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Rapid Identification of Drug-Resistant Tuberculosis Using Nanopore Targeted Next-Generation Sequencing from Sputum and Culture Isolates: Accuracy and Limitations

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Purpose: Drug-resistant tuberculosis (DR-TB) complicates treatment and requires diagnostic approaches capable of comprehensive resistance profiling of *Mycobacterium tuberculosis* (MTB). This study evaluated the diagnostic performance of the Oxford Nanopore Technologies (ONT) custom TB-DR sequencing assay, a targeted Next-Generation Sequencing (tNGS) using Nanopore sequencing technology, in sputum and culture isolates. The assay targets resistance-associated variants across 24 genes covering 13 anti-tuberculosis drugs and integrates the *hsp65* gene and direct repeat (DR) region for species identification and lineage determination.

Methods: DNA was extracted from 88 clinical samples, comprising 30 uncultured sputum specimens (10 MTB-positive, 10 non-tuberculous mycobacteria, and 10 mycobacteria-negative controls) to evaluate species identification, and 58 MTB culture isolates. The culture isolates represented diverse phenotypic resistance profiles, including mono-drug resistant, multidrug-resistant, and pre-extensively drug-resistant strains. tNGS profiles were compared with pDST to evaluate diagnostic performance for drug-resistance profiling, including sensitivity, specificity, and test agreement.

Results: Profiling success in sputum samples was dependent on mycobacterial load. Among MTB-positive sputum specimens, 6 (60%) produced results, including 2 complete resistance profiles and 4 partial profiles limited to species identification; the remaining specimens failed due to low mycobacterial load (smear-negative and high Ct values). All NTM samples were correctly identified, and all mycobacteria-negative controls tested negative. In contrast, 57 of 58 (98.3%) culture isolates yielded complete resistance profiles. Compared with pDST, sensitivity and specificity exceeded 90% for most drugs (except streptomycin, 85.7% sensitivity), with very strong agreement ($\kappa > 0.8$).

Conclusion: The ONT custom TB-DR sequencing assay provides comprehensive resistance profiling with high concordance to pDST in samples yielding complete sequencing profiles and enables species and lineage identification with a shorter analytical turnaround time compared with phenotypic testing. Performance in sputum specimens was influenced by mycobacterial load. Further studies involving larger and more diverse cohorts are needed to validate clinical applicability.

Keywords: *Mycobacterium tuberculosis*, Nanopore sequencing, tNGS, drug-resistant tuberculosis, rapid diagnostics

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), persists as a formidable global health threat. Data from the *Global Tuberculosis Report 2025* confirm that TB remains the leading cause of death from a single infectious agent, following the COVID-19 pandemic period, and presents a heavy burden worldwide.[1] Despite extensive global efforts, the incidence of drug-resistant tuberculosis (DR-TB) remains a critical concern.[1] In this context, accurate and timely drug susceptibility testing (DST) is critical, not only for guiding effective treatment regimens but also for curbing the transmission of resistant strains.[1]

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A major challenge in TB diagnostics is the gap between speed and comprehensiveness. While phenotypic drug susceptibility testing (pDST) remains the gold standard, it is labor-intensive and delayed by the requirement for mycobacterial culture. Rapid molecular assays like Xpert MTB/RIF, line probe assays (LPA), and MTB real-time PCR panels have revolutionized turnaround times.[2,3] However, these molecular methods are primarily limited to detecting common resistance-associated variants (RAVs) for specific drugs, often resulting in incomplete resistance profiles.[4] Whole genome sequencing (WGS) offers a more comprehensive approach, providing detailed genomic data for resistance profiling and phylogenetic analysis. However, WGS is typically performed on cultured MTB isolates and requires biosafety level 3 facilities, delaying treatment decisions by weeks. While WGS enrichment from uncultured sputum is a promising development,[5] the high costs and advanced bioinformatics expertise required to make it inaccessible to many low- and middle-income countries.[6] However, bypassing culture to perform sequencing directly on sputum introduces a practical trade-off in diagnostic performance. Sequencing performed on cultured isolates has been shown to provide reliable drug-resistance profiling, with good concordance to pDST. In contrast, studies of direct sputum sequencing indicate that not all specimens yield sufficient data for complete resistance prediction, with performance influenced by the amount of mycobacterial DNA present in the specimen.[7,8]

