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mgrB Mutations Mediating Polymyxin B Resistance in *Klebsiella pneumoniae* Isolates from Rectal Surveillance Swabs in Brazil

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We aimed to investigate polymyxin B (PMB) resistance and its molecular mechanisms in 126 *Klebsiella pneumoniae* isolates from rectal swabs in Brazil. Ten isolates exhibited PMB resistance with interruption of *mgrB* gene by insertion sequences or missense mutations. Most of the PMB-resistant isolates harbored bla_{KPC-2} ($n = 8$) and belonged to clonal complex 258 (CC258) ($n = 7$). These results highlight the importance of monitoring the spread of polymyxin-resistant bacteria in hospitals, since few options remain to treat multidrug-resistant isolates.

Polymyxin has been widely used to treat infections caused by multidrug-resistant (MDR) Gram-negative bacteria, including *Klebsiella pneumoniae*. However, reports of polymyxin-resistant *K. pneumoniae* (PRKP) isolates have increased worldwide, becoming a great public health concern (1).

Most studies on PRKP have focused on patients with infections. However, there have been few reports assessing data on PRKP carriage in patients around the world (2). Some studies have described a remarkable and concerning number of patients who developed infection by PRKP after previous colonization, resulting in elevated mortality rates (3, 4). Colonization by KPC-producing *K. pneumoniae* and polymyxin therapy are considered important risk factors for PRKP infection (5, 6).

Studies have demonstrated that modifications of PmrA/PmrB and PhoP/PhoQ two-component systems and inactivation of the *mgrB* gene (a regulator of the PhoP/PhoQ system) led to polymyxin resistance by modification of the lipopolysaccharide target (7). Recently, the plasmid-mediated transferable polymyxin resistance *mcr-1* gene, causing resistance by modification of lipid A, was identified in China in *Escherichia coli* and *K. pneumoniae* strains (8).

Here, we searched for molecular mechanisms associated with polymyxin resistance in *K. pneumoniae* isolates from Brazil. A first-step screening for polymyxin B (PMB) resistance was conducted using Etest (bioMérieux, France) in 126 isolates randomly selected from approximately 850 *K. pneumoniae* isolates with reduced susceptibility to carbapenems recovered from rectal swabs from 11 Brazilian states during 2007 to 2013. The bacterial identification was confirmed by conventional biochemical techniques. Considering the PRKP strains showing a MIC of >2.0 mg/liter (9), 10 (8%) PRKP isolates were observed and included in this study. These 10 PRKP isolates were collected between 2009 and 2013 from five Brazilian states (Fig. 1).

To confirm the resistance phenotype, the PMB MIC was retested in duplicate by microdilution with cation-adjusted Mueller-Hinton broth (10). The isolates showed a MIC_{50} of 64 mg/liter, a MIC_{90} of >128 mg/liter, and a MIC range of 16 to >128 mg/liter (Table 1). Concordant Etest and microdilution results were found for only three isolates. The Etest MICs tended to be 1.3-fold to 4.0-fold lower than the microdilution MICs. Discrepancy between the two methodologies demonstrated that the Etest provided a

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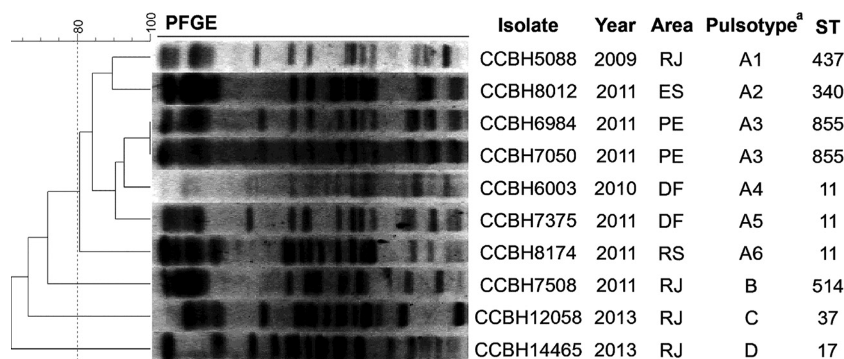

FIG 1 Epidemiological and molecular typing of polymyxin B-resistant *K. pneumoniae* isolates analyzed in this work. DF, Distrito Federal; ES, Espírito Santo; PE, Pernambuco; RJ, Rio de Janeiro; RS, Rio Grande do Sul; ST, sequence type. The PFGE pulsotypes and subtypes (indicated by a superscript "a") were defined as strains with at least 80% and 95% similarities, respectively.

