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GyrA Gene Mutations of *M. tuberculosis* and Previous Use of Ciprofloxacin and Ofloxacin in Quinolone Resistance

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ABSTRACT

Objectives: This study assesed quinolone resistance in MDR and non-MDR cases. It also analyzed the duration of ciprofloxacin (Cfx) and ofloxacin (Ofx) use in resistance and sensitive isolates. The position and type of *gyrA* gene mutations in quinolone resistant isolates were also evaluated. **Methods:** This was a combined analytic observation with cross-sectional timed and biomolecular qualitative study using a total of 35 *M. tuberculosis* clinical isolates between 2007 and 2009. The susceptibilities of isolates to Cfx and Ofx were determined by using MIC methode on LJ medium. Amplification of *gyrA* gene was done using PCR with touch-down program to all resistant-isolates. PCR product was then futher used as a template for DNA sequencing. **Results:** Quinolone resistance among the MDR isolates was significantly higher than non-MDR (p=0,008). Compared to sensitive isolates, the duration of previously used Cfx was 10,46 weeks longer in resistant isolates (p=0,021). There was no significant difference in the duration of Ofx between resistant and sensitive isolates with 22,75 weeks and 17,02 weeks of mean rank respectively (p=0,218). Quinolone resistance exihibited point mutations at Asp89Val (16.7%), Asp94Gly (50%), Asp94Ala (16.7%), Asp94Asn (16.7%), Ser95Thr (83.3%), Ser95Asn (16.7%). **Conclusion:** Quinolone resistance among MDR isolates is high in rate. All of quinolone-resistant isolates shows missense mutations on *gyrA* gene with high-level resistance. Cfx has been used more frequently and longer than Ofx in MDR and non-MDR cases.

Keywords: Quinolones, *M. tuberculosis*, *gyrA*

INTRODUCTION

Indonesia is the fourth country with the highest number of patients with tuberculosis (TB) patients.[1] Anti-tuberculosis drug resistance has emerged in tuberculosis control. Multidrug-resistant TB (MDR-TB) which has not yet been overcomed is now followed by extensively drug-resistant TB (XDR-TB). WHO formulated XDR-TB as a resistance to rifampicin (R) and isoniazid (H) as well as resistance to any one of fluoroquinolones (FQ) and to at least one of three injectable second-line drugs: capreomycin, kanamycin and amikacin.[2] The resistance prevalence of ciprofloxacin (Cfx) and ofloxacin (Ofx), the two largest FQ used in TB, in Indonesia has not yet been clearly known because it does not have a routine examination.[3] WHO recommended second-line and injection drug susceptibility test of MDR isolates to determine the proportion of XDR-TB among MDR-TB.[4]

FQ is one of second-line drug used to treat MDR, besides, it is considered as a broad spectrum antibiotic which is commonly used in infectious diseases. The widespread use of this drug in the tuberculosis treatment without properly diagnostic criteria increases the risk of resistance because of inadequate therapy.[5]

Generally, the causes of antibiotic resistance are due to microbial aspects of gene mutation and clinical aspects of drug use. It is known that *gyrA* gene mutation of *M. tuberculosis* in quinolone-resistance-determining region (QRDR) is responsible for FQ resistance. Missense mutations possibly change the structure and function of *GyrA* protein in DNA gyrase in which FQ binds. Thes lead FQ to fail in DNA gyrase negative supercoil activity. Consequently, *M. tuberculosis* DNA is not damaged and remains alive.[6, 7, 8] From the clinical aspect, the irrational use of Cfx and Ofx in both indications and duration, exposes microbes to sub-lethal drug concentration leading to resistant strains which are potentially dominant in population. The epidemiology studies found that MDR cases have a higher risk in anti-tiberculosis drug resistance affecting FQ therapy outcomes.[4, 5, 9] The detection of *gyrA* gene mutation rapidly predict the sensitvity of FQ especially in MDR case.

This study aimed to analyze *gyrA* gene mutations in Cfx and Ofx resistance. It included the number of mutation,

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the position of codon and the type of mutation. The previous use of FQ and FQ resistance in MDR and non-MDR cases were also evaluated.

