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Preservation of Acquired Colistin Resistance in Gram-Negative Bacteria

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Colistin-resistant mutants were obtained from 17 colistin-susceptible strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. The stability of colistin resistance in these mutants was investigated. Three of four colistin-resistant *P. aeruginosa* mutants recovered colistin susceptibility in colistin-free medium; however, colistin-susceptible revertants were obtained from only one strain each of *A. baumannii* and *E. coli*. No susceptible revertants were obtained from *K. pneumoniae* mutants.

Colistin resistance has been observed in Gram-negative pathogens (1–3). Colistin resistance is mediated by mutations in the PmrAB or PhoPQ two-component regulatory systems, the loss of lipopolysaccharide, or MgrB inactivation (4). Colistin resistance is described as a type of adaptive resistance with the rapid development of resistance in the presence of antibiotics and reversal to susceptibility in the absence of the same (5). This suggests that resistance to colistin may diminish in the absence of colistin or by limiting the extracellular concentration of divalent cations. In this study, we developed colistin resistance *in vitro* in four Gram-negative bacteria—*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. We also examined the stability of the resistant strains.

Seventeen strains, which were randomly isolated from patients suffering from bacteremia or urinary tract infections in South Korea, were used in this study (Table 1). The patients had not received intravenous or inhaled colistimethate. For all isolates, multilocus sequence typing (MLST) was performed as described previously (6–9). MICs were determined by a broth microdilution method using cation-adjusted Mueller-Hinton broth and interpreted according to CLSI breakpoints (10) for *A. baumannii* and *P. aeruginosa* and EUCAST breakpoints (11) for *E. coli* and *K. pneumoniae*.

Colistin-resistant mutants were developed from the colistin-susceptible wild-type strains. Starting with a single colony of each wild-type strain, colistin-resistant mutants were chosen by serial passage, using progressively increasing concentrations of colistin (12). At the end of the induction period, the spontaneous mutants growing in Luria-Bertani (LB) medium containing 16 μg/ml colistin were reinoculated on LB agar plates containing 32 μg/ml colistin in order to obtain single resistant populations.

To investigate the stability of the colistin resistance developed, the mutants were repeatedly subcultured in the absence of colistin. Overnight cultures of all induced colistin-resistant mutants were diluted 1:1,000 in fresh LB medium without colistin and incubated with vigorous shaking (220 rpm) at 37°C for 24 h. Colistin MICs for the pooled populations diluted in saline were estimated for all serially transferred cultures. For *E. coli* and *P. aeruginosa*, the maximum number of passages was 32 days, and *A. baumannii* and *K. pneumoniae* cells were transferred serially for 62 and 42 days, respectively.

Heteroresistance to colistin was identified by population analysis profiling by spreading a 0.1-ml aliquot from a 24-h culture of parental susceptible strains (13). Heteroresistance was defined as the presence of colonies more than the limit of quantification (LOQ) (400 CFU/ml) on the agar plate containing 10 μg/ml colistin (13, 14). Mutation frequency was investigated using cultures that were subjected to several serial passages in antibiotic-free LB broth medium. Mutation frequency was defined as the ratio of the CFU on a plate containing 4 μg/ml colistin to that on an antibiotic-free plate for each strain.

Amino acid substitutions were identified in *pmrAB* for *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, *phoPQ* for *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, and *mgrB* for *K. pneumoniae* using primers described previously (12, 15, 16).

In this study, colistin-resistant mutants were obtained from all susceptible parental strains (Table 1). Colistin-resistant mutants were selected *in vitro* from all cultures grown in medium containing 0.5 to 16 μg/ml colistin, which indicates that colistin resistance can be readily developed under antibiotic pressure. The colistin-resistant mutants had a colistin MIC of ≥64 μg/ml. Rapid development of colistin resistance in some bacterial species has previously been reported (12, 17, 18). A previous mutant prevention concentration study also indicated that colistin resistance can be readily induced during drug therapy by single-step mutation in *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* (19). While MgrB mutations were readily found in other colistin-resistant *K. pneumoniae* strains or mutants (20–23), no mutations of MgrB were identified in this study.

