MgrB inactivation can be found also in colistin susceptible KPC-producing *Klebsiella pneumoniae* of clinical origin

Galani I[1], Lekkakou D[2], Karaiskos I[2], Galani L[2], Souli M[1], Giamarellou H[2]

[1] 4th Dept of Internal Medicine, Athens University School of Medicine, Athens, Greece
[2] 6th Dept of Internal Medicine, Hygeia General Hospital, Athens, Greece

P1006
25th ECCMID COPENHAGEN, DENMARK 25-28 APRIL 2015

HELLENIC REPUBLIC
National and Kapodistrian University of Athens

hygeia hospital

Abstract

Objectives: Insertional inactivation of the *mgrB* gene, encoding a negative feed-back regulator of the PhoQ/PhoP signalling system, has been reported to be responsible for colistin resistance in KPC- *Klebsiella pneumoniae*, due to the resulting up regulation of the Pmr lipopolysaccharide modification system. In this work we have investigated seven pairs of colistin-susceptible and colistin-resistant sequential isolates obtained from stool samples of seven patients with a KPC-*Klebsiella pneumoniae* infection before and after colistin treatment, respectively.

Methods: Colistin resistant *K. pneumoniae* strains isolated in surveillance specimens of 7 patients, who were on colistin treatment, as well as sensitive strains isolated from the same patients before colistin use, were included in the study. Exact MICs for colistin were determined by the E-test. Sensitive and resistant isolates were epidemiologically studied by repetitive extragenic palindromic (REP)-PCR methodology. The *mgrB* and *pmrB* genes from the respective resistant and susceptible isolates were amplified by PCR and sequenced. Expression of the *phoP*, *phoQ* and *pmrK* genes was measured by qRT-PCR.

Results: Only clonally related pairs of isolates (susceptible and resistant) were included in the study. MICs of the colistin resistant isolates ranged between 4 and 256 mg/L while susceptible isolates had MICs of 0.125 to 2mg/L. All fourteen strains carried a bla_{KPC} gene. The *pmrB* gene was identical in all isolates, while *mgrB* was interrupted by an IS5-like insertion at nucleotide 75 in a forward or reverse orientation, in three colistin resistant isolates and in their clonal related susceptible ones. Transcriptional up regulation of the *phoP, phoQ* and *pmrK* genes was observed in three colistin-resistant isolates (MIC range 16-256mg/L) compared to their susceptible pairs (MIC 0.125mg/L). Two of those isolates harbored an interrupted *mgrB,* identical to that found on their isogenic susceptible strain. The third isolate and its isogenic susceptible one harbored an intact *mgrB* with an Asn42→ Lys substitution.

Conclusion: Our findings shows that up regulation of the PhoQ/PhoP system and activation of the *pmrHFIJKLM* operon, which eventually leads to resistance to polymyxins by modification of the lipopolysaccharide target is not always associated with the inactivation of the *mgrB* gene. Other molecular factors mediating colistin resistance in *K. pneumoniae* are likely to be the focus of future studies.

Material and Methods

- Colistin resistant *K. pneumoniae* strains isolated in surveillance specimens of 7 patients, who were on colistin treatment, as well as susceptible strains isolated from the same patients before colistin use, were included in the study.
- Species identification and MIC determinations were performed with the Vitek® 2 (bioMérieux, Marcy-l'Etoile, France).
- Exact MICs for colistin were determined by the E-test (AB Biodisk), in accordance with the manufacturer's instructions.
- Susceptible and resistant isolates were epidemiologically studied by repetitive extragenic palindromic (REP)-PCR methodology and Pulsed Field Gel Electrophoresis (PFGE).
- The *mgrB* and *pmrB* genes from the respective resistant and susceptible isolates were amplified by PCR and sequenced.
- Expression of the *phoP*, *phoQ* and *pmrK* genes was measured by qRT-PCR.
- The presence of carbapenemase was confirmed by polymerase chain reaction (PCR) with specific primers and conditions.

