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3 **CMI – RESEARCH NOTE**

4

5 **The Inoculum Effect of *Escherichia coli* expressing *mcr-1* or not on Colistin Activity in a**
6 **Murine Model of Peritonitis.**

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20 Running title: Colistin in murine peritonitis

21 Key words: *Escherichia coli*; peritonitis; colistin; antibiotic resistance; MCR-1;
22 pharmacodynamics.

23 **Abstract**

24 **Objectives.** Colistin often remains the last resort antibiotic active against carbapenemase-
25 producing Enterobacteriaceae. However, while *in vitro* inoculum effect has been reported,
26 therapeutic relevance of this phenomenon remains questioned.

27 **Methods.** Ten *E. coli* strains were used that included the wild-type CFT073 and its
28 transconjugant CFT073-MCR-1 and eight clinical isolates harbouring a plasmid-encoded
29 polymyxin-resistance *mcr-1* gene. Mice with peritonitis were treated for 24 h with colistin
30 sulfate. Bacterial loads were determined in peritoneal fluid (PF) and spleen and colistin-
31 resistant mutants were detected.

32 **Results.** MICs of colistin against the eight MCR-1 positive clinical strains and CFT073 ranged
33 from 0.125 to 0.5 mg/L with an inoculum of 10^5 CFU/mL and from 2 to 4 mg/L with a 10^7
34 CFU/mL inoculum; 5/9 strains with an MIC of 4 mg/L were considered resistant according to
35 EUCAST breakpoint (resistance, > 2 mg/L). When the bacterial load of wild-type CFT073
36 inoculated in mice increased from 10^7 to 10^8 CFU: i) mean \log_{10} CFU reduction generated by
37 colistin in PF and spleen decreased from 5.8/mL and 3.1/g, respectively, ($p < 0.01$) to 0.9/mL
38 and 0.8/g, respectively (NS); ii) mice survival rate decreased from 15/15 (100%) to 6/15
39 (40%) ($p = 0.017$); and iii) proportion of mice with selection of colistin-resistant mutants
40 increased from 4/15 to 15/15 ($p < 0.01$). These results were comparable to those obtained
41 when peritonitis was produced with a 10^7 CFU bacterial load of *E. coli* CFT073 expressing
42 *mcr-1*, for which the mean \log_{10} CFU reductions were 3.5/mL and 0.6/g in PF and spleen,
43 respectively (NS), and survival rate was 8/15 (53%) ($p < 0.01$ versus survival of mice infected
44 with wild-type CFT073).

45 **Conclusions.** Phenotypic colistin resistance in wild-type *E. coli* due to an increase in inoculum
46 size had a therapeutic impact in mice with peritonitis that was comparable to that observed
47 when the *mcr-1* gene was expressed.

48

49

50 INTRODUCTION

51 Therapeutic options against carbapenemase-producing Enterobacteriaceae are scarce since
52 such strains often harbour plasmid-mediated genes conferring resistance to other
53 antimicrobial classes [1]. In this context, colistin is increasingly used as last-resort antibiotic
54 to treat infections with carbapenem-resistant bacteria [2]. On a pharmacodynamic point of
55 view, colistin causes rapid bacterial killing in a concentration-dependant manner [3].
56 However, rapid emergence of resistant mutants and reduced *in vitro* activity in the presence
57 of a high bacterial inoculum, the so called “inoculum effect”, are potential major limiting
58 factors for clinical use [4]. Since the *in-vivo* relevance of the inoculum effect has been
59 questioned [5], we investigated the therapeutic impact of an increase in the size of bacterial
60 inoculum on colistin activity in a murine peritonitis model due to *Escherichia coli*.

61

62 MATERIALS AND METHODS

63 **Bacterial strains.** Nine unrelated susceptible *E. coli* clinical isolates (including *E. coli* CFT073,
64 a wild-type uropathogenic B2 strain), and one resistant strain, CFT073-MCR-1, a
65 transconjugant obtained by conjugation from the clinical strain *E. coli* Af31 harbouring *mcr-1*
66 plasmid into *E. coli* CFT073 [6], were used (Table 1).

