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Defining threshold values in microscopic examination and qPCR for optimal performance of line probe assays for resistance detection in *Mycobacterium tuberculosis* complex

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ABSTRACT

Line probe assays enable rapid detection of drug-resistant *Mycobacterium tuberculosis* directly from clinical specimens. This study shows that defining threshold values in microscopic examination or PCR detection of *M. tuberculosis* optimizes line probe assay efficiency, thereby avoiding costs and loss of time due to unnecessary molecular testing.

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1. Note

Tuberculosis remains one of the world's deadliest communicable diseases. The emergence of drug-resistant *Mycobacterium tuberculosis* presents a threat for global tuberculosis control efforts (WHO 2017). Phenotypic drug susceptibility testing (DST) requires primary culture and is time consuming with a turnaround time of at least 4 weeks. Molecular-based tests like line probe assays from Autoimmun Diagnostika (AID, Strassberg, Germany) or Hain Diagnostics (Hain, Cookeville, TN) rapidly detect drug-resistant *M. tuberculosis* directly from clinical specimens, thereby significantly shortening time to result to 1–2 days. The sensitivity of line probe assays in paucibacillary specimens is limited (Luetkemeyer et al. 2014; Ritter et al. 2014; Molina-Moya et al. 2015; Nathavitharana et al. 2017). The objective of this study was to define a threshold value in microscopic examination or qPCR detection of *M. tuberculosis* (i.e., cell count in microscopy or cycling threshold value in qPCR) to optimize line probe assay performance in order to avoid costs and loss of time due to unnecessary molecular testing.

From January 2016 to December 2017, *M. tuberculosis* DNA was detected in 161 clinical specimens (117 respiratory and 44 nonrespiratory specimens) by COBAS™ TaqMan™ MTB qPCR (MTB qPCR; Roche Diagnostics, Rotkreuz, Switzerland). Subsequently, these specimens were subjected to the tuberculosis resistance line probe assay (TB LPA) module 1 from AID that detects specific mutations in the *inhA* promoter (positions −16, −15, −8), in *katG* (codon 315), and in *rpoB* (codons 516, 526, 531) to predict isoniazid and rifampin resistance (Ritter et al. 2014; Molina-Moya et al. 2015). Clinical specimens were analyzed microscopically by auramine–rhodamine staining and if determined positive subjected to Ziehl–Neelsen staining for confirmation. Microscopic slides were examined according to the criteria proposed by Kent (1985). The clinical specimens were inoculated into MGIT 960 liquid broth medium (Becton Dickinson, Allschwil, Switzerland) to grow liquid cultures for phenotypic DST using the MGIT 960 system (Becton Dickinson) (Springer et al. 2009; Cambau et al. 2014; Deggim-Messmer et al. 2016).

For 107 of the 161 MTB qPCR-positive specimens, both phenotypic DST and TB LPA results were available (Table 1); for 50 specimens, no TB LPA result was achieved, and 4 specimens were culture negative due to ongoing treatment. Ninety-five of the 107 *M. tuberculosis* isolates lacked mutations in the *inhA* promoter and *katG* and *rpoB* regions, which correlated with phenotypic susceptibility for rifampin and isoniazid. In 1 *M. tuberculosis* isolate, low-level isoniazid resistance was phenotypically detected, but no mutation was found by TB LPA in the *inhA* promoter (positions −16, −15, −8). TB LPA detects the most prevalent *inhA* promoter and *katG* mutations, but it is not able to cover all rarely

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Table 1
Comparison of phenotypic (culture) and molecular (TB LPA) DST results ($n = 107$ clinical *M. tuberculosis* isolates).

