Intestinal Carriage of Colistin Resistant Enterobacteriaceae at Saint Georges Hospital in Lebanon

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2   **Lebanon**

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Abstract:

Objectives

The increase in resistance to antibiotics has led to the revival of colistin, as the last option for treatment, which automatically led to an increase of colistin-resistant Gram-negative bacteria. In this study, we report the presence of clinical colistin-resistant *Enterobacteriaceae* isolated from a Lebanese hospital.

Method

From twenty-three rectal swabs, eight colistin-resistant clinical strains (5 *Escherichia coli*, 2 *Enterobacter cloacae,* and 1 *Klebsiella pneumoniae*) were isolated. Antibiotic susceptibility testing was performed using the disk diffusion method and E-test. Broth microdilution method was performed to determine colistin susceptibility. RT-PCR, standard PCR, and sequencing were used to investigate genes encoding for extended-spectrum β-lactamases, carbapenemases, and colistin resistance. Genotyping of these isolates was conducted by MLST.

Results

Results of antibiotic susceptibility testing revealed that all isolates were resistant to colistin. They have MICs for colistin that ranged from 8 mg/L to 32 mg/L. Real-time PCR results showed that 5 strains harbored $bla_{\text{TEM-1}}$ and one strain harbored $bla_{\text{TEM-163}}$. Moreover, 4 strains were positive for $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-103}}$, and $bla_{\text{CTX-M-189}}$, and *K. pneumoniae* harbored $bla_{\text{SHV-1}}$. Observed colistin resistance was linked to amino acid substitutions into protein sequences of pmrA/B, phoP/Q, and mgrB. Interestingly, we report here mutation in the *mgrB* regulator and pmrA/B, phoP/Q in colistin resistant *E. cloacae* and *E. coli* clinical isolates for the first time in Lebanon.

Conclusion

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46   This study highlights the presence of colistin-resistant Gram-negative bacteria in a Lebanese

47   hospital, which is worrisome. An urgent strategy needs to be adopted in order to avoid the spread

48   of such bacteria.

1. Introduction

Resistance to antibiotics in Gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* has become a field of interest over the last decade. Those bacteria develop high resistance to most available antibiotics such as beta-lactams, aminoglycosides, and fluoroquinolones [1]. This growing infection caused by multidrug-resistant (MDR) Gram-negative bacteria has led to the renewal of colistin as a last-resort treatment option especially for patients in intensive care units (ICUs) [2]. Unfortunately, the resistance to polymyxins has been increasingly reported worldwide [1]. Polymyxin E (colistin) and polymyxin B have been used in humans and are bactericidal toward Gram-negative bacteria except for the bacteria that are intrinsically resistant to colistin such as *Burkholderia*, *Edwardsiella, Proteus, Providencia, Morganella* and *Serratia,*. From 1959, colistin was available to treat infections caused by Gram-negative bacteria. Conversely, its utilization is limited due to the daily dose that cannot be increased because it will lead to nephrotoxicity [3]. Between the late 1990s and early 2000s, colistin was re-introduced into clinical medicine due to the emergence of resistance to beta-lactams and carbapenems. This led to the emergence of colistin-resistant bacteria among patients treated with this compound [1]. The bacterial cell membrane is the main target for the antimicrobial activity of colistin: colistin binds to the anionic lipid A moiety of lipopolysaccharide (LPS) and disrupts the cell membrane [1,3]. Several strategies are used by Gram-negative bacteria to escape from polymyxin. The major mechanism of resistance occurs by the alteration of the negatively charged lipopolysaccharide (LPS), by the addition of phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), to the lipid A moiety of the LPS. This can be accomplished by specific mutations of the two-component systems (TCSs) (*pmrA*/*pmrB*, *phoP*/*phoQ*), or its negative regulator *mgrB.* The modified LPS with this

72 positive charge reduces its binding to polymyxins and produces this resistance [1]. Moreover, it

73 has been demonstrated that resistance to colistin can also be due to the loss of LPS production

