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## **Inactivation of mgrB gene regulator and resistance to colistin is becoming endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: A nationwide study from 2014 to 2017**

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1 **Inactivation of *mgrB* gene regulator and resistance to colistin is becoming**  
2 **endemic in carbapenem-resistant *Klebsiella pneumoniae* in Greece: a**  
3 **nationwide study from 2014 to 2017**  
4

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41 **Running title:** Carbapenem and colistin resistant *Klebsiella pneumoniae*

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43

44

45 **Abstract**

46 **Introduction:** In Greece, the spread of carbapenem-resistant Enterobacteriaceae in humans  
47 has led to the reintroduction of colistin as a therapeutic agent. Unfortunately, resistance to  
48 colistin has emerged with different mechanisms involved. The present work aims to determine  
49 the prevalence of carbapenem and colistin resistance and the corresponding mechanisms in  
50 *Klebsiella pneumoniae* clinical isolates from Greece.

51 **Methods:** From 2014 to 2017, 288 carbapenem-resistant *K. pneumoniae* clinical strains were  
52 collected among a collection of 973 isolates from eight different hospitals in Greece.  
53 Antibiotic susceptibility testing was performed using three different methods. Screening of  
54 carbapenem and colistin resistance genes was conducted using PCR amplification and  
55 sequencing.

56 **Results:** among the 288 (29.6 %) carbapenem-resistant isolates, 213 (73.9%) were colistin-  
57 resistant (MIC>2 mg / L). The KPC type was the most common carbapenemase gene (116;  
58 40.3%), followed by VIM (41; 14.2%), NDM (33; 11.5%) and OXA-48 (22; 7.6 %).  
59 Moreover, 44 (15.3%) strains co-produced two types of carbapenemases. For colistin  
60 resistance, no *mcr* genes were detected, but rather mutations in chromosomal genes were  
61 found. These included inactivation of the *mgrB* gene for 148 (69.5%), including insertion  
62 sequences 94 (44.1%) as well as nonsense 4 (1.9%) and missense mutations 24 (11.3%).  
63 Moreover, PCR amplification of *mgrB* gene was negative for 26 (12.2%) strains. Finally, 65  
64 (30.5%) colistin-resistant strains exhibited a wild-type *mgrB*, the mechanisms of which  
65 remain to be elucidated.

66 **Conclusion:** Our study shows that *K. pneumoniae* clinical strains in Greece are resistant to  
67 both carbapenems and colistin which becomes endemic and likely chromosomally encoded.

68

69 **Keywords:** *Klebsiella pneumoniae*; Greece; carbapenems; colistin resistance; *mgrB* gene.

## 70 1. Introduction

71 Carbapenems have long been considered the treatment of choice for human infections caused  
72 by multidrug-resistant Gram-negative bacteria (GNB), their use has increased due to the  
73 spread of extended spectrum  $\beta$ -Lactamases (ESBLs) among the Enterobacteriaceae family <sup>1</sup>.  
74 Nevertheless, their efficacy has been challenged by the emergence of carbapenem-resistant  
75 Enterobacteriaceae, which have spread worldwide and to Greece <sup>2</sup>.  
76 In Greece, a national outbreak of carbapenem-resistant *Klebsiella pneumoniae* carrying the  
77 VIM gene was reported in 2000s <sup>3</sup>, followed by the emergence of KPC-producing *K.*  
78 *pneumoniae* isolated for the first time in August 2007 <sup>4</sup>. Since then, these KPC-producing  
79 strains have spread rapidly, causing initial outbreaks, evolving into an endemic situation <sup>5</sup>. A  
80 first isolate of NDM-1-producing *K. pneumoniae* was reported in 2012, since several  
81 outbreaks of NDM-producing Enterobacteriaceae have been reported in different hospitals in  
82 Greece <sup>6</sup>. The isolation of OXA-48 producing Enterobacteriaceae is still uncommon <sup>3</sup>. In 2017  
83 the surveillance data from ECDC reports a carbapenem resistance rate of 64.7% in *K.*  
84 *pneumoniae* strains recovered from blood samples in Greece, the highest in Europe <sup>7</sup>. A  
85 European survey of carbapenemase-producing Enterobacteriaceae conducted from 2013 to  
86 2014 in 35 European countries reported an average of 1.3 patients per 10,000 hospital  
87 admissions in Europe carrying carbapenemase <sup>8</sup>. In Greece, this incidence was 5.78 patients  
88 per 10,000 admissions, second only to Italy (5.96 / 10.000) <sup>8</sup>. Cassini et al. have estimated that  
89 33,000 deaths/year in Europe could be attributable to multi-drug resistance bacteria (MDR)  
90 due to their high prevalence of resistance in GNB especially in Greece and Italy <sup>9</sup>. However,  
91 true deaths and attributability to antibiotic resistance is currently highly dubious <sup>10</sup>. The high  
92 prevalence of carbapenem resistance in Greece may lead to difficult-to-treat (DTR) infections  
93 <sup>11</sup>.