		Phenotypic DST	
		Resistant	Susceptible
Line Probe Assay (AID TB resistance module 1)	Rifampin (*rpoB*)		
	Resistant	6[a]	0
	Susceptible	0	101
	Isoniazid (*inhA*, *katG*)		
	Resistant	11[b]	0
	Susceptible	1[c]	95

[a] Six isolates showed a *rpoB* S531L mutation.
[b] Four isolates showed a C-15T mutation in the *inhA* promoter; 7 isolates showed a *katG* S315T mutation.
[c] One isolate was phenotypically resistant at 0.1 mg/L isoniazid and susceptible at 1.0 mg/L; no mutation was detected by sequencing of *inhA* promoter and *inhA*, *katG*, and *oxyR'-aphC* intergenic regions.

occurring mutations conferring isoniazid resistance (Torres et al., 2015). Prediction of isoniazid monoresistance in 5 specimens (*inhA* promoter or *katG* mutation) and multidrug-resistant tuberculosis (*katG* and *rpoB* mutations) in 6 specimens by AID TB LPA was confirmed by phenotypic DST (Table 1).

The 11 drug-resistant *M. tuberculosis* specimens were subsequently subjected to AID TB LPA module 2 (streptomycin, amikacin, capreomycin) and module 3 (fluoroquinolones, ethambutol). Three of 11 specimens showed a mutation in *embB* associated with ethambutol resistance. No resistance to fluoroquinolones and second-line injectable agents was detected.

These results clearly show that the combination of MTB qPCR with TB LPA can be used for genotypic detection of drug-resistant *M. tuberculosis* and separate drug-susceptible *M. tuberculosis* from any strain with therapeutically relevant drug resistance. However, the analytical sensitivity of TB LPA was lower compared to MTB qPCR; 31% of MTB qPCR-positive clinical specimens did not yield a conclusive TB LPA result in this study. Therefore, we investigated whether definition of a specific cell count in microscopic examination or a cycling threshold (Ct) value in the MTB qPCR would improve the diagnostic yield of *M. tuberculosis* resistance detection by TB LPA.

As the analytical sensitivity of line probe assays is relatively low, it is generally recommended to only analyze smear-positive clinical specimens (WHO 2008, Nathavitharana et al. 2017, Singh et al. 2017). If only clinical specimens with a positive detection of bacilli in microscopic examination were analyzed by TB LPA in this study, 78% of TB LPAs would be interpretable, but at the same time, 21 TB LPAs that would deliver interpretable results would be missed (Fig. 1A). As expected, the ratio of interpretable TB LPAs increased with cell count from 71% of interpretable TB LPA results (all clinical specimens included also smear negative ones) to 94% of interpretable TB LPA results if just clinical specimens with numerous bacilli (+++) in microscopic examination were analyzed.

One could also use the Ct value of the MTB qPCR to decide whether clinical specimens should be subjected to TB LPA. Thus, when subjecting all MTB qPCR-positive clinical specimens (Ct value ≤45) to TB LPA, we observed a high proportion (31%) of uninterpretable TB LPA results. If only clinical specimens with a Ct value ≤35 would be considered for TB LPA, 90% of the analyzed LPAs would give an interpretable result, but 32 interpretable TB LPAs would be missed (Fig. 1B). A cutoff Ct value of ≤30 would result in 96% interpretable TB LPA results but would miss 65 interpretable TB LPAs.

We found that definition of a cell count in microscopic examination was not well suited to determine if clinical specimens should be subjected to TB LPA as a high proportion of TB LPAs showed interpretable results despite negative microscopic examination. In our diagnostic mycobacteriological laboratory, we therefore decided to use a Ct value of 35 in the MTB qPCR as threshold to decide if a clinical specimen should be subsequently subjected to TB LPA. As an alternative for clinical specimens above the threshold (i.e., Ct value >35), more sensitive, but

