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Original Article

Comparison of Genotypic (t-NGS) and Phenotypic Results for *Mycobacterium tuberculosis* Identification and Drug Susceptibility Testing (DST) against Tuberculosis

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Abstract

Background : Accurate identification and drug susceptibility testing are crucial for tuberculosis eradication and treatment, but conventional methods require over four weeks to complete. Targeted Next Generation Sequencing (t-NGS) is a promising alternative that provides results in just four days, potentially replacing traditional methods. However, the concordance between genotypic and phenotypic methods has not been widely reported.
Aims : This study aims to see the concordance between phenotypic and genotypic methods for identifying *Mycobacterium tuberculosis* and determining drug susceptibility.
Methods : Sputum samples were collected from Balai Besar Laboratorium Kesehatan Masyarakat (BBLKM) Makassar from June 2024 until July 2024. *M. tuberculosis* DNA was extracted using the Qiagen DNA mini kit, amplified with Deeplex® Myc-TB by Genoscreen, and prepared with Illumina DNA Prep. t-NGS was performed on the MiSeq Illumina platform, and sequencing results were analyzed with Deeplex® Myc-TB by Genoscreen. A comparison of genotypic and phenotypic results (Culture and Drug Sensitivity Test) was conducted using SPSS.
Results : Discrepancies were noted between phenotypic and genotypic results for two samples (Samples 16 and 18), where phenotypic results indicated non-tuberculous mycobacteria (NTM) and genotypic results identified *M. tuberculosis*. These discrepancies were not statistically significant (p>0.05). Additionally, a minor discrepancy was observed in isoniazid results for one sample (Sample 2), but the statistical result is not significance (p>0.05).
Conclusion : t-NGS is a promising alternative to conventional methods due to its shorter testing time and capability to identify novel mutations, with discrepancies compared to phenotypic results being statistically insignificant. However, its higher cost and the need for specialized expertise limit its accessibility to some laboratories.

Keywords: genotypic, *Mycobacterium tuberculosis*, phenotypic, sequencing, t-NGS

INTRODUCTION

The accurate identification of Mycobacterium tuberculosis *(M. tuberculosis)* and testing for drug susceptibility are crucial components in the diagnosis, treatment, and management of tuberculosis (TB).[1–3] TB remains one of the most pressing public health challenges globally. According to the World Health Organisation (WHO), an estimated 10 million people fell ill with TB every year, and about 1.5 million people died from the disease.[4] Indonesia is among the top two countries in the world with the highest TB burden, contributing significantly to the global case load with an estimated 969,000 new TB cases in 2022 alone.[5]

Conventional methods for TB identification and drug susceptibility testing, such as culture and phenotypic drug susceptibility testing (DST), often require significant time, usually exceeding 4–13 days.[6] This lengthy process can hinder timely diagnosis and treatment initiation, potentially worsening patient outcomes and contributing to the spread of the disease. In contrast, novel approaches such as *targeted Next-Generation Sequencing* (t-NGS) offer a promising alternative, drastically reducing diagnosis time to just 3–4 days.[6,7] This accelerated method has the potential to replace conventional techniques, providing healthcare professionals with crucial information more quickly.

Although genotypic methods such as t-NGS have shown great potential, inconsistencies remain regarding their concordance with phenotypic methods in identifying resistance to various anti-TB drugs. Previous studies have reported variable levels of agreement, often affected by mutation diversity, heteroresistance, lineage differences, and drug-specific resistance mechanisms. Moreover, most existing research has been conducted in high-resource settings or in populations with specific resistance patterns, limiting the generalizability of their findings to countries with a high TB burden such as Indonesia.

This highlights a critical research gap: comprehensive comparative data evaluating the concordance of phenotypic and genotypic DST specifically within the Indonesian context––where the burden of TB and drug-resistant TB is substantial––remain limited. The novelty of this study lies in its systematic assessment of both phenotypic and genotypic results using t-NGS within a high-burden, real-world clinical setting, providing context-specific evidence that is currently lacking in the literature. By examining concordance patterns across multiple drug classes, this study offers new insights into the reliability, strengths, and limitations of t-NGS for routine diagnostic use, particularly in settings where rapid and accurate DST results are urgently needed.

