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Correlations between Gene Resistant Markers and Second-Line Anti-TB Drug Resistance in Pre-XDR and XDR-TB Patients

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Abstract

Background: Extensively drug resistant tuberculosis (XDR-TB) is a serious problem in public health and XDR-TB patients usually develop from multi-drug resistance tuberculosis (MDR-TB) and pre-XDR-TB. The rapid molecular test for drug susceptibility testing (DST) can be used for early detection to prevent XDR-TB. **Methods:** We examined 34 clinical *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates from MDR/XDR-TB patients in the upper north of Thailand that were identified with drug susceptibility profiles by indirect agar proportion method from 2005-2012. Our study investigated the genetic mutations in *gyrA* for ofloxacin resistance and *rrs* for kanamycin resistance. The genetic mutations and drug susceptibility test results were analyzed using the exact test. **Results:** The majority of the ofloxacin resistance was detected in *gyrA* 21, *gyrA* 70, *gyrA* 87, *gyrA* 102, *gyrA* 162, and *gyrA* 187 were at 0%, 12.5%, 37.5%, 0%, 50.0% and 25.0% sensitivity, respectively, and at 96.2, 96.2%, 20.1%, 96.2%, 57.7% and 61.5% specificity, respectively. Kanamycin resistance was found in *rrs* 512, *rrs* 241, *rrs* 223, *rrs* 414 and *rrs* 408 at 16.7%, 0%, 0%, 16.7% and 16.7% sensitivity, respectively, and at 96.4%, 92.9%, 82.1%, 82.1% and 71.4% specificity, respectively. This study found no significant correlation between *gyrA* mutations and ofloxacin resistance and also no correlation between the *rrs* gene and kanamycin resistance. **Conclusion:** These primer sequences and PCR products in our study such as *gyrA* and *rrs* might be unsuitable to detect ofloxacin and kanamycin resistance in the upper

north of Thailand.

Keywords

XDR-TB, Pre-XDR-TB, Ofloxacin, *gyrA*, Kanamycin, *rrs*

1. Introduction

The emerging and increasing extensively drug resistant tuberculosis (XDR-TB) is a serious problem in public health. XDR-TB is caused by the *Mycobacterium tuberculosis* (*M. tuberculosis*), which resists up to at least four drugs such as isoniazid (INH), rifampicin (RIF), any fluoroquinolone and at least one of three second-line drug injections: capreomycin, kanamycin and amikacin [1]. The emergence of XDR-TB is caused by many factors, in conjunction with the low laboratory capacity to diagnose XDR-TB; thus, resulting in ineffective control of the spread of the disease and ineffective initial treatment [1] [2] [3]. Treatment of XDR-TB is very complicated and limited because first and second-line anti-TB drugs cannot be used, leading to a long duration, expensive costs, less effective outcomes and many side effects [4] [5].

XDR-TB normally develops from multi-drug resistant tuberculosis (MDR-TB) to pre-extensively drug resistant tuberculosis (pre-XDR-TB), and then turns to XDR-TB. The definition of pre-XDR-TB is a disease caused by the *M. tuberculosis* strain that resists isoniazid and rifampin and either a fluoroquinolone or a second-line injectable drug, but not both [6]. Rapid diagnosis of resistance to second-line anti-TB strains is extremely important leading to effective treatment and prevention of the spread of pre-XDR/XDR strains [7]. Molecular assays, such as MTBDRsl, are rapid diagnosis tests to detect second-line drug resistance based on drug resistant gene mutations [4]. Analyzing the mutation types and frequencies among local strains are fundamentally important for evaluating the usefulness of these molecular tools.

Today, commercially available molecular tests, endorsed by the WHO, can detect only rifampicin and isoniazid, which means they can detect MDR-TB but not pre-XDR and XDR-TB [1] [8]. Related studies have shown specificities of gene mutations in predicting kanamycin [9] [10] [11] and ofloxacin resistance [11] [12] [13] [14]. Many studies have been conducted on genotypic and phenotypic characteristics of drug resistant *M. tuberculosis*; however, this kind of study is rare in Thailand [15]. Better understanding of the molecular mechanisms of drug resistance in pre-XDR-TB and XDR-TB strains can contribute to the improvement of existing techniques in detecting drug resistance and help to explore new target sites and develop new diagnostic tools.

