國立臺灣大學公共衛生學院全球衛生碩士學位學程

碩士論文

Master of Global Health Program

College of Public Health

National Taiwan University

Master Thesis

利用全基因定序預測結核病之抗藥性

Predicting Drug Resistance in *Mycobacterium Tuberculosis* Using Whole Genome Sequencing

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中華民國111年6月

June, 2022

doi:10.6342/NTU202200927

Certificate of Thesis Approval from the Oral Defense Committee

國立臺灣大學碩士學位論文
口試委員會審定書

National Taiwan University
Verification Letter from the Oral Examination Committee for Master's Students

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Using Whole Genome Sequencing

本論文係 Winston Lie, 賴思騰 (R09853002) 在國立臺灣大學全球衛生碩士學位學程完成之碩士學位論文，於民國 2022 年6月9日承下列考試委員審查通過及口試及格，特此證明。

This Thesis is written by Winston Lie (R09853002) studying in the graduate program in the Global Health Program. The author of this thesis is qualified for a master's degree through the verification of the committee.

(指導教授簽名 Advisor Signature)

口試委員 Committee Members:

doi:10.6342/NTU202200927

Abstract (Chinese Version):

結核病是其中一個導致死亡的主要傳染病之一，且抗藥性菌株的傳播使問題更惡化，改善抗藥性結核病的診斷將能成功幫助全球結核病的預防與治療。此研究以培養為主的藥敏性測試為基準來評估全基因定序診斷到的表現。我們分析來自台灣高雄市於 2019 年 1 月至 2021 年 7 月之結核菌培養陽性通報個案中超過 85％，將近 2000 隻 TB 菌株，發現全基因定序能達到接近 90%的敏感性及高於 95%的特異性及診斷正確率。我們也使用全基因定序來預測多種結核病藥物之盛行率，發現與傳統藥敏性測試之表現型結果有很高的一致性。整體而言，研究結果提供使用全基因定序診斷抗藥性結核病以及未來如何將其應用於臨床和公共衛生中的重要支持。

Keywords (Chinese Version)

- 結核病
- 全基因定序
- 藥物敏感性測試

doi:10.6342/NTU202200927

Abstract (English Version)

Tuberculosis (TB) is one of the largest contributors to mortality among all communicable diseases, and the spread of drug-resistant strains will only exacerbate this problem. Diagnostic methods in detecting drug-resistant tuberculosis (DR-TB) need to improve if global prevention and treatment efforts are to be successful. In this study, we evaluate the performance of whole genome sequencing (WGS) in detecting DR-TB compared to culture-based, drug susceptibility testing (DST). To do this, we prospectively analyzed nearly 2,000 TB isolates from Kaohsiung, Taiwan, between January 2019 to July 2021 and achieved a coverage rate of 85% for all TB cases in the city. We found sensitivity as high as 90% for certain anti-TB drugs and greater than 95% for specificity and diagnostic accuracy. Additionally, we used WGS to predict the resistance prevalence for various drugs and found great agreement with what was determined by phenotypic DST. Overall, our results provide greater support on the use of WGS in diagnosing DR-TB, and how it may be applied in both clinical and public health settings.

Keywords (English Version)

- tuberculosis
- whole genome sequencing
- phenotypic drug susceptibility testing

doi:10.6342/NTU202200927

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Introduction

Every year, nearly half a million people globally become sick with drug-resistant tuberculosis (DR-TB).[1] Whereas drug-susceptible TB has a cure rate greater than 90%, success in treating drug-resistant TB is substantially lower at 50-60%.[2] A large reason for why DR-TB is difficult to curb lies with the problem of diagnosis.[3] Historically, drug resistance has been determined through culture-based, phenotypic drug susceptibility testing (DST). Although highly effective, phenotypic DST is time and labor intensive, taking weeks to months for trained technicians to complete.[4] In most places around the world, phenotypic DST is not carried out for every confirmed TB case, and rarely does it occur at a patient's first point of care.[5,6] All of these factors severely limit the usefulness of phenotypic DST in both clinical care and public health.

Molecular testing offers the potential to revolutionize how TB drug resistance is identified. Various, automated systems are already available that perform genotypic DST such as Xpert MTB/RIF, MTBDR*plus*, and MTBDR*sl*.[7,8] These *in vitro* diagnostic tests work by probing for specific mutations in TB isolates known to confer drug immunity.[9] As a result, molecular DSTs can provide rapid results, often in a matter of hours.[10] Despite the advantage in fast turnaround time with genotypic testing, phenotypic DST still remains the standard for characterizing drug resistance.[11] Presently, molecular testing can only screen for a small subset of anti-TB drugs at any given time and it is limited in using PCR-based approaches to search narrow regions of DNA.[12,13] However, with whole genome sequencing (WGS), it becomes possible to analyze the entire genetic code and create a comprehensive diagnostic profile into all known drug resistances for a given patient within a single test.[14] In recent years, large

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online databases and bioinformatics pipelines have been created to catalogue drug resistance markers in TB and facilitate the processing of WGS data.[15,16,17,18] Various papers have gone to investigate the usefulness of WGS in predicting drug resistance.[19,20,21,22] However, most of this work relied on clinical datasets aggregated from various, different countries and are highly enriched for drug-resistant TB strains; as a result, this limits the overall generalizability of their findings for a more localized context. In the few studies that do assess the diagnostic performance of WGS in a targeted setting, that is to say within a country or city's borders, they are often hindered in their statistical power by the small sample sizes their results are based on.[23,24,25,26] Therefore there is an urgent and pressing need for large-scale but localized research, that is representative of a particular population and which evaluates WGS in detecting anti-TB drug resistance in real-world settings.[27]

In this multi-center, multi-year-long study we used whole genome sequencing to identify drug-resistant TB in nearly 2,000 patients living in Kaohsiung, Taiwan, a city with medium-to-high TB disease burden. There were three main objectives for this research project. First, we aimed to assess the diagnostic performance of WGS in detecting anti-TB drug resistance by comparing its classification results with those made by culture-based DST. Second, we applied WGS to predict the community-wide, resistance prevalence for several, first-line and second-line anti-TB therapeutics. Lastly, we explored the distribution patterns of drug resistance-conferring mutations in our study population. Ultimately, we hope this research provides greater insights on the merits and limitations of WGS in TB prevention and treatment programs. Additionally, we believe the findings provided in this study will nonetheless help physicians and public health officials better care for individuals with DR-TB, in Taiwan and abroad.

doi:10.6342/NTU202200927

Methods

Inclusion Criteria and Sample Collection

Under the established city-wide procedure for TB case detection, samples were collected from all suspected patients for culture, smear, and phenotypic drug susceptibility testing. We prospectively collected this data from various medical centers and public health laboratories (i.e. Kaohsiung Medical University Hospital, Taiwan CDC National TB Reference Laboratory at Kunyang, Kaohsiung Chang Gung Memorial Hospital, Eda Hospital, Kaohsiung Veterans General Hospital, Tainan Chest Hospital, and Kaohsiung Department of Health) between the periods of January 2019 to July 2021. Sensitive patient data was concealed and each individual was given a unique project ID number to protect their privacy. Kaohsiung was primarily chosen as the focus of our study as it has the highest incidence of TB for all the major cities in Taiwan.

Processing of TB Isolate and Phenotypic Drug Susceptibility Testing

TB samples were isolated, cultured, and tested by phenotypic DST locally, on site, at each medical center following protocols outlined in the Clinical Microbiology Procedures Handbook, 3rd Edition 2007 and DR-TB testing guidelines by Taiwan CDC.[28,29] Culturing of TB isolates was done by MGIT, LJ culture medium, or 7H11 agar medium. Specimens were derived mainly from sputum but also from tissue samples and other sources. Phenotypic drug susceptibility testing was done for four anti-TB drugs, some at different doses: isoniazid (0.2 ug/mL and 1.0 ug/mL), rifampicin (1.0 ug/mL), ethambutol (5.0 ug/mL and 10.0 ug/mL), and streptomycin (2.0 ug/mL and 10.0 ug/mL). A binary classification, “R” for drug-resistant or “S” for drug-susceptible, was given for each isolate depending on whether the percentage of colonies grown on

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the drug-containing media over those on drug free media exceeded the critical proportion for that drug.

