

Accuracy of whole genome sequencing versus phenotypic (MGIT) and commercial molecular tests for detection of drug-resistant *Mycobacterium tuberculosis* isolated from patients in Brazil and Mozambique

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ABSTRACT

Background: The fast and accurate diagnosis of drug-resistant tuberculosis (DR-TB) is critical to reducing the spread of disease. Although commercial genotypic drug-susceptibility tests (DST) are close to the goal, they are still not able to detect all relevant DR-TB related mutations. Whole genome sequencing (WGS) allows better comprehension of DR-TB with a great discriminatory power. We aimed to evaluate WGS in *M. tuberculosis* isolates compared with phenotypic and genotypic DST.

Methods: This cross-sectional study evaluated 30 isolates from patients with detected DR-TB in Brazil and Mozambique. They were evaluated with phenotypic (MGIT-SIRE™) and genotypic (Xpert-MTB/RIF™, Genotype-MTBDRplus™, and MTBDRsl™) DST. Isolates with resistance to at least one first- or second-line drug were submitted to WGS and analyzed with TB profiler database.

Results: WGS had the best performance among the genotypic DST, compared to the phenotypic test. There was a very good concordance with phenotypic DST for rifampicin and streptomycin (89.6%), isoniazid (96.5%) and ethambutol (82.7%). WGS sensitivity and specificity for detection resistance were respectively 87.5 and 92.3% for rifampicin; 95.6 and 100% for isoniazid; 85.7 and 93.3% for streptomycin while 100 and 77.2% for ethambutol. Two isolates from Mozambique showed a Val170Phe *rpob* mutation which was neither detected by Xpert-MTB/RIF nor Genotype-MTBDRplus.

Conclusion: WGS was able to provide all the relevant information about *M. tuberculosis* drug susceptibility in a single test and also detected a mutation in *rpob* which is not covered by commercial genotypic DST.

1. Introduction

In 2016, globally there were an estimated 10.4 million incident cases of tuberculosis (TB), 1.3 million TB-related deaths among HIV-negative and 374,000 deaths among HIV-positive people [1]. Drug-resistant tuberculosis (DR-TB) is increasing and may undermine efforts to eradicate TB in many countries [2]. Global data from 2016 estimated 490,000 new MDR-TB cases and around 50% related deaths. But even so, information about DR-TB in Brazil and Mozambique is scarce, mainly due to the lack of laboratory facilities for drug susceptibility tests (DST) [1]. From 1998 to 2015, the concept of a high-burden

country (HBC) became familiar and widely used and widely used in the context of TB, TB-HIV, and multi-drug resistant TB (MDR-TB). In 2015, Mozambique was among the three lists and Brazil in the former two [1]. New accurate and rapid diagnostic strategies for drug-resistance are urgently required to ensure that patients are diagnosed early and initiated onto appropriate therapy to improve outcomes and prevent the spread of DR bacilli [3–6]. Currently, phenotypic DST is the gold standard for diagnosis of drug resistance. However they are time-consuming, require expensive laboratory facilities, are not available in many HBC and are not standardized for all anti-TB drugs [7–9].

Recently World Health Organization (WHO) endorsed three rapid

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Abbreviation list

BWA	Burrows-Wheeler Alignment Tool
DR-TB	drug-resistant tuberculosis
DST	drug-susceptibility tests
E	ethambutol
Fq	fluoroquinolone
H	isoniazid
HBC	high burden countries
iSLD	injectable second-line drugs
MDR-TB	multidrug-resistant tuberculosis

