

Molecular Surveillance of Antimicrobial Resistance of *Mycobacterium leprae* from Leprosy Patients in Zhejiang Province, China

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Purpose: Reports on antimicrobial resistance (AMR) of *Mycobacterium leprae* (*M. leprae*) in Zhejiang Province are limited. Thus, this study aimed to investigate the drug resistance of new leprosy cases within several years and analyse the emergence of AMR mutations from Zhejiang Province.

Methods: This study enrolled 34 leprosy cases in Zhejiang Province, China, from 2018 to 2021. Gene mutation of WHO-recommended DRDRs (*folP1*, *rpoB* and *gyrA*) and genes of compensatory AMR-associated DRDRs, including *nth*, *rpoA*, *rpoC*, *gyrB* and 23S rRNA, were detected by amplification. Clinical data analysis was performed to investigate the epidemiological association of leprosy.

Results: Of the 34 samples, 2 (5.9%) strains showed drug resistance, which were mutated to dapsone and ofloxacin, separately. Two single mutations in *gyrB* were detected in different strains (5.9%), whereas one of the *rpoC* mutation was also detected in one strain each (2.9%), which were proved to be polymorphs. No correlation of drug resistance proportion was identified in male vs female, nerve vs no nerve involvement, deformity vs no deformity and reaction vs non-reaction cases.

Conclusion: Results showed well control of leprosy patients in Zhejiang Province. Gene mutations of WHO-recommended DRDRs *folP1* and *gyrA* confirmed the resistance to dapsone and ofloxacin. Compensatory AMR-associated mutations confirmed to be polymorphs still require further study to determine their phenotypic outcomes in *M. leprae*. The results demonstrated that drug-resistant strains are not epidemic in this area. Given the few cases of leprosy, analysing the AMR of *M. leprae* in Zhejiang Province more comprehensively is difficult. However, regular MDT treatment and population management in the early stage may contribute to the low prevalence of leprosy.

Keywords: *M. leprae*, antimicrobial resistance, gene mutation

Introduction

Leprosy, which is caused by *Mycobacterium leprae* (*M. leprae*), remains a public health problem associated with disability, deformity, stigma and discrimination in untreated individuals. Based on the official World Health Organization (WHO) records, a total of 127,396 new leprosy patients were reported globally in 2020,¹ which indicates an endemicity of leprosy, particularly in underdeveloped countries. The current strategy for leprosy control is multidrug therapy (MDT), which is recommended by WHO in 1982.² The combination therapy with dapsone, rifampicin and clofazimine has decreased the prevalence of leprosy globally, which is recommended for the treatment of multibacillary

(MB) leprosy, whereas therapy with dapsone and rifampicin should be used for paucibacillary (PB) leprosy. In addition, ofloxacin is a key drug in the treatment of single-lesion leprosy cases and drug-resistant cases.³ Studies in lepromatous leprosy patients have demonstrated bactericidal activity killing 99.99% of viable bacteria but not acceptable as a single drug due to side effects in human.^{4,5} However, dead *M. leprae* are cleared from tissue very slowly, and the bacterial carcasses may therefore remain visible in biopsies for years after they have been killed. The antigens of these dead organisms may continue to elicit immunological and inflammatory responses.^{6,7}

Since 1964, the resistance of *M. leprae* strains to dapsone was reported,⁸ and drug resistance cases increased gradually. Apart from single-drug resistance, an increasing number of resistances to multiple drugs has been reported in recent years. Based on previous studies, drug resistance detection of drug resistance-determining regions (DRDRs) containing *folP1*, *rpoB* and *gyrA* associated with dapsone, rifampicin and ofloxacin resistance,^{9–11} respectively, has been well established and incorporated in the WHO guidelines for AMR surveillance. For example, position 53, 55 of *folP1* gene are known to confer drug resistance to dapsone, while position 89, 91 of *gyrA* gene conferring to ofloxacin and position 441, 451, 456, 458 of *rpoB* gene conferring to rifampicin.¹² In addition to mutations in WHO-recommended DRDRs, compensatory AMR-associated mutations, including *nth* (DNA repair), *rpoA* (rifampicin), *rpoC* (rifampicin), *gyrA*, *gyrB* (ofloxacin) and 23S rRNA (clarithromycin), have been reported in *M. tuberculosis* and *M. leprae*.^{13–15} However, the roles of these mutations in *M. leprae* are yet to be confirmed.

