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1 **Impact of the Timing of Antibiotic Administration on Digestive Colonization with**
2 **Carbapenemase-Producing Enterobacteriaceae in a Murine Model**

3 **Author's names & affiliations :**

4 **Rémi Le Guern^{1,2}, Teddy Grandjean¹, Marvin Bauduin¹, Martin Figeac³, Guillaume**
5 **Millot¹, Aurore Loquet¹, Karine Faure^{1,4}, Eric Kipnis^{1,5}, Rodrigue Dessein^{1,2}**

6 1 : Univ. Lille, EA 7366 - Recherche translationnelle: relations hôte pathogènes, F-59000
7 Lille, France

8 2 : CHU Lille, Institut de Microbiologie, F-59000 Lille, France

9 3 : Univ de Lille, Plate-forme de Génomique Fonctionnelle et Structurale, F-59000 Lille,
10 France

11 4 : CHU Lille, Unité de Maladies Infectieuses, F-59000 Lille, France

12 5 : CHU Lille, Service de Réanimation Chirurgicale, F-59000 Lille, France

13 **Corresponding Author:**

14 Dr. Rodrigue Dessein, Univ. Lille, EA 7366 - Recherche translationnelle: relations hôte
15 pathogènes, F-59000 Lille, France; CHU Lille, Institut de Microbiologie, F-59000 Lille,
16 France; e-mail: rodrigue.dessein@chru-lille.fr

17

18 **Abstract: (75 words)**

19 While antibiotic use is a risk factor of carbapenemase-producing *Enterobacteriaceae*
20 (CPE) acquisition, the importance of timing of antibiotic administration relative to CPE
21 exposure remains unclear. In a murine model of gut colonization with NDM-1-producing
22 *Klebsiella pneumoniae*, a single injection of clindamycin within at most one week before
23 or after CPE exposure induced colonization persisting up to 100-days. Timing of
24 antibiotic administration relative to CPE exposure may be relevant to infection control
25 and antimicrobial stewardship approaches.

26 **Running title:** Timing of antibiotics relative to CPE exposure

27 (49 characters with spaces)

28 **Keywords:**

29 Carbapenemase, Gut Microbiota, NDM-1, *Klebsiella pneumoniae*, Murine model

30 **Abbreviations:**

31 CPE = carbapenemase-producing *Enterobacteriaceae*

32 Carbapenemase-producing *Enterobacteriaceae* (CPE) are an emerging public health
33 issue, considered a critical priority by the World Health Organization (1). Among CPE,
34 New Delhi metallo-beta-lactamase-1-(NDM-1)-producing *Enterobacteriaceae* are
35 particularly preoccupying. Indeed, NDM-1 confers resistance to most β -lactams,
36 including carbapenems, and has spread worldwide (2), raising fears of severe infections
37 without therapeutic options (3).

38 In the hospital setting, contact with a CPE-colonized patient or prior antibiotic use are
39 major risk factors for CPE acquisition (4, 5). Among antibiotics, anti-anaerobes (e.g.,
40 piperacillin-tazobactam or clindamycin) seem particularly at risk (6, 7).

41 While antibiotics are a known risk factor, the role of the timing of CPE exposure relative
42 to antibiotic administration is unclear. We describe a murine model of gut colonization
43 with NDM-1-producing *Klebsiella pneumoniae* following a single administration of
44 clindamycin and assess the effects of timing of clindamycin administration relative to
45 CPE exposure on effective CPE colonization.

46 The French Ethical Committee for Animal Experimentation approved this study (APAFIS
47 #7166). Seven-week-old C57BL/6 male mice housed under specific pathogen-free
48 conditions and a clinical isolate of *Klebsiella pneumoniae* producing NDM-1-
49 carbapenemase (8) were used.