94

95 Under these circumstances, colistin has been reintroduced as last resort treatment for  
96 infections caused by multidrug-resistant carbapenemase producers <sup>12</sup>. However, the  
97 emergence of colistin resistance in GNB has been reported in several countries mediated via  
98 different genetic variations represented by chromosomal mutations in genes involved in  
99 lipopolysaccharide synthesis, namely *phoP / phoQ*, *pmrA / pmrB* or *crrA / crrB* as well as on  
100 the *mgrB* regulatory gene <sup>12</sup>. These mutations lead to the overexpression of these systems and  
101 to an increase in the synthesis of phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-  
102 arabinose (LAra4N) <sup>12</sup>. Moreover, colistin resistance can also be plasmid-mediated, the first  
103 colistin resistance gene involving a conjugative plasmid named *mcr-1* (mobilized colistin  
104 resistance) has been reported by Yi-Yun Liu et al. in China in December 2015 <sup>13</sup>. The latter  
105 has been identified in GNB isolates of human, animal and environmental origin <sup>13</sup>. The  
106 location of the plasmid gives it the potential to be laterally transferable, which explains its  
107 wide diffusion confirmed by numerous studies <sup>14</sup>. Since then seven other *mcr* variants have  
108 been reported worldwide <sup>15</sup>. The use of an efflux pump and the formation of capsules may  
109 also be involved in the resistance to colistin <sup>14</sup>.

110 Studies conducted in Greece on *K. pneumoniae* isolates from different clinical samples have  
111 reported a significant increase in colistin resistance from 3.5% (4/115) in 2010 to 20%  
112 (25/120) after 2010 <sup>16</sup>, a recent study reported a 19% resistance rate to last-line antibiotics,  
113 including colistin in human clinical isolates <sup>3</sup>. This rapid increase, coupled with the lack of  
114 molecular data, motivated this study project, which consists of evaluating the prevalence of  
115 colistin and carbapenem resistance among a collection of *K. pneumoniae* clinical strains  
116 isolated between 2014 and 2017 from eight hospital sites in Greece, and to understand the  
117 molecular mechanisms involved.

118

119

120 **2. Materials and methods**

121 **2.1 Bacterial isolates**

122 Between 2014 and 2017, 973 *K. pneumoniae* clinical strains were collected from eight Greek  
123 hospitals, six in Athens, one in Thessalia (central Greece) and one in Peloponnese (Figure  
124 S1). Preliminary, the strains were tested against carbapenems (meropenem) by E-test  
125 (BioMérieux, France), the resistant strains with an MIC > 8 mg/L were selected and stored for  
126 further analysis. Identification at the species level was performed by Matrix Assisted Laser  
127 Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonik,  
128 Bremen, Germany) <sup>17</sup>.

129

130 **2.2 Antimicrobial susceptibility test**

131 Antimicrobial susceptibility testing was performed on meropenem-resistant isolates using the  
132 disk diffusion method on a Mueller-Hinton agar (MHE BioMérieux, France) and interpreted  
133 according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)  
134 guidelines except for cyclines that were interpreted according to CLSI recommendations. The  
135 imipenem MIC determination was performed by the E-test method (BioMérieux, France),  
136 data were interpreted according to the criteria of EUCAST. MICs of colistin were determined  
137 by broth microdilution method and the results were interpreted according to the guidelines  
138 described by the EUCAST.

139 DTR bacteria were defined as previously described <sup>11</sup>, strains were considered as DTR when  
140 having an intermediate or resistant status to three antibiotic classes ( $\beta$ -lactams, carbapenems,  
141 and fluoroquinolones).

142

143

144

## 145 **2.3 Molecular characterization**

146 DNA extraction was performed on BioRobot EZ1 (Qiagen, Venlo, Netherlands) using a  
147 commercial EZ1 DNA extraction kit (Qiagen) according to the manufacturer's instructions.

148

### 149 **Carbapenemase encoding genes**

150 All strains were screened for the presence of *bla<sub>KPC</sub>*, *bla<sub>NDM</sub>*, *bla<sub>VIM</sub>*, *bla<sub>OXA-48</sub>*, and *bla<sub>IMP</sub>* by  
151 quantitative polymerase chain reaction (qPCR) (table S\_1).

152

### 153 **Colistin resistance genes**

154 All strains were screened for the presence of plasmid-mediated colistin resistance genes  
155 represented by *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* and *mcr-8* by PCR using specific primers  
156 and probes (table S\_1). Those strains were also investigated to detect possible genetic  
157 alterations associated with colistin resistance in the *mgrB* genes. The *mgrB* genes were  
158 sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster  
159 City, California, United-States). Sequences of the colistin resistance genes were recovered  
160 and compared to those of the reference strain *K. pneumoniae* MGH78578 carrying wild-type  
161 genes (GenBank accession number NC\_009648). The PROVEAN software  
162 (<http://provean.jcvi.org/index.php>) was used to check whether amino acid sequence changes  
163 could induce an alteration of the protein function<sup>18</sup>. In addition, the ISfinder database was  
164 used to identify the insertion sequences types (<https://www-is.biotoul.fr/>)<sup>19</sup>.

