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.

E. Ardizzoni1*, E. Ariza2, D. Mulengwa3, Q. Mpala3, R. de La Tour4, G. Maphalala5, F. Varaine6, B. Kerschberger4, P. Graulus1, A. L. Page7, S. Niemann8,9, V. Dreyer8,9, A. Van Deun7, T. Decroo7,10, L. Rigouts1,11, B. C. de Jong1

1 Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium
2 Infectious Diseases Service, Hospital Clinic-IDIBAPS, Barcelona, Spain
3 Médecins Sans Frontières, Mbabane, Eswatini
4 Médecins Sans Frontières, Rue de Lausanne 78, 1202, Geneva, Switzerland
5 Ministry of Health (NRL), Mbabane, Eswatini
6 Médecins Sans Frontières, 34 Avenue Jean Jaurès, 75019 Paris, France
7 Épicentre14 - 34 Avenue Jean Jaurès, 75019 Paris, France
8 Molecular and Experimental Mycobacteriology, Research Center Borstel, Germany
9 German Center for Infection Research (DZIF), Partner Site Hamburg-Borstel-Lubeck D-23845 Borstel Germany
10 Research Foundation Flanders, Brussels, Belgium
11 University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

Running title: TLA detects rifampicin resistance in Eswatini

Keywords: Resistance detection, TLA, Xpert®MTB/RIF, tuberculosis, MDR, XDR, rpoB I491F mutant

*Corresponding author: eardizzoni@itg.be

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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 MTBDRplus, (Hain, Lifescience, Germany), for isoniazid (INH) and RMP, and MTBDRsl 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].
TLA has previously been described as an affordable method to detect MTB [13][14]. This technique has limited costs [15] and can provide results considerably faster than indirect pDST methods. Direct Thin Layer Agar DST (referred as TLA below) is not among the non-commercial methods recommended by WHO [16]. However, when TLA is used for simultaneous MTB and drug resistance detection, clinical samples are inoculated immediately after decontamination on the medium with and without antibiotics [17] avoiding the intermediate step of MTB isolation, which eliminates the need for high-level biohazard containment such as needed for indirect pDST [18].

These characteristics make this test suitable for low resource settings [19], although studies on its applicability in routine practice are still limited. In settings where the prevalence of \textit{I491F} mutation is low, TLA has shown similar overall performance compared to indirect MGIT-DST in terms of sensitivity, specificity and turnaround time [17][15]. However, the ability of TLA to detect RR due to mutations outside the RRDR is unknown.

The primary objective of this study was to investigate the use of TLA as direct pDST test for RR-TB detection in a peripheral laboratory, applied to smear-positive (Sm+) and smear-negative (Sm-) sputum samples in a setting with high prevalence of the \textit{rpoB I49F} mutation. In addition, we describe the test performance for detection of MTB and resistance to ofloxacin (OFX), as indicator for FQ resistance, key class of drug in the treatment of RR-TB. DST results were evaluated in comparison to a composite reference standard that included genotypic plus phenotypic testing.

\textbf{Materials and Methods}

\textbf{Study patients and test flow}

The study was conducted in the Nhlangano TB laboratory (NTBL), Eswatini, by Médecins Sans Frontières-Switzerland and the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The
catchment area included 22 health clinics and 3 health centres (including Nhlangeko) 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
125 drops were inoculated per each well.

126 During incubation at 5% CO₂, plates were read at day 5, 7, 10, 13, 15, 20, 25, 30, 35, 40, as
127 previously described [15] with few modifications. In addition, the drug-containing wells were
128 read on the day of GC positivity and on the next scheduled reading.

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

133 **Laboratory methods at the reference laboratory**

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

137 All isolates with a discordant RMP result between any two of three tests (Xpert®MTB/RIF,
138 TLA or indirect-DST) were further tested by MTBDRplus, had pDST done in MGIT (RMP 1.0
139 μg/ml), and the minimal inhibitory concentration (MIC) for RMP was determined on LJ (10-
140 160 μl/ml). All isolates showing RR on any test and 100 isolates showing RS on all tests were
141 investigated by Sanger sequencing of the rpoB target at ITM and/or by whole genome
142 sequencing (WGS) performed at the Borstel Research Center (Germany), as previously
143 described [21][22]. To constitute a composite reference standard for RMP resistance, MIC
144 prevailed on pDST, and resistance found on any of the genotypic tests overrode any
145 phenotypic result.

