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# Colistin Resistance Mechanisms in *Klebsiella pneumoniae* Strains from Taiwan

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**Colistin is one of the antibiotics of last resort for the treatment of carbapenem-resistant *Klebsiella pneumoniae* infection. This study showed that capsular type K64 (50%) and ST11 (53.9%) are the prevalent capsular and sequence types in the colistin-resistant strains in Taiwan. The interruption of transcripts (38.5%) and amino acid mutation (15.4%) in *mgrB* are the major mechanisms contributing to colistin resistance. In addition, novel single amino acid changes in MgrB (Stop48Tyr) and PhoQ (Leu26Pro) were observed to contribute to colistin resistance.**

*Klebsiella pneumoniae* is an important human pathogen that causes several hospital-acquired and community-acquired diseases (1, 2). Although carbapenem is generally used to treat infections caused by extended-spectrum β-lactamase (ESBL)-carrying *K. pneumoniae* (3), *K. pneumoniae* strains carrying carbapenemases or ESBL strains combined with the loss of porins can result in carbapenem-resistant *K. pneumoniae* (CRKP) (4–6). To eradicate CRKP, colistin and tigecycline are typically used to treat patients (7). Unfortunately, resistance to colistin and tigecycline has also been reported, with a 17% resistance rate of CRKP to colistin in Taiwan (8). A surveillance study also revealed that 43% of carbapenemase-producing *K. pneumoniae* isolates were resistant to colistin in Italy (9).

Colistin, also called polymyxin E, is a cationic antimicrobial peptide that targets bacterial lipopolysaccharide (LPS), causing cell membrane leakage (10). Previous studies have demonstrated that the modification of lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine neutralizes the negative charge and reduces susceptibility to colistin in *Enterobacteriaceae* (11–15). Modification of Ara4N is achieved by the *pmrHFIJKLM* operon (13), and the two-component systems PhoPQ and PmrAB with connector PmrD are involved in the regulation of the *pmrHFIJKLM* operon (16, 17). Moreover, MgrB is a negative regulator that influences PhoQ-PhoP phosphorylation (18–20). In this study, we analyzed capsular type and multilocus sequence type (MLST) distribution of colistin-resistant *K. pneumoniae* in Taiwan and attempted to define the mechanisms of resistance to colistin.

Colistin-resistant *K. pneumoniae* strains were retrospectively collected from patients in the Taipei Veterans General Hospital (VGH) from February to August 2013. All 26 strains were clinical isolates and were isolated from different patients. Among the 26 clinical isolates, the Col14 and Col40 strains were CRKP, and only the Col14 strain harbored the carbapenemase KPC. Colistin was used to treat infections in 16 of the 26 patients prior to the isolation of strains, and the other patients did not receive colistin in the VGH (Table 1). However, we could not trace the colistin usage of these patients in other hospitals. Previous studies also showed that some colistin-resistant strains are isolated from healthy individuals (21). Resistance to colistin in strains that were not exposed to colistin might be due to spontaneous mutations of genomic DNA or transmission from other individuals. The occurrence of colistin-resistant isolates from bovine mastitis was reported (22), and colistin is also used to treat animals in Taiwan. Therefore, livestock that received colistin might be possible sources of transmission. The MICs to colistin of these 26 strains were determined by agar dilution according to the instructions of the Clinical and Laboratory Standards Institute (CLSI), and ATCC 25922 was used as a quality control. Unexpectedly, the MICs to colistin of 30.8% (8/26) of the strains were ≥512 μg/ml (Table 1).

To investigate the epidemiology of colistin-resistant *K. pneumoniae* strains at the Taipei VGH, the capsular types of these strains were determined using *wzc* genotyping, as in our previous studies (23, 24). The sequences of 7 loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) were amplified using PCR, and sequence types (STs) were determined by sequence alignment using the Pasteur Institute database (http://www.pasteur.fr/mlst/Kpneumoniae.html). The results showed that half of the strains (13/26) were K64, 11.5% (3/26) were K24, and 7.7% (2/26) were K54 (Table 1). According to the MLST results, 53.9% (14/26) of the strains were ST11, 15.4% (4/26) were ST15, and 7.7% (2/26) were ST421 (Table 1). Although most of the colistin-resistant strains were related clones, except for Col4 and Col22, our strains did not consist of identical strains (Table 1; see also Table S2 in the supplemental material). As in a previous study (25), this study also showed correlations between capsular type and ST. The predominant capsular type of colistin-resistant *K. pneumoniae* in Taiwan was K64, and the MLST results revealed that about half of the colistin-resistant isolates were ST11, similar to the results of a previous report in Spain (26). The same results for capsular types and STs were also ob-

