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

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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]. 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

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 $10^9$ CFU: i) mean log$_{10}$ CFU reduction generated by colistin in PF and spleen decreased from 5.8/mL and 31.1/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.
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 \textit{Escherichia coli}.

\textbf{Materials and methods}

\textbf{Bacterial strains}

Nine unrelated susceptible \textit{E. coli} clinical isolates (including \textit{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 \textit{E. coli} AS31 harbouring \textit{mcr-1} plasmid into \textit{E. coli} CFT073) [6], were used (Table 1).

\textbf{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].

\textbf{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 \textit{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 with 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.

\textbf{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].

\textbf{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 fAUC/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].

\textbf{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.

\textbf{Results}

\textbf{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 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 \textit{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).

\textbf{Colistin pharmacokinetics and pharmacodynamics in plasma}

fAUC0–24 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).

\textbf{Impact of in vivo inoculum on colistin activity}

When mice were inoculated with a bacterial load of 10^7 CFU of wild-type \textit{E. coli} CFT073, colistin was highly effective in reducing

\textbf{Table 1}

\textit{In vitro inoculum effect for the 10 \textit{Escherichia coli} study strains}

<table>
<thead>
<tr>
<th>\textit{E. coli} strains</th>
<th>Colistin MICs (mg/L) according to inoculum size (CFU/mL):</th>
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<tbody>
<tr>
<td></td>
<td>10^3</td>
</tr>
<tr>
<td>CFT073</td>
<td>0.125</td>
</tr>
<tr>
<td>CFT073-MCR-1</td>
<td>8</td>
</tr>
<tr>
<td>W1–W8 (n = 8), median (ranges)</td>
<td>0.125 (0.125–0.25)</td>
</tr>
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\textsuperscript{a} According to EUCAST guidelines [7].
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 10^5 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 10^6 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 x 10^{-3} 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 10^6 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 10^6 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 fAUC0–24 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 x 10^{-7}) and with the size of the local bacterial concentration in peritoneal fluid at the start of treatment (10^5 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**

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