Contact Information:
Irene Galani, University General Hospital "ATTIKON", Rimini 1, 124 62 Chaidari, Greece.
Tel : ++321055831984, Fax : ++32105326446,
E mail: egalani@med.uoa.gr

Results

- Only clonally related pairs of isolates (susceptible and resistant) were included in the study.
- MICs of the colistin resistant isolates ranged between 4 and 256 mg/L while susceptible isolates had MICs of 0.125 to 2mg/L.
- Six pairs of strains (1, 2, 3, 4, 6 and 7) carried the bla_{KPC} gene while the seventh pair (5) carried the bla_{VIM}.
- The *pmrB* gene was identical in susceptible and resistant isolate of all pairs, similar to the "sensor protein for *bas*R" *K. pneumoniae* NTUH-K2044 strain.
- Only the VIM-producing *K.pneumoniae* pair of isolates (5) had a T→G substitution at nucleotide 418 corresponding to a Thr(T)140→Pro(P) amino acid change to both susceptible and resistant isolate, compared to *K. pneumoniae* NTUH-K2044.
- The *mgrB* gene was identical in susceptible and resistant isolate of all pairs.
- In three colistin resistant KPC-producing isolates and in their clonal related susceptible strains (2, 6 and 7), *mgrB* was interrupted by an IS5-like insertion at nucleotide 75 in a forward orientation.
- In the colistin resistant VIM-producing isolate and in its clonal related susceptible strain (5), *mgrB* was interrupted by the same IS5-like insertion at nucleotide 75 in a reverse orientation.
- In the other three KPC-producing pairs of isolates (1, 3 and 4), *mgrB* exhibited a A→T substitution at nucleotide 126 corresponding to an Asn(N)42→ Lys(K) amino acid change compared to *mrgB* of *K.pneumoniae* strain 342.
- Transcriptional up regulation of the *phoP, phoQ* and *pmrK* genes was observed in three (1, 5 and 7) colistin-resistant isolates (MIC range 16-256mg/L) compared to their susceptible pairs (MIC 0.125mg/L). Two of those isolates (5 and 7) harbored an interrupted *mgrB,* identical to that found on their isogenic susceptible strain in a reverse or forward orientation respectively. The third isolate and its isogenic susceptible one (1) harbored an intact *mgrB* with an Asn42→ Lys substitution.

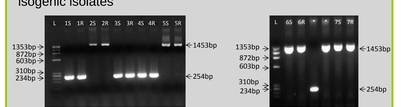
Table 1. Primers used in PCR experiments

Gene	Primer sequence (5'→3')	PCR product	Reference
REP	5'-III GCG CCG ICA TCA GGC-3'	variable	1
	5'-ACG TCT TAT CAG GCC TAC-3'		
pmrB	5'-TCA ATG GGT GCT GAC GTT CT-3'	1096bp	2
	5'-TGG CTC TGT TTG CAA CTG-3'		
mgrB	5'-AAG GCG TTC ATT CTA CCA CC-3'	254bp	3
	5'-TTA AGA AGG CCG TGC TAT CC-3'		
bla_{VIM}	5'-ATG GTG TTT GGT CGC ATA TC-3'	509bp	4
	5'-TGG GCC ATT CAG CCA GAT C-3'		
bla_{KPC}	5'-ATG TCA CTG TAT CGC CGT CT-3'	893bp	5
	5'-TTT TCA GAG CCT TAC TGC CC-3'		

Table 2. Primers used in qRT-PCR experiments (Cannatelli et al, 2013)

