

Extended-Spectrum β -Lactamases and Plasmid-Mediated AmpC Enzymes among Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* from Seven Medical Centers in Taiwan

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Production of extended-spectrum β -lactamases and plasmid-mediated AmpC enzymes was investigated among 291 *Escherichia coli* and 282 *Klebsiella pneumoniae* isolates that showed decreased susceptibilities to extended-spectrum cephalosporins from seven Taiwanese medical centers. CTX-M-type and SHV-type enzymes were the most prevalent extended-spectrum β -lactamases. CMY-2-like and DHA-1-like β -lactamases were the most prevalent AmpC-type enzymes.

The increasing prevalence of extended-spectrum β -lactamases (ESBLs) and plasmid-mediated AmpC β -lactamases in members of the family *Enterobacteriaceae* is a matter of great concern worldwide (1, 2, 10). In Taiwan, TEM-, SHV-, and CTX-M-type ESBLs and CMY- and DHA-type AmpC β -lactamases have been reported in *Escherichia coli* and *Klebsiella pneumoniae* isolates from a few individual institutions (3, 17–20). The present multicenter study was conducted to determine the distribution of ESBLs and AmpC β -lactamases among clinical isolates of *E. coli* and *K. pneumoniae* that showed decreased susceptibilities to extended-spectrum cephalosporins in Taiwan.

Isolates that were suspected of ESBL production by the CLSI (formerly NCCLS) screening method (8) with disks of ceftazidime, cefotaxime, ceftriaxone, aztreonam, and/or cefpodoxime were considered to exhibit decreased susceptibilities to extended-spectrum cephalosporins and consecutively collected between March and August 2003 from seven medical centers in Taiwan. These hospitals were chosen because they represent the largest referral medical centers in each region of Taiwan. A total of 291 *E. coli* and 282 *K. pneumoniae* isolates were obtained (Table 1). Each isolate came from a unique patient.

All isolates were tested for ESBL production by the CLSI recommended disk diffusion confirmatory test (8), and 171 *E. coli* and 260 *K. pneumoniae* isolates were considered ESBL producers. All putative non-ESBL producers were tested further by the double-disk synergy test with ceftazidime, cefo-

taxime, aztreonam, and cefepime disks placed 20 mm from an amoxicillin-clavulanate disk (13). The method has been shown to be capable of detecting false negatives which are caused by coproduction of different classes of β -lactamases in the CLSI method (20). Five *E. coli* and five *K. pneumoniae* isolates gave a positive result in the double-disk synergy test. Thus, ESBL production was detected in 176 (60.5%) of the 291 *E. coli* isolates and 265 (94.0%) of the 282 *K. pneumoniae* isolates.

The expression of β -lactamases was detected by isoelectric focusing as described previously (6, 18). PCR was performed with the previously reported oligonucleotide primers to detect *bla*_{TEM} (5), *bla*_{SHV} (9), and *bla* genes related to *bla*_{CTX-M-1} (12), *bla*_{CTX-M-9} (12), *bla*_{CMY-1} (17), *bla*_{CMY-2} (16), *bla*_{DHA-1} (4), and *bla*_{OXA-10} (14). The PCR-NheI method was used to discriminate between *bla*_{SHV-ESBL} and *bla*_{SHV-non-ESBL} genes (9).

Overall, the production of ESBLs and AmpC-like enzymes was confirmed in 60.5% (176 isolates) and 43.6% (127 isolates), respectively, of the 291 *E. coli* isolates, and in 94.0% (265 isolates) and 14.5% (41 isolates), respectively, of the 282 *K. pneumoniae* isolates (Table 1). Thirty-five (12.0%) *E. coli* isolates and 46 (16.3%) *K. pneumoniae* isolates harbored two or three β -lactamases involved in extended-spectrum cephalosporin resistance. CTX-M-type ESBLs and CMY-2-related AmpC enzymes were predominant in *E. coli*, while SHV- and CTX-M-type ESBLs, and DHA-1-like AmpC enzymes were most prevalent in *K. pneumoniae* (Table 1). Among the CTX-M-type ESBLs, the CTX-M-1 and CTX-M-9 groups were predominant in *K. pneumoniae* and in *E. coli*, respectively. All the above enzymes showed a nationwide distribution.

