Ribaxamase, an Orally Administered β-Lactamase, Diminishes Changes to Acquired Antimicrobial Resistance of the Gut Resistome in Patients Treated with Ceftriaxone

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Introduction: Intravenous (IV) β-lactam antibiotics, excreted through bile into the gastrointestinal (GI) tract, may disrupt the gut microbiome by eliminating the colonization resistance from beneficial bacteria. This increases the risk for Clostridium difficile infection (CDI) and can promote antimicrobial resistance by selecting resistant organisms and eliminating competition by non-resistant organisms. Ribaxamase is an orally administered β-lactamase for use with IV β-lactam antibiotics (penicillins and cephalosporins) and is intended to degrade excess antibiotics in the upper GI before they can disrupt the gut microbiome and alter the resistome.

Methods: Longitudinal fecal samples (349) were collected from patients who participated in a previous Phase 2b clinical study with ribaxamase for prevention of CDI. In that previous study, patients were treated with ceftriaxone for a lower respiratory tract infection and received concurrent ribaxamase or placebo. Extracted fecal DNA from the samples was subjected to whole-genome shotgun sequencing and analyzed for the presence of antimicrobial resistance (AMR) genes by alignment of sequences against the Comprehensive Antibiotic Resistance Database. A qPCR assay was also used to confirm some of the results.

Results: Database alignment identified ~1300 acquired AMR genes and gene variants, including those encoding β-lactamases and vancomycin resistance which were significantly increased in placebo vs ribaxamase-treated patients following antibiotic exposure. qPCR corroborated the presence of these genes and supported both new acquisition and expansion of existing gene pools based on no detectable copy number or a low copy number in pre-antibiotic samples which increased post-antibiotics. Additional statistical analyses demonstrated significant correlations between changes in the gut resistome and clinical study parameters including study drug assignment and β-lactamase and vancomycin resistance gene frequency.

Discussion: These findings demonstrated that ribaxamase reduced changes to the gut resistome subsequent to ceftriaxone administration and may help limit the emergence of AMR.

Keywords: oral β-lactamase, ceftriaxone, gut resistome, whole-genome shotgun sequencing, clinical study, antimicrobial resistance

Introduction
Perturbation of the human gut microbiome has been associated with numerous diverse disease states including obesity, autoimmune diseases and Clostridium difficile infection (CDI).1–3 The human gut microbiome can be influenced by many factors including age, diet, geographic location and exposure to infectious
diseases, but the most damaging are antibiotics. The broad spectrum $\beta$-lactam antibiotics, such as penicillins and cephalosporins, are particularly disruptive as their use significantly reduces certain families of bacteria, including obligate anaerobes, which are considered an essential component of a healthy gut ecosystem.

Antibiotics are a major cause of gut microbiome disruption, and broad-spectrum antimicrobials are strongly associated with an increased risk for CDI, including clindamycin, fluoroquinolones and $\beta$-lactam antibiotics (penicillins, cephalosporins and carbapenems). All intravenous (IV) $\beta$-lactam antibiotics are at least partially processed in the liver and excreted through the bile into the intestine as active antimicrobials. Biliary excretion of $\beta$-lactams can range from a small percentage of the input dose to up to 40% for antibiotics like ceftriaxone, resulting in high intestinal concentrations of up to 1 mg/mL. $\beta$-lactams are the most commonly used broad spectrum antibiotics and are especially harmful to the commensal organisms of the gut microbiome, particularly the beneficial anaerobes that play a role in colonization resistance.

Exposure of the gut microbiome to antibiotics has the additional adverse consequence of propagating antimicrobial-resistant (AMR) organisms. As an antibiotic exerts its selective pressure on the gut microbiota, susceptible organisms are eliminated, allowing antimicrobial-resistant organisms to proliferate. Many AMR genes are found on mobile genetic elements facilitating their spread from one bacterial strain to another. For example, following antibiotic treatment for C. difficile, enterococci resistant to vancomycin commonly emerge. Vancomycin resistance can result from either transfer of resistance from commensal organisms to enterococci or from colonization by exogenous enterococci that are resistant to vancomycin. Vancomycin-resistant enterococci (VRE) are also observed following treatment with antibiotics other than vancomycin, due to antibiotic-mediated microbiome damage resulting in diminished competition within the gut leaving the host susceptible to colonization from the environment. This outcome is particularly devastating for immunocompromised patients, such as those receiving bone marrow transplants, where VRE is a leading cause of infection and mortality.

Another consequence of antibiotic use, and in particular $\beta$-lactam use, is the emergence of extended spectrum, $\beta$-lactamase-producing gram negative bacilli (ESBL-GNB). These bacteria, in the family Enterobacteriaceae, include Escherichia, Klebsiella and Salmonella organisms which are well adapted to the environment of the human gut and have become resistant to most antibiotics. These organisms can also carry $\beta$-lactamases such as Klebsiella pneumoniae carbapenemase and New Delhi metallo-$\beta$-lactamase that are active against all classes of $\beta$-lactam antibiotics including carbapenems. $\beta$-lactamase genes are often carried on plasmids with multiple resistance genes facilitating their spread to other organisms within the gut. Some of these bacteria are resistant to most classes of antibiotics therefore limiting treatment options to older antibiotics like colistin that have high toxicity profiles. Notably, recent reports of colistin-resistant strains of ESBL-GNB found in the United States have raised the specter of having no antibiotics available to treat these devastating infections.