This research therefore aims to bridge the existing knowledge gap and contribute evidence that may support the broader implementation of genotypic DST approaches in national TB control strategies.

METHODS

Only sputum samples that had undergone phenotypic testing were collected. To be eligible, the samples had to meet several criteria: they had to be purulent, have a minimum volume of 500 µL, and must have undergone identification and drug susceptibility testing using conventional methods, as recorded in secondary data from the SITB database. Samples were excluded if they contained insufficient DNA, indicated by a quantification result of less than 0.1 ng/µL.

Sputum that had undergone phenotypic testing were collected from the Tuberculosis Laboratory at Balai Besar Laboratorium Kesehatan Masyarakat (BBLKM) Makassar from June 2024 until July 2024. Out of 24 sputum samples extracted, only 13 were suitable for testing (DNA quantification ± 0.1 ng/µl). Of these, 11 sputum samples were positive by microscopy, while 2 were negative by microscopy but positive by culture for non-*Mycobacterium tuberculosis* (NTM).

Five hundred microliters of sputum were placed in a microtube, then this tube was placed on a heat block at 95°C for 30 minutes. After that, the sputum was centrifuged at 3000 g for one minute. The supernatant was discarded, and the pellet was extracted to obtain the pure bacterial genomic DNA. The extraction was performed using a Qiagen DNA Mini Kit (Qiagen) according to the manufacturer's instructions, with the final elution of 60 µl and stored at -20°C until use.

DNA quantification was performed using the Qubit® 1x dsDNA HS Assay Kit by Invitrogen. The Qubit 1x dsDNA working solution was prepared at room temperature. Standard measurements were carried out by adding 190 µL of reagent to a 200 µL tube, followed by the addition of 10 µL of the standard (for standards 1 and 2). For measuring DNA samples, 198 µL of reagent was added to the tube, followed by 2 µL of the sample. The solution was homogenised by gently mixing, then incubated at room temperature for two minutes. Readings were taken using the Qubit Flex Fluorometer from Invitrogen. Quantification must be performed on all extracted samples before proceeding with sequencing. The samples with DNA quantification above 0.1 ng/mL can proceed to sequencing, whereas those below this threshold will be excluded.

Polymerase chain reaction (PCR) was performed using Deeplex Myc-TB by Genoscreen, with the following composition: 15.5 µL of Deeplex Myc-TB Master Mix, 0.2 µL Internal control, and 9 µL DNA template. Positive control, Negative control, and Internal control were followed in this step. The thermal cycler was conducted using a Bio-Rad C1000 with the following conditions: 95 °C for 2 min; 35 cycles of 95 °C for 1 min, 55 °C for 30 sec,

and 72 °C for 30 sec; 72 °C for 10 min; and hold at 4 °C.

The PCR product was purified using Agencourt AMPure XP® by Beckman CoulterTM. Seventy-five microliters of 10 mM Tris-HCl pH 7,8 was added to the PCR product, then 65 µL was added. This mix was homogenised and then incubated at room temperature for 5 minutes. The mix was placed on a magnetic rack for 5 min. The supernatant was discarded, and the beads were washed twice with 80% ethanol. The beads were resuspended using 26 µL of 10 mM Tris-HCl pH 7.8. Then the mix was homogenised and then incubated at room temperature for 2 minutes. The mix was placed on a magnetic rack for 5 min. Twenty-five microliters of suspension were transferred to a new tube. The purified amplification product was quantified again and diluted to 0.2 ng/µL in PCR-grade water for library preparation.

Library preparation and sequencing were performed using the Illumina DNA Prep and MiSeq Reagent Micro Kit V2 (Box 1 and 2) according to the manufacturer's instructions.

