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## High Heterogeneity of Multidrug-Resistant Enterobacteriaceae Fecal Levels in Hospitalized Patients Is Partially Driven by Intravenous $\beta$ -Lactams

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1 **High heterogeneity of multidrug-resistant Enterobacteriaceae fecal levels in**  
2 **hospitalized patients is partially driven by intravenous beta-lactams.**

3

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8

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36 Short Running title

37 IV beta-lactams impact MRE fecal levels

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55 **ABSTRACT**

56 Multidrug-resistant Enterobacteriaceae (MRE) colonize the intestine asymptotically  
57 from where they can breach into the bloodstream and cause life-threatening infections,  
58 especially in heavily colonized patients. Despite the clinical relevance of MRE colonization  
59 levels, we know little about how they vary in hospitalized patients and the clinical factors  
60 that determine those levels. Here we conducted one of the largest studies of MRE fecal  
61 levels by tracking longitudinally 133 acute leukemia patients and monitoring their MRE  
62 levels over time through extensive culturing. MRE were defined as Enterobacteriaceae  
63 species that acquired non-susceptibility to  $\geq 1$  agent in  $\geq 3$  antimicrobial categories. In  
64 addition, due to the selective media used, the MRE had to be resistant to third-generation  
65 cephalosporins. MRE were detected in 60% of the patients, but their fecal levels varied  
66 considerably among patients and within the same patient ( $>6$  and 4 orders of magnitude,  
67 respectively). Multivariate analysis of clinical metadata revealed an impact of intravenous  
68 beta-lactams (i.e. meropenem and piperacillin-tazobactam), which significantly  
69 diminished the fecal MRE levels in hospitalized patients. Consistent with a direct action of  
70 beta-lactams, we found an effect only when the patient was colonized with strains  
71 sensitive to the administered beta-lactam ( $p < 0.001$ ) but not with non-susceptible strains.  
72 We report previously unobserved inter and intra-individual heterogeneity in MRE fecal  
73 levels, suggesting that quantitative surveillance is more informative than qualitative  
74 surveillance of hospitalized patients. In addition, our study highlights the relevance of  
75 incorporating antibiotic treatment and susceptibility data of gut colonizing pathogens for  
76 future clinical studies and in clinical decision-making.

77

78 **INTRODUCTION**

79 Resistant Enterobacteriaceae, most notably those that are multidrug-resistant (MRE:  
80 acquired non-susceptibility to  $\geq 1$  agent in  $\geq 3$  antimicrobial categories) (1), are a major  
81 threat for hospitalized patients. Infections by Enterobacteriaceae such as *Klebsiella*

82 *pneumoniae* frequently begin by colonization of the intestinal tract (2), from where they  
83 can disseminate to the bloodstream and endanger patients' lives (3). Intestinal  
84 colonization by multidrug resistant pathogens can promote their dissemination to other  
85 patients through fecal contamination of the environment (4). Understanding what  
86 influences MRE intestinal colonization is key to prevent MRE infections.

87 The clinical variables associated with intestinal carriage of resistant  
88 Enterobacteriaceae in hospitalized patients have been analyzed before (5-10), but  
89 previous studies based on culture analysis defined carriage as the detectable presence of  
90 resistant bacteria in fecal samples irrespective of their levels: the question of how MRE  
91 levels varied within and between patients remained open. Intestinal levels, and not just  
92 detectable presence, are crucial for pathogen dissemination from the gut to the  
93 bloodstream (intra-dissemination) and dissemination between patients (inter-  
94 dissemination) (11-13). Leukemia patients carrying higher levels ( $>10^6$  colony forming  
95 units - CFUs/g of fecal sample) of resistant Enterobacteriaceae have 5-fold higher risk of  
96 developing bacteremia provoked by these Enterobacteriaceae (11). In addition, patients  
97 carrying higher relative abundance of *Enterococcus* or *Klebsiella pneumoniae* in feces, as  
98 determined through microbiota sequencing analysis, have increased risk of developing  
99 bloodstream infections by these organisms (12, 13). On the other hand, studies with  
100 vancomycin-resistant *Enterococcus* (VRE) showed that high intestinal pathogen loads  
101 ( $>10^4$  CFUs/g) enhance contamination of a patient's environment (4), which facilitates  
102 dissemination to other patients (14).

103 While it is important to identify and understand the factors that impact intestinal  
104 levels of resistant pathogens, few clinical studies have sought to quantify pathogen loads.

105 Two studies—performed in a single medical center—concluded that the  
106 administration of antibiotics with activity against anaerobic bacteria can increase the fecal  
107 density of VRE and Gram-negative resistant bacilli (4, 15). This effect may have been  
108 caused by depletion of anaerobic commensal microbes, thought to be important in

109 providing colonization resistance (16, 17). However, it is not clear if all antibiotics with  
110 antianaerobic activity will increase to the same extent MRE intestinal colonization levels.  
111 For example, antibiotics administered intravenously (IV) to treat bloodstream infections  
112 must be excreted through the bile in order to reach the intestinal tract. Thus, certain IV  
113 antianaerobic therapies may not reach high enough concentrations in the gut to promote  
114 MRE intestinal colonization. Besides, while certain antianaerobic therapies mainly affect  
115 anaerobes (i.e. metronidazole, clindamycin), others—such as beta-lactams—could impact  
116 the growth of MRE directly if they are excreted into the gut in sufficient amounts and that  
117 particular MRE is sensitive to the antibiotic.

118 Here we conducted an extensive prospective study to examine the effect of specific  
119 antibiotics, as well as other factors such as antifungal treatments or neutropenia, on the  
120 gut expansion of MRE. We focused on acute leukemia patients, which are frequently  
121 colonized by resistant Enterobacteriaceae (11), to evaluate the range of colonization levels  
122 within and between patients, and to further elucidate the impact of IV-administered  
123 antibiotics and other clinical factors on the intestinal levels of MRE.

## 124 **RESULTS**

### 125 **Study population**

126 We initiated a study of 133 acute leukemia patients (Supplementary Tables 1-2), which  
127 lasted 18 months. Patients were tracked over multiple hospitalizations (median  
128 hospitalizations per patient=3; median hospitalization length=26 days; median number of  
129 days a patient was followed=70). The most frequent causes of hospital admission were  
130 chemotherapy, infection and transplantation (Supplementary Tables 3,5).

131 Acute leukemia patients received multiple antibiotic treatments during their  
132 hospitalization periods (Supplementary Tables 3, 4, 6). All antibiotics were IV-  
133 administered except ciprofloxacin, which was administered orally. Ciprofloxacin,  
134 administered as prophylaxis immediately upon hospital admission for chemotherapy or  
135 transplantation, was the most frequently received antibiotic. Other frequently used

136 antibiotics administered for empiric treatment of suspected bacteremia included beta-  
137 lactams, mainly piperacillin-tazobactam (PTZ) and meropenem, the aminoglycoside  
138 amikacin and the glycopeptide vancomycin. Similar results were obtained when days of  
139 therapy (DOT) per 1000 patient days were calculated (Supplementary Table 4). Here,  
140 ciprofloxacin was the therapy with the most DOT, followed by meropenem, PTZ,  
141 vancomycin and amikacin.

