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Accuracy of an amplicon-sequencing nanopore approach to identify variants in tuberculosis drug-resistance-associated genes

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Abstract

A rapid and accurate diagnostic assay represents an important means to detect *Mycobacterium tuberculosis*, identify drug-resistant strains and ensure treatment success. Currently employed techniques to diagnose drug-resistant tuberculosis include slow pheno-typic tests or more rapid molecular assays that evaluate a limited range of drugs. Whole-genome-sequencing-based approaches can detect known drug-resistance-conferring mutations and novel variations; however, the dependence on growing samples in culture, and the associated delays in achieving results, represents a significant limitation. As an alternative, targeted sequencing strategies can be directly performed on clinical samples at high throughput. This study proposes a targeted sequencing assay to rapidly detect drug-resistant strains of *M. tuberculosis* using the Nanopore MinION sequencing platform. We designed a single-tube assay that targets nine genes associated with drug resistance to seven drugs and two phylogenetic-determining regions to determine strain lineage and tested it in nine clinical isolates and six sputa. The study's main aim is to calibrate MinNION variant calling to detect drug-resistance-associated mutations with different frequencies to match the accuracy of Illumina (the current gold-standard sequencing technology) from both culture and sputum samples. After calibrating Nanopore MinION variant calling, we demonstrated 100% agreement between Illumina WGS and our MinION set up to detect known drug resistance and phylogenetic variants in our dataset. Importantly, other variants in the amplicons are also detected, decreasing the recall. We identify minority variants and insertions/deletions as crucial bioinformatics challenges to fully reproduce Illumina WGS results.

DATA SUMMARY

Table 1 describes the accession numbers of fastq files from Illumina whole-genome sequencing.

INTRODUCTION

Tuberculosis (TB) remains a significant health problem worldwide and represents the principal cause of death by a single infectious agent, *Mycobacterium tuberculosis* complex (MTBC). Approximately 10 million new cases and 1.5 million deaths were reported in 2019 [1]. TB has been successfully treated since the late 1940s thanks to the development of the first anti-tubercular drugs. However, the number of drug-resistant cases has increased 13-fold in the last decade, complicating the global control of TB and delaying its eradication [1–3].

Standard TB treatment consists of a combination of four different drugs administered daily for up to 4 months. Treatment of drug-resistant TB (DR-TB) represents a more complex task, comprising a combination of second-line antibiotics, which requires more extended treatment times and is more expensive [4, 5]. Multidrug-resistant TB (MDR-TB) strains are resistant to both rifampicin (RIF) and isoniazid (INH), two key first-line drugs. MDR-TB is treatable by second-line drugs including oral and injectable drugs. Since 2019, injectable drugs are being replaced by all-oral regimens following World Health Organization (WHO) guidelines [6]. Consequently MDR- and XDR-TB definitions are being revisited [7]. However, injectable drugs are still being used in many regimes and countries including those that have not yet approved bedaquiline, an oral drug, to treat MDR-TB cases [8].

The current reference technique for DR-TB diagnosis is based on phenotypic tests that evaluate the susceptibility of MTBC strains to different antibiotics (drug susceptibility tests, DSTs). DSTs assess the ability of mycobacteria to grow in a solid or liquid medium containing antimicrobial drugs [9]. Despite being a commonly used technique, phenotypic diagnosis methods are laborious, time-consuming due to the slow growth rate of mycobacteria and require dedicated facilities [10]; furthermore, the results are not entirely reproducible in some cases [11]. Such limitations can be overcome with rapid molecular tests; however, these approaches target only a reduced number of genomic positions located in hot-spot regions for the most frequent drug-resistance-associated mutations [12–16]. This limitation increases false-negative results due to silent mutations and other mutations located outside the hot-spot regions [17, 18]. As an alternative, whole-genome sequencing (WGS) provides a maximum level of detail regarding the complete genomic sequence. Published catalogues

Impact Statement

Nanopore long-amplicon sequencing represents an under-used technology for diagnosing tuberculosis despite its potential to predict drug resistance and geno-type at almost real-time. This work describes a proof-of-concept study in which a single-tube PCR assay to identify drug resistance and phylogenetic-determining mutations has been designed and evaluated with clinical cultures and diagnostic sputa. In addition, this study demonstrates the viability of the long-amplicon sequencing strategy regardless of the sample type. Our bioinformatics analyses establish a high degree of congruence to Illumina short-reads (as the gold-standard technique); however, we note the challenges associated with interpreting heteroresistance from Nanopore low-frequency variants.

of mutations robustly associated with drug-resistant phenotypes can be used to design targeted approaches against a wide number of regions involved in drug resistance. However, obtaining the whole-genome sequence from sputum samples is challenging and expensive (see, for example, Goig *et al.* [19]).

Compared to WGS, targeted sequencing of drug-resistance-associated genes represents a cheaper approach that can be routinely applied to sputum samples. Two assays based on amplicon sequencing have been developed by next generation sequencing (NGS) technologies – the Deeplex Myc-TB assay

Table 1. Accession numbers of samples included in this study

	Illumina		MinION	
Sample	**Study accession**	**RUN accession**	**Study accession**	**RUN accession**
G841	PRJEB22237	ERR2099789	PRJEB44496	ERS6293816
N0067	PRJEB3223	ERR233356	PRJEB44496	ERS6293819
G1335_W27	PRJEB37609	ERR3994128	PRJEB44496	ERS6293807
G1646_W19	PRJEB37609	ERR3994105	PRJEB44496	ERS6293808
G2103_W23	PRJEB37609	ERR3994108	PRJEB44496	ERS6293812
G2267_W20	PRJEB37609	ERR3994106	PRJEB44496	ERS6293813
G2284_W26	PRJEB37609	ERR3994111	PRJEB44496	ERS6293814
G107	PRJEB38719	ERR4195665	PRJEB44496	ERS6293806
G1800	PRJEB38719	ERR4195305	PRJEB44496	ERS6293809
G1961	PRJEB38719	ERR4195372	PRJEB44496	ERS6293811
G770	PRJEB38719	ERR4195357	PRJEB44496	ERS6293815
G870	PRJEB38719	ERR4195619	PRJEB44496	ERS6293817
G981	PRJEB38719	ERR4195376	PRJEB44496	ERS6293818
182320_M20	PRJEB44496	ERS6337728	PRJEB44496	ERS6293805
G1952	PRJEB44496	ERS6337729	PRJEB44496	ERS6293810

Table 2. Description of the dataset included in this study

Sample	Sample type	Run MinION	Resistance profile	Resistance agreement	Lineage	Lineage agreement
182320_M20	Sputum	Run1	Susceptible	100	Lineage4	100
G107	Culture	Run1	Susceptible	100	Lineage3	100
G1800	Culture	Run1	FQ, RMP, INH, KAN, EMB	100	Lineage2	100
G1952	Culture	Run1	Susceptible	100	Lineage6	100
G1961	Culture	Run1	RMP, EMB	100	Lineage5	100
G770	Culture	Run1	Susceptible	100	Lineage4	100
G841	Culture	Run1	RMP	100	Lineage4	100
G870	Culture	Run1	FQ, RMP, INH, KAN, EMB	100	Lineage2	100
G981	Culture	Run1	Susceptible	100	Lineage2	100
N0067	Culture	Run1	Susceptible	100	Lineage1	100
G1335_W27	Sputum	Run2	Susceptible	100	Lineage2	100
G1646_W19	Sputum	Run2	Susceptible	100	Lineage4	100
G2103_W23	Sputum	Run2	Susceptible	100	Lineage4	100
G2267_W20	Sputum	Run2	Susceptible	100	Lineage4	100
G2284_W26	Sputum	Run2	Susceptible	100	Lineage4	100

TP, true positive variants; FN, false negative variants; FP, false positive variants; TN, true negative variants; TPR, true positive rate; TNR, true negative rate.