165

## 166 **2.4 Whole genome sequencing**

167 Genomic sequencing was performed with the MiSeq sequencer (Illumina Inc., San Diego,  
168 CA, USA) in a pair-end in order to study certain atypical phenotypes. The genome was

169 assembled using the A5 pipeline, then reorganized by mapping on a reference genome *K.*  
170 *pneumoniae* MGH785 using MAUVE.

171

## 172 **2.5 Statistical analyses**

173 Statistical analyses were performed using the Chi-squared test. A p-value <0.05 was  
174 considered as statistically significant.

175

176 **3. RESULTS**

177 **3.1 Bacterial identification**

178 The 973 *K. pneumoniae* strains were isolated from 196 (20.1%) blood, 569 (58.5%) urines,  
179 153 (15.7 %) respiratory samples, and the remaining 55 (5.6%) were from other sources. The  
180 288 meropenem-resistant *K. pneumoniae* strains were isolated from 148 (44%) blood, 89  
181 (27%) urines, 44 (13%) respiratory samples and the remaining 7 (2.43%) were from other  
182 sources. The majority of strains (213, 74%) were isolated in 2017.

183 The MSP dendrogram of 288 meropenem-resistant *K. pneumoniae* strains did not reveal any  
184 distinct groups or clusters (year, origin, hospital), (phenotype of carbapenem or colistin  
185 resistance) (Figure 1).

186

187 **3.2 Phenotypic profile of the bacteria studied**

188 Antibiotic results from the 288 strains of *K. pneumoniae* reported a very remarkable  
189 resistance to the  $\beta$ -lactam family (97%) (Figure 2). *K. pneumoniae* strains were resistant to  
190 aminoglycosides with 57% and 69% for gentamicin and amikacin, respectively. Resistance to  
191 quinolones, sulfamides, nitrofurans and cyclines was observed in 96%, 78%, 75% and 74% of  
192 strains, respectively. However, fosfomycin was the most active antibiotic on the majority of  
193 strains tested (28%). Out of the 288 strains, 226 (78%) of the strains were DTR.

194 The imipenem E-test showed that out of 288 *K. pneumoniae* strains, 256 (88.8%) were  
195 resistant to imipenem with MICs ranging from 3 mg/L to > 32 mg/L: 156 (16%) strains had  
196 an MIC > 32 mg/L. The 32 remaining strains were resistant to both ertapenem and meropenem  
197 but not to imipenem. Colistin MIC revealed that out of 973 *K. pneumoniae* strains, 213  
198 (21.9%) were resistant to colistin, with MICs ranged from 4 mg/L to >256 mg/L.

199 Statistical analyses showed that resistance to colistin was significantly associated with the  
200 resistance to carbapenems for the meropenem resistant strains (n=288) ( $p < 0.05$ ).

### 201 **3.3 Molecular analyses**

#### 202 **Molecular mechanisms of carbapenem resistance**

203 Of the 288 meropenem-resistant *K. pneumoniae* strains, 256 (88.9%) carried a gene encoding  
204 a carbapenemase. 32 (11.1%) strains were carbapenem-resistant and negative for all tested  
205 carbapenemase genes in this study. The KPC enzyme was the most common carbapenemase  
206 and was identified in 116 strains (40.3%), followed by the VIM and NDM detected in 41  
207 (14.2%) and 33 (11.5%) of the strains, respectively. OXA-48 enzymes were found in 22  
208 strains (7.6%) (Table 1). Four OXA-48 positive strains remain susceptible to imipenem  
209 (MICs  $\leq 2$  mg/L). The IMP enzyme was not detected in any strain. On the other hand, 44  
210 (15.3%) strains harboured two types of carbapenemases, with five combinations namely  
211 *bla<sub>KPC</sub> + NDM*, *bla<sub>KPC</sub> + OXA-48*, *bla<sub>KPC</sub> + VIM*, *bla<sub>NDM</sub> + OXA-48*, *bla<sub>NDM</sub> + VIM* (Table 1).

212 Sequence analysis of the *bla<sub>KPC</sub>*, *bla<sub>NDM</sub>*, *bla<sub>OXA-48</sub>* positive strains revealed that these strains  
213 belonged to the KPC-2, NDM-1, and OXA-48 variants. On the other hand, of the VIM  
214 positive strains, five different variants were identified namely VIM-1, VIM-2, VIM-19, VIM-  
215 52 and VIM. -55. The VIM 55 and VIM 52 being variants of VIM-1.

216

#### 217 **Molecular mechanisms of colistin resistance**

##### 218 **Plasmid-mediated colistin resistance genes**

219 PCR screening for plasmid-mediated colistin resistance genes (*mcr-1*, to *mcr-8*) was  
220 performed on the 288 *K. pneumoniae* strains. Results show that no positive *mcr* isolates were  
221 detected.

222

##### 223 **Genetic alterations of the *mgrB* gene**

224 The entire *mgrB* gene of the 213 colistin-resistant *K. pneumoniae* strains was amplified by  
225 standard PCR and sequenced. Among these isolates, 187 were *mgrB* positive and 26 were

226 negative. Of the 187 positives, 94 (50.3 %) strains generated amplicons larger than the normal  
227 size of the *mgrB* gene, suggesting the presence of sequence insertions at the coding region for  
228 the *mgrB* gene.