PAS	para-aminosalicylic acid
R	rifampicin
RRDR	rifampicin-resistance-determining region
SAM	sequence alignment map
Sm	streptomycin
TB	tuberculosis
WGS	whole genome sequencing
WHO	World Health Organization
WT	wild-type
XDR-TB	extensively drug-resistant tuberculosis
Z	pyrazinamide

genetic-based DST: Xpert-MTB/RIF, MTBDR_{plus}, and MTBDR_{sl} line-probe assays [1]. Although these tests are fast and easy to perform, they are not able to detect all the mutations associated with DR-TB [10–12]. Whole genome sequencing (WGS) allows the analysis of *Mycobacterium tuberculosis* genome enabling the identification of mutations which confer resistance, mutations compensating for fitness cost and has an extremely high discriminatory power that to measure the transmission of *M. tuberculosis* [13–15]. The development of next-generation sequencing technologies has reduced costs and time required to sequence the *M. tuberculosis* genome, making it progressively more affordable to study the epidemiology of disease as well as to describe the mechanisms of drug resistance [16]. However, the development and standardization of robust platforms that enable reliable analysis and interpretation of WGS data remain to be achieved [8,17,18].

We used WGS to characterize the mutations conferring resistance in clinical isolates of *M. tuberculosis* from Brazil and Mozambique and to compare these results to those obtained by phenotypic and commercial genotypic DST.

2. Materials and methods

2.1. Study design and population

This is a cross-sectional study evaluating DR *M. tuberculosis* isolated from different patients in Southeastern Brazil (São Paulo state) and Central Mozambique (Sofala province). All the isolates were tested with phenotypic, commercial genotypic DST and were submitted to WGS if resistance was detected to at least one first- or second-line drug by one of these tests.

2.2. Drug-susceptibility tests

Phenotypic DST was conducted using MGIT-960 SIRE kit (MGIT-960; Becton Dickinson Diagnostic Systems, Sparks, MD). The critical concentrations being 0.10 g/mL for isoniazid (H), 1.0 g/mL for rifampicin (R), 5.0 g/mL for ethambutol (E) and 1.0 g/mL for streptomycin (Sm) [19].

Molecular DST was performed using the Genotype-MTBDR_{plus} 2.0 and MTBDR_{sl} 2.0 (Hain Lifesciences, GmbH, Germany) according to the manufacturer's instructions. MTBDR_{plus} evaluates mutations in the *rpoB* (R resistance); *katG* and *inhA* genes (H resistance) [20,21], while the MTBDR_{sl} detects mutations related to resistance to fluoroquinolones (*gyrA* and *gyrB* genes mutations) and second-line injectable drugs as capreomycin, amikacin, and kanamycin (*rrs* and *eis* gene mutations) [22]. In addition, all the isolates were also tested with Xpert-MTB/RIF (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions [23].

2.3. Genomic DNA extraction and whole genome sequencing

The cetyltrimethylammonium bromide (CTAB)-lysozyme method was used for genomic DNA extraction and purification in sub-cultured

isolates [24]. The DNA concentrations were measured using Nanodrop and then checked by agarose gel electrophoresis. The WGS was performed using Illumina MiSeq Sequencing System MiSeqV2-500 cycles (Illumina, San Diego, CA, USA). DNA library was prepared using Nextera XT library preparation kit (Illumina, San Diego, CA, USA). Sequencing was performed using MiSeq sequencer reagent kit V2 as per manufacturer's protocol (Illumina, San Diego, CA, USA) [25], producing paired-end read 2 × 250, with a read length of 500bp. Whole genome sequences were deposited to the European Nucleotide Archive and are available under accession number: PRJEB23648.

2.4. WGS data analysis

Reads with phred quality score ≥ 30 were mapped with BWA v 0.7.5a (Burrows-Wheeler Alignment Tool) using the *M. tuberculosis* H37Rv reference genome. Conversion from sequence alignment map (SAM) format sorted, indexed binary alignment map (BAM) files was done using SAM tools (version 0.1.19)²⁶. PCR-duplicates were removed using the Mark Duplicates option of the Picard software tools (version 1.61). The variants were identified with SAMtools/BCFtools v 0.1.18 and annotated with SnpEffv 4.0.