(GenoScreen, Lille, France) [20] and the Next Gen-RDST assay (Translational Genomics Research Institute, Phoenix, AZ, USA). Both assays target specific genomic regions known to be hot-spots for drug-resistance-conferring variants. Due to its flexibility, custom panels can incorporate other target regions, such as those that allow *M. tuberculosis* genotyping. Importantly, strain type plays an important role in disease outcome, transmission, drug-resistance emergence, host responses to the pathogen, disease epidemiology and virulence [21, 22]. Amplicon sequencing can

be directly performed on patient samples, forgoing the need for long *in vitro* culture times, and obtaining high depth of coverage [23]. In addition, an increasing body of evidence has suggested the role of heteroresistance to some drugs, in which drug-resistance subpopulation undetected during *in vitro* culture may impact treatment outcomes [24].

Nanopore (Oxford Technologies) released MinION, a portable device that allows real-time sequencing and analysis. MinION

Table 3. Genes and phylogenetic regions included in the gene panel, their corresponding positions in the MTBC genome and the antibiotic they confer resistance to

Amplicon MTBC genomic positions		Gene	Prediction
Start	**End**		
4193	7279	*gyrB*	Fluoroquinolones
7300	9882	*gyrA*	Fluoroquinolones
759779	763387	*rpoB*	Rifampicin
1471177	1474319	*rrs*	Streptomycin
1673088	1675890	*inhA*	Isoniazid
2153843	2156356	*katG*	Isoniazid
2287571	2290686	*pncA*	Pirazinamid
2713324	2716363	*eis*	Kanamycin
4246440	4249901	*embB*	Ethambutol
4356331	4359445	*Rv3878/Rv3879c*	L1, L2, L5
1279773	1282976	*Rv1155*/intergenic region *Rv1156-Rv1157c*	L3, L4, L6
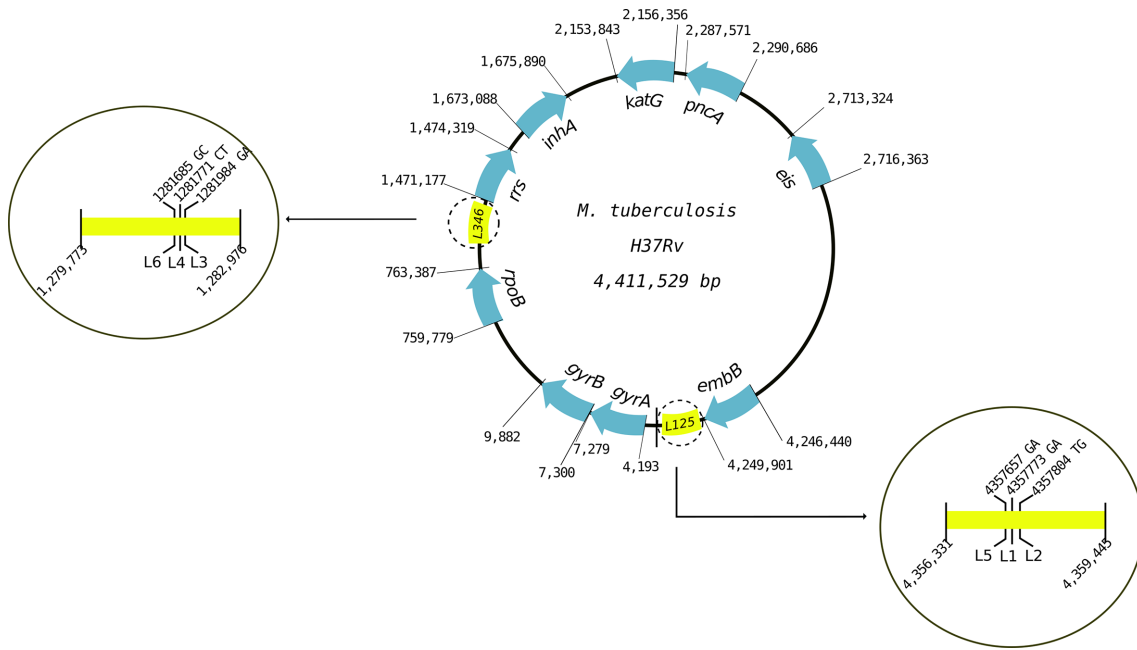
Fig. 1. Diagram of the target regions of the gene panel. The lengths of regions are not scaled to genome size.

Fig. 2. Workflow diagram summarizing the methodology.

Fig. 3. qPCR of the multiplex PCR product. Each colour represents a different gene. Two replicates per gene and negative controls were included.

