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B. Fantin, J. Poujade, N. Grégoire, F. Chau, A. Roujansky, et al.. The inoculum effect of *Escherichia coli* expressing *mcr-1* or not on colistin activity in a murine model of peritonitis. *Clinical Microbiology and Infection*, 2019, 25 (12), pp.1563.e5-1563.e8. 10.1016/j.cmi.2019.08.021 . hal-02477412

HAL Id: hal-02477412

<https://hal.science/hal-02477412>

Submitted on 21 Dec 2021

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FOR AA publication CLM-19-15351 REVISED-3

CMI – RESEARCH NOTE

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

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Running title: Colistin in murine peritonitis

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

Abstract

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

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

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

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INTRODUCTION

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

MATERIALS AND METHODS

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

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

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

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

Bacterial strains used in this animal were wild-type *E. coli* CFT073 and its transconjugant CFT073-MCR-1. Pellets of overnight cultures were mixed 1:1 with porcine mucin 10% (Sigma-Aldrich, Saint-Quentin, France). In order to investigate the impact of the increase in inoculum size, mice were inoculated with a 250-μL intraperitoneal injection of bacteria/mucin mix, corresponding to a final inoculum of 10⁷ CFU or with 10⁸ CFU. The 10⁷ CFU bacterial load has been shown lethal in 97% of the animals within 24 h in the absence of treatment [10]. Two hours after inoculation, colistin treatment was started and start-of-treatment control mice were sacrificed. Peritoneal wash was performed by intraperitoneal injection of 2 mL of sterile saline solution followed by gentle massage of the abdomen and opening the peritoneum to collect 1 mL fluid. Spleen was extracted and homogenized in 1 mL of sterile saline solution. In mice that did not survive to the infection despite antibiotic treatment, only spleen (not peritoneal fluid) was extracted to avoid sample contamination. Ten-fold dilutions of samples were plated onto agar for quantitative culture, containing or

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

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

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

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

RESULTS

***In-vitro* inoculum effect.** MICs of colistin against the nine colistin-susceptible strains ranged from 0.125 to 0.5 mg/L with an inoculum of 10⁵ CFU/mL and from 2 to 4 mg/L with an inoculum of 10⁷ CFU/mL (Table 1). With the inoculum of 10⁷ CFU/mL, colistin MIC was 4 mg/L for five strains including wild-type *E. coli* CFT073 that would thus be considered as

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

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

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

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

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

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

DISCUSSION

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

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

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

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

177 **ACKNOWLEDGMENTS.** We are indebted to Sara Dion and Philippe Letteron for their
178 excellent technical assistance

179 **FUNDING.** This work was partially supported by a grant from INSERM, Laboratoire Européen
180 Associé "Emerging antibioresistance in Gram negative bacteria".

181 **TRANSPARENCY DECLARATIONS.** All authors: None to declare

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.

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<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.

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