#### 142 **MRE prevalence in fecal samples from acute leukemia patients**

143 A total of 802 fecal samples were collected from 133 patients during the study period (6  
144 samples on average per patient). MRE could be detected in 221 samples (27.6%) collected  
145 from 80 (60.1%) of the analyzed patients. In 67.8 % of the colonized patients, MREs could  
146 be isolated in one or more consecutive samples obtained during the same hospital  
147 admission period. The most frequently isolated MRE were *Escherichia coli*, *Citrobacter*  
148 *freundii* and *K. pneumoniae* (Figure 1A). Polymicrobial MRE colonization was relatively  
149 frequent: 20.4% of the MRE positive fecal samples were colonized by more than one MRE  
150 species (Figure 1B). In addition, MRE strains belonging to the same species but with  
151 different antibiotic resistance pattern were identified in 31.7 % of the MRE positive  
152 samples. A summary of the detected resistances is shown in Figure 1C and Supplementary  
153 Tables 7-8. As expected, considering the media used for MRE isolation, the majority of MRE  
154 isolates were resistant to penicillins and third generation cephalosporins (i.e. 100%  
155 resistant to ampicillin and 93.9% resistant to cefotaxime). Consistent with the use of  
156 ciprofloxacin as prophylactic agent, most MRE isolates were resistant to ciprofloxacin  
157 (86.6%). In addition, the majority of isolates were resistant to PTZ (82.3%) and  
158 approximately 50% of the isolates were resistant to carbapenems (i.e. imipenem and/or  
159 ertapenem). The proportion of resistance varied considerably among different species  
160 (Supplementary Table 7).

#### 161 **MRE fecal levels differ significantly within and between patients**

162 The levels of MRE (number of CFUs per gram of feces) varied significantly among different  
163 samples from the same patient (acquired at different days) and also between patients  
164 (Figure 2, Supplementary Table 9). Detected MRE reached high levels in some patients  
165 ( $1.43 \times 10^8$  CFUs/g) while in others it stayed scarcely within the limit of detection (50  
166 CFUs/g of feces). We saw no statistical significant differences in colonization levels among  
167 different MRE species ( $p=0.2$ ; Figure 2B). In order to analyze the dynamics of MRE  
168 colonization levels, we studied the change in MRE levels in pairs of fecal samples. The total  
169 number of analyzed pairs was 135, obtained from 59 patients. The analysis showed that  
170 the levels of MRE changed over time (Figure 2C, D, Supplementary Figure 1) with a pattern  
171 suggesting that once an MRE had colonized the intestinal tract, its levels tended to  
172 decrease (Figure 2C, D,  $p=0.004$ ).

173 **Antibiotic intravenous therapies that include beta-lactams PTZ and/or meropenem**  
174 **decrease MRE fecal levels.**

175 We next studied clinical factors that could explain the detected variability in MRE levels,  
176 focusing first on antibiotics. Based on previous studies, that have detected an effect of  
177 antianaerobic therapies on the intestinal levels of VRE and resistant gram-negative Bacilli,  
178 including MRE (4,15), we first asked whether the introduction of an antibiotic with activity  
179 against anaerobes between two consecutive samples acquired from the same patient  
180 changed the MRE levels (see in Supplementary Table 10 the antibiotic administered in  
181 each pair of samples). As previously described (15), we did not include in this analysis  
182 those pairs of samples that had received a therapy with antianaerobic antibiotics in the  
183 previous week. Interestingly, introduction of antibiotics with activity against anaerobes  
184 between two consecutive samples (21 pairs of samples from 18 patients) diminished on  
185 average significantly more the intestinal levels of MRE (Figure 3A,  $p=0.026$ ), as compared  
186 to pairs of samples in which an antianaerobic treatment was not administered (38 pairs of  
187 samples from 21 patients).



188 In our hospital unit, the most frequently used antibiotics with activity against  
189 anaerobes were IV beta-lactams (mainly PTZ and meropenem), which can also directly  
190 inhibit the growth of some MRE strains. Indeed, in the 21 pairs of samples included in the  
191 group that received antianaerobic therapies, the patient had received at least one of these  
192 two beta-lactams (Figure 3B), suggesting a major role for these antibiotics in the detected  
193 reduction of MRE levels.

#### 194 **Impact of IV beta-lactams (PTZ and meropenem) on MRE fecal levels depends on the** 195 **MRE resistance profile**

196 The role of IV beta-lactams (i.e. PTZ and meropenem) in decreasing MRE levels  
197 could be indirect (e.g. through changes in the microbiome that promote MRE depletion) or  
198 it could be direct (by inhibiting MRE growth). To test if IV beta-lactam administration was  
199 directly reducing MRE levels, we analyzed the effect of these antibiotics, taking into  
200 account the resistance pattern of the MREs isolated (see in Figure 4 and Supplementary  
201 Table 11 the antibiotic administered in each pair of samples and the resistant pattern of  
202 the MREs isolated). Notably, beta-lactam administration did not affect MRE levels when the  
203 MREs isolated in a pair of samples were non-susceptible to the administered antibiotic  
204 (Figure 4A,  $p=0.77$ ; 14 pairs of samples from 9 patients). In contrast, beta-lactam  
205 administration significantly diminished MRE fecal levels when all the strains isolated  
206 within a pair of samples were susceptible to the beta-lactam administered (Figure 4A;  
207  $p=0.002$ ; 16 pairs of samples from 15 patients). We carried out the same analysis but now  
208 excluding the patients who had received in the previous week another beta-lactam therapy  
209 and saw a similar effect: the levels of MRE strains susceptible to the antibiotic  
210 administered decreased ( $p=0.001$ ), but we detected no change in non-susceptible strains  
211 ( $p=0.33$ ) (not shown). Notably, this inhibitory effect of beta-lactams on susceptible MRE  
212 strains was not associated with treatment length (Supplementary Figure 2A).

213 We noticed that the proportion of MRE species within each group of analyzed  
214 samples differed (Figure 4A; Supplementary Table 11). For example, *C. freundii* was

215 common in samples not receiving beta-lactams but was undetected in samples receiving  
216 beta-lactams that contained beta-lactam-susceptible strains from other species. To  
217 corroborate that the inhibition of susceptible strains was due to the beta-lactams  
218 administered and not to confounding differences in their composing MRE species, we re-  
219 analyzed the samples exclusively colonized with *E. coli* MRE strains, the most abundant  
220 MRE species in our cohort. We saw a similar inhibitory effect of beta-lactams in samples  
221 exclusively colonized with *E. coli* (Supplementary Figure 3;  $p=0.035$ ; 9 pairs of samples  
222 from 9 patients).

223 The cultivation media used for MRE isolation selects for strains that are producers  
224 of extended-spectrum beta-lactamases (ESBL). We confirmed that most of the isolated  
225 strains pre and post beta-lactam administration were ESBL producers (80% of analyzed  
226 strains; Supplementary Table 11). Most importantly, a similar impact of beta-lactams on  
227 MRE levels was detected when we included in the analysis only those pairs of samples  
228 containing ESBL+ strains (Supplementary Figure 4).

229 Consistent with the inhibitory effect of IV beta-lactams on MRE susceptible strains,  
230 MRE could not be detected after beta-lactam administration in 75% of the cases in which  
231 the patient was colonized with MRE strains susceptible to the administered beta-lactam  
232 (Table 1;  $N=16$  pairs of samples from 15 patients). In contrast, MRE colonization persisted  
233 in the majority of the cases in which the patient was not receiving a beta-lactam (only  
234 26.1% clearance in 46 pairs of samples analyzed from 27 patients) or when the patient  
235 was colonized with strains non-susceptible to the administered beta-lactam (28.5%  
236 clearance in 14 pairs of samples analyzed from 9 patients; Table 1).

