Amplification of Antimicrobial Resistance in Gut Flora of Patients Treated with Ceftriaxone

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Amplification of Antimicrobial Resistance in Gut Flora of Patients Treated with Ceftriaxone

Short title: Ceftriaxone and amplification of resistance genes

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ABSTRACT

Although antibacterial therapy has an impact on human intestinal flora and emergence of resistant bacteria, its role on amplification of antimicrobial resistance and the quantitative exposure-effect relationship is not clear. An observational prospective study was conducted to determine whether and how ceftriaxone exposure is related to amplification of resistance in non-ICU patients.Serial stool samples from 122 ESBL(+) hospitalized patients were analyzed with quantitative real-time PCR to quantify the resistant gene $bla_{CTX\text{-}M}$. Drug exposure was calculated for each patient using a population pharmacokinetic model. Multi- and univariate regression and classification regression tree (CART) analysis was used to explore relationships between measures of exposure and amplification of $bla_{CTX\text{-}M}$ genes. Amplification of $bla_{CTX\text{-}M}$ was observed in 0% (0/11) patients with no treatment and 33% (20/61) of patients treated with ceftriaxone. Stepwise regression analysis showed a significant association between amplification of $bla_{CTX\text{-}M}$ and plasma $f\text{AUC}_{0\text{-}24}$, fCmax and the duration of ceftriaxone therapy. Using CART analysis, amplification of $bla_{CTX\text{-}M}$ was observed in 11/16 (69%) of patients treated >14d and in 9/40 (23%) of patients treated ≤14d (p=0.0019). In the latter group, amplification was observed in 5/7 (71%) of patients with $f\text{AUC}_{0\text{-}24}$ ≥222 mg.h/l and in 4/33 (12%) of patients with lower drug exposures (p=0.0033). A similar association was found for fCmax ≥30 mg/l (63% vs. 13%, p=0.0079). A significant association was found between amplification of $bla_{CTX\text{-}M}$ resistance genes and exposure of ceftriaxone. Both duration of treatment and degree of ceftriaxone exposure have a significant impact on of amplification of resistance genes.

43 **Introduction**

44 The likelihood of acquisition of nosocomial resistant bacteria during or shortly after
45 therapy with different antibiotic agents is still unclear and is often confounded by scarce data on
46 antibiotic usage at the individual patient level (1). Historically bacteria were susceptible to a
47 wide range of antibiotics. However, plasmid mediated multi-drug antimicrobial resistance has
48 emerged and become widespread among the Enterobacteriaceae (2). In particular, β -lactamases
49 have become an important mechanism of antibiotic resistance in Enterobacteriaceae and
50 intestinal carriage of β -lactamase-producing organisms is an important source of transmission
51 (3). In order to understand the contributing factors of the emergence of antimicrobial resistance,
52 bacteria-, drug- and patient-level investigations are necessary to better elucidate the time-varying
53 and heterogeneous role of antibiotic selection pressure on emergence and selection of
54 antimicrobial resistance.

55 While drug exposure has been associated with clinical and bacteriological outcome in
56 pharmacokinetic/pharmacodynamic studies (4) and is used for dose optimization and the setting
57 of clinical breakpoints (5), its role on the development of antimicrobial resistance is under
58 intense investigation (6). Antibiotics, particularly those excreted into the intestinal tract, may
59 promote amplification of resistance if sufficient exposures are not attained (7). As the human
60 colon flora is a diverse ecosystem with many and different bacteria, culture of specific species is
61 not quantitatively related to the total numbers of individuals of each of those species. It is not
62 practical to follow up every different colony form –if cultured at all - in a sample of feces. In
63 addition, there is the strong potential for a shift in gene copy numbers during the culturing
64 process. Therefore quantitative PCR techniques are important for studying the bacterial and gene
65 composition and the effect of antibiotics in situ (8). We here studied the effect of antibiotic

66 therapy and exposure on the amplification of resistance among identified carriers of enteric
67 resistant bacteria in a prospective study. A previously developed and validated quantitative real
68 time PCR (qPCR) (9) was used to quantitate resistance genes in rectal samples of non-ICU
69 patients colonized with bacteria carrying genes encoding ESBL enzymes and β -lactamases.