Reads were analyzed with a customized pipeline comprised of open source software as described previously [27]. Briefly, trimomatic was used to remove adapters and low-quality bases (phred quality score of less than 20) using a sliding window approach, and filtering for a minimum read length of 36 [28]. Trimmed and filtered reads were aligned to the *M. tuberculosis* H37Rv (Genbank: AL123456.3) reference genome using three different alignment tools: the BWA [26], Novoalign (Novocraft) and SMALT [29]. The alignment files were subjected to local realignment around insertions and deletions (indels) and de-duplication using the Genome Analysis Toolkit (GATK) [30] and Picard Tools [31], respectively. Genomic variants (single nucleotide polymorphisms and indels) in coding and non-coding regions were identified from each alignment file using GATK [30] and SAMTools [26]. Variants identified in all three alignments by both GATK and SAM Tools were used for further analysis. Variants were annotated using annotation data from TubercuList [32].

2.5. Genotypic drug resistance prediction from WGS

TB profiler was used to identify mutations known to cause drug resistance [33].

2.6. Phylogenetic analysis

Concatenated sequences containing all high-confidence variable sites (coding and non-coding single nucleotide variants) were used to create a maximum likelihood phylogeny with RaxML using 1000 bootstrap pseudo-replicates [34]. The General time reversal nucleotide substitution model was applied for phylogenetic inference.

Table 1
Lineage, resistance profile and mutations evidenced by susceptibility tests and WGS of isolates from Mozambique.

N	MZB Isolate ID	Lineage	MTBDR plus		MTBDR sl		Xpert Phenotypic test			Whole genome sequencing								
			Mut R	Mut H	Mut Fq	Mut iSLD	R	R	H	H	Sm	Mut R	Mut H	Mut Z	Mut E	Mut Sm	Mut iSLD	Mut Fq
1	227	4.3	S531L	WT, S315T2 (kat G)			Yes	Yes	No	No	rpoB Ser450Leu	KatG Ser315Thr		embC Thr270Ile, embB Glu378Ala embB Met306Val				
2	243	1	WT8, WT6	WT2 (inhA)			No	No	No	No								
3	751	4.3		WT, S315T2 (kat G)			No	Yes	Yes	Yes	rpoB Val170Phe	KatG Ser315Thr	pncA Lys96Arg		rpsL Lys43Arg			
4	894	4.3		WT, S315T2 (kat G)			No	No	Yes	No	Yes	KatG Ser315Thr			rpsL Lys43Arg			
5	964	2.2	WT7, HS26D				Yes	Yes	No	No	rpoB His445Tyr							
6	2075	4.3					No	No	Yes	No	Yes	KatG Ser315Thr			rpsL Lys43Arg			
7	2078	4.3		WT, S315T2 (kat G)			No	Yes	Yes	Yes	rpoB Val170Phe	KatG Ser315Thr	pncA Lys96Arg	embB Met306Val	rpsL Lys43Arg			
8	2368	4.1.1.3	WT8, S531L	WT1, T8C (inhA)	WT2, A90V (gyr A)	WT2, C14T (eis)	Yes	Inv	Inv	Inv	rpoB Ser450Leu	KatG Ser315Thr	pncA Arg154Gly	embB Met306Ile	rpsL Lys43Arg		gyrA Ala90Val	
9	2721	4.3	WT7	WT, S315T2 (kat G)	WT2, A90V (gyr A)		Yes	Yes	Yes	Yes	rpoB His445Tyr	KatG Ser315Thr	pncA Leu19Arg	embB Gln497Arg	rpsL Lys43Arg		gyrA Ala90Val	
10	2937	3					No	No	Yes	No	Yes	kasA Gly269Ser		embB Met306Ile	rpsL Lys88Arg			
11	3033	4.3	S531L	WT, S315T2 (kat G)			Yes	Yes	Yes	Yes	rpoB Ser450Leu	KatG Ser315Thr		embB Gln497Arg	rpsL Lys43Arg			
12	3150	1					No	No	No	No	Yes			embC Thr270Ile, embB Glu378Ala embB Gln497Arg				
13	3185	4.3	WT7	WT, S315T2 (kat G)	WT2, A90V (gyr A)		Yes	Yes	Yes	Yes	rpoB His445Tyr	KatG Ser315Thr	pncA Leu19Arg		rpsL Lys43Arg		gyrA Ala90Val	