TABLE 1 Phenotypic and molecular characterization of polymyxin B-resistant *K. pneumoniae* isolates analyzed in this work

Isolate	PMB MIC (mg/liter)[a]		Modification in protein[b]:					Additional susceptibility profile[c]		
	Etest	Broth dilution	MgrB	PmrB	PmrA	PhoP	PhoQ	Nonsusceptible to:	Susceptible to:	Resistance genes[g]
CCBH5088[d]	192	>128	Gene disrupted by IS*903B*	T246A[f], R256G[e]	WT	A30S[f]	WT	FOT, CAZ, ATM, FEP, CTX, TZP, GEN, CIP, SXT, CHL, MEM, ERT, IPM	AMK, TGC	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH6003[d]	64	128	Gene disrupted by IS*903B*	Gene deletion (570 bp)	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, CIP, SXT, MEM, ERT, IPM	FOT, AMK, GEN, CHL, TGC	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, *qnrS*, *aadA*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH6984[d]	32	128	Gene disrupted by IS*903B*	T246A[f], R256G[e]	WT	WT	WT	FOT, CAZ, ATM, FEP, CTX, TZP, AMK, GEN, CIP, SXT, CHL, MEM, ERT, IPM, TGC		bla_{KPC-2}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH7050[d]	32	>128	Gene disrupted by IS*903B*	T246A[f], R256G[e]	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, AMK, GEN, CIP, SXT, CHL, ERT, TGC	FOT, MEM, IPM,	bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH7375[d]	24	64	Gene disrupted by IS*10L*	T246A[f], R256G[e]	WT	WT	WT	FOT, CAZ, ATM, FEP, CTX, TZP, AMK, CIP, SXT, MEM, ERT, IPM	GEN, CHL, TGC	bla_{KPC-2}, bla_{SHV}, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH7508	16	16	Gene disrupted by IS*5*	WT	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, AMK, GEN, CIP, MEM, ERT, IPM	FOT, SXT, CHL, TGC	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH8012[d]	64	64	C28R[b]	T246A[f], R256G[e]	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, AMK, GEN, CIP, SXT, CHL, MEM, ERT, IPM	FOT, TGC	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrA*, *aadA*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH8174[d]	48	128	Gene disrupted by IS*Kpn26*	T246A[f], R256G[e]	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, AMK, GEN, CIP, SXT, CHL, MEM, ERT, IPM	FOT, TGC	bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *aadA*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH12058	12	16	Q30stop[b]	T246A[f], R256G[e]	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, SXT, CHL, MEM, ERT, IPM	FOT, AMK, GEN, CIP, TGC	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*
CCBH14465	24	32	Gene disrupted by IS*102*	T246A[f]	E57G[f]	WT	WT	CAZ, ATM, FEP, CTX, TZP, AMK, CIP, SXT, CHL, MEM, ERT, IPM, TGC	FOT, AMK	bla_{KPC-2}, bla_{TEM}, bla_{SHV}, *qnrB*, *qnrS*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*

[a] PMB, polymyxin B.
[b] WT, wild type.
[c] ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; TZP, piperacillin-tazobactam; ERT, ertapenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; FOT, fosfomycin/trometamol; MEM, meropenem; IPM, imipenem; TGC, tigecycline.
[d] Isolates belonging to CC258.
[e] Mutation predicted as deleterious by PROVEAN.
[f] Mutation predicted as neutral by PROVEAN.
[g] The resistance genes searched were related to polymyxin resistance (*mcr-1*), plasmid-mediated quinolone resistance (*qnrA*, *qnrB*, *qnrS*), aminoglycoside resistance [*aadA*, *aadB*, *aac(3′)IIa*, *aac(6′)-Ib*], and β-lactam resistance (bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{IMP}, bla_{VIM}, bla_{SHV}, bla_{TEM}, bla_{CTX-M}).

conservative estimate (11). Furthermore, the cation concentration variability of culture media was correlated to the low accuracy and discrepancies in Etest results (12); thus, the use of a dilution method to confirm the PMB susceptibility is recommended (13).

