

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

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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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Short Running title

IV beta-lactams impact MRE fecal levels



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## ABSTRACT

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


## INTRODUCTION

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



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

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

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

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



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

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

## RESULTS

### Study population

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

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



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

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

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

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



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The levels of MRE (number of CFUs per gram of feces) varied significantly among different samples from the same patient (acquired at different days) and also between patients (Figure 2, Supplementary Table 9). Detected MRE reached high levels in some patients ($1.43x10^8$ CFUs/g) while in others it stayed scarcely within the limit of detection (50 CFUs/g of feces). We saw no statistical significant differences in colonization levels among different MRE species (p=0.2; Figure 2B). In order to analyze the dynamics of MRE colonization levels, we studied the change in MRE levels in pairs of fecal samples. The total number of analyzed pairs was 135, obtained from 59 patients. The analysis showed that the levels of MRE changed over time (Figure 2C, D, Supplementary Figure 1) with a pattern suggesting that once an MRE had colonized the intestinal tract, its levels tended to decrease (Figure 2C, D, p=0.004).

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

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



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

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

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

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



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

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

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

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



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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



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

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

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

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



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

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

**DISCUSSION**

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

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



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

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

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



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

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



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

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



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

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

## MATERIALS AND METHODS

### Ethics



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

**Sample and metadata collection**

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

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

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

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

For analyzing the dynamics of MRE colonization levels, we calculated the $\log_2$FC in MRE levels in pairs of fecal samples. The pairs were defined as two samples consecutively



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

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

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

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

1. Effect of antianaerobic antibiotics on MRE dynamics.

The $log_2FC$ obtained from pairs of samples in which a therapy with an antianaerobic antibiotic had been initiated was compared with the $log_2FC$ of the group of pairs of samples that had not received such therapy. The group of pairs of samples that had received an antianaerobic therapy was defined, based on previous studies (4, 15), as those in which the MRE levels from the first sample of the pair were available within two weeks before the initiation of the regimen, no antianaerobic antibiotic had been administered within seven days before the regimen was initiated, the level of the second sample within the pair was determined at least two days after the initiation of the treatment but no more than seven days after the completion of the antibiotic course. Although we decided to follow this previously established criteria dictating that the second sample in a pair shouldn't have been collected more than seven days after the cessation of the treatment, it is worth mentioning that in our study all the second samples of analyzed pairs were collected while the treatment was still ongoing. The length of the treatment was not accounted as a variable for this type of analysis. The MRE $log_2FC$ detected in this group of



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

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

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

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

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



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

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



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

The same comparisons as with beta-lactams were performed to assess the effect on MRE dynamics of other antibiotics (i.e. glycopeptides and aminoglycosides), antifungal treatments and initiation of parenteral feeding, mucositis or neutropenia. In the case of neutropenia, mucositis or parenteral feeding, the $\log_2$FC of pairs of samples in which these events were initiated was compared with pairs of samples collected during periods in which the patient did not have neutropenia, was not fed through the parenteral route or did not develop mucositis.

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

**Statistical analysis.**

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

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



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

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

P values < 0.05 were considered statistically significant.

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**TRANSPARENCY DECLARATIONS**

None to declare

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## FIGURE LEGENDS

**Figure 1. MRE positive samples show diverse compositions and antibiotic resistance patterns of detected MRE isolates.** MRE could be detected in 221 samples collected from 80 of the analyzed patients. **(A)** The most frequently isolated MRE belonged to the species *Escherichia coli*. Other detected MRE in order of prevalence were *Citrobacter freundii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Morganella morganii*, *Citrobacter amalonaticus*, *Raoultella spp.* and *Escherichia hermanii*. Other MRE species, including *Citrobacter braakii, Proteus vulgaris, Serratia marscescens, Enterobacter aerogenes* and *Escherichia fergusonii* were detected in only one sample. **(B)** In 45 of the 221 MRE positive samples (20.4%), MRE belonging to more than one bacterial species could be identified, and in 70 of the MRE positive samples (31.7%), MRE strains belonging to the same species but with different antibiotic resistance pattern were identified. **(C)**



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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 log10 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 $\log_2$ fold
733 change – FC; N=39) or a decrease in MRE levels (< -1 $\log_2$ 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 $\log_2$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-