The FASTQ file from the machine was retrieved and analysed using Deeplex Myc-TB by the Genoscreen application.

Identification and Drug Susceptibility Testing of the sample were taken from the database of the Tuberculosis Laboratory BBLKM Makassar on Sistem Informasi Tuberkulosis (SITB) Indonesia, Ministry of Health.

All data collected from laboratory testing were statistically analysed by SPSS. Chi square test will be choose for nominal data from this research.

RESULTS

Thirteen sputum samples were sequenced using the MiSeq Illumina platform and analysed with Deeplex Myc-TB. The results are shown in Table 1. The analysis included several antibiotics recommended by WHO, such as Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), Ethambutol (EMB), Streptomycin (SM), Kanamycin (KAN), Amikacin (AMI), Capreomycin (CAP), the Fluoroquinolone class (FQ), including Levofloxacin (LEV), Ofloxacin (OFX), and Moxifloxacin (MOX), Ethionamide (ETH), Linezolid (LZD), Bedaquiline (BDQ), and Clofazimine (CFZ). Two samples (15.38%) did not show identification or DST data (undetermined). According to the genotypic results, the sensitivity of FQ, LZD, AMI, CAP, and KAN was higher than that of BDQ, CFZ, SM, ETH, EMB, and PZA (Table 1). However, BDQ, CFZ, SM, ETH, EMB, and PZA showed higher sensitivity than RIF and INH (Table 1).

The phenotypic results for 13 sputum samples are shown in Table 2. Two samples were identified as Nontuberculous Mycobacteria (NTM) despite microscopy result were negative (Table 2). The antibiotics tested at the Tuberculosis Laboratory of BBLKM

TABLE 1
Genotypic result of Drugs Sensitivity Testing

Antibiotic	t-NGS result		
	% Sensitive	% Resistant	% Undetermined
RIF	0	84.62	15.38
INH	23.08	38.46	38.46
PZA	61.54	15.38	23.08
EMB	53.85	30.77	15.38
FQ	76.92	0	23.08
LZD	84.62	0	15.38
BDQ	69.23	7.69	23.08
CFZ	69.23	7.69	23.08
AMI	84.62	0	15.38
SM	53.85	15.38	23.08
ETH	53.85	15.38	23.08
CAP	76.92	0	23.08
KAN	84.62	0	15.38

% Sensitivity : showed the number of sample from 13 sample that showed the sensitivity against the antibiotics that have tested using tNGS. Undetermined : Machine can't not read the result

TABLE 2
Phenotypic result of Drugs Sensitivity Testing

Antibiotic	Identification and DST		
	% Sensitive	% Resistant	% NTM
MFX High	92.31	7.69	15.38
INH High	53.85	46.15	
INH Low	53.85	46.15	
BDQ	100	0	
CFZ	100	0	
LZD	100	0	
LFX	92.31	7.69	

% Sensitivity : showed the number of sample from 13 sample that showed the sensitivity against the antibiotics that have tested using tNGS. Undetermined : Machine can't not read the result

TABLE 3
Identification Result of 13 Sputum Sample

	Phenotypic	Genotypic
Identification result		
M. tuberculosis	84,62 %	84,62%
NTM	15,38 % (sample number 16 and 18)	0 %
Not detected	0 %	15,38% (sample number 13 and 14)
Acid Fast Bacilli (AFB) Microscopic result		
Negative	2 (Sample 16 and 18)	–
+1	4	–
+3	7	–

Makassar included moxifloxacin, isoniazid, bedaquiline, clofazimine, linezolid, and levofloxacin. These antibiotics exhibited varying sensitivities (Table 2). BDQ, CFZ, and LZD demonstrated complete (100%) sensitivity against *M. tuberculosis*, whereas both low and high concentrations of INH exhibited significantly lower sensitivity, at only 53.85% (Table 2).

