Effectiveness of Genotype MTBDR*sl* to exclude drug-resistance of *Mycobacterium tuberculosis* in a clinical trial

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Running title: Effectiveness of the LPA*s*/ in STREAM Trial

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Abstract (199 words)

Objectives: In this study, we assessed the ability of the Genotype MTBDR*sl* line probe assay Version 1 (V1.0) (LPA*sl* V1.0) to exclude baseline fluoroquinolones (FQ) and second-line injectable drugs (SLI) resistance in the STREAM 1 trial.

Methods: Direct sputum LPA*sl* results in the site laboratories were compared to indirect phenotypic drug-susceptibility testing (pDST) results in the central lab, with DNA sequencing as a reference standard.

Results: Of 413 multidrug-resistant tuberculosis (MDR-TB) patients tested with LPA*sl* and pDST, 389 (94.2%) were FQ susceptible, and 7 (1.7%) FQ resistant, while 17 (4.1%) had an inconclusive LPA*sl* result. For SLI, 372 (90.1%) were susceptible, 5 (1.2%) resistant and 36 (8.7%) inconclusive. There were 9 (2.3%) FQ discordant pDST/LPA*sl* results, of which 3 revealed a mutation, and 5 (1.3%) SLI discordant pDST/LPA*sl* results, none of which were mutants by sequencing. Among the 17 FQ and SLI LPA*sl* inconclusive samples, sequencing showed 1 FQ- and zero SLI-resistant results, similar to frequencies among the conclusive LPA*sl*. The majority of inconclusive LPA*sl* were associated with low bacillary load samples (AFB smear-negative or -scantily positive) compared to conclusive results ($p<0.001$).

Conclusion: LPA*sl* can facilitate the rapid exclusion of FQ and SLI resistances for enrolment in clinical trials.

Keywords: Clinical trial; fluoroquinolones; line probe assay; *M. tuberculosis*; resistant; second-line injectables

Background

Despite the availability of curative anti-TB therapy for nearly half a century, the emergence and spread of MDR strains is a major public health concern and threatens global control of the disease [1–4]. In 2019, according to World Health Organization (WHO) [5], an estimated 465 000 people developed rifampicin-resistant TB (RR-TB).

The evaluation of new drugs and new regimens requires randomized clinical trials [6–8]. STREAM 1 was a phase III, multicentre, open-label, randomized controlled trial that enrolled patients between 2012 to 2015 to evaluate safety, efficacy, and cost-effectiveness of a standardized shorter regimen for MDR-TB similar to a regimen described by Van Deun et al. 2010 [7] compared to long regimen recommended in 2011 by WHO [9]. Enrolment criteria and trial procedures have been previously reported elsewhere [8,10] (STREAM 1, ISRCTN78372190 and clinicaltrials.gov NCT02409290). The trial enrolled adults with rifampicin-resistant (RR)/MDR-TB and no evidence of resistance to fluoroquinolones (FQ) or second-line injectable drugs (SLI) by line probe assays (LPA*s*/) (Genotype MTBDR*s*/, Hain Lifesciences, Germany) in four weeks prior to randomization [8,11].

Second-line drug LPAs are the fastest and most commonly used genotypic drug susceptibility testing (gDST) method available although still requiring an adequate level of molecular technical expertise [12,13]. The MTBDR*s*/ LPA Version 1 (LPA*s*/) detects the most frequent

mutations conferring resistance to FQ in *gyrA* and *gyrB* gene, and SLI in 16S rRNA (*rrs*) and *eis* promoter gene [14]. A systematic review from Cochrane database showed that LPA*s*/ had sensitivities and specificities of 85.1% and 98.2% and 76.9% and 99.5% for detection of FQ and SLI resistance from clinical samples, respectively [14]. At the time STREAM 1 was planned, it was not clear whether this would lead to sufficiently high negative predictive values (NPV) to reliably exclude FQ- and/or SLI-resistant cases from enrolment [15]. Moreover, indeterminate results have been reported in 7.1% of FQ bands and 13.5% for SLI by LPA*s*/ [15,16].