Strategies are needed to protect the gut microbiome from disruption by antibiotics and to prevent AMR phenotypes from emerging and propagating as a consequence of the selective pressure caused by antibiotic use. Ribaxamase is a recombinant $\beta$-lactamase designed to be orally administered with intravenous (IV) $\beta$-lactam antibiotics, specifically, most penicillins and cephalosporins, and functions in the proximal gastrointestinal (GI) tract to degrade excess antibiotics excreted into the intestine through the bile. Thus, antibiotic inactivation is expected to prevent disruption of the gut microbiome and reduce exposure of the gut bacteria to the selective pressure of the antibiotics. Using a pig model of $\beta$-lactam-induced dysbiosis, ribaxamase was demonstrated to protect the gut microbiome and prevent the emergence of AMR.

Ribaxamase was evaluated in a Phase 2b proof-of-concept study performed in 412 patients who were admitted to the hospital for treatment of a lower respiratory tract infection (LRTI) with IV ceftriaxone. Patients were randomized one-to-one to receive co-treatment with either ribaxamase or placebo and could also receive macrolides as required by the attending physician. Patients were monitored for diarrhea and then tested for CDI during and for 6-weeks after the primary course of ceftriaxone. The study met its primary endpoint of significantly reducing the incidence of CDI in patients that received ribaxamase compared to placebo. A total of 3.4% of the placebo patients were diagnosed with CDI, while only 1.0% of ribaxamase patients developed CDI (risk reduction 2.4%, 95% CI –0.6 to 5.9; one-sided P=0.045), a 71% relative reduction in the incidence of CDI including CDI caused by highly pathogenic 027 strains.

Fecal samples, collected at three prescribed
points, were analyzed to determine the composition of the fecal microbiome and compared to evaluate changes to the gut microbiome following antibiotic exposure. This analysis demonstrated that ribaxamase significantly diminished ceftriaxone-induced changes to the gut microbiome and allowed the microbiome to recover more quickly than placebo in patients treated with ceftriaxone.

In the present study, a set of the DNA samples, extracted from the fecal samples collected during the previous Phase 2b clinical study, were analyzed by whole-genome shotgun sequencing and alignment against the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/home). Some of the samples were also analyzed by qPCR and additional statistical analyses using clinical metadata and a linear model were performed to compare the change in the number of hits for AMR genes prior to post-antibiotics. Here we report the findings of these analyses and demonstrate, consistent with its intended mechanism of action, ribaxamase appeared to reduce changes in the AMR gene profile (the resistome) in the GI tract of patients treated with ceftriaxone. Five-fold more AMR genes were significantly changed in terms of hits in placebo-treated patients as compared with ribaxamase-treated patients, including genes relevant to the clinical study such as β-lactamase, vancomycin resistance, and macrolide resistance genes. A qPCR assay confirmed these results for two representative genes.

Materials and Methods

Samples Sequenced and Analyzed

During the Phase 2b clinical study with ribaxamase, conducted in 84 hospitals in North America and Eastern Europe from Sept. 2016-Sept. 2017, 862 fecal samples for microbiome analysis were collected from patients who were receiving ceftriaxone for a LRTI. Of these samples, DNA was extracted with the MoBio PowerMag isolation kit on an automated KingFisher platform and 16S rRNA sequences were determined for 676 samples. Sample selection was based on the criteria that a patient had to have a fecal sample collected at screening so that they could serve as their own baseline prior to study-related treatments. Based on the funding contract with the Centers for Disease Control (CDC), a set of 349 of the extracted fecal DNA samples from the Phase 2b study were included in the present study (Supplemental Table S1). These samples represented 118 patients out of 412 from the study (66 ribaxamase and 52 placebo). Sample selection for analysis was based on the following criteria, 1) patients that had a full set of three longitudinal fecal samples (ie, screening [T0], 72 hours post-antibiotics [T1] and 4 weeks post-antibiotics [T2]), 2) patients that had a screening sample and a T1 sample only, or 3) select samples from patients without a screening sample. For criteria 3, these samples were chosen from patients who met the Phase 2b clinical study endpoints of a) C. difficile infection (CDI), b) non-C. difficile antibiotic-associated diarrhea (AAD) or c) microbiologically confirmed colonization with one of two select pathogens, VRE or ESBL-GNB.

Whole-Genome Shotgun Sequencing and Change in Number of Hits per AMR Gene Variant

DNA extracted from fecal samples for microbiome analysis was shipped frozen to Diversigen, Inc. (Houston, TX) and libraries were prepared as per internal Diversigen standards. Briefly, fecal DNA (10 ng to 500 ng) was sheared into fragments of approximately 300–400 bp in a Covaris E210 system (96 well format, Covaris, Inc., Woburn, MA) followed by purification using AMPure XP beads. DNA end repair, 3′-adenylation, ligation to Illumina (San Diego, CA) multiplexing PE adaptors, and ligation-mediated PCR (LM-PCR) were all completed using automated processes. Current standard library methods utilized KAPA HiFi polymerase (KAPA Biosystems Inc., Wilmington, MA) for PCR amplification (6–10 cycles). Prepared libraries were pooled according to the experimental plan and subjected to Illumina HiSeq 2000 sequencing.