Among the 10 ESBL-producing isolates negative for the CLSI confirmatory method, all five *E. coli* isolates coproduced a CMY-2-related enzyme and an SHV-5-related ESBL, and all

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TABLE 1. Distribution of ESBLs and AmpC enzymes among 291 *E. coli* and 282 *K. pneumoniae* isolates that showed decreased susceptibilities to extended-spectrum cephalosporins from seven medical centers in Taiwan

| Species and ESBL or AmpC | pI of β -lactamase | No. of isolates from hospital ^a : | | | | | | | Total no. (%) of isolates |
|--------------------------|--------------------------|--|----|----|----|----|----|----|---------------------------|
| | | N1 | N2 | C1 | C2 | C3 | S | E | |
| <i>E. coli</i> | | 78 | 33 | 84 | 41 | 18 | 19 | 18 | 291 |
| CMY-2-like | 9.0 | 57 | 0 | 40 | 13 | 8 | 1 | 8 | 127 (43.6) |
| CTX-M | | 21 | 28 | 47 | 28 | 8 | 17 | 9 | 158 (54.3) |
| CTX-M-1 group | 8.4 or 8.6 | 5 | 4 | 19 | 5 | 2 | 2 | 4 | 41 (14.1) |
| CTX-M-9 group | 7.9 or 8.0 | 16 | 24 | 28 | 23 | 6 | 15 | 5 | 117 (40.2) |
| SHV-5-like | 8.2 | 10 | 3 | 5 | 4 | 2 | 3 | 0 | 27 (9.3) |
| None ^b | | 1 | 3 | 4 | 1 | 3 | 0 | 2 | 14 (4.8) |
| <i>K. pneumoniae</i> | | 58 | 59 | 78 | 18 | 23 | 37 | 9 | 282 |
| CMY-2-like | 9.0 | 4 | 1 | 4 | 0 | 0 | 1 | 0 | 10 (3.5) |
| DHA-1-like | 7.8 | 17 | 1 | 5 | 1 | 3 | 4 | 0 | 31 (11.0) |
| CTX-M | | 21 | 21 | 67 | 15 | 16 | 10 | 5 | 155 (55.0) |
| CTX-M-1 group | 8.4 | 12 | 7 | 44 | 8 | 14 | 8 | 5 | 98 (34.8) |
| CTX-M-9 group | 7.9 or 8.0 | 9 | 14 | 23 | 7 | 2 | 2 | 0 | 57 (20.2) |
| SHV | | 34 | 39 | 18 | 1 | 7 | 32 | 4 | 135 (47.9) |
| SHV-2-like | 8.2 | 0 | 5 | 1 | 1 | 0 | 2 | 0 | 9 (3.2) |
| SHV-5-like | 7.6 | 34 | 34 | 17 | 0 | 7 | 30 | 4 | 126 (44.7) |
| None | | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 5 (1.8) |

^a The seven medical centers were Triservice General Hospital (N1) and National Taiwan University Hospital (N2) in northern Taiwan, Taichung Veterans General Hospital (C1), China Medical College Hospital (C2), and Chung Shan Medical University Hospital (C3) in central Taiwan, Chi-Mei Medical Center (S) in southern Taiwan, and Buddhist Tzu Chi General Hospital (E) in eastern Taiwan. Hospitals C1 and S are district general hospitals, and the remainder are university hospitals.

^b ESBLs and AmpC-like enzymes not detected.

five *K. pneumoniae* isolates coproduced a DHA-1-related enzyme and an SHV-5-related ESBL. The presence of additional β -lactamases (non-ESBL, non-AmpC-like) was also suggested by PCR and isoelectric focusing. They were pI 5.4 TEM-1-like (238 *E. coli* isolates and 194 *K. pneumoniae* isolates), pI 6.1 OXA-10-like (5 *K. pneumoniae* isolates), and pI 7.6 SHV-1-like (all 282 *K. pneumoniae* isolates) β -lactamases. No β -lactamase was detected in three *E. coli* isolates, and no CMY-1-related enzymes were detected in any isolate.

PCR products from randomly selected isolates were sequenced on both strands. The sequences of selected amplicons of *bla*_{DHA-1}, *bla*_{CMY-2}, and *bla*_{OXA-10}-related genes were all identical to those of *bla*_{DHA-1} (23 isolates), *bla*_{CMY-2} (16 isolates), and *bla*_{OXA-10} (5 isolates), respectively. The results of sequence analysis of 251 *bla*_{CTX-M}-positive isolates are shown in Table 2. Seven *bla*_{CTX-M} subtypes were identified, which were *bla*_{CTX-M-3}, *bla*_{CTX-M-9}, *bla*_{CTX-M-14}, *bla*_{CTX-M-15}, *bla*_{CTX-M-17}, *bla*_{CTX-M-19}, and an unpublished *bla*_{CTX-M-9}-related gene, *bla*_{CTX-M-38} (GenBank accession no. AY822595). *bla*_{CTX-M-3} and *bla*_{CTX-M-14} were the most common *bla*_{CTX-M} subtypes in *K. pneumoniae* and *E. coli*, respectively.

