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Distribution of fluoroquinolone MICs in *Helicobacter pylori* strains from Korean patients

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***Objectives*: The aim of this study was to assess the prevalence rate of primary fluoroquinolone resistance in *Helicobacter pylori* isolates from Korean patients over the past 16 years.**

***Methods*: One hundred and thirty-five strains of *H. pylori* (34 strains in 1987, 36 in 1994 and 65 in 2003) were isolated from antral gastric mucosal biopsy specimens. The determination of MICs for the *H. pylori* isolates of ciprofloxacin, levofloxacin and moxifloxacin was examined by using the serial 2-fold agar dilution method. DNA sequences of the *gyrA* gene in fluoroquinolone-resistant strains were determined.**

***Results*: The distribution of fluoroquinolone MICs (ciprofloxacin, levofloxacin and moxifloxacin) shifted to higher concentrations during 1987–2003. All of the levofloxacin- or moxifloxacin-resistant strains were resistant to ciprofloxacin. Sequence analysis in fluoroquinolone-resistant strains showed point mutation of the *gyrA* gene at A272G and G271A, indicating mutations of the codon Asp-91 in the fluoroquinolone-resistance-determining region of the DNA gyrase.**

***Conclusions*: These results suggest that resistance to fluoroquinolones has been increasing in the Korean population and the resistance is most likely mediated through point mutation in *gyrA*.**

Keywords: *gyrA*, mutation, QRDR, resistance

Introduction

Helicobacter pylori infection is recognized as one of the causes of chronic gastritis, peptic ulcer and gastric cancer. Thus, *H. pylori* colonization should be eradicated in patients with peptic ulceration, as eradication not only accelerates ulcer healing but also prevents long-term ulcer relapse. Treatment regimens containing a proton pump inhibitor (PPI) and a combination of two or more antibiotics, including amoxicillin and clarithromycin, are considered to be most efficacious, but drug resistance is a growing problem. Therefore, alternative regimens have been developed as components of a triple PPI-based regimen, including ciprofloxacin, levofloxacin or moxifloxacin.[1,2] The resistance, however, of *H. pylori* strains to fluoroquinolones in Korea has not yet been reported. The aim of this study was to assess the prevalence rate of primary fluoroquinolone resistance, against ciprofloxacin, levofloxacin and moxifloxacin, in *H. pylori* isolates from Korean patients over the past 16 years.

Materials and methods

Isolation of H. pylori strains

We isolated 135 strains of *H. pylori* from antral gastric mucosal biopsy specimens obtained in Seoul, Korea, in 1987 (Hanyang University Hospital, 34 strains), in 1994 (Seoul National University Hospital, 36 strains) and in 2003 (Seoul National University Hospital, 65 strains).[3] None of the patients had taken antibiotics, PPI or non-steroidal anti-inflammatory drugs during the preceding 3 months. The distance between the two hospitals (Hanyang University Hospital and Seoul National University Hospital) located in Seoul is less than 5 km and these two are general and tertiary hospitals, indicating that the distribution of patient diseases is similar. The *H. pylori* strains were cultured under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) as previously described.[3] All stock cultures were maintained at –70°C in Brucella broth supplemented with 15% glycerol. These preparations were thawed and subcultured for experiments.

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Determination of MIC

The susceptibilities of the *H. pylori* isolates to ciprofloxacin (Sigma Chemical Co., St Louis, MO, USA), levofloxacin (Sigma) and moxifloxacin (Bayer AG Pharmaceuticals, Germany) were examined by using the serial 2-fold agar dilution method as described previously.[3,4] Briefly, the bacteria were subcultured on Mueller–Hinton agar supplemented with 5% defibrinated sheep blood for 48 h. Bacterial suspension adjusted to 1×10^7 cfu was inoculated directly on each antibiotic-containing agar dilution plate. After incubation for 72 h, the MIC of each antibiotic was determined. The breakpoints for fluoroquinolone resistance were provisionally defined as >1.0 mg/L.