The t-NGS results for 13 sputum samples obtained from the Tuberculosis Laboratory at BBLKM Makassar show both concordance and discordance with the phenotypic results. In the phenotypic results, two samples were identified as NTM, whereas the genotypic results identified 11 sputum samples as *M. tuberculosis* and 2 samples showed not detected results (Table 3).

In several samples, the genotypic drug sensitivity test results showed good concordance with the phenotypic results. However, a few samples did not exhibit good concordance because genotypic results showed uncharacterized (Table 4).

Figure 1 shows a comparison of drug resistance between genotypic and phenotypic methods, where the sample results that showed NTM, not detected, and uncharacterized were excluded.

A statistical test was conducted to evaluate the significance of both concordance and discordance across all data. The Chi-square test was chosen as the method of analysis because the data collected were categorical (nominal). The results of the Chi-square test, with a 95% confidence level, showed a significance value greater than 0.05 for the differences in phenotypic and genotypic results for tuberculosis identification and drug susceptibility testing (Table 5).

DISCUSSION

All sputum samples based on genotypic results were identified as *Mycobacterium tuberculosis* (Table 3), except for two samples (Samples 13 and 14), which were not

TABLE 4
The Drug Sensitivity Test based on Phenotypic and Genotypic Result

Code of sample	Drugs									
	BDQ		INH		CFZ		LZD		FQ as MFX	
	Phen	tNGS	Phen	tNGS	Phen	tNGS	Phen	tNGS	Phen	tNGS
1	S	S	S	S	S	S	S	S	S	S
2	S	U	R	R	S	U	S	S	S	S
5	S	S	S	S	S	S	S	S	S	S
6	S	S	R	R	S	S	S	S	S	S
8	S	S	R	R	S	S	S	S	S	S
9	S	S	S	U	S	S	S	S	S	S
11	S	S	R	R	S	S	S	S	S	S
12	S	S	S	U	S	S	S	S	S	S
13	S	ND	R	ND	S	ND	S	ND	S	ND
14	S	ND	R	ND	S	ND	S	ND	R	ND
15	S	S	R	S*	S	S	S	S	S	S
16	NTM	R*	NTM	U	NTM	R*	NTM	S*	NTM	S*
18	NTM	S*	NTM	R*	NTM	S*	NTM	S*	NTM	U

S: sensitive; R: resistant; NTM: Non Mycobacterium tuberculosis; ND: Not Detected; U: uncharecterized; *dis-concordance; Phen: Phenotypic

detected. Meanwhile, in the phenotypic results (Table 3), two samples (Samples 16 and 18) were identified as non-*Mycobacterium tuberculosis*. This indicates a discrepancy in identification between the phenotypic and genotypic results. The confirmation of phenotypic identification of *Mycobacterium tuberculosis* was based on the TB Ag MPT64 Rapid Test results by SD Bioline. The *Mycobacterium tuberculosis* complex secretes this protein, whereas NTM and Bacillus Calmette-Guerin strains with the RD2 deletion do not.[8,9] Although this rapid test has a high sensitivity (almost 100%) and specificity (almost 100%) for sample culture,[8,10,11] it has limitations. TB Ag MPT64 rapid test by SD Bioline has a limit detection on 6.90×10^4 CFU/ml.[12] Based on the microscopic results (Table 3), the two samples (NTM 16 and 18) were negative for acid-fast bacilli. Sari and Aryati demonstrated a concordance rate of 70.8% between the microscopic result and MPT64.[13] This means that the TB Ag MPT64 test may not detect *M. tuberculosis* if the bacterial load is below the limit of detection. However, in the genotypic results, *M. tuberculosis* was still identified because the amplification step in the testing process allows detection down to a limit of 200 CFU/mL.[3,14]