Raw sequences with average raw reads per sample of 147,251,799 were trimmed of all primer sequences using BBDuK version 36.92, and filtered for human DNA and PhiX (Illumina control) with Bowtie2 version 2.3.0 against a prebuilt hg38-PhiX database (Illumina). Subsequently, reads were individually mapped against the CARD database (https://card.mcmaster.ca/home) using default parameters of 90% read coverage on a perfect match. Reads were aligned to CARD without prior assembly, therefore reads from complete genes were not distinguished from truncated or partial genes. A set of AMR hits was produced using the protein homolog model which detects proteins conferring antibiotic resistance based on similarity to the curated reference sequence. A “hit” was defined as each time the determined sequences of DNA within...
a sample matched the accessioned reference sequence for a specific entry within the CARD database. Further alignment of these protein homolog data were performed with linear discriminant analysis (LDA) effect size (LEfSe) analyses, an algorithm for high-dimensional biomarker discovery. Mann–Whitney (two-sample Wilcoxon) tests were used to compare between screening (T0) and post-antibiotics (T1) time points across treatment groups. Sequencing and resistome analyses data were submitted to the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra), accession PRJNA589866.

qPCR

Quantitative PCR of two representative genes (cfxA and vanRD), identified during sequence analysis as demonstrating a significant increase in number of hits in the placebo group vs the ribaxamase group from T0 to T1, was performed on a subset of 100 fecal DNA samples (Supplemental Table S2). The analyzed samples represented an equal number from each treatment group.

For each gene, primer sets (Table 1) were designed based on available sequences from the CARD database using Primer Express v3.0.1 and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primers were purified by standard desalting, and for qPCR reactions, 10μM working stocks were prepared. Synthetic DNA gene fragments were created using IDT’s gBlocks Gene Fragments for positive controls and standard curves. A fragment was designed from each gene sequence, synthesized to 500ng, and concentrated to a stock of 10ng/μL.

For each qPCR experiment, a standardized thermocycling profile of initial denaturation of 95°C for 10 minutes, followed by 40 cycles of 10 seconds of denaturation at 95°C and 30 seconds of annealing/extension at 60°C, and a final dissociation stage to produce a melt curve were used. For each reaction, 5μL of PerfeCta SYBR Green FastMix, Low Rox 2x (QuantaBio, Beverly, MA), 1μL of primer set, and 5ng of fecal DNA template was added. All experiments and analyses were performed using an Applied Biosystems (Foster City, CA) QuantStudio 7 Real-Time PCR machine with v1.2 software. A no-template, negative control (NTC) was included in all sample runs using Applied Biosystems MicroAmp Fast Optical Reaction plates.

Samples for analysis were concentrated to 1.25ng/μL for a total of 5ng DNA per reaction. Each sample was tested in triplicate, against each of the two designed assays. Each assay was run individually, with a standard curve ranging from 10^–10^8 copies using the gBlocks, with NTC included. The standard curve was determined by quantifying the concentration of the gBlock, and using the molar mass per base (650 g/mol/bp) and fragment length to calculate the proper copy number. Using linear regression analysis, copy numbers of 1 to 10^10 were determined from the five point standard curve with R^2 values consistently 0.99 in agreement with Diversigen internal standard operating procedures. Data are presented as copy number per 5ng of total DNA. If a sample did not produce a copy number above 1.0, as determined using linear regression, it was considered to be a false positive.

The copy numbers were also compared with the number of hits as determined by sequence alignment. Quartiles for number of hits and copy numbers were established using Microsoft Excel and the two parameters were compared for each sample to determine correlation between the two methods. Further, qPCR results across the longitudinal samples for each patient were examined to assess whether changes in the copy numbers of cfxA and vanRD over the course of the study were consistent with new acquisition of organisms carrying these antibiotic resistance genes or expansion of existing pools of organisms carrying the genes following selection by antibiotics.

<table>
<thead>
<tr>
<th>Table 1 Primer Sequences Used for qPCR Analysis</th>
</tr>
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<tbody>
<tr>
<td><strong>cfxA</strong></td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
</tr>
<tr>
<td><strong>vanRD</strong></td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
</tr>
</tbody>
</table>

Statistical Correlation Between Resistome Data and Clinical Study Data

Four classes of acquired antibiotic resistance genes, β-lactamase, vancomycin resistance, macrolide resistance, and multidrug efflux pump component genes, were chosen for analysis with respect to clinical metadata from the Phase 2b ribaxamase study. These classes were selected based on their medical importance as well as their relevance to the antibiotics administered during the study. A series of clinical parameters were also selected for statistical analysis (Supplemental Table S3) based on relevance to the clinical study design (20) and being known risk factors for gut microbiome disruption and CDI including, advanced age, use of macrolides, and use of proton pump inhibitors (PPI). Two demographic parameters,
age and gender, three treatment-based parameters; study drug assignment (ribaxamase vs placebo), use of macrolides, and use of PPI, and one care-based parameter, whether at any time the patient was classified as no-food-by-mouth (NPO, as a surrogate for sicker patients), were investigated. CDI was not included as there were only seven post-antibiotic fecal samples available from CDI patients, an insufficient number for meaningful analysis.

A linear model was used to compare the change in the number of hits for each AMR gene in each class of genes from the T0, pre-ceftriaxone treatment, to the T1 collection point, 72 hrs after ceftriaxone was stopped. Analyses were conducted based on the log transformed values. For each gene, both the t-test and Wilcoxon test were used for the comparisons between the groups, and nominal P-values were provided from these tests without multiplicity adjustment. A linear regression was used to examine the impact of the identified clinical parameters on resistome changes from baseline (T0) to post-antibiotics (T1) for each gene in each subset. In the regression model, the baseline value and age were included as continuous variables, and the treatment group, gender, use of PPI, NPO and use of a ceftriaxone/macrolide combination were included as categorical variables.