50 First, we validated the murine model of CPE gut colonization. Mice were divided into
51 four groups with or without 24-hour CPE exposure in drinking water (10^7 CFU/mL at day
52 0) and/or intraperitoneal clindamycin (200 μ g) (Figure 1). CPE load was evaluated by
53 plating stool samples onto selective medium (lysogeny broth agar with 32 mg/L

54 cefotaxime and 6 mg/L vancomycin). In mice exposed to both CPE and clindamycin, 8.3
55 to 8.7 log CPE/g stools were recovered at Days 7 through 14 (Figure 1A). In mice
56 exposed to CPE without clindamycin, CPE load in stools briefly peaked at 5.6 log of
57 CPE/g of stool at Day 2 but fell below detection threshold from Day 7 onwards. Without
58 clindamycin administration, there was no effective colonization.

59 Microbiota alterations observed with CPE exposure and/or clindamycin injection were
60 analyzed by next-generation sequencing (n=2 per group) of ileal samples taken at Day 7
61 by 16S rDNA gene amplification using the Ion 16STM Metagenomics kit and sequencing
62 on the Ion PGM System (Life Technologies, Carlsbad, USA). Bioinformatic analyses
63 were performed using QIIME2 (9) and R phyloseq package (10). CPE exposure without
64 clindamycin did not alter gut microbiota compared to controls: more than 90% of 16S
65 rDNA sequences were classified as *Firmicutes*, either *Clostridiales* or *Lactobacilliales*
66 (Figure 1B, 1C). Clindamycin administration without CPE exposure led to an increase in
67 *Bacteroidetes* (approximately 45%) and *Proteobacteria*, mainly *Enterobacteriales* (up to
68 26%). Clindamycin administration with CPE exposure resulted in a major increase in
69 *Proteobacteria* (up to 84%), mostly *Enterobacteriales* (up to 76%).

70 To determine CPE colonization localization along the intestinal tract, intraluminal
71 contents from terminal ileum, caecum, colon, and stool samples were taken at Day 7 in
72 five CPE-exposed and clindamycin-treated mice. A higher load of CPE/g of digestive
73 contents was found in the caecum (8.2 log \pm 0.5), colon (8.0 log \pm 0.4) and feces (8.1 log
74 \pm 0.2) compared to ileum (5.0 log \pm 0.8, $p < 0.0001$) (Figure 1D).

75 Finally, to determine the effects of timing of clindamycin administration relative to CPE
76 exposure on effective CPE colonization, CPE exposed mice received clindamycin at
77 different days before or after CPE exposure (figure 2). Stool samples were collected
78 twice weekly for a month, then once weekly for two months to assess CPE load.
79 Clindamycin injection at most one week before or after CPE exposure was necessary to
80 achieve gut colonization (Figure 2). Indeed, when clindamycin was injected at either
81 Days-21/-14 or Days+14/+21, the mice were not durably colonized, despite CPE
82 exposure at Day-0.

83 The timing of antibiotics relative to CPE exposure is a key factor of effective CPE
84 colonization. In our murine model, a single clindamycin injection within a week before or
85 after CPE exposure induced gut colonization for at least 100 days. Remarkably, in a
86 study predating the “omics” era, van der Waaij *et al.* also demonstrated 2-week selective
87 window for persistent digestive colonization with streptomycin/neomycin resistant Gram-
88 negative bacteria following oral administration of these antibiotics (11). Other murine
89 models of digestive CPE colonization have been described. In one model, when mice
90 were exposed to KPC-producing *Klebsiella pneumoniae* three days after the first
91 administration of clindamycin, CPE load initially attained 10 log CFU/g of stool, then
92 decreased to 5-6 log CFU/g of stool five days after the last clindamycin injection (6). In
93 another model, in which mice were exposed to NDM-1-producing *Escherichia coli* four
94 days after the first administration of vancomycin, metronidazole, and ceftriaxone (12),
95 CPE load in stools was 2-3 log CFU/g (close to the detection limit) twenty days after
96 ending antimicrobials. In our model, with a single injection of clindamycin, CPE load over
97 7 log CFU/g of stool persisted for 100 days. Furthermore, our study is original in

98 assessing not only the window of opportunity for colonization after administration of
99 antibiotics, but also before and showing that this opportunity exists both one week
100 before and after antibiotics.

