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Title

MgrB mutations mediating polymyxin B resistance in *Klebsiella pneumoniae* isolates from rectal surveillance swabs in Brazil.

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Running Title

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Key Words

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Abstract

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 PMB resistant isolates harbored bla_{KPC-2} (N=8) and belonged to 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) 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 of 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 on PmrA/PmrB and PhoP/PhoQ two-component systems and inactivation of the *mgrB* gene (a regulator of PhoP/PhoQ system) leads to polymyxin resistance by modification of the lipopolysaccharide target (7). Recently, the plasmid-mediated transferable polymyxin resistance *mcr-1* gene was identified in *Escherichia coli* and *K. pneumoniae* causing resistance by modification of lipid A in China (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 randomly selected isolates of approximately 850 *K. pneumoniae* isolates with reduced susceptibility to carbapenems recovered from rectal swabs from 11 Brazilian States during 2007-2013. The bacterial identification was confirmed by biochemical conventional techniques. Considering PRKP strains showing MIC >2.0 mg/L (9), ten PRKP isolates (8%) were observed and included in this study. These ten PRKP isolates were collected between 2009 and 2013 from five Brazilian states (Fig. 1).

To confirm the resistance phenotype, the MIC for PMB was retested in duplicate by microdilution with cation-adjusted Müeller-Hinton broth (10). The isolates showed MIC_{50} = 64 mg/L, MIC_{90} = >128 mg/L and MIC range 16 to >128 mg/L (Table 1). Concordant Etest and microdilution results were found only in three isolates. The Etest MIC tend to be 1.3 to 4.0-fold lower than the microdilution MICs. Discrepancy between the two methodologies demonstrated that Etest provided a conservative estimate (11). Furthermore, the cation concentration variability of culture media was correlated to the low accuracy and discrepancies in Etest (12), thus, the use of a dilution method to confirm the PMB susceptibility is recommended (13).

The MIC for meropenem, imipenem and tigecycline were also determined by Etest and the susceptibility to other antimicrobial agents was determined by agar diffusion (Table 1). Most of the isolates were non-susceptible 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 (2008-2010) showed 3.2% of PRKP isolates in Brazil (14). This rate increased to 6.6% in ESBL producing *K. pneumoniae* from medical centers in Latin America in 2011 (15) and 9.7% for KPC-2-producing *K. pneumoniae* from Brazil in 2010 (16), showing clear association between PMB resistance and other acquired resistance mechanisms.

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

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

Regarding polymyxin resistance, the presence of *mcr-1* gene was not detected. All the ten PRKP strains exhibited alterations in *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 were observed in *mgrB* (C28R and Q30stop). Mutations on these same amino acid positions were also reported in PRKP isolates from Europe (2,22,26).

Alterations in *phoQ* gene were not detected. However, a partial deletion of PmrB encoding gene was identified in one isolate and a deleterious mutation (R256G) was found in 70% of the isolates (Table 1). This specific mutation was not related to polymyxin resistance as previously reported (27). The mutations T246A, E57G and A30S detected in PmrB, PmrA and PhoP, respectively, were not considered deleterious by PROVEAN. Furthermore, we suggest the PmrB (T246A) and PmrA (E57G) specific mutations found in this study were not capable to produce PMB resistance alone, since these mutations were also found in PMB susceptible isolates (data not shown).

In this study, the disruption of the *mgrB* was associated with PMB resistance in *K. pneumoniae*. It is worrisome the spread of PRKP in Brazil be associated with KPC-producing

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strains belonging to the epidemic CC258. Present findings alert to a broad and effective monitoring of PMB-resistant Gram-negative bacteria in order to follow the evolution of PMB resistance in the country, as well the screening of PMB resistance in colonized nosocomial patients in order to prevent possible infection by these pathogens.

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Conflict of interests

None to declare.

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

Isolate	PMB MIC (mg/L)[a]		Modification in proteins[b]					Additional susceptibility profile[c]		Resistance genes[g]
	Etest	Broth dilution	MgrB	PmrB	PmrA	PhoP	PhoQ	Non-susceptible	Susceptible	
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	$bla_{\text{KPC-2}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, *aadB*, *aac(3')IIa*, *aac(6')-Ib*
CCBH6003[d]	64	128	Gene disrupted by IS*903B*	Gene deletion 570pb	WT	WT	WT	CAZ, ATM, FEP, CTX, TZP, CIP, SXT, MEM, ERT, IPM	FOT, AMK, GEN, CHL, TGC	$bla_{\text{KPC-2}}$, 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	-	$bla_{\text{KPC-2}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, *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}, $bla_{\text{CTX-M}}$, *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	$bla_{\text{KPC-2}}$, 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	$bla_{\text{KPC-2}}$, 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	$bla_{\text{KPC-2}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, *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}, $bla_{\text{CTX-M}}$, *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	$bla_{\text{KPC-2}}$, 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	$bla_{\text{KPC-2}}$, 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 - gentamicina; 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.

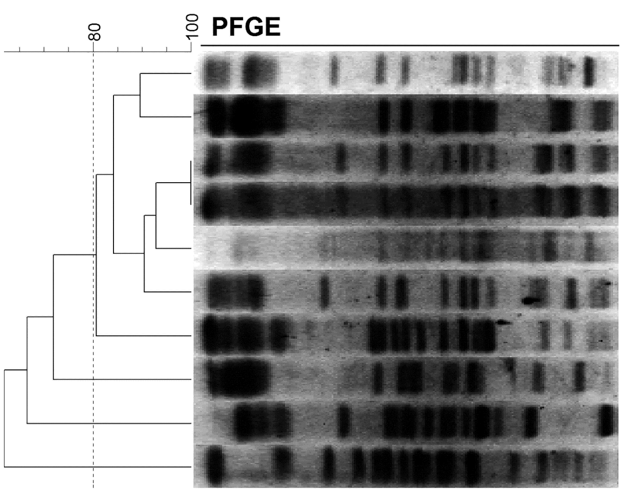
[f] Mutation predicted as neutral by PROVEAN.

[g] The resistance genes searched were related with polymyxins resistance (*mcr-1*), plasmid-mediated quinolones resistance (*qnrA, qnrB, qnrS*), aminoglycosides resistance (*aadA, aadB, aac(3')IIa, aac(6')-Ib*) and β-lactams resistance ($\textit{bla}_{\text{KPC}}$, $\textit{bla}_{\text{NDM}}$, $\textit{bla}_{\text{OXA-48}}$, $\textit{bla}_{\text{IMP}}$, $\textit{bla}_{\text{VIM}}$, $\textit{bla}_{\text{SHV}}$, $\textit{bla}_{\text{TEM}}$, $\textit{bla}_{\text{CTX-M}}$).

Figure 1. Epidemiological and molecular typing of polymyxin B-resistant *K. pneumoniae* isolates analyzed in this work.

Legend: DF - Distrito Federal; ES - Espírito Santo; PE - Pernambuco; RJ - Rio de Janeiro; RS - Rio Grande do Sul; ST - sequence type.

[a] The PFGE pulsotypes and subtypes were defined as strains with at least, 80% and 95% of similarities, respectively.



Isolate	Year	Area	Pulsotype[a]	ST
CCBH5088	2009	RJ	A1	437
CCBH8012	2011	ES	A2	340
CCBH6984	2011	PE	A3	855
CCBH7050	2011	PE	A3	855
CCBH6003	2010	DF	A4	11
CCBH7375	2011	DF	A5	11
CCBH8174	2011	RS	A6	11
CCBH7508	2011	RJ	B	514
CCBH12058	2013	RJ	C	37
CCBH14465	2013	RJ	D	17