70

71 **Material and Methods**

72 **Study.** An observational prospective study was conducted in 3 hospitals 1 in each of
73 Italy, Serbia and Romania with a high prevalence of ESBL at hospital admission as part of the
74 FP7 EU project SATURN (NO241796; clinical trials.gov NTC01208519). The project aimed to
75 identify the prevalence and molecular background of ESBLs, rate of acquisition, duration of
76 colonization, emergence of resistance by way of appearance of β -lactamase genes not present at
77 admission and amplification of resistance, and whether antimicrobial exposure could be related
78 to emergence of resistance. All adult patients admitted to medical and surgical wards were
79 screened for ESBLs using standard culture-based surveillance techniques (10). Overall, 10.035
80 patients were screened at hospital admission and up to four times within the first month of
81 hospitalization for ESBL producing Enterobacteriaceae carriage in rectal swabs. Of 1.102
82 patients screened positive at admission, 122 patients consented to be included in the longitudinal
83 study where serial (every 2-3 days) rectal samples were collected and analyzed using qPCR for
84 detection of resistance genes. Before starting the study, specific forms were developed for
85 collection of demographic and other variables to allow population pharmacokinetic models to be
86 used to predict patient specific exposures. More details of the study can be found in (10).

Sampling method. At the sampling point the swab was taken and put into the E-swab medium and transported to the local lab. After arrival to the local lab, the tubes are vortexed for 1 min. The E-swab medium suspension (~ 600µl) was transferred into new tubes and mixed with an equal volume of 50% glycerol solution. The samples were stored at -80°C until transport to UA. Once arrived at UA, the samples were kept at -80°C until analysis. Prior to the DNA extraction the total volume of each sample (16000xg, 10min) was centrifuged and the supernatant was discarded. The remaining pellet was subjected to the total DNA extraction with QIAamp® DNA Stool Mini Kit (Qiagen). 4 µl of such DNA was used as a template in qPCR analysis.

qPCR assay. A quantitative real-time PCR was performed as a measure for resistance as described before (9). Briefly, two singleplex assays were performed, one for assessing the total bacteria using the 16S rRNA gene primers set 16S_E939F (GAATTGACGGGGGCCCGCACAAG) and 16S_1492R (TACGGYTACCTTGTTACGACTT) (length 597bp), and the other for assessing colonic CTX-M producers with *bla*$_{CTX-M}$ primers CTX-M-A6 (TGGTRAYRTGGMTBAARGGCA) and CTX-M-A8 (TGGGTRAARTARGTSACCAGAA) (length 175bp) (11–13). Samples were included in the analysis only if (i) the CTX-M copies were ≥1 and the CTrepeat1-CTrepeat2 values were ≤1; (ii) the 16S rDNA copies were ≥1000 and the CTrepeat1-CTrepeat2 values were ≤1; and (iii) CTX-M copies of ≤1 and the counterpart number of 16S rDNA copies were ≥1000. Samples were considered as non-interpretable if the 16S rDNA copies were ≤1000 and CTX-M and/or 16S rDNA samples had only one repeat. The results were expressed as % of the number of copies of the *bla*$_{CTX-M}$ gene over the number of copies of 16S rRNA genes (100% x *bla*$_{CTX-M}$/16S rRNA genes, bla/16S ratio) as previously described (9). A linear correlation between bla/16S

ratios and the ratio of CFU/ml of resistant bacteria over total culturable aerobic bacteria was previously found in stool samples and rectal swabs (9). The bla/16S ratio was used in order to overcome the problem of unknown quantities in fecal material among different samples (9).

Exposure parameters. Since plasma sampling was not feasible in this study, a population pharmacokinetic model was used to estimate exposure parameters for individual patients, using their demographic characteristics and other relevant parameters such as creatinine clearance. The plasma PK parameters *f*AUC_{0-24}, *f*Cmax and *f*Cmin based on notional patient-specific protein binding were calculated for each patient using the KinFun1.07 software (Maastricht, NL) using a previously published population 2-compartment pharmacokinetic model (14). The input parameters for the KinFun software were Vc=10.1 L, k12=0.51h^{-1}, k21=0.71 h^{-1} whereas the k10 was individualized based on the estimated ceftriaxone clearance of each patient as CL=0.56+0.32x(CLcr/4.26) where CLcr was the creatinine clearance calculated with Cockcroft-Gault Equation based on the gender, age, weight and serum creatinine (15). In order to calculate the free fraction the following equation was used fC=0.5*{-(nP+1/Kaff)-tC)+ √[(517+1/Kaff -tC)^2+4tC/ Kaff]} where fC and tC are the free and total concentrations in μM, nP is the total concentration of protein binding sites 517 μM and Kaff is the binding affinity constant 0.0367 μM.

