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TB-EFI, a novel 18-Plex microbead-based method for prediction of second-line drugs and ethambutol resistance in *Mycobacterium tuberculosis* complex

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ABSTRACT

Several diagnostic tests are being developed to detect drug resistance in tuberculosis. In line with previous developments detecting rifampicin and isoniazid resistance using microbead-based systems (spoligoriftyping and TB-SPRINT), we present here an assay called TB-EFI detecting mutations involved in resistance to ethambutol, fluoroquinolones and the three classical injectable drugs (kanamycin, amikacin and capreomycin) in *Mycobacterium tuberculosis*. The proposed test includes both wild-type and mutant probes for each targeted locus. Basic analysis can be performed manually. An upgraded interpretation is made available in Excel 2016®.

Using a reference set of 61 DNA extracts, we show that TB-EFI provides perfect concordance with pyrosequencing. Concordance between genotypic resistance and phenotypic DST was relatively good (72 to 98% concordance), with lower efficiency for fluoroquinolones and ethambutol due to some untargeted mutations. When compared to phenotypical resistance, performances were similar to those obtained with Hain MTBDR*sl* assay, possibly thanks to the use of automatized processing of data although some mutations involved in fluoroquinolone resistance could not be included.

When applied on three uncharacterized sets, phenotype could be predicted for 51% to 98% depending on the setting and the drug investigated, detecting one extensively drug-resistant isolate in each of a Pakistan and a Brazilian set of 91 samples, and 9 XDR among 43 multi-resistant Kazakhstan samples.

By allowing high-throughput detection of second-line drugs resistance and of resistance to ethambutol that is often combined to second-line treatments, TB-EFI is a cost-effective assay for large-scale worldwide surveillance of resistant tuberculosis and XDR-TB control.

1. Introduction

The mortality rate of tuberculosis dropped by 47% between 1990 and 2015, still the World Health Organization estimated 10.4 million of new cases, 1.3 million deaths caused by TB only, an additional 0.37 million deaths resulting from TB among HIV–positive people in 2016, and cases of antibiotic resistance are growing with increasing primary resistance (Brites and Gagneux, 2012; Eldholm et al., 2015; WHO, 2017; Zager and McNerney, 2008).

Multidrug-resistant (MDR) TB defines TB cases resistant to the two main first-line drugs, Rifampin (RIF) and Isoniazid (INH). Extensively drug-resistant (XDR) TB refers to resistance to RIF and INH, plus a

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fluoroquinolone (FLQ, such as levofloxacin LVX, Ofloxacin OFX, and Moxifloxacin MXF) and at least one second-line injectable drug (SLID) (kanamycin, KAN; amikacin, AMK; or capreomycin, CAP). One hundred and twenty-three (123) countries reported XDR-TB at the end of 2016 (WHO, 2017). The average proportion of MDR-TB cases with XDR-TB was around 6.2% in 2016, a rate lower than in preceding years (9 to 9.7%) but absolute number of XDR-TB still increases. Highest levels have been observed in Belarus (29% in 2014) and Lithuania (25% in 2013). The five countries that reported the largest numbers of cases in 2016 were China (n = 525), Belarus (n = 572), South Africa (n = 967), Ukraine (n = 1195) and India (n = 2464) (WHO, 2015). These increasing levels of resistance to second-line drugs appeal to resistance detection improvement to provide adequate treatment.

Recommended treatment of MDR-TB is composed of at least five likely effective drugs as per recent WHO updates: one FLQ, one second-line injectable drug (SLID), at least two drugs from group C (Ethionamide/Protionamide, cycloserine, linezolid, clofazimine), and pyrazinamide (PZA). It is also recommended to strengthen the regimen with high-dose INH and/or ethambutol (EMB) (WHO, 2016).

Resistance to second-line drugs and EMB is conferred by mutations in drug targets or genes involved in drug metabolism (Avalos et al., 2015; Chernyaeva et al., 2013; Georghiou et al., 2012; Kambli et al., 2015; Malik et al., 2012; Starks et al., 2009; Takiff et al., 1994; Zaunbrecher et al., 2009; Zhao et al., 2015; Zhu et al., 2012). Relative frequency of these mutations is similar in different studies suggesting that some mutations confer higher level of resistance and are therefore most frequently selected under antibiotic pressure (Table 1).