*bla*_{CTX-M-17}, *bla*_{CTX-M-19}, and *bla*_{CTX-M-38} were first identified in Taiwan. The sequence of *bla*_{CTX-M-38} first deposited in the GenBank database was obtained from a *K. pneumoniae* strain from China. *bla*_{CTX-M-38} differs from *bla*_{CTX-M-14} by a C-to-G change at position 669 of the structural gene, leading to a Ser220Arg substitution. In the susceptibility test by the standard agar dilution method (7), our *bla*_{CTX-M-38}-positive isolate showed high-level resistance to cefotaxime (MIC, >128 μ g/ml) and decreased susceptibilities to ceftazidime (MIC, 8 μ g/ml), aztreonam (MIC, 32 μ g/ml), and cefepime (MIC, 16 μ g/ml) and was susceptible to cefoxitin (MIC, 16 μ g/ml) and imipenem (MIC, 1 μ g/ml). The resistance phenotype is similar to those of reported CTX-M-14-producing strains (1).

Isolates carrying *bla*_{CTX-M-3}, *bla*_{CTX-M-14}, or *bla*_{CMY-2} were randomly selected from the seven hospitals for enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) analysis with primer ERIC2 (15). Each unique pattern was defined as an ERIC-PCR type. Thirteen, 11, and 10 types were observed among 20 CTX-M-14-producing isolates, 16 CTX-M-3-producing isolates, and 15 CMY-2-producing isolates, respectively, in *E. coli*, and 11 and 14 types were observed among 16 CTX-M-14-producing isolates and 21 CTX-M-3-producing isolates, respectively, in *K. pneumoniae*. Clonal relationships be-

TABLE 2. *bla*_{CTX} subtypes detected in randomly selected *E. coli* (*n* = 128) and *K. pneumoniae* (*n* = 123) isolates from seven medical centers in Taiwan

| Species and <i>bla</i> _{CTX-M} | No. of isolates from hospital ^a : | | | | | | | Total no. of isolates |
|---|--|----|----|----|----|----|---|-----------------------|
| | N1 | N2 | C1 | C2 | C3 | S | E | |
| <i>E. coli</i> | | | | | | | | |
| <i>bla</i> _{CTX-M-1} group | | | | | | | | |
| <i>bla</i> _{CTX-M-3} | 4 | 3 | 15 | 4 | 2 | 2 | 3 | 33 |
| <i>bla</i> _{CTX-M-15} | | | 1 | 1 | | | | 2 |
| <i>bla</i> _{CTX-M-9} group | | | | | | | | |
| <i>bla</i> _{CTX-M-9} | | 1 | | | 1 | 2 | | 4 |
| <i>bla</i> _{CTX-M-14} | 12 | 17 | 21 | 14 | 3 | 14 | 7 | 88 |
| <i>bla</i> _{CTX-M-19} | | | 1 | | | | | 1 |
| <i>K. pneumoniae</i> | | | | | | | | |
| <i>bla</i> _{CTX-M-1} group | | | | | | | | |
| <i>bla</i> _{CTX-M-3} | 8 | 6 | 41 | 4 | 14 | 5 | 4 | 82 |
| <i>bla</i> _{CTX-M-9} group | | | | | | | | |
| <i>bla</i> _{CTX-M-14} | 5 | 7 | 12 | 4 | 2 | 3 | | 33 |
| <i>bla</i> _{CTX-M-17} | 1 | 2 | 1 | 1 | | | | 5 |
| <i>bla</i> _{CTX-M-19} | | 2 | | | | | | 2 |
| <i>bla</i> _{CTX-M-38} | | | | 1 | | | | 1 |

^a See Table 1, footnote a.