METHODS

M. tuberculosis isolates. The total of 35 clinical isolates from culture collection of the year 2007-2009 were derived from patients having medical record of Cfx and Ofx use in tuberculosis treatment. All were recultured on Lowenstein-Jensen (LJ) medium for *M. tuberculosis* identification and drug susceptibility test.

Catalase and Nitrate Test. These tests chemically indentified *M. tuberculosis* which was negative in 68 ºC catalase test and positive in nitrate reduction test. On the catalase test, one loopfull of bacteria was transferred to a tube containing PBS pH 7.0. Each tube was then incubated at 68 ºC for 20 minutes. After the mixture of H_2O_2 and fresh Tween-80 10% were added, the forming of bubble was identified. It was considered negative when the buble did not form within 20 minutes. In the nitrate test, one loopfull colonie was added to a solution of 0.85% NaCl and nitrate reagent and then incubated in a water bath at 37 ºC for 2 hours. The existence of pink color with minimum standard of +3 was identified as positive after the addition of HCl 50%, sulfanilamide 0.2% and N-Naphtylen 0,1%.

Drug Susceptibility Test (DST). The DST to rifampicin (R), isoniazid (H), kanamycin (Km) referred to WHO standards. FQ susceptibilities were determined by proportion method on LJ medium. After an inoculums source was made through standard suspension and dilution, it was then inoculated on LJ medium containing Cfx (Bayer) and Ofx (Bayer) with each concentration of 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 4 µg/mL and LJ medium without drug as a control. The culture media were incubated at 37 ºC and the colony growth reading were recorded for two days, on day 28th and 42nd. The MIC was determined by colony forming unit (cfu) counting. The strain of H37Rv was tested as a control isolate which was sensitive to all tested drugs.

DNA Isolation of *M. tuberculosis*. One colony cultured on LJ medium was transferred into an aliquot containing 100 µL lysis solution (Sigma Molecular Biology). It was then heated on boiling water for 15 minutes. After that, the aliquot was centrifuged at 10.000 rpm for 10 minutes. The supernatant was transferred to a new aliquot. DNA concentration was then measured with a spectrophotometer at 260m and 280nm.

GyrA Gene Amplification. Amplifying 320 bps region of *gyrA*, the Polymerase Chain Reaction (PCR) method with touch-down program used primers gyrA-f5'-CAGCTACATCGACTATGCGA-3 'and gyrA-r5'-GGCTTCGGTGTACCTCAT -3' from the AlphaDNA. A total of 25 µL PCR mixture contained 10x PCR Buffer, 6 µM MgCl 2, primers (each) 4µM, 50 µM dNTPs, H2O PCR, 1 IU Taq polymerase, and 50 µM DNA. The mixture was then put in the thermal Cycler (Hybaid Om-E) with following PCR program of denaturation, annealing and extension:
95 C for 3 minutes
2 cycles: 95 C for 1 minute, 58 C for 1 minute, 72 C for 1 minute
2 cycles: 95 C for 1 minute, 57 C for 1 minute, 72 C for 1 minute
2 cycles: 95 C for 1 minute, 56 C for 1 minute, 72 C for 1 minute
2 cycles: 95 C for 1 minute, 55 C for 1 minute, 72 C for 1 minute
Phase extension: 72 C for 5 minutes
6µL of PCR amplification product was mixed with 1 mL loading dye (Promega) then they were poured into ethibium bromide colored 3% agarose gel (Invitrogen). The DNA Ladder used a bench top PCR (Promega). Electrophoresis was performed for 30 minutes in 1x TAE buffer at 100V at room temperature. Gel was visualized in a Dark Reader Transiluminator (Vilbert Loumart).

DNA Sequencing. PCR product was sent to Macrogen Inc.. Korea for purification and single sequencing of *gyrA* gene using the same forward and reverse primer in the PCR. The result was then analyzed with the Genious program. Alignment was then performed with the BLAST (Basic Local Alignment Search Tools) program from the NCBI (National Center for Biotechnology Information). The difference of nuncleotide sequence was analyzed to identify the position and the type of *gyrA* gene mutation.