Using data and *M. tuberculosis* isolates from the STREAM 1 trial, we assess this LPA*s*/ effectiveness and investigate the most appropriate interpretation of inconclusive LPA*s*/ results directly from sputum, including their association with sputum bacillary load.

Ethical statement

This study used microbiological data and isolates from STREAM 1 clinical trial. The isolates tested to resolve discordant LPA*s*l/DST results were identified using the central laboratory accession number, without any patient identifiers. The Institutional Review Board of Institute of Tropical Medicine (ITM), Antwerp, was notified of these analyses. Full protocol review was not requested, in line with the low-risk nature of microbiological analyses.

Materials and methods

Study design and population

This study was a retrospective comparison of LPA*s* results obtained from baseline clinical samples (defined as participant's sputum specimens collected before initiation of MDR-TB treatment) in seven STREAM 1 sites; Ethiopia, Mongolia, South Africa, and Vietnam from patients randomized between July 2012 and June 2015 with phenotypic DST (pDST) on baseline isolates [8,11].

Microscopy

Sputum smear microscopy was conducted at each STREAM trial site following WHO standard protocol [17]. The site TB laboratories currently participated in External Quality Assessment (EQA) programs ensuring the quality of AFB-smear microscopy results. Additionally, the reference laboratory at ITM, Antwerp, warranted the quality of the site results through periodical monitoring visits.

Drug susceptibility testing (DST)

Phenotypic drug susceptibility testing (pDST) of *M. tuberculosis* isolates received from three countries (Ethiopia, South Africa, and Vietnam) was performed at ITM, Antwerp, using proportion method on Middlebrook 7H11 agar at critical concentrations recommended by WHO [18], whereas pDST of *M. tuberculosis* isolates from Mongolian site was conducted in

National Tuberculosis Reference Laboratory (NRL), Ulaanbaatar following WHO standard protocol for indirect proportion method on Löwenstein-Jensen for first and second-line drugs [18].

GenoType line probe assay (LPA*s*/)

The GenoType LPA*s*/ V1.0 was performed and interpreted according to the manufacturer instructions (Hain Lifescience, Nehren, Germany) for clinical samples [19]. The results were recorded inconclusive based on presence of overall weak bands or absence of WT and MUT bands, together with the absence of the loci control bands for one or more of the genes tested. The DNA extracted from the reference *M. tuberculosis* H37Rv strain and molecular-grade water were used as positive and negative controls for each run.

DNA sequencing

To resolve discordant results of FQ LPA*s*/ and pDST, extracted DNA was amplified using gyrA/B specific primers and sequenced by Sanger method as previously described [20]. Sequences were compared to that of H37Rv reference strain (NCBI, GenBank accession number NC_000962) using CLC Sequence Viewer software. For SLIs, next-generation sequencing (NGS) was used to resolve discordance between pDST and LPA*s*/ methods. Illumina NGS short sequencing was performed by TGen/C-Path platform in which the whole genome is analyzed for variants that

142 are known to SLI resistance-conferring genes [21]. TB-profiler (version 2.6.0) was used to analyze
143 raw fastq data.

144

145 **Statistical analysis**

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147 Statistical analysis was performed using STATA version 15.0 (STATA Inc., USA). LPA*s/* results
148 discordant with those of pDST were resolved by sequencing as the reference standard. As
149 evidence of FQ or SLI resistance excluded patients from the trial, only a small fraction of the
150 strains obtained at the screening visit that had LPA*s/* resistant results also had pDST done at the
151 reference laboratories, only the predictive value of a susceptible, but not that of a resistant
152 result in the population screened could be determined. Logistic regression was used for analysis
153 of the association between inconclusive results and a low bacillary load. The Chi-square test
154 was used to compare proportions.

155

156 **Results**

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158 A total of 689 patients were screened from the seven sites, of whom 579 patients with both
159 LPA*s/* and smear microscopy results were used to investigate the association between
160 inconclusive LPA*s/* results and bacillary load. Only 413 patients with both LPA*s/* and pDST results
161 available were also included for the assessment of LPA*s/* performance (Figure 1).

