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Trends in fluoroquinolone resistance of *Mycobacterium tuberculosis* complex in a Taiwanese medical centre: 1995–2003

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***Objectives*: Fluoroquinolones are being used more frequently for the treatment of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* complex (MTB). This study was designed to determine the frequency of the emergence of fluoroquinolone-resistant strains in Taiwan and to assess whether this might be due to use of fluoroquinolones for treatment of patients with MDR or because of increased use of fluoroquinolones in the community for treatment of other infections. We also sought to determine whether there might be clonal spread of fluoroquinolone resistance.**

***Methods*: A total of 3497 clinical isolates of *M. tuberculosis* complex were obtained during 1995–2003, of which 141 were selected. They consisted of 62 isolates fully susceptible to four first-line drugs, 33 isolates resistant to rifampicin and isoniazid (MDR), and 46 isolates with a variety of any drug resistant patterns other than MDR (combination group). The MICs were determined for ciprofloxacin, ofloxacin and levofloxacin.**

***Results*: An increase in the MIC_{90} and rates of resistance to ciprofloxacin, ofloxacin and levofloxacin were noted only in the MDR group. The rates were higher among strains isolated between 1998–2003 compared with those obtained between 1995–1997 (rate of resistance, 20% versus 7.7%; MIC $\geq$ 4 mg/L versus 1–2 mg/L). Among the 10 fluoroquinolone-resistant isolates, five (50%) possessed mutations other than S95T in the *gyrA* gene. No *gyrB* mutation was found in any of the clinical isolates.**

***Conclusions:* These findings suggest that fluoroquinolone resistance is the result of treatment of patients with MDR strains rather than from use in the general community in Taiwan. The emergence of fluoroquinolone resistance among MDR strains reinforces the need for routine fluoroquinolone susceptibility testing whenever these drugs might be used.**

Keywords: TB, treatment, fluoroquinolone resistance

Introduction

Tuberculosis (TB) is one of the major causes of death worldwide. Few antimicrobial agents are as highly active as isoniazid and rifampicin against *Mycobacterium tuberculosis* complex (MTB). The global emergence of multidrug-resistant (MDR) strains resistant to isoniazid and rifampicin has made treatment of TB even more difficult. Fluoroquinolones are now being used more often for treatment of patients infected with MTB resistant to both isoniazid and rifampicin.[1–3] Ciprofloxacin, ofloxacin and levofloxacin are highly active *in vitro* against MTB.[4–8] They have excellent pharmacokinetic profiles, achieve good tissue and cellular distribution and have few adverse effects.[8–10] They have been shown to be effective against experimental MTB infection in mice.[11,12] Clinical trials have demonstrated efficacy in combination with other drugs for treatment of TB, including multidrug-resistant TB strains (MDR-TB).[8,13] It is therefore critically important to determine the potential for emergence of resistance to this class of drugs.

Acquisition of fluoroquinolone drug resistance has been shown to be mainly due to mutations in specific resistance-determining regions of the genetic targets or activating enzymes. The *gyrA*[14–17]

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and *gyrB* genes[18] of the A and B subunits in the quinolone resistance determining regions (QRDRs) of gyrase have been reported to be associated with fluoroquinolone resistance.

Fluoroquinolones were introduced in Taiwan in 1986 for treatment of mycobacterial and other bacterial diseases. There are numerous reports on susceptibility of bacteria other than MTB.[19–25] However, little is known about changes in the susceptibility of MTB to fluoroquinolones.

This study was designed to determine the frequency of the emergence of fluoroquinolone-resistant strains in Taiwan and to assess whether this might be due to use of these drugs for treatment of patients with MDR or because of increased use in the community for treatment of other infections. We also examined the locus of resistance at the *gyrA* and *gyrB* genes of the A and B subunits of the QRDRs of gyrase by DNA sequencing of resistant strains.

Materials and methods

Selection of isolates

Kaoshiung Veterans General Hospital is a 1200-bed tertiary medical centre located in southern Taiwan. MTB was identified either by the *p*-nitro-α-acetyl-amino-β-hydroxypropiophenone (NAP) test or the BDProbeTec CTB assay (Becton Dickinson Microbiology Systems, Maryland, USA).

The fluoroquinolone susceptibility of the isolates obtained from respiratory specimens was analysed in three consecutive 3 year time periods. The strains were further subdivided according to susceptibility to standard drugs. These consisted of strains that were (i) susceptible, fully susceptible to the four first-line drugs (isoniazid, rifampicin, streptomycin and ethambutol); (ii) MDR, resistant to rifampicin and isoniazid;[26] (iii) combinations, other combinations of resistance patterns.

Because the MDR and the combination groups accounted for only 2.4–4.5% and 12.3–15.5%, respectively, of the strains collected during the 9 year study period, the isolates were enriched by adding approximately every one in fifty isolate within the susceptible group, every fourth MDR isolate and every tenth isolate within the other resistant group. A total of 141 isolates were selected. These included 62 fully susceptible isolates, 33 MDR and 46 combinations.