long-read sequencing is based on nanopores that act as biosensors, measuring the changes in voltage produced when a nucleotide passes through them [25]. This sequencing technology has been recently applied 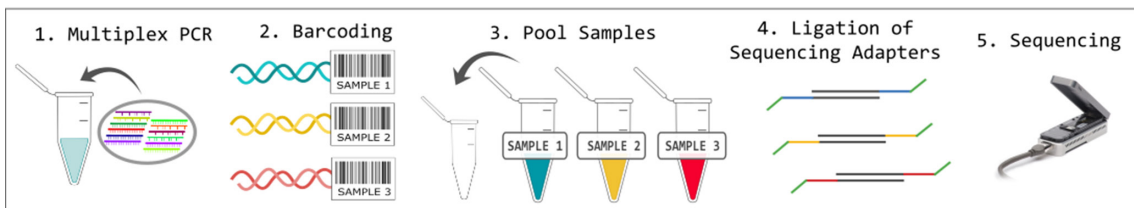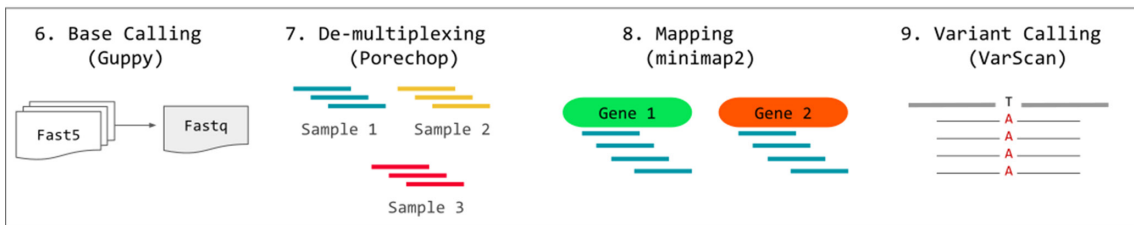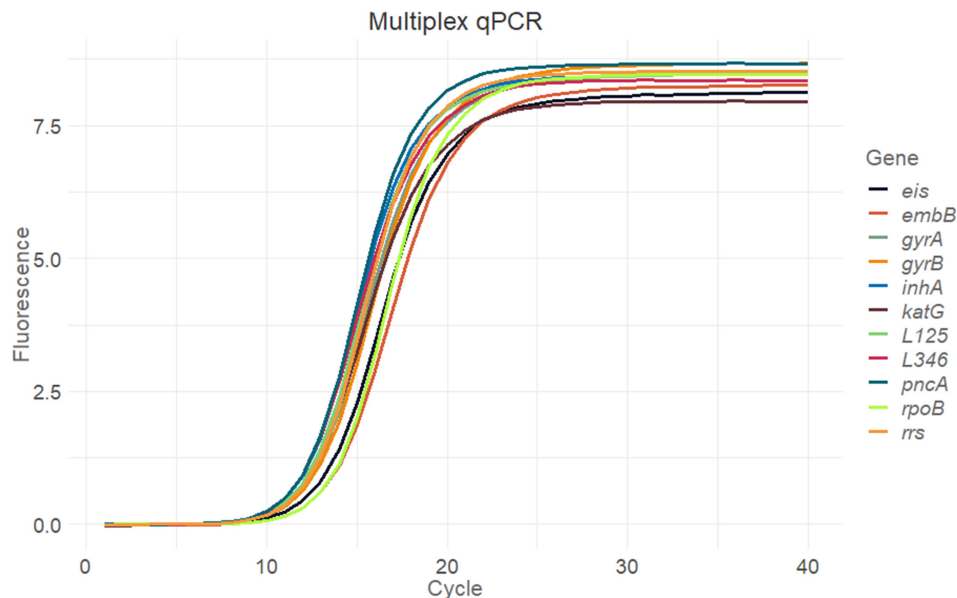to the point-of-care diagnosis of viral and bacterial infections [26, 27]. In MTBC research, MinION technology has been employed to assemble *M. tuberculosis* Beijing XDR strains and detect DR-TB in Madagascar [28, 29]. The Nanopore MinION platform presents certain limitations, including the generation of noisy reads and a high error rate [30]. However, these limitations are balanced by the higher depth of coverage obtained compared to Illumina sequencing technology, which makes free-culture sequencing and shorter diagnostic turnaround times possible [31].

This study aims to develop a single-tube PCR amplification protocol to sequence a panel of genes of interest using Nanopore technology and calibrate MinION variant-calling parameters for prospective sequencing by comparing them to paired Illumina samples. We analyse recall and true negative rate to identify known drug-resistance-associated variants in culture and sputum samples. We also analyse accuracy in identifying new variants and the source of errors associated with Nanopore MinION amplicon sequencing by comparing results with Illumina MiSeq WGS technology, the current gold-standard technique.

METHODS

Samples and DNA extraction

Fifteen samples were barcoded and sequenced in two MinION runs. In the first run, nine samples extracted from solid pure cultures were sequenced, including lineage control strains (L1-L6) and one sample extracted from smear-positive sputum (no smear grading was provided for sample 182320_M20). In the second

run, five smear-positive sputa extracted directly from respiratory specimens of TB culture-positive patients were sequenced. Acid-fast bacilli (AFB) smear results were four 3+ sputa (G2267_W20, G2103_W23, G2284_W26, G1335_W27), and one 1+ sputum (G1646_W19). Cultures from all samples were previously whole-genome sequenced in our laboratory using the Illumina MiSeq platform (Table 2).

DNA from culture samples was extracted following the standard CTAB protocol [32], while DNA from sputum samples was extracted following a differential cell lysis-based protocol to remove, as much as possible, all the contaminant non-MTBC DNA, according to Goig *et al.* [19]. All steps performed before library preparation were carried out in a BSL-3 laboratory.

Culture and sputum samples included in this study were derived from an ongoing surveillance programme of communicable diseases by the General Directorate of Public Health of the Comunitat Valenciana (Spain); therefore, the samples fall outside the mandate of the corresponding Ethics Committee for Biomedical Research. All personal information was anonymized, and no data allowing individual identification were retained.

Primer design

The gene panel consisted of nine resistance genes and two phylogenetic-determining regions. We selected nine genes known to be associated with first- and second-line drug resistance in MTBC (*rpoB, inhA, katG, pncA, embB, rrs, gyrA, gyrB* and *eis*) and two regions containing phylogenetic-determining SNPs (one region for lineages 1, 2 and 5 and another region for lineages 3, 4 and 6) (Table 3, Fig. 1) [33]. Primers were designed to amplify the entire genomic regions, obtaining products between 2500–3600
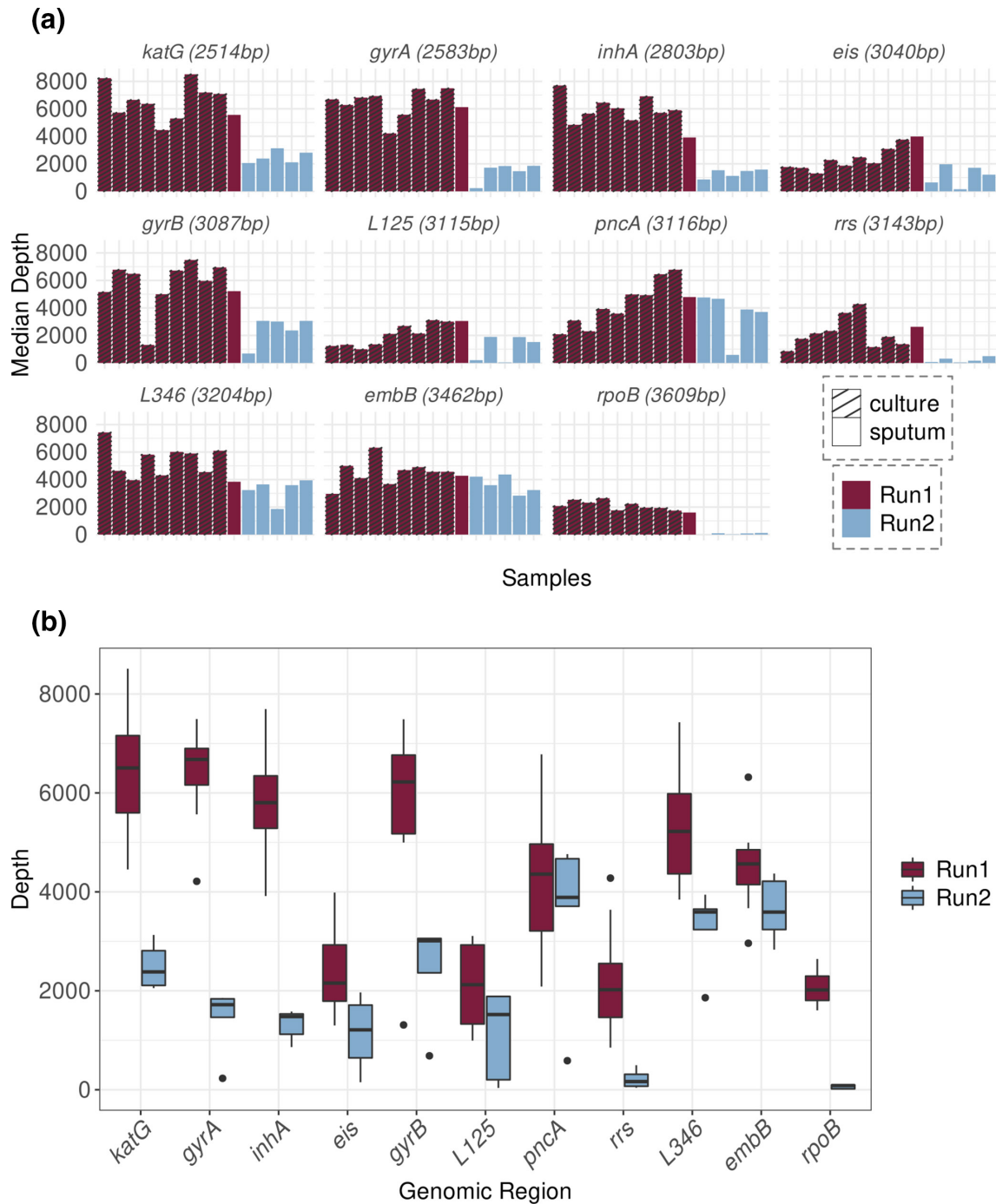
Fig. 4. Depth of coverage by gene in each MinION sequencing run. Genes are ordered by length (from the shortest to the longest). (a) Bar plot representing the median depth by sample and by region of the gene panel. Colours represent the sequencing run and the pattern of the bars represent sample type. (b) Box plot representing the distribution of sequencing depths of each genomic region in the 15 samples. Plots are coloured according to the MinION sequencing run (purple for the first run, blue for the second run).