Statistical Analysis. This study used univariate and bivariate analysis. The difference of quinolone resistance between MDR and Non-MDR isolates was analyzed by the Fisher Exact Test. The Mann-Whitney Test was used to identify significant differences of duration of quinolones use between resistant and sensitive isolates.[10] Data analysis was performed by SPSS for windows version 13.0 on 95% of CI with significant difference of p value ≤ 0.05.

RESULTS

Clinical Characteristics of M. tuberculosis Isolates.

Clinical characteristics of *M. tuberculosis* isolates which include the type of TB patients and the previous use of Cfx and Ofx in the non-MDR, MDR and fluoroquinolone-resistance group can be seen in Table 1.

Cfx and Ofx were mostly used on chronic cases (41.2%) in MDR group. Meanwhile, in non-MDR group they were mostly used on drop out cases (33.3%). Surprisingly, these drug were also widely used on new cases in both of the group. Cfx only was the most frequent drug used in all cases (57.2%), followed by Cfx and Ofx combination (37.1%) and Ofx only was the least common drug used (5.7%). Most of MDR isolates (58.8%) had Cfx and Ofx combination prior use while the majority of FQ resistant isolate used Cfx only in tuberculosis treatment.

Table 1. Clinical Characteristics of *M. tuberculosis* Isolates

Group	Overall (n=35)	Non MDR (n=18)	MDR (n=17)	Resistant to FQ (MIC > 2µg/ml) (n=6)
Patients Type*				
New	5 (14.3%)	3 (16.7%)	2 (11.8%)	2 (33.3%)
Relapse	9 (25.%)	4 (22.2%)	5 (29.4%)	0
Failed	1 (2.9%)	0	1 (5.9%)	1 (16.7%)
Drop Out	8 (22.9%)	6 (33.3%)	2 (11.8%)	0
Chronic and other	12 (34.3%)	5 (27.8%)	7 (41.2%)	3 (50%)
Use of Fluoroquinolones				
CiprOfx Only	20 (57.2%)	14(77.8%)	6 (35.3%)	2 (33.3%)
Ofx Only	2 (5.%)	1 (5.6%)	1 (5,.%)	1 (16.7%)
CiprOfx and Ofx	13 (37.1%)	3 (16.6%)	10 (58,.%)	2 (33.3%)

MDR, multi-drug resistance; FQ, fluoroquinolones; MIC, minimum inhibitory concentration

Tabel 2. MIC of Ciprofloxacin and Ofloxacin

Drug	No. (%) of Sensitive Isolates with MIC (µg/ml)				Cut-off Resistant
	0,5	1	2	4	
Ciprofloxacin	4 (11.4%)	10 (28.6%)	20 (57.1%)	29 (82.9%)	>2
Ofloxacin	5 (14.3%)	14 (40%)	28 (80%)	29 (82.9%)	>2

MIC, minimum inhibitory concentration

DST of Ciprofloxacin and Ofloxacin

The inhibition of *M. tuberculosis* by Cfx and Ofx at different concentration is shown in Table 2. Using >2 cut-off resistant, a total of 29 (82.9%) isolates were sensitive to the concentration of 4 µg/ml. Ofx had higher antimicrobial activity than Cfx at concentration ≤ 2 4 µg/ml. The standard strain of H37Rv was sensitive to all concentrations.

The Duration of Cfx and Ofx Previous Use in FQ Resistance

Of 35 isolates, there were 6 (17.1%) of FQ resistance, and MDR group had 0.647 higher risk of FQ resistant than Non-MDR group (p=0,008) as shown on Table 3. Table 4 shows that the duration (mean rank of week) of Cfx exposure was

associated with Cfx resistance (p <0.05), but there was no correlation between Ofx duration and Ofx resistance (p> 0.05). Compared to Ofx, Cfx was used longer with maximum duration of 44 weeks and 144 weeks respectively and the most frequent duration for Cfx was 4 weeks.