To bridge this gap, targeted next-generation sequencing (tNGS) has emerged as a practical alternative recommended by the WHO for patients requiring comprehensive DST with shorter turnaround times.[9] By amplifying and sequencing only specific drug-resistance regions, tNGS offers a balance between the depth of WGS and the sensitivity required for direct specimen analysis. Accordingly, the *WHO Consolidated Guidelines on Tuberculosis (2025)* have endorsed this class of diagnostics, including platforms such as the Deeplex® Myc-TB assay (GenoScreen) and TBSeq (ShengTing Medical Technology Co).[10] These amplicon-based approaches allow for simultaneous comprehensive profiling without the high cost and infrastructure demands of WGS.[11]

The ONT custom TB-DR sequencing assay (Oxford Nanopore Technologies) is a tNGS platform utilizing Nanopore sequencing technology, specifically designed for TB diagnostics. The assay detects RAVs in 24 target genes of MTB linked to 13 anti-TB drugs: rifampicin (*rpoB*), isoniazid (*katG, inhA, fabG1*), pyrazinamide (*pncA*), ethambutol (*embB, embA*), streptomycin (*rrs, gidB, rpsL*), kanamycin (*eis, rrs*), amikacin (*rrs*), capreomycin (*tlyA, rrs*), fluoroquinolones (*gyrA, gyrB*), ethionamide (*ethA, inhA, fabG1*), linezolid (*rplC, rrl*), bedaquiline and clofazimine (*rv0678*), and delamanid (*fgd1, ddn, atpE, fbiA, fbiB, fbiC*). It also includes the *hsp65* gene for differentiating MTB from non-tuberculous mycobacteria (NTM) and the direct repeat (DR) locus for spoligotyping, enabling genotyping of the *Mycobacterium tuberculosis* complex (MTBC) to track strains and infer lineages. Notably, the platform can process up to 22 samples per flow cell, along with positive and negative controls, making it suitable for moderate-throughput settings. Therefore, this study aimed to validate this assay on both uncultured sputum and culture isolates to assess its diagnostic performance, including sensitivity, specificity, accuracy, and agreement with pDST as the gold standard.

Materials and Methods

Study Population and Samples

This study was conducted retrospectively using leftover DNA extracted from a total of 88 clinical samples stored between 2022 and 2024 at the routine Mycobacterium Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The archived samples were obtained from patients presenting with symptoms of pulmonary tuberculosis at Siriraj Hospital, a tertiary referral center.

We strategically selected archived samples to validate the performance of the assay across different clinical contexts. The study population was structured to address two specific objectives. First, to evaluate diagnostic accuracy using direct clinical specimens, the cohort included 30 DNA samples extracted from uncultured sputum, stratified into 10 confirmed *M. tuberculosis* (MTB), 10 non-tuberculous mycobacteria (NTM), and 10 mycobacteria-negative samples to strictly assess specificity of the assay. Second, to evaluate the accuracy of drug resistance prediction, the remaining 58 DNA samples derived from culture isolates were selected to maximize the diversity of phenotypic resistance profiles. This enriched cohort included 10 isoniazid-resistant (IR-TB), 11 rifampicin-resistant (RR-TB), 16 multidrug-resistant TB (MDR-TB), 4 pre-extensively drug-resistant TB (pre-XDR), and 17 pan-susceptible isolates, aiming to challenge the

assay against both first- and second-line anti-TB drugs. Drug susceptibility profiles for the 58 MTB isolates were determined using standard phenotypic DST methods (MGIT 960 and agar proportion), which served as the gold standard for evaluating the genotypic predictions of the tNGS assay.

All samples underwent routine diagnostic identification serving as the reference standard, including acid-fast bacillus (AFB) staining, liquid medium culture, Löwenstein-Jensen (LJ) culture, and Anyplex™ MTB/NTM Real-time Detection (Seegene Inc., Republic of Korea). Specifically, NTM were identified using the Anyplex™ MTB/NTM assay, while the mycobacteria-negative group consisted of clinical sputum specimens confirmed negative for both MTB and NTM by real-time PCR (true negative samples).

Specimen Processing and DNA Extraction

Sputum Samples

Single uncultured sputum samples were collected, typically in volumes of 4–5 mL (or the maximum available volume from a single collection). To decontaminate the samples, an equal volume of NALC-NaOH solution (2% w/v NALC, 1% v/v NaOH) was added to the sputum in a 50 mL tube. The mixture was shaken for 5–20 seconds to liquefy the sputum and ensure thorough contact with the decontaminating solution. The tubes were then allowed to stand for 15 minutes, followed by adding Phosphate Buffer Saline (PBS) pH 6.8 or distilled water to a final volume of 45 mL. The tubes were capped tightly and inverted several times to mix. Subsequently, the samples were centrifuged at 3000g for 15 minutes. The supernatant was discarded, and 500 µL of PBS was added to the pellet. The mixture was resuspended by pipetting up and down. For culture purposes, 250 µL of the suspension was spread on Löwenstein–Jensen (LJ) medium. The remaining 250 µL was used for genomic DNA extraction using the automated magLEAD® 12gC system (Precision System Science Co., Ltd., Japan) in accordance with the manufacturer's instructions[12] for further analysis.