237 Interestingly, pairs of samples colonized with MRE susceptible strains that were  
238 not cleared after IV beta-lactam administration were associated with shorter antibiotic  
239 treatment, compared to those pairs of samples where the clearance was detected ( $p=0.11$ ).  
240 (Supplementary Figure 2B).

241 A separate analysis of the two most frequently administered beta-lactams (i.e.  
242 meropenem and PTZ) provided similar results. As compared to pairs of samples in which a  
243 beta-lactam was not administered (46 pairs of samples from 27 patients), introduction of  
244 meropenem significantly reduced the MRE levels of strains sensitive to this antibiotic  
245 ( $p=0.005$ ; Figure 4B; Table 1; 13 pairs of samples from 13 patients). PTZ also reduced the  
246 levels of MRE strains susceptible to this antibiotic (Figure 4C;  $p=0.03$ ), although the  
247 number of pairs of samples available for this analysis was low (3 pairs of samples from 3  
248 patients). PTZ did not significantly modify the levels of MRE strains non-susceptible to this  
249 antibiotic (Figure 4C;  $p=0.3$ ; 10 pairs of samples from 8 patients). Interestingly, the levels  
250 of non-susceptible strains increased, on average, with meropenem treatment (Figure 4B;  
251  $p=0.017$ ; 4 pairs of samples from 3 patients). Nevertheless, the number of pairs of samples  
252 available for this last analysis was low and the MRE expansion only occurred in 2 out of the  
253 4 analyzed cases (Figure 4B). A similar impact of individual beta-lactams on MRE  
254 dynamics was detected in those pairs of samples containing exclusively ESBL producing  
255 strains (Supplementary Figure 4).

#### 256 **Other drugs showed no impact on MRE colonization**

257 Consistent with its poor biliary excretion, (18, 19) other frequently administered  
258 antibiotics such as aminoglycosides (i.e. amikacin) or glycopeptides (i.e. vancomycin and  
259 teicoplanin) did not induce observable changes in MRE levels (Supplementary Figure 5,  
260  $p>0.05$ ). We also saw no impact for amikacin on strains sensitive to this antibiotic  
261 ( $p=0.485$ , not shown). The effect of amikacin on non-susceptible strains was not evaluated  
262 due to the low number of pairs of samples available ( $N=1$ ). The effect of quinolones on  
263 MRE dynamics could not be evaluated since they were administered immediately after  
264 hospital admission, before the first fecal sample could be collected.

265 Administration of anti-fungals such as caspofungin or amphotericin did not impact  
266 MRE levels either ( $p>0.25$ ). In addition, initiation of neutropenia ( $p=0.9$ ), mucositis

267 (p=0.83) or starting parenteral feeding (p=0.79) did not affect MRE dynamics between  
 268 pairs of consecutive samples.

269 We next applied multivariate statistical analysis, taking into account all clinical  
 270 variables previously analyzed and other possible confounding factors (i.e. gender, age, type  
 271 of admission, leukemia type). This analysis confirmed IV-administered beta-lactams to be  
 272 an independent variable associated with MRE reduction when the MRE is sensitive to the  
 273 administered beta-lactam (p=0.001).

274 **Beta-lactams (i.e. meropenem/PTZ) could select for MRE clones resistant to the**  
 275 **administered antibiotic**

276 The results obtained suggest that IV beta-lactam therapy (i.e. meropenem or PTZ)  
 277 could diminish the intestinal loads of MRE sensitive to the administered beta-lactam.  
 278 However, beta-lactam therapy has the implied risk of selecting for resistant strains that  
 279 could occupy the niche left by the sensitive ones. To investigate this possibility, we focused  
 280 on patients that were initially colonized with an MRE that was sensitive to the  
 281 administered beta-lactam and asked whether their levels of MRE and their resistance  
 282 patterns changed in time (Figure 5, Supplementary Figures 6 and 7). As previously  
 283 described, meropenem diminished the intestinal loads of meropenem-sensitive strains.  
 284 During meropenem administration, MRE levels decreased in 12 out of 14 analyzed cases  
 285 and MRE levels were below the detection limit in 10 cases. Moreover, MRE could no longer  
 286 be detected in any sample collected after clearance and during the treatment (8 samples  
 287 from 4 patients that were collected in weeks following MRE elimination). Notably, in 1 out  
 288 of 4 events in which we could detect MRE during the meropenem treatment, both sensitive  
 289 and resistant strains to this antibiotic were detected, although the resistant strain was  
 290 from a different species than the original susceptible strain. Introduction of PTZ decreased  
 291 MRE levels in the 3 cases in which patients were colonized with strains susceptible to this  
 292 antibiotic. However, strains non-susceptible to PTZ arose and expanded during PTZ  
 293 treatment in 2 out of 3 analyzed cases. In this particular case, the non-susceptible strains

294 belong to the same species as the original susceptible ones. Thus, although administration  
295 of meropenem and PTZ can overall reduce the levels of MRE susceptible strains,  
296 occasionally, non-susceptible MRE strains may arise.

297 Finally, we asked whether cessation of beta-lactam treatment would enable the  
298 expansion of MRE strains in those cases in which we observed MRE clearance during  
299 treatment. We only had samples from two patients treated with meropenem for this type  
300 of analysis (Supplementary Figure 6). In one of these patients (i.e. 105), an MRE from a  
301 different species than the one eliminated during meropenem treatment was detected in  
302 fecal samples collected 21 days after meropenem cessation. Interestingly, in the patient 75,  
303 an MRE (from the same species and with the same antibiotic resistant pattern as the one  
304 eliminated during meropenem treatment) was detected 9 days after meropenem  
305 cessation, indicating that MRE re-expansion could occur after meropenem withdrawal.

## 306 **DISCUSSION**

307 We conducted an extensive survey on MRE intestinal colonization levels in patients  
308 hospitalized to receive cancer treatments, including the effect of a range of clinical factors  
309 on MRE dynamics. This is, to our knowledge, the first study to investigate quantitatively,  
310 through extensive culturing, MRE colonization in such a large cohort of patients, allowing  
311 us to investigate the factors associated with temporal changes of MRE levels in the  
312 intestinal tract of the same patient. The extensive data generated in this study is publically  
313 available (see supplementary material) so that the scientific and clinical community can  
314 potentially investigate other factors influencing MRE dynamics within hospitalized  
315 patients besides the effect of beta-lactams that we revealed here.

316 Our analysis showed that MRE levels varied significantly between patients and  
317 between samples collected from the same patient during the same hospital admission  
318 period. Therefore, based on these results and previous studies indicating that higher levels  
319 of resistant bacteria increased the risk of pathogen dissemination to the bloodstream or to

320 the environment (4, 11), it may be useful for clinical practice to accurately measure fecal  
321 MRE levels, beyond determining the presence or absence of fecal carriage.

