The inoculum effect of *Escherichia coli* expressing *mcr-1* or not on colistin activity in a murine model of peritonitis

B. Fantin [1, 2, 3, *], J. Poujade [1], N. Grégoire [4], F. Chau [1, 2], A. Roujansky [1], N. Kieffer [5], M. Berleur [1], W. Couet [4], P. Nordmann [1, 5, 6]

[1] *IAME, INSERM UMR 1137, F-75018, Paris, France*
[2] *Université Paris Diderot, Sorbonne Paris Cité, F-75018, Paris, France*
[3] *AP-HP, Groupe Hospitalier Paris Nord Val de Seine, Service de médecine interne, F-92210, Clichy, France*
[4] *University of Poitiers, School of Medicine and Pharmacy, INSERM UMR1070, France*
[5] *Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, INSERM European Unit (LEA, IAME), University of Fribourg, Switzerland*
[6] *University Hospital and University of Lausanne, Lausanne, Switzerland*

Keywords:
Antibiotic resistance
Colistin
Escherichia coli
MCR-1
Peritonitis
Pharmacodynamics

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

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]. From 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

* Corresponding author. B. Fantin, Hôpital Beaujon, Service de médecine interne, 100 boulevard du Général Leclerc, F-92210, Clichy, France.
E-mail address: bruno.fantin@aphp.fr (B. Fantin).

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 sulphate 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 5.10^5 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 × 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 (no. 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). 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 10^7 CFU or with 10^8 CFU. The 10^7 CFU bacterial load has been shown to be lethal in 97% of the animals within 24 hr in the absence of treatment [10]. Two hours after inoculation, colistin treatment was started and start-of-treatment control mice were killed. 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 of 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. Tenfold dilutions of samples were plated onto agar for quantitative culture, containing or not concentrations of colistin fourfold the MIC to detect for selection of resistant mutants *in vivo*.

Colistin treatment

Colistin sulphate was injected subcutaneously with a dose of 10 mg/kg every 6 hr (four injections), to obtain free area under the concentration–time curve (AUC) in the range of that obtained in humans [3,11].

Colistin pharmacokinetic and pharmacodynamic analysis

Colistin concentrations were determined in plasma from infected mice 0.5, 1, 2, 4 and 6 hr 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 (2–50 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 < 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 10^5 CFU/mL and from to 2 to 4 mg/L with an inoculum of 10^7 CFU/mL (Table 1). With the inoculum of 10^7 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

*f*AUC0-24 h/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

Table 1
In vitro inoculum effect for the 10 *Escherichia coli* study strains

E. coli strains	Colistin MICs (mg/L) according to inoculum size (CFU/mL):					
	10^3	10^4	10^5	5.10^5[a]	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 (ranges)	0.125 (0.125–0.25)	0.125 (0.125–0.5)	0.25 (0.125–0.5)	0.5 (0.25–0.5)	1 (0.5–1)	4 (2–4)

[a] According to EUCAST guidelines [7].

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[a]
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)

*$p < 0.005$ vs. standard inoculum.
**$p < 0.01$ vs. start-of-treatment control mice.
[a] Peritoneal fluid could not be sampled in mice dying during colistin therapeutic treatment.

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 eight of 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 four of 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 (per hr), 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, vs. 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: (a) colistin MIC not only increased with the inoculum, but reached values that corresponded to resistance according to current breakpoints; (b) 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; (c) this effect was similar to the impact of the expression of a low-level resistance in a reference strain that produced MCR-1 (Table 2); (d) in terms of PK/PD, the *f*AUC0-24 hr/MIC reported target for colistin to kill 1 $\log_{10}$ CFU is of 3.7–28 [13]. This target was achieved only for CFT073 (8.19) with an MIC was tested according to EUCAST inoculum of 5.10^5 CFU/mL), but not when tested with an inoculum of 10^7 CFU/mL (1.02) and not for CFT073-MCR-1 (0.51).

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, which lasted 24 hr 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 similar 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.

