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Molecular Epidemiology of Extended-Spectrum β-Lactamase-Producing, Fluoroquinolone-Resistant Isolates of *Klebsiella pneumoniae* in Taiwan

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Strains of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) have emerged worldwide. Concomitant ciprofloxacin resistance with ESBL production in *K. pneumoniae* isolates would severely restrict treatment options. Among 39 (18.5%) of 211 ESBL-KP isolates resistant to ciprofloxacin (MIC, ≥4 μg/ml), 37 (95%) were high level resistant (MIC, ≥16 μg/ml). These isolates were also cross resistant to the newer fluoroquinolones, including levofloxacin, gatifloxacin, gemifloxacin, and garenoxacin (BMS 284756). Sitafloxacin was most active against these ciprofloxacin-resistant ESBL-KP isolates with MICs for 67% of the isolates being ≤2 μg/ml. The molecular epidemiology of these multiresistant isolates was investigated by automated ribotyping and pulsed-field gel electrophoresis (PFGE). Ribotyping identified 18 different strains among the 39 ciprofloxacin-resistant ESBL-KP isolates. The majority (67%) of these isolates were contained in six ribogroups which were further confirmed by PFGE. The distribution of the six major strains of ciprofloxacin-resistant ESBL-KP within Taiwan included one (ribogroup 255.3-PFGE type E) with a nationwide distribution and several institution-specific strains. Interhospital cooperation appears necessary, with strict infection control practices coupled with restriction of fluoroquinolone and extended-spectrum β-lactam use to control both the major epidemic strain and the more diverse strains observed within individual institutions.

Strains of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) have appeared worldwide (7). In Taiwan the frequency of ESBL-KP has increased steadily in recent years with reports of 10 to 30% frequency among clinical isolates of *K. pneumoniae* (6, 12, 13). Quinolones are potential therapeutic options for treatment of infections caused by ESBL-KP; however, resistance to quinolones among *K. pneumoniae* strains has been increasingly reported (1, 2, 4, 6, 10, 14) and ranges from 5 to 33% in Taiwan (6). The main quinolone resistance mechanisms include genetic mutations in DNA gyrase and/or topoisomerase IV, caused by prior usage (3, 5). Clonal spread of quinolone-resistant strains has only rarely been reported for *K. pneumoniae* (4, 10, 14).

Paterson et al. (10) described the epidemiology of ciprofloxacin resistance and its relationship to ESBL production among *K. pneumoniae* strains. They found 15 (18%) of 83 ESBL-KP isolates having concomitant resistance to ciprofloxacin. Furthermore, they reported that 33% of ESBL-KP strains from Taiwan were ciprofloxacin resistant. An epidemiological link between ESBL production and ciprofloxacin resistance among *Klebsiella* spp. was also reported by Brisse et al. (4). Clearly the spread of *K. pneumoniae* with resistance to both quinolones and broad-spectrum β-lactams would severely limit treatment choices. Although earlier data suggested that ciprofloxacin-resistant ESBL-KP may be a problem in Taiwan (10), the sample was not representative of the entire nation. We have addressed this potential problem in the context of a nationwide survey of ESBL-KP in Taiwan (12) and describe herein the frequency and molecular epidemiology of ciprofloxacin-resistant ESBL-KP in that country.

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MATERIALS AND METHODS

Bacterial isolates. A total of 211 clinical isolates of *K. pneumoniae* producing probable (9) ESBLs were collected at 24 hospitals in Taiwan between January 1998 and June 2000 (12). Among the participating hospitals, 11 were located in the northern area (N01 to N11), 5 were located in the middle area (M01 to M05), 4 were in the eastern area (E01 to E04), and 4 were in the southern area (S01 to S04) of Taiwan.