Contrary to the nature of development of colistin resistance, the stability of colistin resistance differed between strains. Colistin-susceptible revertants were obtained from only 5 of the 17 colistin-resistant mutants: one *A. baumannii* and three *P. aeruginosa* strains and one *E. coli* strain (Table 1 and Fig. 1). None

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TABLE 1 Gram-negative rod-shaped bacterial strains used in this study, their MICs for colistin, heteroresistance, mutation frequency, and amino acid alterations in colistin-resistant mutants and colistin-susceptible revertants

Species	Strain	ST	Colistin MIC (μg/ml) for:			Heteroresistance[a]	Mutation frequency[b]	Amino acid alteration in:				
			Parent	Resistant mutant	Susceptible revertant			Colistin-resistant mutants				Colistin-susceptible revertants[c]
								PmrA	PmrB	PhoP	PhoQ	
A. baumannii	H07-988	220	1	>64		HR	8.69×10^{-7}		H263R, V444A			NA
	H05-513	20	1	>64	1	HR	5.58×10^{-6}		I235T, G390V			T235I, V390G in PmrB
	H09-673	92	1	>64			6.64×10^{-7}		H263R			NA
	H09-968	138	1	>64			1.84×10^{-7}	M12R				NA
	C095	110	0.5	>64			2.72×10^{-7}					NA
P. aeruginosa	P5	235	1	>64	1		5.74×10^{-6}				K123Q V260G	R117L in PhoP, Q123K in PhoQ
	P6	1340	2	64	0.5	HR	8.29×10^{-7}		A67T			T67A in PmrB
	P33	641	1	>64			4.81×10^{-7}		V15I			NA
	P155	17	0.5	>64	0.5	HR	3.41×10^{-6}		L167P			P167L in PmrB, A110V in PhoP, Q411* in PhoQ
K. pneumoniae	B0608-134	730	1	>64		HR	2.94×10^{-6}					NA
	B0704-039	11	0.5	>64		HR	4.28×10^{-6}					NA
	08-B063	23	0.5	>64		HR	2.94×10^{-6}				Y268S, $del_{14–18}$	NA
	B0701-068	152	0.5	>64		HR	2.16×10^{-6}					NA
E. coli	E015	405	0.25	64	0.25	HR	3.23×10^{-7}		$del_{133–136}$			V24E and $del_{162–165}$ in PmrB
	E139	131	0.25	>64			4.23×10^{-7}		P94L			NA
	E154	38	0.25	64			8.06×10^{-8}		A159V			NA
	E188	410	0.125	64			1.03×10^{-7}		V125E			NA

[a] Heteroresistance (HR) was defined as the presence of colonies more than the LOQ on the agar plate containing 10 μg/ml colistin.
[b] The ratio of the CFU on a plate containing 4 μg/ml colistin to that on an antibiotic-free plate.
[c] NA, not available; *, premature termination.