Table 3. Quinolone Resistance on MDR and non-MDR Isolates

	Resistant		Sensitive		Total		p value
	n	%	n	%	n	%	
MDR	6	35.3%	11	64.7%	17	100%	0,008*
Non-MDR	0	0	18	100%	18	100%	
Overall	6	17,1%	29	82,9%	35	100%	

*Significance difference (p<0.05) in Fisher's Exact test

MDR, multi-drug resistance

Table 4. Exposure Duration on Ciprofloxacin and Ofloxacin Resistance

Variable	Mean Rank	SR	p values	N
Ciprofloxacin				
Resistant	26.67	160	0.021*	6
Sensitive	16.21	470		29
Ofloxacin				
Resistant	22.75	136.50	0.218	6
Sensitive	17.02	493.5		29

* Significance difference (p<0.05) in Mann-Whitney test

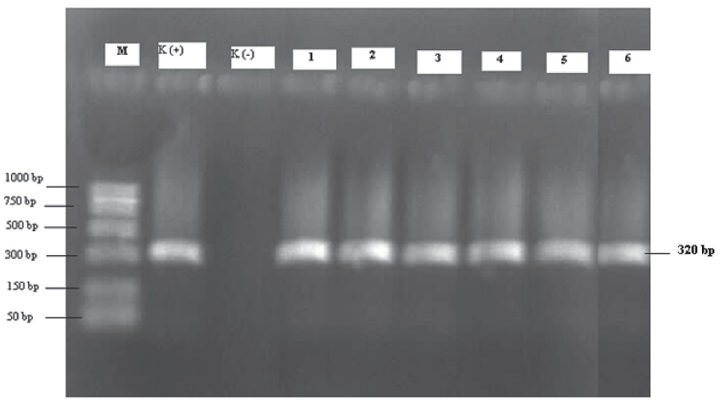

Figure 1. *GyrA* gene PCR product of M. tuberculosis
Lane 1 : Marker
Lane 2 : Positive Control
Lane 3 : Negative Control
Lane 4-8 : PCR Product of 320 bp

Amplification of M. tuberculosis gyrA gene by PCR.

PCR amplification was performed on six resistant isolates and one sensitive isolate of wildtype H37Rv. PCR product showed one single band of *gyrA* gene at 320 bp as shown in Figure 1. This product included QRDR on gyrA. PCR results were then used as templates for DNA sequencing.

Sequencing results.

The position and the type of mutations are shown on Table 5. Along QRDR hotspot regions, all resistant isolates had mutations at codon 94 and codon 95. Substitute point mutations in all position generally changed amino acid and caused missense mutations. Of 6 resistant isolates, the mutation patterns were Asp89Val (16.7%), Asp94Gly (50%), Asp94Ala (16.7%), Asp94Asn (16.7%), Ser95Thr (83.3%), Ser95Asn (16.7%).

Table 5. *GyrA* Gene QRDR Mutation Patterns of M. tuberculosis Isolates Resistant to Ciprofloxacin and Ofloxacin

Codon	Nucleotide Mutation	Type	Amino Acid Change	Mutation Frequency (isolate)/%
76	TCG → TTG	Substitution	Ser → Leu	1 (16,7%)
77	GTT → GTC	Substitution	Val → Val	1 (16,7%)
78	GCC → GCG	Substitution	Ala → Ala	1 (16,7%)
89	GAC → GTG	Substitution	Asp → Val	1 (16,7%)
94	GAC → GGC	Substitution	Asp → Gly	2 (33,3%)
94	GAC → GCC	Substitution	Asp → Ala	1 (16,7%)
94	GAC → AAC	Substitution	Asp → Asn	1 (16,7%)
94	GAC → GGA	Substitution	Asp → Gly	1 (16,7%)
95	AGC → ACC	Substitution	Ser → Thr	5 (83,3%)
95	AGC → AAC	Substitution	Ser → Asn	1 (16,7%)
96	CTG → CCG	Substitution	Leu → Pro	1 (16,7%)
97	GTG → GGG	Substitution	Val → Gly	1 (16,7%)
99	ATG → AAG	Substitution	Met → Lys	1 (16,7%)

DISCUSSION

This study shows a high rate of FQ resistance and the MDR. FQ resistance rate among all isolates were 17.3%, two times lower than MDR isolates (35.3%). Compared to previous studies, these were higher than the rate in Taiwan but lower than the rate in the Philippines.[11, 12] The prevalence of FQ resistance in Taiwan was 3.3% in general and 19% in MDR isolates. Another study in Taiwan found 6.2% of FQ resistance incidence and 22.2% among MDR isolates.[11] In the Philippines, FQ resistance was 35.3% in general and 51.4% in MDR isolates.[10] However, fortunately, we found that there was no XDR-TB because all isolates were sensitive to kanamycin, a second-line antituberculosis injection drug,which WHO showed 2% of resistance.[1] This study suggests that kanamycin is empirically effective for treating MDR.