Culture Isolates

For mycobacterial colonies grown on LJ medium, DNA extraction was performed using the thermolysis method.[13] Using a sterile inoculation loop, 2–3 loopfuls of the colonies were transferred to a 2 mL tube containing 0.5 mL of distilled water. The suspension was then heated at 100°C for 3 minutes. After heating, the samples were centrifuged at 13,000g for 5 minutes at 4°C. The supernatant (300 µL) was transferred to a new 1.5 mL tube, and DNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The extracted DNA was stored at −20°C until subsequent analysis.

Anyplex™ MTB/NTM Real-Time Detection Assay

The Anyplex™ MTB/NTM Real-time Detection assays (Seegene Inc., Republic of Korea) were conducted according to the manufacturer's instructions, as described previously.[14] Available results from the routine Mycobacterium Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital were retrieved for analysis. Of the 15 samples with quantitative data, 10 were from sputum samples identified as MTB-positive, and 5 were from sputum samples classified in the NTM group. The Cycle threshold (Ct) values obtained from these assays were used to assess the impact of mycobacterial load on tNGS performance in sputum samples.

Phenotypic Drug Susceptibility Testing by Agar Proportion and MGIT

Drug susceptibility testing (DST) for all culture-positive isolates was conducted using the MGIT 960 system (Becton Dickinson Diagnostic System, Sparks, MD, USA) at the routine Mycobacterium Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital. The results were retrieved from the laboratory for analysis in this study. The assays utilized critical concentrations recommended by the World Health Organization (WHO): 0.1 mg/L for isoniazid (INH), 1 mg/L for rifampicin (RIF), 5 mg/L for ethambutol (EMB), and 2 mg/L for streptomycin (STR), using BD BACTEC™ MGIT™ 960 SIRE kits. Additionally, pyrazinamide (PZA) susceptibility was tested using the BD BACTEC™ MGIT™ PZA kit with a critical concentration of 100 mg/L.[15] For the remaining drugs, DST was conducted using the agar proportion method on Middlebrook 7H10 agar. The critical concentration applied was 2 mg/L for ofloxacin (OFX). For the injectable agents, the critical concentrations were 4 mg/L for amikacin (AMK), 5 mg/L for

kanamycin (KAN), and 5 mg/L for ethionamide (ETH). These concentrations were based on WHO and CLSI guidelines, ensuring accurate identification of drug resistance across all tested isolates.[15,16]

Library Generation for Targeted Next-Generation Sequencing

The amplification, purification, and quantification of the ONT custom TB-DR sequencing assay amplicons were performed in accordance with the Oxford Nanopore Tuberculosis Drug Resistance test's user manual. In brief, 5 µL of DNA extract from clinical samples underwent PCR amplification using the ONT custom TB-DR sequencing assay, including primer sets designed and developed by Nanopore (Oxford Nanopore Technologies, UK). Libraries were then prepared using the Rapid Barcoding Kit SQK-RBK110.96 (Oxford Nanopore Technologies, UK), following the manufacturer's instructions.[17] Post-barcoding, the samples were pooled and purified using AMPure XP Beads (Beckman Coulter, USA) in accordance with the manufacturer's protocols.[17] The quantification of the purified libraries was performed using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA) on a Qubit fluorometer (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. For progression to the sequencing step, the library concentration had to reach a minimum of 50 ng/µL.[18]

Oxford Nanopore Targeted Next-Generation Sequencing (tNGS)

The sequencing adapter was attached to the qualified library in accordance with the manufacturer's instructions prior to initiating the sequencing step. The MinION flow cell (Oxford Nanopore Technologies, UK) was primed with the designated priming buffers as per the manufacturer's guidelines.[17] Subsequently, 75 µL of the library mixture, consisting of a batch of 22 pooled samples and 2 controls, was loaded onto the flow cell following the prescribed protocols.[17] The sequencing configuration was strictly set as per the Oxford Nanopore Tuberculosis Drug Resistance test's user manual, using the high-accuracy base-calling mode in the MinKNOW software. Sequencing was conducted using the MinION Mk1B Oxford Nanopore sequencer (Oxford Nanopore Technologies, UK), which was connected to a high-performance computer. Sequencing aimed for an approximate depth of 100X to accurately detect genetic resistance markers in the specified regions.