Whole Genome Sequencing, Drug Resistance Prediction, & Genetic Lineage Tracing

TB isolates for all culture-confirmed cases were sent to Kaohsiung Medical University Hospital for WGS. Subculturing was done by MGIT and genomic DNA from each isolate was extracted by CTAB.[30] A cutoff ratio of 1.5 or higher for OD_{260}/OD_{280} was determined so that only samples with sufficient DNA concentrations would be sequenced. DNA libraries were generated by NEXTFLEX Rapid XP DNA-Seq Kit and pair-end sequencing was done by Illumina NovaSeq 6000 SPX platform with the reading length of 150bp. More than 200 samples were conducted for each sequencing run with a coverage rate of 100X to ensure detection of low frequency mutations. Raw sequence files were then uploaded to TB Profiler, an online bioinformatics pipeline for WGS data. Reads were aligned to the H37Rv reference genome using bowtie2, BWA, or minimap and selects variants using bcftools. These variants sequences are compared to a database to identify if they contain mutations known to confer drug resistance.[31,32] In total 1,357 unique mutations were screened for drug resistance to 17 commonly known first-line and second-line anti-TB drugs. The number of mutations predictive for resistance varied amongst different drugs: pyrazinamide possessed the most at 387 mutations and linezolid had the least at 3 mutations (Table 1). If the sequence from a TB isolate was determined to have at least one mutation associated with drug resistance, that isolate would be classified as drug-resistant “R”, otherwise it would be deemed drug-susceptible “S”. Additionally, using TB Profiler, the genetic lineage of TB isolates in our dataset were also characterized and classified based off of a 90 SNP variant barcode, as to explore transmission dynamics.[33]

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Drug	*Abbreviation*	*Mutations*	*Genes*	*Type*	*Class*
Isoniazid	Inh	318	*ahpC, fabG1, inhA, kasA, katG*	1st	N/A
Rifampicin	Rif	135	*rpoB, rpoC*	1st	N/A
Ethambutol	Emb	188	*embA, embB, embC, embR*	1st	C
Pyrazinamide	Pza	387	*pncA, panD, rpsA*	1st	C
Fluoroquinolones	Fq	45	*gyrA, gyraB*	2nd	A
Bedaquiline	Bdq	7	*Rv0678*	2nd	A
Linezolid	Lzd	3	*rplC, rrl*	2nd	A
Clofazimine	Cfz	7	*Rv0678*	2nd	B
Cycloserine	Cs	15	*ald, alr*	2nd	B
Delamanid	Dlm	9	*ddn, fgd1, fbiA*	2nd	C
Ethionamide	Eto	95	*ethA, inhA, ethR, fabG1*	2nd	C
Para-aminosalicylic Acid	Pas	45	*folC, ribD, thyA, thyX*	2nd	C
Aminoglycosides	Ag	6	*rrs*	2nd	C
Amikacin	Am	7	*eis, rrs*	2nd	C
Streptomycin	Str	70	*gid, rrs, rpsL*	2nd	C
Kanamycin	Km	14	*eis, rrs*	2nd	N/A
Capreomycin	Cm	34	*tylA, rrs*	2nd	N/A

Table 1. Complete List of First-Line and Second-Line Anti-TB Drugs Analyzed by TB Profiler

Data Cleaning and Statistical Analysis

All results were processed on R version 4.1.3. Statistical analysis and other calculations were carried out using R packages “pubh” and “Threshold RoC”.

doi:10.6342/NTU202200927

Results

Detection of Drug Resistance by Phenotypic DST and Whole Genome Sequencing

A total of 1,939 TB isolates were tested by both phenotypic DST and whole genome sequencing for drug resistance to isoniazid (Inh), rifampicin (Rif), ethambutol (Emb), and streptomycin (Str). These isolates were collected and derived from 1,926 unique patients, all with culture-confirmed diagnoses of TB. The coverage rate of our study represented ~85% of all new TB cases in Kaohsiung, Taiwan between January 2019 to July 2021. From phenotypic DST, it was determined that 163 isolates (8.41%) and 80 isolates (4.13%) were resistant for isoniazid at 0.2 ug/mL and 1.0 ug/mL; 29 isolates (1.50%) were resistant for rifampicin at 1.0 ug/mL; 23 isolates (1.19%) and 3 isolates (0.15%) were resistant for ethambutol at 5.0 ug/mL and 10.0 ug/mL; and 147 isolates (7.58%) and 86 isolates (4.44%) were resistant for streptomycin at 2.0 ug/mL and 10.0 ug/mL. Between 160 to 269 isolates (8.25 – 13.87%) are expected to be resistant to at least one type of drug (i.e., isoniazid, rifampicin, ethambutol, or streptomycin) and between 14 to 21 isolates (0.72 – 1.08%) are multi-drug resistant tuberculosis (MDR-TB), that is TB immune to both isoniazid and rifampicin.

To predict drug immunity in our TB isolate dataset with whole genome sequencing, TB Profiler was used to analyze raw, variant sequence files for resistance-conferring mutations. Based off of TB Profiler, 318 mutations were predictive for drug resistance to isoniazid, 135 mutations for rifampicin, 188 mutations for ethambutol, and 70 mutations for streptomycin. In total it was estimated from WGS that 155 isolates (7.99%) were resistant to isoniazid, 31 (1.60%) for rifampicin, 25 (1.29%) for ethambutol, and 120 (6.19%) for streptomycin. The number of TB isolates predicted to

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be MDR is 18 (93%, 95% CI: 0.55 – 1.46%). In general, there was good agreement between phenotypic DST and WGS on the number of TB isolates deemed to be drug-resistant. The exact distribution in drug resistance assignments between the two tests in our study population can be seen below from the 2x2 contingency tables (Figure 1).

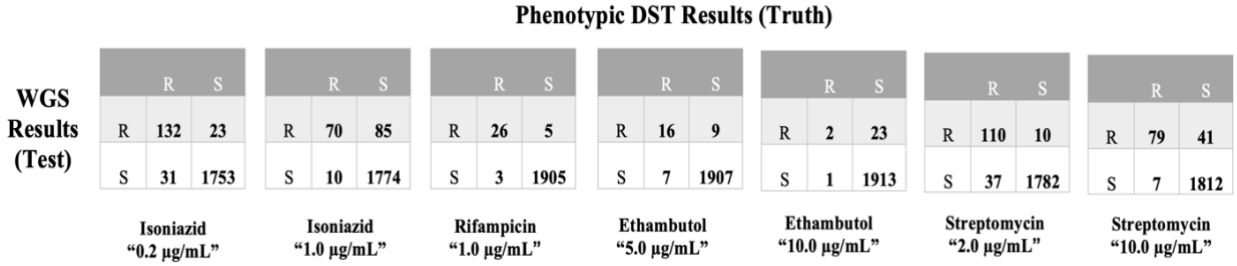

Figure 1. Contingency Tables Comparing DST Results and WGS Prediction for Drug Resistance