Fluoroquinolones

The drugs were obtained as pure chemicals from their manufacturers or supply houses: ciprofloxacin (Bayer, Wuppertal, Germany), ofloxacin (Sigma-Aldrich Co., St Louis, MO, USA), and levofloxacin (Daiichi Pharmaceutical Taiwan Ltd).

MICs

The range of concentrations tested was 0.03–4 mg/L. The MICs were determined by serial dilution on agar plates. Bacteria from Lowenstein-Jensen slants were subcultured in Middlebrook 7H9 broth (Becton Dickinson and Company, Sparks, MD, USA). Once growth had reached a turbidity equivalent to that of a 0.5–1 McFarland standard, the broth was diluted 1:100 to provide an inoculum of 10^4 cfu to drug-containing solid Middlebrook 7H11 agar medium (Becton Dickinson). Controls were diluted 1:10 000 in 0.02% Tween/0.1% albumin for inoculation onto antibiotic-free medium. Plates were incubated for 3 weeks. The MIC was defined as the lowest concentration showing growth <1% of that of the initial inoculum on the drug-free plate. Resistance was defined as an MIC of >2 mg/L for ciprofloxacin and ofloxacin, and >1 mg/L for levofloxacin.[27,28]

DNA sequencing

DNA was extracted with the Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and stored at 4°C. Regions of *gyrA* and *gyrB* were amplified by PCR with the forward and reverse primers as published by Siddiqi *et al*.[29] A GeneAmp system 9600 thermocycler (Perkin-Elmer Corp., Foster City, CA, USA) was used for target amplification with the following parameters: 5 min at 4°C, followed by 30 cycles of 60 s at 94°C, 30 s at 58°C for *gyrA* and 56.5°C for *gyrB*, and 60 s at 72°C and terminated with a final extension step at 72°C for 10 min. The PCR products were purified with the Qiagen MinElute PCR purification kit, according to the manufacturer's instructions. Amplified PCR products were sequenced with the use of forward and reverse primers, the Taq Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA), and an automated sequencer, ABI PRISM 310 Genetic Analyzer (ABI). Sequence data were compared with those published.

Statistical analysis

The confidence intervals for the proportion resistant to fluoroquinolones in each group in the 3 year periods were determined by the exact confidence limits for the population proportion *p* method.[30]

Results

MDR-TB and susceptibility to fluoroquinolones

A total of 3497 isolates of MTB (one isolate per patient) were obtained from clinical specimens during 1995–2003. The sites included the respiratory tract, body fluids, tissues, wound, pus and skin. There were 2883 susceptible strains, 122 MDR strains and 492 strains with other resistance patterns.

The experience in this hospital for all MTB isolated over the 9 year period from 1995–2003 according to drug susceptibility pattern is shown in Table 1. There were no significant differences in the frequency of strains that were susceptible, MDR or combinations during the 3 year intervals.

The trends in MIC_{90}s of the fluoroquinolones according to the categories of susceptible, MDR and combinations during the 3 year periods are shown in Table 2. The only significant increase in MIC_{90} was seen for the MDR strains. This was noted between the baseline period of 1995–1997 and the two subsequent 3 year periods. There was no further increase in MIC_{90} from 1998–2000 to 2001–2003. The trends in the proportion of fluoroquinolone-resistant strains, according to the same categories, are shown in

Table 1. *M. tuberculosis* complex isolated in 1995–2003 (one single isolate per patient)

Year (no.)	No. of isolates (%)		
	susceptible[a]	MDR[b]	combinations[c]
95–97 (1066)	863 (81.0)	48 (4.5)	155 (14.5)
98–00 (1191)	978 (82.1)	28 (2.4)	185 (15.5)
01–03 (1240)	1042 (84.0)	46 (3.7)	152 (12.3)

[a]Susceptible, fully susceptible to isoniazid, rifampicin, streptomycin and ethambutol.
[b]MDR, resistant to isoniazid and rifampicin.
[c]Combinations, other combinations of resistance patterns.

Table 2. Trends in the MIC_{90}s of fluoroquinolones for *M. tuberculosis* complex

	MIC_{90} (mg/L)								
	ciprofloxacin			ofloxacin			levofloxacin		
Year (no.)	susceptible[a] (*n* = 62)	MDR[b] (*n* = 33)	combinations[c] (*n* = 46)	susceptible (*n* = 62)	MDR (*n* = 33)	combinations (*n* = 46)	susceptible (*n* = 62)	MDR (*n* = 33)	combinations (*n* = 46)
95–97 (38)	2	2	2	2	1	1	1	1	1
98–00 (43)	2	>4	2	2	>4	2	1	>4	1
01–03 (60)	2	>4	2	2	4	2	1	4	1

[a]Susceptible, fully susceptible to isoniazid, rifampicin, streptomycin and ethambutol.
[b]MDR, resistant to isoniazid and rifampicin.
[c]Combinations, other combinations of resistance patterns.