In China, leprosy is still at a relatively low endemic level, primarily in Yunnan, Sichuan, Hunan and Guizhou. A total of 406 new cases were detected in China in 2020, with a case detection rate of 0.032 per 100,000 people. The registered cases were 704 by the end of 2020, accounting for a prevalence rate of 0.054 per 100,000 population.¹ Zhejiang is one of the leprosy-endemic provinces in China with a population of 57.37 million, about 1% of China.¹⁶ A total of 16,000 cases have been registered until the end of 2019. Since the implementation of MDT recommended by WHO in the 1980s, Zhejiang achieved leprosy elimination by Chinese level (less than 1 per 100,000) in 1995. Nevertheless, as an important economic province, Zhejiang has a large number of floating populations, which has become the main source of newly detected cases. A total of 167 new leprosy cases were identified in Zhejiang Province from 2011 to 2019, including 128 cases of floating population in other provinces (accounting for 76.65%). In recent years, a few new cases still can be detected in Zhejiang every year. However, almost no studies on the drug resistance of Zhejiang leprosy cases have been reported.

In the current study, we collected 34 newly diagnosed leprosy cases from Zhejiang Province and analysed the AMR of *M. leprae* by direct PCR sequencing of the WHO-recommended DRDRs containing *folP1*, *rpoB* and *gyrA*. We also investigated the sequence of regions and genes of compensatory AMR-associated DRDRs, including *nth*, *rpoA*, *rpoC*, *gyrB* and 23S rRNA, to evaluate the necessity to complement the current surveillance system for AMR mutations. Finally, combined with clinical characteristics of leprosy cases, the prevalence of leprosy resistance in Zhejiang Province was obtained.

Materials and Methods

Sample Collection

During the study period, 34 newly diagnosed cases of leprosy in Zhejiang Province were isolated from skin biopsy (75% alcohol-soaked tissues), including eight local cases and 26 floating population from other provinces from 2018 to 2021. Of the 34 cases, 85.3% were MB, whereas the remaining cases were PB. All patients were examined by experienced dermatologists to ensure the quality and uniformity of these procedures. Skin biopsies were collected based on standard recommendations. Diagnosis and prescription of PB and MB-MDT regimen were performed in accordance with the WHO guidelines.

PCR and Sequencing of DRDRs

The presence of *M. leprae* genomic DNA was confirmed by PCR detection of the 16S rRNA gene. PCR and nested PCR were performed to amplify the WHO-recommended DRDRs (*folP1*, *rpoB* and *gyrA*) and genes of compensatory AMR-associated DRDRs, including *nth*, *rpoA*, *rpoC*, *gyrB* and 23S rRNA, by using primers and conditions listed in

[Supplementary Tables 1](#) and [2](#). The quality of amplified products was assessed using 1–2% agarose gel. PCR products were purified and sequenced at a local commercial sequencing company (Tsingke Biological Technology Co., Ltd., Nanjing, China). Alignments to NCBI nucleotide databases were performed using a basic local alignment sequence tool (NCBI-BLAST) to detect mutations.

Results

Clinical Details of Collected Cases

The detailed study profile is shown in [Figure 1](#). Clinical data confirmed that 34 patients were newly diagnosed from 2018 to 2021, and their characteristics are summarised in [Table 1](#) and [Supplementary Table 3](#). Twenty-nine of the patients (85.3%) were MB, whereas four cases (11.8%) were PB, and one case (2.9%) was not defined. The initial bacilloscopy index of the MB cases ranged between 0 and 5.5, with an average of 2.96. Gender analysis showed that males (67.6%) were more affected than females (32.4%) with age ranging from 17 to 83. Of the 34 cases, eight (23.5%) were born in Zhejiang Province, and 26 (76.5%) were floating population (from other provinces in China). The average age of all the patients was 46.9 years old, whereas floating population cases (33.8 years old) were generally younger than native cases (60 years old) based on the data. All patients had different time delays in diagnosis, ranging from 1 month to 13 years. However, most delay time of all cases was within 24 months (85.3%). Based on the MDT scheme, patients diagnosed from 2018 to 2020 completed the official treatment regimen, whereas cases in 2021 were still under treatment.

