for concordant OFX tests were only partially available. In our study,
330 OFX was considered as an indicator for resistance to the class of FQs. The full performance
331 of TLA for detection of FQ resistance, possibly including other FQs than OFX, should be
332 assessed in settings with higher rates of FQ-R, or when TLA is used after Xpert RR diagnosis.
333 Nevertheless, our study shows that TLA provides numerous advantages.
334 In settings with high prevalence of I491 mutations, TLA could be very useful for patients with
335 presumed RR-TB yet Xpert®MTB/RIF RS-TB. Especially in patients with INH-R and missed RR-
336 TB, the use of a FQ-strengthened regimen as recommended by WHO guidelines [40], would

result in ethambutol and pyrazinamide as sole effective drugs, increasing the risk of developing XDR-TB [41].

The relative gain for MTB detection by TLA when used after Xpert was limited, confirming that Xpert should be the first test for diagnosis[15][17]. However, TLA could be used for monitoring patients during treatment, also to detect amplification of RR-TB in patients failing first-line treatment, especially if missed at baseline. Validation of TLA for new- and repurposed drugs would be the logical next step, given the WHO recommended pDST, as TLA could replace in the flow chart indirect-DST in patients found to have molecular evidence of RMP and/or INH resistance. The level of phenotypic resistance to bedaquiline that is conferred by *Rv0678* mutations is for instance largely unknown, and TLA could save weeks over indirect pDST, at a lower biosafety level.

Conclusions

TLA provides a relatively rapid diagnostic approach for detecting viable TB bacilli and simultaneous susceptibility testing for RMP showing excellent sensitivity for the detection of RR due to the I491F mutation outside of the RRDR that plagues Southern Africa. In the diagnostic algorithm of this setting, TLA could be used to test presumed RR patients yielding an RS result by Xpert®MTB/RIF.

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523 Table 1 Detection of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria, for TLA
524 versus Xpert®MTB/RIF

	TLA										
	Neg		**Pos**		**NTM**		**Cont**		**Unknown**		**Total**
	N	%	N	%	N	%	N	%	N	%	N
Total	**3857**	**84.8**	**322**	**7.1**	**11**	**0.2**	**255**	**5.6**	**102**	**2.2**	**4547**
Xpert											
Neg	3536	91.2	32	0.8	10	0.3	218	5.6	83	2.1	**3879**
Pos	161	35.3	274	60.1	1	0.2	18	3.9	2	0.4	**456**
High	24	15	124	77.5	1	0.6	11	6.9	0	0	**160**
Medium	39	35.1	70	63.1	0	0	2	1.8	0	0	**111**
Low	39	39.4	56	56.6	0	0	3	3	1	1	**99**
Very Low	59	68.6	24	27.9	0	0	2	2.3	1	1.2	**86**
Inconclusive	76	81.7	2	2.2	0	0	12	12.9	3	3.2	**93**
Unknown	84	70.6	14	11.8	0	0	7	5.8	14	11.8	**119**

525 TLA=thin layer agar; Xpert=Xpert®MTB/RIF; MTB=Mycobacterium tuberculosis complex; NTM = non-tuberculous
526 mycobacteria; Cont=contamination; Inconclusive= error, invalid, no result