162

163 **MTBDR*s/* line probe assay (LPA) results**

Of 413 patients, LPA*sl* identified 396 (95.9%) patients with FQ conclusive, and 17 (4.1%) inconclusive results (Table 1). LPA*sl* also identified 377 (91.3%) SLI conclusive, and 36 (8.7%) with inconclusive results (Table 1).

Among all 579 patients, 503 (86.9%) were reported as FQ-susceptible (FQ-S), 26 (4.5%) FQ-resistant (FQ-R), and 50 (8.6%) as having inconclusive results. For SLI LPA*sl* results, 478 (82.6%) were susceptible, 25 (4.3%) resistant and inconclusive for 76 (13.1%) patients. The inconclusive LPA*sl* results were significantly more common in South Africa compared to other trial sites ($p<0.001$ and $p<0.001$ respectively) (Supplement Table 1).

Phenotypic drug susceptibility testing (pDST) versus LPA*sl* results and sequencing

Of the total 413 pDST results (Figure 1 and Table 1), 403 (97.6%) and 406 (98.3%) *M. tuberculosis* isolates were FQ-S and SLI-S, respectively.

For 396 patients with conclusive LPA*sl* and pDST results for FQ, nine patients showed discordant results: 5 LPA-S/pDST-R and 4 LPA-R/pDST-S (Table 1). For 377 patients with conclusive results for SLI, 5 showed discordant results: 2 LPA-S/pDST-R and 3 LPA-R/pDST-S (Table 1). gyrA/B sequencing of the discordant results showed wildtype gyrA/B for all 4 LPA-R, whereas 2 wildtypes and 3 resistance-conferring mutations (2 Ala90Val, 1 Asp94Gly) were observed for the LPA-S. Together with the 3 concordant resistant cases, this brings the total FQ-

186 R to 6 (1.5%) (Supplement Table 2). At this very low prevalence, the negative predictive value
187 (NPV) of LPA*s*/ for FQ resistance was very high, 99.2% (95%CI, 0.98-1.00; Table 2). For the
188 strains with SLI discordance between LPA*s*/ and pDST, sequencing did not show SLI-R-conferring
189 mutations. Hence, only two strains were finally classified as SLI resistant (0.5%; Supplement
190 Table 2). The NPV for the exclusion of SLI-R by LPA*s*/ was thus 100% (95%CI: 0.99-1.00; Table 2).
191 As patients identified as FQ or SLI resistant by LPA*s*/ screening were excluded from the trial no
192 pDST is available from strains for most of these patients. Hence, we could not evaluate false
193 resistance in the screened population. Sensitivity, specificity, and PPV of the FQ or SLI resistant
194 results could thus not be determined in this study.

195

196 **Resistance missed by inconclusive MTBDR*s*/ LPA results**

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198 pDST identified 15 FQ-S and 2 FQ-R samples among the 17 with FQ-inconclusive LPA*s*/ results
199 (Table 1). Sequence analysis could confirm only 1 *gyrA* Ala90Val resistance-conferring mutation
200 among the 2 phenotypic FQ-R, all other samples being wildtype (Supplement Table 2). Thus,
201 among the 17 inconclusive LPA*s*/, 1 FQ-R had not been identified by LPA. Considering the 26
202 (4.9%) FQ-R cases identified at the initial screening of 529 patients with conclusive results, the
203 1/17 (5.9%) confirmed by the reference standard from FQ inconclusive LPA*s*/ results is very
204 similar.

205

206 The pDST identified 3 (8.3%) SLI-R patients among 36 with SLI-inconclusive LPA*s*/ results (Table
207 1). Only half (17) of those - and among them, only one pDST SLI-R - were available for
208 sequencing. No SLI resistance-conferring mutations were detected for these 17 (Supplement

Table 2). Due to insufficient confirmatory testing, it was not possible to compare the proportions of resistance for inconclusive versus conclusive SLI LPA*s*/ results.