Assessing Diagnostic Performance of WGS to Phenotypic DST for Drug Resistance

In order to get more quantitative assessment for how well WGS identifies drug resistance compared to traditional, culture-based DST, several performance measures were calculated (Table 2). Depending on which phenotypic DST dose was used as the reference standard, the sensitivity of WGS in predicting drug resistance varied somewhat. For isoniazid, sensitivity was 81% (95% CI: 74.1 – 87.7%) and 87.5% (95% CI: 78.2 – 93.8) at 0.2 ug/mL and 1.0 ug/mL, respectively. Meanwhile, for streptomycin, the sensitivity was 74.8% (95% CI: 67.0 – 81.6%) and 91.9% (95% CI 83.9 – 96.7%) at 2.0 ug/mL and 10.0 ug/mL. The sensitivity of WGS for rifampicin was slightly higher than the two values seen for isoniazid at 90% (95% CI: 72.6 – 97.8%). Out of the four anti-TB drugs for which both phenotypic DST and WGS data were available, identification of true positives for ethambutol resistance was the worst. Sensitivity was 69.6% (95% CI: 47.1 – 86.8%) and 66.7% (95% CI: 9.43 – 99.2%) at

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5.0 ug/mL and 10.0 ug/mL. Despite testing almost 2,000 TB isolates in our analysis, only a small number of cases, between 3 and 23, were confirmed to be ethambutol-resistant. Because of this, and the large spread in the confidence intervals, making any definitive claim on the sensitivity of WGS in detecting ethambutol resistance by WGS is difficult. Nonetheless, when we compare our drug specific sensitivities with those from another study by Coll et. al, our findings align very well (Figure 2). At times, Coll et. al do report narrower confidence intervals and higher point estimates than us. However, in the case of sensitivity for streptomycin resistance, our results do outperform theirs. Diagnostic accuracy for WGS ranged between 95.1% to 99.6% across all drugs and conditions (Table 2).

	Isoniazid "0.2 µg/mL"	Isoniazid "1.0 µg/mL"	Rifampicin "1.0 µg/mL"	Ethambutol "5.0 µg/mL"	Ethambutol "10.0 µg/mL"	Streptomycin "2.0 µg/mL"	Streptomycin "10.0 µg/mL"
Sensitivity	**81.0%** *(74.1 – 87.7)*	**87.5%** *(78.2 – 93.8)*	**90.0%** *(72.6 – 97.8)*	**69.6%** *(47.1 – 86.8)*	**66.7%** *(9.43 – 99.2)*	**74.8%** *(67.0 – 81.6)*	**91.9%** *(83.9 – 96.7)*
Specificity	**98.7%** *(98.1 – 99.2)*	**95.4%** *(94.4 – 96.3)*	**100%** *(99.4 – 100)*	**99.5%** *(99.1 – 99.8)*	**98.8%** *(98.2 – 99.2)*	**99.4%** *(99.0 – 99.7)*	**97.8%** *(97.0 – 98.4)*
PPV	**85.2%** *(78.6 – 90.4)*	**45.2%** *(37.2 – 53.3)*	**84.0%** *(66.3 – 94.5)*	**64.0%** *(42.5 – 82.0)*	**8.00%** *(1.00 – 26.0)*	**91.7%** *(85.2 – 95.9)*	**65.8%** *(56.6 – 74.2)*
NPV	**98.3%** *(97.5 – 98.8)*	**99.4%** *(99.0 – 100)*	**99.8%** *(99.5 – 100)*	**99.6%** *(99.2 –99.9)*	**100%** *(99.7 – 100)*	**98.0%** *(97.2 – 98.6)*	**99.6%** *(99.2 – 99.8)*
PLR	**62.5** *(41.4 – 94.5)*	**19.1** *(15.3 – 23.9)*	**342.5** *(141 – 829)*	**148** *(73.1 – 300)*	**56.1** *(22.9 – 138)*	**134** *(71.8 – 251)*	**41.5** *(30.5 – 56.6)*
NLR	**0.19** *(0.14 – 0.26)*	**0.13** *(0.07 – 0.23)*	**0.10** *(0.04 – 0.30)*	**0.31** *(0.16 –0.57)*	**0.34** (0.07 – 1.67)	**0.25** *(0.19 – 0.33)*	**0.08** *(0.04 –0.17)*
RR	**49.0** *(34.4 – 69.9)*	**80.6** *(42.4 –153)*	**533.4** *(170 – 1670)*	**175** *(79.0 – 388)*	**153** *(14.4 – 1634)*	**45.1** *(32.6 – 62.3)*	**171** *(80.8 – 362)*
OR	**324** *(184 – 573)*	**146** *(72.7 – 293)*	**3302** *(750 –14545)*	**484** *(1.61 – 1460)*	**166** *(14.6 –1900)*	**530** *(257 – 1093)*	**499** *(217 – 1147)*
Prevalence	**8.41%** *(7.21 – 9.73)*	**4.13%** *(3.28 – 5.11)*	**1.34%** *(0.88 – 1.96)*	**1.19%** *(0.75 – 1.77)*	**0.15%** *(0.03 – 0.45)*	**7.58%** *(6.44 – 8.85)*	**4.44%** *(3.56 – 5.45)*
Accuracy	**97.2%** *(96.4 – 97.9)*	**95.1%** *(94.0 – 96.0)*	**99.6%** *(99.2 – 99.8)*	**99.2%** *(98.7 – 99.5)*	**98.8%** *(98.2 – 99.2)*	**97.6%** *(96.8 – 98.2)*	**97.5%** *(96.7 –98.2)*

Table 2. Diagnostic Performance Measures for WGS in Predicting Drug Resistance

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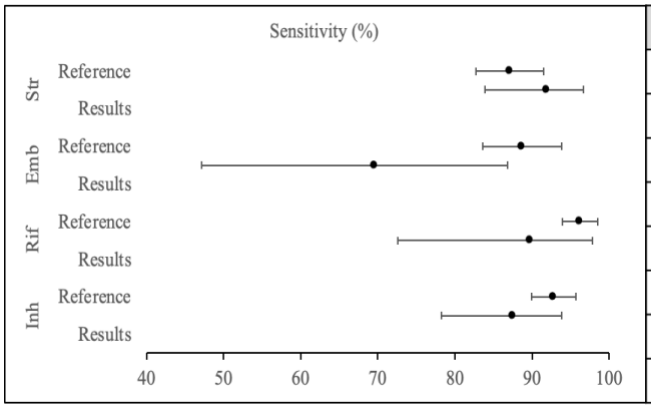

		Point Est. (%)	Low Est. (%)	High Est. (%)
Str	Reference	87.1	82.7	91.5
	Results	91.9	83.9	96.7
Emb	Reference	88.7	83.6	93.8
	Results	69.6	47.1	86.8
Rif	Reference	96.2	93.9	98.5
	Results	90.0	72.6	97.8
Inh	Reference	92.8	89.9	95.7
	Results	87.5	78.2	93.8

Figure 2. Forest Plot of WGS Sensitivity for Detecting Drug Resistance in Inh, Rif, Emb, and Str

Overall, results for specificity were slightly higher and less varied than those for sensitivity (Table 2). When looking at all four anti-TB drugs across different phenotypic DST concentrations, specificity in WGS resistance prediction was always greater than 95%. The lowest specificity seen was for isoniazid at 1.0 ug/mL, which yielded a value of 95.4% (95% CI: 94.4 – 96.3%); the highest specificity was for rifampicin at 1.0 ug/mL, which saw a score of 100% (95% CI: 99.4 – 100%). From our results, it appears WGS performs better at identifying cases of true negatives over true positives; this seemingly agrees with other findings made in the literature. When we compare our results specifically to those from Coll et. al, we see that our specificities for rifampicin, ethambutol, and streptomycin are actually much stronger (Figure 3). In the particular case of isoniazid, however, their reported specificity was slightly higher, at 100% (95% CI: 100 – 100%); while ours was only ~95%, albeit, still very high. When assessing our sensitivity and specificity results to those from Coll et. al, labeled as reference in Figures 2 and 3, we decided to use the findings from the higher dose phenotypic DST in the comparison as this gives a stricter estimate than the low dose phenotypic DST and better accounts for possible classification errors that may have occurred due to