Significant amplification and analysis. To determine the threshold for significant amplification of resistance, a CART analysis was performed on amplification results for treated patients and controls. None of the controls had a >20% absolute increase in bla/16S ratios indicating that this level represents an increase in bla/16S ratio not associated with antimicrobial therapy. In subsequent analyses, patients were categorized as those with ≤20% and >20% increase bla/16S ratios at any time point compared to the initial screening. A univariate analysis

(Fisher exact test) was used to test whether amplification of resistance genes was associated with antibiotic therapy as such and a multivariate stepwise logistic regression analysis was used to associate different variables with amplification of resistance (JMP10, SAS Institute Inc., Cary, NC). In order to assess the significance of time above the MIC in emergence of resistance, %fT>MIC was calculated using the resistant breakpoint of *Enterobacteriaceae* of 4 mg/l. CART analysis was performed to determine relationships between the highest increase in resistance genes and the PK parameters or duration of therapy until the time that highest increase in resistance genes was observed (JMP10, SAS Institute Inc., Cary, NC). The significance between groups with PK parameters lower or higher that the cutoff PK parameter determined with CART analysis was analyzed with Fisher's exact test (GraphPad Software 4.0, San Diego, CA).

Results

Patients. Of the 122 patients who consented to be included in the study 55 were from medical and 67 were from surgical wards. Non-interpretable or qPCR data from one swab only were obtained for 4 and 5 patients, respectively. For the remaining 113 patients the median (range)/25th-75th percentile number of samples with qPCR results was 5 (2-15)/4-7 per patient over a period of 15 (6-57) days. Only 14 patients were sampled before antibiotic treatment was initiated.

Treatment. Of the 113 patients available for analysis, 11 did not receive any antimicrobial treatment whereas 102 patients were treated with one (N=31) or more (N=71) antibiotics (Table 1). Of 31 patients who received only one drug, 20 were treated with ceftriaxone, 2 with vancomycin, 2 with ciprofloxacin and 7 patients with another 7 different

antibiotics. Of 71 patients treated with a combination of drugs, 36 patients received ceftriaxone, 25 patients ciprofloxacin, 17 metronidazole, 11 meropenem, 11 cefuroxime, 11 levofloxacin, 10 vancomycin and another 29 received different antibiotics (<10 patients per drug). Of 46 treated patients who did not receive ceftriaxone alone or in combination with other drugs, 9 were treated with one drug (2 vancomycin, 3 ciprofloxacin, 1 meropenem, 1 cefazolin, 1 amoxicillin, 1 oxacillin) and 37 with a combination of drugs with the most common drug being ciprofloxacin (12) followed by vancomycin (19), levofloxacin (8), meropenem (7), metronidazole (7) and 26 other antibiotics (<5). The details of the 56 patients treated with ceftriaxone (20 as monotherapy and 36 as combination therapy) are shown in Table 1.

qPCR. Of 113 patients with more than one interpretable qPCR data, the *bla*$_{\text{CTX-M}}$ gene was detected in 97 patients at the initial screening with median(range)/25$^{\text{th}}$-75$^{\text{th}}$ percentile bla/16S ratios 3%(0.01%-206%)/0.1%-18% resistance genes, of whom 20 patients were considered significant with >20% bla/16S ratios; the *bla*$_{\text{CTX-M}}$ gene was detected in 110 patients at any time point with 16%(0.01%-1263%)/3%-48% bla/16S ratios (46 patients had >20% bla/16S ratios) and the *bla*$_{\text{CTX-M}}$ gene was detected in 93 patients at the final screening with 4% (0.01%-1263%)/0.7%-19% bla/16S ratios (22 patients had >20% bla/16S ratios) (Figure 1).