bp. Their specificity for MTBC was verified with Primer-BLAST (NCBI). The primer concentration for PCR reactions was optimized for an equimolar amplification of all genes (Methods S1, available in the online version of this article). Table S1 contains all primer concentrations and sequences.

Single-tube multiplex PCR

A single-tube multiplex PCR reaction was performed for each sample using the Kapa HiFi HS polymerase kit (Kapa Biosystems). The final reaction volume was adjusted

depending on the DNA concentration of the samples to optimize yield (50 µl for samples above 10^{-3} ng µl^{-1}, and 100 µl for samples below 10^{-3} ng µl^{-1}). The optimum concentration of primers was previously set-up (see Methods S1 for the evaluation of optimal PCR conditions). PCR conditions were 3 min of DNA denaturation at 95 °C followed by 30 cycles of amplification as follows: 20 s at 98 °C for denaturing, 15 s at 65 °C for primer annealing, 2 min at 72 °C for extension, followed by 5 min at 72 °C for a final extension. The PCR product was purified with AMPure XP magnetic beads at 0.6X volume (following the Agencourt AMPure XP purification protocol). The pure PCR product was eluted with 10 mM Tris (pH=8.5) and quantified with Qubit to evaluate reaction yield. Fig. 2 summarizes the steps of the workflow.

MinION library preparation and sequencing

Samples were barcoded by ligation using customized barcodes (see Methods S2) and pooled in an equimolar manner. An additional PCR enrichment step after barcoding was performed in samples that failed to reach 500 ng of DNA after the first PCR (see Methods S3).

The library was prepared using the 1D SQK-LSK108 Ligation Sequencing Kit (Oxford Nanopore Technologies) following the recommended protocol with modifications to increase process efficiency (see Methods S4).

Analysis of nanopore reads

After sequencing, Fast5 files were base-called with Guppy version 2.3.5 (Oxford Nanopore Technologies) using the 'flip-flop' model described by Wick *et al.* [34], which is slow but provides high read accuracy (see Methods S5 for the code). The resulting fastq file was demultiplexed with Porechop version 0.2.3 (https://github.com/rrwick/Porechop), generating a

fastq file for each sample containing its corresponding reads (Methods S6). The source code of Porechop was modified by substituting the default sequences of Oxford Nanopore barcodes with the sequences of our custom barcodes (Table S2). Reads were mapped to the MTBC reference sequences of target genes included in the gene panel using minimap2 version 2.5-r607-dirty [35] with default parameters (Methods S7) [reference sequences were obtained from an inferred MTBC ancestral genome (NC_000962.3) [36]]. Aligned reads with a map quality under 60 (MAPQ60) were discarded. The remaining BAM file was sorted using samtools [37] (see Methods S7 for the mapping code). Variant calling and indel (insertion/deletion) calling were performed by running VarScan v2.3.7 [38] (https://github.com/dkoboldt/varscan) (see Methods S8 for the variant-calling parameters).

Illumina sequencing and analysis

DNA libraries for WGS were prepared according to the standardized NexteraXT protocol and loaded into the Illumina MiSeq sequencer. Reads were analysed following our tested and publicly available analysis pipeline (https://gitlab.com/tbgenomicsunit/ThePipeline) for mapping and variant calling (http://tgu.ibv.csic.es/?page_id=1794). This pipeline includes a first step to filter out low-quality and non-MTBC reads with fastp (https://github.com/OpenGene/fastp) and Kraken [39]. Filtered MTBC reads were aligned to an inferred MTBC ancestral genome [40] with BWA-MEM version 0.7.10 [41] and applied the MAPQ60 filter. Next, SNP calling and indel calling were performed with VarScan v2.3.7 [42] considering only positions covered at least by six reads, a minimum depth of coverage of 10, a minimum base quality of 25, and that present a minimum frequency of the variant allele above 0.1 (for variant positions, vSNPs) and 0.9 (for fixed variants, fSNPs). Additional information and details of the pipeline can

Table 4. Median depth of coverage for each gene per MinION run

Region	Run 1			Run 2		
	Median depth	**IQR**	**Min–max**	**Median depth**	**IQR**	**Min–max**
gyrA	6677	737.25	4213–7493	1719	373	231–1856
katG	6505	1561	4453–8510	2383	702	2056–3129
inhA	5802	1059.25	3916–7695	1479	410	863–1580
gyrB	6222	1590	1310–7489	3011	695	686–3058
embB	4564	801	2962–6318	3592	976.5	2833–4370
pncA	4358	1754.75	2086–6780	3888	960	587–4763
eis	2156	1135.75	1299–3985	1211	1067	152–1967
rpoB	2016	2011.75	1605–2643	84	78	3–118
rrs	2022	1088	852–4278	166	241	40–495
L125	2124	1597.5	994–3109	1520	1684	37–1896
L346	5222	1618	3844–7428	3593	3930.625	1860–3943