The objective of this study was to determine the association (relationship) between mutation in *gyrA* and resistance to ofloxacin as well as mutation in *rrs* and resistance to kanamycin in MDR, pre-XDR and XDR TB patients in the

northern region of Thailand.

2. Materials and Methods

2.1. Bacterial Isolates and Drug Susceptibility Testing

A total of 34 MDR and XDR *M. tuberculosis* isolates were collected by the Office of Disease Prevention and Control Region 10 (DPC 10) from patients with MDR/XDR-TB in the upper north of Thailand from 2005 to 2012. The identification of MDR/XDR-TB isolates was performed conventionally using the indirect proportion method on agar based medium with concentrations of isoniazid (0.2 μg/ml), rifampicin (40 μg/ml), etambutol (2 μg/ml) and streptomycin (4 μg/ml) as shown in **Figure 1** [16]. When they resisted isoniazid and rifampicin, then ofloxacin (2.0 μg/ml) and kanamycin (30 μg/ml) susceptibility was tested on middle brook medium using the proportion method. All 34 isolates were subcultured in 5 ml of 7H9 broth with PANTA supplementation and 3% Ogawa-based medium. In all, 34 isolates were re-grown and tested for their phenotypes using first and second-line drugs (isoniazid, rifampicin, ofloxacin and kanamycin) by the proportion method with LJ medium as shown in **Figure 1** [17].

Drug susceptibility testing (DST) was conducted by the indirect agar proportion method on two of LJ medium and bacilli suspension of 10^{-2} and 10^{-4} mg/ml for inoculation. The LJ medium were inoculated with following drugs isoniazid (0.2 μg/ml), rifampicin (40.0 μg/ml), ofloxacin (2.0 μg/ml), and kanamycin (30

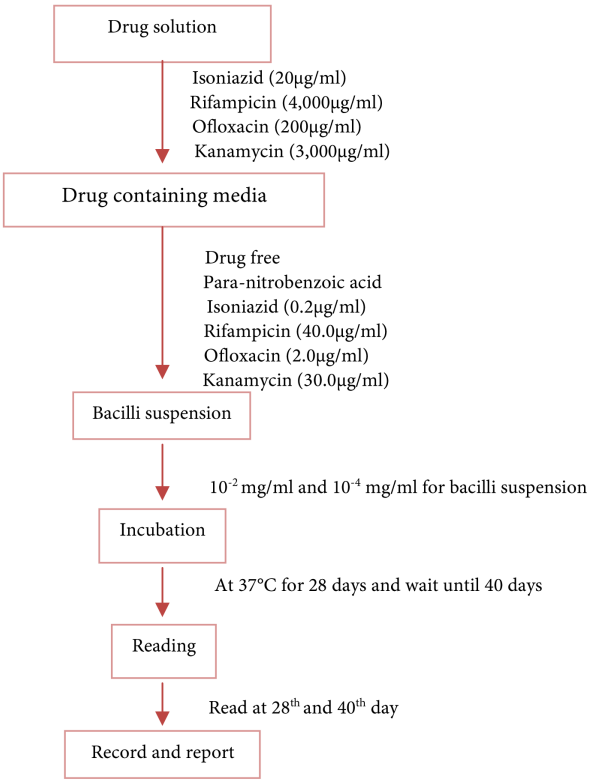

Figure 1. Flow chart for Drug Susceptibility Test.

μg/ml), para-nitrobenzoic acid and no drug (drug-free).