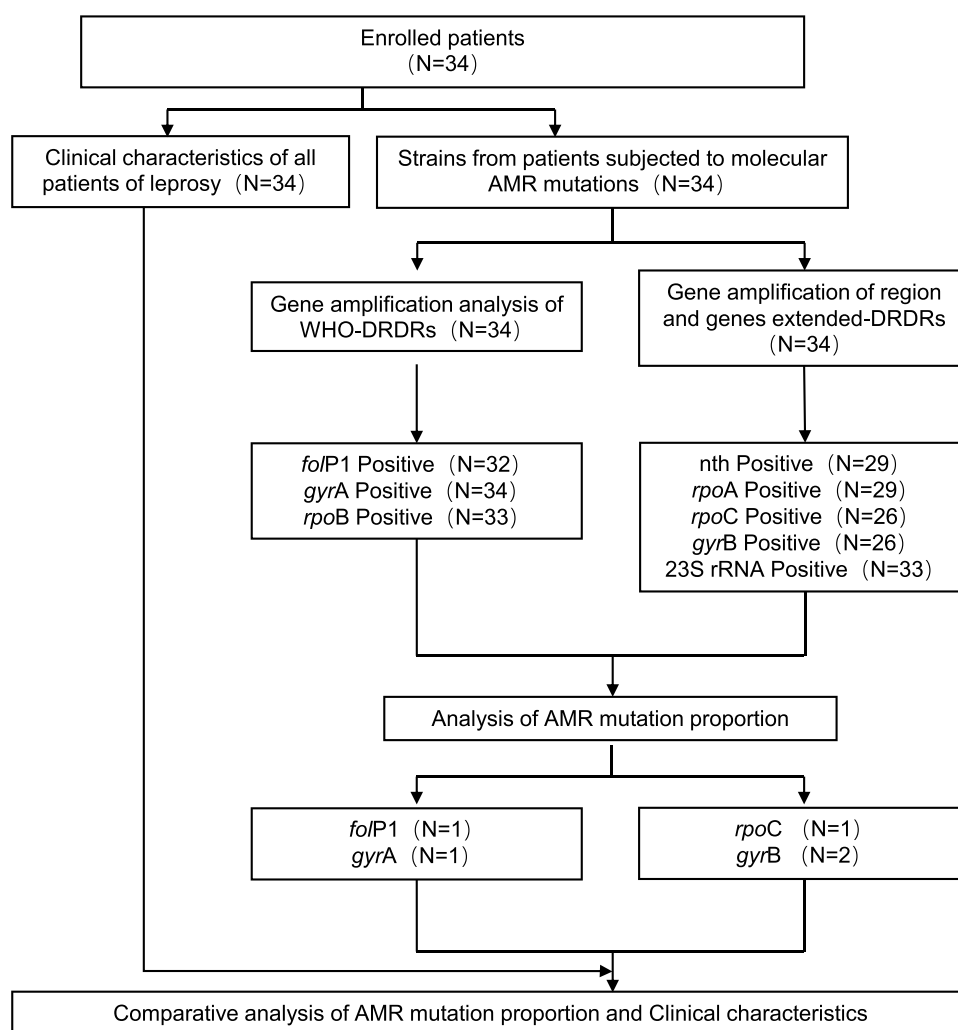


Figure 1 Schematic procedure and structure of the study.