Association of inconclusive MTBDR*s*/ LPA results with bacillary load

Additionally, we analyzed the association between the 579 LPA*s*/ results and sputum bacillary load as determined by smear microscopy (Table 3). Among 140 (24.2%) with low-bacillary load, 94 (16.3%) were smear-negative and 46 (7.9%) had scanty-positive smear microscopy results, while the 439 (75.8%) high-bacillary load comprised of 146 (25.2%) 1+, 118 (20.4%) 2+, and 175 (30.2%) 3+ smear-positive results. Higher proportion of patients with Inconclusive FQ LPA*s*/ and SLI LPA*s*/ had a lower bacillary load compared to those with conclusive results ($p<0.001$), (Table 3).

Discussion

Rapid and accurate diagnostic tools to exclude pre-XDR and XDR-TB patients are essential to decide on the eligibility of patients for enrolment in some MDR clinical trials, and also for timely management of MDR-TB. In this study, we evaluate the performance of LPA*s*/ in such a context, the STREAM 1 trial. The agreement between LPA*s*/ and pDST to detect true susceptibility was excellent for both FQ and SLI. Although DNA sequencing showed most of the discordant results for LPA*s*/ FQ and/or SLI resistance were false, as a clinical trial screening tool, LPA*s*/ still did very well. Similar to results achieved in other settings [14,22], in STREAM, LPA*s*/ performance for the exclusion of resistance was very good, with a NPV over 99% for both FQ and SLI. In addition to minimizing testing delay, LPA*s*/ were almost 100% effective in identifying FQ- and/or SLI-resistant TB patients in trial settings with a low prevalence of resistance and thus a low pre-test probability such as Ethiopia and Vietnam (Supplementary Table 1). Others have also evaluated LPA*s*/ V2.0 and reported the specificity of the LPA*s*/ to be close to 99% [23]. Therefore provided that DNA sequencing is used to resolve discordances with pDST, in high-prevalence settings, the NPV should still be good enough in a trial setting; only five FQ-resistant cases would be missed when screening 1000 patients at 50% prevalence. Although the proportion of patients with SLI resistance missed among the inconclusive could not be determined in this study, these test failures may constitute a serious problem where SLI resistance prevalence is high, since they were not rare, and not as strongly associated with the low bacillary load.

In addition to overall prevalence, the distribution of specific FQ-R mutations might vary geographically [24–26] partly explaining LPA*s*/ performance variation. In the study by Pantel et al. [27]

244 compensatory mutations that restore FQ susceptibility in *M. tuberculosis* strains have been also
245 described in the screening of MDR-TB.

246

247 The assay's good performance in all STREAM 1 sites for exclusion of resistance to FQ and SLI is
248 similar to that reported in a feasibility study describing the molecular assay for screening of
249 patients in TB clinical trials [28] except for some false LPA*s/* results, particularly in South Africa.
250 The WHO expert Group also noted that given high assay NPV for detecting resistance to FQ and
251 SLI, the results of the LPA*s/* could be used for screening, pending the results of pDST results [22]
252 while avoiding placing patients who have resistance to FQ and SLI on the regimen and start
253 eligible patients on treatment [11,29].

254

255 Our analysis showed an overall rate of inconclusive LPA results of 8.6% for FQ and 13.1% for SLI,
256 which is within the reported range from other studies [16,30]. Although not fully understood, poor
257 test execution or suboptimal amounts of DNA in low bacillary burden samples are obvious
258 possible reasons [16]. However, in principle, inconclusive results could also be associated with
259 mutations or deletions in the locus control region, as well as the complete or partial deletion of
260 a target gene [30,31]. A significantly higher proportion of inconclusive LPA*s/* was seen in patients
261 from South Africa, where the majority had no or only scanty bacilli on sputum microscopy,
262 possibly due to a higher proportion of patients enrolled with HIV co-infection. Previous studies
263 have also documented more inconclusive results for smear-negative and lower bacillary load
264 specimens [32,33] and in those who are HIV positive [32,34,35]. WHO recommends that direct use of
265 sputum for the LPA*s/* test is not suitable for smear-negative clinical specimens [34,35]. Ongoing DR-
266 TB trials increasingly enroll larger proportions of patients with low bacillary burdens, e.g. those

who test Xpert very low, AFB negative. As shown in our study, such samples are more likely to yield inconclusive LPA results, lowering LPA utility for rapid exclusion of baseline resistance.