IQR, interquartile range.
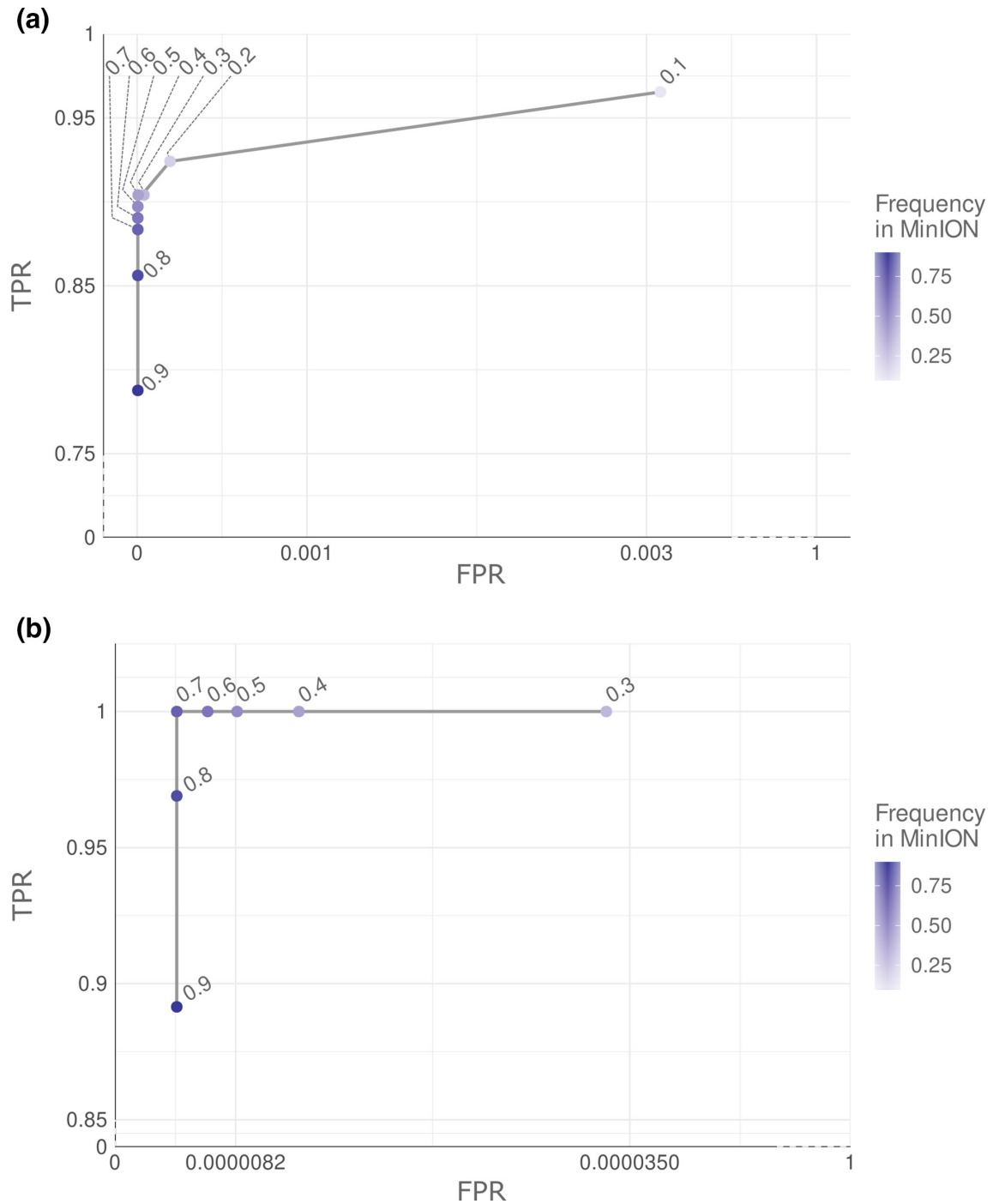
Fig. 5. ROC curve used to set the frequency threshold employed to call variants in MinION. Points represent the values for recall and false positive rate obtained when applying different frequency values in MinION variant calling. Both axes are truncated. (a) ROC curve used to set the frequency threshold to call variable SNPs in MinION. Recall and false positive rate value obtained using different variant calling frequency cut-offs for MinION (from 0.1 to 0.9 using increments of 0.1) and comparing with Illumina variant calls at a 0.1 fixed threshold. (b) ROC curve used to determine the frequency threshold to call fixed SNPs in MinION. Both axes are truncated. Recall and false positive rate value obtained using different variant calling frequency cut-offs for MinION (from 0.3 to 0.9 using increments of 0.1) and comparing with Illumina variant calls at a 0.9 fixed threshold. For a detailed result see Fig. S1.

(a)

(b)
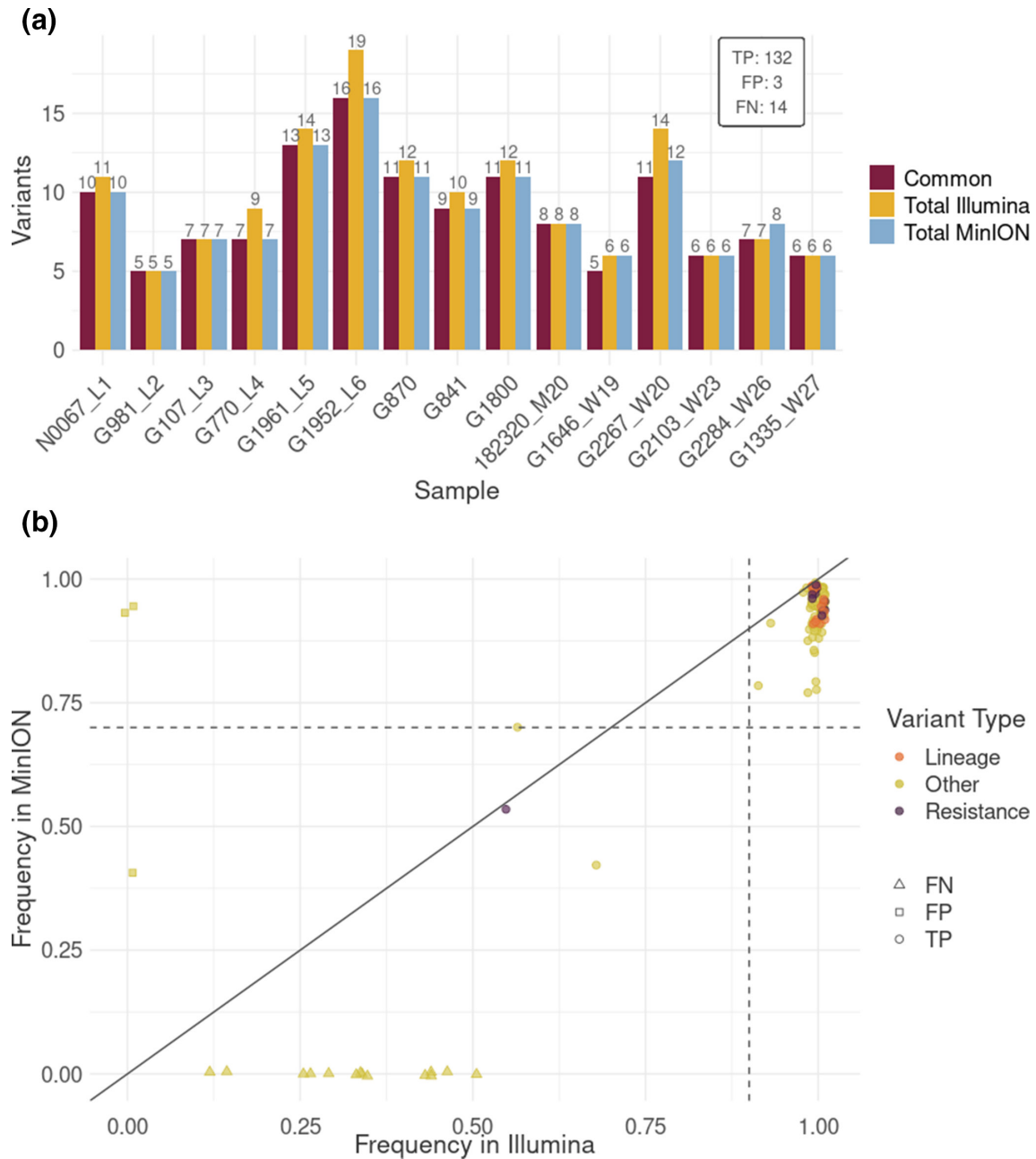
Fig. 6. Comparison of variants found in Illumina and MinION. (a) Number of variants by sample obtained in MinION and Illumina that passed all quality filters for credible variants defined in Methods. Blue bars represent all variants detected in MinION reads, yellow bars represent variants obtained in Illumina reads, and purple bars represent common variants detected by both sequencing platforms. TP, FP and FN values in the inset box were obtained considering 15 samples. (b) Variant frequency correlation between Illumina and MinION reads. Only SNPs that passed all filters are represented. Points represent variants that appear in both platforms, squares represent discrepant variants only present in MinION reads, and triangles represent variants only present in Illumina. Orange represents phylogenetic variants, purple represents variants associated with antibiotic resistance, and yellow represents other variants. Dashed lines represent the threshold values to define fixed variants for MinION (horizontal) and Illumina (vertical).