**Results**

**Whole-Genome Shotgun Sequencing and Identification of Acquired AMR Genes**

Whole-genome shotgun sequences obtained from DNA extracted from the 349 fecal samples were aligned against CARD to identify the total number of hits for each acquired AMR gene present in each sample. This analysis identified DNA reads (hits) mapping to 1297 AMR genes or gene variants which ranged from one hit to five million hits per gene with a median of 64, and a range of 2001 to 277,184 hits per sample with a median of 49,378. The top ten AMR genes in terms of overall hits included five tetracycline resistance genes (tetQ, tetW, tetX, tetO and tet32), one erythromycin resistance gene (ermF) and four variants of the cfxA β-lactamase gene (cfxA2, cfxA3, cfxA4 and cfxA6).

**Comparison of the Change in AMR Gene Hits from T0 to T1**

Statistical analysis of the change in the number of hits from pre-ceftriaxone (screening [T0]) to post-ceftriaxone (72 hours post-antibiotics [T1]) for each of the AMR genes (or variants) and comparison of the change from T0 to T1 of placebo-treated versus the ribaxamase-treated patients were performed using LEfSe. This analysis identified 94 genes or gene variants in the placebo group and 19 genes or gene variants in the ribaxamase group that changed significantly (LDA score >2) from T0 to T1, an almost five-fold difference in the number of genes affected between treatment groups (Figure 1). Based on changes in hits, 68 genes in the placebo group and 11 genes in the ribaxamase group were significantly reduced and 26 genes in placebo and eight genes in ribaxamase were significantly increased in number of hits from pre- to post-antibiotics. The genes demonstrating significant decrease in both groups included several tet and erm genes as well as AMR genes representative of most other resistance classes. Interestingly, AMR genes demonstrating a significant increase in hits in the placebo group, but not in the ribaxamase group, included a family of cfxA β-lactamase gene variants and vancomycin resistance genes from the vanD operon.

A subset of the identified AMR genes significantly increased in the placebo group vs the ribaxamase group was chosen for further statistical analysis based on AMR phenotype and overall number of hits (>500). This subset included 69 genes or variants (Supplemental Table S4), representing three classes of AMR chosen based on their relevance to the study including, β-lactamase, macrolide resistance and vancomycin resistance genes. An additional selection criterion included only genes that were represented in ≥25% of the samples. AMR genes found in <25% of the samples were excluded from further analysis because it was observed that those genes tended to be predominantly found in few samples with high hits thus skewing the results. In general, genes encoding β-lactamases had a greater number of hits and broader distribution across samples than vancomycin and macrolide resistance genes. The final analysis set included 17 AMR genes (or variants), eight β-lactamase, seven vancomycin resistance, and two macrolide resistance genes (Table 2). Statistical analysis of these genes of interest confirmed that seven genes demonstrated a significant increase in number of hits (P<0.05) from T0 to T1 in placebo compared to ribaxamase samples. The identified genes included, five cfxA β-lactamase variants (cfxA-A5) and two vanD operon accessory genes (vanRD and vanSD), while the vanD gene itself demonstrated a trend towards an increase in hits in the placebo group (Table 2).
qPCR Analysis of Two Representative AMR Genes

To support the AMR sequencing analysis data demonstrating increased hit frequency of AMR genes in the placebo vs ribaxamase groups following antibiotic exposure, two representative genes, *cfxA* and *vanRD*, were selected for qPCR analysis. A subset of 100 extracted DNA samples from 35 patients (18 placebo and 17 ribaxamase) were analyzed by qPCR. For *cfxA*, 89 out of the 100 samples had at least one copy of the gene present by qPCR with a mean of 146,958, a median of 52,776 and a range of 0 to 2,325,389 copies. For *vanRD*, 46 out of the 100 samples had at least one copy, with a mean of 76, a median of 1 and a range of 0 to 1149 copies.

In general, there was a positive correlation between the number of hits for *cfxA* as determined by database alignment and copy number as determined by qPCR. Out of the 100 samples subjected to both analyses, 84 of the samples had a copy number in the same or an adjoining quartile of the data set as compared to the hit frequency data (Figure 2). Only 16 samples had either a lower hit frequency and higher copy number (eight samples) or vice versa (eight samples).

The correlation for the *vanRD* gene was also positive with 75 of the samples aligning in terms of quartiles of hit frequency and copy number (Figure 2) with those having at least one copy number of *vanRD* also displaying detectable hits for *vanRD* (second quartile for each). Of the 25 samples that did not align, 15 had no sequence reads mapping to *vanRD* but had at least one copy number of *vanRD* detected by qPCR, while 10 showed *vanRD* hits but were undetectable by qPCR.

Examination of the change in copy number of *cfxA* over the three collection points from the clinical study indicated that there was an overall increase in the mean copy number of *cfxA* in placebo-treated patients from T0 to T1 which remained elevated through T2 (4-weeks post-antibiotics), while there was an overall decrease in mean copy number in ribaxamase-treated patients from T0 to T1 which remained lower through the T2 collection point (Table 3). Similar results were observed with *vanRD* at the three collection points with the mean copy number increasing in the placebo group at T1 before returning to
the starting level at T2, while the mean copy number trended lower at T1 for the ribaxamase samples before returning to the starting level at T2.

