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MYCOBACTERIUM TUBERCULOSIS AND *gyrA* VARIATION IN ZAMBIA

SATOSHI MITARAI[1], LYNDON M KAFWABULULA[1], CHARITY HABEENZU[1],
HIROSHI TERUNUMA[2], DAVID LUBASI[1], FRANCIS C KASOLO[3],
HARUMI SHISHIDO[4], and YOSHIO NUMAZAKI[3]
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Abstract: *M. tuberculosis* strains were isolated from clinically and bacteriologically confirmed patients to evaluate the susceptibility of clinical *M. tuberculosis* isolates to fluoroquinolone and to obtain molecular epidemiological information in Zambia,. The pathogens were subjected to susceptibility testing with isoniazid, rifampicin, ethambutol and streptomycin. The minimum inhibitory concentrations to ciprofloxacin, sparfloxacin and levofloxacin were also evaluated. The *gyrA,* fluoroquinolone resistance-determining region (QRDR), was sequenced and analysed. As a result, three of the 16 strains examined were resistant to isoniazid, rifampicin and/or streptomycin. All of the strains were susceptible to ciprofloxacin, levofloxacin and sparfloxacin. However, a unique *gyrA* gene variation of *M. tuberculosis* was identified in the isolates. One strain had a mutation (T73A) in QRDR. Additionally, 81.25% (13/16) of the strains tested had Thr at codon 88. Several variations of *gyrA* gene have been reported in relation to drug resistance. The *gyrA* variation data will be useful as epidemiological information. It may be important to monitor fluoroquinolone susceptibility even in developing countries for use against resistant *M. tuberculosis* infection, even though no fluoroquinolone resistance was observed in this study.

INTRODUCTION

Multi-drug resistant *Mycobacterium tuberculosis* (MDRTB) is one of the major obstacles to effective control of tuberculosis [1,2] in both developed and developing countries. The emergence of MDRTB is believed to be due, in part, to incomplete anti-tuberculosis treatment. In Sub-Saharan African countries, the incidence of tuberculosis has rapidly increased mainly due to the human immunodeficiency virus epidemic [3]. Thus, the incidence of resistant *M. tuberculosis* infections can be expected to increase in these countries.

It is thought that some drugs, i.e., rifamycin derivatives and fluoroquinolones (FQs), are potentially effective treatments for MDRTB. Even in Zambia, it is relatively easy to obtain ciprofloxacin (CIP) and other FQs in general/private clinics. Therefore, as part of a drug resistance investigation in Zambia, we evaluated the drug susceptibility of *M. tuberculosis* to FQs.

MATERIALS AND METHODS

Clinical isolates and drug susceptibility tests

A total of 16 *M. tuberculosis* strains were isolated from 16 patients with pulmonary tuberculosis at the University Teaching Hospital Chest Clinic in Lusaka, Zambia. All strains were recovered on Lowenstein-Jensen (L-J) medium from sputum specimens. The drug susceptibility of each strain to isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and streptomycin (STR) was determined by the resistant ratio method using L-J media. The critical concentrations for each drug were as follows: INH 0.05, 0.1 and 0.2 μg/ml; RIF 12.5, 25 and 50 μg/ml; EMB 0.8, 1.6 and 3.2 μg /ml; STR 10, 20 and 40 μg/ml.

Minimum inhibitory concentrations of fluoroquinolones

FQs were kind gifts from Bayer (ciprofloxacin: CIP),

1. TB Laboratory in University Teaching Hospital, P/Bag RW1, Lusaka 10101, Zambia
2. Department of Microbiology, Yamanashi Medical College, 1110 Shimogato, Tamaho, Nakakoma, Yamanashi 409-3821, Japan
3. Virology Laboratory in University Teaching Hospital, P/Bag RW1, Lusaka 10101, Zambia
4. Marumori Hospital, Nagamachi, Taihaku, Sendai 982-0012, Japan
Corresponding Author: Satoshi MITARAI, M.D., Ph.D.
Present institution:
Bacteriology division, the Mycobacterium Reference Centre, the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association
3-1-24 Matsuyama, Kiyose, Tokyo 203-8533, Japan
Tel: +81-424-93-5711 Fax: +81-424-92-8258 E-mail: mitarai@jata.or.jp