Table 5. Comparison of common and discrepant fSNPs

Frequency in MinION	TP	FP	FN	TN	TPR	TNR
0.1	129	1489	0	478248	1	0.9968962
0.2	129	98	0	479592	1	0.9997957
0.5	129	4	0	479678	1	0.9999917
0.4	129	6	0	479676	1	0.9999875
0.3	129	16	0	479666	1	0.9999666
0.6	129	3	0	479679	1	0.9999937
0.7	**129**	**2**	**0**	479680	**1**	**0.9999958**
0.8	125	2	4	479680	0.9689922	0.9999958
0.9	115	2	14	479679	0.8914729	0.9999958

TP, true positive; FN, false negative; FP, false positive; TN, true negative; TPR, true-positive rate; TNR, true-negative rate.

be found at http://tgu.ibv.csic.es/wp-content/uploads/2020/03/ThePipeline_flowchart.pdf.

Resistance profile and phylogenetic classification

The resistance profile of each strain was obtained using a customized script (Python 2.7.5) that searched for drug-conferring variants in the target genes of the panel using a catalogue of well-known variants obtained from two databases [PhyResSe (http://www.phyresse.org/) and ReSeqTB (https://platform.reseqtb.org/), 9 July 2019]. In addition, lineage-defining variants (shown in Table S3 and described in a previously published study [33]) were searched to determine the major MTBC lineage of each strain. The customized script for resistance prediction and genotyping is available at GitLab https://gitlabcom/tbgenomicsunit/paneltb_scripts/.

Table 6. Indels' comparison

Frequency in MinION	Indels MinION	Indels Illumina	TP
Freq 10	14413	16	6
Freq 20	3735	16	6
Freq 30	1159	16	5
Freq 40	356	16	5
Freq 50	81	16	5
Freq 60	49	16	5
Freq 70	19	16	5
Freq 80	17	16	4
Freq 90	1	16	1
Freq 100	0	16	0

TP, true positive.

Calibration of variant calling in MinION

A receiver operating characteristic (ROC) curve analysis was performed to determine the optimum variant allele frequency to reduce false-positive calls by calling variants at different frequencies (from 0.1 to 0.9 using increments of 0.1) taking Illumina MiSeq variants (Illumina variants from now to the end) at 0.1 frequency as the reference. These analyses focused on positions that pass all variant-calling thresholds, as described above in the case of Illumina. For Nanopore MinION variants (MinION variants from now), stricter filtering was applied to reduce false-positive calls that increase background noise and decrease the recall. Positions appearing in all 15 samples sequenced by MinION were considered systematic errors and discarded. Triallelic positions with more than one nucleotide change were also discounted from downstream analysis. In addition, only positions that obtained a good depth value in both sequencing platforms were compared (positions above 10× depth in Illumina and 50× depth in MinION). Illumina was selected as the gold standard. Thus, true-positive variants (TP) were defined as variants detected in both sequencing technologies, false-positive variants (FP) as variants detected in MinION but not in Illumina, false negative (FN) as missing variants in MinION, and true negatives (TN) as sites that contained the wild-type allele in both technologies. According to these definitions, true-positive rate (TPR) or recall, true-negative rate (TNR), precision, agreement, error rate and F1-score were obtained (see Methods S9)

Insertion/deletion analysis

We performed an indel calling in MinION at different frequencies (from 0.1 to 1 with 0.1 increment) and validated with indels called by Illumina at 0.1 frequency. Two different comparisons were performed, one considering all indels regardless of the length and the other one filtering out indels longer than 50 bp.

RESULTS

Selection of genes, samples and qPCR assay set-up

We selected nine culture samples from an ongoing TB population study of the Comunitat Valenciana (Spain), while the Hospital Clínico Universitario de Valencia (Spain) provided six sputum samples. Samples were selected to maximize the number of MTBC lineages and antibiotic resistance profiles for this proof-of-concept study. All the 15 samples were barcoded and then sequenced in two MinION runs. In the

first run, we sequenced nine samples extracted from solid pure cultures, including lineage control strains (L1–L6) and one sample extracted from smear-positive sputum. In the second run, we sequenced five samples extracted directly from respiratory specimens of TB culture-positive patients. All 15 samples were previously WGS by Illumina MiSeq.