146 In case of discordance between TLA and indirect-DST for OFX, isolates were investigated by
147 LPA MTBDRsl and sequencing (target genes and/or WGS). As composite reference standard,
148 any resistance related to high confidence mutation found on any of the genotypic DST
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% (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
relatively high positivity rate for MTB detection, albeit below the ≥83% between smear positive samples reported by other studies [13][14]. In our study culture positivity was slightly affected by delay in sample processing, while contamination rate did not significantly increase. These results suggest that TLA may be suitable in laboratories at peripheral levels, where samples collected from remote areas are transported for testing.

TLA performed excellent to detect RR after a median of 18.2 days.

In our study, more than 80% of the discordances between initial Xpert®MTB/RIF and TLA were resolved in favor of TLA, which also correctly detected all I491F mutations, which accounted for almost half of all discordances.

In Eswatini the prevalence of I491F is reason of concern. This mutation is regularly missed by Xpert®MTB/RIF, limitation that persists in the new version Xpert®MTB/RIF Ultra [24], with patients misdiagnosed as having RS-TB and receiving repeated rounds of ineffective first line treatment. The national drug-resistance survey carried out in Eswatini in 2017-2018 [7] has shown that the prevalence of I491F in MDR isolates has reached 56% compared to 30% detected by the survey from 2009[9][11]. In our study, I491F caused 28.6% of all RR, although our findings may not be representative for the entire country. Indeed, WGS analysis showed that isolates with I491F mutations belong to a particular outbreak clone that evolved over time and acquired further resistances to first- and second-line drugs [25][26]. Thus, isolates with this particular mutation, not detected by standard diagnostic tests, is an enormous public health problem. To improve rapid detection of these missed RR cases, the new algorithm proposed by the National TB Program in 2019 includes starting empirical MDR-TB treatment for all detected INH-R cases, while pursuing pDST on solid medium and sequencing of the rpoB gene to determine RR [27]. This approach is supported by our results, where all the isolates with I491F mutations carried also mutations in katG gene, correlated to INH
resistance. In this algorithm, TLA could play a role to rapidly detect these RR cases at peripheral laboratory equipped for moderate hazardous containment (BSL2 level) [18], while waiting for sequencing results.

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

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

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

Surprisingly two isolates, phenotypically RR by indirect-DST, MGIT and LJ-MIC and in one case by TLA, were WT \textit{rpoB} Sanger sequencing while WGS showed presence of mutation P631S in
Polymorphisms in this region, that encodes for a protein involved in mycobacterial growth and cell wall synthesis, seem to constitute an advantage for growth in presence of rifampicin and modifies susceptibility to this drug [32][33]. Even if rare, the role of this mutation and other non-rpoB resistance-conferring mechanisms, should be further investigated.

Of the three TLA-OFX isolates found OFX-R on indirect-DST yet WT by sequencing, two (paired isolates from the same patient), were borderline resistant by the indirect-DST, as they showed the same number of colonies in the drug-free and containing tubes, one of them positive only with three colonies. Although these borderline results could be the cause of discordances, indirect-DST could not be repeated. For the third, no obvious explanation for the discrepancy could be found.

Laboratories in peripheral settings usually lack high-risk TB facilities with stable power supply, negative pressure and complex equipment that is regularly maintained, which limits the implementation of phenotypic testing, especially indirect-DST [34]. TLA direct-DST, similarly to MODS, poses less biosafety risk, lowering requirements to moderate-risk facilities to contain biohazard potentially created by samples centrifugation and vortex [18]. In a head-to-head comparison, TLA showed to be superior to MODS in detecting resistance due to I491F mutation [12].

Our study is the first to test TLA for RMP and OFX in a high endemic setting at district level, although numbers did not suffice for full assessment of OFX performance. A drawback pointed out for direct-TLA DST is the lack of standardization of the inoculum [35], although we did not find an association of bacterial load (as determined by smear microscopy) and false DST results. Secondly, the direct nature of TLA testing complicates appropriate quality controls, for which fresh sputum is needed, as manipulation of strains...
would increase biosafety requirements. However the use of samples spiked with avirulent strains, compatible with BSL2 hazard containment, could be considered for this purpose [36]. For 4 isolates (2.0%), discordant results could not be explained, and for this reason considered as administrative errors. These errors are not infrequent in diagnostic laboratories [37] that handle high amount of tests.