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

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

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



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when the MRE could be detected. Nº of samples in which an MRE could not be detected on each specific day is indicated. Note that the first sample contains always MRE strains susceptible to the beta-lactam received. S= susceptible, R= resistant, I=intermediate phenotype. Colors indicate the taxonomy of the MRE identified in each sample. Days are relative to the date of the first sample included in the figure for each patient. Graphs for each individual patient and samples collected after beta-lactam cessation are shown in Supplementary Figures 6 and 7. N= 14 patients for meropenem group and 3 patients for the PTZ group. Note that as compared to Figure 4, there is one more patient receiving meropenem in this Figure. This is because in Figure 4 a pair of samples was included in the group of pairs sensitive to meropenem only if all MRE strains characterized in the pair were sensitive to the antibiotic.

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

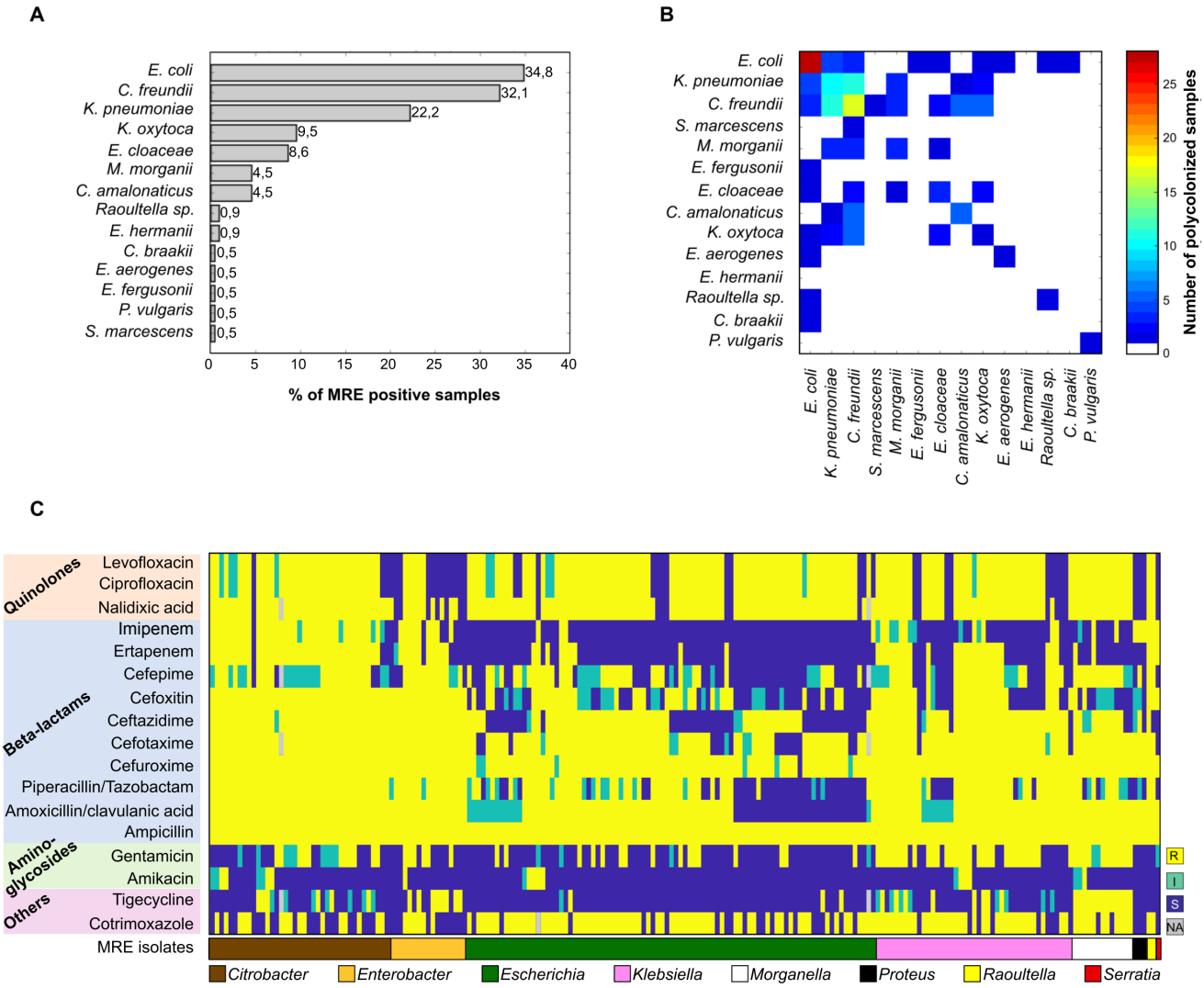
| β-lactam[a] | Susceptibility[b] | MRE clearance rate[c] | Significance[d] |
|---|---|---|---|
| None | NA | 12/46 (26.1%) | |
| PTZ/Meropenem | S | 12/16 (75%) | *** |
| PTZ/Meropenem | R/I | 4/14 (28.5%) | NS |
| PTZ | S | 2/3 (66.6%) | NS |
| PTZ | R/I | 4/10 (40%) | NS |
| Meropenem | S | 10/13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