322 When we started our investigation we expected to find confirmation that  
323 antibiotics with antianaerobic activity promote higher intestinal colonization levels of  
324 multidrug resistant pathogens such as VRE or resistant gram-negative bacilli (4, 15).  
325 However, we found instead that the antibiotics with antianaerobic activity used in our  
326 hospital unit diminished, and not increased, the fecal levels of MRE. A separate analysis of  
327 specific antibiotics revealed that the beta-lactams (PTZ and meropenem) had the greatest  
328 effect. Beta-lactams, although administered intravenously, are partially excreted through  
329 the bile to the intestinal tract (18), and could directly affect the growth of MRE. Indeed, we  
330 were able to demonstrate that IV administration of PTZ and meropenem diminished the  
331 levels of MRE when the strain was sensitive, and not when it was resistant to the  
332 administered antibiotic, pointing to the direct effect of these antimicrobials. In some cases,  
333 the impact of these beta-lactams was substantial, clearing MRE from the intestinal tract in  
334 patients previously colonized with more than  $10^6$  CFU/g of feces. This result agrees with  
335 previous observations in two patients that were highly colonized with Enterobacteriaceae  
336 strains sensitive to meropenem, and that were undetectable after IV meropenem  
337 treatment (15). Notably, for a couple of patients, in which MRE had been cleared during  
338 meropenem treatment, fecal samples could be analyzed after meropenem cessation. In  
339 these two patients MRE could be detected again after meropenem withdrawal, suggesting  
340 that the inhibitory effect of meropenem was only effective during its administration.  
341 Additional studies should be performed in order to evaluate this in a higher number of  
342 patients. Since meropenem withdrawal usually occurred at the end of the hospitalization  
343 period, this type of analysis would require the collection and analysis of samples beyond  
344 the hospitalization period, which was not performed in this study.

345 The effectiveness of PTZ therapy against certain MRE strains (i.e. those producing  
346 ESBL) differs significantly depending on the study. Thus its utility to treat infections by

347 these pathogens remains controversial (20). Indeed, in the only randomized clinical trial  
348 performed, PTZ was shown to be less effective than meropenem in the treatment of  
349 bloodstream infections produced by Enterobacteriaceae ESBL producer strains (21).  
350 Reasons that explain different PTZ treatment outcomes include the site of infection and  
351 susceptibility of the ESBL producing strain to PTZ. Our results are consistent with these  
352 differential effects of PTZ on ESBL producing strains. We found that PTZ decreased the  
353 fecal levels of ESBL producing Enterobacteriaceae that are susceptible to the treatment,  
354 while it did not affect the levels of those non-susceptible to this antibiotic. Nevertheless,  
355 the obtained results should be confirmed in an extended study since only a few pairs of  
356 samples could be included in this analysis. Our data also show occasional selection for  
357 strains resistant to PTZ (2 out of 3 cases) and meropenem (1 out of 14 cases). Thus,  
358 although both IV PTZ and meropenem decrease the intestinal levels of susceptible strains,  
359 their usage should be restricted to the treatment of suspected cases of infection, rather  
360 than decontamination of MRE susceptible strains from the intestinal tract.

361         The administration of beta-lactams and more specifically PTZ, apparently did not  
362 promote large expansion of MRE strains non-susceptible to the administered beta-lactam  
363 in acute leukemia patients. However, our analysis did suggest that the levels of MRE strains  
364 non-susceptible to meropenem might increase after meropenem administration. This  
365 result, despite passing the common threshold of statistical significance, was based on only  
366 four pairs of samples and a closer inspection shows that MRE expanded in only half of  
367 those cases. Thus, additional studies should be performed to validate this result.  
368 Nevertheless, this is consistent with the result obtained in a study in which carbapenem  
369 administration was associated with a higher relative abundance of carbapenem-resistant  
370 *K. pneumoniae*, measured by 16s rRNA sequencing, which was associated with increased  
371 risk of bloodstream infections (13). Thus, IV meropenem can reduce the fecal levels of  
372 susceptible strains, while it may promote the growth of resistant ones.

373 It is worth to mention that our hospital unit rarely uses clindamycin and  
374 metronidazole, two antibiotics with a major effect against anaerobic bacteria but with no  
375 direct effect against MRE. These antibiotics, in addition to PTZ, were some of the most  
376 frequently administered in a previous study showing higher levels of resistant gram-  
377 negative bacilli during antianaerobic treatment (15). The different therapies administered  
378 in both studies may explain the different results obtained regarding the effect of  
379 antianaerobic antibiotics on the growth of resistant pathogens. Indeed, studies performed  
380 in mice have shown that parenteral administration of clindamycin consistently promoted  
381 high levels of MRE intestinal colonization. In contrast, intestinal density of MRE upon PTZ  
382 administration varied depending on the dose of the antibiotic administered, the pathogen  
383 inoculum concentration and its level of antibiotic resistance (22). Future studies should  
384 elucidate how these variables influence the capacity of beta-lactams to promote intestinal  
385 colonization by MRE in patients.

386 We acknowledge several limitations of our study. First, our study was performed in a very  
387 specific human population (acute leukemia patients) that may already display an altered  
388 microbiota due to prior treatments. Nevertheless, we would expect that beta-lactams effect  
389 on susceptible MRE strains would be the same in other types of hospitalized patients since  
390 this effect is direct and probably not dependent on the microbiota. Moreover, acute  
391 leukemia patients are one of the cohorts with higher risk of infection due to intestinal  
392 colonization by MRE and could therefore benefit the most from this type of studies.  
393 Second, our study was restricted to the subset of MRE that were able to grow on plates  
394 containing a 3<sup>rd</sup> generation cephalosporin, designed for the detection of ESBL producing  
395 strains. Indeed, most of the analyzed strains were ESBL producers. In addition, most of the  
396 isolated strains were resistant to quinolones probably because ciprofloxacin is  
397 administered in our hospital unit as prophylaxis immediately upon patient's hospital  
398 admission. Additional work should be carried out in order to validate our findings on  
399 strains with a different resistant background. In addition, further work should be



400 performed in order to study if the effect of beta-lactams on MRE levels will depend on the  
401 different mechanisms of cephalosporin or carbapenem resistance, which were not studied  
402 here. Third, our results did not allow us to evaluate the impact of clinical factors on low  
403 abundant MRE populations since the MRE antibiotic resistant pattern and taxonomy was  
404 only characterized in a few representative isolates from each sample. Fourth, we were able  
405 to validate the inhibitory effect of specific beta-lactams on a particular individual MRE  
406 species (i.e. *E. coli*). However, the effect on other individual MRE species of interest (e.g. *K.*  
407 *pneumoniae*) could not be evaluated due the limited number of samples containing  
408 exclusively these other MREs. Additional studies should be performed to confirm the effect  
409 of beta-lactams on other MRE species of interest. Finally, we were not able to evaluate the  
410 effect of MRE levels on the risk of bacteremia due to the low number of samples available  
411 for this analysis (i.e. N=6 fecal samples collected before bacteremia detection containing  
412 the same MRE species as the one isolated in the bloodstream). Nonetheless, previous  
413 studies have already pointed to a link between high intestinal levels of drug-resistant  
414 bacteria and increased risk of bloodstream infections provoked by these bacteria (11, 13).  
415 Additional work should be performed to corroborate those results using quantitative  
416 measurements to evaluate if a particular threshold leads to higher risk of bloodstream  
417 infections.