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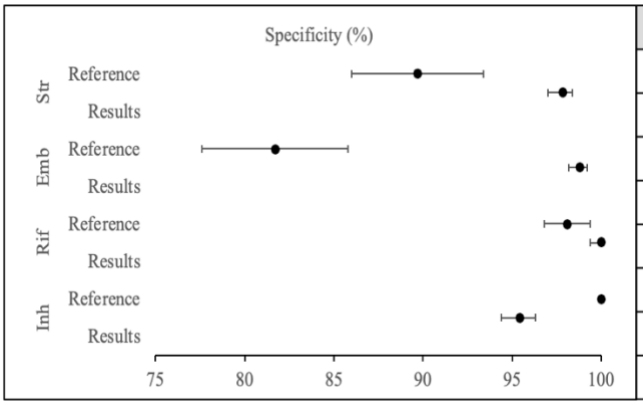
heteroresistance or dose-dependent resistances (i.e., low-level resistance mutations) present in our TB isolates. However, in the unique case of ethambutol (Emb), the higher concentration DST result was not chosen for the comparison of sensitivity and specificity due to the limited numbers of culture-confirmed resistance cases available. Therefore, the low concentration phenotypic DST result was ultimately selected.



		Point Est. (%)	Low Est. (%)	High Est. (%)
Str	Reference	89.7	86.0	93.4
	Results	97.8	97.0	98.4
Emb	Reference	81.7	77.6	85.8
	Results	98.8	98.2	99.2
Rif	Reference	98.1	96.8	99.4
	Results	100	99.4	100
Inh	Reference	100	100	100
	Results	95.4	94.4	96.3

Figure 3. Forest Plot of WGS Specificity for Detecting Drug Resistance in Inh, Rif, Emb, and Str

Predicting Drug Resistance Prevalence by Whole Genome Sequencing

We next wanted to compare the drug resistance prevalence predicted by WGS to what was determined by phenotypic DST for which data on isoniazid, rifampicin, ethambutol, and streptomycin were available (Figure 4). As we wanted to capture all potential cases of drug resistance, the low dose phenotypic DST result was used for the comparison so as to give more flexibility in the analysis. Overall, there was great consensus in the prevalence values between the two methods. For isoniazid resistance, the DST prevalence was 8.4% (95% CI 7.2 – 9.7%) and the WGS prevalence was 8.0% (95% CI: 6.8 – 9.3%). For rifampicin resistance, the DST prevalence was 1.3% (95% CI: 0.9 – 2.0%) and the WGS prevalence was 1.6% (95% CI: 1.1 – 2.3%). For ethambutol resistance, prevalence was 1.2% (95% CI: 0.8 – 1.8%) by DST and 1.3% (95% CI: 0.8 – 1.9%) by WGS. For streptomycin, prevalence was 7.6% (95% CI: 6.4 –

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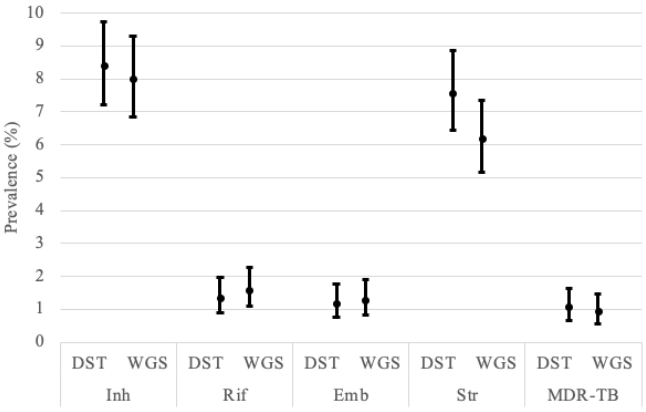
8.9%) and 6.2% (95% CI: 5.2 – 7.4%) from DST and WGS, respectively. Additionally, the prevalence of MDR-TB was assessed and also showed high agreement. The DST prevalence was 1.1% (95% CI: 0.7 – 1.7%) and the WGS prevalence was 0.9% (95% CI: 0.6 – 1.5%).



		Pt Est. (%)	Low Est. (%)	High Est. (%)
Inh	DST	8.4	7.2	9.7
	WGS	8.0	6.8	9.3
Rif	DST	1.3	0.9	2.0
	WGS	1.6	1.1	2.3
Emb	DST	1.2	0.8	1.8
	WGS	1.3	0.8	1.9
Str	DST	7.6	6.4	8.9
	WGS	6.2	5.2	7.4
MDR-TB	DST	1.1	0.7	1.7
	WGS	0.9	0.6	1.5

Figure 4. Comparing Inh, Rif, Emb, and Str Resistance Prevalence from DST and WGS

Unlike for isoniazid, rifampicin, ethambutol and streptomycin, many anti-TB drugs are not routinely assessed by DST in Taiwan. As a result, there is no official figure on the true level of resistance for these drugs in the population. Because of the strong concordance between DST and WGS results, this gave us confidence in using WGS and TB Profiler to predict the resistance for lesser tested, anti-TB drugs. A total of 13 others drugs were assessed in our dataset: pyrazinamide, fluoroquinolones, bedaquiline, linezolid, clofazimine, cycloserine, delamanid, ethionamide, para-aminosalicylic acid, aminoglycosides, amikacin, kanamycin, and capreomycin. The predicted point prevalence for resistance varied from 0% to 5.5% amongst the drugs examined (Figure 5). For drugs such as bedaquiline and delamanid there is no expected drug resistance. Additionally, there were no predicted resistance to linezolid, clofazimine, or cycloserine. We saw that for fluroquinolones, which includes drugs

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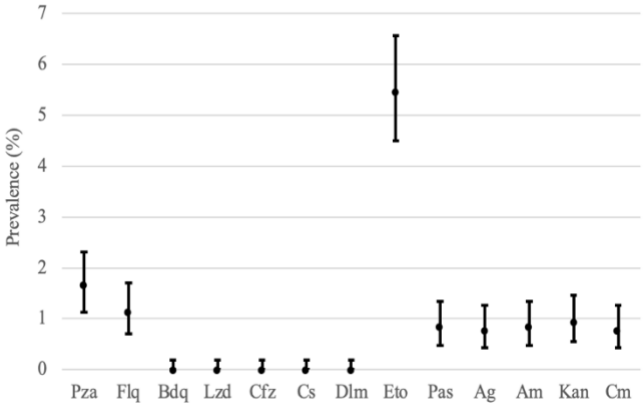
ciprofloxacin, levofloxacin, moxifloxacin, and ofloxacin, the predicted resistance prevalence is 1.1% (95% CI: 0.7 – 1.7%). Resistance to fluroquinolones is one of the markers for extensively drug-resistant tuberculosis (XDR-TB); so far, however, 0% (95% CI: 0 – 0.19%) were predicted to be XDR-TB. In fact, not only for fluoroquinolones, but also many of the non-commonly tested anti-TB drugs (i.e., para-aminosalicylic acid, aminoglycosides, amikacin, kanamycin, and capreomycin) had predicted resistance prevalence as high as those seen for rifampicin and ethambutol when assessed by WGS. With ethionamide, predicted resistance prevalence (5.5%, 95% CI: 4.5 – 6.6%) was even greater than for both rifampicin and ethambutol, and almost as large as isoniazid and streptomycin, which are known to have high levels of drug immunity in the TB population.