Amplification of resistance genes and antimicrobial therapy. Amplification of resistance genes compared to initial screening levels in four patients groups is shown in Figure 2. By definition, no amplification was observed in any of the 11 patients that did not receive any antibiotic (controls). Of the remaining 102 patients treated with antibiotics, amplification of resistance genes were observed in 20/56 (36%) patients treated with ceftriaxone alone or in combination (3/20 patients treated with ceftriaxone alone, 17/36 patients treated with ceftriaxone and another antibiotic) and 10/46 (22%) patients treated with other antibiotics (6/10 were treated

with ciprofloxacin (N=5) or levofloxacin (N=1) together with other antibiotics). Of 30 pts where amplification of resistant genes was observed, 20 were colonized with ESBL+ *E. coli*, 8 with ESBL+ *K. pneumoniae* and 2 with other ESBL+ species. Of the 72 patients that no amplification of resistance was found, 45 were colonized with ESBL+ *E. coli*, 21 with ESBL+ *K. pneumoniae*, 2 with both species, 4 with other ESBL+ species.

To determine whether an association existed between amplification of resistance genes and antimicrobial therapy or any demographic variable, a stepwise (mixed direction with p enter=0.25 and p leave=0.1) multivariate regression analysis was performed with the following nominal (n) and continuous (c) variables: country (n), wardtype (n), gender (n), age (c), weight (c), height (c), previous hospitalization (n), previous ABx (n), ABx on admission (n), any ABx during study (n), number of antibiotics (c), duration of ABx (c), ABx including ceftriaxone (n), ABx only with ceftriaxone (n), ABx without ceftriaxone (n). Of these, the only significant associations with amplification of resistance genes found were ceftriaxone treatment alone (p=0.010, OR=1.8) and ceftriaxone treatment alone or in combination with other drugs (p=0.015, OR=2.7).

Ceftriaxone exposure. The median (range) dose in patients receiving ceftriaxone was 1000 (1000-4000) mg dosed every 12h (40 patients) and 24h (16 patients). Using the individual patient characteristics and the population pharmacokinetic model, the estimated ceftriaxone clearance, *f*AUC_{0-24}, *f*Cmax, *f*Cmin in these patients (median (range)) were 0.89 (0.67-1.36) l/h, 97 (50-667) mg.h/l, 14.8 (5.8-134.5) mg/l and 3.4 (1.3-12.4) mg/l, respectively. The median (range) % free drug levels at *f*Cmax and *f*Cmin were 9% (7-34%) and 6% (5-9%), respectively.

Amplification of resistance genes and ceftriaxone therapy. To assess the association between amplification of resistance genes and ceftriaxone PK parameters, duration of ceftriaxone

201 therapy and their interaction, a stepwise multivariate regression analysis was performed for the
202 56 patients treated with ceftriaxone alone or in combination. A significant association was found
203 between amplification of resistance (used as a categorical variable based on the cutoff of 20%
204 bla/16rRNA increase) and *f*AUC$_{0\text{-}24}$, duration of therapy and their interaction (Chi square
205 ranging between 3.7-7.11 for each of three terms, p=0.02-0.08).

206 CART analysis confirmed these findings (Table 2). Amplification of *bla*$_{CTX\text{-}M}$ genes was
207 found in 11/16 (69%) patients treated >14d with ceftriaxone and in 9/40 (23%) patients treated
208 with ≤14d with ceftriaxone (p=0.0019). In the latter group, a strong association was found for
209 *f*AUC$_{0\text{-}24}$ for which amplification of resistance genes was observed in 5/7 (71%) of patients with
210 *f*AUC$_{0\text{-}24}$ ≥221.9 mg/l and in 4/33 (12%) of patients with lower *f*AUC$_{0\text{-}24}$s (p=0.0033). A
211 significant association was also found with *f*Cmax with amplification of *bla*$_{CTX\text{-}M}$ genes observed
212 in 5/8 (63%) of patients with *f*Cmax ≥29.3 mg/l and in 4/32 (13%) of patients with a lower
213 *f*Cmax (p=0.0079). The *f*AUC$_{0\text{-}24}$ was highly correlated with *f*Cmax (Pearson correlation
214 coefficient r=0.95, p<0.0001). No significant associations were found for *f*Cmin or %*f*T>MIC.

215 The bla/16S ratios were plotted over time for selected patients treated with ceftriaxone
216 are shown in Figure 3. More than 14d of ceftriaxone therapy was associated with amplification
217 of resistance independently of *f*Cmax and *f*AUC$_{0\text{-}24}$. For shorter duration of therapy,
218 amplification of resistance was observed at high drug exposures but not at lower drug exposures
219 for which *f*AUC$_{0\text{-}24}$ was more important than *f*Cmax (two bottom right graphs).