67 ***In-vitro* studies.** All experiments were performed with colistin sulfate purchased from Sigma-
68 Aldrich (Saint-Quentin, France). MICs of colistin were determined by the broth microdilution
69 in cation supplemented Mueller-Hinton broth (MHB) with an inoculum size of $5 \cdot 10^5$ CFU/ml,
70 in accordance with the EUCAST guidelines [7]. In addition, in order to investigate for the
71 presence of a potential inoculum effect *in vitro*, MICs were also performed with various
72 inoculum sizes, increasing from 10^3 to 10^7 CFU/mL.

73 Frequency of selection of spontaneous resistant mutants was determined for a colistin
74 concentration of 4 x MIC [8]; stability of resistance after serial passages in antibiotic-free
75 MHB and maximal growth rate (MGR) were determined, as described previously [9].

76 **Experimental murine model.** An experimental peritonitis murine model was performed,
77 using Swiss ICR female mice, as previously described [10]. Animal experiments complied with
78 ARRIVE guidelines and were performed in our laboratory in accordance with prevailing
79 regulations regarding the care and use of laboratory animals and approved by the
80 Departmental Direction of Veterinary Services (Paris, France, agreement no. 75-861). The
81 peritonitis protocol (n° APAFIS#4949-2016021215347422 v5) was approved by the French
82 Ministry of Research and by the ethical committee for animal experiment.

83 Bacterial strains used in this animal were wild-type *E. coli* CFT073 and its transconjugant
84 CFT073-MCR-1. Pellets of overnight cultures were mixed 1:1 with porcine mucin 10% (Sigma-
85 Aldrich, Saint-Quentin, France). In order to investigate the impact of the increase in
86 inoculum size, mice were inoculated with a 250- μ L intraperitoneal injection of
87 bacteria/mucin mix, corresponding to a final inoculum of 10^7 CFU or with 10^8 CFU. The 10^7
88 CFU bacterial load has been shown lethal in 97% of the animals within 24 h in the absence of
89 treatment [10]. Two hours after inoculation, colistin treatment was started and start-of-
90 treatment control mice were sacrificed. Peritoneal wash was performed by intraperitoneal
91 injection of 2 mL of sterile saline solution followed by gentle massage of the abdomen and
92 opening the peritoneum to collect 1 mL fluid. Spleen was extracted and homogenized in 1
93 mL of sterile saline solution. In mice that did not survive to the infection despite antibiotic
94 treatment, only spleen (not peritoneal fluid) was extracted to avoid sample contamination.
95 Ten-fold dilutions of samples were plated onto agar for quantitative culture, containing or

96 not concentrations of colistin 4-fold the MIC to detect for selection of resistant mutants *in*
97 *vivo*.

98 **Colistin treatment.** Colistin sulfate was injected subcutaneously with a dose of 10 mg/kg
99 every 6h (4 injections), to obtain free area under the concentration-time curve (AUC) in the
100 range of that obtained in humans [3, 8].

101 **Colistin pharmacokinetic and pharmacodynamic analysis.** Colistin concentrations were
102 determined in plasma from infected mice 0.5, 1, 2, 4, and 6 hours after colistin injection by
103 liquid chromatography-tandem mass spectrometry [12]. PK indices were calculated using a
104 non-compartmental analysis. PK indices were calculated using a non-compartmental
105 analysis. PK indices and *f*AUC/MIC, the PK/PD index associated with colistin activity *in vivo*
106 [13], were estimated on free concentrations using a protein binding of 90%, as shown for a
107 wide range of concentrations (2 to 50 mg/L) in mice [13].

108 **Statistical analysis.** Comparisons were made using non-parametric tests: Mann-Whitney U
109 test for continuous variables and Fisher's exact test for proportions. A p value of less than
110 0.05 was considered significant.

111

112 **RESULTS**

113 ***In-vitro* inoculum effect.** MICs of colistin against the nine colistin-susceptible strains ranged
114 from 0.125 to 0.5 mg/L with an inoculum of 10^5 CFU/mL and from to 2 to 4 mg/L with an
115 inoculum of 10^7 CFU/mL (Table 1). With the inoculum of 10^7 CFU/mL, colistin MIC was 4
116 mg/L for five strains including wild-type *E. coli* CFT073 that would thus be considered as

117 resistant according to the EUCAST breakpoints (resistance >2 mg/L). MIC of CFT073-MCR-1
118 was 8 mg/L and was poorly influenced by inoculum size (Table 1).