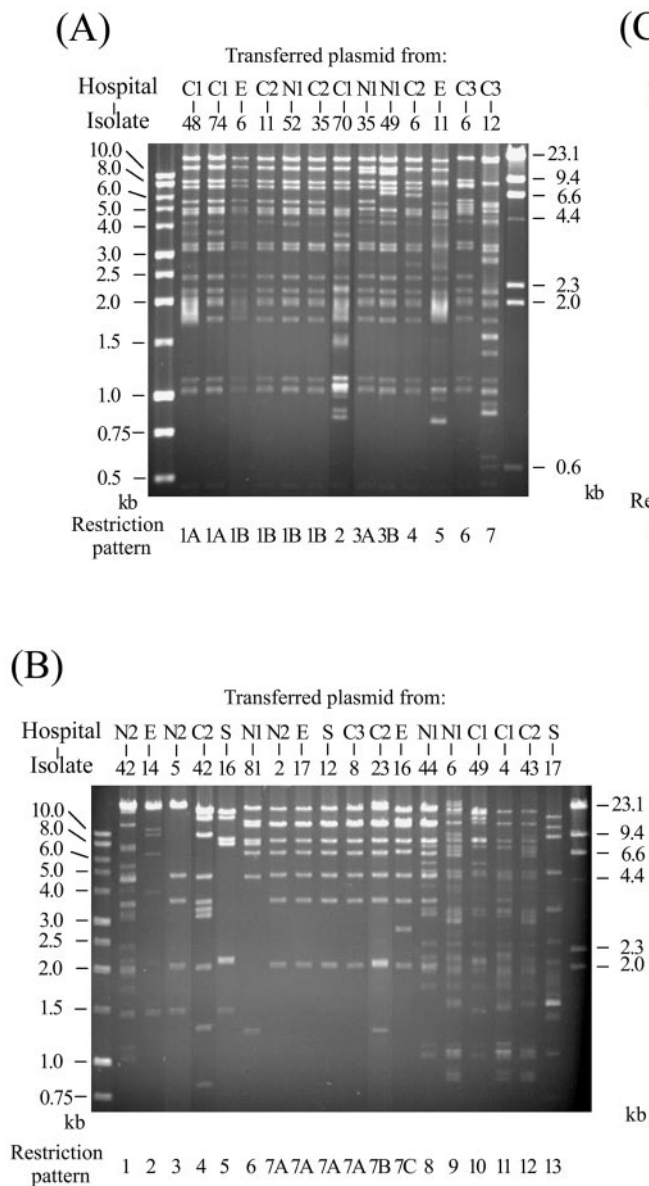


FIG. 1. EcoRI restriction patterns of conjugative plasmids transferred from 13 CMY-2-producing *E. coli* isolates (A), 18 CTX-M-14-producing *E. coli* isolates (B), and 17 CTX-M-3-producing *K. pneumoniae* isolates (C). The numbers designating the donor isolates are shown above the gels, and numbers designating restriction patterns are shown below the gels.

ing. Plasmids from the transconjugants were extracted and analyzed by agarose gel electrophoresis after digestion with the endonuclease EcoRI. Identical or similar restriction patterns were observed among the transferred plasmids from the isolates collected from different hospitals (Fig. 1), suggesting that horizontal transfer of similar resistance plasmids could play an important role in the wide spread of the resistance genes in Taiwan.

In conclusion, this study demonstrated the widespread occurrence of ESBLs and plasmid-mediated AmpC enzymes in *E. coli* and *K. pneumoniae* in Taiwan and particularly points out the high prevalence and increasing diversity of CTX-M-type ESBLs. Our results underline the need for continuous surveillance of the prevalence and evolution of these enzymes in Taiwan.

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tween isolates with the same ERIC-PCR type were further confirmed by pulsed-field gel electrophoresis with chromosomal DNA restricted by XbaI as described previously (18). No interhospital clonal dissemination of strains carrying the three most common resistance genes was observed (data not shown).

Selected isolates belonging to different lineages (as per the results of ERIC-PCR typing) were subjected to the liquid mating-out assay as described previously (11, 19). Transconjugants were selected on tryptic soy agar plates containing streptomycin (512 μ g/ml) and ceftazidime (2 μ g/ml) or cefotaxime (2 μ g/ml). Thirteen, 18, and 17 transconjugants were obtained from 15 CMY-2-producing *E. coli* isolates, 20 CTX-M-14-producing *E. coli* isolates, and 21 CTX-M-3-producing *K. pneumoniae* isolates, respectively.

The presence of transferred relevant β -lactamase genes in transconjugants was confirmed by PCR and isoelectric focus-

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