Genotypic analysis of Drug Sensitivity Testing (DST) was conducted using the Deeplex® Myc-TB by Genoscreen application, based on the extended WHO catalogue, which includes 13 drugs. These drugs are the first-line drugs: Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), and Ethambutol (EMB); the second-line injectables: Streptomycin (SM), Kanamycin (KAN), Amikacin (AMI), and Capreomycin (CAP); the Fluoroquinolone class (FQ), including Levofloxacin (LEV), Ofloxacin (OFX), and Moxifloxacin (MOX); as well as Ethionamide (ETH), Linezolid (LZD), Bedaquiline (BDQ), and Clofazimine (CFZ).[15–17] Meanwhile, phenotypic analysis for drug sensitivity testing was conducted based on national regulations in Indonesia, which have been approved by the US FDA and recommended by the WHO in 2022.[18–20]

There was concordance in the sensitivity of several drugs when comparing phenotypic and genotypic results, except for isoniazid (Figure 1). These concordant results were obtained after excluding uncharacterised, not detected, and NTM samples from the phenotypic analysis. These findings align with recent systematic reviews reporting high overall agreement between sequencing-based DST and culture-based DST, particularly for rifampicin and fluoroquinolones.[21,22]

The genotypic test results for isoniazid indicated a lower level of drug resistance compared to the phenotypic results (Figure 1b). This discrepancy was observed in Sample 15 (Table 4). Resistance to isoniazid typically occurs due to mutations in the *katG* and *inhA* genes; however, molecular analysis showed no