74 due to mutations in *lpxACD* genes [4], in addition to the use of efflux pumps, the capsules

75 formation, and overexpression of OprH, which are all efficiently regulated at the molecular level

76 [1]. Finally, colistin resistance can also be due to the presence of a plasmid-mediated *mcr* gene

77 that encodes for the phosphoethanolamine transferase enzyme which is capable of modifying the

78 lipid A moiety of LPS with the addition of phosphoethanolamine [5]. Finally, the increased use

79 of colistin as a last-resort therapeutic medication to treat patients infected with multidrug-

80 resistant (MDR) Gram-negative bacteria, has been followed by an increase in the number of

81 Gram-negative bacteria resistant to colistin [1]. The present study aims to investigate the

82 prevalence of colistin-resistant Enterobacteriaceae isolated from rectal swabs of patients

83 remaining at ICU of Saint-George Hospital in Beirut, Lebanon for more than one week and who

84 were treated with carbapenem and colistin combination therapy.

2. Materials and methods

2.1 Microbiology procedure:

2.1. Study Design

Between October 2016 and February 2017, a rectal swab was collected for each patient hospitalized in the ICU of Saint-George Hospital in Beirut who received colistin and carbapenem during their stay in ICU for more than one week. These patients were treated with combination therapy because it was given as a precaution for patients to prevent their infections by carbapenem-resistant bacteria that were spreading in the hospital. Only patients of the ICU treated with combination therapy were selected because we believe that patients receiving colistin have a bigger chance to have an intestinal carriage of colistin-resistant isolates. The rectal swab was collected after 7 days of therapy for the analysis of colistin and carbapenem-resistant bacteria. They were kept at -80°C before being transported to the laboratory in Marseille, France.

2.2 Microbiological procedures

2.2.1 Screening of colistin-resistant enterobacteria

Rectal swabs were transferred to an enrichment Tryptic Soy Broth (TSB) medium and incubated overnight at 37°C. After incubation, 100 μL of the enrichment medium were cultivated for 24 hours at 37°C on the selective medium LBJMR for the screening of colistin-resistant organisms, and on MacConkey agar plates supplemented with Ertapenem (2 µg/ml) for the screening of carbapenem-resistant organisms. Species intrinsically resistant to colistin were excluded from this study. Bacterial identification at the species level was performed using the

106 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)

107 method (Microflex; Bruker Daltonics).

2.2.2 Antibiotic susceptibility testing

109 The standard disk diffusion method on Mueller-Hinton agar was performed to determine

110 the antibiotic susceptibility testing of the strains as recommended by the European Committee of

111 Antimicrobial Susceptibility Testing (EUCAST) 2017 (http://www.sfm-

112 microbiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). The minimal

113 inhibitory concentration (MIC) of colistin was determined using the broth microdilution method

114 according to EUCAST 2017. Each strain with a MIC > 2 mg/L for colistin was considered as

115 resistant to this antibiotic.

2.2.3 DNA extraction

117 The automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan) was performed to

118 extract the DNA of the bacteria, with the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany),

119 according to the manufacturer's guidelines and eluted in 200 μL of elution buffer and stored at -

120 20°C.

2.2.4 Multilocus sequence typing (MLST)

122 To determine the genetic relationship between the clinical isolates, genotyping analysis

123 was done using seven housekeeping genes for *E. coli*, *K. pneumoniae* and *E. cloacae,* as

124 described on Institute Pasteur's MLST Web site (www.pasteur.fr/mlst).

2.2.5 Screening of isolates by real-time PCR, standard PCR, and sequencing of the

antibiotic resistance genes

127   To detect the presence of carbapenemase encoding genes, a real-time PCR assay was

128   done, using specific primers for bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, $bla_{NDM,}$ and bla_{VIM} genes. All

129   colistin-resistant isolates were also screened for the presence of extended-spectrum β-lactamase

130   (bla_{TEM}, $bla_{SHV,}$ $bla_{CTX-M,}$) genes and for the *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, and mcr-5 genes.