The TLA technique requires multiple readings of the plates, which is time consuming. Our cumulative data suggests that the workload could be reduced by decreasing the number of readings, as readings at day 28 and 35 provided relatively low incremental yield. For future testing, reading at day 7, 14, 21 and 42 may be logistically more feasible, as well as the use of a redox indicator allowing for macroscopic reading, as suggested by recent studies [38][39].

Our study presented some limitations. It was not possible to link on site multiple samples collected from the same patient, so that a per patient analysis of the results was not possible. In addition, confirmatory tests were performed only on isolates showing discordances. TLA showed to correctly exclude resistance in all susceptible cases, however our samples did not include a sufficient number of isolates resistant to OFX to fully evaluate TLA performance. In addition, molecular tests for concordant OFX tests were only partially available. In our study, OFX was considered as an indicator for resistance to the class of FQs. The full performance of TLA for detection of FQ resistance, possibly including other FQs than OFX, should be assessed in settings with higher rates of FQ-R, or when TLA is used after Xpert RR diagnosis. Nevertheless, our study shows that TLA provides numerous advantages.

In settings with high prevalence of I491 mutations, TLA could be very useful for patients with presumed RR-TB yet Xpert®MTB/RIF RS-TB. Especially in patients with INH-R and missed RR-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 \textit{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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Table 1 Detection of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria, for TLA versus Xpert®MTB/RIF

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</tbody>
</table>

TLA=thin layer agar; Xpert=Xpert®MTB/RIF; MTB=Myobacterium tuberculosis complex; NTM = non-tuberculous mycobacteria; Cont=contamination; Inconclusive= error, invalid, no result
Table 2. Rifampicin test results of TLA and Xpert®MTB/RIF, against composite reference standard

<table>
<thead>
<tr>
<th>CRS</th>
<th>N</th>
<th>rpoB target Sanger/ WGS</th>
<th>LJ</th>
<th>Xpert</th>
<th>TLA RMP DST</th>
<th>MDRTplus LJ-MIC (μg/ml)</th>
<th>MGIT DST</th>
<th>TLA final interpretation</th>
<th>TLA – Xpert concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>M434I; I491F; M434I; I491F</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>na/ H445L</td>
<td>R</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>na/ S450L</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>WT/WT</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>Concordant</td>
</tr>
<tr>
<td>RMP R</td>
<td>(n=28)</td>
<td>H445D/H445D</td>
<td>R</td>
<td>NEG</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>I491F; S450L; M434I; I491F</td>
<td>R</td>
<td>NEG</td>
<td>R</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>I491F; I491F</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>TRUE RR</td>
<td>na</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>I491F/ I491F</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>WT 160, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>H445D + WT/ WT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>WT 160, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>V170F/ V170F</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>WT 160, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>WT/WT</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>WT 160, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>S450L/ S450L</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>delWT, MUT3</td>
<td>160, R</td>
<td>FALSE RS</td>
<td>Discordant</td>
</tr>
<tr>
<td>RMP S</td>
<td>(n=168)</td>
<td>W17/WT</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>WT 160, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>na/WT</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>na</td>
<td>na</td>
<td>TRUE RS</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>WT/nd</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>na</td>
<td>na</td>
<td>TRUE RS</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>na/na</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>na</td>
<td>na</td>
<td>TRUE RS</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>na/na</td>
<td>S</td>
<td>na</td>
<td>S</td>
<td>na</td>
<td>na</td>
<td>TRUE RS</td>
<td>Concordant</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>WT/WT</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>WT 20, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>WT/WT</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>WT 20, &gt;320</td>
<td>S, 2R</td>
<td>Discordant</td>
<td></td>
</tr>
</tbody>
</table>

CRS= composite reference standard; WGS=whole genome sequencing; Xpert=Xpert®MTB/RIF; LJ= Lö wenstein-Jen sen; TLA= thin layer agar; RMP=rifampicin; MIC=minimal inhibitory concentration; MGIT=mycobacterial growth indicator tube; na= not available; §=one result invalid; ¥= isolates with I491F mutations either detected also by Xpert, or without Xpert result; IND= RMP indeterminate; €= paired isolates with rpoB sequencing WT results; *= rpoB H445D+ L452P detected at low frequency mode.
Table 3 Thin layer agar results versus indirect-DST and composite reference standard for resistance to Ofloxacin