119 **Colistin pharmacokinetics and pharmacodynamics in plasma.** $fAUC_{0-24h}/MIC$ was 8.19 for
120 CFT073 and 0.51 for CFT073-MCR-1 when MIC was tested with an inoculum of 10^5 CFU/ml,
121 and decreased to 1.02 for CFT073 when MIC was tested with an inoculum of 10^7 CFU/mL or
122 10^8 CFU/mL (MIC = 4 mg/L for both inocula).

123 **Impact of *in-vivo* inoculum on colistin activity.** When mice were inoculated with a bacterial
124 load of 10^7 CFU of wild-type *E. coli* CFT073, colistin was highly effective in reducing CFU in
125 peritoneal fluid ($p < 0.0001$) and in spleen ($p < 0.001$), and survival rate was 15/15 (100%)
126 (Table 2). When mice were inoculated with a bacterial load of 10^8 CFU of wild-type *E. coli*
127 CFT073, no significant reduction in CFU was observed and survival rate decreased to 6/15
128 (40%) ($p = 0.017$).

129 Against CFT073-MCR-1, using the 10^7 CFU bacterial load, no significant antibacterial effect
130 was achieved in peritoneal fluid and in spleen (Table 2). Mice survival rate was 8/15 (53%).
131 Use of a bacterial load of 10^8 CFU led to the total loss of antibacterial activity and to a
132 mortality rate of 10/10 (100%) (Table 2).

133 **Colistin resistant mutants.** Selection of colistin-resistant mutants occurred *in vitro* at a
134 frequency of 3.5×10^{-7} for CFT073. *In vivo*, this occurred in 4/15 mice inoculated with 10^7
135 CFU of CFT073 and in 15/15 mice inoculated with 10^8 CFU ($p < 0.005$) (Table 2). Resistant
136 clones of CFT073 selected *in vivo* had an MIC ranging from 4 to 32 mg/L ($n = 18$). Resistance
137 was stable after serial passages *in vitro* and did not alter MGR ($\cdot h^{-1}$), as compared with
138 parental strain (median MGR 3.39 [3.38-3.39, $n = 3$] and 3.41 [3.09-3.54, $n = 18$] for *in vitro* and
139 *in vivo* resistant mutants, respectively, versus 3.37 [3.26-3.50, $n = 5$] for CFT073). No mutants

140 with increased level of resistance to colistin were detected *in vivo* with CFT073-MCR-1. This
141 corresponded to a frequency of colistin-resistant mutants $<10^{-10}$ *in vitro*.

142

143 **DISCUSSION**

144 Here we showed a clear *in-vitro* inoculum effect for colistin, as MIC from nine colistin-
145 susceptible *E. coli* strains increased from 0.125-0.5 mg/L to 2-4 mg/L when the inoculum
146 increased from 10^5 to 10^7 CFU/mL (Table 1). This phenomenon is of clinical relevance for
147 several reasons: i) colistin MIC not only increased with the inoculum, but reached values that
148 corresponded to resistance according to current breakpoints; ii) this *in-vitro* phenomenon
149 was translated *in vivo* into an almost total suppression of colistin antibacterial effect in
150 peritoneal fluid and spleen, an increased rate of selection of resistant mutants and to an
151 increased mortality in mice infected with wild-type *E. coli* CFT073; iii) this effect was
152 comparable to the impact of the expression of a low-level resistance in a reference strain
153 that produced MCR-1 (Table 2); iv) in terms of PK/PD, the $fAUC_{0-24h}/MIC$ reported target
154 for colistin to kill 1 \log_{10} CFU is of 3.7 to 28 [13]. This target was achieved only for CFT073
155 (8.19) with an MIC was tested according to EUCAST inoculum of $5 \cdot 10^5$ CFU/ml), but not when
156 tested with an inoculum of 10^7 CFU/ml (1.02) and not for CFT073-MCR-1 (0.51).