Having exposed to Cfx and Ofx, both of non-MDR and MDR groups had similar risk to be resistant. Therefore, it is very important to perform FQ sensitivity test in order to determine the appropriate regiment because Cfx and Ofx has been widely used among non-MDR cases. This means that Cfx which is not recommended for neither sensitive nor resistant tuberculosis [4] is still commonly used in Indonesia. This is because of efficient cost and high availability of Cfx.

The duration of Cfx exposure associated with its resistance as well as previous studies which found that Cfx administration in tuberculosis treatment is rapidly followed by resistance.[13] *M. tuberculosis* isolate which is not

exposed to Cfx have a little opportunity to be resistant with 1CFU per 2 x 10^6CFU of resistance incidence on day 0 and 1 CFU per 7.9 x 10^5CFU of resistance incidence on day 13. Exposing *M. tuberculosis* with 1 CFU per 7.9 x 10^5CFU of Cfx increases rapid resistance from 0.00003% of the total population on the beginning to 0.27% on day 3 and 54.5% on day 7.[13]

FQ resistance is more common in the MDR for the differences of DnaE2 levels between resistant and sensitive *M. tuberculosis*.[14] DnaE2 has an important role in DNA repair which promotes mutation. DNA repair mechanism is the only way for bacteria to survive. *M. tuberculosis* within the host's body gets genotoxic stressors which come from the body's immune response as well as antituberculosis drug exposure causing DNA damage resulting in cell damage and death. Translesion synthesis, one of DNA repair mechanism in *M. tuberculosis* , is a process allowing replication of DNA template damage. This process is performed by the "tend to go wrong" DNA polymerase C enzyme encoded by DnaE2 gene. There is an accumulation of mutations in rpoB and katG genes in MDR *M. tuberculosis*.

We found that all resistant isolate had *gyrA* gene mutation. The frequency of *gyrA* and *gyrB* gene mutation among resistant strains varied from 10.3% in India, 50% in Taiwan, 55.2 -58.8% in Hong Kong, 60% in Thailand, 89.5% in Italy and Abkazia to 100% in Japan.[15] The mutation of codon 94 in this study is similar to previous studies that found hotspot areas of codon 88, 89, 90, 91, and 94.[6, 7, 16, 17] The detection of codon 90, 91 and 94 is an effective way predicting FQ resistance in *M. tuberculosis*. Compared to other mutations, mutation of Asp94 (Gly / Ala) showed high levels of resistance which generally has double missense mutations.[7]

The Asp94Gly mutations (50%) and Ser95Thr mutations (83.3%) potentially change the subunit protein structure of DNA gyrase GyrA 3 4 helix-shaped. Aspartic acid (Asp) is acidic polar amino acid [18], whereas glysin (Gly) is a neutral non polar amino acids. The alteration from Asp to Gly possibly changes structure or function of protein. These may cause FQ resistance through either change binding site of FQ on DNA gyrase-DNA complex or decrease supercoiling activity of DNA gyrase.

There was 83.3% of Ser95Thr mutation which is the most frequent polymorphisms found in some studies. This does not have a direct role in the development of resistance. Polymorphisms of codon 95 found in 15% of strains is a kind of genetic evolution which is not associated with resistance increase.[15]

In regions with high incidence of antituberculosis drug resistance, detection of target gene mutation for resistance identification will improve the diagnosis of MDR and XDR tuberculosis. Identification of gene mutation which is responsible for rifamphicyn and FQ resistance using gene *rpoB*, *gyrA* and *gyrB* is a rapid test to give the most appropriate therapy especially in multi-resistant case.