527 Table 2. Rifampicin test results of TLA and Xpert®MTB/RIF, against composite reference standard

CRS	N	*rpoB* target Sanger/ WGS	LJ	Xpert	TLA RMP DST	MDRTBplus	LJ-MIC (µg/ml)	MGIT DST	TLA final interpretation	TLA – Xpert concordance
RMP R (n=28)	1	M434I; I491F/ M434I; I491F[¥]	R	R	R	na	na	na	TRUE RR	Concordant
	3	na/ H445L	R	R	R					
	8	na/ S450L	R	R	R					
	2	WT/WT[€]	R	R	R					
	1	H445D/H445D	R	NEG	R	na	na	na	TRUE RR	na
	1	I491F;S450L;M434I/ I491F;S450L;M434I[¥]	R	NEG	R					
	1	I491F/I491F[¥]	R	IND	R					
	1	S450L/S450L	R	na	R					
	5	I491F/I491F	R	S	R	WT	160->320[§]	3S, 2R	TRUE RR	Discordant
	1	H445L+WT/WT*	R	S	R	WT	160	S		
	1	V170F/V170F	R	S	R	WT	160	R		
	1	WT/WT°	R	S	R	WT	160	R		
	1	S450L/S450L	R	R	S	delWT,MUT3	160	R	FALSE RS	Discordant
	1	WT/WT°	R	S	S	WT	160	R	FALSE RS	Concordant
RMP S (n=168)	99	na/WT	S	S	S	na	na	na	TRUE RS	Concordant
	6	WT/nd	S	S	S					
	36	na/na	S	S	S					
	24	na/na	S	na	S	na	na	na	TRUE RS	na
	1	WT/WT	S	R	S	WT	20	S	TRUE RS	Discordant
	1	WT/WT	R	S	S	WT	20	S	TRUE RS	Concordant
	1	WT/WT	S	S	R	WT	20	S	FALSE RR	Discordant

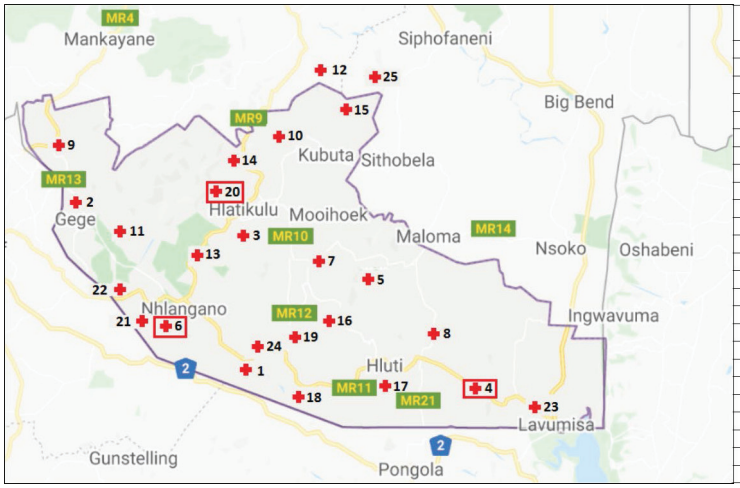
528 CRS= composite reference standard; WGS=whole genome sequencing; Xpert=Xpert®MTB/RIF; LJ= Löwenstein-Jensen; TLA= thin layer agar; RMP=rifampicin; MIC=minimal
529 inhibitory concentration; MGIT=mycobacterial growth indicator tube; na= not available; §=one result invalid; ¥= isolates with I491F mutations either detected also by Xpert, or
530 without Xpert result; IND= RMP indeterminate; €= paired isolates with *rpoB* sequencing WT results; °=P631S mutation in the *ponA1* region;*=rpoB H445L+ L452P detected at
531 low frequency mode.

532 Table 3 Thin layer agar results versus indirect-DST and composite reference standard for resistance to Ofloxacin

OFX result by CRS	N of isolates	*gyrA/B* target Sanger sequencing/WGS	LPA	LJ (µg/ml) OFX 2.0	TLA (µg/ml) OFX 2.0	TLA final interpretation
OFX-R (n=1)	1	na/D94N	na	R	R	TRUE OFX R
OFX-S (n=184)	117	na/WT	na	S	S	TRUE OFX S
	1	na/WT	na	S	R	FALSE OFX R
	62	na/na	na	S	S	TRUE OFX S
	1	WT/na	WT	S	S	TRUE OFX S
	3	WT/WT*	WT	R	S	TRUE OFX S

533 CRS= composite reference standard; TLA=Thin layer agar; OFX=ofloxacin; WGS=whole genome sequencing;
534 na=not available;*= for 1 isolate WGS not done;