229 ISK*pn26* of the IS5 family was the most common insertion sequence found in *mgrB* and was  
230 identified in 42 (19.7%) isolates, followed by ISEc68 (19 strains, 8.9%), ISK*pn14* (13; 6.1%),  
231 IS1R (10; 2.3%), ISK*pn25* (6; 2.8%), IS903 (2; 0.9%) and IS5 (2; 0.9%) (Table 2). The  
232 ISK*pn26* and ISEc68 belonging to the IS5 family had a length of 1,200 bp and 1,199 bp,  
233 respectively (Figure 3). These ISs were inserted at the same position, between nucleotides 69  
234 and 70. In contrast, an ISEc68 was inserted between nucleotide 141 and 142, with a length of  
235 881 bp. An ISK*pn14* belonging to the IS1 family was found inserted at two different positions  
236 with sequence lengths of 780 bp and 778 bp (Figure 3). The IS1R of the IS1 family were  
237 identified at different positions in the gene but at the promoter of the *mgrB* gene in one  
238 isolate. An IS903 was also found inserted at the level of the gene promoter in a strain that also  
239 had a 4-base insertion (GTGC) between nucleotides 80 and 81 (Figure 3). A 394 bp ISK*pn25*  
240 belonging to the ISL3 family was detected between nucleotides 132 and 133.

241

242 ***MgrB* gene sequence changes by nonsense mutations.** In 4 (1.9 %) strains, a single  
243 mutation induced the appearance of a premature stop codon (Table 2), which leads to the  
244 replacement of a glutamine by a stop codon at position 30 (Q30 $\Delta$ ). The resulting protein had  
245 only 29 amino acids long instead of a non-mutated protein of 47 amino acids (Figure 4. A).

246

247 ***MgrB* gene sequence change by missense mutations.** 24 *K. pneumoniae* colistin-resistant  
248 strains had a single nucleotide substitution in the *mgrB* gene (Figure 4. A). Mutations induced  
249 the replacement of phenylalanine (F35I) by isoleucine at position 35 in one isolate and  
250 glycine by serine (G37S) at position 37 in three isolates. Substitution of lysine with valine at

251 position 2 (K2V) was the most frequently encountered mutation (20 isolates) (Table 2). All  
252 these substitutions have been reported deleterious by the PROVEAN software. One isolate  
253 had a four-base insertion (TGCG) between nucleotides 85 and 86, resulting in frame shift and  
254 non-functional truncated protein of 27 amino acids in length.

255

256 **Partial and total deletion of the *mgrB* gene.** For DNAs of 26 (12.2%) colistin-resistant *K.*  
257 *pneumoniae* strains that could not be amplified by standard PCR, additional amplification  
258 using internal primers of the *mgrB* gene was performed (Figure 3). The latter made it possible  
259 to obtain amplicons for 5 of the 26 strains. The five strains had a partial deletion of the gene  
260 as well as significant alterations in the protein sequence of the *mgrB* gene (Figure 4.B). For  
261 the remaining 21 strains, no sequence was obtained, suggesting a complete absence of the  
262 *mgrB* locus.

263 Genomic sequencing of one of those strain revealed that the *mgrB* gene was truncated by an  
264 IS, and the strains lacked a region of 500 bp containing 3 genes including a part a *mgrB* gene  
265 (57 pb). A BLAST search along 7kb region containing the *mgrB* gene against *K.*  
266 *pneumoniae\_MGH 78578* revealed a conserved chromosomal location and genetic  
267 environment of this gene (Figure 5).

268

#### 269 4. DISCUSSION

270 In Greece, since the first report of *K. pneumoniae* producing VIM in 2001, the prevalence of  
271 carbapenem-resistant *K. pneumoniae* has increased and is currently considered endemic <sup>20</sup>.

272 In the present study we found a prevalence of carbapenem resistance in *K. pneumoniae*  
273 clinical strains of about 30% in 8 hospitals Greece. The KPC type (40.3%) remains the most  
274 prevalent carbapenemase encoding gene followed by VIM (12.5%), the latter are also known  
275 for their endemicity in Greece <sup>21</sup>.

276 These results are consistent with a previous national surveillance study carried out between  
277 January 2011 and June 2012, in 119 hospitals in Greece that showed that in *K. pneumoniae*  
278 strains, the KPC enzyme was the most prevalent mechanism of resistance to carbapenems  
279 (82.6%), followed by the VIM in second position (9.7%) <sup>22</sup>. However, a recent study  
280 conducted in a European survey of carbapenemase-producing Enterobacteriaceae from 2013  
281 to 2014 showed for the first time that out of 86 carbapenem-resistant *K. pneumoniae* strains  
282 isolated in Greece, the NDM-type enzyme was the second most prevalent carbapenemase with  
283 a rate of 14% after KPC (10.5%) <sup>8</sup>.

