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Alteration of GyrA Amino Acid Required for Ciprofloxacin Resistance in *Klebsiella pneumoniae* Isolates in China▽

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Resistance to ciprofloxacin was detected in 111 (48.1%) isolates of *Klebsiella pneumoniae* from China. GyrA alterations were identified in the ciprofloxacin-resistant and ciprofloxacin-susceptible isolates. The results, including previously published data, indicate that the single substitution Ser83→Ile and three types of double mutations at Ser83 and Asp87 were required for ciprofloxacin resistance ($P < 0.05$).

Resistance to fluoroquinolones is increasing in *Klebsiella pneumoniae* strains. Mechanisms of resistance to fluoroquinolones in the *Enterobacteriaceae* have been shown to be due primarily to alterations in *gyrA*, which encodes DNA gyrase, a type II topoisomerase (1, 4). The mutations are localized in an area named as the quinolone resistance-determining region (QRDR) (24). DNA sequencing of the GyrA QRDR in clinical isolates showed some alterations associated with fluoroquinolone resistance in *K. pneumoniae* (8, 11, 24).

In this paper, 231 consecutive, nonrepetitive isolates of *K. pneumoniae* were collected from inpatients in three tertiary hospitals in Harbin, the capital city of Heilongjiang Province, between May 2005 and March 2006. The strains were identified with the API 20E system (bioMérieux, Marcy l'Etoile, France) and confirmed as being nonduplicated by randomly amplified polymorphism DNA analysis. MICs of ciprofloxacin and nalidixic acid (Sigma-Aldrich, Inc., St. Louis, MO) were determined by the agar dilution method with Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, MD) as recommended by the CLSI (formerly NCCLS) (15). The MIC_{50} and

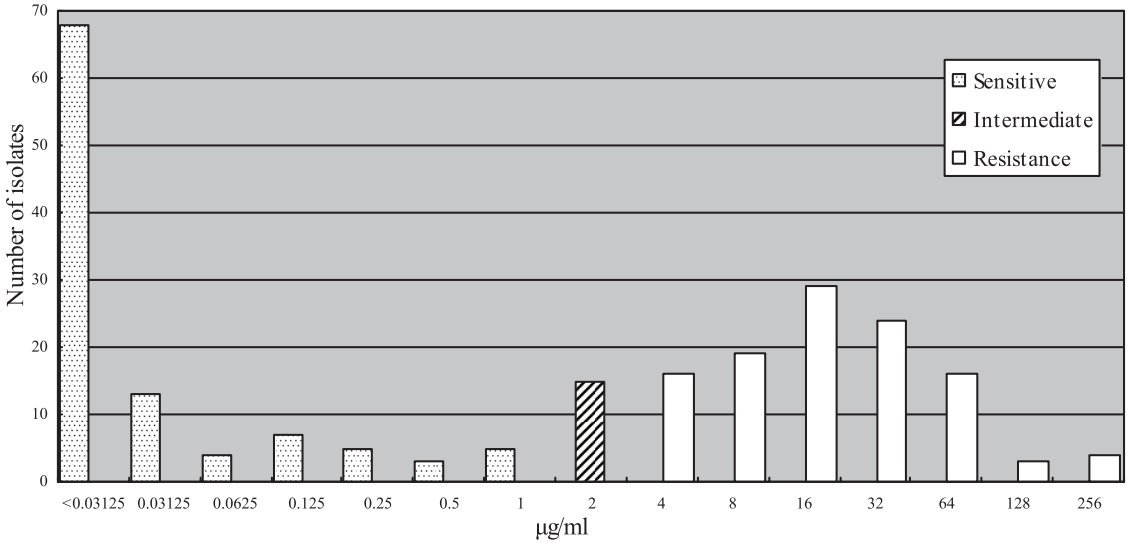

FIG. 1. Distribution of MICs of ciprofloxacin ($n = 231$). MICs were determined by the agar dilution method. One hundred eleven isolates (48.1%) were resistant to ciprofloxacin, among which 16, 19, 29, 24, 16, 3, and 4 isolates had MICs of 4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml, 128 μg/ml, and 256 μg/ml, respectively; 105 isolates (45.5%) were susceptible to ciprofloxacin, with MICs ranging from <0.03125 μg/ml to 1 μg/ml; and 15 (6.5%) were intermediately resistant (MIC, 1 to 4 μg/ml).