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**TABLE 1** Characteristics and amino acid changes in MgrB, PhoP, PhoQ, and PmrB in colistin-resistant strains

| Strain | Source | Colistin usage in VGH | MIC | Capsular type | MLST | mRNA relative fold change (mean ± SD) | | | Insertion sites in the *mgrB* region[a] | MgrB[b] | | PhoP[b] | | PhoQ[b] | | | PmrB[b] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *pmrH*[c] | *pmrK*[d] | *mgrB*[e] | | 28 | 48 | 3 | 86 | 26 | 150 | 258 | 157 | 256 | 280 |
| Col4 | Blood | Yes | >2,048 | K64 | ST11 | 9.38 ± 0.30 | | | | | | | | | | | | Gly | |
| Col7 | Blood | Yes | 1,024 | K64 | ST11 | 4.61 ± 0.16 | | | | | | | | | | | | Gly | |
| Col20 | Sputum | | 2,048 | K64 | ST11 | 4.41 ± 0.08 | | | | | | | | | | | | Gly | |
| Col21 | Sputum | Yes | 512 | K64 | ST11 | 2.61 ± 0.14 | | | | | | | | | | | | Gly | |
| Col22 | Sputum | | 2,048 | K64 | ST11 | 2.73 ± 0.02 | | | | | | | | | | | | Gly | |
| Col36 | Sputum | Yes | 2,048 | K64 | ST11 | 2.92 ± 0.13 | | | | | | | | | | | | Gly | |
| Col44 | Sputum | | 512 | K64 | ST11 | 5.66 ± 0.05 | | | | | | | | | | | | Gly | |
| Col49 | Urine | | 128 | K64 | ST11 | 10.92 ± 0.94 | | | | Tyr | | | | | Gly | | | Gly | |
| Col38 | Urine | | 64 | K64 | ST11 | 25.40 ± 0.67 | | | | | | | | Pro | | | | | |
| Col13 | Sputum | Yes | 128 | K64 | ST11 | 5.33 ± 0.04 | | | Deletion[f] | | | | | | | | | | |
| Col25 | Bile | | 64 | K64 | ST11 | 5.35 ± 0.14 | | | IS10R in coding region (+68 to +76) | | | | | | | | | | |
| Col27 | Sputum | Yes | 128 | K64 | ST11 | 14.68 ± 0.97 | | 0.07 ± 0.02 | IS10R in promoter region (−27) | | | | | | | | | | |
| Col33 | Sputum | Yes | 128 | K64 | ST11 | 16.52 ± 1.12 | | | IS10R in coding region (+68 to +76) | | | | | | | | | | |
| Col11 | Sputum | Yes | 64 | K24 | ST15 | 2.16 ± 0.06 | | 0.01 ± 0.002 | IS5-like in promoter region (−8)[g] | | | | | | | | | | |
| Col37 | Pus | Yes | 16 | K24 | ST15 | 6.17 ± 0.13 | | | | | Tyr | | | | | | | | |
| Col40 | Pus | Yes | 64 | K24 | ST15 | 9.04 ± 0.03 | | | | | Tyr | | | | | Phe | | | |
| Col23 | Urine | Yes | 32 | K54 | ST15 | 2.01 ± 0.02 | | | | | | | | | | | Pro | | |
| Col28 | Urine | | >2,048 | K54 | ST29 | 5.9 ± 0.13 | | | | | | Phe | | | | | | | |
| Col6 | Urine | Yes | 64 | K62 | ST48 | 3.16 ± 0.16 | | | | | | | Leu | | | | Pro | | Leu |
| Col14 | Blood | Yes | 128 | K47 | ST11 | 2.83 ± 0.12 | | | Deletion[f] | | | | | | | | | | |
| Col19 | Urine | Yes | 64 | K28 | ST37 | 3.69 ± 0.06 | | 0.05 ± 0.004 | IS10R in promoter region (−27) | | | | | | | | | | |
| Col26 | Sputum | Yes | 32 | K3 | ST13 | 17.97 ± 0.44 | | 0.19 ± 0.02 | IS5-like in promoter region (−34)[g] | | | | | | | | | | |
| Col32 | Sputum | | 64 | K1 | ST23 | 21.02 ± 1.85 | | | IS5-like in coding region (+75 to +83)[g] | | | | | | | | | | |
| Col24 | Sputum | | 32 | Unknown K-type | ST421 | 0.22 ± 0.01 | 10.63 ± 0.69 | | | | Tyr | | | | | | | | |
| Col31 | Sputum | Yes | 128 | Unknown K-type | ST421 | 0.42 ± 0.07 | 12.72 ± 0.02 | 0.01 ± 0.001 | IS5-like in promoter region (−35)[g] | | | | | | | | | | |
| Col5 | Pus | | 8 | KN3 | ST11-like | 0.34 ± 0.02 | 4.34 ± 1.31 | | | | | | | | | | | Gly | |