284 Although the OXA-48 enzyme has been reported in sporadic cases <sup>3</sup>, we have noted the  
285 emergence of strains carrying OXA-48 gene. The true prevalence of this enzyme is poorly  
286 known in Greece <sup>23</sup>. Usually, OXA-48 confers a lower resistance than other carbapenemases  
287 and is not always detected in antibiotic susceptibility testing <sup>24</sup>. Interestingly we found OXA-  
288 48 positive *K. pneumoniae* strains in our study that were sensitive to carbapenems. We were  
289 not able to detect the IMP enzyme in any of the isolates; indeed, in Greece there are no data  
290 regarding its isolation <sup>25</sup>. The co-production of different carbapenemases by the same strain is  
291 more frequently reported in the latest studies in several countries (India, Egypt, China and  
292 Greece) <sup>23,24,26-29</sup>. In our strain collection, we identified 44 (15.3%) strains of *K. pneumoniae*  
293 co-producing two types of carbapenemases. Recent studies in Greece have reported the

294 presence of *K. pneumoniae* strains producing both KPC-2 and VIM-1 as well as NDM-1 and  
295 VIM-1<sup>28,29</sup>. The KPC-2 and NDM-1 variants remain the most prevalent carbapenemases  
296 among our strains, confirming the previous Greek estimates<sup>21,29</sup>. For the VIM type genes  
297 analysed, several variants were detected. VIM-1, VIM-2 and VIM-19 have already been  
298 described in Greece<sup>21,29</sup>, in contrast to VIM-52 and 55.

299 Colistin has regained a significant part of the therapeutic regimen for the treatment of  
300 carbapenem-resistant bacterial infections. However, it was rapidly followed by the emergence  
301 of resistance. In our strain collection, among the 973 *K. pneumoniae* strains we reported an  
302 overall colistin resistance rate of 21.9% and a rate of 73.9% of resistance among carbapenem-  
303 resistant *K. pneumoniae* strains. The fact that none of the *mcr* genes tested were detected  
304 confirms the data in the literature that show that the prevalence of *mcr* genes worldwide is  
305 much higher in strains of animal origin than in human ones<sup>30</sup>. This assumes that their  
306 reservoir is at least in animals and the environment, following the important use of colistin in  
307 animal production and in agriculture in general<sup>30</sup>. Unlike other countries, in Greece, the  
308 massive use of colistin in clinical practice following the spread of EPCs has led to the  
309 selection of multidrug-resistant bacteria in hospital settings. According to data available on  
310 ECDC, the consumption of colistin was 0.004 DDDs (daily defined dose of antibiotics) / 1000  
311 patients per day in 2000, 0.071 in 2010 and 0.135 in 2016<sup>7</sup>.

312 In the present study, out of the 213 colistin-resistant *K. pneumoniae*, 148 (69.5%) had an  
313 inactivated *mgrB* gene, mediated either by sequence insertions, absence or point mutations of  
314 *mgrB* gene. The *mgrB* gene is a conserved 144 nucleotide gene encoding a small 47 amino  
315 acid transmembrane protein, a strong negative feedback from the *PhoQ / PhoP* regulatory  
316 system<sup>14</sup>. Inactivation by sequence insertions was the predominant cause of colistin resistance  
317 and the ISK<sub>pn26</sub> element was the most prevalent.

318 The interruption of the *mgrB* gene by ISK<sub>pn25</sub>, IS903 and ISCs68, IS5, or ISK<sub>pn14</sub> has  
319 already been reported<sup>31-35</sup>, but has not been described so far in Greece. Avgoulea et al.  
320 reported that insertional inactivation of the *mgrB* gene conferred resistance to colistin in all  
321 isolates tested<sup>31</sup>. The F35I and G30Del substitutions in the *mgrB* gene have been reported,  
322 mutations in the same amino acid position have also been reported in colistin-resistant *K.*  
323 *pneumoniae* isolates by several other studies<sup>36</sup>. This reinforces the hypothesis that these  
324 substitution in the *mgrB* protein is a critical region that is likely to mutate upon the emergence  
325 of resistance. In addition, new amino acid changes in the *mgrB* gene were observed and  
326 probably led to a predicted truncated and non-functional *mgrB* protein are also reported.  
327 The prevalence of colistin resistance remains low in many countries unlike Greece. Overall,  
328 this resistance is closely related to the dissemination of carbapenem-resistant bacteria. Thus,  
329 our statistical analyses have shown that resistance to colistin was significantly associated with  
330 the carbapenem resistance phenotype and correlated with the production of KPC and NDM  
331 carbapenemase genes in *K. pneumoniae*, contributing to their multi-resistant phenotypes.  
332 In conclusion, our study reported a high rate of resistance to colistin (70%) in carbapenem-  
333 resistant *K. pneumoniae* clinical isolates likely due to chromosomal mutations of target genes,  
334 especially inactivation of the *mgrB* gene by sequence insertions and not by spreading of  
335 plasmid-mediated *mcr* colistin resistance genes. This finding clearly supports the notion that  
336 colistin selection pressure in humans and in animals led to the selection of different bacterial  
337 clones in colistin resistant bacteria with *mcr* variants in animals and environment and specific  
338 clones with chromosomal mutations in humans. However, the observed resistance could not  
339 be explained in a large proportion of samples (approximately 31% that harboured a wild type  
340 *mgrB* gene and in about 10% where the *mgrB* gene could not be amplified), thereby limiting  
341 the conclusions that might be drawn. Finally, our study suggests that colistin resistance is  
342 becoming endemic in Greece in carbapenem-resistant *K. pneumoniae* human isolates. The

343 prevalence of resistance to carbapenems and colistin in Greece should be surveyed and new  
344 therapeutic strategies including old drugs should be evaluated and used in Greece. Among the  
345 different antibiotics tested Fosfomycin may be a valid alternative to treat carbapenems and  
346 colistin resistant bacteria in Greece.