	Pt Est. (%)	Low Est. (%)	High Est.(%)
Pza	1.7	1.1	2.3
Flq	1.1	0.7	1.7
Bdq	0	0	0.2
Lzd	0	0	0.2
Cfz	0	0	0.2
Cs	0	0	0.2
Dlm	0	0	0.2
Eto	5.5	4.5	6.6
Pas	0.8	0.5	1.3
Ag	0.8	0.4	1.3
Am	0.8	0.5	1.3
Kan	0.9	0.6	1.5
Cm	0.8	0.4	1.3

Figure 5. Prediction of Resistance Prevalence for Lesser Tested, Anti-TB Drugs by WGS

Investigating Diagnostic Discordance Pairs and Tracing Clinical Outcomes

Although our results did show overall good agreement in drug resistance determination by WGS and phenotypic DST, there were occasions in which the two diagnostic tests disagreed (Figure 1). We wanted to explore the discordance pairs in our 2x2 contingency tables and see what ultimately resulted to the patients from whose TB

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isolates were in this group. For each drug (i.e., isoniazid, rifampicin, ethambutol, and streptomycin), TB isolates could be discordant in either two ways: if WGS reported resistance and phenotypic DST predicted susceptible or if WGS revealed susceptible and phenotypic DST predicted resistance (Table 3). Treatment completion or death were the primary endpoints; because this was a prospective study, some individuals were still ongoing treatment at the time of collection and were excluded from this part of the analysis. Additionally, the total number of discordance pair cases in Table 3 may not necessarily equal the number of discordance pair cases in Figure 1, as some clinical outcomes from patients were not available for certain isolates. From Table 3, we see that for each drug group, the total number of individuals completing treatment, currently undergoing treatment, or who were deceased was always greater when WGS predicted resistance & phenotypic DST determined susceptibility rather than when WGS predicted susceptibility & phenotypic DST determined resistance. When we examine this trend closer, specifically looking into the sources of deaths, we found that 0 individuals died from TB when DST reported resistance and WGS reported susceptibility. However, some patients did pass away from TB-related complications when DST reported susceptibility & WGS reported resistance. For the isoniazid category, 4 males individuals died from TB whose age ranged between 57 –83 years. In the ethambutol group, 2 male individuals succumbed to TB who were 62 and 97 years old. For streptomycin, 3 males and 1 female died from TB who ages ranged from 69 – 96.0. For the rifampicin category, there were no TB deaths reported.

doi:10.6342/NTU202200927

	Discordance Pairs	Completed Treatment	Ongoing Treatment	Deceased	Total Cases
Isoniazid	WGS R / DST S	42	15	15	72
	DST R / WGS S	1	2	3	6
Rifampicin	WGS R/ DST S	3	2	0	5
	DST R / WGS S	0	1	0	1
Ethambutol	WGS R / DST S	2	3	3	8
	DST R / WGS S	5	0	1	6
Streptomycin	WGS R / DST S	10	16	9	35
	DST R / WGS S	0	5	1	6

Table 3. Clinical Outcomes of Discordant Testing Pairs for Inh, Rif, Emb, and Str

Exploring the Distribution of Drug Resistance-Conferring Mutations in Kaohsiung

Although it was apparent from WGS that drug-resistant TB isolates were present in our dataset, it was not readily obvious which mutations were the largest drivers in contributing to drug resistance. To explore this question, for each drug predicted to have TB isolates resistant to it, we examined which resistant-conferring mutations appeared for that particular drug. 12 of the 17 anti-TB drugs we investigated had at least one TB isolate with predicted resistance to it; bedaquiline, linezolid, clofazimine, cycloserine and delamanid were the exceptions (Figure 6).

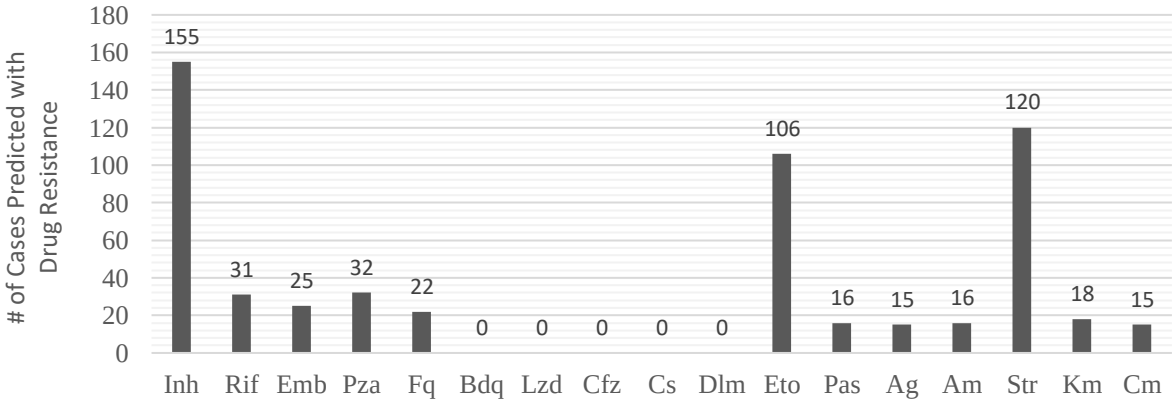

Figure 6. Predicted Drug Resistance Case Counts for Various Anti-TB Drugs by WGS

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Looking at the distribution in resistance-conferring mutations, one of the most striking takeaways is the lack of mutational diversity (Figure 7). From TB Profiler, we screened for over 1,357 mutations which could have possibly contributed to anti-TB drug resistance, but the vast majority of predicted cases could be explained by a relatively few number of mutations that appeared frequently. 10 of the 12 anti-TB drugs had less than 15 unique mutations detected, and the exact range lied between 2 and 21. In fact, for every anti-TB drug we examined, 3 or fewer unique mutations were responsible for over 50% of all predicted resistance cases for that particular drug. It's been reported in the literature that certain mutations in clinical TB isolates do appear more often than others, but these mutations and their relative abundancies were not exactly what we saw. For instance, out of all isoniazid-resistant TB isolates tested globally, the *katG315* and *inhA-15* mutations accounted for 64% and 19%, respectively.[34] However in our study we found that the *katG315* mutation only amounts to 35%, while *inhA* mutations were even less, at only 8%. The most abundant mutation seen for isoniazid resistance was actually *fabG1c.15C>T,* which accounted for 47% of all detected mutations. In the case of rifampicin resistance, it's been reported that 95% of all resistant strains have mutations between codons 507 and 533 of *rpoB* gene.[35] Although in our study, we found that none of our TB isolates had mutations in this specific DNA region, instead 61% had the specific *rpoB_p.Ser450Leu* mutation and the rest had mutations randomly dispersed amongst codons 170, 430, 445, 452, and 491.