Table 3. Trends in resistance of *M. tuberculosis* complex to fluoroquinolones

	Resistant no./total no. (%)								
	ciprofloxacin			ofloxacin			levofloxacin		
Year (no.)	susceptible[a]	MDR[b]	combinations[c]	susceptible	MDR	combinations	susceptible	MDR	combinations
95–97 (38)	1/16 (6.3)	1/13 (7.7)	0/9 (0.0)	1/16 (6.3)	1/13 (7.7)	1/9 (11.1)	1/16 (6.3)	1/13 (7.7)	1/9 (11.1)
98–00 (43)	1/23 (4.3)	2/9 (22.2)	1/11 (9.1)	0/23 (0.0)	2/9 (22.2)	0/11 (0.0)	0/23 (0.0)	2/9 (22.2)	0/11 (0.0)
01–03 (60)	0/23 (0.0)	2/11 (18.2)	0/26 (0.0)	0/23 (0.0)	2/11 (18.2)	0/26 (0.0)	0/23 (0.0)	2/11 (18.2)	1/26 (3.8)
Total (141)	2/62 (3.2)	5/33 (15.2)	1/46 (2.2)	1/62 (1.6)	5/33 (15.2)	1/46 (2.2)	1/62 (1.6)	5/33 (15.2)	2/46 (4.3)

[a]Susceptible, fully susceptible to isoniazid, rifampicin, streptomycin and ethambutol.
[b]MDR, resistant to isoniazid and rifampicin.
[c]Combinations, other combinations of resistance patterns.

Table 3. Once again an increase in resistant strains was statistically significant only in the MDR group. The proportion resistant to fluoroquinolones was 7.7% (95% CI, 7.14–14.3%) in the 1995–1997 group and 22.2% (95% CI, 20.0–30.0%) in the 1998–2000 group. There was cross-resistance among the three fluoroquinolones.

Sequence studies

Regions of the *gyrA* and *gyrB* genes of fluoroquinolone-resistant strains as well as susceptible strains were analysed. Almost all isolates tested, regardless of their MIC, had an S95T substitution in the *gyrA* gene. Four fluoroquinolone-resistant isolates had undergone an additional point mutation (one A90V and three D94G). One isolate had a double mutation (G88A and B94Y). Among the 10 fluoroquinolone-resistant isolates, five (50%) possessed mutations other than the S95T substitution in the *gyrA* gene. No mutations in the *gyrB* loci were found in the clinical isolates in this study.

Discussion

Some investigators[31–33] have noted slightly higher fluoroquinolone activity, against fully susceptible MTB isolates than resistant (MDR) strains. Others[7,34] have disputed these findings. These differences might be explained by when the studies were conducted in relation to the time fluoroquinolones were introduced for treatment of TB and to differences in the geographical regions from which the strains were isolated.

In the current study, we found that the MICs of fluoroquinolones were increased only in MDR-TB isolates. Therefore, exposure to fluoroquinolones for treatment of other bacterial diseases in the community does not appear to be responsible for the increasing trend of resistance. The emergence of MDR-TB is strongly associated with poor or intermittent compliance with anti-TB therapy and AIDS.[35]

The possibility of clonal spread of MDR-fluoroquinolone-resistant strains appears to be remote based on the findings of different antimicrobial resistance patterns and sequencing of the resistant isolates. We observed that almost all isolates tested, regardless of their MIC, carried an S95T substitution in the *gyrA* gene. This is consistent with the previously reported finding that S95T is a marker for evolutionary genetics and does not correlate with drug resistance.[36] Five of the resistant isolates had undergone additional point mutations or double mutation. This finding does not exclude the presence of any other additional resistance mechanisms. Gyrase B mutation was reported to be associated with a low level of resistance, but was only found in a laboratory-selected fluoroquinolone-resistant mutant of an MTB H37Ra isolate.[18] No mutations in the gyrase B loci were found in

the clinical isolates in this study. For the five fluoroquinolone-resistant isolates without *gyrA* or *gyrB* mutations that correlate with drug resistance, it appears that they have acquired resistance to fluoroquinolones due to mutations elsewhere in the target genes or via other mechanisms, e.g. active efflux which has been reported in a quinolone-resistant isolate of *Mycobacterium smegmatis* mc^2-155.[37] Further studies are needed.

Several studies provide the relationship between the mutations and the phenotypic resistance profiles of clinical MTB isolates. The rate of mutations in the *gyrA* gene found in fluoroquinolone-resistant MTB was 10.3% in India,[29] 55.2–58.2% in Hong Kong[38,39] and up to 89.5% in Italy and Abkhazia.[40] Our results (50%) were similar to the rates reported from Asia.

Although fluoroquinolone-resistant MTB isolates are still rare in Taiwan, it is alarming that the rate of resistance to fluoroquinolones significantly increased with time, indicating the capability of these isolates to survive under selection pressure. Thus we suggest fluoroquinolones should be preserved as the last-line therapy when no other agents are active or tolerable. By doing so, emergence of resistance could be delayed. In addition, susceptibility tests to fluoroquinolones should be performed before treating patients infected with MDR-TB, so that the most optimal agents can be prescribed.

TB caused by drug-resistant isolates of MTB poses a therapeutic challenge to select the most appropriate antimicrobial agents. The trend in fluoroquinolone resistance among MDR-TB isolates emphasizes the need for susceptibility testing particularly for MDR strains.

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Transparency declarations

None to declare.

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