tNGS Bioinformatic Analysis

Following the sequencing run, raw fastq files from each sample were processed for species identification, drug resistance prediction, and lineage analysis using the EPI2ME software (https://epi2me.nanoporetech.com) and its associated pipeline (wf-tb-amr-v2.0.0-alpha.4), which are provided free of charge by the ONT company. In brief, MTB and NTM were identified using the *hsp65* gene. Sequencing reads from MTB were aligned to the NC_000962.3 reference genome using minimap2.[19] The base composition of predefined variants was determined with bcftools[20] for variant calling. Indels were called using clair3.[21] Variants were phased with whatshap software.[22] Lineage classification was inferred from spoligotyping by mapping reads to the direct repeat locus. The results were compiled and reported in the EPI2ME software. tNGS profile completeness classification was based on amplicon coverage, as implemented in the wf-tb-amr workflow executed within the EPI2ME software: a complete profile required coverage of 15 out of 24 drug-resistance genes, the *hsp65* gene, and the internal control, all with a median depth >20x. Partial profiles met only *hsp65* coverage, while failed profiles lacked sufficient coverage across all targets. Drug-resistance profiling was specific to MTB, as this platform is not designed for NTM resistance detection.

To verify the accuracy and reliability of the ONT pipeline in identifying and predicting drug-resistant TB, results were cross-validated using TB-profiler[23] v6.2.1 (CLI version with the –platform nanopore parameter), applied directly to the targeted sequencing products to generate resistance profiles derived strictly from the drug-resistance gene amplicons, and a manual variant calling pipeline incorporating freebayes[24] v1.3.6 for variant calling and snpEff[25] v5.2 for annotation.

Statistical Analysis

Statistical analyses were conducted using RStudio (v2024.04.2 build 764) with the epiR package (v2.0.75). Genotypic resistance data for each drug were compared with the corresponding phenotypic drug susceptibility testing (pDST) results across all samples. Diagnostic performance indicators, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, were computed using the epi.tests function. The agreement between

genotypic and phenotypic resistance profiles was assessed using Cohen's Kappa statistic, calculated via the epi.kappa function. The strength of agreement was interpreted based on the Kappa coefficient (κ), following the guidelines set by Landis and Koch:[26] κ values below 0.00 indicate poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, strong agreement; and 0.81–1.00, very strong agreement.

Results

tNGS Performance on Sputum Samples

A total of 30 DNA samples extracted directly from uncultured sputum were analyzed. Among the 10 confirmed *M. tuberculosis* (MTB) samples, species identification was successful in 6 samples (60%), all of which were correctly identified as MTB. Two of these (20%) produced complete profiles (Lineage 2-Beijing and Lineage 1 EAI2-Nonthaburi) and were correctly identified as fully susceptible to all anti-tuberculosis agents. The other four samples (40%) yielded partial profiles sufficient only for species identification, providing insufficient data for drug resistance profiling, while the remaining 4 samples (40%) failed to generate sufficient data for analysis.

Sequencing success was strongly associated with mycobacterial load. Complete profiles (20%) were obtained exclusively from samples with high bacterial loads (AFB grades 2+ and 1+; Ct values 26.81–27.76) and achieved a median sequencing depth >100x. In contrast, samples yielding partial profiles (40%), including one scanty AFB sample, were associated with lower loads (Ct >31) and variable depth coverage (27x–570x). The failed profiles (40%) generally had very low bacterial loads (AFB-negative; Ct >35).

Regarding assay specificity, 20 control samples (10 NTM and 10 mycobacteria-negative) were evaluated. The assay correctly identified NTM in 3 samples (specifically *M. iranicum* and *M. abscessus*). However, similar to the low-load MTB samples, 7 NTM samples with high Ct values failed to yield species-level identification. Importantly, no false-positive MTB detection was observed in the 10 mycobacteria-negative samples, confirming 100% specificity for MTB detection. Detailed results for the control samples are provided in Supplementary Table S1.

tNGS Performance on Culture Isolates

Of the 58 culture isolates sequenced, 57 (98.3%) were successfully profiled. One sample failed to profile due to internal control failure, indicating that the PCR failed and no reads were generated, even after repeat attempts. All 57 successfully profiled samples provided "complete profiles" (100%), with a mean depth >200x for most drug-resistance genes and 200x–500x for *hsp65*. Lineage analysis using EPI2ME (wf-tb-amr) software from the Nanopore tNGS platform found the majority to be Lineage 2-Beijing (78.0%, n=45). Lineage 1 EAI accounted for 13.6% (n=7), Lineage 4 Euro-American made up 5.1% (n=3), and one sample belonged to Manu-ancestor, while one sample failed lineage analysis (Table 1).