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#### 351 **Declarations**

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358 **Ethical Approval :** Not required

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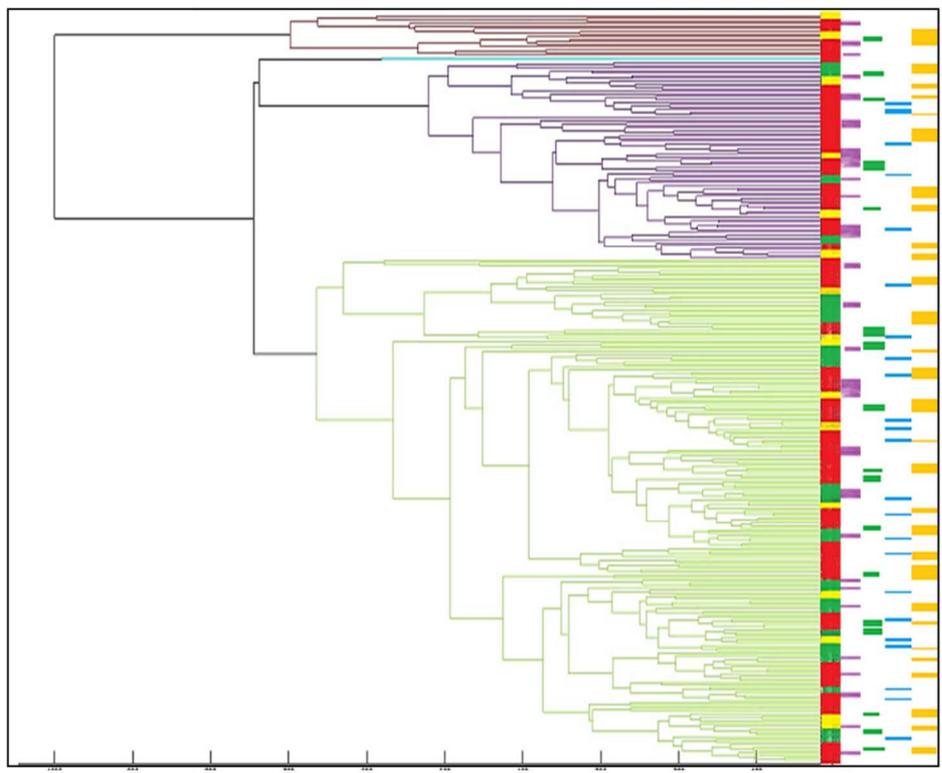
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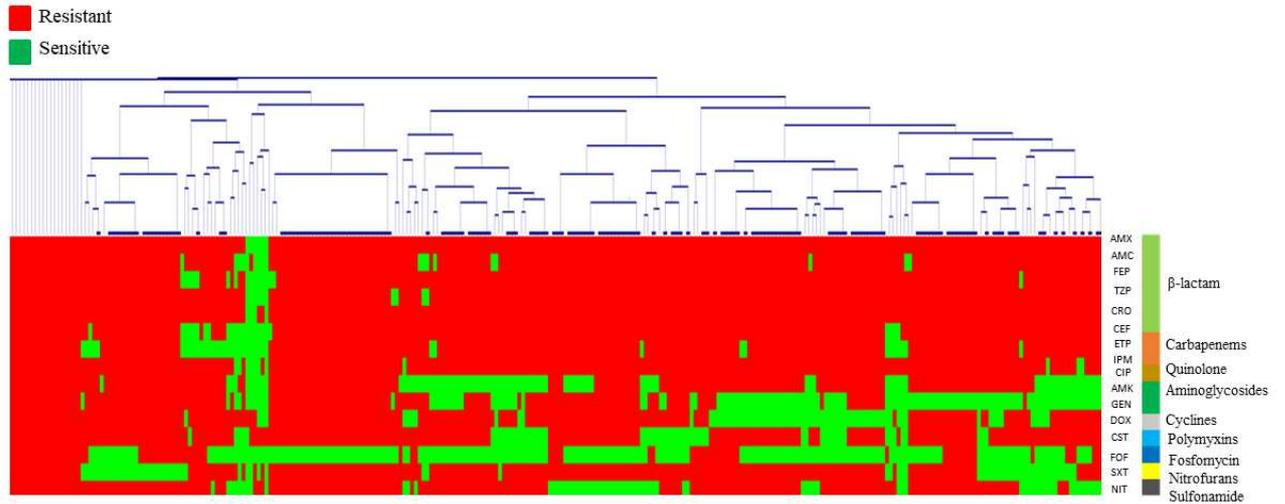


- Strains (ColR and CarbaR)
- Strains (ColR and CarbaS)
- Strains (ColS and CarbaS)
- Sequence insertion
- Amino acid substitution
- MgrB* not detected
- Strains isolated in 2017

12 **Figure 1.** MALDI-TOF MS Dendrogram of 288 meropenem-resistant *K. pneumoniae* clinical  
13 strains, 2014 to 2017.