Fecal samples collected from 19 patients, who were determined to be colonized with VRE by microbiologic methods during the Phase 2b ribaxamase study, were included in the qPCR analysis set. Of these 19 patients, 15 were positive for the vanRD gene both by sequencing and qPCR at one or more of the collection points, while three were detectable by only one method (two by sequencing only and one by qPCR only). Only one VRE colonized patient was negative for vanRD by both detection methods. Of the 16 patients included in the qPCR analysis set who did not have microbiologically detectable VRE colonization during the clinical study, seven also did not have detectable vanRD by either method at any collection point. For the other nine patients, five were positive by both sequencing and qPCR, three were positive by sequencing only and one was positive by qPCR only.

Detection of the vanRD accessory gene by qPCR was correlated with detection of the vanD ligase gene by sequence analysis. Of the 35 patients represented by these qPCR samples in 32 cases, the presence or absence of vanRD by qPCR corresponded to the detection, or lack of detection, of vanD by sequence alignment. In only three cases, vanD was not detected by sequence alignment when

Table 2 Comparison of the Frequency of Antibiotic Resistance Genes at T0 vs T1 in the Ribaxamase vs Placebo-Treated Patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>P-value</th>
<th>Resistance Phenotype</th>
<th>Total Hits</th>
<th>Data Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanRD*</td>
<td>0.0053</td>
<td>Vancomycin</td>
<td>3387</td>
<td>≥25%</td>
</tr>
<tr>
<td>vanSD *</td>
<td>0.0130</td>
<td>Vancomycin</td>
<td>539</td>
<td>≥25%</td>
</tr>
<tr>
<td>cfxA5</td>
<td>0.0153</td>
<td>β-lactam</td>
<td>177,247</td>
<td>≥95%</td>
</tr>
<tr>
<td>cfxA</td>
<td>0.0201</td>
<td>β-lactam</td>
<td>158,315</td>
<td>≥95%</td>
</tr>
<tr>
<td>cfxA2</td>
<td>0.0217</td>
<td>β-lactam</td>
<td>171,730</td>
<td>≥95%</td>
</tr>
<tr>
<td>cfxA3</td>
<td>0.0248</td>
<td>β-lactam</td>
<td>199,467</td>
<td>≥95%</td>
</tr>
<tr>
<td>vanD</td>
<td>0.0849</td>
<td>Vancomycin</td>
<td>1114</td>
<td>≥25%</td>
</tr>
<tr>
<td>cba-l</td>
<td>0.1758</td>
<td>β-lactam</td>
<td>78,578</td>
<td>≥90%</td>
</tr>
<tr>
<td>cfaA6</td>
<td>0.4533</td>
<td>β-lactam</td>
<td>535,181</td>
<td>≥90%</td>
</tr>
<tr>
<td>vanXYG</td>
<td>0.5832</td>
<td>Vancomycin</td>
<td>866</td>
<td>≥25%</td>
</tr>
<tr>
<td>vanWG</td>
<td>0.6375</td>
<td>Vancomycin</td>
<td>1446</td>
<td>≥25%</td>
</tr>
<tr>
<td>mefa</td>
<td>0.6929</td>
<td>Macrolide</td>
<td>91,609</td>
<td>≥90%</td>
</tr>
<tr>
<td>vanTG</td>
<td>0.7378</td>
<td>Vancomycin</td>
<td>2333</td>
<td>≥25%</td>
</tr>
<tr>
<td>vanG</td>
<td>0.7624</td>
<td>Vancomycin</td>
<td>863</td>
<td>≥25%</td>
</tr>
<tr>
<td>mel</td>
<td>0.8011</td>
<td>Macrolide</td>
<td>70,445</td>
<td>≥75%</td>
</tr>
<tr>
<td>cepA</td>
<td>0.8253</td>
<td>β-lactam</td>
<td>6721</td>
<td>≥25%</td>
</tr>
</tbody>
</table>

Notes: *β-lactamase, vancomycin resistance or macrolide resistance genes selected based on Phase 2b clinical study. *P-value is based on Mann Whitney test. Total hits across sample set. Only those with >500 total hits were included in the analysis. *Percentage of samples in the 349 sample set which included at least one hit for the gene of interest. *Also identified by linear discriminant analysis effect size (LEfSe) analyses

Figure 2 Comparing Number of Hits per Sample vs Copy Number per Sample. The graph compares data quartiles for the number of hits per sample with the copy number of the gene for each sample for, (A) cfxA and (B) vanRD. The data were separated into quartiles for the 100 samples that were subjected to qPCR analysis and compares the number of hits per sample, as determined by database alignment, vs the copy number per sample, as determined by qPCR analysis. The quartiles were as follows, cfxA Hits, Q1: 0–2658, Q2: 2659–52,776, Q3: 52,777–175,384, Q4: >175,384; Copy Number, Q1: 0–62, Q2: 63–410, Q3: 411–1244, Q4: >1244, vanRD Hits, Q1 & Q2 = 0, Q3: 1–33, Q4: >33, Copy Number, Q1 & Q2 = 0, Q3: 1–4, Q4: >4. Quartiles 1 and 2 were combined for both parameters on the figure for vanRD due to the abundance of samples with zero values in the sample set. The size of each sphere represents the number of samples, based on the copy number, which corresponded to the indicated quartile for hits. The red lines are added for emphasis.