Table 1 Clinical Characteristics of Patients Involved in the Study

Year of Diagnosis		2018	2019	2020	2021	Total
Number of cases	Male	6	6	6	5	23(67.6%)
	Female	2	2	3	4	11(32.4%)
Ethnicity	Han	7	5	9	9	30(88.2%)
	Ethnic minority	1	3	0	0	4(11.8%)
Number of cases	Local residents cases	2	1	3	2	8(23.5%)
	Floating population cases	6	7	6	7	26(76.5%)
WHO classification	TT	1	0	1	0	2(5.9%)
	BT	1	1	2	0	4(11.8%)
	BB	0	0	1	1	2(5.9%)
	BL	3	3	0	6	13(35.3%)
	LL	3	3	4	2	11(35.3%)
	I	0	1	1	0	2(5.9%)
Delay of diagnosis	≤24 months	7	7	7	8	29(85.3%)
	>24 months	1	1	2	1	5(14.7%)
Nerve involvement	Yes	5	3	6	3	17(50%)
	No	3	5	3	6	17(50%)
Deformity	Yes	4	1	6	3	14(41.2%)
	No	4	7	3	6	20(58.8%)
Completion of MDT	Yes	8	8	9	0	25(73.5%)
	No	0	0	0	9	9(26.5%)
Reaction	Yes	1	1	1	2	5(14.7%)
	No	7	7	8	7	29(85.3%)

Abbreviations: TT, tuberculoid leprosy; BT, borderline tuberculoid leprosy; BB, borderline borderline leprosy; BL, borderline lepromatous leprosy; LL, lepromatous leprosy; I, indeterminate leprosy.

Molecular AMR Analysis

In this study, all the DRDR-associated genes were based on the annotations of *M. leprae* TN, relative accession numbers of *M. leprae* TN and codon numbers are listed in [Supplementary Table 4](#). The results of amplification and DNA sequencing of WHO-recommended DRDRs containing *folP1*, *rpoB* and *gyrA* are presented in [Table 2](#). All the 34 leprosy samples (100%) yielded sequence results for at least one gene fragment; informative sequences were obtained in 32 cases (94.1%) for *folP1*, 33 (97.1%) for *rpoB* and 34 (100%) for *gyrA*. Of the 34 samples, two (5.9%) strains showed drug resistance, which had a mutation in *folP1* gene (linked with dapsone resistance) with CCC-to-CTC transversion (Proline to Leucine) at amino acid position 55 and a mutation in *gyrA* gene (linked with ofloxacin resistance) with GCA-to-GTA transversion (Alanine to Valine) at amino acid position 91. No correlation of drug resistance proportion was identified in male vs female, nerve vs no nerve involvement, deformity vs no deformity and reaction vs non-reaction cases. Single-drug resistance to dapsone was detected in LL cases ([Tables 1 and 2](#)).

Table 2 Results of PCR and DNA Sequencing of WHO-Recommended AMR Genes

Type of Cases	Samples, n	folP1 (Dapsone)			rpoB (Rifampicin)		gyrA (Ofloxacin)		Mutation	16srRNA	RLEP
		PCR-Positive Cases, n (%)	Samples with Mutation, n	Mutation	PCR-Positive cases, n (%)	Samples with Mutation, n	PCR-Positive Cases, n (%)	Samples with Mutation, n		PCR-Positive Cases, n (%)	PCR-Positive Cases, n (%)
TT	2	1(50)	0		1(50)	0	2(100)	0		0	0
BT	4	3(75)	0		4(100)	0	4(100)	0		2(50)	3(75)
BB	2	2(100)	0		2(100)	0	2(100)	0		2(100)	2(100)
BL	13	13(100)	0		13(100)	0	13(100)	0		12(92.3)	12(92.3)
LL	11	11(100)	1	CCC-CTC at codon 55 (Pro-Leu)	11(100)	0	11(100)	1	GCA-GTA at codon 91 (Ala-Val)	11(100)	11(100)
I	2	2(100)	0		2(100)	0	2(100)	0		1(50)	1(50)