Dainippon Pharmaceutical (sparfloxacin: SPX) and Daiichi Pharmaceutical (levofloxacin: LVX). These drugs were tested for minimum inhibitory concentration of each strain in Middlebrook 7H9 broth with albumin-dextrose-catalase (ADC). The isolates were sub-cultured in Middlebrook 7H9 with ADC broth and prepared in the same medium to the turbidity of McFarl and 1. The suspension was further diluted 10^2 to 10^3 times resulting in a concentration of approximately 10^5 CFU/ml. One ml of each suspension was inoculated into 1 ml of Middlebrook 7H9 broth with either no antibiotic or with serial two-fold dilutions of each antibiotic, with the final concentrations ranging from 0.1 to 25 μg /ml. The tubes were incubated at 37℃ for 2 to 4 weeks until sufficient bacterial growth was evident in the control tubes.

PCR and sequencing of *gyrA* region

The tubercle bacilli were harvested from the colonies on L-J medium. DNA extraction was performed using 100 μg/ml of proteinase K and 2% SDS, followed by ordinary solvent assay with phenol, phenol/chloroform and chloroform. After re-suspension of each DNA sample, the reaction mixture was added to 1 μg of DNA template to a final concentration of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM of each of the dNTPs, 25 pmole of each of primers and 2.5 units of *Taq* DNA polymerase in a total volume of 50 μl and subjected to 30 cycles of PCR. The primers for *gyrA* region were as follows: gyrAF1; 5' G GTGCTCTATGCAATGTTCG 3' and gyrAR1; 5' GGATAT TGGTTGCCATGCC 3'. DNA specimens were incubated at 94℃ for one minute to melt the DNA, cooled to 62℃ for one minute to allow for annealing, and incubated for one minute at 72℃ for DNA amplification. The PCR product was recovered from gels and subjected to direct sequencing. The amino acid sequences of GyrA deduced from nucleotide sequence were numbered in the system used for E.coli. The complete nucleotide sequence has been deposited in GenBank under accession number AF400983.

RESULTS

Susceptibility to anti-tuberculosis drugs

One strain (no. 99-1049) was resistant to INH, RIF, and STR by the resistant ratio method. Two strains (no. 99-979 and 99-1201) were solely resistant to INH. No tested strain showed resistance to CIP, SPX or LVX (Table 1). SPX showed the lowest MIC, followed by LVX and CIP.

Sequence analysis of *gyrA* region

A 387 bps of PCR product from *gyrA* involving the quinolone resistance-determining region (QRDR) was amplified and sequenced. The sequences were analysed as shown in Figure 1. Strain 99-1183, which showed significant susceptibility to FQs, was found to have a mutation resulting in a Thr to Ala substitution at codon 73. Furthermore, a Ser > Thr substitution at codon 88 was found in 13 out of the 16 strains examined, all of which were susceptible to FQs.

Table 1. Drug susceptibilities of clinical *M. tuberculosis* isolates

Strain no.	Resistant ratio method				MIC (μg/ml)		
	INH	RIF	EMB	STR	CIP	SPX	LVX
H37Rv	1 (S)	1 (S)	1 (S)	1 (S)	0.39	0.1	0.2
99-794	2 (S)	1 (S)	1 (S)	2 (S)	0.2	<0.1	<0.1
99-800	1 (S)	1 (S)	1 (S)	1 (S)	<0.1	<0.1	0.2
99-804	1 (S)	1 (S)	1 (S)	1 (S)	ND	<0.1	<0.1
99-978	2 (S)	1 (S)	1 (S)	1 (S)	0.2	0.1	0.2
99-979	8 (R)	1 (S)	1 (S)	1 (S)	0.2	<0.1	<0.1
99-980	2 (S)	2 (S)	1 (S)	1 (S)	0.39	<0.1	0.2
99-983	1 (S)	1 (S)	1 (S)	1 (S)	ND	ND	ND
99-995	2 (S)	2 (S)	1 (S)	2 (S)	0.2	ND	0.2
99-999	2 (S)	1 (S)	1 (S)	1 (S)	0.2	<0.1	0.2
99-1049	4 (R)	4 (R)	1 (S)	8 (R)	0.39	0.2	0.2
99-1053	2 (S)	2 (S)	1 (S)	1 (S)	ND	<0.1	<0.1
99-1058	2 (S)	2 (S)	1 (S)	2 (S)	ND	ND	ND
99-1128	2 (S)	1 (S)	1 (S)	1 (S)	0.2	<0.1	0.2
99-1136	2 (S)	1 (S)	1 (S)	1 (S)	ND	<0.1	0.2
99-1183	1 (S)	1 (S)	1 (S)	1 (S)	0.2	<0.1	0.2
99-1201	4 (R)	1 (S)	1 (S)	1 (S)	0.39	<0.1	0.2