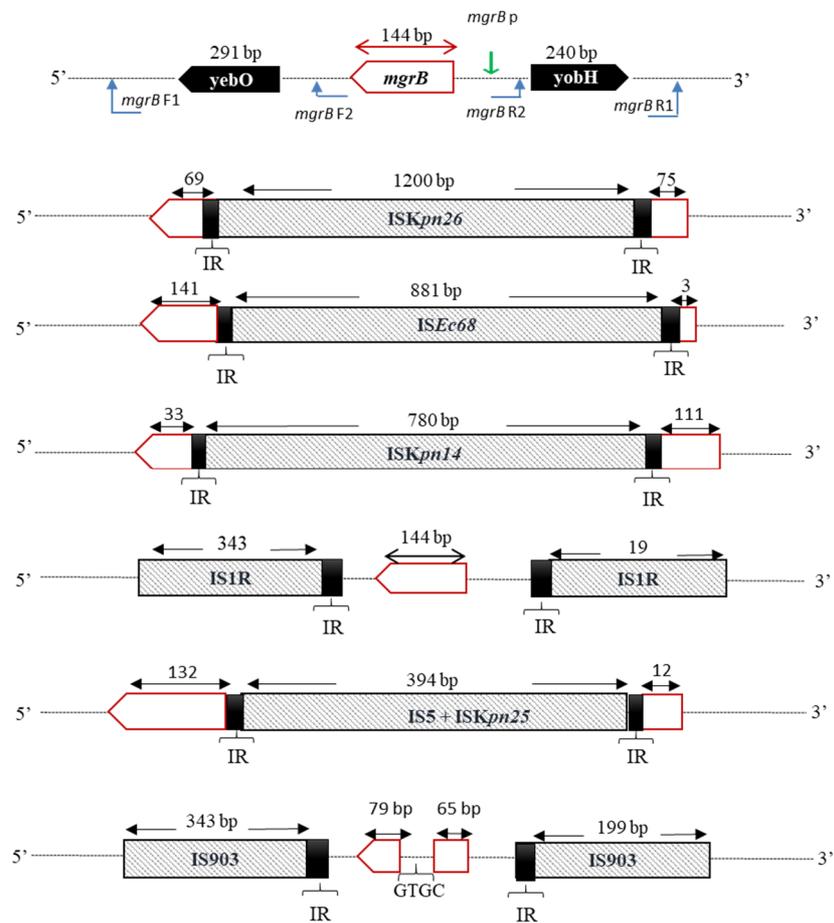
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**Figure 2.** Hierarchical clustering of antibiotic susceptibility profiles (disk diffusion method) representing the activity of the 16 antibiotics tested against the 288 meropenem-resistant *K. pneumoniae* strains isolated in this study. AMX: Amoxicillin; AMC: Amoxicillin + clavulanic acid; FEP: Cefepime; TPZ: Piperacillin + Tazobactam; CRO: Ceftriaxone; CEF: Cefalotin; ERT: Ertapenem; IMP: Imipenem; CIP: Ciprofloxacin; AMK: Amikacin; GEN: Gentamicin; DOX: Doxycycline; CST: Colistin; FOF: Fosfomycin; SXT: Trimethoprim / sulfamethoxazole; NIT: Nitrofurantoin.