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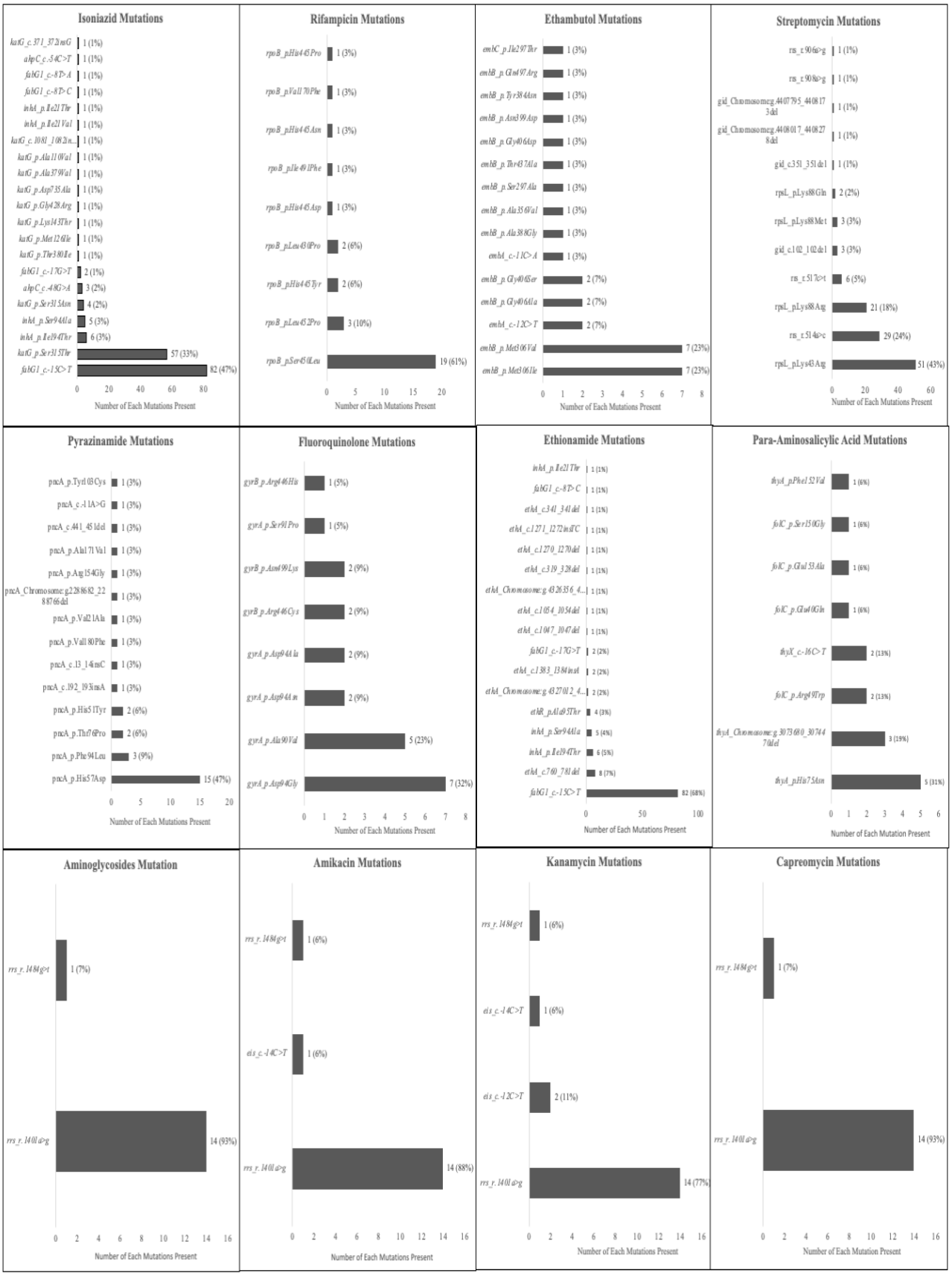

Figure 7. Distribution in Resistance-Conferring Mutations Across Various Anti-TB Drugs

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Assessing the Genetic Lineages of TB Isolates with Same Resistance Mutations

It was not a large surprise that a skewed distribution among resistance-conferring mutations would be observed. What was unexpected is that this distribution would be so heavily concentrated towards a limited number of mutations. Moreover, these select mutations weren't the same as those reported in the literature that typically accounted for the preponderance of drug resistant cases. DR-TB could arise from two different mechanisms: primary resistance, which is the passing of drug resistant TB from one individual to another, or secondary resistance, which is when drug resistance develops naturally due failed prior treatment. The average mutation rate for *Mycobacterium tuberculosis* has been calculated to be less than 1 single nucleotide change per genome per year.[36] Based on how conservative the TB genome is and how rare it is for any spontaneous change to result in drug resistance, it's possible that the high aggregation of resistance-conferring mutations we see towards specific genetic loci, that is unique to this study, is indication of a linked pattern of spread in Kaohsiung as oppose to a random process.

To explore the question of whether primary or secondary resistance is the main driver of DR-TB in our study population, we investigated the genetic lineage of TB isolates that carried the same drug resistance-conferring mutations. We looked specifically into mutations that were most commonly found in our distribution analysis: *fabG1_c.-15C>T*, *katG_p.Ser315Thr*, *rpoB_p.Ser450Leu*, and *rpsL_p.Lys43Arg*. If chain transmission was truly the main reason leading to the high aggregation of these mutations in DR-TB isolates, we would expect one type of strain to overwhelmingly be more representative than all others. However, this was not seen. Instead, the proportion of isolates with *fabG1_c.-15C>T*, *katG_p.Ser315Thr*, *rpoB_p.Ser450Leu*, or

doi:10.6342/NTU202200927

rpsL_p.Lys43Arg mutations were all fairly dispersed among the TB lineages frequent in Taiwan: Lineage 1 (Indo Oceanic), Lineage 2 (East Asia), and Lineage 4 (Euro-American) (Figure 8). There were slight differences in the proportions observed, but this is expected considering certain TB lineages are known to have larger overall mutation rates.[37]

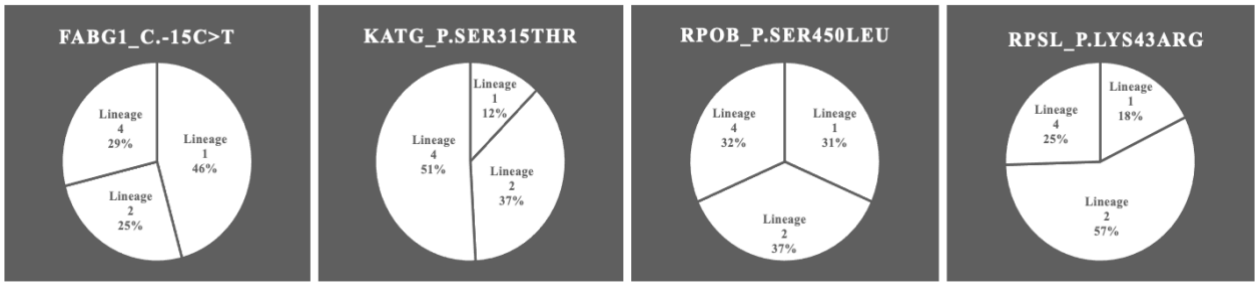

Figure 8. Genetic Lineage of TB Isolates Carrying Certain Drug Resistance-Conferring Mutations

Analysis of Intermediately Drug Resistant TB Isolates and Their Mutations

It's been well reported that resistance to certain anti-TB drugs can be intermediate and could be overcome at sufficiently high doses.[5,38] From our own phenotypic DST results, we saw that at higher doses of isoniazid, rifampicin, and streptomycin there were noticeable decreases in the number of TB isolates reported to be drug resistant. To be precise, 62 TB isolates switched from being drug resistant to drug susceptible when isoniazid concentrations increased from 0.2 ug/mL and 1.0 ug/mL; 14 isolates became susceptible when ethambutol concentrations increased from 5.0 ug/mL and 10.0 ug/mL; and 31 isolates became susceptible when streptomycin concentrations increased from 2.0 ug/mL and 10.0 ug/mL (Figure 1). Using WGS data in tandem with results from phenotypic DST, we explored the specific mutations that were present in these intermediately drug-resistant TB isolates to identify which were responsible (Figure 9).