In our study, the proportion of FQ-resistant organisms among LPA*s*/ inconclusive results did not appear to differ from that observed among conclusive results, but we could not arrive at any conclusion on this point for SLI because of insufficient confirmatory testing. At least for FQ, enrolment of patients with an inconclusive LPA*s*/ result can be fully justified at low FQ-resistance prevalence, and it would seem reasonable to believe this to be true for SLI.

This study has limitations. We could not review the strips for each LPA*s*/ performed in the sites, so we were not able to investigate banding profiles suggestive of heteroresistance or errors of reading at these laboratories as possible causes of the false results observed. Exclusion of FQ and SLI resistant LPA*s*/ samples from a trial further limits our ability to estimate the level of false resistance in the screened population and causes incapable of determining sensitivity and PPV of resistant results. Besides, we could only apply the reference standard, sequencing, to half the samples with inconclusive results for SLI, leaving some uncertainty regarding their importance and most suitable interpretation for clinical trial patient screening.

Conclusion

In this study, LPA*s*/ proved a good screening tool for rapid exclusion of resistance to FQ and SLI using a composite reference standard of pDST plus DNA sequencing to resolve discordant

289 results. Although the prevalence of both FQ and SLI resistance was low to moderate in our
290 study populations, the high specificity suggests that also in high prevalence settings LPA*s*/ can
291 facilitate screening for FQ and SLI resistance. Relatively frequent inconclusive results,
292 particularly for SLI, may constitute a larger problem, especially when enrolling patients with
293 AFB smear-negative disease, such as those diagnosed by Xpert. Inconclusive results may
294 conceal a proportion of resistance proportional to the prevalence of FQ or SLI resistance in the
295 test setting.

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297 **Competing interests**

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299 The authors stated that they have no conflict of interest.

300

301 **Author Contributions**

302

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Table 1. Cross-tabulation of LPA*s*/ versus pDST results

		Phenotypic DST				Phenotypic DST		
		FQ Susceptible (n=403)	FQ Resistant (n=10)	Total (n=413)		SLI Susceptible (n=406)	SLI Resistant (n=7)	Total (n=413)
FQ LPA*s*/	Conclusive	388 (98.0%)	8 (2.0%)	396	SLI LPA*s*/	373 (98.9%)	4 (1.1%)	377
	Susceptible	384 (98.7 %)	5 (1.3 %)	389		370 (99.5%)	2 (0.5%)	372
	Resistant	4 (57.1%)	3 (42.9%)	7		3 (60.0%)	2 (40.0%)	5
	Inconclusive	15 (88.2%)	2 (11.8%)	17		33 (91.7%)	3 (8.3%)	36

FQ= fluoroquinolone; SLI = second-line injectable; LPA*s*/ = second-line drugs Line Probe Assay; pDST = phenotypic drug-susceptibility testing

435 Table 2. LPA*s*l effectiveness for exclusion of resistance

LPAsl Conclusive results		#DNA sequencing			Negative predictive value % (95% CI)
		Resistant	Susceptible	Totals	
Fluoroquinolone	Resistant	3	4	7	
	Susceptible	3	386	389	99.2 (0.98-1.00)
Second-line injectable	Resistant	2	3	5	
	Susceptible	0	372	372	100 (0.99-1.00)

436 CI = Confidence Interval; LPAsl = second-line drugs Line Probe Assay; pDST = phenotypic drug-
437 susceptibility testing
438 #DNA sequencing used as a reference standard to resolve discordant between pDST and LPA*s*l
439 results
440

441 Table 3: Sputum bacillary load stratified for FQ- or SLI- LPA*sl* result

LPA*sl* (N)	Total	Direct smear-microscopy		**p-value	[95%CI]
		Low-bacillary load*	High-bacillary load#		
Fluoroquinolone					
Inconclusive	50	37 (74.0%)	13 (26.0%)	**<0.001**	**[0.04-0.16]**
Conclusive	529	103 (19.5%)	426 (80.5%)		
Total	579	140 (24.2%)	439 (75.8%)		
Second-line injectable					
Inconclusive	76	46 (60.5%)	30 (39.5%)	**<0.001**	**[0.09 – 0.25]**
Conclusive	503	94 (18.7%)	409 (81.3%)		
Total	579	140 (24.2%)	439 (75.8%)		