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TABLE 1. Alterations in GyrA and MICs of quinolones in 33 Chinese clinical isolates of *K. pneumoniae*

Strain	MIC (μg/ml)		Amino acid change at position:						
	Ciprofloxacin	Nalidixic acid	Ser83	Asp87	Arg154	Ala171	Gly177	Leu187	Val198
Resistant to ciprofloxacin									
95	128	>512	Leu	Asn		Ser			Ile
863	128	>512	Leu	Asn		Ser			Ile
149	64	>512	Leu	Asn		Ser			Ile
103	64	>512	Leu	Asn		Ser			Ile
663	64	>512	Leu	Asn		Ser			Ile
94	32	>512	Leu	Asn		Ser			Ile
779	32	>512	Leu	Asn		Ser			Ile
234	16	>512	Leu	Asn		Ser			Ile
3	16	>512	Leu	Asn		Ser			Ile
27	8	>512	Leu	Asn		Ser			Ile
719	8	>512	Leu	Asn		Ser			Ile
205	4	>512	Leu	Asn		Ser			Ile
769	32	>512	Ile				Arg		
828	32	>512	Ile				Arg		
827	32	>512	Ile						
772	32	>512	Ile						
721	8	>512				Ser		Ile	Ile
836	4	>512							
Intermediate resistant to ciprofloxacin									
753	2	>512	Ile						
21	2	16							
715	2	16							
Susceptible to ciprofloxacin									
840	1	16				Ser		Ile	Ile
685	0.125	>512				Ser		Ile	Ile
212	<0.03125	16				Ser		Ile	Ile
517	<0.03125	8				Ser		Ile	Ile
838	1	256	Tyr			Ser		Ile	Ile
49	0.5	>512		Asn	Ser	Ser			
182	<0.03125	4	Thr			Ser			
737	<0.03125	8	Leu						Ile
724	<0.03125	8	Ile						
738	1	8							
577	0.125	4							
760	<0.03125	4							

MIC_{90} of ciprofloxacin were 2 μg/ml and 32 μg/ml, respectively. According to CLSI criteria, 105 (45.5%) and 15 (6.5%) isolates were susceptible (MIC ≤ 1 μg/ml) and intermediately resistant (MIC, 1 to 4 μg/ml) to ciprofloxacin, respectively; 111 isolates (48.1%) had an MIC greater than the breakpoint (MIC ≥ 4 μg/ml) (Fig. 1). Rates of isolation of ciprofloxacin-resistant *K. pneumoniae* strains in the United States increased from 12.9% in 1991 to 35.6% in 2005 (21). Decreased susceptibility was also found in Europe (1). In China, it was reported that the percentage of ciprofloxacin resistance rose from 2% in 1994 to 18% in 2000 (25). However, a higher percentage of ciprofloxacin resistance was found in this study.

To investigate the characteristics of GyrA alterations, *gyrA* gene fragments were amplified and sequenced in 33 randomly selected isolates representing a range of ciprofloxacin MICs. Primers gyrA-F (5′-TGCGAGAGAAATTACACC), corresponding to positions 299 to 316, and gyrA-R (5′-AATATGT TCCATCAGCCC), complementary to nucleotides 906 to 923 of the *K. pneumoniae* sequence (GenBank accession number X16817), were used to amplify the *gyrA* gene fragments with bacterial lysate as a template as described previously (24). PCR products were then sequenced in both directions by use of an ABI 373 automated DNA sequencer (Applied Biosystems, Foster City, CA) with the same primers used for PCR amplification. The nucleotide sequences and the deduced amino acid were compared with that of *K. pneumoniae* ATCC 13883 (GenBank accession number DQ673325) using the online ClustalW2 multiple sequence alignment program.

Among 33 isolates selected, 27 were revealed to have amino acid alterations in GyrA (Table 1). Isolates that were resistant to ciprofloxacin were also resistant to nalidixic acid, while 3 out of 12 ciprofloxacin-susceptible isolates displayed resistance to nalidixic acid. Twenty-one isolates presented Ser83 changes. The most common mutation was Ser83→Leu, which was present in 13 isolates. A Ser83→Ile substitution was found in six isolates; also, one Tyr substitution and one Thr substitution were found in ciprofloxacin-susceptible isolates. It is notable that almost all the ciprofloxacin-resistant isolates had substitutions at Ser83 by Leu or Ile, and all of the Ser83→Leu