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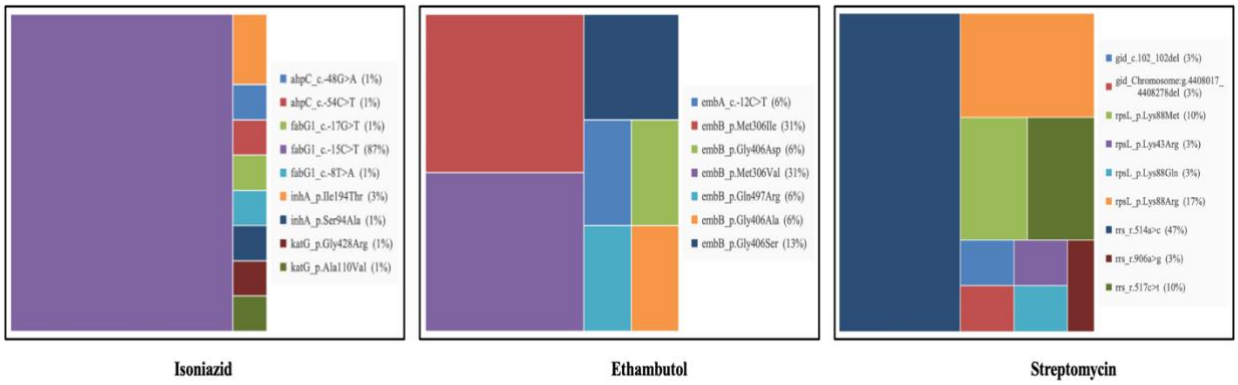

Figure 9. Tree Maps of the Mutations in TB Isolates with Dose-Dependent, Drug Resistance

Out of the 62 TB isolates that had dose-dependent drug resistance to isoniazid, the vast majority, 87%, had the *fabG1_c.-15C>T* mutation. Recall that this mutation was also responsible for the largest proportion, 47%, of all predicted isoniazid resistance cases from WGS in our study population (Figure 7). As for the TB isolates with dose-dependent resistances to ethambutol and streptomycin, the mutations involved were much more equally apportioned. For ethambutol, mutations embB_pMet306Ile and embB_p.Met306Val were the most prevalent, at 31% each, and they similarly made up the highest representation of all TB isolates resistant to the drug (Figure 7 and Figure 9). In the case of streptomycin, the most commonly seen mutation for all resistant TB isolates was rpsL_p.Lys43Arg, at 43%. However among TB isolates with intermediate drug resistance, this specific mutation only accounted for 3%.

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Discussion

The use of whole genome sequencing could revolutionize how diagnostic testing for TB drug resistance is conducted, enabling for more comprehensive and timely results. In this study, we investigated the effectiveness of WGS in detecting DR-TB cases using it to analyze nearly 2,000 TB isolates collected from Kaohsiung, Taiwan, a city with medium-to-high TB disease burden (Figure 1). Overall, we found that WGS performs very well compared to culture-based DST, reporting values as high as 90% for sensitivity and greater than 95% for specificity and accuracy across most of the drugs assessed (Table 2). The main exception was sensitivity for ethambutol resistance which reported values closer to 70%. Many WGS studies have also reported a weaker sensitivity in detecting ethambutol drug resistance[15]; we believe this finding is unique for ethambutol, likely due to a lack of reliable drug resistance markers relative to other anti-TB drugs, rather than problems inherent within our study. When we measured our results against findings from other similar studies, such as those from Coll et. al, they agreed very well (Figure 2 and 3). On most cases, however, Coll et. al's study did show larger point estimates and tighter confidence intervals for sensitivity than ours, but we demonstrated greater point estimates and tighter uncertainty intervals for specificity. In both our studies, we used the same bioinformatics pipeline, TB Profiler, to analyze WGS results. Although, the work by Coll et. al did use an earlier version of this program. Perhaps the slight discrepancy in our diagnostic results is due to the newer versions of TB Profiler better balancing the tradeoffs between achieving high sensitivity and specificity.

doi:10.6342/NTU202200927

When we compared the prevalence of drug resistance predicted by WGS to what was determined from DST, we saw great agreement between the two sets of results (Figure 4). Furthermore, our findings also align very well to a review paper on official studies that measured DR-TB levels in Taiwan. Specifically, Hsueh et. al reported resistance prevalence of 4.7-12% for isoniazid, 0.7-5.9% for rifampicin, 1-6% for ethambutol, and 4-11% for streptomycin.[39] In our WGS study we found resistance prevalence between 6.8-9.3% for isoniazid, 1.1-2.3% for rifampicin, 0.8-1.9% for ethambutol, and 5.2-7.4% for streptomycin. In the case of MDR-TB prevalence, Lee et. al reported values of 1.0 - 1.4%, and similarly we found values of 0.6 to 1.5%.[40] Because of the great concurrence in these results, this suggests that WGS could be used not only in clinical settings but also by public health systems to better gauge the level of drug immunity in their communities; this could be especially helpful for rarer drugs where it may not be practical to manually screen and test every suspected case for resistance. In this study, we applied WGS to do just this, and assessed the levels of resistance to several, first-line and second-line anti-TB drugs that aren't routinely tested by culture-based DST in Taiwan. We discovered that while many drugs, such as bedaquiline and delamanid, have little to no resistance, other drugs such as ethionamide and pyrazinamide have prevalence as high as commonly screened drugs like isoniazid and rifampicin (Figure 5). For most of these drugs, the predicted resistance levels are very reasonable. For instance, streptomycin has been commonly used since the 1950s so a resistance prevalence of 6.2% is possible. Pyrazinamide is a first-line anti-TB drug, so a resistance prevalence of 1.7% is also sensible. Additionally, fluoroquinolones are widely used to treat many pulmonary diseases, like pneumonia, in Taiwan. Although not much is known about population wide resistance to fluroquinolones, a predicted immunity prevalence of 1.1% is not completely unthinkable. However, there may also

doi:10.6342/NTU202200927

be certain caveats to these WGS findings for some other drugs. In the case of bedaquiline resistance in Taiwan, it's been suggested that drug immunity prevalence is actually around 3%.[41] Although bedaquiline is a newer drug, only having been introduced to Taiwan in 2014, there may already be noticeable population immunity to bedaquiline due to cross resistance with other widely used drugs. Additionally, for ethionamide, we saw resistance prevalence with a low 95% CI of 4.5% to high 95% CI of 6.6% as determined from WGS. This is a bit surprising considering that ethionamide is not a drug that is widely used in Taiwan and is only available at health centers specifically trained to treat MDR-TB cases. The predicted ethionamide resistance cases were mainly from TB isolates carrying the *fab_G1.-15C>T* mutation, which also predicted resistance to isoniazid. It's unknown at this time whether resistance prevalence for ethionamide in Kaohsiung is truly this high due to a potential cross resistance effect with isoniazid or maybe if this is just an artificial result from the analysis pipeline. Nonetheless, this all suggest that routine, surveillance screening for drug resistance needs to be expanded to a broader array of anti-TB drugs than what is currently done in Kaohsiung. Furthermore, physicians caring for TB patients need to be aware that mono-drug resistance outside of isoniazid and rifampicin are less rare than they previously thought.

Isoniazid and rifampicin are considered the two most important anti-TB drugs; they are the most effective bactericidal agents and are some of the first drugs prescribed to any TB patient.[4] In most of the world, and in Taiwan, isoniazid immunity is the most prevalent drug resistance for TB. For this reason, when individuals test positive for resistance to rifampicin, clinicians typically view the patient as an MDR-TB case. Resistance to rifampicin is easier to diagnose than for other anti-TB drugs as PCR-based

doi:10.6342/NTU202200927

diagnostic methods have been shown to perform just as well as phenotypic DST, but with the added benefit of rapid results.[13] From our WGS findings, we saw that of the 31 TB isolates with predicted rifampicin resistance, ~60% were also isoniazid resistant. This suggests that the presumption of MDR-TB, given confirmation of rifampicin resistance, is not completely unwarranted and may be permissible by clinicians if there is a lack of time or resources to conduct phenotypic DST for isoniazid. Using WGS, we further analyzed what other drug resistances were reported for these TB isolates with rifampicin resistance and discovered that 11 isolates also had predicted immunity to ethambutol, 8 to pyrazinamide, 5 to streptomycin, 3 to fluoroquinolone, and 1 to kanamycin. This result indicates that most second-line anti-TB drugs (e.g., bedaquiline, delamanid, etc.) will still likely work in treating patients with confirmed mono-drug resistance to rifampicin and whom likely have MDR-TB.