Compared to sputum samples, the culture isolates showed significantly higher success rates and more consistent depth coverage. While sputum samples displayed variability due to differences in *MTB* DNA quantity (as indicated by AFB grades and Ct values), all successfully profiled culture isolates reached complete profiles with high mean depth. This indicates that DNA extracted from culture isolates yielded consistently higher quality and quantity of MTB DNA, enabling more reliable profiling across drug-resistance genes and lineage determination.

The Accuracy of Nanopore tNGS Assay Compared with pDST

The accuracy of the nanopore tNGS platform was assessed by comparing its performance with pDST as the gold standard. A total of 59 samples (57 culture isolates and 2 sputum samples) were included in the analysis. However, pDST for pyrazinamide, streptomycin, and second-line drugs was performed only upon clinical indication; therefore, concordance analyses for these drugs were based on isolates with available paired phenotypic and genotypic data. Detailed phenotypic and genotypic profiles of these isolates are provided in Supplementary Table S2 and Supplementary Figure S1. The results showed high concordance between nanopore tNGS and pDST across both sample types (over 85%). Diagnostic metrics, including sensitivity, specificity, PPV, and NPV, were calculated for each drug assessed (Table 2), with genotypic resistance profiles demonstrating concordance with pDST across both sample types.

Table 1 Lineage Identification of Clinical Samples Using Nanopore tNGS

Lineage Identified Using Nanopore tNGS	Frequency
Lineage 2	
Beijing	46*
Lineage 1	
EAI2-nonthaburi	4*
EAI6-BGD1	2
EAI2-Manila	1
EAI1-SOM	1
Lineage 4	
T family	1
T2 family	1
LAM5	1
Manu-ancestor	1

Note: *Indicates that the frequency includes the samples from uncultured sputum.
Abbreviations: EAI, East-African Indian; LAM, Latin American-Mediterranean.

First-line drugs, including isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA), all showed high sensitivity and specificity (>90%). Specifically, INH had 96.6% sensitivity and 96.7% specificity, RIF had 96.7% sensitivity and 93.1% specificity. While both EMB and PZA exhibited 100% sensitivity and specificity. Streptomycin (SM) showed 85.7% sensitivity and 100% specificity. For second-line injectable drugs (SLIs), amikacin (AMK) and kanamycin (KAN) both showed 100% sensitivity and specificity. Ethionamide (ETH) exhibited 100% sensitivity and 95.7% specificity, while fluoroquinolones (OFX/LFX/MFX) had 100% sensitivity and 96.3% specificity. (Table 2). Overall, sensitivity, specificity, PPV, NPV, and accuracy were consistently high across the drugs analyzed. The concordance results were evaluated using Cohen's kappa coefficients (κ) to assess the level of agreement between tNGS and pDST. For first-line drugs, Cohen's kappa coefficients were 0.93 for INH, 0.9 for RIF, 1 for EMB, 0.91 for SM, and 1 for PZA, all indicating very strong agreement. In SLIs, kappa coefficients were 0.9 for ETH, 0.84 for FQs, and 1 for AMK and KAN, also indicating very strong agreement (Table 2).

Despite the high concordance between tNGS and pDST, discordant results were observed, including five false-positive and three false-negative calls. These discrepancies primarily involved RIF and INH, with single discordant cases observed for SM, ETH, and FQs. Notably, while perfect agreement (κ = 1) was achieved for EMB, PZA, AMK, and KAN, these findings should be interpreted with caution due to the limited number of resistant isolates, particularly for the second-line injectables, where only a single resistant isolate was detected.

Distribution of Resistance-Associated Variants (RAVs)

The distribution of mutations associated with drug resistance in the clinical samples was summarized in Table 3, providing an overview of the most common resistance-associated variants across the genes analyzed. The most frequently detected variants were the *katG* S315T, *rpoB* S450L, *rpoB* H445D, and *embB* M306V, including *embB* G406D. Additionally, the *inhA* promoter mutations, such as −777C>T (−15C>T for *fabG1*) and −154G>A, were observed. Pyrazinamide resistance was commonly associated with *pncA* mutations, including −11A>C, observed. Streptomycin resistance was predominantly associated with *rpsL* K43R.

In this study, we identified a deletion mutation in *rpoB* (c.1327_1335delTTGACCCAC) linked to rifampicin resistance, along with other *rpoB* variants (Table 3), which illustrates the diversity of mutations contributing to drug resistance. Moreover, multiple *rpoB* mutations Q432L, D435A, M434T, and L430P were found co-harboring in specific isolates, as indicated by symbols (* and **), suggesting complex resistance mechanisms. Mutations in second-line drug resistance genes were also observed, including notable mutations linked to fluoroquinolone and ethionamide resistance.