101 Gut microbiota analysis confirms that CPE exposure alone does not lead to colonization
102 since *Enterobacteriales*, which CPE belong to, remained undetected seven days after
103 exposure, similar to unexposed controls. Seven days after clindamycin administration,
104 there was a marked decrease in *Clostridiales* from over 75% to 30% at most, an
105 increase in *Bacteroidetes* (45%), and an appearance of *Enterobacteriales* (23%), even
106 without CPE exposure. *Enterobacteriales* are resistant to clindamycin; therefore their
107 expansion can be enhanced by clindamycin, as described previously in a murine model
108 of *Clostridium difficile* infection (13). Interestingly, in their pre-“omics” demonstration of a
109 selective window for digestive colonization induced by antibiotics, van der Waaij *et al.*,
110 using germ-free mice recolonized with the flora from mice in which colonization was no
111 longer possible late after antibiotic exposure, found (in preliminary experiments) that this
112 flora conferring what was termed “colonization resistance” was characterized by a major
113 proportion of *Clostridiales* (11). A more recent study using conventional culture methods
114 showed that antibiotics allowed colonization by the ESBL *E. coli* strain ST 131
115 regardless of effect on *Bacteroidales* (clindamycin) or not (cefuroxime, dicloxacillin) (14).
116 These results suggest that the timing of antimicrobials relative to CPE exposure in
117 providing ecologic space for implantation and expansion is an important parameter to
118 consider.

119 Clinical studies focusing on healthy travelers at high risk of being exposed to CPE or
120 other multidrug-resistant *Enterobacteriaceae* found a high rate of acquisition of

121 multidrug-resistant *Enterobacteriaceae* (15). Although initially at 51%, colonization was
122 short-lived as only 5% remained colonized three months after their return. In this
123 population of healthy travelers, only 10% reported antibiotic use during their trip. While
124 studies of acquisition and persistence of colonization by NDM-1-producing
125 *Enterobacteriaceae* after travel to endemic countries concern few subjects, larger
126 studies concentrating on extended spectrum β -lactamase-producing *Enterobacteriaceae*
127 show that a significant risk factor is the use of antimicrobials during travel (16). These
128 studies suggest that a healthy gut microbiota protects travelers exposed to CPE or other
129 multidrug-resistant *Enterobacteriaceae* from long-term colonization (17).

130 Antibiotic use is a major risk factor of CPE colonization (5). In a context of up to 50% of
131 antibiotic misuse (18, 19), avoiding an epidemic spread of CPE requires antimicrobial
132 stewardship approaches. Our study suggests that more than the general notion of prior
133 antibiotic use, it is the timing of antibiotics relative to CPE exposure that may be the
134 main factor explaining colonization with CPE. This could be a crucial parameter to take
135 into account in infection control and antimicrobial stewardship strategies. Indeed, our
136 study shows there may be a specific window of opportunity for CPE colonization relative
137 to antibiotics administration.