2.2. DNA Extraction and Sequencing Method

Totally, 34 isolates were cultured on solid media (Löwenstein-Jensen and OGAWA). Chromosomal DNA was extracted by commercial kit using the MolecuTech REBA MTB-MDR 2011 method. The purified DNA pellets were stored at 4˚C until sending to Macrogen, Korea for nucleotide sequencing. After that, the two loci were amplified by PCR: *gyrA* (ofloxacin) and *rrs* (kanamycin). The primers for *gyrA* PCR amplification followed that of Leung Kl, *et al.* in 2010 that were designed using *gyrA* including the QRDRs of this gene: forward, 5'-AAGAGCGCCACCGACATC-3' and reverse, 5'-CAGCATCTCCATCGCCAA-3'. The following steps were performed: one denature cycle at 95˚C for 2 min, followed by 30 cycles of 1 min at 95˚C, 1 min at 65˚C and 1 min at 72˚C, followed by elongation at 72˚C for 10 min. PCR product comprised 320 base pairs in size(11). To detect kanamycin resistance, the primers were designed to identify mutations in codon 1401 of *rrs* gene: 16s-1F (5'-CGTGGCCGTTTGTTTTGTC-3') and 16s-1F (5'-TGGTGCTCCTTAGAAAGGAGG-3') following the method described by Leung Kl, *et al.* in 2010. PCR product comprised 665 base pair fragments, according to the following steps: beginning denaturation at 95˚C for 2 min, followed by 35 cycles of 94˚C for 1 min, 60˚C for 1min, 68˚C for 2 min, followed by elongation at 68˚C for 10 min [11]. The amplified products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA) and DNA sequencing data were produced by the ABI 3730xl DNA analyzer at Macrogen.

3. Analysis

3.1. DNA Sequencing Analysis

The sequencing data were used to determine the confidence level with scanners and ABI sequence chromatograms. The analysis was used to determine the presence and/or absence of gene mutations using the H37Rv sequence to make comparison.

3.2. Statistical Analysis

The resistance gene, genetic site mutation data and DST results were compiled using excel sheet within a Microsoft Office Excel 2010 database. Statistical analysis was performed using STATA version 11.0. Univariate comparison of drug resistance and resistance gene was performed using Fisher's exact test.

4. Results

4.1. Drug Susceptibility Testing and Resistance Gene

We analyzed 34 isolates in total: 24 MDR-TB isolates (70.5%), 9 Pre XDR-TB isolates (26.5%) and 1 XDR-TB isolate (3.0%). Phenotypic DST and DNA sequencing were studied in all isolates for two drugs, ofloxacin and kanamycin, to

predict pre-XDR and XDR-TB [1] [6]. DNA sequencings were performed for two resistance genes: *gyrA*, and rrs. The distribution of genetic drug resistance with the second line anti-tuberculosis drug resistance was shown in Table 1. The frequency of *gyrA* and *rrs* gene mutation was found among patients with MDR, Pre-XDR and XDR TB in Table 2.

Table 1. Distribution of Mycobacterium tuberculosis genetic mutations with drugs resistant.

Gene mutation		Drug resistant					
		Ofloxacin			Kanamycin		
		S (%)	R (%)	Total (%)	S (%)	R (%)	Total (%)
gyrA	No (%)	5 (19.2%)	2 (25.0%)	7 (20.6%)	6 (21.4%)	1 (16.7%)	7 (20.6%)
	Yes (%)	21 (80.8%)	6 (75.0%)	27 (79.4%)	22 (78.6%)	5 (83.3%)	27 (79.4%)
	Total (%)	26 (76.5%)	8 (23.5%)	34 (100%)	28 (82.4%)	6 (17.6%)	34 (100%)
rrs	No (%)	16 (61.5%)	3 (37.5%)	19 (55.9%)	15 (53.6%)	4 (66.7%)	19 (55.9%)
	Yes (%)	10 (38.5%)	5 (62.5%)	15 (44.1%)	13 (46.4%)	2 (33.3%)	15 (44.1%)
	Total (%)	26 (76.5%)	8 (23.5%)	34 (100%)	28 (82.4%)	6 (17.6%)	34 (100%)

Table 2. Frequency distribution of loci mutation *gyrA*, and *rrs* genes among patients with MDR-TB, and Pre-XDR-TB or XDR-TB.