Along with assessing the diagnostic performance of WGS, we also found various novel results that require further research. For instance, in our analysis on the distribution of resistance-conferring mutations, we discovered that for all of the drugs examined, 3 or fewer mutations were responsible for over 50% of predicted resistance cases, and that these mutations were different from those reported in global WGS studies (Figure 7). *FabG1* mutations were the most common for isoniazid resistance in our dataset, but in other multi-country studies, *katG* mutations are the most frequent. While this is surprising, other local studies have found similar results to ours showing differences in the type of drug resistance mutations in TB isolates by region. For example, a paper looking into DR-TB in Mongolia found that *inhA mutations*, which is a gene functionally related to and spatial connected with *fabG1*, are the most prevalent in conferring isoniazid resistance for that population.[42] This all goes to emphasize how

doi:10.6342/NTU202200927

important it is to conduct high quality, local DR-TB studies to know exactly what mutations are most numerous in a particular area.

Originally, we thought that this unique skewness in mutation distribution could be indication of linked transmission of DR-TB in Kaohsiung, but a preliminary analysis examining TB lineage in our study found mixed results (Figure 8). The high frequency of certain, unique drug resistance-conferring mutations is likely not explained by primary resistance. However, it is also not completely random. Perhaps, as reported for other organisms, there are biases in the TB genome that lead some mutations to arise more prominently than others.[43,44] Additionally, it's been theorized that some resistance conferring mutations have a greater fitness costs/disadvantages in transmission than others which may impair their presence in a population.[45] More research, perhaps using SNP distance analysis, should be done in the future to investigate whether mutational biases and fitness selection or linked TB transmission explains why some mutations are more often seen in a population. Regardless, knowing which drug resistance-conferring mutations are most frequent and why will be key in improving molecular testing for all DR-TB.

Using both our phenotypic DST and WGS results, we also examined TB isolates that exhibited low-level drug immunity. During which we implicated several mutations as more reliable predictors for drug resistance than others. For example, the *rpsL_p.Lys43Arg* mutation was present in 43% of all TB isolates immune to streptomycin, but only accounted for 3% in isolates with intermediate resistance to the drug (Figure 7 and 9). In contrast, the *fabG1_c.-15C>T* mutation is likely less predictive for drug resistance. *fabG1_c.-15C>T* accounted for a large proportion of TB

doi:10.6342/NTU202200927

isolates with any immunity to isoniazid (47%), but it was also highly abundant in TB isolates with intermediate resistance to isoniazid (87%) (Figure 7 and 9). Generally speaking, *inhA* gene mutations have been well established in the literature to confer low-level drug resistance to isoniazid, while mutations in the *katG* are known to offer high-level of drug resistance;[46] the *fabG1* gene hasn't been so well defined in either direction. Our study helps suggest that mutations in *fabG1*, similar to *inhA*, may just confer low-level drug resistance to isoniazid when compared to *katG*. This would make sense as the degree of drug resistance is associated by where the mutation is located and how it impairs biological function.[5] In the TB genome, *fabG1* and *inhA* are directly adjacent and together constitute the *fabG1-inhA* regulatory region. Perhaps there is something special with this segment of the TB genome that allows mutations there to have a more limited effect on drug resistance. Alternatively, the way isoniazid kills *Mycobacterium tuberculosis* is through inhibiting mycolic acid formation and cell wall synthesis.[5] The *katG* gene functions in this biochemical pathway at a more upstream position than the *fabG1-inhA* regulatory region[47]; because of this, if *katG* were to be altered, it would have larger outstanding effect on signal cascade and lead to greater impact on cell wall synthesis than more downstream effector elements like *fabG1* and *inhA*. This could be another possible reason why *fabG1* and *inhA* mutations confer lower level isoniazid resistance than mutations in *katG*. This weakened effect on drug resistance was also seen somewhat too in TB isolates carrying intermediate immunity for ethambutol, with mutations specifically in codon 306 of the *embB* gene responsible. What all this suggests is that if TB patients are diagnosed for a particular drug resistance by molecular testing, it may still be appropriate to use that said drug at a larger dose, depending on the specific mutation they have. This could avoid having to use more toxic, second-line drugs to accomplish the same bactericidal or bacteriostatic activity.

doi:10.6342/NTU202200927

Additionally, using the same drugs at a higher dose may be necessary if other second-line TB drugs aren't available. Ultimately it depends on the physician, the patient, and the unique clinical context involved that determines the correct recourse for DR-TB treatment. If WGS is to one day replace culture-based testing for good, greater knowledge needs to be known on which mutations confer definite versus intermediary drug resistance.

Lastly, in examining some of the patient outcomes in our study, we found that in scenarios where there is discordance between testing results, WGS may be able to detect clinically relevant drug resistances missed by phenotypic DST. In particular we found that 10 individuals died to TB when DST determined susceptibility and WGS predicted resistance (Table 3). It could very well be possible that these individuals died because their treatment plans were ineffective due to having other undiagnosed drug resistances. Although this finding is certainly exciting, whether this discovery is substantial is still unknown given the limited information we have. More work should be done to investigate discordant WGS/DST results in the future and to see if they are truly significant for clinical outcomes.

There have been few published research specifically evaluating the application of WGS for DR-TB case detection in Taiwan.[27,48] These studies either investigated resistance for only a single anti-TB drug or screened for several drug resistances but use a very limited sample size, well below 100 clinical isolates. To our knowledge, our research is the first to truly examine this question in Taiwan at a comprehensive, population-wide level, carrying with it high statistical power and great generalizability. The strengths of this research are numerous: it is a prospective multi-year study,

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investigates the use of WGS in a local setting, contains a large representative sample size with high coverage rate, analyzes various resistance mutations in depth, and explores clinical outcomes in discordant test results. The main limitations of this work are that certain drug resistances are better characterized than others by WGS and that we primarily utilize a binary classification for drug resistance. The findings of this paper provide important insights into DR-TB for both physicians and public health practitioners. When it comes to medical treatment, our results demonstrate that using WGS can be helpful in guiding clinical practice and should be considered for greater adoption. More often than not, when individuals first come to a healthcare facility with TB-like symptoms, they are not immediately tested for drug susceptibility. Even when they do, the results don't arrive quickly enough to inform patient-care decision. Instead, the presumption is to just prescribe HREZ, a combination therapy containing isoniazid, rifampicin, ethambutol, and pyrazinamide, even though resistance to one or more of these medications is possible.[49] By incorporating WGS information, physicians can have greater confidence that their treatment plan will include effective bactericidal and bacteriostatic medications, leading to better patient outcomes. This is not to say that WGS should be the only or even primary data source guiding a clinical decision, but it is a valuable input nonetheless. Ideally, a physician would have information from a variety of tests results, including WGS, and along with his/her experience treating the condition and knowledge of the local setting, can adequately make an informed treatment decision. Table 2 of our results may be particularly helpful as using it in conjunction to a WGS test and a Bayes' nomogram can give the physician a clear idea on the probability of drug resistance for an individual. Some studies have already investigated the use of WGS in real-world, healthcare settings showing great success.[50] In terms of public health, it's been well described by researchers that Taiwan needs to

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greatly improve it DR-TB surveillance infrastructure.[39] By employing WGS in disease epidemiology, more individuals could be tested and more types of drug resistances could be screened for at the population level. Wide-spread adoption of WGS and greater advancement of this technology would mean a complete paradigm shift in how detection for DR-TB is carried out: converting from passive surveillance to active case finding. This kind of comprehensive information on population health would allow public health officials to better prevent, identify, and manage outbreaks by surging resources and services to needed areas. A 2019 paper by Lee et. al have already demonstrated the clear benefits of rapid and universal TB testing in Taiwan, and many places around the world already include WGS in some parts of their TB surveillance strategy.[5,40] Including WGS as both a public health tool and clinical diagnostic test will likely improve DR-TB rates and better patient outcomes over time in Taiwan.

doi:10.6342/NTU202200927

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