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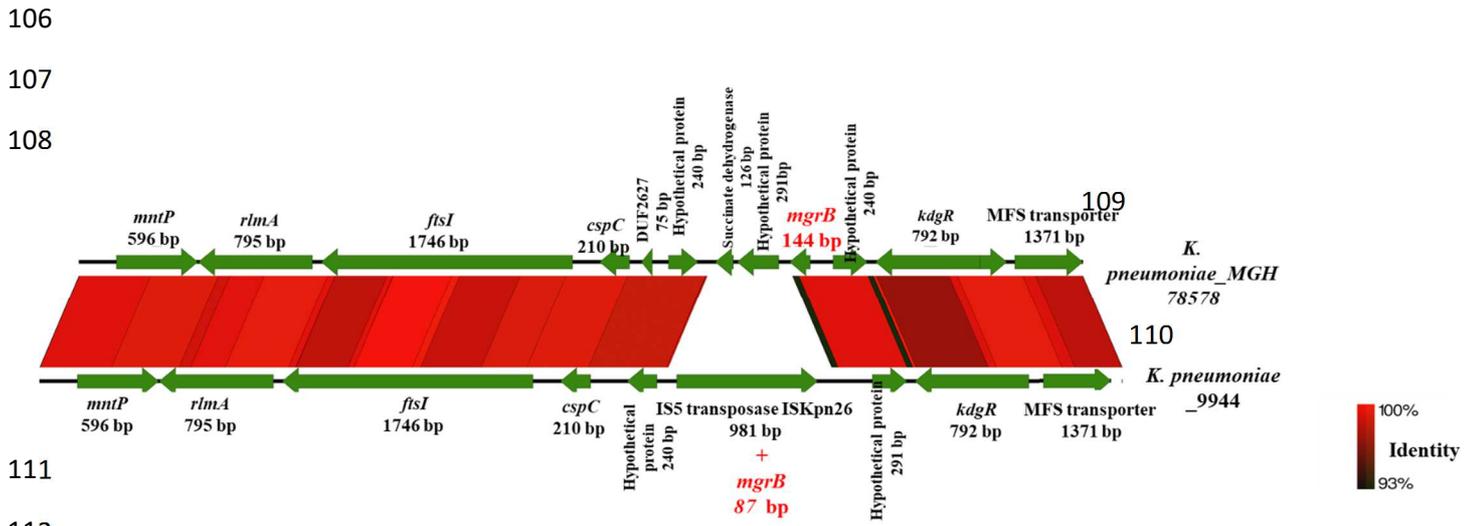
**Figure 3.** Schematic representation of the *mgrB* gene of *K. pneumoniae* strains resistant to colistin, showing the different integration sites of sequence insertions (IR: inverted repeat; *mgrB* p: *mgrB* promoter).

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(a) > KP reference VKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFSSGICTINKFIPW
> KP1(K2V) -VKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW
> KP2 (G37S) VKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFS GICTINKFIPW
> KP3 (F35I) VKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFSSGICTINKFIPW
> KP4 (Q30del) VKKLRWVLLIVIIAGCLLLWTQMLNVMCD-----
> KP5 (TGGGins)VKKLRWVLLIVIIAGCLLLWTQMLNVM-----

(b) >KP reference VKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW
>KP1 -KKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFAAAFALLINLFRGK
>KP2 -KKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFAAAFALLINLFRGK
>KP3 -KKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFAAAFALLINLFRGK
>KP4 -KKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFAAAFALLINLFRGK
>KP5 -KKITVGFTDSSHRLPVAVTQMLNVMCDQDVQFLAAFTLLINLFRG
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**Figure 4.** Alignment of the protein sequences of the *mgrB* gene of *K. pneumoniae* colistin resistant strains (a): using the external primers (the underlined areas indicate the presence of a mutation); (b): using the internal primers; KP: *Klebsiella pneumoniae*.



113 **Figure 5:** Genetic location of the 7kb containing the *mgrB* gene of the reference *K.*  
114 *pneumoniae\_MGH* 78578 and *K. pneumoniae* 9944 using the EasyFig software. The arrows  
115 indicate the positions and directions of the ORFs, direction indicate the gene orientation.

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131 **Table 1:** Carbapenemases detected among carbapenem-resistant *K. pneumoniae* strains  
 132 isolated from Greek hospitals, 2014 to 2017. (\* Several isolates contain more than one  
 133 carbapenemase). KPC: *Klebsiella pneumoniae* carbapenemase; NDM: New Delhi metallo-  
 134 beta-lactamase; VIC: Verona integron-mediated metallo-beta-lactamase; OXA-48:  
 135 Oxacillinase-48.

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Species	Carbapenem resistance genes	Positive results for carbapenemases N (%)		MIC imipenem Median value
		N	Percentage (%)	
<i>Klebsiella pneumoniae</i>	<i>bla<sub>KPC</sub></i>	116	40.3	32
	<i>bla<sub>NDM</sub></i>	33	11.5	
	<i>bla<sub>OXA-48</sub></i>	22	7.6	8
	<i>bla<sub>VIM</sub></i>	41	14.2	12
	<i>bla<sub>KPC+NDM</sub></i>	12	4.2	32
	<i>bla<sub>KPC+OXA-48</sub></i>	9	3.1	12
	<i>bla<sub>KPC+VIM</sub></i>	10	3.5	
	<i>bla<sub>NDM+OXA-48</sub></i>	6	2.1	32
	<i>bla<sub>NDM+VIM</sub></i>	7	2.4	
	Negative	32	11.1	
<b>Total</b>		288*	100	32

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145 **Table 2:** Table summarizing the different genetic alterations of the *mgrB* gene in the 213 *K.*  
 146 *pneumoniae* colistin resistant strains isolated from Greek hospitals, 2014 to 2017.

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Alterations of the <i>mgrB</i> gene		N	Percentage (%)
94 sequence insertions	ISK <sub>pn26</sub> - IS5	42	19.7
	ISEc68 - IS5	19	9
	ISK <sub>pn14</sub> - IS1	13	6.1
	IS1R - IS1	10	4.6
	ISK <sub>pn25</sub> - ISL3	6	2.8
	IS903 - IS5	2	1
	IS5 - IS5	2	1
Amino acid substitutions		24	11.3
Premature stop codon		4	1.9
Intact		65	30.5
Not detected	Total deletion	21	9.8
	Partial deletion	5	2.3
Total		213	100

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