[a] The nucleotide position aligned with genomic DNA of NTUH-K2044 and IS element-inserted sites.
[b] The amino acid positions of mutations in MgrB, PhoP, PhoQ, and PmrB.
[c] The *pmrH* mRNA relative fold changes (against NTUH-K2044 strain) of the other 7 colistin-susceptible strains were 0.6 ± 0.02, 1.11 ± 0.02, 1.68 ± 0.11, 0.59 ± 0.04, 1.65 ± 0.07, 0.37 ± 0.01, and 0.9 ± 0.04.
[d] The *pmrK* mRNA relative fold changes (against NTUH-K2044 strain) of the other 7 colistin-susceptible strains were 4.74 ± 0.69, 0.79 ± 0.28, 1.14 ± 0.51, 6.98 ± 0.82, 0.85 ± 0.08, 3.77 ± 0.78, and 2.01 ± 0.18.
[e] The *mgrB* mRNA relative fold changes (against NTUH-K2044 strain) of the other 7 colistin-susceptible strains were 0.41 ± 0.06, 1.19 ± 0.17, 1.67 ± 0.64, 0.52 ± 0.11, 1.04 ± 0.13, 0.51 ± 0.03, and 0.66 ± 0.11.
[f] No PCR product was detected, and the deletion site is not known.
[g] The IS5-like elements of Col11, Col26, Col32, and Col31 were 95%, 99%, 95%, and 95% identical to the IS5 element.

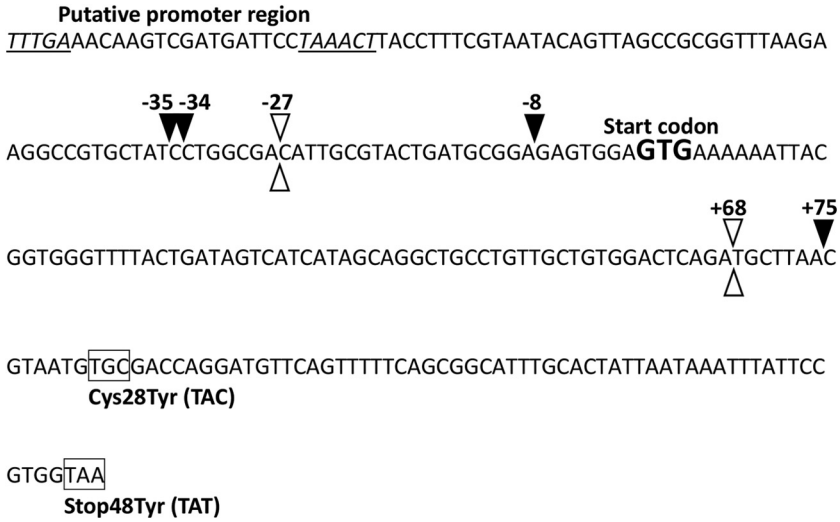


**FIG 1** IS inserted sites and mutations in MgrB were found in this study. In the alignment with sequences of the NTUH-K2044 strain, the open triangle indicates the target sites of IS10R, and the closed triangle indicates the target sites of IS5-like elements. The nucleotides in the open box represent the amino acid changes Cys28Tyr and Stop48Tyr of MgrB. The start codon of MgrB is in bold.

served for CRKP in Taiwan (24). According to our recent study, K64-specific bacteriophage and capsule depolymerase could be alternative choices for eradicating CRKP and colistin-resistant *K. pneumoniae* (24).

