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High-Level Resistance to Colistin Mediated by Various Mutations in the *crrB* Gene among Carbapenemase-Producing *Klebsiella pneumoniae*

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ABSTRACT Mutations in *crrAB* genes encoding a two-component regulator involved in modifications of lipopolysaccharide were searched for among a collection of colistin-resistant *Klebsiella pneumoniae* isolates. Four isolates, respectively, producing carbapenemases NDM-1, OXA-181, or KPC-2 showed mutated CrrB proteins compared with those in wild-type strains. Complementation assays with a wild-type CrrB protein restored the susceptibility to colistin in all cases, confirming the involvement of the identified substitutions in the resistance phenotype.

KEYWORDS polymyxin, rapid polymyxin NP test, susceptibility testing, resistance mechanisms, CrrAB, *Klebsiella pneumoniae*

The emergence and spread of carbapenemase-producing *Klebsiella pneumoniae* isolates worldwide has forced clinicians to reintroduce colistin as last-resort therapy (1). Besides the plasmid-mediated *mcr-1* and *mcr-2* genes in *K. pneumoniae* (2, 3), the chromosomally encoded alterations of the *mgrB* gene and the PmrAB and PhoPQ two-component systems are currently the most commonly reported mechanisms of acquisition of polymyxin resistance in this enterobacterial species (4). Recently, mutations in the *crrB* gene, belonging to a third two-component system (named CrrAB for *c*olistin *r*esistance *r*egulation) and involved in lipopolysaccharide (LPS) modifications, were associated with colistin resistance (5, 6). Mutations in the *crrB* gene are responsible for the increase in *crrC* gene transcription, which in turn regulates the expression of the *pmrC* gene and the *pmrHFIJKLM* operon, through the PmrAB two-component system (6). Expression of these genes leads to the addition of cationic groups on the LPS and consequently to colistin resistance.

In previous studies, the colistin resistance mechanisms of a collection of 185 *K. pneumoniae* isolates recovered from human samples worldwide (Europe, Turkey, Colombia, South Africa) were analyzed. Chromosomally encoded modifications of LPS through alterations (mutation, truncation) of the *mgrB* gene (7–9), the *pmrAB* genes (10), or the *phoPQ* genes (11) were identified in 152 isolates. However, 33 isolates did not present any substitutions in these genes, and they were negative for the plasmid-mediated resistance genes *mcr-1* and *mcr-2*.

We analyzed the genes encoding the CrrAB system in these 33 isolates. Amplification of the *crrA* and *crrB* genes was performed using primers crrAB-extF (5′-GTGAGGCCATCAAAT

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TCTCG-3′) and crrAB-extR (5′-AAGTCCCAAAAGAGGCAAAC-3′) located on each extremity of this operon. No amplification was obtained with the primers for 19 of the 33 isolates. We obtained the same result by using internal primers annealing into the *crrB* gene, namely, crrB-intF (5′-GTGACTATCTTACGTGGGAG-3′) and crrB-intR (5′-CACTCAGCATCA AGGAGTAC-3′). This absence of amplification suggested an absence of the *crrAB* operon in these strains, which is in accordance with the variability of the lateral acquisition of the *crrAB* operon in *K. pneumoniae* (5). Amplification of the *crrAB* gene was obtained for the 14 remaining isolates. Subsequent sequencing identified mutations leading to amino acid changes (F84S, N141Y, P151L, and G183V) in the CrrB protein in 4 of the 14 colistin-resistant *K. pneumoniae* isolates (Table 1).

Three of the CrrB amino acid substitutions were located in the histidine kinase A (HisKA) phosphoacceptor domain (amino acids 136 to 200) (Table 1). Previously, four mutations in this HisKA domain were found to be involved in colistin resistance (6) (Table 1). Among them, two were at the same position as the mutations observed in our strains (amino acids 141 and 151), but the amino acid changes were different. The fourth strain presented a mutation in the HAMP domain of CrrB, whereas a single mutation in this domain has been shown (5). All of the substitutions in the CrrB protein, known to be responsible for colistin resistance, are shown in Table 1.

Determination of the colistin MICs by use of the reference broth microdilution method showed a high level of colistin resistance (MIC of colistin, $>$128 μg/ml for all four isolates with mutated *crrB* genes) (Table 1). The high MICs of colistin in the *K. pneumoniae* strains are in accordance with results reported by Cheng et al. (6) (Table 1). Furthermore, Wright et al. (5) reported lower MICs of colistin (16 μg/ml) for two isolates exhibiting CrrB mutations. However, MICs were determined by use of the Etest strip technique, which is known to underestimate colistin MIC values (12).