220

221 **Discussion**

222 A significant association was found between ceftriaxone exposure and amplification of
223 *bla*$_{CTX-M}$ resistance genes in the GI tract. An increased risk of resistance amplification was found
224 when ceftriaxone therapy lasted >14d independent of drug exposure. In addition, for shorter
225 periods of treatment the risk of amplification was increased in patients with a *f*Cmax >30 mg/l or
226 a *f*AUC$_{0-24}$ >222 mg.h/l.

227 We utilized a quantitative PCR assay to measure CTX-M resistance genes.
228 Bacteriological techniques are not sensitive enough to detect small amounts and changes in
229 subpopulations of resistant bacteria (16). Previous studies showed that rectal swabs are suitable
230 for quantifying the concentration of beta lactamase-producers and that qPCR demonstrated
231 higher correlation between rectal swabs and stool specimens than the culture-based method. The
232 bla$_{CTX-M}$/16S RNA ratio reflect changes in the number of resistant bacteria as a proportion of
233 total bacteria, because all the subpopulations can potentially change during therapy. Previous
234 studies found a relationship between the qPCR ratio and surveillance cultures indicating that this
235 ratio reflects colonization by resistant bacteria. Antibacterial treatment may select for a wide
236 range of cephalosporin resistance genes from different families and micro-organisms (17, 18). In
237 the present study we studied the effect of antibiotic treatment on *bla*$_{CTX-M}$ genes normalized by
238 the total bacterial load using the 16S rRNA genes using a method developed earlier (9). This
239 method was found to give comparable results between rectal swabs and stool specimens by
240 culture methods. Moreover, given that previous findings showed that anaerobic flora plays an
241 important role on emergence of resistant bacteria (19), a PCR method allows quantification of
242 all resistance genes originating from diff erent bacteria including non-culturable ones that
243 would be missed by culture methods. However, since there was considerable variation in
244 positivity, and there were some very high *bla*$_{CTX-M}$/16rRNA ratios that were difficult to explain,

245 the analysis was performed with a qualitative parameter of amplification of resistance genes
246 using the cutoff of 20%, which represents the increase in bla/16S ratio associated with
247 antimicrobial therapy.

248 Antibiotic therapy has a severe impact on GI flora and this effect depends on antibiotic
249 used, its exposure and duration of treatment (20). However, although the use of broad spectrum
250 cephalosporins has been implicated in emergence of resistance (21, 22) few studies demonstrated
251 a clear association between amplification of resistance and drug exposure. Animal studies in pigs
252 found an increase in the prevalence of resistant *E. coli* within the first week of treatment with
253 ceftiofur and amoxicillin (17). Similar studies in calves showed that a 14% increase of fecal
254 bacteria resistant to ceftriaxone within three days after treatment. This response remained stable
255 up to 13 days whereas a further increase was found at day 17 (23). Goessens et al. demonstrated
256 a clear relationship between duration of exposure as well as the % time within the selection
257 window for ceftazidime in a rat model (24). Ceftriaxone is a highly protein bound drug with
258 high volume of distribution and excellent penetration in fluids (25). Up to 67% of the drug is
259 excreted unchanged in urine and the remaining is excreted in bile with average concentrations of
260 581-898 mg/l found 1-3h after dosing when the concurrent plasma concertation was 62.1 mg/l
261 (26). In addition, median ceftriaxone concentrations in feces of healthy volunteers treated with 7
262 days with 2g ceftriaxone IV were 2.4 mg/l on day 4 and 161 mg/l on day 8 indicating
263 accumulation in GI tract although a wide variation among individuals was found (0-806 mg/l)
264 (27). When intestinal flora was quantitated in the latter study, the number of enterococci
265 increased whereas the number of *E. coli* decreased during the ceftriaxone treatment (27). This
266 accumulation is compatible with the strong association that we found in the present study

between emergence of resistance and >14d of ceftriaxone treatment. For shorter period of treatment, drug exposure plays more important role on emergence/amplification of resistance.

As cephalosporin's activity is time-dependent, the driving PK-PD index is time above the MIC and 60-70% of the dosing interval is commonly thought to be associated for maximal bactericidal activity in vivo (28, 29). However, no significant association was found between amplification of resistance with %fT>MIC in the present study. This is in agreement with previous studies in rats where intestinal colonization by ceftazidime-resistant *Enterobacter cloacae* isolates were associated with the time within the mutation selection window, AUC/MIC and *f*Cmax/MIC rather than %*f*T>MIC (24). Although concentrations in feces are not the same as in plasma, it could well be that the location were selection occurs (close to the gut wall) is correlated to plasma concentrations.