Mut R: mutations in rpoB gene related to rifampicin resistance; Mut H: mutations related to isoniazid resistance.
 Mut Fq: mutations related to fluoroquinolones resistance; Mut iSLD: mutations related to injectable second-line drugs resistance; WT: absence of wild-type band; Xpert R: rifampicin resistance detected by Xpert MTB/RIF; R: rifampicin.
 H: isoniazid; E: ethambutol; Sm: streptomycin; Z: pyrazinamide; PAS: para-aminosalicylic acid; NV: not valid phenotypic test.

Table 2
Lineage, resistance profile and mutations evidenced by susceptibility tests and WGS of isolates from Brazil.

N	BRA Isolate ID	Lineage	MTBDR plus		MTBDR sl	Phenotypic test				Whole genome sequencing										
			Mut R	Mut H		Mut Fq	Mut ISLD	R	R	H	H	Sm	Mut R	Mut H	Mut Z	Mut E	Mut Sm	Mut iSLD	Mut Fq	Mut PAS
1	241	4.3	WT, S315T1 (kat G)	WT, S315T1 (kat G)		No	No	Yes	No	No	KatG									
2	536	2.2	WT8, S531L	WT8, S531L		No	Yes	Yes	No	No	KatG, Kasa, Gly269Ser									
3	868	4.9	WT8, S531L	WT2, A90V (gyr A)		Yes	Yes	No	No	No	rpoB Ser450Leu								gyrA Ala90Val	
4	1028	4.9	WT, S315T1 (kat G)	WT, S315T1 (kat G)		No	No	Yes	No	No	KatG Ser315Thr									
5	1245	4.9	WT7	WT, S315T1 (kat G), WT (inh A)		Yes	Yes	Yes	No	No	rpoB His445Leu									
6	1662	4.9	WT8, S531L	WT, S315T1 (kat G)		Yes	Yes	Yes	No	Yes	rpoB Ser450Leu		pncA Ala3Pro	embB Met306Val	rpsL Lys43Arg					
7	1696	4.9	WT3	WT, S315T1 (kat G)		Yes	No	No	No	No										
8	1804	4.3	WT, S315T1 (kat G)	WT, S315T1 (kat G)		No	No	Yes	No	No	KatG Ser315Thr, Kasa, Gly269Ser inhA CI5T									
9	1874	4.3	WT1, C15T (inh A)	WT1, C15T (inh A)		No	No	Yes	No	Yes										
10	2080	4.3.2	WT, S315T1 (kat G)	WT, S315T1 (kat G)		No	No	Yes	No	Yes	KatG Ser315Thr									
11	2370	4.3	WT8, S531L	WT3, D94 N/Y (gyr A)		Yes	Yes	Yes	Yes	Yes	rpoB Ser450Leu		pncA Ser104Arg	embB Met306Leu, rpsL Lys88Arg, rrs intragenic	rpsL Lys88Arg, rrs intragenic	rrs intragenic	gyrA Asp94Asn	folC Arg49Gln		
12	2591	4.3	WT8, S531L	WT, S315T1 (kat G)		Yes	Yes	Yes	No	No	rpoB Ser450Leu		pncA Arg140His, pncA Phe94Val							
13	2782	4.9	WT2	WT, C15T (inh A)		Yes	No	Yes	No	No										
14	2943	4.3	WT3	WT, C15T (inh A)		Yes	No	No	No	Yes	rpoB Ser431Thr									
15	3052	4.3	WT, S315T1 (kat G)	WT, S315T1 (kat G)		Yes	Yes	Yes	No	No	KatG Ser315Thr									
16	3200	4.9	WT8, S531L	WT, S315T1 (kat G)		Yes	Yes	Yes	Yes	Yes	rpoB Ser450Leu		pncA Ala3Pro	embB Met306Val	rpsL Lys43Arg					