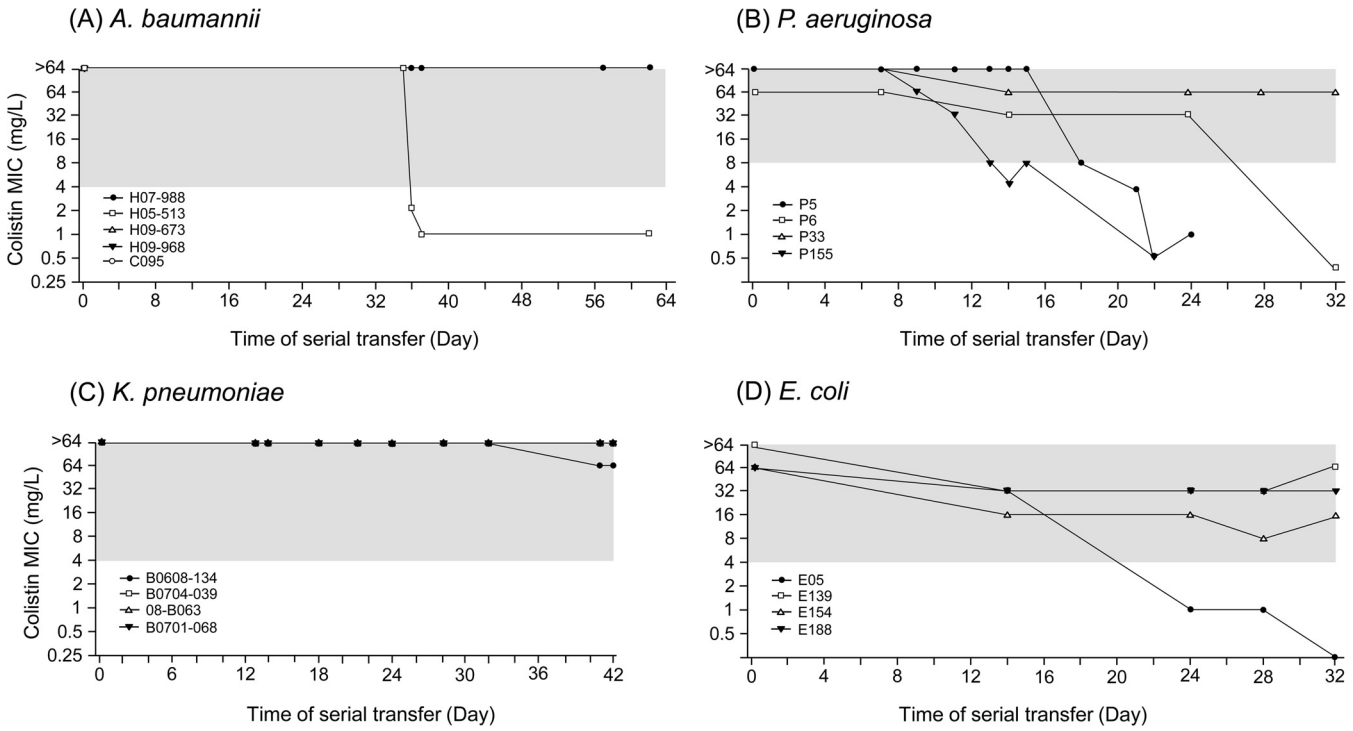

FIG 1 Change in colistin MIC of resistant mutants obtained by serial passage in colistin-free medium. (A) *A. baumannii*, (B) *P. aeruginosa*, (C) *K. pneumoniae*, and (D) *E. coli* colistin-resistant mutant strains. The *y* axis represents the colistin MIC in the $\log_2$ scale. Colistin-susceptible revertants were obtained from three *P. aeruginosa* strains and one strain each of *A. baumannii* and *E. coli* resistant mutants. Dashed lines indicate the breakpoint of colistin resistance for each species.

of the *K. pneumoniae* mutants produced any colistin-susceptible revertants.

Heteroresistance to colistin was identified in all four *K. pneumoniae* strains, and two *P. aeruginosa* and two *A. baumannii* strains and one *E. coli* strain were heteroresistant to colistin (Table 1). The correlation between colistin heteroresistance and stability of colistin resistance may not be supported because the heteroresistant *K. pneumoniae* strains did not lose colistin resistance in antibiotic-free medium. In addition, *A. baumannii* H07-988 showed heteroresistance to colistin, but it did not develop a colistin-susceptible revertant, and *P. aeruginosa* P5 showed a completely opposite nature. Furthermore, mutation frequency might not be associated with the heteroresistance and stability of colistin resistance (Table 1).

We identified several mutations in PhoPQ and PmrAB in colistin-resistant mutants. However, it was not proven that the mutations are associated with colistin resistance. In colistin-susceptible revertants of *P. aeruginosa* P5 and P155 and *E. coli* E015, additional mutations were found compared to their colistin-resistant progenitors (Table 1). However, such compensatory mutations were not observed in colistin-susceptible revertants of *A. baumannii* H05-513 and *P. aeruginosa* P6, in which only genetic reversions were identified. Such genetic reversion was also identified in *P. aeruginosa* P5 and P155.

The induced colistin resistance was eliminated in most *P. aeruginosa* strains in a colistin-free medium, but it remained stable in the other species tested (*A. baumannii*, *K. pneumoniae*, and *E. coli*). Therefore, the principle of adaptive resistance can be applied to *P. aeruginosa* but not to the others. The stability of colistin resistance has already been observed in *A. baumannii* (18). However, this stability is a major concern in the other three Gram-negative species, as newly emerged resistance in these species can be preserved and disseminated even in the absence of antibiotic pressure. Many studies have discussed the factors affecting the fitness cost of colistin resistance, such as increased susceptibility to other antibiotics, growth retardation, and reduced virulence (15, 24, 25), which may prevent an increase in the cases of colistin resistance in hospitals. However, compensatory mutations can change this situation, making it more difficult to treat the infections caused by Gram-negative pathogens.

The colistin resistance developed in patients treated with colistin for Gram-negative pathogenic infections may be preserved is a valid concern in the public health domain, with respect to preventing further development of resistance to the antibiotic. In addition, the mechanisms underlying the stability of colistin resistance, which has marked implications for the therapeutic options, need to be investigated.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been submitted to the GenBank database under accession no. KT716084 to KT716131, KT716132 to KT716179, KT719393, and KT719394.

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