There are several limitations in our study. In this study we only explored the association between ceftriaxone exposure and amplification of CTX-M genes and therefore the effect on ceftriaxone therapy for other resistant genes is unknown. It may therefore well be that the effect of ceftriaxone therapy on resistance amplification is even stronger than demonstrated. A second limitation is the fact that pharmacokinetic parameters was estimated using a population pharmacokinetic model based on the creatinine clearance calculated on Cockcroft-Gault equation using patient demographic data rather than actual measurement as this was not feasible (14). Although this model well described the serum concentrations of ceftriaxone it may not capture the entire variation within our patient population and it does not predict ceftriaxone concentrations in the gut. However, concerning the latter, preclinical studies also showed correlations with plasma levels rather than concentrations in the gut (24). Therefore, even though the quantitative association may be different in the gut, the association does exist, either directly

or indirectly. Finally, the study was a prospective observational cohort study comparing the effects of various treatments of ceftriaxone, rather than a randomized controlled trial, which may have resulted in both bias as well as confounding. The analysis of specifically ceftriaxone exposure presented here rather than that of a specific antibiotic was not defined in advance, since the study ran in several centres and it was not known beforehand for which antibiotics the analysis could be performed. Nevertheless, the data to be collected were defined in advance, and there was an anticipation that only few drugs would be taken by a sufficient number of patients to perform the analysis here. Ceftriaxone was the only drug that was taken by a sufficient number of patients to allow for the present evaluation. Even with the relatively low number of patients included in this analysis we were able to show a significant result, indicating that the effects may be quiet strong. Nevertheless, the association should be confirmed in a prospective randomized trial.

Amplification of a particular known resistance gene by ceftriaxone therapy may have important clinical implications. Amplification of this gene may indicate amplification of the resistant bacteria over susceptible ones or transmission of this gene across different species. Heavily colonized patients are at risk in developing difficult to treat bloodstream infections. Thus, identifying patients with low ceftriaxone exposure and therefore at risk in developing such infections may promote rigorous surveillance studies and high alert of the clinicians. Interventional strategies could be adopted by optimizing ceftriaxone dosing regimens in order to minimize the risk of amplification of $bla_{CTX\text{-}M}$ genes. Thus, in addition to optimizing therapy in terms of efficacy, exposure could or should be optimized with regard to resistance amplification.

311 In conclusion, amplification of CTX-M resistance genes were observed during
312 ceftriaxone therapy with strong association found with the duration of therapy and drug
313 exposure. Long treatment and high drug exposures increased the risk of amplification.
314

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Transparency declaration

None

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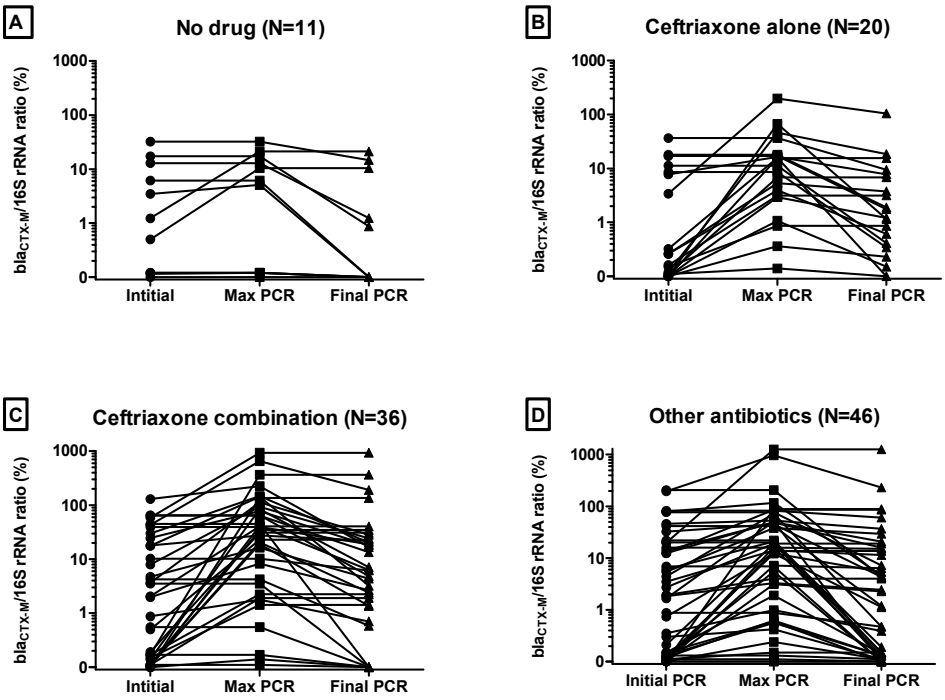