The gene panel consisted of nine genes (*gyrA, gyrB, rpoB, rrs, inhA, embB, eis, pncA* and *katG*) associated with resistance to first- and second-line drugs such as isoniazid, rifampicin,

Table 7. Phylogenetic and drug-resistance-associated variants detected by the gene panel

Sample	Variant	Gene	Antibiotic or lineage	Frequency MinION	Frequency Ilumina	Resistance profile
G1961_L5	761155CT	*rpoB*	RMP	96.33	100	
G1961_L5	1673425CT	*inhA*	INH	52.94	55.67	
G1961_L5	4247431GA	*embB*	EMB	89.12	100	MDR
G1961_L5	4357657GA	L125	Lineage 5	95.37	100	
G1800	7570CT	*gyrA*	FQ	87.44	100	
G1800	761095TC	*rpoB*	RMP	92.72	100	
G1800	2155168CG	*katG*	INH	98.06	100	
G1800	2715344GA	*eis*	KAN	96.54	100	pre-XDR
G1800	4247429AG	*embB*	EMB	98.49	100	
G1800	4357804TG	L125	Lineage 2	98.17	100	
G870	6750CT	*gyrB*	FQ	95.26	100	
G870	761155CT	*rpoB*	RMP	95.76	99.17	
G870	2155168CG	*katG*	INH	98.11	100	
G870	2715346GA	*eis*	KAN	92.68	100	pre-XDR
G870	4247429AG	*embB*	EMB	98.28	100	
G870	4357804TG	L125	Lineage 2	98.49	100	
G841	761277AT	*rpoB*	RMP	98.67	100	
G841	1281771CT	L346	Lineage 4	93.07	100	RIF–R
N0067_L1	4357773GA	L125	Lineage 1	94.77	100	Susceptible
G1335_W27	4357804TG	L125	Lineage 2	97.43	100	Susceptible
G981_L2	4357804TG	L125	Lineage 2	98.4	100	Susceptible
G107_L3	1281984GA	L346	Lineage 3	97.62	100	Susceptible
182320_M20	1281771CT	L346	Lineage 4	92.89	100	Susceptible
G1646_W19	1281771CT	L346	Lineage 4	90.75	100	Susceptible
G2103_W23	1281771CT	L346	Lineage 4	89.69	100	Susceptible
G2267_W20	1281771CT	L346	Lineage 4	90.18	100	Susceptible
G2284_W26	1281771CT	L346	Lineage 4	90.96	100	Susceptible
G770_L4	1281771CT	L346	Lineage 4	90.9	100	Susceptible
G1952_L6	1281685CG	L346	Lineage 6	84.47	100	Susceptible

FQ, fluoroquinolones; RMP, rifampicin; SM, streptomicin; INH, isoniazid; PZA, pyrazinamide; KAN, kanamycin; EMB, ethambutol.

pyrazinamide, ethambutol, streptomycin, kanamycin and fluoroquinolones; and two phylogenetic regions containing lineage determining SNPs (L1, L2 and L5 in the first region and L3, L4 and L6 in the second region) (Fig. 1). We designed this panel to cover the whole region of interest and as a one-tube test. We carried out qPCR of the multiplex PCR product as a quality-control step to assay the proportion of each amplicon before sequencing. Observed Cq values from 9.81 (L346 region) to 11.42 (*rpoB*) suggested that all target regions were amplified at the same ratio (Fig. 3).

Nanopore MinION sequencing

Samples were loaded in two Nanopore MinION R9 flow cells with 764 (first run) and 1375 (second run) active pores. In the first run, 848583 reads passed base calling with an N50 read length of 3123 bp, median read length of 2941 bp, median read quality of 11.4, and median depth of 4194.5× per gene. In the second run, a total number of 623989 reads passed base calling with an N50 read length of 3153 bp, median read length of 3102 bp, median read quality of 10.6, and median depth 1846× per gene. We hypothesize that the quick depletion of the library prompted the lower yield in the second run.

Porechop demultiplexed 65.9 and 61.6% of reads in the first and second runs, respectively; however, we failed to detect barcodes for around 22% of reads in both runs due to problems in the barcode ligation step. In addition, 11.4 and 16.2% of reads in the first and second runs, respectively, could not be demultiplexed as they did not pass the default threshold used in Porechop (see demultiplexing parameters in Methods S6).

Regarding the depth of coverage, it was higher in the first run, perhaps due to the sample type, the library or flow-cell differences. The first run included DNA extracted from cultures (only one sputum sample), while the second run included only sputum samples. The greater heterogeneity of sputum samples generally associates with lower DNA quality and DNA fragmentation. Fig. 4 depicts median depth-coverage values for each gene per sample; we found that the *rpoB* and *rrs* genes had the lowest depth of coverage in both runs (Table 4).

Calibrating variant-calling parameters in MinION

A ROC analysis was performed to determine the frequency threshold for MinION Nanopore variant-calling optimization, taking Illumina variants as a reference (those passing the filters and at least 0.1 frequency) to set the parameters for future applications. The nature of the vSNPs obtained in MinION and Illumina in all 15 samples was compared after filtering (i) variants detected in all MinION samples (systematic errors), (ii) triallelic positions (more than one nucleotide change), and (iii) by depth threshold under 50×. While whole-genome sequences were available in the case of Illumina, only positions belonging to the gene panel regions were included in the comparison study. This analysis highlighted 0.4 as the optimum frequency threshold to obtain the highest TPR and TNR to call variants with >0.1 frequency in Illumina (Fig. 5a, Table S4). Based on these cut-offs, calls from MinION were

classified as TP if they were detected in both sequencing platforms, FP if they were detected only in MinION reads, and FN if they were detected in Illumina reads and not in MinION. A total of 132 TP, 3 FP and 14 FN were obtained, which resulted in a 90.41% TPR, 99.99% TNR, 88.59% agreement, 97.77% precision, 99.99% accuracy, 12.59% error rate and a 0.94 F1-score. All FN positions were Illumina low-frequency variants missed in MinION. Globally, we found a lower variant frequency in MinION than Illumina (Wilcoxon test, *P*-value<0.001) with a median of 4.44 [IQR=4.51] change in frequency (Fig. 6b). This difference explains the loss of low-frequency variants in MinION.

Regarding the identification of variants fixed in Illumina (>0.9 frequency) that are typically used for phylogenetic and epidemiological investigations, ROC analysis identified a cut-off value of 0.7 in MinION to maximize TPR and TNR (Fig. 5b). A strong concordance between both platforms was obtained with an F1-score of 0.99, 98.47% agreement, 99.99% accuracy, 100% TPR, 99.99% TNR, and 1.53% error rate (Table 5). Of note, the 0.7 threshold in MinION involved the identification of two FPs.

Same ROC analysis was performed classifying SNPs by sample type for vSNPs and fSNPs obtaining the same cut-off frequencies mentioned above (see Tables S7 and S8, Fig. S1).

Indels' analysis

We tried to assess the capability of MinION to call insertions and deletions by calling indels at different frequencies (0.1 to 1 with 0.1 increments) and comparing the results with indels called by Illumina at 0.1. The total amount of indels detected by each sequencing platform is shown in Table 6. TPs are considered as indels detected by both sequencing platforms. We observed a very low agreement in indel calling between both sequencing platforms suggesting that most of them could be due to sequencing or mapping errors. So that, indel calling still remains a limitation for drug-resistance prediction from MinION data.