The MICs for meropenem, imipenem, and tigecycline were also determined by Etest, and susceptibility to other antimicrobial agents was determined by agar diffusion (Table 1). Most of the isolates were nonsusceptible to β-lactams (n = 10), ciprofloxacin (n = 9), sulfamethoxazole-trimethoprim (n = 9), gentamicin (n = 7), chloramphenicol (n = 7), and amikacin (n = 6) and remained susceptible to fosfomycin/trometamol (n = 7) and tigecycline (n = 7). Data from SENTRY (2008 to 2010) showed a 3.2% incidence of PRKP isolates in Brazil (14). This rate increased to 6.6% for extended-spectrum-β-lactamase (ESBL)-producing *K. pneumoniae* isolates from medical centers in Latin America in 2011 (15) and 9.7% for KPC-2-producing *K. pneumoniae* isolates from Brazil in 2010 (16), showing a clear association between PMB resistance and other acquired resistance mechanisms.

We performed PCR to detect resistance genes to β-lactams, quinolones, and aminoglycosides and performed sequencing when required. Genetic determinants associated with resistance to those classes were observed in all isolates (Table 1), with $bla_{\text{KPC-2}}$ observed in eight strains. The pulsed-field gel electrophoresis (PFGE) (17) and multilocus sequence typing (MLST) (18) analyses (Fig. 1) revealed four pulsotypes (A [A1 to A6], B, C, and D) and seven sequence types (ST), which may indicate independent events of PMB resistance acquisition. A total of seven PRKP isolates belonged to clonal complex 258 (CC258), the most important CC associated with KPC production (19). Corroborating our findings, the expansion of PRKP isolates belonging to ST11 (CC258) and harboring $bla_{\text{KPC-2}}$ was previously reported in Brazil in 2014 (20). The data raise concerns about the surveillance of PRKP spread, since PMB is one of the limited number of treatment options against infections caused by the endemic KPC-2-producing *K. pneumoniae* strains in Brazil (21).

To investigate the presence of *mcr-1* and related variants and mutational events affecting *mgrB*, *pmrA*, *pmrB*, *phoP*, and *phoQ* genes, PCR and DNA sequencing were conducted. Mutation analysis of genes involved in polymyxin resistance was performed using Geneious (6.1.8) software and the BLASTN (NCBI) tool. The online platform ISfinder (https://www-is.biotoul.fr) was employed to identify insertion sequences (IS) and the PROVEAN platform (http://provean.jcvi.org/index.php) to predict alterations of biological functions of the proteins by the use of *K. pneumoniae* MGH 78578 (CP000647.1) as a reference.

Regarding polymyxin resistance, the presence of the *mcr-1* gene was not detected. All of the PRKP strains exhibited alterations in the *mgrB* gene (Table 1), including disruption by IS*903B*, IS*5*, IS*102*, IS*Kpn26* (IS*5* family), and IS*10L* (IS*4* family). Previous studies (2, 22, 23, 24) have already shown the interruption of the *mgrB* region by IS*10-like* and IS*5-like* elements. This mechanism seems to be common in *K. pneumoniae*, including KPC-producing isolates of CC258 (22). In Brazil, disruption of *mgrB* by IS*903* was already reported in a BKC-1 (Brazilian *Klebsiella* carbapenemase-1)-producing *K. pneumoniae* isolate from São Paulo (25). In addition, deleterious mutations (C28R and Q30stop) were observed in *mgrB*. Mutations at these same amino acid positions were also reported in PRKP isolates from Europe (2, 22, 26).

Alterations in the *phoQ* gene were not detected. However, a partial deletion of the PmrB-encoding gene was identified in one isolate and a deleterious mutation (R256G) was found in seven isolates (Table 1). This specific mutation was not related to polymyxin resistance as previously reported (27). The T246A, E57G, and A30S mutations detected in PmrB, PmrA, and PhoP, respectively, were not considered deleterious by PROVEAN. Furthermore, we suggest that the specific PmrB (T246A) and PmrA (E57G) mutations found in this study were not capable of producing PMB resistance alone, since these mutations were also found in PMB-susceptible isolates (data not shown).

In this study, the disruption of the *mgrB* gene was shown to be associated with PMB resistance in *K. pneumoniae*. It is worrisome that the spread of PRKP in Brazil is associated with KPC-producing strains belonging to the epidemic CC258 variant. The present findings indicate the importance of broad and effective monitoring of PMB-resistant Gram-negative bacteria in order to follow the evolution of PMB resistance in Brazil, as well the importance of screening for PMB resistance in colonized nosocomial patients in order to prevent possible infection by these pathogens.

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