131   Probes and primers used were described previously [6,7]. Positive PCR amplification targeting

132   antibiotic resistance genes were sequenced using BigDye terminator chemistry on an automated

133   ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). ARG-ANNOT database was

134   used to analyze the sequenced genes and was compared to other genes using the BlastN and

135   BlastP analysis. The described colistin-resistant genes including *pmrA, pmrB, phoP, phoQ,* and

136   *mgrB* were amplified and sequenced [2,8–10]. Sequenced genes were compared with those of the

137   reference strains including *E. coli* K-12 MG 1655 (NCBI GenBank accession no.**CP000647**), *K.*

138   *pneumoniae* MGH 78578 (NCBI GenBank accession no.**CP000647**) and *E. cloacae* ATCC

139   13047 (NCBI GenBank accession no.**CP000647**) using the nps alignment software

140   (https://prabi.ibcp.fr/htm/site/web/home). We used PROVEAN (Protein Variation Effect

141   Analyser) software (http://provean.jcvi.org/seq_submit.php), to predict whether the identified

142   amino acids substitutions that result from missense mutations would affect the function of the

143   proteins. If the protein variant has a score below or equal to a predefined threshold (-2.5), it is

144   predicted to have a "deleterious" effect. If it is above this threshold, it is predicted to have a

145   "neutral" effect.

3. Results

23 rectal swabs were collected from 23 different patients treated with colistin and carbapenem combination therapy. No carbapenem-resistant *Enterobacteriaceae* have been isolated from MacConkey agar medium supplemented with Ertapenem. Of the 23 patients, 12 patients have no colistin-resistant *Enterobacteriaceae* (ColiRE) whereas 11 patients carried at least one ColiRE. The mean carriage of ColiRE was 1 /sample except one patient carries 2ColiREs/sample. In summary, twelve ColiRE were collected with the LBJMR medium. Among the isolates, 8 colistin-resistant strains were identified as: (5 *Escherichia coli*, 2 strains as *Enterobacter cloacae,* and 1 strain as *Klebsiella pneumoniae*). In addition, 4 isolates that were intrinsically resistant to colistin were also isolated (1*Proteus mirabilis* and 3 *Morganella morganii)* but were excluded from this study. The antibiotic resistance profile of all the isolates is presented in Table 1. Results showed that all isolates were resistant to amoxicillin, amoxicillin/clavulanic acid, cefalotin, doxycycline, and colistin. Colistin MICs ranged from 8 to 32 mg/L. Inversely, all isolates were susceptible to ertapenem, imipenem, amikacin, gentamicin, and nitrofurantoin. In addition, six strains were resistant to the third generation cephalosporins. Real-time PCR and standard PCR results showed that *E. coli* (EC-5, EC-10, and EC-23), *E. cloacae* (Eclo-14A and Eclo-14B), and *K. pneumoniae* (KP-16), were positive for bla_{TEM} gene. Sequence analysis of detected penicillinase identifies bla_{TEM-1} gene in positive strains except for *E. coli* EC-23 that was identified as $bla_{TEM-163}$. Moreover, 4 out of the 5 *E. coli* strains harbored $bla_{CTX-M-15}$, $bla_{CTX-M-103}$, and $bla_{CTX-M-189}$ (Table 1). Single *K. pneumoniae* KP-16 harbored bla_{SHV-1} gene. None of the strains harbored mobile colistin resistance genes (from *mcr*-1 to *mcr*-5). MLST analysis reveals that five *E. coli* isolates belonged to five different sequence types (STs) including ST131, ST6174, ST405, ST162 and ST1451. *K. pneumoniae* has ST45, and the

169   two *E. cloacae* have new sequence types ST924 and ST925 (Table 1). Due to the absence of *mcr*

170   genes, the associated colistin resistance genes including *mgrB, pmrA, pmrB, phoP,* and *phoQ*