Antimicrobial susceptibility testing. MIC results for selected antimicrobials were determined by broth microdilution as defined by the National Committee for Clinical Laboratory Standards (NCCLS) (8, 9). The ESBL phenotype was confirmed by using the Etest ESBL strips (AB Biodisk, Solna, Sweden) with a reduction of ≥ 3 $\log_2$ dilutions for either cefotaxime or ceftazidime by clavulanic acid (4 μg/ml) as a positive test according to criteria defined by the NCCLS (9). Isolates for which MICs of ciprofloxacin were >2 μg/ml were selected for additional testing with the following Etest strips: ciprofloxacin, levofloxacin, gatifloxacin, gemifloxacin, garenoxacin (formerly BMS 284756 or T-3811), and sitafloxacin. Interpretive criteria for the marketed agents tested were those published by the NCCLS (9). Quality control was performed with *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212. All results were within published control limits (9).

Ribotyping. All isolates with resistance to ciprofloxacin (MIC, ≥4 μg/ml) were ribotyped with the RiboPrinter microbial characterization system (Qualicon, Inc., Wilmington, Del.) as described by Pfaller et al. (11). Briefly, this automated

TABLE 1. MIC distribution of different fluoroquinolones against 39 ESBL-KP isolates resistant to ciprofloxacin (MIC, ≥4 μg/ml)

Quinolone	No. of isolates for which the MIC (μg/ml) was:							
	≤0.5	1	2	4	8	16	32	>32
Ciprofloxacin	0	0[a]	0	1	1	4	0	33
Levofloxacin	0	0	0[a]	1	4	3	1	30
Gatifloxacin	0	0	0[a]	4	9	6	2	18
Gemifloxacin	0	0	0	3	2	2	4	28
Garenoxacin	0	0	0	2	4	1	2	30
Sitafloxacin	3	6	17	8	2	1	0	2

[a] Susceptible breakpoint concentration (9).

process included bacterial cell lysis, cleavage of DNA with the restriction enzyme *Eco*RI, size separation by gel electrophoresis, and modified Southern blotting. A pattern of the restriction fragments containing rRNA genetic information was created through hybridization with a chemiluminescently labeled DNA probe containing the rRNA operon (*rrnB*) from *E. coli*. The chemiluminescent patterns were then electronically imaged and stored in the computer. The ribotype pattern for each isolate was compared to other patterns in the database. The isolates were thereby characterized and assigned to a specific ribogroup based on both band position and signal intensity. Isolates were considered to belong to the same ribogroup if their ribotype patterns yielded a similarity coefficient of ≥0.93.

PFGE. Pulsed-field gel electrophoresis (PFGE), as a second strain typing method, was used to confirm genetic relatedness of representative isolates within specific ribogroup clusters as described by Pfaller et al. (11). Whole chromosomal DNA in agarose was digested with *Spe*I, and the restriction fragments were separated in a CHEF DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, the gels were stained with ethidium bromide, illuminated under UV light, and photographed. All bands had to match exactly to classify isolates as indistinguishable. Patterns differing by one to three bands were designated as highly related subtypes. Isolates with more than six bands different were considered different PFGE types. Among isolates subjected to PFGE, none differed by four to six bands (possibly related). Isolates were designated nontypeable if repeated attempts to prepare DNA failed.

RESULTS AND DISCUSSION

A total of 39 of 211 (18.5%) ESBL-KP isolates were resistant to ciprofloxacin (MIC, ≥4 μg/ml). These 39 isolates were obtained from 14 hospitals including hospitals N02 (6 isolates), N03 (6 isolates), N04 (6 isolates), M02 (10 isolates), and M04 (2 isolates), and there was one isolate each from hospitals N09, M01, M03, M05, E01, and S01 to S04. The frequency of ciprofloxacin resistance among hospitals submitting more than five ESBL-KP strains was 6 of 9 isolates submitted from N03 (67%), 6 of 22 from N02 (27%), 1 of 5 from M03 (20%), 1 of 6 from E01 (17%), 10 of 100 from M02 (10%), and 1 of 23 from M01 (4%).