CONCLUSION

Based on the previous use, Cfx was the most frequently used drug among FQ. Cfx and Ofx were used in all type of TB patients from new case to chronic case. For this widely use, as well as WHO guidelines, Cfx is not recommended in neither sensitive nor resistant TB. All resistant isolates had *gyrA* gene mutation at codon 94 which potentially changes structure or function of of DNA gyrase as FQ target. Detection of gyrA gene mutation codon 94 targeted should be performed for rapid diagnostic test of FQ resistance.

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REFERENCES

[1] World Health Organization, Global Tuberculosis Control : WHO Report 2011. Switzerland. 2011.

[2] Aditama TY. XDR-TB.Indonesia Tuberculosis Journal. 2006; 3(2): 20-2.

[3] Sjahrurachman A .Culture and Sensitivity Test Module of *M. tuberculosis* Againts Anti-Tuberculosis First Line Drugs. Jakarta. Departemen Kesehatan RI, 2008.

[4] World Health Organization. Guidelines for the Programmatic Management of Drug-Resistant Tuberculosis Emergency, 2008.

[5] Aditama TY, Kamso S, Basri C, Surya A. National Guidelines for Tuberculosis Control. Second Ed. Departemen Kesehatan Republik Indonesia, 2007.

[6] Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, Ramasoota P. Mutations in The GyrA and GyrB Genes of Fluoroquinolone-Resistant Mycobacterium tuberculosis from TB Patients in Thailand. Southeast

Asian J Trop Med Public Health. 2005; 36 (4): 228-37.

[7] Aubry A, Veziriz N, Cambau E, Pernot C, Jarlier V, Fisher M. Novel Gyrase Mutations in Quinolone-Resistant and Hypersusceptibile Clinical Isolates of Mycobacterium tuberculosis: Functional Analysis of Mutant Enzymes. Antimicrob. Agents Chemother, Jan. 2006; 50(1): 104-12.

[8] Bernard FM, Maxwell A. Interaction between DNA Gyrase and Quinolones: Effects of Alanine Mutations at GyrA Subunit Residues Ser^{83} and Asp^{87}. Antimicrob Agents Chemother. 2001; 45(7): 1994-2000.

[9] Gaba PD, Haley C, Griffin MR *et al*. Increasing Outpatient Fluoroquinolone Exposure Before Tuberculosis Diagnosis and Impact on Culture-Negative Disease. Arc Intern Med. 2007; 167(21): 2317-22.

[10] Lameshow S, Hosmer Jr DDW, Wanga SK. Samples Size in Health Research: Translation by Pramod Dibyo. Yogyakarta. Gajah Mada University Press, 1997.

[11] Wang JY, Lee L, Lai H-C *et al*. Fluoroquinolon resistance in Mycobacterium tuberculosis isolates: Associated genetic mutations and relationship to antimicrobial exposure. JAC. 2007; 59: 860-5.

[12] Singh M, Chauhan DS, Gupta P *et al*. In Vitro Effect of Fluoroquinolones Against Mycobacterium tuberculosis isolates from Agra & Kanpur Region of North India. Indian J Med Res. 2009; 129: 542-7.

[13] Gumbo T, Louie A, Deziel M, Drusano G. Pharmacodynamic Evidence that CiprOfx Failure against Tuberculosis is not due to microbial kill but to rapid emergence of resistance. AAC. 2005; 49(8): 3178-81.

[14] Tanoerahardjo FS. The role of M. Tuberculosis dna E2 genes in the rpoB and katG gene mutations as the basis for diagnosis and new drug development efforts in cases of multi-tuberculosis drugs resistance. Doctoral Thesis. Universitas Padjadjaran. Indonesia, 2009.

[15] Jaktaji RP, Mohiti E. Study of Mutations in DNA gyrase gene of Escherichia coli. IJPR. 2010; 9(1): 43-8.

[16] Takiff H, Salazar L, Guerrero C *et al*. Cloning and nucleotide Sequence of Mycobacterium tuberculosis gyrA and gyrB Genes and Detection of Quinolone Resistance Mutations. Antimicrob Agents Chemother. 1994; 38(4): 773-80.

[17] Groll AV, Martin A, Jureen P *et al*. Fluoroquionole resistance in mycobacterium tuberculosis and mutation in gyrA and gyrB. AAC. 2009; 53(10): 4498-500.

[18] Yuwono T.Molecular Biology. Jakarta. Erlangga, 2005.