171   were amplified and sequenced. As shown in Table 2, sequence analysis of these latter revealed

172   that the two *E. cloacae* isolates exhibited non-synonymous mutations in *mgrB*, *pmrA, pmrB*, and

173   *phoP* genes leading to amino acid changes. These mutations in *mgrB* were detected for the first

174   time in lebanon and were considered as deleterious by the PROVEAN software. Moreover,

175   sequence analysis of *E. coli* strains revealed that colistin resistance of *E. coli* EC-5 was due to

176   different missense mutations in *pmrAB* operon resulting in amino acids substitutions. For *E. coli*

177   EC-10, the missense mutations occurred in *phoPQ* proteins. *E. coli* EC-12 exhibited missense

178   and deletion mutations in *pmrB* and *phoPQ*. *E. coli* EC-21 showed *pmrA* and *phoP* genes

179   affected by missense mutations, and for *E. coli* EC-23 colistin resistance was due to missense

180   and deletions mutations in *pmrA* and *phoPQ*. Analysis of *K. pneumoniae* KP-16 revealed that

181   there were two genetic changes linked to colistin resistance: firstly, missense mutations leading

182   to a premature stop codon of the *mgr*B and secondly, a missense in the *pmrB* gene that results in

183   amino acid substitutions (Table 2). All these mutations were predicted as deleterious by the

184   PROVEAN software.

4. Discussion

The increase of multidrug-resistant Gram-negative bacteria, especially carbapenem-resistant bacteria, is a worldwide clinical problem. Indeed, this has led to the re-introduction of colistin into clinical treatment against infections caused by MDR bacteria [5]. This study describes the presence of intestinal carriage of colistin-resistant enterobacteria in patients who received colistin and carbapenem for treatment. We found that these patients carried only colistin-resistant bacteria but no carbapenem-resistant bacteria, suggesting that the use of these molecules may select colistin resistance but not carbapenem resistance. One limitation of our study is that no sample was collected before the instauration of the treatment. It is possible that these patients already carried these colistin-resistant bacteria, before the use of colistin. Different studies, in different countries such as France, Columbia, Spain, Laos, Thailand, USA, Nigeria, and Saudi Arabia have described the presence of colistin-resistant enterobacteria in humans without prior colistin exposure [11]. Moreover, a study done by Nakayama T et al. showed that even a short-term trip to some countries may result in the spread of *strains* that harbored *mcr-1* and that are carried by international travelers where their numbers continue to increase, thereby increasing the risk of spreading *of colistin-resistant* to developed countries [12].

Genotyping results showed that our strains belonged to different sequence types (ST) suggesting, therefore, no link between strains isolated in the same hospital. It has been shown that the clone ST131 is a globally important pathogen among multidrug resistance *E. coli* and is linked to nosocomial and acquired infections [13]. Moreover, the clones of other *E. coli* ST405, ST162, ST1451, and *K. pneumoniae* ST45 have been reported in hospitalized patients in different counties, such as Brazil, Spain, Sweden and Uruguay [14–17]. In addition, *E. coli* ST405 has also been reported in animals in Algeria [18].

208   In addition, all strains in this study were resistant to amoxicillin, amoxicillin-clavulanic

209   acid, and cephalosporins including cefalotin, cefepime, and ceftriaxone except the *E. cloacae*

210   isolates*,* which are sensitive to ceftriaxone and cefepime. All isolates harbored β-lactamase genes

211   such as TEM, SHV, and CTX-M. *bla*$_{TEM-163}$, *bla*$_{CTX-M-103,}$ *bla*$_{CTX-M-189}$, and *bla*$_{SHV-1}$ detected in

212   this present study have never been previously reported in Lebanon.