Gene mutation codon	n	%
gyrA		
No mutation	7	20.6
gyrA 70	1	2.9
gyrA 87	5	14.7
gyrA 162	2	5.9
gyrA 187	1	2.9
gyrA 87 and *gyrA* 162	6	17.7
gyrA 87 and *gyrA* 187	4	11.8
gyrA 87, *gyrA* 162 and *gyrA* 187	7	20.6
gyrA 21, *gyrA*70, *gyrA* 87 and *gyrA* 102	1	2.9
rrs		
No mutation	19	55.9
rrs 512	1	2.9
rrs 223	1	2.9
rrs 408	4	11.8
rrs 414	3	8.8
rrs 408 and *rrs* 512	1	2.9
rrs 241 and *rrs* 408	1	2.9
rrs 223 and *rrs* 408	1	2.9
rrs 223 and *rrs* 414	1	2.9
rrs 223, *rrs* 408 and *rrs* 414	1	2.9
rrs 223, *rrs* 241, *rrs* 408 and *rrs* 414	1	2.9

4.2. Ofloxacin and *gyrA*

Eight isolates (23.5%) resisted to ofloxacin. Twenty isolates (79.4%) were positive for any *gyrA* mutation (Table 2). Overall sensitivity and specificity of any *gyrA* mutation to detect ofloxacin resistance were 75% and 19.2%, respectively (Table 3). No significant correlation was found between mutation in *gyrA* gene and ofloxacin resistance. *gyrA* 162 resistance was more sensitive (50%) to ofloxacin resistance than *gyrA* 21 (0%), *gyrA* 70 (12.5%), *gyrA* 87 (37.5%), *gyrA* 102 (0%), and *gyrA* 187 (25%).

4.3. Kanamycin and *rrs*

Six isolates (17.6%) resisted to ofloxacin. Fifteen isolates (44.1%) were positive for any *rrs* mutation in (Table 2). The overall sensitivity and specificity of any *rrs* mutation to detect kanamycin resistance were 33.3% and 53.6%, respectively (Table 4). No significant correlation was observed between the *rrs* gene and kanamycin drug resistance (Table 4).

5. Discussion

To early diagnose pre-XDR and XDR TB, identifying the marker of the genetic site mutation is required. Mutations in select genes of *M. tuberculosis* have been used as markers for anti-TB drug resistance: the *gyrA* gene for ofloxacin and *rrs* gene for kanamycin. However, in our study, the results showed no significant association between ofloxacin resistance and gy*rA* mutations, and also, kanamycin resistance and *rrs* mutations. Based on the correlation studies among these primers, genetic and the geographic distances should be evaluated in a future study [14].

Table 3. Association between *gyrA* loci mutation gene and ofloxacin resistance.

Loci of mutation		Ofloxacin resistance (n = 34)		p-value
		Susceptible	Resistant	
gyrA 70	No	25 (96.2)	7 (87.5)	0.421
	Yes	1 (3.8)	1 (12.5)	
gyrA 102	No	25 (96.2)	7 (100.0)	1.000
	Yes	1 (3.8)	0 (0.0)	
gyrA 21	No	25 (96.2)	8 (100.0)	1.000
	Yes	1 (3.8)	0 (0.0)	
gyrA 87	No	6 (23.1)	5 (62.5)	0.079
	Yes	20 (76.9)	3 (37.5)	
gyrA 162	No	15 (57.7)	4 (50.0)	1.000
	Yes	11 (42.3)	4 (50.0)	
gyrA 187	No	16 (61.5)	6 (75.0)	0.681
	Yes	10 (38.5)	2 (25.0)	

Table 4. Association between *rrs* loci mutation gene and kanamycin resistance.