Table 2 Diagnostic Indicators of Nanopore tNGS Compared with pDST of Clinical Samples

Drug(s)	Resistance Classified By		Discordance Results		Test Agreement (Cohen's Kappa)	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Test accuracy% (95% CI)
	pDST	gDST (tNGS)	FP	FN	(95% CI)					
Isoniazid	29/59	29/59	1	1	0.93 (0.68–1)	96.6% (82.2–100)	96.7% (82.8–100)	96.6% (82.2–100)	96.7% (82.8–100)	96.6% (88.3–99.6)
Rifampicin	30/59	31/59	2	1	0.9 (0.64–1)	96.7% (82.8–100)	93.1% (77.2–99.2)	93.5% (78.6–99.2)	96.4% (81.7–100)	94.9% (85.9–98.9)
Ethambutol	6/59	6/59	0	0	1 (0.74–1)	100% (54.1–100)	100% (93.3–100)	100% (54.1–100)	100% (93.3–100)	100% (93.9–100)
Pyrazinamide	3/9	3/59*	0	0	1 (0.35–1)	100% (29.2–100)	100% (54.1–100)	100% (29.2–100)	100% (54.1–100)	100% (66.4–100)
Streptomycin	7/45	6/59*	0	1	0.91(0.62–1)	85.7% (42.1–99.6)	100% (90.7–100)	100% (54.1–100)	97.4% (86.5–99.9)	97.8% (88.2–99.9)
Amikacin	1/30	1/59*	0	0	1 (0.64–1)	100% (2.5–100)	100% (88.1–100)	100% (2.5–100)	100% (88.1–100)	100% (88.4–100)
Kanamycin	1/30	1/59*	0	0	1 (0.64–1)	100% (2.5–100)	100% (88.1–100)	100% (2.5–100)	100% (88.1–100)	100% (88.4–100)
Ethionamide	6/29	7/59*	1	0	0.9 (0.54–1)	100% (54.1–100)	95.7% (88.1–100)	85.7% (42.1–99.6)	100% (84.6–100)	96.6% (82.2–99.9)
Fluoroquinolones OFX/LFX/MFX	3/30	5/59*	1	0	0.84 (0.49–1)	100% (29.2–100)	96.3% (81–99.9)	75% (19.4–99.4)	100% (86.8–100)	96.7% (82.8–99.9)

Note: *Indicates concordance analysis was performed using the number of isolates with available pDST results.

Abbreviations: OFX, Ofloxacin; LFX, Levofloxacin; MFX, Moxifloxacin; pDST, phenotypic Drug Susceptibility Testing; gDST, genotypic Drug Susceptibility Testing; FP, False Positive; FN, False Negative; PPV, Positive Predictive Value; NPV, Negative Predictive Value; CI, Confidence Interval.

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Table 3 The Distribution of Resistance-Associated Variants (RAVs) Identified in Clinical Samples Using Nanopore tNGS

Anti-Tuberculosis	Gene	Variant Detected	Frequency	WHO Confidence Grading
Isoniazid	*katG*	S315T	26	Group 1: Assoc w R
Isoniazid/Ethionamide	*inhA*	−777C>T (*fabG1* −15C>T)	2	Group 1: Assoc w R
		−154G>A	1	Group 1: Assoc w R
Ethionamide	*ethA*	S214fs (c.639_640delGT)	5	Group 2: Assoc w R - Interim
Rifampicin	*rpoB*	S450L	17	Group 1: Assoc w R
		H445D	6	Group 1: Assoc w R
		H445R	1	Group 1: Assoc w R
		D435A*	2	Group 2: Assoc w R - Interim
		M434T**	1	Group 2: Assoc w R - Interim
		Q432L**	1	Group 1: Assoc w R
		Q432H	1	Group 2: Assoc w R - Interim
		L430P*	1	Group 1: Assoc w R
		V170F	1	Group 1: Assoc w R
		c.1327_1335 delTTGACCCAC	1	Not listed[+]
Ethambutol	*embB*	M306V	3	Group 1: Assoc w R
		M306I	1	Group 1: Assoc w R
		G406D	2	Group 1: Assoc w R
Pyrazinamide	*pncA*	−11A>C	3	Group 1: Assoc w R
		L116R	1	Group 2: Assoc w R - Interim
		Y34D	1	Group 1: Assoc w R
		T76I	1	Group 1: Assoc w R
Streptomycin	*rpsL*	K43R	8	Group 1: Assoc w R
Amikacin	*rrs*	1401A>G	1	Group 1: Assoc w R
Kanamycin	*rrs*	1401A>G	1	Group 1: Assoc w R
Fluoroquinolones	*gyrA*	A90V	3	Group 1: Assoc w R
(OFX/LFX/MFX)		D94G	1	Group 1: Assoc w R
		S91P	1	Group 1: Assoc w R