Gene	Primer sequence (5'–3')	Conditions (temperature/time)	PCR product
phoP	ATTGAAGAGGTTGCCGCCCGC	95°C/30s–52°C/30s–72°C/30s	135bp
	GCTTGATCGGCTGGTCATTCACC		
phoQ	ATATGCTGGCGAGATGGGAAAACGG	95°C/30s–52°C/30s–72°C/30s	138bp
	CCAGCCAGGGAACATCACGCT		
pmrK	GCGGGCCATCAGGATCGACAGCG	95°C/30s–65°C/30s–72°C/30s	223bp
	CGTTCTGGTACTACATCCCGTTCCTGA		
rpsL	GCCGTACTTGGAGCGAGCCTG	95°C/30s–52°C/30s–72°C/30s	108bp
	CCGTGGCGGTCGTGTTAAAGA		

Figure 1. PCR of *mgrB* in susceptible (S) and resistant (R) isogenic isolates



L: DNA ladder, 1S/1R, 2S/2R, 3S/3R, 4S/4R, 5S/5R, 6S/6R, 7S/7R: isogenic pairs of isolates, * pair not included in the study (not identical PGFE type.

Figure 2. Pulsed Field Gel Electrophoresis of susceptible (S) and isogenic resistant (R) isolates

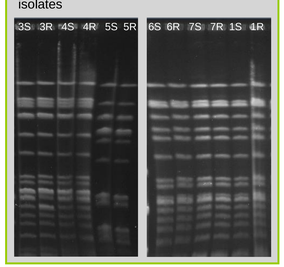


Table 3. Colistin MICs, molecular features, and genotyping analysis of the colistin-resistant and their isogenic colistin susceptible *K. pneumoniae* strains studied.

a/a	Colistin MIC (mg/L)	PFGE similarities	Sequencing of *mgrB* compared to *K.pneumoniae* 342	Sequencing of *pmrB* compared to *K.pneumoniae* NTUH-K2044	qRT-PCR results *Compared to isogenic susceptible isolate*		
					phoP	*phoQ*	*pmrK*
1	0,125	Clone A1	**126 (a→ t) (Asn42→ Lys)**	**none**	1	1	1
	24		**126 (a→ t) (Asn42→ Lys)**	**none**	3,24	5,1	6,56
2	0,5	Clone A	**Insertion of an IS5-like at nt75 (FW)**	**none**	1	1	1
	16		**Insertion of an IS5-like at nt75 (FW)**	**none**	0,53	0,67	1,74
3	2	Clone A2	**126 (a→ t) (Asn42→ Lys)**	**none**	1	1	1
	8		**126 (a→ t) (Asn42→ Lys)**	**none**	2,16	1,65	1,63
4	1,5	Clone A2	**126 (a→ t) (Asn42→ Lys)**	**none**	1	1	1
	4		**126 (a→ t) (Asn42→ Lys)**	**none**	0,22	0,49	0,26
5	0,125	Clone B	**Insertion of an IS5-like at nt75 (RW)**	**418 (t→g)(Thr140→Pro)**	1	1	1
	16		**Insertion of an IS5-like at nt75 (RW)**	**418 (t→g)(Thr140→Pro)**	12,9	12,7	20,8
6	0,125	Clone A	**Insertion of an IS5-like at nt75 (FW)**	**none**	1	1	1
	24		**Insertion of an IS5-like at nt75 (FW)**	**none**	1,02	1,06	1,53
7	0,125	Clone A	**Insertion of an IS5-like at nt75 (FW)**	**none**	1	1	1
	256		**Insertion of an IS5-like at nt75 (FW)**	**none**	33,3	0,18	125

Conclusion

- The truncation of the *mgrB* gene, encoding a negative feed-back regulator of the PhoQ/PhoP signaling system, was recently identified as a source of acquired colistin resistance in *K. pneumoniae* by activation of the Pmr system responsible for modification of the lipopolysaccharide polymyxin target.
- Our findings shows that the insertion of the IS5-like at nt 75 of *mgrB* , or the amino acid change of Asn42 to Lys, were not associated with the increase in colistin MIC, as they were present also in the isogenic susceptible isolates.
- Other molecular factors mediating colistin resistance in *K. pneumoniae*, as promoter mutations leading to a reduction of *mgrB* expression or to mutations in other loci are likely to be the focus of future studies.

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