Drug resistance and lineage prediction

Regarding phylogenetic and drug-resistance-associated variants, 100% of the variants found in Illumina reads were also called by MinION when applying the cut-off frequency obtained in the ROC analysis (0.4). All samples (lineage control samples, sputa and cultures) could be classified correctly in their correspondent lineage by evaluating lineage-specific variants (Table S3). From 15 samples, we obtained one lineage 1, four lineage 2, one lineage 3, seven lineage 4, one lineage 5, and one lineage 6 strains (Table S5). We determined resistance profiles by evaluating known variants in panel genes associated with drug-resistant phenotypes [43, 44] resulting in one sample resistant to RMP (G841), one MDR strain with heteroresistance to INH (G1961), two pre-XDR-TB strains (G1800, G870) and 11 drug-susceptible strains (Table S5). Phylogenetic and drug-resistance SNPs detected are shown in Table 7. Both lineage classifications and antibiotic resistance predictions by MinION agree with the results obtained

with Illumina WGS, when identifying known DR associated SNPs (0.4 frequency), giving a 100% agreement between both sequencing technologies (see Table S9). However, the agreement decreased for some regions when examining all variants. For RMP, the agreement was 50 % in sputa and 90% in cultures; for SM it was 60% in cultures; for KAN it was 75% in sputa; for L125 the agreement was 54 and 60% in sputa and culture, respectively; and for L346 it was 60% in sputa (see Table S9). These findings provide robust evidence that the gene panel sequenced using MinION can predict resistance profiles and strain lineages associated with known variants, but if novel variants are found additional validation will be required.

DISCUSSION

The rapid and accurate detection of drug-resistant mycobacterial strains represents a crucial means to ensure treatment effectiveness; however, conventional diagnostic techniques are often time-intensive due to the requirement for *in vitro* culture steps. Amplicon-based approaches coupled with MinION sequencing are a promising strategy towards culture-free characterization of TB bacilli. Amplicons can detect resistant strains in culture-free samples with very low limit-of-detection meaning that drug-resistance results can be obtained in around a week, which is faster than culture-dependent strategies that can last up to a month. In addition, Illumina MiSeq takes around 72 h to produce 20 GB of output while MinION takes between 24 and 48 h and the main advantage is that the user can stop it when the output is enough for downstream analysis, as it allows real-time base calling. Here we design an amplicon-based approach aimed to take advantage of MinION long reads. Current targeted amplicon-sequencing techniques to identify DR-TB are based on short-read NGS technologies (i.e. Deeplex Myc-TB), which mainly focus on small amplicons (around 300 bp) within regions containing known drug-resistance-associated mutations [20]. Low DNA concentrations and high levels of fragmentation drive the need for small amplicons, particularly in complex samples, but this precludes certain advantages associated with long-read sequencing technologies like Nanopore. MinION long-read technology can sequence entire genes for variant phasing when genomic DNA has high integrity. Notably, the high error rate (as the main associated limitation) has decreased with time [45–47] through improvements in base-calling and variant-calling steps [34].

We present a long-amplicon alternative for research applications and demonstrate its utility using diagnostic samples. The strategy proposed in this study involves sequencing entire genes (around 2000–3000 bp), which is an advantage to identify all variants in each genomic region but it could compromise the efficiency of the multiplex PCR for some amplicons. We observed differences in depth of coverage between the genes included in our panel being *rpoB* and *rrs* the ones with the lowest depth value. Our data suggests that the length of the amplicon could represent an important bias during multiplex PCR amplification as shorter fragments are amplified preferently (as seen

in Fig. 4 and reported by Tafess *et al.* [48]). In addition, we can not discard that sample type and between-run differences could also be responsible for the depth inconsistency. In any case, our results showed that the sequencing depth obtained was enough to detect drug-resistance-associated variants.

For clinical diagnosis, calling susceptibility is as important as calling resistance. This study shows the potential of a long-amplicon panel in predicting susceptibility and drug-resistance from both culture and sputum samples when scanning known markers. We designed a gene panel targeting nine genes (*gyrA, gyrB, rpoB, rrs, inhA, embB, eis, pncA* and *katG*) associated with resistance to commonly used first-line drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) and injectable anti-TB drugs (streptomycin, kanamycin and fluoroquinolones). Since 2019, the WHO has updated the treatment for MDR-TB patients by recommending all-oral regimens including new drugs like bedaquiline over injectable drugs and also, some antibiotics, such as streptomycin, fell into disuse in some countries. In this assay, Streptomycin was included for historical reasons, but we have to emphasize the flexibility of the amplicon approaches to include or drop amplicons for different regions both for legacy drugs and new drugs by re-setting up the conditions of the multiplex PCR. The major obstacle however, is that many clinically relevant regions for new drugs remain unknown [49].

Beyond the limit of detection of our design, which would require a large number of samples, in this proof-of-concept study we focused on the bioinformatic challenges to identify variation, especially SNPs, associated with drug resistance, when using the Nanopore MinION sequencing technology. Reducing the high number of FP variant calls required a high cut-off frequency (0.4) compared with Illumina (0.1), which compromised recall in our study. Other studies have also shown 100% of agreement in the prediction of drug-resistance-associated variants between Illumina and MinION Nanopore when variants detected by MinION at an allele frequency below 0.4 are excluded from the analysis [50, 51]. Similarly, two recent studies showed complete concordance between both sequencing platforms when applied a threshold 0.8 to call fixed variants [52, 53]. Accurate identification of low-frequency variants is relevant for clinical management [11, 54] especially for drugs such as fluoroquinolones, for which frequencies ranging from 14–38 % have been reported [55].

Thus, the identification of heteroresistance below 0.4 remains challenging for MinION. MinION is also currently limited to call insertions/deletions due to the high error rate. Insertions/deletions is a type of variation that has been associated with resistance to diferencrent drugs. Our analysis shows the limited correlation between indel calls in MinION compared to WGS. Fortunately, Nanopore has recently released new flow cells with an improved chemistry that increases the variant-calling accuracy and, therefore, the resolution in low-frequency variant studies [56]. Our proof-of-concept study demonstrates the power of single-molecule sequencing to determine the resistance profile and lineage regardless of sample type and points out the need to overcome variant calling challenges to fully

take advantage of its potential for the point-of-care prediction of drug resistance (especially heteroresistance and insertions/deletions).

Author contributions

Author contributions are described with CRedit taxonomy. Conceptualization: I.C. Investigation: M.T.P., G.A.G., L.M.V., L.Z.I., C.M.L. Methodology: M.T.P., G.A.G., L.M.V. Resources: M.T.P., L.M.V., R.B., D.N., I.C. Software: G.A.G., C.M.L. Formal analysis: L.Z.I., C.M.L. Data curation: C.M.L. Supervision: A.C.O., I.C. Funding acquisition: I.C. Validation: I.C., M.T.P. Visualization: C.M.L., A.C.O., I.C. Writing original draft: C.M.L., A.C.O., I.C. Writing - reviewing and editing: M.T.P., A.C.O., I.C. Project administration: I.C.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Culture and sputum samples included in this study came from an ongoing surveillance programme of communicable diseases by the General Directorate of Public Health of the Comunitat Valenciana (Spain), and therefore fall outside the mandate of the corresponding Ethics Committee for Biomedical Research. All personal information was anonymized, and no data allowing individual identification was retained.

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