Table 3 Change in Mean Copy Number Over Time for cfxA and vanRD in Placebo vs Ribaxamase-Treated Patients

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>169,373 ±24,137</td>
<td>279,822 ±58,886</td>
<td>237,738 ±70,114</td>
</tr>
<tr>
<td>Ribaxamase</td>
<td>237,012 ±40,149</td>
<td>118,367 ±35,227</td>
<td>150,350 ±46,667</td>
</tr>
</tbody>
</table>

Notes: *Mean copy number. *Standard error.
vanRD was detected by qPCR, and in two of these cases vanRD was also detected by sequence alignment.

The change in copy number for cfxA and vanRD over the longitudinal collection points for individual patients appeared to follow one of three patterns. The first was an initially lower copy number followed by a higher copy number, consistent with expansion of existing AMR during ceftriaxone use (cfxA: 19 patients, vanRD: six patients). The second pattern was a higher copy number that decreased by T1 and then rebounded by T2 (cfxA: eight patients, vanRD: four patients). A variation was observed in two patients for whom the cfxA copy number started high and continued to decrease across the three collection points. For vanRD, three patients had a high copy number at screening and maintained this high copy number through T2. The final pattern was an undetectable copy number at screening with increasing copy numbers at T1 and T2 (cfxA: five patients, vanRD: seven patients), consistent with new acquisition of resistant organisms carrying these genes.

**Correlation of AMR Data with Clinical and Demographic Data**

Statistical analyses were conducted comparing the number of hits for a set of AMR genes and clinical data from the Phase 2b ribaxamase study. Six parameters were selected for this analysis (Supplemental Table S3). These parameters were demographic (age and gender), drug treatments (placebo vs ribaxamase, macrolide use and PPI use) and health status (no-food-by-mouth [NPO] as a surrogate for overall health). A linear regression model was used to determine correlation between these study data and changes in hit frequency of four classes of AMR genes, β-lactamase, vancomycin resistance, and macrolide resistance genes and multidrug efflux pumps. Significant correlations were observed with treatment group, macrolide or PPI use, and NPO.

**β-Lactamases and β-Lactam Resistance Genes**

A total of 781 genes or gene variants which confer β-lactam resistance were identified in the sample set, but ten genes accounted for the bulk of the hits in this class, the cfxA variant family (A-A6), cblA-1, PBP2a, OXA-347 and cepA. Linear regression analysis confirmed that five cfxA β-lactamase variants correlated with a statistically significant increase in the number of hits from T0 to T1 in the placebo group vs the ribaxamase group (Table 4). There was also a correlation between the use of PPI and changes in hit frequency from T0 to T1 for several β-lactamase genes (Table 5), with five of the cfxA family of variants and cblA-1 correlating with the use of PPI, while cfxA6 correlated with patients who did not receive PPI during the study.

**Vancomycin Resistance Genes**

During the Phase 2b ribaxamase study, a significantly greater number of patients in the placebo group became newly colonized with VRE (P=0.0001) at the T1 collection point as compared with the ribaxamase group. A total of 34 vancomycin resistance genes were identified in the 349 fecal samples analyzed. These included genes from the vanA, vanB, vanC, vanD and vanG operons. Three van genes demonstrated a significant increase in total number of hits from pre-antibiotic (T0) to post-antibiotic exposure (T1) in placebo-treated patients vs ribaxamase-treated patients. These genes included vanRD and vanSD, previously identified by LEfSe analysis, and vanD (Table 6).

**Macrolide Resistance Genes**

During the Phase 2b study with ribaxamase, patients could also receive macrolides for their LRTI. Eight macrolide resistance genes were identified within the fecal sample set, and all but four of the 349 samples carried at least one

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**Table 4 β-Lactamase Gene Variants Demonstrating a Significant Increase in Placebo vs Ribaxamase-Treated Patients from T0 to T1**

<table>
<thead>
<tr>
<th>β-Lactamase Gene Variant</th>
<th>Placebo</th>
<th>Ribaxamase</th>
<th>P-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0 (54)*</td>
<td>T1 (52)</td>
<td>T0 (65)</td>
</tr>
<tr>
<td>cfxA</td>
<td>42,067 (779a, 0–4951a)</td>
<td>113,364 (2,180, 0–20,881)</td>
<td>53,202 (818, 0–9161)</td>
</tr>
<tr>
<td>cfxA2</td>
<td>53,205 (985, 0–6591)</td>
<td>140,885 (2709, 0–23,657)</td>
<td>66,670 (1025, 0–11,581)</td>
</tr>
<tr>
<td>cfxA3</td>
<td>47,742 (864, 0–5075)</td>
<td>147,920 (2845, 0–17,942)</td>
<td>88,257 (1357, 0–22,534)</td>
</tr>
<tr>
<td>cfxA4</td>
<td>44,115 (816, 0–6372)</td>
<td>119,014 (2289, 0–18,212)</td>
<td>56,108 (863, 0–11,261)</td>
</tr>
<tr>
<td>cfxA5</td>
<td>47,325 (876, 0–5678)</td>
<td>131,480 (2528, 0–26,382)</td>
<td>60,249 (926, 0–10,968)</td>
</tr>
</tbody>
</table>

**Notes:**

- *Number of samples analyzed (≥95% of the samples had at least one hit each of the five genes).
- P-value is from linear regression model.
- Total hits, ie, the total number of matches for this gene or variant in the sample set.
- Average hits.
- Range of hits per sample.
- Was above statistical significance by this analysis, but was identified in other analyses.
sequence match for a macrolide resistance gene. Four genes accounted for the bulk of the macrolide resistance gene hits, *mefA*, *mel*, *mrx* and *mphA*, but only *mefA* demonstrated a significant correlation to any parameter examined in this analysis, a positive correlation with the use of PPI (P=0.0292). A trend toward correlation with macrolide treatment was also observed but did not reach significance (P=0.0785) for this gene.