Table 3 Results of PCR and DNA Sequencing of Compensatory AMR-Associated DRDRs

Type of Cases	Samples, n	Nth	rpoA	rpoC			gyrB			23srRNA
		PCR-positive Cases, n (%)	PCR-Positive Cases, n (%)	PCR-Positive Cases, n (%)	Samples with Mutation, n	Mutation	PCR-Positive Cases, n (%)	Samples with Mutation, n	Mutation	PCR-Positive Cases, n (%)
TT	2	1(50)	2	0			2	1	CCA-CTA at codon 368 (Pro-Leu)	2
BT	4	4	1(25%)	1(25)			0			4
BB	2	2	2	2			2	1	AAG-AAC at codon 215 (Lys-Asn)	2
BL	13	11(84.6)	13	12(92.3)			12(92.3)			13
LL	11	10(90.9)	10(90.9)	10(90.9)	1	GTG-ATG at codon 879 (Val-Met)	9(81.8)			10(90.9)
I	2	1(50)	1(50)	1(50)			1(50)			2

Among 34 (100%) cases, at least one gene fragment of sequence results was obtained, and informative sequences were found in 29 cases (85.3%) for *nth*, 29 (85.3%) for *rpoA*, 25 (73.5%) for *rpoC*, 24 (70.6%) for *gyrB* and 33 (97.1%) for 23S rRNA. No mutations of associated DRDRs were identified in *nth*, *rpoA* and 23S rRNA. The two single mutations in *gyrB* were detected in different strains (5.9%), whereas one *rpoC* mutation was detected in each strain (2.9%, Table 3).

Discussion

In 1982, WHO recommended that all registered patients with leprosy should receive combination therapy with three antibiotics, including rifampicin, clofazimine and dapsone, which remedied many of the problems associated with monotherapy.¹⁷ Moreover, further studies have demonstrated that ofloxacin-containing MDT is both safe and effective for treatment of leprosy.^{18,19} WHO declared that MDT treatment can effectively control leprosy incidence and contribute to the elimination of leprosy burden in several countries, including China. The emergence of drug resistance in leprosy is

a major concern for the implementation of disease intervention programmes. As limited studies described the prevalence of gene mutations associated with AMR in Zhejiang Province, in this study, we analysed the prevalence of drug-resistant leprosy cases by sequencing WHO-recommended DRDRs containing sites in three genes and potential compensatory AMR-associated DRDR genes, using samples of 34 patients with leprosy from 2018 to 2021.

All the cases were newly diagnosed leprosy patients, and they finished MDT treatment based on the WHO guidelines. A mutation at codon 55 (Pro-Leu) of *folP1* (Figure S1A), known to cause resistance to dapsone among 34 samples, was identified.^{9,20} The frequency of drug resistance to dapsone (2.9%) was lower than that in Hunan (10.7%) but higher than that in Sichuan (0%), Guizhou (1.7%) and Yunnan (1.0%).²¹ The *gyrA* sequence also showed a single mutation at codon 91 with amino acid changes from Alanine to Valine (Figure S1B), which demonstrated ofloxacin resistance.²² Whatever, the frequency (2.9%) was lower than that in Yunnan (3.8%) for ofloxacin. In the present study, no mutation associated with rifampicin (*rpoB* gene) was confirmed. Both mutations found in the *folP1* and *gyrA* gene are single mutations. No significant correlation in AMR mutations in *M. leprae* was found in male vs female, nerve vs no nerve involvement, deformity vs no deformity, and reaction vs non-reaction. Although dapsone monotherapy has been used in the pre-MDT era across China,²³ the irregular use of this drug resulted in high dapsone resistance. Based on previous reports, these resistant strains were continuously transmitted in the community.²⁴