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

CRS= composite reference standard; TLA=Thin layer agar; OFX=ofloxacin; WGS=whole genome sequencing; na=not available; *= for 1 isolate WGS not done;
Figure 1 Sample collection sites in Shiselweni region

<table>
<thead>
<tr>
<th>Site name</th>
<th># of samples collected</th>
<th>Distance (km)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Xhobene</td>
<td>323</td>
<td>78.2</td>
<td>76</td>
</tr>
<tr>
<td>2. Gagie</td>
<td>287</td>
<td>33.2</td>
<td>46</td>
</tr>
<tr>
<td>3. Nelirini</td>
<td>71</td>
<td>22.8</td>
<td>31</td>
</tr>
<tr>
<td>4. Matshini</td>
<td>256</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5. Nhlanhlo</td>
<td>554</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6. Nhlangano</td>
<td>555</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7. Nkwe Haven</td>
<td>238</td>
<td>51.9</td>
<td>52</td>
</tr>
<tr>
<td>8. K3</td>
<td>24</td>
<td>90.9</td>
<td>77</td>
</tr>
<tr>
<td>9. Magwedini</td>
<td>536</td>
<td>42.0</td>
<td>57</td>
</tr>
<tr>
<td>10. Methweni</td>
<td>43</td>
<td>22.6</td>
<td>30</td>
</tr>
<tr>
<td>11. Mashephini</td>
<td>353</td>
<td>207.3</td>
<td>38</td>
</tr>
<tr>
<td>12. Mati</td>
<td>23</td>
<td>39</td>
<td>55</td>
</tr>
<tr>
<td>13. Maweni</td>
<td>356</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>14. Mlaweni</td>
<td>79</td>
<td>47.3</td>
<td>39</td>
</tr>
<tr>
<td>15. Mphungu G4V</td>
<td>37</td>
<td>40.6</td>
<td>66</td>
</tr>
<tr>
<td>16. Motlhe</td>
<td>95</td>
<td>38.7</td>
<td>53</td>
</tr>
<tr>
<td>17. Mbeli</td>
<td>103</td>
<td>21.5</td>
<td>18</td>
</tr>
<tr>
<td>18. Mpho</td>
<td>60</td>
<td>35.5</td>
<td>29</td>
</tr>
<tr>
<td>19. Mircho</td>
<td>79</td>
<td>35.9</td>
<td>45</td>
</tr>
<tr>
<td>20. Mthethu</td>
<td>72</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21. Mahendir</td>
<td>87</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>22. Malintwani</td>
<td>264</td>
<td>35.4</td>
<td>18</td>
</tr>
<tr>
<td>23. Mpetha</td>
<td>89</td>
<td>17.4</td>
<td>14</td>
</tr>
<tr>
<td>24. Mphofu</td>
<td>36</td>
<td>49</td>
<td>40</td>
</tr>
<tr>
<td>25. Mphitha</td>
<td>10</td>
<td>40.1</td>
<td>66</td>
</tr>
</tbody>
</table>

(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)

<table>
<thead>
<tr>
<th>Total samples</th>
<th>4547</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xpt pos</td>
<td>456</td>
</tr>
<tr>
<td>Xpt neg</td>
<td>3879</td>
</tr>
<tr>
<td>Xpt RR</td>
<td>52</td>
</tr>
<tr>
<td>Xpt RS</td>
<td>39</td>
</tr>
<tr>
<td>Xpt RMP IND/NA</td>
<td>10</td>
</tr>
<tr>
<td>Xpt INC/NA</td>
<td>212</td>
</tr>
</tbody>
</table>

**Samples on site**

- **NEG 3536**
- **CONT 218**
- **GC<10 1**
- **NTM 10**
- **ND 83**

**TLA results**

- **VALID-DST 31**
  - **RR 2**
  - **RS 29**
- **VALID-DST 32**
  - **RR 23**
  - **RS 9**
- **VALID-DST 238**
  - **RR 16**
  - **RS 222**
- **VALID-DST 1**
  - **RR 1**
  - **RS 1**
- **VALID-DST 16**
  - **RR 1**
  - **RS 6**

**Indirect-DST in NTM**

- **RR 2**
- **RS 18**
- **NA 2**

**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.**