Antimicrobial susceptibility. The MIC distribution of different quinolones for the 39 ciprofloxacin-resistant ESBL-KP isolates is shown in Table 1. Among the 39 isolates, 37 exhibited high-level ciprofloxacin resistance (MIC, ≥16 μg/ml). Cross-resistance between ciprofloxacin and levofloxacin, gatifloxacin, and gemifloxacin (resistant, ≥1 μg/ml), as well as garenoxacin (resistant, ≥8 μg/ml), was common. MICs of all of these agents were ≥4 μg/ml, and the MICs at which 50% of the isolates tested were inhibited were all ≥32 μg/ml. Sitafloxacin was the most active of the fluoroquinolones against these ciprofloxacin-resistant ESBL-KP isolates with MICs being ≤2 μg/ml for 67% of isolates (possible susceptibility).

Among other classes of antimicrobial agents, essentially complete cross-resistance was observed to trimethoprim-sulfamethoxazole, gentamicin, and tobramycin (Table 2). Amikacin remained active against 62% of isolates, and the carbapenems, imipenem and meropenem, were active against 100% of the ciprofloxacin-resistant ESBL-KP isolates (data not shown).

Molecular epidemiology. Ribotyping identified 18 distinct ribogroups among the 39 ciprofloxacin-resistant ESBL-KP isolates. Further characterization by PFGE identified six major strains (ribogroup-PFGE types were 255.3-E [nine isolates], 691.5-H [four isolates], 746.6-F [two isolates], 96.3-J [two isolates], 109.3-L [two isolates], and 95.5-M [two isolates]) encompassing 21 isolates (Table 2) (Fig. 1 and 2). The remaining 18 isolates represented unique ribogroup-PFGE types.

Among the six major strain types of ciprofloxacin-resistant ESBL-KP, strain 255.3-E was detected in the middle (hospital M02), northern (hospitals N02, N04, and N09), and southern (hospitals S02 and S04) regions, indicating spread not only within hospitals and regions but across regions as well. Likewise, strains 691.5-H (N04, M04, and M05) and 109.3-L (E01 and S03) were found in more than one hospital and more than one region, whereas strains 746.6-F (M02) and 95.5-M (N02) were localized to single hospitals. Notably, hospitals N02, N04, and M02 harbored at least two different strains of ciprofloxacin-resistant ESBL-KP. All of these isolates exhibited a multidrug-resistant phenotype.

The geographic distribution of ciprofloxacin-resistant ESBL-KP in Taiwan is striking and encompasses the entire country.

TABLE 2. Geographic distribution and antimicrobial resistance profile of the six major molecular strain types of ciprofloxacin-resistant ESBL-KP in Taiwan

Isolate[a]	Site[b]	Ribogroup	PFGE	Antibiogram[c]	MIC (μg/ml) of drug[d]:			
					CRO	CAZ	CIP	GAT
99	M02	255.3	E	RRRRR	>32	8	>32	>32
102	M02	255.3	E	RRRRR	>32	8	>32	>32
133	N02	255.3	E	RRRRR	>32	8	>32	16
140	N02	255.3	E	RRRRR	>32	8	>32	>32
112	M02	255.3	E	SRRRS	>32	>16	>32	>32
166	N04	255.3	E	SRRRS	>32	>16	>32	32
202	N09	255.3	E	SRRRS	16	>16	>32	>32
216	S02	255.3	E	RRRRS	16	>16	>32	>32
218	S04	255.3	E	RRRRS	>32	>16	>32	>32
171	N04	691.5	H	RRRRR	>32	>16	>32	16
188	N04	691.5	H	SRRRS	16	>16	>32	>32
126	M04	691.5	H	RRRRS	32	>16	>32	32
127	M05	691.5	H	RRRRS	32	>16	>32	12
26	M02	746.6	F	RRRRR	>32	8	>32	12
58	M02	746.6	F	SRRRR	>32	8	8	4
158	N03	96.3	J	SRRRS	32	>16	16	8
159	N03	96.3	J	SRRRS	16	>16	16	8
208	E01	109.3	L	RRRRS	8	>16	>32	>32
217	S03	109.3	L	RRRRR	>32	>16	>32	>32
136	N02	95.5	M	SRRRS	>32	>16	>32	>32
147	N02	95.5	M	SRRRS	32	>16	>32	4