442 FQ= fluoroquinolone; SLI= second-line injectable; LPAsl= second-line drug Line Probe Assay
443 *Low-bacillary load included negative and scanty smear microscopy results
444 #High-bacillary load included 1+, 2+, and 3+ smear microscopy results
445 **Univariable logistic regression analysis (LPA*sl* result versus Smear-microscopy result)
446

447 Supplement Table 1. Analysis of FQ- and SLI- LPA*s*l results by country (STREAM stage 1 sites).

STREAM sites		FQ LPA*s*l				
		Conclusive		Conclusive, Total	Inconclusive, Total	**p-value
	Total	Susceptible	Resistant			
Ethiopia	159	147 (92.5%)	4 (2.5%)	151 (95%)	8 (5%)	0.062
Mongolia	40	38 (95%)	0 (0.0%)	38 (95%)	2 (5%)	0.403
South Africa	278	216 (77.7%)	22 (7.9%)	238 (85.6%)	40 (14.4%)	**<0.001**
Vietnam	102	102 (100%)	0 (0.0%)	102 (100%)	0 (0.0%)	-
Total	579	503 (86.9%)	26 (4.5%)	529 (91.4%)	50 (8.6%)	
		SLI LPA*s*l				
		Conclusive		Conclusive, Total	Inconclusive, Total	**p-value
	Total	Susceptible	Resistant			
Ethiopia	159	128 (80.5%)	6 (3.8%)	134 (84.3%)	25 (15.7%)	0.256
Mongolia	40	31 (77.5%)	5 (12.5%)	36 (90%)	4 (10%)	0.546
South Africa	278	217 (78.1%)	14 (5%)	231 (83.1%)	47 (16.9%)	**0.010**
Vietnam	102	102 (100%)	0 (0.0%)	102 (100%)	0 (0.0%)	-
Total	579	478 (82.6%)	25 (4.3%)	503 (86.9%)	76 (13.1%)	

448 FQ = Fluoroquinolones; SLI = second-line injectable; LPAsl = second-line drug Line Probe Assay
449 **Univariable logistic regression analysis (LPA*s*l result versus STREAM site (Country, factor
450 variable))
451

Supplement Table 2. Final results applying the reference standard comparing conclusive and inconclusive LPA*sl* results