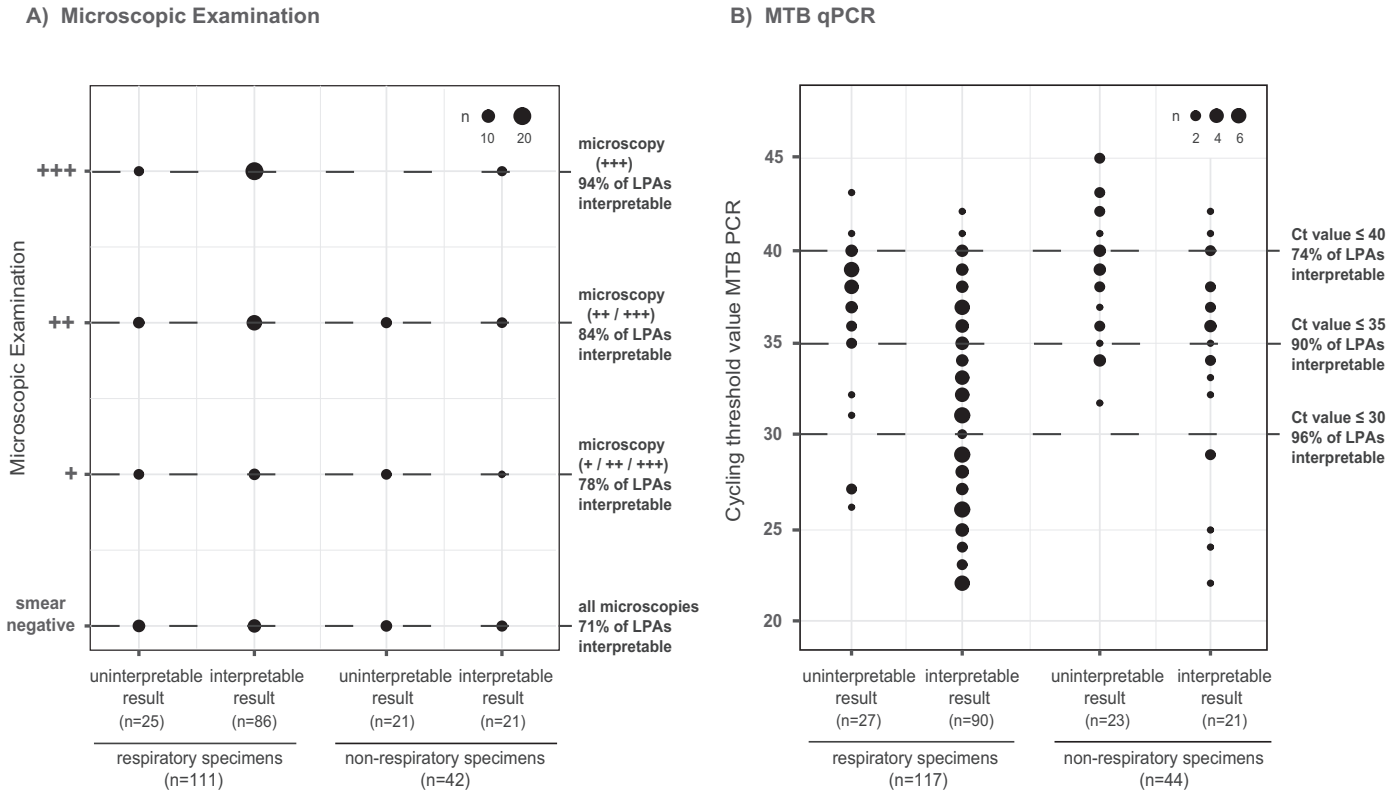

Fig. 1. Analysis of TB LPA results (interpretable vs. uninterpretable test results) when applying different threshold values in microscopic examination (A) and MTB qPCR (B).

mostly also more expensive, molecular DST systems can be used, such as the GeneXpert MTB/RIF Ultra test, targeted qPCR assays, or deep sequencing (Van Doorn et al. 2008; Colman et al. 2015; Chakravorty et al. 2017; Van Rie and De Vos 2017).

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Declarations of interest

None.

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