418 In summary, our results revealed a wide diversity of intestinal MRE levels and  
419 highlighted the relevance of quantitatively analyzing these levels in surveillance and/or  
420 clinical studies. Certain IV-administered antibiotics had a direct effect on the intestinal  
421 growth of MRE, and this effect depended on the sensitivity of the pathogen to the given  
422 antibiotic. Overall, our study highlights the relevance of incorporating antibiotic treatment  
423 and susceptibility data of gut colonizing pathogens for future clinical studies and in clinical  
424 decision-making.

## 425 **MATERIALS AND METHODS**

### 426 **Ethics**

427 This study was approved by the Ethics Committee of CEIC Dirección General de Salud  
428 Pública and Centro Superior de Investigación en Salud Pública (20130515/08). All  
429 included patients gave their consent to participate in the study.

#### 430 **Sample and metadata collection**

431 Fecal samples were collected weekly from November of 2013 until April of 2015 from all  
432 acute leukemia patients hospitalized at the Hospital La Fe (Valencia, Spain) who agreed to  
433 participate in this study. Procedures of sample collection and metadata acquisition are  
434 described in detail in the supplementary data.

#### 435 **Assessing MRE colonization levels and characterization of MRE isolates.**

436 MRE levels, defined as the number of MRE CFUs detected per gram of feces, were  
437 determined by culturing 10-fold dilutions of fecal samples in Brilliance ESBL agar plates  
438 (Oxoid), which contain cefpodoxime, a third generation cephalosporin as selective agent.  
439 These plates allow for isolation of Extended Spectrum Beta-Lactamase (ESBL) producing  
440 organisms, while inhibiting the growth of non-ESBL Enterobacteriaceae and most AmpC  
441 organisms, allowing mainly for identification of ESBL-producing *E. coli* and the *Klebsiella*,  
442 *Enterobacter*, *Serratia* and *Citrobacter* group (known as KESC). The number of colonies in  
443 each plate was normalized by dilution and fecal weight in order to calculate the levels of  
444 colonization of MRE per gram of feces. To confirm that the detected colonies were MRE,  
445 the taxonomy and resistance pattern of 5 isolated colonies per sample were determined  
446 through the Maldi-TOF and VITEK system (supplementary methods). In addition,  
447 production of extended spectrum beta-lactamases was analyzed in a subset (11%) of the  
448 characterized MRE isolates (Supplementary Table 11, Figure 4) as described in the  
449 supplementary methods.

#### 450 **Defining pairs of samples and calculating the fold change (FC) among pairs of** 451 **samples to study MRE dynamics.**

452 For analyzing the dynamics of MRE colonization levels, we calculated the  $\log_2FC$  in MRE  
453 levels in pairs of fecal samples. The pairs were defined as two samples consecutively

454 collected from the same patient and same hospital admission period. The first sample of  
455 the pair was always positive for MRE. Taking into account the weekly sampling strategy  
456 performed in this study, in most cases the two samples were collected one week apart  
457 from each other.

458 Sometimes, MRE could not be detected in the second sample of a pair of samples; we  
459 define those cases as MRE clearance. To avoid infinite values that would arise by dividing  
460 by 0, the limit of the detection (i.e. 50 CFUs/g) was added to the values obtained in each  
461 sample as pseudo-counts before calculating the FC.

462 The MRE clearance rate was defined as the number of pairs of samples from the total  
463 analyzed in which MRE could not be detected in the second sample of the pair.

#### 464 **Description of the comparisons performed to evaluate the effect of clinical factors** 465 **on MRE dynamics.**

##### 466 1. Effect of antianaerobic antibiotics on MRE dynamics.

467 The log<sub>2</sub>FC obtained from pairs of samples in which a therapy with an antianaerobic  
468 antibiotic had been initiated was compared with the log<sub>2</sub>FC of the group of pairs of  
469 samples that had not received such therapy. The group of pairs of samples that had  
470 received an antianaerobic therapy was defined, based on previous studies (4, 15), as those  
471 in which the MRE levels from the first sample of the pair were available within two weeks  
472 before the initiation of the regimen, no antianaerobic antibiotic had been administered  
473 within seven days before the regimen was initiated, the level of the second sample within  
474 the pair was determined at least two days after the initiation of the treatment but no more  
475 than seven days after the completion of the antibiotic course. Although we decided to  
476 follow this previously established criteria dictating that the second sample in a pair  
477 shouldn't have been collected more than seven days after the cessation of the treatment, it  
478 is worth mentioning that in our study all the second samples of analyzed pairs were  
479 collected while the treatment was still ongoing. The length of the treatment was not  
480 accounted as a variable for this type of analysis. The MRE log<sub>2</sub>FC detected in this group of

481 samples was compared with the MRE  $\log_2FC$  obtained in pairs of samples in which no  
482 antibiotic with antianaerobic activity was received neither between the two samples of a  
483 pair (including the days in which the samples were collected) nor in the week before the  
484 date of collection of the first sample of the pair.

485 Antibiotics included in the analysis with activity against intestinal anaerobes were: PTZ,  
486 amoxicillin clavulanic, ampicillin, meropenem, ertapenem, imipenem, linezolid,  
487 metronidazole, tigecycline. Antibiotics included in the analysis that were considered not  
488 active against intestinal anaerobes were: gentamicin, amikacin, cloxacillin, aztreonam,  
489 cotrimoxazole, colistin, ciprofloxacin, levofloxacin, daptomycin, vancomycin, teicoplanin  
490 (23-30).

491 Daptomycin, despite its *in vitro* effect against gram-positive anaerobic bacteria (27), was  
492 included within the group of antibiotics with no activity against intestinal anaerobes. We  
493 made this choice because in mice the parenteral administration of this antibiotic does not  
494 disrupt the anaerobic flora and does not promote the intestinal colonization of Extended-  
495 Spectrum- $\beta$ -Lactamase-producing *K. pneumoniae* (31). In addition, glycopeptides such as  
496 vancomycin and teicoplanin were included in the group with no effect against intestinal  
497 anaerobes because they were administered intravenously and their biliary excretion is low  
498 (18, 19). Nevertheless, similar results as those shown in Figure 3 were obtained if  
499 glycopeptides were included within the group of antibiotics with activity against anaerobic  
500 bacteria (not shown).

501 Patients did not receive antibiotic prophylaxis while outpatient. Moreover, in case the patient  
502 required an antibiotic for other indications (fever or other symptoms), the patient would be  
503 first hospitalized. For this reason, we did not account for the impact of outpatient antibiotic  
504 exposure on MRE levels and we focus our analysis on those antibiotics received during the  
505 hospitalization period.

506 2. Effect of beta-lactam therapy and other clinical variables on MRE dynamics.

507 A comparison was performed between the  $\log_2FC$  obtained from pairs of samples in which  
508 a therapy with a beta-lactam antibiotic had been initiated and the group of pairs of  
509 samples that had not received such therapy. The group of pairs of samples that had  
510 received the beta-lactam therapy was defined as those in which the MRE levels from the  
511 first sample of the pair were available within two weeks before the initiation of the  
512 regimen, the levels of the second sample within the pair were determined at least two days  
513 after the initiation of the treatment but no more than seven days after the completion of  
514 the antibiotic course. As described above, although we decided to follow the criteria  
515 established by previous studies (4, 15), in our study, all the second samples of analyzed  
516 pairs were collected while the beta-lactam treatment was still ongoing. The length of  
517 antibiotic treatment was not accounted as a variable for this type of analysis. To study if  
518 the effect of beta-lactams on MRE change was direct (i.e. direct inhibition of MRE on  
519 susceptible strains), the pairs of samples selected were divided into two subgroups: (i)  
520 pairs of samples containing exclusively strains susceptible to the beta-lactam  
521 administered; (ii) pairs of samples containing exclusively strains non-susceptible to the  
522 beta-lactam administered. The MRE  $\log_2FC$  detected in these two groups of pairs of  
523 samples was compared with the MRE  $\log_2FC$  obtained in pairs of samples in which no beta-  
524 lactam was received between the two samples of a pair (including the days in which the  
525 samples were collected).