We performed complementation assays to confirm the involvement of the mutated *crrB* gene in the colistin resistance phenotype. A recombinant plasmid (pTRIC) was built by cloning a triclosan resistance gene (*mFabI*) (13) into the low-copy-number plasmid pBR322. The wild-type *crrB* gene from the colistin-susceptible *K. pneumoniae* strain Af44a (MIC of colistin, 0.25 μg/ml) was amplified by PCR and cloned into this plasmid. The recombinant crrB-pTRIC plasmid and the pTRIC plasmid were separately transformed by electroporation into the four resistant strains presenting the mutations in the *crrB* gene. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with triclosan (1 μM), and the recombinant clones were checked by PCR and sequencing. MICs of colistin for the transformants revealed that production of a wild-type CrrB protein (crrB-pTRIC plasmid) restored the susceptibility to colistin in all isolates (MIC, $\leq$0.5 μg/ml) (Table 1), confirming that the different substitutions in CrrB were, respectively, responsible for the resistant phenotypes. As expected, transformation with the pTRIC plasmid used as a negative control did not restore any susceptibility to colistin.

All isolates presenting a CrrB amino acid change produced a carbapenemase (NDM-1, OXA-181, or KPC-2) (Table 1). They were recovered from patients who had been treated with colistin in Colombia, France, Greece, and South Africa. For the patient harboring the colistin-resistant isolate Af44b, one colistin-susceptible isolate (Af44a) was recovered before the colistin treatment, and pulsed-field gel electrophoresis analysis confirmed the clonal relationship of the two isolates (data not shown). Sequencing of the *crrB* gene of the Af44a isogenic susceptible strain identified a wild-type CrrB protein, reinforcing the hypothesis that the *in vivo* emergence of colistin resistance under colistin pressure might be related to CrrB mutations in some instances.

In conclusion, four novel mutations in the CrrB protein were identified at the sources of acquisition of high-level colistin resistance among carbapenemase-producing *K. pneumoniae*.

TABLE 1 Features of colistin-resistant *K. pneumoniae* clinical isolates

Isolate[a]	Origin of isolate	MIC of colistin[b] (μg/ml)	Colistin susceptibility[c]	CrrB amino acid change in:									MIC of colistin after complementation[f] (μg/ml)	Carbapenemase	Reference/source
					TM[d]	HAMP[d]		HisKA[d]							
				10[e]	31[e]	84[e]	94[e]	140[e]	141[e]	151[e]	183[e]	195[e]			
Af44a[g]	South Africa	0.25	S	Q	Y	F	L	W	N	P	G	S	-	OXA-181	This study
UHKPC28	United States	16	R	L										KPC-2	5
Col21	Taiwan	512	R	L										No	6
Col28	Taiwan	>2,048	R	L										No	6
Col44	Taiwan	512	R		H									No	6
20.70	France	>128	R			S							0.25	NDM-1	This study
UHKPC26	United States	16	R				M							KPC-2	5
Col36	Taiwan	2,048	R					R						No	6
G104	Greece	>128	R						Y				0.5	KPC-2	This study
Col4	Taiwan	>2,048	R						I					No	6
Col20	Taiwan	2,048	R						I					No	6
Af44b	South Africa	>128	R							L			0.25	OXA-181	This study
Col7	Taiwan	1,024	R							S				No	6
C7	Colombia	>128	R								V		0.5	KPC-2	This study
Col22	Taiwan	2,048	R									N		No	6

[a]Isolates from this study are indicated by shading.

[b]MICs of colistin were determined using the manual broth microdilution reference method for this study, Etest for the study by Wright et al. (5), and agar dilution for the study by Cheng et al. (6).

[c]S, susceptible (MIC, ≤2 μg/ml); R, resistant (MIC, >2 μg/ml), according to EUCAST breakpoints (http://www.eucast.org/).

[d]Domains of the CrrB protein predicted by SMART software are indicated as follows: TM, transmembrane domain (amino acids 12–34); HAMP, histidine kinase, adenylyl cyclase, methyl binding protein, and phosphatase domain (amino acids 81–135); HisKA, histidine kinase A (phosphoacceptor) domain (amino acids 136–200).

[e]Amino acid positions where mutations have been detected.

[f]MICs of colistin after complementation with a wild-type CrrB protein (with plasmid crrB-pTRIC).

[g]The colistin-susceptible isolate Af44a is the isogenic colistin-susceptible counterpart of Af44b.

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