Notes: WHO confidence grading followed the WHO mutation catalogue (2023):[27] Group 1, associated with resistance; Group 2, associated with resistance (interim, limited evidence). [+]indicates variants not listed in the catalogue but reported in the TB-Profiler database from RR-TB isolates. Symbols (*, **) indicate co-occurring variants within the same isolate. *denotes one isolate carrying both *rpoB* D435A and L430P, while ** denotes a different isolate carrying both M434T and Q432L.
Abbreviations: OFX, Ofloxacin; LFX, Levofloxacin; MFX, Moxifloxacin.

Fluoroquinolone resistance was linked to *gyrA* mutations, including A90V and D94G. Mutations in the *ethA* gene, specifically the S214fs frameshift (c.639_640delGT) mutation, were associated with ethionamide resistance (Table 3).

Discussion

Conventional molecular diagnostics such as Xpert MTB/RIF, LPAs, and MTB real-time PCR panels have substantially improved TB detection[28–30] but remain limited by their narrow targets, often failing to provide complete profiles for second-line or repurposed drugs.[4,31] While WGS offers the detailed genomic analysis missing from these assays,[32,33] its routine application is hindered by the need for high-quality DNA from culture, which reintroduces diagnostic delays and demands specialized infrastructure.[5,23,34] In this context, tNGS has emerged as a pragmatic alternative, offering broader resistance coverage than conventional molecular assays while reducing turnaround time, particularly when a comprehensive drug resistance profile is required.[2,35]

In this study, we evaluated the accuracy of nanopore-based tNGS using the ONT custom TB-DR sequencing assay for predicting drug resistance directly from uncultured sputum and culture isolates. The tNGS approach demonstrated over 90% test accuracy for most first-line and second-line drugs. Specifically, accuracy exceeded 90% for first-line drugs such as INH, RIF, EMB, and PZA, consistent with previous findings.[36–39] For second-line drugs, the assay achieved 100% accuracy for AMK and KAN and 96.7% for FQs. The high accuracy observed for SLIs was based on a single positive

resistance case, emphasizing the need for larger-scale studies with more resistant cases to validate these findings comprehensively. Additionally, Cohen's kappa coefficient (κ) was within the range of very strong agreement (0.8–1) across all tested drugs, reflecting the concordance between tNGS and pDST in detecting drug-resistant TB.

Regarding the discrepancies observed in some resistance profiles, primarily in isoniazid and rifampicin, including false-negative and false-positive cases. False negatives were primarily attributed to the amplicon-based design of the ONT custom TB-DR sequencing assay, which could miss resistance-conferring mutations located outside the targeted regions. Detailed analysis of amplicon coverage using the Integrative Genomics Viewer (IGV) confirmed that the assay focuses on hotspot regions of genes where resistance mutations are most commonly found. However, rare mutations outside these targeted areas were occasionally undetected, contributing to discrepancies. For instance, one false-negative case involved a *rpoB* deletion (c.1327_1335delTTGACCCAC), which was not identified by the assay analyzing software (EPI2ME) but was detected through cross-validation with TB-profiler and manual variant calling. This discrepancy highlights the platform's reliance on the WHO DR-TB mutation catalogue,[27] which lacks this specific mutation. In contrast, the TB-profiler database includes additional mutations, demonstrating the critical role of database selection in the accuracy of resistance prediction.

False positives were also identified, such as the *rpoB* L452P mutation, which did not result in phenotypic resistance. This disputed variant has been associated with low-level rifampin resistance but may not consistently manifest phenotypically, as reported by Miotto et al.[40] Variants like L452P can result in minimal inhibitory concentrations (MICs) below the phenotypic resistance threshold, leading to classification as susceptible despite clinical associations with treatment failures. These findings suggest that integrating genotypic data from the ONT custom TB-DR sequencing assay with pDST results could provide a more accurate understanding of resistance, particularly for disputed mutations.