[a] β-lactam that was administered between a pair of samples. PTZ/Meropenem: piperacillin/tazobactam and/or meropenem were administered. Exclusively piperacillin/tazobactam (PTZ) or meropenem therapy was initiated between a pair of samples.
[b] Susceptibility of the MRE to the administered antibiotic. S= susceptible; R/I=non-susceptible (resistant/intermediate phenotype). NA: non-applicable.
[c] Number of pairs of samples from the total analyzed in which the MRE was not detected in the second sample of the pair. Numbers in parenthesis indicate % of clearance.
[d] Significance. *P<0.05; ***P<0.001; NS=P>0.05; Fischer test comparing with the group in which no β-lactams were administered.



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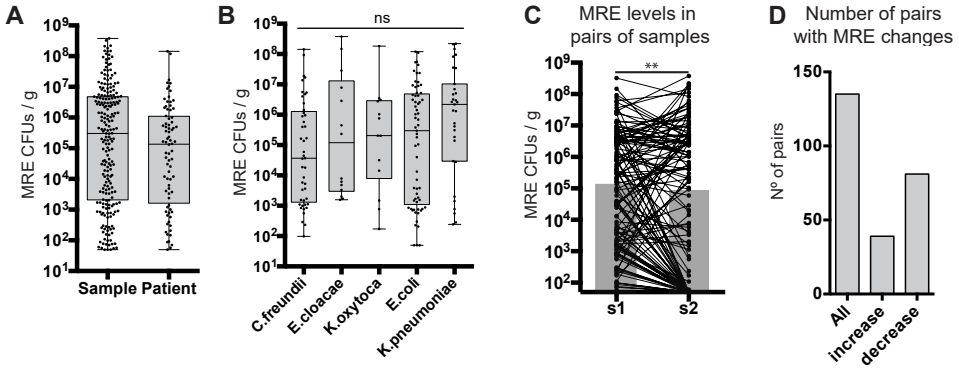
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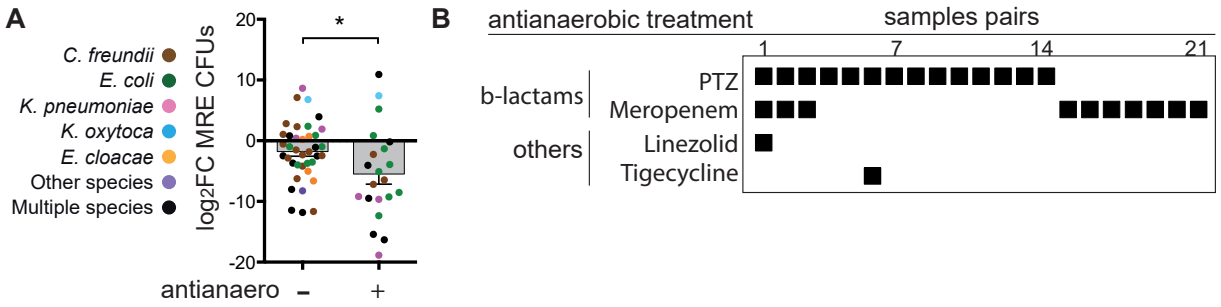
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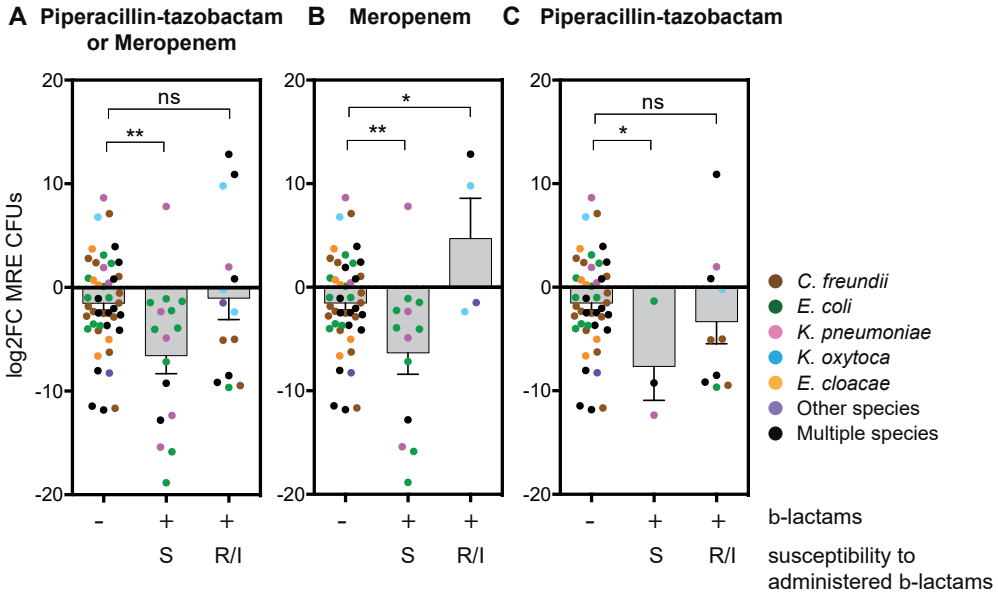
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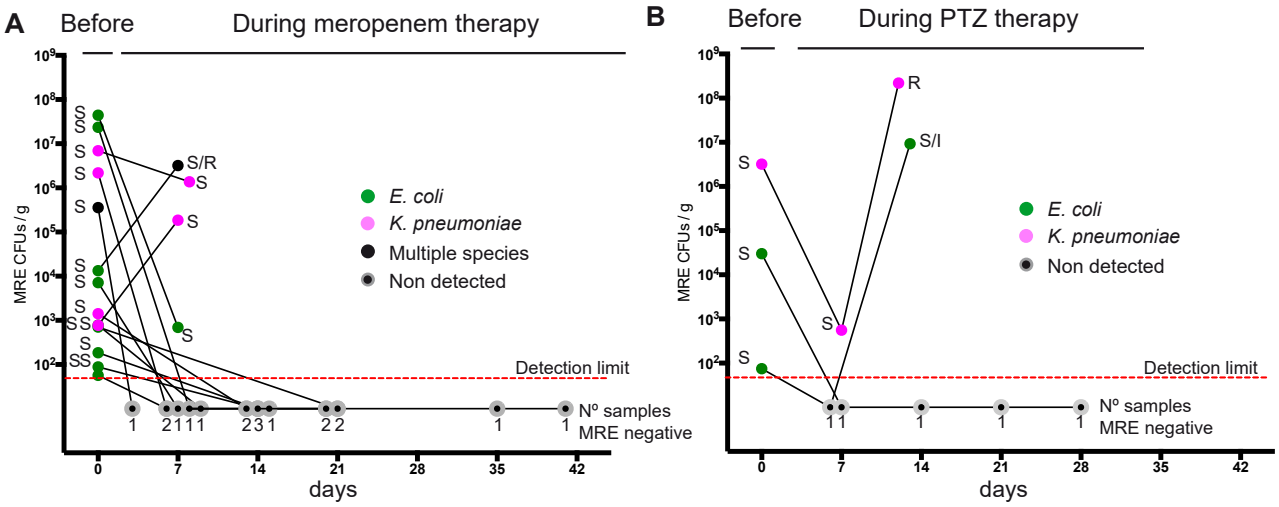
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