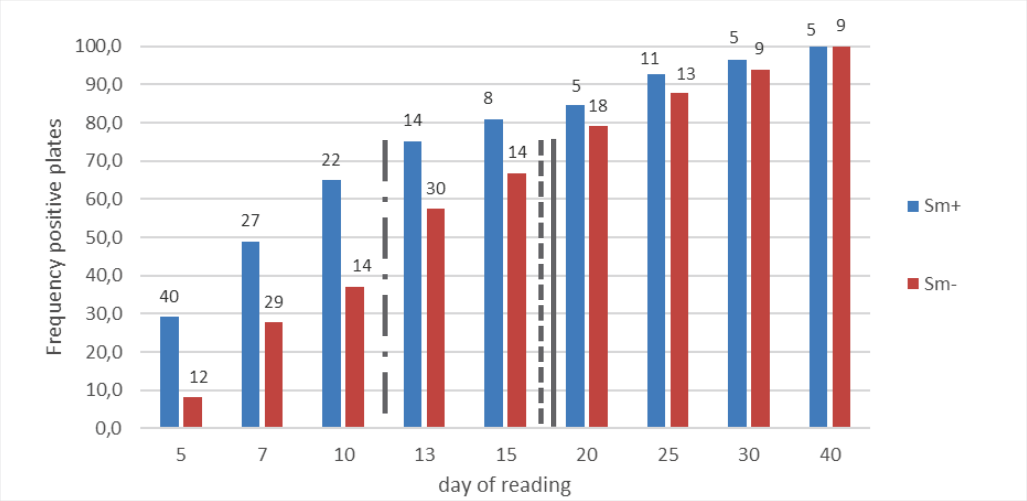
535 Figure 1 Sample collection sites in Shiselweni region



	Site name	N of samples collected	Distance (Km)	Time (min)
1	Zombodze	372	79.2	76
2	Gege	287	31.2	48
3	Nhletjeni	71	22.8	31
4	Matsanjeni	256	0	0
5	Ntshanini	150	48.1	70
6	Nhlangano HC	855	0	0
7	New Haven	238	37.9	52
8	JCI	24	90.3	77
9	Magubheleni	516	42.0	67
10	Mfishane	43	22.6	30
11	Mashobeni	353	20.7	38
12	Moti	23	39	55
13	Dwaleni	356	16	20
14	Nkwene	76	47.3	39
15	Kaphunge GVT	17	40.6	66
16	Silele	91	38.7	53
17	Hluti	101	21.5	18
18	Olos	80	31.5	29
19	Jericho	79	35.9	45
20	Hlathikhulu	72	0	0
21	Mahlandle	87	20	25
22	Tfokotoani	264	15.4	18
23	Lavumisa	89	17.4	14
24	Mhlosheni	36	49	40
25	Kaphunga	10	40.6	66

536
537 (Google map, modified). Distance and time of driving from the site of collection to the closest of the three TB laboratories (in red squares)