213   Interestingly, many different gene mutations including missenses and deletions have been

214   identified in genes associated with colistin resistance in our clinical isolates. However, some of

215   these mutations have been reported earlier such as R81S found in PmrA of a colistin-resistant

216   strain isolated from animal samples under the Spanish Surveillance Network of Antimicrobial

217   Resistance in Bacteria [9,19]. R81S is also similar to the missense mutation R81H which was

218   found in PmrA of *S. Typhimurium* [20]. The D150W mutation in PhoQ of our *E. coli* EC-10

219   isolate was previously reported in Taiwan in a *K. pneumoniae* isolate collected from patient at

220   the Taipei Veterans General Hospital [21]. Mutation T92H found in *pmrB* of *E. coli* EC-12

221   isolate, were similar to T92A described in PmrB of *S. Typhimurium* [4,20]. D191Y mutation in

222   PhoP of *E. coli* EC-21 was reported in South Africa in clinical *K. pneumoniae* isolate [22].

223   Olaitan et al. have also identified mutation L96P in *K. pneumoniae* isolated from patients and

224   healthy individuals in Nigeria, Thailand, Lao PDR, and France [2] which is similar to L96S

225   found in PhoQ of our study. Regarding colistin-resistant *K. pneumoniae* KP-16 isolate, different

226   missense mutations have been found, such as G37M, C39P and N42Y that were similar to the

227   mutations identified in the *mgrB* of *K. pneumoniae* described by Poirel et al. In Lebanon, there is

228   no report describing mechanism of colistin resistance of *Enterobacteriaceae* clinical isolates

229   except a study reported recently by one of our group that describe three colistin-resistant *K.*

230   *pneumoniae* isolated from Lebanese Hospital in Beirut in 2015. The resistance of those strains

231 was due to mutations of *mgrB, pmrA, pmrB,* and *phoQ* genes [5]. Those strains were isolated

232 from patients without previous use of colistin. On the other hand, two studies were done by

233 Dandachi et al., which described the mechanism of colistin resistance in animals. In these studies,

234 colistin resistance was due to the presence of a plasmid-mediated *mcr-1* gene isolated from *E.*

235 *coli* strain from poultry in Lebanon [23] and from 23 *E. coli* strains isolated from Lebanese

236 swine farms [23]. In fact, carriage of *mcr-1* in farms could constitute a potential key for the

237 introduction of this gene into the community as well as to clinical settings in Lebanon through

238 horizontal transfer from animals to humans. Due to the spread of carbapenemase producers in

239 hospitals in Lebanon, it is expected that once *mcr-1* would be introduced, this latter will be

240 selected by the frequent use of colistin.

241 Few studies have described the mechanism of colistin resistance in *E. cloacae*. It seems

242 that the PhoP/PhoQ two-component system may play a role in this colistin resistance. The *mgrB*

243 gene acts as a repressor of this two-component system and inactivation of this gene leads to

244 colistin resistance in this species. However, a previous study on colistin-resistant *E. cloacae* has

245 detected no alteration of the *mgrB* gene, leading the mechanism still unknown [10].On the other

246 hand, a recent study had detected a missense mutation in the *mgrB* gene (I10V), in addition to

247 many missense mutations in the pmrA/B genes which may contribute to the colistin resistance

248 [24]. In our study, we have detected for the first time in Lebanon mutations in the two

249 components system pmrA/B, phoP/Q and *mgrB* genes in *E cloacae.* Moreover, different

250 missense mutations that have been found, such as C39G, N42S, I45Y, W47V, W47S, *48K and

251 *48Y were similar to the mutation identified in the *mgrB* of *K. pneumoniae* described by Poirel

252 et al [19].

253   In conclusion, this study reports the emergence of colistin resistance in Gram-negative

254   bacteria in Lebanon. The use of polymyxins to treat patients has probably contributed to the

255   emergence of colistin-resistant strains in this hospital since no relationship between our strains

256   has been observed. An urgent strategy must be implemented to prevent the spread of these

257   resistant microorganisms among hospitalized patients.