[a] Isolate identification number.
[b] Participating hospitals in the middle (M), northern (N), southern (S), and eastern (E) regions of Taiwan.
[c] Antibiotype (S, susceptible; R, resistant) in the order tetracycline, trimethoprim-sulfamethoxazole, gentamicin, tobramycin, and amikacin.
[d] Abbreviations: CRO, ceftriaxone; CAZ, ceftazidime; CIP, ciprofloxacin; GAT, gatifloxacin.

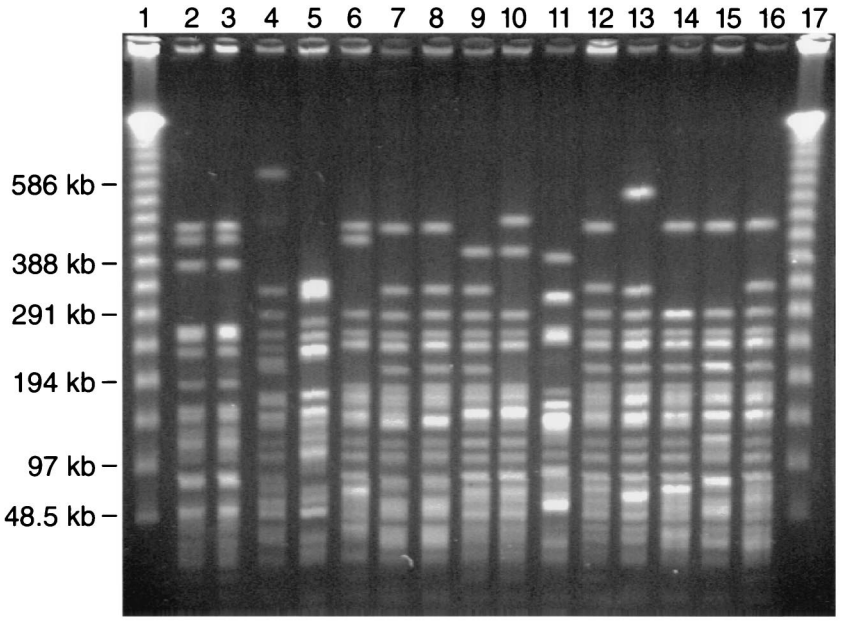

FIG. 1. *Spe*I-digested PFGE gel photo of ESBL-KP isolates within ribogroup 255.3 in Taiwan. Lanes 1 and 17, molecular size markers of lambda DNA ladder (in kilobases). Isolates, except in lanes 2, 5, and 11, were resistant to ciprofloxacin. PFGE type E patterns are shown in lanes 7 to 10 and 12 to 16. The isolates represented in this gel are 123, 122, 154, 5, 6, 99, 102, 133, 140, 135, 112, 166, 218, 216, and 202 (lanes 2 to 16, respectively).

The molecular typing studies indicate that the majority of the isolates with this multiresistant phenotype (ESBL and quinolone resistance) fall into one of six major molecular strain types. Furthermore, the clustering of these strain types indicates the presence of one epidemic strain with nationwide distribution (255.3-E) and other strains with more limited dissemination. Overall, the molecular and phenotypic data indicate the emergence of fluoroquinolone-resistant ESBL-KP in Taiwan that is likely the consequence of high utilization of quinolones coupled with breaks in local infection control prac-