TABLE 2. Amino acid changes in the GyrA QRDR of *K. pneumoniae*

Susceptibility to ciprofloxacin	Amino acid change at position:		No. of isolates	Reference(s) and/or source
	83	87		
Resistant			9	2, 18; this study
	Tyr		15	1, 8, 11, 14, 20, 24
	Phe		10	1, 2, 24
	Ile		7	1, 2, 20; this study
	Phe	Asn	17	1, 2, 4, 5, 6, 11
	Leu	Asn	13	20; this study
	Tyr	Asn	10	20, 24
	Phe	Tyr	4	1, 11
	Phe	Ala	3	4, 5, 6
	Phe	Gly	3	4, 5, 24
	Tyr	Tyr	2	1, 2
	Ile	Asn	1	20
Intermediate resistant			1	18
	Tyr		14	1, 4, 5, 8, 20
	Phe		5	1, 4, 5
	Ile		1	This study
		Gly	3	4, 5; this study
	Phe	Gly	1	4, 5
Susceptible			37	4, 5 1, 4, 5, 11, 14, 18, 25; this study
	Tyr		17	4, 5 4, 5, 8, 11, 20; this study
	Phe		7	4, 5 4, 5, 11, 18, 24
	Leu		1	This study
	Ile		1	This study
	Thr		1	This study
		Gly	1	4, 5
		Asn	1	This study
Total			185	

changes were combined with Asp87→Asn, which is consistent with data from previous reports (1, 2, 20).

However, comparable with the mutations involving substitutions of Ser83 with Phe, Tyr, or Ile and Asp87 alterations reported in Japanese (4), American (24), and European (11) isolates, a large proportion (12 out of 18 [66.7%]) of Chinese fluoroquinolone-resistant *K. pneumoniae* isolates demonstrated Ser83→Leu together with Asp87→Asn (Table 1). Although Ser83→Leu is frequently displayed in *Escherichia coli* (17), the results from China (this study) and Singapore (20) suggested the existence of this alteration in *K. pneumoniae*. Moreover, most of the isolates with this predominant alteration were highly resistant to ciprofloxacin (MIC $\geq$ 8 μg/ml), which may be related to the higher prevalence of ciprofloxacin resistance in China. Besides, changes outside the QRDR, such as Ala171→Ser and Val198→Ile, were found in both ciprofloxacin-susceptible and -resistant isolates (Table 1).

DNA sequencing of GyrA in clinical strains has revealed some mutations in the QRDR associated with fluoroquinolone resistance. However, QRDR alterations were also found in isolates susceptible to ciprofloxacin in this study and others (8, 11, 13, 19, 24). In order to explore the role of individual alteration types found in *K. pneumoniae* in ciprofloxacin resistance, alterations in Ser83 and Asp87 of GyrA were reviewed, based on articles found in the PubMed database, and the association between ciprofloxacin resistance and the individual alteration was analyzed by means of the SPSS 13.0 statistical package using Fisher's exact test or Pearson chi-square test. In total, types of GyrA alterations carried by 138 strains were found among 185 isolates of *K. pneumoniae* with an exact MIC, which included 152 strains in 11 published articles and 33 isolates in this study (Table 2).

Although seven types of single alterations were detected in 84 strains, only Ser83→Ile was distributed differently between the ciprofloxacin-resistant and ciprofloxacin-susceptible isolates ($P < 0.005$), with the Ile substitution occurring more frequently in the former group. The distribution of other single substitutions such as Ser83→Tyr, Ser83→Phe, and Ser83→Leu showed no statistical differences between the two groups. Eight types of double mutations involving both Ser83 and Asp87 were found exclusively in 54 ciprofloxacin-resistant isolates; however, only three types of double mutations, Ser83→Phe plus Asp87→Asn, Ser83→Leu plus Asp87→Asn, and Ser83→Tyr plus Asp87→Asn, were associated with ciprofloxacin resistance ($P < 0.05$). Thus, the three types of double mutations and the single mutation Ser83→Ile are required for ciprofloxacin resistance in *K. pneumoniae*. Also, most of the isolates carrying such mutations had MICs exceeding 16 μg/ml, which indicates that these alterations in GyrA are prone to conferring high-level resistance to ciprofloxacin. The resistance phenotype of isolates with the "silent" alterations (mutations having no statistical association with ciprofloxacin resistance) may be attributed to other factors affecting antibiotic susceptibility, such as a change in the penetration of agents resulting from energy-dependent efflux and porin loss (11, 12), differential expression of

a resistant gene (7), and activities of regulatory loci like *mar* and *sox*, which induce decreased porin expression and increased efflux (9, 10, 16).

In summary, we found the single mutation Ser83→Ile and the double mutation Ser83→Leu plus Asp87→Asn to be associated with ciprofloxacin resistance in China. By reviewing all the alterations in the GyrA QRDR, we demonstrated that a single change, Ser83→Ile, and three types of double alterations, Ser83→Phe plus Asp87→Asn, Ser83→Leu plus Asp87→Asn, and Ser83→Ile plus Asp87→Asn, were required for ciprofloxacin resistance. These results suggest that effectivity of a certain mutation should be considered when studying the alterations of GyrA associated with ciprofloxacin resistance.

Nucleotide sequence accession numbers. The partial sequences of the variant *gyrA* genes in clinical isolates of *K. pneumoniae* have been submitted to the GenBank database under accession numbers EU430280 through EU430289.

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