258

263

264   **Competing interests:**

265   No conflicts of interest or financial disclosure for all authors

266

267   **Ethical approval:**

268   Not required.

269

272

273   **Authors' affiliations**

274   TND and ED collect samples and isolate strains. TND, CAB, SC, LH, SB, ED, and SMD

275   performed experiments and analyzed the data. TND, SMD, ED, and JMR draft and proofreading

276   the manuscript. JMR and ED conceived the study, participated in its design and coordination. All

277   authors read and approved the final version of the manuscript.

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Table 1: Phenotypic and genotypic features of the eight colistin-resistant strains isolated at the Saint Georges hospital in Lebanon

Strain name	AMX	AMC	TZP	CF	CRO	FEP	ERT	IPM	AK	GEN	CIP	FF	F	DO	SXT	CS	Colistin MIC (µg/ml)	bla_{SHV}	bla_{TEM}	$bla_{CTX-M-A}$	ST
E. coli **EC-5**	R	R	S	R	R	R	S	S	S	S	S	S	S	R	S	R	16	-	TEM-1	-	1451
E. coli **EC-10**	R	R	S	R	R	R	S	S	S	S	R	S	S	R	S	R	16	-	TEM-1	CTX-M-15	131
E. coli **EC-12**	R	R	S	R	R	R	S	S	S	S	S	S	S	R	S	R	16	-	-	CTX-M-189	6174
E. coli **EC-21**	R	R	R	R	R	R	S	S	S	S	R	S	S	R	S	R	16	-	-	CTX-M-15	405
E. coli **EC-23**	R	R	S	R	R	R	S	S	S	S	R	S	S	R	R	R	8	-	TEM-163	CTX-M-103	162
E. cloacae **Eclo-14A**	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	8	-	TEM-1	-	924
E. cloacae **Eclo-14B**	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	16	-	TEM-1	-	925
K. pneumoniae **KP-16**	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	R	32	SHV-1	TEM-1	-	45

359   amoxicillin (**AMX**), amoxicillin-clavulanic acid (**AMC**), piperacillin-tazobactam (**TZP**), Cefalotin (**CF**), ceftriaxone (**CRO**), cefepime (**FEP**),

360   ertapenem (**ERT**), imipenem (**IPM**), amikacin (**AK**), gentamicin (**GEN**), ciprofloxacin (**CIP**), fosfomycin (**FF**), nitrofurantoin (**F**), doxycycline

361   (**DO**), trimethoprim-sulfamethoxazole (**SXT**), colistin (**CS**). **R,** resistant; **S,** sensitive, sequence type (**ST**)

Table 2: Mutations of the associated colistin-resistance proteins

Strain names	MgrB	PmrA	PmrB	PhoP	PhoQ
E. coli EC-5	Not tested	L11Q R81S	A65del	No mutation	No mutation
E. coli EC-10	Not tested	No mutation	No mutation	R163P, N165del	I88T, L95P, P111T, W113R, L114S, S116W, R145W, D150W, H157P, L218F
E. coli EC-12	Not tested	No mutation	V77A, L81P, T92H R93P, L95A, E97G L98A, Q99A, L102S E103W	Q113P, K171R, H198del	L231R, N235I, E246del
E. coli EC-21	Not tested	G15E, R81S D82del, D86R, K87del	No mutation	K171R, R185P, S187I, D191Y, K200R, I210S, V213G, Y218H	L96S, Q99A, I109H, W113M, F119S
E. coli EC-23	Not tested	R81S	No mutation	N119I, E154del	K46I, del$_{56-70}$
K. pneumoniae KP-16	S36G, G37D, I38A, C39A, I41T, N42Y	No mutation	D150N, S205I, G207del	No mutation	No mutation
E. cloacae Eclo-14A	G37_V38insG V38S, C39G, A40K I41M, N42S, K43G, I45Y, P46G	S64C, L216W, E217I	L38S	No mutation	No mutation

	W47V, ins48K				
E. cloacae Eclo-14B	V38I, W47S, ins 48V	No mutation	No mutation	D46V, I47F, I49F, E140del, F141del, I143D, N144A, del$_{148-163}$	No mutation

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