DNA sequencing is the standard and most effective method to detect mutations, and tools to ease Next Generation Sequencing raw data analyses are being developed (Bradley et al., 2015; Feuerriegel et al., 2015; Phelan et al., 2016; WHO, 2017). However, processes are still relatively tedious, expensive, necessitating at least basic bioinformatic skills and relatively poorly explicit regarding resistance (Feuerriegel et al., 2015). For the time-being, such solutions are rather suited to reference laboratories with large financing capacities and not for low-income countries. For routine detection of first-line drug-resistance the most commonly used methods are Genotype MTBDR*plus* (Hain Lifescience, Germany), and the GeneXpert MTB/RIF (Cepheid, USA) assays. The recent TB-SPRINT (based on Dual-Priming Oligonucleotide), TB-SNPID (based on Multiple-Ligation Probe dependent Amplification), and TRIOL (based on Nested-PCR) are examples of alternative multiplexed assays, all being based on multiplexed PCR and on microbead-based arrays, a well suited format for efficient and cost-effective nucleic acid amplification tests (NAATs) (Bergval et al., 2012; Brossier et al., 2016; Cabibbe et al., 2015; Gomgnimbou et al., 2013; Hillemann et al., 2009; Sengstake et al., 2014; WHO, 2016; Yin et al., 2016). Many recent systems use chips or Line-Probes to investigate first-line drug resistance or second-line-drug resistance (Table 1): VerePLEX (Cabibbe et al., 2015) Genotype MTBDR*sl* V2.0 (Brossier et al., 2016), microbead-based ASPE assay (Kim et al., 2018; Lee et al., 2015), Anyplex II MTB/MDR/XDR (Molina-Moya et al., 2015; Perez-Garcia et al., 2017).

The goal of this study was to develop a new add-on multiplexed method to TB-SPRINT for detecting the main mutations involved in resistance to second-line drugs (FLQ and injectable drugs) also including EMB, one of the first line drug. We named this system TB-EFI (Ethambutol, Fluoroquinolones, Injectables). It complements our previous TB-SPRINT and shows the advantage of allowing large sample screening and rapid analysis; it has a reasonable cost (in combination

Table 1
Mutations conferring resistance to EMB and second-line drugs and Excel 2016® comparison of various NAAT SNP panels.

Drug	Resistance loci/prediction tool	Prevalence among resistant isolates	Beamedex TB-EFI	HAIN MTBDRsl V 1.0	HAIN MTBDRsl V 2.0	Beamedex MLPA (TB-SNPID)*
FLQ	gyrA_90–91 wt		wt90	WT1,2,3	WT1,2,3	x
	gyrA90_mut_GTG(A90V)	13–29%	mut90	MUT1	MUT1	
	gyrA91_mut_CCG(S91P)	2–7%		MUT2	MUT2	
	gyrA 94_wt_GAC		wt94			x
	gyrA 94_mut_GCC(D94A)	7–17%		MUT3A	MUT3A	
	gyrA94_mut_AAC(D94N)	5–8%	mut2_94	MUT3B	MUT3B	
	gyrA94_mut_TAC(D94Y)	3–14%		MUT3B	MUT3B	
	gyrA94_mut_GGC(D94G)	21–36%	mut1_94	MUT3C	MUT3C	
	gyrA94_mut_CAC(D94H)	0–7%		MUT3D	MUT3D	
	gyrB_WT				WT	
	gyrB_500	4%				
	gyrB_538	1%			MUT1	
	gyrB_540	4%			MUT2	
Injectable drugs	rrs 1401_wt_A		wt1401	WT1	WT1	x
KAN/ AMK/ CAP	rrs1401_mut_G	38–80%/78–80%/50–80%	mut1401	MUT1	MUT1	
	rrs1402_wt_C		wt1402			x
	rrs1402_mut_T	1% /1%/ 2%	mut1402			
	rrs1484_wt_G		wt1484	WT2	WT2	
	rrs1484_mut_T	1–5%/ 1%/ 1%	mut1484	MUT2	MUT2	
	eis WT (G-37)				WT1	
	eis G-37T	0–19%/ 0%/ 0%				
	eis _wt(-10to-14)_CACAG		wt-10-14		WT2	
	eis WT -2				WT3	
	eis_(-10A)_CACAA	5–33% / 0%/ 0%	mut1			x
	eis_(-12T)_CATAG	3% / 3–6%/ 0%	mut2			
	eis_(-14T)_TACAG	5–24% / 2–9%/ 0%	mut3		MUT1	x
EMB	embB306_wt_ATG		wt	WT1		
	embB306_mut-ATA	0–23%	mut2	MUT1A		
	embB306_mut-GTG	34–42%	mut1	MUT1B		
Diagnostic tool references		This work	Hilleman et al. 2009	Brossier et al. 2016	Sengstake et al. 2014	

NAAT = Nucleic Acid Amplification Test.