157 Increase in inoculum size was also associated with selection of resistant mutants in all mice
158 infected with a 10^8 bacterial load of wild-type CFT073 (Table 2). This result was in line with
159 the spontaneous frequency of colistin-resistant mutants in CFT073 (3.5×10^{-7}) and with the
160 size of the local bacterial concentration in peritoneal fluid at the start of treatment (10^7 to
161 10^9 CFU/mL) (Table 2). This represents an ecological threat as resistant mutants were stable
162 and did not show any growth defect suggestive of decreased fitness.

163 A potential limitation of the study was the duration of colistin treatment, that lasted 24 h for
164 ethical reasons. More prolonged treatment may have produced a more selective advantage
165 for the colistin-resistant subpopulations that emerged during treatment and allow them to
166 become dominant among the surviving bacterial population responsible for peritonitis.

167 In conclusion, clinicians should be aware that, at least in *E. coli*, colistin phenotypic
168 resistance due to an increase in inoculum size had a negative therapeutic impact that was
169 comparable to that observed with expression of *mcr-1* gene. These results suggest, in case of
170 severe infection such as peritonitis, the need for drainage of abscesses and for peritoneal
171 lavage before colistin usage, to improve efficacy and limit the selection of colistin resistant
172 mutants. Combination with another antibiotic might be another way to prevent this
173 phenomenon.

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182 **AUTHOR CONTRIBUTIONS.** BF designed the trial. JP and FC performed in vitro studies. JP,
183 AR and MB performed animal studies. NK and PN constructed and provided the resistant
184 strain. NG and WC did the antibiotic assays and the PK study. BF, WC, NG and PN wrote the
185 article.

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243 **Table 1.** *In vitro* inoculum effect for the 10 *E. coli* study strains.

<i>E. coli</i> strains	Colistin MICs (mg/L) according to inoculum size (CFU/mL) :					
	10 ³	10 ⁴	10 ⁵	5.10 ⁵ *	10 ⁶	10 ⁷
CFT073	0.125	0.5	0.5	0.5	2	4
CFT073-MCR-1	8	8	8	8	8	16
W1-W8 (n=8)						
Median	0.125	0.125	0.25	0.5	1	4
[ranges]	[0.125-0.25]	[0.125-0.5]	[0.125-0.5]	[0.25-0.5]	[0.5-1]	[2-4]

244 *: according to EUCAST guidelines [7]

245 **Table 2.** Bacterial counts in peritoneal fluid (log₁₀ CFU/mL) and spleen (log₁₀ CFU/g),
 246 selection of resistant mutants and survival in mice with peritonitis according to strain and
 247 inoculum size.

248

<i>E. coli</i> strains	Number of mice with mutants/Total number of mice			
	(% survival after 24h of treatment)			
	Median [min-max] log ₁₀ CFU/mL in peritoneal fluid and log ₁₀ CFU/g in spleen			
	10 ⁷ CFU bacterial load		10 ⁸ CFU bacterial load	
	Start-of-treatment controls	Colistin	Start-of-treatment controls	Colistin
CFT073	0/10 mutants	4/15 mutants (15/15, 100% survival)	0/10 mutants	15/15 mutants* (6/15, 40% survival)
Peritoneal fluid	8.2 [8.0-8.4]	2.4 [1.3-5.2]**	9.0 [7.8-9.5]	8.1 [2.5-9.9]
Spleen	7.2 [6.6-7.6]	4.1 [1.9-5.3]**	8.0 [7.0-8.5]	7.2 [4.6-8.5]
CFT073-MCR-1	0/9 mutants	0/15 mutants (8/15, 53% survival)	0/10 mutants	0/10 mutants (0/10, 0% survival)
Peritoneal fluid	8.0 [7.5-8.5]	4.5 [2.3-9.6]	8.5 [7.6-8.7]	Not determined §
Spleen	6.9 [6.7-7.1]	6.3 [4.8-8.3]	8.5 [7.6-8.8]	8.7 [8.5-8.8]

249 * p<0.005 versus standard inoculum

250 **p<0.01 versus start-of-treatment control mice

251 § : Peritoneal fluid could not be sampled in mice dying during colistin therapeutic treatment.

252

253