PCR amplification and nucleotide sequence

The extraction of *H. pylori* genomic DNA was performed as reported previously.[3] To detect gene mutation of the quinolone resistance-determining regions (QRDRs) of the A subunit of the DNA gyrase (*gyrA*), we used the oligonucleotide primers: 5′-TTT AGC TTA TTC AAT GAG CGT-3′ and 5′-GCA GAC GGC TTG GTA GAA TA-3′.[5] The size of the amplified fragments of *gyrA* was 429 bp. The PCR profile consisted of 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 57°C and 1 min extension at 72°C. Sequencing was performed on both strands of the non-restricted amplicons, using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Transformation of H. pylori

Transformation of *H. pylori* was accomplished by using a modified version of the method previously described.[6] *H. pylori* HP99, a clinical isolate, was chosen as a recipient for transformation experiments on the basis of prior experiments that revealed that it was readily transformed with DNA. The recipient strains (HP99, ciprofloxacin MIC 0.125 mg/L) were grown for 3 days on charcoal agar and then subcultured to a new plate in a 1 cm^2 area. The plate was incubated overnight; donor DNAs [10 ng of PCR-amplified *gyrA* fragments of Korean resistant strain HP13 (ciprofloxacin MIC 32 mg/L, Asp-91→Gly) and HP11 (ciprofloxacin MIC 32 mg/L, Asp-91→Asn) in Table 1] in a volume of 10 µL were applied to the cells, and the plate was incubated for a further 24 h. The cells were then scraped off the plate into PBS, pH 7.2, diluted, and plated onto chocolate agar containing ciprofloxacin 4 mg/L. After 3 days the colonies were counted, and five colonies from each transformation were purified and maintained on medium containing ciprofloxacin.

Results and discussion

The resistance rates for fluoroquinolones in *H. pylori* strains in Korea have not yet been reported. Rates of resistance lower than 10% have been reported from several countries: France,[5] Japan,[7] The Netherlands,[8] Portugal[9] and Germany.[10] However, the official breakpoints of fluoroquinolone resistance have not been designated for *H. pylori* isolates by the National Committee for Clinical Laboratory Standards (NCCLS). Therefore, we cannot assign any provisional breakpoint to fluoroquinolones. Nevertheless, many reports have provisionally defined the cut-off value as an MIC higher than 1.0 mg/L.[5,7–10] In the present study, *H. pylori* strains with ciprofloxacin MICs > 1.0 mg/L were not found in the 1987 isolates. However, those with ciprofloxacin MICs > 1.0 mg/L increased from 13.9% in 1994 to 33.8% in 2003 (Figure 1a). The distribution of levofloxacin and moxifloxacin MICs demonstrated a continuous spectrum ranging between 0.0625 and 0.5 mg/L in 1987 and 1994, and showed a definite shift to high concentration in 2003 (Figure 1b and c). Thus, the prevalence of *H. pylori*