Compensatory mutations in *rpoA* and *rpoC*, encoding the alpha and beta-prime subunits of RNA polymerase, can occur in rifampicin-resistant *M. tuberculosis*,¹³ whereas only *nth* and *rpoC* mutations were found in *M. leprae*.¹⁴ We identified only one mutation in *rpoC* gene at codon 879 (Val-Met) (Figure S1C), which seems to be a polymorph other than compensatory mutation since no rifampicin-resistant strains was identified here. A hypothesis was proposed that compensatory mutations may be associated with mutations in rifampicin resistance-determining regions (RRDRs)^{25,26} in rifampicin-resistant *M. tuberculosis*. Rifampicin-susceptible isolates or rifampicin-resistant strains without *rpoB* mutations can hardly occur compensatory mutations.¹³ However, further study is required to determine the relationship between phenotype and genotype. As mentioned by previously reported literature, two single mutations of *gyrB* gene were also identified at codon 368 (Pro-Leu) and 215 (Lys-Asn) (Figure S1D and E). The substitutions in *gyrB* are based on the gene numbering system of *M. tuberculosis gyrB*.²² The Quinolone-Resistance Determining Region (QRDR) of *gyrB* stretches from amino acid 426 to 464, from 461 to 499 and from 464 to 504 in *E. coli*, *M. tuberculosis* and *M. leprae*, respectively, while an extension of the *M. tuberculosis* QRDR to amino acid 501 has been proposed previously.²⁷ In our findings of *gyrB*, position 215 and 368 are not included in the QRDR, which might indicate polymorphs only, and drug resistance to ofloxacin cannot develop. Endonuclease III (Nth) is central to the base excision repair pathway in bacteria.²⁸ Mycobacterial genomes usually contain a single *nth* gene and two *fpg/nei* genes, but *M. leprae* has lost *fpg/nei* orthologues and retained the *nth* gene. It has been reported that all *nth* mutants were also drug-resistant so Nth loss likely favors emergence of drug resistance, and *nth* mutations might serve as a surrogate marker for potential drug resistance and treatment failure.^{29,30} However, no *nth* mutation was identified among 34 leprosy cases. 23S rRNA mutation conferring clarithromycin resistance was also not found in Zhejiang leprosy patients. Furthermore, the role of these mutations still requires further study to determine their phenotypic outcomes in *M. leprae*.

In general, mutations of *folP1* gene and *gyrA* gene were showed single-drug resistance to dapsone and ofloxacin, respectively, among all the strains with mutations. Although the substitution of amino acid in *rpoC* was a non-synonymous mutation, while no compensatory was occurred rifampicin resistance was not involved. Two mutations in *gyrB* were also confirmed to be independent of drug resistance since outside the DRDRs. No cases of multidrug resistance were found. Since the establishment of national policies and socioeconomic factors, the transmission of AMR and non-AMR cases of leprosy has been reduced in China. In addition, rifampicin drug resistance is low in Chinese population. Irregular MDT may be the main factor causing drug resistance.³¹ Notably, standardised treatment can achieve good therapeutic effects based on our study. Fluoroquinolones are promising and widely used antibiotics, which are introduced to routine clinical practice to treat infectious diseases in China. Given the easy availability and inappropriate use of these drugs, AMR to fluoroquinolones has emerged in China.³² Clarithromycin is another antibiotic used to treat various bacterial infections^{33,34} and it is used as an alternative or combinational drug that can result in clarithromycin resistance of leprosy.³⁵

The movement of people within nations and around the globe has always been a factor in shaping the epidemiological portrait of leprosy. Migration has unique challenges to leprosy control in the 21st century. Among the 34 cases enrolled in this study, 26 (76.5%) were immigrants from other provinces. Given the movement of people affected by leprosy to provinces that have relatively few cases of the disease, services for leprosy treatment have become scarce.³⁶ General practitioners and dermatologists may be unlikely to diagnose the disease in its early stages. When they associate person's symptoms with leprosy, they may have little knowledge of where to begin with treatment, thereby leading to misdiagnosis and delayed diagnosis.

Conclusion

This study is the first systematic analysis of AMR surveillance of leprosy in Zhejiang Province in recent years. Two single mutations related to dapson and ofloxacin resistance separately were identified and no multidrug resistance was found among 34 leprosy patients. The results presented in our study demonstrate that drug-resistant strains are not epidemic strains in this area. Given the few cases of leprosy, analysing the AMR of *M. leprae* in Zhejiang Province more comprehensively is difficult. However, regular MDT treatment and population management in the early stage may contribute to the low prevalence of leprosy.

Ethics Approval and Informed Consent

This study was approved by the institutional ethical committee of the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, China (2014-KY-003) and this study was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

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Disclosure

All authors declare that they have no conflicts of interest.

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