Previous studies have demonstrated that elevated expression of the *pmrHFIJKLM* operon increased modification of LPS and contributed to colistin resistance (19, 27). In the present study, the expression of *pmrH* mRNA in 26 colistin-resistant strains and 8 colistin-susceptible strains was quantified four times using reverse transcription-quantitative PCR (RT-qPCR), and primers for RT-qPCR are listed in Table S1 in the supplemental material. The relative fold change in RNA expression against a colistin-susceptible strain, NTUH-K2044, was calculated based on the $\Delta\Delta C_T$ value. The results revealed that 88.5% (23/26) of the colistin-resistant strains (all except Col5, Col24 and Col31) had significantly higher expression levels of *pmrH* mRNA than the colistin-susceptible strains (Table 1). The expression of *pmrK*, another gene of the *pmrHFIJKLM* operon, was also quantified in strains Col5, Col24, and Col31. The mRNA expression of *pmrK* in Col24 and Col31 was significantly higher than in the colistin-susceptible strains (Table 1). These data suggest that LPS modification by PmrHFIJKLM is a major (25/26) mechanism of colistin resistance in Taiwan. However, *pmrH* mRNA levels in the Col24 and Col31 strains were not consistent with the *pmrK* mRNA levels, indicating that this operon may have internal promoters or transcription termination sites and that modification of LPS depends on an alteration of the structural conformation. Conversely, the Col5 strain did not exhibit increased expression of the *pmrHFIJKLM* operon, and a lack of LPS was also reported to decrease the susceptibility to colistin in *Acinetobacter baumannii* (28, 29). Therefore, the LPS of the Col5 strain was isolated by the hot phenol method and detected using silver staining as in a previous study (30, 31). However, LPS was still detectable in the Col5 strain (data not shown), and the resistance mechanism(s) of the Col5 strain are still unclear.

Recent studies have demonstrated that the insertion sequence (IS) into *mgrB* disinhibits PhoQ phosphorylation, resulting in increased expression of *pmrHFIJKLM* mRNA and leading to reduced susceptibility to colistin in *K. pneumoniae* (18, 19). Therefore, we used PCR to examine whether the *mgrB* gene in our colistin-resistant strains was interrupted by IS elements. Among the 26 colistin-resistant strains, IS elements in the *mgrB* promoter region and coding region were detected in 5 and 3 strains (DDBJ accession no. LC016697 to LC016704), respectively (Fig. 1). As in previous studies (18, 20, 21, 32, 33), the *mgrB* region of these 8 strains was interrupted by IS10R and IS5-like elements. To demonstrate an effect on *mgrB* expression after IS element insertion in the promoter, the expression of *mgrB* mRNA was quantified by RT-qPCR, and the expression of *mgrB* was significantly reduced in those 5 strains (Table 1). In addition, *mgrB* was not detected in the Col13 or Col14 strains by PCR and Southern blotting (see Fig. S1 in the supplemental material). Therefore, IS elements or the deletion of *mgrB* was associated with resistance to colistin in 38.5% (10/26) of the isolates. This result is consistent with that of a previous study that reported a similar rate of colistin-resistant strains with IS element insertion or deletion of *mgrB* (18, 21).

MgrB regulates the *pmrHFIJKLM* operon through a signaling cascade of PhoPQ, PmrD, and PmrAB (34). Previous studies demonstrated that Leu82Arg and Thr157Pro mutations in PmrB and Leu24His, Cys28Tyr, and Gly37Ser mutations in MgrB can reduce colistin susceptibility in *K. pneumoniae* (18, 27, 33). To recognize which mutations in the regulators of the *pmrHFIJKLM* operon contribute to resistance to colistin, sequences of genes encoding regulators (MgrB, PmrA, PmrB, PmrD, PhoP, and PhoQ) in 26 colistin-resistant strains were analyzed (the primers used for PCR mapping and sequencing are listed in Table S1 in the supplemental material) and compared with sequences of 5 colistin-susceptible strains isolated from the National Taiwan University Hospital, excluding polymorphisms of colistin-susceptible strains. After comparing the sequences, identical amino acid sequences for PmrA and PmrD were observed, and possible substitutions in MgrB, PmrB, PhoP, and PhoQ that may be responsible for colistin resistance were defined (Table 1). As in previous studies (18, 27), PmrB Thr157Pro was detected in the Col6 and Col23 strains, and MgrB Cyc28Tyr was detected in the Col49 strain.