526 In addition to this type of analysis (Figure 4), we performed the same analysis but  
527 excluding from the beta-lactam group those pairs of samples that had received another  
528 beta-lactam in the week before the initiation of the beta-lactam therapy and we compared  
529 it with the groups of samples that had not received beta-lactams neither between the  
530 samples of a pair (including the days in which the samples were collected) nor in the week  
531 before the date of collection of the first sample of the pair. This type of analysis led to  
532 similar results as indicated in the result section, although the number of samples included

533 in this analysis was lower. For this reason and to avoid redundancy, only the first type of  
534 analysis is shown in the Figure 4.

535 The same comparisons as with beta-lactams were performed to assess the effect on MRE  
536 dynamics of other antibiotics (i.e. glycopeptides and aminoglycosides), antifungal  
537 treatments and initiation of parenteral feeding, mucositis or neutropenia. In the case of  
538 neutropenia, mucositis or parenteral feeding, the  $\log_2FC$  of pairs of samples in which these  
539 events were initiated was compared with pairs of samples collected during periods in  
540 which the patient did not have neutropenia, was not fed through the parenteral route or  
541 did not develop mucositis.

542 Besides the analysis described above on the effect of antibiotics on MRE changes, previous  
543 studies have also compared the levels of resistant pathogens identified during the  
544 administration of different groups of antibiotics. Nonetheless, that approach had  
545 limitations: (i) it did not take into account those samples in which the pathogen  
546 was unable to colonize because of the presence of the antibiotic, and (ii) it did not consider  
547 those samples in which the pathogen was eliminated upon the introduction of the  
548 antibiotic. As seen in this study, some of the most frequently administered antibiotics in  
549 our hospital unit (i.e. meropenem and PTZ) often do eliminate MRE when the strain is  
550 sensitive to the antibiotic administered. For this reason, and to make our study  
551 more comprehensive, we focused our analysis on the changes in MRE after the  
552 introduction of specific antibiotics, which allowed us to account for cases in which the  
553 effect of the antibiotic was the complete elimination of the pathogen.

#### 554 **Statistical analysis.**

555 In the case of this descriptive study without hypothesis to be tested, the comparisons and  
556 p-values calculated were descriptive and exploratory.

557 Two-tailed Student's t-test was applied in order to analyze if the  $\log_2$  Fold Change MRE  
558 levels between groups of samples were significantly different. Fischer's exact test was

559 applied to define if MRE clearance rate (Table 1) was significantly different among groups  
560 of samples.

561 Multivariate Lasso regression analysis was performed as described in supplementary  
562 methods.

563 P values < 0.05 were considered statistically significant.

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571

572

#### 573 **TRANSPARENCY DECLARATIONS**

574 None to declare

575

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701

702 **FIGURE LEGENDS**703 **Figure 1. MRE positive samples show diverse compositions and antibiotic resistance**704 **patterns of detected MRE isolates.** MRE could be detected in 221 samples collected from705 80 of the analyzed patients. **(A)** The most frequently isolated MRE belonged to the species706 *Escherichia coli*. Other detected MRE in order of prevalence were *Citrobacter freundii*,707 *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Morganella morganii*,708 *Citrobacter amalonaticus*, *Raoultella spp.* and *Escherichia hermannii*. Other MRE species,709 including *Citrobacter braakii*, *Proteus vulgaris*, *Serratia marscescens*, *Enterobacter*710 *aerogenes* and *Escherichia fergusonii* were detected in only one sample. **(B)** In 45 of the

711 221 MRE positive samples (20.4%), MRE belonging to more than one bacterial species

712 could be identified, and in 70 of the MRE positive samples (31.7%), MRE strains belonging

713 to the same species but with different antibiotic resistance pattern were identified. **(C)**

714 Antibiotic resistance pattern of MRE isolates detected in 221 positive samples. When two  
 715 isolates from the same sample have exactly the same resistance pattern and taxonomy,  
 716 only one of the two isolates is shown. Columns and rows are grouped based on MRE  
 717 taxonomy and antibiotics' class. S: susceptible; I: intermediate resistant phenotype; R:  
 718 resistant. NA: not-analyzed.

719

720 **Figure 2. MRE fecal levels differ significantly between patients but also within a**  
 721 **patient. (A)** MRE levels identified in all colonized fecal samples included in the study  
 722 (N=221 samples) or the mean (obtained from log<sub>10</sub> CFU data) of the MRE levels identified  
 723 in colonized samples from each patient (N=80 patients). Whiskers represent minimum and  
 724 maximum values. Horizontal lines represent the median and 25-75 percentiles. **(B)** MRE  
 725 levels in fecal samples colonized exclusively with the indicated species (low abundant  
 726 species: N≤5, are not included). No significant (ns) differences in MRE levels were  
 727 detected between different species (Kruskal-Wallis test). N= 45 (*C. freundii*); 14 (*E.*  
 728 *cloacae*); 13 (*K. oxytoca*); 65 (*E. coli*) and 31 (*K. pneumoniae*) samples. **(C)** Changes in MRE  
 729 levels among 135 pairs of consecutive fecal samples (see Methods for definition) collected  
 730 from 59 patients. The grey bar represents the median of MRE levels. \*\*p<0.01; Two-tailed  
 731 paired Wilcoxon test. **(D)** Number of the total pairs of consecutive samples included in the  
 732 study (all) and number of pairs of samples in which an increase in MRE levels (> 1 log<sub>2</sub> fold  
 733 change - FC; N=39) or a decrease in MRE levels (< -1 log<sub>2</sub> FC; N=81) was detected.

734

735 **Figure 3. Antibiotic therapies including the beta-lactams piperacillin-tazobactam**  
 736 **and meropenem decrease MRE fecal levels. (A)** MRE log<sub>2</sub>FC among pairs of consecutive  
 737 samples between which a therapy with an antibiotic with activity against anaerobic  
 738 bacteria was initiated (+), as compared to pairs of samples in which no antianaerobic  
 739 antibiotics were administered (-). N=38 pairs of samples from 21 patients and 21 pairs of  
 740 samples from 18 patients for the antianaero (-) and (+) groups respectively. \*p<0.05; Two-

741 tailed t-test. Bars represents the mean, whiskers represent the SEM. **(B)** Antibiotics with  
 742 antianaerobic activity that were administered between each pair of samples shown in (A).  
 743 Colors in (A) indicate the taxonomy of the MRE identified in each pair of samples. Detailed  
 744 taxonomy and antibiotic resistant pattern of all the MREs identified within each pair of  
 745 samples is shown in Supplementary Table 10. PTZ=piperacillin-tazobactam.