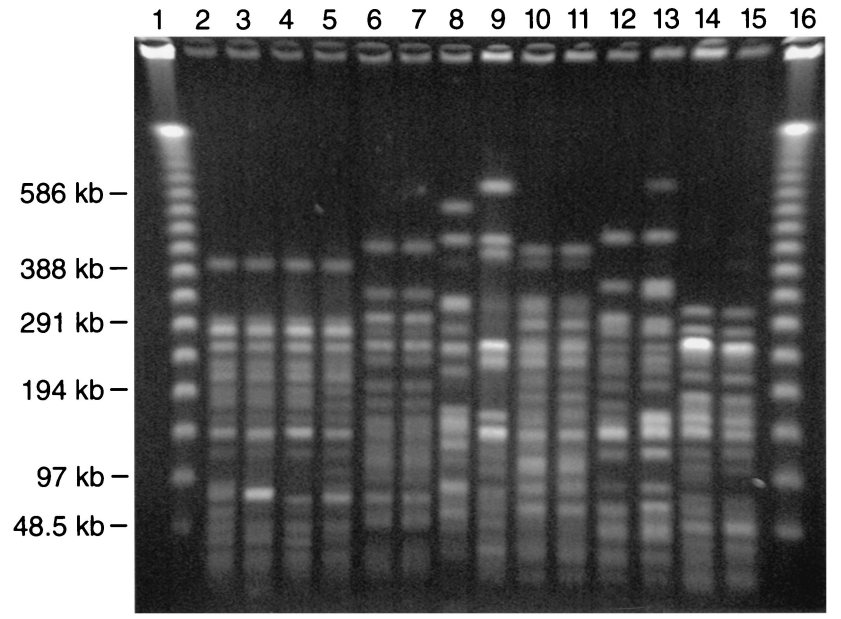

FIG. 2. *Spe*I-digested PFGE gel photo of ESBL-KP isolates within ribogroups 746.6 (lanes 2 to 5), 96.3 (lanes 10 and 11), 109.3 (lanes 12 and 13), and 95.5 (lanes 14 and 15). Lanes 1 and 16, lambda ladder molecular mass standards (in kilobases). Isolates, except in lanes 2, 5, 6, and 7, were resistant to ciprofloxacin. Strains of four PFGE types are shown: type F (lanes 2 to 5), type J (lanes 10 and 11), type L (lanes 12 and 13), and type M (lanes 14 and 15). Isolates in lanes 8 and 9 represent unique PFGE types. The isolates represented in this gel are 7, 26, 58, 190, 8, 9, 155, 157, 158, 159, 208, 217, 136, and 147 (lanes 2 to 15, respectively).

tices. Additionally, one must also consider the fact that clonal spread of ESBL-KP without quinolone resistance has also been described previously for Taiwan (12), and thus, the spread of ESBLs alone may also play a role in the emergence of quinolone resistance in *K. pneumoniae* as has been suggested by Brisse et al. (4) and Yuan et al. (14). Similar to our findings, intra- and interhospital spread of ciprofloxacin-resistant ESBL-KP has been reported before (4, 10, 14).

Although molecular evidence for nationwide distribution of an epidemic clone of ciprofloxacin-resistant ESBL-KP isolates has been presented, it is notable that, in the hospital with the highest frequency of ciprofloxacin resistance among ESBL-KP isolates (N03 [67%]), no evidence of clonal spread was detected. This pattern is highly suggestive of the emergence of resistance within several different strains of the same species due to the pressure of local quinolone use rather than faulty infection control. Thus, the key to controlling this nationwide epidemic of multiresistant *K. pneumoniae* must involve (i) improved infection control in all Taiwanese hospitals and (ii) limiting quinolone and extended-spectrum β-lactam usage to specific indications in an effort to minimize drug selection pressure.

In conclusion, emergence of fluoroquinolone-resistant ESBL-KP was identified in Taiwan. Molecular and phenotypic characterization of isolates has identified a nationwide epidemic clone, as well as institution-specific strains and unique strains. Given the clinical importance and expanding indications of the quinolones in the treatment of serious infections, it is imperative that this expansion of resistant strains be limited. These findings suggest not only that control efforts must not be confined to individual hospitals but also that interhospital cooperation in ensuring rigorous infection control efforts be combined with restricted quinolone and extended-spectrum β-lactam usage. The emergence of quinolone resistance among already broadly resistant *K. pneumoniae* (ESBL) highlights the importance of ongoing resistance surveillance at the local and national level.

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