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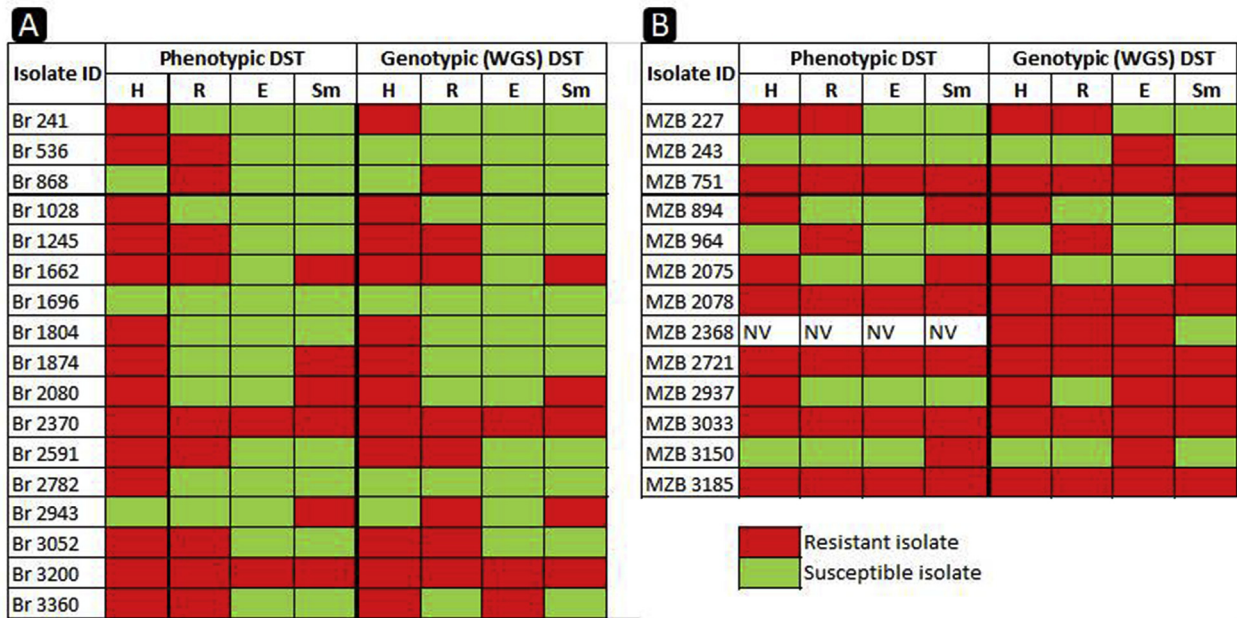


Fig. 1. Heat-map of the resistance profile comparing the phenotypic and WGS DST results. Red color indicates drug resistance and green color indicates drug sensitivity. H: isoniazid; R: rifampicin; E: ethambutol; Sm: streptomycin; NV: not valid phenotypic test. Br: isolates from Brazil, MZB: isolates from Mozambique. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

A		Rifampicin resistance (RR) in Phenotypic DST			Sensitivity= 87.5%	
RR in WGS		Yes	No		Specificity= 92.3%	Concordance= 89.7%
	Yes	14	1	15		
	No	2	12	14		
		16	13	29		
		Isoniazid resistance (HR) in Phenotypic DST			Sensitivity= 95.6%	
HR in WGS		Yes	No		Specificity= 100%	Concordance= 96.5%
	Yes	22	0	22		
	No	1	6	7		
		23	6	29		
B		Rifampicin resistance (RR) in Phenotypic DST			Sensitivity= 82.3%	
RR in Genotype MTBDRplus		Yes	No		Specificity = 66.7	Concordance = 75.8%
	Yes	14	4	18		
	No	3	8	11		
		17	12	29		
		Isoniazid resistance (HR) in Phenotypic DST			Sensitivity= 88%	
HR in Genotype MTBDRplus		Yes	No		Specificity = 75%	Concordance = 86.2%
	Yes	22	1	23		
	No	3	3	6		
		25	4	29		
C		Rifampicin resistance (RR) in Phenotypic DST			Sensitivity= 75%	
RR in Xpert MTB/RIF		Yes	No		Specificity = 76.9%	Concordance = 75.8%
	Yes	12	3	15		
	No	4	10	14		
		16	13	29		