746 **Figure 4. Impact of IV beta-lactams (piperacillin-tazobactam and meropenem) on**  
 747 **MRE fecal levels depends on the MRE resistance profile. (A)** MRE  $\log_2$ FC among pairs  
 748 of consecutive samples between which a beta-lactam (i.e. piperacillin-tazobactam or  
 749 meropenem) was administered (+) or not (-). MRE strains detected in the consecutive  
 750 pairs of samples analyzed were either susceptible (S), or non-susceptible (R/I) towards  
 751 the administered beta-lactam. (B, C) Same as in (A) but only including pairs of samples in  
 752 which the beta-lactam therapy initiated between the pair of samples was exclusively  
 753 meropenem (B) or exclusively piperacillin-tazobactam (C). \* $P < 0.05$ ; \*\* $P < 0.01$ ; two-tailed t-  
 754 test compared with the group not receiving beta-lactams. The results show that beta-  
 755 lactams (i.e. meropenem and piperacillin-tazobactam) reduce the levels of MRE strains  
 756 susceptible to the beta-lactam administered. Colors indicate the taxonomy of the MRE  
 757 identified in each pair of samples. Detailed taxonomy and antibiotic resistant pattern of all  
 758 the MREs identified within each pair of samples is shown in Supplementary Table 11. The  
 759 number of pairs of samples (S) and patients (P) included in each group are: no beta-lactam  
 760 (S=46, P=27); susceptible to beta-lactams (S=16, P=15); non-susceptible to beta-lactams  
 761 (S=14, P=9); susceptible to meropenem (S=13, P=13); non-susceptible to meropenem  
 762 (S=4, P=3); susceptible to PTZ (S=3, P=3); non-susceptible to PTZ (S=10, P=8).

763 **Figure 5. Meropenem and piperacillin-tazobactam decrease fecal levels of**  
 764 **susceptible MRE but occasionally resistant strains emerge.** MRE levels and sensitivity  
 765 pattern to the beta-lactam received before and during therapy with meropenem (A) or  
 766 piperacillin-tazobactam (PTZ) (B). Consecutive samples collected from the same patient  
 767 and admission period are connected with a line. Sensitivity to the antibiotic is indicated

768 when the MRE could be detected. N<sup>o</sup> of samples in which an MRE could not be detected on  
 769 each specific day is indicated. Note that the first sample contains always MRE strains  
 770 susceptible to the beta-lactam received. S= susceptible, R= resistant, I=intermediate  
 771 phenotype. Colors indicate the taxonomy of the MRE identified in each sample. Days are  
 772 relative to the date of the first sample included in the figure for each patient. Graphs for  
 773 each individual patient and samples collected after beta-lactam cessation are shown in  
 774 Supplementary Figures 6 and 7. N= 14 patients for meropenem group and 3 patients for  
 775 the PTZ group. Note that as compared to Figure 4, there is one more patient receiving  
 776 meropenem in this Figure. This is because in Figure 4 a pair of samples was included in the  
 777 group of pairs sensitive to meropenem only if all MRE strains characterized in the pair  
 778 were sensitive to the antibiotic.

779

780 **Table 1. MRE clearance after beta-lactam (piperacillin-tazobactam or**  
 781 **meropenem) administration.**  
 782

$\beta$ -lactam <sup>a</sup>	Susceptibility <sup>b</sup>	MRE clearance rate <sup>c</sup>	Significance <sup>d</sup>
None	NA	12/46 (26.1%)	
PTZ/Meropenem	S	12/16 (75%)	***
PTZ/Meropenem	R/I	4/14 (28.5%)	NS
PTZ	S	2/3 (66.6%)	NS
PTZ	R/I	4/10 (40%)	NS
Meropenem	S	10/13 (77%)	*
Meropenem	R/I	0/4 (0%)	NS

783

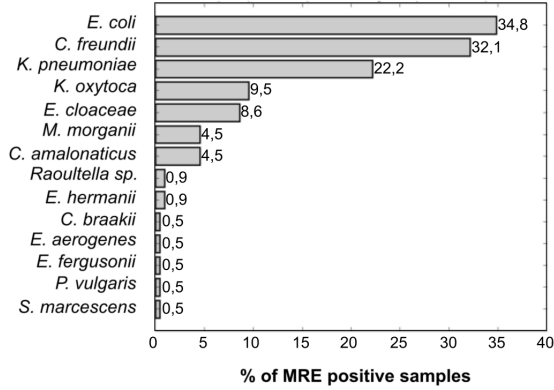
784 <sup>a</sup>  $\beta$ -lactam that was administered between a pair of samples. PTZ/Meropenem:  
 785 piperacillin/tazobactam and/or meropenem were administered. Exclusively  
 786 piperacillin/tazobactam (PTZ) or meropenem therapy was initiated between a pair of samples.

787 <sup>b</sup> Susceptibility of the MRE to the administered antibiotic. S= susceptible; R/I=non-susceptible  
 788 (resistant/intermediate phenotype). NA: non-applicable.

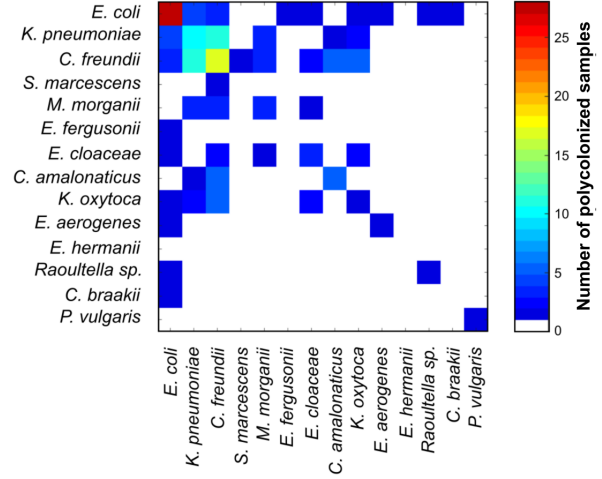
789 <sup>c</sup> Number of pairs of samples from the total analyzed in which the MRE was not detected in the  
 790 second sample of the pair. Numbers in parenthesis indicate % of clearance.

791 <sup>d</sup> Significance. \*P<0.05; \*\*\*P<0.001; NS=P>0.05; Fischer test comparing with the group in which no  
 792  $\beta$ -lactams were administered.  
 793