**Efflux Pumps**

Efflux pumps were also selected for statistical analyses because of their involvement in AMR for many classes of antibiotics. Alignment of the sample sequences against CARD identified 39 efflux pump genes known to confer resistance to multiple drugs. All of the samples had at least one efflux pump gene present, but seven genes accounted for most of the hits, *evgS*, *mdfF*, *acrF*, *acrD*, *mdfB*, *mdtC* and *tolC*. Thirty-one efflux pump genes demonstrated a correlation with the treatment assignment (ribaxamase vs placebo), with 30 significantly increasing in hit frequency in ribaxamase-treated patients (Supplemental Table S5), while only one, *adeC*, demonstrated a significant increase in the number of hits correlating with placebo (P<0.0001). Notably, all 30 of these efflux pump genes demonstrated a positive correlation with macrolide exposure, while *adeC* did not. There was also a positive correlation in increased hit frequency following antibiotic exposure for 29 efflux pump genes in patients who were NPO at any time during the study, the exceptions being *adeC*, *mdtO* and *CRP*.

**Discussion**

In a Phase 2b clinical study, ribaxamase significantly reduced the incidence of CDI in patients treated with ceftriaxone for a LRTI. Consistent with this finding, ribaxamase protected the gut microbiome from damage caused by ceftriaxone as changes in microbiome diversity were significantly less in ribaxamase- vs placebo-treated patients by all measures examined (P<0.01), and appeared to allow the microbiome
Therefore, reducing exposure of B. uniformis and genes are associated with Capnocytophaga and in this Prevotella.

Notably, the relative by sequence analysis also displayed low β and cfxA2 and and suggesting that operon. that was in concordance with the hit frequency for β over and two E. avium two fi and Gene was correlated to cepha-

The two vancomycin resistance genes, vanSD and vanRD, that displayed increased frequency in placebo vs ribaxamase patients, are variants of the vanS/vanR two component regulator system that result in constitutive expression of vanD and confer high-level vancomycin resistance in E. faecium, E. faecalis and E. avium. Increased frequencies of vanSD and vanRD genes in the placebo group following antibiotic exposure are consistent with microbiologic culture data which demonstrated a significant increase in new colonization by VRE (P=0.0001) in placebo vs ribaxamase-treated patients from T0 to T1. Samples which were negative for VRE by microbiologic culture but positive for vanRD in this study may be due to colonization below the level of detection for culturing or that the detected vanRD gene was not part of an active vanD operon.

Since alignment of DNA sequences against a database is only semi-quantitative, yielding total hits based on DNA sequence matches within a sample, a qPCR strategy was pursued to corroborate these data. Two representative genes, one from each class of antibiotics discussed above, were selected for this work, cfxA and vanRD. The qPCR analysis yielded copy number data for cfxA and vanRD that was in concordance with the hit frequency for each gene. In general, samples with a low number of hits for cfxA or vanRD by sequence analysis also displayed low copy numbers, although there were some cases where these data did not correspond. This discordance could have been due to sequencing errors or other methodology shortcomings. Other possibilities include sequence variations affecting primer annealing efficiency or sequence divergence from the CARD database. It is also possible that samples that appeared to have high a high number of hits, but low copy number by qPCR, may be due to gene fragments or truncated genes that were detected by sequencing analyses but not amplifiable by qPCR. Regardless, these few discordances did not affect the overall conclusion that the qPCR data support the findings from sequencing analyses.

The change in copy number of cfxA and vanRD over the three sample collection points across treatment groups also reflected the overall conclusions from the ribaxamase clinical study. Placebo-treated patients had a general increase in copy number of both genes at T1 which
dropped back to near baseline levels by T2 for vanRD but remained elevated for cfxA, while ribaxamase-treated patients displayed a decline in copy number of both genes at T1 and T2. These findings were also consistent with the significant increase in the hit frequency of cfxA and vanRD from T0 to T1 in placebo vs ribaxamase groups.

Examination of the copy number data from individual patients can also be helpful in determining whether changes in the hit frequency of certain AMR genes represented new gene acquisition or expansion of existing genes. For cfxA and vanRD, both scenarios may have occurred. Observations of low initial AMR gene frequency that increased following antibiotic exposure are consistent with propagation of existing AMR gene pools, while undetectable baseline levels of AMR genes that then became detectable post-antibiotics are consistent with new acquisition of organisms carrying these genes. Another AMR gene pattern observed here was a high copy number at baseline which decreased at T1, and rose again by T2. This observation is consistent with an initial loss of organisms harboring these AMR genes during antibiotic exposure that then recolonized following cessation of antibiotic therapy.

A linear regression model was used to compare the change in the hit frequency of AMR genes from T0 to T1 for four antibiotic classes of interest, with specific clinical and demographic data from the study. This analysis confirmed the correlation between placebo treatment and increased hit frequency of the cfxA β-lactamase variants and the vanRD and vanSD vancomycin resistance genes that was also observed with LEfSe analysis. In contrast, increased abundance of efflux pump genes correlated with ribaxamase treatment, rather than placebo, suggesting that organisms carrying these genes were not resistant to ceftriaxone and could persist only when ceftriaxone was degraded by ribaxamase thus preserving these bacteria.