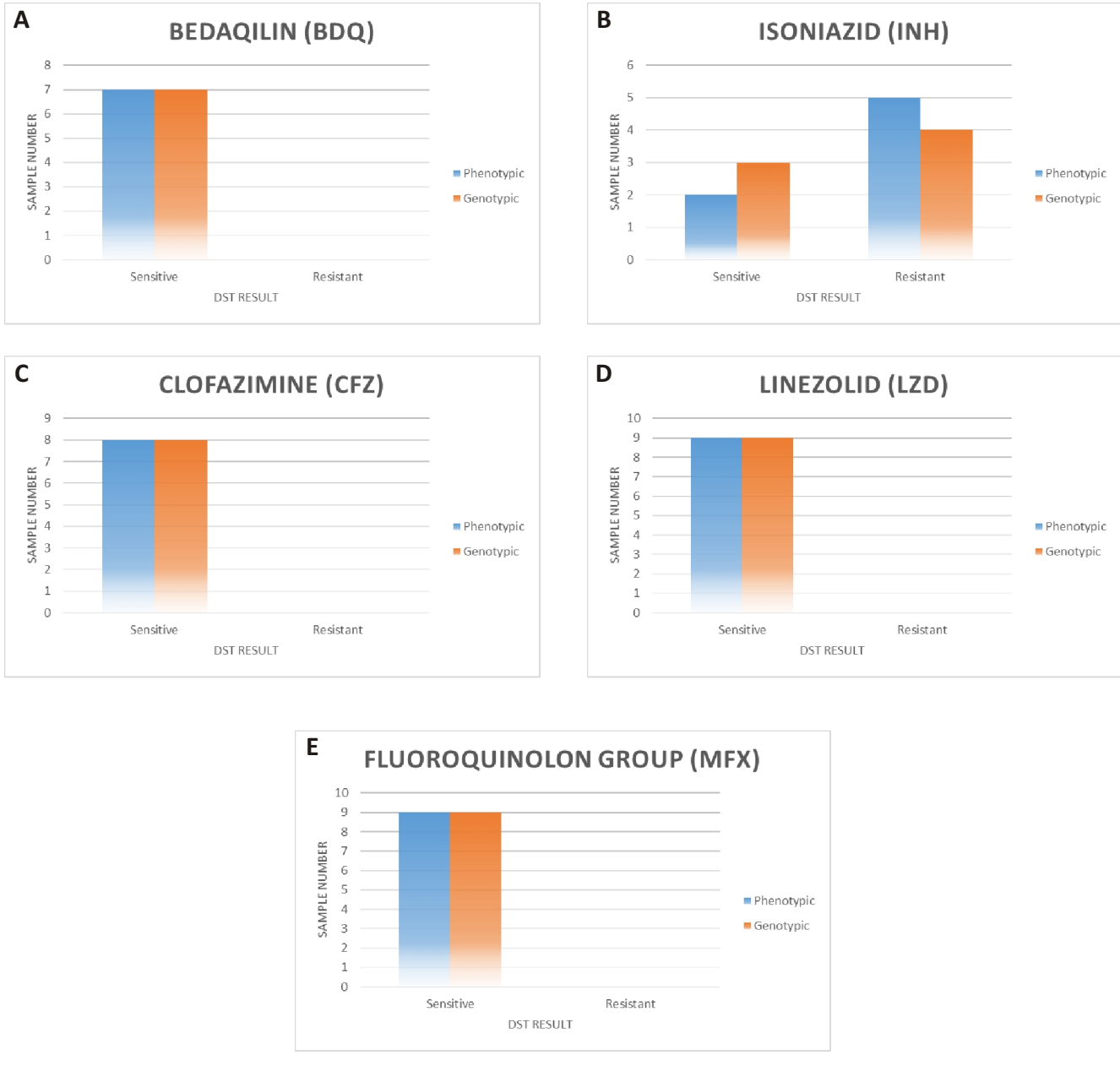

Figure 1. Comparison of the phenotypic and genotypic properties of several drugs.

mutations in these genes. Despite this, the sample exhibited phenotypic resistance to INH. This suggests that other resistance mechanisms may be involved, such as efflux pumps, slow metabolism, or impermeable cell walls.[23,24] Recent genomic studies further support the role of efflux pump mutations *(efpA, iniA)* in low-level INH resistance.[25,26]

The statistical analysis in this study revealed that the differences between phenotypic and genotypic methods for identifying *M. tuberculosis* and assessing drug susceptibility were not statistically significant, with a significance value greater than 0.05 (Table 5). This suggests a strong overall agreement between the two methods, supporting the use of genotypic approaches, such as t-NGS, as a reliable alternative to conventional phenotypic methods. These findings are consistent with results reported by previous researchers.[6,27,28] However, specific instances of discordance highlight the need for further investigation and careful interpretation of genotypic results, particularly when novel or uncharacterized mutations are involved.

Several results were identified as uncharacterized, including BDQ for Sample 2, INH for Samples 9, 12, and 18, CFZ for Sample 2, and FQ for Sample 18 (Table 4).

TABLE 5
Statistical result of Chi Square Test with 95% confidence level

	Significant value (*p*)
Identification	0.135
DST Test:	
BDQ	0.185
INH	0.113
CFZ	0.185
FQ as MFX	0.282
LZD	0.135

Uncharacterised variants refer to sequence variations whose association with drug resistance or susceptibility has not yet been established. Variants of uncertain significance are those that cannot yet be classified as drug-resistant or drug-susceptible according to current WHO confidence grading.[15] For instance, Sample 2 has a mutation in the rv0678 gene (Supplement 1), associated with BDQ resistance, resulting in an amino acid change from L95 to F (L95F), but this mutation remains uncharacterized. Despite this, phenotypic results indicated sensitivity to *M. tuberculosis* (Table 4).

Moreover, for samples not detected in the genotypic results (Samples 13 and 14, Table 4), the Deeplex Myc-TB analysis showed low coverage (Supplement 1). Factors such as operational techniques, including the manual extraction and library preparation processes, could have influenced these results. Therefore, professional and trained technicians are required to perform this method. While t-NGS offers faster results (within 3 days) compared to conventional methods, it is more costly and requires expertise to analyse uncharacterized samples.[27]

CONCLUSION

T-NGS shows promise as a replacement for conventional methods because the discrepancies with phenotypic results are not statistically significant, and it reduces testing time. Additionally, the genotypic method can identify promising mutations that have not been widely reported. However, not all laboratories are equipped for this method due to its higher cost and the need for experts to analyse uncharacterized samples.

CONFLICT OF INTEREST

We declare no conflict of interest

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