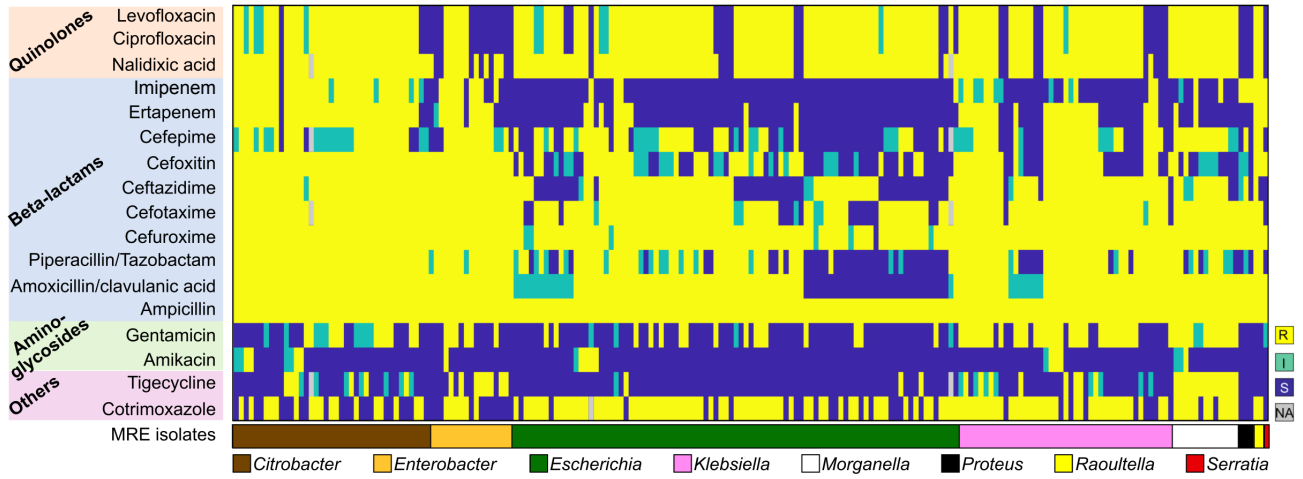
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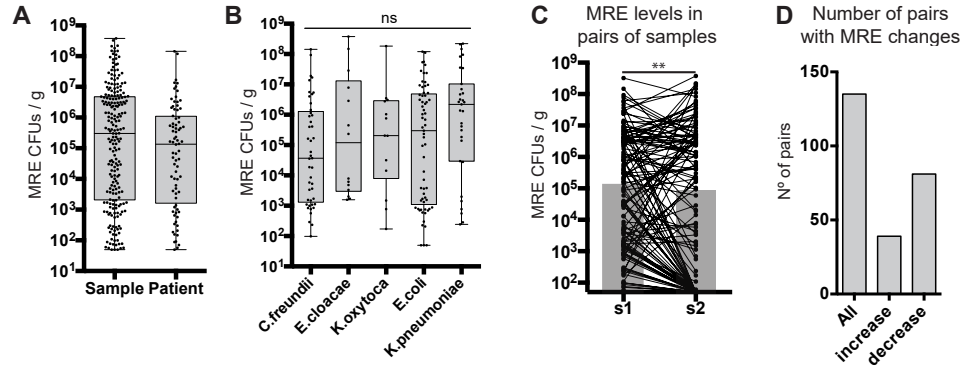


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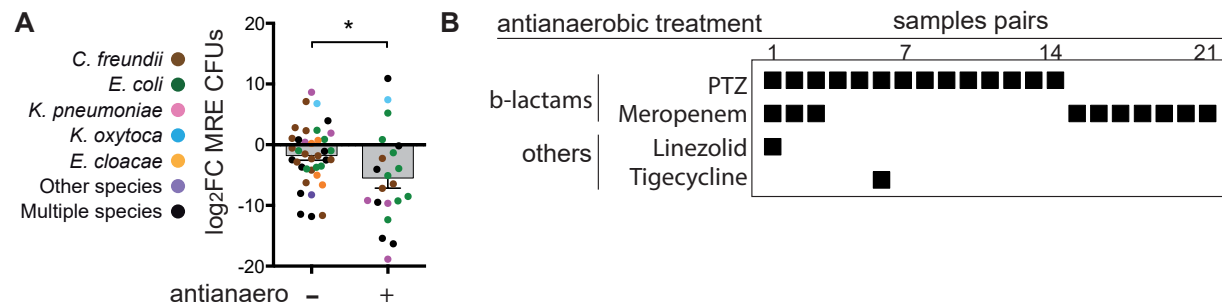
**C**





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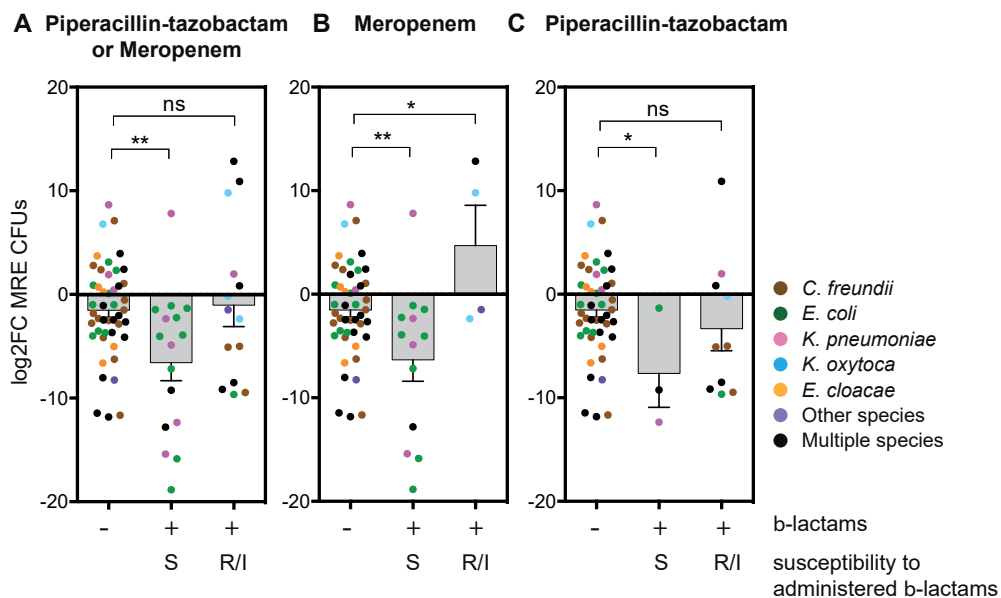
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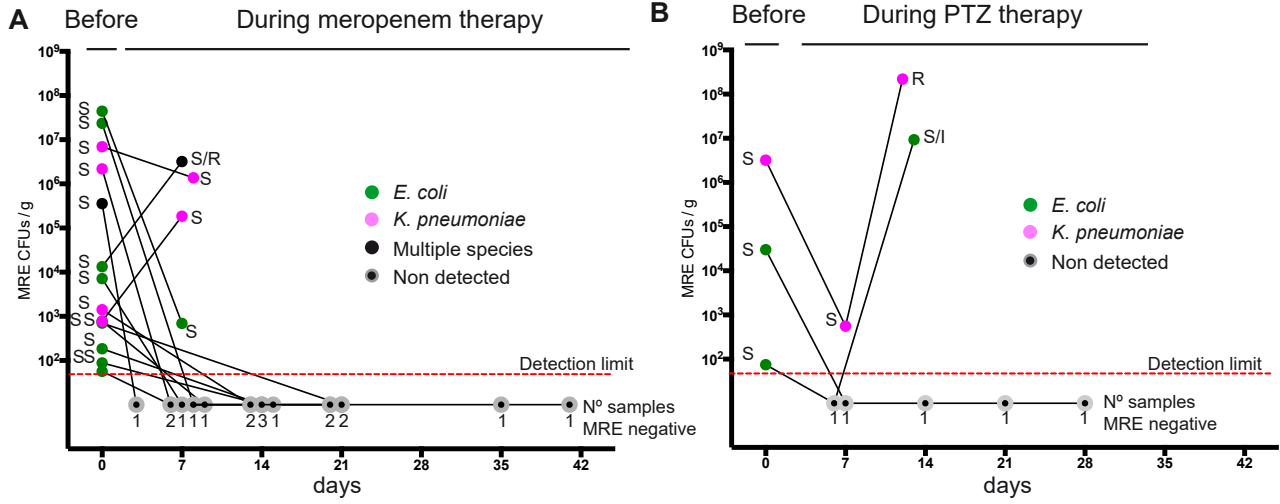
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