**TABLE 2** MICs of colistin in site-directed mutants of NTUH-K2044

| Strain | MIC to colistin | *pmrH* mRNA relative fold change (mean ± SD) |
|---|---|---|
| NTUH-K2044 | | |
| Wild type | 1 | 1 |
| MgrB Stop48Tyr | 32 | 5.30 ± 1.32 |
| PhoP Val3Phe | 1 | |
| PhoP Ser86Leu | 1 | |
| PhoQ Leu26Pro | 32 | 39.64 ± 6.64 |
| PhoQ Asp150Gly | 1 | |
| PhoQ Val258Phe | 1 | |
| PmrB Arg256Gly | 1 | |
| ATCC 25922[a] | 1 | |

[a] ATCC 25922 served as a quality control.

Rescue with a plasmid carrying functional (wild-type) PmrB and MgrB was used to examine whether mutations in PmrB and MgrB contribute to colistin resistance as in previous studies (18, 27, 33); however, high-copy-number plasmid rescue may not reflect the real situation. Here, we speculate that PmrB Val280Leu may not be a critical mutation for colistin resistance because both leucine and valine are nonpolar and branched-chain amino acids. To confirm whether PmrB (Arg256Gly), PhoQ (Leu26Pro, Asp150Gly, and Val258Phe), PhoP (Val3Phe and Ser86Leu), and MgrB stop codon changed to tyrosine (Stop48Tyr) contribute to resistance to colistin, site-directed mutations in a colistin-susceptible strain, NTUH-K2044 (MICs = 1), were generated by the pKO3-km plasmid (35). These DNA fragments, including point mutation sites and flanking regions, were amplified by PCR (primers are listed in Table S1 in the supplemental material) and were cloned into the pKO3-km plasmid. The resulting plasmid was used to generate point mutants as in the previous study, and the mutants were confirmed by sequencing (36). Both the PhoQ Leu26Pro and MgrB Stop48Tyr mutations increased the MICs to colistin by 32-fold (Table 2). These two substitutions enhanced *pmrH* mRNA expression in wild-type NTUH-K2044 by 39.6-fold and 5.3-fold, respectively (Table 2). However, replacement of the other amino acids did not alter the MICs in NTUH-K2044, possibly due to different genomic backgrounds between strains, and secondary factor(s) are required in addition to these amino acid alterations, thus contributing to colistin resistance.

PhoPQ is a two-component system, and PhoQ can sense $Mg^{2+}$ signals. The homolog of PhoQ has been well studied in *Escherichia coli*. According to an amino acid alignment, substitutions at position 26 of PhoQ in *K. pneumoniae* may be located in the transmembrane domain, and substitutions in this domain may influence protein conformation or oligomer stability, affecting PhoQ autokinase activity, phosphate transfer, and phosphatase ability (37–39). MgrB is a 47-amino acid-long peptide, and position 47 is located in the periplasmic domain, which is important for interacting with PhoQ (40). The mutation of a stop codon and the resulting 15-amino-acid prolongation of MgrB in 3 strains (DDBJ accession no. LC016506 to LC016508) may interfere with the PhoQ interaction.

MICs to colistin of 8 strains were unexpectedly ≥512 μg/ml in this study (Table 1). Nonetheless, the expression of *pmrH* mRNA was significantly higher than in colistin-susceptible strains. However, the mechanisms of colistin resistance and increased *pmrH* mRNA expression in these strains were not clarified in this study. A recent study showed that the newly described two-component system CrrAB was involved in colistin resistance, and the results implied that pathways in addition to the PhoPQ-PmrAB pathway can reduce susceptibility to colistin (41). Multiple resistance mechanisms might be combined to induce high resistance to colistin in *K. pneumoniae*.

In conclusion, capsular type K64 and ST11 are the prevalent capsular and sequence types in colistin-resistant strains of Taiwan, and interruptions in *mgrB* that increase expression of the *pmrHFIJKLM* operon are major mechanisms contributing to colistin resistance. Moreover, this is the first observation of the single amino acid changes MgrB Stop48Tyr and PhoQ Leu26Pro, which cause colistin resistance.

**Nucleotide sequence accession numbers.** Sequences have been deposited in DDBJ under accession numbers LC016506 to LC016508 and LC016697 to LC016704.

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