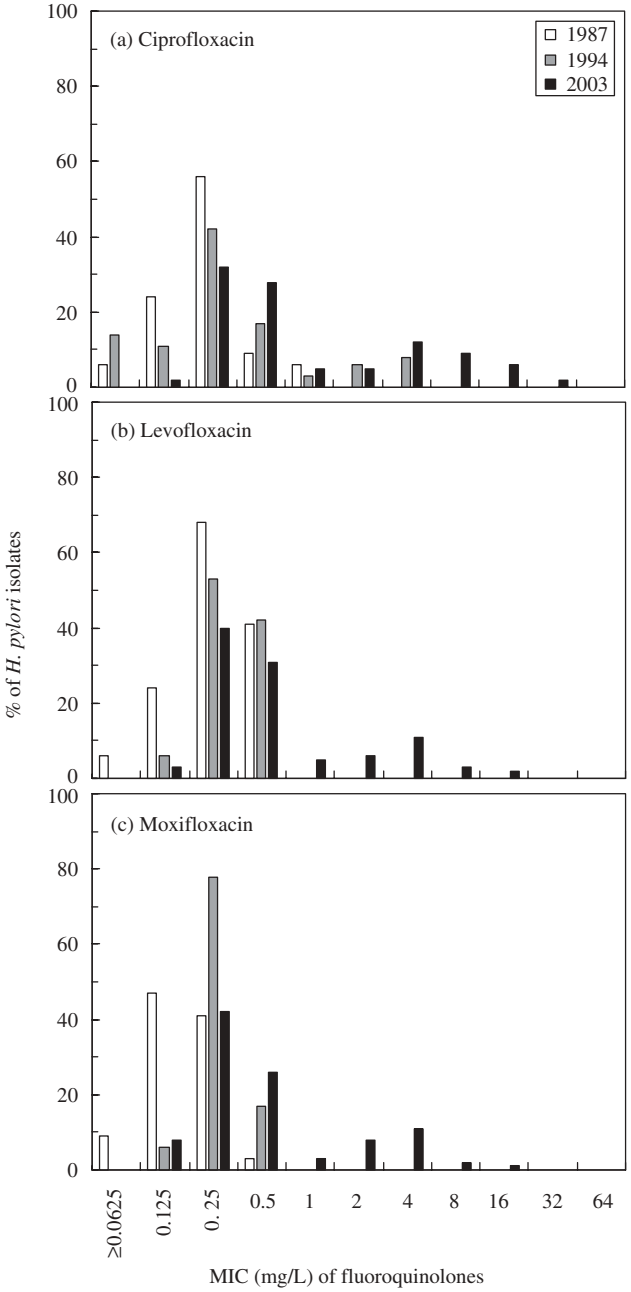

Figure 1. MIC distributions of fluoroquinolones for *H. pylori* over a 16 year period. We evaluated 135 total strains: 34 strains were examined in 1987, 36 in 1994 and 65 in 2003. MICs were determined by agar dilution. (a) ciprofloxacin; (b) levofloxacin; (c) moxifloxacin.

strains with levofloxacin MICs >1.0 mg/L increased rapidly from 0% (1987 and 1994) to 21.5% (2003) and that with moxifloxacin MICs >1.0 mg/L increased from 0% (1987 and 1994) to 21.5% (2003). Interestingly, the fluoroquinolone MICs showed a bimodal distribution in 2003. Since the production of fluoroquinolones in 1998 was ∼2.1 times higher than in 1994 in Korea, a rise in the prevalence of fluoroquinolone resistance may be associated with the increasing use of fluoroquinolones in clinical practice.

In the present study, all of the levofloxacin- or moxifloxacin-resistant strains showed ciprofloxacin resistance (>1.0 mg/L of

Table 1. Phenotypic and genotypic characteristics of fluoroquinolone-resistant strains of *H. pylori*[a]

Strains	MIC (mg/L)			Substitution in GyrA
	ciprofloxacin	levofloxacin	moxifloxacin	
01	2	2	2	Asp-91→Gly
02	2	2	2	Asp-91→Gly
03	4	2	2	Asp-91→Asn
04	4	4	2	Asp-91→Asn
05	4	4	2	Asp-91→Gly
06	4	4	2	Asp-91→Gly
07	8	2	2	Asp-91→Gly
08	8	4	2	Asp-91→Gly
09	16	2	2	Asp-91→Asn
10	16	4	4	Asp-91→Gly
11	32	2	4	Asp-91→Asn
12	32	2	2	Asp-91→Gly
13	32	16	8	Asp-91→Gly
14	64	4	8	Asp-91→Gly

[a]MICs were determined by agar dilution method.

each antibiotic), suggesting cross-resistance among fluoroquinolones. Mutations in *gyrA* genes play a critical role in fluoroquinolone resistance of *H. pylori*: amino acid 87 (Asn to Lys), 88 (Ala to Val), 91 (Asp to Gly, Asn or Tyr) and a double substitution at 91 and 97 (Ala to Val).[5,7] We examined *gyrA* mutations using 14 strains with all fluoroquinolone MICs > 1.0 mg/L. As shown in Table 1, the mutation in *H. pylori* was at Asp-91: 10 strains showed A272G (Asp-91 to Gly) and four strains showed G271A (Asp-91 to Asn). However, we found no *gyrA* double mutants. To confirm that the mutations detected in the amplified fragment caused ciprofloxacin resistance, the PCR products were used to transform ciprofloxacin-susceptible strains of *H. pylori*. The results showed that the amplified fragments from resistant strains could transform the susceptible recipient to a resistant phenotype. Control experiments, in which the recipient received an aliquot of H_2O or DNase-treated DNA, yielded no resistant colonies. To test that the transformed cells contained the mutation present in the donor DNA, PCR was used to amplify the QRDR region from the transformed recipients and the nucleotide sequence was determined. The recipient cells harboured the same mutation carried by the donor DNA. These results demonstrate that the mutations in the PCR fragment could account for resistance to fluoroquinolone.

In conclusion, these results demonstrated that the prevalence of primary resistance to fluoroquinolones has been increasing in the Korean population and the resistance is most likely mediated through point mutation in *gyrA*.

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