Our analysis identified several clinically relevant mutations associated with resistance to first- and second-line drugs. The *katG* S315T mutation was the most frequently observed, confirming its established role in high-level INH resistance.[41,42] Mutations in the *inhA* promoter, such as −777C>T (also known as fabG1 −15C>T or *inhA* −15C>T), and −154G>A were also detected, supporting their roles in low-level INH resistance and potential ethionamide cross-resistance.[43] These findings align with prior reports showing high frequencies of *katG* S315T and *inhA* promoter mutations in resistant strains.[44] Additionally, an ETH-resistant variant (*ethA* S214fs, c.639_640delGT), which disrupts ethionamide activation, was detected, further highlighting the utility of the assay in identifying diverse resistance mechanisms.[45,46]

Mutations in the *rpoB* gene, such as the well-known S450L, were frequently observed and are strongly associated with high-level RIF resistance.[47] The *rpoB* L430P mutation, a disputed variant, was also identified. Interestingly, L430P was detected alongside D435A, a mutation associated with high-level RIF resistance, suggesting that the presence of D435A may reinforce the phenotypic resistance linked to L430P.[40] Other significant mutations included *embB* M306V, associated with EMB resistance,[48] and *pncA* mutations, such as −11A>C and L116R, linked to PZA resistance.[49,50] The *rrs* 1401A>G mutation, a marker for high-level resistance to AMK and KAN, was also identified, consistent with previous findings.[51] FQ resistance was commonly associated with *gyrA* mutations A90V and D94G, which are well-established markers for this drug class.[52]

The assay includes primers targeting resistance genes for drugs in the BPaL regimen[53] (eg, *rplC* and *rrl* for linezolid,[54] *rv0678* for bedaquiline and clofazimine,[55,56] and *fgd1, ddn, atpE, fbiA, fbiB*, and *fbiC* for delamanid and pretomanid).[57] However, the absence of phenotypically resistant isolates for these drugs limited our ability to fully evaluate the assay's accuracy for these agents. Further validation with resistant reference samples is needed to strengthen its clinical application. Additionally, the high prevalence of Beijing lineage isolates (approximately 80%) observed in this study suggests lineage-specific resistance profiles, consistent with prior findings.[58]

Sequencing depth was a key limitation; specifically, complete resistance profiles were obtained in only 2 of 10 (20%) MTB-positive sputum samples, strictly associated with high mycobacterial loads. Samples with higher Ct values (>35) and negative AFB grades often failed to produce complete profiles, reinforcing the importance of sample quality for reliable sequencing. This finding aligns with previous studies indicating a relationship between Ct values and sequencing success.[11] Enhancing preprocessing steps, such as mycobacterial DNA enrichment or host DNA depletion, could improve sequencing performance for sputum samples.[4] Although a major advantage of tNGS is the potential to bypass culture requiring BSL-3 facilities, our results show that culture isolates remain superior to sputum for sequencing quality. DNA from culture isolates consistently yielded complete resistance profiles; in contrast, direct sputum sequencing was

inconsistent and dependent on mycobacterial load. Speed is a clear benefit of tNGS, but culture remains an essential backup for samples with low bacterial load to ensure accuracy.

The absence of WGS for verification presents another limitation, as resistance mutations outside the targeted regions may have been missed. Specifically, the accuracy of lineage classification requires further validation; for instance, the detection of the Manu-ancestor lineage, which is rarely reported in Thailand,[59] may represent either true identification or misclassification by the analysis pipeline. Furthermore, we recognize that the overall sample size was relatively small (n=88), and the diversity of resistance profiles was constrained by the low prevalence of certain strains in our setting. Additionally, incorporating a broader range of resistant isolates, particularly for second-line and repurposed drugs like bedaquiline, delamanid, and clofazimine, in future studies will help to comprehensively evaluate the platform's utility in MDR-TB treatment planning.

Conclusion and Perspectives

The ONT custom TB-DR sequencing assay provides a comprehensive approach to drug-resistance profiling and shows high concordance with phenotypic drug susceptibility testing for first-line drugs in samples yielding complete sequencing profiles, while also supporting species identification and lineage determination. The sequencing workflow offers a shorter turnaround time during the analytical phase compared with conventional phenotypic testing, particularly for comprehensive resistance profiling; however, when culture is required, the overall diagnostic timeline remains constrained by culture incubation. Reduced sequencing depth in sputum samples with low mycobacterial load highlights the importance of specimen quality and identifies areas for further technical optimization, including DNA enrichment strategies. Future studies involving larger and more diverse cohorts, particularly those including isolates resistant to newer anti-tuberculosis drugs, are required to better define diagnostic performance and clinical applicability.

Data Sharing Statement

The sequencing data generated in this study are available in the NCBI Sequence Read Archive (SRA) repository under the BioProject accession number PRJNA1196685.

Ethics Approval and Consent to Participate

This study was approved by the Siriraj Institutional Review Board (SI-IRB), Mahidol University, Bangkok, Thailand (COA No. SI 583/2024), in accordance with the ethical standards of the Declaration of Helsinki and applicable national regulations. A waiver of informed consent was granted by the SI-IRB, as the study used leftover DNA samples and corresponding laboratory data from routine diagnostic procedures without any direct patient contact or identifiable information.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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