Loci of mutation		Kanamycin resistance (n = 34)		p-value
		Susceptible	Resistant	
rrs 512	No	27 (96.4)	5 (83.3)	0.326
	Yes	1 (3.6)	1 (16.7)	
rrs 241	No	26 (92.9)	6 (100.0)	1.000
	Yes	2 (7.1)	0 (0.0)	
rrs 223	No	23 (82.1)	6 (100.0)	0.559
	Yes	5 (17.9)	0 (0.0)	
rrs 408	No	20 (71.4)	5 (83.3)	0.559
	Yes	8 (28.6)	1 (16.7)	
rrs 414	No	23 (82.1)	5 (83.3)	1.000
	Yes	5 (17.9)	1 (16.7)	

5.1. Ofloxacin Resistance

The genotypic distribution of *gyrA* mutations in both ofloxacin resistant and ofloxacin susceptible groups were not different. We found that the *gyrA* primer can detect mutation in *gyrA* 21, *gyrA* 70, *gyrA* 87, *gyrA* 102, *gyrA* 162, and *gyrA* 187 at 0%, 12.5%, 37.5%, 0%, 50.0% and 25.0% sensitivity, respectively, and at 96.2%, 96.2%, 20.1%, 96.2%, 57.7% and 61.5% specificity, respectively. Mutations in *gyrA* have been found in ofloxacin resistance at codons 88, 90, 91, and 94 [18] [19] [20]. One study presented that ofloxacin susceptibility was associated with *gyrA* mutations at codon 88 to 94 in East Asian strains (55.6%) and Indo-Oceanic strains (44.4%), but these mutations could not be found in Euro-American strains [20]. One related study showed that *gyrA* mutations at positions 88 to 94 had a high level of ofloxacin resistance [20], especially *gyrA* 94 that exhibited high level of resistance to moxifloxacin and gatifloxam [21]. Some studies found that mutations in *gyrA* at codons 21, 95, and 668 were not associated with ofloxacin resistance [14]. Only *gyrA* mutation at codon 95 could be detected by commercial molecular tests [20]. Further studies are required to determine real lineage-specific differences regarding the ability of ofloxacin resistance in pre-XDR and XDR-TB strains to develop commercial molecular testing.

5.2. Kanamycin Resistance

The *rrs* mutation were found in *rrs* 512, *rrs* 241, *rrs* 223, *rrs* 414, and *rrs* 408 at 16.7%, 0%, 0%, 16.7% and 16.7% sensitivity, respectively, and 96.4%, 92.9%, 82.1%, 82.1% and 71.4% specificity, respectively, in this study. A systematic review showed that the combination of M401G and C517T mutation sites in *rrs* could predicted kanamycin resistance with high sensitivity and specificity [9]. One study in 2001 reported that streptomycin resistance was related to mutations in the 500 region of *rrs* [22]. Our study followed primer patterns from one related study in Hong Kong that could predict 96.8% specificity and 64.2% sensitivity for kanamycin from *rrs* on gene marker 1401 [11]. The most frequently

reported kanamycin associated *rrs* mutations included A1401G, C1402T and G1484T [11] [23] [24]. The *rrs* A1401G mutation varied from 50% to 90% in these groups [10] [11] [23] [25] [26] [27] [28]. A study from Pakistan also showed 78% of kanamycin resistance correlated to SNPs in *rrs* at nt1401 [28] and concluded that the commercial diagnostic tool determining the aminoglycoside drug resistance target at *rrs* nt 1401 could detect 78% of kanamycin resistance in XDR-TB isolates [28]. One related study in Thailand showed that the designed *rrs* primer using 3 positions and 1680 PCR product size could predict 100% specificity and 72.4% sensitivity of kanamycin resistant M/XDR-TB [29]. However, we found that the ability of *rrs* 1401 mutation to detect kanamycin resistance was quite low with sensitivity of 33.3% and specificity of 53.6%. From the result, this *rrs* primer is not useful to detect kanamycin resistance in this area.

6. Limitations of This Study

One limitation of the current study was the small sample size. Due to the retrospective study, MDR TB isolates could be re-cultured in only 34 isolates from 261.

7. Conclusion

The primer sequences for detecting *gyrA* and *rrs*1401 mutations in this study are not able to detect ofloxacin and kanamycin resistance in the upper north of Thailand. Further studies of other mutation sites are needed.

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Conflict of Interest

No conflict of interest was perceived and none was declared.

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