411

412 **Figure 1.** Time-course of quantitative PCR expressed as % *bla*$_{CTX-M}$ of 16S rRNA in patients
413 without any treatment (A), in patients treated with ceftriaxone alone (B) and in combination with
414 other drugs (C) and in patients treated with other drugs (D) at the time of screening (initial), the
415 time of the maximum (max PCR) and the last sample (final PCR). The % *bla*$_{CTX-M}$/16S rRNA
416 ratios are the absolute measurement calculated by dividing *bla*$_{CTX-M}$ copy numbers over 16S
417 rRNA copy numbers.

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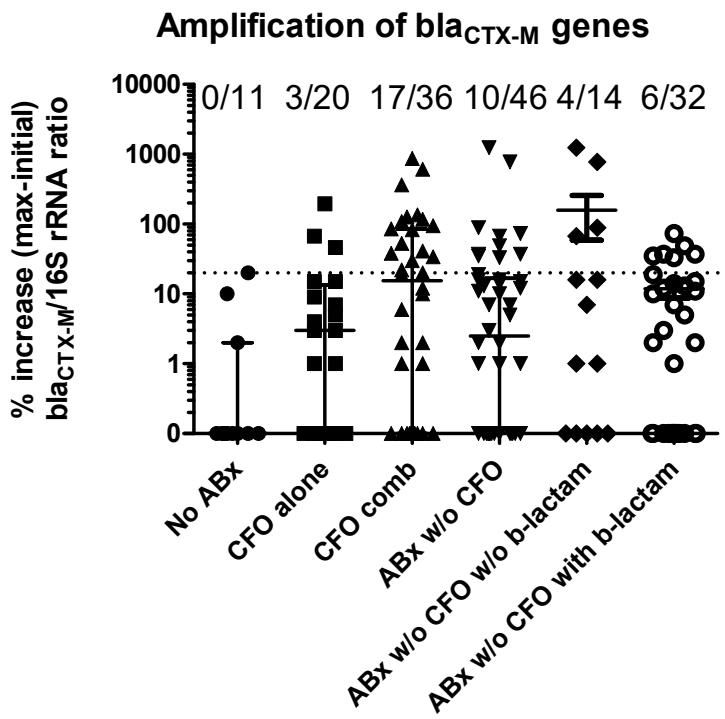

419

420 **Figure 2.** Amplification of resistance genes (% increase calculated as maximum bla$_{CTX-M}$/16S
421 rRNA ratio at any time compared to initial ratio) in different patients groups. On the top of the
422 graph, the number of patents with >20% increase in bla/16S ratios over the total number of
423 patients per group is shown for each group. Error bars represent the median and interquartile
424 range. *;p<0.05 compared to No Abx group. The horizontal broken lines represent a relative
425 increase of 20% of *bla*$_{CTX-M}$/16S rRNA ratios over the initial screening.