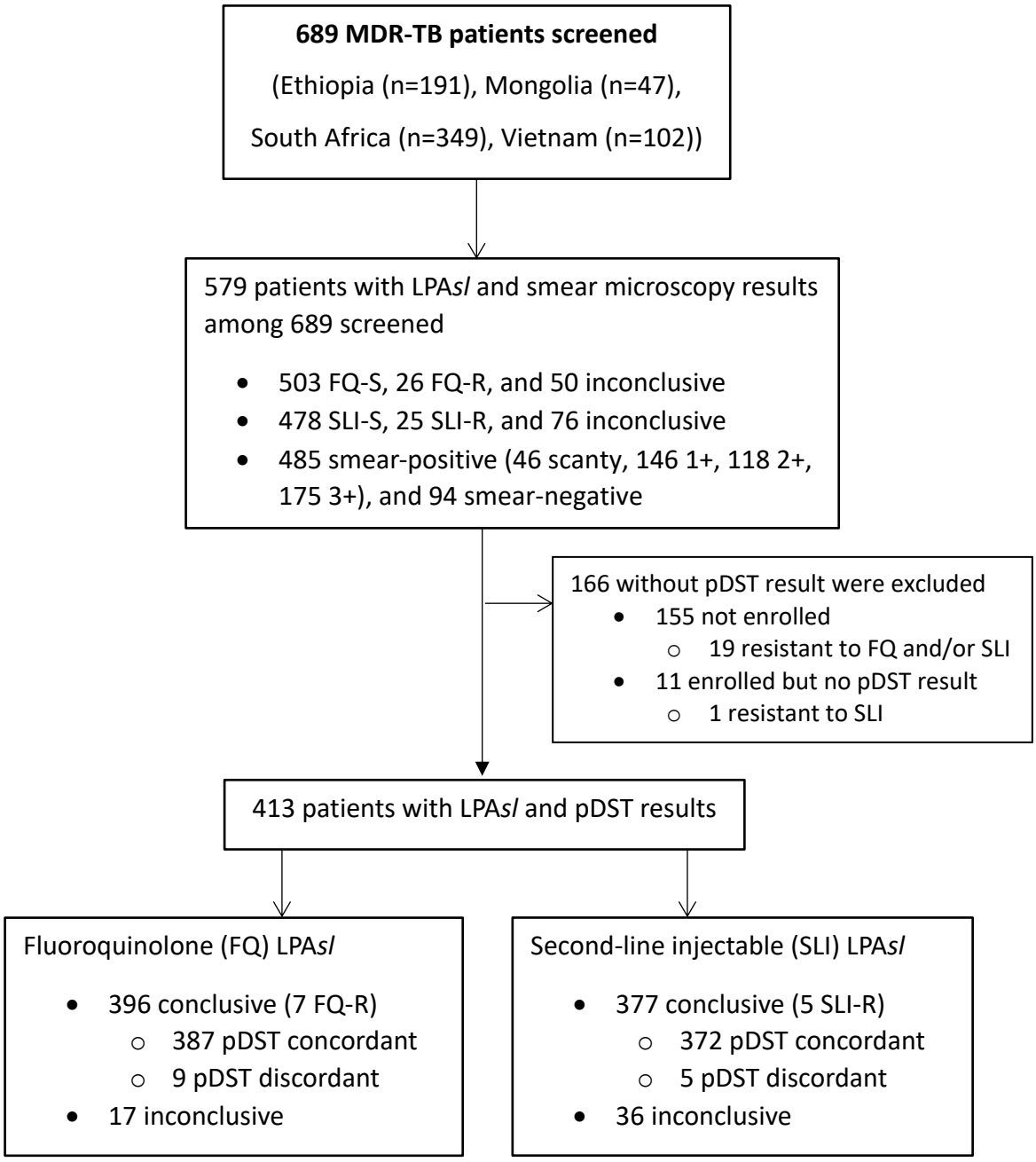
	Reference standard (Sequencing)			
LPA*sl*	Resistant	Susceptible	Total	**p-value
	Fluoroquinolones			0.26
Conclusive	6 (1.5%)	390 (98.5%)	396	
Inconclusive	1 (5.9%)	16 (94.1%)	17	
Total	7 (1.7%)	406 (98.3%)	413	
	Second-line injectables[1]			1.000
Conclusive	2 (0.5%)	375 (99.5%)	377	
Inconclusive	0 (0%)	17 (100%)	17	
Total	2 (0.5%)	392 (99.5%)	394	

LPAsl = second-line drugs Line Probe Assay; pDST = phenotypic drug-susceptibility testing;
1 19/36 SLI inconclusive were not sequenced of which 18-S and 1-R by pDST
**Chi-squared test (Comparison between the reference standard (sequencing) and LPA*sl* results)

459 Figure 1. Flow diagram displaying patients and samples included in the study

460

461


689 MDR-TB patients screened
(Ethiopia (n=191), Mongolia (n=47),
South Africa (n=349), Vietnam (n=102))
579 patients with LPAsl and smear microscopy results among 689 screened
503 FQ-S, 26 FQ-R, and 50 inconclusive
478 SLI-S, 25 SLI-R, and 76 inconclusive
485 smear-positive (46 scanty, 146 1+, 118 2+, 175 3+), and 94 smear-negative
166 without pDST result were excluded
155 not enrolled
19 resistant to FQ and/or SLI
11 enrolled but no pDST result
1 resistant to SLI
413 patients with LPAsl and pDST results
Fluoroquinolone (FQ) LPAsl
396 conclusive (7 FQ-R)
387 pDST concordant
9 pDST discordant
17 inconclusive
Second-line injectable (SLI) LPAsl
377 conclusive (5 SLI-R)
372 pDST concordant
5 pDST discordant
36 inconclusive