138 **Conflict of Interest**

139 No conflict of interest

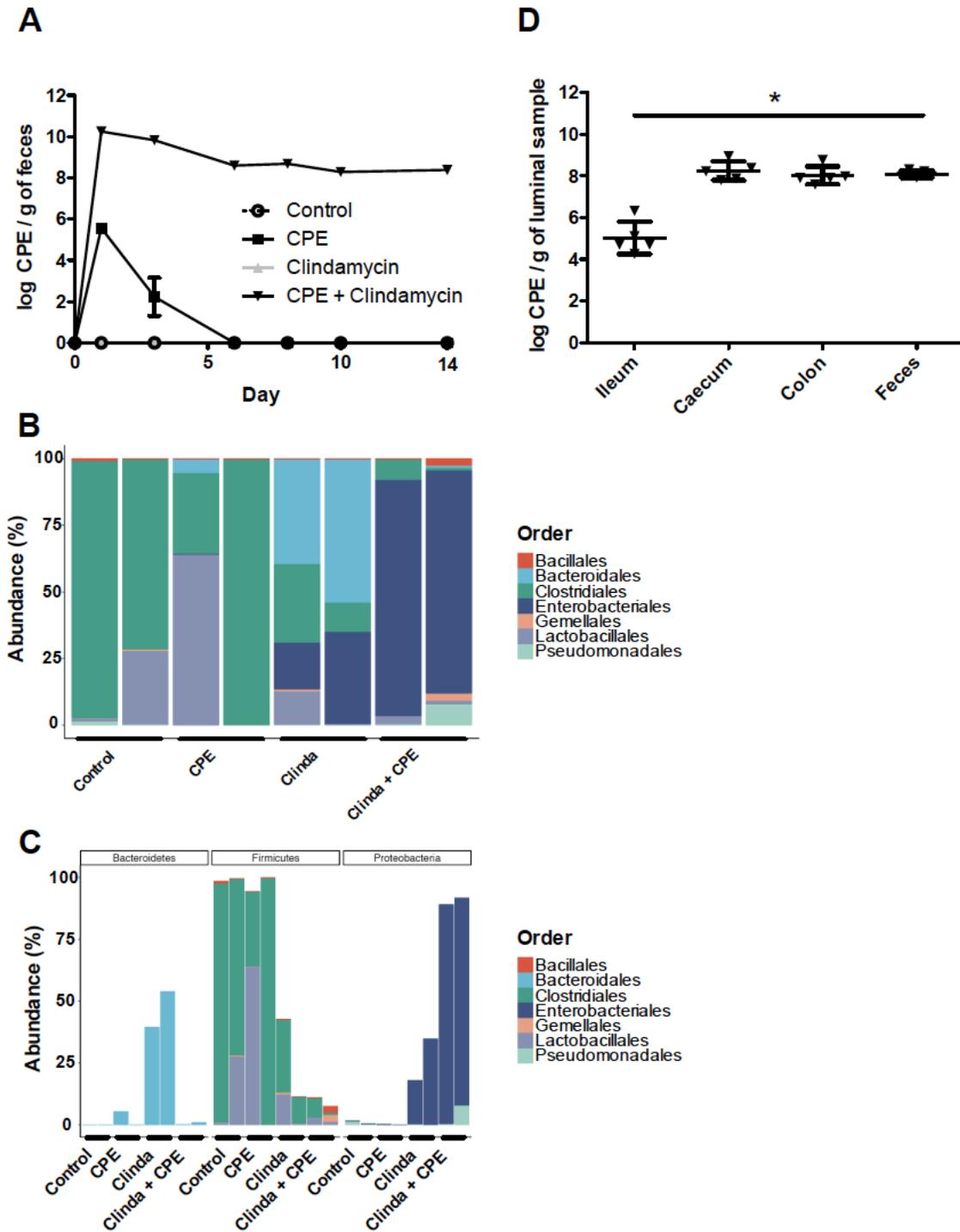
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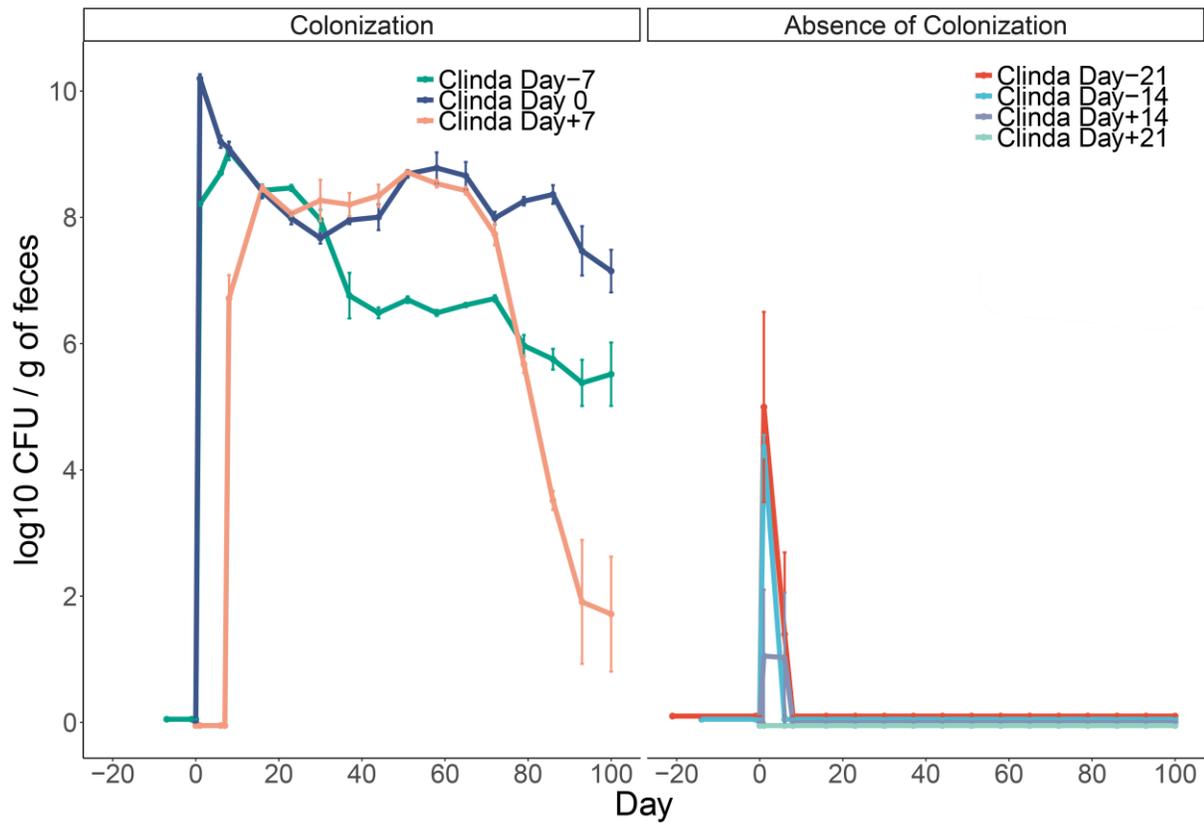
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146
 147 **Figure 1. Gut microbiota alteration using clindamycin is a prerequisite for durable**
 148 **colonization with carbapenemase-producing enterobacteria (CPE) alongside the**
 149 **intestinal tract. (A) Quantification of CPE load in stools depending on clindamycin**