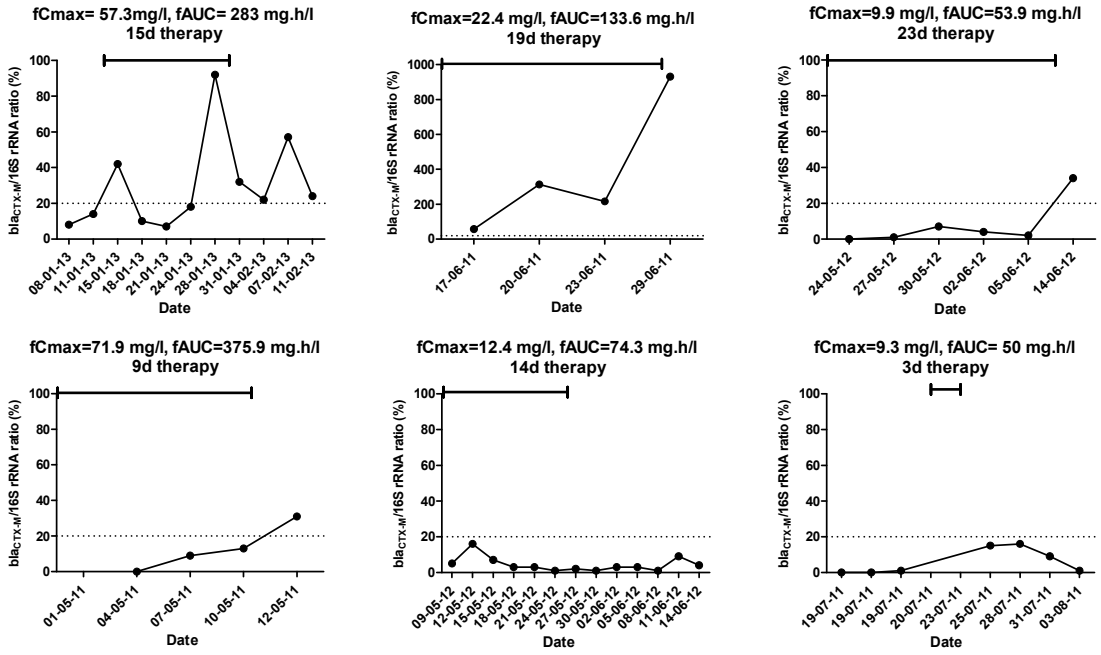

Figure 3. Amplification of CTX-M resistance genes in rectal samples over time for 6 different patients treated with ceftriaxone >14.3d (top graphs) and <14.3 (bottom graphs) attaining low (right graphs), intermediate (middle graphs) and high (left graphs) *f*Cmax. The duration of ceftriaxone therapy and the *f*Cmax together with the $fAUC_{0\text{-}24}$ are shown for each patient. The horizontal line represents the threshold of CTX-M gene amplification observed in non-treated patients.

435 **Table 1.** Details of patients included in the present study with a focus on patients treated with

436 ceftriaxone alone or in combination with other drugs

Patient groups	**Number of pts**
No treatment	11
Treatment	102
E.coli	65
K. pneumoniae	30
Mixed	2
Other	5
One drug	31
Ceftriaxone	20
Other drugs	11
>1 drug	71
Ceftriaxone+another drug	36
Ceftriaxone treated patients	56
Admitted from home	47
Previous hospitalization	9
Catheters (urinary, iv)	12
Underlying disease	
Malignancy	56
Cardiovascular	11
Diabetes	10
CV	29
Renal disease	2
Primary diagnosis	
muscoskeletal system	31
nervous system	14
endocrine system	7
respiratory system	2
digestive system	1
infectious and parasitic disease	1
Surgical/Medical	42/14
Age (y), median (range)	63 (25-88)
Weight (kg), median (range)	74 (50-158)
Height (cm), median (range)	168 (55-187)
Male/female	30/26
ESBL Colonization	56
E.coli	40
K. pneumoniae	13
Mixed	1
Unidentified	2

437

438 **Table 2.** Association between amplification of bla_{CTX-M} genes and duration of therapy as well as
439 exposure of ceftriaxone therapy. The first column indicates the thresholds as determined by
440 CART analysis.

	N of Pts with Amplification[a]		% Pts with Amplification		Fisher exact test
All	**Yes**	**No**	**Yes**	**No**	**P value**
>14 days	11	5	69%	31%	0.0019
≤14 days	9	31	23%	77%	
***f*Cmax≥29.3[b]**	5	3	63%	37%	0.0079
***f*Cmax<29.3[b]**	4	28	13%	87%	
***f*AUC_{0-24}≥221.9[b]**	5	2	71%	29%	0.0033
***f*AUC_{0-24}<221.9[b]**	4	29	12%	88%	

441 [a] "Yes" for amplification was assigned to patients with max bla_{CTX-M}/16rRNA ratio - initial
442 bla_{CTX-M}/16rRNA ratio >20%

443 [b] These cutoffs values were derived from CART analysis for patients treated with ceftriaxone for
444 ≤14 days. Steady state *f*AUC_{0-24}s were estimated using the population pharmacokinetic model of
445 ceftriaxone.