S: Sensitive R: Resistant ND: No data

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H37Rv   5'- GCCCGGTCGG TTGCCGAGAC CATGGGCAAC TACCACCCGC ACGGCGACGC GTCGATCTAC
99-983      ---------- --------A- ---------- ---------- ---------- ----------
99-995      ---------- --------A- ---------- ---------- ---------- ----------
99-1049     ---------- --------A- ---------- ---------- ---------- ----------
99-1053     ---------- --------A- ---------- ---------- ---------- ----------
99-1058     ---------- --------A- ---------- ---------- ---------- ----------
99-1183     ---------- --------G- ---------- ---------- ---------- ----------
99-1201     ---------- --------A- ---------- ---------- ---------- ----------
Amino acid  AlaArgSerV alAlaGluAl aMetGlyAsn TyrHisProH isGlyAspAl aSerIleTyr
                           *Th r

H37Rv       GACAGCCTGG TGCGCATGGC CCAGCCCTGG TCGCTGCGCT ACCCGCTGGT GGACGGCCAG -3'
99-983      ----G----- ---------- ---------- ---------- ---------- ----------
99-995      ----G----- ---------- ---------- ---------- ---------- ----------
99-1049     ----G----- ---------- ---------- ---------- ---------- ----------
99-1053     ----C----- ---------- ---------- ---------- ---------- ----------
99-1058     ----C----- ---------- ---------- ---------- ---------- ----------
99-1183     ----C----- ---------- ---------- ---------- ---------- ----------
99-1201     ----C----- ---------- ---------- ---------- ---------- ----------
Amino acid  AspThrLeuV alArgMetAl aGlnProTrp SerLeuArgT yrProLeuVa lAspGlyGln
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Figure 1. Nucleotide and amino acid sequences of QRDR in *gyrA* gene by direct sequencing. Sequences of other strains that are not shown here were completely consistent with 99-1201 in the sequenced region.

DISCUSSION

Many mutations in residues 81 to 87 (88 to 94 in other systems) have been identified in quinolone resistant *M. tuberculosis* isolates [4-11], suggesting that this region of the genome is associated with susceptibility to FQs. The mutation at codon 73 in strain 99-1183, which may be involved in the susceptibility to FQs, has not been registered in GenBank as far as the authors could determine.

The patients had no a history of frequent and/or continuous use of any FQs for the treatment of either tuberculosis or more common bacterial infections. The strain was susceptible to all four first line anti-tuberculosis drugs. It is known that certain natural variations exist in *M. tuberculosis* with some probability [12]. Strain 99-1183 seemed to be one such strain, but further investigation is required to confirm the role of this mutation. The new mutation identified in this report will provide additional information for analysis of the effects of quinolone on *M. tuberculosis*, and it may also serve as an epidemiological marker. The amino acid at codon 88 was mainly Thr in these strains. It is thought that *M. tuberculosis* is a relatively new species and evolutionarily has a few silent variations. Codon 88 is one of the genetic markers and Thr is assumed to be common in ancestral strains. Here, the higher ratio of S88T strains will be informative for future epidemiological research [13,14].

There is intense interest in the FQs as anti-tuberculosis drugs because of the emergence of MDRTB. The use of FQs for the treatment of MDRTB infection has been shown to be effective in some reports [15,16]. The FQs must be used carefully because resistance may develop soon after clinical application as an anti-tuberculosis drug. The *gyrA* variation found in this study in pathogenic strains may provide useful epidemiological information for future studies implemented to investigate the use of fluoroquinolone to treat resistant *M. tuberculosis* infection.

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