150 administration and CPE exposure, 5 mice per group. Control group: no CPE exposure
151 and no clindamycin; CPE exposure group: CPE in drinking water for the first 24 hours
152 (Day 0) and no clindamycin; clindamycin group: no CPE exposure and intraperitoneal
153 injection of clindamycin at Day 0; CPE + clindamycin group: CPE in drinking water for
154 the first 24 hours (Day 0) and intraperitoneal injection of clindamycin at Day 0. (B-C)
155 Relative abundance of bacterial phyla in terminal ileum samples using next generation
156 16S rDNA sequencing. Same groups than in experiment 1A, 2 mice per group. (D)
157 Quantification of CPE load along the gastro-intestinal tract. Five mice were exposed to
158 CPE in drinking water for the first 24 hours (Day 0) associated with intraperitoneal
159 injection of clindamycin at Day 0. Luminal samples were taken at Day 14.

160



162

163 **Figure 2. Effective CPE colonization depends on the timing of clindamycin**
 164 **administration in relation to CPE exposure.** Twenty-one mice were all exposed to
 165 CPE for 24-hours in drinking water (Day 0). Clindamycin was injected once at one of the
 166 following timepoints before or after CPE exposure: Day-21, Day-14, Day-7, Day-0,
 167 Day+7, Day+14 or Day+21.

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