Transparency declaration

All authors: None to declare. This work was partially supported by a grant from INSERM, Laboratoire Européen Associé “Emerging antibioresistance in Gram negative bacteria”.

Acknowledgements

We are indebted to Sara Dion and Philippe Letteron for their excellent technical assistance.

References

[1] Rodríguez-Baño J, Gutiérrez-Gutiérrez B, Machuca I, Pascual A. Treatment of infections caused by extended-spectrum-beta-lactamase-, AmpC-, and carbapenemase-producing Enterobacteriaceae. Clin Microbiol Rev 2018;31. https://doi.org/10.1128/CMR.00079-17. pii: e00079-17.
[2] Grégoire N, Aranzana-Climent V, Magréault S, Marchand S, Couet W. Clinical pharmacokinetics and pharmacodynamics of colistin. Clin Pharmacokinet 2017. https://doi.org/10.1007/s40262-017-0561-1.
[3] Tsuji BT, Pogue JM, Zavascki AP, Paul M, Daikos GL, Forrest A, et al. International consensus guidelines for the optimal use of the polymyxins: endorsed by the American College of Clinical Pharmacy (ACCP), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Infectious Diseases Society of America (IDSA), International Society for Anti-Infective Pharma-

[4] Bulitta JB, Yang JC, Yohonn L, Ly NS, Brown SV, D'Hondt RE, et al. Attenuation of colistin bactericidal activity by high inoculum of Pseudomonas aeruginosa characterized by a new mechanism-based population pharmacodynamic model. Antimicrob Agents Chemother 2010;54:2051–62. https://doi.org/10.1128/AAC.00881-09.
[5] Craig WA, Bhavnani SM, Ambrose PG. The inoculum effect: fact or artifact? Diagn Microbiol Infect Dis 2004;50:229–30.
[6] Dénervaud Tendon V, Poirel L, Nordmann P. Transferability of the *mcr-1* colistin resistance gene. Microb Drug Resist 2017. https://doi.org/10.1089/mdr.2016.0191.
[7] EUCAST. European committee on antimicrobial susceptibility testing - breakpoint table for interpretation of MICs and zone diameters, version 6.0, 2016-01-01. 2016.
[8] Berleur M, Guérin F, Massias L, Chau F, Poujade J, Cattoir V, et al. Activity of fosfomycin alone or combined with temocillin in vitro and in a murine model of peritonitis due to KPC-3- or OXA-48-producing Escherichia coli. J Antimicrob Chemother 2018;73:3074–80. https://doi.org/10.1093/jac/dky283.
[9] Bleibtreu A, Gros PA, Laouénan C, Clermont O, Le Nagard H, Picard B, et al. Fitness, stress resistance, and extraintestinal virulence in Escherichia coli. Infect Immun 2013;81:2733–42. https://doi.org/10.1128/IAI.01329-12.
[10] Alexandre K, Chau F, Guérin F, Massias L, Lefort A, Cattoir V, et al. Activity of temocillin in a lethal murine model of infection of intra-abdominal origin dur to KPC-producing *Escherichia coli*. J Antimicrob Chemother 2016;71:1899–904.
[11] Nation RL, Li J, Cars O, Couet W, Dudley MN, Kaye KS, et al. Framework for optimisation of the clinical use of colistin and polymyxin B: the Prato polymyxin consensus. Lancet Infect Dis 2015;15:225–34.
[12] Gobin P, Lemaître F, Marchand S, Couet W, Olivier JC. Assay of colistin and colistin methanesulfonate in plasma and urine by liquid chromatography-tandem mass spectrometry. Antimicrob Agents Chemother 2010;54:1941–8.
[13] Cheah S-E, Wang J, Nguyen VTT, Turnidge JD, Li J, Nation RL, et al. New pharmacokinetic/pharmacodynamic studies of systemically administered colistin against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in mouse thigh and lung infection models: smaller response in lung infection. J Antimicrob Chemother 2015;70:3291–7.