Figure 2 Number of positive plates and cumulative percentage per day of reading



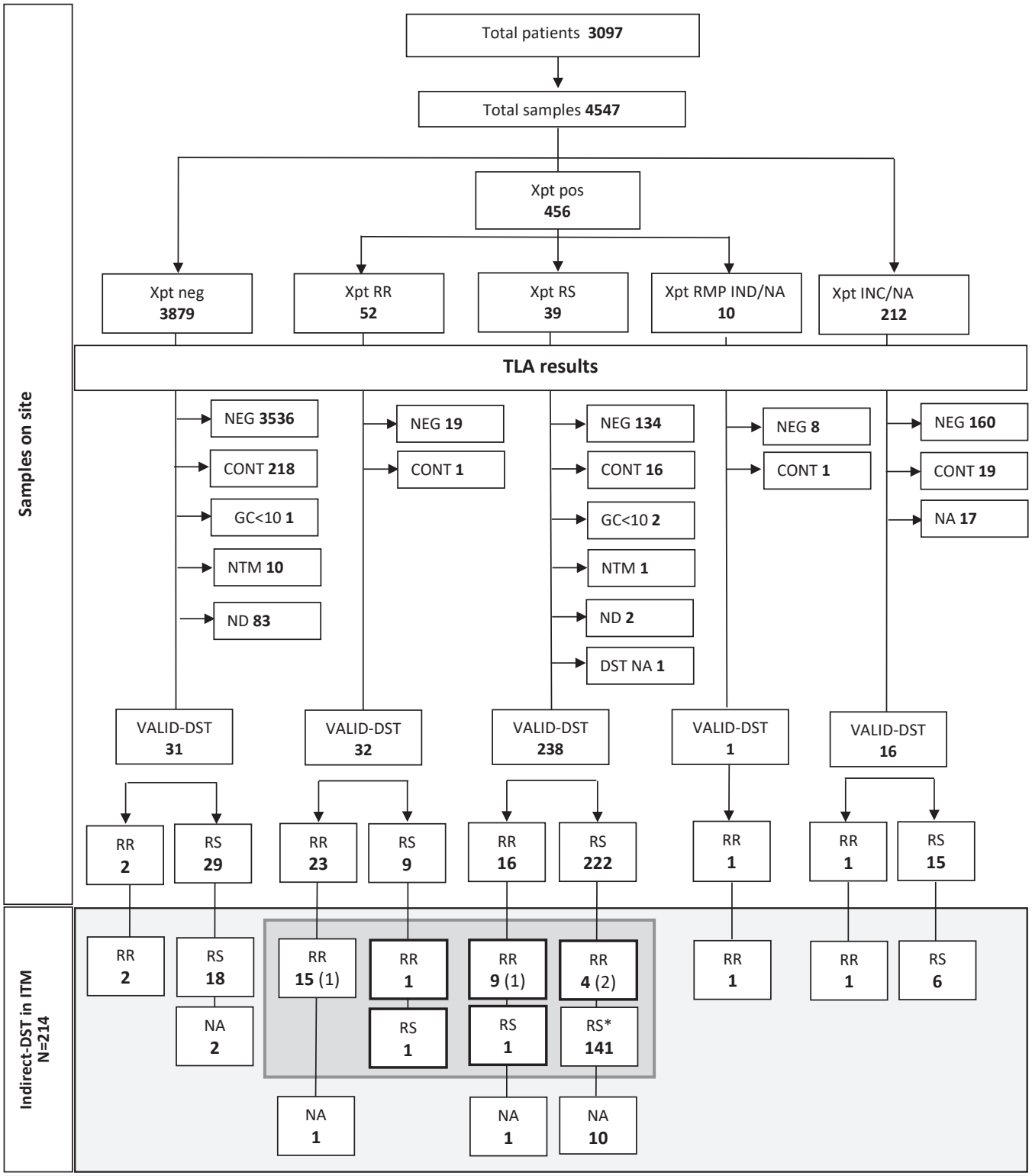
Sm+ = sputum smear microscopy positive; Sm- = sputum smear microscopy negative. Indication of median positivity and drug-susceptibility results for:

rifampicin-susceptible S isolates

rifampicin-resistant isolates with mutations different from I491F

rifampicin-resistant isolates with I491F MUT only

Figure 3 Phenotypic rifampicin-resistance testing results from the site (direct- TLA) and the reference laboratory (indirect LJ)



Xpt=Xpert®MTB/RIF; INC= result inconclusive (error, invalid, no result); RMP= rifampicin; GC=growth control; NTM= nontuberculous mycobacteria; IND=indeterminate; NA= not available; (n)=administrative errors excluded; for details refer to Table S1. In the gray area, results for 172 isolates with indirect-DST, Xpert and TLA results available, including administrative errors; * = 100 isolates selected for WGS, in addition to all RR detected by any method