Fig. 2. The sensitivity, specificity and concordance between phenotypic DST and WGS (A); phenotypic DST and Genotype MTBDRplus (B); phenotypic DST and Xpert-MTB/RIF (C). RR: rifampicin resistance; HR: isoniazid resistance; WGS: whole genome sequencing.

Mozambique (89.4%) compared to the Brazilian isolates (59.5%) [42].

The molecular mechanism of pyrazinamide resistance is largely associated with mutations in the *pncA* gene. These resistance-causing mutations are distributed throughout the gene, but not all mutations detected in this gene confer resistance. In this study, we identified five different *pncA* mutations. All of these have been previously reported to confer resistance [43–45]. However, the WHO guidelines suggest the use of pyrazinamide irrespective of DST results given the challenge of phenotypic DST because of the intracellular activity of pyrazinamide against intracellular pathogens and low confidence on the phenotypic DST [14,46].

The main mutations associated with streptomycin resistance occur in the *rpsL* gene, which confers moderate to high-level drug resistance. Mutations in the *rrs* gene are associated with moderate levels of resistance [47–50]. Among eight isolates from Mozambique with detected resistance to streptomycin in the phenotypic DST, seven (87.5%) also had resistance to isoniazid, both detected by WGS. Among Brazilian isolates it was similar, with five out of six (83.3%) of the isolates with streptomycin resistance in the phenotypic DST had also resistance to isoniazid, as reported previously [51]. This is probably due to the extensive use of streptomycin when DR-TB was suspected in previous decades.

Most mutations that have been already recognized as associated with resistance to fluoroquinolones are located in a conserved region of the *gyrA* gene, in codons 90 and 94, which are the most frequently mutated [52]. A previous study of our group reported that 43.7% of 16 DR-TB isolates from Mozambique showed resistance to fluoroquinolones (100% with the mutation *gyrA* Ala90Val) [53].

As far as we know, this is the first study using WGS in DR *M. tuberculosis* isolates in Mozambique. In this context, the use of WGS should be of great relevance considering that it allows the simultaneous detection of drug-resistant mutations to the most important existing drugs as well as phylogenetic studies that may help to understand and manage DR-TB. Our group had sequenced two isolates from a single patient who evolved from an MDR-TB status to a pre-XDR profile during treatment [54]. Brazil is a big country and there are many regional differences in circulating *M. tuberculosis* strain types, so there is a need for further studies using WGS in other regions and populations across the country.

This study amplifies the sample of clinical isolates, and we were able to show that WGS had an excellent performance and should be considered to be used in high burden TB and DR-TB areas around the world. Besides, WGS has other advantages as the turnaround time and the possibility to identify the presence of heteroresistance within the isolates from a single patient [35].

The main limitations of this study are the absence of phenotypic DST for pyrazinamide and second-line drugs which precluded comparisons with WGS, and the low number of tested isolates.

5. Conclusion

To investigate DR-TB in clinical practice we start with phenotypic DST for first-line drugs and only if resistance is detected, injectable second-line drugs (iSLD) and fluoroquinolones are tested. The same flow is followed for DR-TB investigation with genotypic DST. If rifampicin resistance is detected with Xpert or Genotype MTBDRplus, another genotypic DST for fluoroquinolones and iSLD susceptibility evaluation is required. WGS offers all the information about drug resistance in a single test. In this study, WGS also detected Val170Phe *rpoB* mutation not covered by commercial genotypic DST, which is the great differential for the diagnostic strategy.

Conflict of interest statement

We, the authors (CSF; EN; JRP; KP; AD; RMW; WASJ and VRB) state there is no conflict of interest for the authorship of this paper.

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