The linear regression analysis also revealed that the use of PPI correlated with changes in the gut resistome specifically leading to significant increases in several β-lactamase genes as well as the macrolide resistance gene, mefA. PPI are a known risk factor for CDI as they disrupt the balance of the gut microbiome. Notably, the cfxA family of β-lactamase genes is found in oral anaerobes, including Prevotella and Bacteroides species. Thus, it is interesting to speculate that the use of PPI may have allowed these oral anaerobes to survive stomach acidity and colonize the gut in the presence of β-lactams. The finding that the cfxA6 gene demonstrated a negative correlation with the use of PPI may be indicative of why this gene did not increase in hit frequency in placebo patients while the rest of the variant family did.

While all patients in the Phase 2b clinical study were to be treated with IV ceftriaxone for their LRTI, patients could also receive macrolides as deemed necessary by the treating physician. About a third of the patients in each group received macrolides. A previous study found that the use of macrolides significantly correlated with an increased relative abundance of numerous efflux pump genes, many of which confer macrolide resistance. Interestingly, in the present work, only one macrolide resistance gene, mefA, was associated with macrolide treatment, but did not reach significance (P=0.0785). Instead, mefA was significantly associated with the use of PPI (P=0.0292). The mefA gene is an erythromycin-inducible efflux pump found in S. pneumoniae. Since the patients in the Phase 2b study were admitted with a LRTI, most of which were pneumonias, it is possible that in the presence of PPI, lung bacteria carrying this gene survived passage through the stomach to at least transiently colonize the lower gut.

LEfSe is a tool developed to find biomarkers between two or more groups using relative abundances. LEfSe consistently provides lower false-positive rates and can effectively aid in explaining the biology underlying differences in microbial communities. This analysis indicated that genes encoding multidrug efflux pump components displayed a significantly decreased hit frequency in the placebo group and a significantly increased hit frequency with ribaxamase treatment. There was also a correlation of increased frequency of efflux pump genes with macrolide treatment. These observations are consistent with the hypothesis that bacteria harboring these genes were sensitive to ceftriaxone and had resistance to macrolides. An exception was the adeC gene, part of the AdeABC multidrug transporter from A. baumannii, that displayed a highly significant positive correlation with placebo-treatment but not with macrolide exposure. Acinetobacter relative abundance, detected with 16S rRNA sequencing, was extremely low in all fecal samples making the significance of this finding elusive.

There were shortcomings to the methodology used for this study. The total sample set was limited to 349 samples based on the contracted budget with the CDC, and it is possible that a larger set may have yielded stronger and/or additional correlations. For example, only seven post-antibiotic samples were available from patients with CDI. In addition, the fecal DNA sequencing analysis was performed by alignment with the CARD database. Limitations to this approach include 1) constraint by the
accuracy and breadth of the CARD, 2) a sequence match indicates the presence of at least a fragment of an AMR gene but does not signify that the entire gene is present, 3) sequences with homology to several genes may read as multiple hits to one gene, 4) total number of hits is only semi-quantitative and thus the findings must be qualified, and 5) detection of an AMR gene does not necessarily mean that the gene is functional and the AMR phenotype expressed. However, in this study a good correlation between the sequencing and qPCR data was observed. Assembling the sequences prior to alignment may have eliminated some of these concerns, but would also eliminate the semi-quantitative nature of the analysis. As with any comparative study, innumerable additional analyses, such as metagenomics comparison of these AMR data to our previously reported microbiome composition findings, are warranted and thus additional discoveries are likely to be uncovered from such future investigations.

Conclusions
This study demonstrated that exposure of the gut microbiome to ceftriaxone resulted in significant changes in the gut resistome. AMR genes, such as those encoding β-lactamases, increased significantly and this finding is consistent with the mechanism of antibiotic-mediated selection of resistant organisms. Ceftriaxone treatment also significantly increased the hit frequency of certain vancomycin resistance genes which correlated with changes in VRE colonization that were seen in the Phase 2b ribaxamase clinical study. Additional factors including the use of PPI and macrolides also appeared to precipitate changes in the gut resistome. The Phase 2b clinical study with ribaxamase demonstrated a reduction in the relative risk of CDI by 71% and a significant reduction in new colonization by VRE. Here, we show ribaxamase also attenuated changes to the gut resistome suggesting that ribaxamase has the potential to reduce the emergence of AMR in patients treated with IV β-lactam antibiotics. Complementary to antibiotic stewardship, ribaxamase administration would allow the continued use of a highly effective class of broad-spectrum antibiotics, the β-lactams, with diminished concern over microbiome damage and the emergence of AMR.

Clinical Trial Data
The Phase 2b clinical with ribaxamase which was the source for the samples for this study was published previously (ClinicalTrials.gov, number NCT02563106). Individual, de-identified participant data and additional trial and participant information regarding the clinical study are available and will be shared through the Lancet Infectious Diseases portal. Fecal microbiome sequencing data and antimicrobial resistance gene sequencing data are available through the SRA, accession PRJNA589866. These data will be accessible for at least five years from the time of publication.

Ethics Approval and Patient Consent to Participate
The Phase 2b ribaxamase study protocol and informed consent forms were approved by the appropriate institutional review boards or ethics committees (Supplemental Table S6), and the clinical trial which was the original source of the samples for this analysis was conducted in accordance with the Declaration of Helsinki. All study participants provided written consent to participate in the study and have analysis of their fecal samples conducted.

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References


